ABSTRACT The banana aphid, *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae), is a major pest of cultivated bananas (*Musa* spp., order Zingiberales), primarily due to its role as a vector of *Banana bunchy top virus*, the most severe viral disease of banana worldwide. Here, we generated a highly complete genome assembly of *P. nigronervosa* using a single PCR-free Illumina sequencing library. Using the same sequence data, we also generated complete genome assemblies of the *P. nigronervosa* symbiotic bacteria *Buchnera aphidicola* and *Wolbachia*. To improve our initial assembly of *P. nigronervosa* we developed a k-mer based deduplication pipeline to remove genomic scaffolds derived from the assembly of haplotigs (allelic variants assembled as separate scaffolds). To demonstrate the usefulness of this pipeline, we applied it to the recently generated assembly of the aphid *Myzus cerasi*, reducing the duplication of conserved BUSCO genes by 25%. Phylogenomic analysis of *P. nigronervosa*, our improved *M. cerasi* assembly, and seven previously published aphid genomes, spanning three aphid tribes and two subfamilies, reveals that *P. nigronervosa* falls within the tribe Macrosiphini, but is an outgroup to other Macrosiphini sequenced so far. As such, the genomic resources reported here will be useful for understanding both the evolution of Macrosiphini and for the study of *P. nigronervosa*. Furthermore, our approach using low cost, high-quality, Illumina short-reads to generate complete genome assemblies of understudied aphid species will help to fill in genomic black spots in the diverse aphid tree of life.

KEYWORDS Hemiptera genome assembly insect vector plant pest phylogenomics

Genome Sequence of the Banana Aphid, *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae) and Its Symbionts

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Aphids are economically important plant pests that cause damage to crops and ornamental plant species through parasitic feeding on plant sap and via the transmission of plant viruses. Of approximately 5,000 aphid species, around 100 have been identified as significant agricultural pests (Van Emden and Harrington 2017). Despite their economic importance, little to no genomic resources exist for many of these species or their relatives, hindering efforts to understand the evolution and ecology of aphid pests. To date, genome sequencing efforts have focused on members of the aphid tribe Macrosiphini (within subfamily Aphidinae), including the widely studied aphids *Acrystosiphon pisum* (pea aphid) (International Aphid Genomics Consortium 2010; Li et al. 2019; Mathers et al. 2020) and *Myzus persicae* (green peach aphid) (Mathers et al. 2017, 2020), as well as other important pest species such as *Diuraphis noxia* (Russian wheat aphid) (Nicholson et al. 2015). Recently, additional genome sequences have become available for members of the tribe Aphidini (also in the subfamily Aphidinae) (Wenger et al. 2020; Thorpe et al. 2018; Chen et al. 2019; Quan et al. 2019; Mathers 2020) and the subfamily Lanchinae (Julca et al. 2020), broadening the phylogenetic scope of aphid genomic resources. However, many clades of the aphid phylogeny are still missing or underrepresented in genomic studies.

The banana aphid, *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae), is a major pest of cultivated bananas (*Musa* spp., order Zingiberales) and is widely distributed in tropical and subtropical...
regions where bananas are grown (Waterhouse 1987). Like other aphid species, *P. nigrornervosa* feeds predominantly from the phloem of its plant host. Intensive feeding can kill or affect the growth of young banana plants. However, direct feeding damage to adult plants is often negligible. Instead, the banana aphid causes most economic damage as a vector of plant viruses, some of which induce severe disease symptoms and substantial yield loss of banana (Dale 1987; Sharman et al. 2008; Savory and Ramakrishnan 2015). In particular, *P. nigrornervosa* is the primary vector of the *Banana bunchy top virus* (BBTV), the most severe viral disease of banana worldwide (Dale 1987).

