

***F1000Research* Evaluating Metagenome Assembly on a Complex Community**

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Abstract

Metagenome assembly is a challenging problem due to the biodiversity of the microorganisms. Most assemblers are designed for whole genome assembly and not capable of dealing with metagenomic samples. However, in order to decide which assembler works best for metagenome, we need to evaluate metagenome assembly generated by each assembler.

In this paper, we used three assemblers ; IDBA-UD, SPAdes, and Megahit to assemble metagenome mock community data and evaluate the assembly process in terms of resources utilization, assembly quality, genome fraction covered, duplication ratio, misassemblies and partial alignments.

The results show only small differences in content recovery between assemblers. However, Megahit is much faster and produces shorter contig lengths than IDBA-UD and SPAdes.

Introduction

Metagenomics refers to sequencing of DNA from a mixture of organisms, often from an environmental or uncultured sample. Unlike whole genome sequencing, metagenomics targets more than one genome, which introduces challenges in the analysis of metagenomes. Most approaches of analyzing metagenomic data rely on mapping assembly to reference genomes. However, not all microbial diversity of many environments are covered by reference databases. Hence, the need for de novo assembly of complex metagenomic data rises. Several assemblers exist that can be used for de novo assembly. We evaluated these assemblers on a mock community, in which individual organisms were grown in culture and their DNA extracted, and then the DNA was combined at defined abundance ratios and shotgun sequenced.

Moya et al. in [1] evaluated metagenome assembly using simulated two 454 viral metagenome and six assemblers. The assemblies were evaluated based on several metrics including N50, percentages of reads assembled, accuracy when compared to the reference genome. In addition to, chimeras per contigs and the effect of assembly on taxonomic and functional annotations.

Mavromatis et al. in [2] provided a benchmark study to evaluate the fidelity of metagenome process methods. The study used simulated metagenomic data sets constructed at different complexity levels. The datasets were assembled using Phrap v3.57, 'Arachne v.2 [3] and JAZZ. [4]

The study evaluates assembly, gene prediction, and binning methods. However, the study did not evaluate the assembly quality against a reference genome.

Rangwala et al. in [5] presented an evaluation study of metagenome assembly. The study used a de Bruijn graph based assembler ABYSS [6] to assemble simulated metagenome reads of 36 bp. The data set is classified at different complexity levels. The study compares the quality of the assembly of the data sets in terms of quality measures of contigs length, assembly accuracy. The study also took into consideration the effect of kmer size and the degree of chimericity. However, the study evaluated the assembly based on one assembler, and did not evaluate assembly against several assemblers. Also, both previous studies did not use real data sets and hence did not capture the real complexity of real metagenome.

Lindgrees et al in [7] presented a benchmark study for metagenome analysis tools. The authors compared between several metagenome classification tools in terms of run time, ease of use, information provided, reads and shuffled reads mapped, non existing phyla, divergence of real distribution, and correlation with known community composition. However, the paper did not consider metagenome assembly tools in the study.

In this paper, we propose an evaluation for metagenome assembly using three assemblers; IDBA-UD [8], SPAdes [9], and MEGAHIT [10].

SPAdes [9] is an assembler for both single-cell and standard (multicell) assembly. SPAdes generates single-cell assemblies and provides information about genomes of

uncultivable bacteria that vastly exceeds what may be obtained via traditional metagenomics studies.

IDBA-UD [8] is a de Bruijn graph approach for assembling reads from single cell sequencing or metagenomic sequencing technologies with uneven sequencing depths. IDBA-UD uses multiple depth-relative thresholds to remove erroneous k-mers in both low-depth and high-depth regions. It also uses paired-end information to solve the branch problem of low-depth short repeat regions. It applies an error correction step to correct reads of high-depth regions that can be aligned to high confident contigs.

MEGAHIT [10] is a new approach that constructs a succinct de Bruijn graph using multiple k-mers, and uses a novel "mercy k-mer" approach that preserves low-abundance regions of reads. It also uses GPUs to accelerate the graph construction.

