Rapid low-cost assembly of the *Drosophila melanogaster* reference genome using low-coverage, long-read sequencing

Edwin A. Solares[1,*], Mahul Chakraborty[1,*], Danny E. Miller[2,3], Shannon Kalsow[1], Kate E. Hall[2], Anoja G. Perera[2], J.J. Emerson[1,+], and R. Scott Hawley[2,4,+]

[1] Department of Ecology and Evolutionary Biology, University of California Irvine, Irvine, CA, USA
[2] Stowers Institute for Medical Research, Kansas City, MO, USA
[3] MD-PhD Physician Scientist Training Program, University of Kansas Medical Center, Kansas City, KS, USA
[4] Department of Physiology, University of Kansas Medical Center, Kansas City, KS, USA

[*] These authors contributed equally to this work.
[+] To whom correspondence should be addressed: jje@uci.edu or rsh@stowers.org

ORCID:
0000-0002-3220-4927 (EAS)
0000-0001-6096-8601 (DEM)
0000-0002-6478-0494 (RSH)
0000-0003-2414-9187 (MC)
0000-0001-9474-0891 (JJE)

ABSTRACT

Accurate and comprehensive characterization of genetic variation is essential for deciphering the genetic basis of diseases and other phenotypes. A vast amount of genetic variation stems from large-scale sequence changes arising from the duplication, deletion, inversion, and translocation of sequences. In the past 10 years, high-throughput short reads have greatly expanded our ability to assay sequence variation due to single nucleotide polymorphisms. However, a recent *de novo* assembly of a second *Drosophila melanogaster* reference genome has revealed that short-read genotyping methods miss hundreds of structural variants, including those affecting phenotypes. While genomes assembled using high-coverage long reads can achieve high levels of contiguity and completeness, concerns about cost, errors, and low yield have limited the widespread adoption of such sequencing approaches. Here we resequence the reference strain of *D. melanogaster* (ISO1) on a single Oxford Nanopore MinION flow cell run for 24 hours. Using only reads longer than 1 kb, or 30x coverage, we *de novo* assemble a highly contiguous genome. The addition of inexpensive paired reads and subsequent scaffolding using an optical map technology achieved an assembly with completeness and contiguity comparable to the *D. melanogaster* reference assembly. Surprisingly, comparison of our assembly to the reference assembly of ISO1 uncovered a number of structural variants, including novel LTR transposable element insertions and duplications affecting genes with developmental, behavioral, and metabolic functions. Collectively, these structural variants provide a rare snapshot of the dynamics of metazoan genome evolution. Furthermore, our assembly and comparison to the *D. melanogaster* reference genome demonstrates that reference-quality *de novo* assembly of metazoan genomes and comprehensive variant discovery using such assemblies are now possible for under $1,000 USD.

INTRODUCTION

Characterization of comprehensive genetic variation is crucial for discovery of mutations affecting phenotypes. In the last 10 years, the exponential decline in cost and explosion of throughput for short-read sequencing has revolutionized our ability to assay genome-wide sequence variation. Short reads excel at identifying single nucleotide polymorphisms (SNPs) and short indels in unique genomic regions. However, the majority of the sequence difference between individuals is caused by duplications, deletions, inversions, or translocation of sequences—collectively known as structural variants (SVs) (Feuk, Carson, and Scherer 2006). SVs are often longer than short sequencing reads (generally 50–150bp), meaning genotyping of SVs is indirect, relying on features of alignments to a reference genome such as divergent read mappings, split reads, or elevated read coverage (Medvedev, Stanciu, and Brudno 2009; Alkan, Coe, and Eichler 2011). However, comparisons of extremely contiguous *de novo* genome assemblies from humans (Huddleston and Eichler 2016; M. J. P. Chaisson et al. 2017) and *Drosophila melanogaster* (Chakraborty et al. 2018) revealed that short reads miss 40–80% of SVs. Consequently, methods that are not susceptible to the shortcomings of short read sequencing are essential to obtain a more complete view of genome variation (Alkan, Coe, and Eichler 2011). We propose one approach—comparison of contiguous and accurate *de novo* genome assemblies—that would overcome these limitations and dramatically improve our understanding of genetic variation.

Although short reads have been used extensively for *de novo* genome assembly, they fail to resolve repetitive regions in genomes, leaving errors and gaps while assembling such regions (Paszkiewicz and Studholme 2010; Treangen and Salzberg 2011; Bradnam et al. 2013). Such fragmented draft-quality assemblies are therefore poorly suited for identification of SVs (Alkan, Coe, and Eichler 2011) and lead to incomplete and/or missing gene models (Gordon et al. 2016). Theoretical considerations of the genome assembly problem predict that, with sufficient read depth and length, genome assemblies can resolve even difficult regions (Motahari, Bresler, and Tse 2013; Lam, Khalak, and Tse 2014; Bresler, Bresler, and Tse 2013; Shomorony, Courtade, and Tse 2016). Consistent with this, long reads produced by Single Molecule Real-Time sequencing from Pacific Biosciences (PacBio) and Nanopore sequencing

from Oxford Nanopore Technologies (ONT) provide data capable of achieving remarkably contiguous *de novo* genome assemblies (Kim et al. 2014; Berlin et al. 2015; Michael et al. 2018; Jain et al. 2018). However, due to high error rates (~10–15%), generation of reliable assemblies with these reads require non-trivial coverage (generally 30x or greater) (Koren et al. 2017; Chakraborty et al. 2018). Until recently, long-read methods have required prohibitively expensive reagents, and technologies like PacBio also require substantial capital investment related to the housing and maintenance of equipment necessary to perform the sequencing. Combined with concerns about high error rates, widespread adoption of long-molecule sequencing for *de novo* assembly and variant detection has been tentative.

Sequencing using ONT may produce reads that are hundreds of kilobases in length (Jain et al. 2018), though application to *de novo* assembly of reference-grade metazoan genomes is not yet routine. To understand how effective assembly using ONT is when applied to metazoan *de novo* genome assembly, we measured the contiguity, completeness, and accuracy of a *de novo* assembly constructed with ONT reads. To accomplish this, we resequenced the *D. melanogaster* reference genome strain (ISO1) using the ONT MinION and compared the resulting assembly with the latest release of the *D. melanogaster* reference assembly (Hoskins et al. 2015), arguably the best metazoan reference genome available. We followed a hybrid assembly approach (Chakraborty et al. 2016) to combine modest long-read coverage from a single ONT MinION flow cell (30x depth of coverage with an average read length of 7,887 bp) and Illumina short-read data, which resulted in a highly contiguous *D. melanogaster* genome assembly that was of reference quality. Notably, using this approach, the majority of the euchromatin of each chromosome arm is represented by a single contiguous sequence (contig) that collectively recovered 97.7% of Benchmarking Universal Single-Copy Orthologs (BUSCOs), similar to the 98.3% BUSCO recovered in the most recent release of the *D. melanogaster* genome (version 6.15). Scaffolding of the assembly with Bionano optical maps led to further improvements in contiguity. Finally, we examined the structural differences between our assembly and the published assembly of the same strain and observed several candidate SVs, the majority of which are transposable element (TE) insertions and copy-number variants

(CNVs). These are mutations that must be either: 1) recent mutations that occurred in the genome strain since it was sequenced, 2) segregating in the genome strain due to incomplete isogeny, or 3) errors in one of the assemblies.

Overall, we show that high-quality *de novo* genome assembly of *D. melanogaster* genomes is feasible using low-cost ONT technology, enabling an assembly strategy that can be applied broadly to metazoan genomes. This strategy will make high-quality reference assemblies obtainable for species lacking reference genomes. Moreover, *de novo* assemblies for population samples of metazoan species is now feasible, opening the door for studying evolutionary and functional consequences of structural genetic variation in large populations.

