Vast differences in strain-level diversity in the gut microbiota of two closely related honey bee species

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Abstract

Most bacterial species encompass strains with vastly different gene content. Strain-level diversity in host-associated microbial communities is therefore considered to be of functional importance. Yet, little is known about strain-level diversity differences in phylotypes shared among related host species, or the underlying mechanisms that may constrain and maintain diversity. Here, we used shotgun metagenomics to characterize and compare the gut microbiota of two closely related honey bee species, *Apis mellifera* and *Apis cerana*. While both host species are colonized by largely overlapping bacterial 16S rRNA phylotypes, our metagenomic analysis revealed that their communities are host-specific at the species- and strain-level with few between-host transfers being detected. Strikingly, *A. mellifera* displayed a much higher extent of strain-level diversity and functional gene content in the microbiota than *A. cerana,* per colony and per individual bee. In particular, the gene repertoire for polysaccharide degradation was massively expanded in the microbiota of *A. mellifera* relative to *A. cerana*. Our results illustrate that the gut microbiota of two closely related animal hosts, with similar ecology and overlapping geographic distribution, can undergo dramatic changes in composition and diversity within a timespan of only 6 million years of evolution, with likely consequences for gut microbiota functioning and host-symbiont interactions. Human bee management, divergent ecological adaptation, or habitat size may have contributed to the observed differences in microbiota composition of these key pollinator species. Further studies are needed to evaluate both the underlying causes and functional consequences for honey bee health and bee management.

Significance

Little is known about how gut bacterial communities evolve and adapt across closely related host species, because it requires genome-wide approaches that go beyond classical marker gene analysis and provide insights at the resolution of individual strains and genomic content. We show that the gut microbiota of two closley related honey bee species has undergone major changes in strain-level diversity and funtional gene content, despite the relatively recent common ancestry. Western honey bees harbor much more diversity per bee and per colony than Eastern honey bees, with a larger and more diverse repertoire of functions for sugar breakdown. This is likey to have consequences for the functioning of the gut microbiota, raising important questions about the impact of the gut microbiota on pollinator health.

Introduction

Most bacteria live in complex communities, composed of hundreds to thousands of species, which in turn encompass strains with highly variable gene content (1, 2). Also in host-associated bacterial communities, strain-level diversity can be substantial, despite the general assumption that genetic diversity destabilizes mutualistic interactions (3). For example, multiple strains of a sulfur-oxidizing endosymbiont were found to co-colonize individual hosts of deep-sea mussels, presumably because they encode complementary functions (4). In contrast, strains of the human gut microbiota have been shown to segregate among individuals, resulting in host-specific genetic profiles (5-7). However, despite the increased awareness of the existence and functional importance of strain-level diversity in host-associated bacterial communities, little is known about differences in strain-level diversity across host species, or the underlying mechanisms which constrain and maintain diversity within and among hosts. This is largely due to the technical challenges associated with the deep-sequencing of bacterial genomes from complex natural communities, and the generation of comparable datasets across host organisms.

Unlike most animals, eusocial corbiculate bees (honey bees, stingless bees, and bumble bees) have been shown to harbor a specialized gut microbiota with a simple and highly consistent taxonomic composition, consisting of up to 10 phylotypes, as based on 16S rRNA gene analyses (8). Considering the common occurrence of these phylotypes across bees, it is plausible that they were acquired around the time when eusociality evolved in the bees (8). Interestingly, previous studies on bacterial isolates have demonstrated an impressive amount of genomic diversity within

86 phylotypes, where strains isolated from different hosts often represent divergent
87 phylogenetic sub-lineages (9-12). Moreover, a recent metagenomic analysis of the
88 gut microbiota of the Western honey bee, *Apis mellifera,* found that even within the
89 same host species divergent sub-lineages can be present (5). We refer to these sub-
90 lineages as sequence-discrete populations (SDPs), since metagenomic data
91 demonstrated that they were discrete from each other, in addition to being sufficiently
92 divergent to be considered as different bacterial species (13). Given these results, it
93 is possible that bee species with similar phylotype-level composition in their gut
94 microbiota, including very closely related species of honey bees, harbor very different
95 communities. However, comparative community-wide analyses, based on data with
96 strain- and genome-level resolution, are still lacking.
97
98 In the current study, we perform a comparative metagenomic analysis of the gut
99 microbiota of two closely related species of honey bees, *Apis mellifera* and *Apis*
100 *cerana*. Based on molecular data, their last common ancestor has been dated to
101 approximately 6 million years ago (14-17), and previous 16S rRNA-based studies
102 have shown that they harbor largely overlapping gut microbiota phylotypes (>97%
103 16S rRNA identity) (8). With the exception of *A. mellifera*, all extant species of honey
104 bees (genus *Apis*) are confined to Asia, pointing towards an Asian origin of the *Apis*
105 genus (15). Based on molecular analysis, *A. mellifera* expanded into its native range
106 (Africa, Europe, and Western Asia) approximately 300.000 years ago (14). However,
107 *A. mellifera* was recently re-introduced to Asia by humans (18, 19), thereby bringing
108 not only the bees, but also their associated bacterial communities into close
109 proximity, potentially resulting in a homogenization of their gut microbiota.
110

111 In order to compare the composition, diversity, and evolution of the gut microbiota of
112 *A. mellifera* and *A. cerana* at the strain-level, we analyzed shotgun metagenomes of
113 individual bees, using a common DNA extraction protocol and comparable
114 sequencing depth. We find that each host species harbors a highly distinct bacterial
115 community, largely composed of different SDPs, with occasional transfers among
116 sympatric bees. Quantitative analysis revealed that the gut microbiota diversity of *A.*
117 *mellifera* is much higher than for *A. cerana*, resulting in a larger metabolic flexibility at
118 both the individual and colony level. These results represent the first comparative
119 genome-wide analysis of strain-level diversity between related host-associated
120 microbial communities, raising new questions regarding underlying mechanisms and
121 functional consequences.
122

Results

Metagenomic data reveals that the gut microbiota of *A. mellifera* and *A. cerana* are distinct.

A total of 40 shotgun metagenome samples were collected from individual bees, with 20 bees per host. Two colonies were sampled from each host, all from different apiaries, no more than 100km apart, close to Tsukuba (Japan). In order to perform a metagenomic characterization of the community composition across samples, we first established a genomic database of isolated strains (**Dataset S1**) representative of both hosts. In total, 10 new genomes isolated from *A. cerana* were sequenced and added to a previously established, non-redundant honey bee gut microbiota database (5), together with previously published genomes isolated from more distantly related bee species. Approximately 90% of the host-filtered reads mapped to the new database, regardless of the host affiliation of the samples, indicating that the database is highly and equally representative of the gut microbiota of both hosts (**Fig. S1**).

To make an initial broad comparison of the gut microbiota composition, the relative abundance of all phylotypes in the database was quantified, based on mapped read coverage to single-copy core genes. Consistent with previous studies employing amplicon sequencing of the 16S rRNA gene (8), the five phylotypes constituting the core microbiota of corbiculate bees were found to colonize both hosts, although the relative abundance profiles were distinct between the hosts (**Fig. S2**). Other phylotypes associated with *A. mellifera* (*Bartonella apis*, *Frischella perrara*, *Commensalibacter* sp.) were not detected in any of the *A. cerana* samples.

