Nanopore Sequencing as a Surveillance Tool for Plant Pathogens in Plant and Insect Tissues

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Abstract

Plant pathogens are constantly emerging and spreading into new areas and there are often limited postdiagnosis treatment options for infection, making surveillance key to their control. Here we present results from a study testing the efficacy of a portable nanopore-based massively parallel sequencing (MPS) technology for use in the detection of diverse plant pathogens in selected samples. The Oxford MinION device was coupled with whole transcriptome amplification (WTA) to sequence the metatranscriptome of plant and insect tissues infected with either *Candidatus Liberibacter asiaticus* or plum pox virus. Results showed that this methodology is useful for detecting

unsuspected viral and bacterial pathogens in plant and insect tissues. The percentage of generated reads assigned to plum pox virus was 95% from infected tissue and 3% from the viruliferous insect, *Myzus persicae*. *Diaphorina citri* sequencing led to 22% of the reads mapping as *Ca. L. asiaticus*. Plum pox virus and *Ca. L. asiaticus* were detected in both tissue and insect samples near the beginning of each sequencing run, demonstrating the capability of this methodology to obtain results rapidly. This approach also proved the capability of this system to determine the major components of the insect vector's microbiome and the specific strain of small-genome, high-titer pathogens.

As the global population steadily increases and the threat of climate change rises, our ability to combat plant pathogens promises to become an important factor in the fight for food security. For many plant pathogens, there are few postexposure options for intervention. As a result, the detection of the pathogen plays a key role in containing their spread. Although several technologies exist for molecular detection of plant pathogens, nearly all currently feasible methods require previous knowledge of the pathogen involved, i.e., the assays are inherently targeted against specific pathogens. Recently, massively parallel sequencing (MPS), or next-generation sequencing, has been shown to be useful in identifying pathogens that are not anticipated to be in the sample, or that may even be unknown to science (Adams et al. 2009; Stobbe et al. 2013). While this approach is promising, two main hurdles limit its use in remote settings: 1) the sequencing hardware is not portable, and 2) querying the sequences produced can require sophisticated bioinformatics and timely analyses of a substantial dataset may require high performance computational hardware. Several studies have shown that techniques such as electronic-probe diagnostic nucleic acid analysis, or EDNA, can be used to overcome the second hurdle mentioned above. This technique minimizes and ignores irrelevant sequence data and thereby focuses on specific pathogen-associated sequences (Espindola et al. 2015; Stobbe et al. 2013, 2014).

To assess one possible means of overcoming the lack of equipment portability, we tested the effectiveness of the first portable MPS device as a surveillance tool for detection of viral and bacterial plant pathogens in both infected plant and insect tissues. Specifically, an Oxford Nanopore Technology MinION sequencer was used (Oxford, U.K.). MinION is a portable, single molecule genome-sequencing

instrument that works by taking frequent electrical measurements while a single strand of DNA passes through a protein nanopore. This device is the size of a smart phone, is USB powered, and can stream up to 100 MB of sequence data over a 24-h period. The library preparation is also uncomplicated, and the whole protocol can be executed in less time than when using other comparable MPS technologies. This system was previously demonstrated to be promising for surveillance of viral and bacterial pathogens of humans (Kilianski et al. 2015; Quick et al. 2014, 2015), and single-nucleotide-variant detection in clonal haploid samples (Jain et al. 2015). In order to simulate a diversity of plant pathogens for this initial study, a viral and a bacterial plant pathogen that are each vectored by hemipteran insects were selected. *Plum pox virus* (PPV) is a major viral pathogen of stone fruit around the world (García et al. 2014). For PPV, we tested both infected plant and insect samples in separate trials. The second pathogen is *Candidatus Liberibacter asiaticus*, the causative agent of citrus greening, the most destructive citrus pathogen worldwide. As *Diaphorina citri* is the principal insect vector responsible for infection and spread of this disease and insect surveillance is a major factor in monitoring citrus greening's spread, we decided to focus on the infected insect for this experiment (Gottwald 2010; Ukuda-Hosokawa et al. 2015). This insect was also selected because its bacterial microbiome is well known, and it was therefore simple to assemble a reference dataset that included all predicted bacterial genomes (Fagen et al. 2012). We also developed a computational pipeline for pathogen detection; specifically, querying error-prone MinION output against genome-based reference datasets. This pipeline uses currently available open source software packages to base-call, align, and conduct statistical analyses on these data.