We evaluate the performance of the three assemblers using real mock community data. The performance of each assembler is compared in terms of resources utilization, covered genome fraction, duplication ratio, misassemblies, and contig length. This helps decide which assembler to use when we lack a reference.

Table 1. Running Time and Memory Utilization

(1) IDBA-UD	
Running Time	17:12:43
Memory Utilization (GB)	149.12
(2) SPAdes	
Running Time	42:14:06
Memory Utilization (GB)	391.45
(3) MEGAHIT	
Running Time	56:04.43
Memory Utilization (GB)	34.40

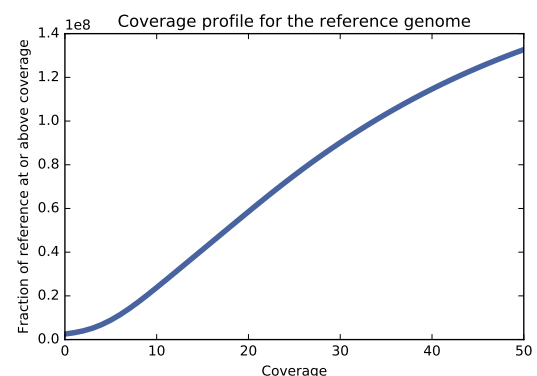


Figure 1. Coverage profile for the reference metagenome, based on read mapping.

Datasets

We used a diverse mock community data set constructed by pooling DNA from 64 species of bacteria and archaea and sequencing them with Illumina HiSeq. The raw

Table 2. Reference Genome Coverage and Duplication Ratio

(1) Best hit Approach		
(1) IDBA-UD		
99.0	Genome Coverage	56.89 %
	Duplication Ratio	0.38 %
95.0	Genome Coverage	58.00%
	Duplication Ratio	0.59 %
(2) SPAdes		
99.0	Genome Coverage	63.79 %
	Duplication Ratio	0.15 %
95.0	Genome Coverage	64.68 %
	Duplication Ratio	0.26%
(3) MEGAHIT		
99.0	Genome Coverage	68.47 %
	Duplication Ratio	0.37 %
95.0	Genome Coverage	68.96 %
	Duplication Ratio	0.45%
(2) Ambiguous Approach		
(1) IDBA-UD		
99.0	Genome Coverage	89.79 %
	Duplication Ratio	0.94%
95.0	Genome Coverage	95.46 %
	Duplication Ratio	1.90 %
(2) SPAdes		
99.0	Genome Coverage	89.42 %
	Duplication Ratio	1.00 %
95.0	Genome Coverage	95.12 %
	Duplication Ratio	1.98 %
(3) MEGAHIT		
99.0	Genome Coverage	91.16 %
	Duplication Ratio	0.55 %
95.0	Genome Coverage	94.22 %
	Duplication Ratio	1.48 %
(3) No Misassemblies Approach		
(1) IDBA-UD		
99.0	Genome Coverage	34.60 %
	Duplication Ratio	0.37%
95.0	Genome Coverage	35.22 %
	Duplication Ratio	0.56 %
(2) SPAdes		
99.0	Genome Coverage	35.92%
	Duplication Ratio	0.16 %
95.0	Genome Coverage	36.42 %
	Duplication Ratio	0.21 %
(3) MEGAHIT		
99.0	Genome Coverage	45.81 %
	Duplication Ratio	0.39%
95.0	Genome Coverage	46.03 %
	Duplication Ratio	0.48 %

