The tiniest genomes shrink much further: extreme reductive evolution in planthopper symbionts

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One-sentence summary: We present the smallest bacterial genomes known and evaluate the patterns and processes of their reductive evolution

Abstract

Endosymbiotic bacteria that provide limiting nutrients to sap-sucking hemipteran insects are known for their highly reduced genomes conserved in organization and function, a product of long-term co-diversification with hosts. Here, through genomics exploration of ancient *Sulcia* and *Vidania* endosymbionts from across 19 families of planthoppers (Hemiptera: Fulgoromorpha), we present multiple departures from that genomic stability. We show that gene have been lost gradually during ~263 my of host-symbiont co-diversification, with more dramatic changes promoted by co-infecting additional microbes and host ecological switches. This has resulted in the convergent evolution, in two planthopper superfamilies, of bacterial genomes at barely 50-52 kb, strikingly similar in gene contents and the ability to produce a single essential amino acid, phenylalanine. Our work identifies key drivers and limits of symbiont reductive genome evolution.

Key words: endosymbiosis, genome evolution, organelle, genome reduction

Introduction

The smallest artificial, carefully designed bacterial genome capable of axenic growth in a rich medium is only 531 kilobases [kb], an order of magnitude less than that of *Escherichia coli* (Hutchison et al. 2016). However, for nearly two decades, we have known that the genomes of obligatorily intracellular insect-symbiotic bacteria that rely on host support mechanisms can be

much smaller (Nakabachi et al. 2006). Specifically, in the genomes of ancient, strictly heritable bacterial symbionts of sap-sucking hemipteran insects, their gene sets have been reduced to only encompass the biosynthesis of essential nutrients and some key cellular functions (McCutcheon and Moran 2012; Moran and Bennett 2014; Campbell et al. 2017; Garber et al. 2021; McCutcheon et al. 2024). The resulting genomes are among the smallest known, sometimes barely exceeding 100 kb (Bennett and Moran 2013, 2015; McCutcheon et al. 2019, 2024). Nevertheless, after the presumed turbulent period of genomic degeneration, several symbionts have reached the stage where the remaining genes are indispensable and their loss slows to nearly a halt (McCutcheon et al. 2019). The genomic comparisons among divergent strains of *Buchnera*, *Tremblaya*, or *Sulcia*, which have co-diversified with their respective hemipteran hosts for well over 200 my, have revealed striking conservation of gene sets and order (Husnik and McCutcheon 2016; Chong and Moran 2018). However, stability has limits, and surveys encompassing a greater range of host lineages have revealed lost genes and occasional rearrangements (Chong and Moran 2018; Deng et al. 2023). The cicada symbiont *Hodgkinia* is perhaps the most striking example of repeated, extreme, and rapid degeneration (Campbell et al. 2017; Łukasik et al. 2018). Unfortunately, taxonomically limited sampling within most insect clades has prevented us from identifying broader patterns and key drivers of further genomic evolution and reduction.

Here, we uncover the genomic evolutionary patterns in ancient nutritional endosymbionts of planthoppers (hemipteran infraorder Fulgoromorpha). The last common ancestor of extant planthoppers that lived ca. 263 mya hosted two specialized, strictly heritable bacterial lineages with reduced genomes - a Bacteroidetes bacterium *Candidatus* Sulcia muelleri (thereafter *Sulcia*) and a Betaproteobacterium *Candidatus* Vidania fulgoroidea (thereafter *Vidania)* (Urban and Cryan 2012; Deng et al. 2023, 2024). Here, using metagenomic data for 149 planthopper species representing 19 families, we ask about the processes, limits, and drivers of genomic evolution of these ancient symbionts.

Results

1. *Sulcia* and *Vidania* have co-diversified with planthoppers for ~263 my

Sulcia and *Vidania* infections are broadly distributed across planthopper taxonomic diversity (Fig. 1). Based on rRNA sequences reconstructed from metagenomes, we have detected *Vidania*, usually accompanied by *Sulcia*, in 87 species from 149 sequenced, representing 15 of 19 surveyed families, on both sides of the deepest split in the planthopper phylogeny (Fig. 1, Table S1). This agrees with past reports that both these symbionts were present in the last common ancestor of extant planthoppers (Urban and Cryan 2012; Deng et al. 2023). Indeed, *Sulcia* infection is known to have pre-dated the divergence of Fulgoromorpha from its sister clade Cicadomorpha (which includes cicadas, spittlebugs, leafhoppers, and treehoppers (Moran et al. 2005). In contrast, the initial infection with *Vidania* seems to have occurred only after Fulgoromorpha and Cicadomorpha separated (Deng et al. 2023). Both symbionts have co-diversified strictly with their planthopper hosts (Fig. S1), as expected given their transovarial transmission (Michalik et al. 2009, 2021).

Besides *Sulcia* and *Vidania,* planthoppers often harbor other microbes. Where both *Sulcia* and *Vidania* are missing, we always observe Hypocreales fungi - members of an insect-

pathogenic clade known to have repeatedly replaced ancient bacterial nutritional endosymbionts of diverse Auchenorrhyncha (Matsuura et al. 2018; Michalik et al. 2023; Siehl et al. 2024). Further, Gammaproteobacteria (*Sodalis*, *Arsenophonus*) and Alphaproteobacteria (*Wolbachia*, *Rickettsia*, Bartonellaceae, Acetobacteraceae), acquired independently by different host lineages (Michalik et al. 2021), are known to provide vitamins and other complementary nutrients (Bennett and Mao 2018; Michalik et al. 2023) but also influence reproduction, defense, or other traits (Kaur et al. 2021; Davison et al. 2022). Together, these data show the relative stability of ancient bacterial associations while highlighting the evolutionary dynamics of more recently acquired microbes.