METHODS

Stocks

The ISO1 *D. melanogaster* reference stock used for both Nanopore and Illumina sequencing was obtained from the BDGP in 2014 (Hoskins et al. 2015). All flies were kept on standard cornmeal-molasses medium and maintained at 25°C.

DNA isolation and quantification

DNA for Nanopore sequencing was isolated from males and females using the Qiagen Blood & Cell Culture DNA Mini Kit. Briefly, 60–80 flies were placed in two 1.5-mL Eppendorf Lo-Bind tubes and frozen in liquid nitrogen before being homogenized using a pestle in 250 µL of Buffer G2 with RNAse. 750 µL of Buffer G2 and 20 µL of 20 mg/mL proteinase K was then added to each tube and incubated at 50°C for 2 hr. After 2 hr, each tube was spun at 5,000 RPM for 5 min, and the supernatant was removed and placed in a new 1.5-mL Lo-Bind tube and vortexed for 10 sec. The supernatant from both tubes was then transferred onto the column and allowed to flow through via gravity. The column was washed 3x with wash buffer and eluted twice with 1 mL of elution buffer into two 1.5-mL Lo-Bind tubes. 700 µl of isopropanol was added and mixed via inversion before being spun at 14,000 RPM for 15 min and 4°C. The supernatant was removed and the pellet was washed with 70% ethanol, then spun at 14,000 RPM for 10 min at 4°C. The supernatant was removed and 25 µL of ddH_2O was added to each tube and allowed to sit at room temperature for 1 hr. Both tubes were then combined into one. DNA was quantified on a Nanodrop and Qubit. DNA for Illumina sequencing was isolated from males and females using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions and quantified on a Qubit.

Library preparation, sequencing, and basecalling

For Nanopore sequencing, 45 µL of DNA was used to prepare a 1D sequencing library (SQK-LSK108) according to the manufacturer's instructions, including the FFPE repair step. 75 µL library was then immediately loaded onto an R9.5 flow cell prepared

according to the manufacturer's instructions and run for approximately 24 hr. Basecalling was completed using ONT Albacore Sequencing Pipeline Software version 2.0.2. Reads collected during the mux phase of sequencing were not included.

For Illumina sequencing, ~600-bp fragments were generated from 500 ng of DNA using a Covaris S220 sonicator. Fragments of 500–700 bp were selected using a Pippin and libraries were prepared using a KAPA High Throughput Library Preparation kit and Bioo Scientific NEXTflex DNA Barcodes. The library was pooled with others and run as a 150-bp paired-end run on a single flow cell of an Illumina NextSeq 500 in medium-output mode using RTA version 2.4.11. bcl2fastq2 v2.14 was then run in order to demultiplex reads and generate FASTQ files.

Genome assemblies

Canu (Koren et al. 2017) release v1.5 was used to assemble the ONT reads. Canu was run with default parameters in grid mode (Sun Grid Engine) using ONT reads >1 kb and a genome size of 130 Mb. To generate the De Bruijn graph contigs for the hybrid assembly, we used Platanus (Kajitani et al. 2014) v1.2.4 with default settings to assemble 67.4x of Illumina paired-end reads obtained from the DPGP (http://www.dpgp.org/dpgp2/DPGP2.html) (Pool et al. 2012). The hybrid assembly was generated with DBG2OLC (Ye et al. 2016) using contigs from the Platanus assembly and the longest 30x ONT reads. DBG2OLC settings (options: k 25 AdaptiveTh 0.01 KmerCovTh 2 MinOverlap 35 RemoveChimera 1) were similar to those used for PacBio hybrid assembly of ISO1 (Chakraborty et al. 2016), except that the kmer size was increased to 25 and the MinOverlap to 35 to minimize the number of misassemblies. The consensus stage of DBG2OLC was run with PBDAG-Con (Chin et al. 2013) and BLASR (M. J. Chaisson and Tesler 2012). Separately, minimap v0.2-r123 (using a minimizer size window of 5, FLOAT fraction of minimizers of 0, and min matching length of 100) and miniasm v0.2-r123 (using default settings) were also used to assemble only the ONT reads (Li 2016).

The Canu and DBG2OLC assemblies were merged using quickmerge (Chakraborty et al. 2016). First, the two assemblies were merged using the DBG2OLC assembly as the query and the Canu assembly as the reference. Thus, the first

quickmerge run filled gaps in the DBG2OLC assembly using sequences from the Canu assembly, giving preference to the Canu assembly sequences at the homologous sequence junctions. The contigs that are unique to the Canu assembly were incorporated in the final assembly by a second round of quickmerge. In the second quickmerge run, the merged assembly from the previous step was used as the reference assembly, and the Canu assembly was used as the query assembly (Figure 1).

Assembly polishing

Assembly polishing was performed two ways. First, nanopolish version 0.7.1 (Loman, Quick, and Simpson 2015) was run using the recommended settings along with reads longer than 500 bp, as the inclusion of shorter reads created coverage that was too high, resulting in segmentation faults. Prior to running nanopolish, the merged genome assembly was indexed using bwa, and ONT reads were aligned to the genome using bwa mem. The resulting bam file was then sorted, indexed and filtered for alignments of size larger than 1 kb using samtools 1.3. Nanopolish was then run with a minimum candidate frequency of 0.1. Following nanopolish, we polished the assembly twice with Pilon (Walker et al. 2014) v1.16. For Pilon, we aligned 44x 150-bp and 67x 100-bp Illumina paired-end reads to the assembly using bowtie2 (Langmead and Salzberg 2012) and then ran Pilon on the sorted bam files using default settings.

Bionano scaffolding

Bionano optical map data was collected following Chakraborty *et al*. (2018). ISO1 embryos less than 12 h old were collected on apple juice/agar Petri dishes, dechorionated using 50% bleach, rinsed with water, then stored at -80°C. DNA was extracted from frozen embryos using the Animal Tissue DNA Isolation kit (Bionano Genomics, San Diego, CA). Bionano Irys optical data was generated and assembled with IrysSolve 2.1 at Bionano Genomics. We then merged the Bionano assembly with the final merged assembly contigs using IrysSolve, retaining Bionano assembly features when the two assemblies disagreed.

BUSCO analysis

We used BUSCO v1.22 to evaluate completeness and accuracy of all ISO1 assemblies (Simão et al. 2015). We used the Diptera database, which contains 2,799 highly conserved genes, in order to estimate assembly completeness.

Assembly comparison and error detection

Assemblies were compared using alignment dot plots. For dot plots, assembled genomes were aligned to the reference *D. melanogaster* genome (r6.15) using nucmer (using options: minmatch 100, mincluster 1000, diagfactor 10, banded, diagdiff 5) (Kurtz et al. 2004). The resulting delta alignment files were used to create the dot plots either with mummerplot (using options: --fat --filter --ps). QUAST v4.5.0 was used to compare each generated assembly to the contig reference genome assembly (*D. melanogaster* r6.15) for completeness and errors. QUAST was run in gage mode, on contigs larger than 1 kb, and the reference assembly as fragmented, as it was originally the scaffolded assembly (Salzberg et al. 2012; Gurevich et al. 2013).