Table 3. Contigs Analysis

(1) Best hit Approach	
(1) IDBA-UD	
No. of Contigs	19,988
Totally Aligned Contigs %	72.96% (97,138,779)
Partial Aligned Contigs %	10.97% (20,261,669)
Unaligned Contigs %	16.07% (61,421,243)
(2) SPAdes	
No. of Contigs	15,254
Totally Aligned Contigs%	76.52% (109,342,809)
Partial Aligned Contigs%	12.08% (22,008,234)
Unaligned Contigs%	11.40% (34,176,209)
(3) MEGAHIT	
No. of Contigs	27,657
Totally Aligned Contigs %	83.07% (128,987,917)
Partial Aligned Contigs%	4.67% (12,325,804)
Unaligned Contigs%	12.26% (50,093,476)
(2) Ambiguous Approach	
(1) IDBA-UD	
No. of Contigs	19,988
Totally Aligned Contigs%	80.59% (161,075,933)
Partial Aligned Contigs%	8.54 % (22,638,415)
Unaligned Contigs %	10.87% (13,378,572)
(2) SPAdes	
No. of Contigs	15,254
Totally Aligned Contigs	81.03 % (154,920,366)
Partial Aligned Contigs%	9.34% (28,028,529)
Unaligned Contigs%	9.62% (12,931,934)
(3) MEGAHIT	
No. of Contigs	27,657%
Totally Aligned Contigs%	87.59 % (169,789,173)
Partial Aligned Contigs%	4.36% (15,658,616)
Unaligned Contigs%	8.04% (12,777,886)
(3) No Misassemblies Approach	
(1) IDBA-UD	
No. of Contigs	19,988
Totally Aligned Contigs%	57.41% (61,874,288)
Partial Aligned Contigs%	6.81%(9,530,232)
Unaligned Contigs %	35.78% (122,899,406)
(2) SPAdes	
No. of Contigs	15,254
Totally Aligned Contigs	62.93% (64,656,150)
Partial Aligned Contigs%	6.84% (9,323,121)
Unaligned Contigs%	30.23% (114,292,858)
(3) MEGAHIT	
No. of Contigs	27,657
Totally Aligned Contigs%	67.62% (87,488,973)
Partial Aligned Contigs%	2.73% (7,069,258)
Unaligned Contigs%	29.65% (102,672,613)

data set consisted of 109,629,496 reads from Illumina HiSeq 101 bp paired-end sequencing (2x101) with an untrimmed total length of 11.07 Gbp and an estimated

fragment size of 380 bp [11].

The original reads are available through the NCBI Sequence Read Archive at Accession SRX200676.

Table 4. Genomes with the most common uncovered bases between the three assemblies.

Genome	Uncovered
Shewanella_baltica_OS223	2,338,136(18.25%)
Fusobacterium_nucleatum	2,170,544(16.95%)
Desulfovibrio_vulgaris_DP4	1,645,406(12.85%)
Enterococcus_faecalis_V583	1,003,172(7.83%)
Thermus_thermophilus_HB27	734,102(5.73%)

Table 5. Comparison between N50 and NG50

Assembler	N50	NG50
IDBA-UD	55,225	52,031
SPAdes	68,189	63,721
Megahit	41,427	39,206

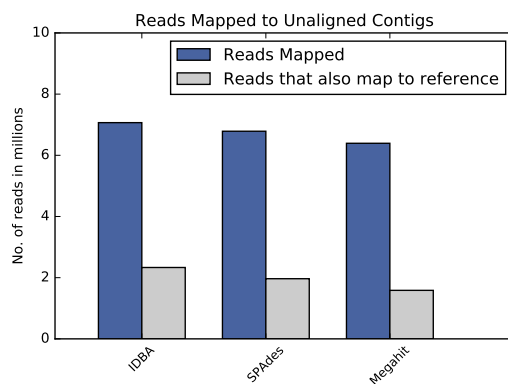


Figure 2. Mapping unaligned reads to reference genome using identity 99%, and Ambiguous Approach

We received the reference genomes from the original authors. They consist of 205.6 Mbp of assembled genomes in 64 contigs, and are available for download at <https://dx.doi.org/10.6084/m9.figshare.1506873.v2>

Methods

The analysis code and run scripts for this paper are available at: <https://github.com/dib-lab/2015-metagenome-assembly/>

Quality Filtering

We removed adapters with Trimmomatic v0.30 in paired-end mode with the Truseq adapters [12].

