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9 **Chromosome-level hybrid *de novo* genome assemblies as an attainable option for non-model**
10 **insects**

11

12 **Running title: *De novo* chromosome-level genome assembly**

13

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25 **Abstract**

26 The emergence of third generation sequencing (3GS; long-reads) is making closer the goal of
27 chromosome-size fragments in *de novo* genome assemblies. This allows the exploration of new
28 and broader questions on genome evolution for a number of non-model organisms. However,
29 long-read technologies result in higher sequencing error rates and therefore impose an elevated
30 cost of sufficient coverage to achieve high enough quality. In this context, hybrid assemblies,
31 combining short-reads and long-reads provide an alternative efficient and cost-effective approach
32 to generate *de novo*, chromosome-level genome assemblies. The array of available software
33 programs for hybrid genome assembly, sequence correction and manipulation are constantly
34 being expanded and improved. This makes it difficult for non-experts to find efficient, fast and
35 tractable computational solutions for genome assembly, especially in the case of non-model
36 organisms lacking a reference genome or one from a closely related species. In this study, we
37 review and test the most recent pipelines for hybrid assemblies, comparing the model organism
38 *Drosophila melanogaster* to a non-model cactophilic *Drosophila*, *D. mojavensis*. We show that
39 it is possible to achieve excellent contiguity on this non-model organism using the DBG2OLC
40 pipeline.

41
42 **Keywords:** de novo assembly; non-model species, long-read, short-read, genomics, merged
43 assembly.

44 1 INTRODUCTION

45 Whole genome sequencing is a major target in evolutionary biology, because it provides the
46 material to study how a species' genome evolves. Notably, whole genome data provides the
47 opportunity to study recombination and large rearrangement events, differential molecular
48 evolution across the genome, and imprints of selection throughout the genome, ultimately
49 improving our knowledge of how species evolve and diverge (Ellegren 2014; Rudman et al.,
50 2018). To increase our understanding of such evolutionary processes, we need to expand the
51 range of studied organisms to non-model organisms, for which the access to well resolved
52 genome assemblies is often lacking.

53

54 Thanks to third generation sequencing (3GS) from platforms such as PacBio (Rhoads & Au
55 2015) and Nanopore (Urban, Bliss, Lawrence & Gerbi 2015), *de novo* genome assemblies of non-
56 model organisms can be obtained, but one drawback from such technologies is the high error rate.
57 *De novo* hybrid assemblies combine long-reads and short-reads (Illumina technology; Bentley et
58 al., 2008) to achieve high contiguity and accuracy while reducing sequencing costs through lower
59 coverage of long-reads data (Ye, Hill, Wu, Ruan & Ma, 2016).

60

61 There is a constantly increasing panel of tools to assemble reads and polish genome assemblies.
62 Identifying the pipeline most optimized to one's needs is one obstacle, and applying it to the
63 actual data is another one, especially in the absence of bioinformatic expertise, since guidelines
64 and practical implementations remain limited. In addition, many of those pipelines are not tested
65 on non-model organisms and assume that the samples are from model organisms where extreme
66 inbreeding and high homozygosity is commonly feasible. In the present study, we reviewed the
67 most recent whole genome assembly pipelines, and selected a promising pipeline relying on
68 hybrid technology (Chakraborty, Baldwin-Brown, Long & Emerson, 2016). We tested it
69 thoroughly with the aim of an optimized assembly, using DNA data from both *Drosophila*
70 *melanogaster* as a model species, and *D. mojavensis* from the Sonora, Mexico population as a
71 non-model species. *Drosophila mojavensis* is a cactophilic North American desert endemic
72 species, ovipositing, developing and feeding as adults on necrotic cactus tissues (Heed 1978).
73 The species is composed of four distinct host populations (Sonoran Desert, Baja California,

74 Mojave Desert and Santa Catalina Island) each utilizing a different cactus species (population
75 from Sonora utilizes the organpipe cactus, *Stenocereus thurberi*). Given its known ecology and
76 ever-growing genomic tools, this species has become a model for the study of the genomics of
77 local ecological adaptation and speciation (Matzkin et al., 2006; Bono, Matzkin, Kelleher &
78 Markow, 2011; Matzkin 2014; Benowitz, Coleman & Matzkin, 2019). Distantly related to *D.*
79 *melanogaster*, *D. mojavensis* has a similar genome size (see Table 1) and six Muller elements,
80 although all its chromosome are acrocentric (Drosophila 12 Genomes Consortium 2007;
81 Schaeffer et al., 2008). Ultimately, this new *D. mojavensis* assembly from Sonora will be used in
82 a much larger upcoming genomic study using *de novo* assemblies of multiple cactophilic species
83 and populations (Matzkin, unpublished data). We provide here an analysis of the effects of
84 different parameters on the quality of the final assembly, assessed by a combination of universal
85 tools (contigs length and N50 as a measure of contiguity; BUSCO score as a measure of quality
86 and completeness (Waterhouse et al., 2017) and a reference-based tool, Quast (Gurevich,
87 Saveliev, Vyahhi & Tesler, 2013) which compares the assembly to a reference genome. We
88 show a significant improvement of assembly quality of *D. melanogaster* compared with results
89 from Chakraborty et al. (2016) simply by tuning parameters and we provide guide parameters for
90 assemblies with similar coverage of non-model organism DNA. Finally, we tested the pipeline
91 on *D. mojavensis* from the Santa Catalina Island, California population using Nanopore long-read
92 data instead of PacBio data.

93

94

95 **2 MATERIALS AND METHODS**

96 **2.1. *Drosophila mojavensis* sequencing**

97 We used flies from a *D. mojavensis* isofemale line (MJ 122) originally collected in Guaymas,
98 Sonora Mexico in 1998, thereafter SON. This isofemale line has been maintained since its
99 collection under laboratory conditions (25°C and 14/10 day/night cycle), transferred every
100 generation (four weeks) into fresh 8-dram vials containing banana-molasses medium (Coleman,
101 Benowitz, Jost & Matzkin, 2018). Prior to DNA extraction the flies were raised on banana-
102 molasses medium with 125 µg/ml ampicillin and 12.5 µg/ml tetracycline to reduce bacteria
103 contamination of the sequencing data. The sequencing methods for short-read data (paired ends

104 and mate pairs) have been described in Allan & Matzkin (2019). Sequencing technologies and
105 coverage for the different data sets are summarized in Table 1.

106

107 *2.1.2. DNA extraction for PacBio Sequencing – Protocol optimization*

108 Due to the long-read potential of PacBio Sequencing Systems, extra care must be taken during
109 extraction to produce high molecule weight DNA. Attempts at using both QIAGEN DNeasy
110 Blood & Tissue Kit and QIAGEN MagAttract HMW DNA Kit failed to produce sufficiently long
111 strands of DNA. As such, a chloroform-based extraction method was used. This relatively
112 simple method is low cost and the only specialized equipment needed is a refrigerated centrifuge.
113 To consistently recover enough DNA for two PacBio libraries, 150 flies of each sex were used
114 for extraction. Flies were starved for two hours in groups of 50 per vial and then frozen at -80° C
115 in 1.5 mL tubes. A lysis solution containing Tris HCl buffer 0.1M pH 8.0, EDTA 0.1 M pH 8.0,
116 and 1% SDS was prepared and stored at room temperature to prevent the SDS from precipitating.
117 While on ice, 500 µL of lysis solution was added to each tube of flies, followed by 2.5 µL of
118 QIAGEN Proteinase K to reduce DNA degradation. Using a plastic pestle, flies in each tube
119 were hand-homogenized by gently grinding them. Hand homogenization resulted in slightly
120 lower amounts of DNA recovered, however the size of DNA fragments were longer compared to
121 when using a battery-operated pestle motor to homogenize. The mixture was incubated at 65 °C
122 for 30 min with gentle mixing halfway through. To further reduce DNA fragmentation, tubes
123 were cooled to 37 °C for three minutes and another 2.5 µL of QIAGEN Proteinase K was added.
124 Tubes were incubated for an additional 30 min at 37 °C. After incubation, 70 µL of 4 M
125 potassium acetate was added, mixed by inversion, and then placed on ice to incubate for 30
126 minutes. In a 4 °C Eppendorf 5920R centrifuge, the tubes were spun for 30 min at 18,000 rcf to
127 pull debris to the bottom of the tubes. For each tube, the supernatant was transferred to new
128 tubes avoiding as much debris as possible. One volume of chloroform:isoamyl alcohol 24:1 was
129 added to each tube and gently inverted 40 times, and then centrifuged at 4 °C for 5 min at
130 10,500 rcf. The upper phase was transferred to a new tube while being careful to not disturb the
131 interface. The DNA was precipitated by adding 350 µL of 2-propanol and gently inverting the
132 tube. At this point visible threads of DNA were apparent. To pellet the precipitate, the tubes
133 were centrifuged at 4 °C for 5 min at 10,500 rcf. The supernatant was discarded and the pellet

134 was washed with 1 mL of room-temperature 70% ethanol. The tube was inverted to insure
135 washing of the pellet and tube. A final 4 °C centrifugation for 2 min at 10,500 rcf was
136 performed. The ethanol was removed with a pipettor as completely as possible and the pellet
137 dried for 10-15 min in a fume hood. 30 µL of Tris-EDTA pH 8.0 was added to each tube to
138 resuspend the DNA. While the pellet normally goes into solution relatively easily, it can be
139 placed at 4 °C overnight to insure resuspension. The six tubes were then combined to a single
140 tube and 3 µL of QIAGEN RNaseA was added and incubated for 30 min at 37 °C. The DNA was
141 delivered as this resuspended solution for PacBio sequencing.

142

143 *2.1.3. PacBio Sequencing*

144 PacBio sequencing was performed at the Arizona Genomics Institute (Tucson, AZ, U.S.A.).
145 DNA was sized in a 1% agarose pulsed field gel electrophoresed at 1-50 sec linear ramp,
146 6 volts/cm, 14 °C in 0.5X TBE buffer for 20 hours (BioRad). The marker used was a lambda
147 ladder Midrange PFG I (New England Biolabs). The resultant DNA smear had a large mass in
148 the 35-65 kb range (Fig. 1). DNA purity was verified using a NanoDrop One Microvolume UV
149 Spectrophotometer with ratios 260/280 and 260/230 over 1.8. Quantity (150 ng/µL in 180 µL =
150 27 µg) was determined by a Qubit Fluorometer (Life Technologies), and was consistently lower
151 than that measured with the Nanodrop. PacBio sequencing libraries were prepared from 6 µg
152 starting material each, following manufacturers protocol for a 20 Kb Template Preparation Using
153 BluePippin™ Size-Selection System (www.pacb.com). The library was size-selected, on a
154 BluePippin, at 20 kb using high pass with S1 Marker (Sage Sciences). The final library was
155 damage-repaired, bead-purified and quantified. Sequencing was performed on a PacBio Sequel
156 instrument following manufacturer's instructions. The sequencing primer annealed was v3, the
157 sequencing kit was v2.1. Two libraries were loaded on two separate SMRT cells with magbeads
158 at concentrations of 25 pmol and 35 pmol, respectively. Sequencing was carried out for
159 collection of 10 hr movies on 1 M SMRT cells.

