PeerJ

Short reads from honey bee (*Apis* sp.) sequencing projects reflect microbial associate diversity

Michael Gerth and Gregory D.D. Hurst

Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom

ABSTRACT

High throughput (or ‘next generation’) sequencing has transformed most areas of biological research and is now a standard method that underpins empirical study of organismal biology, and (through comparison of genomes), reveals patterns of evolution. For projects focused on animals, these sequencing methods do not discriminate between the primary target of sequencing (the animal genome) and ‘contaminating’ material, such as associated microbes. A common first step is to filter out these contaminants to allow better assembly of the animal genome or transcriptome. Here, we aimed to assess if these ‘contaminations’ provide information with regard to biologically important microorganisms associated with the individual. To achieve this, we examined whether the short read data from *Apis* retrieved elements of its well established microbiome. To this end, we screened almost 1,000 short read libraries of honey bee (*Apis* sp.) DNA sequencing project for the presence of microbial sequences, and find sequences from known honey bee microbial associates in at least 11% of them. Further to this, we screened ∼500 *Apis* RNA sequencing libraries for evidence of viral infections, which were found to be present in about half of them. We then used the data to reconstruct draft genomes of three *Apis* associated bacteria, as well as several viral strains *de novo*. We conclude that ‘contamination’ in short read sequencing libraries can provide useful genomic information on microbial taxa known to be associated with the target organisms, and may even lead to the discovery of novel associations. Finally, we demonstrate that RNAseq samples from experiments commonly carry uneven viral loads across libraries. We note variation in viral presence and load may be a confounding feature of differential gene expression analyses, and as such it should be incorporated as a random factor in analyses.

Submitted 4 March 2017
Accepted 11 June 2017
Published 12 July 2017

Corresponding author
Michael Gerth, gerth@liverpool.ac.uk

Academic editor
Sean Rogers

Additional Information and Declarations can be found on page 19

DOI 10.7717/peerj.3529

Subjects Evolutionary Studies, Genomics, Microbiology, Zoology
Keywords Short read sequencing, Contamination, Symbionts, Hologenome, Metagenome, *Spiroplasma*, *Lactobacillus*, Deformed wing virus

INTRODUCTION

Novel DNA sequencing methods have revolutionized biological and medical research in the last two decades (Goodwin, McPherson & McCombie, 2016). High throughput sequencing (or ‘massively parallelized sequencing’, ‘next generation sequencing’,‘NGS’) facilitated the creation of enormous amounts of data for a fraction of the costs associated with traditional Sanger methods (Kircher & Kelso, 2010; Sboner et al., 2011). This ‘genomics revolution’, has not only enhanced our understanding of molecular and genome evolution (Wolfe &

Li, 2003), but also contributed to the recognition that eukaryotes are commonly associated with a plethora of microbial taxa. (For the sake of simplicity, we will in this paper refer to all bacteria, microbial eukaryotes, and viruses as 'microbes'.)

In eukaryote genome sequencing projects, sequences deriving from these microbes may obstruct genome or transcriptome assembly efforts, and measures directed at removing microbial associates are routinely performed. This is achieved either by antibiotic treatment of the target organism prior to sequencing (*Colbourne et al., 2011*), or by removing microbial sequences bioinformatically after sequencing (*Schmieder & Edwards, 2011*). While eliminating microbes may facilitate eukaryotic genome reconstruction, it neglects the recently emerging appreciation of microbes as a biologically important component of all multicellular life. Numerous examples illustrate the impact of microbes on animal and plant biology, including physiology, behavior, and evolution (*McFall-Ngai et al., 2013*). These findings have led to a concept that defines an individual eukaryote with all its associated microbes (microbiome) as an entity (holobiont-hologenome) (*Bordenstein & Theis, 2015*). Although this concept is contentious (*Moran & Sloan, 2015*; *Douglas & Werren, 2016*), it is undisputed that some aspects of organismal biology can only be understood by deciphering interactions with microbial symbionts.

To characterize microbiome composition, three approaches are commonly used. First, microbes may be isolated from the host and cultured axenically. Their properties can then be determined either through traditional microbiological methods or by sequencing (*Browne et al., 2016*). This approach has the benefit of providing both biological and genomic information, but limits discovery to culturable taxa. Second, microbiome taxa may be identified by amplicon sequencing. Specific primers are used to amplify a short informative region from all bacterial taxa in a sample (usually a part of the 16S rRNA gene), and then sequenced (today typically via NGS methods) (*Caporaso et al., 2012*). This mechanism discovers broad patterns of community diversity, but at a coarse scale, and with weaker functional information. Finally, microbiome composition can be determined via metagenomics, i.e., collective genome sequencing of all bacteria present in a sample (*Riesenfeld, Schloss & Handelsman, 2004*). This is unbiased, fine scaled, and provides an assessment of biological potential at a community scale. Approaches to characterize viral sequences found in the environment or hosts are similar to the ones used to detect bacterial microbes described above and metagenomics is now most commonly employed for this task (*Delwart, 2007*; *Mokili, Rohwer & Dutilh, 2012*).

In this study, we examined if the data generated in eukaryotic sequencing projects can be used to identify microbiome taxa and harness genomic data. Previously, this approach was used to recover genomes of heritable microbes that occur in high densities in many arthropod species, and are therefore prone to be retrieved in arthropod sequencing projects. For example, the genomes of multiple *Wolbachia* strains were discovered in *Drosophila* sequencing data, revealing novel *Wolbachia* diversity and patterns of *Wolbachia* evolution (*Salzberg et al., 2005*; *Richardson et al., 2012*; *Siozios, Cestaro & Kaur, 2013*). Furthermore, the software packages such as Glimmer (*Delcher et al., 2007*) and Blobtools/Blobology (*Kumar et al., 2013*) were specifically designed to facilitate and automated the extraction of symbiont genomes from NGS data, and subsequent studies have demonstrated their efficiency in this task (*Blow et al., 2016*; *Denver et al., 2016*; *Fierst et al., 2017*). In bee sequencing projects, (partial) symbiont genomes were previously detected in bumble bees (*Martinson et al., 2014*), a mason bee (*Gerth et al., 2014*), and the *Apis mellifera* genome (*Cox-Foster et al., 2007*).

Here, we systematically examine a large number of short reads of honey bee (*Apis* sp.) sequencing projects to investigate whether this archived data can be used to retrieve a wider set of microbial associates, including pathogens and gut symbionts. We focus on honey bees because: (1) there is a large number of short read sequencing projects targeting *Apis*; (2) the components of healthy and unhealthy *Apis* microbiomes are well established (*Evans & Schwarz, 2011*; *Kwong & Moran, 2016*); (3) managed populations of the economically important honey bees have been in decline worldwide (*Neumann & Carreck, 2010*), and it was hypothesized that certain bacteria and viruses are key players in this decline (*Cox-Foster et al., 2007*). Thus, any novel genomic data on honey bee symbionts may directly contribute to our understanding of bee disease.

To identify 'contaminants', we here use 'bait' sequences of symbionts and pathogens to screen a large number of short read libraries from *Apis* sequencing projects. We demonstrate that the libraries contain non-target sequences from many sources, some of which reflect the natural honey bee microbiome and virome. We further show that highly covered, and possibly novel symbiont genomes can be retrieved from this contamination. Our study highlights the value of database sequences for exploratory symbiont screens and argues against neglecting the filtered 'contaminants' in sequencing projects.

MATERIALS & METHODS

Screening for microbes in Apis short read sequencing libraries

A graphical overview of our screening process can be found in Fig. 1. We compiled two sets of reference sequences to be used as databases for subsequent screens (Fig. 1). First, for 15 common *Apis* associated symbionts and pathogens, we used one short signature sequence each instead of complete genomes, in order to reduce the computational expense of all following steps. These were of slowly evolved ribosomal RNA genes to allow a range of diversity to be recovered through sequence similarity to the bait. Second, for 11 common viral associates, we compiled complete genomes. As all of these were very small (~10,000 bp), this was unlikely to significantly impact computational run times. Fasta files of compiled database sequences are available under https://github.com/gerthmicha/symbiont-sra.

Next, we searched for honey bee sequencing projects in NCBI's short read archive, using the search term '*Apis*', and created lists of the hits for DNA and RNA sequencing projects, which were subsequently processed separately (Fig. 1). The great majority of projects were from *Apis mellifera* samples, but a few RNA sequencing projects from *Apis cerana* and *Apis florea* were also included. We will from here on refer to all of those samples as '*Apis*'. From the list of DNA sequencing projects, we excluded metagenome and amplicon projects, as these are specifically aimed at microbiome taxa. At the time of the search (October 2015), 18 projects matched these criteria. We downloaded all short read libraries associated with