147 Conversely, while *Apibacter* was not detected in any of the *A. mellifera* samples, it
148 appeared to be a prevalent member for *A. cerana* (**Fig. S2**, yellow color).
149
150 To determine whether the five core phylotypes colonizing both hosts are distinct at
151 the SDP level, candidate SDPs were inferred from isolate genomes in the database,
152 based on core genome phylogenies (**Fig. 1A-C,G,H**) and pairwise average
153 nucleotide identities (ANI) (**Dataset S2**). Subsequent metagenomic validation (**Fig.**
154 **S3-S4**) confirmed three new SDPs within the *Gilliamella* phylotype (**Fig.1A**), and one
155 new SDP within the *Lactobacillus* Firm5 phylotype (**Fig. 1B**), all of which were
156 represented exclusively by *A. cerana*-derived isolates. A new SDP was also
157 confirmed for *Snodgrassella* (**Fig. 1C**), based on isolates from *A. cerana*, *A.*
158 *andreiformis* and *A. florea*, suggesting that this SDP may be shared among other
159 species of honey bees than *A. mellifera*. In contrast, for the two remaining core
160 phylotypes *Lactobacillus* Firm4 and *Bifidobacterium*, no new candidate SDPs were
161 inferred (**Fig. 1G-H**), since the *A. cerana*-derived genome isolates had ANI values of
162 up to 95% and 90% to the *A. mellifera* isolates, thus falling within the range of ANI
163 values observed among the *A. mellifera* isolates (**Dataset S2**).
164
165 To further validate the host specificity of the novel SDPs and detect eventual cross-
166 host transfers, we quantified the relative abundance of each SDP across the
167 metagenomic samples, including samples previously collected in Switzerland. All
168 SDPs found to be host-specific in the genomic database displayed a clear host
169 preference across the metagenomic samples, but a small number of transfers were
170 nevertheless detected among the Japanese samples within the *Lactobacillus* Firm5
171 and *Gilliamella* phylotypes (**Fig. 1D-F**). These results therefore suggest that honey

172 bees do get exposed to non-native SDPs, at least in Japan, occasionally resulting in
173 colonization. For the SDPs previously described for *A. mellifera* (5), the abundance
174 patterns were very similar between Swiss and Japanese samples, with co-
175 occurrence of SDPs within individuals being the norm for all core phylotypes except
176 *Gilliamella* (**Fig. 1D-F**, **Fig. S5**). Thus, these distributions likely represent conserved
177 patterns of co-existence.
178
179 For the two core phylotypes harboring SDPs with genome isolates from both hosts
180 (**Fig. 1G-H**), rooting of the core genome phylogenies with related SDPs showed that
181 the *A. cerana*-derived isolates diverged prior to the *A. mellifera*-derived isolates, as
182 would be expected if a more recent host-specialization had occurred. Therefore, to
183 determine whether the communities differ at the strain-level, the fraction of shared
184 single-nucleotide variants was calculated for all sample pairs, and visualized using
185 principal coordinate analysis (**Fig. 1I and J**). Remarkably, the samples were found to
186 cluster strongly by host, indicating that each host is colonized by a distinct population
187 of strains. In contrast, there was no clustering by country or colony affiliation (**Fig. 1I-**
188 **J**). For other community members, clustering by country or colony was observed for
189 only a subset of SDPs (**Dataset S3),** indicating that strains are not necessarily
190 geographically specialized.
191
192 Finally, to obtain a database-independent estimate of the divergence between the gut
193 microbiota of *A. mellifera* and *A. cerana*, we *de novo* assembled the metagenomes
194 and compared the gene contents by clustering all predicted ORFs (open reading-
195 frames) by sequence identity. Metagenomic assemblies and ORFs were inferred
196 individually for each sample, using a subset of 20 million paired-end host-filtered

197 reads per sample, and clustering was done using a range of thresholds (80-95%
198 nucleotide identity). Only a very minor fraction of the clusters contained sequences
199 from both hosts (**Fig. 1K**) further corroborating the small overlap of the gut microbiota
200 between the two honey bee species.
201
202 In conclusion, although the gut microbiota of *A. mellifera* and *A. cerana* appear
203 similar when the community is characterized at the phylotype level, metagenomic
204 analysis clearly demonstrates that the communities are highly distinct, being
205 composed of divergent SDPs and strains, with a low frequency of cross-host
206 transfers.
207
208 **The diversity of the gut microbiota is higher in *A. mellifera* compared to *A.***
209 ***cerana*.**
210 As shown in **Fig. 1K**, the clustering analysis of the metagenomic ORFs resulted in a
211 much larger number of clusters for *A. mellifera*, compared to *A. cerana*. This
212 difference may in part be explained by *A. mellifera* housing a bacterial community
213 composed of more SDPs. While 90% of the host-filtered reads map to the database
214 for both hosts (**Fig. S1**), this fraction represents 12 SDPs for the five core phylotypes
215 in *A. mellifera* (plus up to 4 non-core members), but only 7 in *A. cerana* (plus
216 *Apibacter* sp., and occasionally *Lactobacillus kunkeei*) (**Fig. 1** and **Fig. S1**). Indeed,
217 the number of gene clusters and the total genome assembly size per bee were both
218 approximately twice as large for *A. mellifera* when using comparable subsets of host-
219 filtered reads (**Fig. 2A and Fig. S6**). However, the sharp drop in the number of
220 clusters from 95% to 90% sequence identity observed only for *A. mellifera* (**Fig. 1K**)
221 indicates that strain-level diversity is also a contributing factor.

222

223 Previous analysis of strain-level diversity in *A. mellifera* showed that strains tend to
224 segregate among individuals within colonies (5), in contrast to the SDPs, which
225 mostly co-exist (**Fig.1D-F, Fig. S5**). Therefore, sequence clusters occurring only in a
226 subset of bees are likely to represent strain-level diversity. To determine how the
227 sequence clusters distribute across bees, we plotted the number of clusters relative
228 to the number of samples, using the 95% nucleotide sequence identity threshold.
229 From the cumulative curves (**Fig. 2B**), it is evident that the gene content harbored
230 within individual bees represent a minor fraction of the total gene content present
231 across hosts. Moreover, the number of sequence clusters increased more rapidly
232 with sample size for *A. mellifera* as compared to *A. cerana*, and did not appear to
233 have reached saturation with the current sampling size. This difference was not
234 related to diversity between colonies or countries for *A. mellifera* (**Fig. S7**). Rather,
235 the gene content of the gut microbiota is highly variable among bees, also within
236 colonies, and much more so for *A. mellifera* compared to *A. cerana*. Taken together
237 with the consistent taxonomic profile among bees (**Fig. 1D-F and Fig.S5**), these
238 results indicate that a major fraction of the variation in gene content is related to
239 strain-level diversity.

