Optimizing experimental design for genome sequencing and assembly with Oxford Nanopore Technologies

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Abstract (250 words)

Background

High quality reference genome sequences are the core of modern genomics. Oxford Nanopore Technologies (ONT) produces inexpensive DNA sequences in excess of 100,000 nucleotides but high error rates make sequence assembly and analysis a non-trivial problem as genome size and complexity increases. To date there has been no comprehensive attempt to generate robust experimental design for ONT genome sequencing and assembly. In this study, we simulate ONT and Illumina DNA sequence reads for the model organisms *Escherichia coli*, *Caenorhabditis elegans*, *Arabidopsis thaliana,* and *Drosophila melanogaster* and assemble with Canu, Flye, and MaSuRCA software to quantify the influence of sequencing coverage and assembly approach. Heterozygosity in outbred eukaryotes is a common problem for genome assembly. We show broad applicability of our methods using real ONT data generated for four strains of the highly heterozygous nematode *Caenorhabditis remanei* and *C. latens*.

Results

- ONT libraries have a unique error structure and high sequence depth is necessary to assemble contiguous genome sequences.
- As sequence depth increases errors accumulate and assembly statistics plateau.

- High-quality assembled sequences require a combination of experimental techniques that increase sequence read length and computational protocols that reduce error through correction, read selection and 'polishing' with higher accuracy short sequence reads.
- Our robust experimental design results in highly contiguous and accurate genome assemblies for the four strains of *C. remanei* and *C. latens*.

Conclusions

ONT sequencing is inexpensive and accessible but the technology's error structure requires robust experimental design. Our quantitative results will be helpful for a broad array of researchers seeking guidance for *de novo* assembly projects.

Background

Many factors affect the quality of a *de novo* genome assembly. Genome size increases the size of the "puzzle" to put together, while the size of the pieces (sequence reads) remains the same. Repetitive regions and mobile genetic elements create unique challenges as they are present in more than one location in the genome and without contextual information it is difficult to identify how many copies exist in the complete genome. For example, *Alu* repeat elements reach >1 million copies in the human genome [1].

Third generation sequencing technologies can theoretically solve these problems. The long sequence reads span repetitive regions, potentially allowing for the identification of the exact size and location of repeats on a chromosome. Long sequence reads increase the puzzle piece size for assembly and require less sequencing effort to span the entire genome. Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) are the current front-runners in long-read sequencing platforms; both are capable of average read lengths in the tens of thousands

47 of base pairs and, theoretically, entire chromosomes can be sequenced in a single read [2, 3].
48 Both are also capable of producing quality genome assemblies with reasonable amounts of data
49 [4].

50 ONT offers several advantages over PacBio. Nanopore sequencing relies on running
51 molecular fragments through engineered nanopores and recording the resulting alterations in
52 electrical current. The technology is versatile and can be used for DNA sequencing, mRNA
53 sequencing, amplification-free mRNA quantification [5], and measuring DNA base
54 modifications like methylation [6, 7]. ONT libraries can be readily prepared with low amounts of
55 input DNA, which is an important consideration when studying organisms that are small or
56 difficult to sample. ONT platforms are inexpensive and designed to be used in individual
57 research laboratories and classrooms [8]. For these reasons we have chosen to study ONT and
58 quantify how this inexpensive, accessible technology may be best utilized to produce high-
59 quality assembled reference genome sequences. Similar projects analyzed experimental design
60 for PacBio [9, 10] but the few benchmarking studies for ONT sequencing and assembly are
61 carried out on a narrow scope of organisms [11-13]

62 ONT sequence reads present unique challenges for genome sequence assembly. DNA
63 molecules do not move through protein nanopores at a constant rate and changes in current are a
64 composite signature reflecting 3-5 nucleotides occupying the nanopore (for R9.4.1 flow cells).
65 The signal processing has trouble detecting changes in current with homopolymers (single
66 nucleotide repeats, for example AAAAAA), short tandem repeats and heavily methylated sites
67 [14, 15]. As a result, ONT sequence reads contain small nucleotide stretches that have been
68 incorrectly identified, inserted and deleted (Fig. 1A, C). This error structure results in relatively

69 few large, contiguous stretches of correctly identified nucleotides (Fig. 1B, D) and is uniquely
70 challenging for assembly algorithms.

71 To achieve our results we simulate ONT DNA libraries for the model organisms
72 *Escherichia coli*, *Caenorhabditis elegans*, *Arabidopsis thaliana,* and *Drosophila melanogaster*.
73 For each organism we assemble sequence read sets at different depths (i.e., the average times a
74 nucleotide in the genome is sequenced). We measure the contiguity, completeness, and accuracy
75 of each assembled sequence relative to the current reference genome. Many *de novo* assembly
76 projects target organisms without reference genomes and we also measure the identification of a
77 set of genes thought to be found in single copy in each organism. Pure ONT datasets result in
78 superior assembled genome sequences but can be cost-prohibitive for large genomes where
79 multiple flow cells may be necessary to gather enough data. We also analyze assembled
80 sequences from 'hybrid' DNA read datasets composed of ONT and Illumina sequence reads.

81 For eukaryotes both ploidy and breeding system can influence the assembly of genome
82 sequence. Polyploidy can create scenarios where many sites in a genome look highly similar to
83 one another, making it tough to place these regions within an assembled genome [16, 17]. For
84 non-haploid organisms, there is a potential for heterozygosity and at these sites the effective
85 sequencing coverage is cut in half. For small, non-clonal metazoans sufficient DNA for
86 sequencing cannot be acquired from a single individual. DNA must be extracted from pools of
87 individuals, and for outbreeding organisms there may be substantial individual diversity within
88 the pool. Here, we use results from our simulated sequences to guide experimental ONT
89 sequencing and assembly for four strains of the highly heterozygous *Caenorhabditis remanei* and
90 *C. latens*. We present the experimental and computational protocols that result in high quality
91 assembled genome sequences.

We find that ONT approaches can produce contiguous assembled sequences for the organisms we study with relatively high sequencing coverage of >60x, well above current recommendations [18]. Pure ONT sequencing and assembly outperforms our tested hybrid approach. We find that contiguous assemblies cannot be achieved by solely increasing ONT sequencing depth as errors accumulate and assembly statistics plateau. Pre-assembly filtering and read correction improve contiguity, and post-assembly polishing using short Illumina DNA sequence reads increases accuracy. We also find that the use of Illumina data, even at very low sequencing depths, increases accuracy through iterative polishing. Our results demonstrate that rigorous experimental design can improve our ability to infer a genome sequence and reduce the effort and cost required.

Data Description

Simulated sequence libraries

We obtained ONT sequences (R9 chemistry) from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) on February 2, 2019 [19]. The *E. coli* dataset contained 11,652,330 sequenced bases in 120,151 reads (~2.5x coverage of the 4.64Mb reference sequence), the *C. elegans* dataset contained 8,860,671,330 sequenced bases in 583,466 reads (~87.9x coverage of the 100.8Mb reference sequence), the *A. thaliana* dataset contained 3,421,779,258 sequenced bases in 300,071 reads (~25.22x coverage of the 135.67Mb reference sequence), and the *D. melanogaster* dataset contained 4,617,842,308 sequenced bases in 663,784 reads (~32.39x coverage of the 142.57Mb reference sequence). We obtained the reference genome sequence for *E. coli* strain K12_MG1655 from NCBI; all other reference genome

114 sequences were obtained from Ensembl (Release 95). Accession numbers are provided under
115 'Resource Availability.'
116 We simulated 150bp paired-end Illumina DNA libraries with the software ART [20] and
117 ONT DNA libraries with the software NanoSim (version 2.0.0; [21]). Both software programs
118 utilize an assembled sequence to generate simulated libraries with read profiles similar to an
119 empirically generated DNA library. NanoSim additionally requires real ONT flowcell data to
120 simulate unique, organism-specific mismatch, insertion and deletion rates. ART libraries were
121 simulated to a coverage of 300x with a fragment standard deviation of 50. NanoSim libraries
122 mirror the characteristics of the real ONT flowcells including read length distributions. We used
123 NanoSim to simulate 500,000 *E. coli* reads (943x coverage; read N50 8,158), 2,000,000 *C.*
124 *elegans* reads (336x coverage; N50 21,559), 5,000,000 *A. thaliana* reads (420x coverage; N50
125 19,577) and 10,000,000 *D. melanogaster* reads (219x coverage; N50 11,955). The sequence read
126 N50 indicates 50% of the total sequenced nucleotides are in reads that long or longer.
127
128 C. remanei *sequence libraries*
129 Output for each flow cell used for each strain is summarized in STable 1. Based on an estimated
130 genome size of 125Mb [22] we generated 76x-156x coverage of each strain with sequence read
131 N50 8,203-22,891bp.
132
133 **Genome assembly**
134 *Assembly of Simulated Data*
135 Genome sequences were assembled using three approaches. We first assembled the simulated
136 ONT read sets with Canu v1.9 [18]. Each genome was assembled at decreasing coverage depths

137 until the assembler failed to produce an assembly with the given data. In order to minimize the
138 influence of individual reads and stochastic assembly artifacts, each read set was generated by
139 selecting a random subset of the full simulated dataset. The second approach used the same
140 subsets of ONT data, but with Flye 2.8 [23] as the assembly software.

141 The long-read datasets that performed the best were paired with 2x150 simulated paired-
142 end Illumina data and assembled using MaSuRCA version 3.3.9 [24]; coverage depths were
143 adjusted for both datasets to better understand the effects of increasing or decreasing coverage on
144 the final assembly. Here, we retained the ONT dataset to maximize our ability to draw parallels
145 between assembly approaches. For example, the minimum *C. elegans* ONT dataset that
146 assembled with Canu [18] was an average of ~60x coverage across the genome and this read set
147 was used in the MaSuRCA trials with 50x and 100x Illumina coverage, respectively.

148 We also used Flye [23] to assemble read sets that had been corrected using the 'canu –
149 correct' function. Canu correction uses all-versus-all overlap information to correct each
150 individual read [18]. In addition to the consensus information, Canu employs two filters to avoid
151 biases due to sequence quality or repeats. From read-length estimates, Canu uses the longest
152 available reads up to the user-specified coverage depth for correction [18]. By default, this is set
153 to 40x coverage. Assembling these datasets with both Canu and Flye allowed us to compare the
154 effectiveness of the error correction modules within each software package. We also assembled
155 libraries with reads selected by the Canu correction module but not subject to correction to test
156 the influence of error correction vs. read length on assembly.

157 The most contiguous assemblies from the long-read only and hybrid categories were error
158 corrected using Pilon version 1.23 [25] to determine the effect of short-read polishing on the
159 accuracy of the draft assemblies. Each simulated assembled sequence was polished with the

entire simulated paired-end data set. Four rounds of polishing were completed for each assembly with statistics measured after each round with QUAST v5.0.2 [26] and BUSCO v4.0.3 [27].

Assembly of ONT Nematode Data

Preliminary assemblies were created for each strain by first correcting the complete library with canu –correct (Canu v2.0) [18]. Corrected reads from Canu were then assembled using Flye 2.8 [23]. The draft assembly for each strain was polished using Pilon v1.23 [28] and Illumina paired-end reads. All data sets contained a small sub-set of contaminants from the nematode microbiome that were removed from the final assemblies [29, 30].

Analyses

Assessing the quality of assembled simulated sequences

We measured genome statistics relative to the reference sequence of each organism with QUAST [26]. We assessed contiguity and accuracy of the assembled sequence through eight statistics:

(1) **NG50** is a size median statistic and indicates that 50% of the expected assembled genome sequence (where the expectation is based on a known reference) is contained in contiguous sequences that large or larger.

(2) **NGA50** is a similar size median but indicates that 50% of the expected assembled genome sequence that aligns to the reference genome is contained in contiguous sequences that large or larger.

(3) **LG50** is the number of linkage groups or contiguous assembled sequences containing 50% of the expected assembled genome sequence.

(4) **LGA50** is similar but again measured in the portion of the assembled sequence that aligns to the reference genome.