Structural variant detection

Large (>100 bp) SVs were detected by aligning the Bionano scaffolds to the FlyBase (dos Santos et al. 2015) reference assembly using MUMmer v3.23 (nucmer -maxmatch) (Kurtz et al. 2004) and then annotating the disagreements between the assemblies as indels and duplications using SVMU v0.2beta (commit 4e65e95) (Chakraborty et al. 2018). Insertions overlapping with RepeatMasker (Repeatmasker 4.0.7) annotated TEs were annotated as TE insertions. SVs were validated with at least two ONT reads spanning the entire genomic feature containing the SVs plus 200 bp on both sides of the SV. TEs were inferred to be segregating when corrected long reads supporting the TE insertions were contradicted by other reads showing absence of the TE. For validation with spanning long reads, we aligned Canu-corrected ONT reads to the FlyBase and Bionano assemblies using BLASR (v 1.3.1) (M. J. Chaisson and Tesler 2012) and the sorted alignment bam files were visually examined with IGV (Thorvaldsdóttir, Robinson, and Mesirov 2013).

Mitochondrial genome identification

The mitochondrial genome was identified by using BLAST (Altschul et al. 1990) to compare our final assembly (the Bionano assembly) against the mitochondrial genome from r6.15 of the *D. melanogaster* genome. A single contig was identified (tig00000438_pilon_pilon) with 99% identity to the reference mitochondrial genome. This contig contained two copies of the mitochondrial genome in tandem, therefore the first 16,806 and last 2,104 nucleotides were removed from the contig. We used MITOS (Bernt et al. 2013) with default settings, metazoan reference, and invertebrate genetic code to annotate both the reference mitochondrial genome and our assembled mitochondrial genome.

Data availability

Illumina data generated in this study has been uploaded to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) under bioproject PRJNA433573. Raw Nanopore data is available at ftp://ftp.stowers.org/pub/dem_ncbi/. Genomes assembled in this study are available at https://github.com/danrdanny/Nanopore_ISO1. Release 6.15 of the *D. melanogaster* genome used in this study is available on FlyBase (http://www.flybase.org).

RESULTS

Sequencing results

A 1D sequencing library was loaded onto a release 9.5 flowcell and run for approximately 24 hours (see Methods) generating a total of 663,784 reads (Table 1). Base calling was performed with Albacore 2.0.2 with 593,354 (89%) of all reads marked as “pass” (reads having a quality score ≥7) and an average read length of 7,122 bp (Figure 2). The read N50 for those that passed filter was 11,840 with 41 reads longer than 50 kb and a maximum read length of 379,978 bp (Figure S1).

Genome assembly

ONT-only assembly using minimap

To evaluate ONT data for *de novo* genome assembly, we performed an ONT-only assembly using minimap and miniasm (Li 2016). Together, these programs allow for rapid assembly and analysis of long, error-prone reads. We generated an assembly with a total size of 132 Mb, with 208 contigs, and a contig N50 of 3.8 Mb (Table 2). (Contig N50 is the length of the contig such that 50% of the genome is contained within contigs that are equal to or longer than this contig.) Evaluation of an alignment dot plot between this assembly and the *D. melanogaster* reference genome revealed high correspondence between our assembly and the reference genome (Figure S2). However, BUSCO analysis of the minimap assembly only found 0.5% of expected single-copy genes present, much lower than the BUSCO score of 98.3% obtained from the current release of the *D. melanogaster* genome (Table 3). BUSCO analysis evaluates the presence of universal single-copy orthologs as a proxy of completeness. Such a low BUSCO score as found in the minmap assembly is unlikely to be measuring low completeness, but rather suggests a high rate of errors that disrupt the genes, making them difficult to assay properly.

ONT-only assemblies using Canu

We also generated an ONT-only assembly with Canu using only reads longer than 1 kb. Alignment dot plots for the assembled genome and the *D. melanogaster* reference genome also revealed large collinear blocks between this assembly and the reference,

indicating only one large misassembly on chromosome 2L (this misassembly was broken prior to merging and polishing) (Figure 3A). The Canu assembly was marginally less contiguous (contig N50 = 2.9 Mb) than the minimap assembly, but resulted in a higher BUSCO score of 67.7% (Table S1). The errors in the Canu assembly and the low BUSCO score are consequences of inherently high error rate of ONT reads. However, due to the higher accuracy and completeness of the Canu assembly compared to the *minimap* assembly, we used the ONT-only Canu assembly for the remainder of our analysis.

Hybrid assembly using ONT and Illumina reads

Modest coverage assemblies of PacBio long reads can exhibit high contiguity when a hybrid assembly method involving Illumina reads is used (Chakraborty et al. 2016). Therefore, we examined whether such assembly contiguity improvements also occur when ONT long reads are supplemented with Illumina paired-end reads. We performed a hybrid assembly using DBG2OLC with the longest 30x ONT reads and contigs from a De Bruijn graph assembly constructed with 67x coverage of Illumina paired-end data. To optimize the parameters, we performed a gridsearch on four parameters (AdaptiveTh, KmerSize, KCovTh and MinOverlap), yielding 36 genome assemblies. We used a range of values recommended by the authors for low-coverage assemblies and verified our KmerSize by looking at meryl's kmer histogram and found it coincided with a value that represented a 99% fraction of all k-mers. We selected the best genome based on colinearity and largest N50. The best hybrid assembly was substantially more contiguous (contig N50 = 9.9 Mb) than the ONT-only Canu assembly (contig N50 = 2.9 Mb), had large blocks of contiguity with the reference (Figure 3B), yet had lower BUSCO scores (47.7% compared to the 67.7% observed in the Canu assembly).

Merging of Canu *and DBG2OLC assemblies*

Previous studies have shown that merging of assemblies constructed with only PacBio long reads and hybrid assemblies constructed with PacBio long reads and Illumina paired-end short reads results in a considerably more contiguous assembly than either of the two component assemblies alone (Chakraborty et al. 2016). To examine the

effect of assembly merging on assemblies created with ONT long reads, we merged the ONT-only Canu assembly with the DBG2OLC assembly with two rounds of quickmerge (see Methods). The merged assembly (contig N50 = 18.6 Mb) was more contiguous than both the Canu and the DBG2OLC assemblies alone (Table 2, Figure 3C). As expected, the BUSCO score of the merged assembly (58.2%) fell between the two component assemblies.

Assembly polishing

Low BUSCO scores and a high number of SNP and indel polymorphisms in all of our assemblies are consistent with other *de novo* assemblies created with noisy long reads with high error rates (Loman, Quick, and Simpson 2015; Chakraborty et al. 2016). Many of these errors can be fixed via assembly polishing, and a number of assembly polishing tools exist. We chose to focus on two: nanopolish, which performs consensus-based error correction using ONT reads (Loman, Quick, and Simpson 2015), and Pilon, which performs error correction using Illumina reads (Walker et al. 2014).

We generated three different polished genomes, one using nanopolish alone, one using only two rounds of Pilon, and one using one round of nanopolish followed by two rounds of Pilon (see Methods). Polishing with nanopolish alone recovered only 79%, 79.2%, and 78.5% BUSCOs for the hybrid, ONT-only, and the merged assemblies, respectively, suggesting that polishing with ONT reads alone only partially improved assembly quality. On the other hand, polishing all three assemblies twice with Pilon alone fixed a large number of structural errors as evidenced by improved BUSCO scores of the resulting assemblies (Simão et al. 2015) (Table 3). This suggests that Pilon is a more reliable assembly polishing tool than nanopolish. However, when the merged assembly was polished first with nanopolish and then twice with Pilon, nearly all BUSCOs (97.9%) present in the ISO1 reference assembly (98.3%) were recovered. BUSCO scores of the similarly polished hybrid and ONT-only assemblies showed similar level of completeness (Table 3). Notably, we find that the hybrid assembly recovered a different subset of BUSCOs than the ONT-only assembly. This led to a much higher number of BUSCOs being recovered in the final merged assembly.