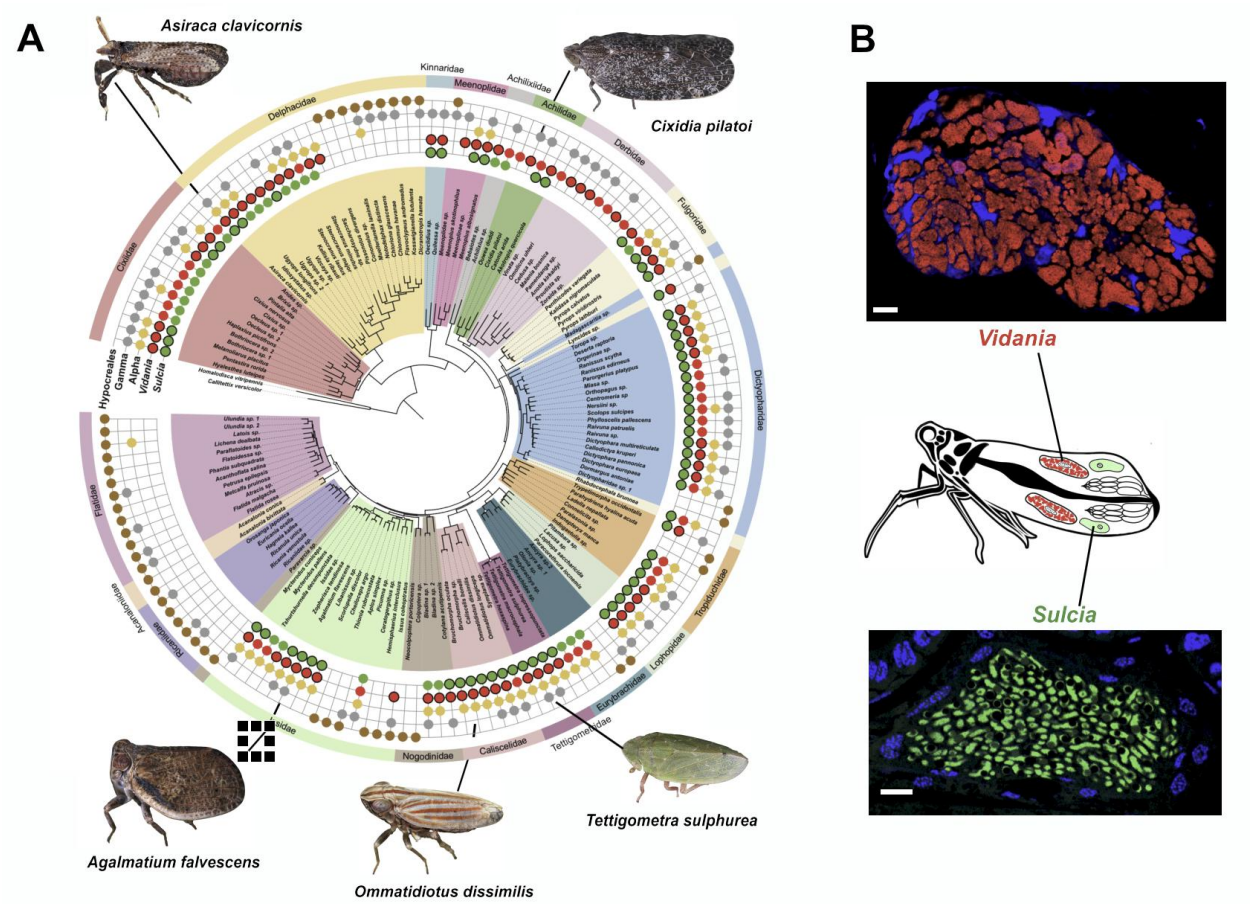

Fig. 1 A. Two ancient symbionts, *Sulcia* and *Vidania*, are broadly distributed across planthopper taxonomic diversity. The phylogeny, based on 1164 nuclear and 13 mitochondrial genes, has been redrawn from (Deng et al. 2024). Circles with bold lines indicate symbiont strains whose genomes were fully assembled and used for subsequent analyses. **B** *Sulcia* and *Vidania* live within the cytoplasm of dedicated cells (bacteriocytes) that form specialized organs (bacteriomes) that retain conserved organization across planthoppers - as visualized using confocal microscopy in *Akotropis quercicola* (Achilidae), scale bar - 10 µm. Photo credit: G. Kunz (insects), A. Michalik (microscopy).

2. *Sulcia* and *Vidania* have tiny genomes

Of 131 genomes of planthopper symbionts (64 *Sulcia*, 68 *Vidania*) that we deemed complete and used for content comparisons, most were circular. The two exceptions included the genome of *Sulcia* from *Trypetimorpha occidentalis*, previously confirmed to comprise two distinct circularly mapping contigs (Michalik et al. 2024), and *Sulcia* from *Brixia* sp., showing the same organization. The analysis of read mapping and the remaining contigs in the assemblies assured us of the completeness of genomes, as did syntheny comparisons. Specifically, all but one of *Vidania* genomes, including two smallest, were synthenic relative to the ancestral state (ref - Deng 2023)(Fig. 2, SX).

In *Sulcia*, one or more inversions relative to the ancestral state were seen in 13/63 (20.6%) genomes representing six families (Fig. 2, Fig. S3).

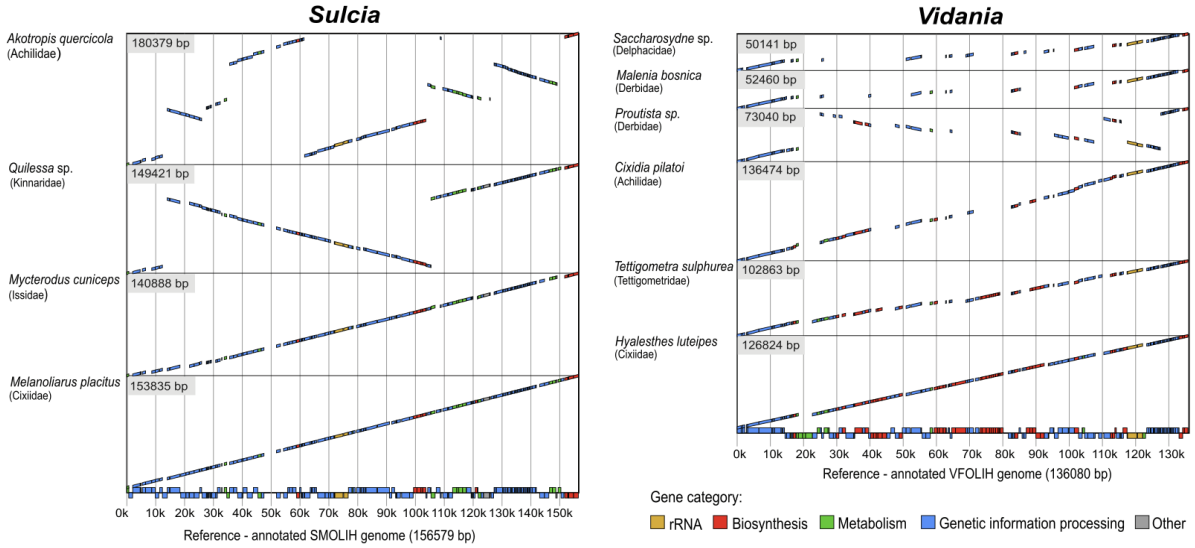

Fig. 2. The comparison of selected *Sulcia* and *Vidania* genomes against those from *Oliarus filicicola* OLIH (Cixiidae), some of the most complete, shows the conservation of genomic organization over >260 my years of evolution: the genomes are usually colinear, but with gaps corresponding to gene losses. Occasionally, however, one or more inversions occurred.