183 (5) **Genome fraction (%)** is the fraction of reference genome captured in the assembled
184 sequence.
185 (6) **Duplication** measures the fraction of reference genome found multiple times in the
186 assembled sequence.
187 (7) **Mismatches** is the number of mismatched nucleotides per 100,000 nucleotides or base pairs
188 (bp).
189 (8) **Indels** are the number of insertions and/or deletions per 100,000 nucleotides.
190 For our metazoan organisms we also used the software package BUSCO (version 4.0.3;
191 released February 2020) to search for a set of unique genes expected to be conserved in single
192 copy in an evolutionarily related group of organisms [27]. Below, we describe the analyses of
193 each organismal dataset.
194
195 *Escherichia coli*
196 The *E. coli* genome is relatively small at 4.64Mbp and contains few repeats (87.8% of the
197 genome codes for proteins; [31]). Assembly of 50,000 ONT sequences (~62x coverage; N50
198 8,350bp) with Canu [18] and Flye [23] resulted in circular contigs with high contiguity and few
199 mismatches, insertions, and deletions (Fig. 2A-D). At low ONT coverage (~19x) the assemblies
200 produced by Canu and Flye were contained in 6 and 9 contigs, respectively. Both assembled
201 sequences had high rates of mismatches, insertions and deletions when compared with sequences
202 assembled from high coverage libraries (Fig. 2C-D). Increasing ONT coverage beyond 62x
203 resulted in poor contiguity for Canu assembled sequences (Fig. 2A) and increased mismatches
204 for Flye assembled sequences (Fig. 2C). Assembly of hybrid Illumina-ONT libraries with
205 MaSuRCA [24] resulted in sequences with variable contiguity and accuracy. Two of the tested

206 hybrid sets were able to assemble the genome into a single contig but the library with the highest
207 Illumina coverage (300x) and highest ONT coverage (227x) assembled into two contigs.
208 MaSuRCA was unable to perform as well as either Canu or Flye when given the same long-read
209 dataset. For instance, the top performing Canu and Flye runs used 50,000 ONT reads (~62x
210 coverage); the same reads passed through MaSuRCA produced 2 contigs, regardless of Illumina
211 coverage (Fig. 2B).
212 After polishing the assemblies with Pilon [25], the Canu-assembled sequence had fewer
213 mismatches and indels per 100 Kbp than the Flye assembled sequence or an assembly produced
214 by Flye using reads that had first been corrected with Canu (SFig. 1A-C). Despite its dependence
215 on higher accuracy Illumina sequences the MaSuRCA assembled sequence had higher numbers
216 of mismatches, insertions and deletions both pre- and post-polishing (SFig 1B). We used
217 MaSuRCA to assemble a set of paired end Illumina sequences to test the influence of adding
218 ONT data to the assembly process. The resulting sequence was 98.76% of the *E. coli* genome
219 and was fragmented into 74 contiguous pieces. The accuracy, 1.7 mismatches and 0.04 indels per
220 100 Kbp, was improved when compared with the hybrid assembled sequences. This indicates
221 that the inclusion of ONT data can introduce errors in hybrid approaches that cannot be corrected
222 with post-assembly polishing.
223
224 *Caenorhabditis elegans*
225 The *C. elegans* genome is 100.8 Mbp [32] contained in a single X chromosome and five
226 autosomes [33]. *C. elegans* is a diploid self-fertile hermaphrodite with low levels of genetic
227 diversity [34] and approximately 16% of the genome is repetitive [35]. Canu, Flye, and
228 MaSuRCA assembled sequences plateaued in contiguity and accuracy above ~70x coverage

229 (Fig. 3A-D). The read N50 for these datasets ranged from 20,742 to 22,840. Both Canu and Flye
230 assembled chromosome-scale contigs with low rates of mismatches, insertions and deletions.
231 The MaSuRCA [24] hybrid assembly approach did not perform as well, even with high ONT
232 coverage (Fig. 3A,B).

233 To improve these assemblies we experimented with polishing the Canu and Flye
234 assembled sequences, Additionally, we used Flye to assemble the Canu corrected dataset
235 ("Corrected" in Fig. 4) and used Flye to assemble the reads selected by the Canu correction
236 module but not corrected ("Selected" in Fig. 4). The dataset corrected with Canu and assembled
237 with Flye had a read N50 of 28,641bp and produced 6 chromosome-scale contigs with low
238 numbers of mismatches, insertions and deletions that further decreased after polishing (Fig. 4A-
239 D). Flye assembly of the same dataset prior to correction resulted in an assembly with higher
240 fragmentation (Fig. 4B) and lower accuracy (Fig. 4C-D). This dataset had a read N50 of
241 29,404bp, indicating that both read length and correction contribute to assembly quality. The
242 assembly graph for the Canu corrected, Flye assembled dataset (Fig. 5) shows 6 chromosome-
243 scale contigs with smaller disjunct assembled sequences [36].

244 We used QUAST [26] to identify annotated features in our assembled sequences to
245 compare each software's ability to assemble different classes of genomic elements. We used the
246 WS277 annotations provided by WormBase Parasite and identified exons, introns, pseudogenes,
247 repeats, small RNAs, transposable elements (TEs) and untranslated regions (UTRs). The worst
248 performing assembly had >99.67% representation of exons, introns, small RNAs and UTRs and
249 >98.85% representation of repetitive sequences like pseudogenes, repeats and TEs (Table 1). The
250 Flye assembled sequences had the best representation of exons, introns, small RNAs and UTRs

251 while the Canu assembled sequences had the best representation of repetitive pseudogenes,
252 repeats and TEs.
253
254 *Drosophila melanogaster*
255 The 180Mb genome of the diploid fruitfly *D. melanogaster* presents multiple challenges for
256 sequencing and assembly including approximately 60Mb of repetitive heterochromatin [37]. The
257 genome sequence is contained in a large X chromosome, a small ("dot") Y chromosome and 3
258 autosomes. Assemblies of the simulated ONT *D. melanogaster* libraries repeated patterns seen
259 with the *C. elegans* dataset; the most contiguous Canu assembly was produced with ~113x ONT
260 coverage and a read N50 of 11,679 (~16Gbp of data; Fig. 6A,B). This assembly produced 145
261 contiguous pieces but many of these were small and the LGA50 was 4 contigs (Fig. 6B). The
262 Flye assembly of the same dataset was much less contiguous, producing 482 contigs. When the
263 dataset was corrected with Canu then assembled with Flye the assembled sequences had high
264 accuracy (SFig. 2).
265 The top performing Canu assembly contained 91% of the metazoan genes expected to be
266 conserved in single copy with BUSCO [27] prior to polishing and 94% after four rounds of Pilon
267 with paired-end reads (SFig. 3). In comparison, the *D. melanogaster* reference sequence contains
268 94.1% of the expected single copy metazoan genes. Additional data decreased contiguity with a
269 slight increase in accuracy and NGA50 (Fig. 6 A,D). Hybrid MaSuRCA [24] assemblies for *D.*
270 *melanogaster* performed markedly worse than Canu assemblies of ONT data (Fig. 6B). The top
271 performing MaSuRCA assembly produced 220 contigs with 93.9% of the expected metazoan
272 genes identified [27].
273

274 *Arabidopsis thaliana*

275 The 135Mbp genome sequence of the self-fertile plant *A. thaliana* is contained in 5 autosomes
276 [38]. *A. thaliana* has undergone at least two rounds of whole genome duplication [39] and
277 contains large tracts of highly similar genomic regions. Assembly of the ONT libraries with
278 Canu [18] and Flye [23] resulted in sequences with lower contiguity and higher fragmentation
279 when compared with the other model organisms, even when the library contained ~420x
280 coverage and a read N50 of 19,577bp (56.7Gbp of sequenced nucleotides). Canu produced 30
281 contiguous pieces (LGA50 5 chromosomes) with 98.8% of the expected Viridiplantae genes
282 (Supplementary Data) identified prior to polishing [27] and Flye produced 26 contigs and 98.8%
283 of the expected Viridiplantae genes. The Flye assembly contained many more mismatches and
284 indels than its Canu counterpart, but this discrepancy was alleviated with Pilon polishing (SFig.
285 5). Following polishing with Pilon [25], the Flye assembled sequences contained 99.1% of the
286 expected Viridiplantae genes [27], matching that of the TAIR10 reference genome for *A.*
287 *thaliana*. Interestingly, our combined approach of long-read methods did not work with *A.*
288 *thaliana* datasets and neither Flye nor Canu was able to assemble a draft genome with <40x
289 coverage.

290 The hybrid MaSuRCA assemblies for *A. thaliana* performed the best of the three
291 eukaryotes. The top performing MaSuRCA [24] assembly produced 45 contiguous pieces with
292 98.8% of the expected Viridiplantae genes identified in the sequence [27]. This was also brought
293 up to 99.1% after polishing with Pilon (Supplementary Data).

294

295 *Caenorhabditis remanei* and *Caenorhabditis latens*

296 We used our simulated data to create a protocol for *de novo* sequencing and assembly for 3
297 strains of the nematode *C. remanei* [40] and one of the closely related nematode *C. latens* [41].
298 Both *C. remanei* and *C. latens* are obligate outcrossing species with high levels of nucleotide
299 diversity [42] that have hobbled previous assembly attempts [35, 43]. The worm's small size
300 means that pools of individuals harboring both individual and population-level diversity must be
301 sacrificed for DNA sequencing and assembly. We prepared high-molecular-weight DNA extracts
302 and generated ONT libraries for an outbred *C. remanei* strain EM464, two inbred laboratory
303 strains PX356 and PX439 and the inbred laboratory *C. latens* strain PX534. We tested library
304 preparation and computational protocols with the inbred *C. remanei* strain PX356 and used these
305 to generate assembled sequences for the other *C. remanei* and *C. latens* strains.

306 The most contiguous *C. remanei* PX356 assembly was achieved by using Canu [18] to
307 correct the entire ~156x coverage ONT dataset prior to assembly with Flye [23]. After
308 correction, the ~40x coverage dataset had a read N50 of 21,340bp and resulted in an assembled
309 sequence contained in 163 contigs with 89.1% of the expected conserved nematode genes [27].
310 Decontamination with Blobtools2 [29] and SIDR [30] resulted in a final assembly for PX356
311 with 124,499,812bp contained in 70 contigs with an N50 of 6.2Mbp and an N90 of 1.93Mbp
312 contained in just 18 large contigs, approximately 3 per chromosome. We compared our results
313 (Fig. 7A-D) with a recently published sequence for the related *C. remanei* strain PX506 [22]
314 generated with Pacific Biosystems sequencing and chromatin conformation capture to produce
315 chromosome-scale sequences [44].

316 Canu-only and Flye-only assemblies contained more contigs and a smaller N50 than the
317 combined approach described above (Fig. 7A-D). The assembly graph (Fig. 8) shows several
318 large contiguous fragments but also multiple regions difficult to disentangle and smaller disjunct

319 fragments. Analyses with GenomeScope [45] suggested ~0.7% of the genome remains
320 heterozygous (SFigure 6). Assemblies with subsamples of the long-read data were increasingly
321 fragmented with decreasing coverage. Flye assemblies with initial coverages of 102x, 70x, and
322 52x produced 183, 242, and 267 contigs respectively. A MaSuRCA-hybrid approach [24], using
323 102x ONT coverage and 450x paired-end coverage yielded 336 contigs and 96.6% of BUSCO
324 single-copy genes.

325 BUSCO identified 97.7% (increased from 89.1%) of the expected conserved single copy
326 genes following 4 rounds of polishing using Pilon [28] with Illumina paired end reads at ~225x
327 average depth (Fig. 6). We tested the influence of Illumina coverage on polishing and found that
328 97.6% of the expected conserved nematode genes could be identified after polishing with just
329 ~20x Illumina coverage (STable 3), indicating that a large amount of data is not necessary to
330 correct the majority of errors in an assembly. However, this was achieved after three successive
331 rounds of error correction with the Pilon software package [28] utilizing the same Illumina DNA
332 sequence reads. This assembled sequence is less fragmented and contains a higher percentage of
333 conserved nematode genes (Fig. 6) when compared with the previously published assembly for
334 *C. remanei* PX356 produced with Illumina libraries and a genetic linkage map [35].

335 We generated assembled genome sequences for *C. remanei* PX439, *C. remanei* EM464,
336 and *C. latens* PX534 using the 'best practices' protocols developed through simulations and our
337 *C. remanei* PX356 experimentation. Our *C. remanei* PX439 dataset had an ONT coverage depth
338 of ~120x with a read N50 of 18,233bp. After Canu correction the read N50 increased to
339 38,434bp and we assembled a 132,046,643bp genome sequence contained in 53 contigs (N50
340 12.6Mbp). The N90 for this assembled sequence was 2.27Mbp contained in 12 large contigs,
341 approximately 2 per chromosome.