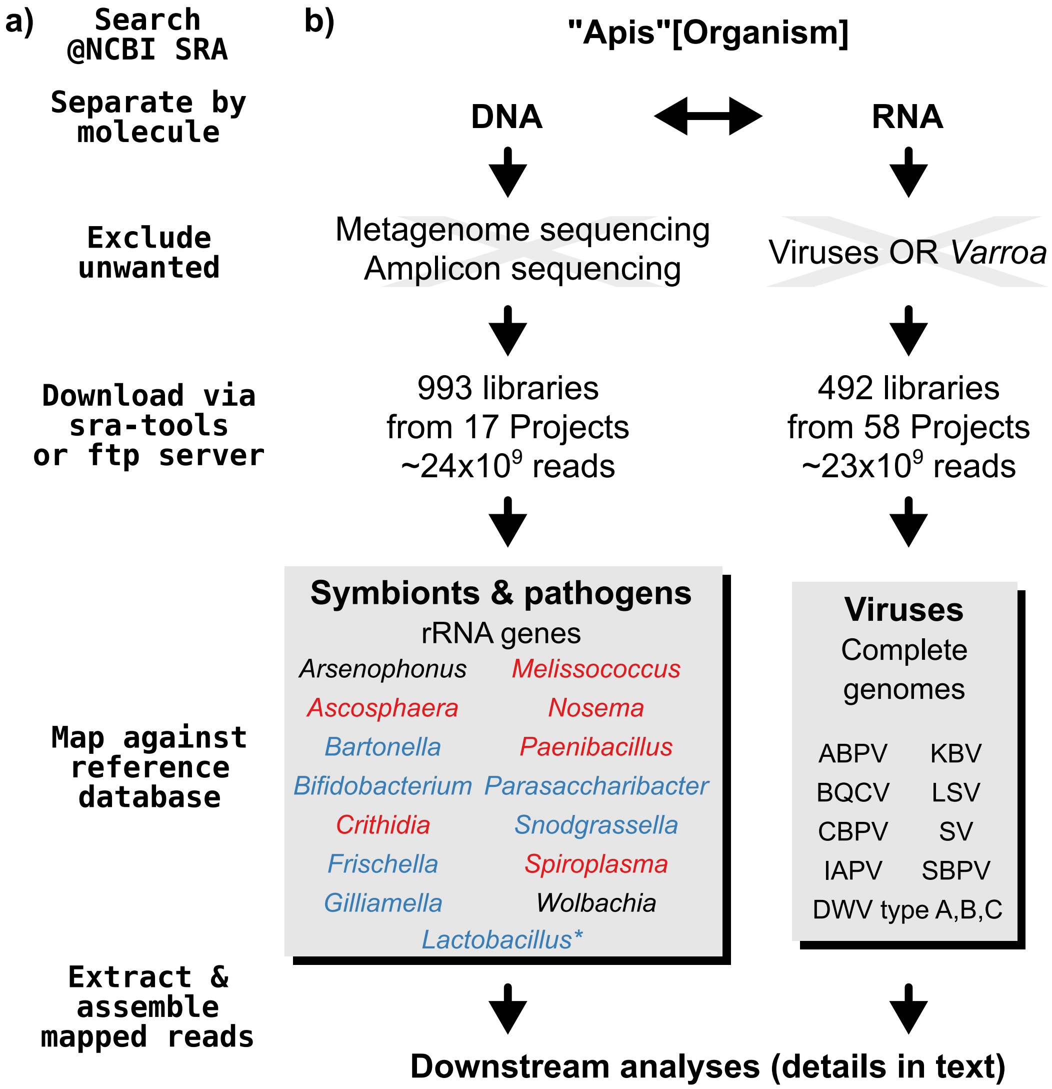


Figure 1 Outline of screening procedure employed in this study. (A) Computational steps involved in screening. (B) Details for each of the steps. For the 'symbionts & pathogens' database, microbial taxa are color-coded as follows: red–pathogens, blue–gut symbionts, black–environmental/opportunistic symbionts. A second, more detailed screen was performed for *Lactobacillus* symbionts only (marked with an asterisk, for details see 'Materials & Methods'). A detailed guide to replicate our analyses can be found under https://github.com/gerthmicha/symbiont-sra.

these projects (993 in total, Table S1) and mapped all reads of each of the libraries to the 'symbionts & pathogens' reference database using NextGenMap version 0.4.12 (*Sedlazeck, Rescheneder & Von Haeseler, 2013*). If at least 1,000 reads of a library were aligned to one or more sequence baits, we extracted the matching reads and assembled them using SPAdes version 3.7 (*Bankevich et al., 2012*). Contigs resulting from this assembly were then subject to taxonomic annotation via BLAST+ (*Camacho et al., 2009*) searches against a local copy of the NCBI 'nt' database, and the Blobtools package (*Kumar et al., 2013*).

For the RNA dataset, we removed any library from projects investigating the effects of viruses or *Varroa* infections, as *Varroa* treatment usually results in viral infections (*Allen & Ball, 1996*). Next, the reads of all remaining RNA sequencing libraries (492 libraries from 58 projects in total, Table S2) were mapped against the viral database as described above, and the number of mapped reads was counted using samtools version 1.4 (*Li et al., 2009*). Mapped reads were then assembled using IDBA-UD (*Peng et al., 2012*) and MEGAHIT version 1.1.1 (*Li et al., 2015*), which were designed to handle highly uneven sequencing depths, as expected from viral data (*Visser et al., 2016*). The 'better' assembly from these two methods was chosen by means of viral contig length comparison, i.e., the assembly that created the single longest contig was kept. This was more appropriate than choosing the highest N50 value, as most libraries contained only a single viral associate. If the two assembly methods produced overlapping contigs, which were identical in the overlap, the contigs were merged. A detailed description of all steps used to screen for microbial associates can be found under https://github.com/gerthmicha/symbiont-sra.

Assessing *Lactobacillus* diversity in *Apis* DNA sequencing libraries

Since our initial screen of DNA sequencing libraries yielded a high number of hits to various *Lactobacillus* species, we repeated the entire procedure using a database of 620 16S bait sequences from *Lactobacillus* only. These sequences were taken from a previously compiled dataset of Lactobacilli associated with *Apis*, other Hymenoptera, and other *Lactobacillus* sequences retrieved from public databases (*McFrederick et al., 2013*). All hits shorter than 250 bp were discarded, and remaining contigs were combined with the reference sequences. We used SSU-ALIGN version 0.1 (*Nawrocki, 2009*) to align and mask this dataset based on conserved secondary structure. Original and masked alignments are available from https://github.com/gerthmicha/symbiont-sra. A maximum likelihood phylogeny was reconstructed from the complete 16S alignment (740 sequences in total) using IQ-TREE version 1.3.10 (*Nguyen et al., 2015*) with automated model selection and 1,000 ultrafast bootstraps (*Minh, Nguyen & Von Haeseler, 2013*) to assess node support. The resulting tree was visualized using the online tool Evolview (*He et al., 2016*). Furthermore, as an approximate measure for the number of *Lactobacillus* OTUs recovered with our approach, we used the average neighbor clustering algorithm as implemented in mothur version 1.34.4 (*Schloss et al., 2009*).

Although our aim was not to recover all, but only the highly covered symbiont data from honey bee short reads, we wanted to test if our screening approach yields comparable results to more commonly used metagenomic approaches. To this end, we screened the reads of a metagenomic dataset created from the pooled DNA of 150 honeybee worker hindguts (*Engel, Martinson & Moran, 2012*; ∼43 M 150 bp paired-end reads, SRA accession: SRR5237156) for *Lactobacillus* in the same way as described above. We found 6 different *Lactobacillus* 16S sequences, all within the Firm-4 and Firm-5 *Lactobacillus* groups (Fig. S1). This was in agreement to the results obtained from taxonomic profiling approaches performed by *Engel, Martinson & Moran (2012)* and thus confirmed the general effectiveness of our approach (Fig. S1).

Reconstruction of symbiont genomes from *Apis* DNA sequencing libraries

Next, we aimed to validate that whole symbiont genomes can in principle be recovered from *Apis* sequencing projects. To this end, we chose one *Apis mellifera intermissa* sequencing library (SRR1046114, ∼85.5 M 100 bp paired-end reads) that contained 'contamination' from two *Lactobacillus* strains (*Lactobacillus kunkeei* & *Fructobacillus* sp.). We performed a *de novo* assembly using all reads with MEGAHIT version 1.1.1 (*Li et al., 2015*). All resulting contigs of this assembly were taxonomically assigned to either *L. kunkeei*, *Fructobacillus* sp. or 'other' based on BLAST searches, GC distributions, and read coverage. Reads matching to contigs from either *Lactobacillus* strain were then separately re-assembled using SPAdes, and all contigs smaller than 500 bp discarded. Completeness and contamination of the novel draft genomes were assessed based on the presence of conserved marker genes using CheckM version 1.0.6 (*Parks et al., 2015*), and annotation performed with PROKKA version 1.12 (*Seemann, 2014*). The annotated draft genomes are available under under https://github.com/gerthmicha/symbiont-sra. To evaluate the evolutionary relationships of newly assembled genomes in a broader taxonomic context, we assessed their phylogenetic placement. Whole-genome datasets were compiled for both strains (13 *L. kunkeei* genomes, 9 *Fructobacillus* & *Leuconostoc* genomes altogether, Table S3). For each of the datasets, single copy orthologs were identified using OrthoFinder version 0.2.8 (*Emms & Kelly, 2015*). Recombining loci were identified by using the pairwise homoplasy index test (*Bruen, Philippe & Bryant, 2006*), and removed from subsequent analyses (window size = 20 amino acid positions, significance cutoff at 0.05). Using IQ-TREE, we performed maximum likelihood analysis of two final supermatrices (947 loci and 290,774 aa for the *L. kunkeii* dataset, 435 loci and 145,069 positions for the *Fructobacillus*/*Leuconostoc* dataset). Prior to this, best-fitting partitioning schemes and models were selected using the 'greedy' scheme implemented in IQ-TREE (*Lanfear et al., 2012*).

Using the same approach, we assembled and annotated a *Spiroplasma melliferum* genome from the *Apis mellifera* library SRR957082, (∼224.5 M 50 bp single end reads). Phylogenetic analysis was performed based on a dataset of 206 concatenated single copy genes (58,950 amino acid positions) shared among 17 *Spiroplasma* strains (Table S3). Furthermore, to assess synteny, the newly assembled draft genome was ordered against and aligned with other *Spiroplasma melliferum* genomes (one genome each of strains IPMB4A and KC3) using the progressiveMauve algorithm of Mauve development snapshot version 2015-02-13 (*Darling et al. 2010*).

Assessing viral diversity in *Apis* RNA sequencing libraries

All potential viral contigs resulting from the assembly steps outlined above were blasted against a local copy of NCBI's 'nt' database. As most contigs were annotated as deformed wing virus (DWV), we reconstructed the phylogenetic relationships between the detected DWV strains. We used only contigs where at least half of the DWV genome was present (5,000 bp minimum length), and aligned all sequences using MAFFT. As a reference, we added complete genomic sequences for each of the three types of DWV (A, B or VDV-1–Varroa destructor virus 1, and C) and one for Kakugo virus (KV), which is a

Table 1 **Number of differentially expressed genes per investigated RNA sequencing project.**

BioProject accession number	Number of investigated RNA sequencing libraries with/without viruses	Number of differentially expressed genes[a]
PRJNA243651	9/12	7
PRJNA292006	3/2	5
PRJNA306498	3/21	5
PRJNA338450	17/19	10

Notes.

[a] Out of 20 candidate loci investigated, see Table 2.

variant of DWV type A (*Mordecai et al., 2016*). The phylogeny was estimated via maximum likelihood with IQ-TREE as described above.

Exploring viral effects on *Apis* gene expression from RNA sequencing libraries

We observed that in some cases, libraries from a single project (i.e., NCBI's BioProject) displayed pronounced differences in viral loads as measured in our screen. We thus hypothesized that these differences should also be detectable through differentially expressed host genes. To test this hypothesis, four projects were investigated (Table 1) in which viral loads were particularly uneven (proportion of viral reads across libraries 0%–37%, 0%–15%, 0%–53%, and 0%–32%, respectively—see Table S4 for details). Several studies have examined differential gene expression of honey bees in response to viral infection, and a set of genes commonly responding to pathogens was recently identified via meta-analyses (*Doublet et al., 2017*). We used the 20 genes that were consistently differentially expressed across multiple studies (first 20 ranks in the category 'differentially-regulated' from the *Doublet et al., 2017* analysis). The relative expression levels of these genes were determined for all libraries of the selected projects by (1) quality trimming of all reads with cutadapt version 1.1.3 (*Martin, 2011*; options: -q 25,25 –minimum-length 50 –pair-filter=both) (2) mapping the reads with NextGenMap (min. identity 99%); (3) counting the reads with samtools; (4) normalizing read counts to reads per kilobase per million mapped reads (RPKM) and log 2 transforming this value, (5) allocating the libraries from each project to either 'virus' or 'virus-free' groups based on the proportion of viral reads in the libraries; (6) testing for differentially expressed genes between these groups using the qCML method and exact tests in the R package 'edgeR' (*Robinson & Smyth, 2007*; *Robinson, McCarthy & Smyth, 2009*; *R Core Team, 2015*). Genes were regarded as differentially expressed for P-values ≤ 0.05.