The size of the reconstructed symbiont genomes ranged from 137,729 bp to 180,379 bp for *Sulcia*, and from 50,141 bp to 136,554 bp for *Vidania*. c Indeed, several *Vidania* genomes are substantially smaller than the tiniest bacterial genomes characterized so far, including *Nasuia* from leafhoppers (>107.8 kb), previously characterized *Vidania* (>108.6 kb), or *Tremblaya* from mealybugs (>138 kb) (Bennett and Moran 2013; Husnik and McCutcheon 2016; Vasquez and Bennett 2022; Michalik et al. 2024). Correspondingly, many *Vidania* strains encode many fewer genes (as few as 63) than almost any other symbiont. Smaller gene sets have so far only been reported from *Hodgkinia* that form unique complexes of cytologically and genetically distinct but complementary lineages that apparently exchange gene products (Van Leuven et al. 2014; Campbell et al. 2017; Łukasik et al. 2018), and from organelles: mitochondria, and some of the most reduced chloroplasts (Kelly 2021).

Ancient insect symbionts have been known for extreme GC-content bias (Mahajan and Agashe 2022; McCutcheon et al. 2024). The values for planthopper endosymbionts are indeed low, with the medians of 25.64% (range 24.21-29.83%) and 20.52% (range 14.41-42.50%) for *Sulcia* and *Vidania*, respectively (Fig. 3B, Suppl. Table S2). Interestingly, while the values for *Sulcia* are relatively consistent, they are much more variable for *Vidania*, especially those with the most reduced genomes.

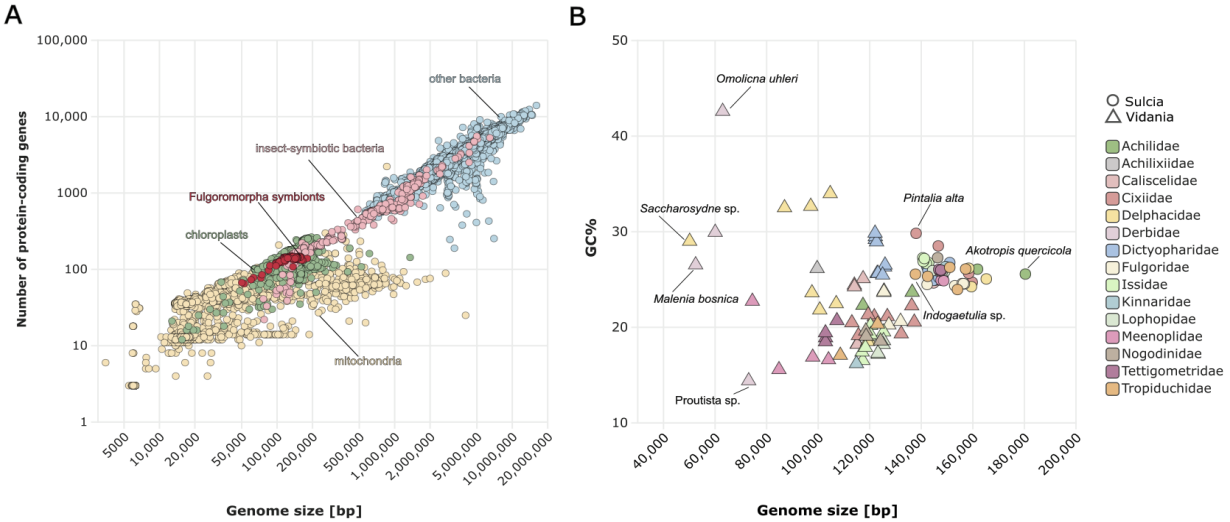

Fig. 3. In terms of genome sizes and gene contents, planthopper symbionts (red) fall among the very smallest insect symbionts (pink), which in turn are among the smallest bacteria (blue) - and among cellular organelles: mitochondria (orange) and chloroplasts (green). **B.** In terms of genome size and GC contents, *Sulcia* genomes tend to be relatively consistent, whereas *Vidania* genomes represent a much broader range on both scales.

3. *Sulcia* and *Vidania* have been systematically losing genes during their co-diversification with hosts

The comparison of curated, custom annotation of these unusual and, in the case of *Vidania*, extremely rapidly evolving genomes (Supplementary Text) enabled the reconstruction of their ancestral gene sets - those with identified functions present in any genome. In the last common ancestor of extant planthoppers, *Sulcia* must have encoded ≥164 total genes. This includes 10 involved in the biosynthesis of four essential amino acids (Leu, Ile, Val, Phe), 132 genes involved in genetic information processing, and 18 involved in metabolism. The ancestral *Vidania* must have encoded ≥169 total genes, including 43 total amino acid biosynthesis genes (Met, Arg, Thr, Trp, Phe, Lys, His), 113 genetic information processing genes, and 13 metabolism-related genes. Additionally, across all *Sulcia* and *Vidania* genomes, we identified 40 and 86 hypothetical protein-coding genes with no significant similarity to any records within the Uniprot database. While some of them were conserved in some host clades, suggesting functionality (Tables S3-S4), we did not include them in subsequent analyses.

Genome comparisons indicated no cases of gene gain by any lineage. However, gene losses were common during the evolution of both *Sulcia* and *Vidania*. With a notably higher rate of loss in the latter symbiont, the patterns in both suggest a gradual, ongoing loss of genes during host-symbiont co-diversification, particularly those involved in genetic information processing and

metabolism. But while some genomes retained >95% of the ancestral gene set, we observe several instances of much more substantial losses, often involving also nutrient biosynthesis pathways (Fig. 4A-B; Fig. S2). For *Vidania*, this includes 49 gene losses on the branch leading to *Sacharosydne* sp. (Delphacidae); 40 losses in the lineage leading to the sole characterized Achilixiidae species, *Bebaiotes* sp., 40 losses in the common ancestor of Derbidae (42), 32 in the ancestor of *Meenoplus skotinophilus* (Meenoplidae), and 25 in the ancestor of *Cixidia pilatoi* (Achilidae). In turn, the branch leading to *Trypetimorpha occidentalis* (Tropiduchidae), and the ancestral branch of Tettigometridae exemplify cases where both *Sulcia* and *Vidania* lost multiple genes (4A-B; Figs. S2-S3). Strikingly, four of the five lineages where *Vidania* lost most genes (all listed above except *Bebaiotes*) have also lost the *Sulcia* symbiont entirely, whereas outside of these lineages, only one additional case of *Sulcia*-but-not-*Vidania* loss, through apparent replacement by a Hypocreales fungus in *Issus coleoptratus* (Issidae) lineage, was seen.