The QUAST output comparing the Canu, DBG2OLC, merged, and Bionano genome assemblies against the *D. melanogaster* reference shows that the quickmerge assembly resulted in intermediate error rates and discordance as compared to the component assemblies (Figure 4). All four assemblies exhibited approximately the same number of SNP and indel errors < 5 bp, whereas the DBG2OLC assembly resulted in approximately 20% more indels > 5 bp in size than the Canu assembly (Figure 4H-I; Table S1). We attribute this difference in indels to the in-depth parameter search performed on the DBG2OLC assembly.

Bionano scaffolding

Because they are substantially longer than the ONT reads, Bionano reads can be used for scaffolding contigs across repetitive regions. We generated 81,046 raw Bionano molecules (20.5 Gb) with an average read length of 253 kb. To use Bionano molecules for scaffolding, we first created a Bionano assembly (509 contigs, N50 = 620 kb, assembly size = 291 Mb) using 78,397 noise-rescaled reads (19.9 Gb, mean read length 253 kb). At 291 Mb, the Bionano assembly was nearly twice the size of the reference assembly (144 Mb), reflecting a diploid assembly size for ISO1. To scaffold the merged contigs using Bionano optical maps, the polished merged contigs were merged with the Bionano assembly. The resulting scaffolded assembly was more contiguous (N50 = 21.3 Mb) than the unscaffolded contigs and contained a comparable number of errors (Figure 3D). Bionano scaffolding of our assembled genome did not improve BUSCO scores (Table 3).

Structural variants

One advantage of high-quality *de novo* assemblies is that they permit comprehensive detection of large (>100 bp) SVs. Highly contiguous assemblies, such as the one generated here, allow comparisons between two or more assemblies, revealing novel SVs and facilitating the study of their functional and evolutionary significance. Although we sequenced the reference strain, structural differences between our stock and the published assembly are expected due to error, but also to new mutations—especially for transposable elements, the most dynamic structural components of the genome. Our

assembly revealed the presence of 34 new TE insertions and 12 TE losses compared to the reference genome assembly. Among the 34 TE insertions, 50% (17/34) are LTR TEs comprising chiefly of *Copia* (5/17) and *Roo* elements (6/17). Interestingly, 29% (10/34) of the TE insertions are defective *Hobo* elements lacking an average of 1.7 kb of sequence (base pairs 905–2,510) from the middle segment of the element encoding the transposase. However, alignment of long reads to the assembly regions harboring the TE insertions revealed that 6/34 insertions are not fixed, but are segregating in the strain (Table S2). The high insertion rate of the LTR and Hobo elements in this single strain mirrors the recent spread of LTR and *Hobo* elements in *D. melanogaster* populations (Pascual and Periquet 1991; Periquet et al. 1994; Bowen and McDonald 2001). As expected, the majority (27/34) of the new TE insertions are located within introns, because insertions within exons generally result in gene disruption. Nonetheless, we found five genes (*Ance*, *Pka-C1*, *CG31826*, *CG43446*, and *Ilp6*) in which new TEs have inserted within exons (Table S2). We also found 12 TEs present in the reference assembly missing from our final scaffolded assembly, among which six are LTR TEs (five *297* elements and one *Roo* element). The high rate of insertion and loss of LTR elements underscores their dynamic evolutionary history in the *D. melanogaster* genome. Because TEs can be locally unique, the presence or absence of such events does not pose a fundamental limitation to assembly. As a result, we predict most of these events to be new mutations rather than errors in assembly.

Additionally, we identified several duplications present in our assembly. For example, a tandem duplication of a 9,086-bp segment (*2L*:5,988,156-5,997,242) has created partial copies of the genes *infertile crescent* (*ifc*) and *little imaginal discs* (*lid*). Another 2,158-bp tandem duplication has created partial copies of the genes *CG10137* and *CG33116* (Table S2). Apart from CNVs affecting single copy sequences, our assembly also uncovered copy number increases in tandem arrays that are of likely functional consequences. For example, we observed copy number increase in a tandem array of a 207-bp segment within the third exon (*2L*:6,152,563-6,153,057) of the gene *Muc26B*, which is predicted to encode a chitin-binding protein. While CNVs such as this have been challenging to identify and validate in the past, at least two Nanopore reads spanning this entire tandem array support the presence of a tandem duplication at this

position (Figure 5). Unlike TEs, classifying such tandem events as errors stemming from shorter Sanger reads or as actual mutations is difficult without access to the original material from which the Sanger data was derived.

Mitochondrial genome identification and identity

After assembly, merging, polishing, and scaffolding, we used BLAST (Altschul et al. 1990) to identify the mitochondrial genome. Using the published *D. melanogaster* mitochondrial genome of ~19,500 bp as the subject, we identified one 38,261-bp contig with nearly 100% identity to the reference mitochondrial genome. The first 16,806 and last 2,100 nucleotides of this contig were 99.6% identical to the reference mitochondrial genome, while the middle 19,228 nucleotides were 98% identical to the reference mitochondrial genome, suggesting that our assembled mitochondrial genome had been duplicated during assembly. For the tandem assembled genomes, nearly all of the SNP and indel polymorphisms occurred in the last 4,000 nucleotides of the reference genome.

To determine if all genes and features were present in our assembled genome that are present in the reference mitochondrial genome, we annotated both genomes using MITOS (Bernt et al. 2013). Both assemblies were annotated nearly identically, with MITOS reporting that both assemblies were missing the origin of replication for the L region (OL) (Figure 6). The Nanopore assembly also resulted in two split genes not observed in the reference genome assembly: *nad4* and *nad6* were both annotated as two continuous genes and not one single gene.

DISCUSSION

Drosophila melanogaster was the first genome assembled using a whole-genome shotgun (WGS) strategy (Myers et al. 2000; Adams et al. 2000). This successful proof-of-principle led to the prevalence of the WGS sequencing approach as a tool in virtually all subsequent metazoan genome assembly projects (Lander et al. 2001; Mouse Genome Sequencing Consortium et al. 2002; Goff et al. 2002; Yu et al. 2002; Aparicio et al. 2002; Gibbs et al. 2004; International Chicken Genome Sequencing Consortium 2004). While improvements in sequencing technology have led to a precipitous drop in the cost of sequencing (https://www.genome.gov/sequencingcosts/, accessed Feb 16, 2018), stagnation and even regression of read lengths resulted in highly fragmented and incomplete assemblies (Alkan et al. 2011). Furthermore, complementary approaches (like hierarchical shotgun sequencing and other clone-based approaches) were required to obtain nearly complete and highly contiguous reference genomes, which add complexity, cost, and time to assembly projects.

Short reads provided by next-generation sequencing technologies present limitations to what a pure WGS assembly approach can accomplish (Alkan, Sajjadian, and Eichler 2011; Narzisi and Schatz 2015). The advent and development of long-read sequencing technologies has led to dramatic increases in read length, permitting assemblies that span previously recalcitrant repetitive regions. Early implementations of these technologies produced reads longer than previous short-read technologies yet still shorter than relatively common repeats, while the cost and error rate remained high compared to the short-read approaches. Continued improvements in read length overcame many of these difficulties, permitting nearly complete, highly contiguous metazoan genome assemblies with only a WGS strategy (Kim et al. 2014; Berlin et al. 2015). Such approaches led to assemblies comparable in completeness and contiguity to release 6 of the *D. melanogaster* genome assembly (Hoskins et al. 2015) for approximately $10,000 USD (Chakraborty et al. 2016; Chakraborty et al. 2018). However, even with the rapid development of these approaches, substantial capital investment in the form of expensive instrumentation and dedicated genome facility staff was required.