Our results suggest that the combination of whole transcriptome amplification (WTA) and nanopore sequencing technologies, or a similar derivative, could be used to detect emergent plant pathogens from infected tissues and also be adapted for use in assaying beneficial plant-associated microbiomes. The speed at which this technology was able to produce pathogen-confirmatory reads suggests that it could also be downscaled in the future to produce more affordable and portable platforms.

Materials and Methods

Material. Three experiments were conducted using plant and insect tissues infected with either of two selected plant pathogens, *Ca. L. asiaticus* or PPV. The insect vectors, *D. citri* infected with *Ca. L. asiaticus*, *Myzus persicae* carrying PPV, and the fruit *Prunus*

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persica infected with PPV, were used as models for plant pathogen detection. Samples were obtained from the greenhouse and insectarium rearing facilities at the USDA-ARS, Foreign Disease-Weed Science Research Unit, Fort Detrick, Maryland. *M. persicae* were reared on *Rumex crispus* (curly dock) in a virus-free insectary and after 3 weeks of colony initiation insects were fed on PPV-infected *P. persica* (cv. GF305). Non-*Ca. L. asiaticus*-exposed *D. citri* were maintained on *Murraya koenigii* (curry leaf) and at 5 days post molt, adults were then fed on *Ca. L. asiaticus*-infected *Citrus sinensis* cv. Pineapple (pineapple sweet orange) for an acquisition access period of 7 days. Plant infection was primarily confirmed via observation of physical symptoms as well as standard PCR analyses.

RNA preparation. After sample collection, nucleic acids were extracted immediately from the tissue samples using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Following RNA extraction, total RNA content was amplified using the TransPlex WTA Kit (Sigma Aldrich, St. Louis, MO) following the manufacturer's instructions.

Sample quality control and quantification. Quality and quantification of the WTA samples were conducted at multiple steps during the library preparation by means of a QIAxcel capillary electrophoresis unit (Qiagen, Valencia, CA) and a Qubit fluorimeter (Thermo, Waltham, MA).

MinION library preparation. Library preparation for amplicon sequencing was achieved using an Oxford SQK-MAP006 ligation sequencing kit following the manufacturer's instructions (Oxford Nanopore Technologies, Oxford, U.K.). Briefly, a total of 1 µg of *D. citri* WTA sample, 800 ng of *M. persicae* WTA sample, and 1 µg of *P. persica* WTA sample were end-repaired using an NEB end-repair module (New England Biolabs, Ipswich, MA), then cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) with 1.8 (v/v) ratio of beads to reaction mixture. End-repaired DNA was A-tailed using the NEB dA-tailing module (New England Biolabs) in a volume of 55 µl, and again cleaned up with AMPure XP beads as above. Following cleaning, 10 µl of the adapter mix and 2 µl of the HP adapter (supplied in the SQK-MAP006 library preparation kit, Oxford Nanopore Technologies) were added to the dA-tailed amplicons along with 50 µl of the NEB-Blunt/TA ligase master mix (New England Biolabs) and incubated for 10 min. The resulting sequencing library was purified using a solution of Dynabeads MyOne Streptavidin C1 (Life Technologies, Carlsbad, CA). Six microliters of the purified library was mixed with 75 µl of running buffer, 65 µl of nuclease-free water, and 4 µl of fuel mix, and loaded on the flow cell.

MinION sequencing configuration. Each sample was run individually in a different flow cell. The flow cell was first primed using 500 µl of the blank buffer solution (500 µl of running buffer, 474 µl of nuclease-free water, and 26.6 µl of fuel mix) and incubated for 10 min at room temperature. The priming process was repeated two times before loading the sample. Library mixture (150 µl) was loaded into the MinION flowcell. The sequencing runs were initiated running MinKNOW (version 0.49.2.9), and the "NC_48Hr-Sequencing_Run_FLO_MIN106_SQK-LSK208.py" script. Read event data were base-called by Metrichor software (version 0.16.37960) using the workflow 2D Basecalling for MAP-006. Each run continued for a total of 24 h.

