Contrasting genomic trajectories of *Bartonellaceae* symbionts of planthoppers

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Abstract

Symbioses with microorganisms have shaped the nutritional biology and evolution of many insects. For example, several ant clades have adapted to nutrient-poor diets through symbiosis with a specific clade of bacteria in the family *Bartonellaceae* (Hyphomicrobiales), notorious for also including virulent vertebrate pathogens. Here we show that *Bartonellaceae* phylogenetically placed within the clade that has only encompassed ant symbionts to date – *Candidatus* genus Tokpelaia – have established as symbionts in four different clades of planthoppers (Insecta: Hemiptera: Fulgoromorpha). Genome size and contents indicate different levels of integration of

these strains into the planthopper host biology and their diverse roles. Symbionts infecting one of the clades have some of the largest genomes among *Bartonellaceae*, at ca. 2 Mb, two others are under 700 kb, and the fourth is reduced to barely 158 kb. The planthopper-associated *Tokpelaia* strains with larger genomes, similarly to ant symbionts, encode multiple amino acid and vitamin biosynthesis genes, complementing the degraded nutritional capabilities of their hosts' ancient heritable endosymbionts. Strikingly, the smallest *Tokpelaia* genome lacks any genes linked to essential amino acid biosynthesis, in contrast to all other known insect-associated bacteria with genomes of comparable size. We identified a single vitamin biosynthesis gene and iron-sulfur cluster assembly genes as its only putative contributions to the host biology.

Our results broaden the host spectrum of non-pathogenic *Bartonellaceae*, indicating that they have contributed to nutrition and symbiotic consortium function in diverse diet-restricted host clades. They also highlight an unexpectedly broad range of evolutionary outcomes for this important bacterial group.

Key words: nutritional endosymbiont, amino acid biosynthesis, vitamins, Auchenorrhyncha, Rhizobiales, *Bartonella*

Significance

Many insects rely on bacterial partners to overcome nutrient-poor diets, yet the diversity and evolutionary trajectories and outcomes for these associations remain unclear. Our discovery of *Bartonellaceae* bacteria in planthoppers improves the understanding of the host range of these broadly relevant bacteria best-known as vertebrate pathogens. The finding that these planthopper symbionts generally have the capacity to produce essential amino acids and vitamins relevant to hosts and likely form long-term symbiotic associations agree with the expectations for sap-sucking hemipteran symbionts. However, the breadth of sizes and functions of the newly assembled

genomes substantially expand the known range of states for *Bartonellaceae*, and their evolutionary trajectories. In particular, the identification of a strain with an ultra-reduced genome of only 158kb with no amino acid biosynthetic functions contrasts with all other known insect-symbiotic bacteria with genomes in a similar size range. This study also indicates how symbionts may enable the degeneration, functional loss, and evolution of other bacteria residing in the same host.

1. Introduction

Microbial symbionts can play dramatically important and highly diverse roles in the biology of their animal hosts, shaping their life history traits, ecological niches, biological interactions, and evolutionary processes (Moran 2007; Douglas 2014; Lukasik and Kolasa 2024). In particular, host long-term evolutionary trajectories have repeatedly been shaped by the microbial contributions to the host nutrition (Moran 2007; Sudakaran, et al. 2017; Brentassi and de la Fuente 2024). Microbial symbionts frequently help address challenges related to the digestion of recalcitrant foods, the neutralization of toxic compounds, and the acquisition of limiting nutrients that animals require but cannot make on their own. For example, plant-based foods typically have low nitrogen contents and specifically lack essential amino acids as well as vitamins, and many herbivores rely on symbiotic bacteria for nitrogen recycling, upgrading, and sometimes fixation, and for the biosynthesis of lacking nutrients (Hansen and Moran 2014). Insects provide many independent examples of associations between heritable symbiont acquisitions and shifts to limiting diets (Cornwallis, et al. 2023).

Ants (Hymenoptera: Formicidae) are often quoted as an example of a clade that has undergone extensive dietary diversification, including multiple independent transitions from more balanced omnivorous diets to herbivory (Russell, et al. 2009; Smith, et al. 2023). These nutritional changes have typically been enabled by bacterial symbionts, most often in the family

73 *Bartonellaceae* (Alphaproteobacteria: Hyphomicrobiales) (Russell, et al. 2009; Bisch, et al. 2018).
74 Genomics and experiments have confirmed *Bartonellaceae* roles in herbivorous genera
75 *Cephalotes* (Hu, et al. 2018), *Dolichoderus* (Bisch, et al. 2018), and *Tetraponera* (Ma, et al. 2025),
76 where they help recycle nitrogenous waste and supplement the host with nitrogen-rich
77 compounds, especially amino acids. On the other hand, *Bartonellaceae* were also found in
78 carnivores, and in the predatory ant *Harpegnathos saltator* they encode genes related to protein
79 degradation, amino acid transport, and riboflavin biosynthesis, apparently assisting the hosts with
80 the digestion of protein-rich diets and using amino acids as energy source, in addition to
81 provisioning vitamins (Neuvonen, et al. 2016; Bisch, et al. 2018). Phylogenomic analyses have
82 shown that the functionally diverse ant symbionts form a monophyletic clade – *Candidatus* genus
83 *Tokpelaia* – that is sister to the genus *Bartonella* that contains notorious human and mammalian
84 pathogens (Segers, et al. 2017; Wagner and Dehio 2019), but also symbionts of bees and other
85 arthropods (Bisch, et al. 2018; Ma, et al. 2025).

86 Planthoppers (Hemiptera: Fulgoromorpha) showcase a very different nutritional history and
87 symbiotic make-up. They have ancestrally fed on phloem sap, and to counteract its imbalanced
88 nutritional contents, have repeatedly formed symbiotic associations with microorganisms that
89 provide the lacking nutrients (Bennett and Moran 2013; Bell-Roberts, et al. 2019; Brentassi and
90 de la Fuente 2024). Specifically, this 263-million-year-old infraorder (Deng, et al. 2025) has
91 ancestrally associated with a Bacteroidetes symbiont *Candidatus* Sulcia muelleri (further referred
92 to as *Sulcia*) and a Betaproteobacteria symbiont *Ca.* Vidania fulgoroidea (thereafter: *Vidania*)
93 (Urban and Cryan 2012; Deng, et al. 2023). These two symbionts, transmitted strictly maternally
94 and co-diversifying with their planthopper hosts (Moran, et al. 2005), jointly synthesize the 10
95 essential amino acids. In most planthoppers, *Sulcia* produces 3 [leucine, valine and isoleucine],
96 and *Vidania* 7 [methionine, histidine, tryptophan, threonine, lysine, arginine and phenylalanine]
97 (Michalik, et al. 2021; Michalik, et al. 2025). However, in several planthopper clades, these
98 microbes have been replaced by Hypocreales fungi (Siehl, et al. 2024; Michalik, et al. 2025), or

else, joined by additional microbes that established residence within host tissues alongside the ancient endosymbionts (Michalik, et al. 2023; Brentassi and de la Fuente 2024). These additional symbionts, representing the genera *Sodalis*, *Asenophonus*, *Serratia*, and others, can complement the amino acid biosynthesis pathways of the ancestral symbionts and frequently encode genes involved in the biosynthesis of B vitamins (Bennett and Mao 2018; Michalik, et al. 2021; Michalik, et al. 2024). However, we do not have a good understanding of how the nutritional functions vary among symbiont or planthopper clades, how they have evolved, and how they complement the functions encoded by other members of the symbiotic partnership. As the genomics-based characterization of these more recently acquired symbionts in Auchenorrhyncha has been largely limited to a few species carrying *Sodalis* (Koga, et al. 2013; McCutcheon, et al. 2019; Michalik, et al. 2021), we know little about other microbes forming relationships with these sap feeders.

In our recent metagenomics study spanning 149 species of taxonomically diverse planthoppers from different geographic areas (Deng, et al. 2025; Michalik, et al. 2025), in some of the surveyed species we identified, for the first time, *Bartonellaceae* symbionts. This included a strain infecting *Saccharosydne* sp., a Delphacidae planthopper, which had lost *Sulcia* and whose *Vidania* had the smallest genome of any known bacteria, barely 50 kilobases, reduced to provisioning a single amino acid, phenylalanine (Michalik, et al. 2025). We thus postulated that in this and other systems, the nutritional functions lost from the ancient symbionts' genomes may have been taken over by the *Bartonellaceae*. This conclusion has prompted a closer investigation into the nature and significance of the associations between *Bartonellaceae,* their planthopper hosts, and co-residing other microbes.