Here, we report an independent re-sequencing and assembly of the *D. melanogaster* reference strain ISO1 for less than $1,000 USD (before Bionano scaffolding) without the need for extensive capital or personnel investment. The resulting assembly before scaffolding is comparable to release 6 of FlyBase in terms of contiguity and completeness (18.9 Mb contig N50 and 97.1% complete single copy BUSCOs). We achieved this using 4.2 Gb of sequence from a single Oxford Nanopore flow cell in conjunction with Illumina short-read data. Such reduction in complexity and cost permits a small team of scientists to shepherd a sequencing project from sample to reference-quality assembly in a relatively short amount of time. The addition of optical mapping data permitted ordering and orientation of the contigs, yielding an assembly nearly as contiguous as the published reference (scaffold N50 of 21.3 Mbp).

Comparing this assembly to the FlyBase reference genome shows that it is both accurate (21 mismatches/100kb, 36 indels/100 kb) and collinear (Figure 3D, Figure 4, Table S2). Most of the small-scale differences are expected to be errors introduced by the noisy Oxford Nanopore reads that escaped correction via polishing. It is possible that some of these errors are SVs, which are expected to accumulate because the ISO1 stock has been maintained in the laboratory for approximately 350 generations (assuming 20 gen/year) since initial sequencing in 2000. This allows for the accumulation of new mutations by genetic drift, including ones reducing fitness (Assaf et al. 2017). Due to the high contiguity in the euchromatic region, our assembly facilitates detection of such euchromatic SVs. Several apparent “assembly errors” in our Bionano assembly are due to TE indels that are supported by spanning long reads. We found 28 homozygous euchromatic TE insertions which are predominantly LTR and defective *Hobo* elements, suggesting a high rate of euchromatic TE insertions (~0.08 insertion/gen). That we observed a predominance of LTR and Hobo elements among the new TE insertions mirrors their recent spread in *D. melanogaster* populations (Pascual and Periquet 1991; Periquet et al. 1994; Bowen and McDonald 2001; De Freitas Ortiz and Silva Loreto 2008). The abundance of defective *Hobo* elements among the new insertions is particularly interesting given that these *Hobo* elements lack the transposase enzyme necessary for mobilization.

Although most novel TEs insertions were found in introns, five were found within exons: the 5' UTR of *Ance*, *CG31826*, and *Ilp6*, the 3' UTR of *Pka-C1*, and the coding region of *CG43446*. Similarly, TE loss primarily involved LTR TEs, including loss of TEs from the 5' UTR of the genes *Snoo* and *CG1358*. We also observed copy number increases in both unique sequences as well as tandem arrays (Table S2), with one duplication creating a new copy of the entire coding sequence of the gene *lid*. Collectively, our assembly provides a snapshot of ongoing genome structure evolution in a metazoan genome that is often assumed to be unchanging for experimental genetics.

A crucial feature of this work is that it is performed in a strain used to generate one of the highest quality reference genomes, ensuring that our inferences can be judged against a high-quality standard. This approach allowed us to demonstrate that assembly with modest amounts of long-molecule data paired with inexpensive short-read data can yield highly accurate and contiguous reference genomes with minimal expenditure of resources. This opens myriad opportunities for high-quality genomics in systems with limited resources for genome projects. Moreover, we can now conceive of studying entire populations with high-quality assemblies capable of resolving repetitive structural variants, something previously unattainable with short-read sequencing alone.

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FIGURES

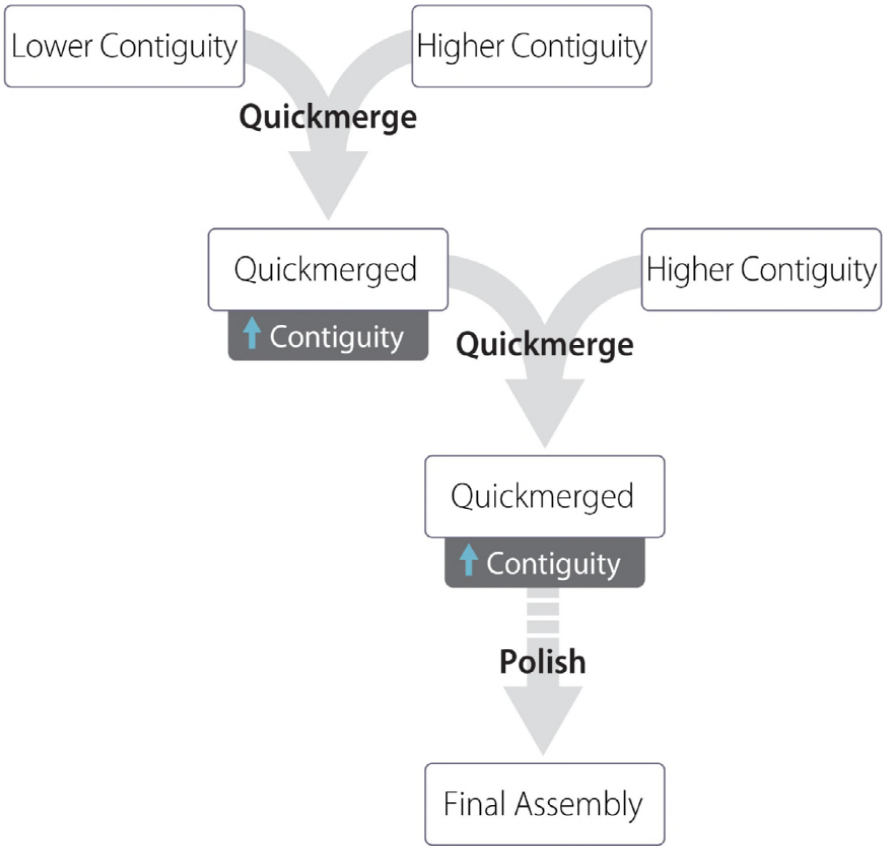

Figure 1. Assembly strategy used in this manuscript. A low-quality assembly (Canu) is merged with a high-quality assembly (DBG2OLC), the resultant assembly is again merged with the DBG2OLC assembly. The genome is then polished one or more times, here with nanopolish followed by Pilon.