RESULTS

Using bait sequences of 15 common *Apis*- associated microbes, we found non-target symbiont sequence data in 105 of the 993 investigated DNA sequencing libraries (∼11%). Assembly of symbiont reads resulted in a total of 782 contigs from these 105 libraries (1–57 contigs per library, Table S5), which were on average 371 bp long (34–1670 bp, Table S5). Taxonomic annotation revealed that these contigs originated from either *Apis*- associated

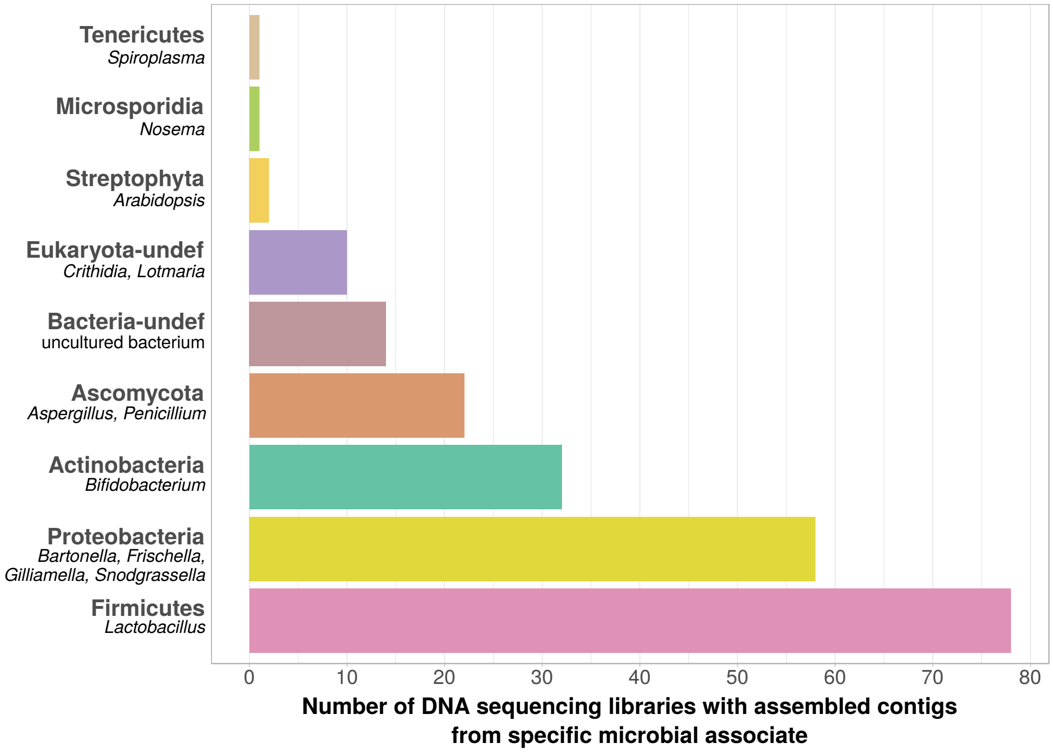


Figure 2 Taxonomic annotation of contigs assembled from 'contaminated' *Apis* DNA short read sequencing libraries. Bar chart shows the frequency of each taxonomic category assigned by best BLAST matches against NCBI's 'nt' database, as the number of libraries in which that taxon was detected among 993 DNA sequencing libraries. Bold categories are 'phyla', as defined in https://www.ncbi.nlm.nih.gov/taxonomy, taxa in italics represent typical genera that were recovered within each phylum. Please note that best blast hits to Arthropods were not included in this plot. See Table S5 for a complete list.

taxa that were targeted with our bait sequences, or from other sources for which there is no current evidence of *Apis* association (Fig. 2). Of the former, we detected most common gut symbionts known to be associated with honey bees (*Kwong & Moran, 2016*), i.e., *Bartonella*, *Bifidobacterium*, *Frischella*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella*, but not *Parasaccharibacter*. Of the pathogens screened for, we detected *Crithidia/Lotmaria* (which are inconsistently annotated in NCBI's nucleotide database and therefore not differentiated here; *Schwarz et al., 2015*), *Nosema*, and *Spiroplasma* (Fig. 2). We did not find evidence for chalkbrood, American foulbrood, or European foulbrood in the investigated DNA libraries (*Ascosphaera*, *Paenibacillus*, and *Melissococcus*, respectively). Sequences from organisms not naturally associated with honey bees included those from fungi (Ascomycota) and plants, that were likely not part of the native microbiome of the sequenced samples. These contaminations were crossed-checked via manual online BLAST searches and were confirmed to represent 'true' hits with high and continuous identities with the respective database sequences.

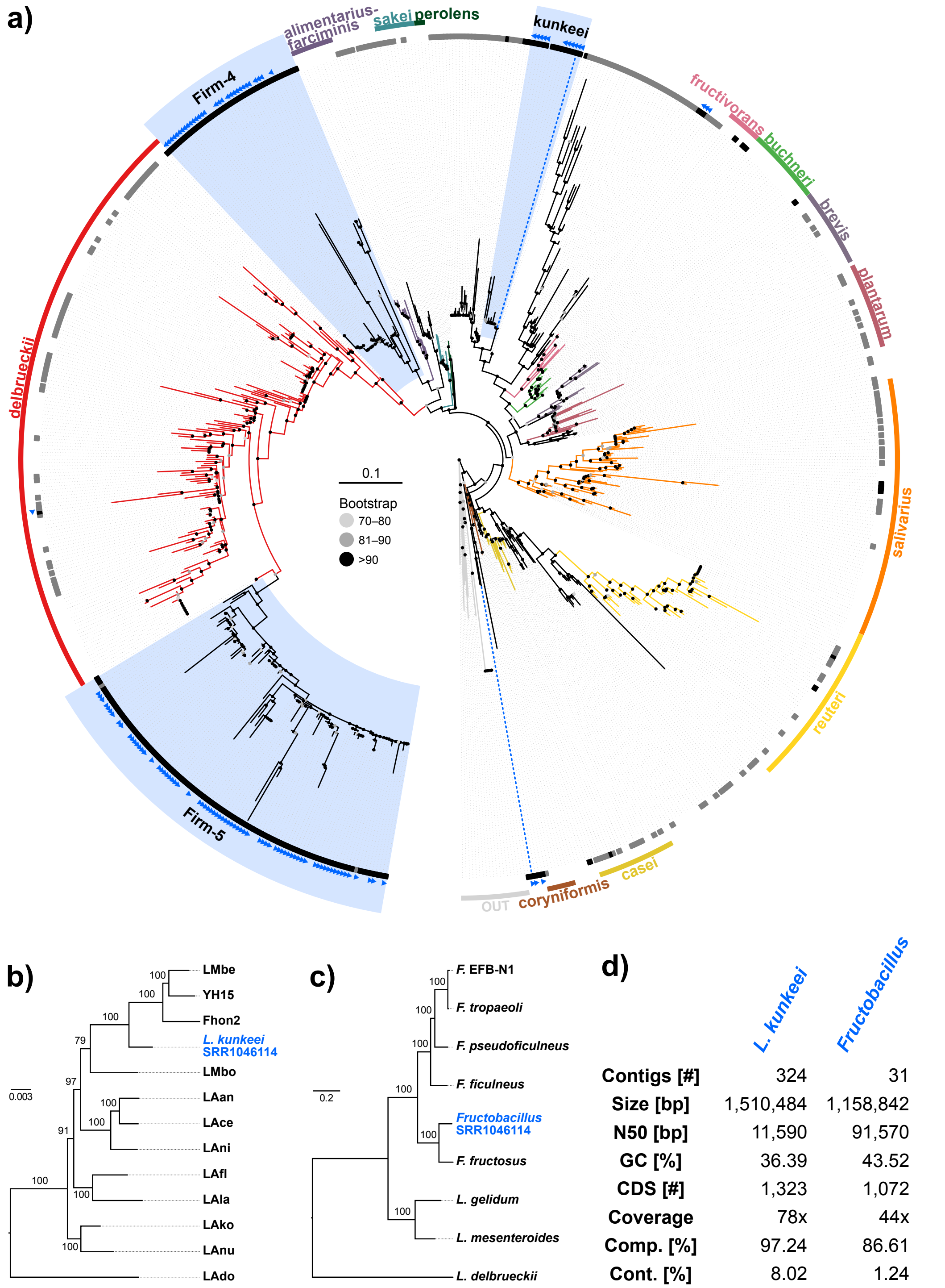
Because the majority of hits in the DNA sequencing libraries were Lactobacilli, we repeated the screening, this time using only *Lactobacillus* 16S sequences as baits. We found 121 *Lactobacillus* sequences in 40 of the 993 investigated libraries, corresponding to 25

Table 2 Evidence for differential gene expression of honey bees in response to viral infections across four RNAseq projects (see Table 1). 20 candidate loci were investigated and differential expression was determined with edgeR (see text for details).

Locus	Description	Number of projects in which differentially expressed*
LOC410087	Uncharacterized LOC410087	4
LOC408807	Uncharacterized LOC408807	3
LOC406114	Alpha-amylase	2
LOC406142	Hymenoptaecin	2
LOC724239	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial-like	2
LOC724367	Protein lethal(2)essential for life-like	2
LOC724654	Cytochrome b5 type B-like	2
LOC726418	Flavin-containing monooxygenase FMO GS-OX-like 3-like	2
Vg	Vitellogenin	2
Apid1	Apidaecin 1	1
Def2	Defensin 2	1
LOC406144	Abaecin	1
LOC725017	UDP-glycosyltransferase	1
LOC725725	Uncharacterized LOC725725	1
Melt	Melittin	1
CYP6AQ1	Cytochrome P450 6AQ1	0
Def1	Defensin 1	0
LOC413908	Cytochrome P450 6AS12	0
LOC552832	Glycine N-methyltransferase-like	0
LOC725158	Peptidoglycan recognition protein S1	0

OTUs (estimated with mothur using a 5% cutoff). In our phylogenetic analysis based on 16S rRNA sequences, most of the detected strains clustered within *Lactobacillus* groups known to be associated with honey bees (Fig. 3A). Of the recovered sequences not clustering within these lineages, three were found to group with other *Apis*- associated Lactobacilli as sister group to the *Lactobacillus coryniformis* group (Fig. 3A). Online BLAST searches revealed *Fructobacillus* species as closest matches based on 16S rRNA sequence.

