The gut microbiome of honey-producing wasps has converged on that of social bees

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Competing interests: The authors declare that they have no competing interests.

Key words: microbiome, honey wasps, paper wasps, honey bees, bumble bees, diet switch

ABSTRACT

Honey-feeding social bees, including honey bees and bumble bees, possess distinctive gut microbiomes that provide benefits to hosts, such as defense against pathogens and parasites. Microbiome members are transmitted through social interactions within colonies. The Mexican honey wasp (*Brachygastra mellifica)* represents an independent origin of honey-storing within a group of social Hymenoptera. Honey wasps feed on and store honey, but they prey on other insects as a protein source, and do not consume pollen as do bees. We surveyed the gut microbiomes of Mexican honey wasps across sites within Texas using 16S rRNA community profiling, and we estimated microbiome size using qPCR. For comparison, we also surveyed non-honey feeding wasps from six families, collected in the same region. We found that honey wasps have large microbiomes dominated by characteristic bacterial species, in contrast to other wasps, which had smaller, more variable microbiomes dominated by environmental bacteria. All honey wasps contained strains of *Bifidobacterium* and *Bombilactobacillus* that were closely related to symbionts of bumble bees and other bees, suggesting their acquisition via host-switching. Some individuals also harbored a close relative of *Candidatus* Schmidhempelia bombi (Orbaceae), an uncultured bumble bee symbiont, again suggesting host-switching. The most prevalent species was an uncultured *Lactobacillus*, related to *L. acetotolerans*, and potentially representing an independent acquisition of environmental *Lactobacillus*. The switch to honey feeding, combined with a highly social life history, appears to have facilitated the establishment of a microbiome resembling those of honey bee and bumble bees.

IMPORTANCE

Honey-feeding social insects such as honey bees and bumble bees have conserved gut microbiomes that are transmitted among nestmates. The bacteria consist of host-associated species that benefit the bee hosts by providing defense against pathogens and parasites, and potentially by contributing to digestion. The microbiomes of wasps are less studied, although some species have bacteria

found in solitary bees. Here, we looked at a group of wasps that have a diet much like that of honey bees, predicting that a diet switch from nectar to stored honey available year-round enabled them to acquire a microbiome like that of honey-feeding bees. Honey wasps (*Brachygastra* spp.) are the only group of wasps that store honey. Their large nests are made from paper, and colonies survive for several years. We found that Mexican honey wasps have a conserved gut microbiome with similarities to those of other honey-feeding bees. Several microbiome members are most closely related to bacteria in bumble bees, suggesting their acquisition via host switching. The most common member was a previously unknown *Lactobacillus*. Solitary wasps and other paper wasps that do not make honey have smaller microbiomes dominated by environmental bacteria, suggesting that honey feeding has shaped the gut bacterial communities of honey wasps.

INTRODUCTION

Gut microbial communities often provide numerous benefits to insect hosts (1). They can protect against pathogens or parasites (2–4), enhance or become essential for physiological development (5), metabolize indigestible substrates consumed by the host (6, 7) or neutralize toxic compounds (8, 9). Depending on the host species, microbiome members can be highly host-specific or can consist of bacteria widespread in the environment. Well-studied examples of host-restricted microbiomes are found in the honey-feeding bees, which belong to a clade consisting of honey bees (genus *Apis*), bumble bees (genus *Bombus*) and stingless bees (tribe Meliponini). These social bees are associated with a microbiome that is relatively consistent among different species, reflecting a shared evolutionary history (10). Most species harbor representatives of five bacterial lineages that appear to have colonized a common ancestor 80 million years ago and to have subsequently diversified with hosts, though a few bee lineages have lost ancestral associations (11). For example, in the best studied host, the western honey bee (*A. mellifera*), adult guts are dominated by these five bacterial groups

(sometimes called the “core” microbiome), with a number of other “accessory” bacteria present more erratically. These bacteria have been implicated in digestion and detoxification of the nectar, honey, and pollen diet of their hosts (8, 12–14). Conversely, solitary bees (such as non-corbiculate bees) have more variable gut microbiomes containing bacteria commonly associated with many bee lineages, flowers (nectar and pollen), and other environmental sources (15–19). The gut microbiomes can vary with pathogens (20) and urbanization (21, 22), although some of these bacteria may also protect against pathogens (23).

Dietary shifts are frequently associated with a corresponding shift in gut microbial communities (24, 25). A new diet may facilitate or necessitate the acquisition of new symbionts including symbionts that can contribute to nutritional processing. Cockroach gut microbiomes reflect the transition to wood feeding: omnivorous, litter-feeding cockroaches have similar gut bacteria regardless of diet composition (26); however, the switch to wood-feeding in *Cryptocercus* cockroaches was accompanied by the acquisition of lignocellulose-degrading protists (27). These symbionts are maintained in the wood-feeding lower termites but were later lost in the soil and litter-feeding higher termites, which possess polysaccharide-degrading fungal and bacterial symbionts that resemble the microbiomes of litter-feeding cockroaches (7, 28–30). Conversely, on a shorter evolutionary timeline, the vulture bee, *Trigona necrophaga*, adopted an obligately necrophagous diet rather than relying on pollen and honey-feeding as in other stingless bees. This diet shift is linked to a higher abundance of environmentally acquired acidophiles and more variable microbiomes compared to related honey-feeding bees (31).

Here, we investigate the gut microbiome of a honey-feeding wasp, representing a convergent independent evolution of honey feeding in a group unrelated to honey bees. Three species of honey wasps (*Brachygastra* spp., Vespidae paper wasps) are known to uniquely store and feed on honey rather than nectar (32, 33). This diet shift is rare among wasps; only *Polistes annularis* is known to store honey to overwinter (34), although *Mischocyttarus* spp. store honey short-term (35). Mexican honey wasps,

Brachygastra mellifica, share other life history traits with honey bees: colonies contain thousands of individuals, can live for several years, and new nests are established by swarming (33, 36). To address how this dietary shift may have affected the microbiome composition of the Mexican honey wasp, we characterized the gut microbiomes of wasps across their range in Texas (Fig. 1). We sequenced the V4 region of the 16S rRNA gene to determine the microbiome composition of 64 wasps from 13 nests. Honey wasp microbiomes were compared to those of *Polistes* paper wasps and several other solitary wasps. Our findings indicate that Mexican honey wasps acquired a unique microbial community composed of bacteria associated with honey-feeding bees plus a novel species of *Lactobacillus*. This composition differs greatly from that observed for other sympatric wasps, including paper wasp relatives and solitary wasps, both of which have smaller and more variable gut microbiomes derived from environmental sources.

RESULTS

Microbiomes of honey wasps

We sampled 64 Mexican honey wasps consisting of three to six workers sampled from 13 nests, and five workers collected from flowers in Austin (Fig. 1). For comparison, 47 other wasps were sampled, but only 26 of these wasp samples (13 *Polistes* paper wasps and 13 solitary wasps) had adequate read counts to be included in the analysis (Table S1-S3, explanation of process in Supplementary materials and methods). As controls, we included blank samples and two honey bee samples. The blanks had no detectable product, and the honey bee samples were dominated by known honey bee gut symbionts (Fig. 2, Table S3).

Honey wasps from all nests possessed *Bifidobacterium* and *Lactobacillus* (Fig. 2), both of which are absent from the *Polistes* and solitary wasps. *Lactobacillus* ASV2 was present in four solitary wasps,

while eight other *Lactobacillus* ASVs (2, 7, 8, 24, 44, 56, 66, 67) and two *Bifidobacterium* ASVs were confined to honey wasps. Honey wasps also possessed *Convivina* (ASV16 and ASV25), *Fructilactobacillus* (ASV13), and *Bombilactobacillus* (ASV19) throughout the Texas range, but these were not present in all individuals (Fig. 2B). Honey wasps from some San Antonio nests had bacteria related to *Ca.* Schmidhempelia bombi (ASV14 and ASV28), an undescribed Neisseriaceae (ASV27), and *Asaia* (ASV34).

