Symbiont loss and gain, rather than co-diversification shapes honeybee gut microbiota diversity and function

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Abstract

Studying gut microbiota evolution across animals is crucial for understanding symbiotic interactions but is hampered by the lack of high-resolution genomic data. Honeybees, with their specialized gut microbiota and well-known ecology, offer an ideal system to study this evolution. Using shotgun metagenomics on 200 honeybee workers from five species, we recovered thousands of metagenome-assembled genomes, identifying several novel bacterial species. While microbial communities were mostly host-specific, we found both specialist and generalist bacteria, even among closely related species, with notable variation between host species. Some generalists emerged host-specific only at the strain level, suggesting recent host switches. Unexpectedly, we found no evidence of codiversification between hosts and symbionts. Instead, symbiont gains, losses, and replacements led to functional differences, such as the ability to degrade pollen-derived pectin. Our results provide new insights into gut microbiota evolution and uncover the functional potential of the previously underexplored gut microbiota of these important pollinators.

Introduction

Animals harbor specialized microbial communities in their gut, which can influence health and disease susceptibility through diverse functional capabilities[1]. These communities are often host-specific, where the gut microbiota of individuals of the same host species are more similar in composition to each other than to those of different host species[2]. In some animal groups, it has also been observed that more closely related host species harbor more similar gut microbiota in composition[3]. This host specificity at the community level emerges from the host-restricted

distribution of individual community members. Dispersal limitation and host-filtering processes have been suggested to contribute to the host-specific distribution of community members in the animal gut[2,4].

Some gut bacteria (*e.g.*, in termites[5] and hominids[6–10]) appear to have codiversified with their hosts, as their phylogenies are congruent with that of the host. However, only a small fraction of the analyzed bacteria showed signs of codiversification in these studies. On the other hand, examples of generalist gut symbionts exist as well[11,12]. This variation in the distribution patterns of different gut bacteria suggests they do not follow the same evolutionary trajectory across various animal species. Indeed, a recent study[13] showed that host specificity could vary across bacterial lineages in the animal gut (*i.e.*, there are generalists and specialists), and this may not be explained by microbial phenotype alone[14], with host ecology playing an important role[2]. This raises the question of why generalist and specialist gut bacteria exist and whether specialist bacteria primarily evolved *via* repeated switches between hosts or through codiversification with hosts.

Our current understanding of the evolution and diversification of gut microbial communities across hosts is limited by at least four technical and conceptual factors. (1) Previous studies have focused on highly diverse and variable systems across host individuals, making it challenging to understand whether microbiota change by acquisition of new members or by *in situ* evolution. (2) Significant differences in the relatedness and ecology of the compared populations hinder the identification of the drivers of community change over evolutionary time scales and the prediction of their functional consequences. (3) Samples from different animals often come from

different geographic regions, making geography an important confounding factor that could explain the host-restricted distribution observed for some gut symbionts. (4) Most comparative studies are based on 16S rRNA analysis, which lacks the resolution to determine the distribution of closely related bacterial species, strains and functional gene content across hosts[15].

Comparative studies at a high genetic resolution of the microbiota of closely related animals with similar phenotypes found in various geographic locations are needed to overcome these limitations. The gut microbiota of honeybee workers is a promising model offering the combined advantage of a simple yet specialized microbial community shared among related host species with similar ecology, social behavior, and overlapping geographic ranges[16–23]. Prominent honeybee gut microbiota members include Lactobacillus, Bombilactobacillus, Bifidobacterium, and several proteobacteria. These bacterial lineages from honeybees are usually monophyletic but belong to host-specific species, of which several can coexist simultaneously in the same host individual[24–26]. Thus, the predominant members of the honeybee gut microbiota have likely been acquired in a common ancestor of social bees and subsequently diversified in honeybees by separation into different host species and distinct ecological niches in the gut[16,27,28]. Co-diversification has been suggested as the primary mode of evolution, but formal tests of co-phylogeny have not yet been conducted. So far, most comparative studies are based on 16S rRNA amplicon sequencing with low genetic resolution or relatively few isolate genomes and biased towards isolates from the managed Western honeybee *Apis mellifera*, providing limited quantitative insights into the distribution of divergent strains and species across hosts. Shotgun metagenomics allows the sequencing of all genomes in a given sample, characterization of the distribution of community members at high resolution (down to the strain level) across hosts, and

assessment of the functional consequences of differences among hosts. However, this approach has only been used to study the Western honeybee (*Apis mellifera*)[20,29–31] and the Asian honeybee (*Apis cerana*)[32,33].

In this study, we applied shotgun metagenomics to individual worker bees of five different species of honeybees representing the most abundant and prevalent species of honeybees described so far, sampled across peninsular Malaysia and South India. From a total of 200 shotgun metagenomes from individual worker bee guts, we generated a comprehensive dataset of metagenome-assembled genomes (MAGs), expanding the catalog of the bacterial genomes available from these important pollinators and model for microbiome research. We reveal striking differences in host specificity across host and bacterial species and identify previously unrecognized but prevalent members of the bee gut microbiota that expand the functional repertoire of these communities in specific host species. Overall, our results show that ancient gut microbiota-host interactions can be maintained in the absence of co-diversification.

Results

An expanded catalog of bacterial genomes from the gut of five honeybee species

We shotgun sequenced (35M ±19M Illumina paired-end reads per sample) the hindguts of 200 individual worker bees from 40 different colonies of five honeybee species covering all three major clades of honeybees Micrapis (dwarf honeybee): *A. florea* and *A. andreniformis*, Megapis (giant honeybee): *A. dorsata*, Apis: *A. mellifera* and *A. cerana* sampled in South India and various locations across peninsular Malaysia **(Fig. 1A, Supplementary Table S1, Supplementary Fig. S1**).

We recovered a total of 1,959 high- and medium-quality metagenome-assembled genomes (MAGs, *i.e.* completeness score > 50% and contamination <5%) (**Supplementary Fig. S2**), including 156 from *A. florea*, 163 from *A. andreniformis*, 869 from *A. dorsata*, 324 from *A. mellifera* and 413 from *A. cerana*) (**Fig. 1B, Supplementary Table S2**). The MAGs were discriminated into 150 species spanning 23 genera (prevalence > 10%) using 95% ANI (average nucleotide identity) clustering (**Supplementary Fig. S3**). A majority of these species belonged to genera previously reported to be predominant in the gut microbiota of honeybees, including *Lactobacillus* (formerly Firm5), *Bombilactobacillus* (formerly Firm4), *Bifidobacterium*, *Gilliamella*, *Snodgrassella*, *Frischella*, *Bartonella* and *Apibacter* (constituted by 91 / 150 species clusters), but also lesser known genera such as *Dysgonomonas* (family Dysgonomonadaceae), *Saezia* (family Burkholderiaceae), *Pectinatus* (family Selenomonadaceae), *Entomomonas* (family Pseudomonadacea), *Pluralibacter* (family Enterobacteriaceae), WRHT01 (Desulfovibrionaceae family), CALYQQ01 (family Enterobacteriaceae) and CALYQJ01 (family Lactobacillaceae) (constituted by 21 / 150 species). Around 47% of the identified bacterial

species (71 / 150 species) have not been cultured to date, with many belonging to the predominant genera (41 / 71 species) and with genomic information currently lacking in public databases (**Fig. 1B**). The established collection of MAGs massively expands the catalog of bacterial genomes from the gut environment of honeybees.