Specifically, we asked about *Bartonellaceae* distribution across planthopper clades and the phylogenetic relationships among the newly identified strains and other members of the family, including mammalian pathogens and ant symbionts. We also asked about the genomic characteristics and contents of these newly identified strains, particularly in relation to nutritional provisioning that had been identified as a key function of previously studied *Tokpelaia* symbionts

of ants. Finally, we asked how these microbes fit into multi-partner symbioses that provide hosts with essential nutrients. We addressed these questions by exploring the extensive recent metagenomics data for diverse global planthoppers, and through comparative genomics analyses of the newly identified planthopper-associated and previously published *Bartonellaceae* strains.

2. Results

2.1. The distribution and phylogenetic relationships of planthopper-associated *Bartonellaceae*

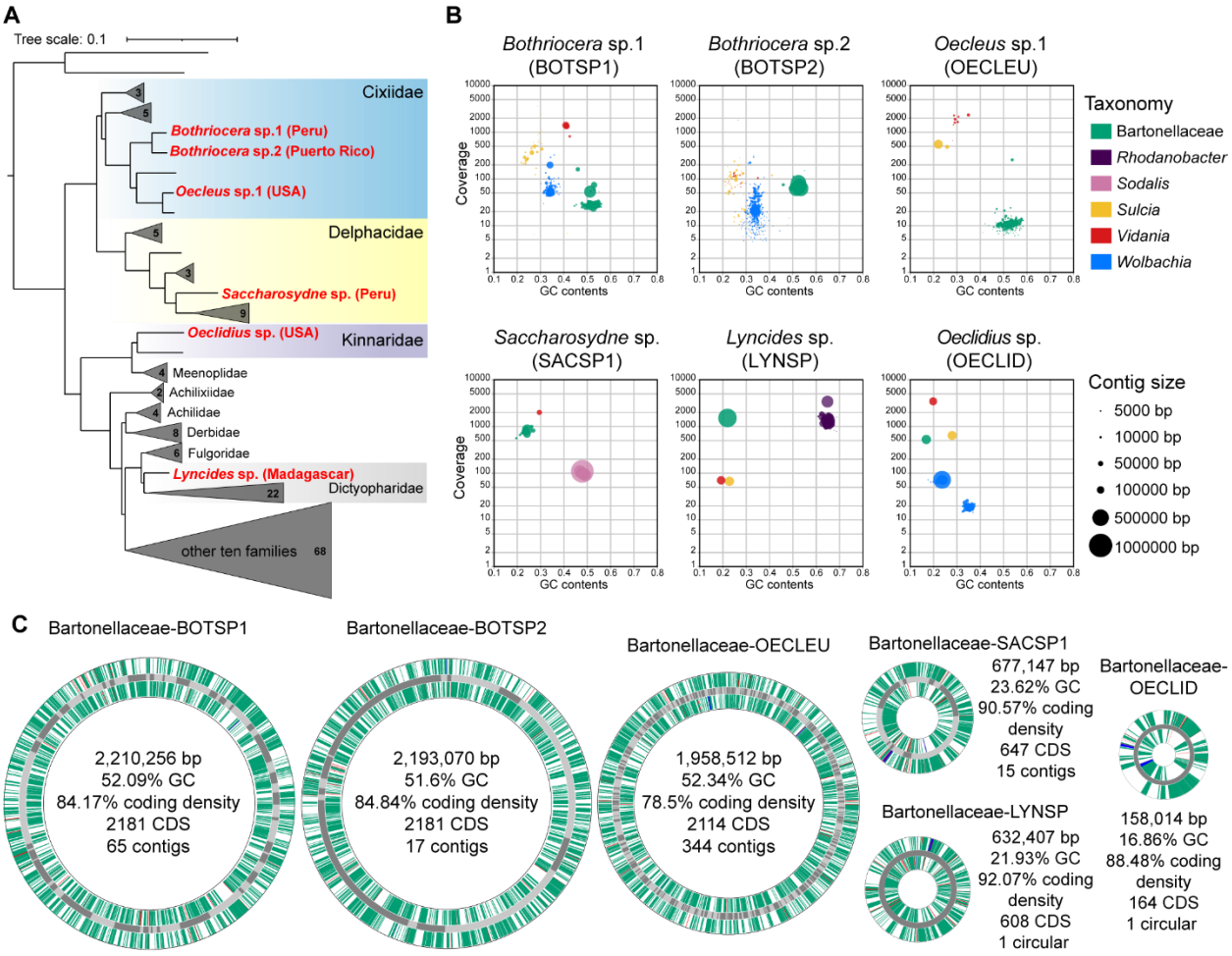


Fig. 1. *Bartonellaceae* symbiont distribution across planthoppers, their representation within metagenomic assemblies, and genomic characteristics. (A) The genome-based phylogeny of 149

135 planthopper species representing 19 families, modified from Deng, et al. (2025), with species harboring
136 *Bartonellaceae* indicated with red font. Clades where these symbionts were not detected were collapsed,
137 with the number of species in these clades shown. **(B)** Taxon-annotated GC content-coverage plots for
138 metagenomic assemblies of six planthopper species where *Bartonellaceae* were identified. Colored blobs
139 represent contigs corresponding to the identified genomic fragments of different symbiotic bacteria. The
140 size of each blob represents contig size. **(C)** Circular diagrams of *Tokpelaia* genomes from six species,
141 showing gene positions on forward and reverse strands and basic genome characteristics. Note that only
142 two genomes were circularized - most comprise multiple contigs, connected for the ease of illustration. The
143 inner and outer circles represent coding sequences (CDS), and the functional categories are shown in
144 green. tRNA, rRNA, and tmRNA genes are shown in red, blue, and purple, respectively. The dark gray and
145 light gray in the middle circle represent different contigs. The size of the first five circles correlates with
146 genome size, the sixth (OECLID) is magnified three times.
147
148 The systematic survey of 149 phylogenetically diverse planthopper species (Deng, et al. 2025;
149 Michalik, et al. 2025) using 16S rRNA sequences reconstructed from metagenomes using
150 phyloFlash (Gruber-Vodicka, et al. 2020), revealed *Bartonellaceae* in six species from four
151 planthopper families, all classified to the genus level only. They included three members of the
152 family Cixiidae: two representatives of the genus *Bothriocera* (BOTSP1, BOTSP2) and *Oecleus*
153 sp. (OECLEU); a Dictyopharidae species *Lyncides* sp. (LYNSP); a Delphacidae planthopper
154 *Saccharosydne* sp. (SACSP1), and a Kinnaridae species *Oeclidius* sp. (OECLID) (Fig. 1A, Tab.
155 S1). This, combined with the phylogenetic distance among *Bartonellaceae*-hosting planthoppers
156 (see below) indicated at least four independent host-microbe associations – one in each family.
157 In turn, the Cixiidae clade that included three *Bartonellaceae*-hosting species also contained two
158 species with no traces of this bacterium, and based on currently available data, we cannot
159 distinguish among scenarios such as independent infections, an ancestral gain followed by
160 subsequent losses in some clades, or a facultative association.