*P. nigrornervosa* carries at least two bacterial symbionts: *Buchnera aphidicola* and *Wolbachia* (De Clerck et al. 2014). *Buchnera aphidicola* is an obligate (primary) symbiont present in almost all aphid species and provides essential amino acids to the aphids (Baumann 1995; Douglass 1998; Hansen and Moran 2011; Shigenobu and Wilson 2011). In contrast, *Wolbachia* is considered a facultative (secondary) symbiont and is found in a few aphid species at low abundance (Augustinos et al. 2011; Jones et al. 2011). Interestingly, *Wolbachia* is found systemically across the *P. nigrornervosa* range (De Clerck et al. 2014) and is also present in the closely related species *P. caladdii* van der Goot (Jones et al. 2011), which rarely colonizes banana, and prefers other plant species of the order Zingiberales (Foottit et al. 2010). Possibly, *Wolbachia* provides essential nutrients and vitamins to the *Pentalonia* spp or and protect them from plant-produced defense molecules such as anti-oxidants or phenolic compounds of banana (Hosokawa et al. 2010).

Here, we generate highly complete genome assemblies of *P. nigrornervosa* and its symbiotic bacteria *Buchnera aphidicola* and *Wolbachia*, using a single PCR-free Illumina sequencing library. Phylogenomic analysis reveals that *P. nigrornervosa* falls within the aphid tribe Macrosiphini, but is an outgroup to other Macrosiphini sequenced so far. As such, the genomic resources reported here will be useful for understanding the evolution of Macrosiphini, and for the study of *P. nigrornervosa*.

**METHODS**

**Aphid rearing and sequencing library construction**

A lab colony of *P. nigrornervosa* was established from a single asexually reproducing female collected initially from the IITA’s banana field at the International Livestock Research Institute (ILRI) Nairobi, Kenya. A single colony of *P. nigrornervosa* was collected from a field-grown banana plant and introduced on an eight-week-old potted tissue culture banana plant in an insect-proof cage, placed in a glasshouse under room temperature and natural light. Pure aphid colonies were propagated by transferring a single aphid from the potted banana plant to another fresh young banana plant in the glasshouse every eight weeks. Aphids from this colony were used for all subsequent DNA and RNA extractions. Genomic DNA was extracted from a single individual with a modified CTAB protocol (based on Marzachi et al. 1998) and sent to Novogene (China), for library preparation and sequencing. Novogene prepared a PCR free Illumina sequencing library using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, USA), with the manufacturers protocol modified to give a 500 bp – 1 kb insert size. This library was sequenced on an Illumina HiSeq 2500 instrument with 250 bp paired-end chemistry. To aid scaffolding and genome annotation, we also generated a high coverage, strand-specific, RNA-seq library. RNA was extracted from whole bodies of 20-25 individuals using Trizol (Sigma) followed by clean-up and on-column DNAse digestion using RNeasy (Qiagen) according to the manufacturers’ protocols, and sent to Novogene (China) where a sequencing library was prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA). This library was sequenced on an Illumina platform with 150 bp paired-end chemistry.

**De novo genome assembly and quality control**

Raw sequencing reads were processed with trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove adapters and then assembled using Discovar de novo (https://software.broadinstitute.org/software/discovar/blog/) with default parameters. The content of this initial assembly was assessed with Benchmarking Universal Single-Copy Orthologs (BUSCO) v3.0 (Simão et al. 2015; Waterhouse et al. 2018) using the Arthropoda gene set (n = 1,066) and by k-mer analysis with the k-mer Analysis Toolkit (KAT) v2.2.0 (Mapleson et al. 2017), comparing k-mers present in the raw sequencing reads to k-mers found in the genome assembly with KAT comp. We identified a small amount of k-mer content that was present twice in the genome assembly but that had k-mer coverage in the reads of a single-copy region of the genome, indicating the assembly of haplotigs (allelic variants that are assembled into separate contigs) (Supplementary Figure 1a). To generate a close-to-haploid representation of the genome, we applied a strict filtering pipeline to the draft assembly based on k-mer analysis and whole genome self-alignment. First, the k-mer coverage of the homozygous portion of the genome was estimated with KAT distanalysis, which decomposes the k-mer spectra generated by KAT comp into discrete distributions corresponding to the number of times their content is found in the genome. Then, for each scaffold in the draft assembly, we used KAT sect to calculate the median k-mer coverage in the reads and the median k-mer coverage in the assembly. Scaffolds that had medium k-mer coverage of 2 in the assembly and median k-mer coverage in the reads that fell between the upper and lower bounds of homozygous genome content (identified by KAT distanalysis), were flagged as putative haplotigs. We then carried out whole genome self-alignment with nucmer v4.0.0beta2 (Marçais et al. 2018) and removed putative haplotigs that aligned to another longer scaffold in the genome with at least 75% identity and 25% coverage. The deduplicated assembly was then checked again with BUSCO and KAT comp to ensure that no (or minimal) genuine homozygous content had been lost from the assembly.