Data handling. After sequencing, data were collected as fast5 files containing raw electrical signals. After processing through Metrichor (Oxford Nanopore), base-called data were obtained in the form of fast5 files containing metadata. Fasta sequences and read event data, including the strand translocation times, were extracted for further analysis using the R package poRe (Watson et al. 2014). Fasta sequence data from each sample was combined into a single fasta archive for downstream alignment analysis.

Alignment analysis. Nucleotide base alignment was carried out with GraphMap Aligner (Sović et al. 2016). GraphMap generates sam files, which were converted into bam files using SAMtools software (Li et al. 2009). Metadata from each bam file containing the alignment information were analyzed using the R package Rsamtools (Morgan et al. 2016), Bamstat 1.25, a C++ program developed to

tabulate mapping statistics, and the BEDtools software (Quinlan and Hall 2010). True alignment was considered for both positive and negative strands, by following the GraphMap parameters for correct alignment. Each read was assigned with a quality indicator (mapQ), and those reads with a quality score ≥ 40 were accepted for inferences, while reads with quality rate ≤ 40 were not considered as a true alignment. All analyses were performed on a standard desktop computer running Linux or Windows depending on software requirements.

Alignments to microbial databases. Each sample dataset was queried against the appropriate portable reference dataset. Reads from *M. persicae* and *P. persica* were tested against the current complete set of high quality, annotated, viral genomes available from NCBI RefSeq (<ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral>). In addition, reads produced from *M. persicae* sample were aligned against a set of four different datasets: the aphid *M. persicae* CDS sequence (*M. persicae* TGAC clone O v1.1 CDS fasta file) data downloaded from AphidBase (<http://bipaa.genouest.org/is/aphidbase/>); two genomes of endosymbiotic bacteria known to be major parts of the *M. persicae* microbiome (*Buchnera aphidicola* [NZ_CP002701] and *Ca. Regiella insecticola* [GL379589]) (von Burg et al. 2008, Jiang et al. 2013); and the plum pox virus genome (NC_001445.1). Data of the *P. persica* tissue was tested by aligning the produced dataset with the *P. persica* genome (AKXU00000000.2) and the PPV genome (NC_001445.1). The *D. citri* dataset was analyzed against a collection of seven datasets, including *D. citri* genomic data (gil645508417), the pathogen known to be infecting the food source for the psyllids used in this study (*Ca. L. asiaticus* NC_020549.1), and five additional genomes from organisms known to comprise the normal endosymbiont bacteria of *D. citri* (Fagen et al. 2012; Nakabachi et al. 2013) (*Candidatus Carsonella rudii* [NC_018417.1], *Candidatus Proffella armatura* [NC_021885.1], *Salmonella enterica* [NC_003198.1], *Klebsiella variicola* [NZ_JUSW01000023], and *Wolbachia wDi* [NZ_AMZJ01000037; NZ_KB223535]).

Viral and bacterial detection. To determine the level of pathogen genome coverage and to identify the pathogen present in the samples, the generated reads from each dataset were mapped against the whole reference genome of the respective pathogens. Reads generated from the psyllid *D. citri* were aligned against the genome of *Ca. L. asiaticus* (NC_020549.1), while reads from the aphid *M. persicae* and from the fruit *P. persica* were aligned against the PPV genome (NC_001445.1).

Results

Alignments to microbial databases. Data produced by the MinION was more than sufficient to correctly identify the source pathogens and discriminate among other microbial genomes tested (Table 1; Fig. 1A to C). During the mapping process, all potential alignments were assigned. To represent the datasets in the most unbiased manner (i.e., alignment to several large-genome and highly genome-plastic pandoraviruses), the mean of the reads mapped to a given reference genome was used as a simple summary statistic. The percentage of reads mapped of both *M. persicae* and *P. persica* datasets showed a high number of reads assigned to the PPV reference genome. The mean of reads mapped, adjusted by target genome size, were significantly higher in both plant and insect tissues when compared with all other viral genomes tested (Table 1; Fig. 1A and B). Considering the mean of reads mapped normalized by genome size of the *D. citri* dataset, reads that were assigned to the *Ca. L. asiaticus* reference genome showed the third highest percentage of reads mapped (Table 1).