Most honeybee species harbor host-specific microbial communities in their gut

We assessed the taxonomic diversity of the gut microbiota across the five honeybee species using our collection of MAGs. Between 7 - 56M reads from each sample mapped to these MAGs (**Supplementary Table S3**), providing sufficient depth of bacterial reads for MAG recovery from individual guts. Rarefaction curves show that our sampling effort prioritizing broad sampling from different colonies of each host species (**Fig. 1A**) was sufficient to cover most of the species-level diversity in each host species (**Supplementary Fig. S4**).

Prevalence of the identified genera varied greatly across host species (**Fig. 1C**). *Lactobacillus*, *Bombilactobacillus*, and *Bifidobacterium* were consistently present across all five honeybee species (**Fig. 1B**). All other genera were only found in a subset of the five host species or occurred sporadically throughout. Notably, samples of *A. dorsata* contained several additional genera in high prevalence, such as *Dysgonomas* (90.9%), *Pectinatus* (70.5%), and *Saezia* (90.9%), which were rarely found in the other host species.

Comparison of the species-level bacterial diversity revealed that *A. dorsata* contained the most diverse microbiota, followed by *A. mellifera*, *A. cerana* and then the two dwarf honeybees (**Fig. 1C**). This pattern holds when considering only species of the shared genera (*i.e.*, genera with a

prevalence of at least 10% in each host), except that *A. dorsata* and *A. mellifera* were not significantly different from each other (**Supplementary Fig S5**). Interestingly, the bacterial biomass per individual gut was the highest in *A. mellifera*, followed by *A. dorsata*, *A. cerana* and the dwarf bees, and hence, it cannot necessarily explain the differences in alpha-diversity (**Fig. 1E**). Most bacterial species displayed significant strain level diversity as measured by the percentage of single nucleotide variants (SNVs) per bacterial species within each bee gut with the possible co-occurrence of several closely related strains (**Supplementary Fig. S6**). The western honeybee *A. mellifera* hosted significantly more strains than other hosts within most bacterial species, while dwarf bees harbored the least strain-level diversity (**Fig. 1F**).

Taxonomic composition was host-specific at the species level as suggested by β-diversity analyses (PERMANOVA Sorenen distance Ω^2 =0.197, p=0.001, N=195), but with no distinction between the two dwarf bee species (**Fig. 1E**) and no influence of geography (Ω^2=0.009, p=0.078, N=131 excluding two host species sampled unequally across locations) (**Supplementary Fig. S7-9**). We found no evidence for phylosymbiosis, as there was no correlation between bacterial community distance and host divergence (Spearman's p-value = 0.49, mean q-value = 0.619±0.204 across 1000 mantel tests, **Fig. 1H**). For example, *A. andreniformis* and *A. mellifera* are the most distant host species but have much smaller distances in bacterial community composition than other pairs of host species.

Host specificity of individual community members varies across bacteria and host species

Specificity at the community level emerges because of the host-specific distribution of individual community members[13]. We calculated the specificity per bacterial species based on Rohde's

Index[34], which considers the prevalence of each community member across samples from different hosts (**Fig. 2A, Supplementary Table S4**).

Overall, we found large differences in the specificity of individual bacterial species both within and between genera. For example, the genus Bombilactobacillus mainly consisted of generalist species found across all five bee species, while the genera Lactobacillus or Bifidobacteria contained specialists and generalists (**Fig. 2A**). Some species showed an intermediate level of specificity as they were shared only among a subset of the five honeybee species and were more prevalent in one host than another (*e.g.*, one of the *Snodgrassella* species). Few shared genera consisted of only specialist species (*Apibacter* and Commensalibacter). Notably, there was a clear difference in the specificity of bacterial species across hosts. The microbiota of *A. dorsata* and *A. mellifera* were mainly composed of specialist species, while the microbiota of the smaller bee species (*A. cerana*, *A. florea*, and *A. andreniformis*) was primarily composed of shared species (**Fig. 2A**)

Bacterial species may be shared between host species but exhibit host specificity at the strain level. To look for such strain-level host specificity, we measured the extent of shared SNVs between samples for each bacterial species shared between two or more host species using popANI[35]. We found both bacterial species that segregate and species that do not segregate by host species in terms of strain-level composition (**Fig. 2B, Fig. 2C**). This pattern was observed across all analyzed genera (**Fig. 2B, Fig. 2C**). Moreover, in some cases, we also found that samples segregated by sampling location (**Fig. 2B, Fig. 2C**). Overall, 75% of the species were host-specific at either strain or species level.

Host switches and species gains and losses interrupted the host-specific evolution of gut microbiota members of honeybees

Codiversification has been described in other host-associated microbiomes as an important driver of host-specificity patterns[6,9,10]. To test for evidence of codiversification in the honeybee gut microbiota, we constructed core genome phylogenies for all prevalent genera using the collection of MAGs assembled from the five honeybee species (**Fig. 3**). We used genus-level phylogenies to test for codiversification because divergence time within genera was roughly equivalent to divergence times in the *Apis* genus (see methods). While most honeybee-derived MAGs from a given bacterial genus were monophyletic relatives to bacteria found in bumble bees and stingless bees, they belonged to distinct clades corresponding to the species clusters determined by ANI (see above). We identified several novel lineages of bacteria, especially in the giant honeybee *Apis dorsata*, that have not been isolated or represented in public databases before. Among these are several species of the genus *Dysgonomonas* forming several monophyletic clades, related to species isolated previously from cockroaches[36] and human stool[37], among other sources. In agreement with the observed differences in host specificity, a subset of the identified species-level clades were exclusive to one host species (24% - 47% of all nodes), while others were mixed. To test for codiversification, we used the Hommola cospeciation test, a generalization of the Mantel test for arbitrary host-parasite interaction graphs[38]. We then carried out second-order permutation tests (N=100) with shuffled tip labels to account for the rate of false discoveries as described before[39]. Overall, we found that in all nine tested genera (trees containing multiple nodes containing host-specific clades from three or more host species), we detected no more significant codiversifying nodes than expected by chance (**Supplementary Table S5 and Supplementary Table S6**). Overall, we show that honeybees and their gut bacteria did not

codiversify despite high host specificity. Instead, as seen in the phylogenies (**Fig. 3**), deep-branching host-specific clades are interrupted by recent generalist clades, and deep-branching generalist clades comprise adjacent host-specific clades (and show more host-specificity at the strain level), indicative of a history of intense shuffling of the associations.

Functional differences in the gut microbiota across honeybees

To test whether the host specificity observed at the taxonomic level translates into functional host specificity of the gut microbiota, we performed a functional analysis of the MAGs. Of 4,759,622 prokaryotic ORFs in our MAG database, more than half (2,685,895 ORFs) were assigned a KO identifier. The rarefaction curve analysis of these KOs indicated that most of the functional diversity across the analyzed samples was recovered with our MAG database (**Supplementary Fig. S10**). Overall, the number of KOs per sample followed a similar trend as the taxonomic diversity, with *A. dorsata* having the most and *A. florea* having the fewest KOs (**Fig. 2A**).