Based on the "core" microbiome analysis, some ASVs of *Lactobacillus*, *Bifidobacterium*, *Bombilactobacilus*, *Fructilactobacillus*, and *Convivina* occur in most honey wasp individuals and in high abundance (Fig. S1). A NMDS ordination of the Bray-Curtis dissimilarity index shows that honey wasps cluster together apart from the other wasps and honey bees studied (Fig. 3A, Table S4, PERMANOVA, F = 9.334, p = 0.001). Pairwise comparisons further support this finding (Table S5 honey wasps:other wasps, F = 15.366, adjusted p = 0.003; honey wasps:honey bees, F = 4.458, adjusted p = 0.006). Beta dispersions significantly differed between the groups (Table S6, betadisper, F = 16.472, p = 0.001).

Microbiomes of *Polistes*, solitary wasps

The microbiomes of *Polistes* and solitary wasps sometimes contained *Bifidobacterium* or *Lactobacillus*, but these ASVs differed from those found in honey wasps (Fig. 2B). *Polistes* and solitary wasps shared Enterobacterales ASVs (ASV4 and ASV6) with honey wasps, and they more often contained *Lactococcus*, *Gluconobacter*, *Gilliamella*, *Acinetobacter*, *Pseudomonas,* and *Atopobiaceae* (Fig. 2). Core microbiome analysis showed that *Polistes* were typified by the presence of Enterobacterales, *Lactococcus*, *Acinetobacter*, and *Exiguobacterium* (Fig. S1). No bacteria were detectable in more than 40% of *Polistes* individuals sampled, indicating that *Polistes* microbial communities are often absent or tiny. Several *Polistes* ASVs were shared with the 13 solitary wasps (Enterobacterales ASV4 and *Acinetobacter* ASV31), but *Gilliamella* (ASV 32) and another Enterobacterales (ASV6) were more

prevalent in *Polistes* samples. Overall, the prevalence of bacterial ASVs was lower in the individuals of other wasp species compared to the honey wasps.

Absolutes sizes of honey wasp microbiomes

Based on qPCR of a region of the bacterial 16S rRNA gene, honey wasps have substantial gut communities, averaging greater than 10^8 gene copies per bee individual, with relatively little variability in community size (Fig. 4). In contrast, the quantity of gut bacteria in *Polistes* and in solitary wasps was lower and highly variable, ranging from below the limit of detection to 10^{10} copies of the bacterial 16S rRNA gene (Fig. 4, Table S7). *Polistes* wasps had a median of 3.1×10^5 copies of 16S rRNA gene, ranging from 10^3 to 10^7 copies, and solitary wasps had a similar wide range of bacterial load (Table S8, Dunn's test, $z = -1.662$, adjusted $p = 0.290$).

Microbiome diversity among honey wasp nests

Honey wasp microbiome composition varied among sample locations and varied significantly among nests (Fig. 3B, Table S4-S5, PERMANOVA, $F = 3.198$, $p = 0.001$; betadisper, $F = 0.973$, $p = 0.498$). No nests had distinctly different microbiomes based on pairwise comparisons of honey wasps from all 13 nests (Table S6, adjusted for multiple comparisons). The primary members of the microbiome, *Bifidobacterium* and *Lactobacillus*, were present in all nests, but there were multiple ASVs for each that were differentially distributed among nests. Honey wasp *Bifidobacterium* was represented by three ASVs. Indicator species analysis revealed that ASV1 was significantly associated with five nests while ASV3 was associated with four different nests (Table S9). *Bifidobacterium* ASV1 and ASV3 rarely cooccurred in the same host, but ASV3 typically occurred with *Bifidobacterium* ASV5 (Fig. 2B). *Lactobacillus* ASV2 was present in all nests, but the indicator species analysis associated ASV2 with five particular nests (Table S9, indicator value = 0.548, $p = 0.022$). Other *Lactobacillus* ASVs were associated

with a subset of nests. For example, *Lactobacillus* ASV24 was associated with San Antonio nest 6 and ASV44 with the Port Lavaca nest (Table S9; ASV24: indicator value = 0.814, p = 0.001; ASV44: indicator value = 0.861, p = 0.001). Some bacteria had a limited association with only a few nests, for example, San Antonio nests 1, 2, and 3 were characterized by the presence of *Ca.* Schmidhempelia (Table S9; ASV14: indicator value = 0.634, p = 0.007; ASV28: indicator value = 0.649, p = 0.004). Additionally, *Wolbachia* and *Lactococcus* were present in the single nests collected from Kingsville and Port Lavaca, while the Austin nest showed high abundance of *Fructilactobacillus* (Fig. 2B, Table S3).

Laboratory isolation of honey wasp bacteria

We isolated pure cultures of *Ca.* Schmidhempelia (wjB12) and several strains of *Bifidobacterium* (efB7, efB4, wjB1) from two San Antonio nests (SA1 and SA7) grown on heart infusion agar (HIA) with 5% sheep's blood under 5% CO_2 at 35°C. A *Fructilactobacillus vespulae* isolate (sjhB6) and additional *Bifidobacterium* isolate (sjhB46) were also cultured in MRS broth under anaerobic conditions at 31°C.

Phylogenetic relationships of key honey wasp bacteria

We constructed phylogenies of three bacterial groups prevalent in honey wasp microbiomes belonging to Orbaceae, *Bifidobacteria*, and Lactobacillaceae. Within Orbaceae, the honey wasps possessed two ASVs and a bacterial isolate (strain wjB12) that form a well-supported clade together with *Candidatus* Schmidhempelia bombi, an uncultured Orbaceae known only from bumble bees (Fig. 5A).

Three *Bifidobacterium* ASVs and numerous *Bifidobacterium* isolates from honey wasps also form a clade with multiple species of *Bifidobacterium* isolated from several species European bumble bees (*B. commune*, *B. bohemicum*, *B. bombi*) (Fig. 5B). This clade is not closely related to the *Bifidobacterium 'asteroides'* clade associated with honey bees, bumble bees, carpenter bees, and *Osmia* mason bees.

Honey wasps also possess a variety of bacteria in the family Lactobacillaceae. Eight ASVs represent an undescribed species of *Lactobacillus* that is closely related to the clade of *Lactobacillus* associated with honey bees, bumble bees, and *Osmia* bees (formerly referred to as "Firm-5") (Fig. 5C). It is also closely related to *Lactobacillus acetotolerans*, an acetic-acid tolerant bacterium isolated from vinegar. This bacterium remains uncultured, and the 16S rRNA gene amplicon variation suggests there is strain diversity within the honey wasps with ASV2 found in 92% of honey wasps sampled. *Bombilactobacillus* (formerly "Firm-4") was sequenced from multiple honey wasps but remains uncultured (Fig. 5C). Previously the genus was known only from honey bees, bumble bees, and carpenter bees. The sequences from honey wasps were close to *Bombilactobacillus apium* isolated from the Asian honey bee, *Apis cerana*. Additionally, honey wasps possess the vespid wasp-associated *Fructilactobacillus vespulae*, a *Lactiplantilactobacillus*, and another Lactobacillaceae (Fig. 5C).

DISCUSSION

Honey wasps (genus *Brachygastra*) represent an independent evolution of honey storage and feeding in a eusocial member of the Hymenoptera, and have also acquired a gut microbiome very similar to that of social bees. Honey wasps are not closely related to honey-feeding bee lineages, yet most members of the honey wasp microbiome are related to bacteria known from bumble and honey bees (Fig. 6). These include *Bifidobacterium* and *Ca.* Schmidhempelia (both related to species solely known from bumble bees), *Bombilactobacillus*, and *Convivina*, though these were not consistently found in workers from all nests (Fig. 2A). Practically all honey wasps in this study possessed a novel *Lactobacillus* in high abundance that is related to other bee-associated *Lactobacillus* species but not known from bumble bees and honey bees. *Fructilactobacillus vespulae* was also present in some colonies, showing they also possess bacteria associated with paper wasps from the subfamily Vespinae. The high microbial abundance (Fig. 4) is comparable to that observed for honey bees, averaging slightly

207 lower, which may reflect the smaller body size of honey wasps (10) (Fig. 1A). The honey wasp
208 microbiome differs from that of other wasps (Fig. 3A); the *Polistes* paper wasps and solitary wasps had
209 microbiomes with lower abundance variable composition, and dominated by environmentally acquired
210 bacteria; they lacked all core members of the honey wasp microbiome. Some environmental bacteria
211 were shared among honey wasps and *Polistes* and solitary wasps, including Enterobacteriacae,
212 *Pseudomonas*, *Lactococcus*, and some others found in environmental sources such as nectar and in
213 other pollinating Hymenoptera (19, 37–39).