PPV and *Ca. L. asiaticus* sequencing and detection. The MinION generated reads that confirmed the presence of each of the pathogens tested (Fig. 1D to I). The raw statistics data are described in Table 2. From the three datasets analyzed, numbers of fast5 reads ranged from 18,960 to 97,906 with a mean read length of 456 to 768 bp. A minority of the data output by MinION aligned well to the pathogen reference genomes when reads were mapped solely to these genomes. The number of reads aligned to the pathogen genomes ranged from 968 to

7,732, with a mean alignment length of 358 to 487 bp. Considering mapQ scores ≥ 40 , the total number of reads aligned ranged from 935 to 7,531 (Table 2). The total number of reads aligned with mapQ scores higher than 40 were less than the total number of reads aligned with mapQ lower than 40. However, for all datasets tested, reads with mapQ ≤ 40 were produced at the beginning and at the end of the runs, demonstrating the capacity of the MinION to produce reads with favorable mapQ during the entirety of a sequencing run. Despite the total number of high quality aligned reads being lower than the number of reads produced by MinION, the proposed method was able to identify all three target pathogens in the samples tested. Considering only the reads with mapQ scores equaling 40, and the total time of the sequencing run, reads that were correctly mapped with the pathogen targets were clearly produced within the first minute of each run (Fig. 1D to F), which demonstrates the capability of this method to identify pathogens in a fast and reliable way. It should be noted that the data were analyzed immediately following the completion of each run, although there is software that would have made it possible to scan each read during the course of the run. We chose this method because we wanted to rely upon open source analysis software and the more sensitive GraphMap alignment algorithm. In all three host-pathogen systems analyzed here, the total time from the end of each run to the positive identification of each pathogen was less than 2 h using a standard desktop computer. The first *Ca. L. asiaticus* sequence that was positively identified in the *D. citri* sample occurred at 38 s into the run. Plum pox virus was identified in the *M. persicae* sample at 41 s into the run, and in the *P. persica* sample at 38 s into the run. In all cases, the initial matches were quickly confirmed by numerous other reads with positive matches to the pathogen reference genome. The distribution of reads aligned across the known reference genomes is shown in Figure 1 (G-I). The *P. persica* dataset had the largest proportion of reads spanning the reference genome, while reads from the *D. citri* and *M. persicae* datasets showed lower coverage. The insect samples showed lower genome coverage due to the lower number of

total reads. In addition, for *Liberabacter*, the target organism has a much larger genome than the viral targets, and therefore the lower observed genome coverage was expected.

Discussion

The development of rapid detection and accurate diagnostic methods for plant pathogens and their vectors plays an important role in fighting the spread of the diseases they cause. The ability to accurately detect multiple pathogens simultaneously is a valuable feature for any diagnostic tool. Regarding vectored pathogens, the ability to detect and discriminate both the pathogen and the vector from a complex background such as an insect trap is highly desirable. Multiplex PCR or immune assays are limited in the number of possible targets to be addressed. In contrast, massively parallel sequencing analysis of metagenomic samples could be used to detect pathogens other than those known to be normally associated with a plant or insect species, provided their titers are high enough. In addition, because RNA viruses are the most common form of plant viruses, the ability to simultaneously detect pathogens with RNA and DNA genomes is highly desirable.

Here we report the first successful use of the Oxford MinION sequencer for use in the detection of plant pathogens from infected plant tissues and insect vectors. In addition, because many plant pathogens such as PPV have RNA genomes, we chose to couple total RNA amplification (WTA) and cDNA library preparation with nanopore analyses. Seeking to simulate a diversity of plant pathogens, a viral and bacterial plant pathogen that are each vectored by hemipteran insects were selected. Although we obtained a high level of reads with low quality score (≥ 40), likely due to using WTA samples, pathogen detection was possible using a reference data set that encoded either all available viral genomes or a compilation of bacterial genomes known to associate with the host organisms. Using this approach, it was possible to identify the pathogens in all samples, demonstrating that this method is an effective surveillance tool for plant pathogen detection and can be widely implemented in the future.