The deduplicated draft assembly was screened for contamination based on manual inspection of taxon-annotated GC content coverage plots (“blobplots”) generated with BlobTools v1.0.1 (Kumar et al. 2013; Laetsch and Blaxter 2017). Genomic reads were aligned to the deduplicated draft assembly with BWA mem (Li 2013) and used to estimate average coverage per scaffold. Additionally, each scaffold in the assembly was compared to the NCBI nucleotide database (nt) with BLASTn v2.2.31 (Camacho et al. 2009). Read mappings and blast results were then passed to BlobTools which was used to create “blobplots” annotated with taxonomy at the order- and genus-level. Using this approach, we were able to identify and remove scaffolds corresponding to bacterial symbionts and scaffolds that had aberrant coverage and GC content patterns that are likely contaminants.

Finally, to further improve contiguity and gene-level completeness, we performed an additional round of scaffolding using our high coverage RNA-seq data with P_RNA_scaffold (Zhu et al. 2018). RNA-seq reads were trimmed for adapters and low-quality bases with trim_galore and aligned to the deduplicated and cleaned assembly with HISAT2 v2.0.5 [-k 3 -pen-noncansplice 1000000] (Kim et al. 2015).
The resulting BAM file was then passed to P_RNA_scaffolder along with the draft assembly, and scaffolding performed with default settings. Gene-level completeness was assessed before and after RNA-seq scaffolding with BUSCO and final runs of KAT comp and BlobTools were performed to check the quality and completeness of the assembly.

**Genome annotation**

Repeats were identified and soft-masked in the frozen genome assembly using RepeatMasker v4.0.7 [-e ncbi -species insecta -a -xsmall -gff] (Smit et al. 2005) with the Repbase (Bao et al. 2015) Insecta repeat library. We then carried out gene prediction on the soft-masked genome using the BRAKER2 pipeline v2.0.4 (Lomsadze et al. 2014; Hoff et al. 2015) with RNA-seq evidence. BRAKER2 uses RNA-seq data to create intron hints and train a species-specific Augustus (Stanke et al. 2006, 2008) model which is subsequently used to predict protein coding genes, taking RNA-seq evidence into account. RNA-seq reads were aligned to the genome with HISAT2 v2.0.5 [-max-intronlen 25000–dta-cufflinks–rna-strandness RF] and the resulting BAM file passed to BRAKER2, which was run with default settings. Completeness of the BRAKER2 gene set was assessed using BUSCO with the Arthropoda gene set (n = 1,066). We generated a functional annotation of the predicted gene models using InterProScan v5.22.61 (Enright et al. 2002; Jones et al. 2014).

**Upgrading Myzus cerasi v1.1**

To demonstrate the usefulness of our k-mer based deduplication pipeline, we applied it to the published short-read assembly of *M. cerasi* (Myzer_v1.1) (Thorpe et al. 2018). We ran the pipeline as for *P. nigronervosa*, using the PCR-free Illumina reads that were originally used to assemble Myzer_v1.1 (NCBI bioproject PRJEB24287) and scaffolded the deduplicated assembly using RNA-seq data from Thorpe et al. (2016) (PRJEB9912) with P_RNA_scaffold. RNA-seq reads were first trimmed for low quality bases and adapters with trim_galore, retaining reads where both members of a pair were at least 75 bp long after trimming. The deduplicated, scaffolded, assembly was ordered by size and assigned a numbered scaffold ID to create a frozen release for downstream analysis (Myzer_v1.2). Myzer_v1.2 was then soft-masked with RepeatMasker using the Repbase Insecta repeat library and protein coding genes predicted with BRAKER2 using the Thorpe et al. (2016) RNA-seq.