161 The metagenome assemblies for these six planthopper species contained a set of contigs,
162 between 1 and 344, with significant sequence similarity to the genome of *Candidatus* Tokpelaia
163 hoelldoblerii (*Bartonellaceae*) from a predatory ant *Harpegnathos saltator* (Neuvonen, et al. 2016),
164 and forming a distinct cluster in GC contents and coverage space (Fig. 1B, Tab. S2, S3). The
165 *Bartonellaceae* symbionts from Cixiidae planthoppers (BOTSP1, BOTSP2, and OECLEU) all
166 possessed relatively large genomes, ranging from 1.96 to 2.21 Mb across 17-344 contigs,
167 predicted to encode 2,114–2,181 proteins, and with the coding density from 78.5% to 84.84%
168 (Fig. 1C). Their genomic GC content values were relatively high, between 51.6% and 52.34%.
169 The *Bartonellaceae* symbionts of SACSP1 and LYNSP had genomes approximately three-fold
170 smaller, 0.68 Mb (15 contigs - SACSP1) and 0.63 Mb (circular - LYNSP), with 647 and 608
171 predicted protein-coding genes, respectively. These reduced genomes also exhibited markedly
172 higher coding densities—90.57% for SACSP1 and 93.2% for LYNSP—as well as much lower GC
173 contents, 23.62% and 21.93%, respectively (Fig. 1C, Tab. S2). In turn, for the Kinnaridae strain
174 OECLID, we have identified a single circularly mapping contig of 158 kb, with a GC content of
175 16.86% and a coding density of 88.48%, encoding predicted 164 genes that included the large
176 majority of those conserved in other extremely reduced bacterial genomes (McCutcheon, et al.
177 2024). Despite the low sequence similarity to any previously known *Bartonellaceae* (84.13 - 86.39%
178 at 16S rRNA gene, amino acid identity across phylogenetically informative genes: 47.03 - 51.47%,
179 Tab. S4, S5) amino acid sequences of protein-coding genes primarily resembled *Bartonellaceae*
180 among all NCBI records. No further contigs within the assembly could be classified to the same
181 genome.

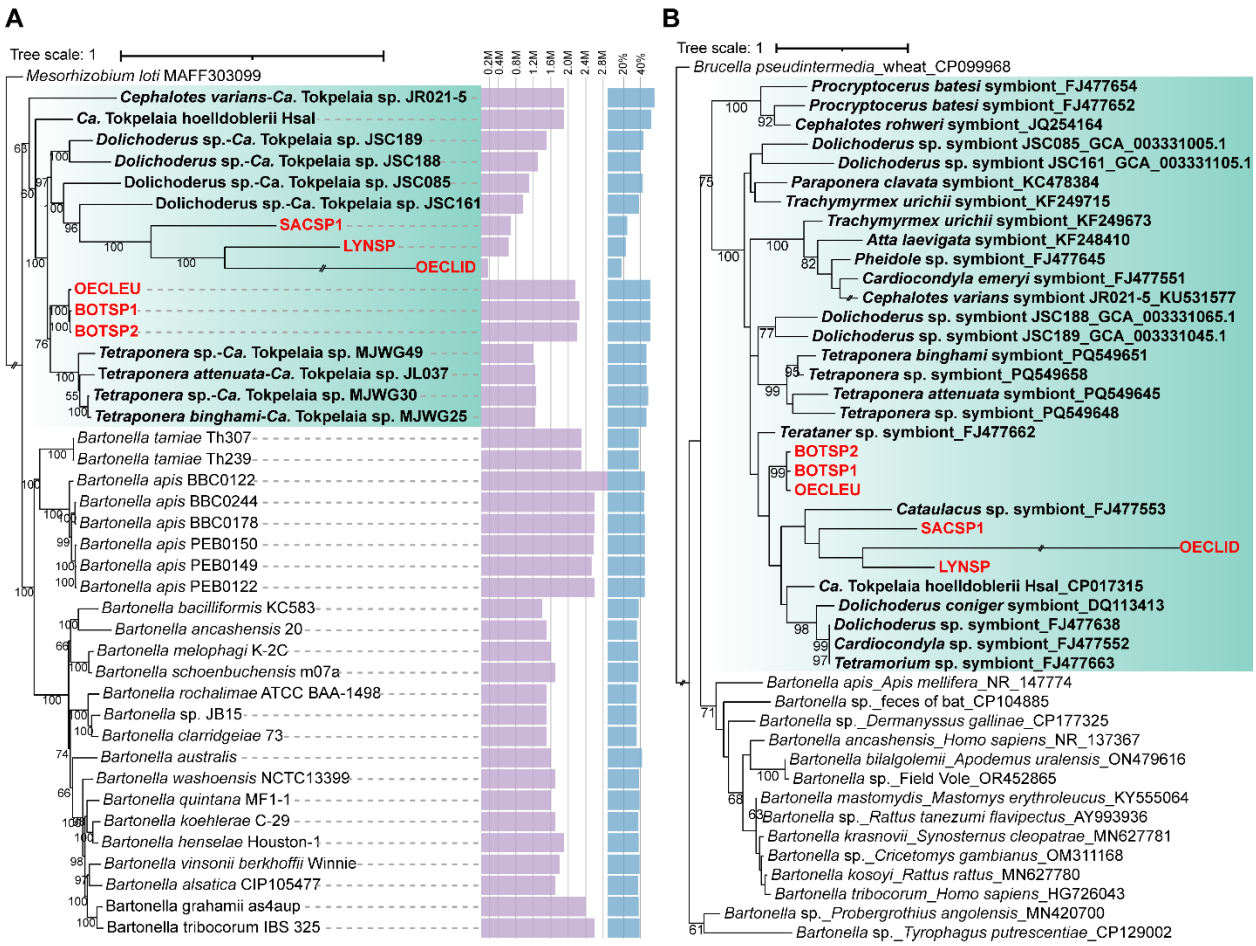


Fig. 2. Phylogenetic placement of *Bartonellaceae* symbionts of planthoppers. (A) Maximum likelihood analysis of *Bartonellaceae* strains with sequenced genomes, based on 68 homologous single-copy genes. The purple bars represent the genome size, and the blue bars - the GC content. **(B)** Maximum likelihood phylogeny of full-length 16S rRNA gene sequences of *Bartonellaceae* associated with planthoppers, ants, and selected other hosts, derived from metagenomes and the NCBI database. In both panels, the *Tokpelaia* clade is indicated with green boxes; bold black labels indicate ant-associated, and bold red – planthopper-associated strains. Bootstrap support values above 50% are shown by the respective nodes. The very long branch leading to the OECLID strain was truncated in both figures.

Phylogenomic analysis of the planthopper-associated *Bartonellaceae* reliably placed them among previously characterized ant symbionts forming the genus *Tokpelaia* (Fig. 2A, S1).

194 Specifically, the planthopper strains form two well-supported subclades within *Tokpelaia*. One of
195 them, on a relatively short branch, comprises symbionts of Cixiidae planthoppers (BOTSP1,
196 BOTSP2, and OECLEU) and forms a sister clade with strains of *Tetraponera* ants. The second
197 clade, monophyletic but with very long branches among strains, comprises symbionts of LYNSP,
198 SACSP1, and OECLID. These small-genome strains show a closer relationship to smallest-
199 genome symbionts of *Dolichoderus* ants, with strong support but long branches. This indicates
200 an accelerated rate of sequence evolution, which combined with a strong AT bias, could have
201 affected the accuracy of phylogenomic reconstructions through systematic problems such as long
202 branch attraction, especially for the extremely reduced OECLID (Susko and Roger 2021). 16S
203 rRNA gene-based phylogeny, including a much broader sampling of *Tokpelaia* sequences,
204 produced a topology broadly consistent with the genome-based analysis, again placing the
205 planthopper-associated symbionts among ant-associated *Tokpelaia* strains and forming two
206 separate clades (Fig. 2B, S2).

207 The genome synteny comparisons did not support phylogenetics-based conclusions (Fig. S3).
208 The highly fragmented assemblies of two of Cixiidae symbiont genomes prevented comparisons
209 within that clade, and there was little similarity among strains comprising the second clade.
210 Surprisingly, large syntenic regions were detected between BOTSP2 and SACSP1, despite them
211 representing different clades. Also, symbionts from BOTSP1 and BOTSP2 shared limited but
212 distinct regions of synteny with *Tokpelaia* from *Tetraponera* ants.

213 2.2. Genomic features and metabolic functions in *Tokpelaia* 214 symbionts from planthoppers and ants

215 The genomic contents comparisons among planthopper and ant symbionts revealed large
216 differences among bacterial strains and host clades (Fig. 3). Generally, *Tokpelaia* strains devote

217 a large portion of their genome contents to the metabolism of carbohydrates, amino acids,
218 cofactors and vitamins, nucleotides, and energy metabolism, as well as to membrane transport.
219 Interestingly, Cixiidae symbiont strains consistently encode much greater numbers of
220 carbohydrate metabolism genes than others. In turn, the numbers of amino acid and
221 cofactor/vitamin biosynthesis genes were relatively consistent except for the genome-reduced
222 planthopper symbiont clade and a single ant strain (JR021), where they were substantially
223 reduced. Interestingly, secondary metabolism genes are consistently much less abundant in
224 planthopper symbionts than in ant symbionts.