160

161 **2.2. *Drosophila melanogaster* and *D. mojavensis* public sequencing data**

162 To generate the *D. melanogaster* assembly (hereafter, Dmel), PacBio data was retrieved from the
163 NCBI Short-read Archive SRX499318 (Kim et al., 2014). This data set contained 42 PacBio RS

164 II SMRT cells from male *D. melanogaster* ISO1 flies. We used data from 20 randomly selected
165 cells only to obtain a coverage similar to our data sets (cell numbers SRR1204085, SRR1204088,
166 SRR1204451, SRR1204466, SRR1204467, SRR1204469, SRR1204471, SRR1204472,
167 SRR1204473, SRR1204481, SRR1204482, SRR1204485, SRR1204486, SRR1204615,
168 SRR1204617, SRR1204690, SRR1204691, SRR1204692, SRR1204693, and SRR1204696). We
169 used the SMRT Illumina HiSeq 2000 100 bp paired-end data from male *D. melanogaster* ISO1
170 flies, which was retrieved from the European Nucleotide Archive ERX645969 (Miller, Smith,
171 Hawley & Bergmann, 2013).

172

173 For the *D. mojavensis* assembly from the Santa Catalina Island, California population (hereafter,
174 CAT), Nanopore sequencing data was kindly provided by Miller, Staber, Zeitlinger & Hawley
175 (2018). Short-read Illumina data of *D. mojavensis* from Catalina was retrieved from the NCBI
176 Short-read Archive SRR6425997 (Miller et al., 2018) and of Sonora from NCBI BioProject
177 PRJNA530196 (Allan & Matzkin, 2019).

178

179 **2.3. Computing resources**

180 All the programs were run on the UA Research Computing High Performance Computing (HPC)
181 at the University of Arizona. The cluster used is composed of 28 core processors with 168 gb
182 RAM per node, and is run via a PBS-Pro grid system. All the programs used were installed
183 under a user python virtual environment (pip). The majority of the programs used are available
184 as Bioconda packages for easy installation in non-cluster environments (Grünings et al., 2018).
185 They are also provided as Docker containers through Bioconda which can be run through
186 *Singularity* (<https://sylabs.io/>) on cluster systems. All command lines are provided in Appendix.

187

188

189 **2.4. Assembly pipelines**

190 *2.4.1. DBG2OLC Pipeline*

191 The DBG2OLC Pipeline is composed of three main steps: (i) the hybrid assembly via the
192 DBG2OLC program, (ii) the long-read assembly only, and (iii) the merging of those two
193 assemblies (Fig. 2).

194

195 (i) *Hybrid assembly*

196 *DBG2OLC* uses contigs from a short-read assembly and maps them to the raw long-reads, which
197 are then compressed into the list of the short-read's contig identifiers (Ye et al., 2016). A best
198 overlap graph is constructed from those compressed long-reads before uncompressing them into a
199 consensus sequence. This method is both highly accurate and extremely fast (Ye et al., 2016).
200 Then, the consensus contigs, or backbones are corrected using *Sparc* (Ye & Ma, 2016). *Sparc*
201 builds a sparse k-mer graph (k-mers in different positions are treated independently) using the
202 contigs identifiers' list associated with each raw long-read. All short-read contigs are then
203 aligned to their associated long-read using the *Blasr* aligner from the PacBio SMRT toolkit
204 (SMRT Link v4.0.0), previously *Pbdagcon*, which is the most time-consuming step. *Sparc*
205 finally uses these alignments to refine the graph and create a polished consensus sequence. In the
206 present study, we tested two competing short-read assemblers, *SparseAssembler* (provided with
207 the *DBG2OLC* installation package) (Ye, Ma, Cannon, Pop & Yu, 2012), and *Platanus* version
208 1.2.4 (Kajitani et al., 2014). We used the March 2019 version of *DBG2OLC* (Ye et al., 2016), the
209 January 2015 version of *Sparc* (Ye & Ma, 2016), and *Blasr* 5.3.5 (b30da0) (SMRT Link v4.0.0).
210 Note that we began working with an older version of *Blasr* which was significantly slower and
211 led to slightly different results. For this reason, and because programs often include third party
212 packages, it is important to keep track of each version used and physically separate the
213 repositories, so the SMRT toolkit was installed in an independent directory with no direct link to
214 the user bin, except for the *Blasr* program. We modified the `split_and_run_sparc.sh` script
215 available from the *Sparc* Github repository so as to call the `split_reads_by_backbone.py` script
216 externally (Appendix I), and to set the number of chores used by *Blasr* from the command line.
217 This way, it is easier to rerun the time-consuming *Sparc* step in case of crash from where it
218 stopped, and after moving the already corrected backbones into another directory.

219

220 The hybrid assembly was then polished using the PacBio tool in the SMRT toolkit (SMRT Link
221 v4.0.0). The version of the PacBio correction tool is frequently updated along with chemistry
222 technology of PacBio sequencing, therefore the version *Quiver* (v2.1.0) was used for
223 *D. melanogaster* (sequenced in 2014 on a PacBio RS II system; Kim et al., 2014), and the version

224 *Arrow* (v2.1.0) was used for *D. mojavensis* (sequenced in 2017 on a PacBio Sequel system
225 installed with SMRT Link v4.0.0, see above). For simplicity, we will thereafter refer to that step
226 simply as '*Quiver*'. *Quiver* aligns the raw PacBio reads to the assembled and corrected contigs
227 output by *Sparc*, and uses a consensus caller to polish them (Chin et al., 2013). Lastly, the hybrid
228 assembly was polished using *Pilon* v1.22 (Walker et al., 2014). *Pilon* uses raw short-reads
229 aligned to the assembly with the *Bowtie2* aligner version 2.2.9 (Langmead, Trapnell, Pop &
230 Salzberg, 2009), to first find and correct SNPs and small indels (base error consensus), and
231 secondly local misassemblies (alignment discrepancies scan) that are reassembled using paired
232 ends and mate pairs (if provided). Parameters were optimized at each step: (a) choice of the
233 short-read assembler (*Platanus* vs. *SparseAssembler* with kmer-size 39 or 53); (b) *DBG2OLC*
234 parameters, based on recommended optimization ranges (Ye et al., 2016): **MinOverlap** in [20;
235 150]; **AdaptiveTh** in [0.002; 0.02]; **KmerCovTh** in [2; 10] and **MinLen** in [200; 2,000]; default
236 values were otherwise used ($k = 17$; $LD1 = 0$); (c) **ContigTh** 0 (default) vs. 1 (recommended for
237 $> 100x$ PacBio coverage only); and (d) *Sparc* one vs. two iterations. These parameters are
238 summarized in Table 2.

239

240 (ii) Long-read assembly only

241 The long-read assembly was created using *Canu* v1.5 (Koren et al., 2017), which significantly
242 outperforms its older version *Celera Assembler* (PbcR) used in Chakraborty et al. (2016) as well
243 as other assemblers, notably by using an adaptive kmer weighting which both improves the
244 efficiency and the quality of the assembly of highly repetitive genomic regions. We tested two
245 parameters for the **correctedErrorRate**: 0.039 vs. 0.055 (low end and middle value of
246 recommended range, Table 3, Koren et al., 2017). Note that this adjustment is limited by
247 coverage, and thus intrinsic to the analyzed data set. We ran the three *Canu* steps (correction,
248 trimming and assembly) separately using the options **-correct**, **-trim** and **-assemble** (see
249 Appendix) to optimize the assembly step without running again the first two steps. Similarly to
250 the hybrid assembly, the long-read only assembly was polished using both *Quiver* and *Pilon*.

251

252 (iii) Assembly merging

253 The hybrid assembly and long-read only assembly were merged after polishing using the
254 *Quickmerge* tool v0.2 (Chakraborty et al. 2016). *Quickmerge* uses *MUMmer* (v 3.0) (Kurtz et al.
255 2004) to align the two assemblies and find the unique best alignment (using the `-delta-filter`
256 option in *MUMmer*). *Quickmerge* then identifies high confidence overlaps between the two
257 assemblies to find seed contigs (i.e., contigs that can be extended at both ends). Finally, it
258 merges the overlapping contigs using sequences from the reference (donor assembly) into the
259 query (acceptor assembly). The optimization consisted of trying both the hybrid and long-read
260 assemblies as reference vs. query, and varying the `l` and `lm` cutoff parameters in *Quickmerge*
261 (Table 3). Lastly, the merged assembly was polished using *Quiver* and *Pilon*.

262

263 2.4.2. Test of the DBG2OLC pipeline with Nanopore long-reads

264 We ran the DBG2OLC pipeline on CAT sequencing data using Nanopore raw reads instead of
265 PacBio raw reads for the long-read only assembly, the hybrid assembly, and the polishing steps.
266 We used the optimal parameter set (2.4.1; P6), except for *Canu*, for which we had to increase the
267 `correctedErrorRate` to 0.055 to recover 97% of the genome, while we could recover only 51%
268 using a `correctedErrorRate` of 0.039. Instead of *Quiver*, we used *Nanopolish* version 0.11.0
269 (Simpson et al., 2017). Similar to *Quiver* and *Pilon*, raw Nanopore reads were first aligned to the
270 target assembly using the *Bwa* aligner version 0.7.17 (Li & Durbin, 2010). *Nanopolish* then
271 generates an improved consensus sequence.

272

273 2.4.3. Alternative Pipelines

274 *DBG2OLC* was identified as the only pipeline, among the most recently published assemblers,
275 allowing the assembly of long-reads prior to correction. Alternatively, long-reads may be
276 corrected prior to assembly, as is the case in the *Canu* pipeline. Other possible correction tools
277 include *LSCplus* (Hu, Sun & Sun, 2016), a modified version of the *MHAP* tool (indexing kmers
278 used to build the assembly graph in *Celera*; (Carvalho, Dupim & Goldstein, 2016), *HALC* (Bao
279 & Lan, 2017), and *FMLRC* (Holt, Wang, Jones & McMillan, 2016), and most recently (not tested
280 here) *MECAT* (Xiao et al., 2017) and *Jabba* (Miclotte et al., 2016). The *LSCplus* package was
281 not available at the time of our study, and we were therefore not able to assess its efficiency. The
282 modified *MHAP* tool was implemented in *Celera* only (the older version of *Canu*), which thus

283 yielded poor results in term of assembly contiguity, and this solution was abandoned. Note
284 however that some aspects of kmers indexing as proposed in the modified *MHAP* tool have now
285 been implemented in *Canu* (Koren et al., 2017), and were therefore implicitly used in our
286 *DBG2OLC* Pipeline. *FMLRC*, although it correctly performed the long-read correction, proved
287 to be non-compatible with *Canu* (Holt et al., 2016). Therefore, this alternative was abandoned as
288 well.