Next, we aimed at recovering draft genome sequences of bee-associated Lactobacilli. We chose an *Apis mellifera intermissa* sequencing library from which 16S sequences of both *L. kunkeei* and *Fructobacillus* isolates were detected in our screen. The contigs of a meta-assembly were taxonomically annotated, and reads matching to the respective target taxa were then assembled and annotated separately. For each assembly, we performed a phylogenetic analysis based on all single copy orthologs shared with related genomes (Figs. 3B and 3C), thus confirming the identity of the strains as *L. kunkeei* (Fig. 3B) and *Fructobacillus* (Fig. 3C). Both genomes were highly covered and mostly complete based on the presence of conserved markers (Fig. 3D). Finally, we recovered the genome of a *Spiroplasma melliferum* strain from another *Apis* sequencing library (Fig. 4). In the meta-assembly, *Spiroplasma* and *Apis* contigs could be clearly separated by coverage



	L. kunkeei	Fructobacillus
Contigs [#]	324	31
Size [bp]	1,510,484	1,158,842
N50 [bp]	11,590	91,570
GC [%]	36.39	43.52
CDS [#]	1,323	1,072
Coverage	78x	44x
Comp. [%]	97.24	86.61
Cont. [%]	8.02	1.24

Figure 3 **'Contamination' from Lactobacilli in *Apis* short read libraries.** (A) Maximum likelihood tree of 720 16S rRNA sequences from Lactobacilli. Branch colors and the color of the outer annotation circle correspond to *Lactobacillus* species groups according to Felis & Dellaglio (2007). Inner circle demarks taxa found Hymenoptera (grey squares) and in corbiculate apids (honey bees and relatives, black squares). *Lactobacillus* sequences recovered in this study from contaminated *Apis* libraries are labeled with blue triangles. The Lactobacilli typically associated with honey bees

(continued on next page...)

Figure 3 (…continued)
(Firm-4, Firm-5, *L. kunkeei*) are further highlighted with a blue background color. Two dotted blue lines denote the taxa of which whole draft genomes were recovered. See text for details. An interactive version of the tree containing all node labels is available under http://www.evolgenius.info/evolview/#shared/wZcKHbwJuT. (B) Phylogeny of *Lactobacillus kunkeei* strains based on maximum likelihood analyses of 947 concatenated single copy orthologs (290,774 amino acid positions). Tree is rooted with *Lactobacillus apinorum* Fhon13 (taxon not shown). Strain names correspond to the names used in Tamarit et al. (*2015*; see Table S3). Blue taxon label corresponds to the *L. kunkeei* strain recovered from 'contaminants' in library SRR1046114. Bootstrap values are given on nodes. See Table S3 for sources of genomes. (B) Maximum likelihood tree of *Fructobacillus* (F.) and *Leuconostoc* (L.) species based on 435 concatenated single copy orthologs (145,069 amino acid positions). Tree is rooted with *Lactobacillus delbruecki*. Numbers on nodes correspond to bootstrap values. Again, blue taxon label denotes the *Fructobacillus* genome recovered from the 'contaminated' library SRR1046114. Note that the phylogenetic distance between *Fructobacillus fructosus* and the novel genome is similar to other between-species distances in this tree. See Table S3 for accession numbers of all genomes used for phylogenetic analysis. (D) Assembly statistics for the two novel draft genomes recovered from library SRR1046114. Abbreviations: CDS, coding sequences predicted with PROKKA; Comp. & Cont., completeness and contamination as estimated with CheckM version 1.0.6 (*Parks et al., 2015*) based on the number of conserved marker loci. Phylogenetic affiliations of the two strains are depicted in Figs. 3B and 3C, respectively.

and taxonomic annotations (Fig. 4B). The refined assembly resulted in a highly covered draft genome of *Spiroplasma melliferum*, which is very similar to the two previously sequenced *Spiroplasma melliferum* strains (*Alexeev et al., 2012*; *Lo et al., 2013*), based on shared ortholog clusters, genome organisation, and phylogeny (Figs. 4A, 4C and 4D).

The second part of our screen was focused on viruses in honey bee RNA sequencing libraries. Here, whole viral genomes were used as baits, and viral reads were found in about half of the investigated libraries (236/492 libraries with 500 or more viral reads). Deformed wing virus type A (DWV A) was by far the most frequently found virus, and was present in almost all libraries that contained viral reads. We found evidence for all other tested honey bee viruses as well, albeit at a lower frequency (Fig. 5A). In many cases, the viral reads made up only a negligible proportion of a sequencing library (Fig. 5B). However, in 75 RNA libraries, more than 5%, and up to 54% of the reads were of viral origin, indicating substantial viral loads in the honey bees from which the samples were originally prepared (Fig. 5B). We next investigated the genetic diversity of DWV variants by assembling draft genomes from the viral reads extracted from RNA sequencing libraries. Our phylogenetic analysis showed a clear separation between A and B types of DWV and considerable genetic variation is evident especially in the DWV A samples (Fig. 6). Furthermore, viruses extracted from libraries of the same project were often phylogenetically closely related (Fig. 6), which is not surprising given that samples in one project were commonly from a single geographic location (Table S2).

Viral infections usually result in a host response that should be measurable by means of differentially expressed genes (e.g., immunity-related genes). We tested this prediction using RNA sequencing libraries from four experiments (NCBI's BioProjects) that showed strong variations in viral loads across libraries, and 20 candidate loci that were previously found to be differentially expressed in response to pathogens (*Doublet et al., 2017*; see Table S6). When comparing RNA sequencing libraries with and without viruses, 15 of these genes were differentially expressed in at least one of the investigated experiments

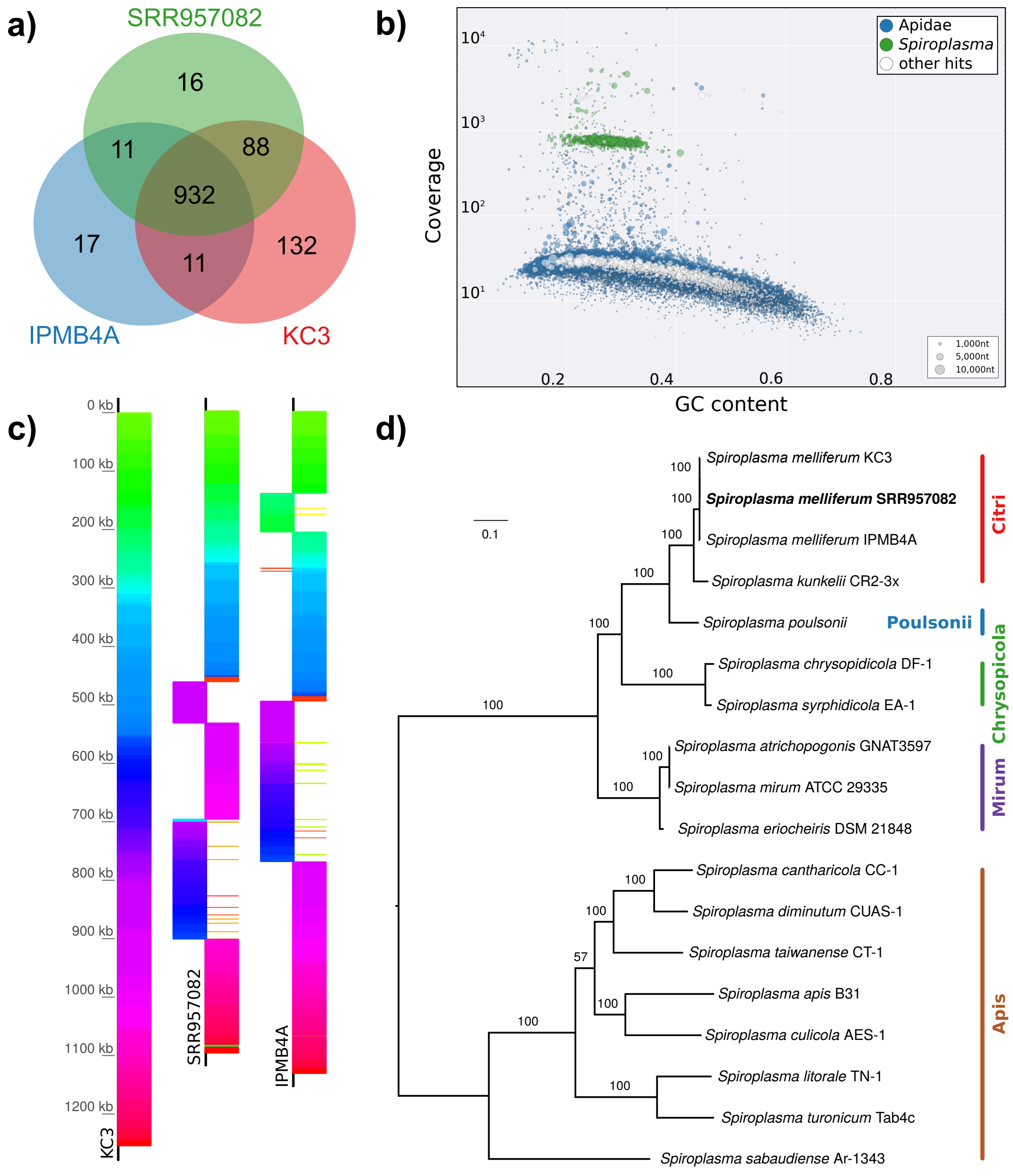


Figure 4 **Characteristics of *Spiroplasma melliferum.* isolated from a 'contaminated' *Apis mellifera* sequencing library (SRR957082).** (A) Venn diagram illustrating the number of ortholog groups shared between the novel strain and its closest sequenced relatives IBMB4A (*Lo et al., 2013*) and KC3 (*Alexeev et al., 2012*). (B) Taxon-annotated GC-coverage plot of SRR951082 metaassembly created with Blobology. *Spiroplasma* and *Apis* contigs can be differentiated by coverage. (C) Synteny across *Spiroplasma melliferum* genomes. Contigs from assemblies SRR957082 and IPMB4A were ordered against KC3, the most complete of the three *S. melliferum* genomes. Identical colors indicate syntenic blocks of the genomes. (D) Phylogenetic relationships within the genus *Spiroplasma*. Maximum likelihood tree is based on 206 concatenated loci (58,950 amino acid positions), numbers on branches correspond to bootstrap values. *Spiroplasma* groups are highlighted with colors. The taxon label of the novel genome is highlighted in bold. Accession numbers for all taxa are listed in Table S3.