225 As the number of translation-related genes is comparable across all but the tiniest genome,
226 their relative proportion negatively correlates with the genome size. In the tiniest *Tokpelaia*-
227 OECLID genome, genetic information processing genes make up 61.8% of the total gene number,
228 as despite it losing many genes in that category, much greater losses occurred in other categories.
229 This included the elimination of all genes involved in essential amino acid biosynthesis, with only
230 two non-essential amino acid metabolism genes (*cysE* and *cysK*) and one vitamin biosynthesis
231 gene (*cobA*) remaining.

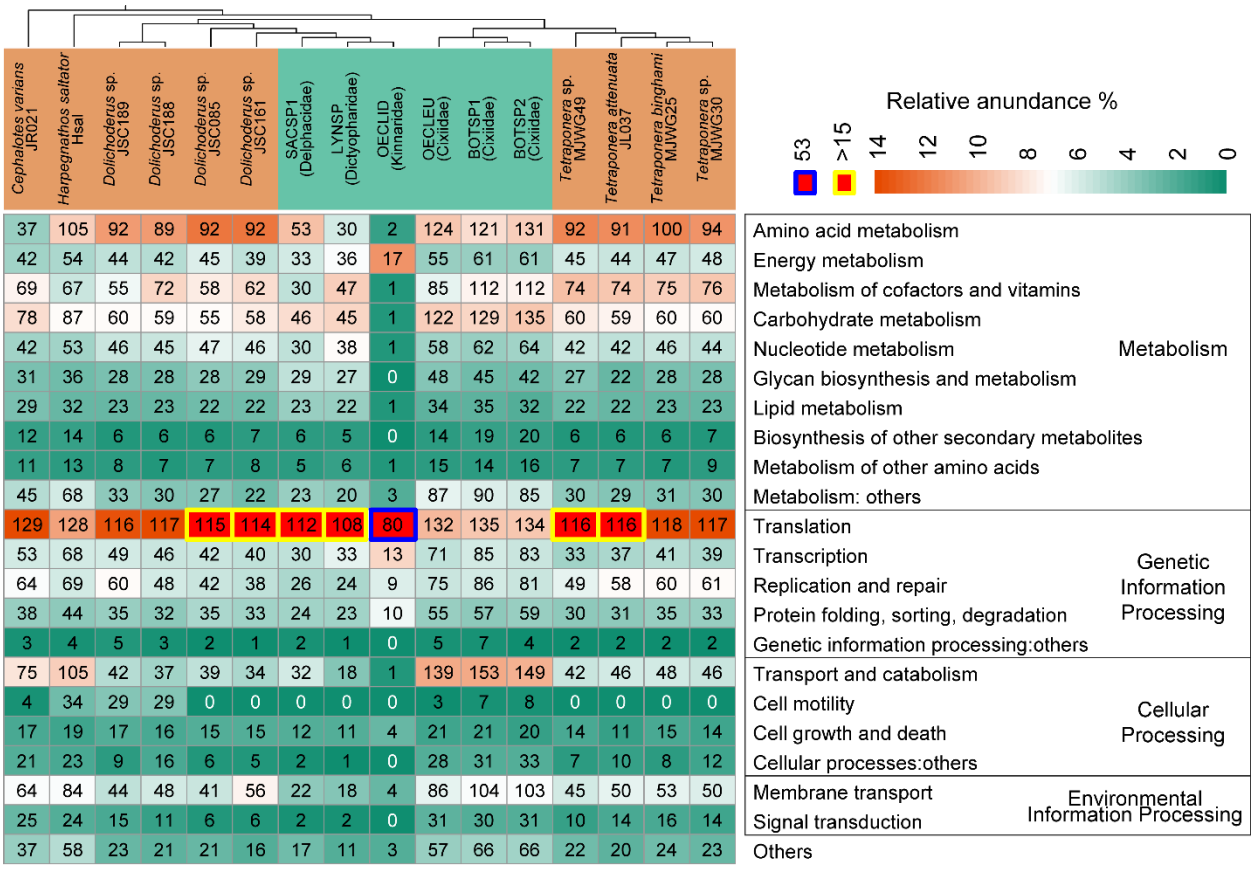


Fig. 3. Relative abundance of KEGG pathways in *Tokpelaia* genomes. For each genome and each functional category, the numbers in the squares represent the number of genes in that category, and the background colors represent the proportion of the genes in that category relative to all annotated genes in the genome.

Comparative analysis of nutrient biosynthesis pathways across the sequenced planthopper *Tokpelaia* genomes indicated a strong similarity among symbionts of Cixiidae (BOTSP1, BOTSP2, and OECLEU) and those of herbivorous *Dolichoderus* and *Tetraponera* ants (Fig 4), whereas strains from LYNSP and SACSP1 had substantially reduced biosynthetic repertoire. Most strikingly, the highly reduced OECLID retained very little nutrient biosynthetic capacity.

Specifically, amino acid biosynthesis pathway comparisons revealed that the Cixiidae planthopper and *Dolichoderus* and *Tetraponera* ant symbionts retain very similar gene sets, spanning nearly complete pathways for the biosynthesis of non-essential and essential amino acids with the exception of alanine-synthesizing transaminase (*alaA*) and asparagine synthase

247 (*asnB*). *Tokpelaia* of BOTSP2 is the only one missing prephenate dehydratase (*pheA2*), which
248 catalyzes the decarboxylation of prephenate to phenylpyruvate in the phenylalanine pathway. The
249 *Tokpelaia* of OECLEU lacks three additional genes: *aspB* and *glyA* for aspartate and glycine
250 synthesis, and *argF* involved in arginine biosynthesis. Notably, however, many of the genes in
251 *Tokpelaia* genomes of BOTSP1, BOTSP2, and OECLEU scattered across pathways, appeared
252 pseudogenized (Fig. 4). In contrast, *Tokpelaia* of LYNSP and SACSP1 have both lost most amino
253 acid biosynthesis genes. The LYNSP strain lacks the complete pathways for any essential amino
254 acid, retaining six of nine genes for lysine biosynthesis and complete pathways for four non-
255 essential amino acids: glutamate, aspartate, glycine, and serine. The SACSP1 strain retains
256 complete pathways for three essential amino acids (lysine, histidine and threonine) and three non-
257 essential amino acids (glutamate, aspartate, and glycine), along with partial pathways for leucine,
258 isoleucine, and valine. This reduced biosynthetic capacity resembles strain JR021, a culturable
259 member of conserved multi-partite microbiota of a herbivorous *Cephalotes* ant (Hu, et al. 2018).
260 The highly reduced OECLID strain encoded only two genes involved in the biosynthesis of one
261 non-essential amino acid, cysteine.