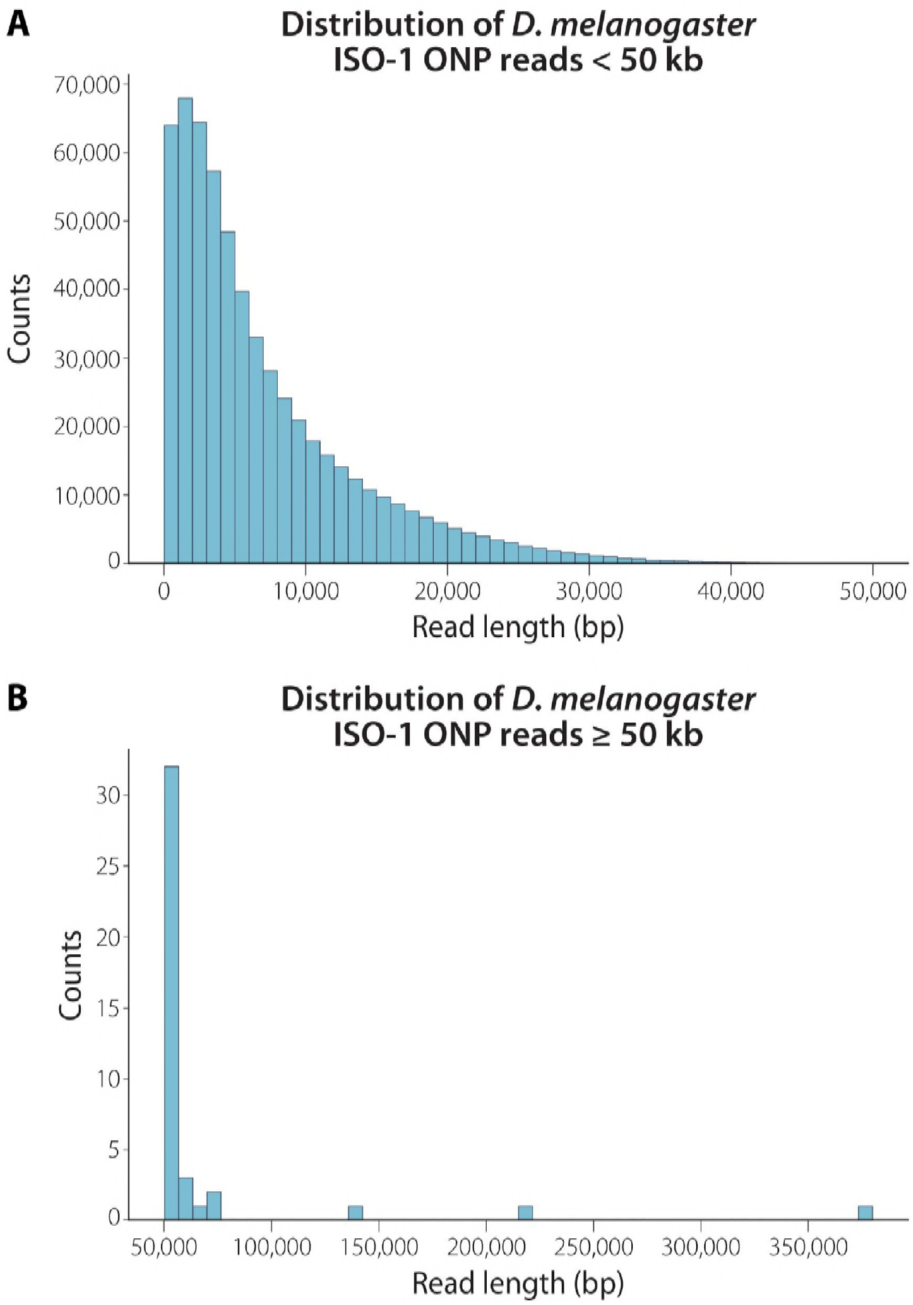

Figure 2. Read length distribution for reads with quality scores greater than or equal to 7. Length is the sequence length after base calling by Albacore, not the length that aligned to the genome. (**A**) Distribution of read lengths less than 50 kb. (**B**) Distribution of reads 50 kb or greater. The longest read that passed quality filtering was 380 kb.

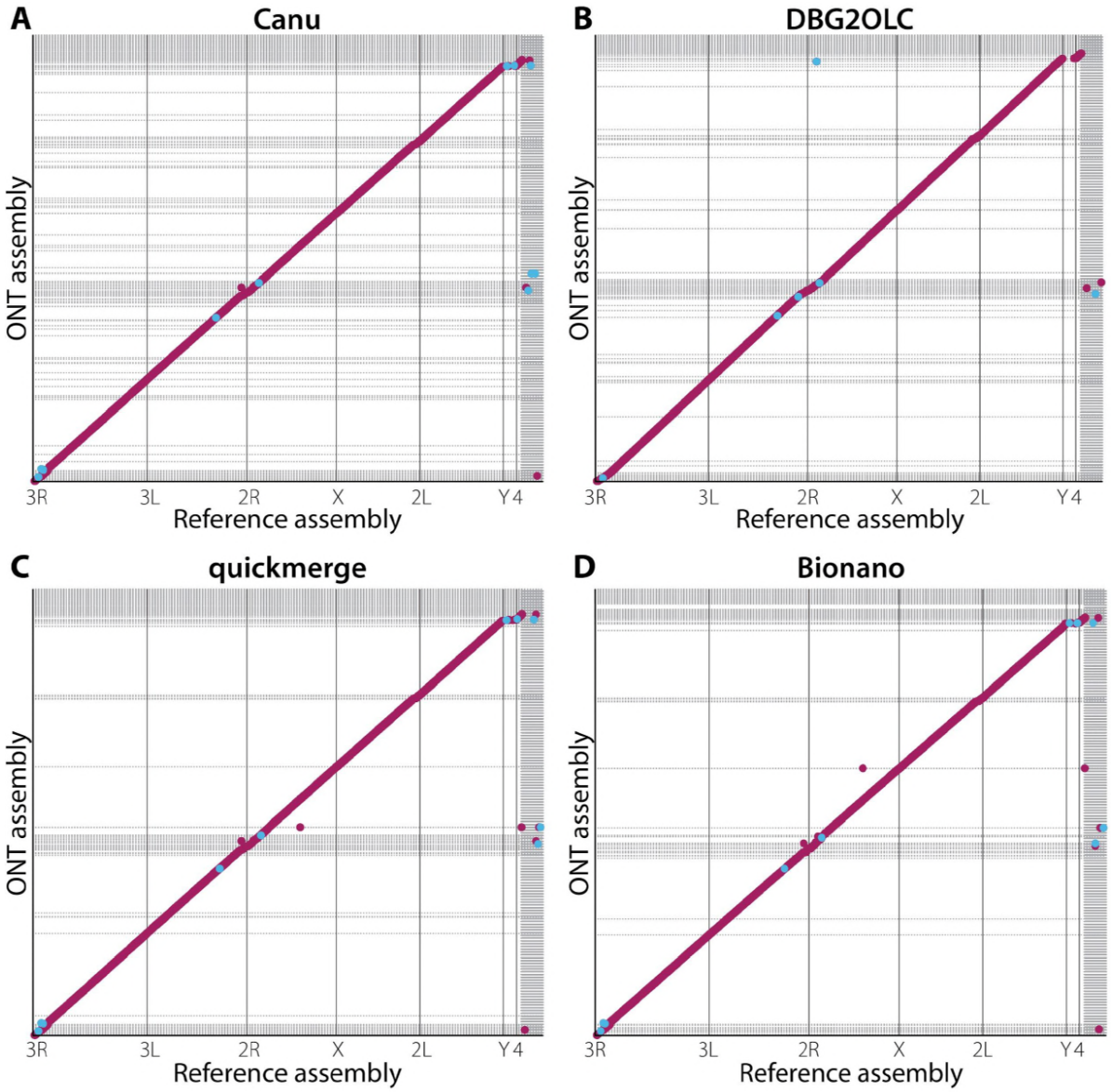

Figure 3. Dot plots showing colinearity of our assembled genomes with the current version of the *D. melanogaster* reference genome. Red dots represent regions where the assembly and the reference aligned in the same orientation, blue dots represent regions where the genomes are inverted with respect to one another. (**A**) Plot of the Canu-only assembly against the reference genome. (**B**) Plot of the hybrid DBG2OLC Nanopore and Illumina assembly against the reference. (**C**) Plot of merged DBG2OLC and Canu assemblies showing a more contiguous assembly than either of the component assemblies. (**D**) Bionano scaffolding of the merged assembly resolves additional gaps in the merged assembly.

