Bacteria in honeybee crops are decoupled from those in floral nectar and bee mouths

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Abstract

Bacteria in the honeybee gut are a well-recognized factor affecting bee health. However, the primary focus of this research has been the hindgut, while the crop, or honey stomach, is assumed to be dominated by environmentally acquired transient taxa that matter little to the bees. To evaluate this assumption, we examined bacterial taxa in the crop and mouth of *Apis mellifera* and *A. cerana japonica* foragers and in the nectar of *Prunus mume* flowers visited by the bees in the Minabe-Tanabe region of Japan. We found that in bacterial composition, the crop was distinct from both the mouth and the nectar, whereas mouth and nectar samples were indistinguishable. Furthermore, the crop remained similar in bacterial composition and diversity, while the mouth showed a sharp drop in alpha diversity and a large increase in beta diversity, from summer to winter. These results refute the conventional assumption, suggesting instead that the crop contains a conserved bacterial community largely distinct from environmental taxa. We also found that strains of a crop-associated species, *Apilactobacillus kunkeei*, could be season- and host species-specific. Together, these findings suggest that crop-associated bacterial communities should be studied further to better understand the relationship between honeybees and their gut bacteria.

Keywords: microbial ecology; honeybee microbiome; nectar microbiome; plant-pollinator-microbe interactions

Introduction

Microbes in the honeybee gut are receiving increasing attention not only as a factor affecting the health of the agriculturally vital insects, but also as a model system for gaining basic understanding of gut-associated microbiota [1, 2]. One component of the honeybee gut is used for the transport of liquids. This organ, which is an expandable portion of the esophagus hereby termed the crop, but also known as the honey stomach or honey sac [3], contains species such as *Apilactobacillus kunkeei* and *Bombella apis* that may affect the survival and pathogen resistance of the foraging adults that host them and the larvae that the adults feed [1, 4-6]. However, microbes in the crop are not as well studied as those in the hindgut, which are generally considered more important to honeybee health [7, 8].

Given its function for temporary storage and transport of floral nectar [9], it seems reasonable to assume that the crop is occupied mainly by the transient microbes acquired from the environment via foraging of nectar [10, 11]. One way to investigate this assumption of environment–crop matching is to compare microbes in the crop of foraging bees and those in their mouth as well as in the floral nectar they forage for. If the assumption is true, the crop should be similar to both the mouth and the floral nectar in bacterial composition [4, 12]. To our knowledge, no study has directly compared bacteria in crop, mouth, and nectar for this purpose, leaving untested the assumption that the crop microbiota is dominated by environmentally derived transients.

Here we test the hypothesis that crop-associated bacteria show species composition that mirror those observed in mouth- and nectar-associated bacteria. To this end, we compare crop-, mouth-, and nectar-associated bacteria in samples that were collected simultaneously at the same locations. Our crop and mouth samples come from actively foraging adults of the introduced *Apis mellifera* and the native *A. cerana japonica* (hereafter *A. cerana*) collected in the Minabe-Tanabe region of Wakayama Prefecture in Japan. In this region, farmers use both species of honeybees to pollinate the winter-blooming Japanese apricot, *Prunus mume* [13]. Our nectar samples come from *P. mume* flowers collected in the winter near the hives of the *A. mellifera* and *A. cerana* bees that we caught for crop and mouth sampling.

Materials and Methods

Study sites

Japanese apricot orchards in the Minabe-Tanabe region are embedded within a mountainous countryside landscape that has recently become internationally recognized. In 2015, the Food and Agricultural Organization designated the region as a Globally Important Agricultural Heritage System for the agricultural practice in the region that embraces both human needs and biodiversity conservation. Many of the apricot orchards are located adjacent to natural and managed forests, which are thought to provide stable habitats for the native *A. cerana*. The two *Apis* species are the main pollinators of the apricot, which blooms from mid or late February to early or mid-March, during which few other flower-visiting insects are available [13]. In the winter, *A. mellifera* colonies are supplemented with a sugar solution, while *A. cerana* colonies are

not. Although there may have been several different blooming species during our collections in winter and summer, our interest in nectar focused on the economically important *P. mume.* All our bee and nectar collection sites were located within this landscape, but at least 1.0 km away from their respective nearest neighboring collection site in each season (Fig. 1a, Table S1).

Bee collection

A total of 12 *P. mume* orchards in the study region were visited in the summer of 2018 and the winter of 2019. Seventy-two and 149 honeybees were collected in the summer of 2018 and the winter of 2019, respectively, from hives in or near these apricot orchards (Table S1). At each collection site, there were about four to 10 *A. cerana* hives or 10 to 40 *A. mellifera* hives. Honeybees were collected as they flew near their hives located within or near the orchards. At orchards where the beekeepers maintained *A. cerana* hives, *A. cerana* bees were collected near their hives whose locations varied from tens of meters to about a hundred meters from the orchard and were often situated at the edge of the local forest. The collected bees were individually placed in a sterile, sealed plastic vial immediately after the collection. The vials were kept for up to two hours in a cool box with ice packs in it for transport to the Japanese Apricot Laboratory located within 100 m from one of the collection sites (Higashi-Honjo) (Fig. 1a).