Assemblers

We assembled the reads using three different assemblers: IDBA-UD [8], SPAdes [9], and MEGAHIT [10]. For IDBA-UD v1.1.1 [8], we used `-pre_correction` to perform pre-correction before assembly and `-r` for the pe files. For SPAdes v3.9.0 [9], we used `-meta -pe1-12 -pe1-s` where `-meta` is recommended when working with metagenomic data sets, `-pe1-12` for file with interleaved reads for the first paired-end library, and `-pe1-s` is for single reads. For MEGAHIT [10], we used `-l 101 -m`

Table 6. Misassembled Contigs using Identity 99%

Assembler	No. of Contigs	No.of Bases
IDBA-UD	4,980 (24.91%)	112,309,828
SPAdes	3,143 (20.60%)	108,969,624
Megahit	5,977 (21.61%)	90,889,558

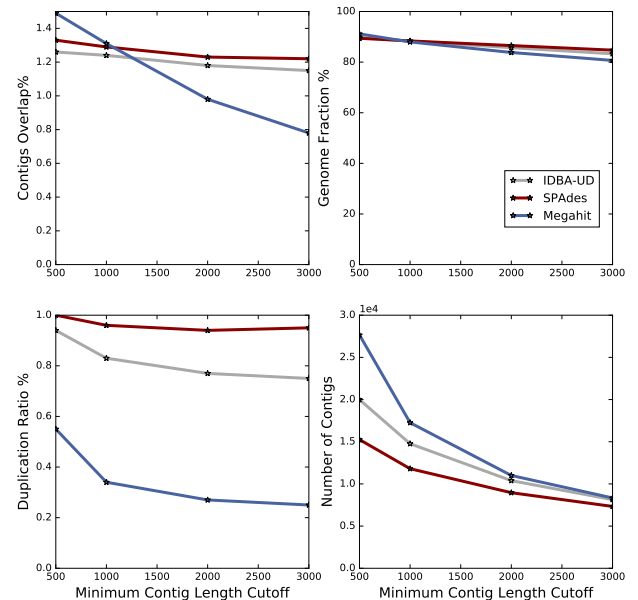


Figure 3. Genome Fraction, duplication ratio, Contigs overlap ratio, and number of contigs using different minimum contig length, identity 99%, and Ambiguous Approach

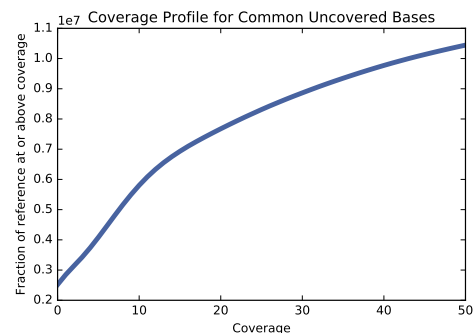


Figure 4. Base Coverage for Common Uncovered Bases

`3e9 -cpu-only` where `-l` is for maximum read length, `-m` is for max memory in bytes to be used in constructing the graph, and `-cpu-only` to use only the CPU and not the GPU. We also used `-presets meta-large` for large and complex metagenomes, and `-12` and `-r` are parameters for inputting paired-end, interleaved-paired-end and single-end files respectively. Unless otherwise mentioned, we eliminated all contigs less than 500 bp from each assembly prior to further analysis.

Assembly analysis using Nucmer

We used the Nucmer tool from MUMmer3.23 [13] to align assemblies to the reference genome with options `-coords -p`. Then we parsed the generated ".coords" file using a custom script `analyze_assembly.py` to calculate several analysis metrics at two alignment identities, 95% and 99%.

Mapping

We aligned all quality-filtered reads to the reference metagenome with `bwa aln` (v0.7.7.r441) [14]. We aligned paired-end and orphaned reads separately using `bwa aln samse`. We then used `samtools` (v0.1.19) [15] to convert SAM files to BAM files for both paired-end and orphaned reads. To count the unaligned reads, we found the records with the "4" flag in the SAM files [15].

