

1 Evaluating Metagenome Assembly on a Simple
2 Defined Community with Many Strain Variants

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4 June 25, 2017

5 **Abstract**

6 We evaluate the performance of three metagenome assemblers, IDBA,
7 MetaSPAdes, and MEGAHIT, on short-read sequencing of a defined
8 “mock” community containing 64 genomes (Shakya et al. (2013)). We
9 update the reference metagenome for this mock community and detect
10 several additional genomes in the read data set. We show that strain
11 confusion results in significant loss in assembly of reference genomes
12 that are otherwise completely present in the read data set. In agree-
13 ment with previous studies, we find that MEGAHIT performs best
14 computationally; we also show that MEGAHIT tends to recover larger
15 portions of the strain variants than the other assemblers.

16 Introduction

17 Metagenomics refers to sequencing of DNA from a mixture of organisms,
18 often from an environmental or uncultured sample. Unlike whole genome
19 sequencing, metagenomics targets a mixture of genomes, which introduces
20 metagenome-specific challenges in analysis [1]. Most approaches to analyz-
21 ing metagenomic data rely on mapping or comparing sequencing reads to
22 reference sequence collections. However, reference databases contain only a
23 small subset of microbial diversity [2], and much of the remaining diversity
24 is evolutionarily distant and search techniques may not recover it [3].

25 As sequencing capacity increases and sequence data is generated from
26 many more environmental samples, metagenomics is increasingly using *de*
27 *novo* assembly techniques to generate new reference genomes and metagenomes
28 [4]. There are a number of metagenome assemblers that are widely used -
29 see [5] for an overview of the available software, and [1] for a review of the
30 different assembler methodologies. However, evaluating the results of these
31 assemblers is challenging due to the general lack of good quality reference
32 metagenomes.

33 Moya et al. in [6] evaluated metagenome assembly using two simulated
34 454 viral metagenome and six assemblers. The assemblies were evaluated
35 based on several metrics including N50, percentages of reads assembled, ac-
36 curacy when compared to the reference genome. In addition to, chimeras per
37 contigs and the effect of assembly on taxonomic and functional annotations.

38 Mavromatis et al. in [7] provided a benchmark study to evaluate the
39 fidelity of metagenome processing methods. The study used simulated
40 metagenomic data sets constructed at different complexity levels. The datasets
41 were assembled using Phrap v3.57, Arachne v.2 [8] and JAZZ [9]. This study
42 evaluates assembly, gene prediction, and binning methods. However, the
43 study did not evaluate the assembly quality against a reference genome.

44 Rangwala et al. in [10] presented an evaluation study of metagenome
45 assembly. The study used a de Bruijn graph based assembler ABYSS [11] to
46 assemble simulated metagenome reads of 36 bp. The data set is classified at
47 different complexity levels. The study compared the quality of the assembly
48 of the data sets in terms of contig length and assembly accuracy. The
49 study also took into consideration the effect of kmer size and the degree of
50 chimericity. However, the study evaluated the assembly based on only one
51 assembler. Also, both previous studies used simulated data, which may lack
52 confounders of assembly such as sequencing artifacts and GC bias.

53 In a landmark study, Shakya et al. (2013) constructed a synthetic com-

54 munity of organisms by mixing DNA isolated from individual cultures of
55 64 bacteria and archaea, including a variety of strains across a range of
56 nucleotide distances [12]. In addition to performing 16s amplicon analy-
57 sis and doing 454 sequencing, the authors shotgun-sequenced the mixture
58 with Illumina. While the authors concluded that this metagenomic sequenc-
59 ing generally outperformed amplicon sequencing, they did not conduct an
60 assembly based analysis. This data set was also used in several other eval-
61 uation studies, including gbtools for binning [13] and benchmarking of the
62 MEGAHIT assembler [14].

63 More recently, several benchmark studies systematically evaluated metagenome
64 assembly of short reads. The Critical Assessment of Metagenome Interpre-
65 tation (CAMI) collaboration benchmarked a number of metagenome assem-
66 blers on several data sets of varying complexity, evaluating recovery of novel
67 genomes and multiple strain variants [3]. Notably, CAMI concluded that
68 “The resolution of strain-level diversity represents a substantial challenge
69 to all evaluated programs.” Another recent study evaluated eight assem-
70 blers on nine environmental metagenomes and three simulated data sets
71 and provided a workflow for choosing a metagenome assembler based on
72 the biological goal and computational resources available [15]. [5] explored
73 metagenome assembler performance on a pair of real data sets, again con-
74 cluding that the biological goal and computational resources defined the
75 choice of assembler. Also see [16] for an analysis of a previously generated
76 HMP benchmark data set; however, the Illumina reads used for this study
77 are much shorter than current sequencing and are arguably not relevant for
78 future studies.

