A Recent (2020) Comparative Analysis of Genome Aligners Shows HISAT2 and BWA are Among the Best Tools

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A Recent (2020) Comparative Analysis of Genome Aligners Shows HISAT2 and BWA are Among the Best Tools

by

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The undersigned state that Ryan Musich, a candidate for the Master of Science degree in Bioinformatics, has submitted his thesis and has satisfactorily defended it.

This completes the requirements for the Master of Science degree in Bioinformatics at Rochester Institute of Technology.

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Abstract

Genome aligners are an important tool in bioinformatics research as they can be used to detect gene variants to create higher crop yields, detect abnormal gene production in cancer cell lines, or identify weaknesses in a newly discovered pathogen. Aligners work by taking sequenced DNA or RNA and mapping these reads to their corresponding location in a reference genome. Although beneficial as a tool, choosing which aligner to use for a project is often a difficult decision due to the large number of tools available and each one claiming to be the best at what it does. The goal of this project is to determine which aligner performs the best in a controlled environment using the default settings for six of the most used genome aligners: Bowtie2 (using both end-to-end and local alignment modes), Burrows-Wheeler Aligner (BWA), Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT2), MUMmer4, Spliced Transcripts Alignment to a Reference (STAR), and TopHat2. Each aligner was run using 48 geographically distinct samples of *Erysiphe necator*, more commonly known as powdery mildew. Alignment results were assessed based on three major criteria: 1) the number of reads successfully mapped to the reference genome, 2) their runtimes using a varying number of cores, and 3) the percentage of the full transcriptome covered. Aligners were further analyzed for potential biases in the types of genes that were unable to be mapped. The results for each aligner were compared against one another to determine the aligner which had the best performance on the provided dataset. The two best performing aligners were BWA, which achieved the highest alignment rate, and HISAT2, which achieved the fastest runtime. Overall, HISAT2 was determined to be the better aligner of the two as both aligners had similar transcriptome coverage regardless of alignment rate.

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Abbreviations in the table appear in order of their appearance in the document.

Abbreviation	Meaning
Indels	Insertion/Deletion Mutations
BLAST	Basic Local Alignment Search Tool
FM-Index	Full-Text Index in Minute Space
BWT	Burrows-Wheeler Transform
BWA	Burrows-Wheeler Aligner
HISAT2	Hierarchical Indexing for Spliced Alignment of Transcripts
STAR	Spliced Transcripts Alignment to a Reference
GFM-Index	Graph-Based FM-Index
SRA	Sequence Read Archive
MUM	Maximal Unique Match
MMP	Maximal Mappable Prefix
SAM	Sequence Alignment/Map
BUSCO	Benchmarking Universal Single-Copy Orthologs
BAM	Binary SAM
GTF	Gene Transfer Format
GFF	General Feature Format
BLASTN	Nucleotide-Nucleotide BLAST
COG	Clusters of Orthologous Groups of Proteins
SNP	Single Nucleotide Polymorphism
RAM	Random Access Memory
CPU	Central Processing Unit

Introduction

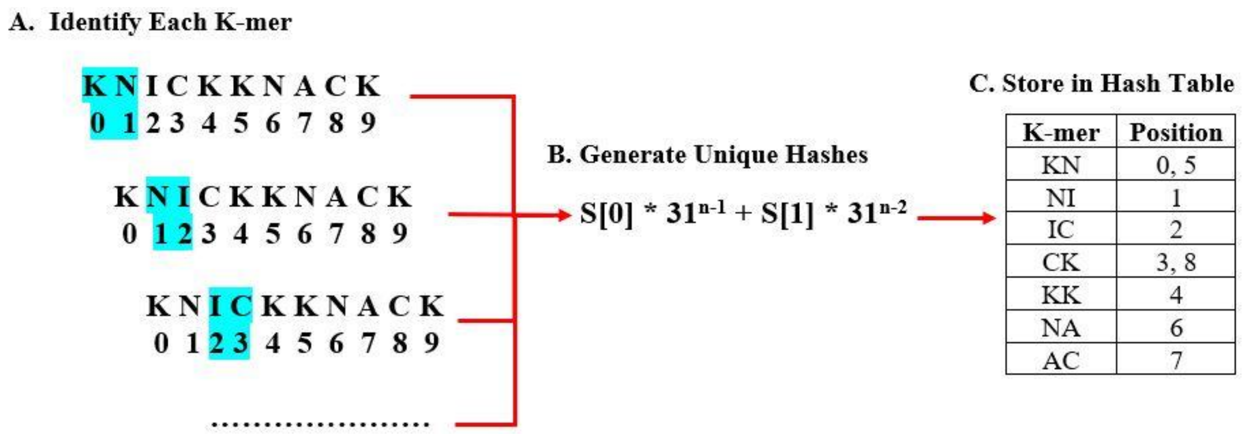
A large portion of biological research today involves the study of genetic mutations and how these changes contribute to the overall chemical makeup of the organism. In its most general form, genetic research comes down to a comparison between the DNA of multiple organisms or subgroups and analyzing which mutations or gene variants are beneficial or disadvantageous to those groups. On the simplest scale, one could study the differences between a single gene that is common between two organisms. This can be done by aligning together the sequences in a pairwise fashion and simply showing which bases are different. Most often though a single gene comparison is not enough as many traits are considered polygenic and would require the analysis and alignment of multiple genes. This leads to the larger scale studies of comparing and aligning whole genomes, which can lead to the discovery of indels, gene duplications, chromosomal inversions, etc. By comparing the genomes of plants of the same species, one could identify which gene variants contribute to greater crop yield or increase photosynthetic rates. The genomes of cancer cell lines can be analyzed to identify mutations throughout multiple genes which are contributing to the accelerated growth of the cells. A newly discovered pathogen's genome could be examined for potential weaknesses to target, such as a lack of antibiotic resistant genes, to protect the host. Through all these analyses, it is the aligning of the genome itself that is often a crucial step in any genomics research as an inaccurate alignment can lead to incorrect conclusions downstream. This step is further hampered by the countless tools available for genome alignment claiming to be the best and most efficient at what they do. In this project, the top genome alignment tools will be tested against one another to determine the fastest and most accurate genome aligner.

After a genome is sequenced, the data obtained are many small reads each containing about 100 base pairs or less. These reads represent the genome itself fragmented into small parts, which essentially means that the current data is of limited utility for any analysis without assembling a new genome or aligning to a reference. If a novel organism has been sequenced, a genome assembler is used to stitch together the fragments based on overlapping segments in each of the reads. This tends to be a time-consuming process as the genome is being created from scratch and fragments may require additional rounds of sequencing to accurately fill in the missing gaps. An aligner takes in a previously assembled reference genome and a set of sequenced reads, often RNA sequencing data, and works to match the reads to their appropriate position in the genome. With an already completed genome to work with, a genome aligner is often preferred to save time. Although they seem to be less complex, there are often two major problems that aligners must overcome: 1) accurately matching a read to its position in the reference genome and 2) quickly processing large genome files.

Seed-and-Extend with Hash Tables

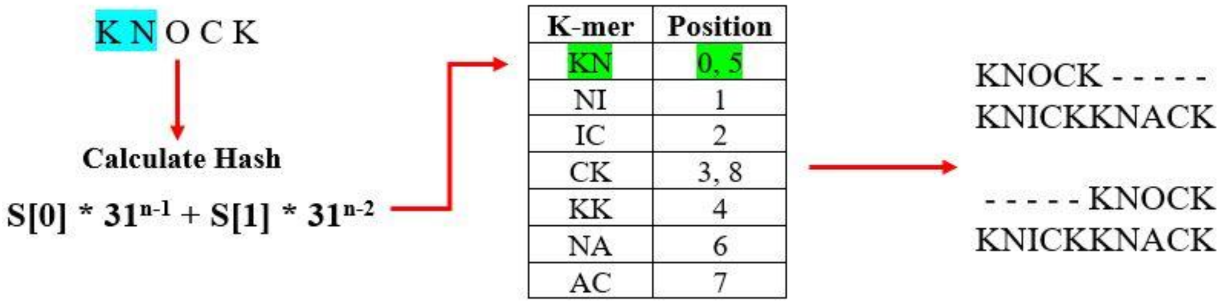
When it comes to matching a fragment to its position, the fragmented sequence will most likely not be an exact match to the reference genome. This often is due to the frequency of mutations in the DNA of the sequenced organism, errors during sequencing, or polymorphisms that occur within a species. In 1990, the basic local alignment search tool (BLAST) was published, which contained an algorithm used to align two sequences through a process called seed-and-extend (Altschul *et al.*, 1990). The algorithm starts by dividing the reference sequence into fragments of a user-determined length k. These fragments are referred to as k-mers and the process of generating these fragments is shown in Figure 1-A. A k-mer is then run through an equation to generate a unique numerical index, called a hash code. A simple example of this is

Java’s hash code calculation which adds the ASCII value of each character in the k-mer multiplied by decreasing exponentials of base 31, shown in Figure 1-B. The generated hash code is then used as an index to a structure called a hash table and used to store the position that the k-mer occurs in the overall reference sequence as shown in Figure 1-C. Once the hash table is created for the reference sequence, the query sequence is now split into k-mers of the same length and inputted into the same hash code calculation to get an index. If the k-mer in the query sequence is an exact match to a k-mer in the reference sequence, the hash table will return the positions in the reference to begin the alignment as shown in Figure 2. The process of finding exact matches between the query and reference sequence is known as seeding and makes up the “seed” portion of the seed-and-extend algorithm. Once the algorithm finds the position of the exact match, an attempt is made to extend the alignment before and after the position of the match. During the extension stage, the algorithm uses a specific scoring system that gives positive scores to exact base matches and negative scores to inexact matches. Once an alignment falls below a specific scoring threshold, the alignment is returned to the user and either the next position of an exact match is used for an alignment if available or the next k-mer from the query sequence is used.



K-mer	Position
KN	0, 5
NI	1
IC	2
CK	3, 8
KK	4
NA	6
AC	7

Figure 1: Building of a Hash Table: This figure shows the process of creating a hash table from the word ‘knickknack’. Part A shows the process of identifying each individual k-mer. Part B shows the calculation used in Java to generate unique hash codes for the k-mer where n represents the length of the k-mer. Part C shows the completed hash table with k-mers as keys and positions as values.



K-mer	Position
KN	0, 5
NI	1
IC	2
CK	3, 8
KK	4
NA	6
AC	7

Figure 2: Lookup using a Hash Table: This figure shows the process of looking up a k-mer in a hash table. The query sequence ‘knock’ is divided into k-mers, which are then used to calculate the unique hash code. The hash code is used to index the hash table, which returns the positions 0 and 5 where the k-mer is an exact match to the reference sequence.

Overall, this algorithm works well for pairwise alignments and was one of the initial algorithms used for genome alignment. By finding small exact matches first, the seed-and-extend algorithm works well to not allow a single base mismatch to hinder an alignment for the overall segment. The use of a hash table for storing the reference sequence data works well for smaller sequences but is most often a slow process when dealing with full genomes. The chosen length of the k-mer causes a tradeoff of speed versus accuracy. If a small value for k is used, there is a greater number of alignments that will be returned due to the large number of exact matches that will be found between the query and reference sequence. For example, it is statistically more likely that a 2-mer, such as AT, will appear more often in a random reference sequence than a 3-

mer, like ATG. However, the smaller k value causes a large amount of k-mer overlap which results in many stored positions in the hash table. This slows down the algorithm as each position requires an alignment to be created in the extension stage. In contrast, a large value for k will not have as many positions stored and ultimately leads to a faster alignment that may not be as accurate due to their needing to be an exact match between longer sequences in both the reference and query.

Suffix Trees

To cut down on time taken for a genome alignment, an alignment tool called MUMmer was released in 1999, which, among other updates, introduced the use of a suffix tree to replace the hash table used in previous tools (Delcher *et al.*, 1999). Suffix trees originated in the field of computer science to search for substrings more efficiently within a large text file. MUMmer's algorithm builds a suffix tree from the reference genome sequence. Before creating a suffix tree, all possible suffixes of the reference sequence are generated with each being linked to an index representing the first character of the suffix's position in the overall sequence as shown in Figure 3-B. A tree is initially created by creating a root node with no children. The first suffix is then added to the tree character by character as child nodes to the root. The final node of the branch stores the position of the suffix in the overall sequence. The next suffix is added to the tree by traversing from the root down to nodes of the same current character. If the character has not been added as a child to a node, a new node for that character is added to the current node. An example of a completed suffix tree is shown in Figure 3-C.

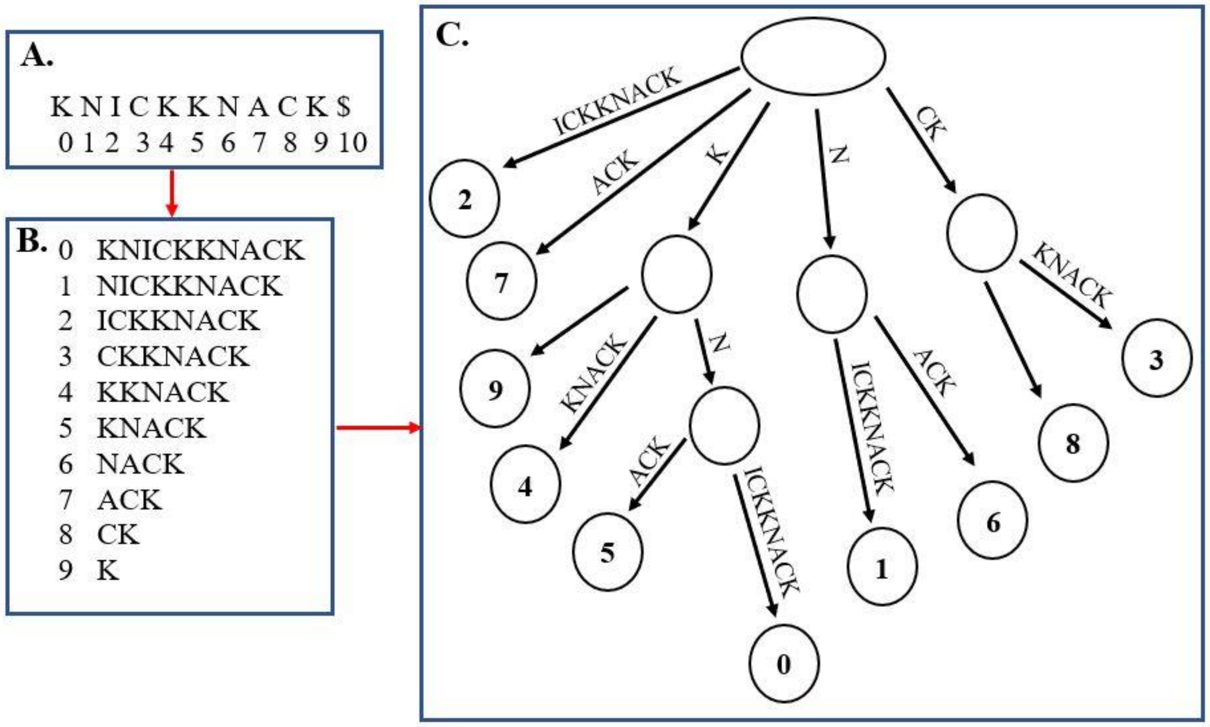


Figure 3: Creating a Suffix Tree: This figure shows the process of creating a suffix tree from the word 'knickknack'. The overall word with its associated indexes is shown in part A. Part B shows all suffixes of the word with their associated indexes. Part C shows the completed suffix tree.

Although building the suffix tree takes time for larger sequences, finding an exact match to a k-mer of the sequenced reads is fast (linear-time) (Homer and Li, 2010). Lookup in a suffix tree begins with finding k-mers of the query sequence and searching for exact matches in the reference sequence by traversing down through the tree starting at the root as shown in Figure 4. The traversed path is chosen by matching characters in the query k-mer to characters in a child node. If a node with a position is met, then only one exact match exists at the indicated position and extension of the alignment carries out as described previously. If traversal ends on an internal node (such as shown in Figure 4), an alignment is automatically generated for each child node off the current node. In this way, more than one alignment is created at the same time, unlike the hash table method which requires each alignment to be processed separately. Extension of the alignment is also made simpler by only having to traverse further down the tree to align the next bases.