240

241 To quantify the extent of strain-level diversity, the total fraction of single nucleotide
242 variants (SNVs) within core genes was determined for all SDPs. At the level of
243 individual bees, only two of the SDPs colonizing *A. cerana* were found to harbor
244 more than 2% SNVs in any of the samples ("Firm5-7", "Bifido-1")(**Fig. 2C**). In
245 contrast, nearly all the SDPs colonizing *A. mellifera* had more than 2% SNVs per bee
246 in a major fraction of the samples (**Fig. 2C**). For example, "Bifido-1", an SDP shared

247 between both bee species, had on average 9.6% SNVs per individual bee in *A.*
248 *mellifera*, while in *A. cerana* the average percentage SNVs was as low as 0.8%.
249
250 To quantify diversity at the colony level, we again generated cumulative curves of
251 SNVs as a function of the number of analyzed bees (**Fig. 2D-E**, **Fig. S8**).
252 Interestingly, the two SDPs occurring in both hosts, "Bifido-1" and "Firm4-1" (**Fig. 1G**
253 **and H**), displayed a clear difference in strain-level diversity between the host species
254 (**Fig. 2D-E**). This difference was not explained by the choice of reference genome,
255 since the pattern persisted after swapping the reference genome with an isolate from
256 the alternate host (**Fig. S8).**
257
258 Based on these results, we conclude that strain-level diversity in *A. mellifera* is
259 substantially higher than in *A. cerana*, both in individual bees and within colonies.
260
261 **The gut microbiota of *A. mellifera* encodes more diverse enzymes for**
262 **polysaccharide degradation.**
263 The observed differences in SDP composition and strain-level diversity raise the
264 question whether the gut microbiota is functionally distinct between the two hosts. To
265 address this question, we annotated metagenomic ORFs using the COG and
266 CAzyme databases. Furthermore, the analysis was restricted to the Japanese
267 metagenomes (20 samples per host), using metagenome assemblies with 20 million
268 host-filtered paired-end reads per sample, in order to facilitate quantitative
269 comparisons.
270

271 Although the number of ORFs per sample was approximately twice as high for *A.*
272 *mellifera* compared to *A. cerana*, the relative COG profiles were indistinguishable
273 among hosts (**Fig. 3A**). Consistent with previous studies (20), carbohydrate
274 metabolism and transport" (COG category "G") was abundant across the
275 metagenomic samples of both host species. In order to identify possible differences
276 within this important functional category, metagenomic ORFs encoding glycoside
277 hydrolases (GHs) and polysaccharide lyases (PLs) were annotated with dbCAN2
278 (21), and the number of annotations per family were counted for each sample.
279 Notably, when calculating the mean number of annotations per family for each host
280 species, the relative abundance of each GH/PL family was highly similar between the
281 hosts, as evidenced by the linear correlation between the mean counts per family
282 (**Fig. 3B**). However, samples from *A. mellifera* harbored approximately twice as many
283 genes per family, as evidenced from the slope of the correlation (**Fig. 3B**).
284
285 Although the annotation of GH/PL families is based on sequence homology, a wide
286 range of specificities have been reported within families (22). Therefore, to estimate
287 the diversity among the genes within GH/PL families, they were clustered separately
288 for each family, using a highly conservative threshold (50% amino acid sequence
289 identity). Even at this threshold, the clustering resulted in an average of 10.4 clusters
290 per family, indicating that a major fraction of the genes annotated to the same family
291 is likely to have distinct functions or substrate specificities. A total of 52 out of 62
292 GH/PL families contained host-specific clusters (**Fig. 3C**). However, only 17 families
293 contained clusters specific to *A. cerana*, whereas all 52 contained clusters specific to
294 *A. mellifera* (**Fig. 3C)**. Moreover, the mean number of host-specific clusters per family
295 was 6.0 for *A. mellifera*, but only 3.1 for *A. cerana*. Taken together, these results

296 therefore indicate that *A. mellifera* has a much larger host-specific repertoire of
297 GH/PL families as compared to *A. cerana.*
298
299 In order to gain further insights into the origin of the GH/PL families, all sequences
300 were blasted against the honey bee gut microbiota database. Overall, 97% of the
301 sequences had significant hits to the database (e-value < 10e-05, > 80% query
302 coverage), with 79% having a close hit (>95% amino acid identity). Among the
303 sequences with close hits, the vast majority of hits were to genomes of the "Bifido-1"
304 SDP, followed by other SDPs of the *Lactobacillus* Firm4 and Firm5 phylotypes (**Fig.**
305 **3D**, upper panel). For the host-specific GH/PL clusters, only 52% of the sequences
306 falling within *A. mellifera*-specific clusters had a close hit to the database, whereas
307 67% of the *A. cerana*-specific clusters had close hits. Thus, although the current
308 database contains fewer genome isolates from *A. cerana* compared to *A. mellifera*, it
309 is more representative of *A. cerana* in terms of GH/PL families, likely as a
310 consequence of GH/PL genes being at least partly associated with strain-level
311 diversity. Among the sequences corresponding to host-specific GH/PL clusters, the
312 majority of blast hits were once again to "Bifido-1" (**Fig. 3D**, lower panel). Strikingly,
313 they all originated from *A. mellifera* metagenomes, suggesting that GH/PL families
314 encoded by "Bifido-1" in *A. cerana* represent a subset of those present in *A. mellifera*.
315 A similar pattern was observed for "Firm4-1", the other SDP shared between the two
316 host species. While GH/PL family genes matching "Firm-4" sequences were found in
317 the gut microbiota of both honey bees, almost all host-specific sequences came from
318 *A. mellifera*. Instead, for the clusters specific to *A. cerana*, most of the hits were to
319 *Apibacter* sp. and *Lactobacillus* "Firm5-7", suggesting that diversity occurring at the

320 phylotype- and SDP-level contribute more to functional specialization than strain-
321 level diversity in *A. cerana*.
322
323 In conclusion, despite the similarity in the general functional profiles, *A. mellifera*
324 harbors a much more diverse repertoire of GH/PL families, with many more host-
325 specific GH/PL clusters compared to *A. cerana*. Moreover, the majority of GH/PL
326 sequences were associated with the "Bifido-1" SDP, which is much more diverse in
327 *A. mellifera* compared to *A. cerana*, suggesting that strain-level diversity in *A.*
328 *mellifera* is a major contributor to functional differences between the two host
329 species.
330
331 ***A. mellifera* and *A. cerana* differ in bacterial community size within individual**
332 **bees.**
333 According to neutral theory, diversity is expected to correlate with habitat size and
334 population size. Therefore, we sought to determine whether *A. mellifera* and *A.*
335 *cerana* differ in terms of the spatial niche they provide for their gut bacterial
336 communities. Based on wet-weight, the hindgut, where most of the bacteria reside,
337 was not significantly different between the hosts (**Fig. 4A**). However, the bacterial
338 community size, as estimated from quantitative real-time PCR with universal 16S
339 rRNA primers (normalized to the copy number of the host gene actin) was still found
340 to be significantly larger for *A. mellifera* compared to *A. cerana* ($p < 0.001$, Mann-
341 Whitney U test) (**Fig. 4B**).
342
343

344 **Discussion**

345 In the current study, we carried out a community-wide metagenomic characterization
346 of the gut microbiota of two closely related honey bee species, *A. mellifera* and *A.*
347 *cerana*. From this analysis, three key results emerged. Firstly, we found that the gut
348 bacterial communities of the two host species were highly divergent, consisting of
349 different SDPs and strains, despite having a very similar phylotype-level composition.
350 Secondly, the two host species displayed major differences in the magnitude of
351 strain-level diversity within their bacterial communities. And thirdly, the gut microbiota
352 of *A. mellifera* harbored a much larger repertoire of enzymes related to
353 polysaccharide breakdown. Thus, in the time since their last common ancestor,
354 approximately 6 million years ago (14, 17), the gut bacterial communities of *A.*
355 *mellifera* and *A. cerana* have undergone substantial changes in composition,
356 genomic diversity, and functionality, with likely consequences for the interaction with
357 their hosts.

