Recovering individual haplotypes and a contiguous genome assembly from pooled long-read sequencing of the diamondback moth (Lepidoptera: Plutellidae)

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Abstract

The assembly of divergent haplotypes using noisy long-read data presents a challenge to the reconstruction of haploid genome assemblies, due to overlapping distributions of technical sequencing error, intra-locus genetic variation and inter-locus similarity within these data. Here we present a comparative analysis of assembly algorithms representing overlap-layout-consensus, repeat graph and de Brujn graph methods. We examine how post-processing strategies attempting to reduce redundant heterozygosity interact with the choice of initial assembly algorithm and ultimately produce a series of chromosome-level assemblies for an agricultural pest, the diamondback moth, *Plutella xylostella* (L.). We compare evaluation methods and show that BUSCO analyses may overestimate haplotig removal processing in long-read draft genomes, in comparison to a k-mer method. We discuss the trade-offs inherent in assembly algorithm and curation choices and suggest that "best practice" is research question dependent. We demonstrate a link between allelic divergence and allele-derived contig redundancy in final genome assemblies and document the patterns of coding and non-coding diversity between redundant sequences. We also document a link between an excess of non-synonymous polymorphism and haplotigs that are unresolved by assembly or post-assembly algorithms. Finally, we discuss how this phenomenon may have relevance for the usage of noisy long-read genome assemblies in comparative genomics.

Introduction

Technical and analytical advances in genomics have dramatically improved the achievable standard of genome projects. The amount of high molecular weight (HMW) DNA required to perform long-read sequencing has reduced significantly and has been accompanied by a steady increase in sequencing read lengths and read accuracy(Kingan *et al.*, 2019). Longer reads have aided genome assembly efforts by providing information linking unique genomic sequences flanking repetitive elements, which represented a challenge to algorithms reliant on short-

read data (Koren *et al.*, 2017). However, early long-read methods, such as Oxford nanopore and SMRT sequencing contained higher error rates in raw sequence reads (Derrington *et al.*, 2010; Chin *et al.*, 2013). Whilst this technical noise can be tolerated by various means (Chin *et al.*, 2013, 2016; Koren *et al.*, 2017; Kolmogorov *et al.*, 2019; Ruan and Li, 2020), it is ultimately confounded with the real biological variation present in the underlying samples. The form this biological variation takes and the way it is distributed across the genome of an organism can influence the accuracy of a reconstructed haploid (or phased diploid) genome assembly (Kajitani *et al.*, 2019). Various assembly pipelines and algorithms have been explicitly designed to overcome challenges of heterozygosity (Chin *et al.*, 2016; Huang et al., 2017; Roach et al., 2018), repeat resolution (Kolmogorov *et al.*, 2019), speed (Ruan and Li, 2020) and integration of multiple data-types(Ye *et al.*, 2016; Zimin *et al.*, 2017). Furthermore, all assembly software has some level of parameterisation available to optimise results, yielding a huge array of possible outcomes.

Alongside these computational innovations, several experimental approaches and supplemental data types can augment existing data. Trio-sequencing can partition heterozygous variation in an F1 individual using information from parental haplotypes (Koren *et al.*, 2018). Linked-reads utilise microfluidics to uniquely barcode reads that derive from discrete large DNA fragments, thereby capturing longer range information than standard short read preparations do not (Zheng *et al.*, 2016). Chromosome conformation capture (Hi-C) data crosslinks in vivo chromatin molecules and recovers pairs of reads that derive from these crosslinks, producing data that reflects the 3D organisation of the nucleus, and also long-range cis-chromosome associations(Ghurye *et al.*, 2018). In addition to the proliferation of supporting data-types, the efficacy and quality of core genomic data has improved. Improvements to data quality predominantly come from platform advancements such as high-fidelity (HiFi) long reads (Nurk *et al.*, 2020; Cheng *et al.*, 2021) and updated nanopore proteins (Karst *et al.*, 2021). Meanwhile concurrent developments in library preparation and whole genome amplification have enabled the use of decreased input DNA amounts(Schneider *et al.*, 2021). A

recent study found that the best lepidopteran genome assembly available at the time utilised a combination of HiFi data and Hi-C (Ellis et al., 2021).