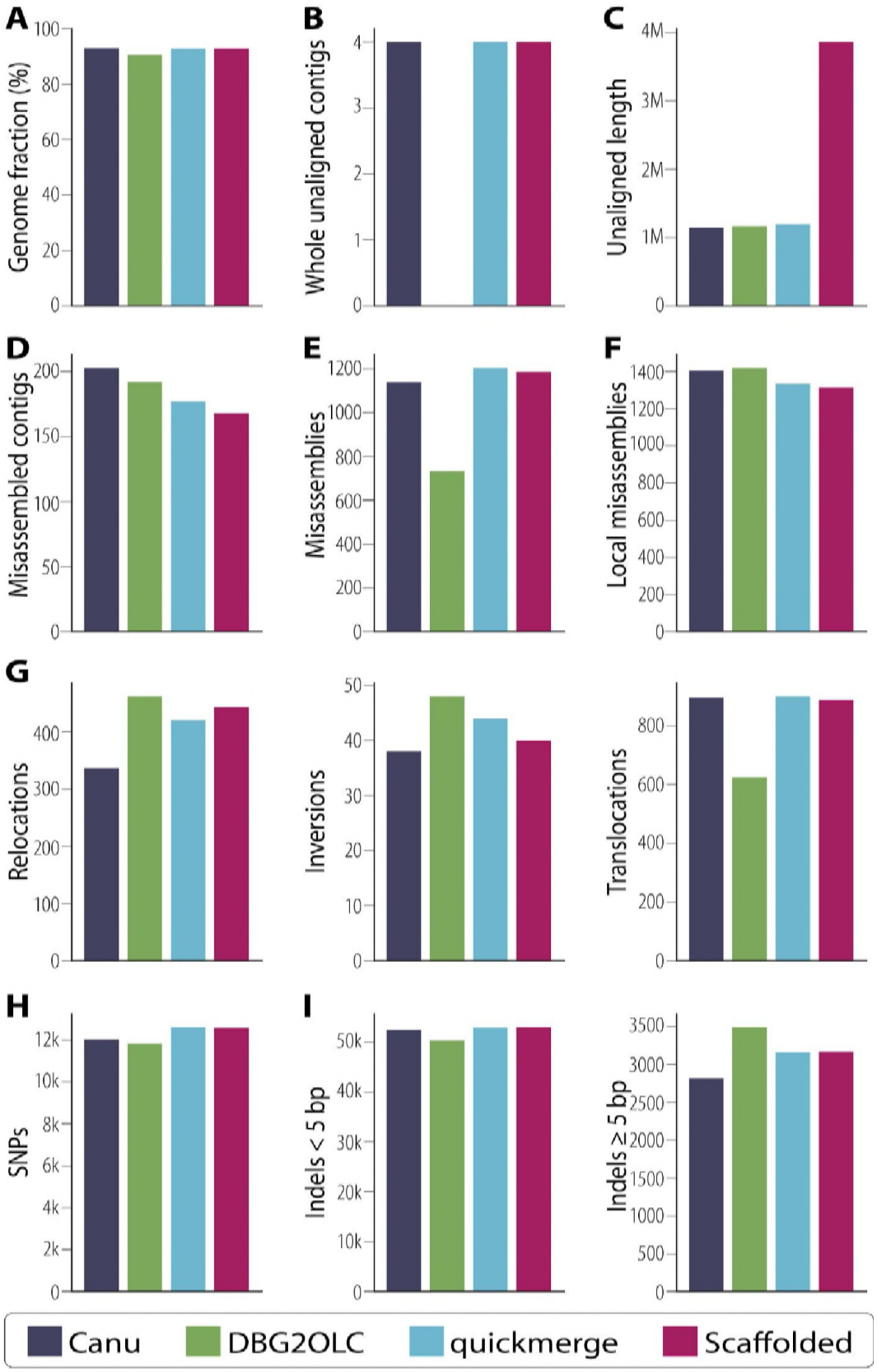

Figure 4. QUAST was used to compare each assembly to the *D. melanogaster* reference genome with selected statistics presented here. (**A**) Greater than 90% of bases in the reference genome were aligned to each of our four assemblies. (**B**) Four contigs failed to align to the reference genome for all assemblies except the DBG2OLC assembly. (**C**) Total unaligned length includes contigs that did not align to the reference as well as unaligned sequence of partially aligned contigs. (**D**) The number of contigs that contain misassemblies in which flanking sequences are ≥1 kb apart, overlap by ≥1 kb, or align to different reference scaffolds. As assembly contiguity increases, the number of misassembled contigs decreases. (**E**) Total count of misassemblies as described in (D). (**F**) Local misassemblies include those positions in which a gap or overlap between flanking sequence is <1 kb and larger than the maximum indel length of 85 bp on the same reference genome scaffold. (**G**) Misassemblies can be subdivided into relocations (a single assembled contig aligns to the same reference scaffold but in pieces at least 1 kb apart), inversions (at least one part of a single assembled contig aligns to the reference in an inverted orientation), or translocations (at least one part of a single assembled contig aligns to two different reference scaffolds). Not all misassemblies are captured in these three categories. (**H**) Total SNPs per assembly are shown and were not significantly different among assemblies. (**I**) Indels per 100 kb can be divided into small (<5 bp) and large (≥ 5bp) indels. Indels >85 bp are considered misassemblies and are shown in panels D, E, or F. Indel numbers were not markedly different using different assembly strategies, suggesting that this variation is real and not an artifact of the assembly.

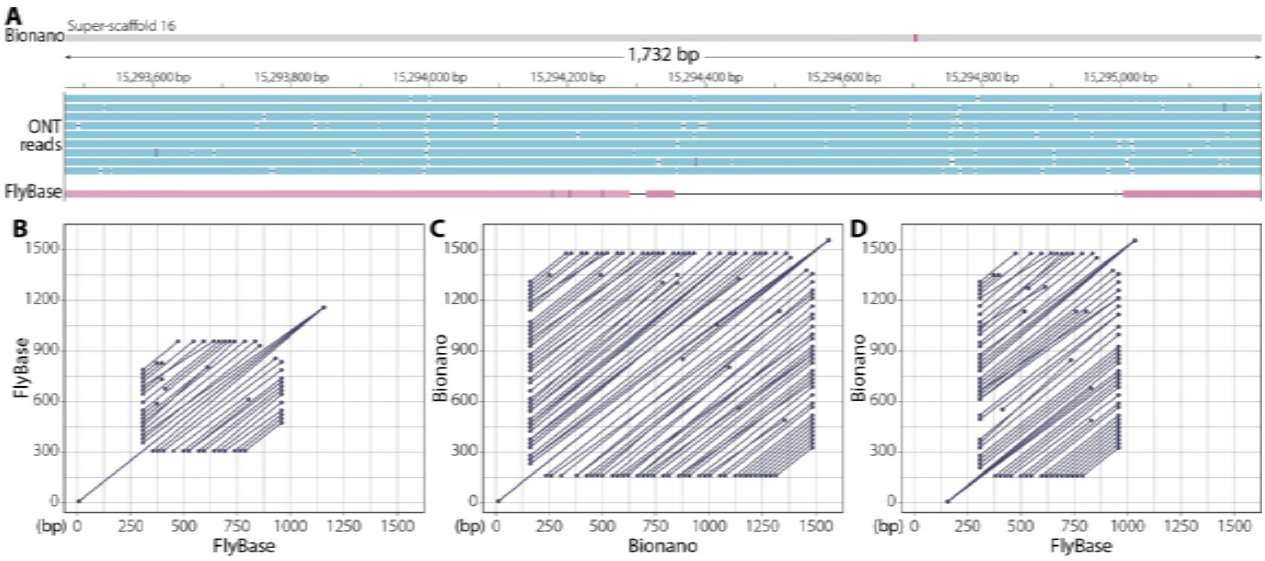

Figure 5. Copy number increase in a 207-bp tandem array located inside the third exon of *Muc26B*. (**A**) Three tracks showing Bionano assembly (gray) with Nanopore long reads (blue) and reference (FlyBase) assembly (red) aligned to it. The alignment gap in the reference assembly is due to the extra sequence copies in the Bionano assembly. (**B**) Alignment dot plot between the reference sequence possessing the tandem array to itself. (**C**) Alignment dot plot between the genomic region possessing the tandem array in the Bionano assembly to itself. As evidenced by the dot plot, the Bionano assembly has more repeats in this region than the reference assembly in panel (B). (**D**) Alignment dot plot between the reference genomic region (*x* axis) shown in (B) and the corresponding Bionano genomic region (*y* axis) shown in (C).