358

359 Based on amplicon sequencing of the 16S rRNA gene, multiple studies have shown
360 that gut microbiota composition is influenced by host phylogeny (23, 24) with the
361 overall observation that closely related host species harbor more similar gut bacterial
362 communities at the phylotype level than more distant ones (25). However, the slow
363 evolutionary rate of the 16S rRNA gene does not permit evolutionary analysis of
364 phylotypes that are shared across related hosts. Therefore, there is currently little
365 data providing insights into the evolution of the gut microbiota for closely related
366 animal hosts. Targeting the fast-evolving *gyrA* gene for three bacterial families
367 colonizing hominids, Moeller *et al.* found evidence of co-diversification in the
368 Bacteroidaceae and Bifidobacteriaceae, but not the Lachnospiraceae (26). Similarly,

369 for honey bees and bumble bees, amplicon-sequencing of the *minD* gene uncovered
370 both host-specific and more generalist clades within the core phylotype
371 *Snodgrassella* (27). Consistently, comparative genome analyses of bacterial isolates
372 have also uncovered several examples of host-specific lineages (9-12). However, the
373 current study is the first to report community-wide patterns of host-specialization
374 using metagenomic data.
375
376 Interestingly, while we found evidence of host-specialization for all of the five core
377 phylotypes colonizing both hosts, the extent of divergence differed widely among
378 them. For three of the phylotypes (*Lactobacillus* Firm5, *Gilliamella*, and
379 *Snodgrassella*), each host was found to be colonized by different SDPs, i.e. bacterial
380 lineages which are sufficiently divergent to be classified as different species (13). In
381 contrast, the host-specialization of the *Bifidobacterium* and *Lactobacillus* Firm4
382 phylotypes only became evident when analyzed with strain-level resolution,
383 consistent with the comparatively short branch lengths observed for the core genome
384 phylogenies. Contemplating a 16S rRNA gene divergence rate of about 1% per 50
385 million years (28, 29), it seems unlikely that any of the SDPs could have emerged
386 within the 6 million years separating *A. mellifera* and *A. cerana*. More likely, the
387 current SDP composition represents a selection of pre-existing SDPs, with
388 secondarily evolved traits resulting in the currently observed host preference. For
389 example, we found that all SDPs within the *Lactobacillus* Firm5 phylotype contributed
390 to highly divergent host-specific glycoside hydrolases, which could potentially allow
391 for dietary specialization and host adaptation. In contrast, the comparatively little
392 sequence divergence between the host-specialized strains of *Lactobacillus* Firm4
393 and the Bifidobacteria could match a time span of 6 million years, and thus be a

394 product of co-diversification. However, a larger genomic dataset from multiple host
395 species will be needed to properly test this hypothesis.
396
397 Remarkably, we also found that the two host species differed widely in terms of the
398 extent of strain-level diversity across the gut microbiota. Whether such differences
399 are commonplace among related animals is unknown, since comparable data is not
400 yet available. Despite a rapid increase in metagenomic studies, quantification of
401 strain-level diversity is still technically challenging and quantitative comparisons
402 across hosts are therefore rare. Differences in sampling, DNA extraction, and
403 sequencing depth can have a strong impact on both composition and diversity,
404 making cross-comparisons between samples and studies particularly difficult (30).
405 For example, for the human gut microbiota, it was estimated that a cumulative
406 genome coverage of at least 1000x would be required to obtain a representative
407 sampling of the strain-level diversity (31), a number which is still well beyond the
408 current norm. Even in simple communities, like the bacterial endosymbionts
409 colonizing deep-sea mussels, quantification of strains can be sensitive to sequencing
410 depth (4). In the current study, technical biases were limited by using a common
411 sampling and DNA extraction protocol, and a comparable sequencing depth. Given
412 the simple taxonomic composition of the honey bee gut microbiota, we were able to
413 obtain deep sequencing of the individual community members, with comparable
414 mapping and *de novo* metagenome assembly efficiencies in both hosts. Furthermore,
415 normalization by sampling and sequencing depth was applied in all analyses. Taken
416 together, we are therefore confident that the observed community-wide differences in
417 strain-level diversity are not due to technical biases.
418

419 Several factors could potentially have resulted in the observed difference in strain-
420 level dviersity, which are not mutually exclusive (**Fig. 4C**). Firstly, although *A. cerana*
421 has long been used for honey production in Asia, *A. mellifera* differs from all other
422 extant honey bee species by having been extensively transported around the globe
423 by humans, for hundreds of years(19). Thus, it is possible that humans have
424 contributed to a mixing of locally adapted strains, thereby increasing diversity within
425 colonies (**Fig. 4C**, "Human influence"). As of yet, large-scale studies on the
426 distribution of strains are still lacking for honey bees, and previous studies have
427 reported mixed results (8, 27). Likewise, in the current study, ordination plots based
428 on shared SNVs clustered by country for only a subset of SDPs. Future studies on
429 wild honey bee populations should provide further insights into this question.
430 Interestingly, if the high strain-level diversity in *A. mellifera* is caused by human
431 interference, it raises the possibility that the gut microbiota of *A. mellifera* is sub-
432 optimal for local conditions, potentially resulting in reduced colony fitness.
433
434 However, based on molecular data, *A. mellifera* has had a large and varied
435 geographic range long before human interference (14, 15). Moreover, despite the
436 occurrence of colony collapse disorder in managed colonies around the world (32-
437 34), *A. mellifera* has been found to succesfully establish feral colonies even outside
438 its native range (i.e. the New World), indicative of a remarkable ability to survive
439 under highly varied conditions (35). Thus, it is possible that *A. mellifera* is more of a
440 generalist than *A. cerana*, for example by having a wider foraging range, and
441 therefore is able to maintain a more varied bacterial community and larger repertoire
442 of sugar breakdown functions (**Fig. 4C,** "Dietary specialization"). Indeed, diet has
443 been shown to have an impact on diversity in the gut microbiota in multiple studies,

444 with for example a reduction of diversity reported for the human gut microbiota as a
445 consequence of westernized diet (36, 37). As of yet, large-scale systematic studies
446 on the foraging preferences of *A. cerana* and *A. mellifera* have not been conducted,
447 but they appear to have largely overlapping foraging ranges in Asia (38, 39). Further
448 studies are therefore needed to determine whether differences in strain-level diversity
449 are related to dietary differences.
450
451 Finally, it is also possible that the difference in strain-level diversity between *A.*
452 *mellifera* and *A. cerana* is driven by neutral processes (**Fig. 4C**, "Species-area
453 relationship"). Specifically, the "species-area relationship", posit a positive correlation
454 between habitat size and diversity, and is widely held to constitute one of the few
455 laws in ecology (40-42). However, it is still debated whether this relationship also
456 applies to bacteria (43-45). The gut microbiota represents an attractive model system
457 for testing the hypothesis, since bacterial populations in this case can be easily
458 delineated, due to the host-association. Interestingly, *A. mellifera* and *A. cerana*
459 exhibit spatial differences at multiple levels (**Fig. 4C**, "Species-area relationship").
460 Firstly, we found that the size of the bacterial communities within individuals was
461 signficantly larger for *A. mellifera* compared to *A. cerana*, consistently with the
462 findings of a previous study (8). Secondly, *A. mellifera* is known to form larger
463 colonies than *A. cerana* (16). And thirdly, the native range of *A. mellifera* is also
464 larger compared to *A. cerana* (19, 46, 47). Our analysis clearly shows that the
465 diversity within individual bees is much lower than the diversity found within their
466 colonies. Thus, it seems plausible that competition among strains is alleviated when
467 strains colonize different hosts. If so, it also follows that larger colonies should be
468 able to support more strain-level diversity, by providing more colonization