We also found major differences among gene functional categories in the overall level of conservation (Fig. 4C). In *Sulcia*, ancestral biosynthesis genes are always retained, with one exception: *aspC*, a gene involved in phenylalanine biosynthesis. *AspC* is found in *Sulcia* from seven species, whereas in most other planthoppers this gene is encoded within the *Vidania* genome instead (Fig. S2). In *Vidania*, despite overall conservation, every biosynthetic pathway has been lost from at least one genome, with Phe pathway most consistently retained. In both symbionts, among genetic information processing and metabolism-related genes, rRNA genes are always retained, and ribosomal proteins and genes involved in protein-folding and degradation are highly conserved. In contrast, aminoacyl-tRNA synthetases are frequently lost from the genomes.

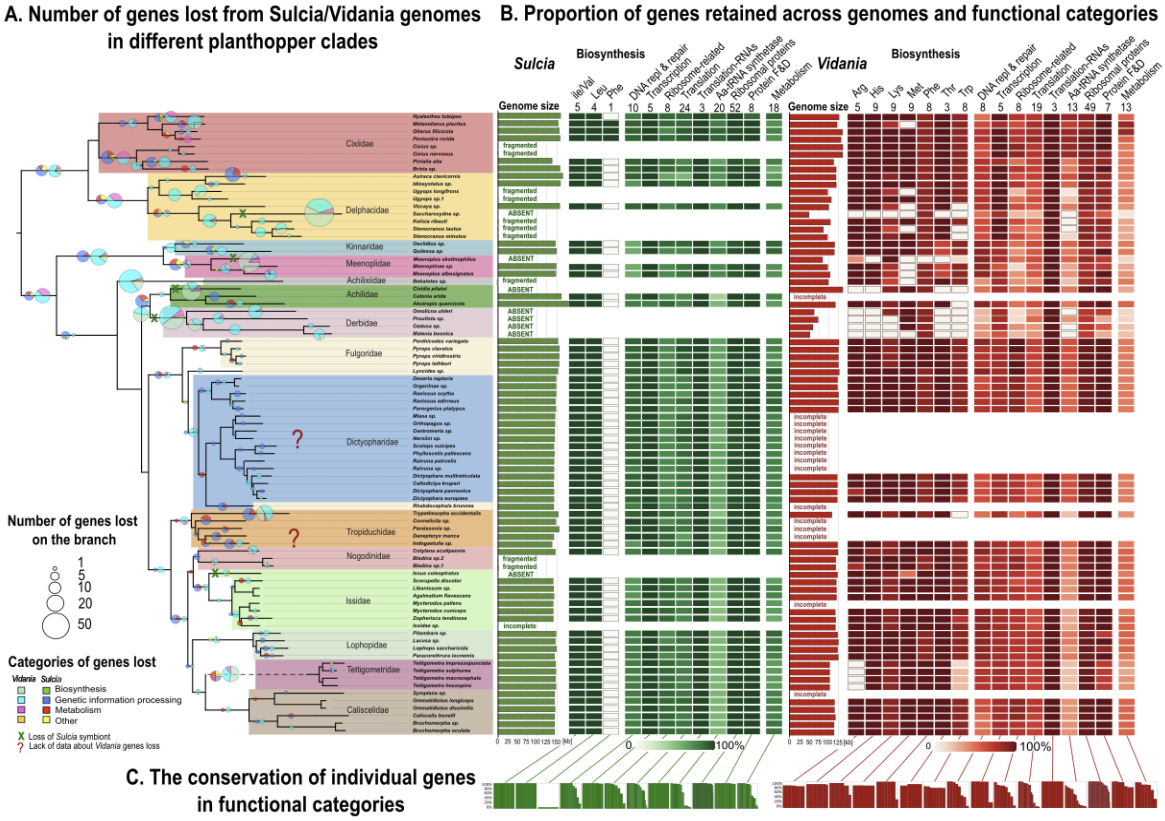

Fig. 4. The reconstructed patterns of *Sulcia* and *Vidania* gene retention across the planthopper phylogeny indicate gradual gene losses throughout host-symbiont co-diversification, and much more dramatic changes in some lineages, with substantial variation among symbionts and gene categories. **A.** The insect phylogenomic tree, trimmed from Fig. 1 to only include species with the genome of at least one symbiont complete, is used to map the reconstructed numbers of genes on all branches, indicated by separate size-scaled pie charts for each symbiont. **B.** For all complete *Sulcia* and *Vidania* genomes and key functional categories, we show the proportion of the ancestral gene set that was retained. **C.** We show how the conservation of individual genes, i.e., the proportion of genomes where the gene is retained, varies among gene categories.

4. The tiniest known genomes belong to symbionts reduced to Phe provisioners

The two smallest bacterial genomes in our collection - and the smallest bacterial (non-organellar) genomes described to date - are only 50-52 kb: that of *Vidania* strain VFSACSP1 from *Saccharosydne* sp. (Dephacidae) has 50,141 bp, and that of VFMALBOS from *Malenia bosnica* (Derbidae) has 52,460 bp (Fig. 5A). The smallest encode just over 60 protein-coding genes with identified functions each (68 in VFSACSP and 62 in VFMALBOS). Notably, the extremely reduced state represented by these two genomes has evolved independently in two superfamilies, on both sides of the deepest split in the planthopper phylogeny.

Despite their independent evolution, these genomes exhibit striking convergence in gene retention patterns and organization compared to their common ancestor, which must have had a genome approximately three times their size (Figs. 5A, B, S3, S5, S6).

In each of them, the set of genes involved in essential cellular processes comprises rRNA genes, a set of 35-38 ribosomal proteins, 3-4 ribosomal DNA and RNA polymerase subunits, 15 other genes involved in genetic information processing (incl. two chaperonins - dnaJ & dnaK), and one or two metabolism-related genes. Interestingly, neither genome retains any aminoacyl-tRNA synthetases, but VFMALBOS and VFMEESP1 retain some tRNA genes, although extreme rates of sequence evolution complicate their detection) (Table S4). Further, both these strains encode the entire ancestral set involved in phenylalanine biosynthesis, while pathways for the biosynthesis of six other essential amino acids have become entirely lost (Fig. 5B). No other significant functions seem to be encoded in these genomes.

