Deep divergence and genomic diversification of gut symbionts of neotropical stingless bees

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Abstract

Social bees harbor conserved gut microbiota that may have been acquired in a common ancestor of social bees and subsequently co-diversified with their hosts. However, most of this knowledge is based on studies on the gut microbiota of honey bees and bumble bees. Much less is known about the gut microbiota of the third and most diverse group of social bees, the stingless bees. Specifically, the absence of genomic data from their microbiota presents an important knowledge gap in understanding the evolution and functional diversity of the social bee microbiota. Here we combined community profiling with culturing and genome sequencing of gut bacteria from six neotropical stingless bee species from Brazil. Phylogenomic analyses show that most stingless bee gut isolates form deep-branching sister clades of core members of the honey bee and bumble bee gut microbiota with conserved functional capabilities, confirming the common ancestry and ecology of their microbiota. However, our bacterial phylogenies were not congruent with those of the host indicating that the evolution of the social bee gut microbiota was not driven by strict co-diversification, but included host switches and independent symbiont gain and losses. Finally, as reported for the honey bee and bumble bee microbiota, we find substantial genomic divergence among strains of stingless bee gut bacteria suggesting adaptation to different host species and glycan niches. Our study offers first insights into the genomic diversity of the stingless bee microbiota, and highlights the need for broader samplings to understand the evolution of the social bee gut microbiota.

Importance

Stingless bees are the most diverse group of the corbiculate bees and represent important pollinator species throughout the tropics and subtropics. They harbor specialized microbial communities in their gut that are related to those found in honey bees and bumble bees and that are likely important for bee health. Few bacteria have been cultured from the gut of stingless bees which has prevented characterization of their genomic diversity and functional potential. Here, we established cultures of major community members of the gut microbiota of six stingless bee species and sequenced their genomes. We find that most stingless bee isolates belong to novel bacterial species distantly related to those found in honey bees and bumble bees and encoding similar functional capabilities. Our study offers a new perspective on the evolution of the social bee gut microbiota and presents the basis to characterize the symbiotic relationships between gut bacteria and stingless bees.

57 **Introduction**

58 The eusocial corbiculate bees (hereafter social bees) comprise more than 700 species
59 distributed in three distinct tribes: honey bees (Apini), bumble bees (Bombini), and stingless
60 bees (Meliponini). Stingless bees and bumble bees form a monophyletic clade, which is sister
61 to the honey bees, with whom they shared a common ancestor 80-100 million years ago (1-
62 3). Like mammals, social bees harbor dense and specialized bacterial communities in their gut
63 that affect bee health and behavior (4-12). The composition of the gut microbiota of social
64 bees is relatively simple typically consisting of <10 bacterial phylotypes, i.e. sequence clusters
65 sharing >97% identity in the 16S rRNA gene (13-20). Five of these phylotypes (*Snodgrassella*,
66 *Gilliamella*, Bombilactobacillus Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium*) have been
67 referred to as the core gut microbiota of the social bees (20), because they are prevalent and
68 abundant across honey bee, bumble bee, and stingless bees. Most members of the bee gut
69 microbiota are culturable and gnotobiotic bees can be generated for several species (5, 21-
70 23). Together, these distinctive characteristics make the social bee microbiota a versatile
71 model system for studying the evolution and ecology of host-associated microbial
72 communities. Moreover, social bees are important pollinators that suffer from severe
73 population declines (24, 25) which makes studies of their microbiota relevant in its own right.

74 Most of what is currently known about the gut microbiota of social bees stems from studies
75 on honey bees and bumble bees. Genomic and experimental approaches have revealed that
76 their gut bacteria are usually saccharolytic fermenters that utilize plant glycans derived from
77 the pollen and nectar/honey diet of the host (22, 23, 26-32). Further, it has been shown that
78 the core members of the honey bee and bumble bee gut microbiota have substantially
79 diversified (27-30, 33-36). They consist of divergent sub-lineages (or species) and contain

80 large extents of strain-level diversity and gene content variation. Most sub-lineages are host-
81 specific (23, 27, 33, 36), and their phylogenetic relationships are to some extent congruent
82 with the phylogeny of the host (16, 33). Therefore, it has been suggested that the core
83 members of the microbiota were acquired in a common ancestor of the social bees (20) and
84 possibly co-diversified with the host (16, 33). In addition, studies in the Western honey bee
85 (*Apis mellifera*) have shown that the diversification of the bee gut microbiota was also driven
86 by adaptation to different spatial and metabolic niches within the gut (27, 30, 33-35). For
87 example, strains of closely related sub-lineages of *Lactobacillus* Firm-5 and *Bifidobacterium*
88 can coexist in individual bees. They carry distinct gene sets for the breakdown and utilization
89 of pollen-derived carbohydrates which allows them to partition the available dietary glycan
90 niches in the gut (27, 30, 34).

91 In contrast to the microbiota of honey bees and bumble bees, much less is known about the
92 gut microbiota of the third group of social bees, the stingless bees (Meliponini). Previous
93 studies have focused on determining the taxonomic composition of the gut microbiota of
94 these bees using 16S rRNA gene sequencing (15, 17, 20, 37-39). However, only a few bacteria
95 have been cultured from stingless bees (40-42) and, except for two strains of
96 *Bombilactobacillus* Firm-4 recently isolated from bees from Australia (43), no genomic data is
97 currently available for core members of the gut microbiota of stingless bees.

98 With >500 described species, stingless bees present the largest and most diverse group of the
99 social bees (44, 45). They are naturally distributed throughout the tropical and subtropical
100 regions of Africa, Asia, Australia, and the Americas and exhibit great variation in morphology,
101 diet, foraging range, social structure, and nesting habits (44, 45). As host phylogeny and
102 ecology are both key determinants of gut microbiota composition (46-51), we hypothesize

103 that genomic studies on bacterial isolates will help us to understand the functional diversity
104 of gut bacteria of stingless bees and provide novel insights into the evolution of these bacteria
105 across social bees, specifically in respect of the possible co-diversification with the host.

106 To address these questions, we looked at the gut microbiota of six neotropical species of
107 stingless bees from Brazil: *Frieseomelitta varia* (Fv), *Scaptotrigona polysticta* (Sp), *Melipona*
108 *fuliginosa* (Mf), *Melipona interrupta* (Mi), *Melipona seminigra* (Ms), and *Melipona lateralis*
109 (Ml). We determined the composition of the gut microbiota of these bees using 16S rRNA
110 gene sequencing, established a comprehensive culture collection of bacterial isolates, and
111 conducted genome sequencing and comparative genomics to determine the phylogenetic
112 placement, genomic diversity, and functional capabilities of these bacteria relative to those
113 previously isolated from honey bees and bumble bees.

114 ## Results

115 **Six neotropical stingless bee species from Brazil harbor distinct gut microbiota dominated**
116 **by nine bacterial families.**

117 We sampled three colonies of six stingless bee species (Fv, Sp, Mf, Mi, Ms, Ml) from a
118 meliponary located in the Amazonian rainforest near Manaus (**Supplementary Table 1**). For
119 each colony, we pooled the guts of 15-60 worker bees (depending on the size of the bee
120 species, see Methods) before DNA isolation. The V4 region of the 16S rRNA gene was
121 amplified and sequenced with Illumina MiSeq 2x250bp resulting in a median depth of 64,713
122 (52,479 to 95,774) reads per sample. In total, we identified 276 amplicon sequence variants
123 (ASVs, 29-63 ASVs per sample), which belonged to 37 different bacterial families
124 (**Supplementary Table 2**). Despite this diversity, only nine families dominated the samples,
125 representing together 97% of all quality-filtered reads (93-99% of the reads per sample):
126 Acetobacteraceae, Bifidobacteriaceae, Enterobacteriaceae, Lactobacillaceae, Neisseriaceae,
127 Orbaceae, Prevotellaceae, Streptococcaceae and Veillonellaceae (**Figure 1A**).

128

129 While Lactobacillaceae and Bifidobacteriaceae were abundant across all samples, there were
130 clear differences in the distribution of some of the other bacterial families (**Figure 1A).**
131 Neisseriaceae were abundant in the samples of Sp and Fv, but were only detected in three
132 out of 12 samples from the genus *Melipona* (**Figure 1A, Supplementary Table 2**). In contrast,
133 Acetobacteraceae and Streptococcaceae were present in most *Melipona* samples but rare
134 across samples of Fv and Sp. Orbaceae and Enterobacteriaceae were mostly detected in the
135 three Fv samples. Intriguingly, a single Enterobacteriaceae ASV constituted the most
136 abundant community member in this bee species (21 – 36% of the reads per sample).
137 According to these compositional differences, non-metric multidimensional scaling (NMDS)

138 separated the samples into three distinct clusters: two clusters comprised all samples of Fv
139 and Sp, respectively, and the third cluster comprised all samples of the four *Melipona* species
140 (Mf, Mi, Ms, Ml) (**Supplementary Figure 1**).
141
142 **Related stingless bee species have overlapping community profiles.**
143 We compared our results to a previously published amplicon sequencing dataset from
144 stingless bees (20) to assess the similarity of the communities to those of other stingless bee
145 species. After discarding samples with <5,000 reads to control for variation in sequencing
146 depths, our dataset comprised 136 samples from 19 different host species and three different
147 countries. We detected 688 ASVs in total with a median of 18 ASVs per sample (3 to 63 ASVs)
148 (**Supplementary Table 3**), spanning 53 bacterial families. Overall, the taxonomic patterns
149 were similar across the analyzed bee species. Apart from one sample of *Tetragonula*
150 *fuscobalteata,* for which 99% of the reads belonged to a single Weeksellaceae ASV, the nine
151 families dominating in the six bee species from our study were also abundant in the
152 microbiota of the samples from the previous study and represented between 34% to 100% of
153 the total number of reads (**Supplementary Figure 2**, **Supplementary Table 3**).
154
155 NMDS based on ASV relative abundances separated the samples by location (i.e. samples
156 from Brazil were different from those from Australia and Malaysia**,** Permutational
157 multivariate analysis of variance (PERMANOVA)**: '**Location' pseudo-F: 20.17, p-value=0.001**)**,
158 and by bee genus (PERMANOVA: 'Genus' pseudo-F=10.49, p-value=0.001), although
159 taxonomy and geography are not independent (**Figure 1B**). In contrast, there was only weak
160 clustering at the species level ('Species' pseudo-F=1.96, p-value=0.03). Notably, only 30% of
161 all ASVs (206 of 688 ASVs) were shared across host species (i.e. 70% of all ASVs are only found

162 in one species), and most of them (83.4%) only between 2-5 species (**Supplementary Figure**
163 **3A**). However, the shared ASVs belonged to the nine predominant bacterial families and
164 represented a large fraction of the total number of reads per sample (53.3-99.7 %; except for
165 the samples of Fv and *P. helleri*: 10.6-15 % of the reads) (**Supplementary Figure 3B**). In
166 particular, bees sampled in the same country or belonging to the same bee genus shared the
167 same ASVs, explaining the clustering of these samples in the NMDS analysis. Together, these
168 results show that despite the large variability observed, the gut microbiota of most stingless
169 bee species is dominated by a few bacterial families and that bee species of the same genus,
170 or with overlapping geographic distribution, have similar community profiles at the 16S rRNA
171 gene level.
172
173 **Establishment of a strain collection of gut bacteria isolated from stingless bees.**
174 To enable genomic and experimental analyses of stingless bee gut bacteria, we established a
175 culture collection of bacteria isolated from Fv, Sp, Mf, Mi, Ms, and Ml. We plated
176 homogenized gut samples of the six bee species on eight different semi-solid media and under
177 three different atmospheres (microaerophilic and anaerobic). This resulted in the cultivation
178 of 98 distinct bacterial isolates (i.e. different 16S rRNA genotype or isolated from a different
179 bee species or colony) from 11 bacterial families (**Figure 2A, Supplementary Table 4**). Most
180 bacteria grew under both microaerophilic or anaerobic condition on generic growth media
181 and formed colonies after 2-4 days of growth. The 16S rRNA genotypes of the isolated strains
182 matched to 32 ASVs, accounting for 16 – 87 % of the overall community of the six stingless
183 bee species and including many shared ASVs (**Figure 2A** and **B**). BLASTN searches of the 16S
184 rRNA gene sequences revealed that many of the isolates (55/98) were related to bacterial
185 strains obtained from the gut of honey bees and bumble bees (such as *Lactobacillus apis*,

186 *Bifidobacterium commune*, *Gilliamella sp.,* or *Snodgrassella alvi*) suggesting that they
187 represent stingless bee isolates of core members of the social bee microbiota. Other isolates
188 had best BLASTN hits to bacteria from other environments, such as *Floricoccus* sp. isolated
189 from flowers, various Enterobacteriaceae (e.g. *Pantoea* sp., *Klebsiella* sp., *Rosenbergiella*
190 *epipactidis*) isolated from humans and water, or *Fructobacillus* isolated from flowers and
191 fruits. The percent identity of many of the blast hits was relatively low (<98%) suggesting that
192 the isolated strains potentially correspond to new bacterial species **(Figure 2A** and **C,**
193 **Supplementary Table 4)**.
194
195 **Stingless bee isolates form deep-branching phylogenetic lineages related to bacteria**
196 **isolated from honey bees and bumble bees.**
197 To assess the phylogenetic placement of the isolated stingless bee gut bacteria relative to gut
198 bacteria from honey bees and bumble bees, we selected 46 strains from 10 different bacterial
199 families for genome sequencing **(Figure 2A, Supplementary Table 4)**. Using a combination of
200 Illumina and Oxford Nanopore sequencing, we obtained 23 complete and 23 draft genomes
201 (2-66 contigs). Genome size of the cultured isolates ranged from 1.2-6.3 Mb. *Fructobacillus*
202 ESL0730 (1.2Mb) and the two Streptococcaceae strains ESL0687 and ESL0729 (1.4 Mb)
203 harbored the smallest, and Leuconostocaceae ESL0723 the largest (6.3 Mb) genomes of the
204 sequenced strains **(Supplementary Table 4)**. Genome comparisons with other bacteria,
205 including strains isolated from honey bees and bumble bees, showed that most stingless bee
206 gut bacteria had 80% average nucleotide identity with previously sequenced strains indicating
207 that we have isolated strains of novel bacterial species or genera (**Figure 2D**).
208 Accordingly, genome-wide phylogenies based on single-copy orthologs showed that most
209 isolates formed deep-branching, stingless bee-specific lineages, exclusive of any previously

210 sequenced strain. However, consistent with the results of the 16s rRNA gene analysis, several
211 of these lineages were related to major phylotypes of the honey bees and bumble bee gut
212 microbiota (**Figure 3A-D, Supplementary Figure 4-12)** such as *Snodgrassella*, *Gilliamella*,
213 *Lactobacillus* Firm-5, *Bifidobacterium*, and *Bombella*. In the case of *Snodgrassella*, *Gilliamella*,
214 and *Lactobacillus* Firm-5, the stingless bee-specific lineages formed a monophyletic clade with
215 lineages of honey bee and bumble bee isolates (**Figure 3A-C** and **3F)**. Notably, in all three
216 cases, the bacteria from stingless bees presented the earliest branching lineages, i.e. the
217 honey bee and bumble bee gut bacteria diverged after the split from the stingless bee gut
218 bacteria. While these results suggest that these bacteria have derived from a common
219 ancestor that was already adapted to social bees, the bacterial phylogenies were incongruent
220 with current phylogenies of the host, which show that the honey bees (Apini) diverged before
221 the split of stingless bees (Meliponini) and bumble bees (Bombini) (**Figure 3E**). A different
222 pattern was observed for *Bifidobacterium*. In this case, strains isolated from stingless bees,
223 honey bees, and bumble bees were not monophyletic. In fact, the stingless bee isolates
224 belonged to a different clade than the honey bee isolates, while the strains isolated from
225 bumble bees belonged to either of them (**Figure 3D** and **3G**). Similarly, the two
226 Acetobacteraceae strains (ESL0695 and ESL0709) were not monophyletic with the honey bee
227 isolates of the genus *Bombella*, although they belonged to the same Hymenoptera-associated
228 clade within this family **(Supplementary Figure 6).** This suggests that in both cases,
229 *Bifidobacterium* and Acetobacteriaceae, bacteria of distinct lineages have independently
230 adapted to the gut environment of social bees.
231
232 Of the 46 strains selected for sequencing, 13 were not directly related to bacteria previously
233 isolated from social bees. Most of these isolates matched to minor ASVs in our community

234 profiling analysis, with the exception of three strains (ESL0689, ESL0687, and ESL0729, **Figure**
235 **2A**). ESL0689 corresponded to the Enterobacteriaceae ASV19 which dominated the
236 communities of all three Fv samples (20-35% of the reads per sample). This isolate was
237 situated on a long branch diverging between the genera *Klebsiella* and *Raoultella*
238 (**Supplementary Figure 7**). ESL0687 and ESL0729 corresponded to ASVs of the family
239 Streptococcaceae which were detected in several stingless bee species in our studies as well
240 as in previous studies (20, 37, 38). They formed a deep-branching sister clade of the flower-
241 associated genus *Floricoccus* (**Supplementary Figure 8**). All three strains seem to be novel
242 species based on their divergence to previously sequenced bacteria and may represent
243 specialized gut symbionts of stingless bees.
244
245 **Stingless bee gut bacteria have diversified into distinct species and reveal a high extent of**
246 **genomic diversity.**
247 Stingless bee isolates belonging to the same lineage were often separated by long branches
248 in our phylogenies indicating substantial genomic divergence (**Figure 3F** and **G**,
249 **Supplementary Figures 4-12**). This was confirmed by comparing pairwise 16S rRNA gene
250 identity to genome-wide average nucleotide identity (ANI) between isolates of the same
251 bacterial family. Despite high similarity in 16S rRNA gene identity, ANI was often <95%
252 suggesting that most lineages of stingless bee gut bacteria contain several divergent species
253 (**Figure 4A**). For example, the 12 sequenced strains of *Lactobacillus* Firm-5 fell into 8 distinct
254 species-level clusters (i.e. ANI <95%, **Figure 4B**). A similar pattern was observed for the 17
255 *Bifidobacterium* strains, which fell into 14 distinct species-level clusters (**Figure 4C**), as well as
256 the two *Gilliamella* and the two Streptococcaceae strains, which both also fell below the
257 species-level ANI cut-off. Notably, some *Bifidobacterium* and *Lactobacillus* Firm-5 strains that