469 opportunities for strains that would otherwise compete. However, given that the
470 diversity was also consistently higher for individual bees of *A. mellifera* as compared
471 to *A. cerana*, the species-area relationship may be equally applicable at this level. In
472 contrast, the inclusion of multiple colonies had very little impact on diversity,
473 suggesting that host geographic range is of less importance for maintenance of
474 strain-level diversity in honey bees.
475
476 In conclusion, the results of the current study brought several fundamental questions
477 regarding the evolution and maintenance of diversity in host-associated bacterial
478 communities to the foreground. While the term “diversity” has an inherently positive
479 connotation, it is not obvious whether diversity in host-associated bacterial
480 communities should be beneficial, and if so, in what sense (48). For example, high
481 strain-level diversity in the gut microbiota of *A. mellifera* may provide more metabolic
482 flexibility, facilitating foraging on more diverse pollen sources, and thereby faster
483 adaptation to changing environmental conditions. On the other hand, high strain-level
484 diversity could also lead to increased competition within the gut microbiota, with
485 resources being diverted towards inter-bacterial warfare, rather than host-symbiont
486 mutualistic interactions. It is also possible that a less diverse gut microbiota
487 consisting of strains adapted to local conditions would be more beneficial than a
488 more diverse one. These possibilities can be experimentally tested in honey bees,
489 thereby providing novel insights into the functional relevance of strain-level diversity
490 in host-associated bacterial communities and for honey bee health.
491

492 **Methods**

493 Detailed protocols are available in the Supplementary Information (**Supplementary**
494 **Materials and Methods**). All bioinformatic tasks were accomplished with custom
495 scripts (perl, R, Bash) unless otherwise indicated. Metagenomic samples were
496 collected from individual honey bees, with 20 samples for each host species (*A.*
497 *mellifera, A.cerana*), each represented by two colonies. All colonies were located in
498 different apiaries, no more than 100km apart, close to Tsukuba, Japan. DNA
499 extraction was done as described previously (5), using a custom protocol for
500 enrichment of bacterial cells. Sequencing was done with the Nextera XT library kit,
501 on an Illumina Hiseq 2500 instrument. A honey bee gut microbiota database was
502 generated for the current study, including previously published genomes and novel
503 genomes isolated from *A. cerana*, in order to be representative of both targeted
504 communities. Candidate sequence-discrete populations (SDPs) were inferred from
505 isolate genomes in the database (based on core genome phylogenies and average
506 nucleotide identities), and subsequently validated by recruitment of metagenomic
507 ORFs to core gene alignments. Quantification of SDPs within and across samples
508 was done as described previously (5), based on mapped read coverage to single-
509 copy core gene families. Profiling of single-nucleotide variants (SNVs) was done with
510 freebayes (49), using a reduced version of the database, with a single representative
511 genome per SDP, and a strict filtering pipeline. Metagenome assemblies were
512 generated from host-filtered reads, based complete datasets and subsets of 20
513 million paired-end reads per sample, using SPAdes (50). Annotation of metagenomic
514 ORFs was done with the eggnog (51) and dbcan2 databases (21). To estimate
515 bacterial loads and wet-weights of honey bee hindguts, new samples were collected,
516 from which total DNA was extracted with a CTAB-based protocol. Bacterial load

517 quantification was done with qPCR, targeting the V3-V4 region of the 16S rRNA

518 gene, and normalized based on the actin gene of the host.

519

520 **Data Availability**

521 The metagenomic data has been deposited under Bioproject PRJNA59809.

522

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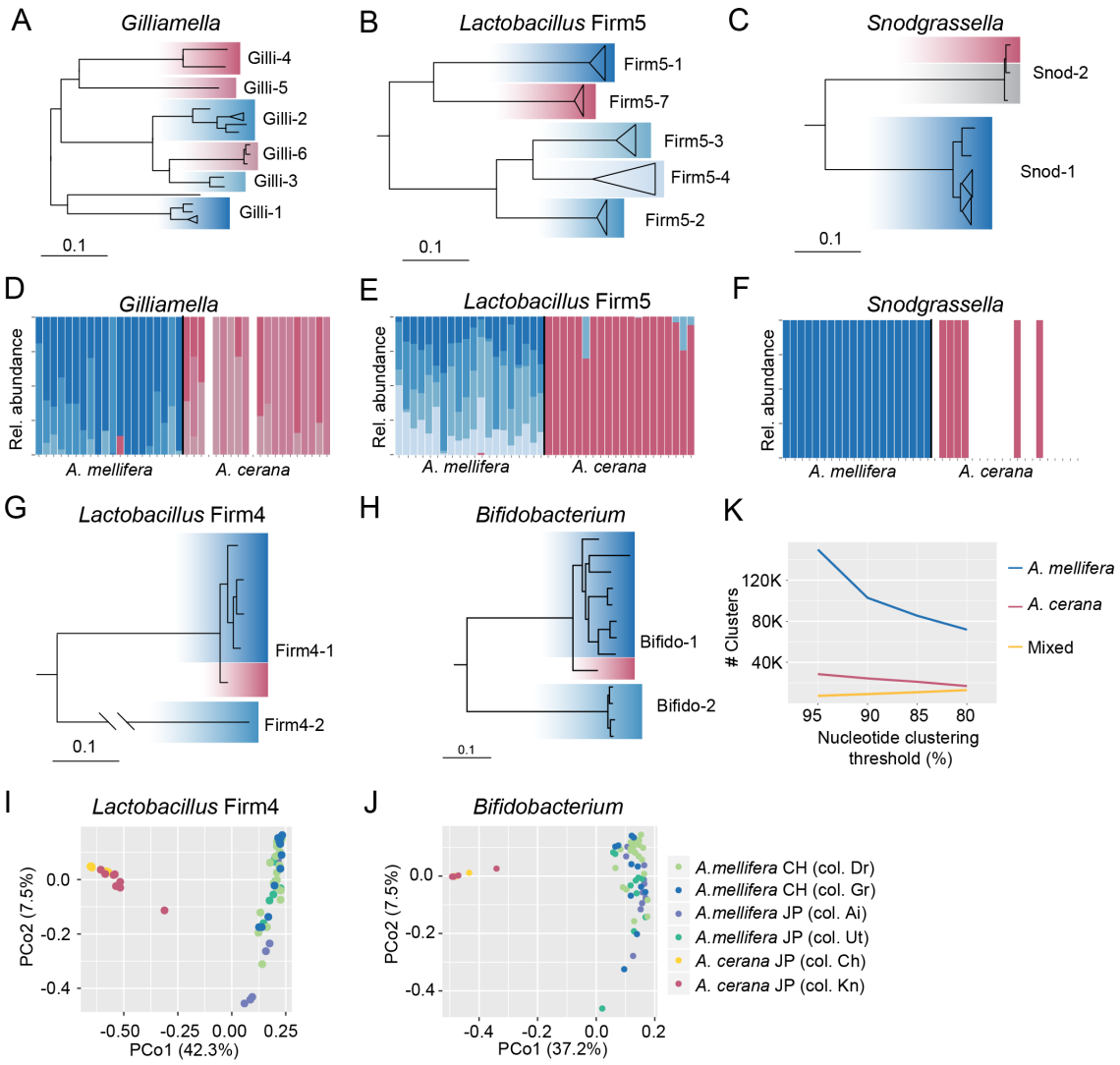
532 **Figures**