79 In this study, we extend previous work by delving into questions of
80 chimeric misassembly and strain recovery in the Shakya et al. (2013) data
81 set. First, we update the list of reference genomes for Shakya et al. to in-
82 clude the latest GenBank assemblies along with plasmids. We then compare
83 IDBA [17], MetaSPAdes [18], and MEGAHIT [19] performance on assem-
84 bling this short-read data set, and explore concordance in recovery between
85 the three assemblers. We describe the effects of “strain confusion” between
86 multiple strains. We also detect and analyze several previously unreported
87 strains and genomes in the Shakya et al. data set. We find that in the ab-
88 sence of closely related genomes, all three metagenome assemblers recover
89 95% or more of known reference genomes. However, in the presence of
90 closely related genomes, these three metagenome assemblers vary widely in
91 their performance and, in extreme cases, can fail to recover the majority of
92 some genomes even when they are completely present in the reads. Our re-

93 port provides strong guidance on choice of assemblers and extends previous
94 analyses of this low-complexity metagenome benchmarking data set.

95 Datasets

96 We used a diverse mock community data set constructed by pooling DNA
97 from 64 species of bacteria and archaea and sequencing them with Illumina
98 HiSeq. The raw data set consisted of 109,629,496 reads from Illumina HiSeq
99 101 bp paired-end sequencing (2x101) with an untrimmed total length of
100 11.07 Gbp and an estimated fragment size of 380 bp [12].

101 The original reads are available through the NCBI Sequence Read Archive
102 at Accession SRX200676. We updated the 64 reference genomes sets from
103 NCBI GenBank using the latest available assemblies with plasmid content
104 (June 2017); updated data is available for download at <https://osf.io/8uxj9/>.

105 Methods

106 The analysis code and run scripts for this paper are written in Python and
107 bash, and are available at: [https://github.com/dib-lab/2015-metagenome-](https://github.com/dib-lab/2015-metagenome-assembly/)
108 [assembly/](https://github.com/dib-lab/2015-metagenome-assembly/). The scripts and overall pipeline were examined by the first and
109 senior authors for correctness. In addition, the bespoke reference-based anal-
110 ysis scripts were tested by running them on a single-colony *E. coli* MG1655
111 data set with a high quality reference genome [20].

112 Quality Filtering

113 We removed adapters with Trimmomatic v0.30 in paired-end mode with
114 the TruSeq adapters [21], using light quality score trimming (`LEADING:2`
115 `TRAILING:2 SLIDINGWINDOW:4:2 MINLEN:25`) as recommended in MacManes,
116 2014 [22].

117 Reference Coverage Profile

118 To evaluate how much of the reference metagenome was contained in the
119 read data, we used `bwa aln` (v0.7.7.r441) to map reads to the reference
120 genome [23]. We then calculated how many reference bases were covered by
121 mapped reads (custom script `coverage-profile.py`).

122 Measuring k-mer inclusion and Jaccard similarity

123 We used MinHashing as implemented in sourmash to estimate k-mer inclu-
124 sion and Jaccard similarity between data sets [24]. MinHash signatures were
125 prepared with `sourmash compute` using `--scaled 10000`. K-mer inclusion
126 was computed by taking the ratio of the number of intersecting hashes with
127 the query over the total number of hashes in the subject MinHash. Jac-
128 card similarity was computed as in [25] by taking the ratio of the number
129 of intersecting hashes between the query and subject over the number of
130 hashes in the union. K-mer sizes for comparison were chosen at 21, 31, or
131 51, depending on the level of taxonomic specificity desired - genus, species,
132 or strain, respectively, as described in [26].

133 When specified, high-abundance k-mers were selected for counting by
134 using the script `trim-low-abund.py` script with `-C 5` from khmer v2 [27,
135 28].

136 Assemblers

137 We assembled the quality-filtered reads using three different assemblers:
138 IDBA-UD [17], MetaSPAdes [18], and MEGAHIT [19]. For IDBA-UD v1.1.1
139 [17], we used `--pre-correction` to perform pre-correction before assembly
140 and `-r` for the pe files.

141 For MetaSPAdes v3.9.0 [18], we used `--meta --pe1-12 --pe1-s` where
142 `--meta` is used for metagenomic data sets, `--pe1-12` specifies the interlaced
143 reads for the first paired-end library, and `--pe1-s` provides the orphan reads
144 remaining from quality trimming.

145 For MEGAHIT v1.1.1-2-g02102e1 [19], we used `-l 101 -m 3e9 --cpu-only`
146 where `-l` is for maximum read length, `-m` is for max memory in bytes to
147 be used in constructing the graph, and `--cpu-only` to use only the CPU
148 and no GPUs. We also used `--presets meta-large` for large and complex
149 metagenomes, and `--12` and `-r` to specify the interleaved-paired-end and
150 single-end files respectively. MEGAHIT allows the specification of a memory
151 limit and we used `-M 1e+10` for 10 GB.