214 Honey wasps have a diverse consortium of Lactobacillaceae that is distinct from that of honey
215 bees or the other wasps included in this study. All honey wasps host an uncultured *Lactobacillus* which
216 belongs to a clade of honey bee-associated *Lactobacillus* (formally Firm-5); multiple ASVs were detected
217 indicating strain diversity in this taxon (Fig. 5C). *Bombilactobacillus* ASVs in honey wasps are closely
218 related to *B. apium* isolated from *Apis cerana* (40). Honey wasps also consistently possess three related
219 *Bifidobacterium* that belong to the same clade as *Bifidobacterium commune*, *Bifidobacterium bombi*,
220 and *Bifidobacterium bohemicum* (Fig. 5B), all isolated from European bumble bees (41, 42). These three
221 *Bifidobacterium* ASVs were found in high abundance in all honey wasp nests. However, individual wasps
222 had either ASV1 or ASV3 in high abundance, not both, suggesting competition or another
223 incompatibility. Several nests of honey wasps in San Antonio had *Candidatus* Schmidhempelia bombi,
224 known from metagenomic samples of *Bombus impatiens* (Fig. 5A) (10, 43). *Ca.* Schmidhempelia is
225 abundant in the colonization phase of *B. impatiens* but decreases as bees age (44). These findings
226 suggest that the core species in guts of Mexican honey wasps were acquired from social bees some time
227 in the past. Honey wasps and bumble bees may have co-occurred in North America for at least several
228 million years based on dated phylogenies of the Vespids (45) and *Bombus* (46) providing ample time for
229 the lineages to interact. Notably, however, several honey wasp bacteria are more similar to honey bee

230 and bumble bee species located in Asia and Europe. This could be attributed to long-term patterns of
231 symbiont acquisition and loss or an incomplete knowledge of symbiont distributions.
232 Also present in the honey wasps were bacteria commonly associated with other bees. These
233 include *Lactococcus, Convivina*, and some *Lactobacillaceae* such as *Lactiplantibacillus*. Three honey
234 wasps collected individually from Austin had high abundances of a bacterium that may be a member of
235 *Candidatus* Stammerula. The short ASV aligns with 16S sequences in the NCBI database with 99%
236 sequence identity to an uncultured symbiont of tephritid flies (47, 48).
237 This study adds to others indicating that vespid wasps have some reliable associations with gut
238 bacteria. The subfamily Vespinae contains four genera, and two have been the focus of microbiome
239 research. *Vespula pensylvanica*, *Vespula vulgaris* and *Vespa velutina* are all associated with several
240 bacteria that were found in the Mexican honey wasp. *Fructilactobacillus vespulae* was found in most
241 honey wasp nests and highly abundant in one nest. This bacterium was isolated from *Vespula vulgaris* in
242 China, and sequenced from *Vespula pensylvanica* in the US (37) and *Vespa velutina* in Belgium, showing
243 an association with vespid wasps on three continents. *Convivina was* detected in about 40% of the
244 honey wasps representing all sampled nests, but not in other wasps. *Convivina intestini* was isolated
245 from *Bombus terrestris* (49) and *Convivina praedatoris* was found in all individuals in a single *V. velutina*
246 nest collected in Belgium (outside of the Korean native range of *V. velutina*) (50). Similarly,
247 *Lactiplantibacillus* was found in about 43% of the honey wasps, and is another possible member of the
248 *V. velutina* microbiome. A study of five *Vespa* species in the South Korean native range did not find
249 *Convivina* and *Lactiplantibacillus* among the top 10 bacterial genera (51), so these associations may be
250 site-specific.
251 *Polistes* wasps did not share many bacteria with honey wasps and were associated with
252 Enterobacteriaceae and *Gilliamella*, *Acinetobacter*, *Gluconobacter,* and *Lactobacillus* (Fig. 2A). These
253 bacteria were found in the four species of *Polistes* inconsistently and are typically associated with flower

254 nectar and nectar-feeding solitary bees (15, 52, 53), and they have been found in *Vespa* spp. (51) and
255 *Vespula pensylvanica* (37). *Gilliamella* sp. (ASV 32) was found in the mutilid, crabronid, sphecid, and
256 several species of *Polistes*. *Gilliamella* is one of the core bacteria in honey bees and bumble bees (39)
257 but some strains have been detected in native bee species and in nectar (52). The amount of gut
258 bacteria based on 16S rRNA gene copies is lower and more variable in *Polistes* and solitary wasps
259 compared to honey wasps. It is unknown why this is, but could be due to conditions of the wasp gut
260 which may make it hard for microbes to colonize or because not all animals require a gut
261 microbiome (1, 54).

262 Flowers are an important hub for the transmission of microbes (15), and bacteria are present in
263 the nectar and pollen (16). The shared food source of nectar may have enabled honey wasps to acquire
264 bacteria from honey-feeding bees. *Polistes* workers also feed on nectar and share it with larvae, as do
265 many solitary wasps, explaining how they acquire some bacteria. However, many adult bees have
266 microbiomes similar to that of their pollen provisions (18, 19, 21, 22, 52, 55), whereas *Polistes* derive
267 protein from insect prey. Overall, it appears that the studied *Polistes* and solitary wasp species contain
268 variable environmentally acquired bacteria with considerable variation among individuals.

269 We note that determining microbiome composition from PCR amplicons of extracted DNA
270 cannot distinguish between DNA from bacteria colonizing the guts, DNA from bacteria that are simply
271 present in the food, and extracellular DNA from dead bacteria. The possibility that profiles represent
272 these other sources is especially great for microbiomes consisting of environmental bacteria, such as
273 those in the *Polistes* and solitary wasps. Since many bees and wasps can visit the same flowers, where
274 they may contaminate nectar, small numbers of live or dead cells from other host species may be
275 ingested in food and stored food provisions.

276 Bumble bees or honey bees appear to have contributed some bacterial lineages to honey wasps,
277 but numerous others were not transferred. Mexican honey wasps do not possess members of the

278 *Bifidobacterium "asteroides"*, *Snodgrassella,* or *Gilliamella* clades, which are found in bumble bees and
279 honey bees (10, 56). Honey wasps also lack the "Firm-5" *Lactobacillus* species associated with honey
280 bees but possess an undescribed relative. *Snodgrassella* and *Gilliamella* form a biofilm along the ileum
281 of honey bees and bumble bees (57, 58). Their absence is not surprising given that they have been lost
282 by *Melipona* stingless bees (11) and that honey wasps do not have a defined ileum gut structure (Fig.
283 1A). Furthermore, multiple *Gilliamella spp.* synthesize pectin-degrading enzymes for the breakdown of
284 cell walls including pollen (12, 13). Mexican honey wasps source protein from insect prey, so pollen-
285 degrading bacteria would be unnecessary. Also missing are the "accessory" bacteria in the honey bee
286 microbiome which include species in the genera *Apilactobacillus*, *Bombella*, *Arsenophonus*, *Bartonella*,
287 *Parasaccharabacter*, *Apibacter*, and *Frischella perrara* (39, 59).
288 Social and gregarious insects can facilitate the transmission of symbionts among group members
289 by direct contact (60). Social spiders, for example, acquire their bacteria from co-nesting females via
290 regurgitation resulting in homogenized microbiomes among members of the same nest (61). Honey
291 wasp nests can house up to 20,000 individuals, share food sources through trophallaxis, and establish
292 new nests by swarming (33, 36), providing frequent opportunities for symbiont transfer among
293 nestmates and to new nests. Sociality, however, is not essential for maintaining a conserved, host-
294 restricted microbiome. Brood provisions also serve as a microbial transmission route for solitary bees
295 such as *Osmia* Mason bees (18, 62), and *Xylocopa* Carpenter bees have conserved microbiomes with
296 only moderate social interactions within communal nests (38, 63, 64). The gut microbiomes of many
297 solitary bees are acquired from environmental sources (pollen and nectar) and brood provisions and as
298 such are heterogeneous (19). *Polistes* paper wasps are eusocial and have a means of bacterial
299 transmission among nestmates and generations, yet their gut microbiomes are composed of
300 environmental bacteria found in nectar and in solitary bees. It appears that solitary wasps acquire
301 bacteria from environmental sources based on the wasps sequenced in this study.