Table 1. Raw statistics for alignment generated against a dataset of different genomes using GraphMap aligner

Dataset	Reference genome	Total of reads aligned	Reads aligned mapQ = 40	Mean of reads aligned adjusted to genome size	SD of reads aligned adjusted to genome size
<i>Diaphorina citri</i>	<i>Candidatus Liberabacter asiaticus</i>	105	13	0.022	0.265
	<i>D. citri</i>	3,306	1,018	0.057	0.138
	<i>Carsonella rudii</i>	10	2	0.012	0.178
	<i>Wolbachia wDi</i>	27	24	0.012	0.115
	<i>Profftella armatura</i>	210	79	0.132	2.019
	<i>Klebsiella variicola</i>	73	25	0.016	0.104
	<i>Salmonella entérica</i>	47	15	0.002	0.049
<i>Myzus persicae</i> ^a	<i>Apis mellifera virus</i>	24	12	0.036	0.189
	<i>Mannheimia phage</i>	80	30	0.058	0.804
	Plum pox virus	45	18	0.741	0.859
<i>M. persicae</i> ^b	Plum pox virus	16	15	0.739	0.828
	<i>Buchnera aphidicola</i>	07	05	0.002	0.053
	<i>M. persicae</i>	464	130	0.136	0.292
	<i>Ca. Regiella insecticola</i>	44	12	0.047	0.15
	Plum pox virus	929	889	41.721	22.027
<i>Prunus persica</i> ^c	<i>Enterobacteria phage</i>	189	186	1.616	13.699
	<i>Pandoravirus inopinatum</i>	254	105	0.008	0.100
	<i>Papiine herpesvirus</i>	227	71	0.041	0.263
	<i>Mollivirus sibericum</i>	22	11	0.011	0.104
	<i>A. mellifera virus</i>	105	40	0.036	0.228
	<i>C. herpesvirus 2</i>	96	29	0.032	0.192
	Plum pox virus	15,372	5,652	36.286	19.933
<i>P. persica</i> ^d	<i>P. persica</i>	807	782	0.105	0.289

^a *M. persicae* generated dataset tested against the viral reference dataset.

^b *M. persicae* generated dataset tested against endosymbiont bacteria of the insect *M. persicae* and PPV reference genomes.

^c *P. persica* generated dataset tested against the viral reference genome.

^d *P. persica* generated dataset tested against *P. persica* and PPV reference genomes.

Fig. 1. Nanopore sequencing of whole transcriptome amplification is capable of identifying plant pathogens from both plant and insect tissues. **A and B,** Alignment of reads produced by MinION from *Prunus persica* and *Myzus persicae* datasets to the total viral genomes currently available from NCBI RefSeq. The inset in A shows the number of total viral reads versus the number of reads mapping to the *P. persica* genome. The inset in B shows the total number of reads for the insect genome versus viral genomes and the genomes of the two major bacterial endosymbionts of *M. persicae*. **C,** Alignment of reads from *Diaphorina citri* dataset aligned to a reference database composed of the known bacterial microbiome of *D. citri*. All data shown in figures from A, B, and C were normalized by microorganism genome size. **D to F,** Time of the run that plum pox virus and *Candidatus* Liberibacter asiaticus were first identified over the 24 h of the sequencing process. Graphs from D to F contain data from the first 20 aligned and unaligned reads to the two pathogens reference genomes. **G to I,** Depth of the genome coverage produced by alignments to the whole reference genome of the two pathogens tested here. As expected, the bacterial sample showed lower coverage as the target organism has a much larger genome than the viral target.