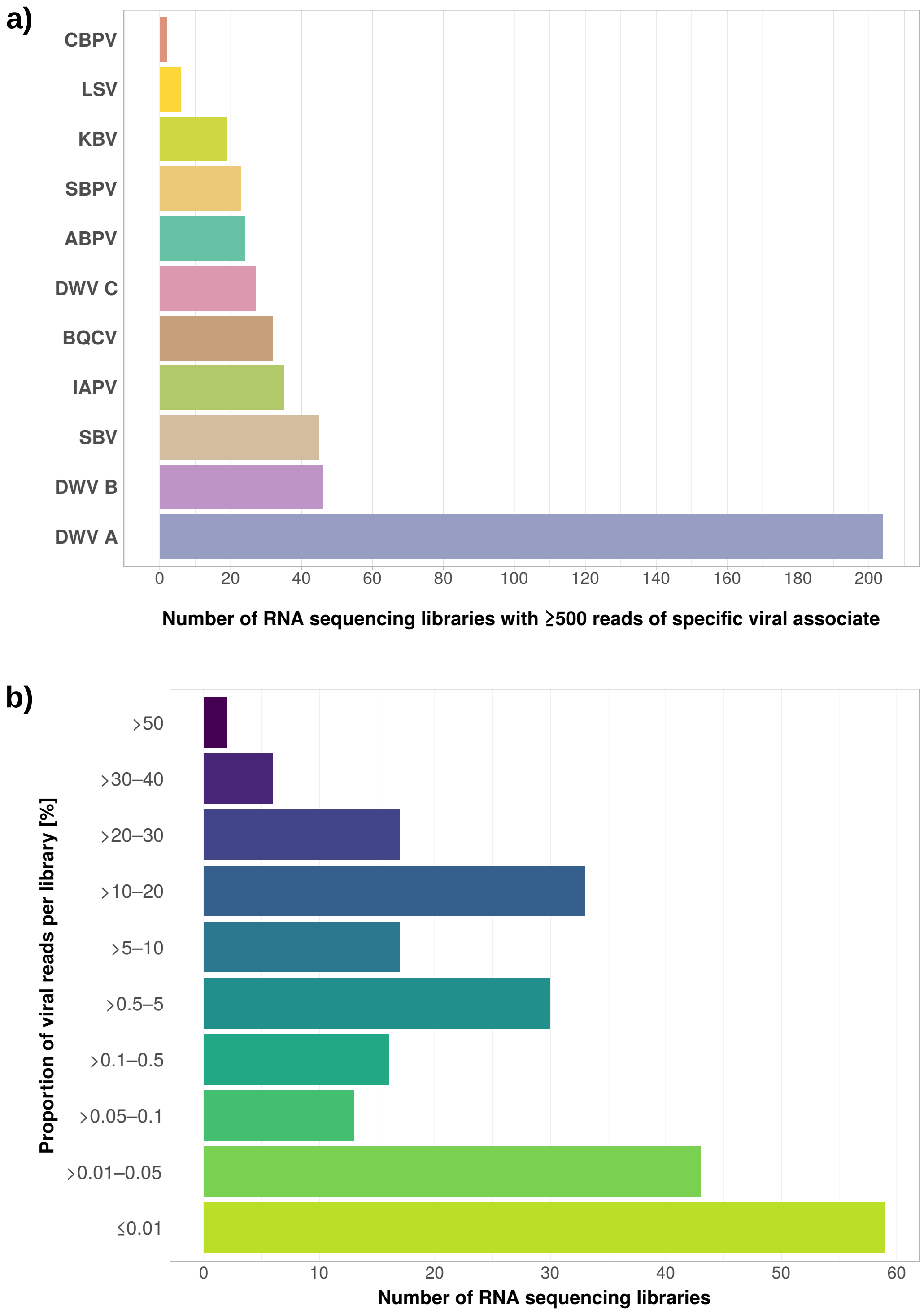


Figure 5 Distribution of viral reads in *Apis* RNA sequencing libraries. (A) Bar chart shows how often each of the viruses associated with honey bees was detected with our approach. Results are only shown for libraries with at least 500 reads of viral origin (236 in total). (B) Bar chart shows the distribution of relative proportions of viral reads for these 236 libraries.

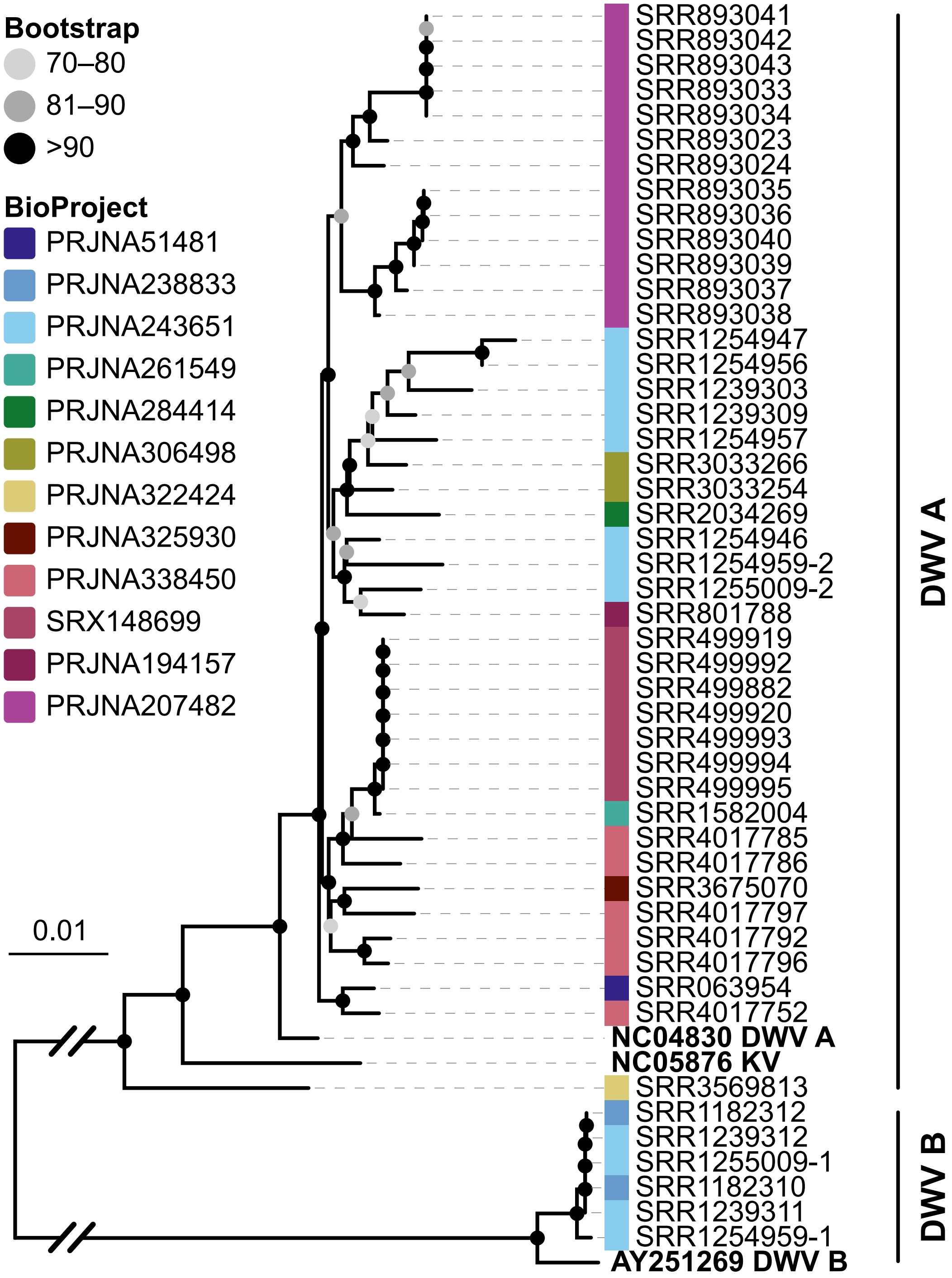


Figure 6 Phylogeny of DWV sequences extracted from *Apis* RNA sequencing libraries. Tree is based on 10,291 aligned positions and only sequences with at least 5,000 bp were included. Reference sequences and corresponding accession numbers are highlighted with bold typeface. Colors at nodes indicate NCBI's BioProjects from which the sequences originated.

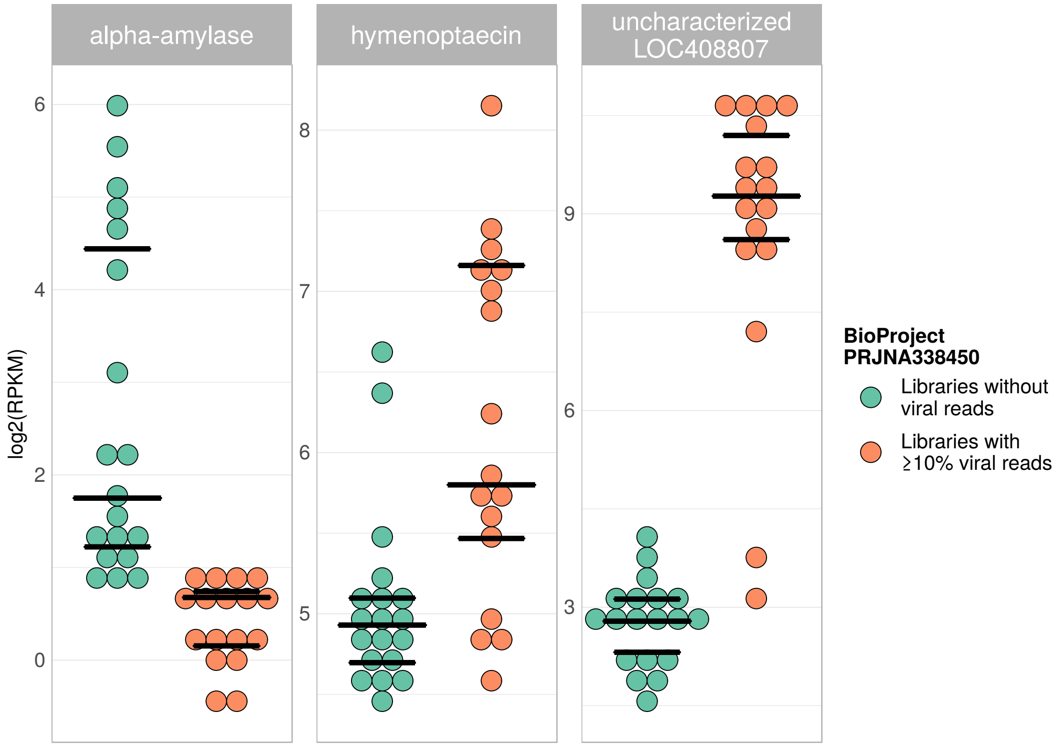


Figure 7 Example of differentially expressed honey bee genes in response to viral infection. The relative expression levels of three genes that were determined to be significantly differentially expressed with respect to viral infection status are plotted for one project. Each library is represented by one dot, and lines correspond to 75% quartile, median, and 25% quartile of all samples of a group, respectively. Expression levels for all investigated genes and projects can be found in Figs. S2–S5 and Table S5.