To extract the reads that contribute to unaligned contigs, we mapped the quality filtered reads to the unaligned contigs using `bwa aln` (v0.7.7.r441) [14]. Then we used `samtools` to retrieve the reads that mapped to the unaligned contigs.

k-mer Presence

In order to examine k-mer presence for a k-mer size of 20, we build a k-mer counting table from the given quality filtered reads using `load-into-counting.py`. Then we calculate abundance distribution of the k-mers in the quality filtered reads using the pre-made k-mer counting table using `abundance-dist.py`. We followed the same approach to examine k-mer presence in assemblies.

Gene annotations using Prokka

We used `prokka` [16] to annotate the reference genome using `-outdir mprokka -prefix testasm -metagenome`. Then we parsed the `testasm.tbl` output file to get the coordinates of CDS genes. We searched the alignments for how many genes is contained in those alignments.

Reference Coverage Profile

To evaluate how much of the reference genome was contained in the read data, we used `bwa aln` to map reads to the reference genome. We then calculated how many reference bases are covered by how many mapped reads (custom script `coverage-profile.py`)

Analyzing Assembly: Ambiguous, Best-Hit, and No misassemblies Approaches

We processed the alignments in three different ways: ambiguous, best-hit, and no-misassemblies.

In the ambiguous approach, we take into account all the alignments of a contig to the reference, even if alignments overlap in the reference or they are aligned to multiple locations in the reference.

In the best-hit approach, among all alignments of a contig, we take into consideration only the alignment with the best score.

In the no-misassemblies approach, we only count contigs that have precisely one alignment to the reference.

In all approaches, we flag a base in the reference genome as "covered" if it is hit by a kept alignment. We define the duplication ratio as the percentages of bases in the reference covered more than once by the kept alignments. We define misassemblies as those contigs that are divided into different parts when mapped to the reference. The number of misassembled contigs is equal to the number of aligned contigs (both totally and partially) in the ambiguous approach minus the number of aligned contigs in the no-misassemblies approach. aligned to the reference that exist in more than one contig. We define the contig overlap ratio as the number of bases aligned to the reference that exist in more than one kept alignment.

All approaches have a non-zero duplication ratio within the reference because we don't explicitly discard contigs that map to the same location in the reference.

Results

The raw data is high quality

We trimmed sequences as described in Methods. We retained 7.1 Gbp in 108,422,358 paired-end sequences, and 36 Mbp in 520,403 orphaned reads.

This quality trimmed ("QC") data set was used as the basis for all further analyses.

98% or more of the reference is present in the read data set

We evaluated the fraction of the reference genome covered by at least one read (see Methods for details). Quality filtered reads cover 203,058,414.0 (98.76%) bases of the reference metagenome (205,603,715 bp total size). Figure 1 shows the cumulative coverage profile of the reference metagenome, and the percentage of bases with that coverage. Most of the reference metagenome was covered at least minimally; only 3.33% of the reference metagenome had mapping coverage <5 and 1.24 % of the bases in the reference aren't covered by any reads in the QC data set.

In order to evaluate reconstructability with De Bruijn graph assemblers, we next examined k-mer presence for a k-mer size of 20. Of the 174m 20-mers in the reference data set, 98.7% were present in the data set and 95.5% of them occurred with abundance 5 or greater in the quality filtered read data set. $SRR606249.qc.dist(1-0.045)*100$, the 98.7 is from the same file but $(1-0.013)*100$

MEGAHIT is the fastest and lowest-memory assembler evaluated

We ran three commonly used metagenome assemblers on the QC data set: IDBA-UD, SPAdes, and Megahit. We recorded the time and memory usage of each (Table 1). MEGAHIT outperformed both SPAdes and IDBA-UD considerably, producing an assembly in one hour – approximately 17 times faster than IDBA and 42 times faster than SPAdes – with 34.40 GB of RAM – 1/5 to 1/11th the memory of IDBA and SPAdes.