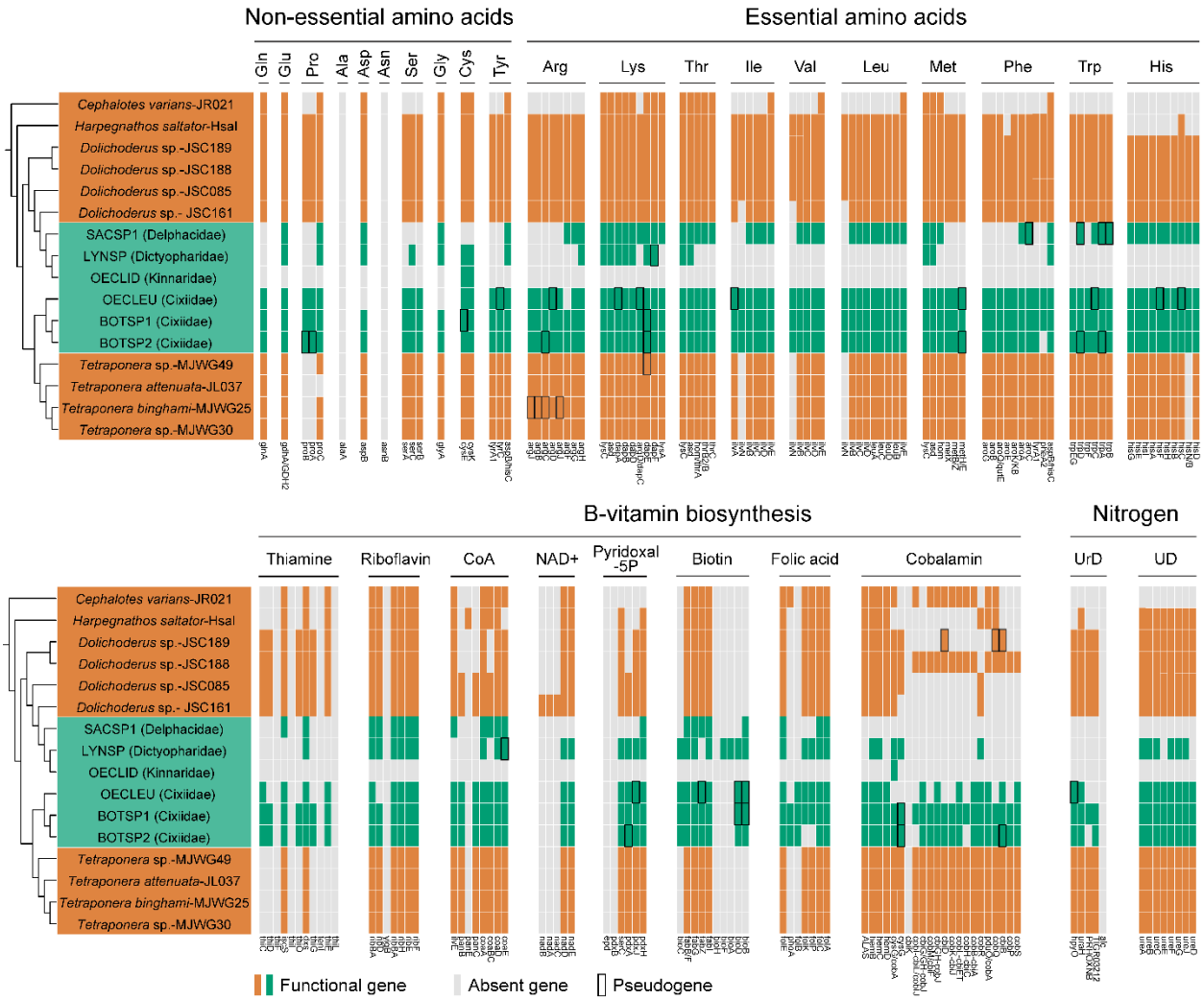


Fig. 4. Summary of *Tokpelaia* biosynthetic functions. Genes associated with nitrogen cycling and cofactor biosynthesis in the genomes of planthopper (green) and ant (orange)-associated *Tokpelaia*. Each bar represents a single gene with an abbreviation at the bottom. Genes are classified as present (green or orange) and absent (light gray); putative pseudogenes, with disrupted open reading frames, are placed in a black frame. A cladogram based on the phylogenomic relationships of *Tokpelaia* is shown on the left, with labels in the same order as in Fig. 2A (where full host names are provided). UrD: Uric acid degradation, UD: Urea degradation.

Besides amino acids, the *Tokpelaia* symbionts display a broad vitamin biosynthetic capability, with substantial variation among strains, and again a substantial similarity among Cixiidae, *Dolichoderus,* and *Tetraponera* symbionts. For thiamine biosynthesis, BOTSP1 and BOTSP2

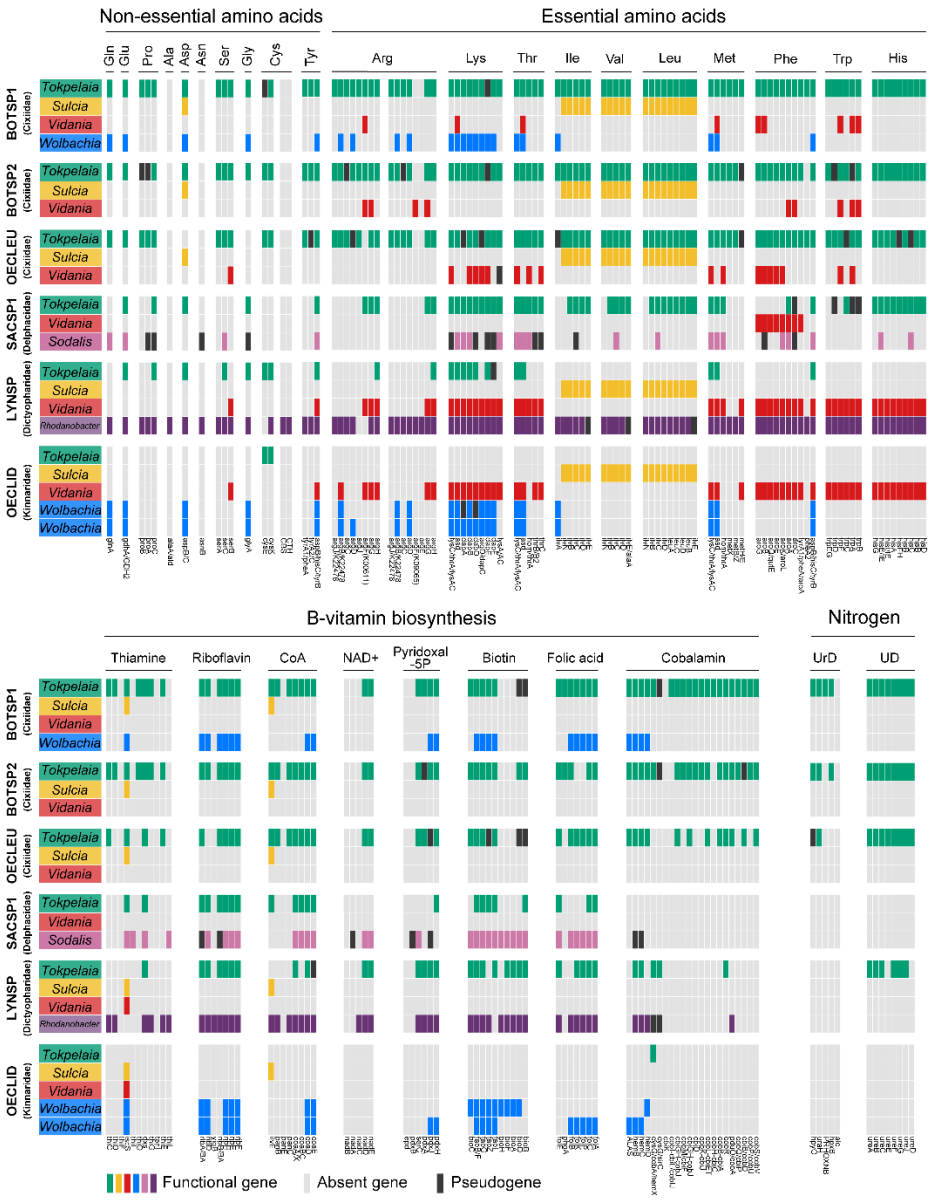
274 symbionts encode the same set of seven genes as *Dolichoderus* strains, whereas all other strains
275 encode a more reduced set. All *Tokpelaia* strains except OECLID symbiont retain genes for
276 riboflavin (vitamin B2) biosynthesis, only missing *yigB* gene, with OECLEU additionally lacking
277 *ribD*. For CoA and pyridoxal-5P, *Tokpelaia* symbionts of Cixiidae planthoppers encode similar
278 gene sets as those of *Tetraponera* ants, with *Dolichoderus* and LYNSP-SACSP1 symbionts
279 encoding fewer. In turn, the set of four biotin (vitamin B7) biosynthesis genes is also highly
280 conserved among *Tokpelaia* strains (with a single gene loss in LYNSP), but all planthopper
281 symbionts encode a set of additional genes involved in the production of this vitamin. Interestingly,
282 BOTSP1's *Tokpelaia* is the only one retaining the full pathway from purines to folic acid, with all
283 other strains having experienced gene losses. Finally, cobalamin, a key coenzyme involved in the
284 metabolism of branched-chain amino acids and methionine, has the longest synthesis pathway
285 of 22 steps, all but one of which (*cbiK*) is encoded by *Tetraponera* and BOTSP1 symbionts. Here,
286 BOTSP2 and one of the *Dolichoderus* strains lost more genes, and all others have a more
287 substantially reduced set. The highly reduced OECLID symbiont only encodes one vitamin
288 biosynthesis gene, *cobA*, in the cobalamin biosynthesis pathway.