152 All three assemblies were executed on the same high-memory buy-in
153 node on the Michigan State University High Performance Compute Cluster,
154 and we recorded RAM and CPU time of each assembly job using the `qstat`
155 utility at the end of each run.

156 Unless otherwise mentioned, we eliminated all contigs less than 500 bp
157 from each assembly prior to further analysis.

158 Mapping

159 We aligned all quality-filtered reads to the reference metagenome with `bwa`
160 `aln` (v0.7.7.r441) [23]. We aligned paired-end and orphaned reads separately.
161 We then used `samtools` (v0.1.19) [29] to convert SAM files to BAM files for
162 both paired-end and orphaned reads. To count the unaligned reads, we
163 included only those records with the “4” flag in the SAM files [29].

164 Assembly analysis using NUCmer

165 We used the NUCmer tool from MUMmer3.23 [30] to align assemblies to the
166 reference genome with options `-coords -p`. Then we parsed the generated
167 “coords” file using a custom script `analyze_assembly.py`, and calculated
168 several analysis metrics across all three assemblies at a 99% alignment iden-
169 tity.

170 Reference-based analysis of the assemblies

171 We conducted reference-based analysis of the assemblies under two condi-
172 tions. “Loose” alignment conditions used all available alignments, including
173 redundant and overlapping alignments. “Strict” alignment conditions took
174 only the longest alignment for any given contig, eliminating all other align-
175 ments.

176 The script `summarize-coords2.py` was used to calculate aligned cov-
177 erage from the loose alignment conditions: each base in the reference was
178 marked as “covered” if it was included in at least one alignment. The script
179 `analyze_ng50.py` was used to calculate NGA 50 for each individual refer-
180 ence genome.

181 Analysis of chimeric misassemblies

182 We analyzed each assembly for chimeric misassemblies by counting the num-
183 ber of contigs that contained matches to two distinct reference genomes. In
184 order to remove secondary alignments from consideration, we included only
185 the longest non-overlapping NUCmer alignments for each contig at a mini-
186 mum alignment identity of 99%. We then used the script `analyze_chimeric2.py`
187 to find individual contigs that matched more than one distinct reference
188 genome. As a negative control on our analysis, we verified that this ap-
189 proach yielded no positive results when applied to the alignments of the
190 reference metagenome against itself.

191 Results

192 The raw data is high quality.

193 The reads contains 11,072,579,096 bp (11.07 Gbp) in 109,629,496 reads with
194 101.0 average length (2x101bp Illumina HiSeq).

195 Trimming removed 686,735 reads (0.63%). After trimming, we retained
196 108,422,358 paired reads containing 10.94 Gbp with an average length of
197 100.9 bases. A total of 46.56 Mbp remained in 520,403 orphan reads with
198 an average length of 89.5 bases. In total, the quality trimmed data contained
199 10.98 Gbp in 108,942,761 reads. This quality trimmed (“QC”) data set was
200 used as the basis for all further analyses.

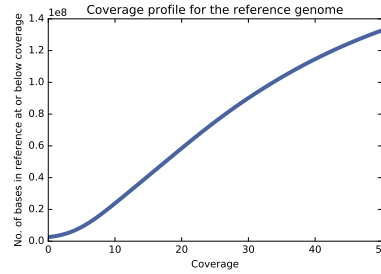


Figure 1: Cumulative coverage profile for the reference metagenome, based on read mapping.

201 The reference metagenome is not completely present in the 202 reads.

203 We next evaluated the fraction of the reference genome covered by at least
204 one read (see Methods for details). Quality filtered reads cover 203,058,414
205 (98.76%) bases of the reference metagenome (205,603,715 bp total size). Fig-
206 ure 1 shows the cumulative coverage profile of the reference metagenome,
207 and the percentage of bases with that coverage. Most of the reference
208 metagenome was covered at least minimally; only 3.33% of the reference
209 metagenome had mapping coverage <5 , and 1.24% of the bases in the ref-
210 erence were not covered by any reads in the QC data set.

211 In order to evaluate reconstructability with De Bruijn graph assemblers,
212 we next examined k-mer containment of the reference in the reads for k of
213 21, 31, 41, and 51 (Table 1). The k-mer overlap decreases from 96.8% to

Table 1: Jaccard containment of the reference in the reads

k-mer size	% reference in reads
21	96.8%
31	95.9%
41	94.9%
51	94.1%

94.1% as the k-mer size increases. This could be caused by low coverage of some portions of the reference and/or variation between the reads and the reference.

Some individual reference genomes are poorly represented in the reads.