**Phylogenomic analysis of aphids**

Protein sequences from *P. nigronervosa*, our upgraded *M. cerasi* genome, and seven previously published aphid genomes (Supplementary Table 1), were clustered into orthogroups with OrthoFinder v2.2.3 (Emms and Kelly 2015, 2019). Where genes had multiple annotated transcripts, we used the longest transcript to represent the gene model. OrthoFinder is a comparative genomics pipeline that reconstructs orthogroups, estimates the rooted species tree, generates rooted gene trees, and infers orthologs and gene duplication events using the rooted gene trees, providing a rich resource for downstream comparative analysis. We ran OrthoFinder in multiple sequence alignment mode [-M msa -S diamond -T fasttree] using MAFFT (Katoh and Standley 2013) to align orthogroups and FastTree (Price et al. 2010) to infer maximum likelihood gene trees for each orthogroup. The species tree was then estimated based on a concatenated alignment of all conserved single-copy orthogroups and rooted using evidence from gene duplications with STRIDE (Emms and Kelly 2017). To confirm the topology recovered by the OrthoFinder–FastTree analysis, we carried out a bootstrapped maximum likelihood phylogenetic analysis based on the concatenated alignment with IQ-TREE v2.0.5 (Nguyen et al. 2015; Minh et al. 2020) and a coalescent analysis using conserved single copy gene trees with ASTRAL-III v5.6.3 (Mirarab et al. 2014; Mirarab and Warnow 2015; Zhang et al. 2018). For the IQ-TREE analysis, we automatically identified the optimum model of protein evolution with ModelFinder (Kalyaanamoorthy et al. 2017) and carried out 1,000 ultrafast bootstrap replicates (Hoang et al. 2018). For the ASTRAL-III analysis, we re-estimated gene trees for all conserved single-copy orthogroups using IQ-TREE with automatic protein model selection and ran ASTRAL-III with default settings.

**Data availability**

Sequence data and genome assemblies (including symbiont genomes) for this project have been deposited in NCBI databases under the project accession number PRJNA628023. The *P. nigronervosa* genome assembly and annotation, the updated *M. cerasi* genome assembly and annotation, orthogroup clustering results and code to run our assembly de-duplication pipeline are available for download from Zenodo ([https://10.5281/zenodo.3765644](https://10.5281/zenodo.3765644)). The *P. nigronervosa* genome assembly and annotation is also available from AphidBase ([https://bipaa.genouest.org/sp/pentalonia_nigronervosa/](https://bipaa.genouest.org/sp/pentalonia_nigronervosa/)). Supplemental material available at figshare: [https://doi.org/10.25387/g3.12251810](https://doi.org/10.25387/g3.12251810).

**RESULTS AND DISCUSSION**

**P. nigronervosa genome assembly and annotation**

In total we generated 23 Gb of PCR-free Illumina genome sequence data (~61x coverage of the *P. nigronervosa* genome) and 18 Gb of strand-specific RNA-seq data from a clonal lineage of *P. nigronervosa* (Supplementary Table 2). Using these data, we generated a de novo genome assembly of *P. nigronervosa* (Penig_v1). Penig_v1 is assembled into 18,348 scaffolds totaling 375 Mb of sequence with an N50 of 104 Kb (contig N50 = 64 Kb, n = 20,873; Table 1). The assembly is highly complete, with little duplicated or missing content (Figure 1a), and has excellent representation of conserved arthropod genes (95% complete and single-copy), meeting or exceeding the completeness of other published aphid genomes (Figure 1b). Furthermore, taxon annotated “blob-plots” show that Penig_v1 is free from obvious contamination (Supplementary Figure 2). Gene prediction using BRAKER2 with RNA-seq evidence resulted in the annotation of 27,698 protein coding genes and 29,708 transcripts. Completeness of the gene set reflects that of the genome assembly with 93% of BUSCO Arthropoda genes present as complete single copies in the annotation (Supplementary Figure 3). We were able to assign functional domains to 12,869 (47%) of the annotated gene models (Supplementary Table 3). Statistics for the final assembly and annotation of *P. nigronervosa* are summarized in Table 1.