289 Similarly, *Tokpelaia* from Cixiidae planthoppers and most ants (*Dolichoderus*, *Tetraponera*,
290 and *Harpegnathos saltator*) harbor a complete urease gene cluster (ureABCDEFGJ) for urea
291 degradation. However, the LYNSP strain lacks two accessory components (ureE and ureJ), and
292 SACSP1 has lost the entire gene cluster, same as the *Cephalotes* ant symbiont JR021 (Fig. 4).
293 On the other hand, while the pathway for uric acid degradation is retained in *Tokpelaia* from
294 *Dolichoderus* and *Tetraponera* ants, BOTSP1 is the only planthopper whose symbiont retains the
295 entire pathway, with partial or complete losses in other species (Fig. 4). Again, none of the
296 nitrogen recycling genes were found in *Tokpelaia*-OECLID.

297 The close inspection of the OECLID symbiont gene set revealed, outside of genetic
298 information processing genes, two substantial functions that were retained (Fig. S4, Tab. S7). The
299 first is energy metabolism, including components of the electron transport chain (17 genes related

to cytochrome c oxidase, ATP synthase, and electron transfer), the second is iron–sulfur cluster assembly. The only two amino acid (cysteine) biosynthesis-related genes present in this genome are also involved in iron-sulfur cluster assembly (Fig. S5).

2.3. The degeneration of ancient symbionts is often associated with the *Tokpelaia* acquisition



306 **Fig. 5. Genomic complementarity in nutritional provisioning among planthopper symbionts.** Amino
307 acid and B vitamin biosynthesis gene distribution among genomes of different symbionts that were identified
308 in metagenomes of six *Tokpelaia*-hosting planthopper species. Genes are classified as functional genes,
309 absent (light gray), and pseudogenes (black).
310
311 Within metagenomic assemblies of the six *Tokpelaia*-hosting planthopper species we also
312 identified other bacteria (Fig. 1B, Tab. S2) (Michalik, et al. 2025). Specifically, within all studied
313 insects, we identified rRNA sequences of *Vidania*, and in five of them (all except SACSP1), of
314 *Sulcia* – two ancestral symbionts of planthoppers. BOTSP1, BOTSP2 and OECLID also contained
315 the facultative symbiont *Wolbachia*. LYNSP carried *Rhodanobacter*, whereas SACSP1 hosted
316 *Sodalis* as an additional symbiont (Fig. 1B). For most of these symbionts, we recovered genomic
317 bins with acceptable completeness and contamination statistics (Tab. S2). Intriguingly, in Cixiidae
318 species, *Vidania* and *Sulcia* genomes were fragmented and apparently incomplete, unlike in the
319 three other *Tokpelaia*-hosting species and the vast majority of previously studied planthoppers
320 (Michalik, et al. 2025). Further, in BOTSP2, *Wolbachia* was not successfully reconstructed
321 through binning; we identified matching contigs via BLASTn, but because of high contamination
322 scores for this set, as computed by CheckM (72.81%), we decided to exclude it from analyses.
323 The ancient nutritional endosymbionts' genome organization and functions varied among the
324 studied species (Fig. 5). Despite its fragmentation in Cixiidae, *Sulcia* nutritional functions were
325 conserved in the five planthoppers that retained it, always comprising full pathways for valine,
326 leucine, and isoleucine biosynthesis. In contrast, *Vidania* genome retained ancestral organization
327 and the ability to produce seven essential amino acids in only two species, LYNSP and OECLID.
328 In the third, SACSP1, *Vidania* genome was reduced to provisioning phenylalanine (Michalik, et al.
329 2025). In turn, in all three Cixiidae species, the unusually fragmented *Vidania* strains only retained
330 a small and variable subset of genes scattered across biosynthetic pathways. In Cixiidae and
331 SACSP1, the gaps in conserved essential amino acid biosynthesis pathways are mostly filled by

332 the genes encoded by other symbionts (Fig. 5). Specifically, in Cixiidae, the largely complete
333 biosynthetic gene sets encoded in *Tokpelaia* genomes seem to fully compensate for the *Vidania*
334 gene losses. With a few exceptions (lysine and phenylalanine), this is also the case in SACSP1;
335 additionally, in this species, 17 genes overlapping with those of *Tokpelaia* are encoded by *Sodalis*.
336 In LYNSP, the conserved gene sets of *Sulcia* and *Vidania* are almost entirely duplicated by
337 *Rhodanobacter*, with *Tokpelaia* providing further scattered functions. Finally, in OECLID, two
338 *Wolbachia* strains encode a set of biosynthetic genes that largely overlap with those of ancient
339 symbionts.

340 Species differed substantially in terms of B vitamin and cofactor biosynthesis gene sets and
341 their distribution across symbiont genomes (Fig. 5). In Cixiidae species, *Tokpelaia* encodes large
342 numbers of genes across different pathways, with *Wolbachia* duplicating these functions in
343 BOTSP1. In SACSP1 and LYNSP, *Sodalis* and *Rhodanobacter*, respectively, encode the greatest
344 number of genes, with *Tokpelaia* duplicating many of them. Finally, in OECLID, its two *Wolbachia*
345 genomes encode multiple functions, and *Tokpelaia* contributes one gene. Across all these
346 species, few vitamin biosynthetic pathways appear complete, but we note that the assembly
347 fragmentation may have prevented the detection of some genes, and they may also be encoded
348 within the host genomes.

349 3. Discussion

350 When famously writing about ‘a veritable fairyland of insect symbiosis’, Paul Buchner (1965)
351 referred to the huge variety of bacterial symbionts of Auchenorrhyncha that accompany, and
352 sometimes have replaced, their ancient, degraded nutritional endosymbionts. Of these, the
353 enterobacterial genus *Sodalis* – apparently, the most broadly distributed across the taxonomic
354 diversity of insects – may be the most widespread but also best understood in terms of genomic
355 evolution and nutritional contributions to the host biology (McCutcheon, et al. 2019; Renoz, et al.

356 2023). Through our research, the family *Bartonellaceae* joins the ranks of Auchenorrhynchan co-
357 symbionts, showing patterns of the distribution, evolution, and function not unlike *Sodalis*, but
358 casting a new perspective on microbial symbioses of hemipterans, ants, and other insects.

359 3.1. Bartonellaceae host range broadens

360 *Bartonellaceae* are primarily known as pathogens of mammals, often vectored by blood-
361 sucking insects (Engel, et al. 2011). As an example, *Bartonella bacilliformis* causes the
362 devastating and often lethal Carrion disease in humans (Pons, et al. 2016), while *Bartonella*
363 *henselae* causes persistent and usually asymptomatic infections in cats, vectored by cat fleas
364 (Chomel, et al. 1996). The discovery of the bee gut symbiont *Bartonella apis* (Segers, et al. 2017)
365 and the identification of more divergent strains colonizing diverse ants, classified to the
366 *Candidatus* genus *Tokpelaia* (Russell, et al. 2009; Bisch, et al. 2018; Ma, et al. 2025), have
367 changed the understanding of the biological spectrum of this bacterial group. Our discovery of
368 planthopper-associated *Bartonellaceae*, fitting right within the *Tokpelaia* clade and with at least
369 four independent original host association events, further broadens the list of confirmed hosts.
370 The plant-based diet of many *Bartonellaceae*-hosting ants and planthoppers, but also the
371 mutualistic interactions among many ants and hemipterans, including planthoppers (Ivens, et al.
372 2017; Bourgoin, et al. 2023), suggest potential avenues for parallel acquisition of related microbes
373 by these groups, or their exchange among groups. However, we already know that
374 *Bartonellaceae* host list is longer: for example, a divergent clade commonly colonizes stored-
375 product mites (Xiong, et al. 2024), and *Bartonellaceae* 16S rRNA sequences were identified in a
376 scorpion and in heteropteran bugs (Matsuura, et al. 2012; Martinez, et al. 2019). We expect that
377 a systematic survey across diverse arthropods will reveal these bacteria in a substantially broader
378 range of hosts. At the same time, as shown recently, systematic screens across related species

379 and within species, microscopy, transcriptomics, and/or experiments, can clarify the nature of
380 their associations with diverse hosts (Xiong, et al. 2024; Ma, et al. 2025).