Table 2: Top uncovered genomes

Genome	Read coverage
<i>Desulfovibrio vulgaris</i> DP4	93.2%
<i>Thermus thermophilus</i> HB27	91.1%
<i>Enterococcus faecalis</i> V583	74.6%
<i>Fusobacterium nucleatum</i>	47.6%

To see if specific reference genomes exhibited low coverage, we analyzed read mapping coverage for individual genomes. Of the 64 reference genomes used in the metagenome, 60 had a per-base mapping coverage above 95%. The remaining four varied significantly (Table 2), with *F. nucleatum* the lowest – only 47.6% of the bases in the reference genome are covered by one or more mapped reads.

We next did a 51-mer containment analysis of each reference genome in the reads; k=51 was chosen so as to be specific to strain content [26]. 99% or more of the constituent 51-mers for 51 of the 64 reference genomes were present in the reads, suggesting that each of the 51 genomes was entirely present at some minimal coverage.

We excluded the remaining 13 genomes (see Table 3) from any further reference-based analysis because interpreting recovery and misassembly statistics for these genomes would be confounding; also see the discussion of strain variants, below.

Table 3: Genomes removed from reference for low 51-mer presence

51-mers in reads	Genome
98.7	<i>Leptothrix cholodnii</i>
98.7	<i>Haloferax volcanii</i> DS2
98.6	<i>Salinispora tropica</i> CNB-440
97.4	<i>Deinococcus radiodurans</i>
97.2	<i>Zymomonas mobilis</i>
97.1	<i>Ruegeria pomeroyi</i>
96.8	<i>Shewanella baltica</i> OS223
95.5	<i>B. bronchiseptica</i> D989
94.5	<i>Burkholderia xenovorans</i>
72.0	<i>Desulfovibrio vulgaris</i> DP4
65.0	<i>Thermus thermophilus</i> HB27
53.4	<i>Enterococcus faecalis</i>
4.7	<i>Fusobacterium nucleatum</i> ATCC 25586

234 **MEGAHIT is the fastest and lowest-memory assembler eval-**
235 **uated**

Table 4: Running Time and Memory Utilization

Assembler	CPU time	Wall time	RAM
MEGAHIT	52hr 25m	4 hr 9m	11.4 GB
IDBA-UD	49h	49h	39.8GB
MetaSPAdes	94hr 43m	94hr 44m	100.7 GB

236 We ran three commonly used metagenome assemblers on the QC data
237 set: IDBA-UD, MetaSPAdes, and MEGAHIT. We recorded the time and
238 memory usage of each (Table 4). In computational requirements, MEGAHIT
239 outperformed both MetaSPAdes and IDBA-UD considerably, producing an
240 assembly in four hours (“wall time”) – approximately 12 times faster than
241 IDBA and 23 times faster than MetaSPAdes. MEGAHIT used only 11.4
242 GB of RAM – 1/3rd to 1/9th the memory used by IDBA and MetaSPAdes,
243 respectively.

244 CPU time measurements (which include processing on multiple CPU
245 cores) show that MEGAHIT and IDBA are competitive in overall process-
246 ing time, but MEGAHIT’s ability to make use of multiple cores results in
247 significantly less overall assembly time; this is particularly relevant given

the increasing availability of manycore processors. Despite a variety of configuration attempts, we were unable to get MetaSPAdes to use threading effectively; however, we note that even with perfectly parallel processing on 16 cores, MetaSPAdes would take 6 hours and still use approximately 9 times as much RAM as MEGAHIT.

The assemblies contain most of the raw data

Table 5: Read and high-abundance (> 5) k-mer exclusion from assemblies

Assembly	Unmapped Reads	51-mers omitted
IDBA	3,328,674 (3.05%)	2.4%
MetaSPAdes	3,844,123 (3.52%)	3.2%
MEGAHIT	2,737,640 (2.51%)	2.8%

We assessed read inclusion in assemblies by mapping the QC reads to the length-filtered assemblies and counting the remaining unmapped reads. Depending on the assembly, between 2.7 million and 3.9 million reads (2.5-3.5%) did not map to the assemblies (Table 5). All of the assemblies included the large majority of high-abundance 51-mers (more than 96.8% in all cases).

Much of the reference is covered by the assemblies.

Table 6: Contig coverage of reference with loose alignment conditions.

Assembly	bases aligned	duplication	51-mers
MEGAHIT	94.8%	1.0%	96.7%
MetaSPAdes	93.1%	1.1%	96.2%
IDBA	93.6%	0.98%	97.2%

We next evaluated the extent to which the assembled contigs recovered the “known/true” metagenome sequence by aligning each assembly to the adjusted reference (Table 6). Each of the three assemblers generates contigs that cover more than 93.1% of the reference metagenome at high identity (99%) with little duplication (approximately 1%). All three assemblies contain between 96.2% and 97.2% of the 51-mers in the reference.

At 99% identity with the loose mapping approach, approximately 2.5% of the reference is missed by all three assemblers, while 1.7% is uniquely covered

268 by MEGAHIT, 0.74% is uniquely covered by MetaSPAdes, and 0.64% is
 269 uniquely covered by IDBA.