Within genome assembly, accounting for genomic variation is largely a technical consideration. However, this variation isn't uniformly or randomly distributed and is shaped by a range of evolutionary and demographic processes. One particularly challenging aspect of genome assembly is the resolution of highly divergent regions (HDRs) (Kajitani *et al.*, 2019), which often cannot be determined as allelic within the assembly process and requires supervised analysis (Roach et al., 2018). Genome assembly projects often aim to pre-emptively avoid this problem by severely inbreeding the source material to increase the proportion of genome homozygosity (The Heliconius Genome Consortium, 2012; Nowell *et al.*, 2017). However, previous studies indicate that high levels of heterozygosity are often counter-intuitively maintained despite multiple generations of sib-sib inbreeding (The Heliconius Genome Consortium, 2012; Nowell *et al.*, 2017). A candidate for such an effect is the presence of overdominant or pseudo-overdominant loci. These loci, by various mechanisms, produce severe fitness consequences in a homozygous state. In the case of pseudo-overdominance, the presence of tightly linked recessive lethal mutations on different alleles prevents either haplotype from becoming homozygous (Charlesworth and Willis, 2009). Alternatively, pseudo-overdominance may be produced by multiple linked mildly deleterious alleles, of which the cumulative effect is functionally equivalent to a single recessive lethal. Whatever the fundamental cause, these phenomena can also accumulate linked neutral variation, particularly in recombination cold-spots (Zhao and Charlesworth, 2016). These features appear to make pseudo-overdominance blocks (PODs) a plausible candidate for the HDRs known to interfere with genome assembly (Waller, 2021).

If HDRs can indicate regions experiencing particular forms of selection, failure to properly resolve them could impact downstream analyses, particularly the detection of balancing selection and overdominant loci. Since this bias is non-random, it may also affect comparative genome analyses, for example in instances

of balancing selection predating speciation, or other forms of trans-species polymorphism, such as the well-studied MHC locus (Azevedo *et al.*, 2015). Similarly, there may be common features of genetic architecture that may be more likely to produce effects like overdominance or pseudo-overdominance at common ancestral regions. Nonetheless, the increasing quality of long-read sequencing, read-lengths and supporting data, should help to mitigate the issue, and enable evaluation of the scale of this problem across historic genome datasets. One method that is used widely in evaluating the completedness of genome assemblies is the use of highly conserved gene sequences that are consistently present as single copy genes (Simão *et al.*, 2015). However, in the context of HDRs and their putative sources, it is possible that these methods may be biased against representing genome regions that are more likely to harbour HDRs. One potential resolution to this is to find genome validation methods that do not rely on such cross-species inferences (Rhie *et al.*, 2020).

Here we investigate the complex trade-offs that are made in the choice of genome assembly algorithm using a long-read genomic dataset for the diamondback moth - *Plutella xylostella,* which was the subject of a previous major genome sequencing effort, culminating in the publication of an assembly in 2013 (GCA_000330985.1) (You *et al.*, 2013). The assembly strategy utilised the sequencing of fosmids, in order to mitigate the short read lengths of Illumina sequencing. The authors report extensive structural variation based on alignments between their assembly and both the fosmids and a previously sequenced BAC (GenBank accession GU058050). The genome of *P. xylostella* therefore represents two distinct challenges to current long-read assembly methods, namely a large proportion of structural variation and a small amount of extractable DNA per individual. Our study includes the additional challenge of sequencing the heterogametic sex, containing the W-chromosome which has been shown to be highly repetitive and intractable to assembly (24).

Methods

Insect material origin and DNA extraction

Starting material was provided by Oxitec Ltd. (Abingdon, U.K.) from a lab colony that has been continuously cultured on artificial diet and is derived from the Vero Beach strain (Martins *et al.*, 2012). Several lines were inbred in parallel by mating sib-sib pairs each generation. A 7 generation inbred family was selected for genome sequencing. DNA was extracted by phenol-chloroform from a pool of 15 sisters of the final inbred generation and a single male and female (Saccheri and Bruford, 1993).

Library construction and sequencing

The pooled DNA was sheared to 7Kbp or 10Kbp. A subset was size selected at 15Kbp on the BluePippin (Sage Science, Inc.). In total 66 SMRT cells were sequenced with P5-C3 chemistry on the RSII platform (Pacific Biosciences, Inc.). Reads were filtered according to subread length (>50bp), polymerase read quality (>75bp) and polymerase read length(>50bp). Extracted DNA from the individual male and female was sheared and used for individual libraries followed by 2x 100bp paired-end Illumina sequencing (Illumina, Inc).

Genome assembly parameters

We performed assembly using canu (version 2.1.1), flye (version 2.8.2-b1689) and wtdbg2 (version 2.5). These assemblies were subsequently polished with the same pacbio read-set for two iterations using quiver (version 2.3.3) As a preliminary step, we applied author recommended parameters for producing separated haplotypes in the presence of heterozygosity and subsequently used the resulting assembly with the highest rate of duplicated BUSCO genes (run as described below). For flye and wtdbg, we selected the default parameter result, for canu we selected the assembly using the parameter set [genomeSize=340m

corOutCoverage=200 correctedErrorRate=0.040 "batOptions=-dg 3 -db 3 -dr 1 -ca 500 -cp 50"].