(Table 2), and one gene was found to be differentially expressed in all of them (Table 1). This gene (LOC410087) is not functionally characterized in honey bees, but likely encodes a protein related to heat shock response, which may be activated during viral infections (*Merkling et al., 2015*). Within each experiment, at least five of the investigated genes were differentially expressed between the two groups (Table 1). Relative expression rates of three exemplary genes that were significantly different between libraries with and without viral reads are plotted for one project in Fig. 7. Expression plots for all genes from all investigated projects are shown in Figs. S2–S5.

DISCUSSION

Apis DNA sequencing libraries can help to reconstruct the honey bee microbiome

We used bait sequences of *Apis* symbionts and pathogens to determine if microbial data can be retrieved from DNA sequencing projects targeting *Apis* (honey bees) and found evidence for the presence of these taxa in 11% of 993 *Apis* short read libraries. This measure of non-target 'contamination' can be considered as conservative, since our approach only reports relatively high levels of contamination (at least 1,000 reads per bait sequence). Our approach revealed that all common gut symbionts of honey bees are also present as 'contamination' in DNA sequencing libraries (Fig. 2). Furthermore, the relative frequency of each of the gut colonizers in focused study roughly corresponds to the frequencies at which we detected them. For example, the microbiome of healthy honey bees is dominated by Lactobacilli (*Kwong & Moran, 2016*), and this is also reflected in our results (Fig. 2, Table S5). Our findings demonstrate that a reasonable understanding of honey bee gut microbiome composition could be gained solely from non-target sequences produced as a by-product of honey bee sequencing projects.

When we next targeted our screen of DNA sequencing libraries only at *Lactobacillus*, our protocol detected 25 taxonomically different *Lactobacillus* strains. Phylogenetic reconstruction of *Lactobacillus* relationships based on 16S rRNA generally reflected the current understanding of this genus' taxonomy (*Felis & Dellaglio, 2007*; *Salvetti & Torriani, 2012*), and revealed that most Lactobacilli known to be associated with honey bees are also present in *Apis* short read libraries. This includes Firm-4 and Firm-5 Lactobacilli, both of which are honey bee hindgut colonizers, and *L. kunkeei*, which is common in nectar and hive material, and sometimes found in honey bee crops (*Kwong & Moran, 2016*). Furthermore, we found *Fructobacillus,* which share an ecological niche with *L. kunkeei*, i.e., they are found in flowers, nectar, and in honey bee guts (*Endo, Futagawa-Endo & Dicks, 2009*; *Endo & Salminen, 2013*). Although not classified as such, recent phylogenomic evidence suggests that *Fructobacillus* (and the closely related *Leuconostoc*) are part of the *Lactobacillus* radiation (*Sun et al., 2015*). Here, we also infer *Fructobacillus* grouping within, rather than outside of *Lactobacillus* (Fig. 2A).

In addition to the gut symbionts, three common honey bee pathogens were detected with our approach: *Nosema*, *Crithidia/Lotmaria*, and *Spiroplasma. Nosema* are microsporidian gut parasites of various honey bee species, and while the sampling of our screen is not representative, this finding corroborates the recognition of *Nosema* as widespread pathogen of honey bee colonies worldwide (*Nixon, 1982*; *Klee et al., 2007*). *Crithidia/Lotmaria* (Trypanosomatidae), another gut pathogen of *Apis* and related bee species (*Schwarz et al., 2015*) was detected at an even higher frequency (Fig. 1, Table S5). We further found *Spiroplasma melliferum* in one of the investigated sequencing libraries. *Spiroplasma* are common symbiotic bacteria of many invertebrates (*Duron et al., 2008*) and have been connected to pathogenicity in honey bees (*Clark, 1977*). The bait sequences of all of these pathogens showed a high coverage in our screen, suggesting that novel genetic variants can be recovered from already available data, or from data that will become available as by-product of future honey bee sequencing projects.

In addition to the targeted microbes, a number of taxa that are likely not part of the natural *Apis* microbiome were detected. For example, we detected *Aspergillus* in several sequencing libraries that originated from museum material, which likely represents post mortem saprophytic growth. We also retrieved hits to plant sequences which might originate from co-amplified and sequenced pollen DNA (Fig. 2). This 'false discovery' illustrates an important caveat in our approach: the differentiation between host-associated microbes and microbes from other sources may not always be possible, and will be particularly difficult for museum specimens. Though not problematic in the examples we present, the situation is likely more complicated for study of host species with a less well-investigated microbiome, or for symbionts that are very similar to environmental taxa. In these cases, the approach will establish candidates that will then require direct validation.

Finally, as demonstrated previously in other taxa with very similar approaches (*Kumar et al., 2013*), we show that draft genomes of microbial symbionts can be recovered from *Apis* short reads. For example, inspecting the non-target components of just a single *Apis mellifera intermissa* sequencing library produced novel, highly covered, and near complete draft genomes of *Lactobacillus kunkeii* and a *Fructobacillus* strain (Figs. 3B–3D). Although the 16S sequence of the *Fructobacillus* strain best matched *F. fructosus*, our analysis suggests it belongs to a species so far not represented by genomic sequences in public databases, or even a novel species (Fig. 3C). Conceivably, many additional *Lactobacillus* variants could be retrieved from the libraries investigated here, potentially providing a more complete picture of the *Apis* microbiome composition and function. It should be noted that draft genomes reconstructed this way must be regarded as 'population consensus' genomes, as opposed to genomes sequenced from cultured bacterial clones. While these genomes cannot be linked to a bacterial clone, they still provide information of metabolic capacities within the *Apis* microbiome.

In summary, our analyses suggest that 'contamination' from honey bee sequencing projects can help to characterize the honey bee microbiome, and inform about its composition, abundance of specific taxa, and through recovery of genomes, about metabolic capacities. Short read repositories thus provide plenty of biological information on known associates, but also on undescribed ones. If, for example, a novel microbial associate of honey bees is discovered, one could potentially use the available short read data (and associated metadata) to learn about its distribution, prevalence, and genome. Or, alternatively, one could confirm the absence of certain taxa with more confidence. *Kwong et al. (2017)* identified *Bombiscardovia* sp. and *Schmidhempelia* sp. as gut microbes of bumble bees (*Bombus* sp.) that are typically not present honey bees. We also did not find evidence for these taxa in *Apis* with our screening approach and thus provide further support for their absence based on a large sample size.

Implications of viral reads as common contaminations in *Apis* RNA sequencing libraries

We found viral reads in about half of the 498 investigated RNA libraries, and DWV type A as the most common viral associate, followed by DWV type B (=Varroa destructor virus-1), and all other viruses we screened for (Fig. 5). DWV has a global distribution (*Genersch & Aubert, 2010*) and its transmission is facilitated by *Varroa* mites, which are also globally distributed (*Martin et al., 2012*; *Wilfert et al., 2016*). Our findings are thus in line with these previous observations. The detection of DWV despite our deliberate exclusion of RNA sequencing experiments which investigated *Varroa* or virus treatments can be explained by the fact that, in many cases, DWV infections are asymptomatic (*Lanzi et al., 2006*). The low proportion of DWV reads found in many samples (as a proxy for viral load) also argues for asymptomatic infections in the bees from which the RNA was extracted (Fig. 5). However, we found a number of RNA sequencing libraries in which DWV read numbers made up a very large proportion of the reads (10–54%, Fig. 5). Very high viral titers are typically found in symptomatic bees (*Chen, Higgins & Feldlaufer, 2005*; *Lanzi et al., 2006*; *Brettell et al., 2017*), and inspection of the metadata associated with RNA libraries showing high proportions of viral reads revealed that many of these were created from heads or brains (out of 58 libraries with >10% viral reads, 25 from heads, 10 from other tissues, 23 undisclosed, Table S4). This is notable because high DWV titres in honey bee heads have been found to correlate with deformed wing phenotypes (*Yue, 2005*; *Zioni, Soroker & Chejanovsky, 2011*). This suggests that for these specific experiments, RNA may have been extracted from DWV infected bees despite obvious symptoms (i.e, crippled wings).

Varroa mites and DWV are considered one of the main factors driving the decline in managed honey bee populations (*VanEngelsdorp et al., 2009*; *Conte, Ellis & Ritter, 2010*), and molecular data have been used to trace the global spread of these pathogens (*Martin et al., 2012*; *Wilfert et al., 2016*). We here showed that almost complete DWV draft genomes can be extracted from short read data, revealing considerable genetic diversity between the strains (Fig. 6). Archived RNA sequencing reads of honey bees are thus a valuable, largely untapped resource for genomic data of viruses. As described above for symbionts in DNA sequencing libraries, these data inform about host association, distribution, and genetic diversity of viruses.

Furthermore, the detection of large amounts of viral reads in honey bee RNA sequencing libraries has other, more direct implications. Differential gene expression analysis was previously used to study e.g., development, learning, caste differences, and other behavioral traits in honey bees (*Whitfield, 2003*; *Liu et al., 2011*; *Liang et al., 2012*; *Cameron, Duncan & Dearden, 2013*; *Naeger & Robinson, 2016*). It has further been established that viral infections lead to transcriptomic responses in honey bees, some of which are conserved and universal for all pathogens, while others are specific with respect to the pathogen present (summarized in *Doublet et al., 2017*). Following from this, the presence of viruses alters transcriptomic profiles of honey bees and therefore incorporated into analyses of differential gene expression, e.g., by adding viral load as one factor in the interpretation. In most experiments analysed here, the presence of viruses was likely not a problem, as viral reads were uniformly low across all samples. However, we did identify some examples in which uneven viral loads very likely had an effect on transcriptomic profiles (Tables 1 and 2, Fig. 7 & Figs. S2–S5). In these examples, the presence of viruses, if unaccounted for, potentially leads to erroneous interpretations of the RNAseq data. Consequently, viruses should be screened for and identified in any RNA sequencing experiment investigating differential gene expression.

CONCLUSION

The biological properties of an individual are a composite of the functions encoded in their genome and that of microbial associates, both symbionts and pathogens. Here we revisited published short read data from *Apis* spp. sequencing projects to investigate if these give insight into the wider set of associates that are commonly disgarded as ‘contaminants’. We found that a large variety of distinct *Apis*-associated microbial symbionts and pathogens can be detected as ‘contamination’ in these data. Further, due to the large depths of today’s sequencing projects, the genomes of some microbial associates (which are typically much smaller than the target genomes) can often be recovered in high quality. Honey bees have a comparatively simple microbiota (*Kwong & Moran, 2016*) and are thus considered suitable models for microbiome-animal interactions and evolution (*Engel et al., 2016*). Their enormous economic importance (*Calderone, 2012*) has driven a large number of honey bee sequencing projects. Our examination of the output of these projects suggests that substantial amounts of genomic information on bee-associated microbes are included in these data. While genomes gained from contaminated bee samples cannot and should not replace focused microbiological and metagenomic investigations, they might still improve our understanding of honey bee microbiome composition and functioning.