In contrast, the smallest genome from the third planthopper superfamily, VFMEESP1 (*Meenoplus skotinophilus*, Meenoplidae), is substantially larger at 84,795 bp and retains an expanded set of genetic information processing and metabolism genes (Fig. 5A). VFMEESP1 preserves the complete pathway for lysine biosynthesis and three genes from the arginine and tryptophan pathways, but interestingly, this is the only *Vidania* genome that has lost the phenylalanine biosynthesis pathway (Fig. 5D).

Notably, all planthoppers hosting these highly reduced *Vidania* strains have also lost *Sulcia:* they represent the few clades in our dataset where that symbiont has been lost while *Vidania* remained (Fig. 4, 5).

The organization and ultrastructure of *M. bosnica Vidania*-hosting bacteriome tissue reveal several differences when compared with those of other planthopper species that we studied before (Michalik et al. 2021, 2023, 2024). Most strikingly, *M. bosnica* bacteriocytes exhibit an unusually high density of mitochondria (Fig. 5C). Additionally, in some specimens we observed

signs of cellular degeneration, including vacuolization of the bacteriocyte cytoplasm and higher bacterial cytoplasm density (Fig. 5C). In the bacteriome, we did not detect any other adjacent bacteria that could serve as an obvious source of targeted genes (Bublitz et al. 2019).

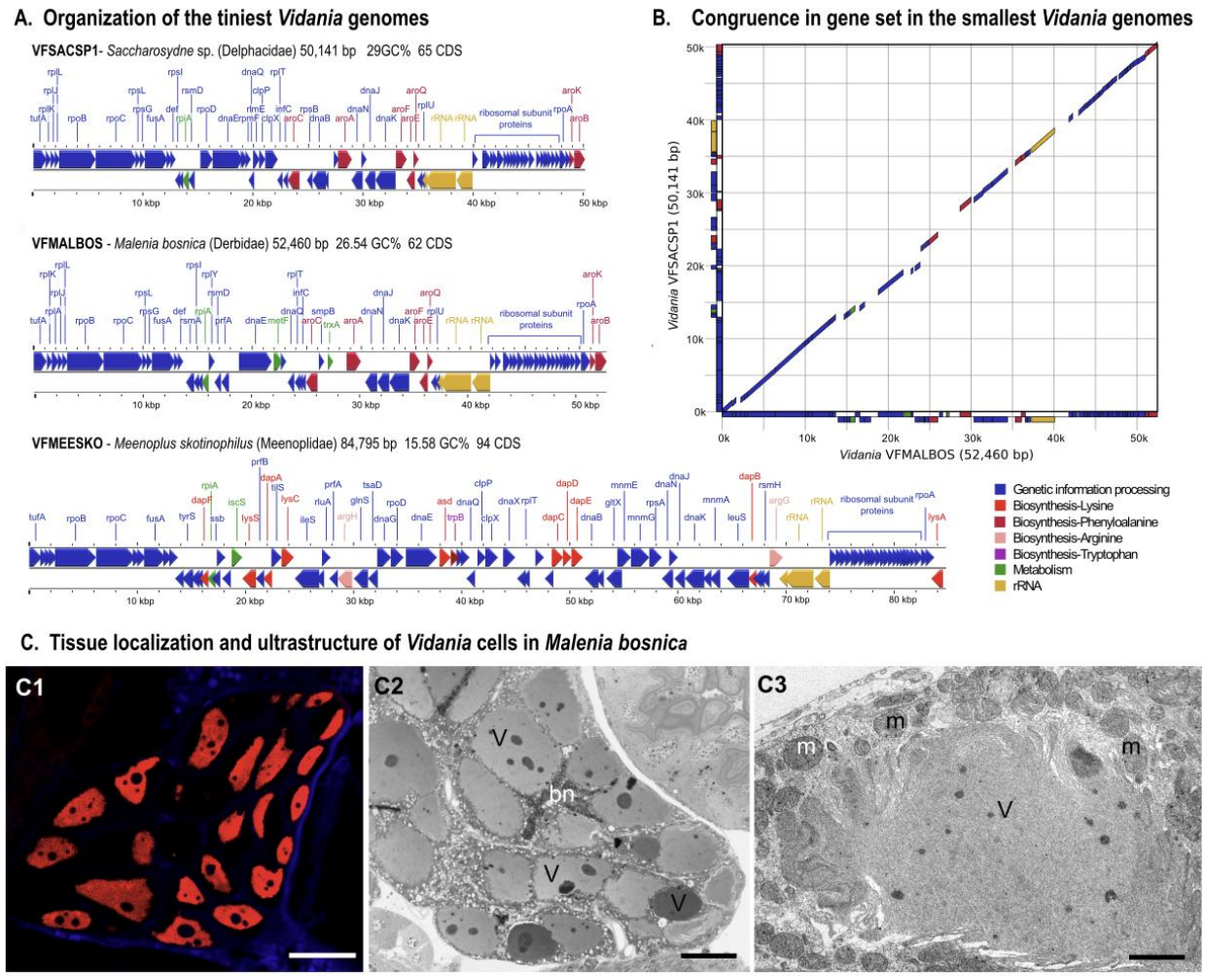

Fig. 5. A. The representation of the smallest *Vidania* genomes from three planthopper superfamilies showcases their strongly reduced gene sets. **B.** The alignment of the two tiniest *Vidania* genomes that evolved independently from an ancestor approximately three times the size shows striking convergence in which genes are retained. Because of very low sequence similarity among genomes, the alignment plot is based on the positions of annotated genes. **C.** Morphology and ultrastructure of *Malenia bosnica* bacteriome tissue and its tiny-genome *Vidania* VFMALBOS, shown using fluorescence (C1), light (C2), and transmission electron microscopy (C3), differs from other planthoppers by particularly high mitochondrial density. bn - bacteriocyte nucleus, m - mitochondrium, V - *Vidania* cell; C1, C2 - 10 um, C3 - 1 um.

5. Nutritional functions explain the evolution of symbiont genomes and multi-partite symbioses

In hemipteran nutritional symbioses, the set of functions related to nutrient biosynthesis is thought to be generally conserved at the level of symbiosis, even when microbial partners change.