Mexican honey wasps occur from central Texas south to Panama, so our collection range represents a small part of the *B. mellifica* range. Furthermore, the Mexican honey wasp was restricted to the USA/Mexico border until around 1995 (33) and recently expanded approximately 500 kilometers north over 3 years. We collected 13 nests across the Texan range and found significant variation in microbiome composition between nests. Individual worker microbiomes are more similar to those of nestmates. Differences between nests are due to the presence and abundance of particular ASVs, indicating strain variation among the core microbiome members, such as the uncultured *Lactobacillus*, with eight ASVs that form a clade (fig. 5c), and the *Bifidobacterium* with 4 ASVs that varied among hosts.

All individuals in all nests had a microbiome dominated by *Lactobacillus* and *Bifidobacterium* with the exception of the Austin nest, where three of the five individuals were dominated by *Fructilactobacillus vespulae*. Additionally, five individual honey wasps were collected from flowers (not nests) in Austin. One had the characteristic microbiome profile, and the four other wasps had higher abundance of Enterobacteriaceae, *Ca*. Stammerula, and *Conviva*. These two Austin collection sites sit close to the northernmost extent of the Mexican honey wasp range, suggesting a change in microbial diversity associated with edge populations, though our sampling is not sufficient for conclusions. Two species of English butterflies have microbiomes with decreased diversity and a shift in composition in the northern part of their range. (65) Similarly, the microbiomes of range-expanding plants were more similar to each other than to those of conspecifics (66). Further research into honey wasps throughout Mexico and Central America would reveal whether the core microbiome is stable throughout its range.

The transition to long-term honey-feeding and storage in a wasp is only known in three species of *Brachygastra* honey wasps (*B. mellifica*, *B. azteca*, and *B. lecheguana*) (32, 33). This diet switch may have facilitated the acquisition of bee-associated bacteria as honey wasps feed on a carbohydrate-rich diet similar to that of honey-feeding bees. However, the carbohydrate profile of honey wasp honey differs from that of honey bee honey, which may influence the composition of gut bacteria. Honey bee

honey is predominantly glucose and fructose whereas, in a study based on seven nests, honey wasp honey contains less glucose and fructose (23% and 30% respectively), more sucrose (~9.8%), with 28.6% of the sugars not identified (33).

Honey wasps routinely collect honeydew from aphids and psyllids (33) which contains large amounts of melezitose and trehalose (67). Honey bees can also collect honeydew when nectar is not available (68), studies in Greece showed that honey can have up to 14% melezitose when honey bees forage directly on honeydew from aphids on fir trees (67, 69). Gut bacteria of honey bees can metabolize a variety of sugars, including some monosaccharides toxic to honey bees (8). *Bifidobacterium* spp. have extensive abilities to degrade plant-derived polysaccharides (70), and bee-associated *Bifidobacterium* can degrade and use hemicelluloses (13). Honey wasps likely do not ingest significant amounts of polysaccharides, since they do not eat pollen, so metabolic capabilities of their microbiomes may be very different from those of honey bees and bumble bees, which rely on pollen. Investigation of the functional capabilities of the *Bifidobacterium* strains isolated from honey wasps. will offer insights into their metabolic capabilities and potential benefits to hosts.

Overall, the Mexican honey wasp gut microbiome differs greatly from microbiomes of related wasps, in both size and composition. It is an amalgamation of bacteria that appear to have originated primarily from bumble bees or honeybees, and from vespid wasps. The switch from feeding on nectar and insect prey to a stable, year-long supply of stored honey may have facilitated (or been facilitated by) the acquisition of bacteria associated with honey-feeding bees.

METHODS

Sample Collection

Honey wasps from 13 nests, individual honey wasps, *Polistes* wasps, solitary wasps, and honey bees were collected across sites in central and south Texas (Fig. 1, Table S1). Guts were dissected from

specimens using sterile technique and stored in 100% ethanol at -80°C. Additional honey wasp guts were preserved in 20% glycerol at -80°C for bacterial isolation.

DNA Extractions, Sequencing, and qPCR

DNA was extracted from whole guts using the CTAB/phenol-chloroform extraction method (71). The full length or V4 region of the 16S rRNA gene was PCR amplified and sequenced on an Illumina MiSeq platform at the Genome Sequencing and Analysis Facility (University of Texas at Austin). Quantitative PCR was performed on the DNA extracts to quantify total 16S rRNA gene copies as an estimate of bacterial abundance.

Bacterial Isolation and Identification

The preserved honey wasp gut homogenates from two nests (SA1 and SA7) and an individual collected in Austin were grown on HIA + 5% sheep's blood or in MRS broth at 35°C in 5% CO_2 or 31°C in anaerobic conditions for 1-3 days. To identify bacterial isolates, the 16S rRNA gene was PCR amplified, and amplicons were sequenced at the DNA Sequencing Facility (University of Texas at Austin).

Data Analysis and Phylogenetic Reconstruction

Illumina sequencing files were processed in R using the dada2 pipeline. Taxonomy was assigned using the SILVA 138.1 database (72) and manually confirmed using NCBI BLASTn. Read counts were checked after each filtering step, resulting in the removal of 21 *Polistes* and solitary wasps from the dataset (see Supplementary methods, Table S2). The remaining samples were rarefied to 90% of the minimum sampling depth (Fig. S2, Table S3). The core microbiomes of honey wasps, *Polistes*, and solitary wasps were identified using the microbiome package. Indicator species analysis was also performed using the indicspecies package to determine if any ASVs were significantly associated with each honey wasp nest. Statistical differences between gut bacterial communities across honey wasp nests and insect types

were analyzed using PERMANOVA (*adonis2*), and assumptions were assessed using beta dispersion analysis. Post-hoc pairwise comparisons with Bonferroni adjustment were performed to directly compare bacterial communities across nests and insect types.

The 16S rRNA gene phylogenies were reconstructed for Lactobacillaceae, Orbaceae, and *Bifidobacterium*, which were commonly amplified ASVs in the honey wasps and isolated bacteria. Full length 16S rRNA gene sequences were obtained from GenBank. These were aligned using MAFFT with ASV sequences and full length 16S rRNA from the isolated bacteria. Maximum likelihood phylogenies were reconstructed using IQtree with auto-generated substitution models and 100 standard bootstrap alignments.

DATA AVAILABILITY

Raw 16*S* rRNA gene amplicon reads are deposited in the National Center for Biotechnology Information Sequence Read Archive under accession PRJNA932820. ASV sequences, taxonomic assignments, read numbers, metadata can be found in the supplement. Scripts for the analysis can be found on GitHub (https://github.com/annatpham/honey-wasp-microbiome), and insect specimens are deposited at the University of Texas Insect Collection (UTIC).

ACKNOWLEDGEMENTS

We thank G. Rankin, H. Koch, S. Bard, D. Fall, S. Biles, J. Hare, M. Reed, B. Freitag, B. Fehrenkamp and for specimen acquisition and collection. Thanks also to A. Santillana for images, A. Wild for specimen ID, and E. Frederick and K. Chow for technical assistance. This work was supported by USDA-NIFA grant (2017-06473), NSF award (NSF 2103208), and the Texas EcoLab program, and undergraduates were supported by the Freshman Research Initiative (UT Austin).

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FIGURE LEGENDS

Figure 1: Specimen images and collecting locations. (A) Mexican honey wasp, *Brachygastra mellifica*, (B) common paper wasp, *Polistes exclamans*, (C) honey bee, *Apis mellifera*. Specimens have their guts removed to show the internal structure. The bottom section of the images were cut and rotated to orientate the midguts to the right and sting to the left. (D) Map of Texas showing the collecting sites of honey wasps, *Polistes* paper wasps and solitary wasps, and honey bees.