258 were isolated from the same sample belonged to different ANI clusters, indicating that
259 divergent bacterial species can co-occur in the same host species and colony. Inversely,
260 strains belonging to the same ANI cluster were sometimes isolated from different bee species,
261 suggesting that these bacterial species clusters are not necessarily host-specific (**Figure 4B**
262 and **C**).
263
264 **Core microbiota members in stingless bees have similar functional capabilities as in honey**
265 **bees and bumble bees.**
266 To assess the functional potential of stingless bee gut bacteria, we determined the genomic
267 completeness of different metabolic pathways and functions in the genomes of the
268 sequenced strains and compared it to related bacteria isolated from honey bees, bumble
269 bees, or from elsewhere, and which were included in our phylogenomic analysis. We
270 specifically looked at energy and carbon metabolism, amino acid, co-factor, and nucleoside
271 biosynthesis, as well as secretion, motility, and adhesion (**Figure 5)**.
272
273 ***Energy and carbon metabolism:*** Many of the sequenced strains (39/46) - including all
274 Lactobacillaceae, the Bifidobacteria, and the two Streptococcaceae and *Gilliamella* strains –
275 were missing key genes of the TCA cycle and for oxidative phosphorylation, but encoded
276 functions for the breakdown (mostly glycoside hydrolases (GH)), and oxidation of sugars
277 (Embden-Meyerhof-Parnas (EMP), pentose phosphate (PPP), and/or Entner-Doudorrof (ED)
278 pathways). A detailed analyses of the enzymes used for carbohydrate breakdown by the core
279 members *Lactobacillus* Firm-5, *Bifidobacterium*, and *Gilliamella* showed that the stingless bee
280 gut bacteria encoded similar glycoside hydrolase family genes as honey bee and bumble bee
281 isolates (**Figure 6**), including enzyme families for cleaving plant-derived glycans. For example,

282 glycoside families GH5, GH30, GH31, GH42, GH43, and GH51 are involved in the degradation
283 of hemicellulose (27, 30, 52). GH78 can be responsible for the cleavage of rhamnose residues
284 from rutin, a major pollen-derived flavonoid that was demonstrated to be deglycosylated by
285 honey bee isolates of *Lactobacillus* Firm-5 which encode GH78 genes (52). Another example
286 is GH13 which includes neopullulanases and α-amylases for the breakdown of plant-derived
287 starch. Together, these results suggest that stingless bee gut isolates of *Lactobacillus* Firm-5,
288 *Bifidobacterium*, and *Gilliamella* are saccharolytic fermenters that breakdown pollen or
289 nectar-derived glycans, similar as previously reported for the corresponding bacteria in the
290 gut of honey bees or bumble bees. Notably, there was substantial variation in the number
291 and type of glycoside hydrolase family genes between divergent strains, which goes in line
292 with the extensive genomic diversity detected between stingless bee gut isolates of these
293 three phylotypes.A complete TCA cycle was only found in the genomes of Neisseriaceae sp.
294 ESL0693, *Acinetobacter* ESL0695, the Enterobacteriaceae, and the Erwiniaceae. The same
295 strains also harbored the most complete gene sets for oxidative phosphorylation. Notably,
296 Neisseriaceae sp. ESL0693 also lacked key genes in the EMP, PPP, and the ED pathways and
297 contained very few GH family genes (**Figure 5**). This suggests that this bacterium cannot utilize
298 sugars and obtains energy via aerobic respiration, as previously found for *Snodgrassella*
299 isolates of honey bees and bumble bees (23).
300
301 ***Amino acid, nucleoside, and co-factor biosynthesis***: Differences between stingless bee gut
302 isolates of different taxonomic groups were also found in terms of their biosynthetic
303 potential. Strains belonging to the *Lactobacillus* Firm-5 clade were auxotrophic for the
304 production of most amino acids (i.e. all except for Lys, Gln, and Asn) as well as purine and
305 several co-factors (e.g. heme, vitamin B6 and B12) **(Figure 5)**. Isolates of the Bifidobacteria,

306 Streptococcaceae, and Leuconostocaceae were also auxotrophic for many co-factors, but for
307 much fewer amino acids than *Lactobacillus* Firm-5. Interestingly, there was variation in
308 auxotrophies among the Bifidobacterial strains, especially for the production of purine, NAD+,
309 Thr, Lys, Arg, Gly, and chorismate. Other strains (such as those of *Gilliamella*, *Snodgrassella*,
310 Acetobacteraceae, Enterobacteriaceae, and Erwiniaceae) had fewer auxotrophies. Similar
311 biosynthetic capability profiles were found in related strains included in our phylogenies,
312 which suggests that these functional profiles are not specific to stingless bee gut bacteria but
313 rather conserved across the entire phylotype (**Supplementary Figures 4-12**).
314
315 ***Secretion, adhesion, motility:*** Secretion systems, pili, and flagella were mostly restricted to
316 the gram-negative bacteria of the isolated strains. Type 1, Type 5, and Type 6 secretion
317 systems were prevalent across these bacteria, whereas Type 2 and Type 4 secretion systems
318 were only present in a few strains **(Figure 5)**. Flagella were detected in the two
319 Acetobacteraceae, all Erwiniaceae, and Enterobacteriaceae strain ESL0689. Tad pili were not
320 detected in any of the analyzed bacteria, while Type IV pili components were mostly found in
321 Neisseriaceae sp. ESL0693 and *Acinetobacter* ESL0695, and to some extent also in Orbaceae,
322 Erwiniaceae, and Enterobacteriaceae ESL0689. Similar gene sets for secretion, adhesion, and
323 motility were also found in related gut bacteria from honey bees or bumble bees, as shown
324 by the functional profiles of all strains included in our phylogenies (**Supplementary Figures 4-**
325 **12**).
326
327 Altogether, this first assessment of the gene content of the stingless bee gut bacteria show
328 that they have similar functional potential as isolates from honey bees and bumble bees
329 suggesting that they occupy similar ecological niches in the gut across social bees.

330 ## Discussion

331 Previous findings suggested that the core members of the bee gut microbiota have been
332 acquired in a common ancestor of the social bees (20) and possibly co-diversified with their
333 hosts over millions of years (16, 33). However, the lack of genomic data from gut bacteria of
334 stingless bees has limited our view on the evolution of these specialized microbial
335 communities. With the establishment of isolate genomes of diverse stingless bee gut bacteria
336 our study fills an important knowledge gap and provides new lines of evidence that rule out
337 strict co-diversification between the core microbiota members and social bees.

338

339 Our genome-wide phylogenies of *Snodgrassella*, *Lactobacillus* Firm-5, and *Gilliamella*
340 revealed that the identified lineages of stingless bee gut bacteria branched off before the
341 divergence of the lineages of honey bee and bumble bee gut bacteria. This pattern is not
342 congruent with the topology of the host phylogeny, in which honey bees diverged before the
343 split of stingless bees and bumble bees (53, 54). The basal split of the stingless bee gut
344 bacterial lineages depends on the rooting of our trees being correct. However, all phylogenies
345 were supported by high bootstrap values at the critical nodes suggesting robust phylogenetic
346 signals in our datasets. Our phylogenies also showed that for both *Gilliamella* and
347 *Snodgrassella*, honey bee isolates were not monophyletic, i.e. some lineages branched before
348 and others after the divergence of the bumble bee clades, which is inconsistent with co-
349 diversification. This was already noted in a previous study (33), and similar results were also
350 obtained for Lactobacillus Firm5 based on the phylogenetic analysis of single protein-coding
351 genes cloned from different social bee species (20). Finally, our phylogenomic analysis
352 showed that honey bee and stingless bee isolates of *Bifidobacterium* belonged to two

353 separate clades of the Bifidobacteriaceae suggesting that these bacteria have independently
354 adapted to the gut environment of social bees.
355
356 Co-diversification can only occur when symbionts and hosts exhibit a high degree of partner
357 fidelity and are transmitted vertically from one generation to the next over many generations
358 (55). While most core microbiota members of social bees indeed seem to have a host-
359 restricted distribution (20), examples of lineages with a broader host range exist as well (56).
360 Moreover, some strains have been experimentally shown to be able to colonize non-native
361 hosts demonstrating that host jumps are possible (20, 27). Our study showed that closely
362 related stingless bee species (i.e. from the same or related genera) can have overlapping
363 community profiles with predominant ASVs being shared across hosts. Similar observations
364 have been made in other 16S rRNA gene profiling studies of stingless bees (20, 37). While
365 such analyses often provide insufficient resolution to discriminate between closely related
366 strains or species, our genomic analyses confirmed that stingless bees isolates of
367 *Bifidobacterium* and *Lactobacillus* do not necessarily cluster by host species. It is possible that
368 the strength of host specificity varies across social bees, or bacterial lineages, depending on
369 the symbiotic function of the gut bacteria, or the hosts' divergence, ecology, or geographic
370 distribution. This would influence the extent to which gut bacteria can co-diversify within
371 certain host lineages. To test this hypothesis, future studies would have to compare the
372 strength of host specificity across multiple bees using representative sets of host species in
373 each of the tree main bee clades.
374
375 Another piece of evidence indicating that the core microbiota across social bees may be more
376 variable than previously assumed comes from our finding that only *Lactobacillus* and

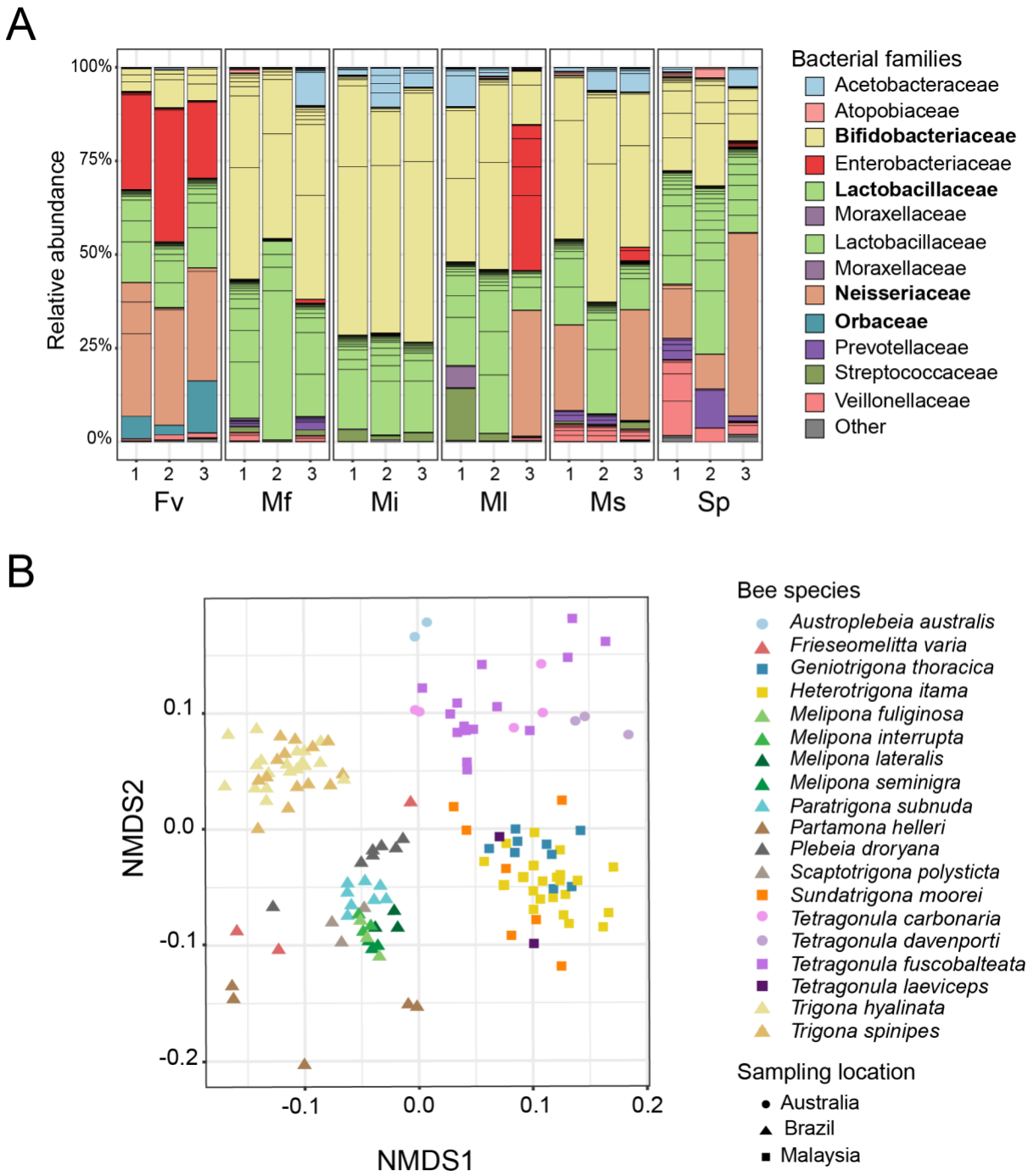
377 *Bifidobacterium* were prevalent across all stingless bee samples. Similar observations have
378 been made in other studies (15, 17, 37, 57). For example, stingless bees of the genus *Melipona*
379 were shown to systematically lack the two core members *Snodgrassella* and *Gilliamella* (37).
380 While both taxa were also rare across the four *Melipona* species analyzed in our study, three
381 out of 12 analyzed colonies had high abundances of *Snodgrassella*. This suggests that this
382 bacterium is not completely absent from this bee genus, but may be occasionally acquired
383 from other bee species, restricted to only the *Melipona* species analyzed in our study, or
384 varies in occurrence depending on season, bee age, or development.
385
386 Finally, representatives of core members of the social bee gut microbiota were recently also
387 found in bees of the distant genus *Xylocopa* (Carpenter bees) (58, 59), suggesting that these
388 bacteria may have been associated with bees before the emergence of sociality, or that they
389 have a broader and less-specific distribution across bees than previously suspected.
390
391 In summary, our results together with previous findings indicate a rather dynamic
392 evolutionary background of the core members of the social bee gut microbiota. Rather than
393 having strictly co-diversified with their hosts extended periods of host-restricted evolution
394 (and likely co-diversification in some lineages) seem to have been interrupted by host
395 switches, and independent symbiont gains and losses. Our observation that the stingless bee
396 isolates repeatedly form the most a sister group to bumble bee and honey bee isolates (for
397 *Gilliamella*, *Snodgrassella*, and *Lactobacillus* Firm-5) is intriguing given the host phylogeny. It
398 may suggest that these core members have an origin in stingless bees and then spread to the
399 other two groups, especially for *Lactobacillus* where two stingless bee isolates clades split
400 before the split between bumble bee and honey bee isolates. However, given the large

401 diversity of social and solitary bees, it is clear that the currently available datasets are
402 insufficient to explain the distribution and phylogenetic relationships of these gut symbionts
403 across hosts. Broader samplings of stingless bees and bumble bees, and genome-resolved
404 approaches are needed to fully understand the evolutionary trajectories of social bee gut
405 bacteria and to accurately reconstruct the ancestral bee microbiome composition; and formal
406 analysis should be applied to test for co-diversification.
407
408 The importance of sampling biases when assessing patterns of co-diversification between
409 hosts and their gut bacteria is highlighted by the analyses of Bacteroidaceae gut symbionts of
410 hominids. While originally reported to have co-diversified with their hosts (60), re-
411 examination with increased sampling disrupted the co-diversification pattern observed
412 earlier (61). In contrast, a recent study identified strong signals of parallel evolutionary history
413 between seven (out of 56 tested) gut bacterial taxa and human populations (62), and
414 phylogenetic congruency has also been found for certain stinkbug insects and their primary
415 gut symbiont (63). This demonstrates that co-diversification has occurred between certain
416 gut bacteria and their hosts.
417
418 Besides offering new insights into the evolution of the social bee gut microbiota, our genomic
419 analysis also revealed the functional potential of major gut symbionts of the analyzed
420 stingless bee species. All isolates of *Lactobacillus* Firm-5, *Bifidobacterium*, and *Gilliamella*
421 carried genes for the saccharolytic fermentation of diet-derived carbohydrates. In contrast,
422 *Snodgrassella* ESL0689 lacked such functions in its genome, but instead harbored genes for
423 aerobic respiration. These results are consistent with findings from honey bees and bumble

424 bees (22, 23, 26-28, 30) and hence suggest that the core microbiota members occupy similar
425 ecological niches across the three groups of social bees.
426
427 Another parallel to findings from honey bees and bumble bees was the extensive genomic
428 divergence present among strains of the core members *Lactobacillus* Firm-5, *Gilliamella*, and
429 *Bifidobacterium*, even when isolated from the same host species. Moreover, we found
430 genomic variation in carbohydrate breakdown and amino acid and nucleoside biosynthesis
431 functions among these strains. This suggests that the diversification of these bacteria has not
432 only been driven by isolation into different host species but also by the adaptation to different
433 ecological niches in the gut, similar as shown for bumble bees and honey bees (28, 33, 35,
434 64). These parallels may not be surprising as the dietary preferences of the analyzed stingless
435 bee species are similar to those of honey bees and bumble bees. In the future, it will be
436 interesting to look at the functional potential of the core microbiota members in bees with
437 different dietary habits, such as the vulture bees (38), i.e. stingless bees that feed on raw meat
438 instead of pollen yet share a subset of the core members with other social bees.
439
440 Some of the isolate genomes we sequenced in our study did not come from any of the core
441 members of the social bee gut microbiota. They may present transient community members,
442 opportunistic pathogens, or host-specific gut symbionts with complementary functions to the
443 core microbiota. Therefore, the established genomes present an important resource for
444 future research. A particular strain that drew our attention was Enterobacteriaceae ESL0689,
445 as it belonged to an ASV that was present at high relative abundance in all three colonies of
446 *Fv.* ESL0689 harbored a complete TCA cycle and a respiratory chain and could also synthesize
447 most amino-acids and co-factors indicating a similar metabolic niche as *Snodgrassella* (23).

In conclusion, our study provides new insights into the evolution of the social bee gut microbiota and presents a first step in characterizing the functional potential of major gut bacteria present in stingless bees. However, given the large diversity of stingless bees with hundreds of different species distributed throughout the tropical and subtropical regions of the world, it is clear that our study only presents the starting point in characterizing their genomic diversity and functional potential. More detailed studies and larger genomic survey combined with experimental analyses will be needed to understand their evolution and assess their impact on the host.