289
290 *HALC* corrects long-reads by (i) aligning them to the contigs from a short-read assembly, (ii)
291 constructing a graph from this alignment, and (iii) finding the best path in the graph to correct
292 each long-read. It relies on *Blasr* (SMRT Link v4.0.0) for the alignment and on *LoRDEC*
293 (Salmela & Rivals 2014) for the correction. We used the one version of *HALC* available, *Blasr*
294 5.3.5 and *LoRDEC* 0.6 with the *GATB* library 1.0.6. After read correction with *HALC*, we ran
295 *Canu* (-assemble option) with a correctedErrorRate = 0.039. The contiguity of the assembly
296 was orders of magnitude worse than when using the *Canu* correction tool and the same assembly
297 parameters (*HALC* correction: N50 = 488,850; total length = 138,021,997; max length =
298 2874227; *Canu* pipeline: N50 = 10,9990,654; total length = 151,043,692; max length =
299 25,950,142). This might have been improved by parameter optimization of both the *HALC*
300 correction step and the assembly step with the *Canu* assembler, but due to the strong difference in
301 contiguity we chose to not utilize *HALC*. Therefore, we focused on optimizing the *DBG2OLC*
302 pipeline only.

303

304 **2.5. Assembly quality check**

305 Comparisons between assemblies and quality assessment were performed based on assembly
306 statistics from *Quast* version 4.6.2 (Gurevich et al., 2013) by comparing each assembly to a
307 reference genome to estimate the number of global and local misassemblies as well as the number
308 of mismatches and indels. For both general statistics (number of fragments, N50) and error rates
309 (presented in Tables 4-6), we used contigs longer than 400 bp only, so as to run the program
310 faster. We also calculated *BUSCO* scores using the diptera (odb9) set of Benchmarking
311 Universal Single-Copy Orthologs (Waterhouse et al. 2017). We used the reference genomes
312 FB2017_01 and FB2015_02 (Consortium DG, 2007) released on FlyBase (Thurmond et al., 2019)

313 for Dmel and CAT, respectively. For SON, we used a template assembly constructed based on
314 the Catalina reference genome (Allan & Matzkin, 2019). For each data set, we extracted only the
315 fragments that have been previously designated to chromosomes (i.e., for Dmel, the four
316 chromosomes; and for SON and CAT the 39 biggest scaffolds), so as to run quality assessment
317 faster. We are aware that using a template assembly as a reference for SON may introduce biases
318 especially in terms of number of misassemblies, due to the evolutionary history of the
319 *D. mojavensis* populations (Matzkin, 2014) therefore the results must be considered carefully.
320 However, this provides a valid guide to make relative comparisons between assemblies created
321 here. *Quast* relies on *MUMmer* v3.23 (nucmer aligner v3.1; Kurtz et al., 2004) to align the
322 assembly to the reference genome, and includes metrics and methods from the *GAGE* assessment
323 tool (Salzberg et al., 2012) and other tools.
324 Finally, assemblies were aligned to their reference genome using MUMmer4 (Marçais et al.,
325 2018) and plotted against the reference genome using Dot (<https://github.com/dnanexus/dot>).

326

327 **2.6. Test of the *DBG2OLC* pipeline with Nanopore long-reads**

328 We tested the *DBG2OLC* pipeline on *D. mojavensis* population Catalina using Nanopore long-
329 reads instead of PacBio long-reads with parameters optimized for SON: *Platanus* short-read
330 assembler; *DBG2OLC* parameters MinOverlap 150; AdaptiveTh 0.020; KmerCov 2; MinLen
331 200; number of *Sparc* iterations 2 or 3 (both tested: P6r” vs. P6fr”). For the *Canu* assembly, we
332 used the correctedErrorRate = 0.055 since lower rates resulted in incomplete genome (0.039:
333 73.7 %; 0.045: 86.7 %; 0.055: 93.8 %). For the merged assembly, we used parameters as
334 optimized for SON: the hybrid assembly as query; l = 1 Mb and lm = 10,000 bp (Table 3).

335

336 **3 RESULTS**

337 **3.1. DNA preparation**

338 The custom chloroform extraction led to a remarkable increase in the sizes of DNA fragments
339 (light band between 30 and 120 Kb, right panel, Fig. 1) compared with standard extraction kits
340 (left and middle panels, Fig. 1) for which the majority of fragments were shorter than 30 Kb.
341 Long fragments in DNA libraries significantly increase DNA quantity output by PacBio
342 sequencing (www.pacb.com).

343

344 **3.2. Optimization of the DBG2OLC Pipeline**

345 *3.2.1. Short-read assembler*

346 *Platanus* and *SparseAssembler* with a kmer size of 53 bp resulted in very similar assemblies;
347 *SparseAssembler* with a kmer size of 39 bp led to reduced contiguity; and applying two
348 successive rounds of *SparseAssembler* 53 bp-Kmer size did not improve the short-read assembly.
349 In the final merged assemblies, the use of *SparseAssembler* always led to slight decrease in
350 contiguity (comparing P3 to S3 and P6 to S6 for both Dmel and SON, Table 4).
351 *SparseAssembler* slightly reduced error rates but also BUSCO scores for Dmel, with limited
352 effects for SON (Fig. 3B). We also observed that differences in P6 and S6 for SON mainly
353 resided in highly repetitive regions.

354

355 *3.2.2. DBG2OLC parameters*

356 We varied the DBG2OLC parameters `MinOverlap`, `AdaptiveTh`, `KmerCovTh` and `MinLen` to
357 simultaneously optimize the contiguity and quality of the final assembly. Misassemblies created
358 during the first steps of the hybrid assemblies were overall not resolved later, which makes that
359 step key to the optimization. P0 corresponds to the reference set of parameters used in
360 Chakraborty et al. (2016).

361

362 `MinOverlap` had a major effect on final assemblies, with a major improvement of contiguity
363 (reduced number of fragments, increased N50, increased length of longest fragment; Fig. 3A,C)
364 and of accuracy (reduced number of global and local misassemblies, reduced number of
365 mismatches and indels; Fig. 3B, 3D) as seen in the P0 vs. P3 and P1 vs. P6 comparisons. This
366 came at a cost of a slight decrease in BUSCO score for Dmel but not SON. Only an increase of
367 `MinOverlap` up to 150 (the maximum recommended value for more than 50x coverage of PacBio
368 reads) led to an optimal lower number of misassemblies (P2 vs. P6).

369

370 `AdaptiveTh` had little influence, except when `MinOverlap` was kept low: it decreased contiguity
371 and accuracy (P2 vs. P0). For assemblies with high `MinOverlap`, we found that P3 was less
372 fragmented than P4, P5 or P6 for SON and P4 was the least fragmented for Dmel. P6 was the

373 best compromise between contiguity and accuracy for SON, with the highest BUSCO score. P4
374 was the best compromise and with the highest BUSCO score for Dmel. Although coverage in
375 both Illumina short-reads and PacBio long-reads was lower in SON than Dmel (Table 1), the
376 quality of PacBio long-reads was higher (longer reads thanks to DNA extraction protocol, and
377 more recent PacBio technology), which might have facilitated the better results with the more
378 stringent *AdaptiveTh*.

379

380 High *KmerCovTh* values resulted in major global misassemblies in SON (assessed with
381 Mummer plots, not shown), with the largest fragment longer than the theoretical longest fragment
382 in the Reference assembly (P4y vs. P4 and P6y,x vs. P6). It also caused a slight increase in error
383 rates and a slight decrease in BUSCO score. In Dmel, no major global misassembly was
384 detected, however error rates were higher and BUSCO scores slightly lower. We recommend to
385 use *KmerCovTh* = 2, especially when using high *AdaptiveTh*. Using *ContigTh* = 1 had similar
386 effects (major global misassemblies; P6g vs. P6 for SON) than high *KmerCovTh* values.

387

388 Increased *MinLen* from 200 to 2,000 resulted in a slight increase in contiguity for both Dmel and
389 SON (PX vs. PXa). Error rates and BUSCO scores were not notably different, unless *MinLen*
390 was increased up to 5,000 in which too many reads were parsed out, leading to higher error rates.

391

392 Increasing the number of *Sparc* iterations from 2 to 3 allowed a higher contiguity of large
393 fragments, although with little effect on overall statistics.

394

395 The following parameters were used throughout the next optimization step: short-read assembler:
396 *Platanus*; *MinOverlap* 150; *AdaptiveTh* 0.020 for SON, 0.010 for Dmel; *KmerCov* 2; *MinLen*
397 200 ; *ContigTh* 0; and number of *Sparc* iterations 3.

398

399 3.2.3. *Canu* parameters

400 Increasing *correctedErrorRate* from 0.039 to 0.055 slightly increased contiguity of the merged
401 assembly (P4q vs. P4r for Dmel and P6q vs. P6r for SON; Table 5). However, it also increased
402 error rates overall, and decreased the BUSCO complete genes score, especially for SON.

403

404 3.2.4. *Quickmerge* parameters

405 Parameters used in *Quickmerge* are shown in Table 3, and results in Table 5. Using the long-read
406 assembly as the Query assembly resulted in a strong decrease in contiguity compared with the
407 opposite (P4s vs. P4q for Dmel and P6s vs. P6q for SON). It also considerably increased error
408 rates for both species, and slightly decreased BUSCO complete gene score for SON.

409

410 We also tested the impact of the *l* and *lm* parameters. Using low *lm* with high *l* resulted in
411 identical assemblies (P4p vs. P4 for Dmel and P6p vs. P6 for SON) since backbones were already
412 parsed out due to high *lm*. Also, using *lm* = N50 or *l* = N50/2 resulted in identical assemblies
413 for Dmel. Otherwise, decreasing *l* resulted in lower number of fragments but higher error rates.
414 However, using a too high *lm* value would prevent smaller fragments from being merged.

415

416 3.2.5. *Polishing*

417 Polishing with both Quiver/Arrow and Pilon did not affect the contiguity (number of fragments,
418 N50, and largest fragment; Table 6, Fig. 3) for either species. Conversely, it significantly
419 reduced the number of indels on hybrid, long-read and merged assemblies. The number of
420 mismatches was also reduced to a lesser extent. One drawback was the increase in number of
421 misassemblies, except for the merged assembly. Finally, polishing increased the BUSCO score,
422 especially on the hybrid assembly.

423

424 3.3. Test of the *DBG2OLC* pipeline with Nanopore long-reads

425 Compared with Miller et al. (2018), the CAT merged assembly was more contiguous and with a
426 higher BUSCO score, but with higher error rates (Table 5, P6r” vs. Ref. Miller), likely due to the
427 multiple polishing steps performed by Miller et al. (2018). Also note that we used raw,
428 uncorrected Nanopore reads for the CAT hybrid assembly similar to the SON hybrid assembly
429 with raw PacBio reads. Read correction prior to the hybrid assembly might help reduce error
430 rates (e.g., using the Nanopore basecall *Guppy* algorithm; Wick, Judd & Holt, 2019). Compared
431 with SON, CAT assemblies were less contiguous but with higher BUSCO scores (CAT-P6r” vs.

432 SON-P6n” and CAT-P6fr” vs. SON-P6fn”). The assignments of SON-P6fn” scaffolds to Muller
433 elements can be found in Supporting Information S1.