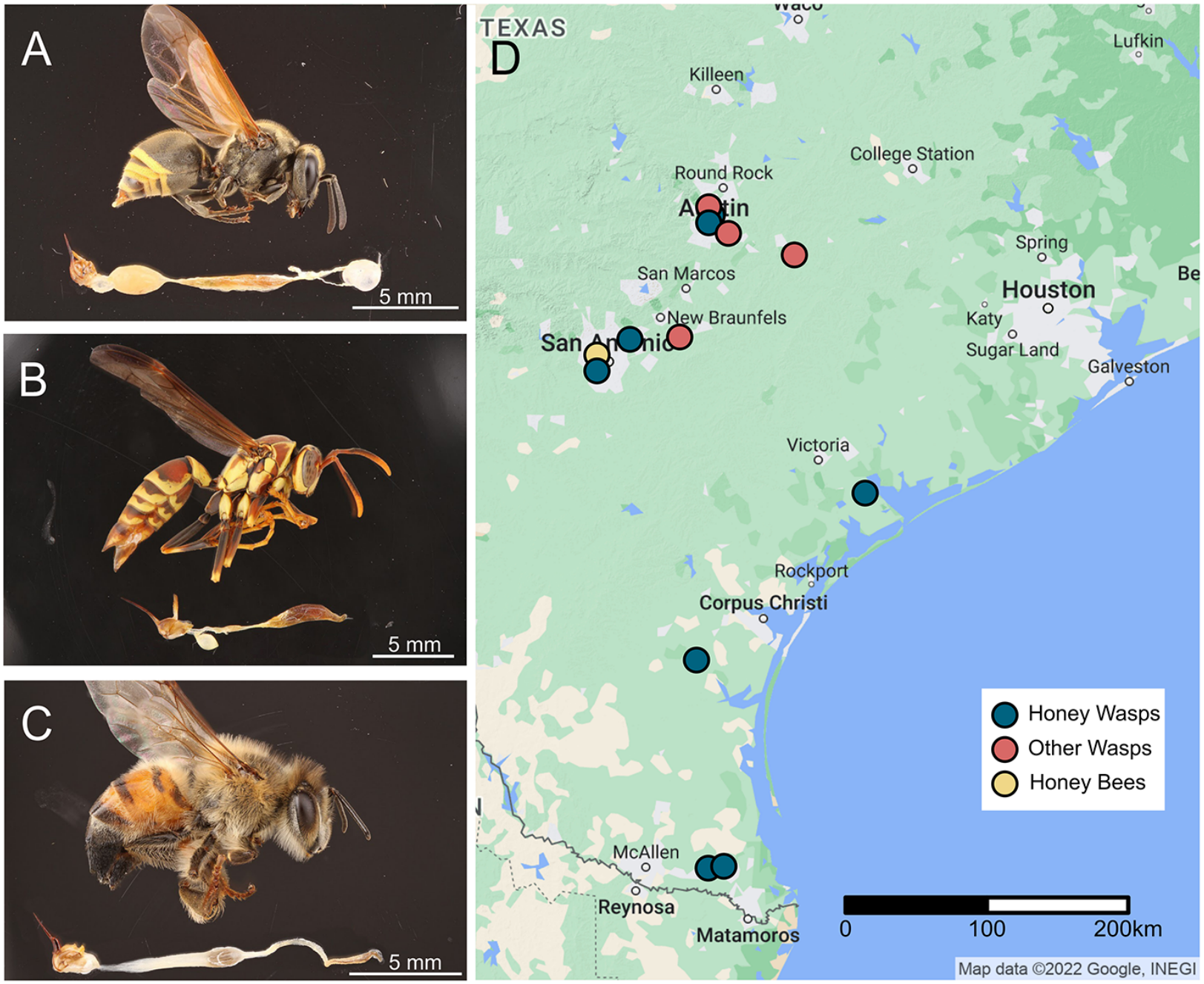
Figure 2: Relative abundance of (A) the top 16 bacteria genera and (B) the top 50 amplicon sequence variants (ASVs) from honey wasps, other wasps, and honey bees. Honey wasps are grouped by nest, with the exception of five individuals (Ind). Nests are named based on their location and organized on the plot from north (top) to south Texas (bottom). Other wasps are grouped by family (Vesp = Vespidae, Mut = Mutillidae, Thynn = Thynnidae, Crab = Crabronidae, Sphec = Sphecidae, Ich = Ichneumonidae). Honey wasps typically possess a characteristic microbiome that distinguishes them from other wasp species.

Figure 3: Nonmetric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarity index. (A) Honey wasp gut microbial communities compared to those of other wasps and honey bees (stress = 0.19). (B) Honey wasp nest gut microbial communities (stress = 0.24).

Figure 4: Gut bacterial abundance of honey wasps, *Polistes* paper wasps, and solitary wasps. Dashed line is the median. The gray shaded area is the region below the detection threshold, and the yellow shaded area represents the range of 16S rRNA gene copies for *Apis mellifera* from Kwong et al. (10). Honey wasps (n = 10) were from nests in San Antonio (SA1 and SA3), *Polistes* samples represent five species (n = 19), and solitary wasps represent 7 families (n = 30, including four solitary eumenine vespid wasps). Honey wasp gut microbial communities significantly differed from those of paper wasps and solitary wasps in all pairwise comparisons (honey wasps:paper wasps, $z = -3.417$, adjusted $p = 0.002$; honey wasps:solitary wasps, $z = -4.990$, adjusted $p = 1.8 \times 10^{-6}$).

Figure 5: Phylogenies of the 16S rRNA gene of the major bacterial taxa from the honey wasps. (A) Orbaceae, (B) Bifidobacteriaceae, and (C) Lactobacillaceae. Phylogenies were inferred by maximum likelihood, bootstrap support indicated by black circles. Colors represent bacterial genera or clades associated with bees, host source listed in parentheses.

Figure 6: Diagram showing the highly abundant bacteria found in the Mexican honey wasp (slate blue) that have host-switched from bumble bees (brown), honey bees (cream), and vespid bees (light blue). Bacterial names in black were found in honey wasps and names in gray are host-restricted bacteria that were not found in honey wasps. Bacteria commonly associated with solitary bees (gray) and the nectar in wild flowers (gray) were not found in honey wasps. The *Lactobacillus** present in honey wasps is likely a new species unknown from other insects.


