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

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Abstract

Bacteria in the honeybee gut have garnered much attention as a factor affecting bee health. However, unlike the hindgut, which has been the primary focus of this research, the crop, or the honey stomach, is often 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 the mouth of foraging adults of *Apis mellifera* and *A. cerana japonica* and the floral nectar of the Japanese apricot, *Prunus mume*, visited by the bees in the Minabe-Tanabe region of Japan. We found that the crop was distinct from both the mouth and the nectar in bacterial composition even though all samples were collected simultaneously at the same locations, whereas mouth and nectar samples were indistinguishable from each other. Furthermore, the crop remained similar in bacterial composition and alpha and beta diversity from summer to winter, whereas 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 that is largely distinct from the environmental taxa that the bees encounter as they forage in the environment. We also found, however, that strains of a representative crop-associated species, *Apilactobacillus kunkeei*, could be season- and host species-specific. Together, these findings suggest that further studies of crop-associated bacterial communities are needed to better understand the relationship between honeybees and their gut bacteria.

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 (1-3). One component of the honeybee gut, the crop, 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 (2, 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 (7-10).

Given its function for temporary storage and transport of floral nectar (11), it seems reasonable to assume that the crop is occupied mainly by the transient microbes acquired from the environment via foraging of nectar (12). 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, 13). 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 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* (14). Our nectar samples come from *P. mume* flowers near the hives of the *A. mellifera* and *A. cerana* bees that we caught for crop and mouth sampling.

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 FAO 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 *Apis 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 (14). 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. 1, 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 colonies in or near these apricot orchards (Table S1). Honeybees were collected as they flew near their hives located within or near the orchards. At orchards where 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. 1).

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 handling. The taped bees were made to drink approximately half of 20 μl of sterile 20% sucrose solution placed on the lid of a sterile 1.5-ml microcentrifuge tube. After the feeding, the remaining solution was placed in the microcentrifuge tube with the lid now closed. These solutions were later used to study the bacteria associated with the mouth. We refer to these solutions as mouth samples. Since we had the bees extend their proboscis into the sterile sucrose solution, our method is likely to have captured mostly

114 the bacteria from the surface of the proboscis rather than those present within the
115 mandibles.
116
117 Immediately after the sampling of mouth-associated bacteria, we dissected the bees by
118 gently pulling the abdomen using tweezers to expose the crop, making sure the crop did
119 not touch other parts of the bee body. We then removed the crop from the bee body
120 using another set of tweezers. Both tweezers were sterilized before each use by dipping
121 in 70% ethanol and then flaming. Each crop sample was separately placed in 40 μl of
122 sterile water within sterile microcentrifuge tubes and homogenized using a sterile plastic
123 pestle. All mouth and crop samples were immediately stored at -80°C until further
124 processing.
125
126 We collected crop and mouth samples not just in winter during the *P. mume* flowering
127 season, but also in summer, to determine whether crop and mouth differed in the way
128 bacterial composition changed from summer to winter. Hives of *A. cerana*, many of
129 which were made of carved tree trunks, were maintained by local beekeepers
130 throughout the year in the region. On the other hand, most *A. mellifera* hives in the
131 region were transported elsewhere in the country while *P. mume* was not in bloom.
132 However, some were kept locally throughout the year, allowing us to collect bees in both
133 seasons. One site (Nishi-Honjo) was available for bee collection in both summer and
134 winter, allowing bacterial taxa from the same site and hives to be compared between
135 seasons. In addition, at two of our sites (Higashi-Honjo and Kamihaya), we were able to

136 collect both *A. cerana* and *A. mellifera*, enabling comparison of the two species
137 collected at the same locations (Table S1).
138
139 *Nectar collection*
140 In addition to the mouth and crop samples, we also collected samples of *P. mume* floral
141 nectar on the same days the bees were collected in the winter of 2019. At each of the
142 nine orchards where we sampled bees that winter (Table S1), we collected 16 nectar
143 samples (except at one site, Kamihaya, where we instead collected 24 nectar samples),
144 for a total of 152 nectar samples. Each sample was collected by probing 10 flowers on a
145 randomly selected branch of an individual *P. mume* tree with a sterile 0.5μl
146 microcapillary tube and dispensing the collected nectar into a PCR tube containing 40 μl
147 of PCR-quality sterile deionized water. The collected nectar samples were immediately
148 placed in a box with ice packs during the transport to the laboratory. Within six hours,
149 the nectar samples were brought back to the Japanese Apricot Laboratory. The samples
150 were kept at -80°C until further processing for DNA extraction, except during a one-day
151 transport to the laboratory at Kyoto University in Shiga, Japan, and another one-day
152 transport from there to Stanford University in California, USA, during which the samples
153 were kept at about -5 °C.
154
155 *Microbial DNA extraction*
156 In our laboratory at Stanford University, the Qiagen DNeasy Blood and Tissue kit and
157 Qiagen Genomic DNA Buffer set (Qiagen, Germantown, MD, USA) were used to extract
158 DNA from all the samples. The extraction followed the gram-positive bacteria protocol

159 along with the following modifications. For each of the crop, mouth, and nectar samples
160 collected, 10 µL of the thawed sample was combined with 2 µL of zymolyase enzyme
161 (MP Biomedicals, Burlingame, CA, USA) within a microcentrifuge tube. These mixtures
162 were incubated at 30°C for 30 minutes. Once the incubation period was complete, 10 µL
163 of a 500:1 G2 (from the Qiagen Genomic DNA Buffer set) buffer to RNAse A (Qiagen)
164 mixture was added to each DNA and zymolyase mixture. 20 µL of ProteinaseK (Qiagen)
165 and 180 µL of Buffer ATL (Qiagen) were added to each sample and vortexed to mix. The
166 samples were then incubated at 56°C overnight. The next morning the samples were
167 mixed and centrifuged to collect any condensation on the caps, and 410 µL of AL-
168 Ethanol (Qiagen) mixture were added to each sample and shaken vigorously to mix.
169 The rest of the protocol was followed as stated in the DNeasy Blood & Tissue Handbook
170 (2018) with the only exception being that the final elution of DNA was done with 50 µL of
171 Buffer AE (Qiagen) rather than 200 µL to increase the final DNA concentration in the
172 eluate. The final extracted DNA from each sample was stored in sterile microcentrifuge
173 tubes at 4°C until library preparation.
174
175 *Bacterial amplicon sequencing*
176 The highly variable (V4) region of the 16S rRNA gene was amplified using primers 515F
177 (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-
178 3´) and 806R
179 (5´GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT
180 -3´) (15). Both primers included an Illumina (San Diego, CA, USA) adapter on the 5´
181 ends used to attach unique Nextera XT indexes for sample identification. Each 10 µL