434

435

436 **4 DISCUSSION**

437 **4.1. Optimized DBG2OLC pipeline**

438 We performed an optimization of the *DBG2OLC* pipeline at each step, using both the model
439 species *D. melanogaster* and a non-model cactophilic *Drosophila*, *D. mojavensis* (population
440 Sonora). Based on our analysis, we make the following recommendations:

441

442 First, we were able to replicate the results by Chakraborty et al. (2016): our P0 assembly had
443 similar contiguity as theirs with ~100x coverage although with lower error rates. The short-read
444 assembler had little impact, but we recommend using *Platanus*, which is especially designed for
445 genomes with high level of heterozygosity (Kajitani et al., 2014), and this is more likely in non-
446 model organisms that have not been raised for many generations in the laboratory. Based on
447 *Quast* results and BUSCO scores, we found the following parameter to be optimal on our
448 assembly for *D. mojavensis*: MinOverlap 150; AdaptiveTh 0.020; KmerCov 2; MinLen 200;
449 ContigTh 0; number of *Sparc* iterations 3 (SON assembly “P6fn”). We also tested these
450 parameters on the genomes of the other two *D. mojavensis* populations (from Baja California and
451 Mojave Desert) and of two sibling species, *D. arizonae* and *D. navojoa* (unpublished data L.
452 Matzkin), and found that the most stringent value for MinOverlap and AdaptiveTh lead to the
453 best results, although AdaptiveTh had a smaller impact and its value may be safely reduced to
454 0.010, to be adjusted based on PacBio coverage. With these two parameters set to high values,
455 we recommend to not increase KmerCovTh, MinLen and ContigTh since this would result in
456 too many long-reads parsed out and we observed major misassemblies. Conversely, increasing
457 *Sparc* iterations, which is supposed to reduce the number of chimeras, had no negative effect.
458 We found that with high PacBio reads coverage, it is best to use the *Canu* correctedErrorRate
459 as low as possible, to 0.039. This increases the contiguity and decreases errors in the long-read
460 only assembly. However, this is not always possible, and can cause the genome size to be shorter
461 than expected, as observed with the Nanopore reads. Finally, and similarly to Chakraborty et al.

462 (2016), we recommend to use the hybrid assembly as query and to adjust the *Quickmerge*
463 parameter *l* to an intermediate value of 1 Mb, to prevent too many chimeric scaffolds while
464 allowing smaller fragments to be merged. Note that BUSCO scores and general statistics can
465 always be calculated even in absence of a reference genome of a closely related species.

466

467 **4.2. Benefits of using the *DBG2OLC* pipeline and demonstration of effectiveness on a non-** 468 **model species**

469 By merging hybrid and long-read only assemblies, we considerably increased the contiguity
470 compared with that of the hybrid assembly or the long-read only assembly (Table 6, Fig. 3), as
471 shown in Chakraborty et al. (2016). Also, error rates were lower than the long-read assembly,
472 especially for Dmel. To obtain such low error rates with long-read data only, a higher coverage
473 would have been necessary representing a significant increase in sequencing cost (discussed in
474 Chakraborty et al., 2016). For this study the *D. mojavensis* Illumina sequencing was performed
475 in 2011, if using current sequencing core prices it will total ~ \$178 (PE 150 HiSeq lane ~\$1,300
476 [only 1/12th of a lane needed for the 160 Mb *D. mojavensis* genome]; quality control of library
477 \$15; library preparation \$50-\$400 [depending if done in-house or by a core]). The PacBio
478 sequencing was performed using a Sequel system, totaling \$3,190 (library preparation \$495 × 2
479 libraries; SMRT cells \$1,100 × 2). Given the recent release of PacBio's Sequel II system the cost
480 for a similar amount of long-reads would be approximately ~\$740 (library preparation ~\$450,
481 SMRT cell \$1,750 [would only need 1/6th of a cell for *D. mojavensis*]), therefore the *de novo*
482 assembly described in this study could be built for less than \$1,000.

483

484 One major improvement of the merged assembly (P6fn") in SON is that the 2q⁵ inversion in the
485 Muller element E (described in Ruiz, Heed & Wasserman, 1990) is now resolved, with the two
486 breakpoints clearly bridging the three chromosome parts (Fig. 4). This was not the case in the
487 hybrid assembly or the long-read only assembly (not shown). The Muller elements B, D and E in
488 our merged assembly P6fn" are in one piece and correspond to 99.24%, 99.11% and 96.67%
489 respectively of the corresponding chromosomes in the CAT reference genome. The Muller
490 element C was composed of three pieces in P6fn" accounting for 99.94% of the length of the
491 Muller element C in the CAT reference, and the Muller element A was more fragmented, as is

492 also the case in the CAT reference genome and all fragment lengths summed up to 94.77% of the
493 total size in the AT reference. However, in the CAT reference genome, D was in two fragments
494 that were joined in our assembly.

495

496 **4.3. Conclusion**

497 In the not too distant past genomic analysis was limited to just a set of a few model laboratory
498 species. Although this has led to unprecedented advances in our understanding of genetics and
499 genomics, in many instances such studies lacked an ecological context. Genome assemblies of
500 non-model species tended to be more fragmented or tended to be built using a genome from a
501 related model species, which is problematic if interested in trait mapping or genome structure
502 evolution. Current sequencing and computational advancements have liberated our dependence
503 on classical laboratory model species. Here we have outlined a widely applicable computational
504 pipeline and sets of parameters to facilitate the construction of chromosome or nearly-
505 chromosome level genomic assemblies in a non-model species. Our PacBio merged assembly
506 performed better than using Nanopore reads, but more work is still needed to assess any
507 differences across multiple species, especially with newer advances to the sequencing platforms.
508 Although it would be ideal to have a single set of parameters that would produce chromosome-
509 level assemblies in all species, genomes are different. Ultimately the most optimal assembly
510 strategy would likely be to create a number of assemblies using multiple parameters, assessing
511 their performance and possibly combining parts of assemblies.

512

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519

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651 **7 DATA AVAILABILITY**

652 Raw PacBio reads are available at NCBI's SRA (<https://www.ncbi.nlm.nih.gov/sra>) under
653 BioProject accession number PRJNA573111. The *D. mojavensis* final assembly P6fn" is
654 available at OSF (doi.org/10.17605/OSF.IO/pvbde).

655

656 **8 AUTHOR CONTRIBUTION**

657 CCJ, CWA and LMM reviewed the literature and selected the pipeline. CWA performed DNA
658 extraction. CCJ performed the bioinformatics work with the help of CWA. CCJ wrote the
659 manuscript with contributions from LMM and CWA.

660

661

662 **Tables**

663

664 **Table 1. Sequencing technology and coverage of each data set.**

| Organism | Genome size | Sequencing technology | Data output | Coverage |
|---|-------------|--|----------------------|----------|
| <i>Drosophila melanogaster</i> | ~ 140 Mb | Illumina HiSeq 2000 | 16.8 Gb ^a | 120x |
| | | PacBio RS II | 16.1 Gb ^b | 115x |
| <i>Drosophila mojavensis</i> (Sonora) | ~ 160 Mb | Illumina HiSeq 2500 100 bp paired ends | 10.1 Gb ^a | 63x |
| | | 2,500 bp mate pairs | 5.9 Gb ^c | 37x |
| | | PacBio Sequel | 10.4 Gb ^b | 65x |
| <i>Drosophila mojavensis</i> (Catalina) | ~ 160 Mb | Illumina NextSeq 500 150 bp paired ends | 9.1 Gb ^a | 55x |
| | | Oxford Nanopore MinION | 15.2 Gb | 95x |

665 ^a calculated as the total number of bases in data files after trimming from *Platanus_trim*.

666 ^b calculated as the total number of bases in data files obtained from converting .bax.h5 files into .bam files, and
667 converting the obtained subreads.bam files into fasta files, with no specific trimming

668 ^c calculated as the total number of bases in data files after trimming from *Platanus_internal_trim*.

669

670

671 **Table 2. DBG2OLC parameters.**

| Assembly | Assembler | MinOverlap | AdaptiveTh | MinLen | KmerCovTh | Number of | |
|----------|------------------------|------------|------------|--------|-----------|----------------------------|----------|
| | | | | | | <i>Sparc</i> iterations | ContigTh |
| P0 | <i>Platanus</i> | 20 | 0.002 | 200 | 2 | 2 | 1 |
| P1 | <i>Platanus</i> | 20 | 0.020 | 200 | 2 | 2 | 1 |
| P2 | <i>Platanus</i> | 100 | 0.020 | 200 | 2 | 2 | 1 |
| P3 | <i>Platanus</i> | 150 | 0.002 | 200 | 2 | 2 | 1 |
| S3 | <i>SparseAssembler</i> | 150 | 0.002 | 200 | 2 | 2 | 1 |
| P4 | <i>Platanus</i> | 150 | 0.010 | 200 | 2 | 2 | 1 |
| P4f | <i>Platanus</i> | 150 | 0.010 | 200 | 2 | 3 | 1 |
| P4g | <i>Platanus</i> | 150 | 0.010 | 200 | 2 | 3 | 2 |
| P5 | <i>Platanus</i> | 150 | 0.015 | 200 | 2 | 2 | 1 |
| P6 | <i>Platanus</i> | 150 | 0.020 | 200 | 2 | 2 | 1 |
| P6f | <i>Platanus</i> | 150 | 0.020 | 200 | 2 | 3 | 1 |
| P6g | <i>Platanus</i> | 150 | 0.020 | 200 | 2 | 3 | 2 |
| S6 | <i>SparseAssembler</i> | 150 | 0.010 | 200 | 2 | 2 | 1 |
| P4a | <i>Platanus</i> | 150 | 0.010 | 2000 | 2 | 2 | 1 |
| P5a | <i>Platanus</i> | 150 | 0.015 | 2000 | 2 | 2 | 1 |
| P6a | <i>Platanus</i> | 150 | 0.020 | 2000 | 2 | 2 | 1 |
| P6b | <i>Platanus</i> | 150 | 0.020 | 5000 | 2 | 2 | 1 |
| P4y | <i>Platanus</i> | 150 | 0.010 | 200 | 10 | 2 | 1 |
| P6y | <i>Platanus</i> | 150 | 0.020 | 200 | 10 | 2 | 1 |
| P6x | <i>Platanus</i> | 150 | 0.020 | 200 | 5 | 2 | 1 |