457 **Figures**



458

459 **Figure 1. Community analysis of the gut microbiota of stingless bees. (A)** 16S rRNA gene-

460 based community profiles of the gut microbiota of three colonies of six different stingless

461 bee species collected in Brazil. Fv – *Frieseomelitta varia,* Ms – *Melipona seminigra*, Ml –

462 *Melipona lateralis*, Mf – *Melipona fuliginosa*, Mi – *Melipona interrupta*, Sp – *Scaptotrigona*

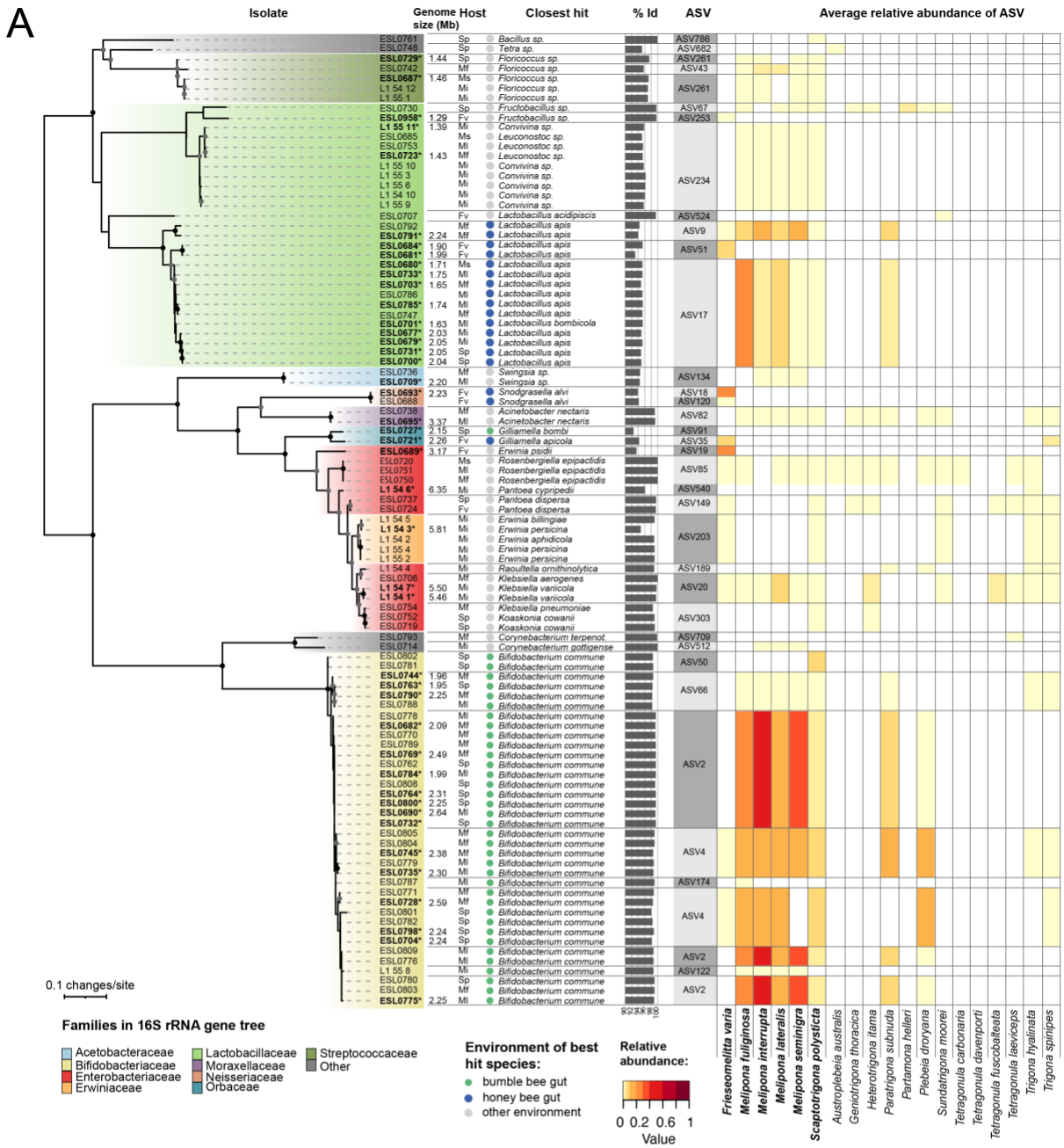
463 *polysticta*. Relative abundance of ASVs is shown. ASVs are ordered and colored at the family

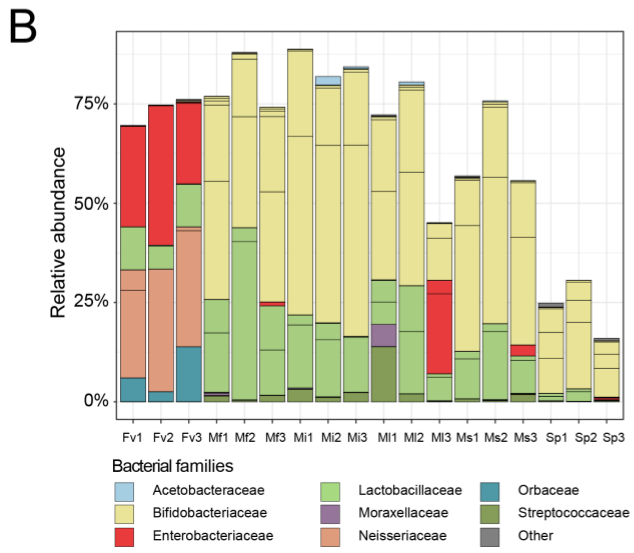
464 level according to the legend, families of core members are displayed in bold. ASVs with

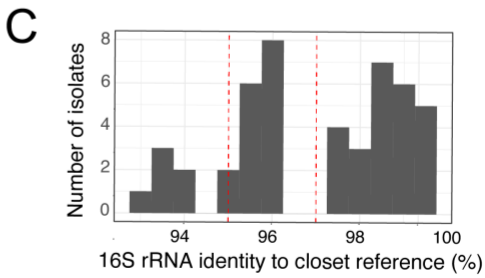
465 <1% relative abundances are summed up as 'others' and shown in grey. **(B)** NMDS based on

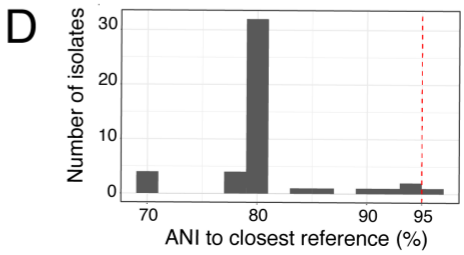
466 ASV relative abundance (Bray-Curtis dissimilarity) across 136 16S rRNA gene amplicon

467 sequence samples including data from a previous study (20) and our study.


A
Isolate
Genome size (Mb)
Host
Closest hit
% Id
ASV
Average relative abundance of ASV
0.1 changes/site
Families in 16S rRNA gene tree
Acetobacteraceae
Bifidobacteriaceae
Enterobacteriaceae
Erwiniaceae
Lactobacillaceae
Moraxellaceae
Neisseriaceae
Orbaceae
Streptococcaceae
Other
Environment of best hit species:
bumble bee gut
honey bee gut
other environment
Relative abundance:
0 0.2 0.6 1
Value
Frieseomelitta varia
Melipona fuliginosa
Melipona interrupta
Melipona lateralis
Melipona seminigra
Scaptotrigona polysticta
Austroplebeia australis
Geniotrigona thoracica
Heterotrigona itama
Paratrigona subnuda
Partamona helleri
Plebeia droryana
Sundatrigona moorei
Tetragonula carbonaria
Tetragonula davenporti
Tetragonula fuscobalteata
Tetragonula laeviceps
Trigona hyalinata
Trigona spinipes

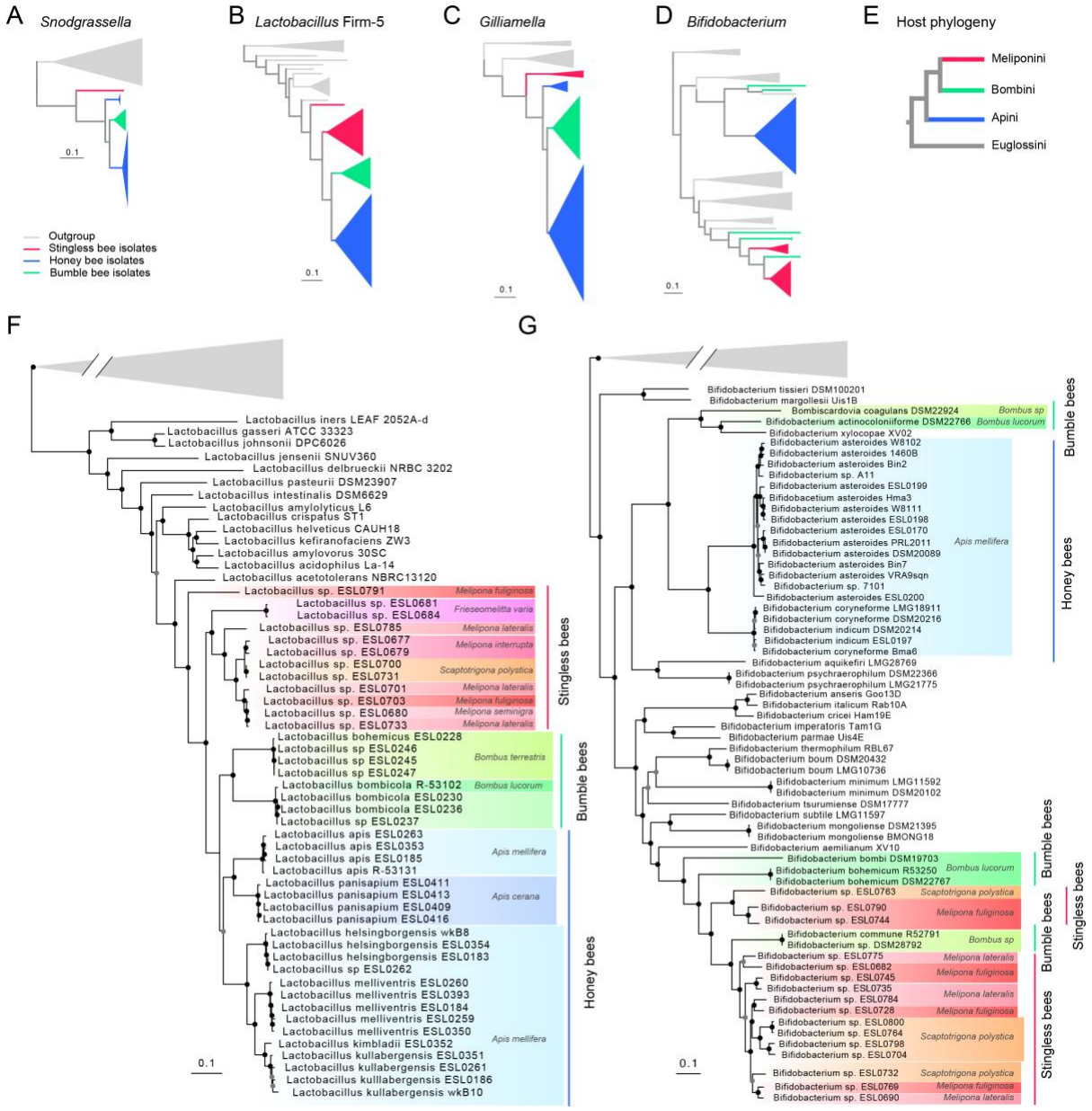

B
Relative abundance
75%
50%
25%
0%
Fv1 Fv2 Fv3 Mf1 Mf2 Mf3 Mi1 Mi2 Mi3 Ml1 Ml2 Ml3 Ms1 Ms2 Ms3 Sp1 Sp2 Sp3
Bacterial families
Acetobacteraceae
Bifidobacteriaceae
Enterobacteriaceae
Lactobacillaceae
Moraxellaceae
Neisseriaceae
Orbaceae
Streptococcaceae
Other


C
Number of isolates
0
2
4
6
8
94
96
98
100
16S rRNA identity to closet reference (%)


D
Number of isolates
0
10
20
30
70
80
90
95
ANI to closest reference (%)

468

469 **Figure 2. Bacterial strains isolated from the gut of six stingless bee species from Brazil. (A)**
470 Maximum likelihood tree was inferred from nearly complete sequences of the 16S rRNA gene
471 of each isolate. Strain names of isolates for which we sequenced the genome are indicated in
472 bold. The genome size of each sequenced strain is noted next to it. The isolate host is
473 indicated as follows: Fv – *Frieseomelitta varia,* Ms – *Melipona seminigra*, Ml – *Melipona*
474 *lateralis*, Mf – *Melipona fuliginosa*, Mi – *Melipona interrupta*, Sp – *Scaptotrigona polysticta*.
475 Closest hit indicates best BlastN hit of the 16S rRNA gene sequence against the 16S ribosomal
476 RNA (NCBI: Bacteria and Archaea type strains) database. Colored circles indicate if the strain
477 of the best hit was isolated from the gut of a bumble bee, a honey bee, or from elsewhere.
478 Bar plots indicate % identity of the best BlastN hit. The matching ASV and its average relative
479 abundance across the nineteen analyzed stingless bee species are indicated. The names of
480 the stingless bee species from our study are given in bold. Note that many isolates matched
481 the same ASV. **(B)** Relative abundances of matching ASVs in each of the 18 samples of the six
482 stingless bees species sampled in our study, i.e. representativeness of the isolates in the
483 amplicon data. **(C)** Distribution of the isolated strains based on best 16S rRNA gene identity
484 to closest reference species. These are the same values as shown in Figure 2A. The dashed
485 lines indicate 95% and 97% identity corresponding to the genus and species threshold,
486 respectively. **(D)** Distribution of the isolated strains based on pairwise ANI with the closest
487 reference genome publicly available. The dashed line indicates the species threshold at 95%
488 ANI.
489



490

491 **Figure 3. Isolates of stingless bee gut bacteria present novel species and belong to deep-**

492 **branching phylogenetic lineages. (A-D)** Simplified genome-wide maximum likelihood

493 phylogenies of *Snodgrassella*, *Lactobacillus* Firm-5, *Gilliamella*, and *Bifidobacterium* based on

494 single copy gene orthologs. All branches shown are supported by >95/100 bootstrap

495 replicates. Most of the branches have been collapsed. **(E)** Dendrogram depicting the topology

496 of the social bee phylogeny (adapted from (53, 54))**. (F)** Detailed genome-wide phylogeny of

497 the genus *Lactobacillus* with bacteria belonging to the social bee-specific phylotype

498 *Lactobacillus* Firm-5 highlighted in different colors according to the host species/group. The

maximum likelihood tree was computed on the concatenated amino acid sequences of 355 single copy core genes using the substitution model LG+F+I+G4. **(G)** Detailed genome-wide phylogeny of the genus *Bifidobacterium* with bacteria belonging to social bee-specific clades highlighted in different colors according to the host species/group. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 151 single copy core genes using the substitution model LG+F+I+G4.

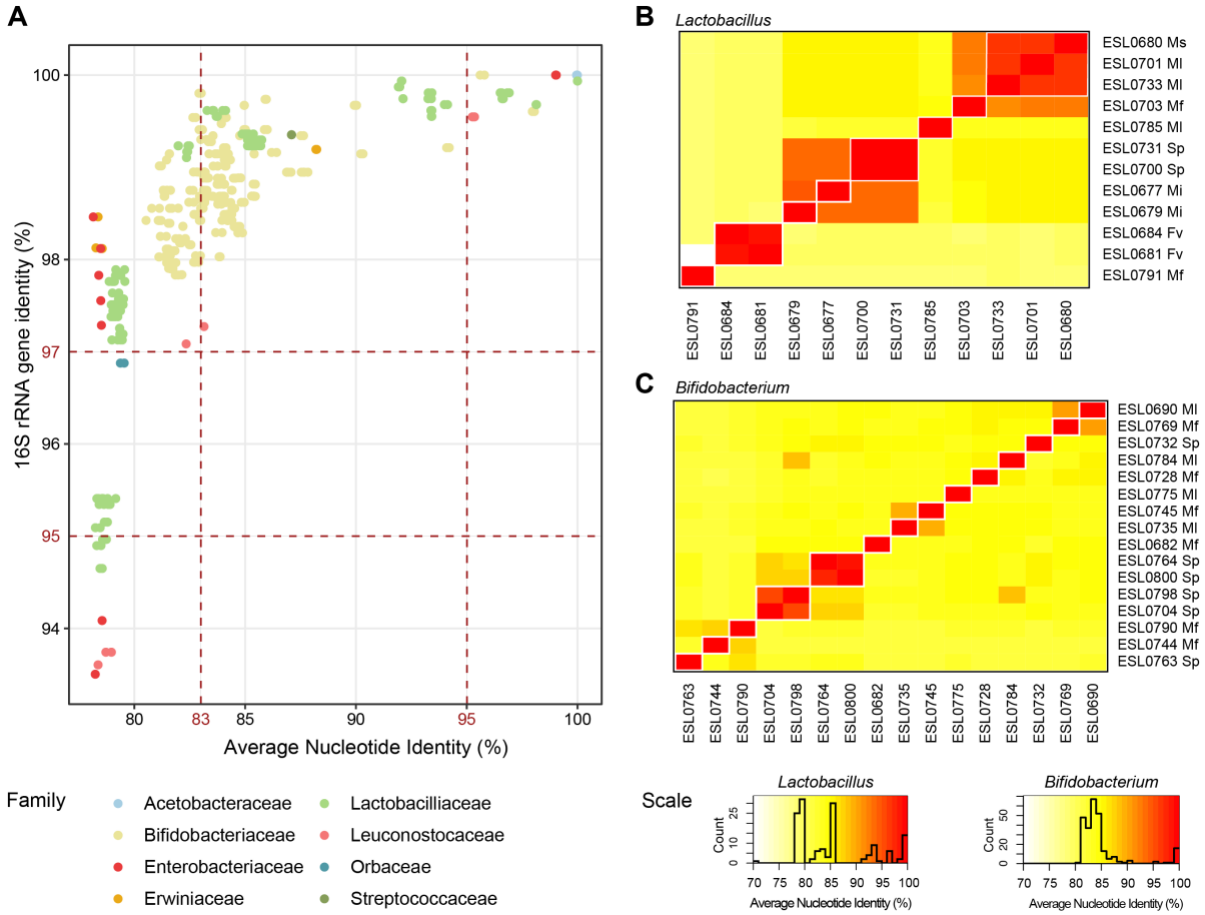


Figure 4. Genomic divergence among isolates with high 16S rRNA gene similarity. (A) The average nucleotide identity (ANI) versus 16S rRNA gene identity was plotted for each pair of isolate genomes belonging to the same bacterial family. The two vertical dashed bars indicate the ANI thresholds of 95% and 83% demarcating the intra-species and inter-species ANI values, respectively (65). The horizontal dashed lines delineate the commonly used species (>97%) and genus (>95%) thresholds for the 16S rRNA gene similarity. Average nucleotide identity heatmaps of **(B)** *Lactobacillus* Firm-5 isolates and **(C)** *Bifidobacterium* isolates. The 95% ANI clusters are delimited in white. The isolate host is indicated, Fv – *Frieseomelitta varia,* Ms – *Melipona seminigra*, Ml – *Melipona lateralis*, Mf – *Melipona fuliginosa*, Mi – *Melipona interrupta*, Sp – *Scaptotrigona polysticta*.