A
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College Station
Round Rock
Austin
Spring
San Marcos
Houston
New Braunfels
Katy
San Antonio
Sugar Land
Galveston
Victoria
Rockport
Corpus Christi
Honey Wasps
Other Wasps
Honey Bees
McAllen
Reynosa
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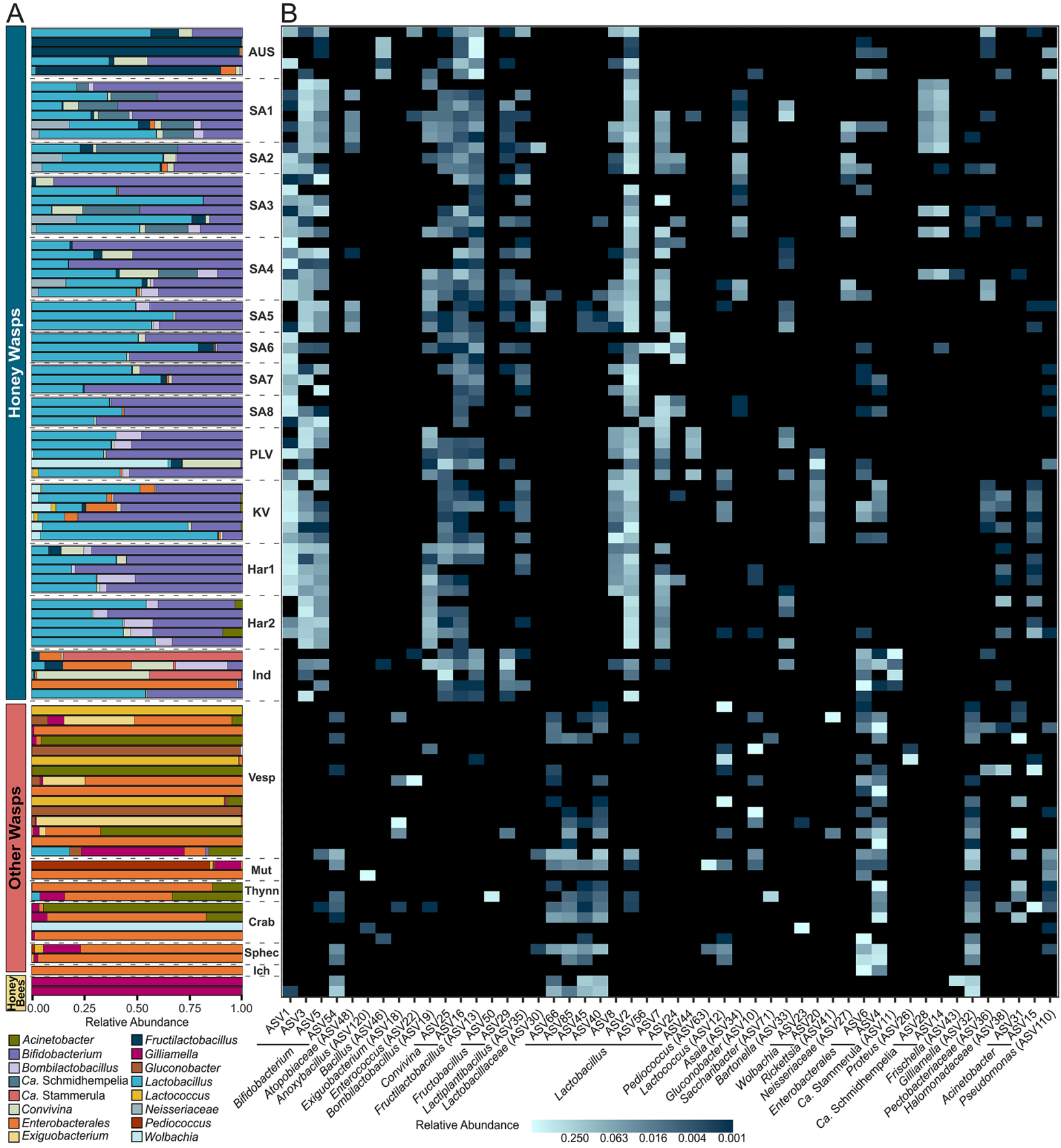
Map data ©2022 Google, INEGI


A
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Honey Wasps
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Har1
Har2
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Vesp
Mut
Thynn
Crab
Sphec
Ich
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Acinetobacter
Bifidobacterium
Bombilactobacillus
Ca. Schmidhempelia
Ca. Stammerula
Convivina
Enterobacterales
Exiguobacterium
Fructilactobacillus
Gilliamella
Gluconobacter
Lactobacillus
Lactococcus
Neisseriaceae
Pediococcus
Wolbachia
ASV1
ASV3
ASV5
ASV54
ASV148
ASV120
ASV46
ASV18
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ASV19
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ASV44
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ASV34
ASV10
ASV71
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ASV23
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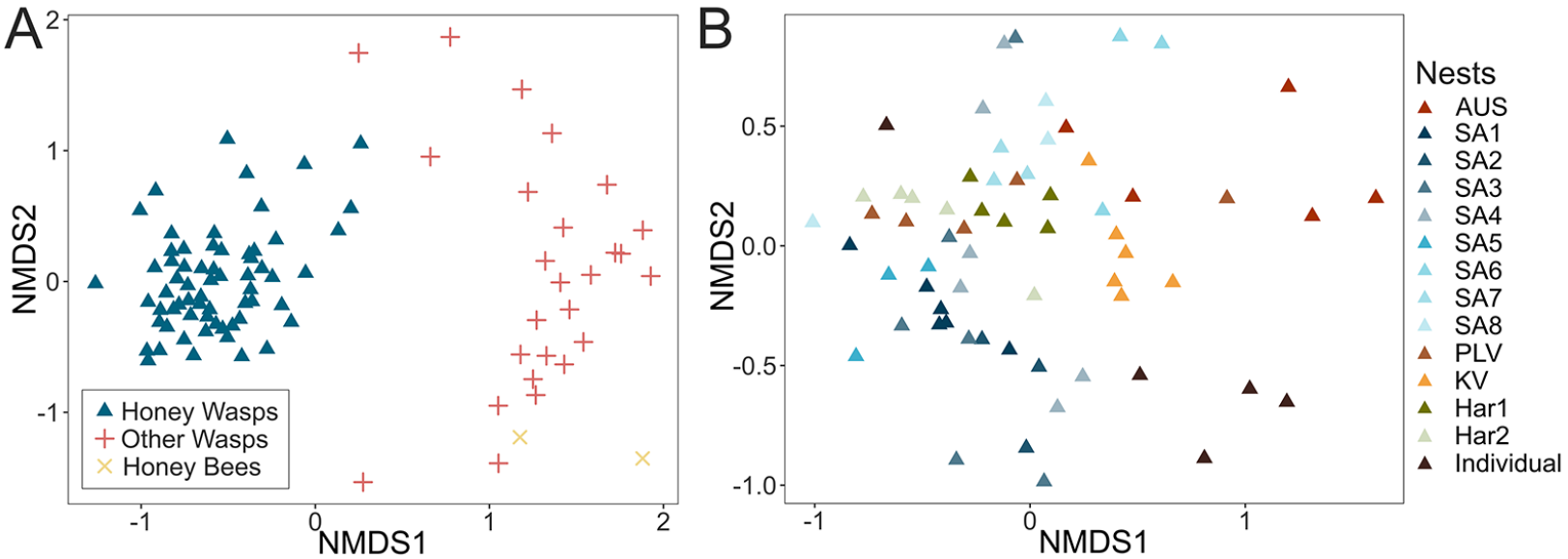
ASV38
ASV31
ASV15
ASV110
Bifidobacterium
Atopobiaceae (ASV148)
Anoxybacillus (ASV120)
Bacillus (ASV46)
Exiguobacterium (ASV18)
Enterococcus (ASV22)
Bombilactobacillus (ASV19)
Convivina
Fructobacillus
Fructilactobacillus (ASV16)
Lactiplantibacillus (ASV13)
Lactobacillaceae (ASV50)
Lactobacillus
Pediococcus (ASV29)
Lactococcus (ASV35)
Asaia (ASV30)
Gluconobacter (ASV66)
Saccharibacter (ASV85)
Bartonella (ASV45)
Wolbachia
Rickettsia (ASV40)
Neisseriaceae (ASV8)
Enterobacterales (ASV2)
Ca. Stammerula (ASV56)
Proteus (ASV7)
Ca. Schmidhempelia (ASV24)
Frischella (ASV44)
Gilliamella (ASV63)
Pectobacteriaceae (ASV12)
Halomonadaceae (ASV34)
Acinetobacter (ASV10)
Pseudomonas (ASV71)
Relative Abundance
0.250
0.063
0.016
0.004
0.001


