Reclassification of seven honey bee symbiont strains as *Bombella apis*

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Abstract

Honey bees are important pollinators of many major crops and add billions of dollars annually to the US economy through their services. Recent declines in the health of the honey bee have startled researchers and lay people alike as honey bees are agriculture's most important pollinator. One factor that may influence colony health is the microbial community. Although honey bee worker guts have a characteristic community of bee-specific microbes, the honey bee queen digestive tracts are colonized predominantly by a single acetic acid bacterium tentatively named *Candidatus Parasaccharibacter apium*. This bacterium is related to flower-associated microbes such as *Saccharibacter floricola*, and initial phylogenetic analyses placed it as sister to these environmental bacteria. We used a combination of phylogenetic and sequence identity methods to better resolve evolutionary relationships among *P. apium*, strains in the genus *Saccharibacter*, and strains in the closely related genus *Bombella*. Interestingly, measures of

genome-wide average nucleotide identity and aligned fraction, coupled with phylogenetic placement, indicate that many strains labeled as *P. apium* and *Saccharibacter sp.* are all the same species as *Bombella apis*. We propose reclassifying these strains as *Bombella apis* and outline the data supporting that classification below.

Key Words

Bacterial classification, honey bee, evolution, phylogenetics, average nucleotide identity

Introduction

The honey bee (*Apis mellifera*) is extremely important economically because of the pollination services it provides to numerous agricultural crops. As a result, there is increasing interest in determining how the microbiome supports and influences bee function. While a honey bee colony is made up of individuals with distinct roles, or castes, the majority of studies on bee microbiomes have focused on workers. The microbial community of worker bees consists of eight to ten core bacterial species (Anderson *et al.*, 2016; Martinson *et al.*, 2011; 2012; Moran, 2015; Moran *et al.*, 2012; Sabree *et al.*, 2012). The characterization of these groups led to speculation about their role in honey bee health and whether or not they provision nutrients (Moran, 2015) or assist in the breakdown of plant-derived carbohydrates, as is the case in other insect-microbe interactions (Douglas, 2013; Gündüz & Douglas, 2009; McCutcheon & Moran, 2007). There has also been speculation as to the role of the microbiome in resistance to pathogens, as microbial communities have been shown to protect the bumble bee (*Bombus terrestris*) from the parasite *Crithidia bombi* (Koch & Schmid-Hempel, 2011). Honey bee-associated microbes interact with each other in diverse ways both *in vitro* and *in vivo*, suggesting that they may interact syntrophically within workers (Martinson *et al.*, 2012; Rokop *et al.*, 2015). While these studies focused on honey bee workers are intriguing, it is surprising that only recently was the microbiome of queen bees throughout development characterized (Tarpy *et al.*, 2015).

Interestingly, the microbial community associated with queen bees is vastly different than that of workers and comprises a large percentage of acetic acid bacteria, a group of bacteria present only at very small percentages in workers. One of the primary bacteria that differentiate queens from workers was tentatively named *Candidatus Parasaccharibacter apium* (Corby-Harris

et al., 2014). This bacterium is in the family *Acetobacteraceae* and occupies defined niches within the hive, including: queen guts, nurse hypopharyngeal glands, nurse crops, and royal jelly, and is only rarely found in high abundance outside of these areas (Anderson *et al.*, 2013; Vojvodic *et al.*, 2013). Evidence suggests that it might play a role in protecting developing larvae and queens from pathogens such as *Nosema* (Corby-Harris *et al.*, 2014; 2016). Given that this bacterium makes up a large proportion of the queen gut microbiome, it is possible that it plays an important role in queen nutrition, protection from pathogens, and possibly modulating queen fertility, fecundity, and longevity (Anderson *et al.*, 2018).

We sought to determine the evolutionary relationships between strains of *P. apium*, strains of the closely related genus *Saccharibacter*, and characterized sequences from strains of the recently named genus *Bombella*, using previously published data (see Table 1 for information on all genomes used in this study). Using a combination of phylogenetic and sequence similarity methods, we found that many genomes labeled as *P. apium* or *Saccharibacter sp.* are actually the same species as strains of the previously described species *Bombella apis*. We reclassify these strains and outline the data supporting this reclassification below.

Materials and methods

16S rRNA phylogeny

To determine relatedness of the strains classified as *P. apium* and *Saccharibacter spp.* compared to existing *Bombella* and *Saccharibacter* species, we compared 16S rRNA gene sequences of these genomes to one another. We first downloaded all 16S sequences from NCBI that were labeled as *Bombella*, *Parasaccharibacter*, or *Saccharibacter* (Table 1). We also included *Gluconobacter oxydans* H24 for use as an outgroup. All sequences were aligned using

the SINA aligner (Pruesse *et al.*, 2012); parameters used were set using the --auto option. A maximum likelihood phylogeny was constructed using RAXML with the GTRGAMMA substitution model and 1000 bootstrap replicates (v8.2.11, (Stamatakis, 2006)). The final tree was visualized using FigTree (v1.4.2, <http://tree.bio.ed.ac.uk/software/figtree/>).