270 **The generated contigs are broadly accurate.**

Table 7: Contig accuracy measured by reference coverage with strict alignment.

Assembly	% covered
MEGAHIT	89.3%
IDBA	87.7%
MetaSPAdes	83.4%

271 When counting only the best (longest) alignment per contig at a 99%
 272 identity threshold, each of the three assemblies recovers more than 87.3% of
 273 the reference, with MEGAHIT recovering the most – 89.3% of the reference
 274 (Table 7).

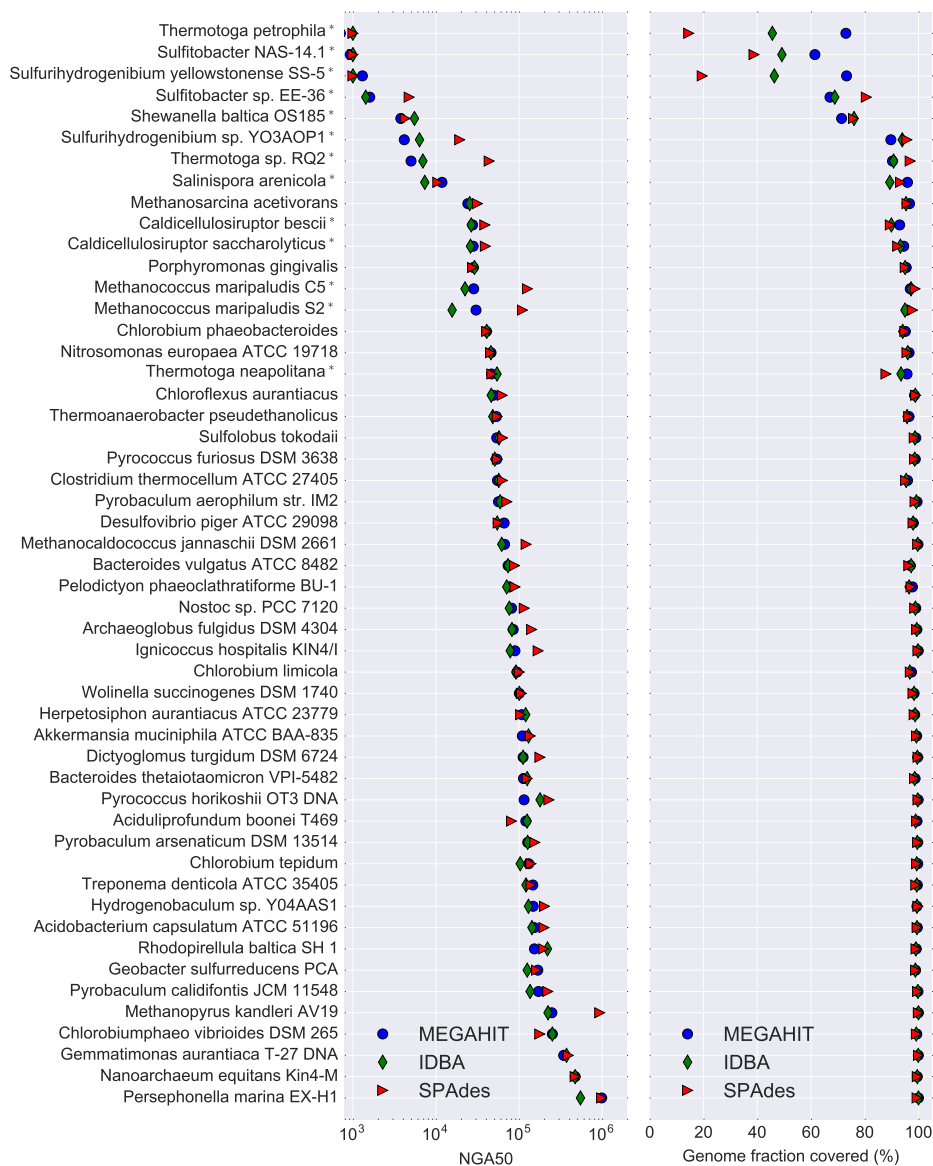


Figure 2: NGA50 and genome fraction covered, by genome and assembler. A '*' after the name indicates the presence of at least one other genome with > 2% Jaccard similarity at k=31 in the community.

275 **Individual genome statistics vary widely in the assemblies.**

276 We computed the NGA50 for each individual genome and assembly in order
 277 to compare assembler performance on genome recovery (see left panel of Fig-
 278 ure 2). The NGA50 statistics for individual genomes vary widely, but there
 279 are consistent assembler-specific trends: IDBA yields the lowest NGA50 for
 280 28 of the 51 genomes, while MetaSPAdes yields the highest NGA50 for 32
 281 of the 51 genomes.