Multivariate analysis of normalized KO counts (and the Robust Aitchison distance) showed that the functional composition of the gut microbiota of five honeybee species was distinct, even between *A. florea* and *A. andreniformis*, which overlapped in composition at the taxonomic level (Ω^2=0.312, p=0.001). To understand which bacteria drive these differences, we performed a similar analysis using counts of all KO families in each high- and medium-quality MAG across all samples. As expected, MAGs of bacterial species from the same genus clustered across all host species, suggesting that they occupy similar functional niches and that the overall functional capacity of the bee microbiota is conserved across hosts. Yet, there were some important differences. Some of the genera only found in *A. dorsata* formed distinct clusters (*e.g.*,

Dysgonomonas), suggesting that these bacteria occupy unique functional niches in the microbiota of this host. Other genera (*e.g.*, Saezia and Entomomonas) clustered with community members prevalent in other honeybee species (*e.g.*, Snodgrassella) but absent from *A. dorsata*, indicating potential functional replacements. Finally, although MAGs from the same genus mostly overlapped in terms of their functional profile, we found some cases where they clustered by host of origin, suggesting host-specific differences in the functional repertoire (and potentially, adaptation) within these genera (**Supplementary Fig. S11)**.

Since pollen-derived glycan (*i.e.*, pectin and hemicellulose) breakdown has been described as one of the significant catabolic functions of the gut microbiota *of A. mellifera*[30,40], we specifically looked at the Carbohydrate-active enzyme (CAZyme) repertoire of the microbiota across the five host species. We detected 19,562 CAZyme families among the prokaryotic ORFs in our MAG database. Overall, the composition of these gene families was host-specific (**Supplementary Fig. S12**). While all five honeybee species harbored a similar set of CAZymes for pectin, hemicellulose, and cellulose degradation, *A. dorsata* contained a more extensive and diverse set of such gene families, especially many of which are involved in pectin degradation (**Fig. 4D**). To understand which bacteria drove these differences, we considered the number of genes belonging to CAZyme families from each bacterial species. Previously recognized members contributed most families to degrade pollen-derived glycans, specifically *Gilliamella* and *Bifidobacterium* (**Fig. 4E**), except in the giant honeybee *A. dorsata, where* the genus *Dysgonomonas* also contributed key enzyme families involved in pectin degradation such as PL1, PL11, GH28, GH78, GH128, or CE12. Interestingly, the CAZyme genes of Dysgonomonas were often organized in large genomic islands reminiscent of specialized PULs (polysaccharide utilization

loci) found in gut symbionts of mammals[41], suggesting that this community member expands the functional capability to degrade plant-derived fibers in this particular honeybee species (**Fig. 4F**).

Discussion

Many animals harbor host-specific bacterial communities in their gut[2–4,13]. However, it is still unclear whether and why host specificity varies among closely related host species or bacterial lineages, which evolutionary processes underlie the observed patterns of specificity and their functional consequences. Our comparative shotgun metagenomic analysis of the gut microbiota of five honeybee species provides novel insights into these questions. It advances our understanding of the evolutionary processes that govern gut microbiota composition across closely related animal species.

We find that the community composition of the gut microbiota of three of the five analyzed honeybee species is host-specific, confirming previous results based on amplicon sequencing[16] or less comprehensive metagenomic datasets[32]. However, in contrast to *A. mellifera*, *A. cerana* and *A. dorsata*, the gut microbiota of the two phylogenetically ancestral dwarf honeybee species, *A. florea* and A. *andreniformis*, were indistinguishable from each other, suggesting that host specificity is not absolute but can vary between closely related host species. Our genome-level data, coupled with our broad sampling design, allowed us to probe further than community-level patterns to identify the host-specificity of individual clades of community members.

We found that 75% of the bacterial species detected were host-specific at the bacterial species or strain level. First of all, we found that host specificity emerges at different taxonomic levels, for example, at the genus (such as *Saezia* and *Dysgonomonas* in *A. dorsata*), species (such as many *Gilliamella*, *Snodgrassella* and *Lactobacillus* species), and strain level (such as *Bombilactobacillus mellis-36_2*). Considering that all of these bacteria belong to clades specific

to social bees, this suggests that the host-specific association between different gut symbionts and their honeybee hosts has been established across different phases in their evolutionary history. These findings also highlight that host-specific associations can be overlooked without strain-level/genomic resolution.

Another key finding of our analysis is that the degree of host-specificity varies across bacteria (i.e., there are generalist and specialist species). This has also been observed in other systems[13,14,42] and linked to bacterial phenotypes, such as aerotolerance and spore-formation, that affect dispersal ability[13,43–46]. However, in addition to previous work, we observed unexpected high variability in host specificity between closely related bacteria, i.e., species belonging to the same genera, suggesting that microbial traits, such as aerotolerance, usually conserved within genera, cannot explain variation in specificity patterns. Instead, we find host identity to be an important determinant of the specificity of individual community members. Most of the highly host-specific bacterial species were found in *A. dorsata* and *A. mellifera*, while the communities of *A. cerana* and the two dwarf honeybees were dominated by shared bacterial species. *A. mellifera* and *A. dorsata* guts may harbor more specific ecological niches than the other bee species, facilitating the colonization of additional 'specialist' bacteria. This is consistent with the observation that *A. cerana* and the two dwarf bees harbor significantly fewer bacterial species per individual bee than *A. dorsata* and *A. mellifera*. The smaller body and colony size of these bees may limit carrying capacity and niche diversity in the gut, as proposed previously[16,32]. Another possibility is that the host control over their gut microbiota is stronger in some bee species than in others, as observed in other animal species[47]. Still, the reason for this variation is unclear.

In contrast to studies of other social bees[16,27,48,49], we did not find any evidence for co-diversification in the honeybee gut microbiota. This might be because previous analyses did not (1) apply extensive tests and corrections[39], (2) did not use genome-scale phylogenies, or (3) did not sample the full breadth of microbiota diversity as they relied on isolate genomes. Our results also contrast with codiversification findings in mammals and, in particular, hominids[5–10]. However, even in hominids, only 16%-36%[6,8,10] of all tested bacterial clades showed statistically significant signals of codiversification, suggesting that the evolution of only a minority of the microbiota is driven by codiversification and that codiversification might be the exception rather than the rule in both mammals and social bees.

Overall, our results show that ancient gut microbiota-host interactions can be maintained in the absence of codiversification. Instead, host switches and symbiont gains and losses between related species have been frequent, suggesting that they govern compositional changes in gut microbiota over evolutionary time. This is surprising given the social lifestyle of bees that facilitate within-host species transmission mode. In contrast, ecological opportunities for spillover and invasion of the gut microbiota are not uncommon, maybe due to the sympatric distribution of *Apis* species. Furthermore, since all five honeybee species have similar dietary preferences, gut structure, and physiology and lack an adaptive immune system, the barrier to colonization between native and non-native bacteria may be low. This is supported by experiments with microbiota-free bees, which showed that bacterial strains isolated from honeybees can colonize a non-native honeybee species and sometimes even outcompete native bacterial strains[16,28].