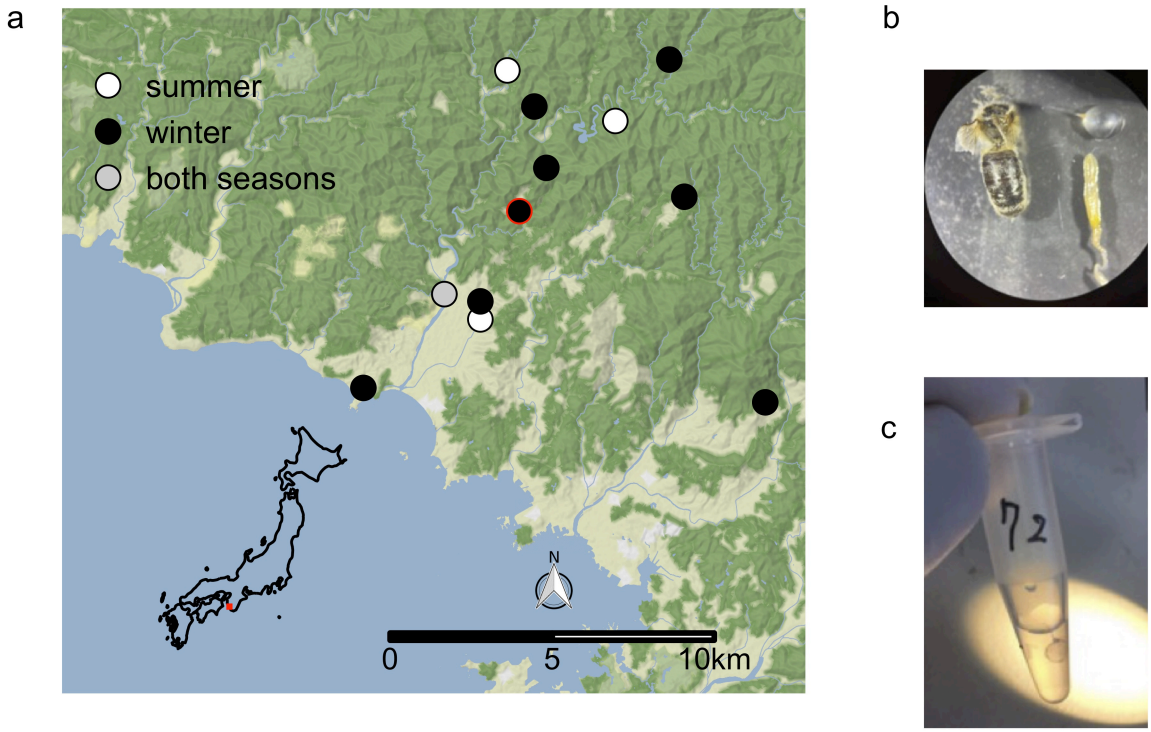

Fig. 1 Collection sites and crop dissection. (**a**) Foraging adults of *Apis cerana* and *A. mellifera* were collected from a total of 12 sites (see Table S1) in or near Japanese apricot orchards in the summer of 2018 and the winter of 2019 in the Minabe-Tanabe region, the location of which is indicated by the red square on the map of Japan. Nectar samples were also collected from apricot flowers at each of the winter collection sites. Sites are labeled with circles colored by collection season (summer = white, winter = black, both seasons = grey). The location of the Japanese Apricot Laboratory is indicated by the red outline of the Higashi-Honjo site. (**b**) The crop was distinctly separated from the rest of the alimentary tract. (**c**) The crop of each honeybee was sterilely dissected and placed in an aliquot of sterile water

After we brought the collected bees into the laboratory in the Japanese Apricot Laboratory facility, the bees were kept cool in the box for up to two additional hours so that the bees would stay sluggish for ease of handling. The captured bees were then taken out of the vials, and their wings were fixated with adhesive tape to facilitate

131 handling. The taped bees were made to drink approximately half of 20 μL of sterile 20%
132 sucrose solution placed on the lid of a sterile 1.5-mL microcentrifuge tube. After the
133 feeding, the remaining solution was placed in the microcentrifuge tube with the lid now
134 closed. These solutions were later used to study the bacteria associated with the mouth.
135 We refer to these solutions as mouth samples. As we had the bees extend their
136 proboscis into the sterile sucrose solution, our method is likely to have captured mostly
137 the bacteria from the surface of the proboscis rather than those present within the
138 mandibles.
139
140 Immediately after the sampling of mouth-associated bacteria, we dissected the bees by
141 gently pulling the abdomen using tweezers to expose the crop, making sure the crop did
142 not touch other parts of the bee body. We then removed the crop from the bee body
143 using another set of tweezers (Fig. 1b). Both tweezers were sterilized before each use
144 by dipping in 70% ethanol and then flaming. Each crop sample was separately placed in
145 40 μL of sterile water within sterile microcentrifuge tubes (Fig. 1c) and homogenized
146 using a sterile plastic pestle. All mouth and crop samples were immediately stored at -
147 80°C until further processing.
148
149 We collected crop and mouth samples not just in winter during the *P. mume* flowering
150 season, but also in summer, to determine whether crop and mouth differed in the way
151 bacterial composition changed from summer to winter. Hives of *A. cerana*, many of
152 which were made of carved tree trunks, were maintained by local beekeepers
153 throughout the year in the region. In contrast, most *A. mellifera* hives in the region were

154 transported elsewhere in the country while *P. mume* was not in bloom. However, some
155 were kept locally throughout the year, allowing us to collect bees in both seasons. One
156 site (Nishi-Honjo) was available for bee collection in both summer and winter, allowing
157 bacterial taxa from the same site and hives to be compared between seasons. In
158 addition, at two of our sites (Higashi-Honjo and Kamihaya), we were able to collect both
159 *A. cerana* and *A. mellifera*, enabling comparison of the two species collected at the
160 same locations (Table S1).
161
162 *Nectar collection*
163 In addition to the mouth and crop samples, we also collected samples of *P. mume* floral
164 nectar on the same days the bees were collected in the winter of 2019. At each of the
165 nine orchards where we sampled bees that winter (Table S1), we collected 16 nectar
166 samples (except at one site, Kamihaya, where we instead collected 24 nectar samples),
167 for a total of 152 nectar samples. Each sample was collected by probing 10 flowers on a
168 randomly selected branch of an individual *P. mume* tree with a sterile 0.5μl
169 microcapillary tube and dispensing the collected nectar into a PCR tube containing 40
170 μL of PCR-quality sterile deionized water. The collected nectar samples were
171 immediately placed in a box with ice packs during the transport to the laboratory. Within
172 six hours, the nectar samples were brought back to the Japanese Apricot Laboratory.
173 The samples were kept at -80°C until further processing for DNA extraction, except
174 during a one-day transport to the laboratory at Kyoto University in Shiga, Japan, and
175 another one-day transport from there to Stanford University in California, USA, during
176 which the samples were kept at about -5 °C.

177

178 *Microbial DNA analysis*

179 Using the nectar and bee samples obtained as above, we conducted microbial DNA
180 extraction, bacterial amplicon sequencing, quantitative real-time PCR (qPCR) of 16S
181 rRNA gene, and processing of the amplicon sequencing data (for details, see Text S1).

182

183 *Statistical analysis*

184 All analyses were conducted using R version 4.0.4 [14]. The ASV_{97} dataset was
185 analyzed with the phyloseq and vegan packages [15, 16]. Venn diagrams of sample
186 types and seasons at the Genus level were created using the MicrobiotaProcess
187 package [17, 18]. Differential abundances of bacteria agglomerated to the Genus level
188 were explored with the DESeq2 package version 1.30.1 [19]. The taxa identified as
189 significantly differentially abundant in the different contrasts (i.e. winter mouth vs. winter
190 crop, winter nectar vs. winter crop, winter mouth vs. winter nectar, and summer crop vs.
191 summer mouth; Fig S1) were aggregated. Of these taxa, those that were present in at
192 least 50% of the sample type groups (Table S2) were further analyzed with a heatmap.
193 The heatmap was created with the ComplexHeatmap package [20]. Differential
194 abundance taxa phylogeny was made with a GTR model using the phangorn package
195 [21]. Sample cladogram was based on a weighted UniFrac distance [22]. Alpha diversity
196 (Shannon index) in each sample was calculated and compared by fitting a linear mixed
197 effects model of Shannon index as the response and site as a random effect with 2000
198 parametric bootstraps [23]. Shannon-Weaver evenness was calculated using the
199 evenness function from the microbiome package [24]. Permutation multivariate analysis