182 first step PCR reaction consisted of 3.2 µL of PCR-grade water, 5 µL of MyTaq HS Red
183 Mix (Bioline, Taunton, MA, USA), 0.4 µL each of the forward and reverse primers, and 1
184 µL of the extracted DNA sample. PCR conditions were 95ºC for 2 minutes, followed by
185 35 cycles of 95ºC for 20 seconds, 50ºC for 20 seconds, 72ºC for 50 seconds, and a final
186 extension at 72ºC for 10 minutes with storage at 4ºC. Amplification was confirmed by
187 running samples on an agarose gel, and the resulting amplicons were purified using
188 Sera-Mag SpeedBeads (Sigma-Aldrich, St. Louis, MO, USA). The second step 10 µL
189 PCR reaction consisted of 3.2 µL of PCR-grade water, 5 µL of MyTaq HS Red Mix
190 (Bioline), 0.4 µL each of the combined Nextera XT index primers 1 and 2, and 1 µL of
191 the first step PCR product. PCR conditions for this second step were 95ºC for 2
192 minutes, followed by 8 cycles of 95ºC for 20 seconds, 50ºC for 20 seconds, 72ºC for 50
193 seconds, and a final extension at 72ºC for 10 minutes with storage at 4ºC. Amplification
194 was confirmed by running samples on an agarose gel, the barcoded amplicons were
195 purified once again using Sera-mag SpeedBeads (Sigma-Aldrich), and the resulting
196 clean product was pooled evenly across samples. The pooled samples were combined
197 to a concentration of 4 nM using a Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA,
198 USA), and then the entire amplicon libraries were pooled together. The final DNA
199 concentration and quality of the pooled amplicon libraries were quantified with a
200 Fragment Analyzer (Agilent, Santa Clara, CA, USA), and then sequenced on an Illumina
201 MiSeq using a 2 X 300 cycle sequencing kit with a 15% PhiX spike-in (16) at the
202 Stanford Genomic Sequencing Service Center.
203
204 *Quantitative real-time PCR (qPCR) of 16S rRNA gene*

205 A subset of the crop and mouth samples was selected for 16S rRNA gene
206 quantification. These 112 samples were made up of mouth and crop samples from 28
207 *Apis mellifera* and 28 *A. cerana* bees, 14 each from the summer and winter sites. To
208 ensure we captured differences in bacterial load in Nishi-Honjo, the one site where we
209 did the crop and mouth sampling in both seasons, we included all samples from this site
210 in this analysis. The remaining *A. cerana* and *A. mellifera* samples were randomly
211 selected from the other sites. The total number of bacterial 16S rRNA genes was
212 quantified in the extracted DNA samples. Targeted genes were amplified using the
213 CFX96™ Real-Time System (C1000TM Thermal Cycler, Bio-Rad, Hercules, CA, USA).
214 All reactions were performed using SsoFast™ EvaGreen® Supermix (Bio-Rad)
215 according to the manufacturer's instructions and in triplicate using 10 µL of SsoFast
216 EvaGreen Supermix, 0.6 µL of each primer, 2 µL DNA samples, and nuclease-free
217 water to a final volume of 20 µL. 16S rRNA genes were amplified using the primer sets
218 1055YF(ATGGYTGTCGTCAGCT) and 1392R (ACGGGCGGTGTGTAC), with the PCR
219 conditions as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles
220 of 15 seconds at 95°C and 1 minute at 58°C (17). The detection limit (1.6×10^2 gene
221 copies per reaction) for quantitative real-time PCR was determined using 55 standard
222 curves for 16S rRNA genes that were constructed using the serial 10-fold dilutions from
223 10^{-1} to 10^{-7} of known concentrations of synthesized oligonucleotides (Integrated DNA
224 Technologies, Inc., Coralville, IA, USA). The gene copy number was determined from
225 CT values equal to or higher than the negative controls were considered as below the
226 detection limit.
227

228 *16S rRNA gene amplicon-sequencing data processing*

229 The raw amplicon sequences were trimmed using the Cutadapt software (18). The
230 Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline (19) was used to merge
231 paired-end sequences, quality filter these sequences, remove chimeric reads, and
232 cluster sequences into amplicon sequence variants (ASVs). Taxonomy assignments for
233 each ASV were assigned using the SILVA database (20). ASVs present in the DNA
234 extraction and first and second step PCR negative controls as well as those identified
235 as chloroplast or mitochondria were removed from the dataset. Any phyla less than 1%
236 abundant among all samples were trimmed (21). This preprocessing resulted in several
237 samples containing no sequence reads. Consequently, these samples were excluded
238 from further analysis, resulting in a total of 218 crop samples, 161 mouth samples, and
239 82 nectar samples used for downstream analysis. Note that the taxon previously called
240 *Parasaccharibacter apium* (5) has been re-named *Bombella apis* (22). ASVs were
241 clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using
242 the DECIPHER and speedyseq R packages (23, 24).

243

244 *Statistical analysis*

245 All analyses were conducted using R version 4.0.4 (25). The OTU dataset was analyzed
246 with the phyloseq and vegan packages (21, 26). Venn diagrams of sample types and
247 seasons at the genus level were created using the MicrobiotaProcess package (27, 28).
248 Differential abundances of bacteria agglomerated to the genus level were explored with
249 the DESeq2 package version 1.30.1 (ref. 29). The taxa identified as significantly
250 differentially abundant in the different contrasts (i.e., winter mouth vs. winter crop, winter