533

534 **Figure 1. The gut microbiota of *A. mellifera* and *A. cerana* are composed of**
535 **divergent SDPs and strains. (A-C, G-H)** Core genome phylogenies of the five
536 shared core phylotypes colonizing *A. mellifera* and *A. cerana*. Confirmed SDPs are
537 indicated by the labels of the clades. Genomes are highlighted with blue shades for
538 isolates from *A. mellifera*, and red shades for isolates from *A. cerana*. Grey shades
539 indicate isolates from other honey bee species. Bars correspond to 0.1 substitutions
540 per site **(D-F)** Barplots displaying relative abundance of the confirmed SDPs shown
541 in panels A-C, across metagenomes from Japan (see Fig. S5 for the same plots
542 including metagenome samples from Switzerland). **(I-J)** PCoA ordination plots based

543 on the pairwise fractions of shared SNVs (jaccard distance) (see **Dataset S3** for plots
544 of other SDPs). Dots represent individual samples, color-coded by host and colony
545 origin as indicated by the legend. **(K)** Number of host-specific and mixed sequence
546 clusters generated from metagenomic ORFs at different clustering thresholds for the
547 complete dataset (Swiss and Japanese samples).
548

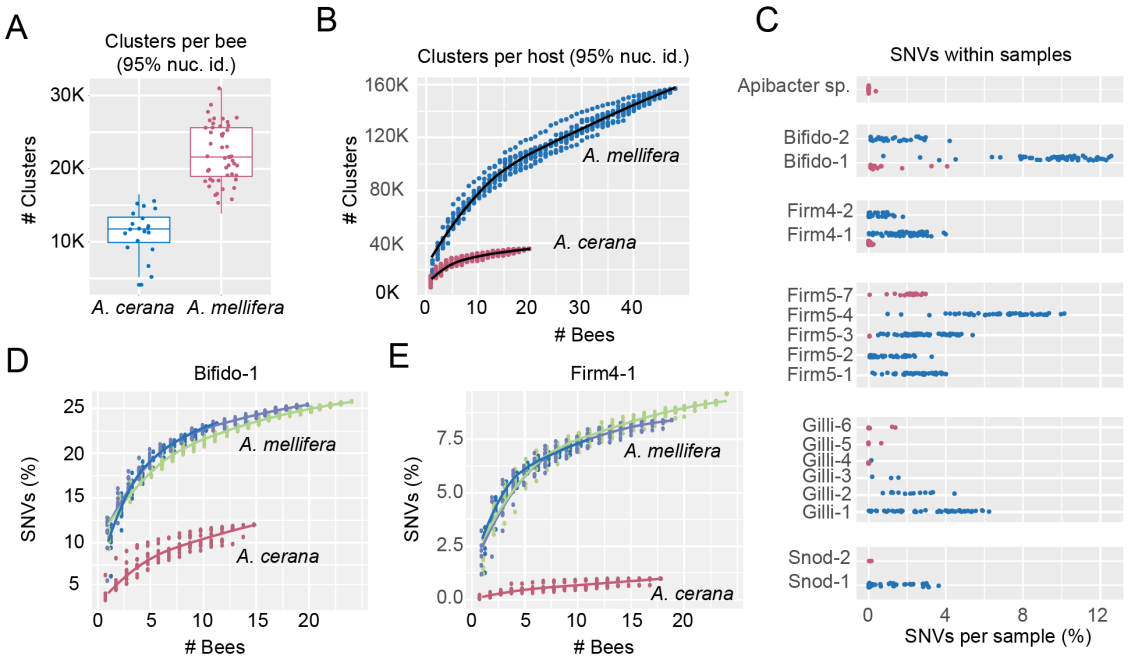


Figure 2. **Major differences in strain-level diversity in the gut microbiota of *A. mellifera* and *A. cerana*. (A)** Number of sequence clusters per sample for each host, based on metagenomic ORFs from assemblies generated with 20 million paired-end host-filtered reads. **(B)** Cumulative number of sequence clusters for each host, relative to the number of bee samples. **(C)** Fraction of single nucleotide variants (SNVs) within core genes in each sample, for SDPs corresponding to core phylotypes, plus *Apibacter* sp.. SDP labels correspond to Fig. 1. **(D-E)** Cumulative fractions of SNVs within core genes relative to the number of samples, for the "Bifido-1" and "Firm4-1" SDPs. Blue-green shades represent different *A. mellifera* colonies, whereas the *A. cerana* samples were pooled due to the smaller number of samples. See **Fig. S8** for curves corresponding to other SDPs and mappings to alternative reference genomes.