282 We also evaluated aligned coverage per genome for each of the three
 283 assemblies (right panel, Figure 2). We found that 13 of the 51 genomes were
 284 missing 5% or more of bases in at least one assembly, despite all 51 genomes
 285 having 99% or higher read- and 51-mer coverage.

286 There are 12 genomes with k=31 Jaccard similarity greater than 2%
 287 to other genomes in the community, and these (denoted by '*' after the
 288 name) typically had lower NGA50 and aligned coverage numbers than other
 289 genomes. In particular, these constituted 12 of the 13 genomes missing 5%
 290 or more of their content, and the lowest eight NGA50 numbers.

291 **Longer contigs are less likely to be chimeric.**

Table 8: Chimeric contigs by contig length.

Assembly	> 50kb	> 5kb	> 500 bp
IDBA	0	1	7 (0.06%)
MEGAHIT	1	4	14 (0.13%)
MetaSPAdes	0	3	30 (0.48%)

292 Chimerism is the formation of contigs that include sequence from multi-
 293 ple genomes. We evaluated the rate of chimerism in contigs at three different
 294 contig length cutoffs: 500bp, 5kb, and 50kb (Table 8). We found that the
 295 percentage of contigs that match to the genomes of two or more different
 296 species drop as the minimum contig size increases, to the point where only
 297 the MEGAHIT assembly had a single chimeric contig longer than 50kb.
 298 Overall, chimeric misassemblies were rare, with no assembler generating
 299 more than 30 chimeric contigs out of thousands of total contigs.

300 **The unmapped reads contain strain variants of reference genomes.**

301 Approximately 4.8 million reads (4.4%) from the QC data set did not map
 302 anywhere in the reference provided by the authors of [12]. We extracted

Table 9: GenBank genomes detected in assembly of unmapped reads

match	GenBank genome
44.1%	<i>Fusobacterium</i> sp. OBRC1
23.0%	<i>P. ruminis</i> strain ML2
18.2%	<i>Thermus thermophilus</i> HB8
7.7%	<i>P. ruminis</i> strain CGMCC
8.2%	<i>Enterococcus faecalis</i> M7
7.3%	<i>F. nucleatum</i> 13_3C
3.7%	<i>F. nucleatum</i> subsp. <i>polymorphum</i>
2.9%	<i>Fusobacterium hwasookii</i>
1.0%	<i>E. coli</i> isolate YS
1.7%	<i>F. nucleatum</i> subsp. <i>polymorphum</i> , alt.
1.9%	<i>F. nucleatum</i> subsp. <i>vincentii</i>

303 and assembled these reads in isolation using MEGAHIT, yielding 6.5 Mbp
304 of assembly in 1711 contigs > 500bp in length. We then did a k-mer in-
305 clusion analysis of this assembly against all of the GenBank genomes at
306 k=31, and estimated the fraction of the k-mers that belonged to different
307 species (Table 9). We find that 51.1% of the k-mer content of these contigs
308 positively match to a genome present in GenBank but not in the reference
309 metagenome.

310 To verify these assignments, we aligned the MEGAHIT assembly of un-
311 mapped reads to the GenBank genomes in Table 9 with NUCmer using
312 “loose” alignment criteria. We found that 1.78 Mbp of the contigs aligned
313 at 99% identity or better to these GenBank genomes. We also confirmed
314 that, as expected, there are no matches in this assembly to the full updated
315 reference metagenome.

316 We note that all but the two *P. ruminis* matches and the *E. coli* isolate
317 YS are strain variants of species that are part of the defined community
318 but are not completely present in the reads (see Table 2). For *Proteiniclas-*
319 *ticum ruminis*, there is no closely related species in the mock community
320 design, and very little of the MEGAHIT assembly aligns to known *P. ru-*
321 *minis* genomes at 99%. However, there are many alignments to *P. ruminis*
322 at 94% or higher, for approximately 2.73 Mbp total. This suggests that the
323 unmapped reads contain at least some data from a novel species of *Proteinic-*
324 *lasticum*; this matches the observation in [12] of a contaminating genome
325 from an unknown *Clostridium* spp., as at the time there was no *P. ruminis*
326 genome.

327 Discussion

328 Assembly recovers basic content sensitively and accurately.

329 All three assemblers performed well in assembling contigs from the con-
330 tent that was fully present in reads and k-mers. After length filtering,
331 all three assemblies contained more than 95% of the reference (Table 6);
332 even with removal of secondary alignments, more than 87% was recovered
333 by each assembler (Table 7). About half the constituent genomes had an
334 NGA50 of 50kb or higher (Figure 2), which, while low for current Illumina
335 single-genome sequencing, is sufficient to recover operon-level relationships
336 for many genes.