Haplotype merging

We trialled two post-assembly haplotype merging procedures, purge_dups and Haplomerger 2. Genomes processed with Haplomerger2 were first masked using windowmasker (version 20120730) . A species-specific scoring matrix was inferred at 95% identity using the lastz_D_Wrapper.pl script included with Haplomerger2 (Huang et al., 2017). The masked genome and scoring matrix were then used to run scripts B1-B5 of the Haplomerger2 pipeline (version 20161205)(Huang et al., 2017).

Scaffolding

The preparation of HiC libraries was performed by Dovetail LLC. using pools of starved larvae. HiC libraries were prepared as described by Kalhor *et al.* (Kalhor *et al.*, 2012). Both library preparations used the restriction enzyme DpnII for digestion after proximity ligation. Scaffolding and misassembly detection was performed by running the 3D-DNA pipeline on each of the haplotype merged assembly versions.

Validation procedures

Here we quantify the the haplotype resolution processes using two independent methods. Firstly, we utilised the gene-based BUSCO score (version 5.0.0) with the "Lepidoptera odb10" database, consisting of 5286 gene groups and augustus species model "heliconius_melpomene1". Secondly we utilised a combination of the stacked k-mer coverage histograms (a.k.a. spectra-cn) plots generated with KAT (version 2.4.2) and the read-based k-mer models produced by the genomescope R script. In brief we used the R function "pmin" to intersect the assembly copy number coverage distributions with the modelled distributions

from genomescope, specifically the error distribution, and the heterozygous and homozygous components of the unique distribution. This provided a quantitative k-mer based comparison of the haplotype resolution processes.

Haplotype divergence assessment

We quantified the divergence between duplicated genes identified by the BUSCO analysis using an alignment-based and a supporting alignment-free method. The amino-acid sequences of duplicated BUSCO gene copies were aligned with MAFFT (version v6.864b), and subsequently translated into codon based alignments with pal2nal.pl (version 14), followed by calculation of synonymous and non-synonymous variants using the biopython function "cal_dn_ds". For the alignment-free comparison we used the full genomic sequence (including introns) between the gene start and end coordinates identified by BUSCO and used the python package "alfpy" with a word-size of two to calculate the Canberra distance (see 28).

Results

Insect materials, sequencing & assembly

The material described in this study was inbred for 7 generations and is derived from a long-term laboratory culture, itself derived from the "Vero Beach" strain (U.S.A.). 15 sisters were pooled to meet minimum HMW DNA input requirements. Initial assemblies showed that substantial genetic variation was retained and was of sufficient complexity to produce multiple allelic sequence contigs from the same locus, inflating the total size way beyond the expected size of 338.7 (+/−1.1) Mbp, reported by Baxter *et al.* (2011). We subsequently trialled two approaches to resolve the redundant heterozygosity Haplomerger2 and purge_dups (Huang et

al., 2017; Guan *et al.*, 2020). After filtering 2,655,788 PacBio subreads remained (mean subread length=7,301bp, N50=10,398bp, total bases 19.4Gbp).

Heterozygosity assessment

All initial assembly strategies resulted in an over-inflated genome size, suggesting differing amounts of redundant haplotig sequence (Fig. 1A). We determined BUSCO results for the initial assemblies as evidence for the levels of allelic redundancy (measured as duplicated BUSCO genes) and overall completeness (Fig. 1B & 1C). We also utilised k-mer based methods. Firstly, a histogram of corrected PacBio reads, provided an initial estimation of genome heterozygosity as approximately 1.11%, which is moderately, but not exceedingly high for a North American sample (see You *et al.,* 2020 for context) (Fig. 2A). Estimates from related individuals (non-pooled) were 0.54% for a related inbred male and 1.00% for a related inbred female (Sup. fig. 3). Stacked histograms coloured by assembly coverage provided a qualitative assessment of genome assembly completeness and redundant allelic variation (Fig. 2B). Secondly, we intersected the modelled distributions of homozygous, heterozygous and sequencing error content from genomescope with stacked histograms in order to make quantitative comparisons of the initial assemblies (methodology illustrated in Fig. 1A, data shown in Fig. 2C) (Vurture *et al.*, 2017).