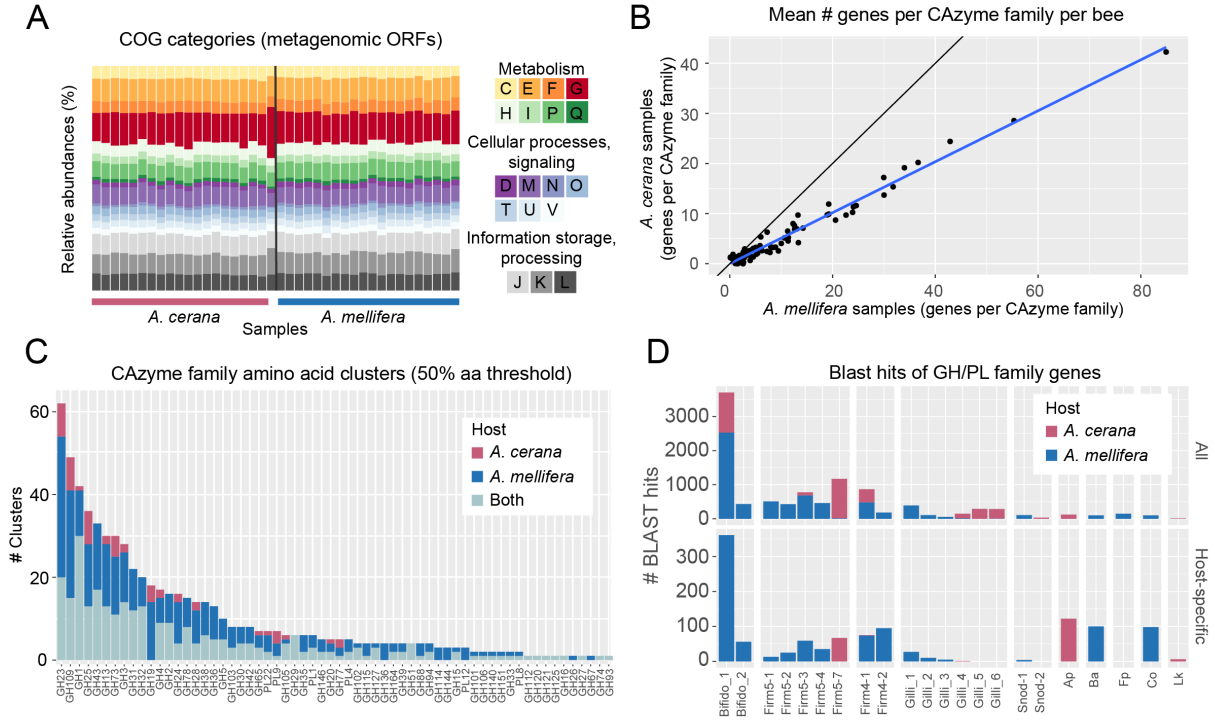
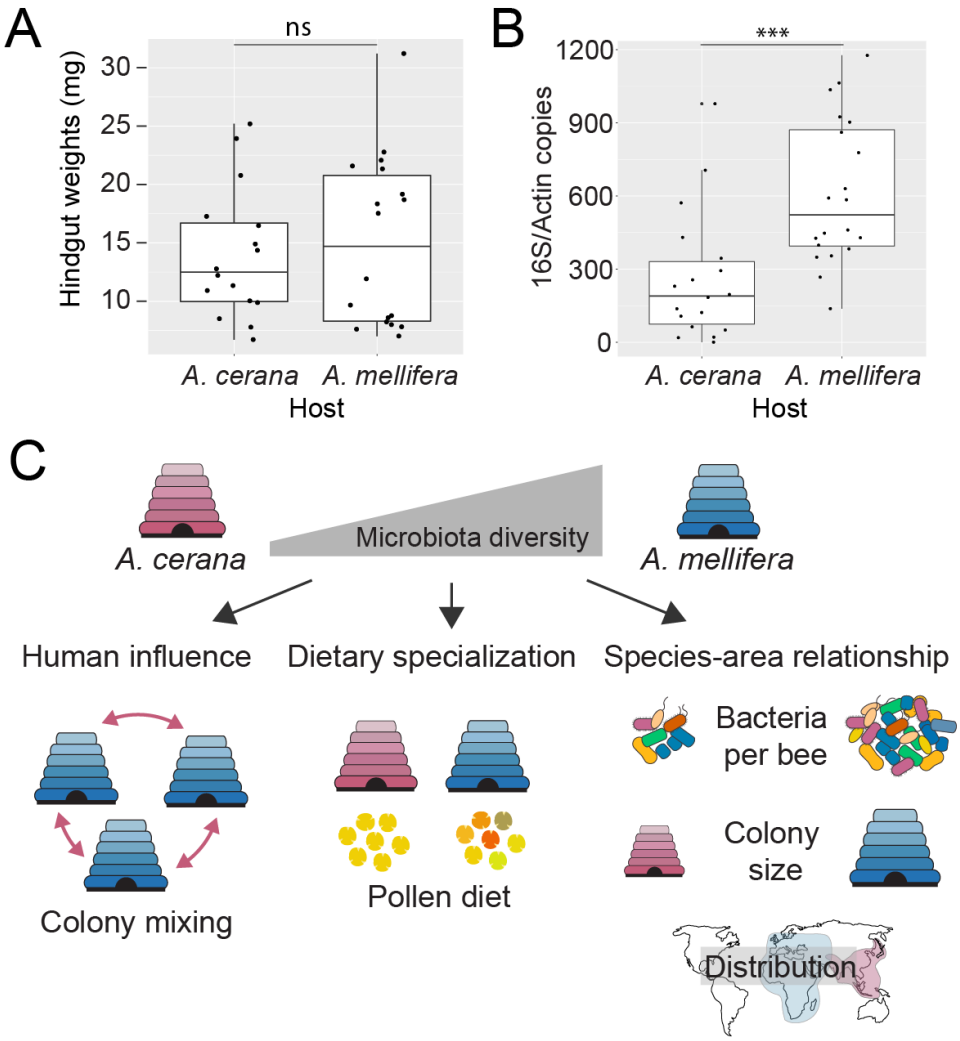


Figure 3. Functional comparison of the gut microbiota of *A. mellifera* and *A. cerana.* (A) Relative abundance of COG annotations according to general functional COG categories. **(B)** Mean number of ORFs assigned to each CAzyme family, calculated separately for each host. As shown by the blue regression line, there was a linear correlation between the counts, indicating that the CAzyme families display similar relative abundance patterns in both hosts. But, the total number of ORFs assigned to each CAzyme family is larger in *A. mellifera* samples as compared to *A. cerana* samples as indicated by the deviation from the black line. **(C)** Number of sequence clusters within each CAzyme family (clustered at 50% amino acid sequence identity), with colors indicating the subsets of clusters specific to each host, and clusters containing ORFs derived from both hosts. **(D)** SDP affiliation of blast hits for all ORF sequencess annotated as GH/PL CAzyme families, in the honey bee gut microbiota database. Results are only shown for close hits (>95% amino acid sequence identity). The upper panel includes all ORFs passing this threshold, the lower panel includes the subset of these occurring within host-specific clusters. All

analyses were carried out on only the Japanese metagenome samples, to facilitate quantitative comparisons (20 samples per host, with assemblies based on 20 million paired-end host-filtered reads per sample). SDP labels correspond to Fig. 1. Species abbreviations: Ap - *Apibacter* sp., Fp - *Frischella perrara*, Co: *Commensalibacter* sp., Lk - *Lactobacillus kunkeei*.



585

586 **Figure 4. Possible factors explaining the difference in gut microbiota diversity**
587 **between *A. mellifera* and *A. cerana*. (A and B)** Wet-weight of the hindgut (where
588 most of the bacteria reside) and qPCR results for estimation of bacterial community
589 size (targeting the 16S rRNA gene, and normalized by copy-number of the host gene
590 actin). Statistical significance was calculated using a Mann-Whitney *U* test (ns - not
591 significant, ***p<0.001). **(C)** Schematic illustration of three possible factors explaining
592 differences in diversity. "Human influence": transportation and mixing of *A. mellifera*
593 colonies and genotypes around the world by beekeepers, resulting in mixing of
594 strains from different geographic origins, and thereby increasing strain-level diversity
595 in *A. mellifera*. "Dietary Specialization": *A. mellifera* may have a more generalist diet

596 (here illustrated by pollen grain diversity) as compared to *A. cerana*, and therby be
597 able to sustain a more diverse community. “Species-Area relationship”: Although
598 previously applied to species-level diversity in animals, this concept may also apply
599 to strain-level diversity in bacteria. For the honey bee gut microbiota, spatial
600 differences are applicable at three levels: The size of the bacterial community within
601 individual bees, the size of honey bee colonies, and the size of the geographic range.
602
603

604 **References**

605 1. Kashtan N*, et al.* (2014) Single-cell genomics reveals hundreds of coexisting
606 subpopulations in wild Prochlorococcus. *Science* 344(6182):416-420.
607 2. Cordero OX & Polz MF (2014) Explaining microbial genomic diversity in light
608 of evolutionary ecology. *Nat Rev Microbiol* 12(4):263-273.
609 3. Mitri S & Foster KR (2013) The genotypic view of social interactions in
610 microbial communities. *Annu Rev Genet* 47:247-273.
611 4. Ansorge R*, et al.* (2019) Functional diversity enables multiple symbiont strains
612 to coexist in deep-sea mussels. *Nat Microbiol* 4(12):2487-2497.
613 5. Ellegaard KM & Engel P (2019) Genomic diversity landscape of the honey bee
614 gut microbiota. *Nat Commun* 10(1):446.
615 6. Zhu A, Sunagawa S, Mende DR, & Bork P (2015) Inter-individual differences
616 in the gene content of human gut bacterial species. *Genome Biol* 16:82.
617 7. Greenblum S, Carr R, & Borenstein E (2015) Extensive strain-level copy-
618 number variation across human gut microbiome species. *Cell* 160(4):583-594.
619 8. Kwong WK*, et al.* (2017) Dynamic microbiome evolution in social bees. *Sci*
620 *Adv* 3(3):e1600513.
621 9. Steele MI, Kwong WK, Whiteley M, & Moran NA (2017) Diversification of Type
622 VI Secretion System Toxins Reveals Ancient Antagonism among Bee Gut
623 Microbes. *MBio* 8(6).
624 10. Kwong WK, Engel P, Koch H, & Moran NA (2014) Genomics and host
625 specialization of honey bee and bumble bee gut symbionts. *Proc Natl Acad*
626 *Sci U S A* 111(31):11509-11514.
627 11. Zheng H*, et al.* (2016) Metabolism of Toxic Sugars by Strains of the Bee Gut
628 Symbiont Gilliamella apicola. *MBio* 7(6).