A
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Honey Wasps
Other Wasps
Honey Bees
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KV
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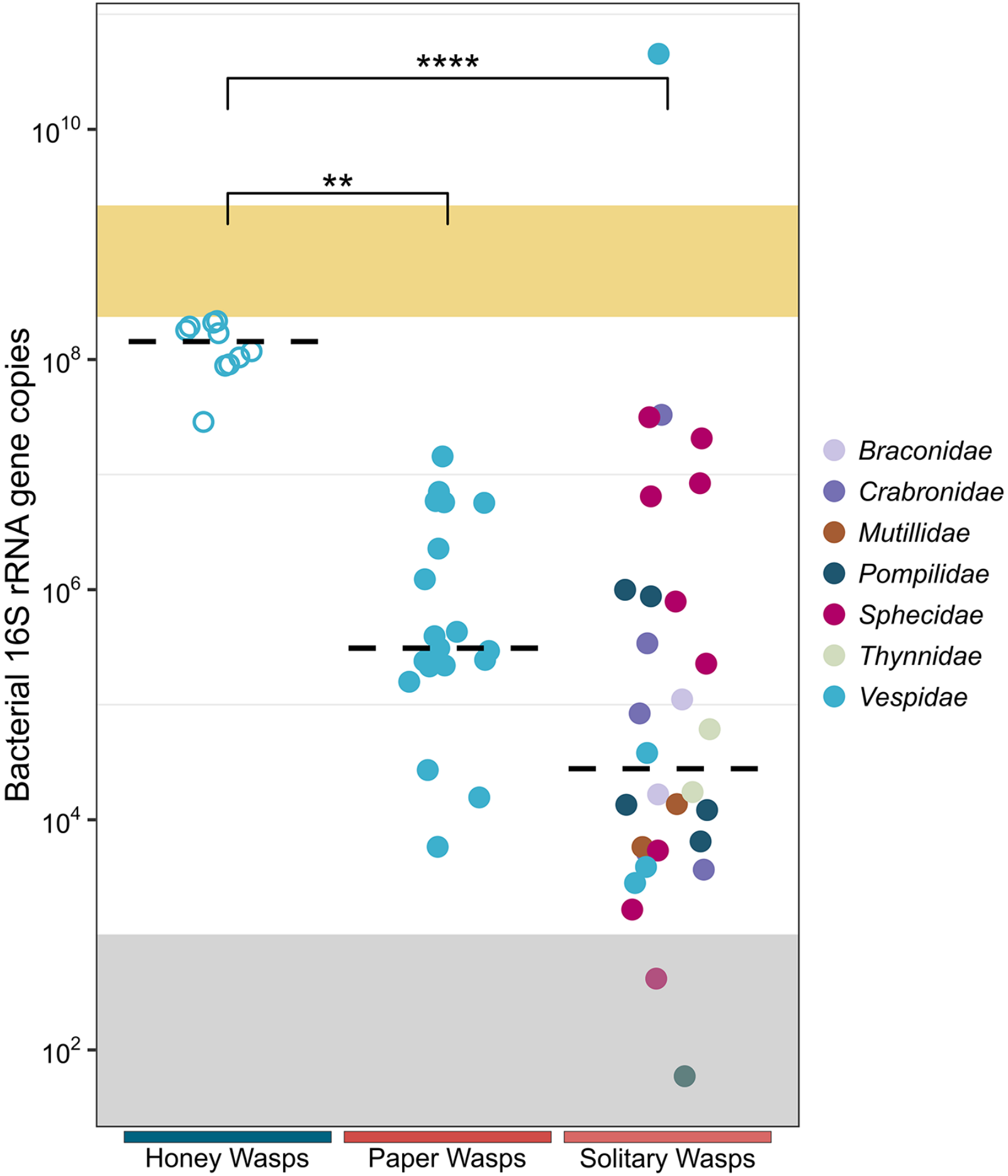
Individual


****
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10^{10}
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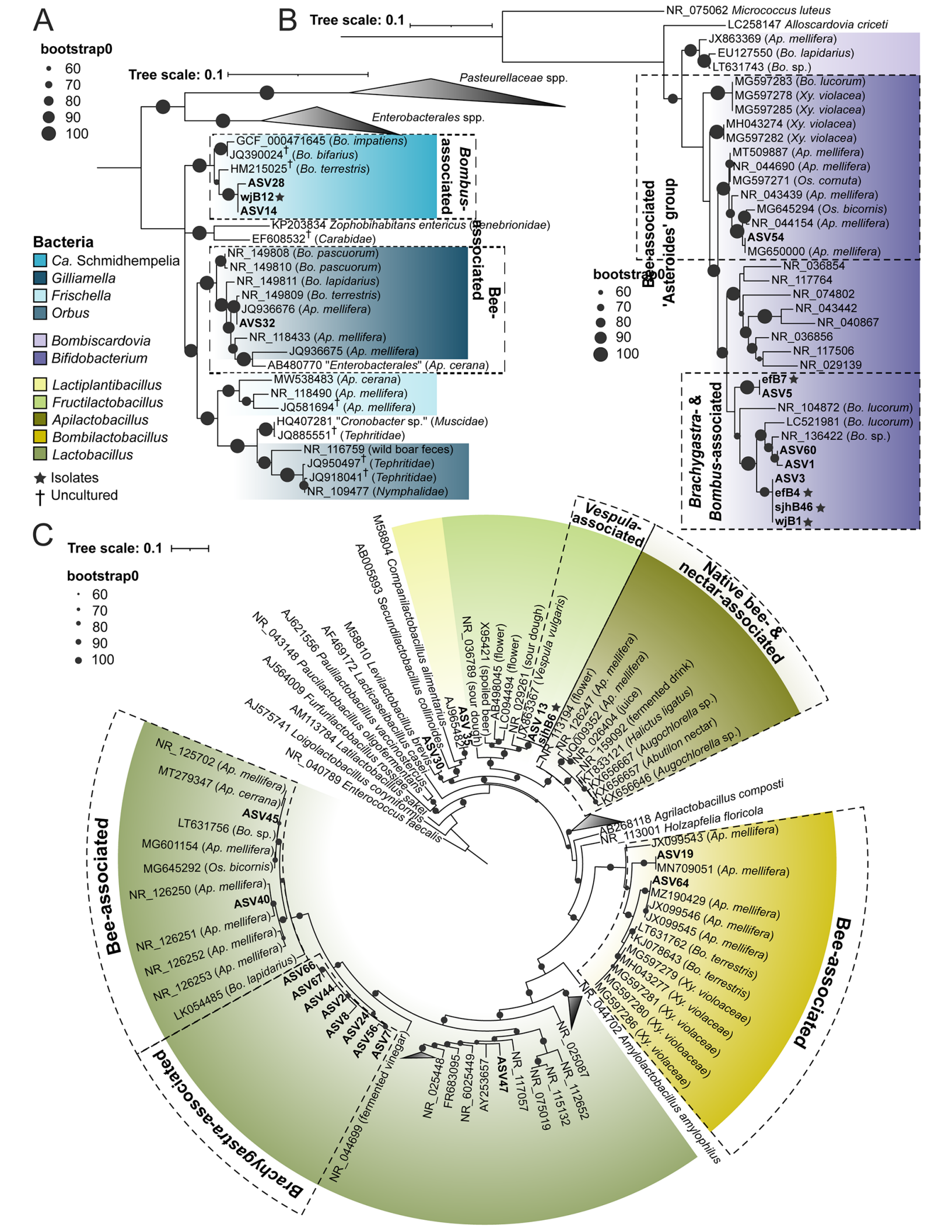
Bacterial 16S rRNA gene copies
Honey Wasps
Paper Wasps
Solitary Wasps
Braconidae
Crabronidae
Mutillidae
Pompilidae
Sphecidae
Thynnidae
Vespidae