Core ortholog phylogeny

To determine genome-wide phylogenetic relationships between strains, we clustered genes from all genomes in Table 1 into orthologous gene clusters using OrthoMCL (v2.0.9, (Li *et al.*, 2003)). Amino acid sequences were downloaded from NCBI and clustering was performed using default OrthoMCL parameters. We then extracted single copy orthologs using a custom Perl script (available at: https://github.com/esmith1032/bombella_apis/blob/master/parse_ortho_out.pl). We constructed a phylogeny using concatenated amino acid alignments of all single-copy orthologs. The amino acid sequences were aligned using the MAFFT L-INS-I algorithm (v7.310, (Katoh *et al.*, 2002)), and alignments were then concatenated, and used to construct a maximum likelihood phylogeny using RAXML with substitution model PROTGAMMALGF and 1000 bootstrap replicates (v8.2.11, (Stamatakis, 2006)). The final tree was visualized using FigTree (v1.4.2, <http://tree.bio.ed.ac.uk/software/figtree/>).

Calculation of genomic similarity

To determine relatedness and proper species assignment, we calculated genome-wide Average Nucleotide Identity (gANI) and aligned fraction (AF) for each pairwise comparison using ANIcalculator (Varghese *et al.*, 2015). Predicted transcript sequences for each pairwise

comparison were passed to the software, which output gANI and AF in each direction for the pairwise comparison. As gANI and AF can vary depending on the direction of comparison due to differences in genome lengths, we report results for comparisons in both directions.

Results

16S rRNA gene phylogeny

To determine phylogenetic relationships of all genomes labeled *Parasaccharibacter*, *Saccharibacter*, and *Bombella* on NCBI, we constructed a maximum likelihood phylogeny using 16S rRNA sequences (Figure 1A). This phylogeny indicates that the 16S sequences from the following genomes are all very closely related and represent a single, distinct clade: *Bombella apis* (16S sequence accession number: NR_157653.1), *Saccharibacter* sp. M18 (NCBI genome accession number: GCA_002150105.1), *Saccharibacter* sp. 3.A.1 (GCA_002150125.1), *Saccharibacter* sp. AM169 (GCA_0007235565.1), *P. apium* G7_7_3c (GCA_002079945.1), *P. apium* A29 (GCA_002917995.1), *P. apium* B8 (GCA_002917945.1), and *P. apium* C6 (GCA_002917985.1). For simplicity, this clade will hereafter be referred to as the “clade of interest”. *P. apium* AS1 is not included in the clade of interest for reasons outlined below. The relationships between sequences within the clade of interest are difficult to determine – as is evidenced by the low bootstrap support for nodes within the clade – owing to the high degree of similarity between the genomes. However, the phylogeny clearly places these accessions with *B. apis*, and sister to *P. apium* AS1. It is interesting to note that, although the sequences all show similar degrees of divergence from *B. intestini* and *B. apis*, bootstrap support is quite high for the separation of *B. intestini* from the clade of interest (Figure 1A).

Core ortholog phylogeny

We used OrthoMCL (v2.0.9 (Li *et al.*, 2003)) to define clusters of orthologous genes (COGs) using the *Parasaccharibacter*, *Saccharibacter*, and *Bombella* genomes listed in Table 1; *Gluconobacter oxydans* H24 was used as an outgroup. In total, 2,214 COGs were defined, with an average of 8.8 genes per COG. Of these, 1,259 COGs were present as single copies in every genome in the analysis; these were then used to construct a core ortholog phylogeny (Figure 1B) to better resolve phylogenetic relationships.

This core orthology phylogeny largely agrees with our 16S rRNA gene phylogeny with one exception: the relationships of *B. intestini* and *P. apium* AS1 have switched such that *B. intestini* is now sister to the clade of interest. Bootstrap support for the nodes in this tree are much higher than in the 16S tree, but are still relatively low within the clade of interest. However, bootstrap support among the nodes making up the main backbone of the tree are all 100, indicating very high support for the basal nodes (Figure 1B).

Calculation of genomic similarity

Given the discrepancy between nomenclature and the phylogeny (the placement of “*Parasaccharibacter*” and “*Saccharibacter spp.*” within the *Bombella* group), and considering the short branch lengths within the clade of interest, we calculated genome-wide Average Nucleotide Identity (gANI) and aligned fraction (AF) to clarify species relationships. The general criteria for two genomes to be considered the same species are $AF > 0.6$ and $gANI > 96.5$ (Varghese *et al.*, 2015). Using these criteria, all genomes within the clade of interest should be considered the same species, and distinct from *B. intestini* and *P. apium* AS1 (Figure 2), though part of the *Bombella* genus. Unfortunately, no genome has been published for *B. apis*.