342 *C. remanei* EM464 is outbred and retains high levels of heterozygosity. We did not
343 expect to assemble a cohesive genome sequence but generated ONT libraries and an assembled
344 sequence to identify the distribution of allelic variants in the population. The ONT library had a
345 coverage depth of ~76x and a read N50 of 22,891bp. The Canu corrected dataset used for Flye
346 assembly had an N50 of 34,951bp. Flye produced a 138,626,018bp genome sequence contained
347 in 320 contigs (N50 3.4Mbp). The flow-cytometry estimated genome size of *C. remanei* is 124-
348 131Mbp and we estimate that 5-12% of the assembled sequences are allelic variants. The *C.*
349 *latens* PX534 dataset had a coverage depth of ~119x and a read N50 of 14,759bp. After Canu
350 correction the read N50 of the dataset 34,591bp and we assembled a 120,367,464bp genome
351 sequence in 97 contigs with an N50 of 13.6Mbp.
352
353 **Discussion**
354 We have found that Canu [18] and Flye software packages [23] produce high-quality contiguous
355 draft assemblies from ONT libraries for a broad range of organisms. In many cases, Flye slightly
356 outperforms Canu while being much faster and using fewer computational resources [13, 46],
357 however there are many instances in our results where the best assembly is produced by first
358 error correcting the data with the Canu correction modules, then assembling the corrected reads
359 with Flye. Given the same set of corrected reads, the Flye assembler regularly creates a better
360 assembly than Canu. This finding highlights the need for performing multiple assembly
361 strategies to identify the top performing strategy for each dataset.
362 We found the hybrid assemblies produced by MaSuRCA [24] contained a higher
363 proportion of expected conserved genes when compared with the unpolished long-read only
364 assemblies and had fewer mismatches and insertion/deletion errors. However, given the same

365 amount of ONT data, both Canu [18] and Flye [23] assembled more contiguous genome
366 sequences. The long-read only assemblies also produced larger NG50 values and contained a
367 larger fraction of the expected genome when compared to hybrid MaSuRCA assemblies. The
368 shortfalls of long-read only assembly can be overcome by 'polishing' with low coverage (~20x)
369 Illumina DNA sequences using a software such as Pilon [28] to error correct the draft
370 assemblies. We found that polishing played a larger role in increasing accuracy of the real-world
371 data compared to the results seen with simulated datasets. These methods improved the accuracy
372 of Canu or Flye assemblies to be on par with, or better than, those produced by MaSuRCA.
373 These highly contiguous assemblies were achieved with relatively high sequencing depth,
374 at least 100x coverage across the genome. Although ONT can theoretically produce megabase-
375 sized reads, in reality, many of the sequence reads in 'real' projects are shorter due to
376 fragmentation that occurs during DNA purification and library preparation. ONT libraries may
377 have many more small reads and >100x 'real' coverage may be necessary to achieve >40x
378 coverage with reads >25 Kbp and highly contiguous assemblies. Increasing read N50 in a library
379 through improvement of DNA isolation and library preparation methods reduces the necessary
380 coverage to produce a high-quality assembly [47]. Therefore, researchers should adjust their
381 coverage goals based on the quality of libraries they are able to reliably produce.
382 Our findings with simulated datasets were supported by all real-world datasets tested.
383 *Caenorhabditis remanei* PX356, PX439 and *C. latens* PX534 all have previously published
384 genome assemblies generated with paired-end Illumina sequences [35]. Our assemblies proved to
385 be at least one order of magnitude more contiguous than any of the previous assemblies,
386 highlighting the ability of ONT long reads to drastically improve contiguity. Outbreeding
387 Caenorhabditis are resistant to inbreeding [48] and previous assembly attempts resulted in over-

assembly due to residual heterozygosity [49] or under-assembly. Despite this, we were able to finalize a highly contiguous and complete assembly for these nematodes. The strains we targeted are closely related and interfertile [50] yet their assembled genome sequences vary up to 10% in length. This level of structural variation between closely related species highlights the need for *de novo* assembled sequences in molecular evolution research.

Our *de novo* assembled *Caenorhabditis* sequences are contiguous and achieved BUSCO scores greater than those of Illumina-only assembled sequences (STable 4). The findings we present here clearly demonstrate existing genome sequences can be improved by adding in ONT datasets. As sequencing and assembly technologies continue to improve and drop in cost, many researchers will find published datasets can be updated to further improve downstream analyses.

Potential Implications

In light of our findings, we suggest that long-read data be prioritized when undertaking *de novo* genome assembly projects. Our results indicate that an assembly with sufficient ONT long read coverage will be highly contiguous and polishing with Illumina data can achieve high levels of accuracy. For situations where high-coverage ONT libraries are not feasible, MaSuRCA-assembled [24] Illumina and ONT read sets can produce reliable draft sequences. However, the quality and contiguity of the assembled sequence is determined by Illumina read depth and effort should be made to increase Illumina read depth, even if it is at the expense of ONT sequences. MaSuRCA-assembled Illumina sequences have fewer mismatches and insertion/deletion errors when compared with MaSuRCA-assembled ONT and Illumina hybrid read sets, indicating that the inclusion of ONT sequences introduces errors. We suggest that error correction with Illumina

410 DNA sequences and the Pilon software package [25] is a necessary finishing step in any
411 assembly project utilizing ONT data.
412 Our results demonstrate that near-chromosome-level genome sequences are achievable
413 with sufficient ONT data. However, chromosome-level genome assemblies are often not
414 necessary to address many research questions, particularly those focused on small numbers of
415 genes or phylogenomic information. Researchers should approach genome sequencing by first
416 determining what genome-completion level will be sufficient for their research goals. To aid in
417 this approach, we hope our study will help researchers determine the amount of sequencing
418 effort, and the sequencing approaches, that will best suit their needs.
419
420 **Resource Availability**
421 *Lead Contact:*
422 Janna L. Fierst: janna.l.fierst@ua.edu
423 *Materials Availability:*
424 This study did not generate new unique reagents.
425 *Data and Code Availability:*
426 *E. coli* genome sequence GCF_000005845.2; ONT read set SRR8154670
427 *C. elegans* genome sequence GCA_000002985.3; ONT read set ERR2092776
428 *A. thaliana* genome sequence GCA_000001735.1; ONT read set ERR2173373
429 *D. melanogaster* genome sequence GCA_000001215.4; ONT read set SRR6702603
430 *C. remanei* PX356 BioProject PRJNA248909
431 *C. remanei* PX439 BioProject PRJNA248911
432 *C. remanei* EM464 PRJNA562722

433 *C. latens* PX534 BioProject PRJNA248912

434 Full datasets are available at https://doi.org/10.5061/dryad.3r2280gd2

435 Bioinformatic scripts and workflows are available at

436 https://github.com/jmsutton2

437

438 **Methods**

439 *DNA Extraction and Sequencing*

440 Nematodes used for genomic sequencing were grown on two 100mm NGM plates [51]

441 seeded with *E. coli* OP50. Worms were harvested by washing plates with M9 minimal media

442 into 15mL conical tubes. Samples were rocked on a tabletop rocker for 1 hour before being

443 centrifuged to pellet worms. The supernatant was removed and tubes refilled with sterile M9,

444 mixed and pelleted by centrifugation again. This process was repeated five times or until the

445 supernatant was clear after centrifugation. The pellet was moved to 2mL tubes and frozen at –

446 20°C until extraction. Worm pellets were allowed to thaw to room-temperature then flash frozen

447 in liquid nitrogen and repeated three times. Worm pellets were placed in 1.2mL of lysis buffer

448 solution (100mM EDTA, 50mM Tris, and 1%SDS) and 20µL of Proteinase K (100mg/mL).

449 Tubes were then placed on a 56°C heat block for 30 minutes with shaking. Genomic DNA was

450 then isolated using a modified phenol-chloroform extraction[52]. DNA concentrations and purity

451 were measured with a Qubit 4 (Life Technologies, Carlsbad, CA USA) and NanoDrop® 1000

452 spectrophotometer, respectively (Thermo Fischer Scientific, Waltham, MA USA). Extracts were

453 visualized on a 0.8% agarose gel to verify high-molecular weight gDNA. DNA size selection

454 was carried out using the Short Read Eliminator Kit from Circulomics Inc. according to

455 manufacturer guidelines (Baltimore, MD USA).

456 DNA libraries were prepared for each sample using the SQK-LSK109 ligation
457 sequencing kit and loaded on to R9.4.1 RevD flow cells. The recommended protocol from ONT
458 was modified by replacing the first AmpureXP bead clean step with an additional treatment with
459 the Short Read Eliminator Kit. Approximately 700ng of gDNA from each library was loaded on
460 to a flow cell sequenced for 48 hours on a GridION X5 platform with basecalling performed by
461 Guppy v.4.0.11. ONT reads for each strain were trimmed of adapters using Porechop
462 (https://github.com/rrwick/Porechop) with the –discard_middle flag to remove chimeric reads.
463
464 *Assembly and Decontamination of Real Datasets*
465 We evaluated contaminants in the *C. remanei* PX356, *C. remanei* PX439, *C. remanei*
466 EM464 and *C. latens* PX534 draft assemblies with Blobtools2 [29] and SIDR [30]. Blobtools2
467 uses phylum-level taxonomic assignment, read coverage depth and GC content to identify non-
468 target contigs within the assembly. To prepare data for visualization with BlobToolKit, the ONT
469 reads were aligned to the initial assemblies using minimap2 v2.17 [53] and sorted using
470 SAMtools v1.8 [54]. A reference database for taxonomic identification of scaffolds was created
471 using blastn 2.2.31 [55] using the nt database from the National Center for Biotechnology
472 Information. An additional database was generated using diamond tblastx [56]. Contigs that were
473 >10,000bp and taxonomically identified as *Nematoda* were retained in the assembly.
474 SIDR [30] utilizes ensemble based machine learning to train a model capable of
475 discriminating target and contaminant contigs based on measured predictor variables. To prepare
476 data for SIDR decontamination Illumina DNA and RNA sequence reads were aligned to the draft
477 assembly with bwa [57] using the BWA-MEM algorithm. We used bbtools/bbmap software [58]
478 to calculate the average sequence coverage, length, GC, bases covered in alignment, RNA

479 average sequence coverage and bases covered in RNA alignment for each contig. We used these
480 data to train a bootstrap aggregated (bagged) decision tree model based on the blast identification
481 of contig origin and used this model to predict the origin, target or contaminant of each contig.
482 SIDR allowed us to assign probable *Nematoda* origin to contigs that lacked blast identification.
483 We discarded contaminants common to *Nematoda* genome assemblies including E. coli,
484 Pseudomonas, Serratia and Stenotrophomonas [59]. We also identified one large 4.57Mb *E. coli*
485 contig in the *C. latens* PX534 that had been labeled *Nematoda* by BlobToolKit and removed it
486 from the *C. latens* PX534 assembled sequences.
487
488 *Heterozygosity analyses*
489 We assessed heterozygosity using the software Jellyfish version 2.2.4 [Marcais 2011] and
490 GenomeScope [Ranallo-Benavidex 2020]. Jellyfish is a fast, memory efficient kmer counting
491 tool that searches for occurrences of unique kmers in raw sequence reads based on user selected
492 size. GenomeScope estimates genome heterozygosity, genome size, and repeat content using a k-
493 mer based statistical approach. Jellyfish was run using the default settings and a kmer size of 21.
494 This kmer size was selected based on recommendation from the maker of Jellyfish and should be
495 sufficient to obtain all unique kmers present. A histogram file of all kmer occurrences was
496 created by Jellyfish and then uploaded into the online interface of GenomeScope. GenomeScope
497 was run using default settings with a kmer length of 21 and a read length consistent with each
498 sample's sequencing parameters.
499
500 *Evaluation*

501 We used the software BUSCO version 4.0.1 [27] to identify conserved gene sets. Briefly,
502 BUSCO searches assembled DNA sequences for a set of unique genes that are expected to be
503 conserved in single copy in an evolutionarily related group of organisms. We used BUSCO
504 version 4.0.1 [27] and the datasets Nematoda_odb10 for *C. elegans*, *C. remanei,* and *C. latens*,
505 Metazoan_odb10 for *D. melanogaster*, and Viridiplantae_odb10 for *A. thaliana*. We also
506 measured BUSCO completeness with the Diptera_odb10 for the *D. melanogaster* assemblies but
507 found that in some instances our assembled sequence contained a greater proportion of
508 conserved genes than the reference sequence. We chose to focus on the Metazoan_odb10 for *D.*
509 *melanogaster* and present both sets of statistics in the Supplementary Data. BUSCO genes in
510 each assembly were classified as single copy, duplicated, fragmented or missing. For single-copy
511 and duplicated classifications, the target gene must be present and >95% identical to the
512 expected size and sequence of the reference in the database [60].
513

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519

520 **Author Contributions**

521 Conceptualization, J.M.S. and J.L.F.; Methodology, J.M.S. and J.L.F.; Formal Analysis, J.M.S.,
522 J.D.M., A.C.M, and J.L.F.; Resources, J.L.F.; Writing – Original Draft, J.M.S., J.D.M. and
523 J.L.F.; Writing – Review & Editing, J.M.S., J.D.M and J.L.F.; Visualization, J.L.F.; Supervision,
524 J.L.F.

Declaration of Interests

The authors declare no competing interests.