In previously characterized planthopper symbioses, *Sulcia* and *Vidania* were thought to fulfill their hosts' nutritional needs, as they retain the conserved set of genes for the biosynthesis of all ten essential amino acids, with genes encoded on the host genome thought to complete the pathways (Fig. 6). However, we find that other bacteria present in these metagenomes contribute amino acid biosynthesis genes. Some of them, including *Sodalis*, *Arsenophonus*, *Symbiopectobacterium*, *Wolbachia*, or *Tisiphia*, represent known heritable endosymbionts previously detected in planthopper tissues (Michalik et al. 2023); others, including *Serratia*, *Klebsiella*, *Sphingomonas*, or *Pseudomonas*, are likely to represent less specialized gut associates. The genes they encode often overlap with those present in *Sulcia* or *Vidania*. However, in many species that had lost *Sulcia* or some *Vidania* biosynthesis pathways, the genes encoded by other microbes often at least partially fill the resulting gaps (Fig. 6). This pattern is most evident in planthopper species where symbionts have the tiniest genomes - *Saccharosydne sp.* or *M. bosnica* - where such 'other' bacteria - *Tokpelaia* and *Sphingomonas*, respectively - encode largely complete sets of biosynthesis genes. In turn, in most other derbids, in *Issus coleoptratus*, and others, for a large share of genes lost from ancient genomes we did not find an alternative within the assemblies. For example, for *Meenoplus skotinophilus*, none of the genes from nine amino acid biosynthesis pathways lost was present in 9Gb metagenome for its dissected abdomen. We note, however, that insufficient sequencing coverage for genomes of other bacteria (or fungus in *I. coleoptratus*) could have prevented the discovery of some functions. Likewise, the reliable identification of biosynthesis genes potentially encoded within the host nucleus requires a more complete assembly of the host genome.

The mapping of major biological transitions onto the phylogeny also suggests the role of host ecology in structuring their nutrition. Specifically, Derbidae and Achilidae feed on fungal hyphae during their immature stages (*Howard et al. 2001*), species in the genus *Tettigometra* engage in obligate trophobiotic associations with ants (Delabie 2001), and *Meenoplus skotinophilus* has adapted to life in caves (Hoch et al. 2025). The resulting alteration of nutrient availability and nutritional demands in these clades is likely to explain changes in biosynthetic gene sets.

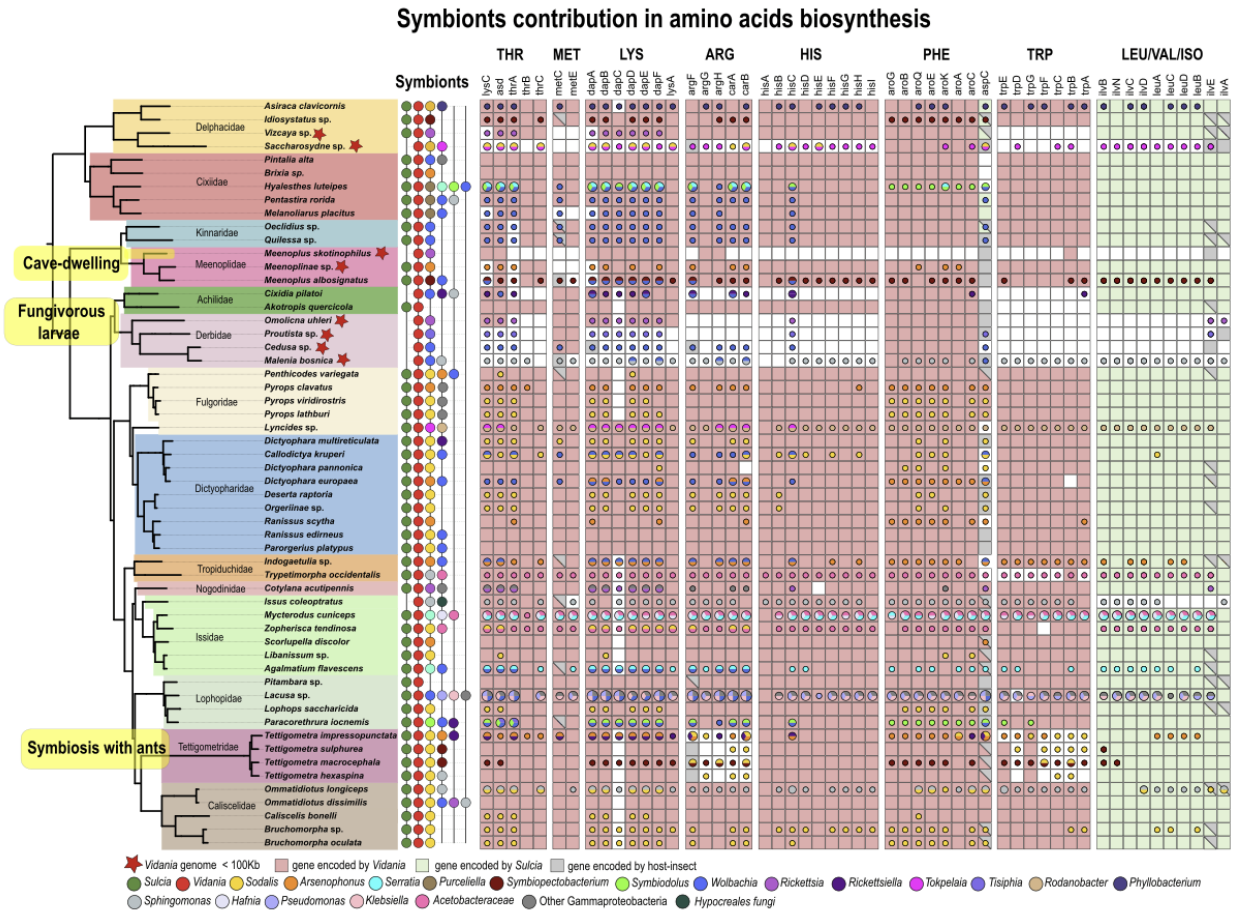

Fig. 6. The distributions of amino acid biosynthesis genes across bacterial genomes in different planthopper species suggest that they may often compensate for the loss of pathways from *Vidania* genomes or the loss of *Sulcia*. Host phylogeny is redrawn from Fig. 1, but trimmed to only include species where *Vidania* and *Sulcia* (if present) genomes are complete. Bacterial presence/genus-level classification is based on the combined 16S rRNA and protein-coding gene reconstruction. Green, red, and grey backgrounds in the main plot panel indicate genes present in *Sulcia*, *Vidania*, and host genomes, respectively, and colored circles represent genes matched to other bacteria, with the circle size proportional to the number of genomes where the gene was found.