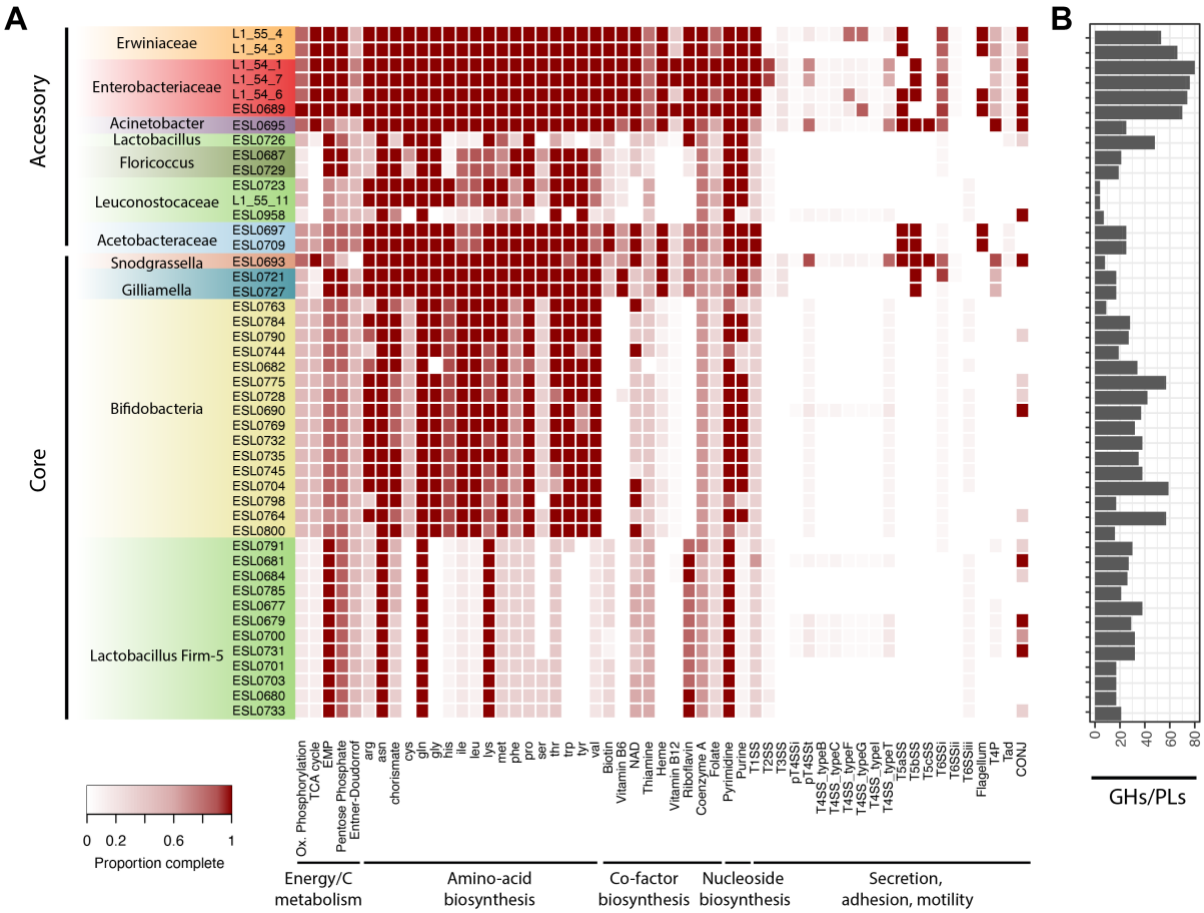
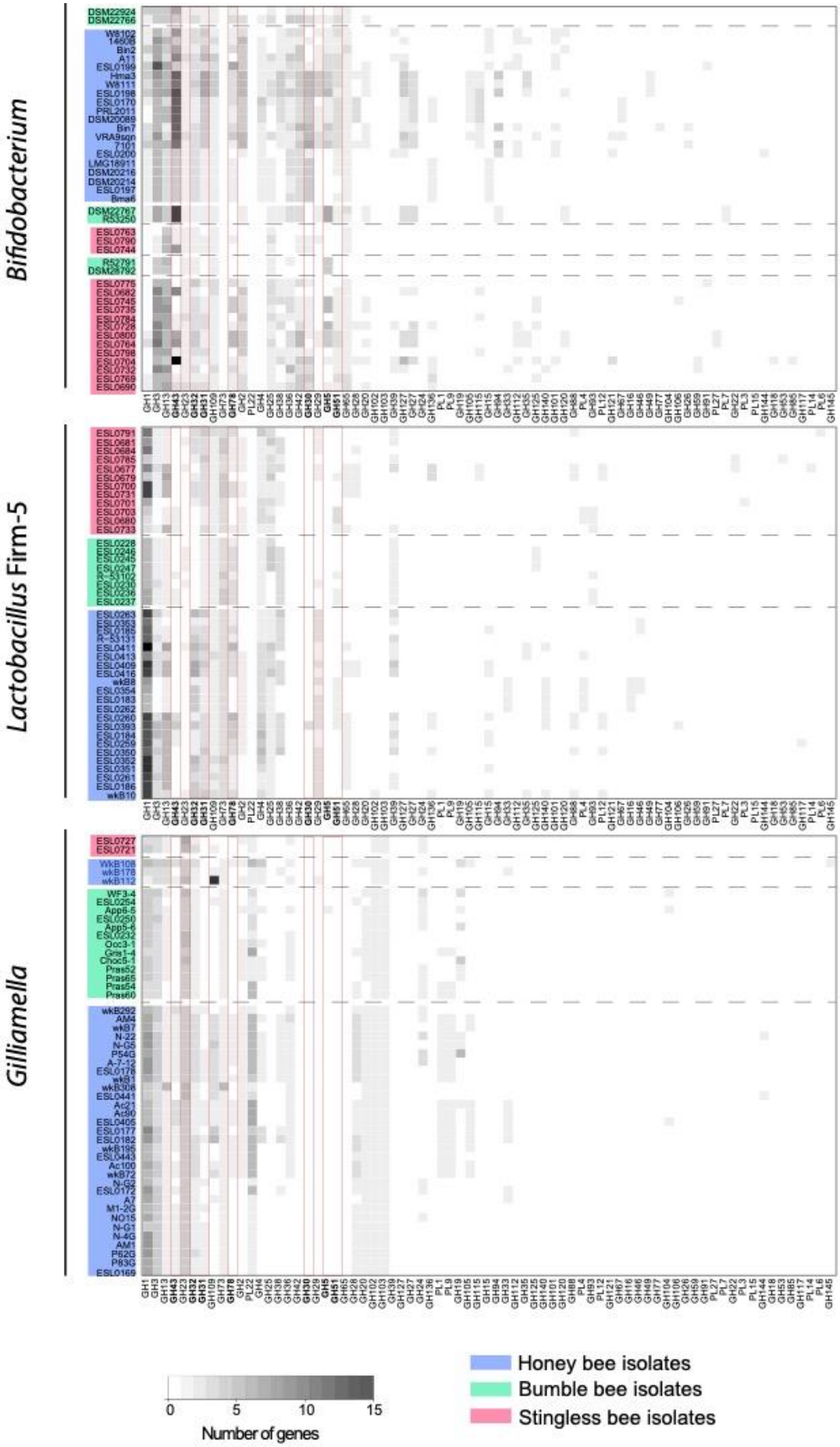


Figure 5. Metabolic capabilities of the sequenced stingless bee gut isolates. (A) The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates. The isolates are ordered into core and accessory members based on whether they are related to bacteria isolated from other social bees. **(B)** Total number of glycoside hydrolase (GH) and polysaccharide lyase (PL) genes in each isolate.


Bifidobacterium
Lactobacillus Firm-5
Gilliamella
Honey bee isolates
Bumble bee isolates
Stingless bee isolates
0
5
10
15
Number of genes

529

530 **Figure 6. Carbohydrate-active enzyme profiles for *Bifidobacterium, Lactobacillus* Firm-5 and**
531 ***Gilliamella* isolates.** Distribution of genes in each GH and PL families respectively for *Bifidobacterium,*
532 *Lactobacillus* Firm-5 and *Gilliamella* isolates. The isolates were sorted according to their position in
533 the phylogenies, the hosts are indicated by the background colors. Glycoside hydrolases families
534 mentioned in the text are outlined with a red square. The color scale indicates the number of genes
535 in each family.
536
537

538 ## Methods

539 **Bee sampling**

540 Bees were collected from three different nests of each of the following six Meliponini species
541 in February and March 2019: *Frieseomelitta varia*, *Scaptotrigona polysticta*, *Melipona*
542 *fuliginosa*, *Melipona interrupta*, *Melipona lateralis* and *Melipona seminigra*. All nests were
543 located in a rural meliponary in the vicinities of Iranduba municipality (Iranduba-AM, Brazil,
544 3°10'52.7"S 60°07'08.5"W), and the sampling was carried out under the SISGEN collection
545 permission A256E82. Bees were sampled at the entrance of each nest and immediately
546 immobilized by cooling at 4 °C. Then the entire gastrointestinal tract was dissected, and the
547 hindgut separated from the anterior gut parts. Hindguts of bees from the same nest were
548 pooled in two separate tubes. One pool per nest was mixed with 1x PBS and glycerol,
549 homogenized using bead-beating, and subsequently cryopreserved at -80°C. This pool was
550 used for bacterial culturing as described below. The other pool was cryopreserved at -80°C
551 without homogenization and was subsequently used for DNA extraction and 16S rRNA gene
552 analysis. For the sample used for sequencing, 20 bees per nest were pooled, while for the
553 sample for culturing, only 3 bees per nest were pooled. For *Frieseomelitta varia* and
554 *Scaptotrigona polysticta*, we pooled 40 and 10 bees, respectively, due to the small size of
555 these bee species.

556

557 **Bacterial culturing**

558 For establishing a culture collection of primary isolates from the gut of the six sampled bee
559 species, serial dilutions of the cryopreserved homogenates were plated on eight different
560 media: CBA (Columbia Blood Agar supplemented with 5% defibrinated sheep blood
561 (Thermofisher)), MRSA (De Man, Rogosa and Sharpe agar) supplemented with Fructose (2%)

562 and L-Cysteine (0.1%), MRSA supplemented with Mannitol (2%), Chocolate Agar, TSA
563 (tryptone soya agar), TYG (tryptone glucose yeast extract Agar), GC, LBA (Luria Bertani agar)
564 without NaCl, BHIA (brain heart infusion agar) and SDA (Sabouraud dextrose agar). Plates
565 were incubated in two different conditions: in a microaerophilic 5% CO_2-enriched atmosphere
566 and in an anaerobic chamber (72% N_2, 8% H_2, 20% CO_2), both at 34°C. After 2-7 days of
567 incubation, colonies of different size and appearance were picked and re-grown on the same
568 media and culturing condition. Cryo-stocks of bacterial strains of interest were prepared by
569 harvesting bacterial biomass in liquid media corresponding to the solid growth media and
570 supplemented with 20% glycerol. For DNA isolation, bacteria were grown from the stocks,
571 and a single colony was picked and re-grown on fresh media before harvesting bacterial
572 biomass.
573
574 **Genotyping**
575 All colonies that were selected for culturing were genotyped by PCR and Sanger sequencing
576 of a 16S rRNA gene fragment. To this end, a small amount of bacterial material was
577 transferred to a lysis buffer (Tris-HCl 1M pH=7.5, EDTA 0.5M, SDS 10%) containing 2.5 µl
578 lysozyme (20mg/mL) and 2.5 µl proteinase K (20mg/mL), and incubated for 10 min at 37°C,
579 20 min at 55°C, and 10 min at 95°C. PCR was performed with universal bacterial primers that
580 amplify the V1-V5 region of the 16S rRNA gene (27F - AGRGTTYGATYMTGGCTCAG and 907R-
581 CCGTCAATTCMTTTRAGTTT) using the following reagents and thermocycler program: initial
582 denaturing at 94°C for 5 min, followed by 32 cycles of denaturing at 94°C for 30 sec, annealing
583 at 56°C for 30 sec, and extension 72°C for 1 min, and a final extension at 72°C for 7 min. PCR
584 results were checked on a 1% agarose gel. PCR reactions selected for Sanger sequencing were
585 purified using ExoSAP-IT™ (1µl ExoSAP 5x, 4µl ddH_2O) with the thermocycler program: 30 min

586 at 37°C followed by 15 min at 80°C. Purified samples were then sent to Eurofins® for
587 sequencing. Sanger sequences were analyzed with Geneious suite (Geneious®) and compared
588 to GenBank at NCBI using BLAST tools (66).
589

590 **DNA isolation and genome sequencing**

591 DNA isolation for Illumina sequencing was carried out using a customized SPRI bead-based
592 extraction method or the FastPure Bacteria DNA Isolation Mini Kit (Vazyme). For the SPRI-
593 bead method, bacteria were harvested and resuspended in tubes containing 200 mg of 0.1
594 mm-sized acid washed glass beads and 200 µl of TER buffer (10 mM Tris-HCl, 1 mM EDTA, 100
595 µg /ml RNAse A, pH8.0). Samples were homogenized using a FastPrep-25 5G instrument (2
596 rounds of 30 sec with the power set to 6) and subsequently centrifuged at max speed for 10
597 min at room temperature. 40 µl of SPRI beads were added to 100 µl of supernatant,
598 immediately mixed thoroughly by repeated pipetting (>20x) and incubated for 5 min at room
599 temperature. After placing the tubes on a magnet stand the liquid was removed and
600 discarded and the beads washed two times with 200 µl 80% ethanol. After air drying the tubes
601 on the magnetic stand 22 µl of 5 mM Tris-HCl pH8 was added. For isolating bacteria with the
602 FastPure Bacteria DNA Isolation Mini Kit the manufacturer's protocol for gram-positive
603 bacteria was followed.
604 DNA isolation for Oxford Nanopore Technologies (ONT) sequencing was carried out using a
605 custom DNA extraction protocol for Gram-positive bacteria. Tubes were prepared with glass
606 beads and 160 µl of buffer P1 (Qiagen). Then bacteria were harvested and resuspended in
607 these tubes by intensive vortexing. Lysozyme (20 µl, 100 mg/ml) was added and after gentle
608 mixing, tubes were incubated at 56°C with shaking at 600 rpm for 30 min. Then 4 µl RNase A

609 (100 mg/ml) were added to the tubes followed by 150 µl of buffer AL (lysis buffer, Qiagen).
610 After mixing by vortexing, tubes were incubated in a thermomixer (37°C, 900 rpm) for 20 min.
611 Tubes were centrifuged 10 min at 14'000 rpm to pellet the beads, the supernatant was
612 transferred to new tubes with 35 µl Na-acetate and 270 µl isopropanol and mixed by
613 inverting. Following an incubation of 1 h at 4°C, DNA was pelleted by centrifugation at 14'000
614 rpm for 10 min at 25°C. The supernatant was discarded, and the pellets were washed with 1
615 ml EtOH 80%. After a second centrifugation (14'000 rpm, 10 min at 25°C) the ethanol was
616 removed, and the pellet left to dry at room temperature. DNA pellets were solubilized with
617 50 µl TER (10 mM Tris-HCl, 1 M EDTA, pH 8.0, 2 mg/ml RNase A) and tubes incubated at 37°C
618 for 15 min. The solution was then transferred to PCR tubes. 40 µl NGClean beads were added,
619 and the solution mixed by repeated pipetting. After a 5 min incubation, PCR tubes were
620 placed on magnetic stands. When the solutions were clear, the liquid was removed, and the
621 beads washed two times with 200 µl of 80% EtOH. Upon complete drying, beads were
622 resuspended with 22 µl of 5mM Tris-HCl (pH 8.0). Finally, tubes were placed again on the
623 magnetic stand and when the solution were clear, the supernatant was transferred to new
624 1.5 ml Eppendorf tubes.
625 Illumina sequencing libraries were prepared using the Nextera DNA Flex Library Preparation
626 kit following instructions of the Illumina Reference Guide. This was followed by indexing,
627 dilution, and denaturation according to the 'Index Adapters Pooling Guide' and the 'MiniSeq
628 System Denature and dilute Libraries guide'. Libraries were checked and quantified using a
629 dsDNA fluorescent dye, before loading them on a MiniSeq High-output flowcell (150PE). ONT
630 libraries were prepared using the ligation-based approach (LSK109). The sequencing was
631 conducted on a ONT MinION for a duration of 72 hours with the high-accuracy base-calling
632 using Guppy (*v5.0.11*).

633

634 **Genome assembly**

635 Forty-six isolates from the stingless bees were sequenced with Illumina MiniSeq (150PE). Raw
636 reads were checked using FastQC *v0.11.9* (67) and trimmed by Trimmomatic v0.39 (68) using
637 parameters: PE -phred33 AllIllumina-PEadapters.fa:3:25:7 LEADING:9 TRAILING:9
638 SLIDINGWINDOW:4:15 MINLEN:60. For *de novo* assembly we used SPAdes (–careful option,
639 *v3.15.2*) (69). For assembly quality control, reads were mapped back against the assembly
640 with BWA *v0.7.17 (70)*, samtools *v1.12* (71) and plotted with R *v4.1.1*. Genomes completeness
641 was evaluated with checkM *v1.0.13* (72).

642 Additionally, 26 isolates were also sequenced with Oxford Nanopore to produce long reads.
643 Nanopore long reads were filtered with Filtlong *v0.2.0* (https://github.com/rrwick/Filtlong)
644 for a minimum length of 7'000 and minimum mean q-score of 10 (min_length 7000,
645 min_mean_q 10, length_weight 10). ONT-based assemblies were computed with Flye *v2.7.1*
646 (73) over 5 iterations. Graphmap *v0.5.2* (74) and Racon *v1.0.1* (75) were used to perform two
647 rounds of polishing. Finally, the racon-corrected assembly and Illumina reads were fed to
648 Pilon *v1.24* (76) for single base and indels corrections.

649

650 **Genome annotation and analysis**

651 Genomes were annotated with Prokka *v 1.13* (77). Phylogenies were computed for the
652 bacterial families for which we had an isolate. For each family, we identified a set of closely
653 strains and outgroup taxa and retrieved their genomes from NCIB and IMG/Mer (78). All
654 genomes were re-annotated with Prokka to ensure annotations consistency. Gene orthology
655 was inferred with OrthoFinder *v2.3.8 (79)*. Single copy ortholog genes were selected and their
656 amino acid sequences were aligned (mafft *v7.453) (80).* An in-house script was used to trim

657 the alignments by removing positions with more than 50% gaps and sequences belonging to
658 the same genome were concatenated to produce a core gene alignment. This alignment was
659 used to infer the maximum likelihood phylogeny using IQTree (*v1.7.beta17* , -st AA -bb 1000
660 -seed 12345 -m TEST) (81). For each phylogeny, the best evolutionary model was chosen
661 according to BIC: *LG+I+G4* – Enterobacteriaceae ; *LG+F+I+G4* – Acetobacteraceae,
662 *Bifidobacterium*, *Lactobacillus*, Leuconostocaceae, Moraxellaceae, Neisseriaceae and
663 Streptococcaceae ; and *JTTDMut+F+I+G4* – Orbaceae. Branch support of the trees were
664 inferred using 1000 ultrafast bootstrap (UFBoot) repetitions. Clades can be trusted when
665 UFBoot values are >95%.
666
667 **16S rRNA community profiling**
668 Region V4 of the 16S rRNA gene was amplified using the primers 515F-Nex and 806R-Nex
669 (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA,
670 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT). The primers
671 include Nextera XT index adapter sequences and the primers for the 16S rRNA V4 region (82)
672 described by Kešnerová et al. (2020). The two step PCR was performed as following: the first
673 PCR was performed using 12.5 µL of 2× Phanta Max Master Mix (Vazyme, Nanjing, China), 5 µl
674 of MilliQ water, 2.5 µl of each primer (5 µM), and 2.5 µl of template DNA for a total volume
675 of 25 µl. The PCR program started with a denaturation step at 98°C for 30sec, followed by 25
676 cycles of amplification (10 sec at 98°C, 20 sec at 55°C, and 20 sec at 72°C) and a 5 min final
677 extension step at 72°C. The PCR products were verified by 2% agarose gel electrophoresis,
678 then purified with clean NGS purification-beads in a 1:0.8 ratio of PCR product to beads and
679 eluted in 27 µl Tris (10mM, pH 8.5). A second PCR step was performed to append the unique
680 dual indexes to each sample in a total volume of 25 µl using 12.5 µL of 2× Phanta Max Master

Mix (Vazyme, Nanjing, China), 5 µl of MilliQ water, 2.5 µl of Nextera XT index primers 1 and 2 (Nextera XT Index kit, Illumina) and 2.5 µl of templated DNA. The PCR program started with a denaturation step at 95°C for 3 min, followed by 8 cycles of amplification (30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C), and a 5 min final extension step at 72°C. The libraries were then cleaned usingClean NGS purification beads (1:1.1 ratio of PCR product to beads) and were eluted in 27.5 µl Tris (10mM, pH 8.5). Prior to sequencing, the PCR product concentrations were quantified by PicoGreen and pooled in equimolar concentrations; the negative controls and blank extractions were pooled in equal volume. Sequencing was performed on an Illumina MiSeq sequencer (2 x 250bp) by the Genomic Technology Facility of the University of Lausanne. We followed DADA2 (83) pipeline to analyze the sequencing data. Sequence quality control was performed with DADA2 integrated function *plotQualityProfile*. Data from the three sequencing runs were processed independently for the filtering, dereplication, the error rates calculation and the sample inference. After merging the denoised forward and reverse pairs, we merged the three sequence tables (*mergeSequenceTables*) and we applied the *collapseNoMismatch* function to unite similar ASVs with shifts or length variation. We selected sequences in range 250-256bp and removed chimeric sequences. Silva non-redundant SSU database v138.1 (83) was used for the taxonomic assignment of the ASVs. We filtered out 62 ASVs for which the taxonomic assignment was matching ‘Eukaryota’, ‘Chloroplast’ or ‘Mitochondria’. Finally, we removed samples with less than 5,000 reads. Non-metric MultiDimensional Scaling plots were computed in R using Bray-Curtis distances (Phyloseq: *ordinate*). The adonis package was used to compute permanovas.