381 Overall, our findings support the view of *Bartonellaceae* as relatively broadly distributed
382 across animals, and perhaps a wider range of environments. We suspect that some strains are
383 relatively versatile and capable of synthesizing a wide range of nutrients, which enable them to
384 move across and colonize new host lineages and then potentially evolve into more specialized
385 associations. Some of these, including the mammal-pathogenic *Bartonella* clade, may retain the
386 ability to switch hosts, while others may become strictly heritable, promoting genomic reduction
387 and the loss of functions not essential to the specific host. The latter evolutionary trajectory agrees
388 with the emerging consensus on the evolution of some other key insect-symbiotic bacteria, as
389 exemplified by the enterobacterial genus *Sodalis* (McCutcheon, et al. 2019). It includes strains
390 such as *Sodalis ligni* that live in dead wood (Tlaskal, et al. 2021)*; Sodalis praecaptivus* isolated
391 from a human wounds following impalement on a branch (Clayton, et al. 2012); and diverse strains
392 that have independently established heritable nutritional symbiotic relationships with a wide
393 variety of insect lineages (Renoz, et al. 2023). Such heritable associations typically lead to
394 rampant genomic changes, increased evolutionary rates, and rapid gene losses. Some of the
395 degenerating symbionts may reach a stable state where gene loss slows to nearly a halt – as
396 evidenced by high similarity among genomes of *Buchnera*, *Blochmannia*, or *Baumannia*, all likely
397 derived from *Sodalis* tens or hundreds of millions years ago (McCutcheon, et al. 2019).

398 3.2. Planthopper symbionts represent contrasting genomic 399 evolutionary outcomes

400 Variation in planthopper-associated *Tokpelaia* genome size and contents indicates their
401 different lifestyles and varying effects on the host biology, paralleling the range of associations of
402 *Sodalis*-allied symbionts.

403 On the one hand, the three *Tokpelaia* strains infecting Cixiidae planthoppers have the largest
404 genomes (~2Mb) among the *Tokpelaia* strains studied so far (0.96-1.9Mb), retaining a broad
405 range of amino acid and vitamin biosynthesis genes. This suggests a substantial contribution to
406 the host nutrition, comparable to that of ant-associated *Tokpelaia* (Bisch, et al. 2018; Ma, et al.
407 2025). Further, these strains' positions on short branches in the *Tokpelaia* phylogeny and high
408 GC contents suggest proximity to the putative ancestral state of other associations with diverse
409 ants and planthoppers. This resembles the versatile *Sodalis praecaptivus*, whose function-rich,
410 high-GC genome is much larger than most insect-symbiotic *Sodalis* strains, and consistently falls
411 near the base of *Sodalis* phylogenies, among variable-length branches representing insect-
412 symbiotic strains that departed from this ancestral state (McCutcheon, et al. 2019).

413 On the other hand, the *Tokpelaia* genomes of LYNSP and SACSP1 planthoppers are
414 substantially reduced (632-677 Kb), to the level comparable with *Sodalis*-derived long-term
415 obligate nutritional symbionts of aphids (B*uchnera*, 412-671 kb), carpenter ants (*Blochmannia*,
416 705-792 kb), sharpshooter leafhoppers (B*aumannia*, 633-759 kb), or tsetse flies (Wigglesworthia,
417 ~700 kb) (Bennett, et al. 2015; Bing, et al. 2017; Chong, et al. 2019). Despite the massive variation
418 in their age of association, tissue localization, transmission mechanisms, and biosynthetic
419 capacity, all these gamma-proteobacterial symbionts provide deficient nutrients that make them
420 virtually indispensable to their hosts (Feldhaar, et al. 2007; McCutcheon, et al. 2009; Bing, et al.
421 2017). We suspect that LYNSP and SACSP1 *Tokpelaia* strains play comparable roles in
422 planthoppers. As both strains have lost multiple nutritional genes that are retained by other
423 symbionts, their specific situation may particularly resemble that of *Baumannia* (Wu, et al. 2006),
424 or *Buchnera* from *Cinara* aphids (Manzano-Marin, et al. 2016), or endobacterial symbionts of
425 mealybugs (Husnik and McCutcheon 2016): all of them are complementary in their amino acid
426 biosynthetic capacity to other symbionts residing within the same host insect.

427 In our view, the most striking discovery is the tiny, 158-kb genome of the *Tokpelaia* symbiont
428 of the Kinnaridae planthopper *Oeclidius* sp. (OECLID), comparable in size to the most reduced
429 known insect endosymbionts: *Sulcia*, *Vidania*, *Nasuia*, *Hodgkinia*, *Tremblaya*, or *Carsonella*
430 (McCutcheon, et al. 2024). All these bacteria colonize sap-sucking hemipterans, where they have
431 well-defined roles as providers of essential amino acids and sometimes vitamins. This does not
432 appear to be the case with *Tokpelaia*-OECLID – even if, as shown in a mealybug, one nutrition-
433 related biosynthetic gene (*cobA* in the current case) might potentially be enough to justify long-
434 term symbiosis (Garber, et al. 2024). But in fact, OECLID symbiont's genetic repertoire resembles
435 much more closely the endosymbionts of certain protists. Specifically, the 158-kb genome of a
436 Verrucomicrobium, *Ca.* Organicella extenuata, symbiont of an *Euplotes* protist inhabiting a
437 hypersaline Antarctic lake, also retains the capacity for genetic information processing but not for
438 the biosynthesis of amino acids or vitamins (Williams, et al. 2021), with fatty acid synthesis and
439 iron-sulfur (Fe-S) cluster assembly identified as its likely contribution to the host biology. Fe-S
440 cluster assembly has not been identified as critical to sap-sucking insects' biology. Thus, the
441 nature of the association, significance, and evolutionary history of this unusual planthopper-
442 associated bacterium that conflicts with the current understanding of insect heritable
443 endosymbioses remains a mystery and, unfortunately, it cannot be resolved based on a single
444 mid-coverage metagenome alone. To further complicate conclusions, from OECLID metagenome,
445 we also reconstructed a low-coverage rRNA sequence representing an Entomophthorales fungus
446 with 96.6% identity to entomopathogens *Pandora delphacis* and *Furia pieris*, and we cannot
447 exclude the possibility that *Tokpelaia*-OECLID actually associates with the planthopper's
448 pathogen (Matsuura, et al. 2018; Siehl, et al. 2024).

449 3.3. *Bartonellaceae* significance in the biology of planthoppers and 450 their other symbionts

451 We know that heritable nutritional endosymbionts in diverse insect systems produce essential
452 nutrients deficient in their hosts' diets, thus being indispensable to the hosts. At the same time,
453 they drive the evolution of hosts' dependence on symbiont nutritional contributions, even as these
454 symbionts degrade and loose efficiency – a process aptly described as 'the evolutionary rabbit
455 hole' (Bennett and Moran 2015). Further, they drive the complementary loss of functions by their
456 co-symbionts (McCutcheon, et al. 2009; Husnik and McCutcheon 2016), forcing the hosts to
457 manage an increasingly specialized symbiotic consortium. We think that these general statements
458 accurately describe the biology and significance of most planthopper-associated *Tokpelaia*
459 strains that we have identified, which in all cases, belong to multi-partite symbiotic consortia with
460 complementary biosynthetic capabilities (Michalik, et al. 2021). Specifically, in a delphacid
461 SACSP1, the *Bartonellaceae* symbiont largely fills up the nutritional gap resulting from *Sulcia* loss
462 and *Vidania* genome reduction to the smallest-known state, while in Cixiidae, they seem to
463 compensate for the apparent degeneration of *Vidania.* While such complementarity cannot be
464 seen in LYNSP, and in OECLID it is limited to a single gene, it is tempting to think of *Tokpelaia*
465 as the common driver of the functional loss by ancient symbioses. At the same time, other
466 symbionts are likely to be driving the gene loss in *Tokpelaia.* We propose that through alternative
467 evolutionary routes, symbiotic complexes involving *Tokpelaia* have reached, or are on a path
468 towards, complementarity in the biosynthesis of largely the same set of nutrients as most other
469 planthoppers. Depending on the order of arrival and the genomic properties of bacteria that were
470 in planthoppers before, the independently acquired *Bartonellaceae* have assumed different
471 positions and roles in the symbiotic consortium.