The host-specific nature of the microbiota of these honeybees translates into host-specific microbial functional potential. In particular, both the specific repertoire of conserved members and the gain of new members with unique potential significantly impacted microbiota functions. For example, honeybee-specific lineages of *Dysgonomonas*, a genus of polysaccharolytic Bacteroidetes also present in the gut of cockroaches, termites, and humans[36,37,50], greatly expanded the repertoire of pectin and hemicellulose degradation genes in the gut microbiota of *A. dorsata* without necessarily replacing another gut symbiont. A. dorsata may be distinct in its broad exposure to more diverse microbes compared to other honeybees, given its highly migratory nature[51]. Interestingly, differences in diet may not explain differences in microbiome diversity, considering that *A. dorsata* tends to forage on fewer mass-flowering plants[52] , and plant-pollinator network analysis suggests that *A.florea*, *A. cerana*, and *A. dorsata* show substantial overlap in the flowers they visit[53]. In summary, the honeybee gut microbial community shows a highly dynamic evolution despite the presence of a few conserved lineages.

Our results form the basis for more detailed functional characterization of diverse bacterial gut symbionts previously not recognized as important members of the honeybee gut microbiome. It vastly expands our knowledge of bee microbiota obtained chiefly from the managed western honeybee. More generally, it advances our understanding of host specificity in gut microbiota-host associations by showing that even ancient gut microbiota-host interactions can be maintained in the absence of co-diversification via dynamic gain, losses and exchanges of microbes. This history of frequent turnover can have a critical impact on microbiome functions

and suggest that apparently conserved ancient gut microbiota–host associations are actually susceptible to change over evolutionary time.

Methods

Sampling and storage

All honeybee workers (10-50 individuals per colony) collected from colonies across locations described in the metadata sheet (**Supplementary Table S1)** were stored in small ventilated boxes and processed for storage within the day. We stored the bees in absolute ethanol at -20°C until dissection and DNA isolation, sufficiently preserving the sample for sequencing the microbiome[54]. Our sampling strategy prioritized covering many colonies across locations to capture the diversity of each host species rather than many individuals from the same colony, which has been done before[29,32].

Dissection and DNA extraction

As the hindgut contains most of the bacterial biomass, we aimed to decrease the ratio of DNA from the host cells to bacterial cells. Hence, we removed the gut tissue by carefully opening the cuticle, removing the stinger and venom glands, and, finally, cutting the tissue near the pylorus, removing the malpighian tubules and sections, including the midgut and above. Then, the tissue was transferred to a bead-beating tube containing 478 µL of sterile PBS and 2.0 mm sterile glass beads and subjected to bead-beating for 30s at 6 m/s in FastPrep-24 5G (MP Biomedicals). Next, 0.1 mm zirconia beads along with 250 µL of 2x CTAB, 2 µL of β-mercaptoethanol and 20 µL of proteinase K (20 mg/mL) was added and bead-beating was carried out for 30s at 6 m/s in FastPrep-24 5G (MP Biomedicals). The homogenate was incubated for 1h at 56°C. Next, 750 µL of Phenol-chloroform isoamyl alcohol (25:24:1, v/v) at pH 8.0 was added and mixed thoroughly, followed by centrifugation for 10 min at 16,000 xg. The upper aqueous layer was transferred to a new tube, and 500 µL of chloroform was added and mixed thoroughly. The mixture was

centrifuged for 10 min at 16,000 xg, and the 300 µL of the upper phase was mixed with 900 µL of chilled absolute ethanol and incubated at -20°C overnight. Next, the precipitated DNA was pelleted by centrifugation for 30 mins at 16,000 xg at 4°C. After two washes with 70% ethanol, it was resuspended in 50 µL of nuclease-free water. Next, it was treated with RnaseA and purified using magnetic beads (Clean NGS). DNA concentration was measured using Qubit™ (Thermo Fischer Scientific). Bacterial absolute abundance was measured using qPCR with primers targeting the 16S rRNA region as described earlier[55].

Metagenomic sequencing

Library preparation was carried out using the Nextera XT library kit (Illumina), according to manufacturer instructions, with the number of PCR cycles chosen to account for the amount of DNA in samples. Illumina sequencing was carried out using Novaseq (2x 150 bp) with a target of 10-50 million microbial reads. Since there was a significant difference between host species in the total gut microbial content as determined by qPCR using universal primers, first, a run of sequencing was carried out without normalization of the amount of samples added to the library to estimate the percentage of host DNA and microbial DNA in the library made from each sample. This was useful in ensuring that there was enough DNA from the bacterial community in all the samples, including those where the library might have been dominated by host DNA due to lower bacterial biomass. Further sequencing was then carried out after pool correction, where the library was adjusted in such a way when possible that at least 15 million microbial reads were obtained (based on expectation from the first shallow sequencing), even for the samples with the lowest percentage of microbial DNA. All library preparation and sequencing was done at the Lausanne Genomic Technologies facility (GTF), UNIL. Details about all further bioinformatic analysis, including the code used, can be found on GitHub

(https://github.com/Aiswarya-prasad/honeybee-cross-species-metagenomics) and Zenodo (https://zenodo.org/doi/10.5281/zenodo.13732977) in the repositories.

Mapping to databases

To assess the amount of bacterial, host, and other reads in each sample, reads were mapped to host and MAG databases. The host database comprised draft or complete genomes of all the four host species (GCA_000184785.2, GCA_000469605.1, GCA_001442555.1, GCF_003254395.2) except *Apis andreniformis* since no genome was available in public databases for this host species. A database was compiled using representative MAGs identified for each 95% ANI cluster or bacterial species with the highest quality score (calculated as described by dRep[56] using various MAG characteristics, including checkM contamination and completeness). Trimmed and quality-filtered reads were mapped to the host database using BWA-mem[57] followed by the MAG database (and *vice versa* to account for non-specific mapping). Three samples were removed from the downstream analysis as their metagenomic reads did not map the host that they were labeled as (**Supplementary Table S2**) and might have faced cross-contamination during sample processing. Samples for which relatively few reads mapped to the MAG database often were also low in bacterial biomass as measured by qPCR (**Supplementary Fig. S13**). Indicating that the sequencing and workflow to detect microbial diversity in each sample was otherwise robust. Initial taxonomic profiling of the reads using mOTUs[58] detected the expected core marker genes from various honeybee gut microbiota members.

Assembly and binning of MAGs

Raw reads were trimmed and quality filtered before assembly using Trimmomatic[59] and bbtools. For some samples that had a large number of reads (19/150 samples) due to which assemblies

were not successful, bbnorm was used to down-sample regions of high depth and for samples with too many host reads due to which assemblies failed (3/150 samples), host-filtered reads were used. The detailed workflow used for assembly and binning into MAGs is described in GitHub (https://github.com/Aiswarya-prasad/honeybee-cross-species-metagenomics). Briefly, contigs were assembled using metaSpades[60]. Filtered reads were mapped against assembled scaffolds (all against all samples), and MetaBAT2 used the coverage information[61] to bin scaffolds into MAGs. The quality and completeness of resulting MAGs were assessed using CheckM[62] and low (< 50% completeness or >5% contamination), medium (50-90% completeness and <5% contamination), high (> 90% completeness and <5% contamination). MAGs were then clustered into groups averaging 95% average nucleotide identity (ANI) using dRep[56].

Taxonomic annotation was carried out using GTDB-tk[63]. No manual curation of MAGs was performed; however, Kaiju[64] (nr database) and Kraken2[65] (custom database including bacteria, viruses and honeybee) classification of scaffolds screened by random sampling further confirmed that scaffolds from the same MAG matched each other and the taxonomic assignment of the MAG. Each cluster of MAGs was named to reflect the GTDB species name or, in cases where none was assigned, a unique identifier, including the genus name. Combined with the inspection of ANI heatmaps (**Supplementary Fig. S13**), a final set of species names was assigned to each of the 95% ANI clusters.