672

673

674 **Table 3. *Canu* and *Quickmerge* parameters.**

| Species | Assembly | <i>Canu</i> | Merge parameters | | |
|---------|----------|--------------------|------------------|------------------|---------|
| | | correctedErrorRate | Query assembly | l (bp) | lm (bp) |
| Dmel | P4 | 0.039 | Hybrid | N50: 10,000,000 | 10,000 |
| | P4f | 0.039 | Hybrid | N50: 10,000,000 | 10,000 |
| | P4n | 0.039 | Hybrid | N50/2: 5,000,000 | 10,000 |
| | P4o | 0.039 | Hybrid | N50/4: 2,500,000 | 10,000 |
| | P4o'' | 0.039 | Hybrid | 1,000,000 | 10,000 |
| | P4fo'' | 0.039 | Hybrid | 1,000,000 | 10,000 |
| | P4p | 0.039 | Hybrid | N50: 10,000,000 | 0 |
| | P4q | 0.039 | Hybrid | 0 | 0 |
| | P4r | 0.055 | Hybrid | 0 | 0 |
| | P4s | 0.039 | Long-read | 0 | 0 |
| SON | P6 | 0.039 | Hybrid | N50: 2,600,000 | 10,000 |
| | P6f | 0.039 | Hybrid | N50: 2,600,000 | 10,000 |
| | P6n | 0.039 | Hybrid | N50/2: 1,300,000 | 10,000 |
| | P6n'' | 0.039 | Hybrid | 1,000,000 | 10,000 |
| | P6fn'' | 0.039 | Hybrid | 1,000,000 | 10,000 |
| | P6o | 0.039 | Hybrid | N50/4: 0,650,000 | 10,000 |
| | P6p | 0.039 | Hybrid | N50: 2,600,000 | 0 |
| | P6q | 0.039 | Hybrid | 0 | 0 |
| | P6r | 0.055 | Hybrid | 0 | 0 |
| | P6s | 0.039 | Long-read | 0 | 0 |
| CAT | P6r'' | 0.055 | Hybrid | 1,000,000 | 10,000 |
| | P6fr'' | 0.055 | Hybrid | 1,000,000 | 10,000 |

675

Table 4. *DBG2OLC* parameter optimization: contiguity and accuracy. Assemblies refer to parameter sets defined in Table 2.

| Species | Assembly | # Fragments | N50 (bp) | Largest fragment size (bp) | # global misassemblies | # local misassemblies | # Mismatches per 100 Kb | # Indels (per 100 Kb) | BUSCO score (%) | | |
|---------|----------|-------------|------------|----------------------------|------------------------|-----------------------|-------------------------|-----------------------|-----------------|------------------|---------------|
| | | | | | | | | | complete genes | fragmented genes | missing genes |
| Dmel | P0 | 792 | 16,084,532 | 25,835,722 | 2,356 | 1,001 | 12.62 | 9.51 | 98.11 | 0.57 | 1.32 |
| | P1 | 900 | 16,023,660 | 24,892,400 | 2,656 | 1,027 | 11.82 | 9.55 | 97.32 | 0.61 | 2.07 |
| | P2 | 292 | 21,449,278 | 24,867,057 | 1,076 | 371 | 10.27 | 7.35 | 97.07 | 0.64 | 2.07 |
| | P3 | 282 | 19,409,490 | 25,811,113 | 890 | 370 | 10.68 | 6.57 | 97.21 | 0.50 | 2.29 |
| | S3 | 314 | 19,674,671 | 24,895,732 | 771 | 312 | 10.03 | 7.03 | 95.64 | 0.79 | 3.57 |
| | P4 | 266 | 21,413,354 | 25,775,485 | 623 | 267 | 8.66 | 6.49 | 98.75 | 0.57 | 0.68 |
| | P4f | 267 | 21,413,185 | 25,776,014 | 919 | 318 | 10.55 | 6.73 | 98.75 | 0.57 | 0.68 |
| | P4g | 227 | 21,412,816 | 25,796,604 | 667 | 263 | 10.05 | 6.47 | 98.39 | 0.57 | 1.04 |
| | P5 | 268 | 19,684,947 | 25,810,399 | 882 | 323 | 10.19 | 6.95 | 97.11 | 0.57 | 2.29 |
| | P6 | 261 | 21,455,994 | 24,861,269 | 801 | 289 | 10.65 | 7.02 | 97.11 | 0.64 | 2.25 |
| | S6 | 290 | 19,674,784 | 24,911,291 | 726 | 331 | 10.19 | 6.84 | 95.39 | 0.82 | 3.79 |
| | P4a | 267 | 21,414,759 | 25,777,026 | 920 | 338 | 10.79 | 6.94 | 98.75 | 0.57 | 0.68 |
| | P5a | 270 | 19,684,947 | 25,810,399 | 892 | 323 | 10.19 | 6.95 | 97.11 | 0.61 | 2.29 |
| | P6a | 258 | 21,455,918 | 24,861,450 | 553 | 282 | 9.11 | 6.36 | 97.11 | 0.64 | 2.25 |
| | P6b | 259 | 21,455,964 | 24,861,284 | 785 | 295 | 10.37 | 6.90 | 97.11 | 0.64 | 2.25 |
| | P4y | 239 | 21,413,104 | 25,789,761 | 633 | 304 | 10.43 | 6.83 | 98.71 | 0.57 | 0.71 |
| P6x | 257 | 21,450,514 | 24,861,357 | 777 | 324 | 10.64 | 7.08 | 97.11 | 0.64 | 2.25 | |

| | | | | | | | | | | |
|------------------------------------|-----|------------|------------|--------|--------|--------|--------|-------|------|------|
| P6y | 242 | 21,455,993 | 24,861,357 | 610 | 298 | 9.15 | 6.35 | 97.03 | 0.64 | 2.32 |
| Ref. genome | 7 | 25,286,936 | 32,079,331 | NA | NA | NA | NA | 98.68 | 0.75 | 0.57 |
| Ref. Chakraborty et al. (P0) | NA | ~23 Mb | NA | ~5,500 | ~3,300 | ~18 | 130 | NA | NA | NA |
| P0 | 348 | 28,767,831 | 34,246,767 | 8,244 | 10,064 | 328.42 | 265.12 | 98.39 | 0.86 | 0.75 |
| P1 | 495 | 21,851,486 | 34,252,641 | 9,066 | 10,330 | 329.18 | 266.49 | 98.39 | 0.82 | 0.79 |
| P2 | 132 | 27,067,002 | 33,145,725 | 7,351 | 9,756 | 326.40 | 264.27 | 98.25 | 0.96 | 0.79 |
| P3 | 80 | 27,092,095 | 34,236,744 | 7,189 | 9,670 | 325.21 | 263.71 | 98.25 | 1.00 | 0.75 |
| S3 | 90 | 18,984,317 | 34,265,435 | 7,214 | 9,687 | 324.97 | 262.72 | 98.36 | 0.93 | 0.71 |
| P4 | 92 | 27,081,921 | 33,130,663 | 7,166 | 9,688 | 324.99 | 264.24 | 98.21 | 1.04 | 0.75 |
| P5 | 97 | 27,073,333 | 34,231,430 | 7,327 | 9,782 | 324.58 | 263.90 | 98.36 | 0.89 | 0.75 |
| P6 | 104 | 27,074,084 | 33,145,389 | 7,168 | 9,753 | 324.69 | 264.16 | 98.39 | 0.93 | 0.68 |
| P6f | 103 | 27,125,966 | 33,144,936 | 7,173 | 9,760 | 324.80 | 264.49 | 98.36 | 0.96 | 0.68 |
| P6g | 80 | 27,068,405 | 37,117,163 | 7,151 | 9,759 | 324.58 | 267.38 | 98.39 | 0.96 | 0.64 |
| S6 | 119 | 11,627,144 | 33,084,645 | 7,280 | 9,737 | 324.83 | 263.96 | 98.25 | 1.00 | 0.75 |
| P4a | 88 | 27,072,608 | 34,217,177 | 7,255 | 9,736 | 324.67 | 263.74 | 98.32 | 0.89 | 0.79 |
| P5a | 97 | 27,081,858 | 34,230,744 | 7,391 | 9,750 | 324.72 | 264.10 | 98.36 | 0.96 | 0.68 |
| P6a | 104 | 27,124,465 | 33,144,017 | 7,179 | 9,761 | 325.07 | 264.57 | 98.43 | 0.89 | 0.68 |
| P6b | 98 | 27,089,768 | 34,232,191 | 7,237 | 9,755 | 324.76 | 264.37 | 98.39 | 0.93 | 0.68 |
| P4y | 98 | 27,058,011 | 37,135,015 | 7,391 | 9,798 | 324.25 | 267.26 | 98.29 | 1.04 | 0.68 |

SON

| | | | | | | | | | | |
|------|-----|------------|------------|-------|-------|--------|--------|-------|------|------|
| P6x | 99 | 27,066,010 | 33,076,049 | 7,211 | 9,740 | 324.72 | 263.24 | 98.32 | 0.89 | 0.79 |
| P6y | 100 | 27,061,534 | 37,152,367 | 7,168 | 9,753 | 324.56 | 267.59 | 98.32 | 1.00 | 0.68 |
| Ref. | 39 | 26,426,104 | 33,738,561 | NA | NA | NA | NA | 98.14 | 0.93 | 0.93 |

679 **Table 5. *Canu* and *Quickmerge* parameter optimization: contiguity and accuracy.** Assemblies refer to parameter sets defined in
 680 Table 3.

| Species | Assembly | # Fragments | N50 (bp) | Largest fragment size (bp) | # global misassemblies | # local misassemblies | # Mismatches per 100 Kb | # Indels per 100 Kb | BUSCO score | | |
|---------|----------|----------------|------------|----------------------------------|---------------------------|--------------------------|----------------------------|------------------------|-------------------|---------------------|------------------|
| | | | | | | | | | complete genes | fragmented genes | missing genes |
| Dmel | P4 | 266 | 21,413,354 | 25,775,485 | 623 | 267 | 8.66 | 6.49 | 98.75 | 0.57 | 0.68 |
| | P4f | 267 | 21,413,185 | 25,776,014 | 919 | 318 | 10.55 | 6.73 | 98.75 | 0.57 | 0.68 |
| | P4o | 265 | 21,413,352 | 25,775,437 | 635 | 275 | 8.57 | 6.42 | 98.75 | 0.57 | 0.68 |
| | P4o'' | 210 | 21,413,344 | 25,775,300 | 638 | 295 | 8.10 | 6.02 | 98.79 | 0.57 | 0.64 |
| | P4fo'' | 211 | 21,413,185 | 25,776,408 | 943 | 340 | 9.90 | 6.20 | 98.79 | 0.57 | 0.64 |
| | P4q | 116 | 21,413,360 | 25,775,323 | 1,561 | 521 | 8.72 | 5.77 | 98.79 | 0.57 | 0.64 |
| | P4r | 113 | 21,450,483 | 25,801,485 | 1,751 | 582 | 8.66 | 5.75 | 98.61 | 0.75 | 0.64 |
| | P4s | 338 | 14,528,003 | 25,770,616 | 3,684 | 1,145 | 12.34 | 7.34 | 98.79 | 0.57 | 0.64 |
| | Ref. | 7 | 25,286,936 | 32,079,331 | NA | NA | NA | NA | 98.68 | 0.75 | 0.57 |
| SON | P6 | 104 | 27,074,084 | 33,145,389 | 7,168 | 9,753 | 324.69 | 264.16 | 98.39 | 0.93 | 0.68 |
| | P6f | 103 | 27,125,966 | 33,144,936 | 7,173 | 9,760 | 324.80 | 264.49 | 98.36 | 0.96 | 0.68 |
| | P6n | 88 | 27,074,290 | 33,145,104 | 7,357 | 9,788 | 324.44 | 263.58 | 98.39 | 0.93 | 0.68 |
| | P6n'' | 67 | 27,074,601 | 33,145,127 | 7,261 | 9,728 | 325.29 | 263.80 | 98.29 | 0.96 | 0.75 |
| | P6fn'' | 66 | 27,125,795 | 33,144,975 | 7,271 | 9,727 | 325.23 | 263.97 | 98.25 | 1.00 | 0.75 |
| | P6o | 65 | 27,074,498 | 33,145,149 | 7,261 | 9,718 | 325.17 | 263.76 | 98.32 | 0.93 | 0.75 |
| | P6q | 62 | 27,074,467 | 33,145,189 | 7,470 | 9,752 | 325.83 | 263.63 | 98.32 | 0.93 | 0.75 |