Functional Profiler

705 We developed a genomic profiler using several software to annotate the genomes and
706 compute the completeness of key pathways and functions. The genomes were re-annotated
707 with GhostKOALA (84) to get KEGG annotations of genes. We created a set of rules to define
708 selected energy and metabolism pathways, and co-factors/nucleoside biosynthesis pathways
709 and computed their completeness (steps present relative to total number of steps needed)
710 with an in-house script. To obtain the completeness of amino acid biosynthesis pathways, we
711 used GapMind (85). Secretion systems and related appendages were detected in the
712 genomes by MacSyFinder's TXSScan module (86). Finally, we ran dbCan (87) for the
713 annotation of carbohydrate-active enzymes.
714

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738 **Data and materials availability:**

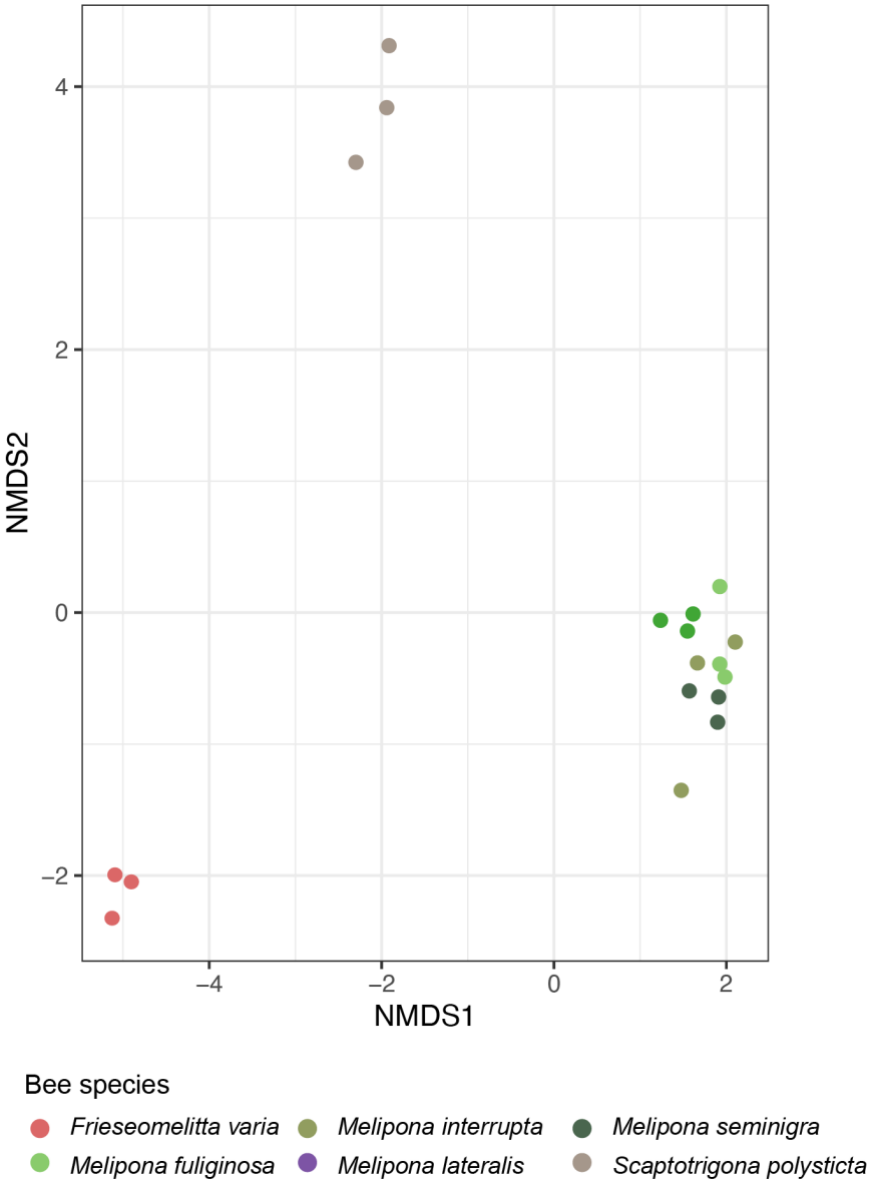
739 The bacterial genomes and sequencing data are available in the NCBI's BioProjects
740 PRJNA906295 and PRJNA897721. Scripts used for the amplicon sequencing analysis, the
741 phylogenies and the metabolic profiler can be found on GitHub
742 https://github.com/gsartonl/Publication_Sarton-Loheac_2022.

743

744

745 **Supplementary Figures and Tables**

746



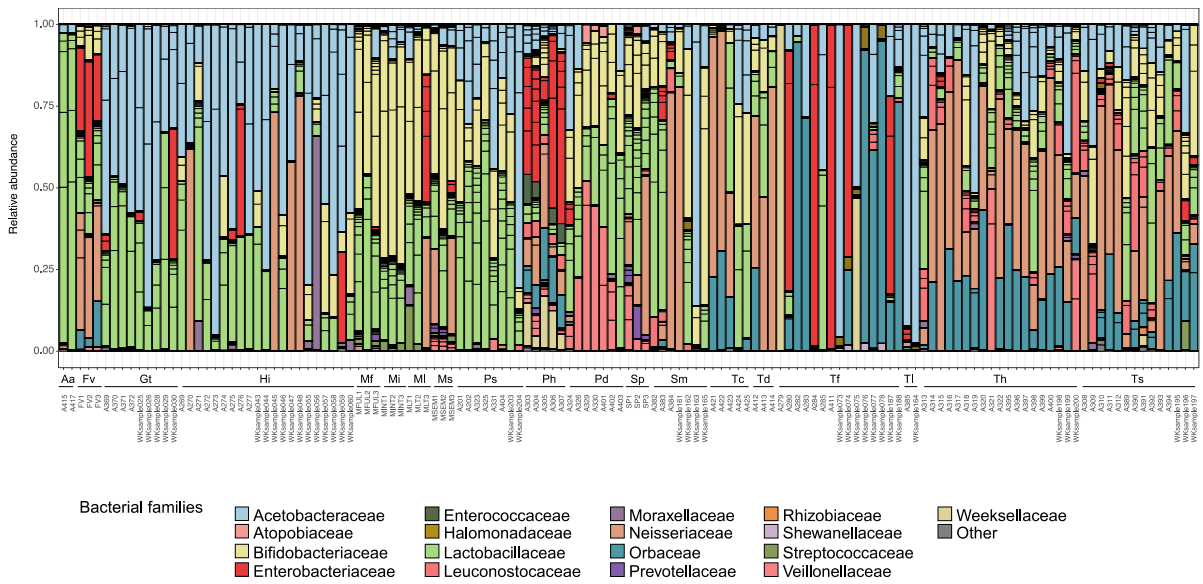
747

748 **Supplementary Figure 1.** NMDS based on ASV relative abundance in the 18 samples

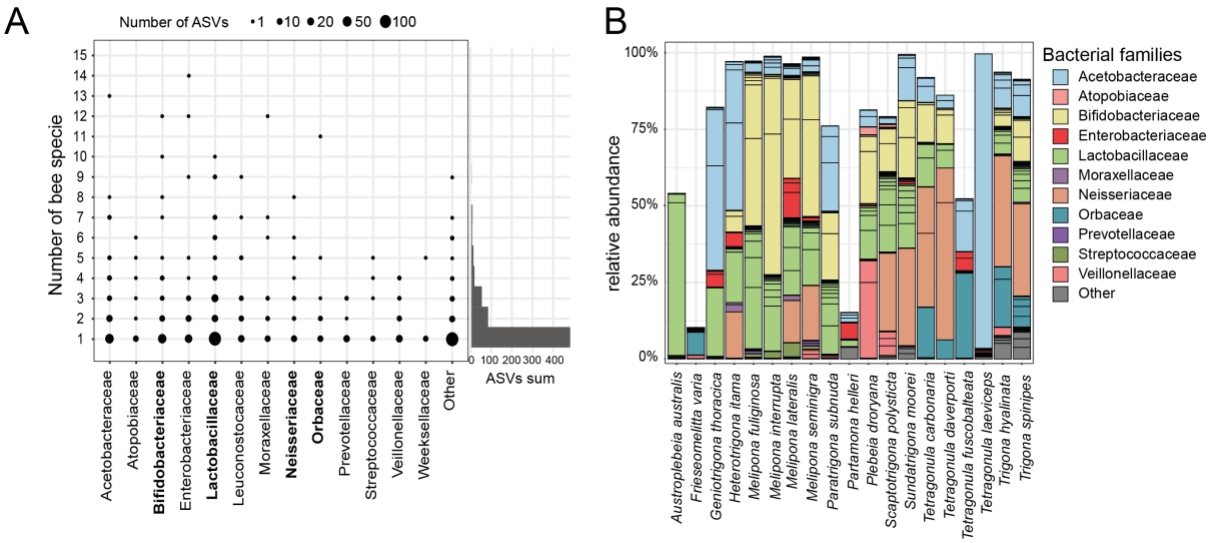
749 collected from six different stingless bee species in Brazil. The samples clustered by bee

750 genus (PERMANOVA**:** 'Genus' pseudo F=11.05, p-value< 0.001).

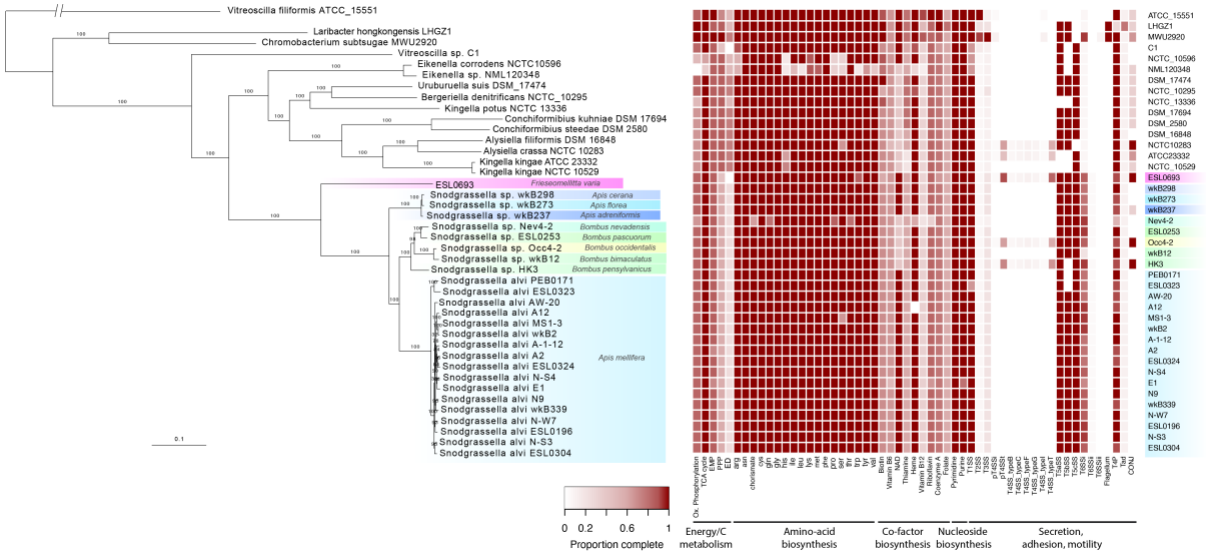
751



Supplementary Figure 2. Community profiles of the gut microbiota of 136 samples from 19 different stingless bee species collected in Brazil, Malaysia, and Australia. Relative abundance of ASVs is shown. ASVs are ordered and colored at the genus level according to the legend. ASVs with <1% relative abundances are summed up as 'others' and shown in grey. This plot includes the datasets from stingless bees from (20) and our study. The samples are ordered by bee species : Aa – *Austroplebeia australis*, Fv – *Frieseomelitta varia*, Gt – *Geniotrigona thoracica* , Hi – *Heterotrigona itama*, Mf – *Melipona fuliginosa*, Mi – *Melipona interrupta*, Ml – *Melipona lateralis*, Ms – *Melipona seminigra*, Ps – *Paratrigona subnuda*, Ph – *Partamona helleri*, Pd – *Plebeia droryana*, Sp – *Scaptotrigona polysticta*, Tc – *Tetragonula carbonaria*, Td – *Tetragonula davenporti*, Tf – *Tetragonula fuscobalteata*, Tl – *Tetragonula laeviceps*, Th – *Trigona hyalinata*, Ts – *Trigona spinipes*.



Supplementary Figure 3. (A) Number of shared ASVs. For each family, we reported how many ASVs were observed (indicated by the dot size) in how many bee species. Families with an average abundance <1% are grouped as 'Other'. The sum of species-specific or shared (by two or more bee species) ASVs is reported on the right side. Core members families are highlighted in bold. **(B)** Relative abundances of the shared ASVs across the 19 stingless bee species (samples from the same species were pooled).



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775

776 **Supplementary Figure 4.** Core genome phylogeny of the Neisseriaceae including phylotype

777 *Snodgrassella*. The maximum likelihood tree was computed with the amino-acid sequences

778 of 428 concatenated single copy core genes using LG+F+I+G4 substitution model. Bee

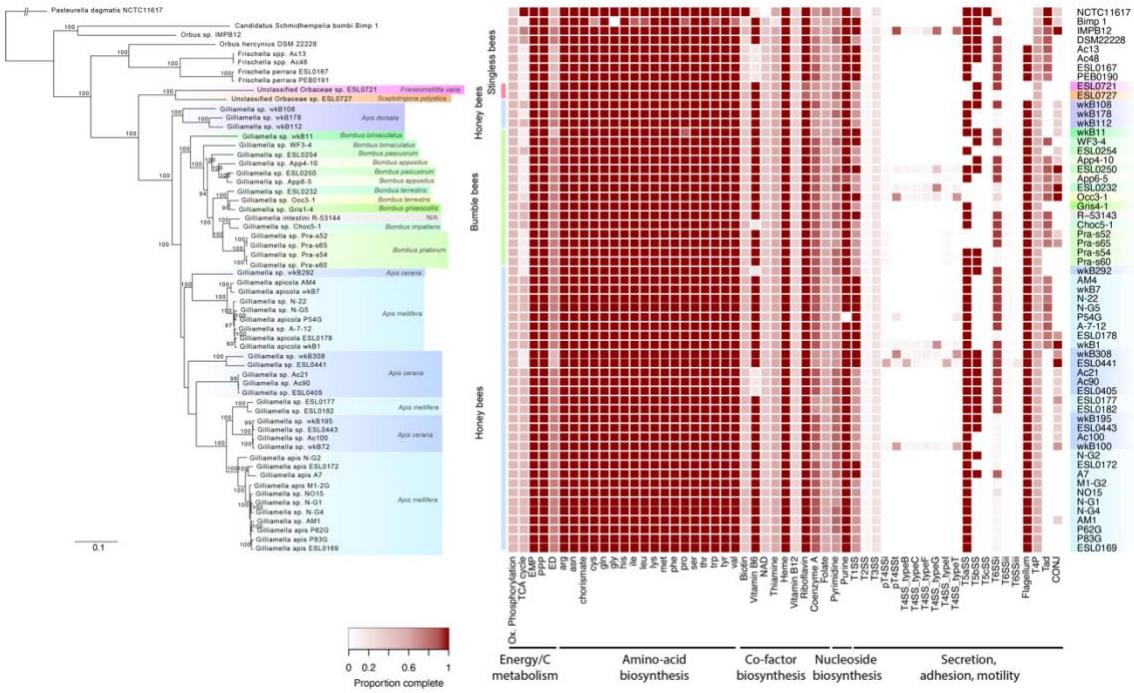
779 isolates form a monophyletic clade. The heatmap indicates the genomic completeness of

780 major metabolic pathways and functions related to energy and carbon metabolism (EMP:

781 Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as

782 well as secretion, adhesion, and motility across the sequenced bacterial isolates.

783



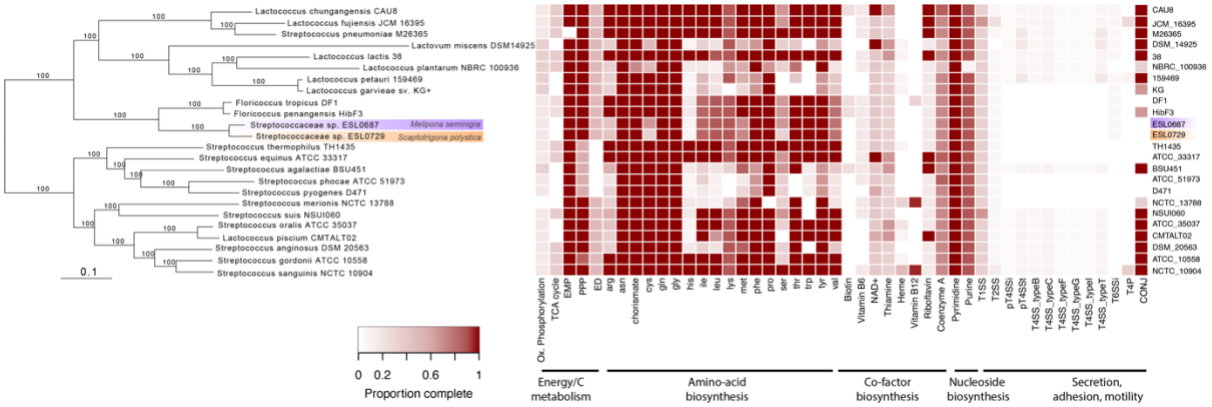
Supplementary Figure 5. Core genome phylogeny of the Orbaceae including *Gilliamella* phylotype. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 782 single copy core genes using JTTDCMut+F+I+G4 substitution model. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.