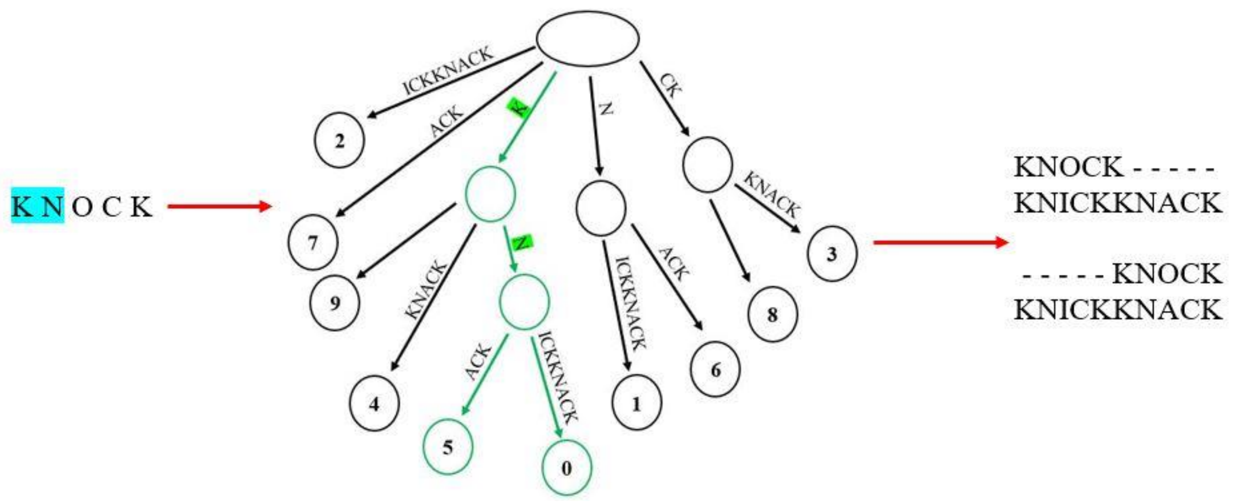
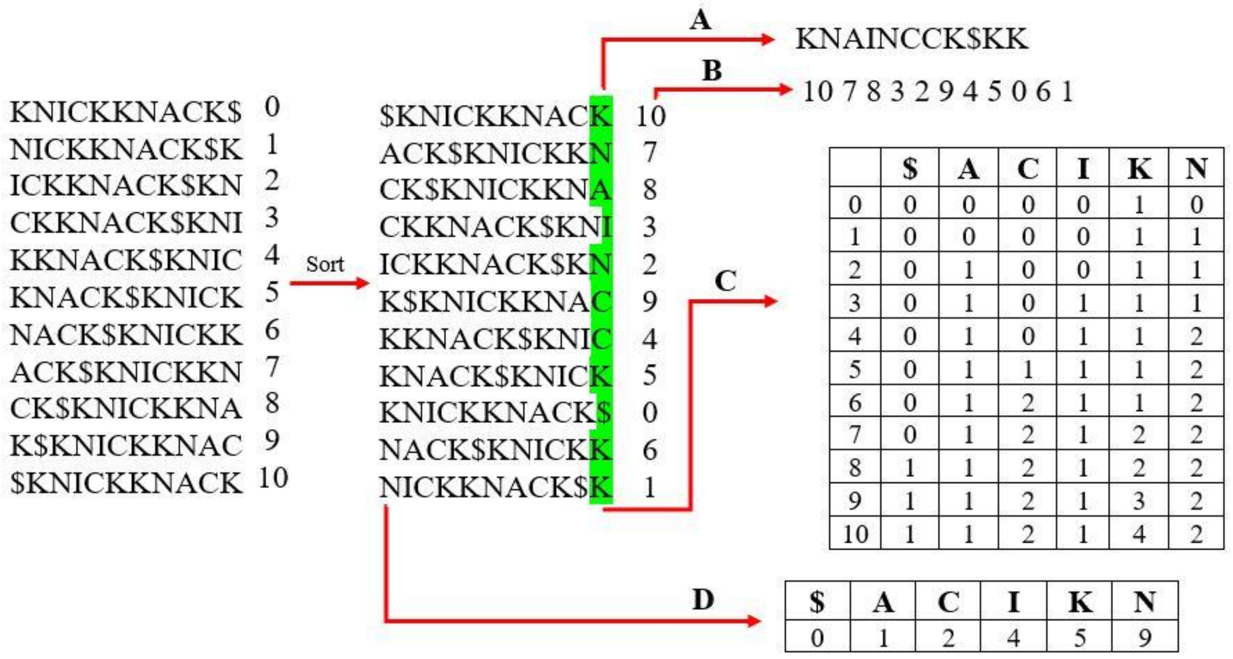


Figure 4: Lookup using a Suffix Tree: This figure shows how lookup works using a suffix tree of the word ‘knickknack’. The query word ‘knock’ is divided into 2-mers which are then used to traverse through the tree starting at the root. As a result of the lookup, there are two exact matches in the reference so there are two alignments created.

A tradeoff to the increased speed of the suffix tree algorithm is its massive memory requirement. A single character requires 1 byte of memory. This means that if an algorithm was to store all 3 billion bases of the human genome in memory, then 3 billion bytes, or 3 gigabytes, of memory space would be required. A suffix tree not only requires memory to store suffixes of the whole reference genome, but also all data involving the structure of the tree. This includes memory used for each individual node of the tree as well as all paths between nodes. This amounts to requiring approximately 15 bytes per base, which would amount to 45 gigabytes of memory to store a suffix tree for the human genome (Kurtz *et al.*, 2004). This massive amount of required memory makes computation impractical for large genomes and led researchers to look elsewhere for more efficient algorithms.

FM-Index with BWT

The need to align larger genomes led to researchers adding a Full-text index in Minute space (FM-Index) into a genome aligner algorithm (Ferragina and Manzini, 2000). An FM-Index was regarded as the next evolution of a suffix tree for its low memory space and ability to be compressed even further. To create an FM-Index, an end of file character, typically $, is appended to the reference string. This character is used later to identify where in the string the end occurs. All rotations of the reference string are then generated in a matrix as shown in the first step of Figure 5. A rotation of a string is simply the removal of the first character in the string and appending it onto the end. A suffix array is also created for the matrix which stores the index of the starting position of each suffix (all characters to the left of $) in the original string. For example, the initial suffix starts at position 0, the first rotation starts at position 1, second rotation at position 2, etc. The matrix is then sorted lexicographically by the first column, which causes the suffix array to sort accordingly as well. The last column of the sorted matrix is referred to as the Burrows-Wheeler transform (BWT) (shown as Figure 5-A) and is stored alongside the sorted suffix array (shown as Figure 5-B). Using the BWT, a rank table is created which contains a column for each unique symbol in the reference string and a row for each character in the BWT. The table is filled starting at the 0th row and counts how many times and in what order each symbol appears in the BWT. A completed example of a rank table is shown in Figure 5-C. Based off the first column in the matrix, a lookup table is created that stores the index/row of the first occurrence of each unique character. An example of this is shown in Figure 5-D, which shows the '$' being in the 0th row of the sorted matrix, the first 'A' in row 1, the first 'C' in row 2, etc. Only the BWT, suffix array, rank table, and lookup table are stored in memory and these four objects make up the completed FM-Index.

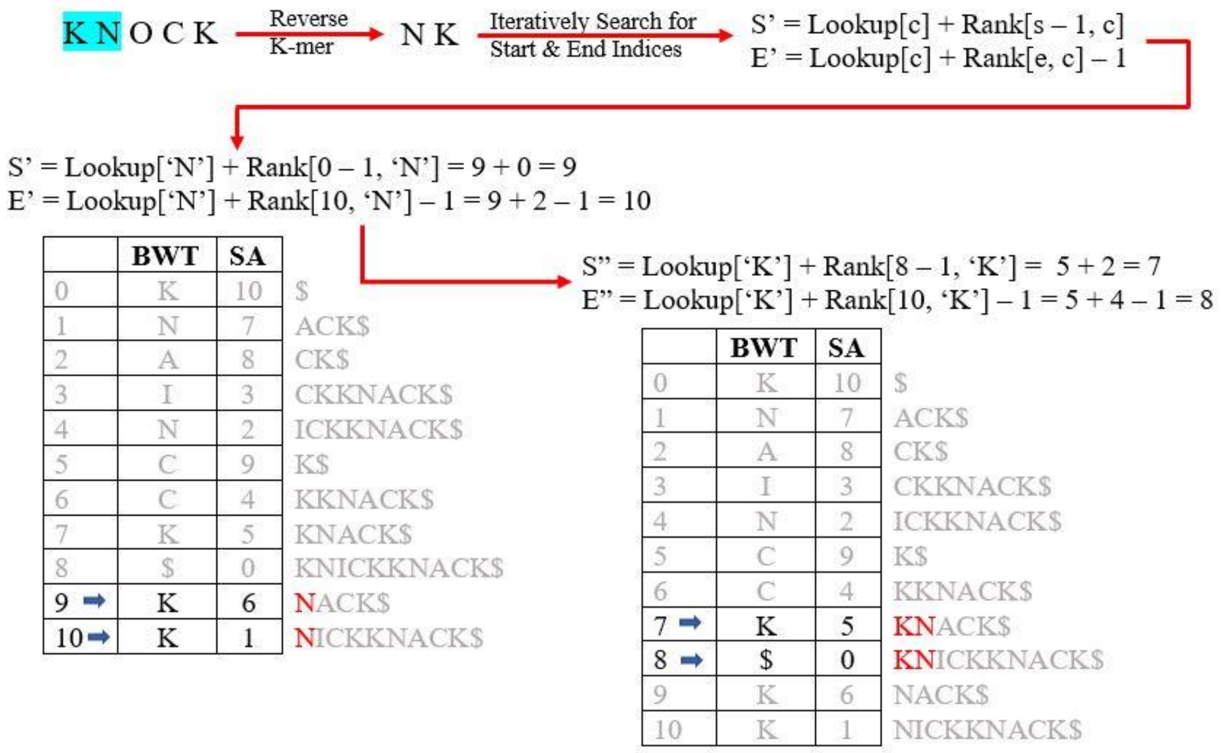


	$	A	C	I	K	N
0	0	0	0	0	1	0
1	0	0	0	0	1	1
2	0	1	0	0	1	1
3	0	1	0	1	1	1
4	0	1	0	1	1	2
5	0	1	1	1	1	2
6	0	1	2	1	1	2
7	0	1	2	1	2	2
8	1	1	2	1	2	2
9	1	1	2	1	3	2
10	1	1	2	1	4	2

$	A	C	I	K	N
0	1	2	4	5	9

Figure 5: Creating an FM-Index: This figure shows the process of creating an FM-Index of the word 'knickknack'. The first step is to generate all rotations of the reference sequence and sort them lexicographically. The last column is stored as the BWT in part A and the corresponding suffix array in part B. A rank table is created from the BWT which lists the occurrence and order of each unique character shown in part C. Part D shows the lookup table, which lists the index of the first occurrence of each character from the first column of the sorted matrix.

Lookup in an FM-Index begins by first dividing the query sequence into k-mers. An FM-Index uses a backwards search technique, which requires the k-mer itself to be reversed before moving onto the next step. The characters in the k-mer are then used to iteratively search for a start and end index in the BWT and suffix array, which represents the range of suffixes that begin with an exact match to the k-mer. The initial start index begins at 0 and the initial end index begins at the length of the BWT minus 1. The start index is calculated by adding the value from the lookup table for the current character to the value from the rank table of the current character at the index of the current start minus 1. In the first calculation, the current start minus 1 would result in a value of -1, so the index remains at 0. The end index is similarly calculated by adding the value from the lookup table for the current character to the value from the rank table of the current character at index of the current end then subtracting 1 from the sum. The algorithm proceeds base-by-base in the sequence until the end of the k-mer is reached and the resulting indices are used in the suffix array to return the positions of the exact matches in the reference sequence. This process of lookup using an FM-Index is shown in detail in Figure 6. Extension of the alignment is carried out as described before.



S' = Lookup[c] + Rank[s – 1, c]
E' = Lookup[c] + Rank[e, c] – 1

S' = Lookup['N'] + Rank[0 – 1, 'N'] = 9 + 0 = 9
E' = Lookup['N'] + Rank[10, 'N'] – 1 = 9 + 2 – 1 = 10

	BWT	SA	
0	K	10	$
1	N	7	ACK$
2	A	8	CK$
3	I	3	CKKNACK$
4	N	2	ICKKNACK$
5	C	9	K$
6	C	4	KKNACK$
7	K	5	KNACK$
8	$	0	KNICKKNACK$
9 ➡	K	6	NACK$
10 ➡	K	1	NICKKNACK$

S" = Lookup['K'] + Rank[8 – 1, 'K'] = 5 + 2 = 7
E" = Lookup['K'] + Rank[10, 'K'] – 1 = 5 + 4 – 1 = 8

	BWT	SA	
0	K	10	$
1	N	7	ACK$
2	A	8	CK$
3	I	3	CKKNACK$
4	N	2	ICKKNACK$
5	C	9	K$
6	C	4	KKNACK$
7 ➡	K	5	KNACK$
8 ➡	$	0	KNICKKNACK$
9	K	6	NACK$
10	K	1	NICKKNACK$

Figure 6: Lookup using an FM-Index: This figure shows the process of finding exact matches using an FM-Index. The query sequence is divided into k-mers which are then reversed. The start and end indices of the BWT and suffix array are then searched for iteratively using the calculations shown above. 'c' represents the current character and 's' and 'e' represent the current start and end indices respectively.

The benefit of using an FM-Index is its ability to massively reduce the memory space used for an alignment. The BWT itself only requires the standard 1 byte per character, which means the entire human genome can be stored in only 3 gigabytes, a significant difference to the suffix tree's size of 45 gigabytes (Li and Homer, 2010). To further reduce memory, the BWT itself has the ability to undergo compression. Due to the lexicographical sorting, the BWT often results in long stretches of like characters (as shown in Figure 5-A with the groupings of 'C' and 'K'). These long stretches can be compressed into a single occurrence of the character because the length of the run can be inferred from a combination of the lookup and rank tables. In most cases, memory is further reduced by not actually creating a suffix array to store alongside the BWT. When the appropriate start and end index are found, the BWT, in column form, is reversed back into the original reference sequence using repeated steps of prepending the BWT and sorting the matrix lexicographically. In some algorithms using an FM-Index, the rank table only stores data for every other row with missing data being inferred from the surrounding data. The low memory space for using an FM-Index allows for the alignment of large genomes to be computationally practical.

Modern Aligners

The aligners being used in this project are Bowtie2, Burrows-Wheeler Aligner (BWA), Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT2), MUMmer4, Spliced Transcripts Alignment to a Reference (STAR), and TopHat2.

Bowtie2

The first iteration of Bowtie was released in 2008 and performed ungapped alignments of short reads (approximately 35 base pairs) (Langmead *et al.*, 2009). The assumption behind not allowing gaps in the alignments was that shorter reads should have a unique place in the genome and not accounting for gaps allows the aligner to run much faster. With longer reads, this assumption results in low alignment rates and does not allow for the alignment of RNA sequences to a genome (as any introns in the gene will automatically add gaps to the RNA alignment). In addition to ungapped alignments, Bowtie only performed alignments in end-to-end mode. This method of alignment requires that an entire read be aligned from one end to the other in order for the alignment to be reported to the user. This often leads to lower alignment scores and forced alignments if the ends of reads are not trimmed properly for quality control. In

2011, Bowtie2 was released which built upon the previous ungapped alignments of Bowtie by accounting for the addition of gaps (Langmead and Salzberg, 2012). Bowtie2 uses an FM-Index to index the genome and seeds a query sequence to find multiple ungapped alignments which are then extended, which is similar to Bowtie. An extra step for Bowtie2 allows for multiple “seeds” to be combined together based on their proximity to each other, which accounts for adding gaps to create larger alignments. Bowtie2 also is able to run in both end-to-end and local mode for generating alignments. In local mode, the aligner is able to trim bases from either end of the read to increase alignment scores and not be hindered by low-quality bases on the end of reads leftover from poor quality control. Regardless of the mode being run, Bowtie2 generates multiple alignments for each read but only reports the single best alignment per read. A downside to Bowtie2 is that the aligner was designed with the intent of aligning DNA reads to a reference genome and does not allow for the addition of a transcript annotation file to aid in the mapping of RNA reads.

BWA

The BWA tool initially released in 2008 as an aligner for mapping short reads under 100 base pairs long. To index the genome, BWA uses a combination of the Burrows-Wheeler transform and an FM-Index to achieve a linear lookup time for finding exact matches to a given k-mer (a sequence of length n is found in O(n) time) (Li and Durbin, 2009). This method of indexing the genome has remained virtually unchanged through the years, however, the alignment algorithm itself has been updated to accommodate longer reads. The BWA-MEM algorithm, released in 2012, is designed to be used for reads of all sizes (Li, 2013). The aligner works by first seeding a query sequence by searching for exact matches in the reference genome using a k-mer size of 19 by default. BWA-MEM then extends a seed until a given cutoff value is reached, which is calculated by a function which does not penalize large gaps in the resulting alignment. Unlike Bowtie2 (which allows the user to choose one mode or the other), BWA-MEM's extension algorithm stores the best alignment score for both an end-to-end (if the end of the query is reached during extension) and local alignment and reports the better alignment (with a bias toward choosing end-to-end alignments for longer alignments). By storing multiple alignments, BWA-MEM also has the ability to report multiple alignments per read in the form of secondary and supplementary alignments (on by default). Like Bowtie2, a downside to the BWA-MEM algorithm is that it was originally created to align DNA reads to a reference genome and therefore does not have the ability to import a transcript file to identify splice junctions in RNA.

HISAT2

HISAT was released in 2014 with the intent to align RNA-seq reads and was created by the same developers of both TopHat and Bowtie (Kim, Langmead, and Salzberg, 2015). HISAT indexes the genome using a method similar to Bowtie's FM-Index. A major difference between these aligners is that HISAT uses one large FM-Index, which covers the entire genome, and many smaller FM-Indexes, where each smaller index accounts for 64,000 base pair portions of the genome with about 1,000 base pairs of overlap between consecutive areas. Not long after the release of HISAT, HISAT2 was created in 2015 which implemented a graph-based FM-Index (GFM) to index the reference genome, the first time an algorithm of this kind was ever developed (Kim *et al.*, 2019). The GFM-Index used by HISAT2 works similarly to HISAT's one larger and multiple smaller indexes, however, using a graph-based approach allows the aligner to take in SRA data to add variants into the index which could occur in the form of insertions, deletions, or mutations. The GFM-Index allows for multiple paths to be created through the genome index with one path being the original reference sequence and other paths in the index representing a different variant. This type of approach works well for most model organisms where variant data is known. If SRA data is not added as input, HISAT2 defaults to creating the FM-Index created by HISAT. HISAT2 also deals with repetitive sequences differently than other aligners by combining repeat sequences in the reference genome into one sequence. This reduces the number of alignments reported by only reporting one alignment for a read aligning to these regions rather than one per repetitive element. As HISAT2 is designed to align both genomic and RNA reads, the ability to input a file of known splice sites allows for better mapping of exons to places in the genome.