| | | | | | | | | | | | |
|-----|----------------------|-----|------------|------------|-------|--------|--------|--------|-------|------|------|
| | P6r | 58 | 27,027,841 | 34,117,449 | 7,245 | 9,165 | 329.97 | 262.71 | 93.25 | 0.93 | 5.82 |
| | P6s | 151 | 27,122,727 | 34,181,614 | 9,159 | 10,205 | 328.59 | 263.06 | 98.14 | 0.93 | 0.93 |
| | Ref. | 39 | 26,426,104 | 33,738,561 | NA | NA | NA | NA | 98.14 | 0.93 | 0.9 |
| CAT | P6n ^o | 79 | 12,454,906 | 23,097,599 | 2,272 | 3,104 | 56.41 | 67.39 | 98.39 | 1.00 | 0.61 |
| | P6fn ^o | 79 | 12,457,238 | 23,102,169 | 2,274 | 2,949 | 56.40 | 65.70 | 98.64 | 0.82 | 0.54 |
| | Ref. | 39 | 26,866,924 | 34,148,556 | NA | NA | NA | NA | 98.11 | 0.93 | 0.96 |
| | Ref. | | | | | | | | | | |
| | Miller et al. (2018) | 122 | 5.0 Mb | NA | NA | NA | 0.22 | 0.052 | 98 | NA | NA |

681 **Table 6. Improvement of contiguity and quality throughout the pipeline.** Abbreviations: Sr: short-read assembly; H: hybrid assembly;
682 Lr: long-read assembly; M: merged assembly; Q: Quiver polishing; P: Pilon polishing. Here MQP corresponds to P4 for Dmel and P6 for
683 SON (Tables 4, 5).

| Species | Assembly | # Fragments | N50 (bp) | Largest fragment (bp) | # global misassemblies | # local misassemblies | # Mismatches per 100 kb | # Indels per 100 kb | BUSCO score | | |
|---------|----------|-------------|------------|-----------------------|------------------------|-----------------------|-------------------------|---------------------|----------------|------------------|---------------|
| | | | | | | | | | complete genes | fragmented genes | missing genes |
| Dmel | Sr | 15,404 | 22,245 | 250,600 | 138 | 22 | 3.05 | 0.68 | 96.96 | 2.07 | 0.96 |
| | H | 302 | 5,369,803 | 20,351,387 | 449 | 391 | 28.34 | 570.44 | 66.02 | 15.51 | 18.47 |
| | HQ | 302 | 5,378,161 | 20,387,636 | 685 | 439 | 8.26 | 13.51 | 95.03 | 2.00 | 2.97 |
| | HQP | 302 | 5,378,529 | 20,385,575 | 689 | 464 | 8.91 | 7.78 | 97.89 | 0.57 | 1.54 |
| | Lr | 426 | 10,086,116 | 24,845,957 | 3,935 | 2,126 | 16.25 | 12.16 | 89.03 | 6.50 | 4.47 |
| | LrQP | 426 | 10,090,934 | 24,862,005 | 4,002 | 2,131 | 12.23 | 8.07 | 98.21 | 0.57 | 1.21 |
| | M | 266 | 21,413,390 | 25,776,101 | 603 | 288 | 8.95 | 7.19 | 98.75 | 0.57 | 0.68 |

| | | | | | | | | | | | |
|-----|------|--------|------------|------------|-------|--------|--------|--------|-------|------|------|
| | MQP | 266 | 21,413,354 | 25,775,485 | 623 | 267 | 8.66 | 6.49 | 98.75 | 0.57 | 0.68 |
| | Ref. | 7 | 25,286,936 | 32,079,331 | NA | NA | NA | NA | 98.68 | 0.75 | 0.57 |
| SON | Sr | 57,046 | 3,385 | 45,376 | 346 | 2,539 | 219.24 | 200.08 | 89.10 | 7.93 | 2.97 |
| | H | 136 | 9,893,295 | 18,894,064 | 6,773 | 9,558 | 331.40 | 457.22 | 88.14 | 8.40 | 3.47 |
| | HQ | 136 | 9,840,048 | 18,808,757 | 6,898 | 9,646 | 324.72 | 271.29 | 98.54 | 0.93 | 0.54 |
| | HQP | 136 | 9,834,752 | 18,808,038 | 6,893 | 9,663 | 324.81 | 265.21 | 98.68 | 0.82 | 0.50 |
| | Lr | 343 | 2,678,315 | 8,942,850 | 8,790 | 9,912 | 332.66 | 316.57 | 96.57 | 1.93 | 1.50 |
| | LrQP | 343 | 2,679,816 | 8,945,441 | 9,055 | 10,035 | 328.02 | 262.04 | 98.14 | 1.04 | 0.82 |
| | M | 104 | 27,077,180 | 33,146,112 | 7,167 | 9,792 | 325.14 | 264.44 | 98.43 | 0.89 | 0.68 |
| | MQP | 104 | 27,074,084 | 33,145,389 | 7,168 | 9,753 | 324.69 | 264.16 | 98.39 | 0.93 | 0.68 |
| | Ref. | 39 | 26,426,104 | 33,738,561 | NA | NA | NA | NA | 98.14 | 0.93 | 0.9 |

685 **Figure legends**

686

687 **Fig. 1. Size profile of DNA from *D. mojavensis* (Sonora) extracted with three different methods.**

688 Images of three gels, corresponding to each method, have been collated here, using the same ladder
689 (sizes shown on the left).

690

691 **Fig. 2. DBG2OLC Pipeline, including the final merging step and the polishing steps**

692

693 **Fig. 3. Contiguity (A, D), error level (B, E) and Busco score (C, F) for Dmel (A-C) and SON (D-F)**
694 **assemblies, at each step of the pipeline.** Significantly larger values are printed above dashed lines.

695 Assembly parameters are described in Table 6.

696

697 **Fig. 4. Alignment of SON merged assembly P6fn” (y-axis) on the *D. mojavensis* (Catalina)**

698 **reference genome (x-axis).** Only fragments longer than 900 Kb are shown. Muller elements

699 (chromosomes) of the reference genome (Catalina) are shown. Yellow boxes represent a single Muller

700 element. Gray horizontal lines indicate the contigs from the SON assembly. The assignments of

701 scaffolds to Muller elements can be found in Supporting Information S1.

702

703 **Supporting information**

704

705 **Table S1.** Assignments of scaffolds to Muller elements

706 **Figures**

707

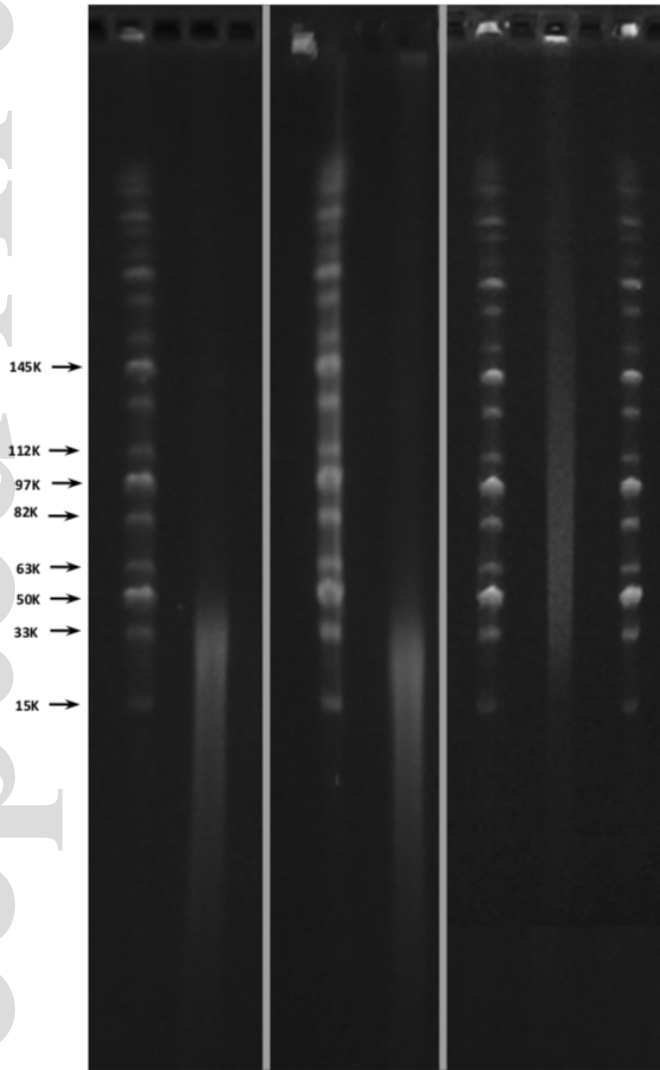
DNeasy Blood
& Tissue Kit

708

709

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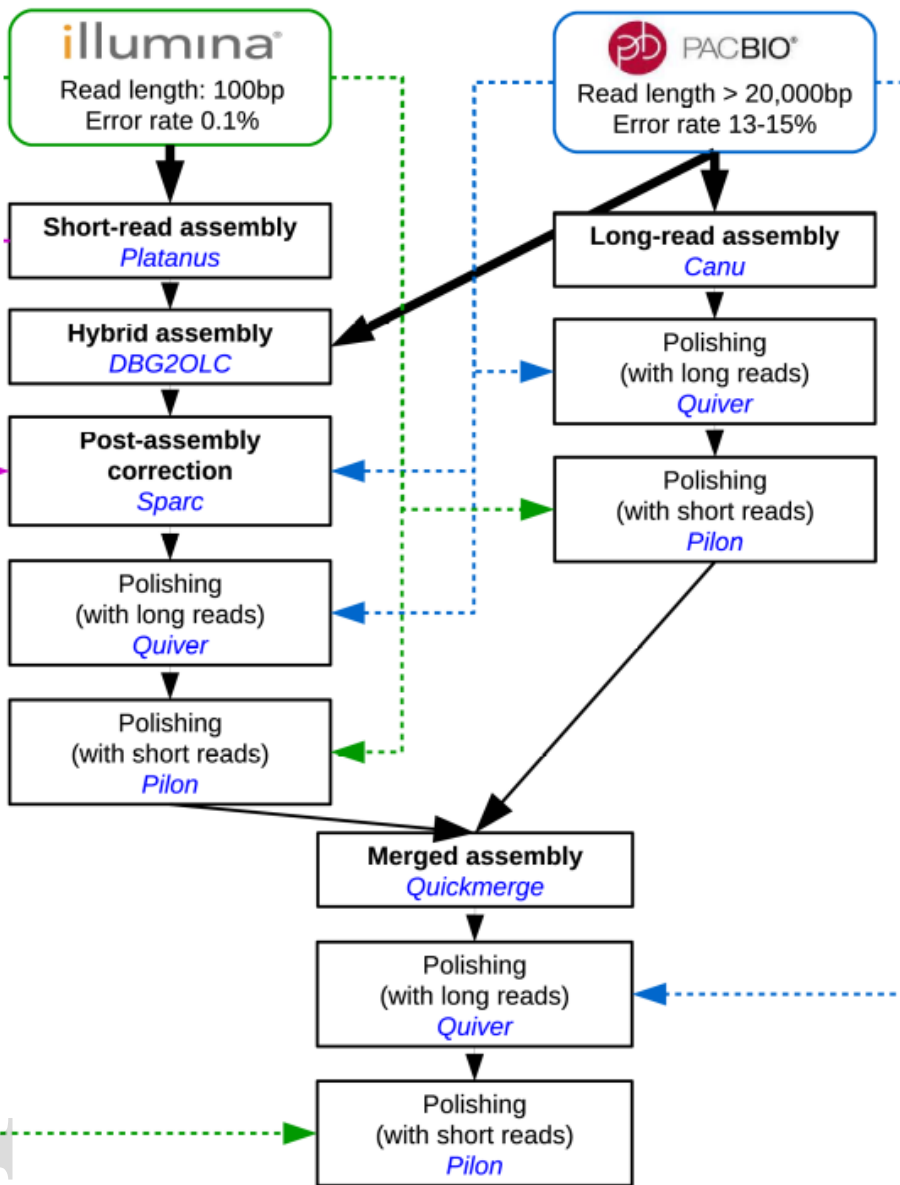
711