251 nectar vs. winter crop, winter mouth vs. winter nectar, and summer crop vs. summer
252 mouth; Fig. S1) were aggregated. Of these taxa, those that were present in at least 50%
253 of the sample type groups (Table S2) were further analyzed with a heatmap. The
254 heatmap was created with the pheatmap package (30). Differential abundance taxa
255 phylogeny was made with a GTR model using the phangorn package (31). Sample
256 cladogram was based on a weighted UniFrac distance (32). Alpha diversity (Shannon
257 diversity index) in each sample was calculated and compared by fitting a linear mixed
258 effects model of Shannon diversity index as the response and site as a random effect
259 with 2000 parametric bootstraps (33). Shannon-Weaver evenness was calculated using
260 the evenness function from the microbiome package (34). Permutation multivariate
261 analysis of variance using a weighted UniFrac distance matrix (32) was used to test the
262 effects of season, sample type, site, and host species on bacterial OTU composition.
263 The beta diversity of samples was visualized with a Principal Coordinate Analysis, and
264 individual sample distances from the centroid and the homogeneity of dispersion of the
265 samples were calculated. We also did Multinomial Species Classification Method
266 (CLAM) tests, using the clamtest package (35), to identify OTUs found more frequently
267 in either winter or summer, those found frequently in both seasons, and those too rare
268 to categorize as summer- or winter-specialists or generalists.
269
270 Apilactobacillus kunkeei *strain-level phylogenetic analysis*
271 Fifty µL of each thawed honeybee crop sample were aliquoted onto De Man, Rogosa
272 and Sharpe (MRS) agar (Sigma-Aldrich), spread thoroughly using a sterile loop, and
273 incubated aerobically at 37°C for approximately 48 hours. Half of each single white

274 colony was picked and placed in 10 μL of MilliQ (EMD Millipore, Burlington, MA, USA)
275 water, and the other half was streaked onto a new MRS agar plate with a sterile loop.
276 The 16S rRNA gene of these colonies was amplified using the universal primers 27F
277 and 1492R in a 25 μL PCR reaction that included 12.5 μL of MyTaq Red Mix (Bioline),
278 8.75 μL of PCR-grade water, 1.25 μL each of the forward and reverse primers, 0.25 μL
279 of dimethyl sulfoxide (DMSO), and 1 μL of each diluted colony. The resulting product
280 was sequenced with Sanger sequencing, and colonies identified as *Apilactobacillus*
281 *kunkeei* were once again isolated and placed in 10 μL of MilliQ (EMD Millipore) water.
282 The variable segments of three housekeeping genes, *lepA*, *recG*, and *rpoB* were
283 amplified using primers described in Tamarit et al. 2015 and Ogier et al. 2019 (ref. 4,
284 36). The 25 μL PCR reaction was made up of 11 μL of PCR-grade water, 12 μL of
285 MyTaq Red Mix (Bioline), 0.5 μL of each forward and reverse primer, and 1 μL of the
286 diluted colony. The annealing temperature for the PCR reaction was set to 47°C for *lepA*
287 and recG and 60°C for *rpoB* for 30 seconds (4, 36). The amplified PCR product was
288 sequenced with Sanger sequencing. The sequences were aligned with MAFFT (37) and
289 trimmed with TrimAl (38) with a penalty for more than 50% gaps. Individual gene trees
290 and the partitioned analysis for the multi-gene alignment were inferred using IQ-TREE
291 (39, 40). Statistical support for the phylogenies was calculated using 1000 replicates of
292 both a Shimodaira-Hasegawa-like approximation likelihood test and ultrafast
293 bootstrapping along with a Bayesian-like transformation of aLRT (41-43). The partition
294 model was selected by the IQ-TREE ModelFinder (Table S3) (44). *Apilactobacillus*
295 *apinorum* Fhon13 (ref. 45; Table S4) served as the outgroup for this phylogenetic tree.
296 Outlier long branches were trimmed with TreeShrink (46).

297

298 **Results**

299 *Crop-mouth-nectar comparisons*

300 Among winter samples, we found more than seven times as many bacterial OTUs in the
301 crop (a total of 657) as in the mouth and the nectar (a total of 77 and 81, respectively)
302 (Fig. 2). Mean OTU diversity per sample was about six times higher in the crop (1.78)
303 than in the mouth (0.37) and the nectar (0.36) (crop-mouth: Welch's $t = 17.1$, $p < 0.001$;
304 crop-nectar: Welch's $t = 15.8$, $p < 0.001$). Similarly, mean evenness was about two
305 times higher in the crop (0.74) than in the mouth (0.42) and the nectar (0.38) (crop-
306 mouth: Welch's $t = 6.8$, $p < 0.001$; crop-nectar: Welch's $t = 7$, $p < 0.001$) (Fig. 3).

307

308 Of sample type (crop, moth, or nectar), bee species (*A. mellifera* or *A. cerana*), and
309 sampling site (12 sites shown in Fig. 1), only sample type was a significant predictor of
310 bacterial OTU composition (PERMANOVA $R^2 = 0.10$ $p = 0.001$). Specifically, mouth and
311 nectar samples were more similar to each other than either mouth and crop samples
312 were, or nectar and crop samples were to each other (Fig. 4; PERMANOVA mouth-
313 nectar $R^2 = 0.03$, $p = 0.005$; mouth-crop $R^2 = 0.11$, $p = 0.001$; nectar-crop $R^2 = 0.08$, $p =$
314 0.001).

315

316 In fact, most OTUs in the crop were distinct from those in the mouth and the nectar, with
317 only 9% (4-7% in *A. cerana* and 6-9% in *A. mellifera*; Fig. S2) of the crop OTUs also
318 present in the mouth or the nectar (Fig. 2). Taxa frequently found in the crop, such as
319 *Bombella, Apibacter, Gilliamella, Lactobacillus*, and *Snodgrassella*, were mostly absent

320 in the nectar and only infrequently present in the mouth (Figs. 5 and S3). Furthermore,
321 some OTUs belonging to *Acinetobacter,* Comamonadaceae, *Flavobacterium*, and
322 *Pseudomonas* were consistently found in the crop at all local sites examined, whereas
323 there were no taxa that were present in mouth or nectar samples at all sites.
324
325 *Summer-winter difference in crop vs. in mouth*
326 Although OTU richness was higher in summer than in winter in both the crop and the
327 mouth, the magnitude of this difference was eight times greater in the mouth than in the
328 crop (Figs. 3 and S4). In terms of OTU composition, most of the crop samples were
329 clustered together regardless of season (winter vs. summer, PERMANOVA $R^2 = 0.03$, p
330 = 0.001), whereas the mouth samples formed two distinct clusters, corresponding to
331 summer and winter (PERMANOVA $R^2 = 0.20$, p = 0.001; Figs. 4 and 5, Fig. S3). These
332 two mouth clusters differed in the amount of variation among samples, with winter
333 showing higher variation than summer (Figs. 4 and S5) despite no significant difference
334 between them in total bacterial load (Fig. S6).
335
336 The CLAM test (35) identified 27% of the OTUs in the crop as generalists, meaning that
337 they were similarly frequent in winter and summer (Fig. 6). In contrast, the CLAM test
338 classified only 2% of the OTUs in the mouth as generalists, with the majority of others
339 identified as either summer- or winter-specialists (76 and 8%, respectively; Fig. 6).
340
341 Apilactobacillus kunkeei *strains in the crop*