Much of the reference is covered by the assemblies

We evaluated the extent to which the assembled contigs recovered the “known/true” metagenome sequence by aligning each assembly to the reference (Table 2). All three assemblers generate contigs that cover more than 89% of the reference metagenome at high identity (99% or greater) with little duplication (0.55 -1.0%) (see “Ambiguous approach” in Table 2). If we relax the identity threshold to 95%, then the assembled contigs cover more than 94% of the reference metagenome.

When we use only the highest-scoring alignment at 99% identity, we find that the reference coverage drops to 57-68%, depending on the assembler (“Best hit approach”, Table 2). The reference coverage from highest-scoring alignments does not substantially increase when the identity threshold is relaxed to 95%.

At 99% identity, approximately 6.23% of the reference is covered by no contig from any of the three assemblies; we discuss this in more detail below.

The generated contigs are broadly accurate

When counting only the best alignment per contig and with a 99% identity threshold, more than 72% of contigs align to the reference completely, i.e. across the whole length of the contig. If we allow multiple alignments per contig, then more than 80% of contigs align completely (although not contiguously) to the reference. Approximately 4 - 9% of the contigs align partially, and the remaining 8 - 10% of contigs do not align at all to the reference (discussed in detail below).

Most genes within the reference metagenome are contained within contigs

The reference genome has 188,880 CDS with 91,806 annotated as genes. Using Ambiguous approach and 99% identity, IDBA-UD kept alignments contained 82,791 (95.20%) of the reference genes coordinates, while SPAdes kept alignments 83,475 (94.44%), and Megahit contained 80,256 (94.59%) of the reference genes coordinates. prokka-analysis.out.

The assemblies contain most of the raw data

The assemblies also represent the majority of the reads (99.7%) and the majority of the abundance-5 20-mers (93.7% of the 198m high-abundance 20-mers in the reads), suggesting that the assemblies represent the underlying content of the reads very well.

The portions of the reference metagenome that are not reconstructed are not present in the read data set

We identified bases in the reference that had no match (at 99% identity with the ambiguous mapping approach) in any of the assemblies, and evaluated their base coverage. 29.2% had coverage < 5 and (19.66%) has no coverage in the reads.

Large portions of several reference genomes are not assembled by any assembler

A number of the genomes in the reference metagenome had many missing bases in the assemblies (Table 4). In three extreme cases, *Shewanella baltica*_OS223, *Fusobacterium nucleatum*, and *Desulfovibrio* contribute, 18.25%, 16.95%, and 12.85% of the common uncovered bases respectively.

Many assembled contigs do not align to the reference metagenome

Depending on assembler, between 8.04% and 10.87% of the assembled contigs do not align anywhere in the reference metagenome.

6.49% of reads mapped to the unaligned contigs of IDBA. Only 33.01% of those reads mapped to the reference. (2.14% of all the reads). 6.23% of reads mapped to the unaligned contigs of SPAdes. Only 28.97% of those reads mapped to the reference. (1.80% of all the reads). 5.87% of reads mapped to the unaligned contigs of Megahit. Only 24.80% of those reads mapped to the reference. (1.45% of all the reads) For each assembly, approximately 5m quality-filtered reads map only to the unaligned contigs (and nowhere in the reference).

For IDBA QC, the reads that aligned to the unaligned contigs but not to the reference has coverage bases 27.21% <5 in the unaligned contigs. 75.72% has coverage >0.

For SPAdes QC, the reads that aligned to the unaligned contigs but not to the reference has coverage bases (3,109,539) 24.04% <5 in the unaligned contigs. 79.10% has coverage >0.

For Megahit QC, the reads that aligned to the unaligned contigs but not to the reference has coverage bases (2,578,896) 20.18% <5 in the unaligned contigs. 83.08% has coverage >0.