The WTDBG2 assembly was the smallest in size (427Mbp) and contig number (4023) (Fig. 1A). It had the lowest number of duplicated BUSCO genes (805) and highest number of missing genes (110) (Fig. 1B). Consistent with the BUSCO results, WTDGB2 had the lowest number of homozygous k-mers duplicated in the assembly (Fig. 2B). But it also had the highest number of modelled error k-mers present (Fig. 2C). In contrast, the Flye assembly was the largest in size (494Mbp) and contig number (5985). It had the most duplicated BUSCO genes (1324) and the least missing genes (88). Again the k-mer results show concordance with the

highest number of homozygous k-mers present duplicated in the assembly. However, the number of modelled error k-mers present in the assembly was comparable with the canu assembly. Canu produced intermediate values in total size (448Mbp) and contig number (5341). Similarly, BUSCO results indicated an intermediate number of duplicated genes (1105) and missing genes (106). k-mer results followed the same pattern except for error k-mers in the final assembly.

Both post-assembly allelic redundancy approaches reduced the overall sizes of the assemblies and appears to follow the patterns observed in the initial assemblies, such that WTDBG2 still retains the lowest number duplicated and highest number of missing genes in contrast with Flye. For each of the three starting assemblies, Haplomerger2 provided a greater reduction in total size and number of contigs compared to purge_dups (Fig. 1A). When applied to the canu assembly we also observe an increase in contiguity (Fig. 1A), due to a tiling effect produced when corresponding redundant heterozygous regions are merged at the ends of contigs (Supp. Fig. 1)

Post-assembly processing resolved most duplicated BUSCO genes to a single copy regardless of the initial assembly algorithm, however in all cases, the numbers of missing BUSCO genes also increased (Fig. 1B & 1C). We observe that purge_dups resolved some duplications that are not resolved by Haplomerger2 and vice versa (Fig. 1C). Similarly, genes that go from complete and single copy in the primary assembly to fragmented or missing after post-processing are not necessarily the same across the two methods (Fig. 1C). This suggests that removal of redundancy is not simply, more or less “aggressive”, and that performance varies by algorithm depending on specific sequence properties.

Comparison of heterozygosity assessments

Using the k-mer intersection approach described in Figure 2, we produce a k-mer proxy of BUSCO genes for comparison. The proxy is calculated using the modelled homozygous k-mers (analagous to single copy genes) and divides the occurrence

of duplicated assembly k-mers by the sum of the single copy and duplicated assembly k-mers (Supp. Tab. 2). Levels of percent duplication in the initial assemblies are remarkably concordant between the genic (BUSCO) and unbiased (k-mer) methods (Supp. Tab. 2), with the exception of flye. This exception is likely due to the relatively higher occurrence of three-copy redundancy observed with the flye assembly algorithm (Fig. 2B), which are not captured in our k-mer proxy measurement (BUSCO duplications do not distinguish 2 copy genes from >2 copy genes). However, after assembly post-processing to remove redundant haplotigs, BUSCO genes appear to overestimate the efficiency of the procedures in comparison to the k-mer proxy. Across all methods and initial starting assemblies, the k-mer proxy shows consistently higher residual duplication than suggested by BUSCO genes (Supp. Tab. 2).

HiC misassembly detection and scaffolding

We observed the greatest overall number of detected misassembled region candidates in 'canu + purge_dups' after two iterations of the HiC scaffolding pipeline 3d-DNA. The least misassembled region candidates detected after two iterations was in 'wtdbg + HM2', followed by 'canu + HM2' (Tab. 1). We find the greatest disparity in total misassembled region candidates between post-processing methods in the canu assemblies. Furthermore, we find that 'canu + purge_dups' produced the lowest final N50 value, whereas all other assemblies produced very similar results, though the metric is limited by karyotype at this resolution (Tab. 1).

Patterns of divergence between redundant alleles

For BUSCO genes that were duplicated in the initial assembly and subsequently reduced to single copy by the post-processing methods, we broadly describe the

variation between the copies using the ratio of non-synonymous and synonymous nucleotide diversity and an alignment free method using the entire genomic region (Zielezinski *et al.*, 2019). We observed that the distributions of genes remaining after the application of purge_dups were more heavily weighted toward a low k-mer based distance and a πN/πS ratio of 0 as compared to the distributions of genes de-duplicated by Haplomerger2 (Fig. 3). We partitioned duplicated BUSCOs depending on whether they are present on the same initial assembly contig or not, as these genes may plausibly be real duplication events rather than haplotypic redundancy. The distribution of πN/πS between putative tandem duplicated BUSCOs (occurring on the same assembly contig) appears to be somewhat inflated in comparison to putative haplotig BUSCO genes. This pattern is also reflected in the overall genomic DNA k-mer distances (Fig. 3).