342 The multi-gene tree of *Apilactobacillus kunkeei* strains suggested potential season- and
343 *Apis* species-specificity. Specifically, the tree had one clade consisting of only isolates
344 from summer *A. cerana* samples and two other clades consisting of isolates from
345 summer and winter *A. mellifera* samples (Figs. 7 and S7). Those associated with *A.*
346 *cerana* originated from five bees, three from Nishi-Honjo and two from Ichiigawa. Those
347 associated with *A. mellifera* originated from nine bees: six summer bees from Oshine 1
348 and three winter bees, one each from Shimanose, Yamauchi, and Kamihaya.
349

350 **Discussion**

351 Based on the conventional assumption that bacterial assemblage in the crop primarily
352 consists of transient microbes acquired via nectar foraging (3, 45, 47, 48), we expected
353 a high degree of overlap among nectar, mouth, and crop samples. We did find high
354 overlap between the nectar and the mouth, but to our surprise, most of the crop taxa
355 were clearly distinct from those found in the nectar and the mouth (Figs. 2 and 5).
356 Several OTUs of *Acinetobacter* and *Pseudomonas* that were relatively prevalent in both
357 crop and mouth samples in our study have been commonly found in nectar as well (49-
358 53). However, most of the taxa that were more prevalent in the crop were rare or
359 entirely absent in nectar and mouth samples (Figs. 5, S1 and S3).
360

361 In addition, our results indicate that the crop maintained a high degree of taxonomic
362 consistency between winter and summer, a pattern that is in sharp contrast to the large
363 compositional changes seen in the mouth (Figs. 3, 4 and 6). Taxa common in the crop
364 in both seasons included Comamonadaceae, *Acinetobacter*, Saprospiraceae,

365 *Flavobacterium*, *Gilliamella*, *Bombella*, *Apilactobacillus*, *Pseudomonas*, *Fluviicola*,
366 *Polynucleobacter*, *Snodgrassella*, *Microbacteriaceae*, and *Rhodococcus* species (Fig.
367 5). *Apilactobacillus* and *Bombella*, which are among the most commonly reported taxa
368 in the crop of foraging adults, have also been found frequently in food stores, larvae,
369 and queens (3-5, 45, 48, 54-56). Although the data we present in this study are
370 correlational, preventing us from establishing causal relationships, we speculate that the
371 crop may serve as a consistent reservoir of these taxa that can influence the health of
372 the adult bees and the larvae that they tend (3, 45).
373
374 We also found evidence for potential strain-level specificity among *Apilactobacillus*
375 *kunkeei* isolates from collected foragers. One clade was made up of summer *A. cerana*
376 isolates from two different sites, and two others contained only *A. mellifera* isolates from
377 both seasons (Figs. 7 and S7). Although a larger sample size than we had in this study
378 as well as full genome sequencing are needed to ascertain the putative strain-level
379 differences, previous studies have shown differences in specific house-keeping genes
380 to suggest strain-level host specificity between *Apis* and *Bombus* and even among *Apis*
381 species (57-59). As in these previous studies, some of our isolates clustered more
382 frequently by host species than by site (60, 61), potentially suggesting host specificity
383 and possibly specific host-symbiont relationships between *Apis* species and *A. kunkeei*
384 strains, even though this possibility remains speculative at this stage.
385
386 A few caveats should be considered in interpreting our findings. First, our analysis is
387 mostly based on relative abundance (60), even though we used qPCR to attempt to

388 estimate at least total absolute abundance in crop and mouth samples (Figs. S5 and
389 S6). Absolute abundance of each taxon, which may be more relevant to bacterial effects
390 on the host (13, 60), can differ even when relative abundance is the same. Second, we
391 had only one site, Nishi-Honjo, where bees were sampled in both summer and winter. A
392 greater and more even number of bees at each site in multi-year seasonal sampling
393 would have made our results most robust. Nevertheless, our analysis of several
394 hundred bee and nectar samples all sampled within the same landscape clearly refutes
395 the hypothesis that crop communities simply reflect what is available in the
396 environment.
397
398 In conclusion, our results suggest that the composition of crop microbes may be more
399 deterministic than generally assumed, questioning the notion that crop microbes are
400 environmentally acquired transients that are unimportant to the bees. Although less well
401 studied than midgut and hindgut microbes, a small but increasing number of recent
402 studies indicate that some crop microbes do alter the health of individuals and hives (6,
403 62-64). Our data reinforce the idea that it would help to study how microbial
404 communities are assembled and maintained not just in the midgut and the hindgut, but
405 also the crop, to better understand the relationship between honeybees and their gut
406 microbes.
407
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420 **Figure legends**

421 **Figure 1**. Map of collection sites. Foraging adults of *Apis cerana* and *A. mellifera* were
422 collected from a total of 12 sites (Table S1) in or near Japanese apricot orchards in the
423 summer of 2018 and the winter of 2019 in the Minabe-Tanabe region, the location of
424 which is indicated by the red square on the map of Japan. Nectar samples were also
425 collected from apricot flowers at each of the winter collection sites. Sites are labeled
426 with circles colored by collection season (summer = white, winter = black, both seasons
427 = grey). The location of the Japanese Apricot Laboratory is indicated by the red outline
428 of the Higashi-Honjo site.

429

430 **Figure 2**. The crop contains more common and constant OTUs than the mouth and the
431 nectar. Only 9% of winter crop OTUs were shared with the mouth or nectar samples
432 while the mouth and nectar shared about 50% of their OTUs. Venn diagrams are
433 colored by sample type with OTU counts in bold.

434

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

443

444 **Figure 4**. Principal Coordinate Analysis of all samples presents similar composition
445 across season in the crop but not the mouth. Winter mouth and nectar samples were
446 highly similar (PERMANOVA sample type: $R^2 = 0.07$, $p = 0.001$, season: $R^2 = 0.05$ $p =$
447 0.001, host species: $R^2 = 0.003$, $p = 0.154$). Each dot represents a sample from an
448 individual bee, colored by sample type. *Apis mellifera* samples are depicted with
449 triangles and the solid ellipse while *Apis cerana* samples are depicted with circles and
450 the dashed ellipse. Ellipses mark the 95% confidence interval, and axes are labeled
451 with corresponding percent variation explained.