Roseomonas gilardii U14-5
Acidiphilium cryptum JF-5
Rhodopila sp. LVNP
Acidisphaera rubrifaciens HS-AP2
Acidocella facilis ATCC35904
Acidibrevibacterium fodinaquatile G45-3
Granulibacter bethesdensis CGDNIH2
Commensalibacter intestini A911 (Drosophila melanogaster isolate)
Commensalibacter sp. MX-MONARCH01 (Danaus plexippus isolate)
Commensalibacter sp. ESL0366
Commensalibacter sp. ESL0379
Commensalibacter sp. ESL0392
Commensalibacter sp. ESL0382
Commensalibacter sp. ESL0390
Commensalibacter sp. ESL0367
Commensalibacter sp. ESL0284
Commensalibacter sp. AMU001
Apis mellifera
Honey bees
Gluconacetobacter diazotrophicus PA1 5
Gluconacetobacter liquefaciens DSM 5603
Komagataeibacter nataicola RZS01
Komagataeibacter medellinensis NBRC 3288
Komagataeibacter saccharivorans JH1
Komagataeibacter cocois WE7
Komagataeibacter hansenii NQ5
Komagataeibacter xylinus ATCC 53582
Acetobacter aceti ATCC 23746
Acetobacter nitrogenifigens DSM 23921
Acetobacter ghanensis LMG 23848
Acetobacter pasteurianus IFO 3283 01
Acetobacter pomorum SH
Acetobacter senegalensis 108B
Acetobacter malorum LMG 1746
Tanticharoenia sakaeratensis NBRC 10319
Acidomonas methanolica DSM 5432
Neoasaia chiangmaiensis NBRC 101099
Asaia platycodi SF2 1
Asaia sp. As1742
Asaia prunellae JCM 25354
Swaminathania salitolerans NBRC 104436
Kozakia baliensis NBRC 16680
Swingsia samuiensis AH83
Gluconobacter albidus TMW2 1191
Gluconobacter oxydans DSM 3504
Gluconobacter morbifer G707
Gluconobacter frateurii NBRC 103465
Neokomagataea thailandica NBRC 106555
Acetobacteraceae sp. AAB2-BBS1
Acetobacteraceae sp. AAB2-BBS3
Oecophyllibacter saccharovorans Ta1
Oecophyllibacter saccharovorans Jb2
Oecophyllibacter saccharovorans Ha5
Acetobacteraceae ESL0697
Melipona lateralis
Stingless bees
Saccharibacter sp. EH60
Saccharibacter sp. EH70
Saccharibacter sp. EH611
Saccharibacter sp. 17LHSD
Saccharibacter floricola DSM 15669
Bombella apis ESL0368
Bombella apis ESL0387
Parasaccharibacter apium G7_7_3c
Bee queen gut isolate ESL0380
Saccharibacter sp. M18
Bombella apis SME1
Parasaccharibacter apium A29
Saccharibacter sp. AM169
Parasaccharibacter apium C6
Saccharibacter sp. 3.A.1
Parasaccharibacter apium B8
Bombella intestini R-52487
Bombus lapidarius
Bombella apis ESL0378
Bombella apis ESL0385
Parasaccharibacter apium AS1
Honey bees
Bumble bees
0.1
Ox. Phosphorylation
TCA cycle
EMP
PPP
ED
arg
asn
chorismate
cys
gly
his
ile
leu
lys
met
phe
pro
ser
thr
trp
tyr
val
Biotin
Vitamin B6
NAD
Thiamine
Heme
Vitamin B1
Riboflavin
Coenzyme A
Folate
Pyrimidine
Purine
T1SS
T2SS
T3SS
pT4SSi
pT4SSt
T4SS_typeB
T4SS_typeC
T4SS_typeF
T4SS_typeG
T4SS_typeI
T4SS_typeT
T5aSS
T5bSS
T6SS
T6SSi
T6SSii
T6SSiii
Flagellum
T4P
Tad
CONJ
Energy/C metabolism
Amino-acid biosynthesis
Co-factor biosynthesis
Nucleoside biosynthesis
Secretion, adhesion, motility
0 0.2 0.6 1
Proportion complete

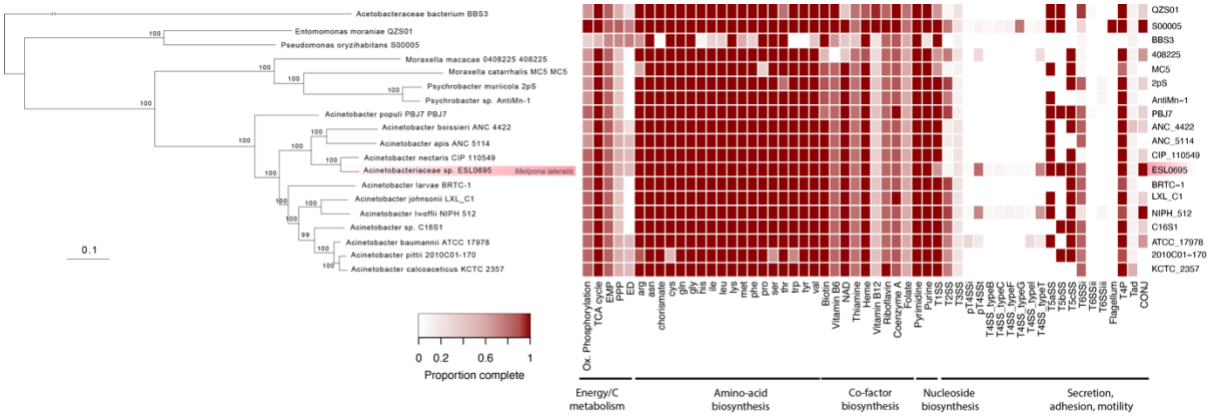
793

794 **Supplementary Figure 6.** Core genome phylogeny of the Acetobacteraceae. The maximum
795 likelihood tree was computed on the concatenated amino-acid sequences of 303 single copy
796 core genes using LG+F+I+G4 substitution model. Bee isolates are distributed in two distant
797 monophyletic clades. The heatmap indicates the genomic completeness of major metabolic
798 pathways and functions related to energy and carbon metabolism (EMP: Embden–
799 Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as
800 secretion, adhesion, and motility across the sequenced bacterial isolates.
801

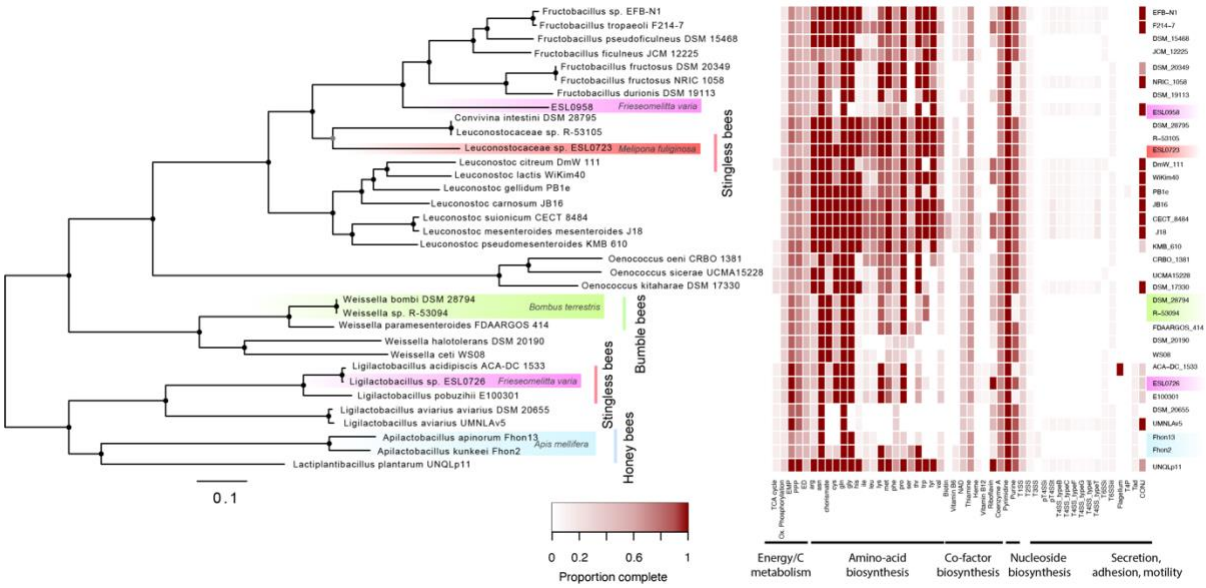
Supplementary Figure 7. Core genome phylogeny of the Enterobacteriaceae. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 303 single copy core genes using LG+F+I+G4 substitution model. Metabolic heatmap of the Enterobacteriaceae isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.



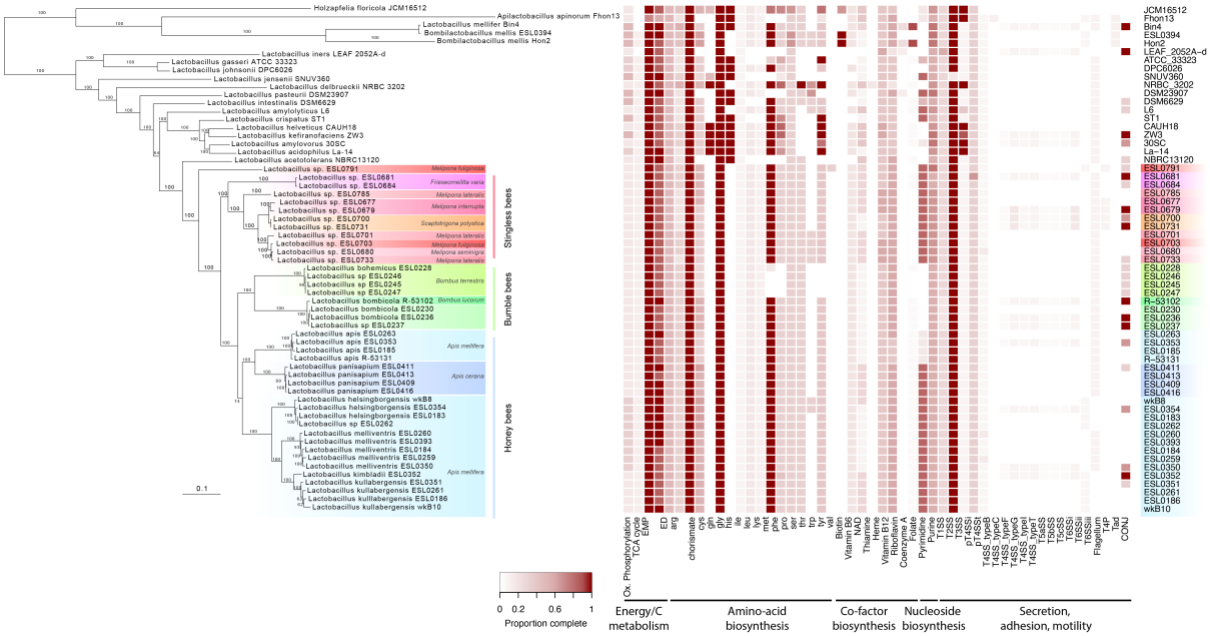
Supplementary Figure 8. Core genome phylogeny of the Streptococcaceae including *Floricoccus* isolates. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 303 single copy core genes using LG+F+I+G4 substitution model. Metabolic heatmap of the Streptococcaceae isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.



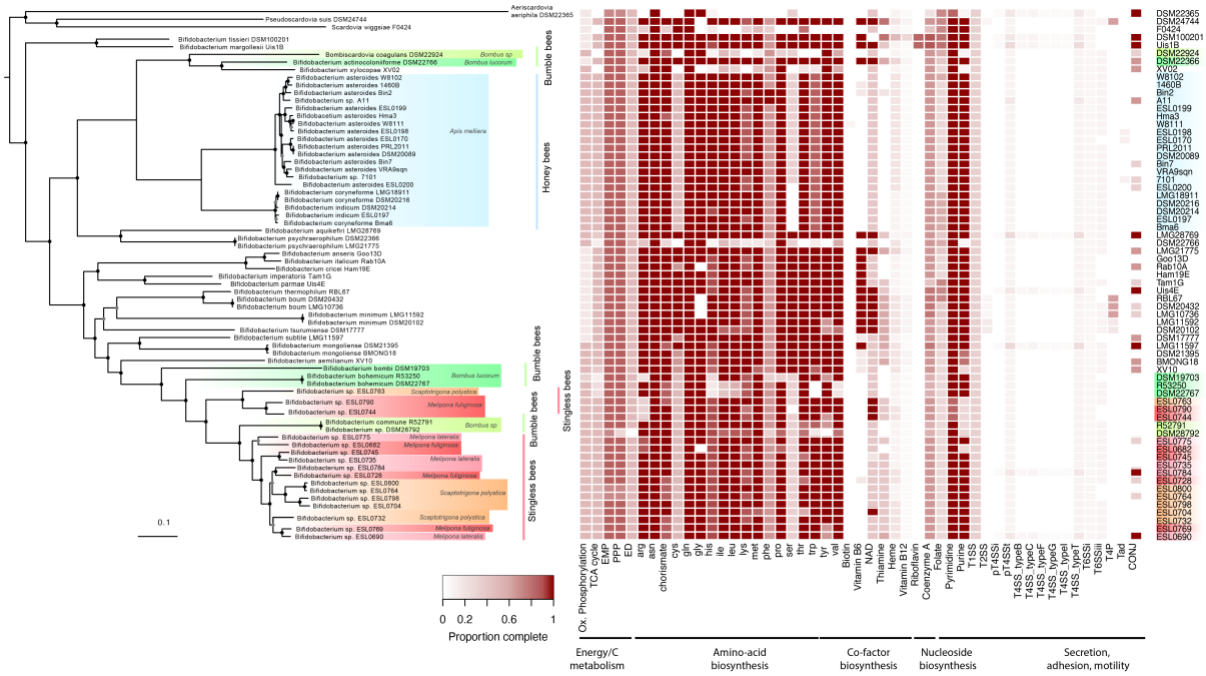
Supplementary Figure 9. Core genome phylogeny of the Moraxellaceae. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 303 single copy core genes using LG+F+I+G4 substitution model. Metabolic heatmap of the Moraxellaceae isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.



Supplementary Figure 10. Core genome phylogeny of the Leuconostocaceae. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 303 single copy core genes using LG+F+I+G4 substitution model. Bee isolates are distributed in two distant monophyletic clades. Metabolic heatmap of the *Leuconostocaceae* isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.



Supplementary Figure 11. Genome-wide phylogeny of the genus *Lactobacillus* including bacteria belonging to the social bee-specific phylotype *Lactobacillus* Firm-5 highlighted in different colors according to the host species/group. The maximum likelihood tree was computed on the concatenated amino acid sequences of 355 single copy core genes using the substitution model LG+F+I+G4. Metabolic heatmap of the *Lactobacillus* isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.



Supplementary Figure 12. (G) Genome-wide phylogeny of the genus *Bifidobacterium* with bacteria belonging to social bee-specific clades. The maximum likelihood tree was computed on the concatenated amino-acid sequences of 151 single copy core genes using the substitution model LG+F+I+G4. Metabolic heatmap of the *Bifidobacterium* isolates, the genomes are ordered according to the phylogeny. The heatmap indicates the genomic completeness of major metabolic pathways and functions related to energy and carbon metabolism (EMP: Embden–Meyerhof–Parnas), the biosynthesis of amino-acid, co-factors, and nucleoside, as well as secretion, adhesion, and motility across the sequenced bacterial isolates.