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700
701 **Table 1.** The proportion of genomic features that QUAST identified in the assembled genome
702 sequence. The best performing software and dataset combination is highlighted in bold for each
703 genomic feature. Flye reliably assembled exons, introns, small RNAs and UTRs while Canu
704 performed best when assembling pseudogenes, repeats and transposable elements. Polishing the
705 assembled genome sequence with Illumina short reads did not affect the inference of genomic
706 features.
707

Assembly Software	Dataset	Exons	Introns	Pseudo-genes	Repeats	Small RNA	TEs	UTRs
Flye	Selected	**0.9988**	**0.9993**	0.9954	0.9967	**0.9985**	0.9941	0.9996
Flye	Corrected	**0.9988**	**0.9993**	0.9954	0.9971	0.9984	0.9942	**0.9997**
Flye	Corrected, Polished	**0.9988**	**0.9993**	0.9954	0.9971	0.9984	0.9942	**0.9997**
Canu	Corrected, Polished	0.9986	0.9986	**0.9986**	**0.9981**	0.9981	**0.9978**	0.9988
MaSuRCA		0.9967	0.9973	0.9885	0.9897	0.9972	0.9887	0.9975

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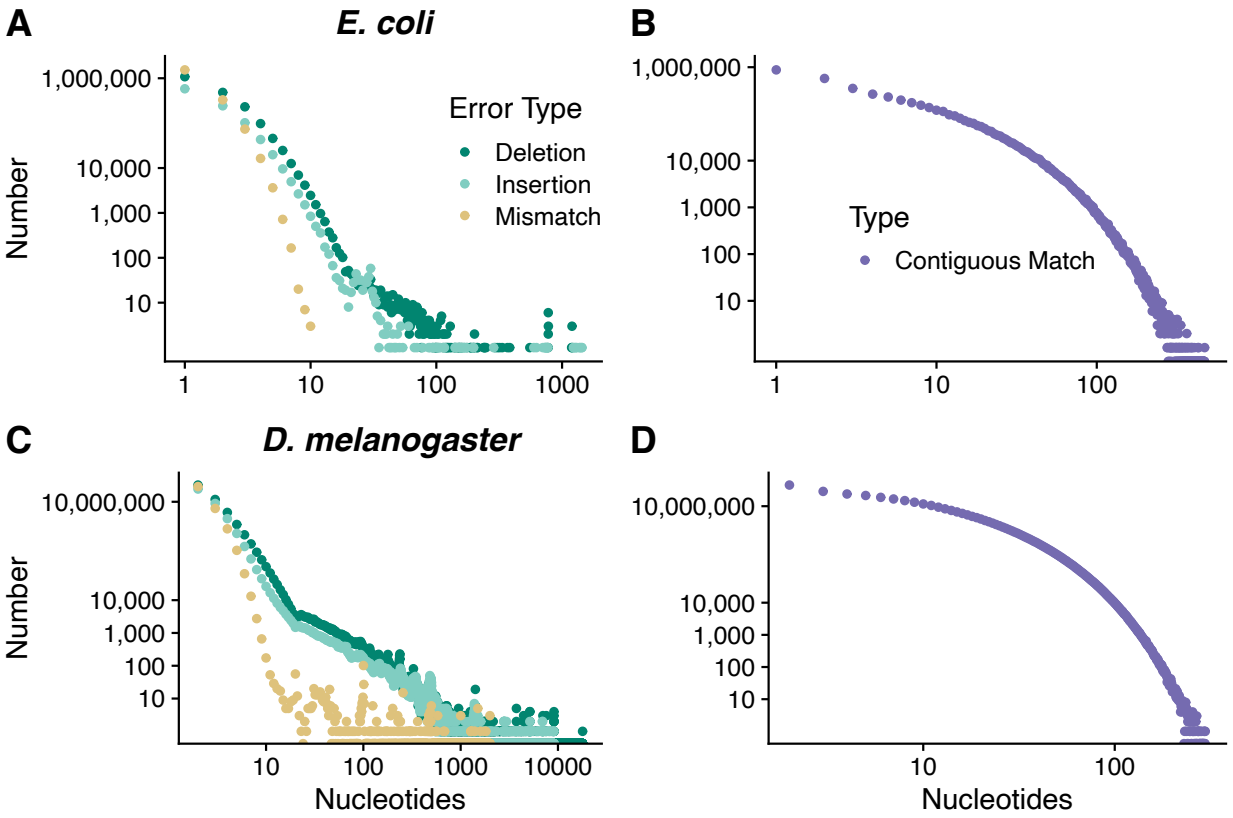
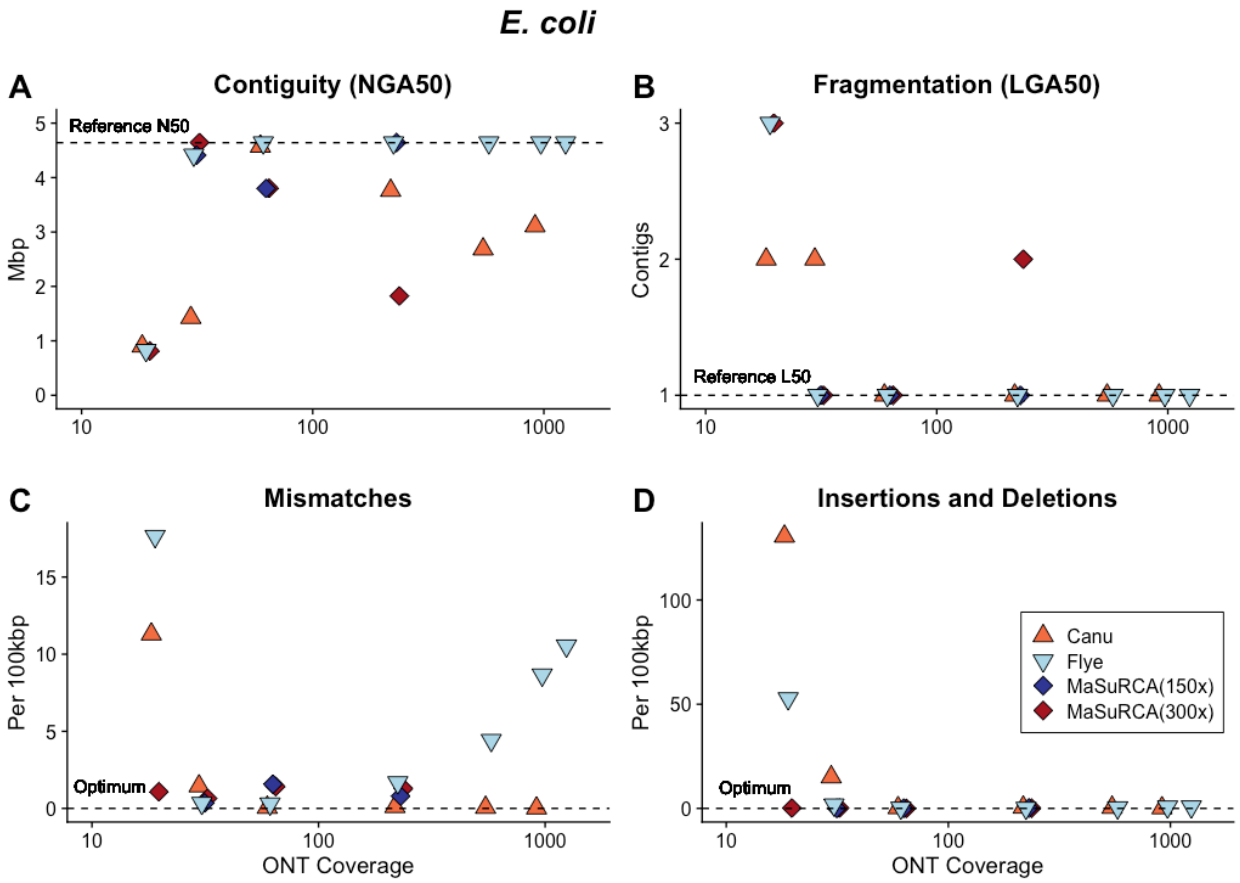


Figure 1. ONT sequence reads contain mixtures of errors including miscalled nucleotides, deletions, insertions and truncated homopolymers. When aligned to the reference genome this results in a large number of single and multi-base deletions, insertions and mismatches for (A) *E. coli* and (B) relatively few stretches of contiguous matching sequence that extend beyond a few nucleotides. For (C) *D. melanogaster* the error profile is similar but the large, repeat-rich genome results in multi-nucleotide deletions and insertions and again (D) few stretches of long, contiguous matching sequence.



725
726
727 **Figure 2.** The *E. coli* genome is relatively small at 4.64Mbp and less complex when compared
728 with metazoan genome sequences. Assembly of ONT libraries at relatively high coverage (>60x
729 average sequence depth) with Canu and Flye results in assembled sequences with (A) high
730 contiguity; (B) low contig number converging on a single chromosome; (C) few mismatches;
731 and (D) few insertion/deletion errors (indels) when compared with the reference sequence. The
732 influence of sequencing coverage on contiguity or fragmentation was not monotonic for Canu or
733 MaSuRCA assembled sequences. The relationship between sequencing coverage and accuracy
734 was not monotonic for Flye assembled sequences.
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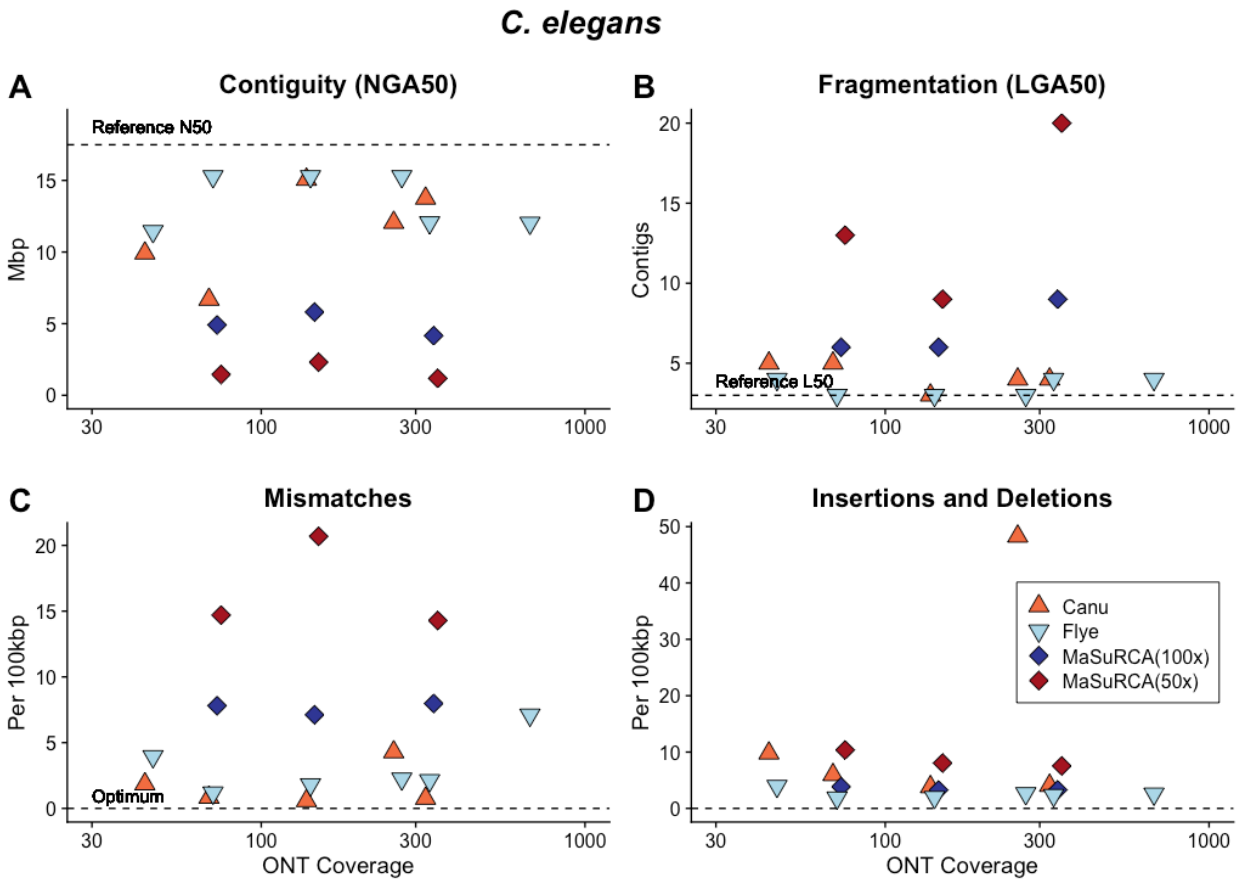


Figure 3. Both Canu and Flye assembled libraries with >70x ONT coverage into genome sequences with A) high contiguity; B) low fragmentation; C) few mismatches; and D) few insertions and deletions when compared with the *C. elegans* reference sequence. In comparison MaSuRCA performed poorly across a range of ONT sequencing coverages.