Table 2. Raw statistics for alignment generated against the references genomes of plum pox virus and *Candidatus* Liberibacter asiaticus using GraphMap aligner

Dataset	Number of fast5 reads	Total number of reads aligned	Min read length aligned (bp)	Max read length aligned (bp)	Mean read length aligned (bp)	Number of reads aligned mapQ ≥40
<i>Diaphorina citri</i>	66,705	4,701	80	13,908	358	2,999
<i>Myzus persicae</i>	18,960	968	83	3,163	389	935
<i>Prunus persica</i>	97,906	7,732	80	19,908	487	7,531

Reads from the *P. persica* dataset showed a large proportion of reads spanning the reference genome. The total number of reads produced for the *P. persica* dataset was also much larger than either insect dataset. There are several reasons to explain why the plant-derived dataset produced more total reads and pathogen positive reads. First, it is technically difficult to acquire RNA from these insect samples, and therefore the quality of our RNA input might have been lower for the insect samples. Second, pathogen titers in insects may be proportionally lower than in plants, as the pathogen may be present in some tissues at certain times within an infected insect host. It is known that *Ca. Liberibacter* is differently distributed in psyllids tissues, with significantly lower titers in the salivary glands than in the rest of the body (Ammar et al. 2011). Fagen et al. (2012) studied the abundance of *Ca. L. asiaticus* in the whole insect body of *D. citri* and also found lower levels of this pathogen when compared with four other endosymbiotic bacteria present in the microbiome of the insect. The authors suggested that the variable effect of *Ca. L. asiaticus* on the endosymbiotic bacteria may be caused by the irregular distribution of *Ca. L. asiaticus* within the host causing certain bacteria to be displaced and not others. Potyviruses such as PPV are non-persistently stylet-transmitted, meaning the only virus present in the insect body are those in the process of moving through the digestive tract. Results from *M. persicae* and *D. citri* datasets from this study are generally in line with these past results, suggesting that the viral and bacterial content in these insects are lower because of the more disperse infection distribution in the insect bodies.

This study shows that nanopore sequencing coupled with WTA, or the development of direct nanopore sequencing of RNA, is a promising alternative means of identifying unexpected pathogens in agricultural settings. The short time in which each assay was able to produce numerous pathogen-confirmatory reads also suggests that the current platform could be improved into an operative format that is more cost-effective and amenable to use in remote settings. Early detection and confirmation of pathogen presence in the sequencing process is advantageous not only because it is more timely, but also because it is extremely useful when data handling is considered, as the large amounts of data usually generated with standard next generation sequencing would potentially be avoided. Oxford Nanopore is constantly upgrading both their flow cells and bioinformatics technologies, and it is expected that this technology will only become more accurate and cost effective in the future. Likewise, Oxford is currently introducing a novel flow cell for the direct sequencing of RNA molecules, which would further streamline our proposed methods. We plan to continue testing this technology in the future by including these and other nanopore-generated data in a cost-benefit analysis of several current MPS platforms and by coupling our nanopore data analysis pipeline with EDNA technology to test for the potential of increased efficiency using basic computational resources (Stobbe et al. 2013). The possibility of eventually downscaling their current platform for more affordable diagnostics will also be explored as the obtained results suggest that a smaller flow cell would likely be effective in the type of scenarios simulated here.

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Literature Cited

Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M., and Boonham, N. 2009. Next-generation

sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Mol. Plant Pathol.* 10:537-545.

Ammar, E. D., Shatters, R. G., Lynch, C., and Hall, D. G. 2011. Detection and relative titer of *Candidatus Liberibacter asiaticus* in the salivary glands and alimentary canal of *Diaphorina citri* (Hemiptera: Psyllidae) vector of citrus huanglongbing disease. *Ann. Entomol. Soc. Am.* 104:526-533.

Espindola, A., Schneider, W., Hoyt, P. R., Marek, S. M., and Garzon, C. 2015. A new approach for detecting fungal and oomycete plant pathogens in next generation sequencing metagenome data utilising electronic probes. *Int. J. Data Min. Bioinform.* 12:115-128.

Fagen, J. R., Giongo, A., Brown, C. T., Davis-Richardson, A. G., Gano, K. A., and Triplett, E. W. 2012. Characterization of the relative abundance of the citrus pathogen *Ca. Liberibacter asiaticus* in the microbiome of its insect vector, *Diaphorina citri*, using high throughput 16S rRNA sequencing. *Open Microbiol. J.* 6:29-33.

García, J. A., Glasa, M., Cambra, M., and Candresse, T. 2014. Plum pox virus and sharka: a model potyvirus and a major disease. *Mol. Plant Pathol.* 15:226-241.