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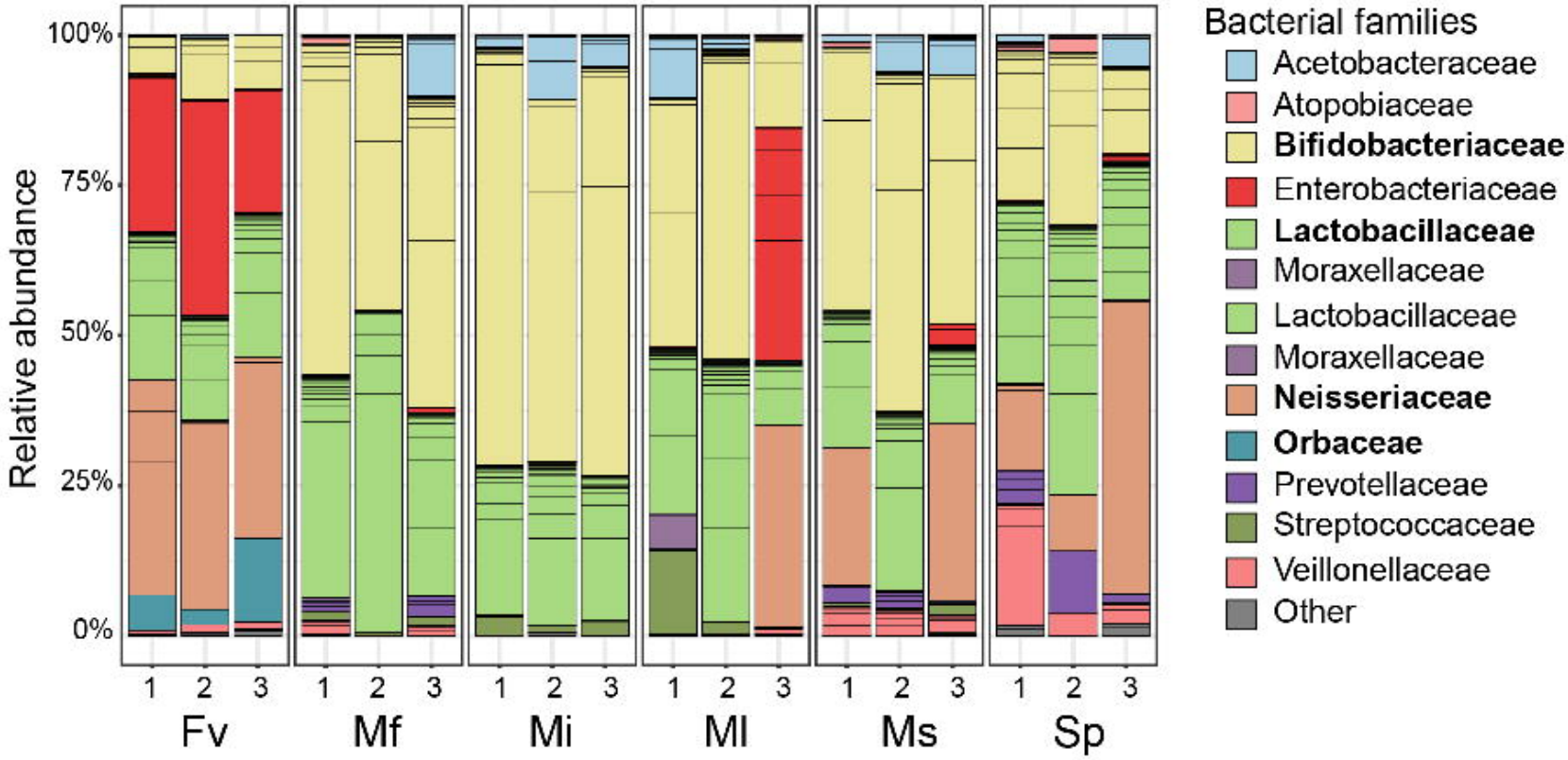
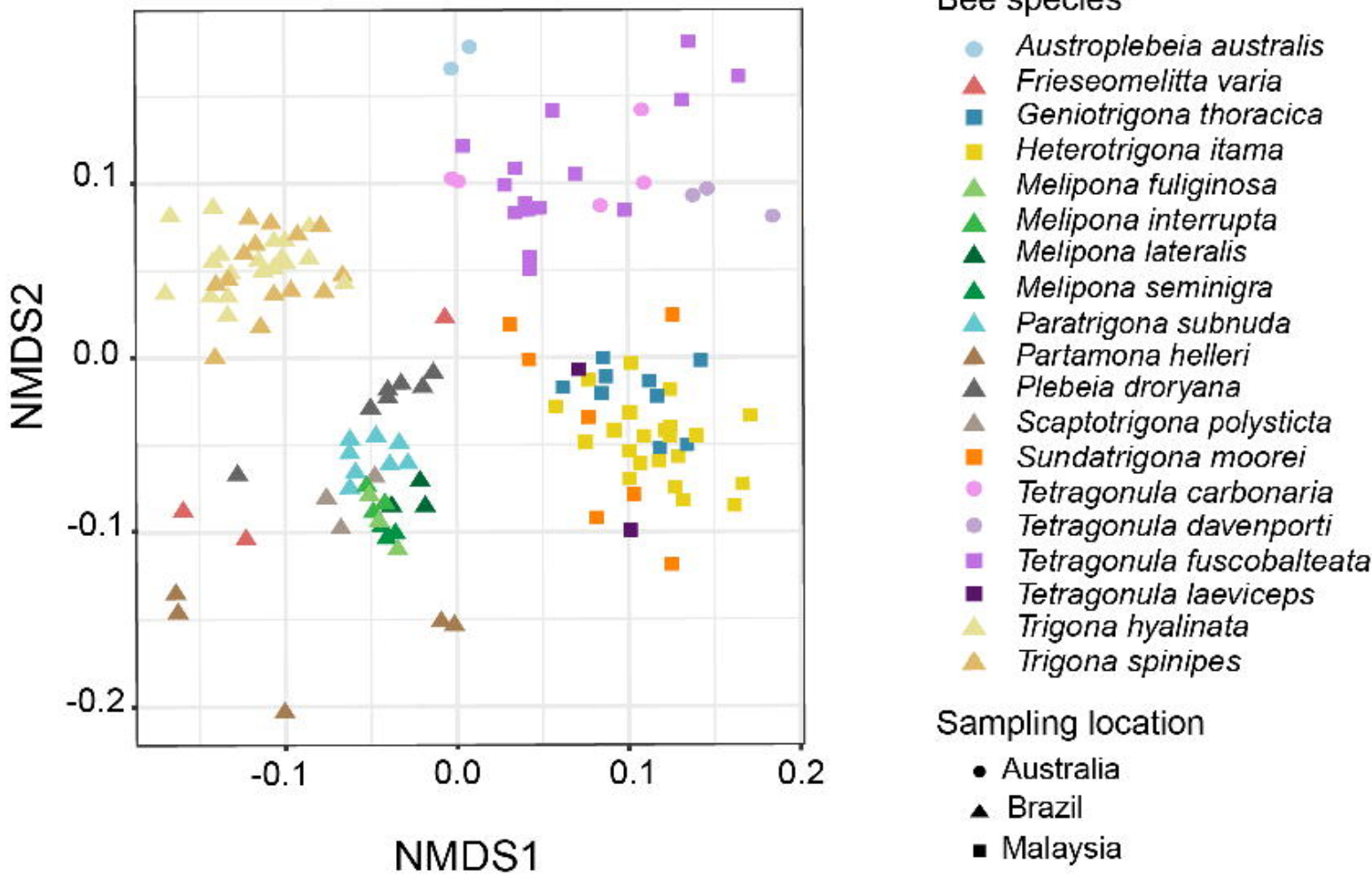
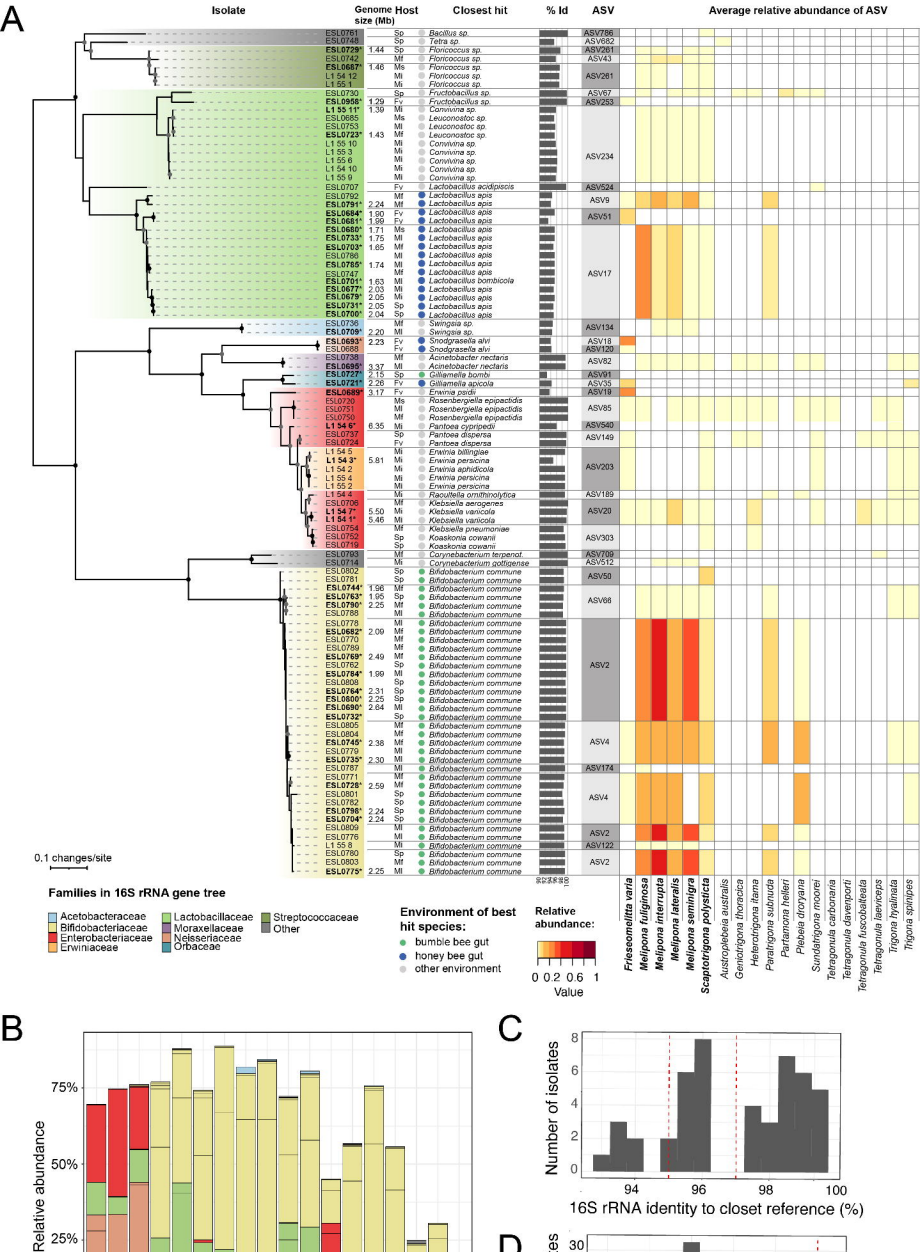
1064

A


Relative abundance
100%
75%
50%
25%
0%
1 2 3
Fv
Mf
Mi
Ml
Ms
Sp
Bacterial families
Acetobacteraceae
Atopobiaceae
Bifidobacteriaceae
Enterobacteriaceae
Lactobacillaceae
Moraxellaceae
Lactobacillaceae
Moraxellaceae
Neisseriaceae
Orbaceae
Prevotellaceae
Streptococcaceae
Veillonellaceae
Other

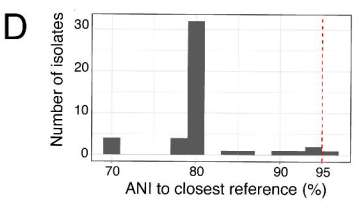
B

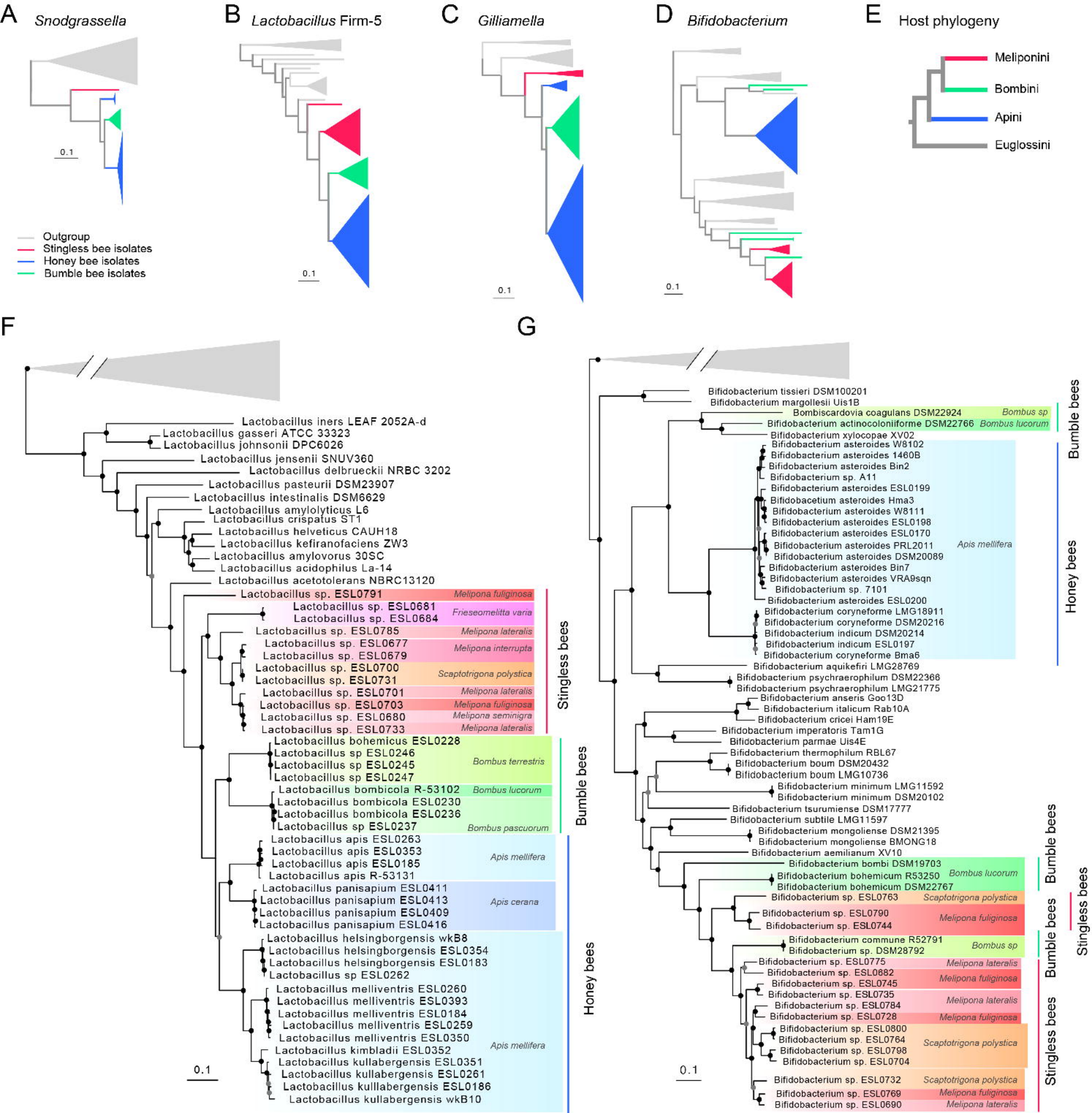

NMDS2
0.1
0.0
-0.1
-0.2
-0.1
0.0
0.1
0.2
NMDS1
Bee species
Austroplebeia australis
Frieseomelitta varia
Geniotrigona thoracica
Heterotrigona itama
Melipona fuliginosa
Melipona interrupta
Melipona lateralis
Melipona seminigra
Paratrigona subnuda
Partamona helleri
Plebeia droryana
Scaptotrigona polysticta
Sundatrigona moorei
Tetragonula carbonaria
Tetragonula davenporti
Tetragonula fuscobalteata
Tetragonula laeviceps
Trigona hyalinata
Trigona spinipes
Sampling location
Australia
Brazil
Malaysia

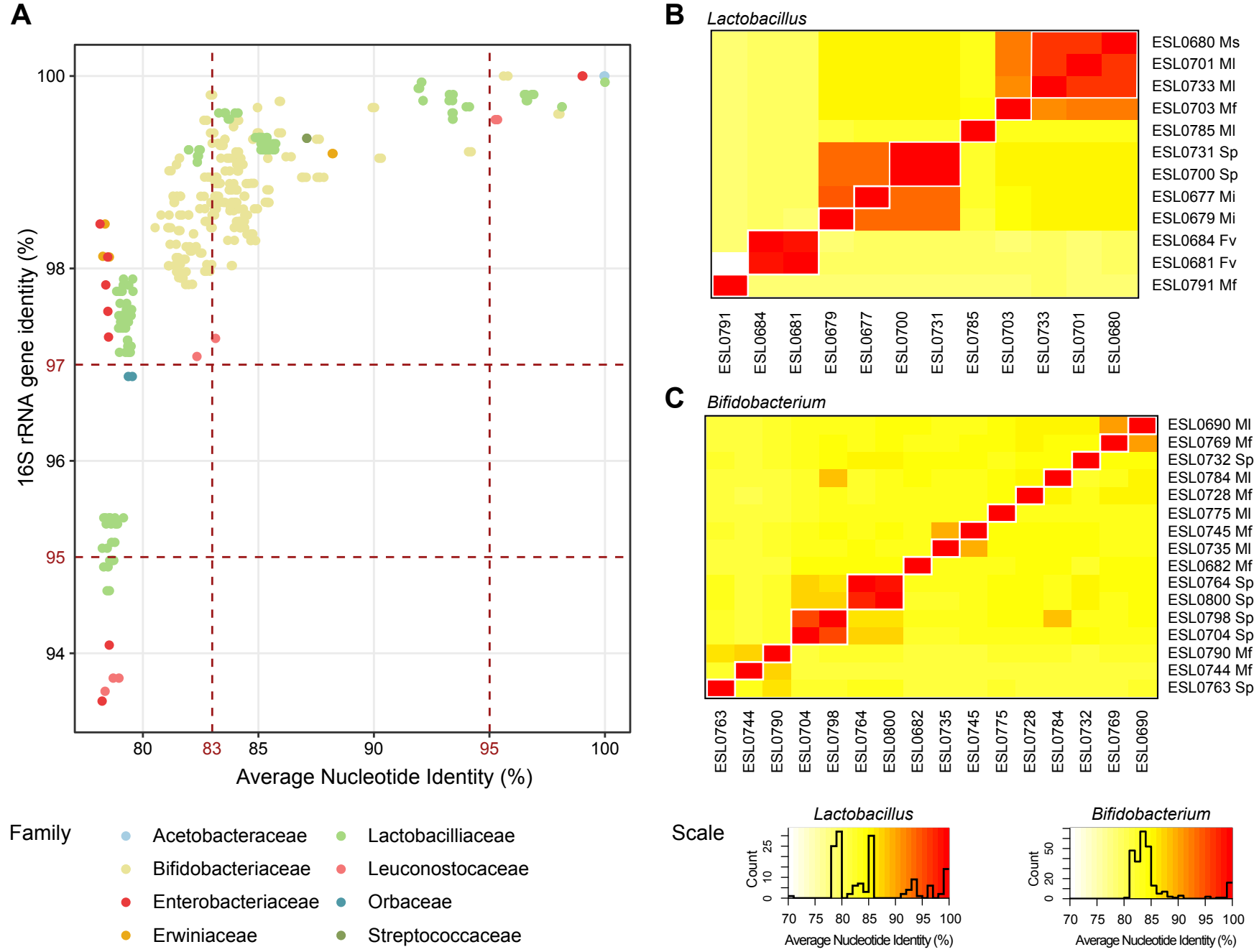

A
Isolate
Genome size (Mb)
Host
Closest hit
% Id
ASV
Average relative abundance of ASV
0.1 changes/site
Families in 16S rRNA gene tree
Acetobacteraceae
Bifidobacteraceae
Enterobacteraceae
Erwiniaceae
Lactobacillaceae
Moraxellaceae
Neisseriaceae
Orbaceae
Streptococcaceae
Other
Environment of best hit species:
bumble bee gut
honey bee gut
other environment
Relative abundance:
0 0.2 0.6 1
Value
Friesomelitta varia
Melipona fuliginosa
Melipona interrupta
Melipona lateralis
Melipona seminigra
Scaptotrigona polysticta
Austroplebeia australis
Geniotrigona thoracica
Heterotrigona itama
Parahrigona subnuda
Partamona helleri
Plebeia droryana
Sundatrigona moorei
Tetragonula carbonaria
Tetragonula davenporti
Tetragonula fuscobaltheata
Tetragonula laeviceps
Trigona hyalinata
Trigona spinipes

B
Relative abundance
75%
50%
25%
0%
Fv1 Fv2 Fv3 Mf1 Mf2 Mf3 Mi1 Mi2 Mi3 Ms1 Ms2 Ms3 Sp1 Sp2 Sp3
Bacterial families
Acetobacteraceae
Bifidobacteraceae
Enterobacteraceae
Lactobacillaceae
Moraxellaceae
Neisseriaceae
Orbaceae
Streptococcaceae
Other

C
Number of isolates
16S rRNA identity to closet reference (%)


D
Number of isolates
ANI to closest reference (%)