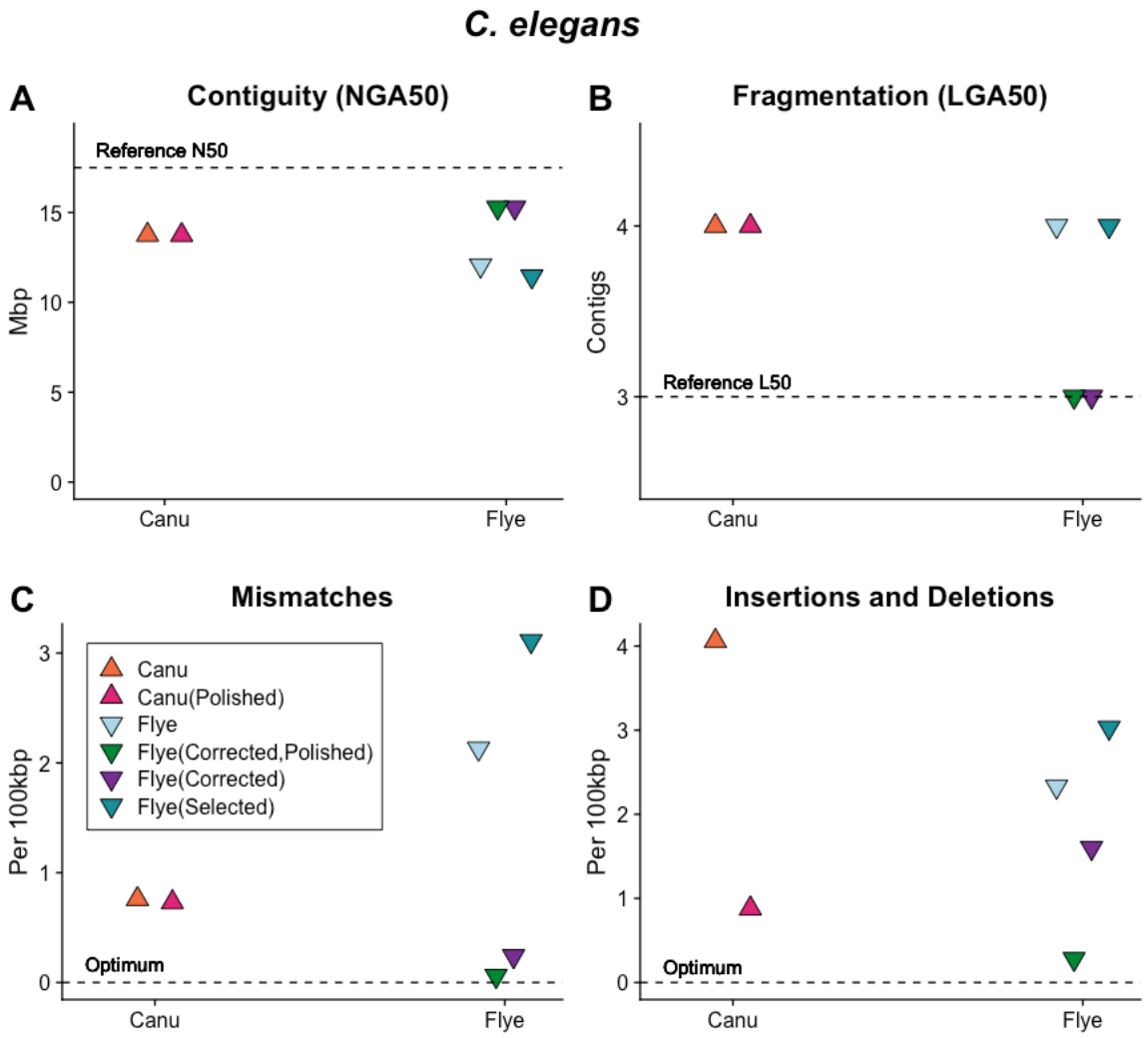


Figure 4. The relative performance of Canu and Flye assembly software with raw datasets, datasets with reads selected for length ('Selected'), reads selected for length and pre-corrected ('Corrected' in the figure legend) and sequences post-assembly polished with Illumina short reads ('Polished' in the figure legend) as measured by A) genome contiguity; B) mismatches and C) insertions and deletions relative to the *C. elegans* reference sequence. Canu assembly of *C. elegans* ONT libraries produces assembled sequences with high contiguity, low fragmentation, few mismatches and relatively more insertions and deletions that are eliminated with post-assembly polishing. The performance of Flye was increased through read selection, correction and post-assembly polishing with the highest-quality *C. elegans* sequence produced through Canu correction, Flye assembly and post-assembly polishing. The dataset with reads selected for length did not assemble as well as the dataset with reads selected and corrected, indicating that correction is an important step in assembly.

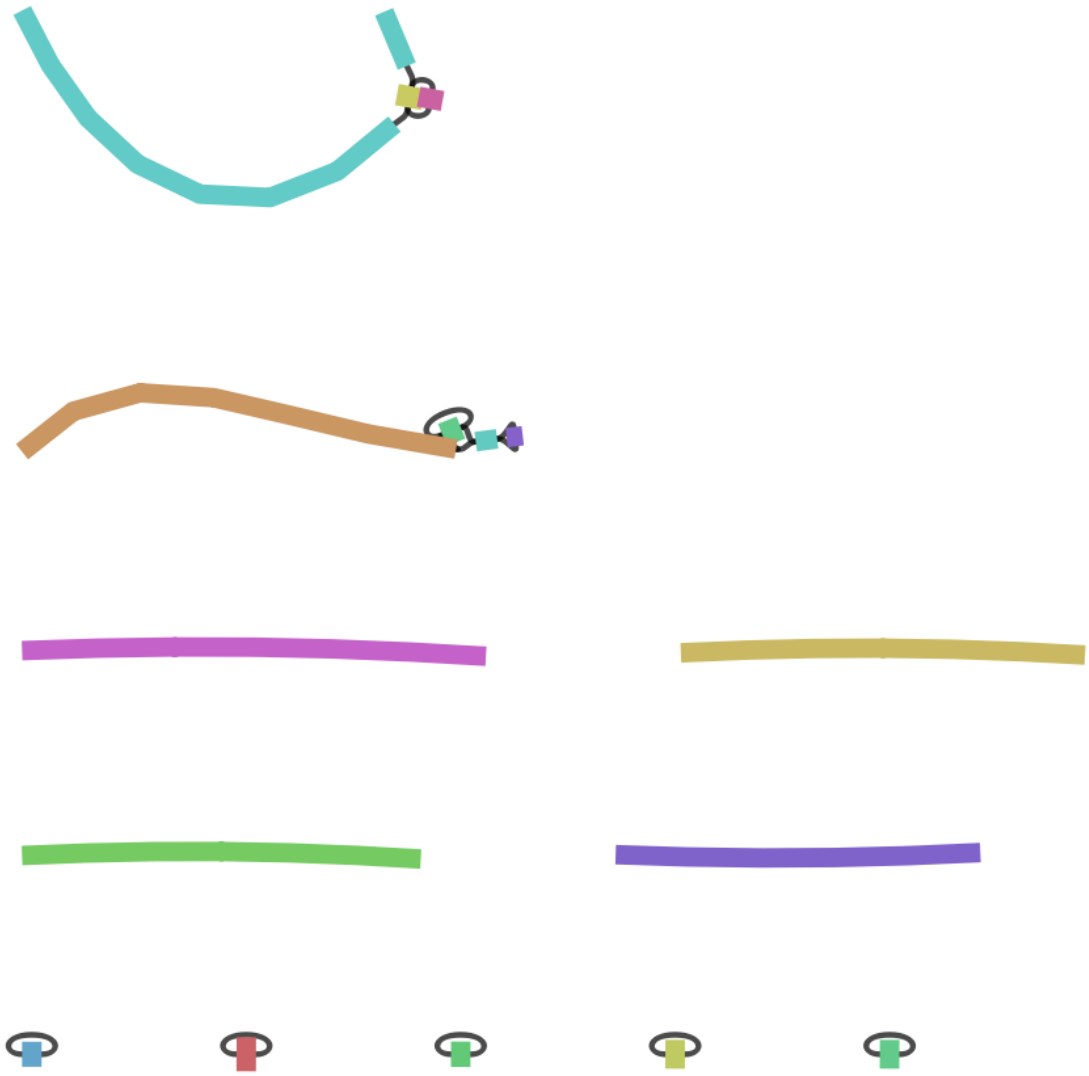


Figure 5. The assembly graph for the Flye assembled Canu selected and corrected *C. elegans* dataset shows 6 large sequences corresponding to the 6 chromosomes of *C. elegans* with 5 small unconnected sequences. Two of the large sequences contain unresolved 'bubbles' in the assembly graph.

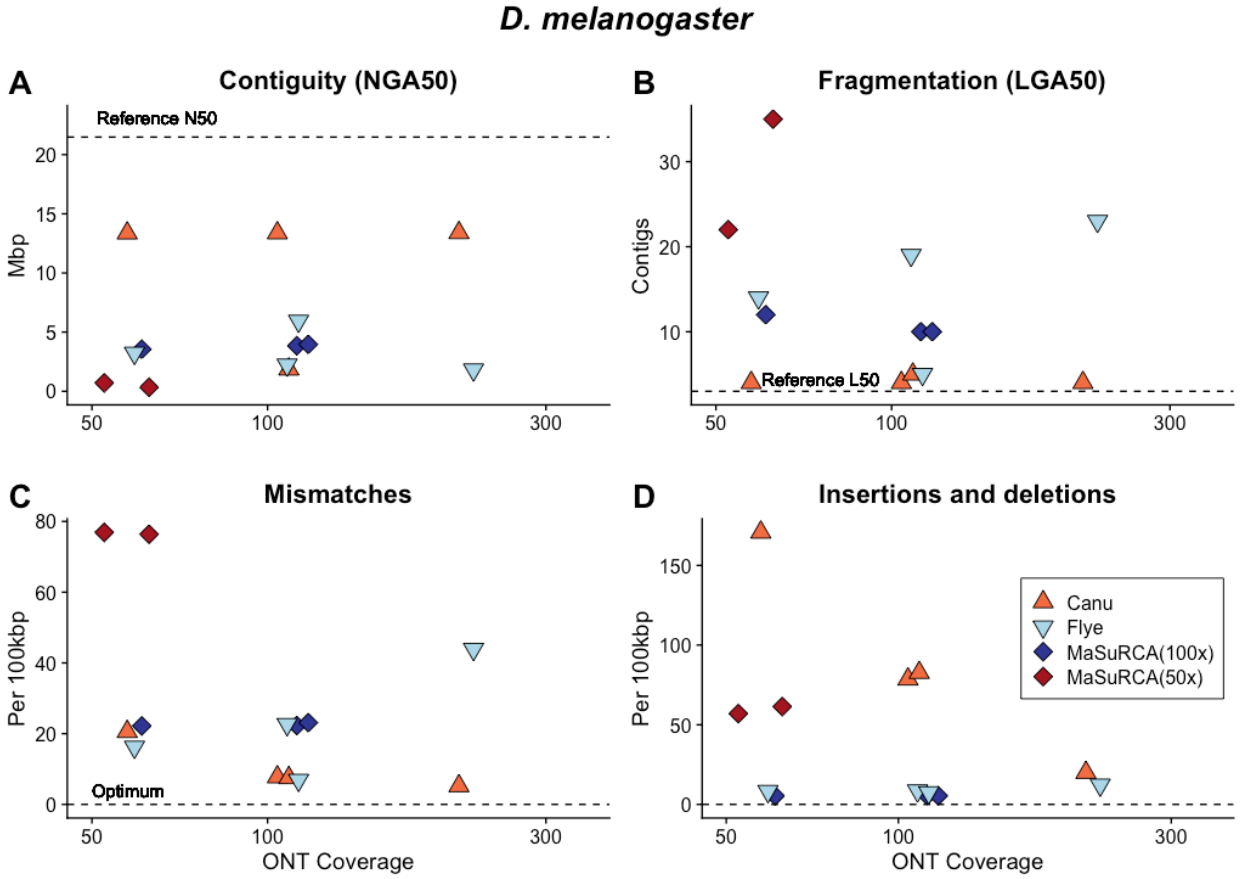


Figure 6. The *D. melanogaster* genome presents multiple challenges for genome sequencing and assembly including approximately 60Mb of repetitive heterochromatin. Canu assembly of ONT libraries with >60x sequencing depth produced a sequence with (A) high contiguity; (B) low contig number; (C) few mismatches and (D) few insertions and deletions (indels) when compared with the reference. The accuracy of the Canu assembled sequence increased with ONT library coverage. Flye assembly of the same ONT libraries produced sequences with relatively less contiguity, more fragmentation, more mismatches and fewer insertions and deletions. MaSuRCA assembly of the same ONT sequences resulted in fragmented sequences with similar error levels with compared with the reference sequence.