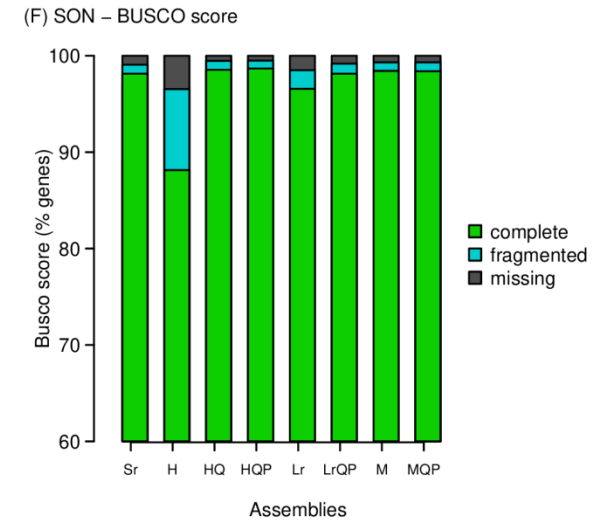
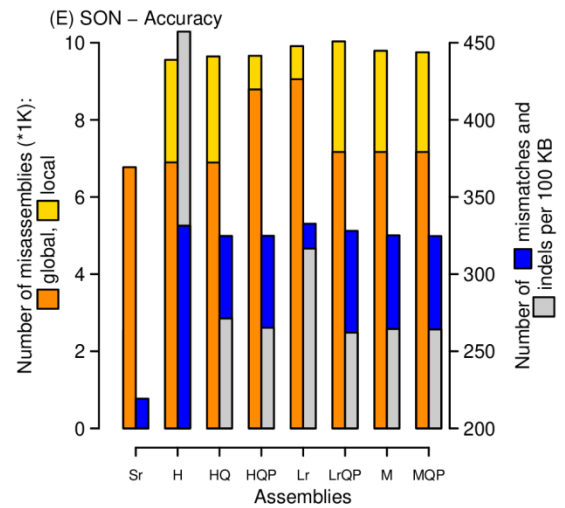
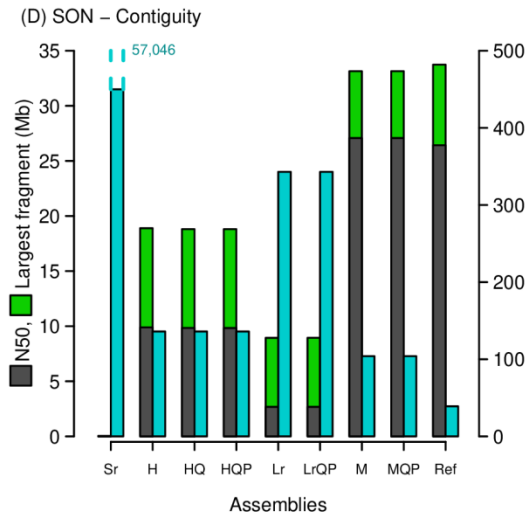
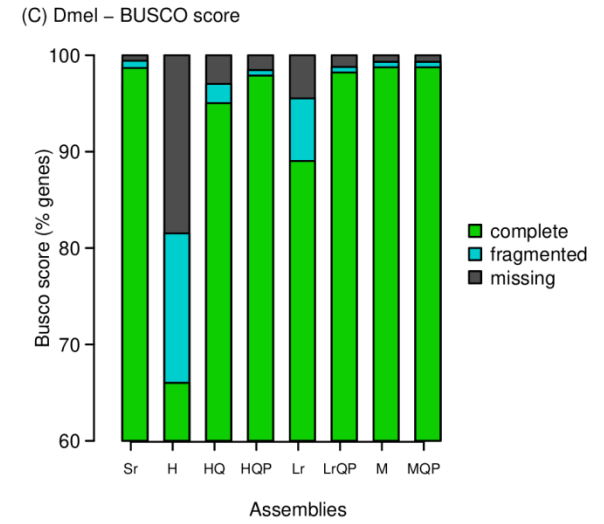
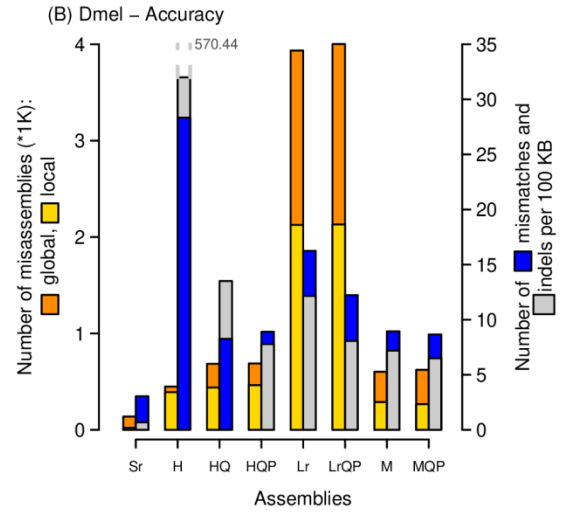
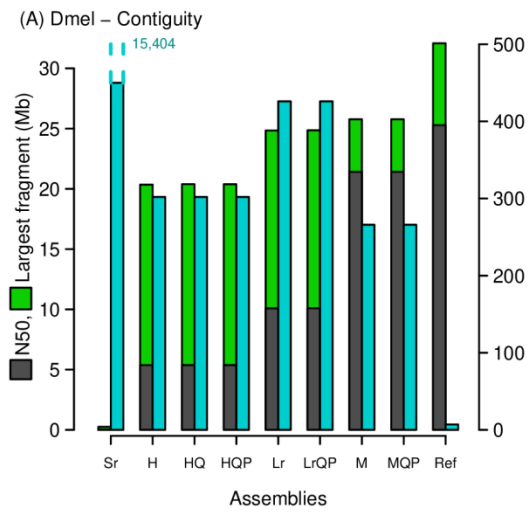


712

713

714



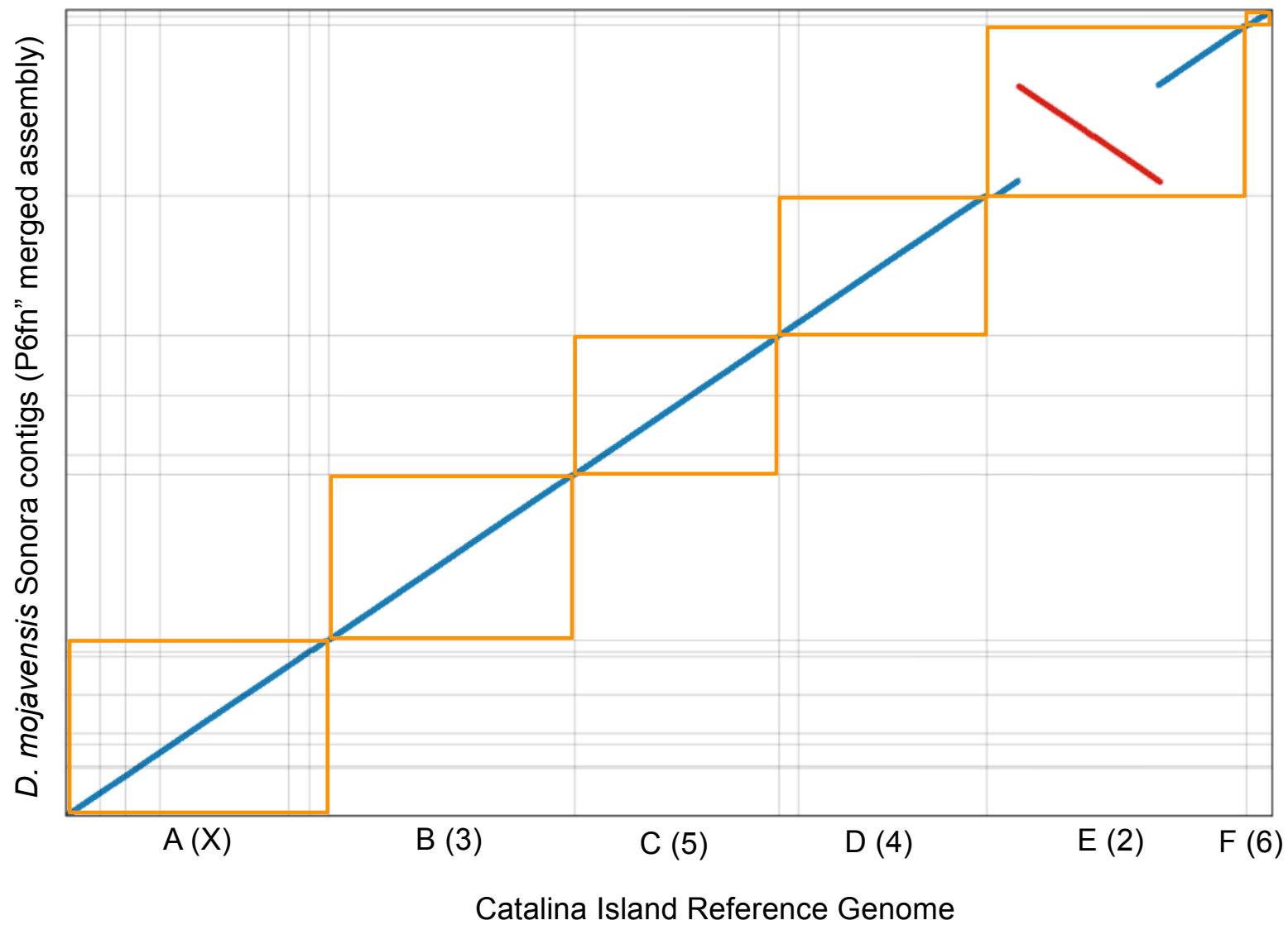


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719

720

721



723 **Appendix. Command lines to call each program used in the study.**

724

725 *(1) Data manipulation*

726 Merge .bam files from several PacBio sequencing cells (node=1:ncpus=1:mem=6gb):

727 bamtools merge -list ListPBreadsFiles.fofn -out PBreads_all.bam

728 Merge two .fasta files: (node=1:ncpus=1:mem=6gb):

729 cat reads1.fasta reads2.fasta > reads1_and_2.fasta

730 Convert .bax.h5 to .bam (node=1:ncpus=1:mem=6gb):

731 bax2bam my_movie.*.bax.h5 -o my_movie

732 Convert .bam to .fastq (node=1:ncpus=1:mem=6gb):

733 samtools fastq my_reads.bam > my_reads.fastq

734 OR

735 bam2fastq my_reads.bam -o my_reads

736 Convert .bam to .fasta (node=1:ncpus=1:mem=6gb):

737 bam2fasta my_reads.bam -o my_reads

738 Convert .fastq to .fasta with the *prinseq-lite tool* (v0.20.4) (node=1:ncpus=1:mem=6gb):

739 perl prinseq-lite.pl -fastq my_reads.fastq -out_format 1

740 Convert .fastq to .fasta with the *FASTX* toolkit (v0.0.13) (node=1:ncpus=1:mem=6gb):

741 fastq_to_fasta -i my_reads.fastq -o my_reads.fasta

742

743 *(2) Short-read assembly*

744 Trimming (with *Platanus* trimmer)

745 (node=1:ncpus=5:mem=30gb;cput=10:00:00;walltime=02:00:00):