Taxonomic profiling

InStrain[35] was used according to its recommended workflow for taxonomic profiling at the bacterial species and strain level. Trimmed and quality-filtered reads were competitively mapped

to the database of representative MAGs using the tool bowtie2[66], which presents mapping scores in a format compatible with InStrain. Among many approaches to detecting microbial species in a dataset, this stands out in its appropriate filters applied to mapped reads, including read ANI, which helps differentiate genomes of closely related species (<95% ANI) and breadth of the genome mapped to avoid falsely identifying species as present due to high coverage of a region shared with others or horizontally transferred. Moreover, genome coverage of a given species can be misleading when comparing communities of varying diversity, as an equally abundant species would have higher coverage in a community with low diversity. A species was considered present if it had a median coverage of at least 1x across all scaffolds and its genome (comprising all scaffolds) was at least 50% covered by reads. Relative coverage, when used, was obtained by dividing the coverage of each species by that of the sum of all and qPCR copy numbers of the bacterial 16S rRNA region were used to estimate absolute abundance for comparison between samples where mentioned. For samples from India (n=50), qPCR values were interpolated based on the median of the host species. The underlying data (presence-absence, relative abundance etc.) and normalization approach are explicitly indicated for each downstream analysis section. After filtering samples as mentioned earlier and removing samples with fewer than 1 million filtered reads mapping to the representative database of MAGs, a total of 195 samples were included in the analysis (*A. mellifera* 34, *A. cerana* 45, *A. dorsata* 44, *A. florea* 42 and *A. andreniformis* 30).

Strain-level composition profiles for each bacterial species in the representative MAG database were generated per sample using InStrain in database mode. The percentage of variant sites was calculated as the percentage ratio of the number of SNVs to the total length of the MAG covered.

A site is counted as an SNV if the site is covered at least 5x and the allele is present at a frequency of at least 0.05. Regions mapping with a read ANI of <0.92 (might be cross-mapping of reads from a different species) or coverage of <1x were not considered. To compare the strain profile of samples, the popANI measure, as comprehensively described by InStrain[35], was used. Briefly, a value from 0 to 1 was calculated for each species for each pair of samples in which the genome was covered at least by 5x and at least half of the genome was covered in both samples. A position of the genome was considered if there was more than one allele with a frequency of at least 0.05. If both samples share no alleles in that position, it is counted as a popANI substitution. The value for that pair of samples was then calculated as the ratio of positions containing popANI substitutions and the number of positions compared. This value was used to create a matrix of Jaccard distances between all pairs of samples.

Phylogeny construction and codiversification test

To gain a rough estimate of the taxonomic level at which to expect codiversification, we computed a core genome phylogeny using the nucleotide sequence of 120 bacterial marker genes for all medium and high-quality MAGs identifying core genes using GTDB-tk, aligning in a codon-aware manner using MACSE[67] and constructed using IQ-TREE[68]. From this tree, the median substitutions per site for MAGs of the same species was 0.014, for different species of the same genus was 0.39, and for different genera was 2.65 (**Supplementary Table S7**). Bacteria evolve at a rate in the order of magnitude of 10^{-3} substitutions per site per million years[69]. Since the *Apis* clade is about 30 million years old[70], gut microbes that diverged during this timeline could be as divergent as different species within a genus or closer, confirming that genus-level trees are appropriate for these tests.

OrthoFinder[71] was used to infer orthologous gene families of all medium and high-quality MAGs for each genus along with previously published genomes of the same genus from other environments as outgroup (details of genomes used in metadata files included in the Github (https://github.com/Aiswarya-prasad/honeybee-cross-species-metagenomics) and Zenodo (https://zenodo.org/doi/10.5281/zenodo.13732977) repository. Phylogenies were constructed using IQ-TREE using the amino acid sequences of genus-level single-copy core genes aligned by MAFFT[72]. Since medium-quality MAGs were included in phylogenies, core genes can be randomly missing in some MAGs. Naively defining core genes as those present in all MAGs would result in the exclusion of most orthologs. Hence, as implemented in OrthoFinder, when <1000 single-copy orthologs are present in all MAGs, it iteratively identifies the orthologs that are found once in N-1 MAGs until either considering one less MAG does not double the number of orthologs or more than half the MAGs are dropped. Core single-copy orthologs identified were in agreement with those orthologs identified as core by mOTUpan[73], which uses a Bayesian approach informed by the completeness score of MAGs. The number of orthologous gene families (OG) used for core genome phylogenies and the minimum percentage of MAGs of the genus in which each OG was detected (*e.g.*, 100% means all OGs were detected in all MAGs and 50% means every OG was found in 50% or more of the MAGs): Apibacter - 1096 (94.5%), Apilactobacillus - 704 (81.8%), Bartonella - 705 (81.5%), Bifidobacterium - 118 (84.0%), Bombilactobacillus - 194 (89.4%), CALYQJ01 - 883 (89.5%), CALYQQ01 - 1221 (84.6%), Commensalibacter - 1120 (96.6%), Dysgonomonas - 223 (86.5%), Entomomonas - 1144 (91.7%), Frischella - 984 (85.9%), Gilliamella - 189 (88.0%), Pectinatus - 1143 (97.3%), Pluralibacter - 1419 (100.0%), Saezia - 1000 (92.3%), Snodgrassella - 799 (91.5%), WRHT01 - 1897 (100.0%)]. Further information about the parameters and best-fit model chosen to compute

the phylogenies can be found in the log files included in the corresponding directory containing IQ-TREE results in the Zenodo (https://zenodo.org/doi/10.5281/zenodo.13732977) repository.

A host phylogeny inferred based on complete mtDNA sequences of honeybee species rooted by a bumble species published before[74] was used for codiversification tests. Tests were carried out using the Hommola cospeciation test[75] (implemented in Python) on all nodes containing at least 7 MAGs from at least three host species and with a maximum tip-tip distance not exceeding 1 for 100 permutations. This was carried out on trees with tip labels artificially permuted randomly (N=100) to estimate the number of nodes displaying significant results by chance, as described in an earlier study[39]. Thresholds were set at various levels for comparison of the number of significant nodes in the original tree and the median number of significant nodes in tip label permuted trees: relaxed ($p<0.05$), medium ($p<0.05$ and $r>0.75$) and strict ($p<0.01$ and $r>0.75$).

Gene functional annotation and profiling

Open reading frames (ORFs) were identified on all assembled scaffolds of each sample using Prodigal[76]. ORFs marked partial or shorter than 300 bp were removed. Further, those ORFs detected on scaffolds identified as being of eukaryotic origin by Whokaryote[77] or unclassified as bacteria by Kaiju (nr database) were also excluded. The remaining ORFs were then annotated using DRAM[78] to obtain KEGG ortholog (KO) identities. Finally, the gene catalog comprising all these ORFs was assigned to CAZyme families as implemented in Cayman[79].