However, the high degree of support for placement of the *B. apis* 16S rRNA gene sequence within the clade of interest strongly suggests that the genomes within the clade of interest belong to the same species and are all *Bombella apis* strains.

Discussion

Here, we used a combination of phylogenetic analysis (16S rRNA and core orthologs) and calculations of aligned fraction and genome-wide average nucleotide identity to determine relationships among symbionts of honey bees, bumble bees, and environmental bacteria. The phylogenetic data largely agree with each other, and with the AF and gANI delimitations of species. The combination of these data indicate that the genomic accessions GCA_000723565.1, GCA_002079945.1, GCA_002917995.1, GCA_002917945.1, GCA_002917985.1, GCA_002150105.1, and GCA_002150125.1 are all the same species, despite being named in NCBI as various strains of *Parasaccharibacter apium* and *Saccharibacter sp.* Furthermore, 16S rRNA sequence data indicates that these genomes are all very closely related to *Bombella apis* and are likely the same species. Given that *P. apium* has been effectively but not validly described (i.e. in accordance with IJSEM standards)(Corby-Harris *et al.*, 2016), while *B. apis* has been validly described (Yun *et al.*, 2017), we propose renaming the above accession numbers to reflect the proper genus and species assignment while maintaining their current strain designations (see Table 1 for full genus, species, and strain designations). We also propose renaming *P. apium* AS1 to *Bombella sp.* AS1.

Given the ever decreasing cost of genome sequencing and ever increasing number of bacterial genomes being published, this study demonstrates the necessity for exercising caution when 1) describing a new species and 2) assigning a new genome a genus/species designation. It

is becoming increasingly clear that 16S rRNA gene sequence data on their own, while useful indicators of approximate phylogenetic placement of a bacterial strain, may not be sufficient for accurate assignment of genus and species to a newly sequenced bacterial strain. Genome-wide data, when available, should be used to determine appropriate genus and species assignments (see Chun *et al.*, 2018).

Authors and contributors

Conceptualization: EAS and ILGN; Formal analysis: EAS; Writing – original draft: EAS; Writing – review and editing: EAS, KEA, VCH, QSM, ILGN

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Maximum likelihood 16S rRNA gene phylogeny (A) and core ortholog phylogeny (B).

Accession numbers are given with current names in parentheses. Numbers at nodes represent bootstrap support from 1000 bootstrap pseudoreplicates. The “clade of interest” is marked by a purple dot.

Figure 2. Pairwise aligned fraction (A) and genome-wide average nucleotide identity (B) of all strains used in this study. Accession numbers are given with current names in parentheses.

Colors in each cell scale with the metric. Aligned fraction scales from 0-100, while gANI scales from 0-1. To be considered the same species, two genomes should have aligned fraction > 0.6 and gANI > 96.5 (Varghese *et al.*, 2015). Both metrics can vary depending on which genome is considered the reference for alignment, and we made the calculations in both directions.

Genomes listed on the horizontal axis were considered as the reference for these calculations.

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306 Tables

307 **Table 1. GenBank accession number, current name, isolation source, genome size, %GC,**
 308 **and proposed new names of the strains used in this study.**

GenBank accession	Current name	Isolation source ¹	Genome size (Mbp)	%GC	Proposed new name
GCA_002079945.1	<i>Parasaccharibacter apium</i> G7_7_3c	<i>Apis mellifera</i> hindgut ^a	2.01	59.42	<i>Bombella apis</i> G7_7_3c
GCA_002917995.1	<i>Parasaccharibacter apium</i> A29	<i>Apis mellifera</i> larva ^a	2.01	59.39	<i>Bombella apis</i> A29
GCA_002917945.1	<i>Parasaccharibacter apium</i> B8	<i>Apis mellifera</i> larva ^a	2.01	59.38	<i>Bombella apis</i> B8
GCA_002917985.1	<i>Parasaccharibacter apium</i> C6	<i>Apis mellifera</i> larva ^a	2.01	59.38	<i>Bombella apis</i> C6
GCA_000723565.1	<i>Saccharibacter</i> sp. AM169	<i>Apis mellifera</i> stomach ^b	1.98	59.32	<i>Bombella apis</i> AM169
GCA_002150105.1	<i>Saccharibacter</i> sp. M18	<i>Apis mellifera</i> stomach ^c	2.01	59.35	<i>Bombella apis</i> M18
GCA_002150125.1	<i>Saccharibacter</i> sp. 3.A.1	Honey ^c	2.01	59.41	<i>Bombella apis</i> 3.A.1
GCA_002592045.1	<i>Parasaccharibacter apium</i> AS1	<i>Apis mellifera</i> larva	1.85	52.64	<i>Bombella</i> sp. AS1
GCA_002003665.1	<i>Bombella intestini</i> R-52487	<i>Bombus lapidarius</i> crop ^d	2.02	54.94	N/A
GCA_000378165.1	<i>Saccharibacter floricola</i> DSM15669	Flower ^e	2.38	51.22	N/A
GCA_000311765.1	<i>Gluconobacter oxydans</i> H24	Industrial sample ^f	3.82	56.24	N/A