452

453 **Figure 5**. There is a greater prevalence of differentially abundant taxa among crop
454 groups than mouth and nectar groups. Samples were grouped by season, sampling
455 site, host *Apis* species, and sample type as illustrated by colored bars above the
456 heatmap and described in Table S2. For ease of visualization, heatmap columns are
457 differentially abundant taxa that were present in at least 50% of one of the sample types
458 e.g., at least 50% of all the mouth groups. The full heatmap with individual samples and
459 all differentially abundant taxa can be found in Fig. S3. The OTUs were agglomerated to
460 genus, colored by prevalence within groups, arranged according to their phylogeny. The
461 y-axis dendrogram depicts the weighted UniFrac distance between samples.

462

463 **Figure 6.** There are bacteria that have high affinity for the crop. Generalist bacteria, i.e.,
464 those with no special affinity to one environment or, in this case, season, are greater
465 between the summer and winter crop samples (about 27% of the OTUs) than the

466 corresponding mouth samples. The mouth presents only about 2% generalists, with
467 most OTUs classified as summer (76%) specialists. Scatterplots depict the log
468 abundance of each OTU, with each point representing a single OTU color coded by its
469 category. OTUs from both *A. mellifera* and *A. cerana* are included. The identity line is
470 plotted in a dotted grey pattern. The proportion of OTUs classified in each category is
471 depicted in the corresponding bar plots below the scatterplots, with categories colored
472 as in the scatterplot.
473
474 **Figure 7.** Multi-gene maximum likelihood tree of *Apilactobacillus kunkeei* isolates shows
475 *Apis* species specific clades. The phylogeny is the result of a multi-gene alignment
476 partitioned analysis of 49 *A. kunkeei* isolates and *A. apinorum* Fhon13 (see Tables S3
477 and S4). In the depicted tree, substitution frequencies are represented by branch length.
478 Isolates are labeled starting with an “S” for summer or “W” for winter, followed by a “C”
479 for *Apis cerana* and an “M” for *Apis mellifera* host species, the host ID number, and
480 lastly the isolate number. Isolates that occurred in both species are colored in grey while
481 those that are phylogenetically similar with high statistical support and associated with a
482 single host species are colored by host species. Statistical support was estimated from
483 1000 Shimodaira-Hasegawa-approximate likelihood ratio test (SH-aLRT) pseudo
484 replicates and ultrafast bootstraps (UFboot). Nodes with an SH-aLRT score greater than
485 0 are labeled with respective SH-aLRT/UFboot values. *Apilactobacillus apinorum* root is
486 not depicted for ease of visualization. Supplementary Figure 7 shows the full tree with
487 statistical support on all nodes and *A. apinorum* branch.