Discussion

The symbioses between planthoppers and their ancestral heritable endosymbionts *Sulcia* and *Vidania* can be remarkably stable, as shown by the similarity in genome organization and contents across the planthopper phylogeny and the previously demonstrated conserved organization of symbiont-containing tissue (Michalik et al. 2023). However, this is not always the case, and during the ~263 my of planthopper evolution, one or both of these symbionts have been replaced by Hypocreales fungi at least 10 independent times (Fig. 1; Siehl et al. 2024), and complemented by other bacteria much more often. While the ancient symbionts have been gradually losing metabolism and genetic information processing genes during co-diversification

with hosts, our data indicate that the combination of contributions by other bacteria and of host ecological shifts can set the arena for a much more dramatic reduction of *Sulcia* and *Vidania* genomes and functions.

Genome size evolution: how small can it get?

In several planthopper lineages, these changes resulted in genomes much smaller than the tiniest characterized to date, which begets the question of the gene set and genome size limits (Moran and Bennett 2014; McCutcheon et al. 2024). To remain viable, a nutritional symbiont needs to provide biosynthetic functions that make it indispensable for the multi-partner symbiosis, and encode a set of genetic information processing and metabolism genes needed for its basic cellular functions that cannot be provided by the host.

We showed that in planthopper symbionts, nutritional functions encoded within a genome can be reduced to a single amino acid biosynthesis pathway. However, in other symbiotic systems, encoding genes involved in a single enzymatic reaction may suffice (Garber et al. 2024). In terms of cellular functions, the symbionts with tiny genomes, just like organelles, are kept alive through extensive host support systems, with host-encoded proteins targeted into the symbiont cells (Mao et al. 2018a; McCutcheon et al. 2024). As a result, only a small fraction of genes need to be encoded on the symbiont's genome. The core gene set universally retained within the *Vidania* genomes includes rRNA genes, about 34 ribosomal proteins, RNA polymerase and DNA polymerase subunits, and two chaperonins. This set is very similar in convergently evolved, tiniest *Vidania* genomes from two planthopper superfamilies, suggesting that they have independently reached the limit of reductive evolution in this system. The conserved gene set may also include tRNAs, but due to extreme evolutionary rates of these genomes, their accurate annotation may require focused efforts (Van Leuven et al. 2019), and in mitochondrial genomes, tRNAs can be lost entirely (Butenko et al. 2024). Other potential sources of cell function and genetic information processing-related gene products also include other symbiont lineages present in the same host. For example, in cicadas, distinct cellular lineages derived from the ancestral *Hodgkinia* symbiont are thought to exchange proteins from the conserved set involved in these fundamental cell functions (Campbell et al. 2017; Łukasik et al. 2018).

Judging from the parallels with organelles, symbiont genomes could potentially become much smaller than even the tiniest *Vidania* strains identified here. The smallest known mitogenomes encode two rRNA genes plus two function-related genes (Butenko et al. 2024), suggesting that with adequate host support, a theoretical gene content limit of three (rRNAs plus one function-related) may be enough to ensure the genome's long-term persistence (Oborník and Lukeš 2015). We anticipate that future genomic surveys across non-model insects will reveal symbionts much closer to that limit.

How the tiniest genomes shrink further?

The mechanisms driving genomic reduction in insect nutritional intracellular endosymbionts include strong mutation and deletional pressure combined with the lack of recombination, no opportunities for gene acquisition, and strong genetic drift associated with symbiont transmission bottlenecks (McCutcheon and Moran 2012; Bennett and Moran 2013;

Moran and Bennett 2014; McCutcheon et al. 2024). Through these processes, non-essential genes are gradually lost, with Mueller's ratchet facilitating the fixation of somewhat detrimental but non-lethal changes (Moran 1996; Pettersson and Berg 2007; Bennett and Moran 2015). The long-term conservation in ancient symbionts' genomes of genes and pathways related to the biosynthesis of essential nutrients lacking in the host diet is linked to their essentiality for the symbiosis. However, host association with additional microorganisms that encode that gene, or perhaps their horizontal acquisition, can enable their loss (Husnik et al. 2013; Husnik and McCutcheon 2016; Mao et al. 2018b; Van Leuven et al. 2019). Also, changes in the host biology, especially switches to diets with different nutritional contents, may render ancient symbiont-encoded nutritional functions unnecessary and prone to loss. For genes associated with basic cellular functions, their loss from the symbiont genome may occur through gene transfer to the host genome, re-targetting of products already encoded in the host genome, or the emergence of mechanisms that allow for the exchange of gene products between co-ocurring symbiotic bacteria (e.g., protein trafficking to organelles and symbionts; apparent tRNA sharing (Van Leuven et al. 2019; McCutcheon et al. 2024)).

Our results support both proposed mechanisms for the loss of nutritional-function genes in planthopper ancient symbionts: compensation by other microbes and host ecological shifts. In planthopper species whose *Vidania* lost amino acid biosynthesis genes or entire pathways (or where *Sulcia* was lost), we frequently identified the same genes within the genomes of other microbes comprising the symbiotic system, and sometimes within the host genome. They do offer a plausible replacement strategy for *Sulcia* or *Vidania* functions, even if their expression, product sharing with the host, or the ecological stability of associations with complementing microbes need to be verified. Dissecting host-encoded mechanisms for protein targeting or transferring among symbionts would require dedicated experimental efforts, including spatial multi-omics, but would substantially expand our knowledge about the mechanisms of host-symbiont-organelle interactions. However, such processes have been convincingly demonstrated in mitochondria and chloroplasts (Gray 2012; Smith and Keeling 2015; Kelly 2021). In turn, insect ecological shifts, especially those linked to nutrition, have been convincingly linked to microbiota changes, and specifically, the formation of heritable symbioses (Sudakaran et al. 2017; Cornwallis et al. 2023). We know of few parallels to Derbidae-Achilidae planthoppers, where the dietary switch enabled the degeneration or dissolution of ancient nutritional symbioses. *Typhlocybinae* leafhoppers, which had switched from feeding on plant sap to feeding on parenchymal cell cytoplasm and in many cases seem to have lost ancient symbionts (Buchner 1965) are one such example.

What are the consequences of extreme symbiont genome reduction?