200 of variance using a weighted UniFrac distance matrix [22] was used to test the effects of
201 season, sample type, site, and host species on bacterial ASV_{97} composition. The beta
202 diversity of relative abundance and absolute abundance samples was visualized with a
203 Principal Coordinate Analysis, and individual sample distances from the centroid and
204 the homogeneity of dispersion of the samples were calculated. We also did Multinomial
205 Species Classification Method (CLAM) tests, using the clamtest package [25], to identify
206 $ASVs_{97}$ found more frequently in either winter or summer, those found frequently in both
207 seasons, and those too rare to categorize as summer- or winter-associated, or
208 generalists.
209
210 Apilactobacillus kunkeei *strain-level phylogenetic analysis*
211 Fifty µL of each thawed honeybee crop sample were aliquoted onto De Man, Rogosa
212 and Sharpe (MRS) agar (Sigma-Aldrich), spread thoroughly using a sterile loop, and
213 incubated aerobically at 37°C for approximately 48 hours. Half of each single white
214 colony was picked and placed in 10 µL of MilliQ (EMD Millipore, Burlington, MA, USA)
215 water, and the other half was streaked onto a new MRS agar plate with a sterile loop.
216 The 16S rRNA gene of these colonies was amplified using the universal primers 27F
217 and 1492R in a 25 µL PCR amplification that included 12.5 µL of MyTaq Red Mix
218 (Bioline), 8.75 µL of PCR-grade water, 1.25 µL each of the forward and reverse primers,
219 0.25 µL of dimethyl sulfoxide (DMSO), and 1 µL of each diluted colony. The resulting
220 product was sequenced with Sanger sequencing, and colonies identified as
221 *Apilactobacillus kunkeei* were once again isolated and placed in 10 µL of MilliQ (EMD
222 Millipore) water. The variable segments of three housekeeping genes, *lepA*, *recG*, and

223 *rpoB* were amplified using previously described primers [4, 26]. The 25 µL PCR
224 amplification was made up of 11 µL of PCR-grade water, 12 µL of MyTaq Red Mix
225 (Bioline), 0.5 µL of each forward and reverse primer, and 1 µL of the diluted colony. The
226 annealing temperature for the PCR amplification was set to 47°C for *lepA* and *recG* and
227 60°C for *rpoB* for 30 seconds [4, 26]. The amplified PCR product was sequenced with
228 Sanger sequencing. As previously described [27], the sequences were aligned with
229 MAFFT [28] and trimmed with TrimAl [29] with a penalty for more than 50% gaps.
230 Individual gene trees and the partitioned analysis for the multi-gene alignment were
231 inferred using IQ-TREE [27, 30]. Statistical support for the phylogenies was calculated
232 using 1000 replicates of both a Shimodaira-Hasegawa-like approximation likelihood test
233 and ultrafast bootstrapping along with a Bayesian-like transformation of aLRT [27, 31,
234 32]. The partition model was selected by the IQ-TREE ModelFinder (Table S3) [27, 33].
235 *Apilactobacillus apinorum* Fhon13 (Table S4) [27] served as the outgroup for this
236 phylogenetic tree. Outlier long branches were trimmed with TreeShrink [34].
237

238 **Results**

239 *Crop–mouth–nectar comparisons*

240 Among winter samples, we found more than seven times as many bacterial $ASVs_{97}$ in
241 the crop (a total of 655) as in the mouth and the nectar (a total of 77 and 81,
242 respectively) (Fig. 2). Mean ASV_{97} diversity per sample was about six times higher in the
243 crop (1.78) than in the mouth (0.37) and the nectar (0.36) (crop–mouth: Welch's t =
244 17.1, *p-value* < 0.001; crop–nectar: Welch's t = 15.8, *p-value* < 0.001). Similarly, mean
245 evenness was about two times higher in the crop (0.74) than in the mouth (0.42) and

246 the nectar (0.38) (crop–mouth: Welch's t = 6.8, *p-value* < 0.001; crop–nectar: Welch's t

247 = 7, *p-value* < 0.001) (Fig. 3).

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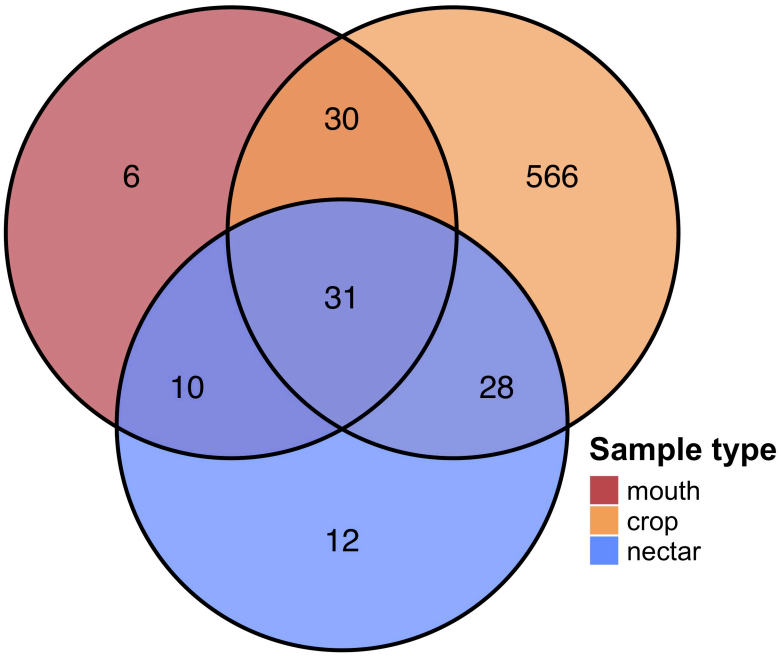

Fig. 2 The crop contains more common and constant $ASVs_{97}$ than the mouth and the nectar. Only 9% of 655 winter crop $ASVs_{97}$ were shared with the mouth or nectar samples whereas the mouth and nectar shared about 50% of their 77 and 81 $ASVs_{97,}$ respectively. Venn diagrams are colored by sample type with ASV_{97} counts in bold