Trimmed and filtered reads from each sample were mapped back to their respective assemblies using bowtie2. Low-quality ($Q<20$) alignments were removed, and coverage per gene/ORF was calculated using BEDtools[80]. Genes were considered detected if >5 reads were mapped to it and

> 90% of the bases in the gene were covered by at least a read. The abundance of each gene was then calculated in terms of reads per kilobase per million (RPKM) calculated as follows: $RPKM = \frac{\#\,Mapped\ reads}{\left(\frac{Gene\ length}{1000}\right) * \#Total\ reads * 10^6}$. For KOs and CAZymes, summed RPKMs of constituent genes were used. To estimate the average count of CAZymes of interest (all families other than GT and AA families) in each genus per individual, the RPKM of all CAZymes in each genus was summed per sample, and then the average of this sum across samples was converted to a relative proportion to represent the relative contribution of each genus on average in each host species and plotted. Only genera with a prevalence >0.05 in the respective host were considered.

Statistical tests and bioinformatic analyses

All details about the specific parameters used for each tool and their versions, along with conda environment specifications, are included in the Github (https://github.com/Aiswarya-prasad/honeybee-cross-species-metagenomics) and Zenodo (https://zenodo.org/doi/10.5281/zenodo.13732977) repositories. In general, bioinformatic workflows were implemented using Snake make (v7.28.1) to parallelize jobs on a computing cluster at UNIL, preprocessing and filtering using Python scripts, visualization using ggplot with several related packages in R and iTOL for phylogenetic trees and arrangement and decoration of plots using Adobe Illustrator. Significance in boxplots was tested using the Wilcoxon Rank Sum test (significant if $p<0.05$). Distance matrices were calculated using the method indicated in their respective plots- The package Betapart[81] was used to estimate the turnover component of the Jaccard and Sorensen distances. Multivariate analyses were visualized using PcoA or NMDS and tested using the adonis2 function implemented in the R package Vegan. The effect size was adjusted for sample size to get Ω^2 using the adonis_OmegaSq function from the package

Russel88/MicEco (https://zenodo.org/badge/latestdoi/83547545). To test for phylosymbiosis, we minimized bias due to pseudo-replication by performing 1000 permutations. Each permutation included five randomly selected samples, one of each host species, tested for correlation between bacterial and host distances using a Mantel test (as implemented in the Vegan package). The p-values were adjusted using the FDR method.

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Author contributions

Conceptualization – PE, SHY, AP, FM; Methodology – AP, FM, PE; Software – AP; Validation – AP; Formal analysis – AP; Investigation – ADP, AP, RS, AS; Resources – PE, SHY, AB; Data curation – AP; Writing - Original Draft – AP; Review and Editing – PE, FM, AP, AB, SHY; Visualization – AP; Supervision – PE, SHY, FM, AB; Project administration – PE, SHY, AB; Funding acquisition – PE, SHY

Competing interests: All authors declare they have no competing interests.

Data availability: Raw metagenomic data has been deposited to the NCBI Sequence Read Archive (SRA) under the Project ID PRJNA1157353. The collection of MAGs, some important

intermediate files, and tables can be found in the Zenodo (https://zenodo.org/doi/10.5281/zenodo.13732977) repository.

Code availability: Code and details of parameters and software used are available at https://github.com/Aiswarya-prasad/honeybee-cross-species-metagenomics archived at https://zenodo.org/doi/10.5281/zenodo.13732977

Figures

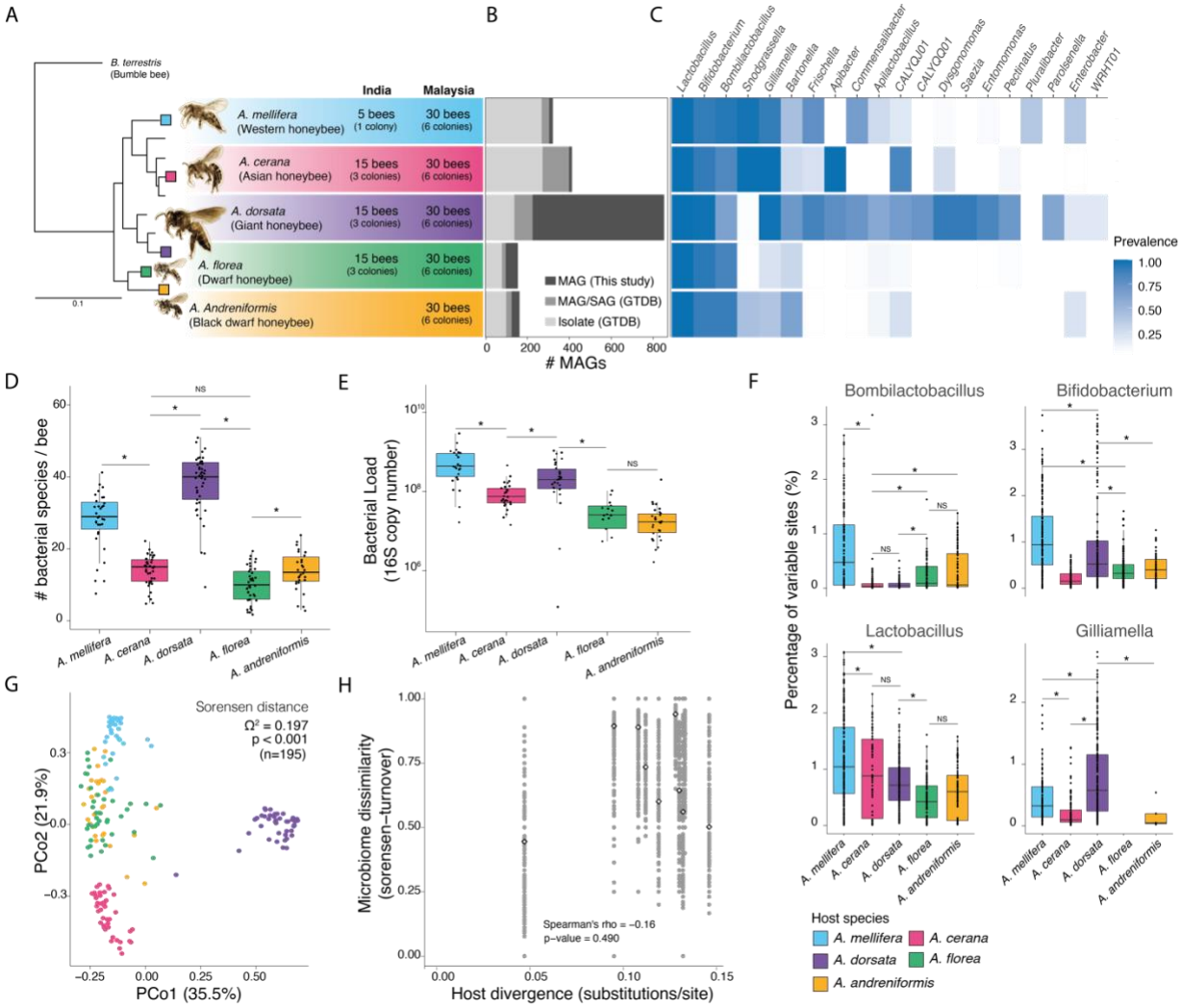

Figure 1. Community-level patterns in the honeybee gut microbiome across taxonomic scales. (A) Apis bee phylogeny with tips indicating extant species and the colored tips species included in this study. Pictures of bees in [82] are to-scale. Numbers and text next to species names indicate the sampling design. (B) Barplot showing the number of MAGs recovered from this study for which no isolate genomes of the same species (>95% ANI) were found in GTDB, those that were represented by MAGs or SAGs in GTDB and those that are similar to published genomes from isolated bacteria represented in GTDB. (C) Heatmap with the prevalence of each

genus within each host species showing genera with prevalence > 10% excluding the following three: *Klebsiella* (*K. pneumonia*) – 10% in *A. mellifera*; Pantoea (P. dispersa) – 17% in *A. mellifera*; *Melissococcus* (*M. plutonius*) – 13% in *A. andreniformis*). (D) Boxplot of the number of bacterial species in each honeybee gut across host species. (E) Boxplot of the number of 16S rRNA gene copies per 0.1μL of individual bee gut homogenate across host species. (F) Boxplot of the percentage of variable sites per individual of each host species per genus (four most prevalent genera shown), with each box including points represented by all species of the respective genus found in individuals of that host species. (G) PCoA plot based on the turnover component of Sorensen distance across host species. (H) Scatter plot of microbiota dissimilarity (as the turnover component of Sorenson distance) as a function of host species divergence based on 16S+12S rRNA[83] with white points with thick black outlines marking the median of values for the comparisons of all pairs of samples from a given pair of host species.