As a consequence of gene losses, symbiont roles in insect biology can change substantially. The biosynthetic capacity of all three most reduced *Vidania* strains is limited to providing two amino acids known to be in high demand for the formation, melanization, and sclerotization of a cuticle - insects' first line of defense against natural enemies and abiotic challenges such as desiccation (Andersen 2010; Suderman et al. 2010). *Vidania* of *Saccharosydne* sp. and *Malenia bosnica* have convergently retained the ability to produce phenylalanine, a substrate for the production of tyrosine (Kaltenpoth et al. 2025), identified as the key symbiotic contribution in beetles and hymenopterans (Vigneron et al. 2014; Anbutsu et al.

2017; Kiefer et al. 2023). In contrast, the lysine-biosynthetic capacity of the *Vidania* from cave-dwelling *Meenoplus skotinophilus*, may reflect its adaptation to an environment where a hardened, melanized cuticle is not necessary. This pattern suggests that in these planthopper systems, the role of ancient nutritional symbionts has become restricted to cuticle formation, whereas host insects' other nutritional needs are fulfilled through changed diets or other microbes' contributions. Systems such as the recently detailed *Trypetimorpha occidentalis* (Michalik et al. 2024), demonstrate how the relatively recent host colonization by a new symbiont with a comprehensive set of functions may have permitted the genomic degeneration in other players within the multi-partite symbiotic systems, with the new symbiont likely becoming the central player in its nutrient-provisioning patchwork. Either way, the reduced reliance of hosts on the ancient symbionts may have facilitated the loss of genes associated with nutritional cellular functions from their genomes, genomic rearrangements, and the complete *Sulcia* loss by some lineages.

Relaxed selective pressure on the ancient symbiont is likely to speed up their evolution and rate of gene loss. The ratchet-like accumulation of negative changes and functional losses in the symbiont genomes will push the symbiosis deeper into what was described as "the evolutionary rabbit hole" - where the host is tied in a mutually obligate relationship with a degenerating partner (Bennett and Moran 2015). A few possible long-term outcomes of this situation are exemplified by planthoppers and other Auchenorrhyncha. First, the reduced symbiosis may reach a new 'stable equilibrium' where the ancient symbiont is at an even more reduced state, with some functions potentially shifted to other microbes or the host. The striking similarity among two *Vidania* genomes independently reduced to ~50kb from a three-fold larger ancestral state showcases that such state may exist, and as evidenced by animal mitochondria, such a state can remain functionally unchanged for a very long time, despite rapid sequence evolution. Second, the symbiosis may not be able to escape the degenerative spiral, ultimately leading to the extinction of the host lineage. The extremely fragmented and very rapidly evolving *Hodgkinia* symbiotic complexes of *Magicicada* periodical cicadas (Campbell et al. 2015, 2017), with their high apparent maintenance costs (Campbell et al. 2018), may exemplify this situation. We suspect that the fragmented symbiont genomes of some planthoppers, which we were not assemble and chose not to characterize here, may represent a similar situation. Third, the replacement of degenerate ancient symbionts by newly arriving and more versatile and efficient bacteria or fungi (Matsuura et al. 2018; Michalik et al. 2023; Siehl et al. 2024) remains a possibility that may enable planthoppers and their symbionts, to take new evolutionary paths. On the other hand, such replacement is very likely to start the new cycle of symbiosis degeneration (McCutcheon et al. 2019), with the "evolutionary rabbit hole" very difficult, if at all possible, to escape permanently.

Methods

We used the recently published set of metagenomic datasets for abdominal tissues of taxonomically diverse global planthoppers (Deng et al. 2024), prepared using different commercial kits and sequenced on various Illumina platforms (Table S1). Previously extracted insect nuclear and mitochondrial gene data and phylogenies (Deng et al. 2024) served as the phylogenomic framework in the current study. *Sulcia* and *Vidania* genomic fragments were

identified in Megahit metagenomic assemblies using BLAST tools and previously curated reference genomes. After connecting circularly mapping scaffolds, we validated them through read mapping, and reorganized to ensure the same start positions of each genome (for further details see Michalik et al., 2021). Annotations of these highly divergent genomes, especially of *Vidania* characterized by extreme sequence evolutionary rates, were done through iterative HMMER searches using collections of hand-curated protein alignments, validated and updated after every round of annotation (Supplementary Text). The final annotations of these genomes, analyzed in phylogenetic context, underlie all figures. Genome organization was compared using *Sulcia* and *Vidania* genomes from *Oliarus filicicola* OLIH, which turned out to be among the most complete (Bennett and Mao 2018), as references. Amino acid biosynthesis gene presence in genomes of symbionts other than *Sulcia* and *Vidania* within translated assemblies was reconstructed through HMMER searches using HMM profiles from the NCBI Protein Family Models database. We taxonomically classified the identified proteins through BLAST-based comparisons against the nr database and curated *Sulcia* and *Vidania* genomes. At that stage, we also filtered out genes of the known reagent-derived contaminant in our data, *Cellulosimicrobium*. Information about genome sizes and contents used for Fig. 2 was based on resources available in the NCBI db on April 2024, filtered to retain complete genomes only (67081 genomes). Data was visualized using iTol (Letunic and Bork 2024), Flourish (https://flourish.studio/), and custom Python and Processing scripts. Panels were assembled, and final details added using Inkscape.

For histological and ultrastructural analyses, insects abdomens were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Sections were stained in methylene blue or contrasted with lead citrate and uranyl acetate and examined under a light microscope Nikon Eclipse i80 and transmission electron microscope Jeol JEM 2100, respectively. For fluorescent *in situ* hybridization (FISH), ethanol-preserved abdomens were fixed in 4% formaldehyde, dehydrated, and embedded in Technovit 8100 resin. Sections were incubated overnight with a hybridization buffer containing *Sulcia* and *Vidania*-specific probes (Supplementary text) and analyzed under a Zeiss Axio Observer LSM 710 confocal microscope.

Data availability

Sequencing data underlying this article and curated genomes can be accessed through NCBI Umbrella BioProject PRJNA684615. Annotation, analysis, and visualization scripts are available through the project GitHub page: https://github.com/AnnaMichalik22/Planthopper-ancient-symbioses---supplementary-materials.git.
Genomic annotations, gene alignments, and protein references used for annotation were deposited in the Figshare repository under the doi 10.6084/m9.figshare.29064272.

Author contributions

AM: Conceptualization (equal), Resources (supporting), Methodology (equal), Investigation (lead), Formal Analysis (lead), Visualization (equal), Validation (equal), Data curation (equal),

Funding Acquisition (equal), Project Administration (lead), Writing – Original Draft Preparation (supporting), Writing – Review & Editing (equal)
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Competing interests

The authors declare no competing interests.

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