Discussion

Plutella xylostella populations harbour large amounts of polymorphism (You *et al.*, 2013, 2020). We observed a relatively low heterozygosity in our pooled data compared to other species, however this individual should not be considered representative of the wild population due to severe inbreeding and prior laboratory domestication. Despite this apparently reduced heterozygosity, a large amount of redundant sequence remains after genome assembly, suggesting that the heterozygosity is largely co-localised in highly divergent alleles. This pattern may suggest regions of low recombination, enabling haplotypes to accumulate linked neutral variation and persist through drift. Alternatively, in the case of associative overdominance, neutral variation can accumulate alongside linked overdominant or pseudo-overdominant loci (linked deleterious recessives with opposing phase) (Ohta and Kimura, 1970). It is important that such regions are represented appropriately in genome assemblies, as downstream analyses involving mapping reads rely on both overall completeness and regions being present in a haploid state (although see (Hickey *et al.*, 2020) for how this is changing).

We tested two post-assembly redundancy reduction procedures (Haplomerger2 & purge_dups) and found that Haplomerger2 generally appears to “resolve” more redundant sequence, at the expense of erroneous removal of non-redundant genome content and erroneous scaffolding of overlapping divergent regions. Both programs utilise a self-alignment step to detect haplotigs, purge_dups then implements a further QC step to these results by assessing the coverage of the identified haplotigs. For self-alignment, Haplomerger2 utilises LASTZ and enables users to calculate and use a sample specific scoring matrix, whilst purge_dups utilises minimap2 with a fixed intra-species scoring parameter (asm5). The parameterisation reflects a balance in differentiating intra-locus divergence, from inter-locus paralogue similarity. To give specific examples; ancient balancing selection vs relatively recent gene duplication or ancient balancing selection vs genetic convergence. The idealised genome assembly or redundancy removal pipeline can accurately differentiate these effects.

Genic analyses of assembly completeness such as BUSCO are widely used and relatively straight forward to apply, however by definition they are limited to genomic regions containing coding sequences (Simão *et al.*, 2015). The genes are highly conserved at the amino-acid level, suggesting that non-synonymous substitutions are largely deleterious. Because of this, the surrounding genomic region (including non-coding variation) may be likely to harbour less variation than a neutral region, due to the action of background selection (Gilbert *et al.*, 2020). In short, BUSCO genes are likely to inhabit (and help maintain) conserved genomic regions. The practical implication is that BUSCO genes, when utilised to assess the removal of redundant haplotigs, may systematically overestimate the effectiveness of the procedure, as they are unlikely to represent HDRs. Indeed, our results support the notion that before and after BUSCO duplication scores overestimate the removal of redundant haplotig sequences when compared to an analogous k-mer estimator. If BUSCO duplication results are liable to overestimate the haploid nature of a given draft genome assembly, it may hamper comparative genomic efforts to identify

balancing selection or overdominance (which may have either common or independent origins).

Despite this potential limitation, BUSCO scores are still useful as a guide to assembly completeness. BUSCO scores also provided insights into assembly post-processing, showing that, despite resolving more duplications than purge_dups, Haplomerger2 results do not completely overlap those of purge_dups. This indicates that both underlying methods are sub-optimal and the results may be complementary. We also note that BUSCO results, particularly missing genes, are dependent on the optimisation of input parameters. For example, the "--long" parameter can increase sensitivity at the cost of greater runtime. Similarly, detection of BUSCO genes may differ between haplotypes, thereby underestimating the number of duplicated genes.

We demonstrate a supporting validation method, providing relative quantification of assembly accuracy, using overlaps between modelled k-mer distributions and the k-mer frequency histogram subdivided by numerical representation in the final assembly version. Whilst this assessment applies to any non-repetitive genome region it only offers a general comparative measure between assemblies from the same read set and cannot determine appropriate representation at specific sites, due to stochasticity in read coverage. However, the ability to confidently determine truly heterozygous k-mers from homozygous will be increased in low-error, high-coverage read datasets, such as those currently being generated by projects like DToL (Darwin Tree of Life). This would offer an independent and unbiased validation method, but only with sufficiently high coverage to accurately partition the different k-mer peaks.

Our initial expectation was that a greater reduction in redundancy may correspond with an increase in detected misassembled region candidates, particularly in the case of Haplomerger2, which can join overlapping contigs (Sup. Fig. 1). Instead, we find the opposite pattern, though this does not necessarily imply more accurate assembly representation, since complex regions may be

absent from a final assembly altogether. For example, the lowest number of misassemblies ('wtdbg + HM2'), occurred alongside both the lowest putative allelic redundancy, but also the greatest values for missing BUSCO genes and het/hom modelled k-mers with 0x assembly coverage).

After an appraisal of the results of haplotig resolution, we compared the overall divergence of duplicated genes from the two methods. The results of purge_dups retained a greater proportion of low divergence haplotigs and this also corresponded to genes with a lower proportion of non-synonymous substitutions. The remaining genes in both sets suggests that both methods did not resolve more greatly diverged sequences and that this divergence corresponded to elevated non-synonymous substitutions relative to synonymous substitutions. Taken together with the high levels of coding sequence conservation intrinsic to BUSCO genes, this pattern would appear consistent with pseudo-overdominant regions generated by the linked arrangement of multiple deleterious non-synonymous substitutions. However, additional investigations with supplementary data will be required to establish this with confidence and determine the processes responsible for these patterns.