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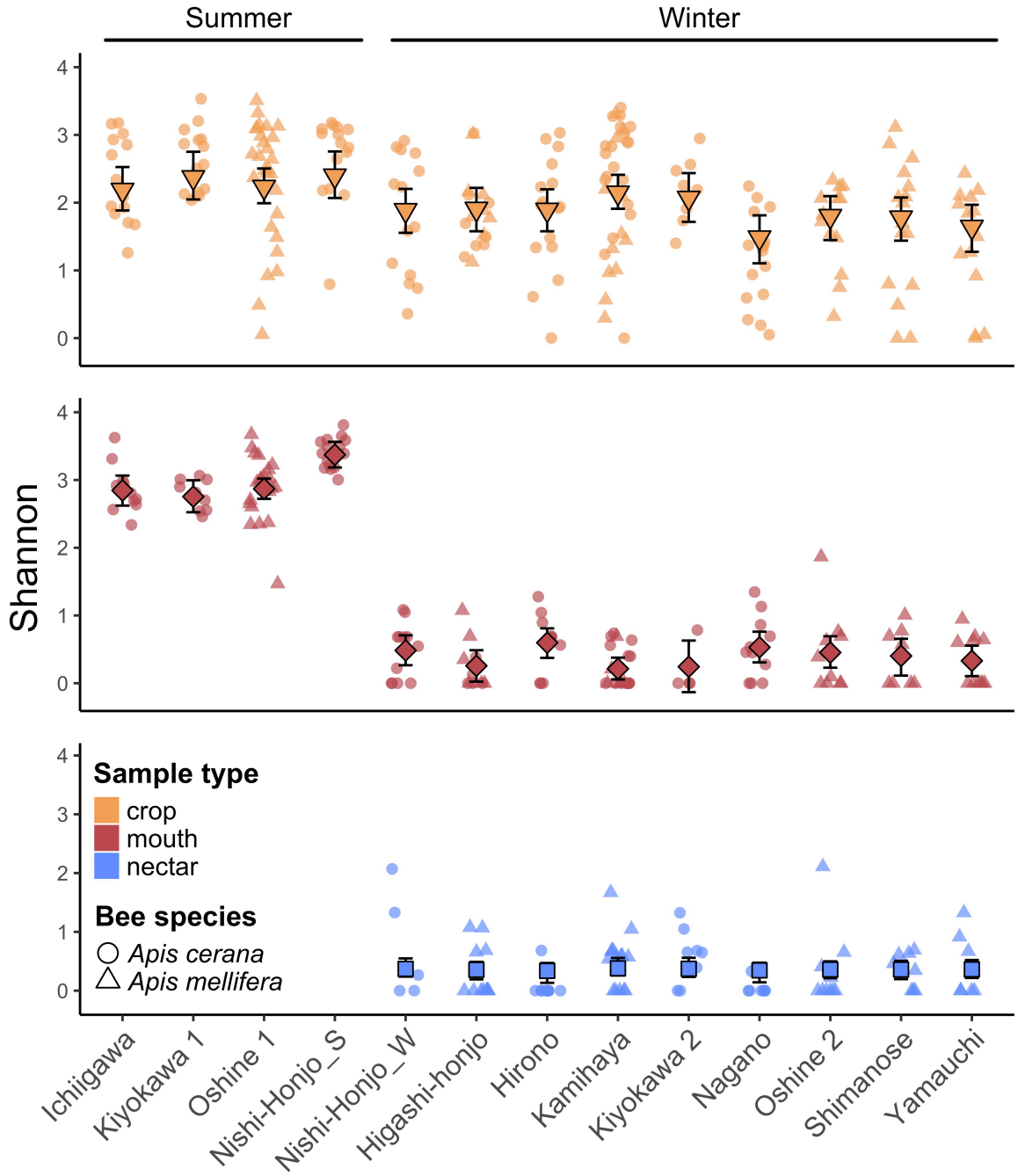
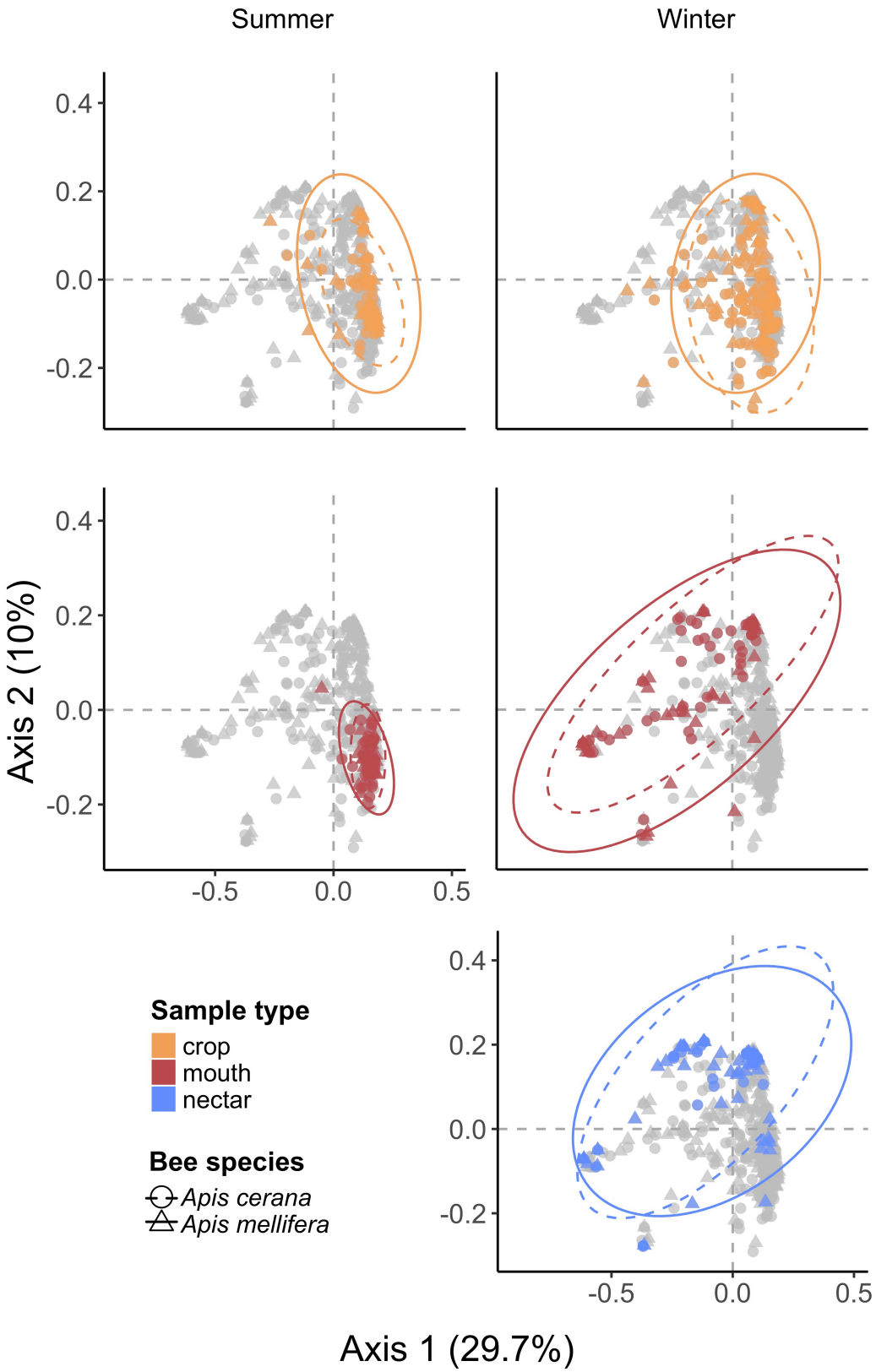


Fig. 3 The crop presents more consistent species richness. Species richness (Shannon index) in the crop varied less from summer to winter than the mouth, and winter mouth and nectar samples had similar species richness across sites. Nishi-Honjo_W and Nishi-Honjo_S are the samples collected at the Nishi-Honjo site (see Table S1) in winter and summer, respectively. Each data point represents a single sample collected from the labeled site, colored by sample type, and shaped by host species. Mean diversity is depicted by the large center point and the error bars depict the bootstrapped 95% confidence interval for samples from each site.