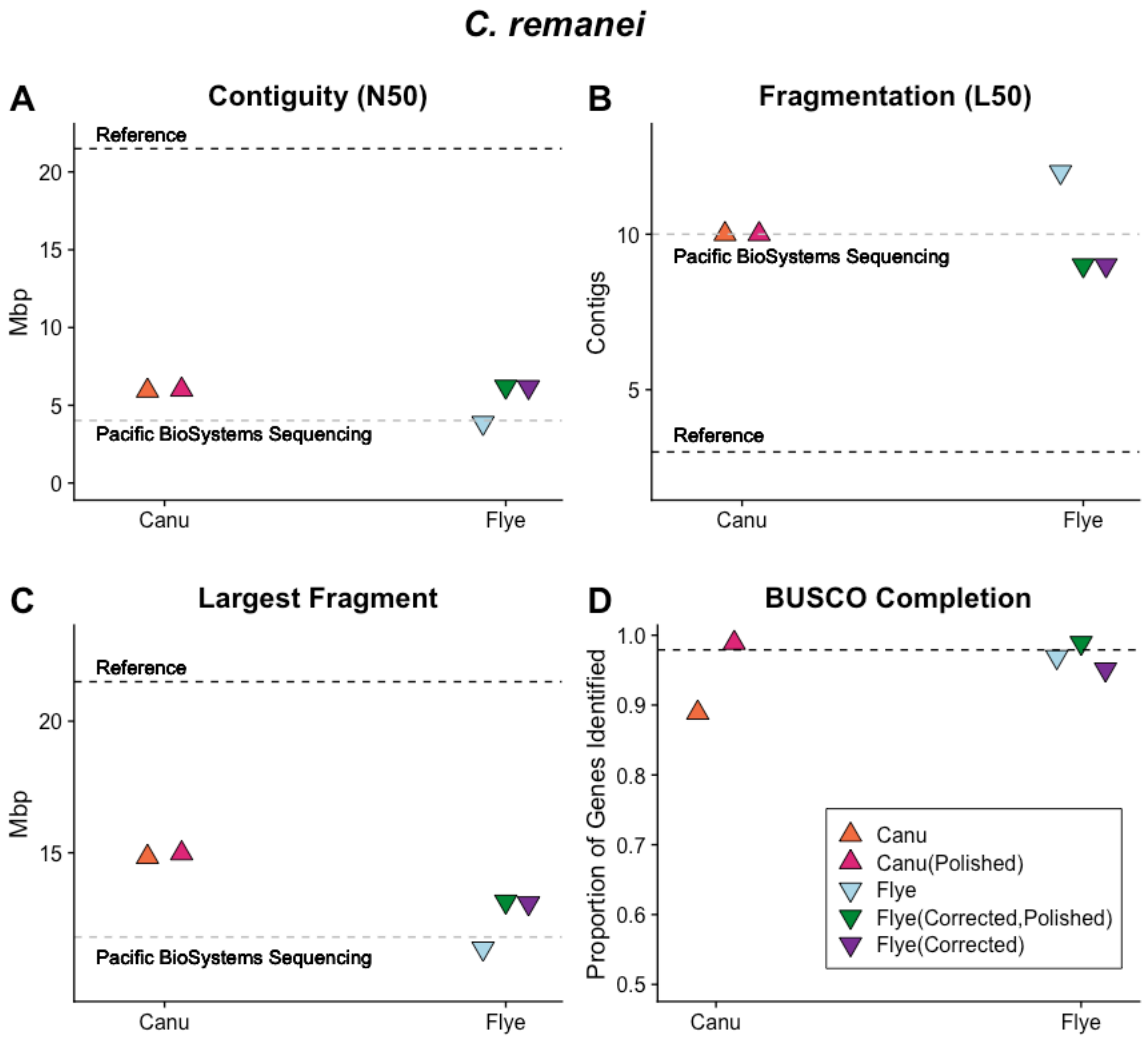


Figure 7. Both Canu and Flye produced assembled sequences with A) contiguity; B) fragmentation; C) the largest fragment and D) BUSCO gene completeness comparable to Pacific BioSystems sequencing after read correction and post-assembly polishing.

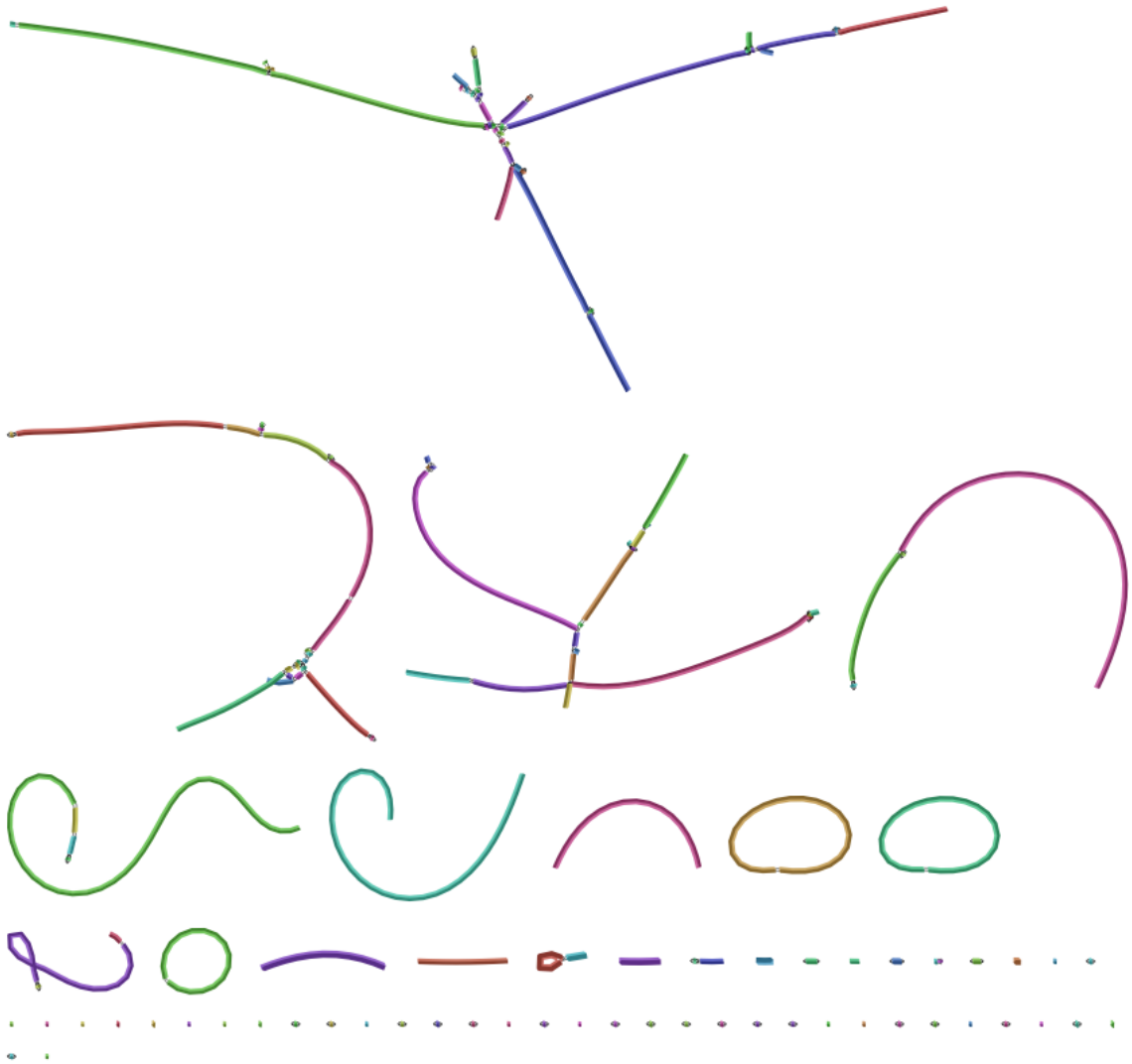
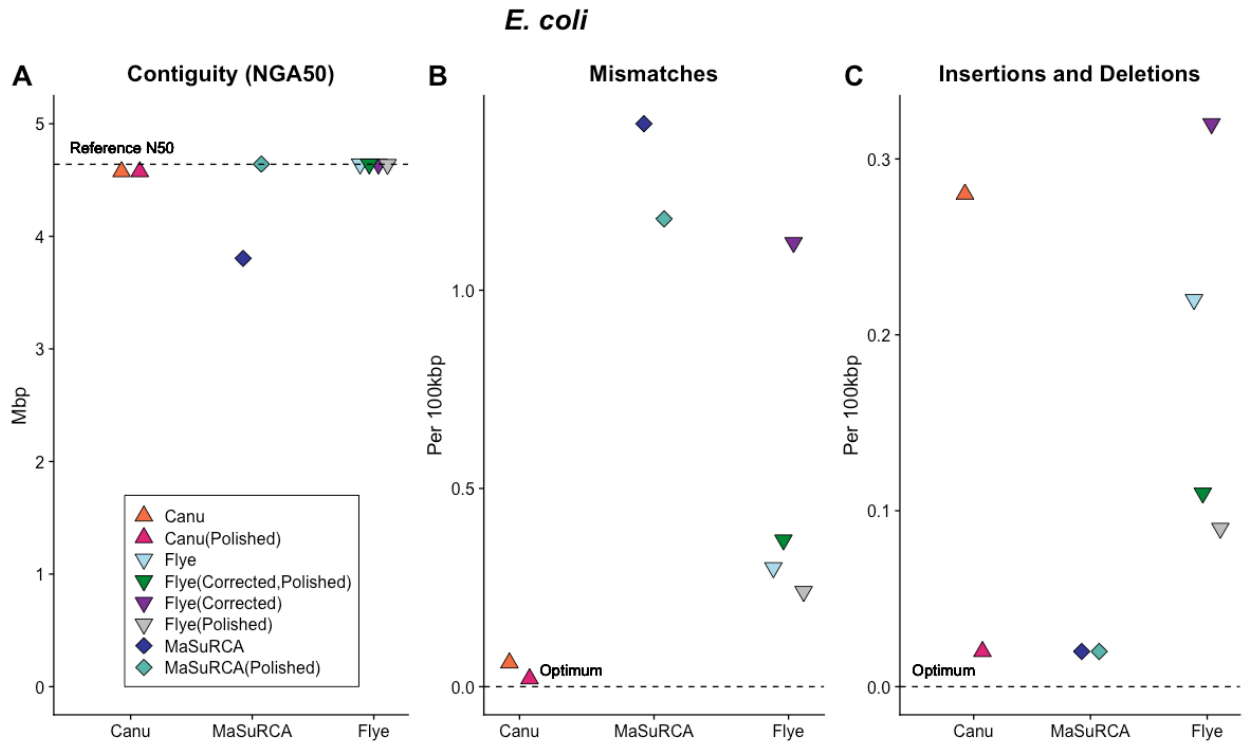
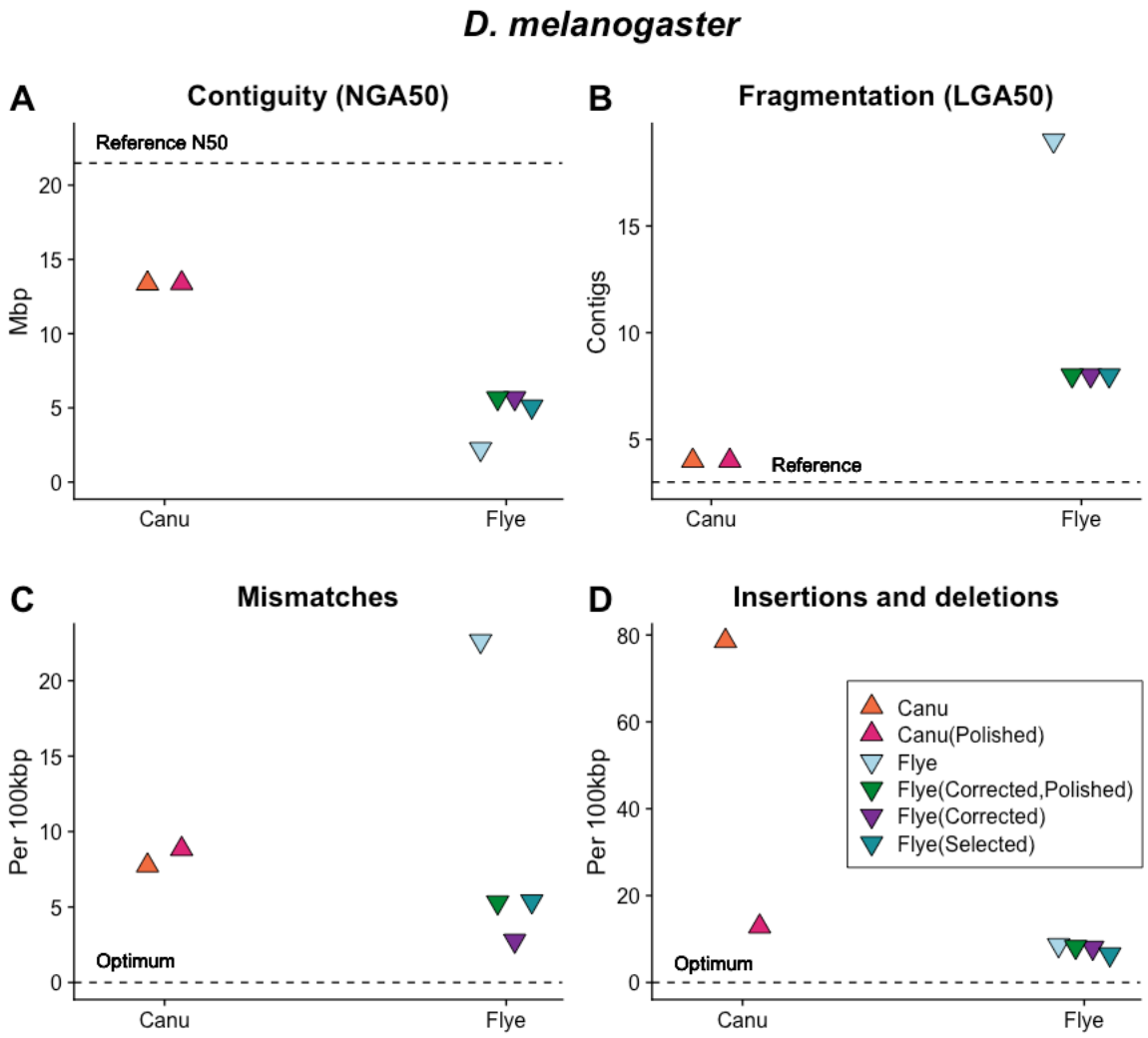