MUMmer4

One of the oldest aligners, MUMmer, was released in 1999 with the goal of aligning whole genomes to one another (Delcher *et al.*, 1999). MUMmer's initial form of genome indexing was the use of a suffix tree alongside the assumption that the genomes are from closely related organisms. Using this assumption, MUMmer then looks for maximal unique matches (MUMs) between the genomes, which is the largest possible subsequence of 100% identity that occurs once in each genome. The alignment is then built from the MUMs that are found. As suffix trees require large amounts of memory in order to be built, MUMmer was redefined over the years to reduce memory space and increase speed with the release of MUMmer2 and MUMmer3 (Delcher *et al.*, 2002; Kurtz *et al.*, 2004). MUMmer3, in particular, added the ability to align non-unique MUMs which are large subsequences that can occur multiple times in the reference but still only once in the query. To further reduce memory space, the release of MUMmer4 replaced the suffix tree index with a suffix array, which allows for the processing of genomes up to 141 trillion base pairs (larger than any known genome by 1000x) (Marçais *et al.*, 2018). To increase runtime, MUMmer4 allowed for the use of multiple cores to process alignments across more than one computing core and the ability to pre-build the reference genome index to then be loaded in later for alignments to the same reference.

STAR

Built primarily to handle mapping of RNA-seq data, STAR was released in 2012 with the intent on tackling the issue of mapping spliced RNA transcripts (Dobin *et al.*, 2013). STAR indexes the reference genome using an uncompressed version of a suffix array, which often takes up a large amount of memory (due to it being uncompressed) and time in order to build. This type of data structure (as is used by MUMmer4 as well) has a fast searching time and can handle nearly any size genome. To cut down on the runtime for successive alignments, STAR allows for the genome to be pre-built once and the resulting indexes to be loaded in for each individual alignment, which drastically decreases the runtime. To identify matches between reference and query, STAR uses a similar approach to MUMmer's maximal unique match approach, but adapts this methodology to spliced RNA. As most RNA transcripts will be made up of multiple exons, one alignment is often not enough for a given transcript as the first half the read may map to location A and the second half to location B with a large intron inbetween the two. To accommodate this, STAR identifies a Maximal Mappable Prefix (MMP), which is simply the longest exact match to the genome starting from the first base of a query read. This results in the first half of the read given a seeded location in the reference genome which is then extended to generate the alignment. This step is then repeated for the next portion of the query sequence that did not belong to the first alignment. This allows for a single RNA transcript to be aligned to multiple locations in the genome and accounting for splice junctions within the read. Further adding to its ability to correctly align spliced transcripts, STAR provides the option of inputting an annotation file of known splice junctions to aid in the process of identifying MMPs.

TopHat2

Building upon the success of Bowtie, TopHat was created in 2009 as a tool to refine Bowtie's alignment of DNA to aligning RNA across splice junctions (Trapnell, Pachter, and Salzberg, 2009). The first iteration of TopHat worked by performing an initial alignment of all reads with Bowtie (using end-to-end mode). Those reads that mapped well from end-to-end were passed through as alignments without splice junctions. An alignment with a splice junction would report relatively low scores due to the large gaps and/or mismatches identified using end-to-end mode. Those reads which were not mapped are then sent through a second round of alignment using a simple seed-and-extend algorithm to identify potential splice junctions that would not have been reported during an end-to-end alignment. The release of TopHat2 in 2012 would incorporate the use of Bowtie2 into the first stage of alignment (Kim *et al.*, 2013). In addition, TopHat2 allowed for the input of a file containing known splice junctions to aid in alignment as well as the ability to directly align reads to an inputted transcriptome rather than an entire reference genome. As TopHat2 uses Bowtie2 under the hood, all of Bowtie2's default parameters are used including the indexing of the genome using an FM-Index. Recently, TopHat2 has been overshadowed by the release of HISAT2, which were created by the same developers.

Project Goal

As genome aligners have evolved over time, the underlying algorithms that are built-in have evolved with them. Although changes are made to fix certain facets of the aligner, the updated algorithms often must sacrifice speed for accuracy or *vice versa*. The goal of this project is to determine which aligner achieves the best balance between runtime and accuracy and ultimately determine the best aligner. This will be done by running each aligner in a controlled enviroment using default settings and the same data sets.

Materials & Methods

Sequence Generation

The query samples used for this project were 48 samples of *Erysiphe necator*, more commonly known as the fungal disease powdery mildew, and were obtained from the lab of Dr. Lance Cadle-Davidson of the United States Department of Agriculture. Samples were grown on the leaves of *Vitis vinifera* in diverse geographical regions (see Supplementary S4). RNA was isolated from the fungal samples by using clear nail polish on the infected leaves to separate the fungal tissue from the leaves followed by RNA extraction (Cadle-Davidson *et al.*, 2009). Samples were sequenced in one single-end run of an Illumina GA HiSeq with 5 base-pair barcodes provided for each isolate. The pooled library of reads was run through the barcode splitter of the FASTX-toolkit (v0.0.13) (Hannon, 2010) to create a separate file of reads for each of the 48 isolates. The reference genome scaffold for *Erysiphe necator* (C-strain) used in this project was obtained from the Cantu Lab at UC Davis (https://cantulab.github.io/e-necator.scaffolds.c.NCBI.fasta.gz; Jones *et al*, 2014).

Quality Control

Raw reads were checked for their initial quality using FastQC (v0.11.7) (Andrews, 2018). Initial quality of any sequencing reads should always be checked to determine if there is a need for any base trimming, sequencing adapters to remove, etc. The following command was used to check quality of the reads using FastQC:

Quality Control for split_A01.fq:

```
1) /path_to_FastQC/fastqc \
2)   initial_seqs/split_A01.fq \
3)   --outdir=qc_initial/
```

Line 1 calls the “fastqc” function from the appropriate directory, followed by the inputted sample FASTQ file on line 2 and an appropriate location for outputting the quality reports (qc_initial/) set equal to the argument ‘outdir’ on line 3. The sample file for input (line 2) was modified to run FastQC on each of the sample files.

The first step for cleaning up the reads was to remove the first 6 bases from the beginning of each read: the 5 base-pair barcodes plus the 6th base as FastQC indicated a high N content for all samples at this position. Leaving the barcode attached to the reads would interfere with downstream tools ability to align the beginning of reads to the reference genome as Illumina barcodes are not naturally occurring and should not be aligned. A high N content at position 6 indicated that the sequencer had trouble identifying a specific base for that position and so the base was removed to avoid any downstream complications. The “fastx_trimmer” function from FASTX-toolkit was used to perform this task with the following command:

Remove Barcodes from 5’ End of split_A01.fq:

```
1) /path_to_FASTX-toolkit/fastx_trimmer \
2)   –i initial_seqs/split_A01.fq \
3)   -o minus_barcodes/split_A01.fq \
4)   -Q33 /
5)   -f 7
```

Line 1 calls the “fastx_trimmer” function from the appropriate directory, followed by the inputted FASTQ file for argument ‘i’ on line 2 and the location for the newly-trimmed reads for argument ‘o’ on line 3. Line 4 requires the program to use Phred+33 encoding: the scoring system for Illumina 1.8+ (by default uses Phred+64 for previous versions of Illumina sequencing). Line 5 passes a value of 7 to the argument ‘f’ which indicates the position of the first base in each read to be kept (removes bases 1 through 6 for the barcode plus base with high N content). Lines 2 and 3 were modified accordingly for each of the 47 other samples.

The next step for cleaning the reads was to trim low-quality bases from the 3’-ends and filtering out reads too small for alignment. Scores below 20 were chosen as “low-quality” as a quality score of 20 indicates a 99% chance that the base listed at the corresponding position is a correct base-call. Reads less than 100 bases in length were chosen as “too small” as previous studies have shown that longer reads are more beneficial for RNA-seq results due to the added benefit of finding unique splice junctions during alignment (Chhangawala *et al.*, 2015). To do this, the “fastq_quality_trimmer” function from FASTX-toolkit was used with the following command:

Remove Low-Quality Bases and Filter Short Reads from split_A01.fq:

```
1) /path_to_FASTX-toolkit/fastq_quality_trimmer \
2)   –i minus_barcodes/split_A01.fq \
3)   -o trimmed_seqs/split_A01.fq \
4)   -Q33 \
5)   -t 20 \
6)   -l 100
```

Line 1 calls the “fastq_quality_trimmer” function from the appropriate directory, followed by the inputted FASTQ file (output from “fastx_trimmer”) for argument ‘i’ on line 2 and the location for the outputted trimmed reads for argument ‘o’ on line 3. Line 4 requires the program to use Phred+33 encoding as previously described for “fastx_trimmer”. Line 5 shows the cutoff value for low quality bases (bases with scores under 20 removed) to be trimmed from the 3’ end of each read and line 6 corresponds to the minimum length for each read (reads under 100 bases after trimming are removed). Lines 2 and 3 were modified accordingly for each of the 47 other samples.

To check the quality post-filtering, the trimmed sample files were used as input into FastQC once more. The output of FastQC indicated sufficient filtering was performed and all samples were ready for use in the subsequent methods. The number of reads pre- and post-trimming were recorded to calculate the percentage of reads leftover from filtering.

Alignment Generation

For all aligners, the first step was to index the reference genome. This step is not required for a run of the aligner, however, building the genome index prior to alignment allows the aligner to call the index for each run. This means that the genome index will not have to be created for individual run. This helps to reduce the runtime of individual runs of the aligner and removes any variability regarding different indexing techniques impacting the timing statistics for each sample. Creating the reference genome index is the only pre-processing step performed to run each of the aligners. After creating the genome index, the aligners can be run on each sample individually by reading in the sample file and the pre-built genome index. All aligners were run using the default parameters with some of these values outlined in Table 1.

Table 1: Default Settings for Aligners: The following table shows some of the default settings used by each of the aligners. The "K-mer Length" column shows the size of k-mer used to search for exact matches between query and genome. The "Mismatch", "Gap Open", and "Gap Extend" columns show the penalties applied to the alignment score during extension. The "Alignment Mode" column lists whether the aligner performs end-to-end or local alignments. The "Annotation File" column lists whether or not an annotated transcript file can be supplied to aid in mapping RNA-seq reads (not default but recommended setting). The "Genome Index Method" column lists the method in which the reference genome is indexed and stored. The "Max. Mismatches" column lists the number of mismatches allowed in a reported alignment. A value of 'S' in this column indicates that the reported alignments are based on the overall alignment score, which is calculated by summing together scoring penalties and matches, and is not directly dependent on the number of mismatches.

Tool	K-mer Length	Alignment Score Penalty			Alignment Mode	Annotation File (Yes/No)	Genome Index Method	Max. Mismatches
		Mismatch	Gap Open	Gap Extend				
Bowtie2 E2E	22	-6	-5	-3	E2E	No	FM-Index	S
Bowtie2 Local	20	-6	-5	-3	Local	No	FM-Index	S
BWA	19	-4	-6	-1	Both	No	FM-Index	S
HISAT2	N/A	-6	-5	-3	Local	Yes	FM-Index	S
MUMmer4	20[a]	N/A	N/A	N/A	Local	No	Suffix Array	S
STAR	MMP	N/A	-2	-2	Local	Yes	Suffix Array	10
TopHat2	22	-6	-5	-3	Both[b]	Yes	FM-Index	2

a. Minimum length of a Maximal Unique Match (MUM)
b. Runs End-to-End first then Local on those that were not aligned

Bowtie2

To create the index from the reference genome for Bowtie2 (v2.3.5.1), the following command was used to run the function "bowtie2-build":

Creating Bowtie2 Genome Index:

```
1) /path_to_Bowtie2/bowtie2-build \
2)   e_necator.fasta \
3)   e_necator
```

Line 1 calls the Bowtie2 function "bowtie2-build" from the appropriate directory. The function requires the reference genome FASTA file on line 2 and a prefix to be used for naming of the outputted bt2 files on line 3. For example, each of the outputted bt2 files will begin with "e_necator" such as "e_necator.1.bt2", "e_necator.2.bt2", etc. This command only needs to be run once as all samples use the same reference genome.

Bowtie2 has two different modes for alignment: End-to-End and Local. When run in End-to-End (default) mode, all characters from a given read are required to be aligned in the reference genome. This requires reads to be fairly cleaned of any adapter sequences or troublesome bases near the 3'-end as these bases will negatively impact the alignment of the read. In Local mode, bases from a given read can be trimmed from either the 5'- or 3'-end if the removal of these bases results in an increase in alignment scores for the read.

To run Bowtie2 in End-to-End mode, the following command was run:

Run Bowtie2 on split_A01.fq in End-to-End Mode:

```
1) /path_to_Bowtie2/bowtie2 \
2)   -x e_necator \
3)   trimmed_seqs/split_A01.fq \
4)   -S /path_to_output/A01.sam \
5)   -p 5 \
6)   --end-to-end
```

Line 1 calls the Bowtie2 function "bowtie2" which runs the Bowtie2 alignment algorithm. To read in the indexed reference genome, the prefix used for the outputted bt2 files of the "bowtie2-build" function ("e_necator") is used as input for argument 'o' as shown on line 2. Line 3 shows the path for the outputted file ("A01.sam") used for argument 'S' which indicates that the outputted alignment file should be in SAM format. To the run the alignment with 5 cores in parallel, 5 was used as input for the 'p' argument which indicates the number of cores to be used for parallel computing. Line 6 indicates that the aligner should be run in End-to-End mode.

Lines 3 and 4 were modified accordingly to be run for each of the other 47 sample files. Line 5 was also modified to run each of the sample files using 1, 2, 3, and 5 cores.

To run Bowtie2 in Local mode, the following command was used:

Run Bowtie2 on split_A01.fq in Local Mode:

```
1) /path_to_Bowtie2/bowtie2 \
2)   -x e_necator \
3)   trimmed_seqs/split_A01.fq \
4)   -S /path_to_output/A01.sam \
5)   -p 5 \
6)   --local
```

All arguments are the same as Bowtie2 being run in End-to-End mode with the only exception being line 6, which changes the algorithm to be run in Local mode. Lines 3 and 4 were changed accordingly to run the aligner for each of the other 47 files and line 5 was modified to run each of the files using 1, 2, 3, and 5 cores.

BWA

To pre-build the genome index for BWA (v0.7.17-r1188), the following command was used:

Creating BWA Genome Index:

```
1) /path_to_BWA/bwa index \
2)   e_necator.fasta \
3)   -p e_necator
```

Line 1 calls the “bwa index” function from the appropriate directory. This function indexes the inputted reference genome on line 2 and outputs the index into files containing the prefix passed in as input for argument ‘p’.

To run the BWA aligner, the following command was used:

Run BWA on split_A01.fq:

```
1) /path_to_BWA/bwa mem \
2)   e_necator \
3)   trimmed_seqs/split_A01.fq \
4)   -t 5 \
5)   > /path_to_output/A01.sam
```

Line 1 calls the function “bwa mem” which runs the alignment algorithm for BWA. The function requires the prefix of the index files on line 2 and the input file to be aligned on line 3. To run the aligner in parallel, the number of cores to be used is passed in as input for the argument ‘t’ shown on line 4. To direct the aligner’s output to a file, the ‘>’ character is used followed by the path to the appropriate output file as shown on line 5. Lines 3 and 5 were changed accordingly to run the aligner on the other 47 samples. Line 4 was modified accordingly to align each sample using 1, 2, 3, and 5 cores.

HISAT2

To pre-build the genome index for HISAT2 (v2.1.0), the following command was used:

Creating HISAT2 Genome Index:

```
1) /path_to_HISAT2/hisat2-build \
2)   e_necator.fasta \
3)   e_necator
```

Line 1 calls the “hisat2-build” function from the appropriate directory. This function indexes the genome given on line 2 and outputs the index into files beginning with the prefix listed in line 3.

To run the HISAT2 aligner, the following command was run:

Run HISAT2 on split_A01.fq:

```
1) /path_to_HISAT2/hisat2 \
2)   -x e_necator \
3)   trimmed_seqs/split_A01.fq \
4)   -S /path_to_output/A01.sam \
5)   -p 5
```

Line 1 calls the “hisat2” function from the appropriate directory. The aligner requires the prefix used for each of the genome index files to be inputted for the ‘x’ argument as shown on line 2 and the file to be aligned as shown in line 3. To direct output to a SAM file, the output file to be created (“A01.sam”) is used as input for the ‘S’ argument as shown on line 4. To run the aligner using multiple cores for parallel computing, the required number of cores is used as input for the ‘p’ argument. Lines 3 and 4 were modified accordingly to run the aligner on each of the other 47 aligners. Line 5 was modified to run the aligner using 1, 2, 3, and 5 cores for each file.