252 Of sample type (crop, mouth, or nectar), bee species (*A. mellifera* or *A. cerana*), and
253 sampling site (Fig. 1a), only sample type was a significant predictor of bacterial ASV_{97}
254 composition (PERMANOVA R^2 = 0.10 *p-value* = 0.001). Specifically, mouth and nectar
255 samples were more similar to each other in bacterial composition than either mouth and
256 crop samples or nectar and crop samples were to each other (Fig. 4; PERMANOVA
257 mouth–nectar R^2 = 0.03, *p-value* = 0.002; mouth–crop R^2 = 0.11, *p-value* = 0.001;
258 nectar–crop R^2 = 0.08, *p-value* = 0.001).
259


Summer
Winter
0.4
0.2
0.0
-0.2
Axis 2 (10%)
-0.5
0.0
0.5
Sample type
crop
mouth
nectar
Bee species
Apis cerana
Apis mellifera
Axis 1 (29.7%)

260

Fig. 4 Principal coordinate analysis of all samples presents similar composition across season in the crop but not the mouth. Winter mouth and nectar samples were highly similar (PERMANOVA sample type: R^2 = 0.07, *p-value* = 0.001, season: R^2 = 0.05 *p-value* = 0.001, host species: R^2 = 0.003, *p-value* = 0.154). Each dot represents a sample from an individual bee, colored by sample type. *Apis mellifera* samples are depicted with triangles and the solid ellipse whereas *Apis cerana* samples are depicted with circles and the dashed ellipse. Ellipses mark the 95% confidence interval, and axes are labeled with corresponding percent variation explained

261 In fact, most $ASVs_{97}$ in the crop were distinct from those in the mouth and the nectar,
262 with only 9% (4-8% in *A. cerana* and 6-9% in *A. mellifera*; Fig. S2) of the crop $ASVs_{97}$
263 also present in the mouth or the nectar (Fig. 2). Taxa frequently found in the crop, such
264 as *Bombella, Gilliamella, Lactobacillus*, and *Snodgrassella*, were mostly absent in the
265 nectar and only infrequently present in the mouth (Figs. 5 and S3). Furthermore, some
266 $ASVs_{97}$ belonging to *Acinetobacter,* Comamonadaceae, and *Flavobacterium* were
267 consistently found in the crop at all local sites examined, whereas *Acinetobacter* was
268 present in only seven of the nine nectar sites and 11 of the 15 mouth sites, and there
269 were no other taxa that were present in mouth or nectar samples at all sites.
270

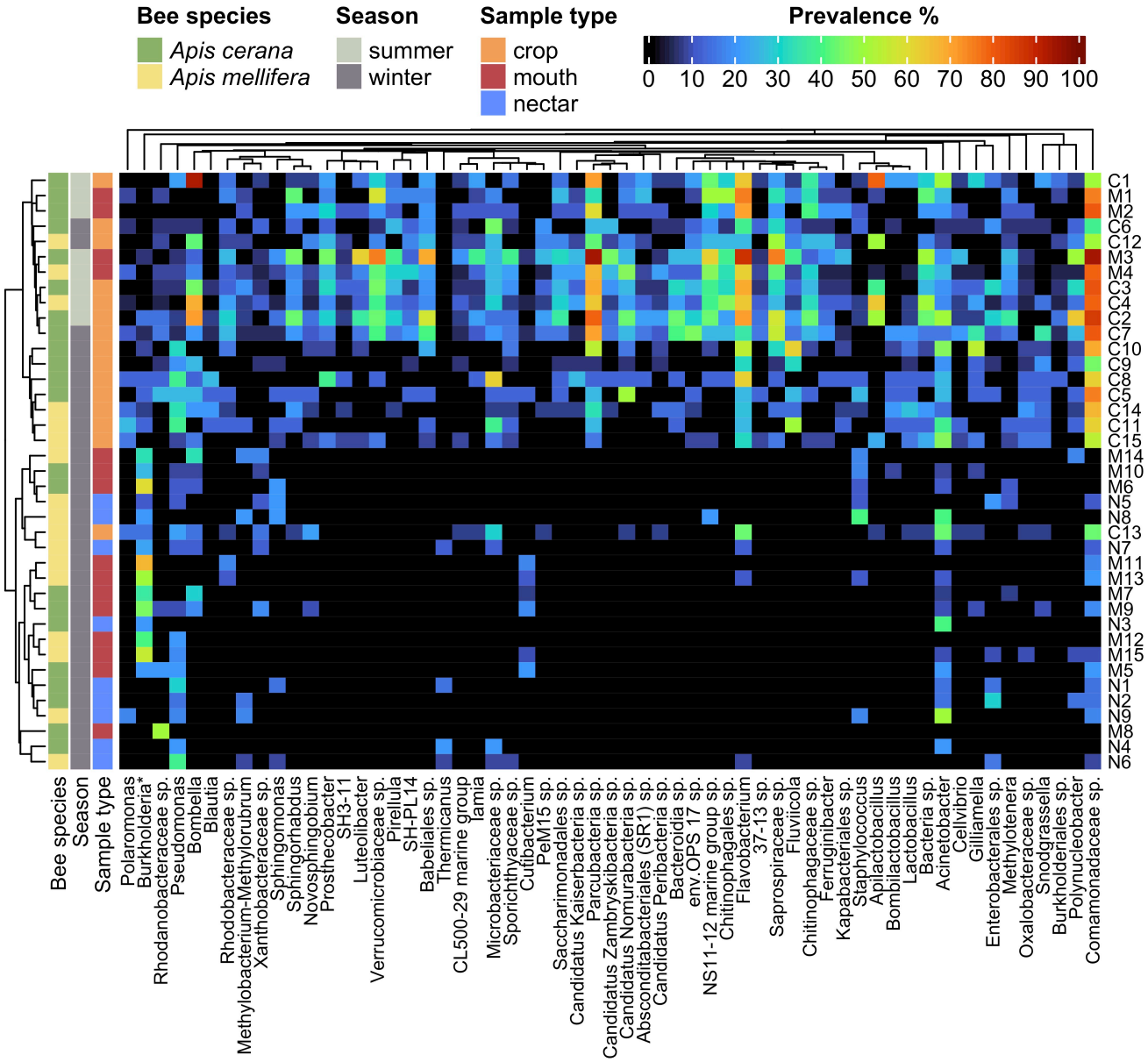


Fig. 5 There is a greater prevalence of differentially abundant taxa among crop groups than mouth and nectar groups. Samples were grouped by season, sampling site, host *Apis* species, and sample type as illustrated by colored bars to the left of the heatmap and described in Table S2. For ease of visualization, heatmap columns are differentially abundant taxa that were present in at least 50% of one of the sample groups e.g., at least 50% of all the mouth groups. Burkholderia* denotes the genus Burkholderia-Caballeronia-Paraburkholderia. The full heatmap with individual samples and all differentially abundant taxa can be found in Figure S3. The $ASVs_{97}$ were agglomerated to genus, colored by prevalence within groups, and arranged according to their phylogeny. The y-axis dendrogram depicts the weighted UniFrac distance between samples

271

These results held true even when we excluded those taxa that are known as the core species of the hindgut bacterial community (*Gilliamella*, *Lactobacillus*, and *Snodgrassella*) from the dataset used for the analysis (Fig. S4).