Although our study was focused on *Apis*, it is conceivable that the amount of non-target ‘contamination’ is similar for other sequencing projects. As a best practice, and potentially rewarding research avenue in any sequencing project, we therefore suggest that all non-target taxa should be identified, and their genomes assembled, annotated, and published alongside the target genome. This requires less effort than it may seem, as de-contamination is already a standard post-processing step. Instead of discarding the contaminated reads, they can be processed with one of many available software solutions that automate the process of identifying and assembling genomes from metagenomes (*Oulas et al., 2015*), thus minimizing the additional workload. Not only would this provide the community with valuable genomic data of symbionts from known host taxa, but it can additionally be argued that this is the most sensible thing to do from a biological point of view. Evidence is mounting that symbiotic microbes influence almost all aspects of their host’s biology (*Douglas, 2014*; *Bordenstein & Theis, 2015*). Taking into account the total genomic information recovered in sequencing projects may therefore provide a more complete picture of the target organism’s biology.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Seth Barribeau for comments on an earlier version of this manuscript, and anonymous reviewers for constructive feedback. We further thank Dr Philipp Engel and Olivier Emery for providing access to short read data.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the European Commission through H2020 funding in the form of an EMBO long term fellowship (ALTF 48-2015, LTFCOFUND2013, GA-2013-609409) and a Marie Curie Fellowship (H2020-MSCA-IF-2015, 703379) to MG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

EMBO long term fellowship: ALTF 48-2015, LTFCOFUND2013, GA-2013-609409.
Marie Curie Fellowship: H2020-MSCA-IF-2015, 703379.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Michael Gerth conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Gregory D.D. Hurst conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

Github: https://github.com/gerthmicha/symbiont-sra.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3529#supplemental-information.

REFERENCES

Alexeev D, Kostrjukova E, Aliper A, Popenko A, Bazaleev N, Tyakht A, Selezneva O, Akopian T, Prichodko E, Kondratov I, Chukin M, Demina I, Galyamina M, Kamashev D, Vanyushkina A, Ladygina V, Levitskii S, Lazarev V, Govorun V. 2012. Application of *Spiroplasma melliferum* proteogenomic profiling for the discovery of virulence factors and pathogenicity mechanisms in host-associated spiroplasmas. *Journal of Proteome Research* **11**:224–236 DOI 10.1021/pr2008626.

Allen M, Ball B. 1996. The incidence and world distribution of honey bee viruses. *Bee World* **77**:141–162 DOI 10.1080/0005772X.1996.11099306.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology* **19**:455–477 DOI 10.1089/cmb.2012.0021.

Blow F, Gioti A, Starns D, Ben-Yosef M, Pasternak Z, Jurkevitch E, Vontas J, Darby AC. 2016. Draft genome sequence of the *Bactrocera oleae* symbiont "*Candidatus* Erwinia dacicola". *Genome Announcements* **4**:e00896–16 DOI 10.1128/genomeA.00896-16.

Bordenstein SR, Theis KR. 2015. Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLOS Biology* **13**:e1002226 DOI 10.1371/journal.pbio.1002226.

Brettell L, Mordecai G, Schroeder D, Jones I, Da Silva J, Vicente-Rubiano M, Martin S. 2017. A comparison of deformed wing virus in deformed and asymptomatic honey bees. *Insects* **8**:Article 28 DOI 10.3390/insects8010028.

Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. 2016. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* **533**:543–546 DOI 10.1038/nature17645.

Bruen TC, Philippe H, Bryant D. 2006. A simple and robust statistical test for detecting the presence of recombination. *Genetics* **172**:2665–2681 DOI 10.1534/genetics.105.048975.

Calderone NW. 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992–2009. *PLOS ONE* **7**:e37235 DOI 10.1371/journal.pone.0037235.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* **10**:Article 421 DOI 10.1186/1471-2105-10-421.

Cameron RC, Duncan EJ, Dearden PK. 2013. Biased gene expression in early honeybee larval development. *BMC Genomics* **14**:Article 903 DOI 10.1186/1471-2164-14-903.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6**:1621–1624 DOI 10.1038/ismej.2012.8.

Chen Y, Higgins J, Feldlaufer M. 2005. Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (Apis mellifera L). *Applied and Environmental Microbiology* **71**:436–441 DOI 10.1128/AEM.71.1.436-441.2005.

Clark T. 1977. *Spiroplasma* sp, a new pathogen in honey bees. *Journal of Invertebrate Pathology* **113**:112–113.

Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, Bauer DJ, Caceres CE, Carmel L, Casola C, Choi J-H, Detter JC, Dong Q, Dusheyko S, Eads BD, Frohlich T, Geiler-Samerotte KA, Gerlach D, Hatcher P, Jogdeo S, Krijgsveld J, Kriventseva EV, Kultz D, Laforsch C, Lindquist E, Lopez J, Manak JR, Muller J, Pangilinan J, Patwardhan RP, Pitluck S, Pritham EJ, Rechtsteiner A, Rho M, Rogozin IB, Sakarya O, Salamov A, Schaack S, Shapiro H, Shiga Y, Skalitzky C, Smith Z, Souvorov A, Sung W, Tang Z, Tsuchiya D, Tu H, Vos H, Wang M, Wolf YI, Yamagata H, Yamada T, Ye Y, Shaw JR, Andrews J, Crease TJ, Tang H, Lucas SM, Robertson HM, Bork P, Koonin EV, Zdobnov EM, Grigoriev IV, Lynch M, Boore JL. 2011. The ecoresponsive genome of *Daphnia pulex*. *Science* **331**:555–561 DOI 10.1126/science.1197761.

Conte YL, Ellis M, Ritter W. 2010. *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? *Apidologie* **41**:353–363 DOI 10.1051/apido/2010017.

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P-L, Briese T, Hornig M, Geiser DM, Martinson V, VanEngelsdorp D, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchison SK, Simons JF, Egholm M, Pettis JS, Lipkin WI. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**:283–287 DOI 10.1126/science.1146498.

Darling AE, Mau B, Perna NT, Zasada IA. 2010. Progressivemauve: multiple genome alignment with gene gain, loss and rearrangement. *PLOS ONE* **5**:e11147 DOI 10.1371/journal.pone.0011147.

Delcher AL, Bratke KA, Powers EC, Salzberg SL. 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* **23**:673–679 DOI 10.1093/bioinformatics/btm009.

Delwart EL. 2007. Viral metagenomics. *Reviews in Medical Virology* **17**:115–131 DOI 10.1002/rmv.532.

Denver DR, Brown AMV, Howe DK, Peetz AB, Zasada IA. 2016. Genome skimming: a rapid approach to gaining diverse biological insights into multicellular pathogens. *PLOS Pathogens* **12**:e1005713 DOI 10.1371/journal.ppat.1005713.

Doublet V, Poeschl Y, Gogol-Döring A, Alaux C, Annoscia D, Aurori C, Barribeau SM, Bedoya-Reina OC, Brown MJF, Bull JC, Flenniken ML, Galbraith DA, Genersch E, Gisder S, Grosse I, Holt HL, Hultmark D, Lattorff HMG, Conte YL, Manfredini F, McMahon DP, Moritz RFA, Nazzi F, Niño EL, Nowick K, Van Rij RP, Paxton RJ, Grozinger CM. 2017. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. *BMC Genomics* **18**:Article 207 DOI 10.1186/s12864-017-3597-6.

Douglas AE. 2014. Multiorganismal insects: diversity and function of resident microorganisms. *Annual Review of Entomology* **60**:1–18 DOI 10.1146/annurev-ento-010814-020822.

Douglas AE, Werren JH. 2016. Holes in the hologenome: why host-microbe symbioses are not holobionts. *MBio* **7**:e02099–e02015.

Duron O, Bouchon D, Boutin S, Bellamy L, Zhou L, Engelstädter J, Hurst GDD. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology* **6**:Article 27 DOI 10.1186/1741-7007-6-27.

Emms DM, Kelly S. 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biology* **16**:Article 157 DOI 10.1186/s13059-015-0721-2.

Endo A, Salminen S. 2013. Honeybees and beehives are rich sources for fructophilic lactic acid bacteria. *Systematic and Applied Microbiology* **36**:444–448 DOI 10.1016/j.syapm.2013.06.002.

Endo A, Futagawa-Endo Y, Dicks LM. 2009. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. *Systematic and Applied Microbiology* **32**:593–600 DOI 10.1016/j.syapm.2009.08.002.

Engel P, Kwong WK, McFrederick Q, Anderson KE, Barribeau SM, Chandler JA, Cornman RS, Dainat J, De Miranda JR, Doublet V, Emery O, Evans JD, Farinelli L, Flenniken ML, Granberg F, Grasis JA, Gauthier L, Hayer J, Koch H, Kocher S, Martinson VG, Moran N, Munoz-Torres M, Newton I, Paxton RJ, Powell E, Sadd BM, Schmid-Hempel P, Schmid-Hempel R, Song SJ, Schwarz RS, Van Engelsdorp D, Dainat B. 2016. The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *MBio* **7**:Article e02164-15.

Engel P, Martinson VG, Moran NA. 2012. Functional diversity within the simple gut microbiota of the honey bee. *Proceedings of the National Academy of Sciences of the United States of America* **109**:11002–11007 DOI 10.1073/pnas.1202970109.

Evans JD, Schwarz RS. 2011. Bees brought to their knees: microbes affecting honey bee health. *Trends in Microbiology* **19**:614–620 DOI 10.1016/j.tim.2011.09.003.

Felis GE, Dellaglio F. 2007. Taxonomy of lactobacilli and bifidobacteria. *Current Issues in Intestinal Microbiology* **8**:Article 44.

Fierst JL, Murdock DA, Thanthiriwatte C, Willis JH, Phillips PC. 2017. Metagenome-assembled draft genome sequence of a novel microbial *Stenotrophomonas maltophilia* strain isolated from *Caenorhabditis remanei* tissue. *Genome Announcements* **5**:e01646–16 DOI 10.1128/genomeA.01646-16.

Genersch E, Aubert M. 2010. Emerging and re-emerging viruses of the honey bee (*Apis mellifera* L). *Veterinary Research* **41**:Article 54 DOI 10.1051/vetres/2010027.

Gerth M, Gansauge M-T, Weigert A, Bleidorn C. 2014. Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. *Nature Communications* **5**:Article 5117 DOI 10.1038/ncomms6117.