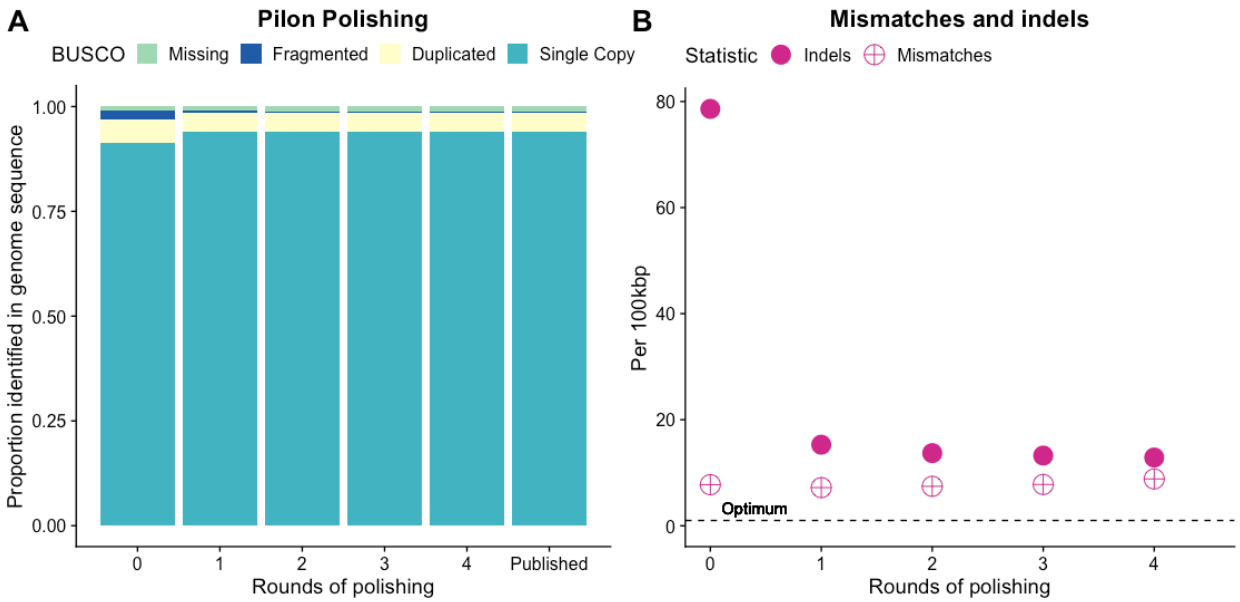
Figure 8. The *C. remanei* PX356 assembly graph shows multiple unresolved regions, bacterial contaminants (visible as circular chromosomes) and small fragments.



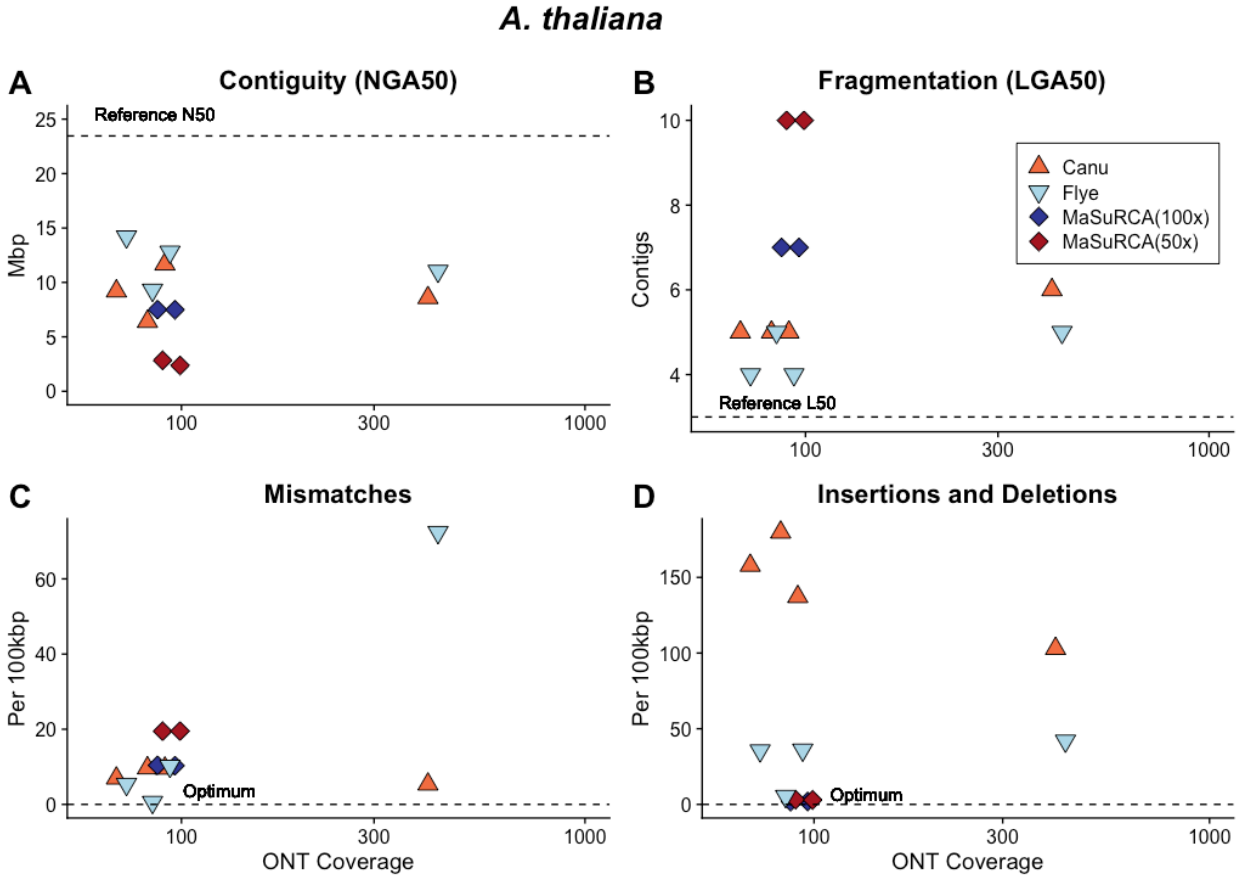
SFigure 1. The relative performance of Canu, Flye and MaSuRCA assembly software with raw datasets, datasets with reads pre-corrected ('Corrected' in the figure legend) and post-assembly polishing with Illumina short reads ('Polished' in the figure legend) as measured by A) genome contiguity; B) mismatches and C) insertions and deletions relative to the *E. coli* reference sequence. Both Canu and Flye assembled highly contiguous sequences for all datasets and the MaSuRCA assembled sequence was highly contiguous post-polishing. The Canu assembled sequences had few mismatches and insertion/deletion errors were eliminated through polishing. The MaSuRCA assembled sequence had numerous mismatches that were not addressed through polishing and few insertions and deletions. The accuracy of all Flye assembled sequences was improved with polishing.



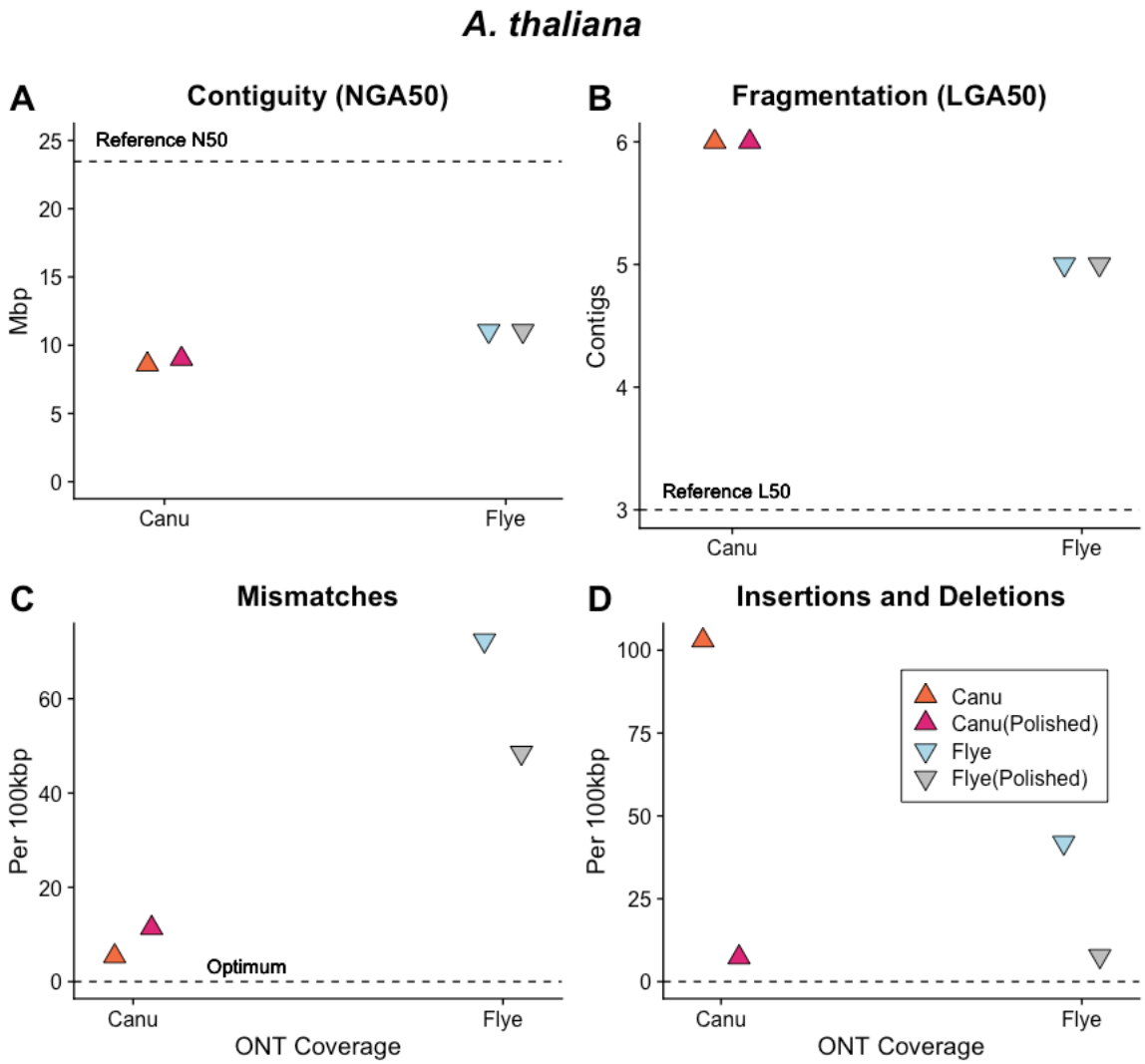
SFigure 2. The relative performance of Canu and Flye assembly software with raw datasets, datasets with reads selected for length ('Selected'), reads selected for length and pre-corrected ('Corrected' in the figure legend) and sequences post-assembly polished with Illumina short reads ('Polished' in the figure legend) as measured by A) genome contiguity; B) mismatches and C) insertions and deletions relative to the *D. melanogaster* reference sequence. Canu assembly of *D. melanogaster* ONT libraries produces assembled sequences with high contiguity, low fragmentation, few mismatches and relatively more insertions and deletions that are eliminated with post-assembly polishing. The performance of Flye can be increased by selecting, correcting and polishing.



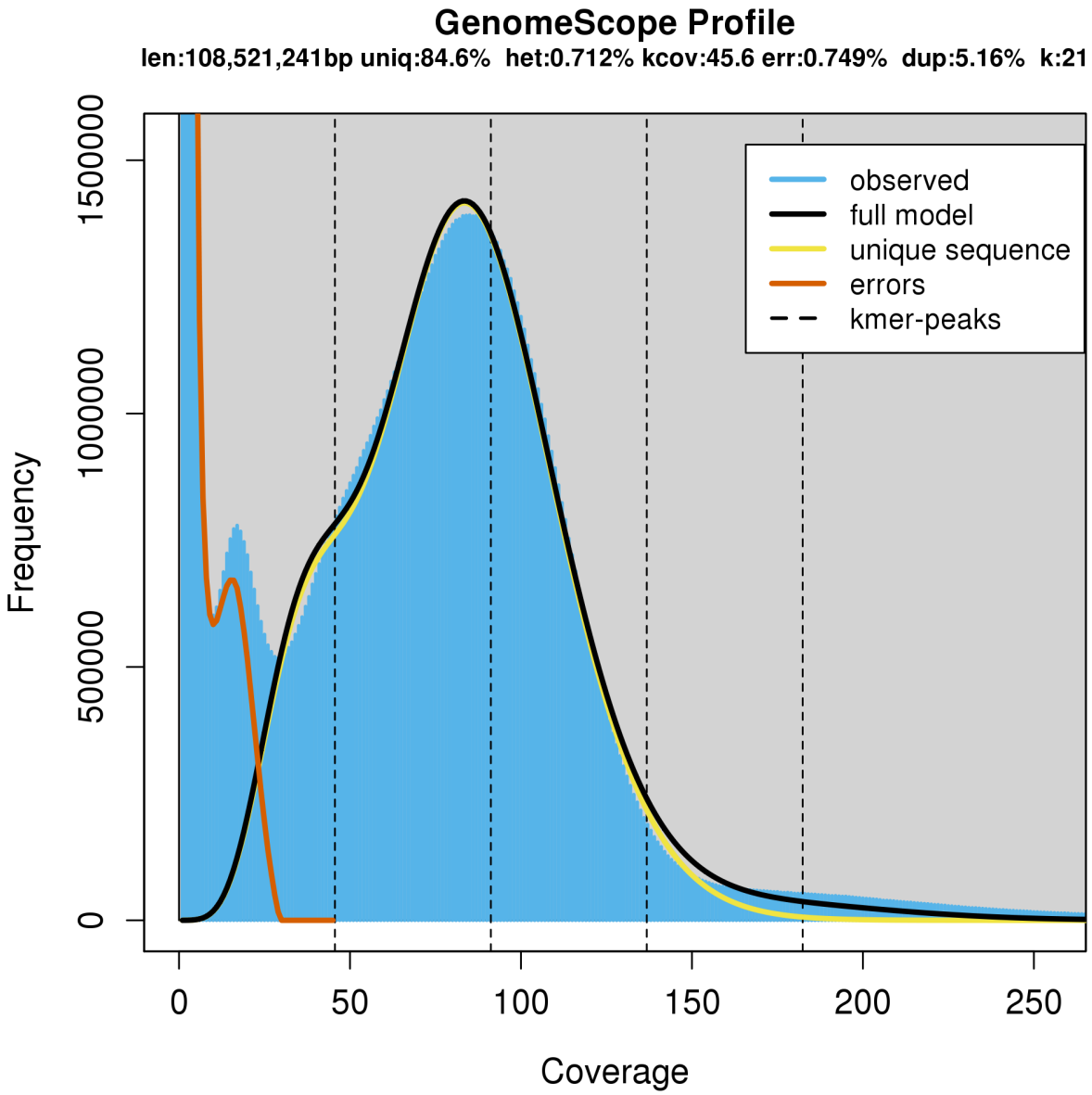
SFigure 3. Polishing the assembled *D. melanogaster* sequence with Illumina libraries and the software package Pilon (REF) increases (A) the number of conserved genes found in single copy; and reduces (B) the number of mismatches and indels compared with the reference sequence. The bulk of this improvement occurs after 1-2 rounds of polishing.