746 platanus_trim my_PE_reads_1.fastq my_PE_data_2.fastq -t 5

747 *Platanus* assembler (node=1:ncpus=14:mem=65gb; cput=28:00:00;walltime=02:00:00):

748 platanus assemble -o Shortread_contigs -f my_PE_reads_[12].fastq.trimmed -t 16 -m 75 2>

749 Plat_SON2.log

750 *SparseAssembler*, with kmer size 53 (node=1:ncpus=1:mem=6gb; cput=02:00:00;

751 walltime=02:00:00):

752 *SparseAssembler* LD 0 k 53 g 15 NodeCovTh 1 EdgeCovTh 0 GS 165000000 p1

753 my_PE_reads_1.fastq.trimmed p2 my_PE_reads_2.fastq.trimmed

754

755 (3) *Hybrid assembly*

756 *DBG2OLC* step (node=1:ncpus=1:mem=6gb; cput=02:50:00:walltime=02:50:00):

757 *DBG2OLC* k 17 KmerCovTh 2 MinOverlap 150 AdaptiveTh 0.002 LD1 0 MinLen 200 Contigs

758 Shortread_contigs.fasta RemoveChimera 1 f my_PBreads.fasta

759 Note: *DBG2OLC* has memory limitations. If run on node=1:ncpus=2:mem=12gb, it will still use

760 one chore only, but more memory as allowed, and run twice as fast.

761 Building list of contigs identifiers (node=1:ncpus=1:mem=6gb;

762 cput=00:05:00;walltime=00:05:00) (requires python2):

763 split_reads_by_backbone.py -b backbone_raw.fasta -o ./Cons_backbones -r

764 Shortread_contigs_and_PBreads.fasta -c *DBG2OLC*_Consensus_info.txt

765 *Sparc* step ((node=1:ncpus=28:mem=90gb; cput=84:00:00;walltime=03:00:00):

766 sh split_and_run_sparc_ncpus_new.sh ./Cons_backbones 2 28 > Sparclog_Part1.txt

767

768 (4) *Long-read only assembly with Canu*

769 canu -p PB_Only_Assembly -d /path_to_curr_dir genomeSize=123m correctedErrorRate=0.039 -

770 useGrid=true -maxThreads=16 -maxMemory=90 -gridEngineThreadsOption="-l

771 select=1:ncpus=16:mem=100gb" -gridEngineMemoryOption="-l walltime=02:00:00" -gridOptions="-W

772 group_list=my_group_ID -q standard" -pacbio-raw /path_to_Preads/Preads.fasta

773 Note: Because the *Canu* pipeline calls a master script, more parameters, normally passed to the

774 PBS script have to be sent to the command line when calling *Canu*. The option -nanopore-raw was

775 used when assembling Nanopore reads.

776

777 (5) *Assembly merging*

778 Using the *Quickmerge* wrapper, and with hybrid assembly as donor and long-read only assembly
779 as acceptor (node=1:ncpus=1:mem=6gb; cput=00:25:00;walltime=00:25:00; requires python/3):
780 merge_wrapper.py Hybrid_assembly.fasta longread_assembly.fasta -l 10000000 -lm 10000

781

782 (6) *Quiver polishing*

783 Align PB reads (in .bam format) to assembly with *Pbalign* (node=1:ncpus=28:mem=168gb;
784 cput=224:00:00;walltime=08:00:00):

785 pbalign --nproc 28 my_PBreads.bam my_assembly.fasta aligned_PBreads.bam

786 Index aligned PB reads (node=1:ncpus=1:mem=6gb; cput=00:10:00;walltime=00:10:00):

787 pbindex aligned_PBreads.bam

788 Index assembly (node=1:ncpus=1:mem=6gb; cput=00:02:00;walltime=00:02:00):

789 samtools faidx my_assembly.fasta

790 Run *Quiver* (node=1:ncpus=28:mem=168gb; cput=154:00:00;walltime=05:30:00):

791 quiver -j 28 -r my_assembly.fasta -o my_assembly_polished.fasta aligned_PBreads.bam

792 Run *Arrow* (node=1:ncpus=28:mem=168gb; cput=154:00:00;walltime=05:30:00):

793 arrow -j 28 -r my_assembly.fasta -o my_assembly_polished.fasta aligned_PBreads.bam

794

795 (7) *Pilon polishing*

796 Index the assembly file (node=1:ncpus=6:mem=6gb; cput=00:05:00;walltime=00:05:00):

797 bowtie2-build my_assembly.fasta my_assembly

798 Align short-reads to assembly with *Bowtie2* (node=1:ncpus=28:mem=168gb;

799 cput=42:00:00;walltime=01:30:00):

800 bowtie2 -x my_assembly -1 my_PE_reads_1.fastq.trimmed -2 my_PE_reads_2.fastq.trimmed -S

801 Aligned_reads.sam -p 28

802 Convert .sam to .bam (node=1:ncpus=1:mem=6gb; cput=00:10:00;walltime=00:10:00):

803 samtools view -bS Aligned_reads.sam > Aligned_reads.bam

804 Sort reads (node=1:ncpus=28:mem=168gb; cput=07:00:00;walltime=00:15:00):

805 samtools sort Aligned_reads.bam -o Aligned_reads_sorted.bam -@ 28

806 Index reads (node=1:ncpus=6:mem=6gb; cput=00:02:00;walltime=00:02:00):
807 samtools index Aligned_reads_sorted.bam -@ 28

808 Run *Pilon* with paired ends only (node=1:ncpus=28:mem=168gb;
809 cput=14:00:00;walltime=00:30:00; requires java/8):
810 java -Xmx64G -jar pilon-1.22.jar --genome my_assembly.fasta --frags Aligned_Pereads_sorted.bam --
811 output ./my_assembly_polished --threads 28

812 Run *Pilon* with paired ends and mate pairs
813 (node=1:ncpus=28:mem=168gbcput=28:00:00;walltime=01:00:00; requires java/8):
814 java -Xmx64G -jar pilon-1.22.jar --genome my_assembly.fasta --frags Aligned_Pereads_sorted.bam --
815 jumps Aligned_MPreads_sorted.bam--output ./my_assembly_polished --threads 14

816 Note: *Pilon* seems to try to use more cpus than allowed with the --thread option and requires more
817 memory to run on both paired ends and mate pairs. To counter this problem, we actually ran it on
818 28 cpus but passed less cpus (just enough to avoid memory issue) to the --thread option.

819

820 (8) *Nanopolish polishing*

821 Index the raw Nanopore reads (node=1:ncpus=1:mem=6gb; cput=02:30:00;walltime=02:30:00):
822 nanopolish index -d ./path_to_Fast5 -f List_summary.fofn -v ./Reads_basecalled_pass.fastq
823

824 Index the assembly file (node=1:ncpus=6:mem=6gb; cput=00:10:00;walltime=00:10:00):
825 bwa index my_assembly.fasta

826 Align short-reads to assembly with *Bwa* (node=1:ncpus=28:mem=168gb;
827 cput=161:00:00;walltime=05:45:00):
828 bwa mem -x ont2d -t 28 my_assembly.fasta /path_to_raw_reads/Reads_basecalled_pass.fastq | samtools
829 sort -o Reads.sorted.bam -@ 28
830 samtools index Reads.sorted.bam -@ 28

831 Run *Nanopolish* (node=1:ncpus=28:mem=168gb; cput=854:00:00;walltime=30:30:00):
832 python nanopolish_makerange.py my_assembly.fasta | cat > Chunks.txt
833 more Chunks.txt | parallel --results ./nanopol.res -P 7 nanopolish variants --consensus --faster -o
834 polished.{1}.vcf -w {1} -r ./Reads_basecalled_pass.fastq -b ./Reads.sorted.bam -g ./my_assembly.fasta -t
835 4 --min-candidate-frequency 0.2

836 Note: *Nanopolish* was unable to handle paths to directory others than the working directory,
837 therefore we placed both raw indexed reads, the draft assembly and the sorted reads in the same
838 directory.

839 Convert *.vcf* to *.fasta* (node=1:ncpus=1:mem=6gb; cput=00:03:00;walltime=00:30:00):
840 `nanopolish vcf2fasta -g ./my_assembly.fasta ./polished.*.vcf > ./my_assembly_polished.fasta`

841

842 (9) Quality assessment

843 *Quast* (node=1:ncpus=5:mem=30gb; cput=05:00:00;walltime=01:00:00;requires python/3)

844 `quast.py -o ./ -R /path_to_reference_genome/Reference.fasta --threads 5 --min-alignment 400 --no-plot --`
845 `no-html --no-icarus --labels My_assembly --eukaryote /path_to_assembly/My_assembly.fasta`

846 Note: walltime will depend on how divergent is the draft assembly from the reference assembly

847 *Busco* (node=1:ncpus=14:mem=84gb; cput=25:40:00;walltime=01:50:00; requires python/3,
848 *augustus/3* and *hmmer/3*)

849 `python run_BUSCO.py -i /path_to_assembly/My_assembly.fasta -o output_directory -l`
850 `~/Programs/busco/diptera_odb9/ -m genome -c 14 -sp fly --blast_single_core`

851

852 (10) Visualization with *Dot*

853 (node=1:ncpus=1:mem=6gb; cput=00:08:00;walltime=00:08:00)

854 `nucmer -c 100 -t 3 -prefix=my_assembly /path_to_reference/Reference_genome.fasta`
855 `/path_to_assembly/My_assembly_100KB.fasta`

856 `python DotPrep.py --delta my_assembly.delta --out outputname --unique-length 10000 --overview 500000`