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653

Figure 1

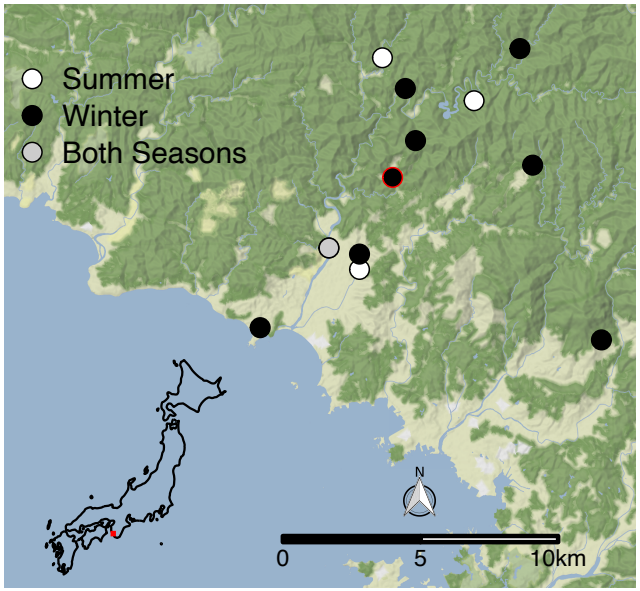


Figure 2

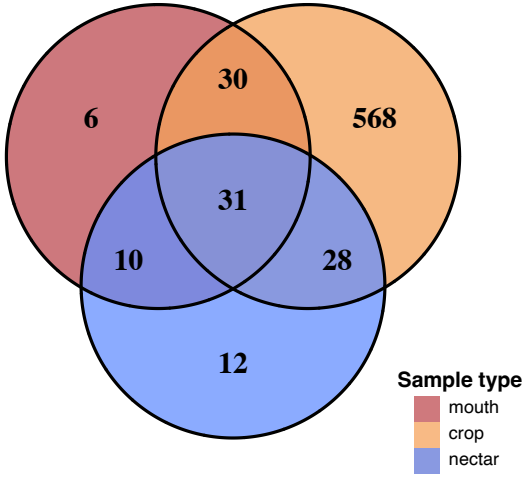


Figure 3

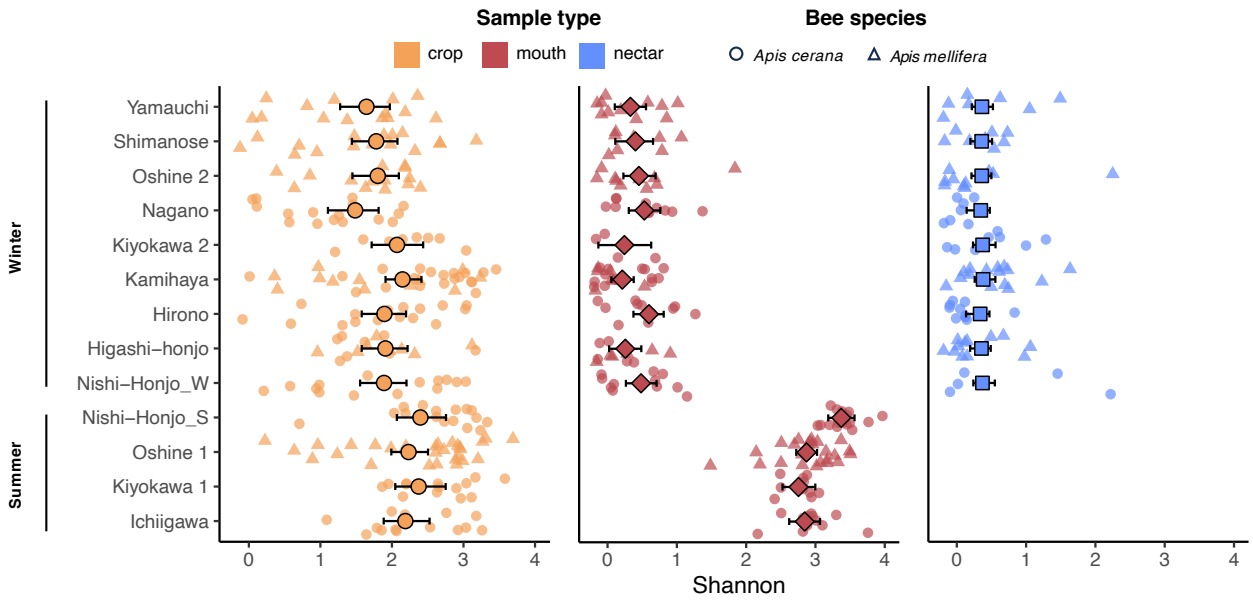

Sample type
crop
mouth
nectar
Bee species
Apis cerana
Apis mellifera
Winter
Summer
Yamauchi
Shimanose
Oshine 2
Nagano
Kiyokawa 2
Kamihaya
Hirono
Higashi–honjo
Nishi–Honjo_W
Nishi–Honjo_S
Oshine 1
Kiyokawa 1
Ichiigawa
0
1
2
3
4
Shannon

Figure 4

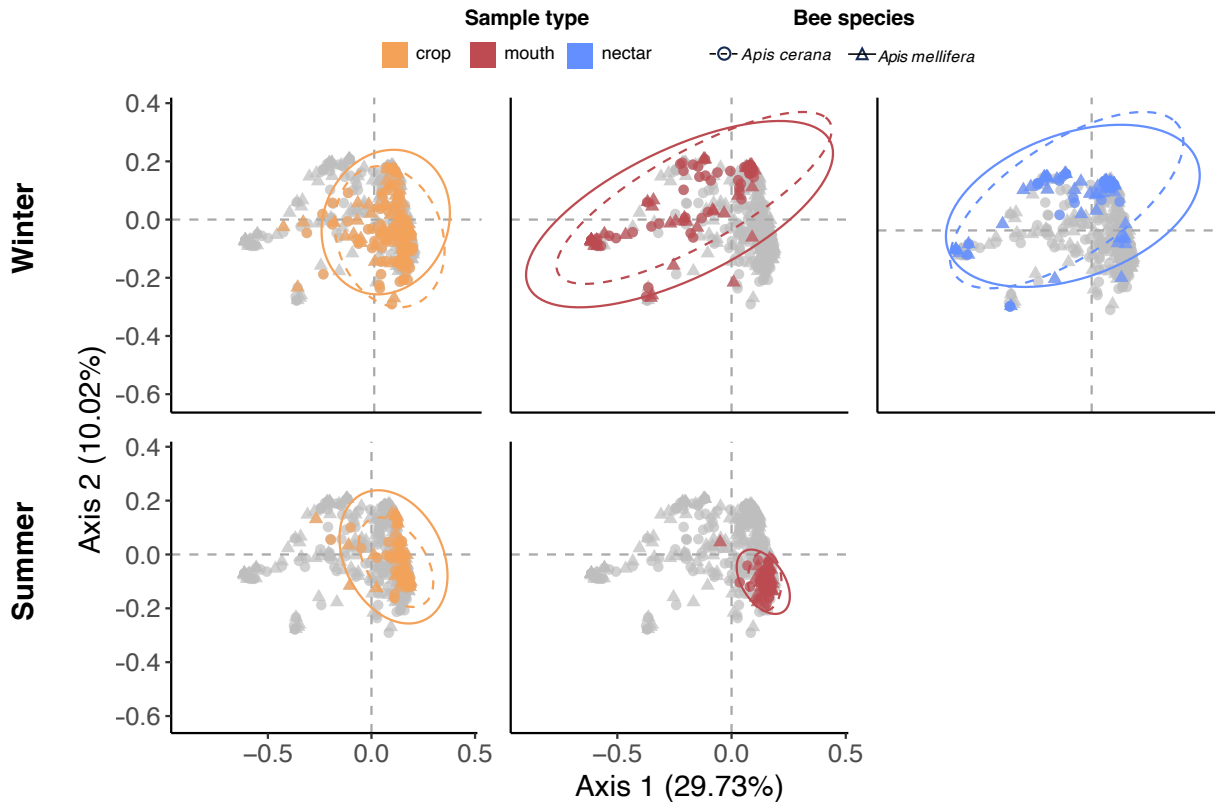

Sample type
crop
mouth
nectar
Bee species
Apis cerana
Apis mellifera
Winter
Summer
Axis 2 (10.02%)
Axis 1 (29.73%)
0.4
0.2
0.0
-0.2
-0.4
-0.6
−0.5
0.0
0.5