337 The presence of multiple closely related genomes confounds 338 assembly.

339 In agreement with CAMI, we also find that the presence of closely related
340 genomes in the metagenome causes loss of assembly [3]. This is clearly shown
341 by Figure 2, where 12 of the bottom 14 genomes by NGA50 (left panel)
342 also exhibit poor genome recovery by assembly (right panel). Interestingly,
343 different assemblers handle this quite differently, with e.g. MetaSPAdes
344 failing to recover essentially any of *Thermotoga petrophila*, while MEGAHIT
345 recovers 73%. The presence of nearby genomes is an almost perfect predictor
346 that one or more assembler will fail to recover 5% or more - of the 13/51
347 genomes for which less than 95% is recovered, 12 of them have close genomes
348 in the community. Interestingly, very little similarity is needed - all genomes
349 with Jaccard similarity of 2% or higher at k=31 exhibit these problems.

350 The *Shewanella baltica* OS185 genome is a good example: there are two
351 strain variants, OS185 and OS223, present in the defined community. Both
352 are present at more than 99% in the reads, and more than 98% in 51-mers,
353 but only 75% of *S. baltica* OS185 and 50% of *S. baltica* OS223 are recovered
354 by assemblers. This is a clear case of “strain confusion” where the assemblers
355 simply fail to output contigs for a substantial portion of the two genomes.

356 Another interest of this study was to examine cross-species chimeric as-
357 sembly, in which a single contig is formed from multiple genomes. In Table 8,
358 we show that there is relatively little cross-species chimerism. Surprisingly,
359 what little is present is length-dependent: longer contigs are less likely to
360 be chimeric. This might well be due to the same “strain confusion” effect
361 as above, where contigs that share paths in the assembly graphs are broken
362 in twain.

363 **MEGAHIT performs best by several metrics.**

364 MEGAHIT is clearly the most efficient computationally, outperforming both
365 MetaSPAdes and IDBA by 3-9x in memory and 12-23x in time (Table 4).
366 The MEGAHIT assembly also included more of the reads than either IDBA
367 or MetaSPAdes, and omitted only 0.4% more of the unique 51-mers from
368 the reads than IDBA. MEGAHIT covered more of the reference genome
369 with both loose and strict alignments (Table 6 and Table 7), with little
370 duplication. This is clearly because of MEGAHIT’s generally superior per-
371 formance in recovering the genomes of closely related strains (Figure 2, right
372 panel). The sum “fraction of genome recovered” is arguably the most im-
373 portant measure of a metagenome assembler (see [5] in particular) and here
374 MEGAHIT excels for individual genomes even in the presence of strain vari-
375 ation.

376 When comparing details of sequence recovery between the assemblers,
377 the assembly content differs by only a small amount when loose alignments
378 are allowed: all three assemblers miss more content (approximately 2.5% of
379 the reference) than they generate uniquely (1.7% or less). In addition to
380 preferring no one assembler over any other, this suggests that combining as-
381 semblies may have little value in terms of recovering additional metagenome
382 content.

383 **The missing reference may be present in strain variants of the**
384 **intended species.**

385 Several individual genomes are missing in measurable portion from the QC
386 reads (Table 2), and many QC reads (4.4% of 108m) did not map to the
387 full reference metagenome. These appear to be related issues: upon anal-
388 ysis of the unmapped reads against GenBank, we find that many of the
389 contigs assembled from the unmapped reads can be assigned to strain vari-
390 ants of the species in the mock community (Table 9). This suggests that
391 the constructors of the mock community may have unintentionally included
392 strain variants of *Fusobacterium nucleatum*, *Thermus thermophilus* HB27,
393 and *Enterococcus faecalis*; note that the microbes used were sourced from
394 the community rather than the ATCC (M. Podar, pers. communication). In
395 addition, we detect what may be portions of a novel member of the *Proteini-*
396 *clasticum* genus in the assembly of these reads - this is likely the *Clostridium*
397 spp. detected through amplicon sequencing in [12].

398 Without returning to the original DNA samples, it is impossible to con-
399 clusively confirm that unintended strains were used in the construction of the

400 mock community. In particular, our analysis is dependent on the genomes in
401 GenBank: the genomes we detect in the contigs are clearly closely related to
402 GenBank genomes not in the reference metagenome, based on k-mer anal-
403 ysis and contig alignment. However, GenBank is unlikely to contain the
404 exact genomes of the actually included strain variants, rendering conclusive
405 identification impossible.

406 Conclusions

407 Overall, assembly of this mock community works well, with good recovery
408 of known genomic sequence for the majority of genomes. All three assem-
409 blers that we evaluated recover similar amounts of most genomic sequence,
410 but (recapitulating several other studies [3, 5, 15]) MEGAHIT is compu-
411 tationally the most efficient of the three. We note that assembly resolves
412 substantial portions of several previously undetected strain variants, as well
413 as recovering a substantial portion of a novel *Proteiniclasticum* spp. that
414 was detected via amplicon analysis in [12], suggesting that assembly is a
415 useful complement to amplicon or reference-based analyses.