Conclusions

Highly divergent alleles can pose a challenge to accurate haploid reconstruction from noisy long read data. Post-processing can mitigate these problems somewhat to produce mosaic resolved sequences for reference purposes, however results in our case are largely imperfect and present a set of complex trade-offs between assembly completeness, redundancy and mis-assembly. Researchers producing or using genomes should be aware of these issues when using genome assembly data derived from noisy long-reads, especially when investigating genomic regions likely to harbour significant linked variation. Our results lead us to the conclusion that unresolved HDRs may be widespread in draft genomes assembled from noisy long-read data and that BUSCO analyses may

overestimate their resolution by post-processing methods. Plausible causes include loci experiencing balancing-selection or overdominance effects that originated prior to speciation events, or that exist within a genetic architecture liable to parallel origins of these processes. This may impact comparative genomic studies that aim to identify or describe these evolutionary processes, however further investigation is required to examine this.

Finally as a recommendation to researchers utilising similar data, we suggest that the optimal strategy is research question dependent. Our data shows that there are complex trade-offs between gene set completeness, the presence and abundance of redundant haplotigs, and overall genome contiguity. We list some recommendations resulting from our dataset: 1) For comparative analyses of large-scale shared-synteny or chromosome-level structures, we would recommend wtdbg2 followed by Haplomerger2, however it should be stressed that for this type of analysis researchers should supplement long-read data with HiC, both to extend contigs into larger-scale scaffolds, but also to correct any erroneous assembly, or post-processing mis-joins. When HiC data is available, the contiguity differences between different assemblers becomes less important, however wtdbg2 followed by Hapolomerger2 should still reduce misleading inter-specific alignment signals produced by residual haplotigs. 2) For comparative analysis of orthologues, the decision is complicated, as there is a trade-off between false-positive paralogues due to redundant haplotigs, vs false-negative missing genome content that is eliminated by redundancy removal procedures. 3) For analysis of a particular gene of interest, researchers can assemble their data with flye or canu, and process the resulting assembly with purge_dups. Reference to both the initial assembly and the post-processed data, should enable researchers to recover their gene of interest and examine whether any allelic variation is present. 4) For researchers wishing to produce a multi-purpose reference assembly, with no specific research question, we would suggest producing detailed and transparent methods, such that subsequent users can understand the limitations and reanalyse for their specific purpose if necessary.

Declarations

Ethics approval and consent to participate:
N/A

Consent for publication:
All authors agreed to the publication of this manuscript.

Availability of data and materials:
The read datasets generated during the current study are available in the ENA database under accession PRJEB34571 (see Supp. Tab. 1). All assembly versions are available at **10.5281/zenodo.5647466.** Code provided at https://github.com/swomics/Plutella_genomes

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Samuel Whiteford's studentship was co-funded by Oxitec
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Authors' contributions:
SW, NIM and ACD designed and planned the project. SW performed analyses with ACD. AH, SH, IS and MG prepared material for sequencing. The manuscript was written by SW, IJS and ACD with input from all authors.

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Figure 1. Contiguity and BUSCO content and of alternative genome assembly methods and the effects of removing putative allelic redundancy. In each panel, "canu", "flye" and "wtdbg" refer to the preliminary assemblies produced by each algorithm. "+ purge_dups + HiC" refers to these same assemblies with the additional application of the purge_dups program followed by HiC scaffolding or, Haplomerger2 followed by HiC scaffolding (A)_depicts the differences in overall contig size and contiguity between the different methods. The dotted curve describes a previously published reference genome (accession: GCA_000330985.1). The dashed straight line indicates the estimated genome size from an independent flow cytometry estimate (Baxter *et al.*, 2011). (B) shows overall BUSCO scores from a database of 5286 genes. BUSCO Scores from the aforementioned accession are also included. (C) details the relationships of genes within these sets. Groups of genes are coloured by BUSCO score in the intial assembly. BUSCO genes that are single-copy and complete in all assemblies are omitted to emphasise differences between assemblies.