MUMmer4

To pre-build the genome index for MUMmer4 (v4.0.0beta2), the following command was used:

Creating MUMmer4 Genome Index:

```
1) /path_to_MUMmer4/nucmer \
2)   --save=e_necator \
3)   e_necator.fasta
```

Line 1 calls the “nucmer” function from the appropriate directory. If an output file prefix is passed in as input to the ‘save’ argument as shown in line 2, the “nucmer” function will automatically run with the purpose of pre-building the reference genome that is entered in on line 3.

To run the MUMmer4 aligner, the following command was used:

Run MUMmer4 on split_A01.fq:

```
1) /path_to_MUMmer4/nucmer \
2)   --load=e_necator \
3)   --sam-long=/path_to_output/A01.sam \
4)   --threads=5 \
5)   e_necator.fasta \
6)   trimmed_seqs/split_A01.fq
```

Line 1 calls the “nucmer” function from the appropriate directory. If the prefix of genome index files is passed in as input to the ‘load’ argument as shown in line 2, the “nucmer” function will automatically load in the required genome index files from the current directory. Line 3 shows the required output file name saved to the ‘sam-long’ argument, which indicates that the output file that is created should be in SAM file format. To run the aligner with multiple cores, the required number of cores to be used is entered as input to the ‘threads’ argument as shown in line 4. Line 5 shows the reference genome file being entered and line 6 is the file to be aligned to the reference. Lines 3 and 6 were modified accordingly to run the aligner on each of the 47 other files. Line 4 was modified to run each of the files using 1, 2, 3, and 5 cores.

After running the alignments, it was discovered that MUMmer4 does not create the correct header for its SAM file alignment. This results in the outputted alignment file being unable to be used as input for downstream analysis. Through further analysis of the SAM file headers, each outputted file was missing the '@SQ' header lines. These lines appear one for each contig in the reference genome and list the name of the contig followed by the length of the contig all in the same line. As these lines refer to the reference genome and do not depend on an individual aligner, the '@SQ' are identical between all outputted alignment files regardless of the aligner. For this reason, lines taken from the MUMmer4 SAM files were combined with the '@SQ' header lines from a correct SAM header from another aligner (the alignment of A01 using BWA) using a series of "grep" (v2.27) commands to correctly assemble a SAM file header usable by downstream tools (see Supplementary B12).

STAR

To pre-build the genome index for STAR (v2.5.4b), the following command was used:

Creating STAR Genome Index:

```
1) /path_to_STAR/STAR \
2)   --runMode genomeGenerate \
3)   --genomeFastaFiles e_necator.fasta \
4)   --genomeDir /path_to_output/STAR_Genome
```

Line 1 calls the "STAR" function from the appropriate directory and line 2 sets the 'runMode' argument to 'genomeGenerate'. The 'genomeGenerate' mode pre-builds the reference genome index from the reference genome listed as input to the 'genomeFastaFiles' argument in line 3 and stores the resulting index files in the directory “STAR_Genome”, which is listed as input to the ‘genomeDir’ argument as shown in line 4.

To run the STAR aligner, the following command was used:

Run STAR on split_A01.fq:

```
1) /path_to_STAR/STAR \
2)   --genomeDir /path_to_index/STAR_Genome \
3)   --readFilesIn trimmed_seqs/split_A01.fq \
4)   --outFileNamePrefix /path_to_output/A01_ \
5)   --runThreadN 5
```

Line 1 calls the “STAR” function from the appropriate directory, which by default uses the ‘alignReads’ mode for the ‘runMode’ parameter so there is no need to update this argument. The aligner takes in the path to the directory where the pre-built reference genome was stored as input to the ‘genomeDir’ argument as shown in line 2. The file to be aligned is entered as input to the ‘readFilesIn’ argument as shown in line 3 and the prefix and output path for the outputted alignment files is used as input to the ‘outFileNamePrefix’ argument as shown in line 4. To run the aligner using multiple cores, the required number of cores to be used is entered as input to the ‘runThreadN’ argument as shown in line 5. Lines 3 and 4 were changed accordingly to run the aligner on each of the 47 other files. Line 5 was modified accordingly to run the aligner on each file using 1, 2, 3, and 5 cores.

TopHat2

Since TopHat2 uses Bowtie2, the commands used to pre-build the genome index for Bowtie2 were the same used for TopHat2 (see ***Bowtie2*** section for the exact command). To run the TopHat2 aligner, the following command was used:

Run TopHat2 on split_A01.fq:

```
1) /path_to_TopHat2/tophat \
2)   -o /path_to_output/A01 \
3)   e_necator \
4)   trimmed_seqs/split_A01.fq \
5)   -p 5
```

Line 1 calls the "tophat" function from the appropriate directory. TopHat2 creates a directory with outputted alignment files and the name/path to this directory can be added as input to the 'o' argument as shown in line 2. The prefix used for the pre-built reference genome index files is entered as input in line 3 followed by the file to be aligned in line 4. To run the aligner using multiple cores, the number of cores to be used is added as input to the 'p' argument in as shown in line 5. Lines 2 and 4 were modified accordingly to run the aligner on each of the 47 other files. Line 5 was change accordingly to run each of the files using 1, 2, 3, and 5 cores.

Alignment Assessment

To assess how well an aligner performed, three major variables were tracked: runtime required, percentage of reads that were mapped to the reference genome (called alignment rate), and total coverage of the transcriptome.

Time

To track the runtime of each aligner, the "time" function was used to track the runtime in seconds for each file's alignment. Runtime was recorded for an aligner during both pre-processing and for each individual sample being aligned. Overall, there were four times recorded for each file run: time for the aligner run using 1 core, 2 cores, 3 cores, and 5 cores. Each samples runtime was normalized by the number of reads by dividing the time taken on X number of cores by the number of reads per sample. Speedup for each aligner was calculated by dividing the average time taken in serial (on 1 core) by the average time taken on X cores. An example run through of the calculation of both “Runtime per Read” for each sample and “Speedup” for each aligner is shown in Figure 7.

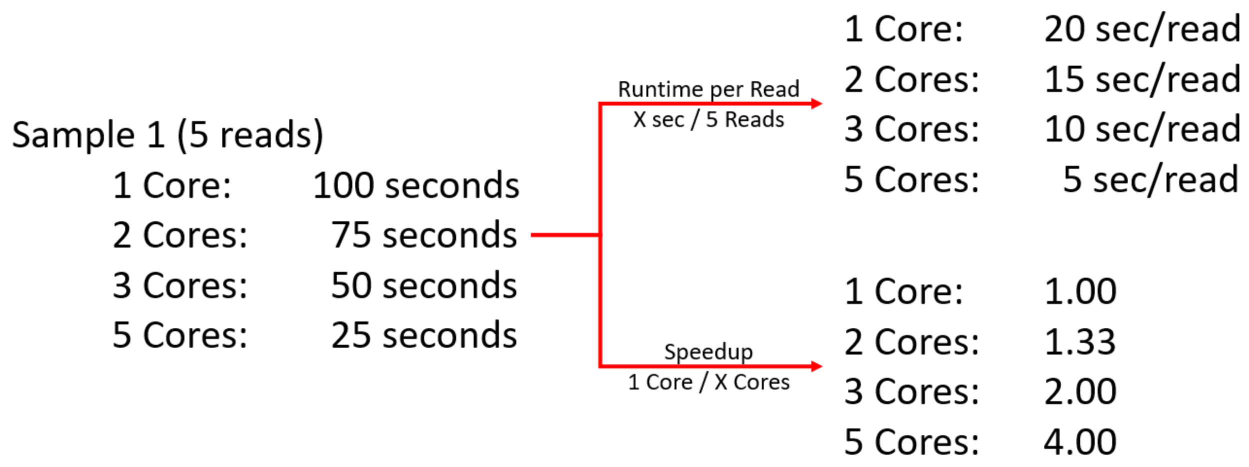


Figure 7: Runtime Calculation Example: This figure shows a quick example of how runtime calculations were handled. The left-hand side shows the raw runtimes of a sample on 1, 2, 3 and 5 cores. The top-right shows the “Runtime per Read” being calculated by dividing the runtime by the total number of reads in the sample. The bottom-right shows the “Speedup” being calculated by dividing the time in serial by the time on X cores.

Alignment Rate

Most of the aligners used output overall alignment rates to the user after each run. Bowtie2 and HISAT2 directly output the alignment rate directly to the user after finishing an alignment. TopHat2 outputs the overall alignment rate in a file called “align_summary.txt” for each sample. STAR outputs the percent of reads aligned to a single location, percent of reads mapped to multiple locations, and percent of unmapped reads among other statistics in a file with the suffix “_Log.final.out”. To get the total alignment rate for STAR, the unmapped read percentage was subtracted from a total of 100%. Both BWA and MUMmer4 do not output any alignment rates for a given alignment so an additional step was necessary to obtain these values. The following commands were used to output the total number of aligned reads using “samtools” (v1.6) given the outputted SAM files from a run of BWA and MUMmer4:

Calculate Alignment Rate for A01.sam from BWA and MUMmer4:

```
1) samtools view \
2)   -F 2308 \
3)   /path_to_SAM/A01.sam
4)   -c
```

Line 1 calls the “samtools” function “view” rate is to convert the SAM file to a BAM file using the “view” function of “samtools” as shown in line 1. Each read in a SAM file has a distinct flag which marks it as one of a variety of alignments. The three relevant types to be excluded for counting were unmapped reads, secondary reads, and supplementary reads (Marshall, Bonfield, and Danecek, 2009). An unmapped read is simply a read that was in the original read file but was unable to be aligned to an area of the reference genome. A secondary read occurs if a single read aligns to two or more areas of the reference genome. In this case, the alignment with the highest score is labelled as the primary read and any additional alignment is labelled as a secondary alignment for the same read. A supplementary alignment is one that arrives due to a potential chimeric read. A chimeric read is one in which one portion of a read aligns to the reference genome and another portion of the same read aligns to a distinctly different area than the rest of the read. One portion of the read is listed as the representative read and all other portions that align to distinctly different areas of the genome are labelled as supplementary reads. As there is only one primary or representative but potentially many secondary or supplementary alignments for a given read, both secondary and supplementary reads (along with unmapped reads) were excluded from the overall count of aligned reads. If these reads were not excluded, reads would be double-counted resulting in much higher read counts than the initial count in the original file. To exclude these files, the specific SAM flag (in hexadecimal form) for each of these types of reads were added together as follows: unmapped reads are 0x4 (corresponds to 4 in decimal), secondary alignments are 0x100 (corresponds to 256 in decimal), and supplementary alignments are 0x800 (corresponds to 2048 in decimal). Adding the resulting decimal values together results in the sum of 2308, which is passed as input for the 'F' argument as shown in line 2, which tells the function to exclude all reads that make up the combination of this summed flag. Line 3 shows the input of the aligned SAM file and line 4 calls the 'c' argument which tells the function to simply return a count of the resulting reads after exclusion. To get the overall alignment rate for a file, the resulting read count from this function call was divided by the total number of reads in the original reads file. An outline of this process is shown in Figure 8. Line 3 was modified accordingly to run this command on all 48 alignment files from both BWA and MUMmer4. This function was tested on SAM files from other aligners (STAR, HISAT2, Bowtie2) which directly output their alignment rates to confirm that the resulting read count was correct.

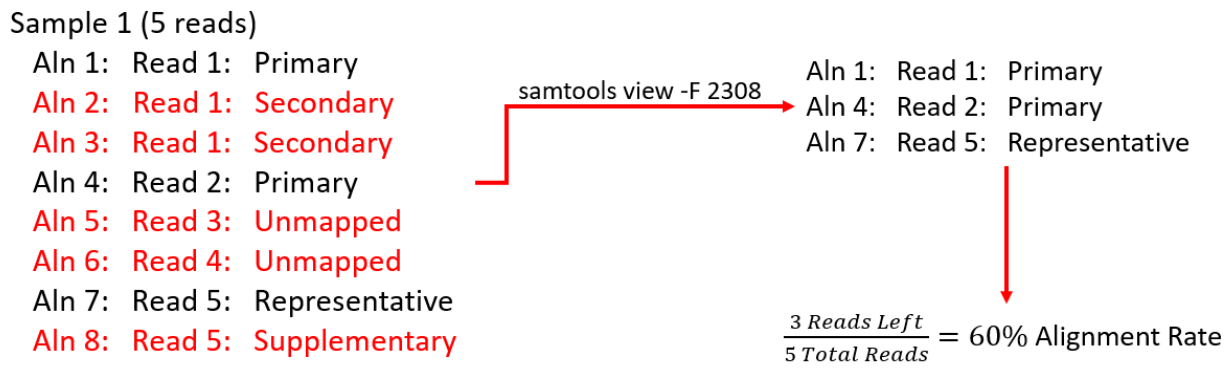


Figure 8: Calculating Alignment Rate: This figure shows an example of how the alignment rate was calculated given the SAM files from BWA and MUMmer4. The left-hand side shows the simplified version of output from a SAM file, which has a new alignment on each line with information regarding the read which was aligned and the type of alignment that was created. Alignments in red show the alignments that must be removed to avoid double counting a read or including unmapped reads in the alignment rate calculation. The top-right section shows the resulting SAM file after filtering with "samtools". The number of alignments leftover (3) is divided by the total number of reads in the initial file (5) to give the overall alignment rate for the sample (60%).

Transcriptome Coverage

Attempts were made using a variety of tools to assess the completeness of the overall transcriptome of *E. necator*. Coverage of the transcriptome was calculated to be used as an extrapolation of the alignment rate variable to determine if aligners had a certain bias as to what type of genes were able to be mapped to the reference genome. The goal for calculating transcriptome coverage was to combine the aligned reads from all 48 samples for a given aligner and use these results to determine an aligners maximum coverage of the transcriptome given reads from 48 “replicates”. This would remove the outside variable of a given sample not containing (or having a low expression) of a particular gene because the sample was grown in a distinctly different geographical location. The following is a breakdown of the attempts at calculating transcriptome coverage for *E. necator*.

BUSCO

The first attempt at determining transcriptome coverage was to use the Benchmarking Universal Single-Copy Orthologs tool (or BUSCO) (Simão *et al.*, 2015). The BUSCO tool works to identify “BUSCOs”, which are often core metabolic genes that exist in nearly all (>90%) species in each phylogenetic clade, that appear in a sequenced sample file. For example, the clade of all *Leotiomycetes*, the class level of which *E. necator* belongs to, has 3,234 BUSCOs that have been identified which are “universal” to all species that are classified under *Leotiomycetes*. A resulting run reports the number of BUSCOs discovered in a given sample that are fully intact (“Complete”), partially found (“Fragmented”), or missing from the sample entirely. BUSCO is often used as a measure for completeness by adding the “Complete” and “Fragmented” percentages to make sure that a newly assembled genome/transcriptome has a majority of the nearly universal core genes. As input, BUSCO requires a FASTA file created from the SAM files given from an alignment. To first convert the SAM files to a sorted BAM file, the "samtools" functions 'view' and 'sort' were used (see Supplementary B13). To convert from BAM to FASTA, the "samtools" function 'fasta' was used. To run BUSCO (v4.0.5), the following commands must be run first:

Adding Configuration Files to Environment Variables for BUSCO:

```
1) export BUSCO_CONFIG_FILE=/path_to_file/myconfig.ini
2) export AUGUSTUS_CONFIG_FILE=/path_to_config/
```

Line 1 adds the 'myconfig.ini' file, which is the configuration file created upon installing BUSCO, to the environment variable "BUSCO_CONFIG_FILE". Line 2 does a similar task by adding the configuration directory for the tool Augustus (installed alongside BUSCO) to the environment variable "AUGUSTUS_CONFIG_FILE". Both calls are required for BUSCO and Augustus to run properly. To run BUSCO on the resulting FASTA files from an alignment, the following command was used:

Running BUSCO on A01.fa:

```
1) /path_to_BUSCO/busco \
2)   -i A01.fa \
3)   -o A01_BUSCO \
4)   -m tran \
5)   -l leotiomycetes_odb10 \
6)   -c 5
```

Line 1 calls the "busco" function from the appropriate directory followed by the inputted FASTA file for argument 'i' on line 2 and the name of the outputted directory and files to be created for argument 'o' on line 3. Line 4 tells the function to run in "transcriptome" mode (as the FASTA files are created from aligned RNA-seq reads) by passing ‘tran’ as input for argument ‘m’. Line 5 chooses the correct database of BUSCOs to compare to, in this case “leotiomycetes_odb10”, and uses it as input for argument ‘l’. To run BUSCO using multiple cores, the number of cores to be used is passed in as input for argument ‘c’.

Exonerate

The second attempt at calculating transcriptome coverage was to use the tool Exonerate (v2.2.0) (Slater and Birney, 2005). Exonerate works by performing pairwise alignments between two samples. The two samples used in this case were the previously converted FASTA files from SAM files and the *E. necator* protein file from the Cantu Lab at UC Davis (https://cantulab.github.io/E.necator.proteins.NCBI.6533.fasta.gz). Transcriptome coverage would be calculated by dividing the number of proteins which had alignments to the transcripts in the SAM file by the total number of proteins for *E. necator*. To run Exonerate, the following command was used:

Running Exonerate on A01.fa:

```
1) /path_to_Exonerate/exonerate \
2)   --model protein2genome \
3)   e_necator.proteins.fa \
4)   A01.fa \
5)   > A01_Exonerate.txt
```

Line 1 calls the “exonerate” function from the appropriate directory and line 2 sets the ‘model’ to be used as ‘protein2genome’, as the comparison is between a protein file and the transcript file is made of genomic sequences. Line 3 takes in the query sequence file, which in this case is the protein file from the Cantu Labs, and line 4 takes in the target sequence file, which is the FASTA file of aligned reads. Line 5 redirects output from Exonerate into a file.