416 The presence of closely related strains is a major confounder of metagenome
417 assembly, and causes assemblers to drop considerable portions of genomes
418 that (based on read mapping and k-mer inclusion) are clearly present. In this
419 relatively simple community, this strain confusion is present but does not
420 dominate the assembly. However, real microbial communities are likely to
421 have many closely related strains and any resulting loss of assembly would
422 be hard to detect in the absence of good reference genomes. While high
423 polymorphism rates in e.g. animal genomes are known to cause duplication
424 or loss of assembly, some solutions have emerged that make use of assump-
425 tions of uniform coverage and diploidy [31]. These solutions cannot however
426 be transferred directly to metagenomes, which have unknown abundance
427 distributions and strain content.

428 An additional concern is that metagenome assemblies are often per-
429 formed after pooling data sets to increase coverage (e.g. [4, 32]); this pooled
430 data is more likely to contain multiple strains, which would then in turn
431 adversely affect assembly of strains. This may not be resolvable within the
432 current paradigm of assembly, which focuses on outputting linear assem-
433 blies that cannot properly represent strain variation. The human genomics
434 community is moving towards using *reference graphs*, which can represent
435 multiple incompatible variants in a single data structure [33]; this approach,
436 however, requires high-quality isolate reference genomes, which are generally

437 unavailable for environmental microbes.

438 Long read sequencing (and related technologies) will undoubtedly help
439 resolve strain variation in the future, but even with highly accurate long-
440 read sequencing, current sequencing depth is still too low to resolve deep
441 environmental metagenomes [34, 35]. It is unclear how well long error-
442 prone reads (such as those output by Pacific Biosciences SMRT [36] and
443 Oxford Nanopore instruments [37]) will perform on complex metagenomes:
444 with high error rates, deep coverage of each individual genome is required
445 to achieve accurate assembly, and this may not be easily obtainable for
446 complex communities. Single-molecule barcoding (e.g. 10X Genomics [38])
447 and HiC approaches [39] show promise but these remain untested on well-
448 defined complex communities and are still challenged by the complexity of
449 complex environmental metagenomes; see [40, 41, 42].

450 Much of our analysis above depends on having a high-quality “mock”
451 metagenome. While computationally constructed synthetic communities
452 and computational “spike-ins” to real data sets can provide valuable controls
453 (e.g. see [15] and [43]) we strongly believe that standardized communities
454 constructed *in vitro* and sequenced with the latest technologies are critical to
455 the evaluation of both canonical and emerging tools, e.g. efforts such as [44].
456 From the perspective of tool evaluation, we must disagree somewhat with
457 Vollmers et al. [5]: good metagenome tool evaluation necessarily depends on
458 mock communities that are as realistic as we can make them. Likewise, from
459 the perspective of bench biologists, actually sequencing real DNA is critical
460 because it can evaluate confounding effects such as kit contamination [45].
461 Large-scale studies of computational approaches systematically applied to
462 mock communities such as CAMI [3] can then provide fair comparisons of
463 entire toolchains (wet + dry) applied to these mock communities.

464 We omitted two important questions in this study: binning and choice
465 of parameters. We chose not to evaluate genome binning because most
466 binning strategies either operate post-assembly (see e.g. [46]), in which
467 case the challenges with assembly discussed above will apply; or require
468 multiple samples (e.g. [47]), which we do not have. We also chose to use
469 only default parameters with all three assemblers, for two reasons. First,
470 we are not aware of any widely used automated approaches for determining
471 the “best” set of parameters or evaluating the output, other than those
472 integrated into the assemblers themselves (e.g. choice of k-mer sizes), and
473 absent such guidance we do not feel comfortable blessing any particular set of
474 parameters; here the choice of default parameters is parsimonious. Second,
475 any parameter exploration pipeline would not only need to be automated

476 but would need to run multiple assemblies, whose time and resource usage
477 should be measured; in this case, any comparison based on runtime of the
478 parameter choice pipeline should naturally favor MEGAHIT because of its
479 substantial advantage in computational efficiency.

480 **Author contributions**

481 SA, LI and CTB developed, tested, and executed the analytical pipeline.
482 SA and CTB created the tables and figures and wrote the paper.

483 **Competing interests**

484 No competing interest to our knowledge.

485 **Grant information**

486 This work is funded by Gordon and Betty Moore Foundation Grant GBMF4551
487 and NIH NHGRI R01 grant HG007513-03, both to CTB.

488 **Acknowledgments**

489 We thank Michael R. Crusoe and Phillip T. Brooks for input on analysis and
490 pipeline development. We thank Migun Shakya, Mircea Podar, Jiarong Guo,
491 Harald R. Gruber-Vodicka, Juliane Wippler, Krista Ternus, and Stephen
492 Turner for valuable comments on drafts of this manuscript.

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