Gottwald, T. R. 2010. Current epidemiological understanding of citrus huanglongbing. *Annu. Rev. Phytopathol.* 48:119-139.

Jain, M., Fiddes, I. T., Miga, K. H., Olsen, H. E., Paten, B., and Akeson, M. 2015. Improved data analysis for the MinION nanopore sequencer. *Nat. Methods* 12: 351-356.

Jiang, Z., Jones, D. H., Khuri, S., Tsinoremas, N. F., Wyss, T., Jander, G., Alex, C. C., and Wilson, A. C. 2013. Comparative analysis of genome sequences from four strains of the *Buchnera aphidicola* Mp endosymbiont of the green peach aphid *Myzus persicae*. *BMC Genomics* 14:917.

Kilianski, A., Haas, J. L., Corriveau, E. J., Liem, A. T., Willis, K. L., Kadavy, D. R., Rosenzweig, C. N., and Minot, S. S. 2015. Bacterial and viral identification and differentiation by amplicon sequencing on the MinION nanopore sequencer. *Gigascience* 4:12.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25:2078-2079.

Morgan, M., Pagès, H., Obenchain, V., and Hayden, N. 2016. Rsamtools: Binary alignment (BAM), FASTA, variant call (BCF), and tabix file import. R package version 1.24.0. <http://bioconductor.org/packages/release/bioc/html/Rsamtools.html>

Nakabachi, A., Ueoka, R., Oshima, K., Teta, R., Mangoni, A., Gurgui, M., Oldham, N. J., van Echten-Deckert, G., Okamura, K., Yamamoto, K., and Inoue, H. 2013. Defensive bacteriome symbiont with a drastically reduced genome. *Curr. Biol.* 23:1478-1484.

Quick, J., Ashton, P., Calus, S., Chatt, C., Gossain, S., Hawker, J., Nair, S., Neal, K., Nye, K., Peters, T., De Pinna, E., Robinson, E., Struthers, K., Webber, M., Catto, A., Dallman, T. J., Hawkey, P., and Loman, N. J. 2015. Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of Salmonella. *Genome Biol.* 16:114.

Quick, J., Quinlan, A. R., and Loman, N. J. 2014. A reference bacterial genome dataset generated on the MinIONportable single-molecule nanopore sequencer. *Gigascience* 3:22.

Quinlan, A. R., and Hall, I. M. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26:841-842.

Sović, I., Šikić, M., Wilm, A., Fenlon, S. N., Chen, S., and Nagarajan, N. 2016. Fast and sensitive mapping of nanopore sequencing reads with GraphMap. *Nat. Commun.* 7:11307.

Stobbe, A. H., Daniels, J., Espindola, A. S., Verma, R., Melcher, U., Ochoa-Corona, F., Garzon, C., Fletcher, J., and Schneider, W. 2013. E-probe diagnostic nucleic acid analysis (EDNA): a theoretical approach for handling of next generation sequencing data for diagnostics. *J. Microbiol. Methods* 94:356-366.

Stobbe, A. H., Schneider, W. L., Hoyt, P. R., and Melcher, U. 2014. Screening metagenomic data for viruses using the E-probe diagnostic nucleic acid assay. *Phytopathology* 104:1125-1129.

Ukuda-Hosokawa, R., Sadoyama, Y., Kishaba, M., Kuriwada, T., Anbutsu, H., and Fukatsu, T. 2015. Infection density dynamics of the citrus greening bacterium "*Candidatus Liberibacter asiaticus*" in field populations of the psyllid *Diaphorina citri* and its relevance to the efficiency of pathogen transmission to citrus plants. *Appl. Environ. Microbiol.* 81:3728-3736.

von Burg, S., Ferrari, J., Müller, C. B., and Vorburger, C. 2008. Genetic variation and covariation of susceptibility to parasitoids in the aphid *Myzus persicae*: no evidence for trade-offs. *Proc. Royal Soc. B.* 275:1089-1094.

Watson, M., Thomson, M., Risse, J., Talbot, R., Santoyo-Lopez, J., Gharbi, K., and Blaxter, M. 2014. poRe: an R package for the visualization and analysis of nanopore sequencing data. *Bioinformatics* 31:114-115.