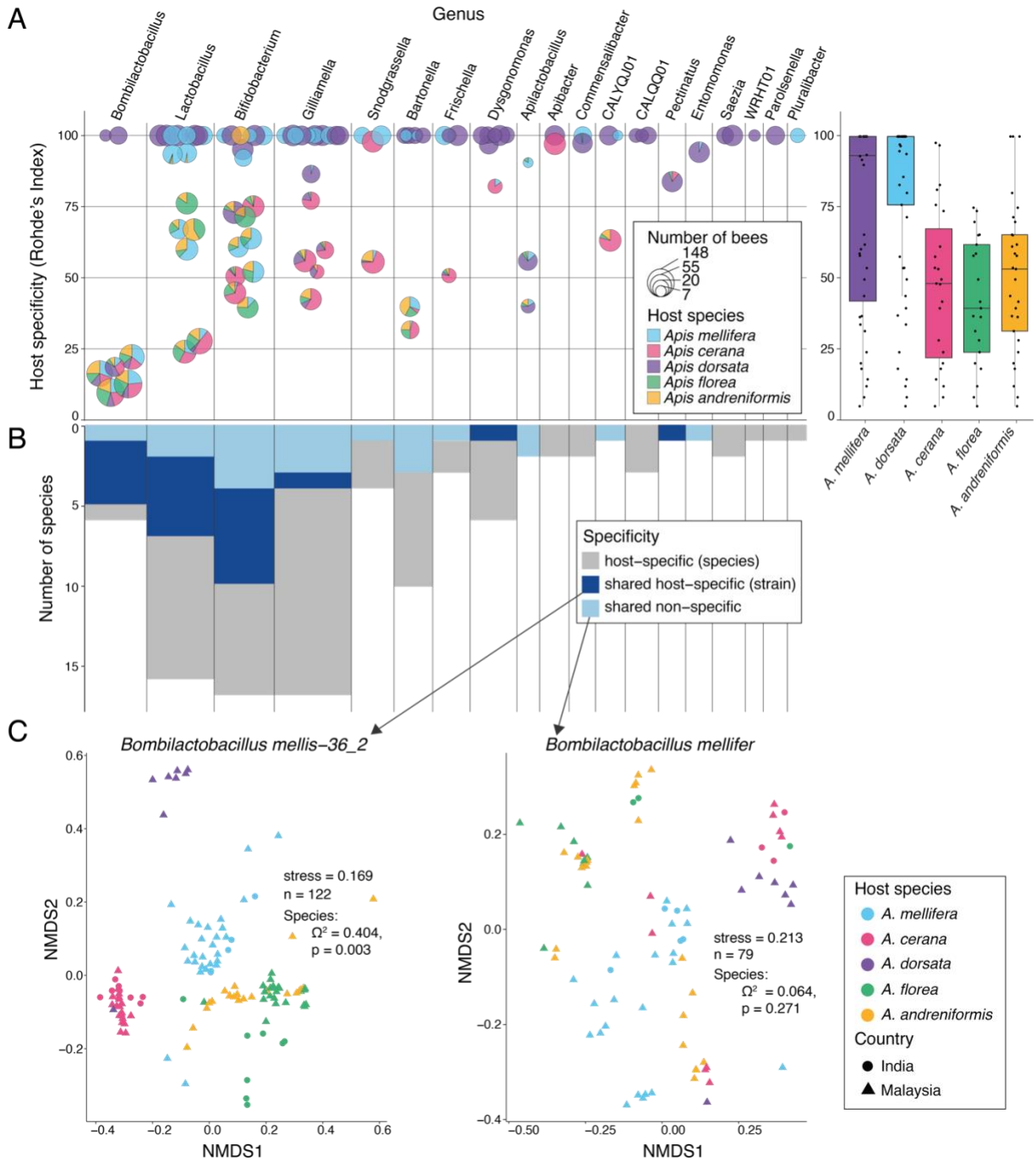

Figure 2. Host-specificity of individual gut microbial lineages across honeybee hosts. (A) Scatter-pie plot of Rohde's index of specificity with each circle representing a bacterial species with the size representing the number of bees in which the species was found, and the pie chart showing the proportion of samples belonging to each honeybee host. The adjacent boxplot shows

Rohde's specificity index for each species found in each honeybee host. (B) Distribution of species richness per genus across three categories of specificity levels: host-specific at the species level (grey), host-specific at the strain level, but the species is detected across hosts (dark blue) or not specific even at the strain level (light blue). (C) NMDS plots from a strain level generalist and specialist bacterial species are included as an example. The Jaccard dissimilarity was calculated in terms of strain level composition (popANI) tested using PERMANOVA (significant if the p-value is < 0.05). Only bacterial species in at least five samples and at least two host species and samples with enough coverage, the bacterial species to recover SNPs, were included.