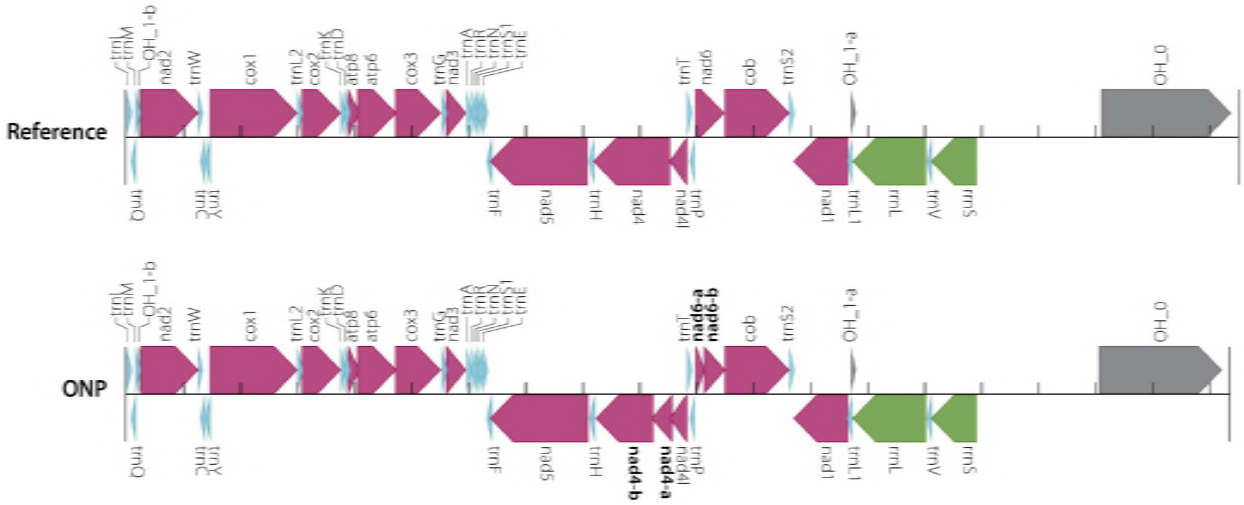

Figure 6. Mitochondrial genome annotations generated by MITOS. (**A**) Annotation of the reference mitochondrial genome. (**B**) Annotation of the mitochondrial genome assembled in this project is identical to the reference with two exceptions: *nad4* was annotated as *nad4-a* and *nad4-b*, while *nad6* was annotated as *nad6-a* and *nad6-b*.

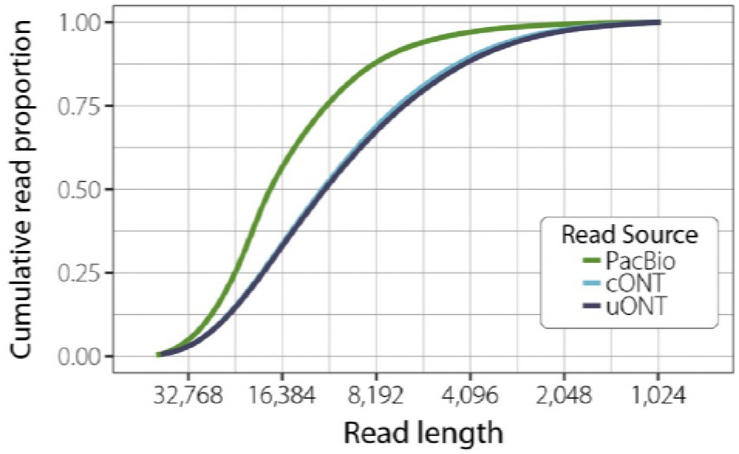

Figure S1. Proportion of corrected (cONT) and uncorrected (uONT) reads >1 kb used in this study compared to PacBio data collected for Chakraborty *et al.* (2018).

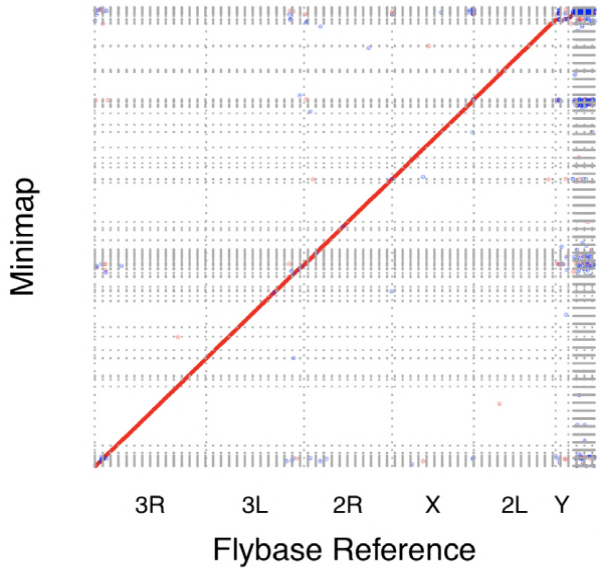

Figure S2. Dot plot of minimap2 assembly (*y* axis) compared to *D. melanogaster* reference assembly (*x* axis).

TABLES

Table 1. Statistics of reads used for genome assembly.*

Total Reads	593,354
Avg Read Length	7,122
Total Bases Sequenced	4,184,159,334
Genome Coverage	30.2x
Reads > 1Kb	530,466
Genome Coverage in Reads > 1Kb	29.9x
Reads > 10Kb	145,634
Genome Coverage in reads > 10Kb	17.5x

*Only reads with quality scores ≥ 7 were used. A genome size of 140 Mb was used for all calculations.

Table 2. Genome assembly statistics.

Name	Genome Size	Contigs	Largest Contig	N50	L50
FlyBase r6.15*	142,573,024	2442	27,905,053	21,485,538	3
MiniMap	131,856,353	208	16,991,501	3,866,686	9
DBG2OLC	131,359,678	339	13,129,070	9,907,720	6
Canu	139,205,737	295	14,326,064	2,971,262	11
QuickMerge 2x	138,130,519	250	25,434,901	18,616,266	4
QM2x Nanopolish	139,303,903	250	25,367,201	18,818,677	4
QM2x NP + Pilon x2	140,153,080	250	25,783,280	18,923,871	4
QuickMerge 2x Bionano	142,817,829	231	28,580,427	21,305,147	3

*Values are for scaffolds, not contigs.

Table 3. BUSCO scores demonstrating genome quality before or after polishing.

Name	Single Copy	Duplicate	Fragmented	Missing	Complete
FlyBase r6.15	2749 (98.2%)	14 (0.5%)	22 (0.8%)	14	2763
MiniMap	14 (0.5%)	0 (0.0%)	31 (1.1%)	2754	14
DBG2OLC	1332 (47.6%)	3 (0.1%)	557 (19.9%)	907	1335
Canu	1884 (67.3%)	11 (0.2%)	557 (19.9%)	347	1895
QuickMerge (QM) 2x	1623 (58.0%)	6 (0.3%)	560 (20.0%)	610	1629
QM 2x Nanopolish (NP)	2189 (78.2%)	8 (0.3%)	400 (14.3%)	202	2197
QM 2x NP + Pilon x2	2726 (97.4%)	14 (0.5%)	39 (1.6%)	20	2740
QM 2x Pilon x2	2718 (97.1%)	14 (0.5%)	45 (1.4%)	22	2732
QM 2x Bionano	2715 (97.0%)	15 (0.5%)	40 (1.4%)	29	2730
QM 2x Bionano All	2720 (97.2%)	16 (0.6%)	40 (1.4%)	23	2736

Table S1. Detailed QUAST output for the Canu, DBG2OLC, Quickmerge, and Bionano assemblies.

Table S2. Detailed position information for SVs identified in this report.

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