A
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60
70
80
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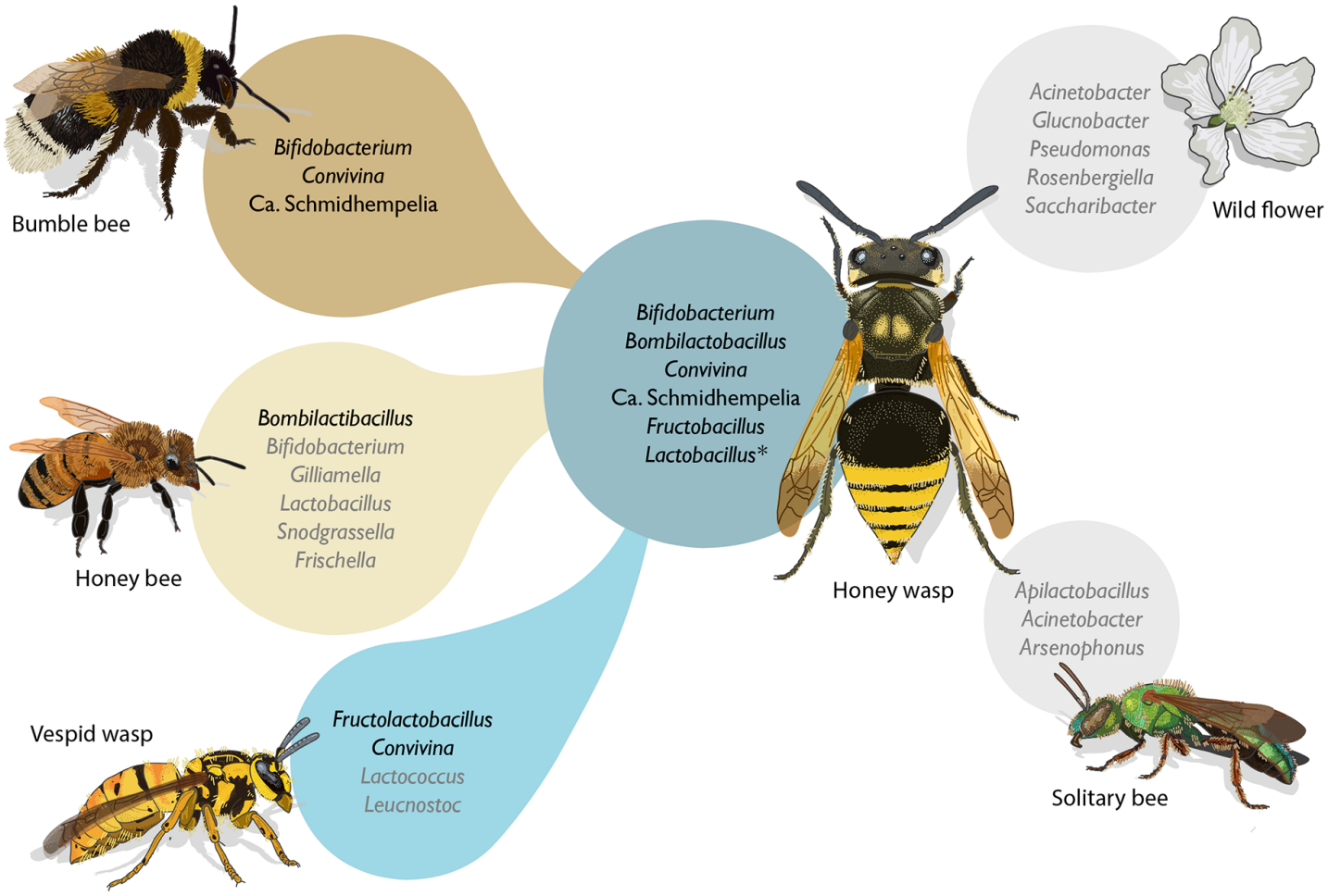
100
Tree scale: 0.1
Pasteurellaceae spp.
Enterobacterales spp.
GCF_000471645 (Bo. impatiens)
JQ390024† (Bo. bifarius)
HM215025† (Bo. terrestris)
ASV28
wjB12★
ASV14
Bombus-associated
KP203834 Zophobihabitans entericus (Benebrionidae)
EF608532† (Carabidae)
NR_149808 (Bo. pascuorum)
NR_149810 (Bo. pascuorum)
NR_149811 (Bo. lapidarius)
NR_149809 (Bo. terrestris)
JQ936676 (Ap. mellifera)
AVS32
NR_118433 (Ap. mellifera)
JQ936675 (Ap. mellifera)
AB480770 "Enterobacterales" (Ap. cerana)
Bee-associated
MW538483 (Ap. cerana)
NR_118490 (Ap. mellifera)
JQ581694† (Ap. mellifera)
HQ407281 "Cronobacter sp." (Muscidae)
JQ885551† (Tephritidae)
NR_116759 (wild boar feces)
JQ950497† (Tephritidae)
JQ918041† (Tephritidae)
NR_109477 (Nymphalidae)
Bacteria
Ca. Schmidhempelia
Gilliamella
Frischella
Orbus
Bombiscardovia
Bifidobacterium
Lactiplantibacillus
Fructilactobacillus
Apilactobacillus
Bombilactobacillus
Lactobacillus
★ Isolates
† Uncultured
B
Tree scale: 0.1
NR_075062 Micrococcus luteus
LC258147 Alloscardovia criceti
JX863369 (Ap. mellifera)
EU127550 (Bo. lapidarius)
LT631743 (Bo. sp.)
MG597283 (Bo. lucorum)
MG597278 (Xy. violacea)
MG597285 (Xy. violacea)
MH043274 (Xy. violacea)
MG597282 (Xy. violacea)
MT509887 (Ap. mellifera)
NR_044690 (Ap. mellifera)
MG597271 (Os. cornuta)
NR_043439 (Ap. mellifera)
MG645294 (Os. bicornis)
NR_044154 (Ap. mellifera)
ASV54
MG650000 (Ap. mellifera)
Bee-associated 'Asteroides' group
bootstrap0
60
70
80
90
100
NR_036854
NR_117764
NR_074802
NR_043442
NR_040867
NR_036856
NR_117506
NR_029139
efB7★
ASV5
NR_104872 (Bo. lucorum)
LC521981 (Bo. lucorum)
NR_136422 (Bo. sp.)
ASV60
ASV1
ASV3
efB4★
sjhB46★
wjB1★
Brachygastra- & Bombus-associated
C
Tree scale: 0.1
bootstrap0
60
70
80
90
100
Vespula-associated
Native bee- & nectar-associated
M58804 Companilactobacillus alimentarius
AB005893 Secundilactobacillus collinoides
M58810 Levilactobacillus brevis
AF469172 Lacticaseibacillus casei
AJ621556 Paulilactobacillus vaccinostercus
NR_043148 Paucilactobacillus oligofermentans
AJ564009 Furfurilactobacillus rossiae
AJ575741 Latilactobacillus sakei
AM113784 Loigolactobacillus coryniformis
NR_040789 Enterococcus faecalis
AJ965482
ASV30
ASV35
NR_036789 (sour dough)
X95421 (spoiled beer)
AB498045 (flower)
LC094494 (flower)
NR_029261 (sour dough)
JX863367 (Vespula vulgaris)
ASV13
sjhB6★
NR_113194 (flower)
NR_126247 (Ap. mellifera)
JQ099352 (Ap. mellifera)
NR_026404 (juice)
NR_159092 (fermented drink)
KT833121 (Halictus ligatus)
KX656667 (Augochlorella sp.)
KX656657 (Abutilon nectar)
KX656646 (Augochlorella sp.)
AB268118 Agrilactobacillus composti
NR_113001 Holzapfelia floricola
JX099543 (Ap. mellifera)
ASV19
MN709051 (Ap. mellifera)
ASV64
MZ190429 (Ap. mellifera)
JX099546 (Ap. mellifera)
JX099545 (Ap. mellifera)
LT631762 (Bo. terrestris)
KJ078643 (Bo. terrestris)
MG597279 (Xy. violaceae)
MH043277 (Xy. violaceae)
MG597281 (Xy. violaceae)
MG597280 (Xy. violaceae)
MG597286 (Xy. violaceae)
NR_044702 Amylolactobacillus amylophilus
Bee-associated
NR_125702 (Ap. mellifera)
MT279347 (Ap. cerrana)
ASV45
LT631756 (Bo. sp.)
MG601154 (Ap. mellifera)
MG645292 (Os. bicornis)
NR_126250 (Ap. mellifera)
ASV40
NR_126251 (Ap. mellifera)
NR_126252 (Ap. mellifera)
NR_126253 (Ap. mellifera)
LK054485 (Bo. lapidarius)
ASV66
ASV67
ASV44
ASV2
ASV8
ASV24
ASV56
ASV7
NR_044699 (fermented vinegar)
Brachygastra-associated
NR_025448
FR683095
NR_6025449
AY253657
ASV47
NR_117057
NR_075019
NR_115132
NR_112652
NR_025087


Bumble bee
Bifidobacterium
Convivina
Ca. Schmidhempelia
Honey bee
Bombilactibacillus
Bifidobacterium
Gilliamella
Lactobacillus
Snodgrassella
Frischella
Vespid wasp
Fructolactobacillus
Convivina
Lactococcus
Leucnostoc
Bifidobacterium
Bombilactobacillus
Convivina
Ca. Schmidhempelia
Fructobacillus
Lactobacillus*
Honey wasp
Acinetobacter
Glucnobacter
Pseudomonas
Rosenbergiella
Saccharibacter
Wild flower
Apilactobacillus
Acinetobacter
Arsenophonus
Solitary bee