HTSeq

The third attempt at calculating transcriptome coverage used the python package HTSeq (v0.11.3) (Anders, Pyl, and Huber, 2014). The "count" function from the HTSeq package requires an alignment file and a feature file in GTF format, which lists the name and position of all features (in this case transcripts) in the reference species. "HTSeq-count" works by counting the number of reads in the alignment file that correspond to a given transcript in the feature file. To calculate transcriptome coverage from these results, the number of transcripts that had a count of at least one would be divided by the total number of transcripts in the feature file. In this case, the feature file being used is the *E. necator* GFF file from the Cantu Lab at UC Davis (https://cantulab.github.io/e-necator.c-strain.gff.gz). To convert the GFF file from the Cantu Lab into GTF format, the "gffread" (Cufflinks v2.2.1) (Trapnell *et al.*, 2010) function from the Cufflinks package was used in the following command:

Convert GFF to GTF:

```
1) /path_to_Cufflinks/gffread \
2)   e_necator.gff \
3)   -T \
4)   -o e_necator.gtf
```

Line 1 calls the "gffread" function from the appropriate directory followed by the inputted GFF file from the Cantu Lab on line 2. The 'T' argument on line 3 tells the function to output in GTF format and line 4 gives the outputted file name to argument 'o'. After converting the GFF file into the correct format, the “count” function from the HTSeq package was run using the following command:

Running HTSeq-count on A01.sam:

```
1) python \
2)   -m HTSeq.scripts.count \
3)   A01.sam \
4)   e_necator.gtf
```

Line 1 runs Python on the current system followed by line 2 which runs the correct module (using argument ‘m’) to run the ‘HTSeq.scripts.count’ function. Line 3 inputs the alignment file to be counted and line 4 gives the feature file in GTF format.

BLASTN

The last tool used for checking coverage was performing BLASTN searches to compare alignments between known *E. necator* transcripts and the aligned RNA-seq reads. BLASTN works by calculating pairwise alignments between two nucleotide sequences and reporting various statistics (such as alignment length and E-values), which can be used to determine the quality of the resulting alignments. To return alignments of the longest possible length, the aligned reads must first be assembled based on their overlap with each other. To assemble the aligned reads into larger ones, the “cufflinks” (v2.2.1) function from the Cufflinks package was used as follows:

Running Cufflinks on A01.sam:

```
1) /path_to_Cufflinks/cufflinks \
2)   -p 5 \
3)   A01.bam \
4)   -o Assembled/A01 \
5)   --library-type fr-firststrand
```

Line 1 calls the “cufflinks” function from the appropriate directory. To run the function using multiple cores, the required number of cores to be used is passed to the ‘p’ argument as shown on line 2. Line 3 shows the alignment file inputted in BAM format (created previously when converting SAM into FASTA format for BUSCO analysis) and line 4 adds the output directory to be created to the argument ‘o’. Line 5 tells the function which library prep strand type was used for the reads. Specifying a strand type was only required for output from BWA, STAR, and HISAT2. Only Lines 3 and 4 were changed accordingly to assemble the transcripts between each of the 48 BAM files for each of the aligners.

The “cufflinks” function results in the reads for a given sample being aligned into the smallest possible number of transcripts. The next step is to combine the assembled reads from all 48 samples together for a given aligner. This can be done using the “cuffmerge” function also from the Cufflinks package. The function works like the “cufflinks” function by assembling reads across multiple samples to create the largest (and smallest number of) transcripts across all 48 samples. To run “cuffmerge” on each of the 48 assembled reads, the following command was used:

Running Cuffmerge:

```
1) export PATH=/path_to_Cufflinks/:$PATH
2) /path_to_Cufflinks/cuffmerge \
3)   -p 5 \
4)   Assembled_File_List.txt
```

Line 1 adds the path to the Cufflinks package to the system's current global path. This is necessary for the "cuffmerge" function to use the "gtf_to_sam" function. Line 2 calls the "cuffmerge" function from the appropriate directory followed by line 3 adding the number of cores to be used to argument 'p'. Line 4 contains the name of a file which contains a list of paths pertaining to the "transcript.gtf" file for each of the 48 samples. The "transcript.gtf" file is one of the files outputted by the "cufflinks" function and is stored in the directory specified as output in the run of the program. The content of the file listed on line 4 was changed accordingly to incorporate the assembled transcripts for each aligner. As output, a directory called "merged_asm" is created and the resulting merged transcript file is stored inside called "merged.gtf".

It was discovered during analysis that a few of the assembled transcripts from "cuffmerge" extended past the boundaries of the contig which they mapped to in the reference genome. To keep the transcripts within the bounds of the reference genome, a Perl script called "cufftrim.pl" was used (aechchiki, 2018). This function requires a FASTA index file of the reference genome, which is created using the "samtools" function "faidx". The outputted file contains the name of each contig followed by the size, location, and other information about the contigs themselves from the reference genome. With this FASTA index file, the following command was used to run the "cufftrim.pl" script:

Running cufftrim.pl on Bowtie2_E2E's merged.gtf:

```
1) perl cufftrim.pl \
2)   e_necator.fa.fai \
3)   merged.gtf \
4)   > Bowtie2_E2E.trim.gtf
```

Line 1 runs the "cufftrim.pl" script in a Perl environment followed by the created FASTA index file on line 2. Line 3 is the merged transcript file outputted by the "cuffmerge" function and line 4 redirects the output of this function to an appropriately named file. The result of this file is a GTF feature file which contains merged transcripts that fall within the appropriate range of the reference genome's contigs.

To align both the Cantu Lab transcripts and assembled reads, both files must be in FASTA format. As the assembled reads are currently in GTF format, the "gffread" function from the Cufflinks package was used once gain to convert the file into a FASTA file. This function took in the assembled GTF file for an aligner, which contained the location in the reference genome where the transcript aligned too and extracted the corresponding read from the same location from the reference genome. The following command was used to perform this file conversion:

Converting GTF to FASTA for Bowtie2_E2E.trim.gtf:

```
1) /path_to_Cufflinks/gffread \
2)   Bowtie2_E2E.trim.gtf \
3)   -g e_necator.fa \
4)   -w Bowtie2_E2E_tran.fa
```

Line 1 calls the “gffread” function from the appropriate directory followed by the inputted GTF transcript file on line 2. Line 3 assigns the reference genome to the argument ‘g’ and line 4 adds the output file to be created to argument ‘w’. Lines 2 and 4 were modified accordingly to create FASTA files for transcripts from each of the aligners.

To align the known transcripts of *E. necator* to the transcripts assembled from an aligner, a database must be created from the assembled transcript file. The “makeblastdb” function from the BLAST+ package (v2.5.0+) (Camacho *et al.*, 2009) was used to create the database with the following command:

Creating a BLAST Database from Bowtie2_E2E_tran.fa:

```
1) /path_to_BLAST+/makeblastdb \
2)   -in Bowtie2_E2E_tran.fa \
3)   -parse_seqids \
4)   -dbtype nucl
```

Line 1 calls the “makeblastdb” function from the appropriate directory followed by the inputted FASTA file added to argument ‘in’ on line 2. Line 3 is required if the input is in FASTA format for the function to correctly pull out the sequence ID’s from the header of each entry. Line 4 tells the function which type of database is to be created by adding ‘nucl’ (for nucleotide) to the argument ‘dbtype’.

After creating the database of aligned transcripts, the BLAST search was performed using the transcript file taken from the Cantu Lab at UC Davis as a query (https://cantulab.github.io/E.necator.transcripts.NCBI.6533.fasta.gz). The BLAST search was performed using the following command:

Running BLASTN with Bowtie_E2E_tran.fa Database:

```
1) /path_to_BLAST+/blastn \
2)   -query e_necator.transcript.fa \
3)   -db Bowtie_E2E_tran.fa \
4)   -out Bowtie_E2E.tsv \
5)   -max_target_seqs 1
6)   -outfmt “6 qacc qlen sacc slen evalue length qcovs”
```

Line 1 calls the “blastn” function from the appropriate directory. Line 2 assigns the FASTA file of transcripts from the Cantu Lab as the ‘query’ argument. Line 3 requires the name of the FASTA file from which the database was created provided as input for the ‘db’ argument’ and line 4 assigns the file to be created as output to the ‘out’ argument. Line 5 tells the function to only output the single best alignment per transcript in the query by adding a 1 to the argument ‘max_target_seqs’. Line 6 tells the function which types of the information to report in the outputted TSV (format notified by the ‘6’): query name, query length, target name, target length, E-value for the alignment, length of the alignment, and query coverage, respectively. Lines 3 and 4 were modified accordingly to run the BLASTN function against databases for the other 6 aligners.

Transcriptome coverage was then calculated from the output of the BLASTN function by counting the number of lines in the output file and dividing by the total number of transcripts in the Cantu Lab transcript file. Each line in the output corresponded to a single alignment and there was no double counting of transcripts as only one alignment was returned per transcript. Additionally, the transcriptome coverage was calculated similarly for various cutoffs based on minimum values for the overall alignment length.

Checking for Gene Bias

BLAST+ results were then used to check whether there was any bias as to which type of genes were unable to be mapped by an aligner. Code was written to extract from the Cantu Lab transcript file those genes that did not have at least a partial hit in the BLAST+ output for an aligner. These genes which were not found were submitted in FASTA file format to eggNOG-mapper v2 to classify the missing genes based on their annotation results (Huerta-Cepas *et al.*, 2017). Bias was determined for each aligner by examining the proportion of missing genes which were reported to belong to each Clusters of Orthologous Groups of proteins (COG) category assigned by EggNOG-mapper (Tatusov *et al*., 2000).

Results

Initial Quality Assessment

Raw reads for all samples were initially checked for overall quality using FastQC. The average number of raw reads per sample was 3,677,174. The initial results from FastQC on these reads displayed poor quality for each of the 48 samples of *E. necator*. Each of the 48 samples had similar distributions for all results outputted by FastQC so Figure 9 shows the output for a representative sample, split_A01.fq. Examining the “Per base sequence quality” resulted in a large proportion of the bases having low quality scores, particularly near the 3’-end of the reads, given the large portions of the box plot distributions (one plot created for each base position) being in the < 20 Q-score area (red in color) of the chart in Figure 9A. As a Q-score of 20 was the desired value and indicates a 99% accurate base call at a given position, any score below this value would have an incorrect base call > 1% of the time and therefore would need to be trimmed. A second important finding from examining the initial FastQC tests was the particularly high N-content at position 6 of all reads as seen in Figure 9B. The results from the N-content distribution showed that in nearly 45% of the reads an ‘N’ was placed at the 6th position due to the sequencer being unable to determine a base for this position. Due to the large ambiguity at this position, it was this finding that resulted in the decision to remove the 6th base from the reads along with the first 5 bases which corresponded to the sample barcodes. Initial results also showed a slight bias across the length of each read in the calling of A’s and T’s as seen in Figure 9C. Ideally, each of the bases should be assigned roughly 25% of the time (a ¼ chance due to their being 4 nucleotides), so there was a slight overrepresentation of A’s and T’s (approximately 30%) throughout all 48 samples. In contrast, bases 1 through 6 all had abnormal base-calling distributions, which was to be expected as the same 5 bases were placed at the beginning of each read for sample identification. This led to a nearly 100% calling of a given base for the first 5 positions, which further solidified the reason to remove these bases from each of the reads. Further, a higher percentage of the reads had low GC content, indicated by the leftmost peak of the red curve in Figure 9D showing the “Per sequence GC content”. This peak most likely came from an overabundance of poly-A tails being represented in the dataset as reads of all (or large portions of) A’s would record GC contents of ~0%, resulting in the peak for a high number of low GC reads. Ideally, the GC content distribution across all reads should follow a normal distribution (displayed by the blue curve in Figure 9D).

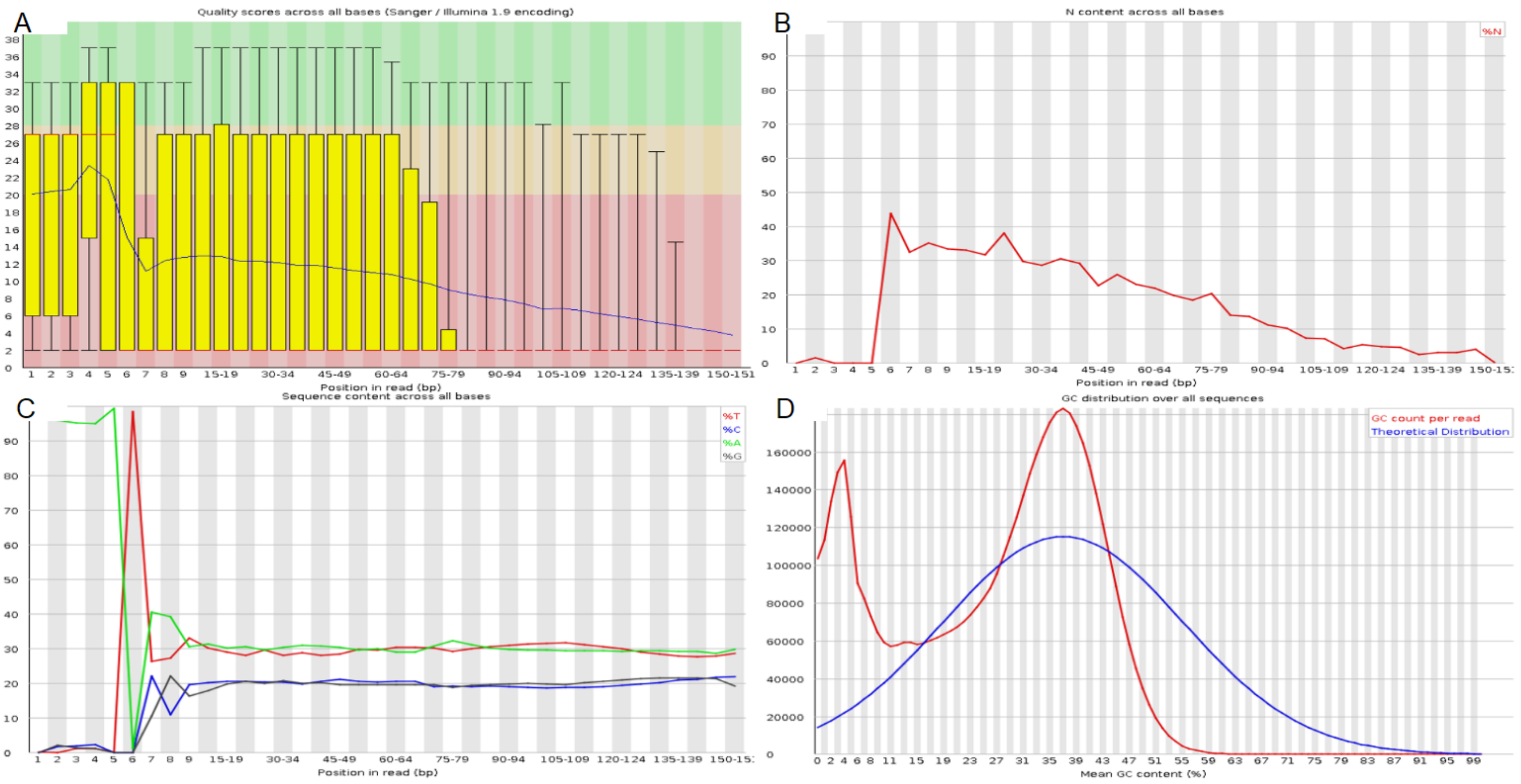


Figure 9: Initial FastQC Results for split_A01.fq: This figure shows the results of running FastQC on the raw reads of split_A01.fq. Figure A shows the "Per base sequence quality" chart, which shows the distribution of quality scores (y-axis) for each base (x-axis) across each of the reads in the sample. Figure B shows the "Per base N content", which shows the percentage of times an 'N' was sequenced (y-axis) for each base (x-axis) across all reads. Figure C shows the "Per base sequence content", which shows the percentage of times a particular base was called (y-axis) for each of the bases (x-axis) across all reads. Figure D shows the "Per sequence GC content", which shows the comparison of the theoretical normal distribution of GC content (blue) with the actual distribution for the sample of the number of reads (y-axis) that have a given GC content percentage (x-axis) (red).

FASTX Clean Up

Raw reads were then trimmed by removing the 5 base pair barcodes and high N-content base at position 6 from the 5'-end as well as removing low quality bases from the 3'-end. After quality trimming, any read below 100 base pairs was removed from the sample. Running FastQC on the trimmed reads showed much cleaner reads for all samples. All samples had the same general results so the FastQC results of a single sample, split_A01.fq, can be seen in Figure 10. The overall distribution of quality scores was significantly improved by removal of low-quality reads as seen in Figure 10A by the boxplot distributions appearing primarily in the "> 20 Q-score"-region. The drastic increase in quality was most likely since FASTX had removed a large percentage of reads from each of the samples. After trimming, the average number of reads remaining across all samples was 741,219. This resulted in an average of 20.04% of reads left after quality control. The percentage of N's appearing throughout the reads was also reduced, shown in Figure 10B, so that no sample even had over 5% of N's at a given base position. The sequence content on the 5'-ends was improved by removing the barcodes (as shown in Figure 10C), which lessened the overrepresented base calling as the same 5 bases were attached to every read for a given sample. The slight bias in A and T overrepresentation was also present post-trimming but was not determined to have much of an impact on alignment results downstream. Further, the GC content across all the reads post-trimming was much more normally distributed as shown in Figure 10D, most likely due to the removal of low-quality poly-A tails. Taking all of these quality control results into consideration, it was determined that the reads were of a good enough quality to continue with alignment.