Figure 5

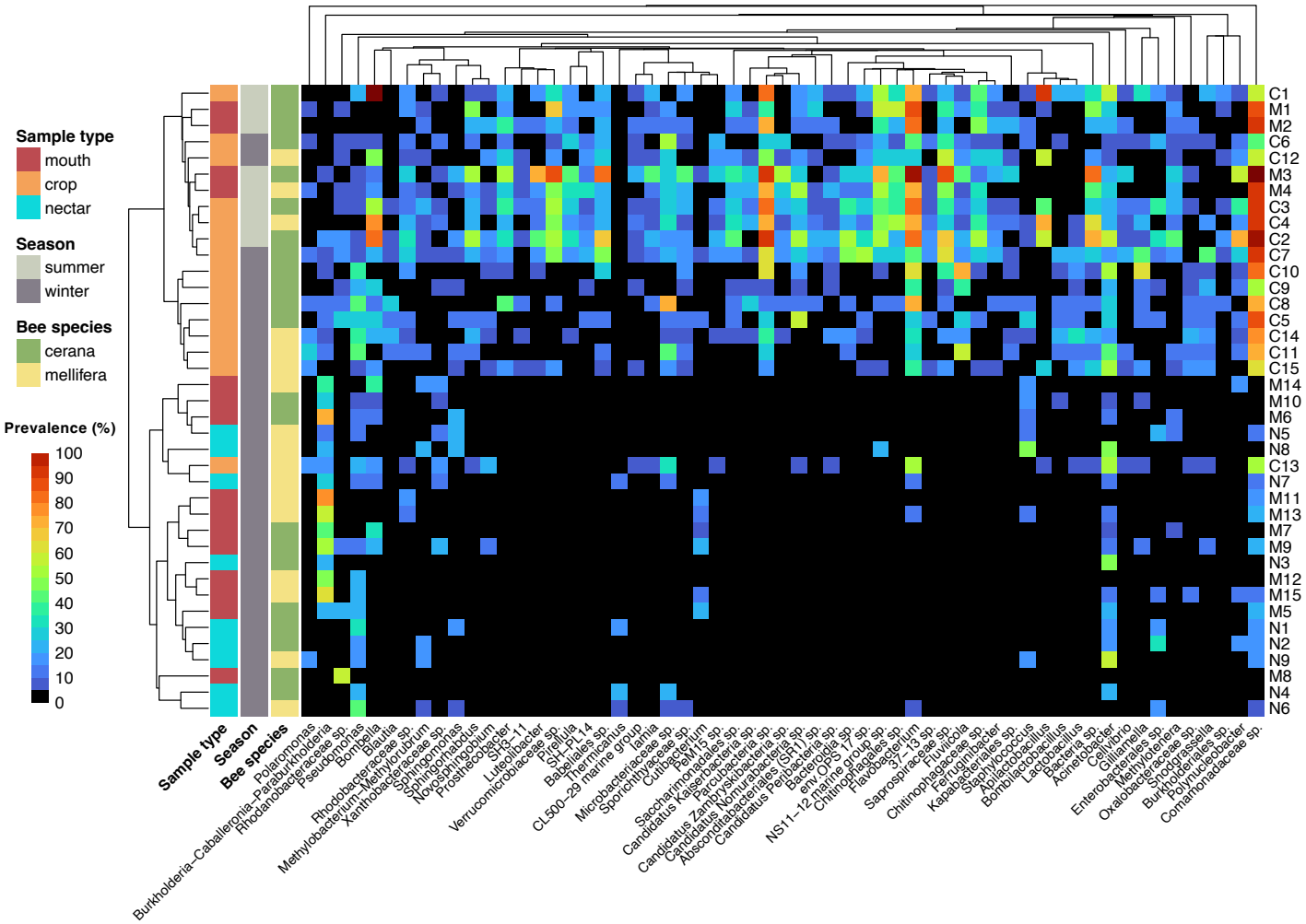

Sample type
mouth
crop
nectar
Season
summer
winter
Bee species
cerana
mellifera
Prevalence (%)
100
90
80
70
60
50
40
30
20
10
0
C1
M1
M2
C6
C12
M3
M4
C3
C4
C2
C7
C10
C9
C8
C5
C14
C11
C15
M14
M10
M6
N5
N8
C13
N7
M11
M13
M7
M9
N3
M12
M15
M5
N1
N2
N9
M8
N4
N6
Sample type
Season
Bee species
Polaromonas
Burkholderia–Caballeronia–Paraburkholderia
Rhodanobacteraceae sp.
Pseudomonas
Bombella
Blautia
Rhodobacteraceae sp.
Methylobacterium–Methylorubrum
Xanthobacteraceae sp.
Sphingomonas
Sphingorhabdus
Novosphingobium
Prosthecobacter
SH3-11
Luteolibacter
Verrucomicrobiaceae sp.
Pirellula
SH-PL14
Babeliales sp.
Thermicanus
CL500-29 marine group
Iamia
Microbacteriaceae sp.
Sporichthyaceae sp.
Cutibacterium
PeM15 sp.
Saccharimonadales sp.
Candidatus Kaiserbacteria sp.
Parcubacteria sp.
Candidatus Zambryskibacteria sp.
Candidatus Nomurabacteria sp.
Absconditabacteriales (SR1) sp.
Candidatus Peribacteria sp.
Bacteroidia sp.
env.OPS 17 sp.
NS11-12 marine group sp.
Chitinophagales sp.
Flavobacterium
37-13 sp.
Saprospiraceae sp.
Fluviicola
Chitinophagaceae sp.
Ferruginibacter
Kapabacteriales sp.
Staphylococcus
Aphelococcus
Bombilactobacillus
Lactobacillus
Bacteria sp.
Acinetobacter
Cellvibrio
Gilliamella
Enterobacteriales sp.
Methylotenera
Oxalobacteraceae sp.
Snodgrassella
Burkholderiales sp.
Polynucleobacter
Comamonadaceae sp.