Summer–winter difference in crop vs. in mouth

Although ASV_{97} richness was higher in summer than in winter in both the crop and the mouth, the magnitude of this difference was eight times greater in the mouth than in the crop (Figs. 3 and S5). In terms of ASV_{97} composition, most of the crop samples were clustered together regardless of season in both the relative and absolute abundance datasets (relative abundance: winter vs. summer, PERMANOVA R^2 = 0.03, *p-value* = 0.001; absolute abundance: winter vs. summer, PERMANOVA R^2 = 0.04, *p-value* = 0.021), whereas the mouth samples formed two distinct clusters, corresponding to summer and winter (relative abundance: PERMANOVA R^2 = 0.20, *p-value* = 0.001; absolute abundance: PERMANOVA R^2 = 0.31, *p-value* = 0.001; Figs. 4-6a). These two mouth clusters differed in the amount of bacterial composition variation among samples, with winter mouth samples showing higher variation than summer mouth samples (Figs. 4 and 6a) despite no significant difference between them in total bacterial load (Fig. 6b). In contrast to the mouth, the bacterial load in the crop was significantly higher in the summer than in the winter in the *A. mellifera* crop (but not in *A. cerana* crop; Fig. 6b).

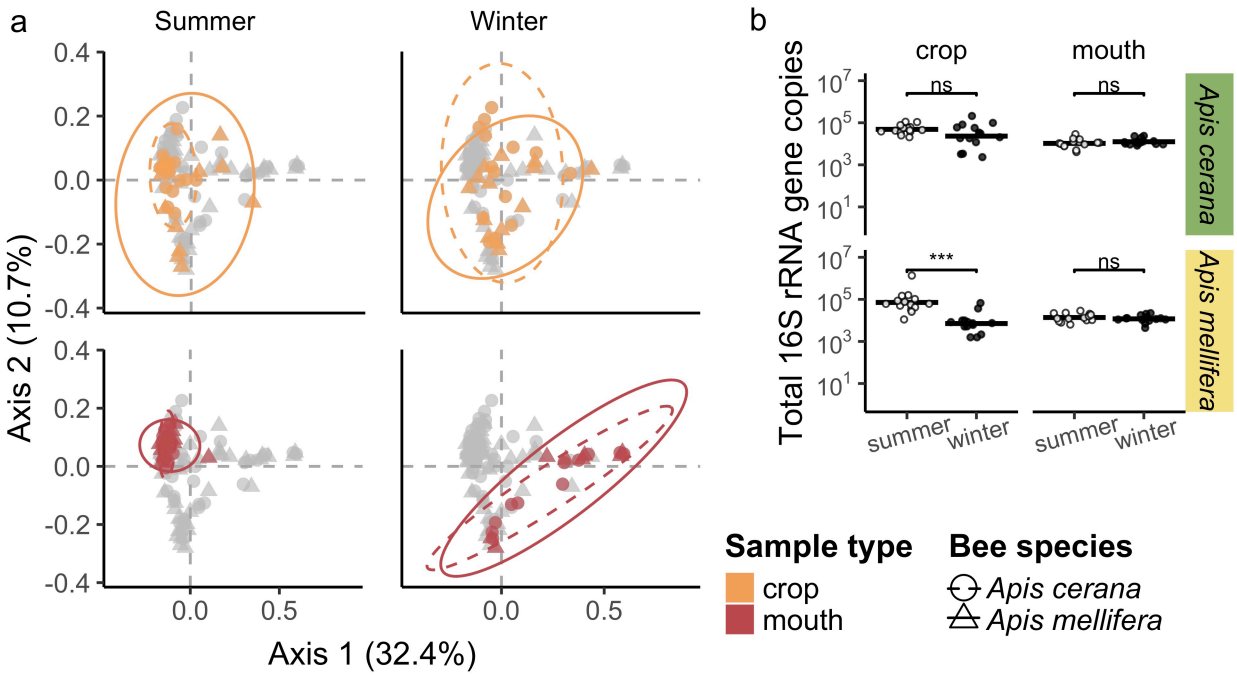


Fig. 6 Consideration of absolute abundance estimates in analysis of bacterial composition. (**a**) As in Figure 4, principal coordinate analysis of samples that takes into account absolute total bacterial abundance indicates greater compositional similarity between crop samples than mouth samples across season. Specifically, mouth samples cluster more closely in the summer and are more dispersed in the winter than the crop samples (betadisper distance to median: summer-mouth = 0.21, winter-mouth = 0.40, summer-crop = 0.26, winter-crop = 0.32; betadisper Tukey HSD summer-winter mouth: *p-value* < 0.001, summer-winter crop: *p-value* = 0.008). Overall, sample type and season but not host species are significant predictors of composition (PERMANOVA sample type: R^2 = 0.03, *p-value* = 0.009, season: R^2 = 0.12, *p-value* = 0.001, host species: R^2 = 0.01, *p-value* = 0.214). Each point represents a single sample, is color coded by sample type, and shaped by bee species. Ellipses represent the 95% confidence interval for each bee species. Percent variation explained by each axis is labeled. (**b**) Mouth samples tended to have a more even, lower bacterial load across season than did crop samples. The *Apis mellifera* but not the *Apis cerana* crop samples had a higher bacterial load in the summer than the winter. The mouth maintained the same bacterial load across seasons. Total 16S rRNA gene copies per 10 µl are presented on the log scale, with each point depicting a sample from an individual bee colored by season (summer = white, winter = black). Wilcoxon test significance is labeled with *** = *p-value* < 0.001 and ns = not significant

295

296 The CLAM test [25] identified 28% of the $ASVs_{97}$ in the crop as generalists, meaning

297 that they were similarly frequent in winter and summer. In contrast, the CLAM test

298 classified only 2% of the $ASVs_{97}$ in the mouth as generalists, with the majority of others

299 identified as either summer- or winter-associated (76 and 8%, respectively; Fig. 7).