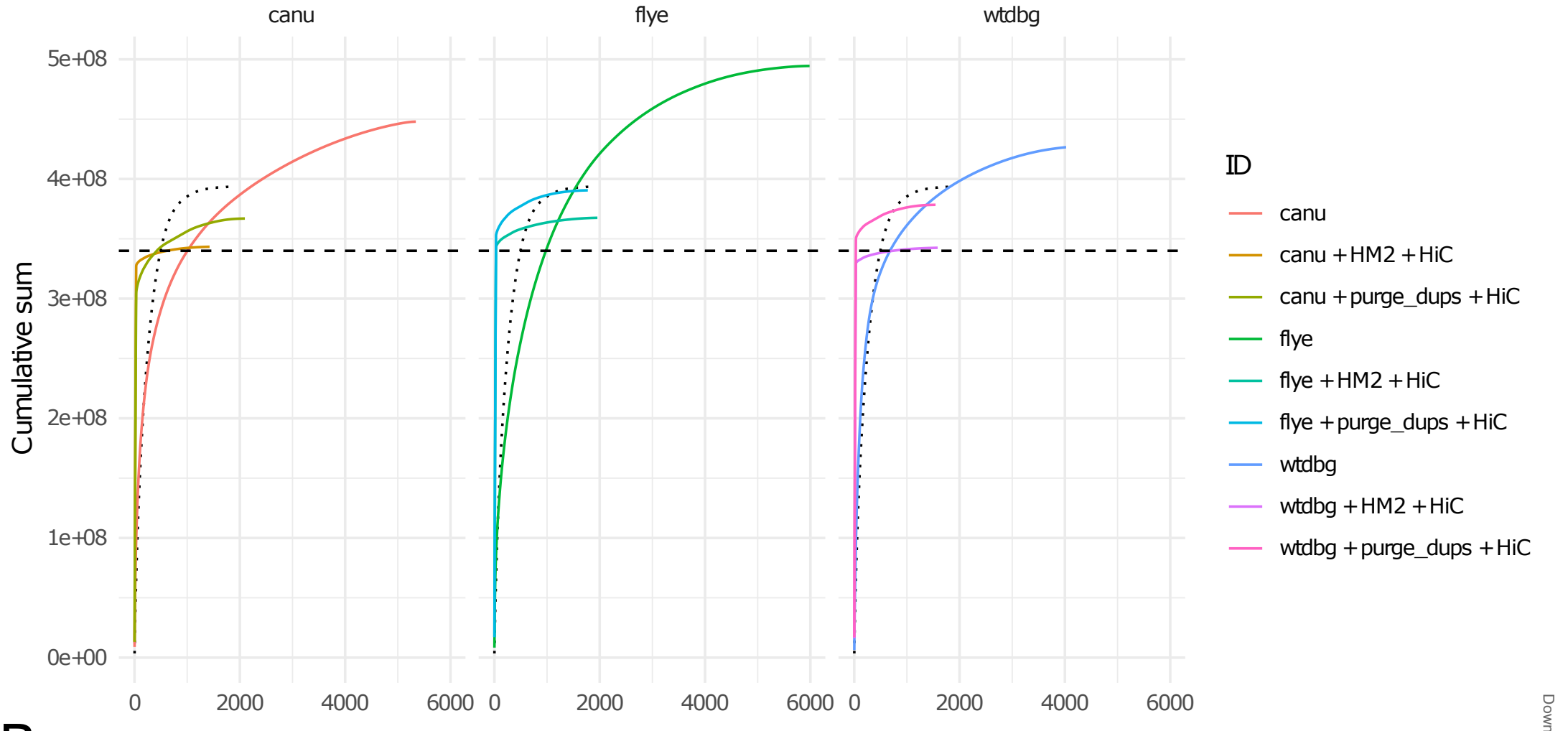
Figure 2. A k-mer based validation of the alternative genome assembly methods and effects of removing putative allelic redundancy. (A) shows an example of stacked k-mer distributions subdivided by assembly representation (spectra-cn plot) and an overlay of the modelled contributions of sequencing errors, heterozygous content and homozygous content (dotted lines from left to right respectively). (B) shows the spectra-cn plots for each of the assembly versions (C) shows the number of k-mers present in the intersections between the modelled k-mer content distributions and individual assembly coverage categories present in the spectra-cn plots.

Figure 3. Quantifying divergence between duplicated BUSCO genes. (A) shows the distribution of πN/πS scores for duplicated (N. copies = 2) BUSCO genes remaining after the application of purge_dups or Haplomerger2. (B) shows and alignment free quantification of the dissimilarity of intronic and exonic sequence between the same duplicated BUSCO genes (see methods for details). Panels labelled "Tandem" indicate that the BUSCO copies were found on the same assembly contig, whereas "Unique" indicates that the copies were found on different assembly contigs.