*P. nigronervosa* in known to harbor the obligate aphid bacterial endosymbiont *Buchnera aphidicola* and a secondary symbiont, Wolbachia, that is found systematically across the species range (De Clerck et al. 2014). We identified both symbiotic bacteria in the initial discovar de novo assembly of *P. nigronervosa* (Figure 1c). *B. aphidicola* BP0 was assembled into a single circular scaffold 617 Kb in length, along with 2 plasmids. The Wolbachia WolPenNig assembly was more fragmented (1.46 Mb total length, 182 scaffolds, N50 = 15.5kb). Despite this, the WolPenNig assembly is likely highly complete as it is similar in size to both a more contiguous long-read assembly of a strain found in the soybean aphid (1.52 Mb total length,
9 contigs, N50 = 841 Kb [Mathers 2020]) and to the reference assembly of Wolbachia wRi (Klasson et al. 2009) from Drosophila simulans (1.44 Mb total length, 1 contig). Furthermore, BUSCO analysis using the proteobacteria gene set (n = 221) reveals that WolPenNig has similar gene-level completeness to these high-quality assemblies, with 81% of BUSCO genes found as complete, single copies (Supplementary Figure 4).

Upgrading the Myzus cerasi genome assembly and annotation

The initial discovar de novo assembly of P. nigronervosa was moderately improved by applying our deduplication pipeline and by scaffolding the assembly with RNA-seq data. Compared to the raw discovar de novo assembly, contiguity increased by 8% (scaffold N50 = 104 kb vs. 96 Kb). Furthermore, the number of fragmented BUSCOs

![Table 1 Genome assembly and annotation statistics for P. nigronervosa and M. cerasi](image)

**Figure 1** The P. nigronervosa genome assembly is complete and free from duplication and contamination. (a) KAT k-mer spectra plot comparing k-mer content of PCR-free P. nigronervosa Illumina reads to k-mer content of the final P. nigronervosa genome assembly (Penig_v1). Colors indicate how many times fixed length words (k-mers) from the reads appear in the assembly. Red indicates k-mers found only once in the assembly, black indicates content present in the reads but missing from the assembly and other colors indicate k-mers that are duplicated in the assembly. The x-axis shows the number of times each k-mer is found in the reads (k-mer multiplicity) and the y-axis shows the count of distinct k-mers in 1x k-mer multiplicity bins. (b) BUSCO analysis of Penig_v1, our updated assembly of M. cerasi (Mycer_v1.2) and published Macrosiphini genome assemblies. Myper_O_v2 = Myzus persicae clone O v2, Acpis_JIC1 = Acythosiphon pisum clone JIC1, Mycer_v1.1 = Myzus cerasi v1.1 and Dnox_v1 = Diuraphis noxia v1. The genomes were assessed using the Arthropoda gene set (n = 1,066). (c) Taxon-annotated GC content-coverage plot of the P. nigronervosa Discovar de novo genome assembly (post deduplication and prior to RNA-seq scaffolding – see Methods) showing co-assembly of the aphid and its symbionts. Each circle represents a scaffold in the assembly, scaled by length, and colored by order-level NCBI taxonomy assigned by BlobTools. The X axis corresponds to the average GC content of each scaffold and the Y axis corresponds to the average coverage based on alignment of P. nigronervosa PCR-free Illumina short reads. Marginal histograms show cumulative genome content (in Kb) for bins of coverage (Y axis) and GC content (X axis). Arrows highlight scaffolds assigned to the symbiotic bacteria Buchnera aphidicola and Wolbachia which were removed from the final assembly (Supplementary Figure 2).
Arthropoda genes was reduced from 11 to 8 indicating improved representation of the gene space in the processed assembly. Because the pipeline removes scaffolds that are predominantly made up of erroneously duplicated k-mers, these improvements were achieved without compromising genuine single-copy genome content (Supplementary Figure 1b). This approach will likely benefit other low-cost aphid genome assembly projects that use short-read sequencing, particularly when heterozygosity is high. To demonstrate this, we attempted to improve the published genome assembly of *Myzus cerasi* (Mcer_v1.1) (Thorpe et al. 2018), using publicly available data. Mcer_v1.1 is made up of 49,286 scaffolds, and k-mer analysis shows high heterozygosity and the presence duplicated content, likely the result of assembly haplotigs (Supplementary Figure 5a). We applied our deduplication and RNA-seq scaffolding pipeline to Mcer_v1.1 to create Mcer_v1.2. In total we removed 12.9 Mb of putatively duplicated content from Mcer_v1.1, reducing the assembly size from 405.5 to 392.6 Mb (Table 1). The updated assembly is 52% more contiguous than Mcer_v1.1 (scaffold N50 = 35 Kb vs. 23 Kb; Table 1) and BUSCO analysis indicates that Mcer_v1.2 better represents the gene space, with fewer duplicated (35 vs. 46) and fragmented (9 vs. 27) BUSCO Arthropoda genes (Figure 1b). As with Pnig_v1, these improvements were achieved without loss of genuine single-copy genome content (Supplementary Figure 5b). We annotated protein coding genes in Mcer_v1.2 with BRAKER2 using RNA-seq evidence, identifying 31,070 protein coding genes with 33,159 transcripts. Again, BUSCO analysis of the updated gene set indicates significant improvement over Mcer_v1.1, with the number of missing and fragmented BUSCO Arthropoda genes reduced from 65 to 20 and 55 to 20 respectively, and overall completeness increased by 8% from 946 to 1,026 BUSCO Arthropoda genes (Supplementary Figure 3).