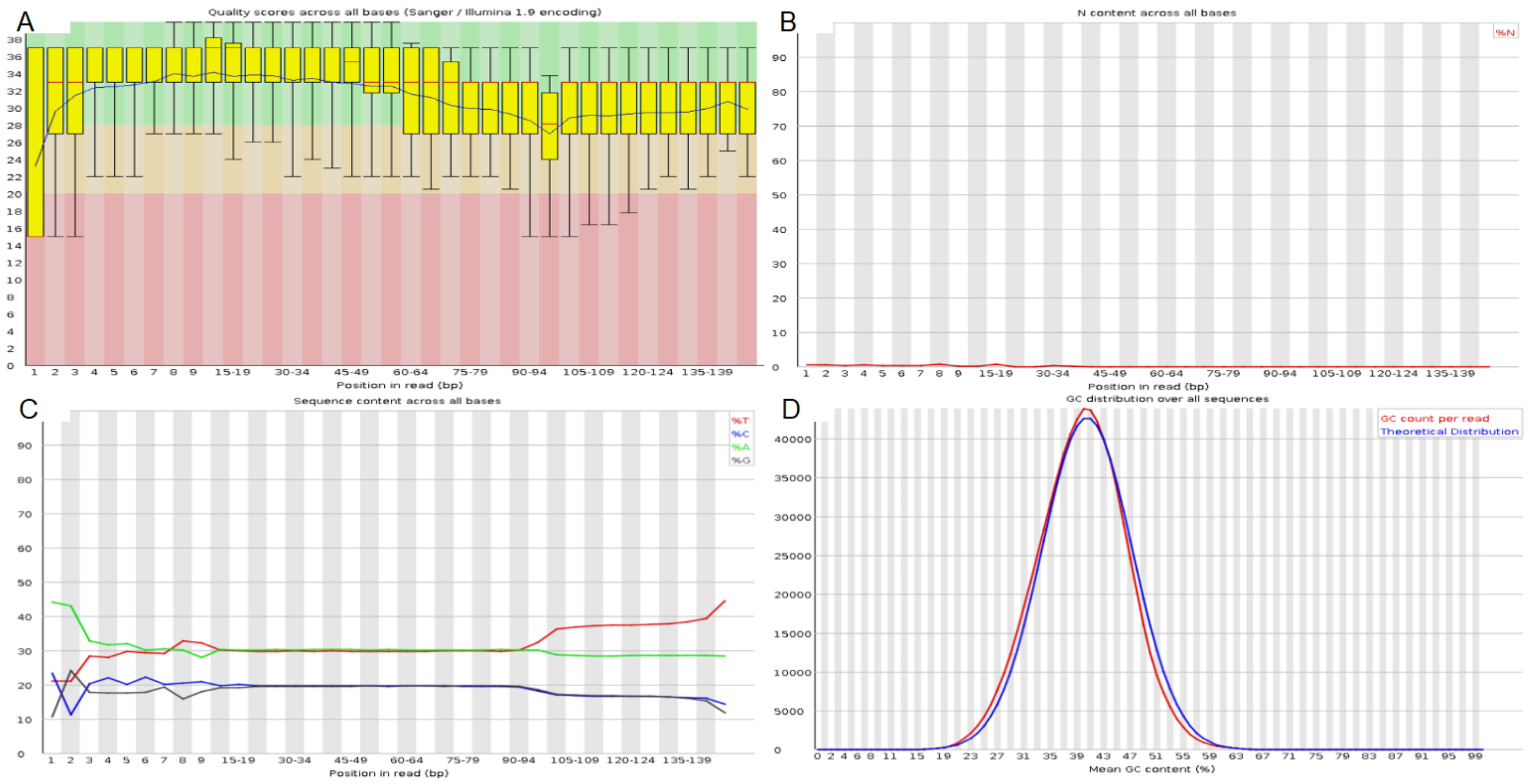


Figure 10: Post-Trimmed FastQC Results for split_A01.fq: This figure shows the results of running FastQC on the trimmed reads of split_A01.fq. Figure A shows the “Per base sequence quality” chart, which shows the distribution of quality scores (y-axis) for each base (x-axis) across each of the reads in the sample. Figure B shows the “Per base N content”, which shows the percentage of times an ‘N’ was sequenced (y-axis) for each base (x-axis) across all reads. Figure C shows the “Per base sequence content”, which shows the percentage of times a particular base was called (y-axis) for each of the bases (x-axis) across all reads. Figure D shows the “Per sequence GC content”, which shows the comparison of the theoretical normal distribution of GC content (blue) with the actual distribution for the sample of the number of reads (y-axis) that have a given GC content percentage (x-axis) (red).

Individual Assessments of Runtime & Alignment Rate

Bowtie2

Bowtie2 has both an end-to-end and a local mode which changes the way that the tool attempts to align a given read to the reference genome. In end-to-end mode, an entire read from 5'- to 3'-end must be aligned to a place in the genome in order to be reported out to the user. Local mode is more flexible with its alignment algorithm and has the ability to trim off bases on either end of the read that do not align well to the reference genome. Due to this extra layer of flexibility, it was hypothesized that local mode would have higher alignment rates than end-to-end mode, but the extra trimming step would result in longer runtimes. To test this hypothesis (alongside determining which tool performs best), Bowtie2 was run in both end-to-end and local alignment mode. Prior to running both modes of alignment, the reference genome was indexed using the same command for both end-to-end and local mode. It took Bowtie2's genome indexing function 34.30 seconds to run to completion on the *E. necator* reference genome (Table 3).

Running Bowtie2 in end-to-end alignment mode resulted in a range of ~32% to ~90% of reads that were successfully mapped to the reference genome (full distribution seen in Figure 11A). Overall, Bowtie2 in end-to-end mode had an average alignment rate of 65.641% (as shown in Table 2). Although the mapping rate was low, Bowtie2 in end-to-end mode had a fast runtime. Using 5 cores, Bowtie2 in end-to-end mode had an average runtime of 20.93 microseconds when normalized by the number of reads per sample (Table 3) with a range of ~15 to ~25 microseconds (full distribution seen in Figure 11B). Using only a single core, Bowtie2 in end-to-end mode had an average runtime of 110.86 microseconds per read. When comparing the runtime per read of a given sample with its resulting alignment rate, 46 of the 48 samples clustered together with a positive correlation (overall R^2 for all 48 samples was 0.6445) (Figure 11C). Additionally, two of the samples with the lowest alignment rates (A06 and D11) also reported the two lowest runtimes per read. Another important aspect of an aligner is how much the runtime scales when allowing the tool to use additional cores. Ideally, one would hope to achieve linear speedup (such as running 3x's faster on 3 cores than 1) although this tends to be rare. Bowtie2 in end-to-end mode was able to achieve super-linear speedup (such as > 3x's speedup on 3 cores) on average when using 2, 3, and 5 cores (linear curve shown in Figure 11D and raw data in Table 4).

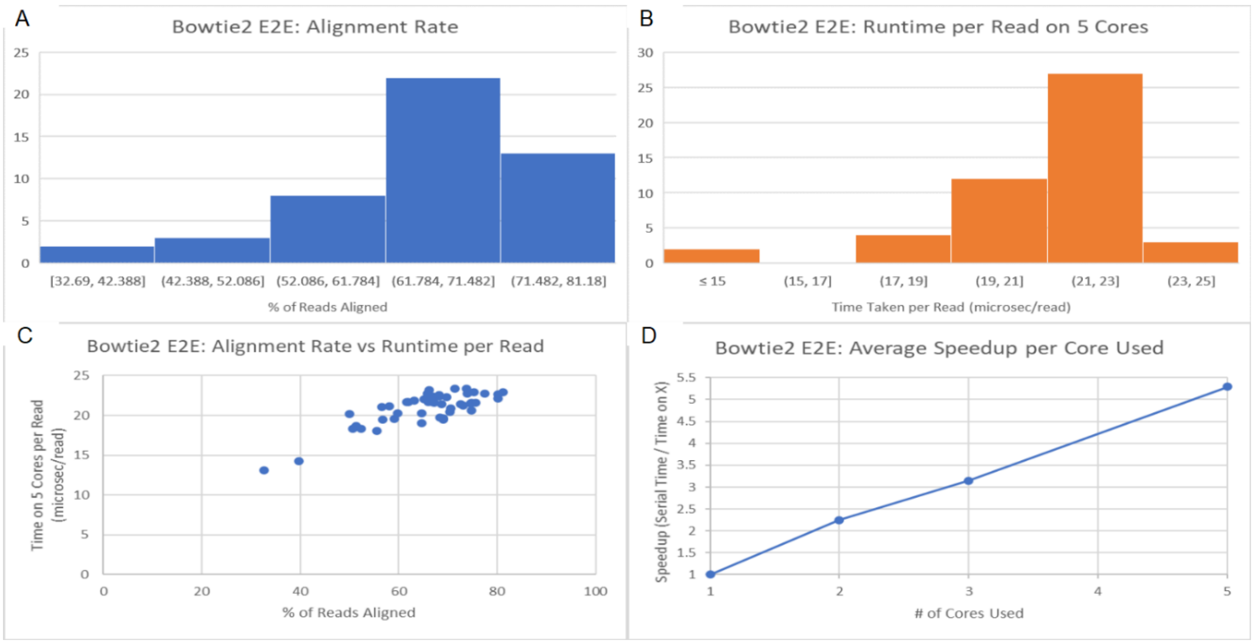


Figure 11: Alignment Results for Bowtie2 in End-to-End Mode: This figure shows a summary of the alignment statistics for Bowtie2 in end-to-end alignment mode. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

When running in local alignment mode, Bowtie2 had an average alignment rate of 87.19% (Table 2) with samples ranging from ~47% to ~99% (full distribution shown in Figure 12A). This mapping rate was considerably higher than the end-to-end alignment mode, however as expected, the runtime per read was nearly doubled with an average of 44.96 microseconds per read using 5 cores (Table 3). This resulted in a range of runtimes from ~30 to ~54 microseconds per read (full distribution seen in Figure 12B). Using only a single core, Bowtie2's local alignment mode had an average runtime per read of 226.63 microseconds (Table 3), also doubled from end-to-end mode's single core runtime. When comparing the runtime per read against the alignment rate, Bowtie2 in local alignment mode also showed tight clustering for 46 of the 48 samples with an a highly positive correlation ($R^2 = 0.9495$) (Figure 12C). Additionally, the two samples in local mode which achieved the lowest alignment rates and lowest runtime per read (A06 and D11) were the same two samples which end-to-end mode had a difficult time mapping to the reference genome. Also, like end-to-end mode, Bowtie2's local alignment mode achieved linear speedup on average when using 2, 3, and 5 cores (linear curve seen in Figure 12D and raw data in Table 4).

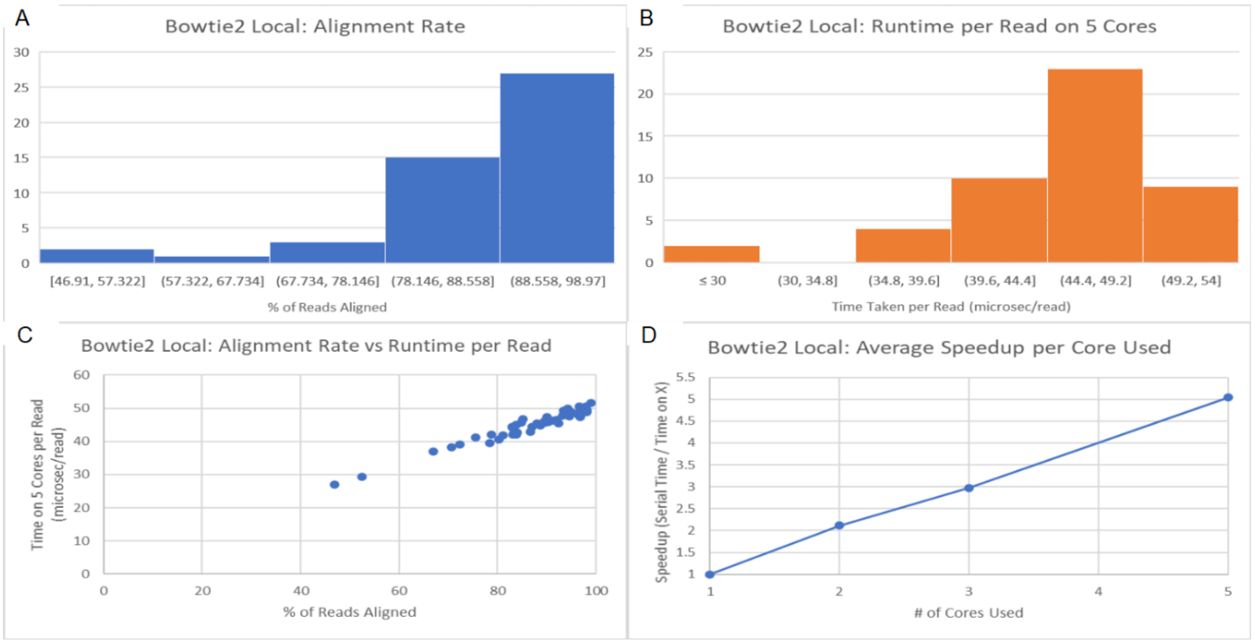


Figure 12: Alignment Results for Bowtie2 in Local Mode: This figure shows a summary of the alignment statistics for Bowtie2 in local alignment mode. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

BWA

Building the reference genome index with BWA took roughly the same amount of time as Bowtie2 with a time of 33.45 seconds (Table 3). Aligning the samples with BWA saw a large percentage of the reads being mapped to the reference genome. On average, 87.41% of a sample's reads were successfully aligned (Table 2) with a range of ~47% to ~99% and a nearly identical distribution as Bowtie2 when run using local mode (Figure 13A). BWA achieved the best average alignment when compared to all other aligners tested. The runtime per read using BWA was fast using on average 24.04 microseconds per read with 5 cores and 104.50 microseconds per read with a single core (Table 3). The range of runtimes on 5 cores had a very tight distribution with a range of ~21 to ~28 microseconds per read (Figure 13B). Like both alignment modes for Bowtie2, BWA had 46 of the 48 samples cluster tightly together when comparing the runtime per read and alignment rates (Figure 13C). Overall, there was only a slightly positive correlation between the two variables (R^2 = 0.2286). Again, the two samples that recorded the lowest alignment rates (A06 and D11) were the same as those identified by Bowtie2. When analyzing the speedup curve, BWA achieved nearly linear speedup on average when using 2 cores, but less than linear using 3 and 5 cores (linear curve seen in Figure 13D and raw data in Table 4).

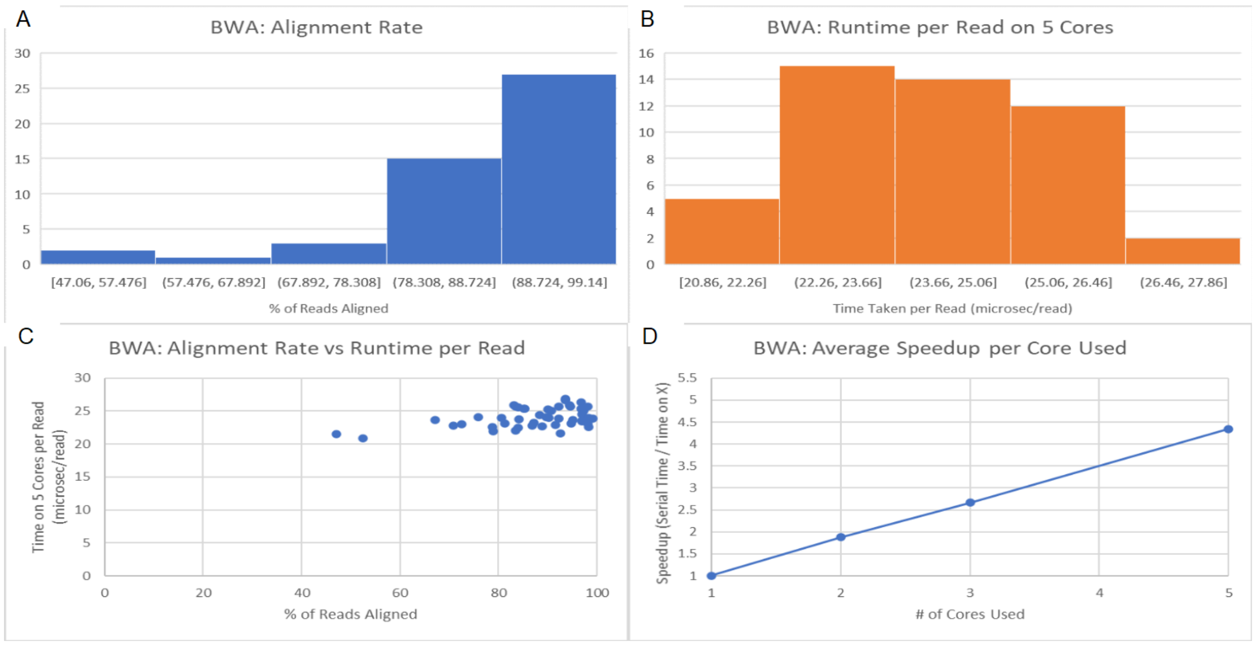


Figure 13: Alignment Results for BWA: This figure shows a summary of the alignment statistics for BWA. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

HISAT2

When compared to the other aligners, HISAT2 had the second fastest pre-processing time by building the reference genome index in 25.82 seconds (Table 3). Aligning with HISAT2 saw extremely fast runtimes paired with lower alignment rates. On average, only 66.43% of a sample's reads were successfully mapped to the reference genome when using HISAT2 (Table 2). Alignment rates ranged from ~32% to ~86% leading to a rather large distribution (Figure 14A). In contrast to this wide spread of alignment rates, the runtimes for HISAT2 were quite fast with a very tight range. On average, the runtime per read using 1 core was 37.81 microseconds and using 5 cores was 8.28 microseconds (Table 3) with a range of ~6 to ~11 microseconds (Figure 14B). The runtimes reported by HISAT2 were among the fastest runtimes for any aligner tested. When comparing the alignment rate against the runtime per read, no correlation (R^2 = 0.0876) was found partially due to the large range of alignment rates obtained (Figure 14C).