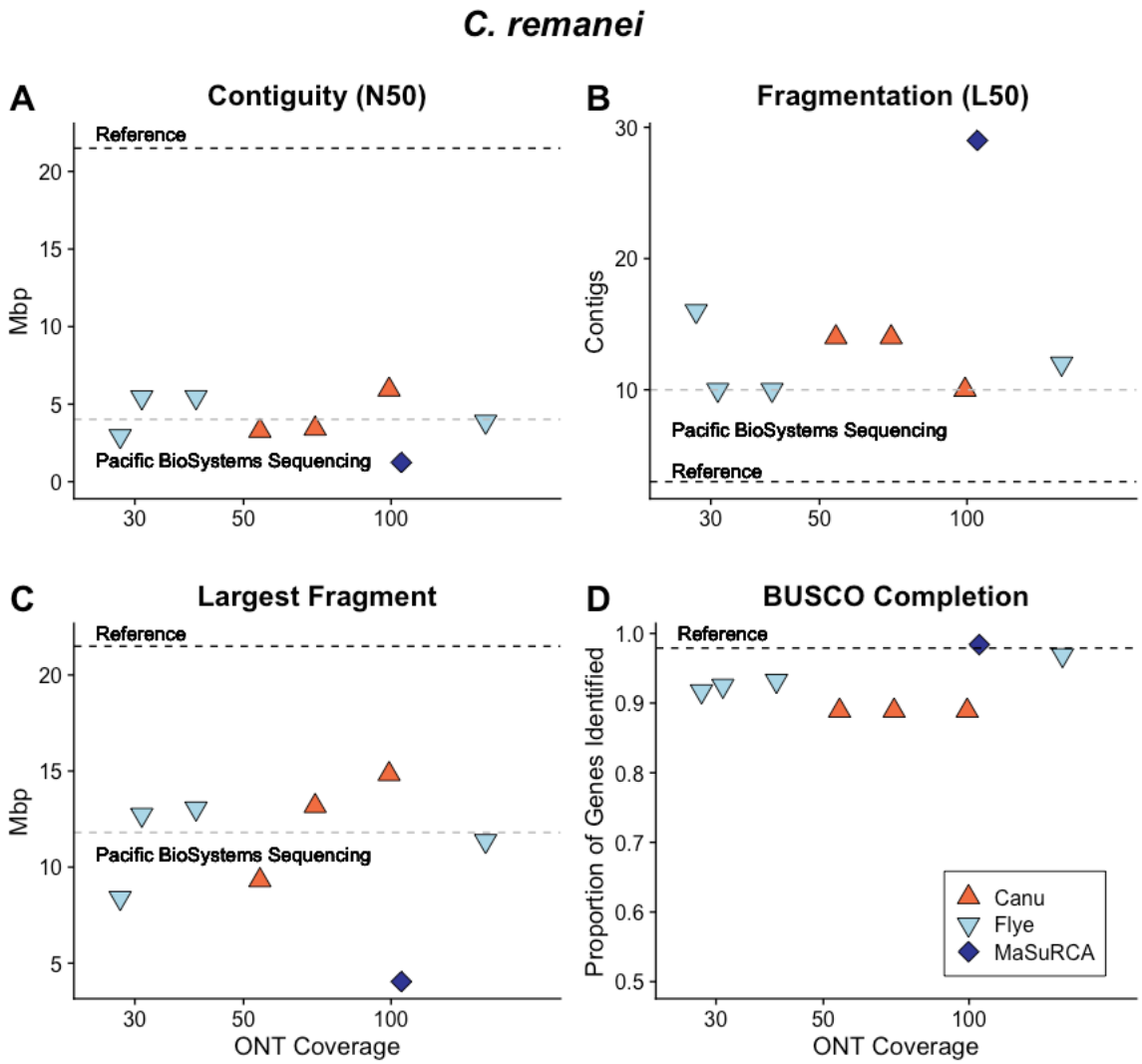
SFigure 4. Canu, Flye and MasuRCA assembled sequences across a range of ONT sequencing coverages had A) low contiguity; B) high fragmentation; C) mismatches and D) insertions and deletions when compared with the *A. thaliana* reference sequence.



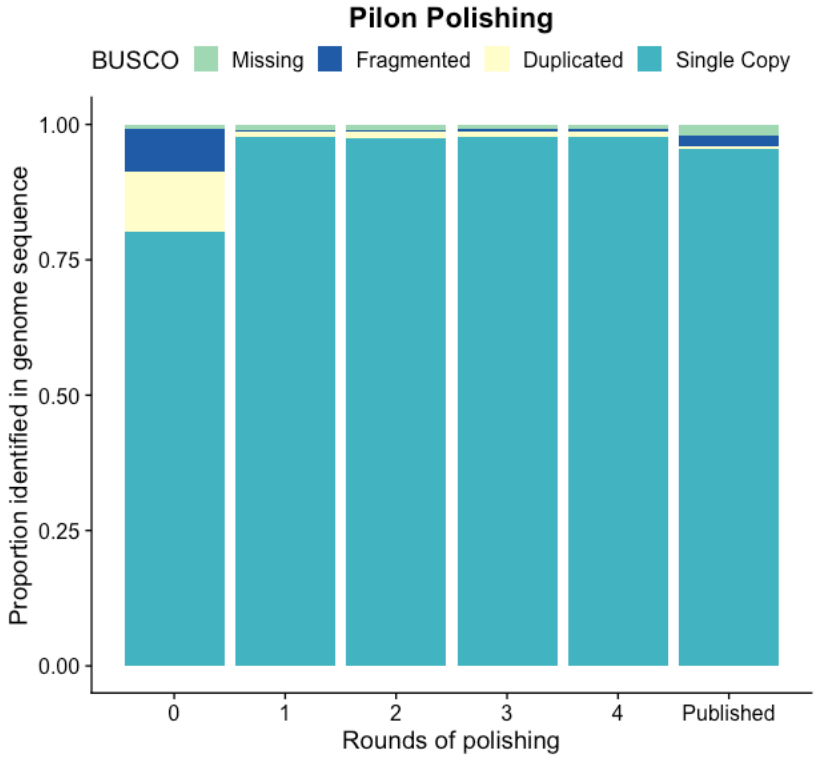
SFigure 5. Post-assembly polishing increased the number of mismatches for Canu assembled sequences but decreased the number of insertions and deletions. Post-assembly polishing decreased mismatches, insertions and deletions for Flye assembled sequences.



SFigure 6. Analysis with the GenomeScope software revealed residual heterozygosity in the C. remanei PX356 sequences (estimated at 0.712%). Here, the residual heterozygosity is visible as a shoulder at ~50x sequencing coverage.



SFigure 7. Canu and MaSuRCA assembled *C. remanei* sequences with A) low contiguity and B) high fragmentation compared with the true chromosome size and number but the sequences were of comparable contiguity and fragmentation when compared with Pacific BioSystems sequences. The C) largest fragment was also comparable to that achieved by Pacific BioSystems and the BUSCO genome completeness was high with assembly of a high coverage ONT library.



SFigure 8. Polishing the *C. remanei* PX356 empirical assembled sequence with Illumina libraries and the Pilon software package (REF) increases the BUSCO completeness with a single round. The assembled sequence has a greater number of conserved genes found in single copy when compared with the published sequence (REF).

Organism	Flow Cell ID	Total # Reads (K)	Total bp passed(Gbp)
C. remanei PX464	464.1	391	4.11
	464.2	457	5.39
C. latens PX534	534.1	545	0.579
	534.2	504	0.784
	534.3	492	2.76
	534.4	824	2.35
	534.5	719	4.35
	534.6	1,020	5.41
C. remanei PX439	439.1	2,080	6.1
	439.2	940	5.3
	439.3	398	5.59
C. remanei PX356	356.1*	2,240	4.31
	356.2	940	5.01
	356.3	1,048	4.19
	356.4	750	0.723
	356.5	1,710	4.16

*Sequenced with ONT SQK-LSK108 Library Prep Kit

STable 2. Genome statistics for MaSuRCA-assembled Illumina paired sequences and error corrected Canu-assembled sequences. Although the Illumina paired end sequence is highly fragmented and incomplete it has fewer mismatches, insertions and deletions when compared with hybrid ONT and Illumina read sets.

	Read Coverage		**BUSCO**					
Sample	ONT	Illumina	Single (%)	Duplicated	Fragmented	Missing	Mismatches per 100Kpb	Indels per 100Kpb
A. thaliana	0	100	98.1	0.9	0.5	0.5	10.99	1.03
	420	polished	99.1	0.5	0	0.4	11.36	7.3
C. elegans	0	200	95.8	0.8	1.6	1.8	4.78	1.02
	336	polished	97.7	0.6	0.4	1.3	0.73	0.88
D. melanogaster	0	100	93.5	1.5	0.4	4.6	10.01	1.7
	108	polished	94	1.2	0.3	4.5	8.84	12.87
E. coli	0	100	-	-	-	-	1.7	0.04
	62	polished	-	-	-	-	0.02	0.02

STable 3: Polishing the *C. remanei* assembled sequence with an Illumina library at 20x depth increases the percentage of conserved genes identified by BUSCO (Simão et al., 2018) after 3 rounds of polishing with Pilon (Walker et al., 2014). Polishing with a higher depth Illumina library (114x average sequencing coverage) produces similar results after 2 rounds of polishing with Pilon. The highest percentage of conserved genes found in single copy is achieved after 3 rounds of polishing with Pilon and a high depth Illumina library at 227x average coverage.

	Polishing		**BUSCO**			
Sample	Rounds	Coverage	Single (%)	Duplicated	Fragmented	Missing
Canu	0	20x	80.3	0.9	7.7	11.1
Assembled	1	20x	97.2	1	0.5	1.3
C. remanei	2	20x	97.3	1	0.4	1.3
	3	**20x**	**97.6**	**1**	**0.2**	**1.2**
	4	20x	97.6	1	0.2	1.2
	1	114x	97.6	1.1	0.3	1
	2	**114x**	**97.6**	**1**	**0.3**	**1.1**
	3	114x	97.5	1	0.3	1.2
	4	114x	97.5	1	0.3	1.2
	1	227x	97.6	1	0.3	1.1
	2	227x	97.5	1	0.3	1.1
	3	**227x**	**97.7**	**0.9**	**0.3**	**1.1**
	4	227x	97.7	0.9	0.3	1.1

STable 4

Organism	Assembly Version	ONT Coverage	Read N50	Post-correction N50	Assembly Size (Mbp)	Contigs	BUSCO Single Copy	Duplicate	Fragmented	Missing
	Published PX356	-	-	-	145.44	1,591				
C. remanei	Draft PX356	156	8,203	21,340	154.64	163				
PX356	Final PX356				124.5	70	97.7	1	0.2	1.1
	Published PX439	-	-	-	124.54	912				
C. remanei	Draft PX439	95	18,233	38,434	144.15	85				
PX439	Final PX439				131.75	53	97.6	0.9	0.3	1.2
	Published EM464	-	-	-	N/A	N/A				
C. remanei	Draft EM464	76	22,891	33,688	154.81	400				
EM464	Final EM464				138.62	320	96.2	2.6	0.3	0.9
	Published PX534	-	-	-	117.12	1,858				
C. latens	Draft PX534	119	14,759	34,951	125.13	132				
PX534	Final PX534				124.94	98	97.6	1.1	0.3	1

Accessions

PX356	GCA_001643735.2
PX439	GCA_002259225.1
EM464	N/A
PX534	GCA_002259235.1