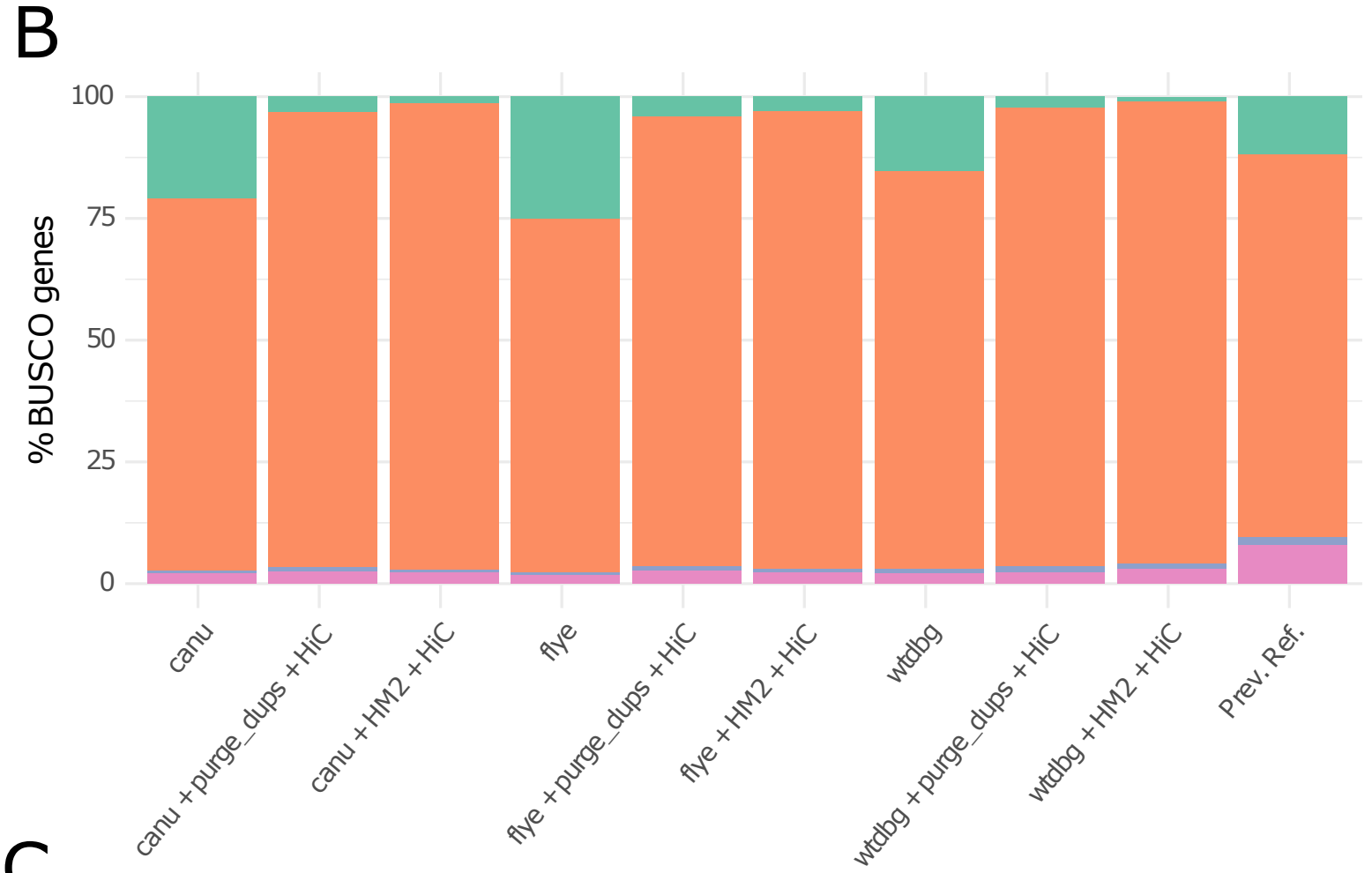
Table 1: Number of misassembly region candidates detected within the 3D-DNA pipeline. We used the default resolution parameters "wide_res = 25000bp" and "narrow_res = 1000bp"

	Total size (initial size) Mbp	Pre-HiC N50 (Mbp)	Post-HiC N50 (Mbp)	Narrow misassemblies		Wide misassemblies		Total iteration 2 misassemblies	Total disparity
				Iteration 1	Iteration 2	Iteration 1	Iteration 2		
canu + HM2	343 (448)	1.14	11.24	719	806	134	107	913	619
canu + purge_dups	367 (448)	0.62	9.49	794	1247	164	285	1532	
flye + HM2	368 (494)	0.41	11.42	1065	1225	185	115	1340	55
flye + purge_dups	391 (494)	0.32	11.44	835	1187	225	208	1395	
wtdbg + HM2	343 (427)	1.83	11.1	673	721	127	92	813	382
wtdbg + purge_dups	378 (427)	0.94	11.49	827	993	204	202	1195	

A



B



C