All three assemblers recover most of the reference

XX% of the reference metagenome is recovered by all three assemblers (“common covered”) with relatively little duplication. (99% identity, ambiguous alignments allowed).

All three assemblers fail to recover 6.23% of the reference

(2,518,234) of the common uncovered bases has zero coverage (19.66%), while (3,739,532) of the common uncovered bases has coverage <5 (29.2%).

At the margins, the three assemblers differ at about 1% in recovery

IDBA, SPAdes, and MEGAHIT each uniquely recover about 1% (0.64 -1.92%) of the reference metagenome, and each uniquely fail to recover about 1% (1.22-1.79%) of the reference.

Pending text: To add or not

Figure 3 shows the number of contigs using different minimum contigs cutoff. The figure shows that Megahit [10] has more fragmented contigs in the assembly. The small contigs size leads to best alignment; Megahit has the highest number of uniquely covered bases, highest genome coverage, and lowest unalignment.

Discussion

Assembly recovers basic content well

The majority of the reference metagenome is recovered by all three assemblers: 89% or more of the reference metagenome is contained within each assembler's output at 99% identity, and 94% can be recovered if we relax the identity threshold to 95%. This is close to the measured maximum reconstructability based on read mapping and k-mer presence: 98.76% of the reference is covered by at least one read, and 98.7% of the genome is present in k-mers of size 20. and 98.7 are from SRR606249.qc.coverage and SRR606249.qc.dist respectively

The contigs generated align well to the reference metagenome: 80% (or more) of contigs align to the reference metagenome across the whole length of the contig, and another 4% (or more) of the contigs are entirely contained within the reference metagenome, although they do not align to only one location in the reference. This could be caused by misassembly (a computational error) or by rearrangements in the source DNA (in which case the reference is incorrect).

More than 6.23% of the reference metagenome is missing from all of the assemblies when 99% similarity is required, and large portions of the missing sequence are from a few genomes – in some cases, close to a third of the source genome is missing from the assemblies.

of the unassembled regions of the source genome is low to nonexistent, while other portions of the source genomes are well represented within the reads; these regions were simply not sequenced, either because they were missing from the input DNA or because they challenge the sequencer. we have only 29.2

Assembly produces content not in the reference metagenome

In addition to recovering most of the content of the reference metagenome, all three assemblers generate many contigs (approximately 10% of the total) that do not map to anything in the reference. While some could be misassemblies, many of the reads that map to these contigs do not map anywhere in the reference, while many of the contigs have coverage >XX, suggesting that they are present within the source DNA at an abundance that is similar to many of the genomes in the mock community. Whatever their true identity, these contigs are not present in the reference metagenome. Because this is a mock metagenome for which isolates were grown and individually extracted prior to being combined for sequencing, we believe that these extra genomic contigs must come

from one or more contaminants. We cannot rule out contamination from kits, but because large amounts of DNA were used to create the mock community, it seems unlikely that these are due to trace contaminants created by PCR (as can happen in amplicon studies); they are most likely from contaminants grown together with the isolates.

Different assemblers differ, but not by much

The three assemblers differ very little in recovery of basic content, as judged by reference metagenome alignments: at 99% identity, XX% is recovered in common, with 0.64 to 1.92% specific to each assembler (and a total of XX% being recovered by one or two, but not all three, of the assemblers). None of the assemblers include all of the sequence produced by the others.

In practice, then, we do not see a significant advantage to one assembler over another in terms of recovering the known reference, and we speculate that (for this data set), there is little advantage to combining assemblies from multiple assemblers, since very little extra content will be recovered.

Conclusions

Assembly works well. There is no big difference between assemblers' performance in terms of assembly quality. In terms of cost, Megahit is much faster and utilizes less memory.

Author contributions

In order to give appropriate credit to each author of an article, the individual contributions of each author to the manuscript should be detailed in this section. We recommend using author initials and then stating briefly how they contributed.

Competing interests

No competing interest to our knowledge.

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