472 We are not aware of any major ecological transitions in planthopper lineages represented by
473 *Tokpelaia*-infected species. Such changes, exemplified by Typhlocybinae leafhoppers that
474 switched to feeding on parenchymal cell cytoplasm (Buchner 1965; Kobiałka, et al. 2025) and in
475 Derbidae and Achilidae planthoppers that switched to feeding on fungi during nymphal stages

476 (Michalik, et al. 2025), seem to have enabled major changes and functional losses in their
477 microbiota.

478 However, in all presented systems, achieving the full understanding of host-microbe
479 associations would require a much broader set of data than what we had available - a combination
480 of within-species microbiome surveys, genomic comparisons among closely related species,
481 microscopy, and, ideally, experiments. Unfortunately, these approaches are not easy to
482 implement on unidentified non-model species sampled on different continents years ago, which
483 was the case here. Nevertheless, this study demonstrates that a broad genomics survey alone
484 can help discover new biological states and processes that change our understanding of biology
485 and evolution of significant clades of organisms. In the age of genomics, such broad surveys
486 should become an important tool for the discovery and characterization of biodiversity and
487 biological interactions that shape it.

488 4. Methods

489 4.1. Study material and data

490 For the study, we used a set of metagenomic datasets for 149 taxonomically diverse
491 planthoppers, presented in our previous work that focused on phylogenomic relationships of these
492 planthoppers and comparative genomics of their *Sulcia* and *Vidania* symbionts (Deng, et al. 2025;
493 Michalik, et al. 2025). Briefly, insects were wild-caught from their natural habitats around the world
494 between 1996 and 2022. Following identifications by taxonomic experts, the DNA extracted from
495 dissected abdominal tissue of single individuals, or in a few cases, dissected bacteriome tissue,
496 was used for metagenomic library preparation and sequencing on Illumina platforms. Collection
497 metadata, reagents, and sequencing platforms for the entire metagenomic collection are provided

498 in the abovementioned publications. For the six specimens within which we detected *Tokpelaia*,
499 listed in Table S1, abdominal tissue was used for DNA extraction using the Bio-Trace DNA
500 Purification Kit (Eurx, Poland), and sequencing libraries were prepared and sequenced by
501 Novogene Ltd. on NovaSeq 6000 platform using a 300-cycle kit.

502 4.2. Bioinformatics analysis

503 To detect *Bartonellaceae* within the sequenced metagenomes, we systematically examined
504 16S rRNA sequences assembled using phyloFlash v3.4.2 (Gruber-Vodicka, et al. 2020),
505 validating all those without close matches to recognized bacteria through phylogenetics and
506 blastn comparisons against NCBI databases. In all cases where these data suggested
507 Hyphomicrobiales infections, we followed with the analysis of metagenomic assemblies obtained
508 using Megahit v1.2.9 (maximum k-mer size = 255, min contig size= 1000) (Li, et al. 2015). To
509 obtain the draft genomes of *Bartonellaceae* and other bacteria present in these samples, we
510 binned the metagenomic assembly data using the metaWRAP pipeline (Uritskiy, et al. 2018),
511 incorporating three binning tools: CONCOCT, MaxBin, and metaBAT. The resulting bins were
512 refined with metaWRAP's "Bin_refinement" module, applying a minimum completeness threshold
513 of 70% and a maximum contamination limit of 10%. To avoid the omission of genomic contigs,
514 assemblies were further examined using BLASTn and BLASTx searches against the NCBI
515 database, and separately against published and unpublished genomes of different insect-
516 symbiotic bacteria. Only contigs consistent with the bin classification, and meeting criteria for read
517 coverage and GC content were retained (Tables S2, S3). Bin completeness and contamination
518 were evaluated using CheckM v1.0.12 (Parks, et al. 2015), and taxonomic classification was
519 performed using the Genome Taxonomy Database via GTDB-Tk v2.1.122 (Chaumeil, et al. 2019).
520 Average amino acid identity (AAI) was calculated using CompareM v0.1.2
521 (https://github.com/dparks1134/CompareM).

522 Contigs of bacteria other than *Sulcia* and *Vidania* were annotated with Prokka v1.14.6
523 (Seemann 2014), and gene functions were assigned to KEGG Orthology (KO) pathways using
524 KofamScan (Aramaki, et al. 2020) and the online tool eggNOG-mapper (Tab. S6) (Cantalapiedra,
525 et al. 2021). Based on these annotations, metabolic pathways of the symbiotic bacteria were
526 manually reconstructed using the KEGG database. Genomes were visualized using Proksee
527 (Grant, et al. 2023). Genome synteny analysis was performed using Promer tool for Mummer
528 v3.07, with default settings (Kurtz, et al. 2004). Pseudofinder v1.1.0 (Syberg-Olsen, et al. 2022)
529 software was used to predict pseudogenes in the genomes of symbiotic bacteria. In turn, all
530 confirmed *Sulcia* and *Vidania* contigs and genomes were annotated using a custom pipeline
531 (Łukasik, et al. 2018) adopted for planthopper symbionts recently (Michalik, et al. 2025). The
532 pipeline first extracted all Open Reading Frames (ORFs) and translated them into amino acid
533 sequences. These ORFs were then searched using HMMER v3.3.1 (Eddy 2011) (hmmer.org)
534 against a custom database containing manually curated protein-coding and rRNA genes from
535 previously characterized *Sulcia* and *Vidania* genomes. Ribosomal RNA genes were detected with
536 nhmmer implemented in HMMER v3.3.1, and tRNA genes were identified with tRNAscan-SE
537 v2.0.7 (Chan, et al. 2021) Based on the relative length to the reference genes, protein-coding
538 genes were classified as functional (>90%), putative pseudogenes (>60%), or pseudogenes
539 (<60%). Any ORFs longer than 300 bp with no significant similarity to any reference genes were
540 further examined by querying UniProt (The UniProt 2021) and NCBI databases and compared
541 carefully to the top hits. Genes lacking any annotations were marked as “hypothetical”.

542 4.3. Phylogenetic analyses

543 To investigate the evolutionary origin of *Bartonellaceae* bacteria associated with
544 planthoppers, we compared phyloFlash-reconstructed 16S rRNA sequences against top hits of
545 insect and environmental origin, especially published ant-associated *Tokpelaia* sequences. All

546 sequences were aligned using MAFFT v7.526 (Rozewicki, et al. 2019), with divergent regions
547 removed using Gblocks v0.91b (Talavera and Castresana 2007). Phylogenetic analysis was
548 performed using RAxML v8.2.12 (Stamatakis 2014) under the GTRGAMMA model, with *Brucella*
549 *pseudintermedia* (accession number: CP099968) designated as the outgroup. The analysis
550 included 1000 bootstrap replicates and was visualized using iTOL (Letunic and Bork 2021).

551 To clarify the evolutionary relationships among the newly identified strains and other
552 *Bartonellaceae*, we conducted a genome-wide phylogenetic analysis that included all currently
553 published *Tokpelaia* or *Bartonella* genomes representing the major recognized clades. The
554 proteins were clustered into families using OrthoFinder v2.5.5 (Emms and Kelly 2019), resulting
555 in a data set of 68 single-copy orthologs. And then, proteins from each family were aligned using
556 MAFFT v7.526 (Rozewicki, et al. 2019), with divergent and ambiguously aligned blocks trimmed
557 using Gblocks v0.91b (Talavera and Castresana 2007) . We concatenated the resulting
558 alignments. Prottest 3.4.2 (Guindon and Gascuel 2003; Darriba, et al. 2011) was used to select
559 the optimal amino acid substitution model, JTT+I+G+F, according to AIC and BIC criterions.
560 Phylogenetic analysis was performed with RAxML v8.2.12 (Stamatakis 2014) using the best
561 model and 1000 bootstrap replicates, and results were visualized and annotated using iTOL
562 (Letunic and Bork 2021). All images were adjusted using Adobe Illustrator CC 2019.

563 5. Data availability

564 All raw data and genomic assemblies used in comparisons have been uploaded to NCBI
565 databases. BioProject and BioSample accession numbers are listed in Tables S1-S2.
566 Supplementary Tables were deposited in the public Figshare repository under the DOI:
567 10.6084/m9.figshare.30010978.

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7. Author Contributions

M.M., A.M., and P.Ł. conceived the study. J.D. and A.M. provided bioinformatics support and additional *Vidania* and *Sulcia* genome annotations. M.M., A.M., J.D., Y.H., and P.Ł. analyzed the data and wrote the manuscript. All authors reviewed and agreed on the manuscript.

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