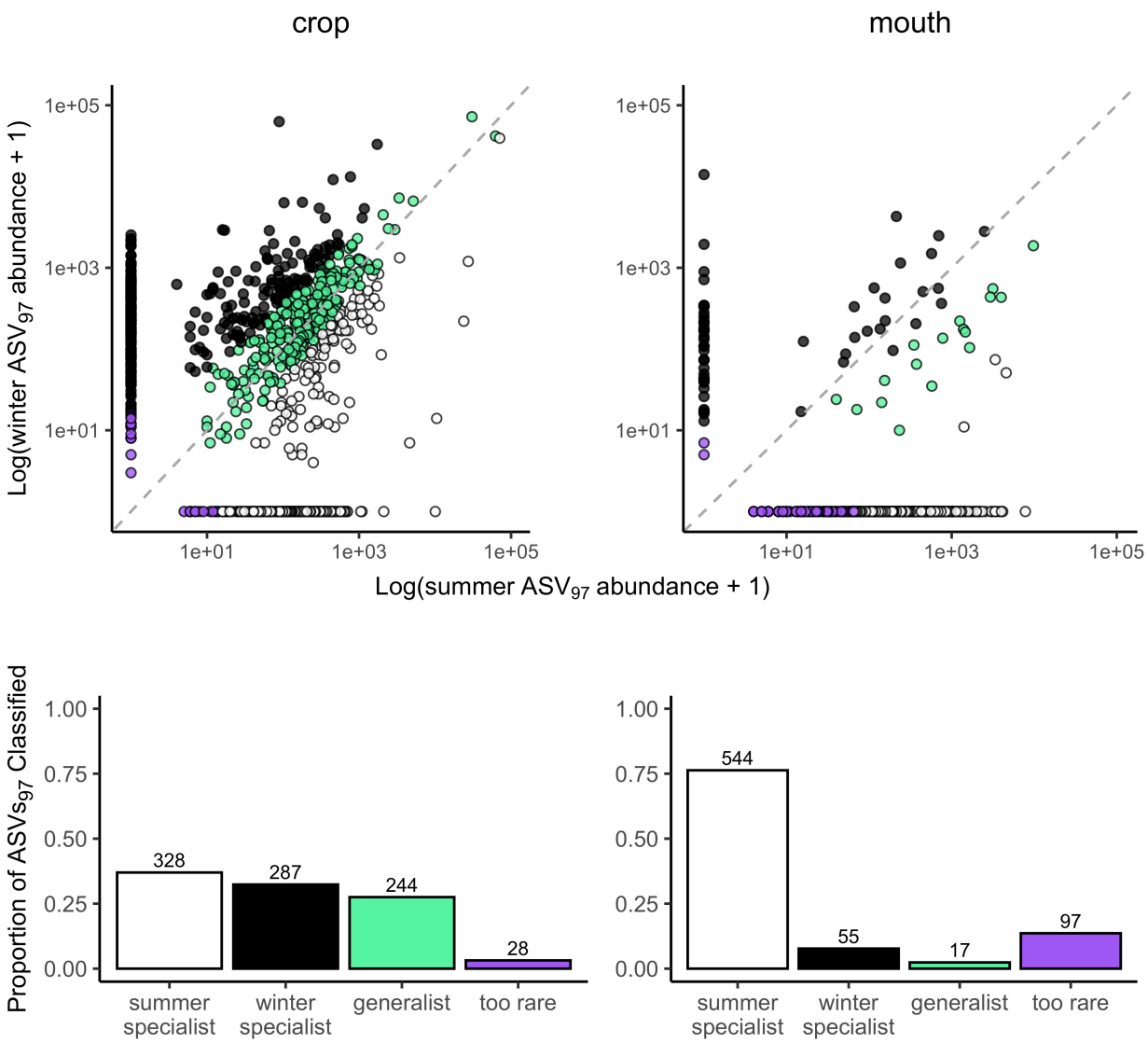


Fig. 7 There are bacteria that have high affinity for the crop. Generalist bacteria, i.e., those with no special affinity to one environment or, in this case, season, are greater between the summer and winter crop samples (about 27% of the $ASVs_{97}$) than the corresponding mouth samples. The mouth presents only about 2% generalists, with most $ASVs_{97}$ classified as summer-associated (76%). Scatterplots depict the log abundance of each ASV_{97}, with each point representing a single ASV_{97} color coded by its category. $ASVs_{97}$ from both *A. mellifera* and *A. cerana* are included. The identity line is plotted in a dotted grey pattern. The proportion of $ASVs_{97}$ classified in each category is depicted in the corresponding bar plots below the scatterplots, with categories colored as in the scatterplot

302 Apilactobacillus kunkeei *strains in the crop*

303 The multi-gene tree of *Apilactobacillus kunkeei* strains suggested potential season- and
304 *Apis* species-specificity. Specifically, the tree had one clade consisting of only isolates
305 from summer *A. cerana* samples and two other clades consisting of isolates from
306 summer and winter *A. mellifera* samples (Figs. 8 and S6). Those associated with *A.*
307 *cerana* originated from five bees, three from Nishi-Honjo and two from Ichiigawa. Those
308 associated with *A. mellifera* originated from eight bees: five summer bees from Oshine 1
309 and three winter bees, one each from Shimanose, Yamauchi, and Kamihaya.