Goodwin S, McPherson JD, McCombie WR. 2016. Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics* **17**:333–351 DOI 10.1038/nrg.2016.49.

He Z, Zhang H, Gao S, Lercher MJ, Chen W-H, Hu S. 2016. Evolview v2: an online visualization and management tool for customized and annotated phylogenetic trees. *Nucleic Acids Research* **44**:W236–W241 DOI 10.1093/nar/gkw370.

Kircher M, Kelso J. 2010. High-throughput DNA sequencing—concepts and limitations. *BioEssays* **32**:524–536 DOI 10.1002/bies.200900181.

Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, Chinh TX, Puerta F, Ruz JM, Kryger P, Message D, Hatjina F, Korpela S, Fries I, Paxton RJ. 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology* **96**:1–10 DOI 10.1016/j.jip.2007.02.014.

Kumar S, Jones M, Koutsovoulos G, Clarke M, Blaxter ML. 2013. Blobology: exploring raw genome data for contaminants, symbionts and parasites using taxon-annotated GC-coverage plots. *Frontiers in Genetics* **4**:Article 237 DOI 10.3389/fgene.2013.00237.

Kwong WK, Moran NA. 2016. Gut microbial communities of social bees. *Nature Reviews Microbiology* **14**:374–384 DOI 10.1038/nrmicro.2016.43.

Kwong WK, Medina LA, Koch H, Sing K-W, Soh EJY, Ascher JS, Jaffé R, Moran NA. 2017. Dynamic microbiome evolution in social bees. *Science Advances* **3**:e1600513 DOI 10.1126/sciadv.1600513.

Lanfear R, Calcott B, Ho SYW, Guindon S. 2012. Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* **29**:1695–1701 DOI 10.1093/molbev/mss020.

Lanzi G, De Miranda JR, Boniotti MB, Cameron CE, Lavazza A, Capucci L, Camazine SM, Rossi C. 2006. Molecular and biological characterization of deformed wing virus of honeybees (*Apis mellifera* L). *Journal of Virology* **80**:4998–5009 DOI 10.1128/JVI.80.10.4998-5009.2006.

Li D, Liu C-M, Luo R, Sadakane K, Lam T-W. 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* **31**:1674–1676 DOI 10.1093/bioinformatics/btv033.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* **25**:2078–2079 DOI 10.1093/bioinformatics/btp352.

Liang ZS, Nguyen T, Mattila HR, Rodriguez-Zas SL, Seeley TD, Robinson GE. 2012. Molecular determinants of scouting behavior in honey bees. *Science* **335**:1225–1228 DOI 10.1126/science.1213962.

Liu F, Li W, Li Z, Zhang S, Chen S, Su S. 2011. High-abundance mRNAs in *Apis mellifera*: comparison between nurses and foragers. *Journal of Insect Physiology* **57**:274–279 DOI 10.1016/j.jinsphys.2010.11.015.

Lo W-S, Chen L-L, Chung W-C, Gasparich GE, Kuo C-H. 2013. Comparative genome analysis of *Spiroplasma melliferum* IPMB4A, a honeybee-associated bacterium. *BMC Genomics* **14**:Article 22 DOI 10.1186/1471-2164-14-22.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal* **17**:10–12 DOI 10.14806/ej.17.1.200.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S, Schroeder DC. 2012. Global honey bee viral landscape altered by a parasitic mite. *Science* **336**:1304–1306 DOI 10.1126/science.1220941.

Martinson VG, Magoc T, Koch H, Salzberg SL, Moran NA. 2014. Genomic features of a bumble bee symbiont reflect its host environment. *Applied and Environmental Microbiology* **80**:3793–3803 DOI 10.1128/AEM.00322-14.

McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Ruby EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ. 2013. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America* **110**:3229–3236 DOI 10.1073/pnas.1218525110.

McFrederick QS, Cannone JJ, Gutell RR, Kellner K, Plowes RM, Mueller UG. 2013. Specificity between lactobacilli and hymenopteran hosts is the exception rather than the rule. *Applied and Environmental Microbiology* **79**:1803–1812 DOI 10.1128/AEM.03681-12.

Merkling SH, Overheul GJ, Van Mierlo JT, Arends D, Gilissen C, Van Rij RP. 2015. The heat shock response restricts virus infection in *Drosophila*. *Scientific Reports* **5**:Article 12758 DOI 10.1038/srep12758.

Minh BQ, Nguyen MAT, Von Haeseler A. 2013. Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* **30**:1188–1195 DOI 10.1093/molbev/mst024.

Mokili JL, Rohwer F, Dutilh BE. 2012. Metagenomics and future perspectives in virus discovery. *Current Opinion in Virology* **2**:63–77 DOI 10.1016/j.coviro.2011.12.004.

Moran NA, Sloan DB. 2015. The hologenome concept: helpful or hollow? *PLOS Biology* **13**:e1002311 DOI 10.1371/journal.pbio.1002311.

Mordecai GJ, Wilfert L, Martin SJ, Jones IM, Schroeder DC. 2016. Diversity in a honey bee pathogen: first report of a third master variant of the Deformed Wing Virus quasispecies. *The ISME Journal* **10**:1264–1273 DOI 10.1038/ismej.2015.178.

Nawrocki EP. 2009. Structural RNA homology search and alignment using covariance models. PhD Thesis, Washington University in Saint Louis.

Naeger NL, Robinson GE. 2016. Transcriptomic analysis of instinctive and learned reward-related behaviors in honey bees. *The Journal of Experimental Biology* **219**:3554–3561 DOI 10.1242/jeb.144311.

Neumann P, Carreck NL. 2010. Honey bee colony losses. *Journal of Apicultural Research* **49**:1–6 DOI 10.3896/IBRA.1.49.1.01.

Nguyen L-T, Schmidt HA, Von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution* **32**:268–274 DOI 10.1093/molbev/msu300.

Nixon M. 1982. Preliminary world maps of honeybee diseases and parasites. *Bee World* **63**:23–42 DOI 10.1080/0005772X.1982.11097850.

Oulas A, Pavloudi C, Polymenakou P, Pavlopoulos GA, Papanikolaou N, Kotoulas G, Arvanitidis C, Iliopoulos I. 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. *Bioinformatics and Biology Insights* **9**:Article 75 DOI 10.4137/BBI.S12462.

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research* **25**:1043–1055 DOI 10.1101/gr.186072.114.

Peng Y, Leung HCM, Yiu SM, Chin FYL. 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* **28**:1420–1428 DOI 10.1093/bioinformatics/bts174.

R Core Team. 2015. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. *Available at http://www.r-project.org*.

Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM. 2012. Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. *PLOS Genetics* **8**:e1003129 DOI 10.1371/journal.pgen.1003129.

Riesenfeld CS, Schloss PD, Handelsman J. 2004. Metagenomics: genomic analysis of microbial communities. *Annual Review of Genetics* **38**:525–552 DOI 10.1146/annurev.genet.38.072902.091216.

Robinson MD, Smyth GK. 2007. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* **9**:321–332 DOI 10.1093/biostatistics/kxm030.

Robinson MD, McCarthy DJ, Smyth GK. 2009. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**:139–140 DOI 10.1093/bioinformatics/btp616.

Salvetti E, Torriani S, Felis GE. 2012. The genus *Lactobacillus*: a taxonomic update. *Probiotics & Antimicrobial Proteins* **4**:217–226 DOI 10.1007/s12602-012-9117-8.

Salzberg SL, Hotopp J, Delcher A, Pop M, Smith D, Eisen M, Nelson W. 2005. Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biology* **6**:R23 DOI 10.1186/gb-2005-6-3-r23.

Sboner A, Mu X, Greenbaum D, Auerbach RK, Gerstein MB. 2011. The real cost of sequencing: higher than you think! *Genome Biology* **12**:Article 125 DOI 10.1186/gb-2011-12-8-125.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Horn DJV. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537–7541 DOI 10.1128/AEM.01541-09.

Schmieder R, Edwards R. 2011. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLOS ONE* **6**:e17288 DOI 10.1371/journal.pone.0017288.

Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, Graaf DC, Evans JD. 2015. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n gen, n sp. *Journal of Eukaryotic Microbiology* **62**:567–583 DOI 10.1111/jeu.12209.

Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**:2068–2069 DOI 10.1093/bioinformatics/btu153.

Sedlazeck FJ, Rescheneder P, Von Haeseler A. 2013. NextGenMap: fast and accurate read mapping in highly polymorphic genomes. *Bioinformatics* **29**:2790–2791 DOI 10.1093/bioinformatics/btt468.

Siozios S, Cestaro A, Kaur R. 2013. Draft genome sequence of the *Wolbachia* endosymbiont of *Drosophila suzukii*. *Genome Announcements* **1**:e00032–13 DOI 10.1128/genomeA.00032-13.

Sun Z, Harris HMB, McCann A, Guo C, Argimón S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF, Liu W, Song Y, Salvetti E, Wrobel A, Rasinkangas P, Parkhill J, Rea MC, O'Sullivan O, Ritari J, Douillard FP, Ross RP, Yang R, Briner AE, Felis GE, De Vos WM, Barrangou R, Klaenhammer TR, Caufield PW, Cui Y, Zhang H, O'Toole PW. 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications* **6**:Article 8322 DOI 10.1038/ncomms9322.

Tamarit D, Ellegaard KM, Wikander J, Olofsson T, Vasquez A, Andersson SGE. 2015. Functionally structured genomes in *Lactobacillus kunkeei* colonizing the honey crop and food products of honeybees and stingless bees. *Genome Biology and Evolution* **7**:1455–1473 DOI 10.1093/gbe/evv079.

VanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R, Tarpy DR, Pettis JS. 2009. Colony collapse disorder: a descriptive study. *PLOS ONE* **4**:e6481 DOI 10.1371/journal.pone.0006481.

Visser M, Bester R, Burger JT, Maree HJ. 2016. Next-generation sequencing for virus detection: covering all the bases. *Virology Journal* **13**:Article 85.

Whitfield CW. 2003. Gene expression profiles in the brain predict behavior in individual honey bees. *Science* **302**:296–299 DOI 10.1126/science.1086807.

Wilfert L, Long G, Leggett HC, Schmid-Hempel P, Butlin R, Martin SJM, Boots M. 2016. Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. *Science* **351**:594–597 DOI 10.1126/science.aac9976.

Wolfe KH, Li W-H. 2003. Molecular evolution meets the genomics revolution. *Nature Genetics* **33**:255–265 DOI 10.1038/ng1088.

Yue C. 2005. RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *Journal of General Virology* **86**:3419–3424 DOI 10.1099/vir.0.81401-0.

Zioni N, Soroker V, Chejanovsky N. 2011. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. *Virology* **417**:106–112 DOI 10.1016/j.virol.2011.05.009.