A
Snodgrassella
0.1
B
Lactobacillus Firm-5
0.1
C
Gilliamella
0.1
D
Bifidobacterium
0.1
E
Host phylogeny
Meliponini
Bombini
Apini
Euglossini
Outgroup
Stingless bee isolates
Honey bee isolates
Bumble bee isolates
F
Lactobacillus iners LEAF 2052A-d
Lactobacillus gasseri ATCC 33323
Lactobacillus johnsonii DPC6026
Lactobacillus jensenii SNUV360
Lactobacillus delbrueckii NRBC 3202
Lactobacillus pasteurii DSM23907
Lactobacillus intestinalis DSM6629
Lactobacillus amylolyticus L6
Lactobacillus crispatus ST1
Lactobacillus helveticus CAUH18
Lactobacillus kefiranofaciens ZW3
Lactobacillus amylovorus 30SC
Lactobacillus acidophilus La-14
Lactobacillus acetotolerans NBRC13120
Lactobacillus sp. ESL0791 Melipona fuliginosa
Lactobacillus sp. ESL0681 Lactobacillus sp. ESL0684 Frieseomelitta varia
Lactobacillus sp. ESL0785 Melipona lateralis
Lactobacillus sp. ESL0677 Lactobacillus sp. ESL0679 Melipona interrupta
Lactobacillus sp. ESL0700 Lactobacillus sp. ESL0731 Scaptotrigona polystica
Lactobacillus sp. ESL0701 Melipona lateralis
Lactobacillus sp. ESL0703 Melipona fuliginosa
Lactobacillus sp. ESL0680 Melipona seminigra
Lactobacillus sp. ESL0733 Melipona lateralis
Stingless bees
Lactobacillus bohemicus ESL0228
Lactobacillus sp ESL0246
Lactobacillus sp ESL0245
Lactobacillus sp ESL0247
Bombus terrestris
Lactobacillus bombicola R-53102 Bombus lucorum
Lactobacillus bombicola ESL0230
Lactobacillus bombicola ESL0236
Lactobacillus sp ESL0237 Bombus pascuorum
Bumble bees
Lactobacillus apis ESL0263
Lactobacillus apis ESL0353
Lactobacillus apis ESL0185
Lactobacillus apis R-53131
Apis mellifera
Lactobacillus panisapium ESL0411
Lactobacillus panisapium ESL0413
Lactobacillus panisapium ESL0409
Lactobacillus panisapium ESL0416
Apis cerana
Lactobacillus helsingborgensis wkB8
Lactobacillus helsingborgensis ESL0354
Lactobacillus helsingborgensis ESL0183
Lactobacillus sp ESL0262
Lactobacillus melliventris ESL0260
Lactobacillus melliventris ESL0393
Lactobacillus melliventris ESL0184
Lactobacillus melliventris ESL0259
Lactobacillus melliventris ESL0350
Lactobacillus kimbladii ESL0352
Lactobacillus kullabergensis ESL0351
Lactobacillus kullabergensis ESL0261
Lactobacillus kulllabergensis ESL0186
Lactobacillus kullabergensis wkB10
Apis mellifera
Honey bees
0.1
G
Bifidobacterium tissieri DSM100201
Bifidobacterium margollesii Uis1B
Bombiscardovia coagulans DSM22924 Bombus sp
Bifidobacterium actinocoloniiforme DSM22766 Bombus lucorum
Bumble bees
Bifidobacterium xylocopae XV02
Bifidobacterium asteroides W8102
Bifidobacterium asteroides 1460B
Bifidobacterium asteroides Bin2
Bifidobacterium sp. A11
Bifidobacterium asteroides ESL0199
Bifidobacetium asteroides Hma3
Bifidobacterium asteroides W8111
Bifidobacterium asteroides ESL0198
Bifidobacterium asteroides ESL0170
Bifidobacterium asteroides PRL2011
Bifidobacterium asteroides DSM20089
Bifidobacterium asteroides Bin7
Bifidobacterium asteroides VRA9sqn
Bifidobacterium sp. 7101
Bifidobacterium asteroides ESL0200
Bifidobacterium coryneforme LMG18911
Bifidobacterium coryneforme DSM20216
Bifidobacterium indicum DSM20214
Bifidobacterium indicum ESL0197
Bifidobacterium coryneforme Bma6
Apis mellifera
Honey bees
Bifidobacterium aquikefiri LMG28769
Bifidobacterium psychraerophilum DSM22366
Bifidobacterium psychraerophilum LMG21775
Bifidobacterium anseris Goo13D
Bifidobacterium italicum Rab10A
Bifidobacterium cricei Ham19E
Bifidobacterium imperatoris Tam1G
Bifidobacterium parmae Uis4E
Bifidobacterium thermophilum RBL67
Bifidobacterium boum DSM20432
Bifidobacterium boum LMG10736
Bifidobacterium minimum LMG11592
Bifidobacterium minimum DSM20102
Bifidobacterium tsurumiense DSM17777
Bifidobacterium subtile LMG11597
Bifidobacterium mongoliense DSM21395
Bifidobacterium mongoliense BMONG18
Bifidobacterium aemilianum XV10
Bifidobacterium bombi DSM19703
Bifidobacterium bohemicum R53250 Bombus lucorum
Bifidobacterium bohemicum DSM22767
Bumble bees
Bifidobacterium sp. ESL0763 Scaptotrigona polystica
Bifidobacterium sp. ESL0790
Bifidobacterium sp. ESL0744 Melipona fuliginosa
Stingless bees
Bifidobacterium commune R52791
Bifidobacterium sp. DSM28792 Bombus sp
Bumble bees
Bifidobacterium sp. ESL0775 Melipona lateralis
Bifidobacterium sp. ESL0682
Bifidobacterium sp. ESL0745 Melipona fuliginosa
Bifidobacterium sp. ESL0735
Bifidobacterium sp. ESL0784 Melipona lateralis
Bifidobacterium sp. ESL0728 Melipona fuliginosa
Bifidobacterium sp. ESL0800
Bifidobacterium sp. ESL0764
Bifidobacterium sp. ESL0798
Bifidobacterium sp. ESL0704 Scaptotrigona polystica
Stingless bees
Bifidobacterium sp. ESL0732 Scaptotrigona polystica
Bifidobacterium sp. ESL0769 Melipona fuliginosa
Bifidobacterium sp. ESL0690 Melipona lateralis
0.1


A
16S rRNA gene identity (%)
100
98
97
96
95
94
80
83
85
90
95
100
Average Nucleotide Identity (%)
Family
Acetobacteraceae
Bifidobacteriaceae
Enterobacteriaceae
Erwiniaceae
Lactobacilliaceae
Leuconostocaceae
Orbaceae
Streptococcaceae
B
Lactobacillus
ESL0680 Ms
ESL0701 Ml
ESL0733 Ml
ESL0703 Mf
ESL0785 Ml
ESL0731 Sp
ESL0700 Sp
ESL0677 Mi
ESL0679 Mi
ESL0684 Fv
ESL0681 Fv
ESL0791 Mf
ESL0791
ESL0684
ESL0681
ESL0679
ESL0677
ESL0700
ESL0731
ESL0785
ESL0703
ESL0733
ESL0701
ESL0680
C
Bifidobacterium
ESL0690 Ml
ESL0769 Mf
ESL0732 Sp
ESL0784 Ml
ESL0728 Mf
ESL0775 Ml
ESL0745 Mf
ESL0735 Ml
ESL0682 Mf
ESL0764 Sp
ESL0800 Sp
ESL0798 Sp
ESL0704 Sp
ESL0790 Mf
ESL0744 Mf
ESL0763 Sp
ESL0763
ESL0744
ESL0790
ESL0704
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ESL0800
ESL0682
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ESL0745
ESL0775
ESL0728
ESL0784
ESL0732
ESL0769
ESL0690
Scale
Lactobacillus
Count
0
10
25
70
75
80
85
90
95
100
Average Nucleotide Identity (%)
Bifidobacterium
Count
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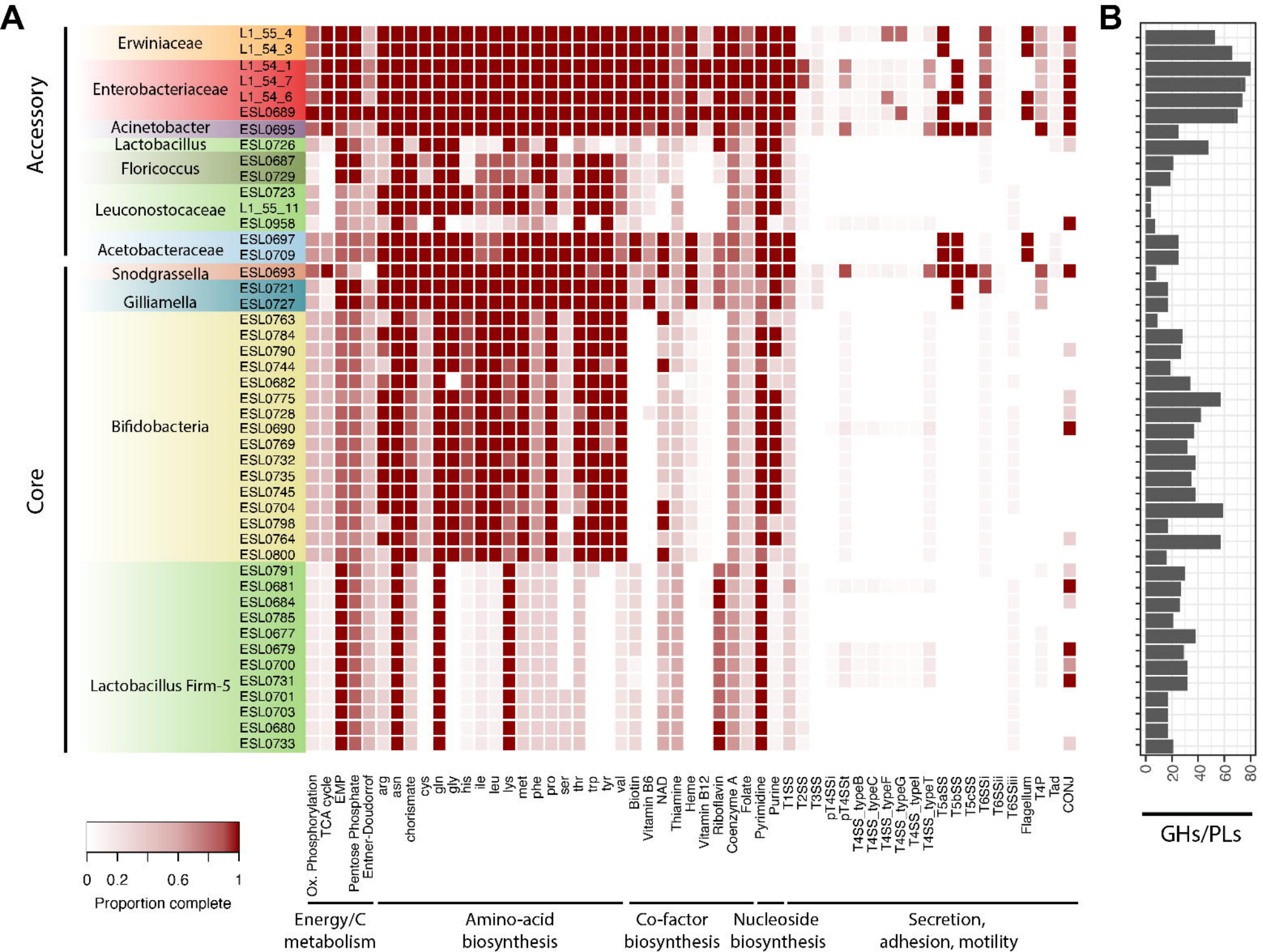
20
50
70
75
80
85
90
95
100
Average Nucleotide Identity (%)


A
B
Accessory
Core
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L1_54_3
Enterobacteriaceae
L1_54_1
L1_54_7
L1_54_6
ESL0689
Acinetobacter
ESL0695
Lactobacillus
ESL0726
Floricoccus
ESL0687
ESL0729
Leuconostocaceae
ESL0723
L1_55_11
ESL0958
Acetobacteraceae
ESL0697
ESL0709
Snodgrassella
ESL0693
Gilliamella
ESL0721
ESL0727
Bifidobacteria
ESL0763
ESL0784
ESL0790
ESL0744
ESL0682
ESL0775
ESL0728
ESL0690
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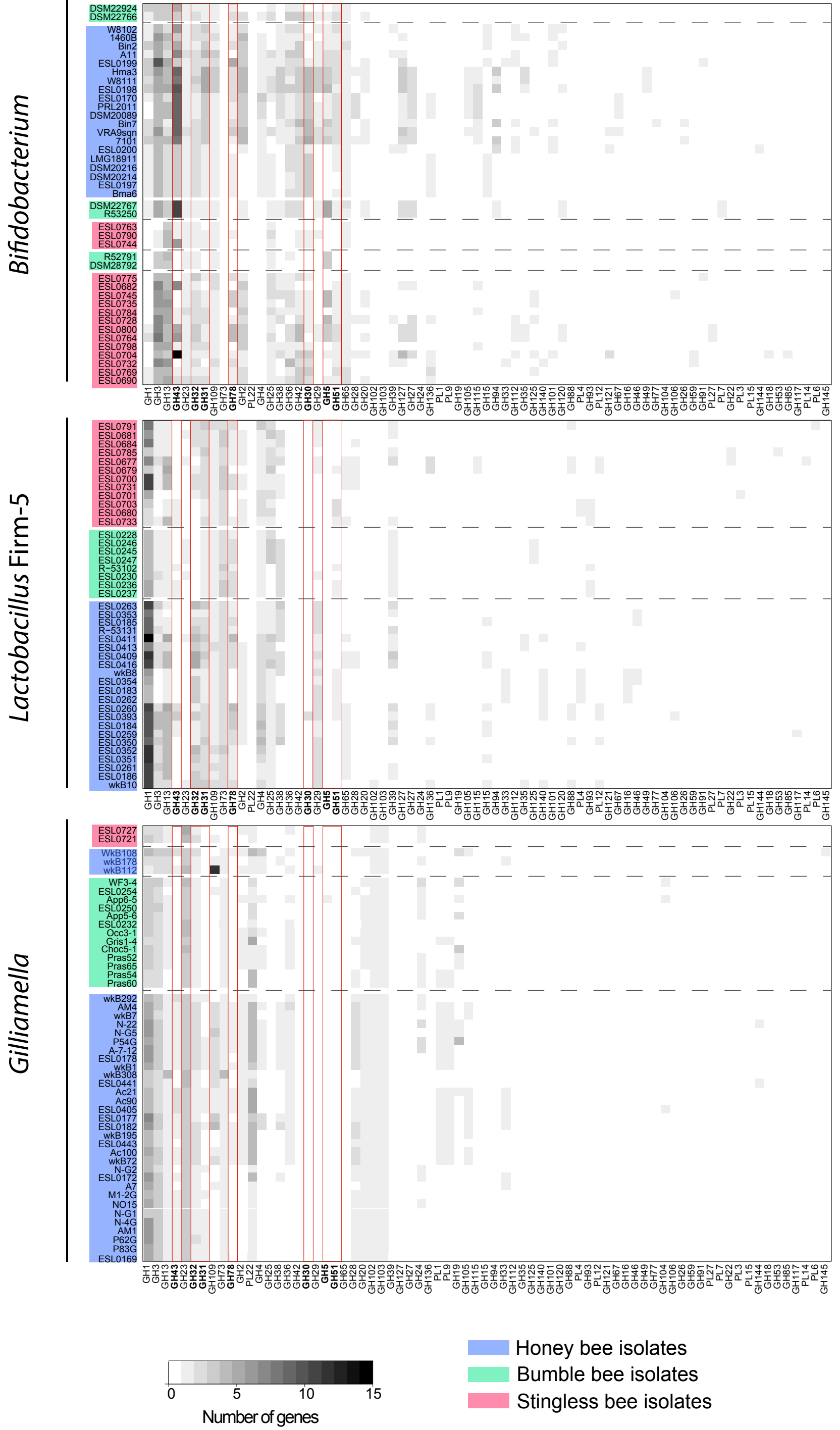
ESL0732
ESL0735
ESL0745
ESL0704
ESL0798
ESL0764
ESL0800
Lactobacillus Firm-5
ESL0791
ESL0681
ESL0684
ESL0785
ESL0677
ESL0679
ESL0700
ESL0731
ESL0701
ESL0703
ESL0680
ESL0733
Ox. Phosphorylation
TCA cycle
EMP
Pentose Phosphate
Entner-Doudorof
arg
asn
chorismate
cys
gln
gly
his
ile
leu
lys
met
phe
pro
ser
thr
trp
tyr
val
Biotin
Vitamin B6
NAD
Thiamine
Heme
Vitamin B12
Riboflavin
Coenzyme A
Folate
Pyrimidine
Purine
T1SS
T2SS
T3SS
pT4SSi
pT4SSt
T4SS_typeB
T4SS_typeC
T4SS_typeF
T4SS_typeG
T4SS_typeI
T4SS_typeT
T5aSS
T5bSS
T5cSS
T6SSi
T6SSii
T6SSiii
Flagellum
T4P
Tad
CONJ
0
0.2
0.6
1
Proportion complete
Energy/C metabolism
Amino-acid biosynthesis
Co-factor biosynthesis
Nucleoside biosynthesis
Secretion, adhesion, motility
0
20
40
60
80
GHs/PLs


Bifidobacterium
DSM22924
DSM22766
W8102
1460B
Bin2
A11
ESL0199
Hma3
W8111
ESL0198
ESL0170
PRL2011
DSM20089
Bin7
VRA9sqn
7101
ESL0200
LMG18911
DSM20216
DSM20214
ESL0197
Bma6
DSM22767
R53250
ESL0763
ESL0790
ESL0744
R52791
DSM28792
ESL0775
ESL0682
ESL0745
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ESL0764
ESL0798
ESL0704
ESL0732
ESL0769
ESL0690
Lactobacillus Firm-5
ESL0791
ESL0681
ESL0684
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ESL0700
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ESL0247
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ESL0236
ESL0237
ESL0263
ESL0353
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R-53131
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ESL0413
ESL0409
ESL0416
wkB8
ESL0354
ESL0183
ESL0262
ESL0260
ESL0393
ESL0184
ESL0259
ESL0350
ESL0352
ESL0351
ESL0261
ESL0186
wkB10
Gilliamella
ESL0727
ESL0721
WkB108
wkB178
wkB112
WF3-4
ESL0254
App6-5
ESL0250
App5-6
ESL0232
Occ3-1
Gris1-4
Choc5-1
Pras52
Pras65
Pras54
Pras60
wkB292
AM4
wkB7
N-22
N-G5
P54G
A-7-12
ESL0178
wkB1
wkB308
ESL0441
Ac21
Ac90
ESL0405
ESL0177
ESL0182
wkB195
ESL0443
Ac100
wkB72
N-G2
ESL0172
A7
M1-2G
NO15
N-G1
N-4G
AM1
P62G
P83G
ESL0169
GH1 GH3 GH13 GH43 GH23 GH32 GH31 GH109 GH73 GH78 GH2 PL22 GH4 GH25 GH38 GH36 GH42 GH30 GH29 GH5 GH51 GH65 GH28 GH20 GH102 GH103 GH39 GH127 GH27 GH24 GH136 PL1 PL9 GH19 GH105 GH115 GH15 GH94 GH33 GH112 GH35 GH125 GH140 GH101 GH120 GH88 PL4 GH93 PL12 GH121 GH67 GH16 GH46 GH49 GH77 GH104 GH106 GH26 GH59 GH91 PL27 PL7 GH22 PL3 PL15 GH144 GH18 GH53 GH85 GH117 PL14 PL6 GH145
0
5
10
15
Number of genes
Honey bee isolates
Bumble bee isolates
Stingless bee isolates