Once again, the two samples that reported the two lowest alignment rates, and the two shortest runtimes per read, (A06 and D11) were the same reported by previous tools. Similar to BWA's speedup curve, HISAT2 performed best using 2 cores (super-linear speedup on average) when compared with using 3 and 5 cores, which both achieved slightly less than linear speedup on average (curve seen in Figure 14D and raw data in Table 4).

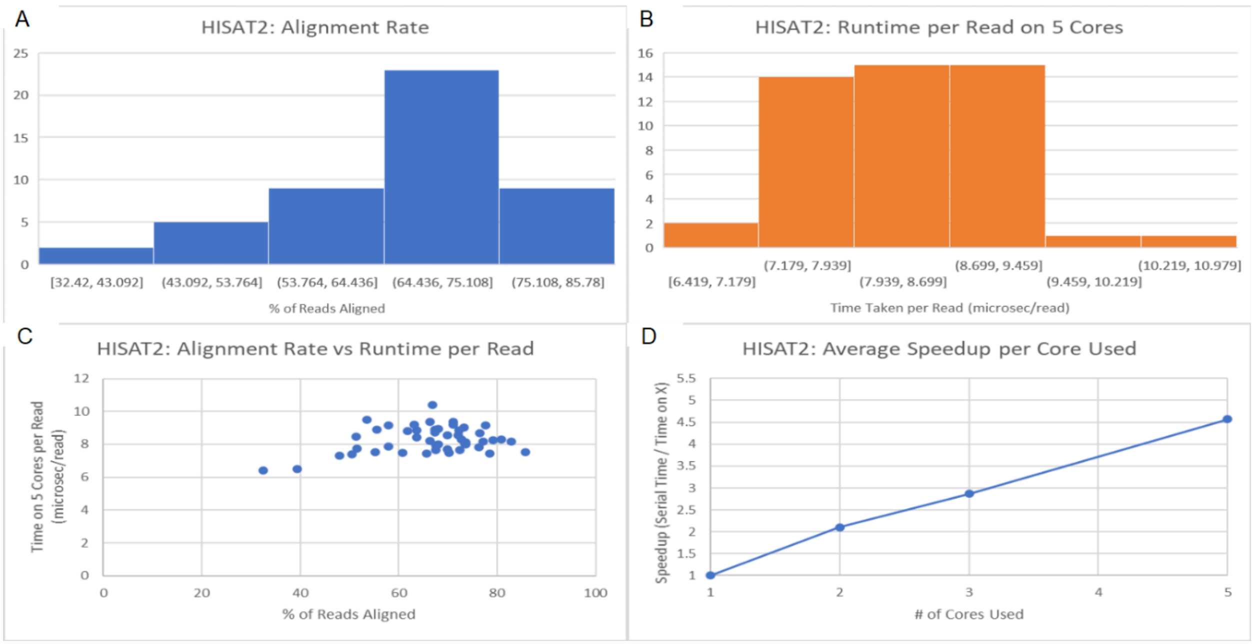


Figure 14: Alignment Results for HISAT2: This figure shows a summary of the alignment statistics for HISAT2. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

MUMmer4

One place where MUMmer4 performed substantially better than other aligners was its ability to build the genome index very quickly. MUMmer4 only required 7.96 seconds to process E. necator's genome, approximately 3x's faster than the second fastest aligner HISAT2 (Table 3). Although being the cause of the most trouble due to having incorrect output formats, MUMmer4 had good results for both alignment rates and runtimes. The average alignment rate with MUMmer4 was 77.94% (Table 2) with a range of ~40% to ~93% (Figure 15A). Although this range is rather large, a large proportion of the samples (46 of the 48) achieved > 60% alignment rates with the two samples < 60% being sample A06 and D11, the same ones reported by previous aligners as being troublesome. When using only a single core, MUMmer4 had an average runtime per read of 186.90 microseconds. Although being one of the longer runtimes on 1 core, MUMmer4 scaled well by using more cores as made evident by the average runtime per read on 5 cores being 37.70 microseconds (Table 3) with a range of ~31 to ~43 microseconds (Figure 15B). In addition, the linear speedup curve for MUMmer4 showed the aligner performing at super-linear speedup using 2 cores and linear speedup using 3 and 5 cores (curve in Figure 15D and raw data in Table 4). MUMmer4 showed tight clustering on both axes and a slightly positive correlation ($R^2 = 0.2722$) between the runtime per read with the alignment rate (Figure 15C). As stated earlier, the two samples with the fastest runtime and lowest alignment rate were A06 and D11.

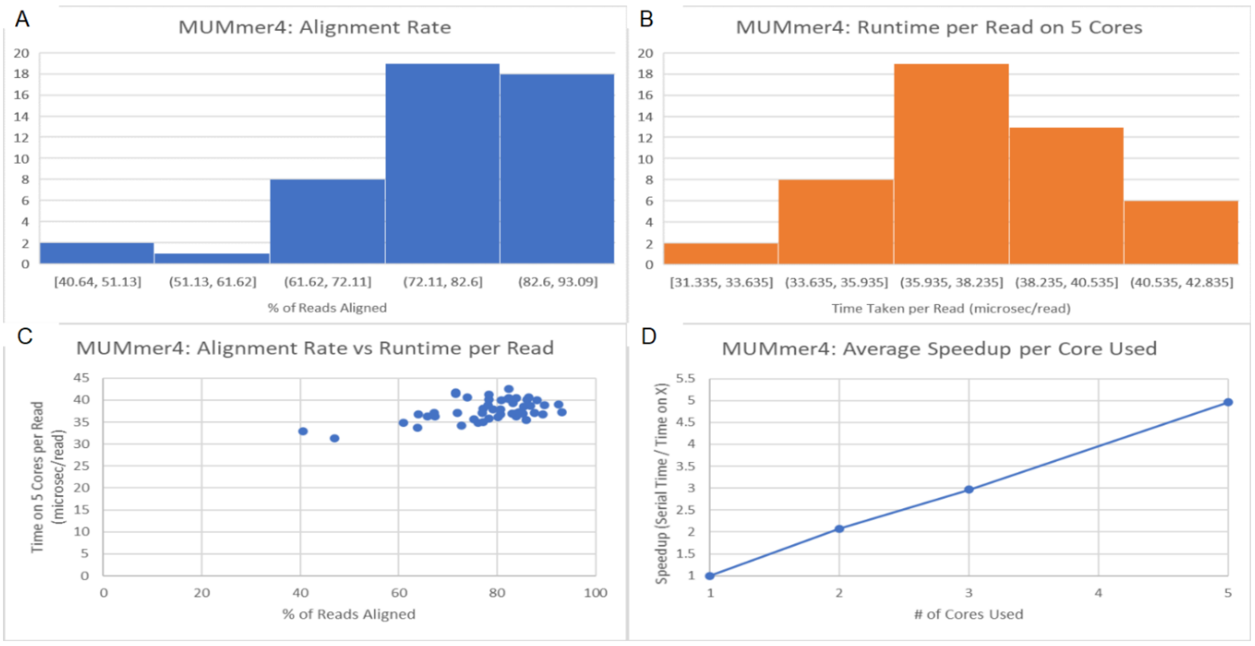


Figure 15: Alignment Results for MUMmer4: This figure shows a summary of the alignment statistics for MUMmer4. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

STAR

Pre-processing with STAR involved building the uncompressed suffix array from the E. necator reference genome, which took 80.42 seconds to build (Table 3). If STAR did not have the option to pre-build genome index, this long runtime would negatively impact overall runtimes as STAR had the slowest pre-processing time of all aligners (2x's the next slowest). However, the ability to only run this command once and call the built index in subsequent alignments resulted in STAR having the second fastest (behind HISAT2) runtime per read using a single core with an average time of 67.45 microseconds (Table 3). Running STAR on 5 cores resulted in an average time of 22.89 microseconds per read (Table 3) and a range of ~12 to ~59 microseconds (Figure 16B). Using an increasing number of cores resulted in a logarithmic speedup curve that was well below the ideal linear curve (Figure 16D and raw data in Table 4). In terms of alignment rates, STAR had an average alignment rate of 78.48% across all samples

(Table 2) with a range of ~40% to ~93% (Figure 16A). Analyzing the correlation between runtime per read and alignment for STAR found an overall negative relationship ($R^2 = 0.3904$) between the two variables (Figure 16C). Like previous aligners, STAR also reported samples A06 and D11 with having the lowest alignment rates.

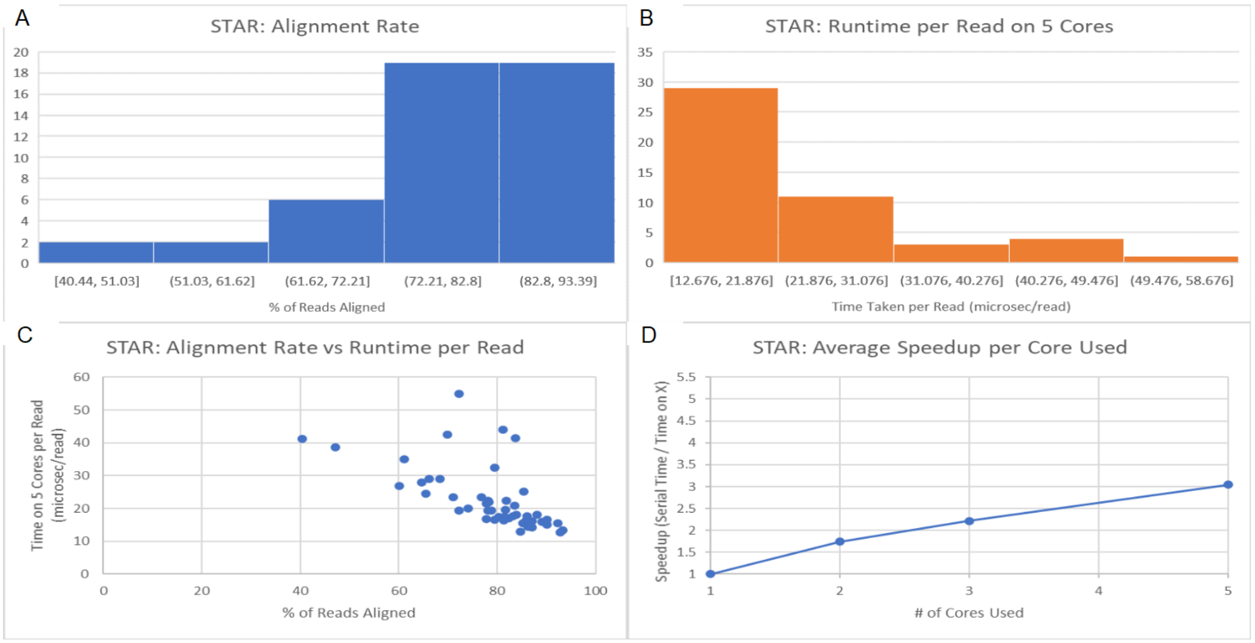


Figure 16: Alignment Results for STAR: This figure shows a summary of the alignment statistics for STAR. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

TopHat2

TopHat2 has been largely replaced with HISAT2 in terms of overall tool usage and the results from TopHat2 on *E. necator* show why any aligner is a much better option. Due to TopHat2 using Bowtie2 to perform initial alignments, the pre-processing time is equivalent to Bowtie2's 34.30 second runtime to build the reference genome index (Table 3). When looking at alignment rates, TopHat2 had the lowest rate among aligners by a substantial amount (3x's less than the next lowest). On average, TopHat2 was able to map 18.33% of a sample's reads to the reference genome (Table 2) with a range of ~7% to ~31% (Figure 17A). In addition to the low alignment rates, TopHat2 also recorded the highest runtimes by a large amount comparatively. Using a single core, the average runtime per read was 508.70 microseconds (Table 3). Using 5 cores, the average runtime per read was 221.07 microseconds (Table 3) with a range from ~195 to ~265 microseconds (Figure 17B). One positive result from TopHat2 was that it was consistent with its low assessment scores as comparing runtime to alignment rate showed tight clustering on both axes (Figure 17C). Overall, there was a slight negative correlation between the two variables ($R^2 = 0.1033$). Further leading to TopHat2's poor assessment was its poor scalability by using more than one core. The speedup curve generated for TopHat2 resulted in a logarithmic curve well below a linear curve and the worst among tested aligners (Figure 17D and raw data in Table 4).

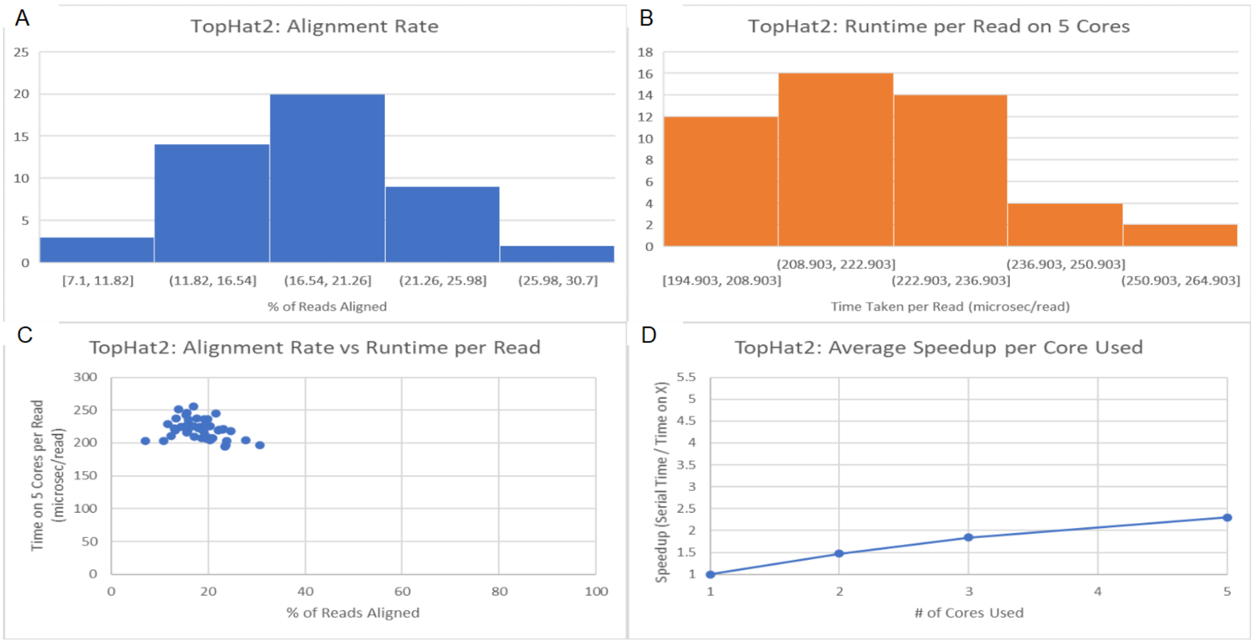


Figure 17: Alignment Results for TopHat2: This figure shows a summary of the alignment statistics for TopHat2. Figure A shows the distribution of alignment rates for all 48 samples. Figure B shows the distribution of runtime (in microsecond) normalized by the number of reads per sample. Figure C shows a comparison between alignment rate (x-axis) and runtime per read (y-axis). Figure D shows the resulting speedup curve when using 1, 2, 3, and 5 cores.

Comparative Assessment of Runtime & Alignment Rate

The best way to determine which aligner performed best is by looking at a comparison of the raw data. When examining the average alignment rate, four of the seven aligners tested had rates greater than 75%: Bowtie2 in local alignment mode, BWA, MUMmer4, and STAR (Table 2). The aligner with the highest average alignment rate was BWA with 87.41%, followed closely behind by Bowtie2 in local alignment mode with 87.192%. On the other end of the spectrum, TopHat2 had the lowest average alignment rate by far with a rate of 18.33% (next lowest was Bowtie2 in end-to-end mode with 65.64%). To further compare the aligners against each other, a comparative boxplot chart was created where each aligner's distribution plot was made from the alignment rates of all 48 samples (Figure 18). A Tukey test (using Tukey's 'Honest Significant Difference' method with 95% confidence level) was also performed on the resulting distributions to determine which of the aligner's average alignment rates were statistically different from another. The Tukey test resulted in four significantly distinct groups between the seven aligners. In one group, BWA and Bowtie2 in local mode had nearly identical distributions with the highest alignment rates. The group with the second highest rates belonged to MUMmer4 and STAR, followed by the third group of Bowtie2 in end-to-end mode and HISAT2. The fourth and final grouping belonged to TopHat2 by itself. All aligners, except TopHat2, identified both samples A06 and D11 as outliers that had alignment rates well-below the rest of the samples. Like the others, TopHat2 identified sample D11 as being an outlier below the others, but, unlike the other aligners, identified sample A12 as an outlier with an alignment rate greater than the rest of the samples.