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310 ¹Isolation source references: ^aCorby-Harris & Anderson 2018, ^bChouaia et al. 2014, ^cVeress et
 311 al. 2017, ^dLi et al. 2016, ^eJojima et al. 2004, ^fGe et al. 2013

312

313

A

B

A

Aligned Fraction (AF)

1	0.96	0.96	0.96	0.96	0.96	0.96	0.91	0.71	0.46	GCA_000723565.1 (<i>Saccharibacter</i> sp. AM169)
0.95	1	0.95	0.95	0.95	0.95	0.95	0.89	0.7	0.45	GCA_002079945.1 (<i>Parasaccharibacter apium</i> G7_7_3c)
0.95	0.95	1	0.99	0.99	0.95	0.95	0.89	0.7	0.44	GCA_002917995.1 (<i>Parasaccharibacter apium</i> A29)
0.94	0.95	0.99	1	0.99	0.95	0.95	0.89	0.7	0.44	GCA_002917945.1 (<i>Parasaccharibacter apium</i> B8)
0.94	0.95	0.99	0.99	1	0.94	0.95	0.89	0.7	0.44	GCA_002917985.1 (<i>Parasaccharibacter apium</i> C6)
0.92	0.92	0.92	0.92	0.92	1	0.94	0.89	0.68	0.45	GCA_002150105.1 (<i>Saccharibacter</i> sp. M18)
0.95	0.95	0.95	0.95	0.95	0.96	1	0.9	0.7	0.46	GCA_002150125.1 (<i>Saccharibacter</i> sp. 3.A.1)
0.89	0.89	0.89	0.89	0.89	0.91	0.89	1	0.71	0.49	GCA_002003665.1 (<i>Bombella intestini</i> R-52487)
0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.77	1	0.54	GCA_002592045.1 (<i>Parasaccharibacter apium</i> AS1)
0.38	0.38	0.38	0.38	0.38	0.39	0.39	0.42	0.43	1	GCA_000378165.1 (<i>Saccharibacter floricola</i> DSM15669)
GCA_000723565.1	GCA_002079945.1	GCA_002917995.1	GCA_002917945.1	GCA_002917985.1	GCA_002150105.1	GCA_002150125.1	GCA_002003665.1	GCA_002592045.1	GCA_000378165.1	

B

gANI

100	99.24	99.27	99.27	99.27	98.82	99.22	82.96	76.03	71.91	GCA_000723565.1 (<i>Saccharibacter</i> sp. AM169)
99.24	100	99.41	99.41	99.41	98.89	99.34	82.97	75.98	71.91	GCA_002079945.1 (<i>Parasaccharibacter apium</i> G7_7_3c)
99.27	99.41	100	100	100	98.93	99.37	82.96	76.02	71.89	GCA_002917995.1 (<i>Parasaccharibacter apium</i> A29)
99.27	99.41	100	100	100	98.93	99.37	82.96	76.02	71.89	GCA_002917945.1 (<i>Parasaccharibacter apium</i> B8)
99.27	99.41	100	100	100	98.93	99.37	82.97	76.02	71.89	GCA_002917985.1 (<i>Parasaccharibacter apium</i> C6)
98.82	98.89	98.93	98.93	98.93	100	98.86	83.07	76.06	71.85	GCA_002150105.1 (<i>Saccharibacter</i> sp. M18)
99.22	99.34	99.37	99.37	99.37	98.86	100	83.02	76	71.87	GCA_002150125.1 (<i>Saccharibacter</i> sp. 3.A.1)
82.96	82.96	82.94	82.94	82.95	83.07	83	100	76.39	71.88	GCA_002003665.1 (<i>Bombella intestini</i> R-52487)
76.03	75.99	76.02	76.02	76.02	76.06	76.01	76.38	100	71.94	GCA_002592045.1 (<i>Parasaccharibacter apium</i> AS1)
71.92	71.9	71.89	71.89	71.89	71.85	71.86	71.88	71.95	100	GCA_000378165.1 (<i>Saccharibacter floricola</i> DSM15669)
GCA_000723565.1	GCA_002079945.1	GCA_002917995.1	GCA_002917945.1	GCA_002917985.1	GCA_002150105.1	GCA_002150125.1	GCA_002003665.1	GCA_002592045.1	GCA_000378165.1	