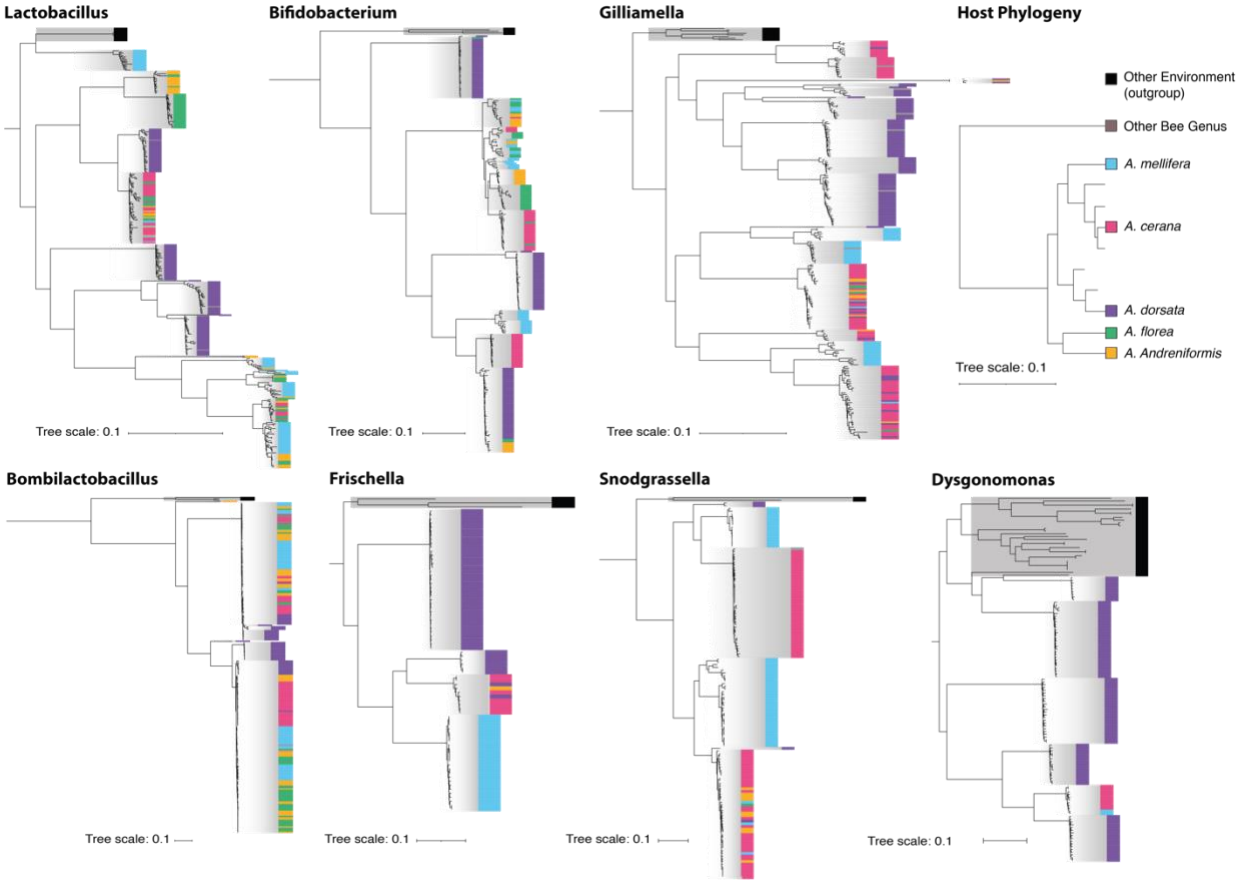

Figure 3. Bacterial genus-level core genome phylogenies of MAGs. Core genome phylogenies of MAGs from various genera found in more than one host species with the color at the leaf tip indicating the host species from which the MAG was recovered and solid grey boxes the set of genomes included to represent outgroup species of the genus from environments other than honeybee guts. The scale bar represents 0.1 amino-acid substitutions per site. The phylogenies are maximum likelihood trees inferred by IQ-TREE (see Methods).

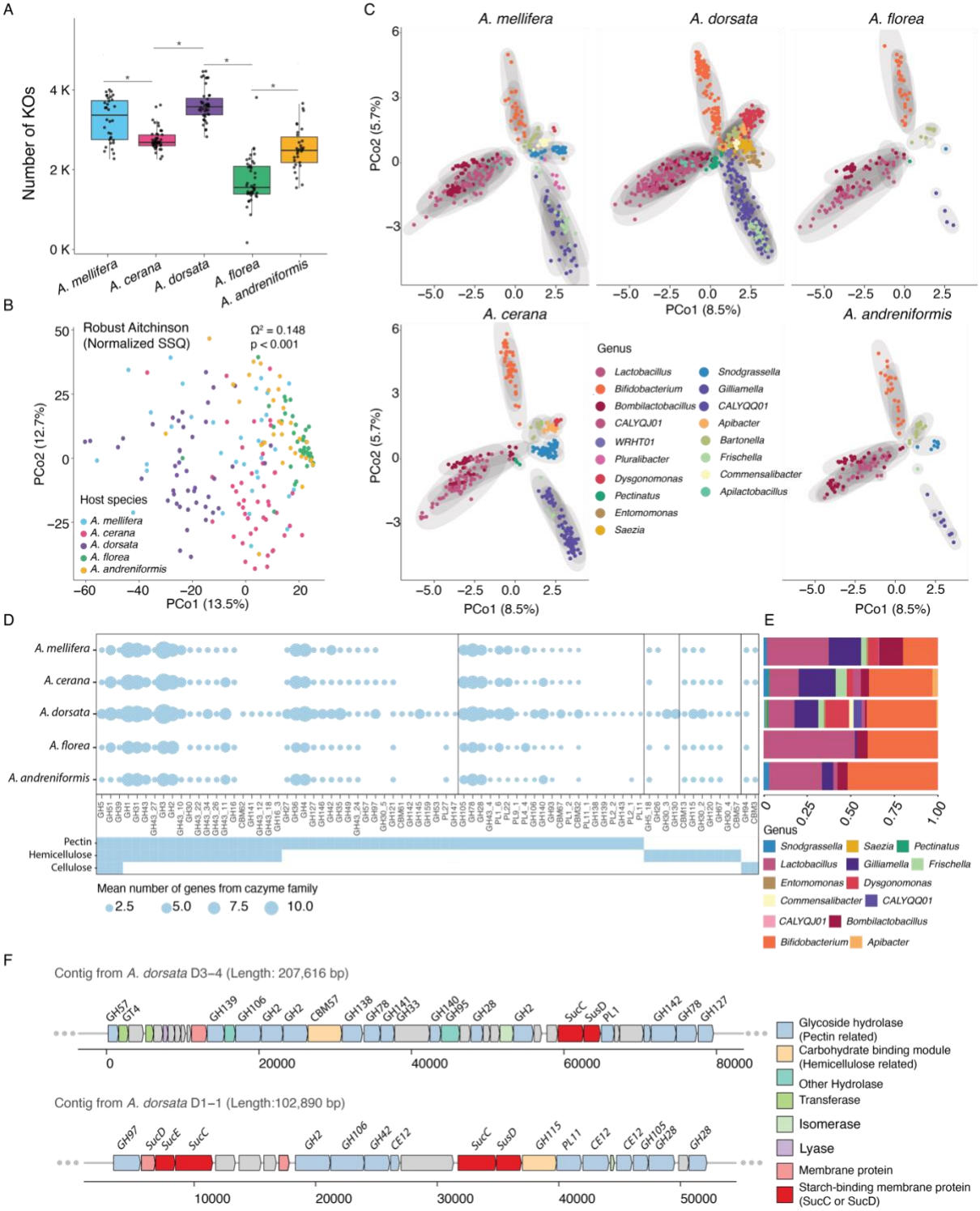

Figure 4. Functional potential of honeybee gut microbiota across host species. (A) Boxplot of the number of KEGG ortholog (KO) families detected per individual of each host species

Wilcox test ($p < 0.05$) indicated on the plot. (B) PCoA of KO family composition across all samples was visualized using the Robust Aitchison distance on a matrix of reads per kilobase per million (RPKM) summed across genes belonging to the respective KO family. Each point on the plot represents an individual bee gut. PERMANOVA test indicated on the plot ($p < 0.05$). (C) PCoA of all KO families from genes annotated within MAGs across all samples from each host species plotted on the same scale across host species using the Robust-Aitchison distance on the RPKM matrix of KOs of MAGs such that each point on the plot represents a MAG recovered from an individual bee. (D) Dotplot of the mean number of genes from each CAZyme family with a potential role in pectin, hemicellulose, or cellulose degradation per individual of each host species. (E) Barplot of the proportion of genes from CAZyme families contributed by each bacterial genus. (F) Loci within Dysgonomonas MAGs encoding putative polysaccharide utilization loci (PULs).

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