629 12. Ellegaard KM*, et al.* (2015) Extensive intra-phylotype diversity in lactobacilli
630 and bifidobacteria from the honeybee gut. *BMC Genomics* 16:284.
631 13. Jain C, Rodriguez RL, Phillippy AM, Konstantinidis KT, & Aluru S (2018) High
632 throughput ANI analysis of 90K prokaryotic genomes reveals clear species
633 boundaries. *Nat Commun* 9(1):5114.
634 14. Wallberg A*, et al.* (2014) A worldwide survey of genome sequence variation
635 provides insight into the evolutionary history of the honeybee Apis mellifera.
636 *Nat Genet* 46(10):1081-1088.
637 15. Han F, Wallberg A, & Webster MT (2012) From where did the Western
638 honeybee (Apis mellifera) originate? *Ecol Evol* 2(8):1949-1957.
639 16. Park D*, et al.* (2015) Uncovering the novel characteristics of Asian honey bee,
640 Apis cerana, by whole genome sequencing. *BMC Genomics* 16:1.
641 17. Chen C*, et al.* (2018) Population genomics provide insights into the evolution
642 and adaptation of the eastern honey bee (Apis cerana). *Mol Biol Evol*.
643 18. Nanzan Takamura YT, Shigeru Nakano, Mitsuyuki Horikiri (1966) Yoho no
644 Seisei. *Chikusan Hattatsu Shi*, ed Livestock Industry Bureau MoAaF (Chuo
645 Kouron Jigyo, Tokyo), pp 1313-1334.
646 19. Requier F*, et al.* (2019) The Conservation of Native Honey Bees Is Crucial.
647 *Trends Ecol Evol* 34(9):789-798.
648 20. Engel P, Martinson VG, & Moran NA (2012) Functional diversity within the
649 simple gut microbiota of the honey bee. *Proc Natl Acad Sci U S A*
650 109(27):11002-11007.
651 21. Zhang H*, et al.* (2018) dbCAN2: a meta server for automated carbohydrate-
652 active enzyme annotation. *Nucleic Acids Res* 46(W1):W95-W101.

653 22. Helbert W*, et al.* (2019) Discovery of novel carbohydrate-active enzymes
654 through the rational exploration of the protein sequences space. *Proc Natl*
655 *Acad Sci U S A* 116(13):6063-6068.
656 23. Nishida AH & Ochman H (2018) Rates of gut microbiome divergence in
657 mammals. *Mol Ecol* 27(8):1884-1897.
658 24. Ley RE*, et al.* (2008) Evolution of mammals and their gut microbes. *Science*
659 320(5883):1647-1651.
660 25. Groussin M*, et al.* (2017) Unraveling the processes shaping mammalian gut
661 microbiomes over evolutionary time. *Nat Commun* 8:14319.
662 26. Moeller AH*, et al.* (2016) Cospeciation of gut microbiota with hominids.
663 *Science* 353(6297):380-382.
664 27. Powell E, Ratnayeke N, & Moran NA (2016) Strain diversity and host
665 specificity in a specialized gut symbiont of honeybees and bumblebees. *Mol*
666 *Ecol* 25(18):4461-4471.
667 28. Ochman H, Elwyn S, & Moran NA (1999) Calibrating bacterial evolution. *Proc*
668 *Natl Acad Sci U S A* 96(22):12638-12643.
669 29. Moran NA, Munson MA, Baumann P, & Ishikawa H (1993) A Molecular Clock
670 in Endosymbiotic Bacteria Is Calibrated Using the Insect Hosts. *P Roy Soc B-*
671 *Biol Sci* 253(1337):167-171.
672 30. Reese AT & Dunn RR (2018) Drivers of Microbiome Biodiversity: A Review of
673 General Rules, Feces, and Ignorance. *MBio* 9(4).
674 31. Schloissnig S*, et al.* (2013) Genomic variation landscape of the human gut
675 microbiome. *Nature* 493(7430):45-50.
676 32. Cox-Foster DL*, et al.* (2007) A metagenomic survey of microbes in honey bee
677 colony collapse disorder. *Science* 318(5848):283-287.

678 33. Stokstad E (2007) Entomology. The case of the empty hives. *Science*
679 316(5827):970-972.
680 34. Oldroyd BP (2007) What's killing American honey bees? *PLoS Biol* 5(6):e168.
681 35. Seeley T (2019) *The Lives of Bees: The Untold Story of the Honey Bee in the*
682 *Wild* (Princeton University Press, New Jersey, USA).
683 36. Sonnenburg ED*, et al.* (2016) Diet-induced extinctions in the gut microbiota
684 compound over generations. *Nature* 529(7585):212-215.
685 37. Fragiadakis GK*, et al.* (2019) Links between environment, diet, and the hunter-
686 gatherer microbiome. *Gut Microbes* 10(2):216-227.
687 38. Tatsuno M & Osawa N (2016) Flower visitation patterns of the coexisting
688 honey bees Apis cerana japonica and Apis mellifera (Hymenoptera: Apidae).
689 *Entomological Science* 19(3):255-267.
690 39. Suryanarayana M, Mohana Rao G, & Singh T (1992) Studies on pollen
691 sources for Apis cerana Fabr and Apis mellifera L bees at Muzaffarpur, Bihar,
692 India. *Apidologie* 23.
693 40. Rosenzweig M (1995) *Species diversity in space and time* (Cambridge
694 University Press, Cambridge, UK).
695 41. Arrhenius O (1921) Species and Area. *Journal of Ecology* 9:95-99.
696 42. Gleason H (1922) On the relation between species and area. *Ecology*
697 3(2):158-162.
698 43. Logue JB*, et al.* (2012) Freshwater bacterioplankton richness in oligotrophic
699 lakes depends on nutrient availability rather than on species-area
700 relationships. *ISME J* 6(6):1127-1136.
701 44. Bell T*, et al.* (2005) Larger islands house more bacterial taxa. *Science*
702 308(5730):1884.

703 45. Koskella B, Hall LJ, & Metcalf CJE (2017) The microbiome beyond the horizon
704 of ecological and evolutionary theory. *Nat Ecol Evol* 1(11):1606-1615.
705 46. Koetz AH (2013) Ecology, Behaviour and Control of Apis cerana with a Focus
706 on Relevance to the Australian Incursion. *Insects* 4(4):558-592.
707 47. Ruttner F (1988) *Biogeography and Taxonomy of Honeybees* (Springer).
708 48. Shade A (2017) Diversity is the question, not the answer. *ISME J* 11(1):1-6.
709 49. Garrison E & Marth G (2012) Haplotype-based variant detetion from short-
710 read sequencing. *bioRxiv*.
711 50. Bankevich A*, et al.* (2012) SPAdes: a new genome assembly algorithm and its
712 applications to single-cell sequencing. *J Comput Biol* 19(5):455-477.
713 51. Huerta-Cepas J*, et al.* (2019) eggNOG 5.0: a hierarchical, functionally and
714 phylogenetically annotated orthology resource based on 5090 organisms and
715 2502 viruses. *Nucleic Acids Res* 47(D1):D309-D314.
716