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311

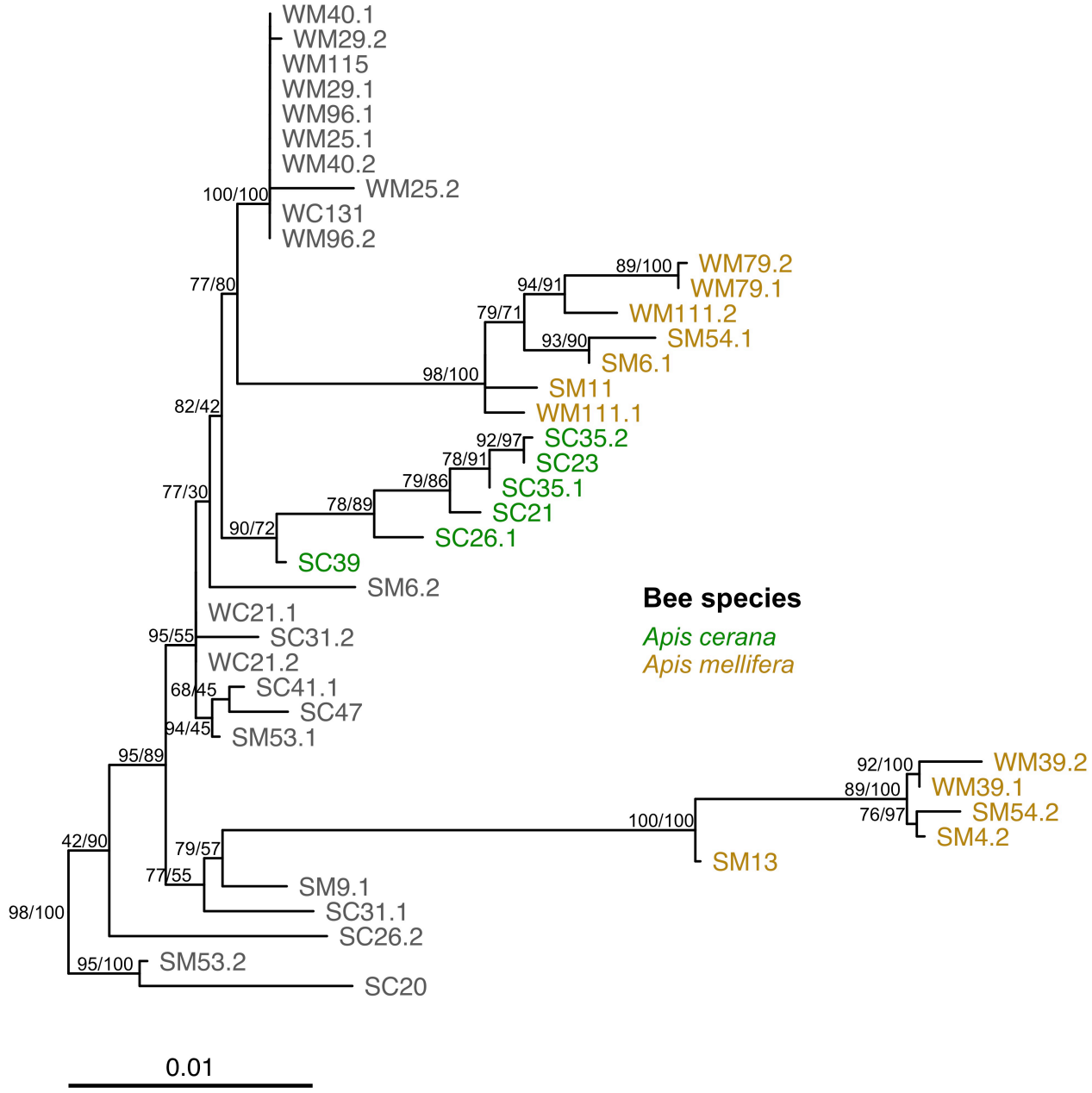

Fig. 8 Multi-gene maximum likelihood tree of *Apilactobacillus kunkeei* isolates contains *Apis* species specific clades. The phylogeny is the result of a multi-gene alignment partitioned analysis of 49 *A. kunkeei* isolates and *A. apinorum* Fhon13 (see Tables S3 and S4). In the depicted tree, substitution frequencies are represented by branch length. Isolates are labeled starting with an “S” for summer or “W” for winter, followed by a “C” for *Apis cerana* and an “M” for *Apis mellifera* host species, the host ID number, and lastly the isolate number. Isolates that occurred in both species are colored in grey whereas those that are phylogenetically similar with high statistical support and associated with a single host species are colored by host species. Statistical support was estimated from 1000 Shimodaira-Hasegawa-approximate likelihood ratio test (SH-aLRT) pseudo replicates and ultrafast bootstraps (UFboot). Nodes with an SH-aLRT score greater than 0 are labeled with respective SH-aLRT/UFboot values. *Apilactobacillus apinorum* root is not depicted for ease of visualization. Figure S6 presents the full tree with statistical support on all nodes and *A. apinorum* branch

312

313 **Discussion**

314 Based on the conventional assumption that bacterial assemblage in the crop primarily
315 consists of transient microbes acquired via nectar foraging [2, 27, 35, 36], we expected
316 a high degree of bacterial overlap among nectar, mouth, and crop samples. We did find
317 high overlap between the nectar and the mouth in the winter, but to our surprise, most of
318 the crop taxa were clearly distinct from those found in the nectar and the mouth (Figs. 2
319 and 5) [11]. Several $ASVs_{97}$ of *Acinetobacter* and *Pseudomonas* that were relatively
320 prevalent in both crop and mouth samples in our study have been commonly found in
321 nectar as well [37-39]. However, most of the taxa that were more prevalent in the crop
322 were rare or entirely absent in nectar and mouth samples (Figs. 5, S2 and S3) [11].

323

324 In addition, our results indicate that the crop maintained a high degree of taxonomic
325 consistency between winter and summer despite differences in bacterial load in *A.*
326 *mellifera* crop samples, a pattern that is in sharp contrast to the large compositional
327 changes seen in the mouth between seasons and between bee species (Figs. 3, 4, 6a
328 and 7). Taxa common in the crop in both seasons included Comamonadaceae,
329 *Acinetobacter*, Saprospiraceae, *Flavobacterium*, *Gilliamella*, *Bombella*, *Apilactobacillus*,
330 *Pseudomonas*, *Fluviicola*, *Polynucleobacter*, *Snodgrassella*, and *Microbacteriaceae*
331 species (Fig. 5). *Apilactobacillus* and *Bombella*, previously called *Parasaccharibacter*
332 *apium* [5, 40], which are among the most commonly reported taxa in the crop of
333 foraging adults, have also been found frequently in food stores, larvae, and queens [2,
334 4, 5, 27, 36, 41-43]. Although the data we present in this study are correlational,

335 preventing us from establishing causal relationships, we speculate that the crop may
336 serve as a consistent reservoir of these taxa that can influence the health of the adult
337 bees and the larvae that they tend [2, 27].
338
339 We also found evidence for potential strain-level specificity among *Apilactobacillus*
340 *kunkeei* isolates from collected foragers. One clade was made up of summer *A. cerana*
341 isolates from two different sites, and two others contained only *A. mellifera* isolates from
342 both seasons (Figs. 8 and S6). Although a larger sample size than we had in this study
343 as well as full genome sequencing are needed to ascertain the putative strain-level
344 differences, previous studies have shown differences in specific house-keeping genes
345 to suggest strain-level host specificity between *Apis* and *Bombus*, and even among *Apis*
346 species [44, 45]. As in these previous studies, some of our isolates clustered more
347 frequently by host species than by site [46, 47], potentially suggesting host specificity
348 and possibly specific host-symbiont relationships between *Apis* species and *A. kunkeei*
349 strains, even though this possibility remains speculative at this stage.
350
351 A few caveats should be considered in interpreting our findings. First, our analysis is
352 mostly based on relative abundance [46], even though we used qPCR to attempt to
353 estimate at least total absolute abundance in crop and mouth samples (Fig. 6b).
354 Absolute abundance of each taxon, which may be more relevant to bacterial effects on
355 the host [12, 46], can differ even when relative abundance is the same. However, we did
356 see similar bacterial composition patterns in our analysis of both relative and absolute
357 abundance (Figs. 4 and 6a). Second, we found that the crop did not consistently have a

358 higher bacterial load than the mouth (Fig. 6b). Although resident populations can be
359 persistent regardless of size, there may be more to the crop community than is
360 suggested by the compositional data. Third, we had only one site, Nishi-Honjo, where
361 bees were sampled in both summer and winter. A greater and more even number of
362 bees at each site in multi-year seasonal sampling would have made our results most
363 robust. Nevertheless, our analysis of several hundred bee and nectar samples all
364 sampled within the same landscape clearly refutes the hypothesis that crop
365 communities simply reflect what is available in the environment.
366
367 In conclusion, our results suggest that the composition of crop bacteria may be more
368 deterministic than generally assumed, questioning the notion that crop bacteria are
369 environmentally acquired transients that are unimportant to the bees. Although less well
370 studied than midgut and hindgut microbes, a small but increasing number of recent
371 studies indicate that some crop microbes do alter the health of individuals and hives [6,
372 48-50]. Our data reinforce the idea that it would help to study how microbial
373 communities are assembled and maintained not just in the midgut and the hindgut, but
374 also the crop, to better understand the relationship between honeybees and their gut
375 microbes.
376

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Competing Interests

The authors declare no competing interests.

Author Contributions

KT, MK, and TF designed the study and collected the samples. MLW did the DNA extraction, sequencing preparation, and bioinformatic analyses. JY and ACH did the quantitative PCR processing and analyses. MLW and LED did the statistical analyses. MLW wrote the first draft of the manuscript. All authors contributed to and approved the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available in GenBank, https://www.ncbi.nlm.nih.gov (BioProject ID no. PRJNA1076572 and accession no. PP412473-PP412519, PP341993-PP342038, PP382363-PP382409, PP382266-PP382314, PP382315-PP382362), or are included in this published article and its supplementary information files. All custom scripts are available on GitHub at https://github.com/mlwarren20/ume_bee_analysis.