**P. nigronervosa** is an outgroup to other sequenced Macrosiphini

To investigate the phylogenetic relationship of *P. nigronervosa* within aphids we carried out orthology clustering of 223,889 protein sequences from *P. nigronervosa*, our improved *M. cerasi* annotation, and seven previously published aphid genomes (Nicholson et al. 2013; Thorpe et al. 2018; Chen et al. 2019; Mathers 2020; Mathers et al. 2020). Although the number of aphid species with sequenced genomes is still low, the included species span three aphid tribes (Macrosiphini, Aphidini and Lachnini) and approximately 100 million years of aphid evolution (Kim et al. 2011; Hardy et al. 2015; Julca et al. 2020). In total, 204,139 genes (85%) were clustered into 22,759 orthogroups, 4,721 of which are conserved and single-copy in all species (Supplementary table 4). Maximum likelihood phylogenetic analysis using a concatenated alignment of the single-copy orthogroups with FastTree produced a fully resolved species tree with 100% support at all nodes (Figure 2). The same fully supported topology was also recovered using maximum likelihood phylogenetic analysis with IQ-TREE (Supplementary Figure 6a) and when using the summary method ASTRAL-III (Supplementary Figure 6b), which performs well in the presence of incomplete lineage sorting (Mirarab et al. 2014). Macrosiphini and Aphidini are recovered as monophyletic groups in agreement with previous analyses based on a small number of genes (von Dohlen et al. 2006; Choi et al. 2018) and a recent phylogenomic analysis of aphids and other insects (Julca et al. 2020). *P. nigronervosa* is placed as an outgroup to other, previously sequenced, members of Macrosiphini (Figure 2).

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**CONCLUSIONS**

Using a single Illumina short-read sequence library and high-cover- age RNA-seq data we have generated a high-quality draft genome assembly and annotation of the banana aphid and simultaneously assembled the genomes of its *Buchnera* and *Wolbachia* symbiotic bacteria, providing an important genomic resource for the future study of this important pest. Furthermore, as an outgroup to other sequenced aphids from the tribe Macrosiphini, the banana aphid genome will enable more detailed comparative analysis of a group that includes a large proportion of the most damaging aphid crop pests (Van Emden and Harrington 2017) as well as important model species such as the pea aphid (Brisson and Stern 2006) and the green peach aphid (Mathers et al. 2017, 2020).

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**Figure 2** Maximum likelihood phylogeny of selected aphid species with sequenced genomes based on a concatenated alignment of 4,721 conserved one-to-one orthologs. All branches received maximal support based on the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) implemented in FastTree (Price et al. 2009, 2010) with 1,000 resamples. Clades are colored by aphid tribe. Branch lengths are in amino acid substitutions per site.
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