Figure 6

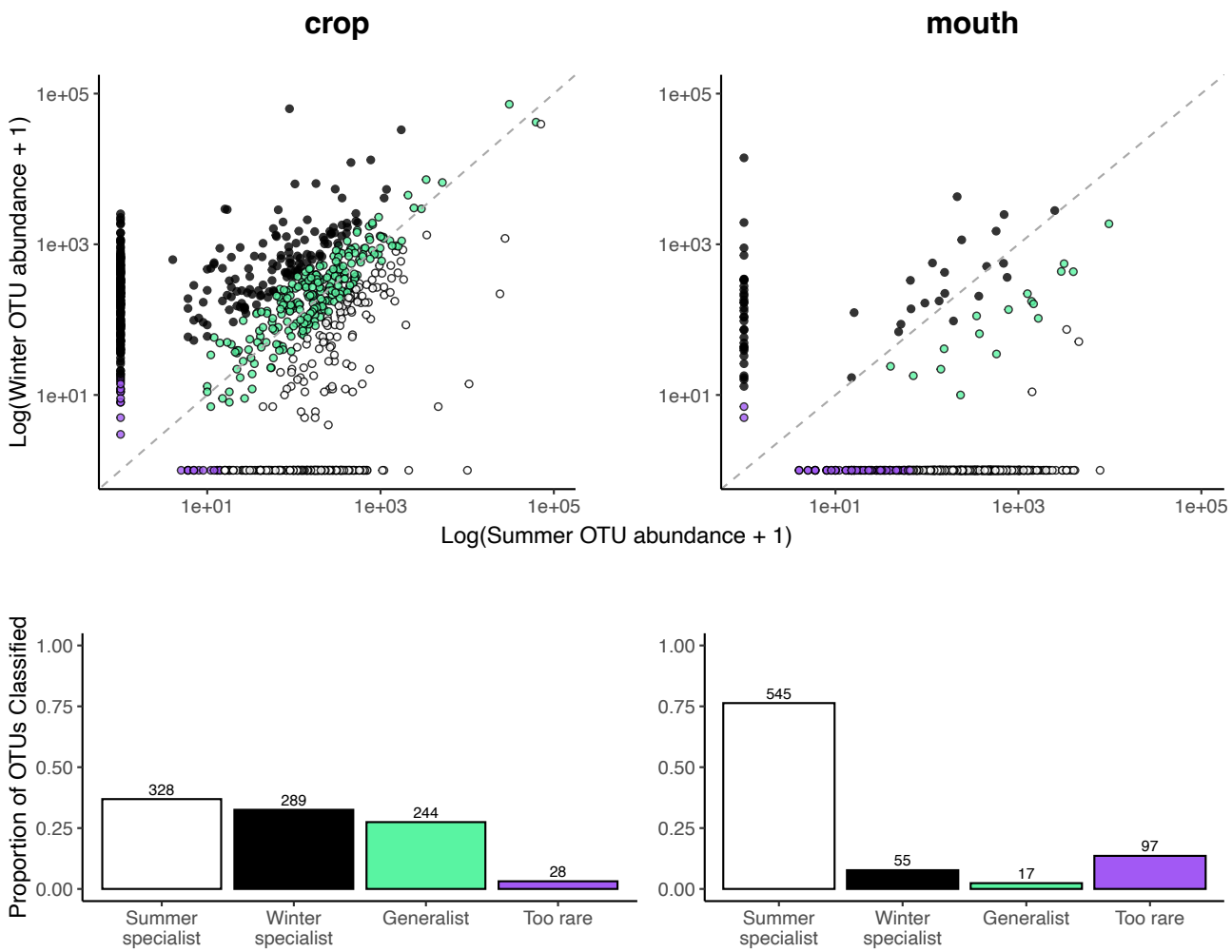
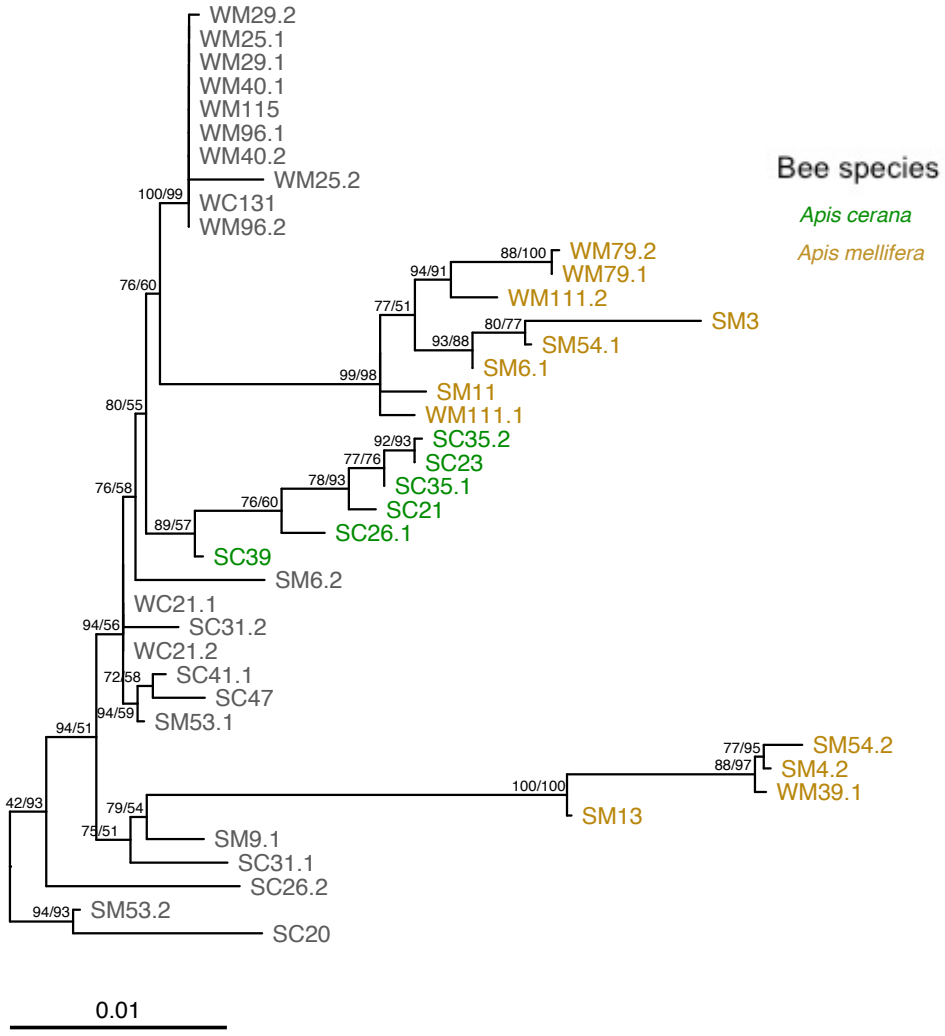

crop
mouth
Log(Winter OTU abundance + 1)
Log(Summer OTU abundance + 1)
1e+01
1e+03
1e+05
Proportion of OTUs Classified
1.00
0.75
0.50
0.25
0.00
328
289
244
28
545
55
17
97
Summer specialist
Winter specialist
Generalist
Too rare

Figure 7


Bee species
Apis cerana
Apis mellifera
WM29.2
WM25.1
WM29.1
WM40.1
WM115
WM96.1
WM40.2
WM25.2
WC131
WM96.2
100/99
76/60
88/100
WM79.2
WM79.1
94/91
WM111.2
77/51
SM3
80/77
SM54.1
93/88
SM6.1
99/98
SM11
WM111.1
80/55
92/93
SC35.2
SC23
77/76
78/93
SC35.1
76/58
76/60
SC21
89/57
SC26.1
SC39
SM6.2
WC21.1
94/56
SC31.2
WC21.2
72/58
SC41.1
SC47
94/59
SM53.1
94/51
77/95
SM54.2
88/97
SM4.2
WM39.1
100/100
42/93
SM13
79/54
75/51
SM9.1
SC31.1
SC26.2
94/93
SM53.2
SC20
0.01