Table 2: Average Alignment Rate per Aligner: This table shows the average alignment rate per aligner. Averages were calculated by summing together the alignment rates for all samples and dividing by the total sample count, 48.

Tool	% of Reads Aligned
Bowtie2 E2E	65.641
Bowtie2 Local	87.192
BWA	87.411
HISAT2	66.432
MUMmer4	77.941
STAR	78.482
TopHat2	18.329

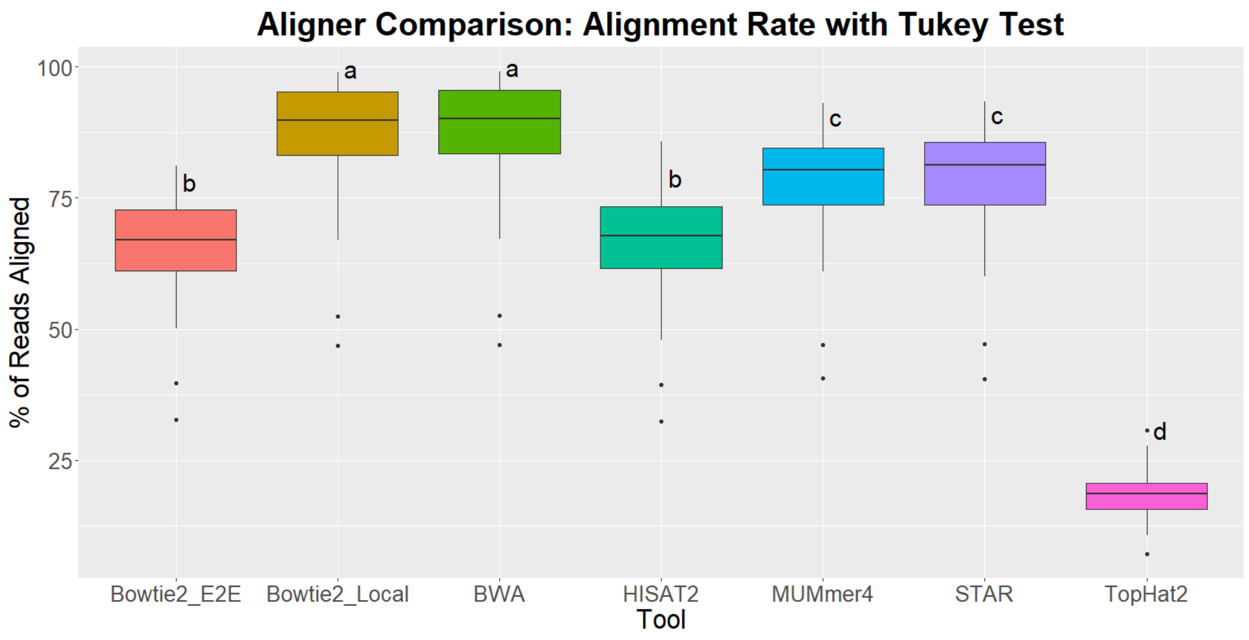


Figure 18: Boxplots for Alignment Rate with Tukey Test: This figure shows a comparative boxplot for the alignment rate distributions for each of the 7 aligners tested. The results of a Tukey Test using Tukey's 'Honest Significant Difference' method with 95% confidence level are shown as single letters ('a', 'b', 'c', or 'd') placed above each boxplot. Boxplots that share the same character have average alignment rates which are indistinguishable from each other ($\mu_1 = \mu_2$). Boxplots that have different characters have average alignment rates which are significantly different from one another ($\mu_1 \neq \mu_2$).

When examining the time taken to build the reference genome index, MUMmer4 finished the fastest with a time of 7.96 seconds which was well above the second fastest pre-processing time of 25.82 seconds achieved by HISAT2 (Table 3). Bowtie2's genome indexing function (which was used for both modes of Bowtie2 and TopHat2) finished within 1 second (34.30

seconds) of BWA's pre-processing time of 33.45 seconds. The slowest genome indexing function belonged to STAR with a time of 80.42 seconds which was over twice as slow as the next slowest time. When examining the average runtime per read using only a single core, HISAT2 achieved the fastest runtime of 37.81 microseconds per read followed by STAR with 67.45 microseconds per read. The slowest runtime per read on one core belonged to TopHat2 with an average time of 508.70 microseconds which was twice as slow as the next slowest (Bowtie2 in local mode with a time of 226.63 second). When upping the number of cores used to five, HISAT2 still achieved the fastest runtime per read with an average time of 8.28 microseconds. Although STAR was the second fastest using one core, Bowtie2 in end-to-end scaled better with the use of more cores to have the second fastest average runtime with a time of 20.93 microseconds per read (STAR was third with 22.89 microseconds). Once again, TopHat2 achieved the slowest runtime of 221.07 microseconds per red, which was almost five times as slow as the next slowest aligner, Bowtie2 in local mode with a time of 44.96 microseconds per read.

Similar to the alignment rates, a comparative boxplot with corresponding Tukey test results was created to directly compare the runtime per reads using 5 cores for all aligners (Figure 19). TopHat2 had by far the slowest distribution of runtimes per read and skewed results in the initial comparison. For this reason, a second Tukey test (and resulting boxplots) was run which excluded TopHat2 from the analysis to better estimate the differences between the other six aligners (Figure 20). The Tukey test resulted in HISAT2 being grouped individually as the fastest aligner, followed by the second fastest group of Bowtie2 in end-to-end mode and STAR. Due to the large distribution of runtimes, STAR was the only aligner to appear in two distinct groups: one as mentioned before and the second with BWA. The two slowest aligners in this subset of aligners were MUMmer4 and Bowtie2 in local mode, both grouped individually with MUMmer4 being faster. Only Bowtie2 (in both end-to-end and local mode) identified samples A06 and D11 as being outliers with runtimes faster than the other 46 samples. STAR identified samples C10, C07, C06, B10, and D11 as outliers having runtimes slower than the other samples and TopHat2 identified sample B10 as an outlier in the same way.

Table 3: Average Runtime per Aligner: This table shows the average runtime per reads using multiple cores as well as the pre-processing time for each aligner. The “Pre-Processing” column shows the time taken to finish building the reference genome index in seconds. Each of the “X Cores” columns show the average runtime per read using X number of cores measured in microseconds per read. Averages were calculated by summing together the runtimes of all samples and dividing by the total number of samples.

Tool	Pre-Processing (sec)	1 Core (usec/read)	2 Cores (usec/read)	3 Cores (usec/read)	5 Cores (usec/read)
Bowtie2 E2E	34.304	110.864	49.362	35.238	20.931
Bowtie2 Local	34.304	226.631	107.188	76.146	44.960
BWA	33.445	104.498	55.604	39.257	24.037
HISAT2	25.815	37.812	17.970	13.187	8.284
MUMmer4	7.958	186.901	89.819	62.898	37.699
STAR	80.416	67.452	38.866	30.412	22.888
TopHat2	34.304	508.699	345.318	276.630	221.069

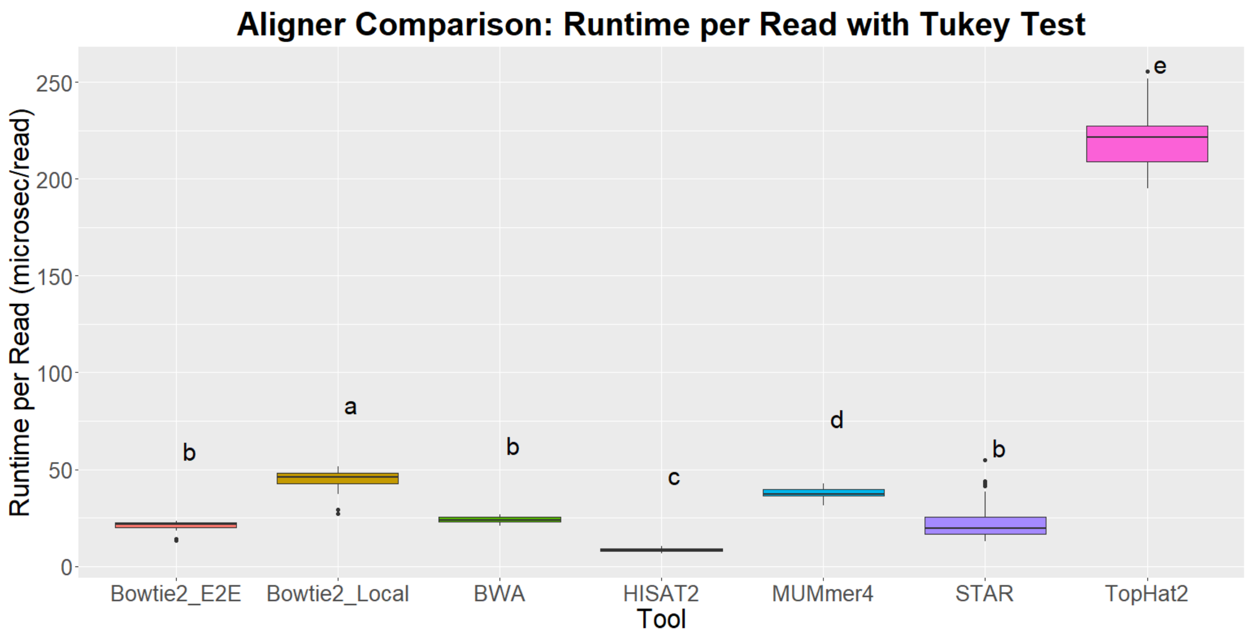


Figure 19: Boxplots for Runtime per Read with Tukey Test: This figure shows a comparative boxplot for the runtime per read distributions using 5 cores for each of the 7 aligners tested. The results of a Tukey Test using Tukey’s ‘Honest Significant Difference’ method with 95% confidence level are shown as single letters (‘a’, ‘b’, ‘c’, ‘d’, or ‘e’) placed above each boxplot. Boxplots that share the same character have average alignment rates which are indistinguishable from each other ($\mu_1 = \mu_2$). Boxplots that have different characters have average alignment rates which are significantly different from one another ($\mu_1 \neq \mu_2$).

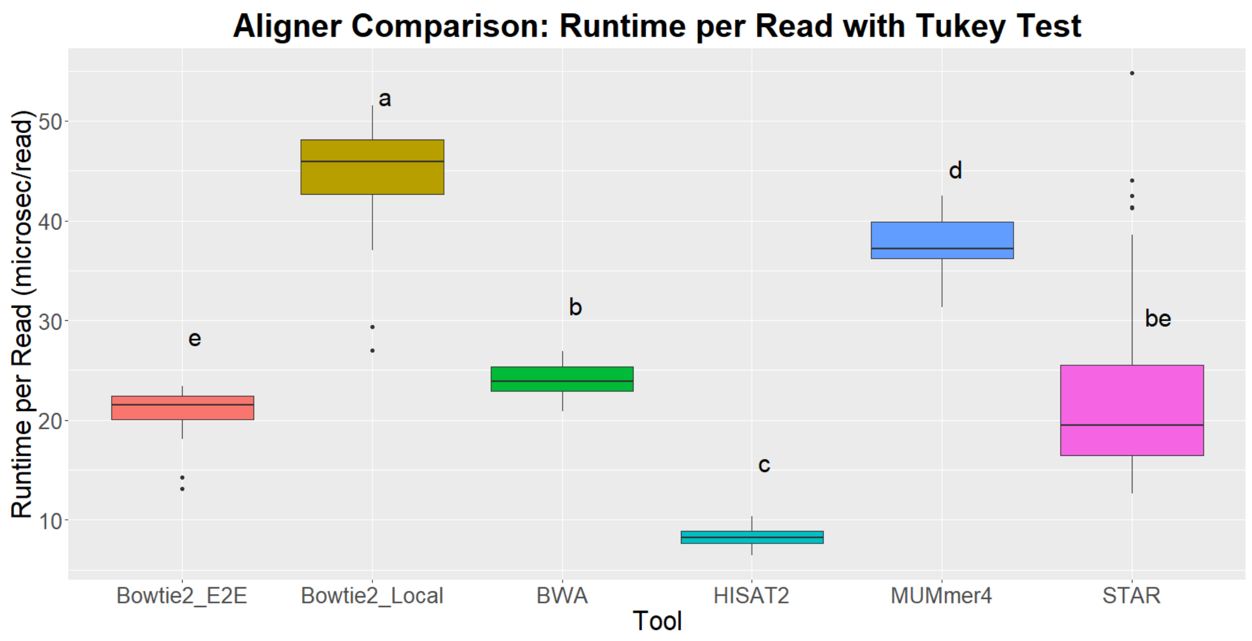


Figure 20: Excluding TopHat2 Boxplots for Runtime per Read with Tukey Test: This figure shows a comparative boxplot for the runtime per read distributions using 5 cores for each of the aligners tested excluding TopHat2 due to its distribution being an outlier. The results of a Tukey Test using Tukey's 'Honest Significant Difference' method with 95% confidence level are shown as single letters ('a', 'b', 'c', 'd', or 'e') placed above each boxplot. Boxplots that share the same character have average alignment rates which are indistinguishable from each other ($\mu_1 = \mu_2$). Boxplots that have different characters have average alignment rates which are significantly different from one another ($\mu_1 \neq \mu_2$).

To determine which aligner would perform best with an even larger number of cores, a comparison of the average speedup using 2, 3, and 5 cores were analyzed to see how well a given aligner's runtime scales with more cores (Table 4). Overall, Bowtie2 in end-to-end was the only aligner to achieve super-linear speedup on all number of cores tested. Although achieving super-linear speedup on both 2 and 5 cores, Bowtie2 in local mode had average values closer to a linear speedup. BWA, HISAT2, and MUMmer4 all achieved nearly linear speedup values overall with HISAT2 and MUMmer4 seemingly performing best when using only 2 cores (average speedup values were super-linear for 2 cores). Both STAR and TopHat2 had logarithmic speedup curves when using 5 cores, which means that the theoretical maximum speedup would be achieved by using close to 5 cores.

Table 4: Average Speedup per Aligner: This table shows the average speedup using 2, 3, and 5 cores. Speedup was calculated for each of the 48 samples by dividing the time taken on one core by the time taken by X cores. Averages were then calculated for X number of cores by summing together the speedups for all samples and dividing by the total number of samples, 48.

Tool	2 Cores	3 Cores	5 Cores
Bowtie2 E2E	2.245	3.146	5.295
Bowtie2 Local	2.114	2.977	5.040
BWA	1.879	2.663	4.348
HISAT2	2.104	2.867	4.568
MUMmer4	2.079	2.970	4.955
STAR	1.742	2.214	3.046
TopHat2	1.472	1.839	2.303

Transcriptome Coverage Assessment

Multiple tools were used in an attempt to analyze how much of the transcriptome was covered with the results for a given aligner with the first tool being BUSCO. After running BUSCO on a single sample for testing, it was determined that both the long runtime (8+ hours) and low percentage of BUSCOs (only 3.4% fragmented BUSCOs found of the 3234 BUSCOs searched for in the *Leotiomycetes* database) found was not sufficient to continue with running on more samples. Although being run with multiple cores, a large portion of BUSCO's runtime is used performing TBLASTN searches between each of the reads in the FASTA file, which involves translating the transcript into a protein sequence for all 6 reading frames. These steps are unable to be spread across multiple cores, so are always run in serial despite telling the program to use multiple cores. For this reason, an additional tool was sought out to calculate transcriptome coverage with this tool being Exonerate.

Running Exonerate on a single sample resulted in the function having a runtime of at least 16+ hours (the process was killed at the 16-hour mark without having finished). This was considered unfeasible to run on all 48 samples for all 7 aligners given the time constraints of the project, so a third tool was sought after for transcriptome coverage with this tool being HTSeq-count.

Running HTSeq-count on a single alignment file completed in under one minute but reported no features found within the SAM file. Upon looking further into the code, it was found that HTSeq-count does not perform any comparisons between the sequence content in the reads from the alignment file, but instead counts the number of times that a given transcript's name (given by the feature file) appears within the SAM file. As the alignment files were aligned using the reference genome and not the transcript file, the names present in the SAM file were those of the contigs in the reference genome file. Therefore, the transcript names did not appear in the alignment, which was the reason HTSeq-count reported all features to be missing. As the number of transcripts found was not available from these results, yet another tool was used for transcriptome coverage with this tool being BLAST+.

BLAST+ was run to align the combined and assembled reads of all 48 samples for a given aligner (for example, all 48 sample's aligned reads using BWA were combined into a single FASTA file and assembled) as a database and the Cantu Lab's *E. necator* transcriptome as a query. Combining the alignment results of all 48 files was done with the hopes to remove any potential bias of a particular sample not having a high enough expression of a gene (or not containing the gene at all), due to the samples coming from various geographic climates. A low expression for a given gene could result in the transcript either not being sequenced initially or being removed during quality control. BLAST+ results reported which of the known *E. necator* transcripts (given by the Cantu Lab's file) were found within an aligner's "database" of samples. These alignment results were then analyzed for how complete the coverage of the full transcriptome was at varying alignment length cutoffs (Figure 21). This analysis was able to compare the aligners in multiple ways: 1) overall transcriptome coverage and 2) how well an aligner was at generating transcripts of larger size (after assembling the reads of 48 "replicates").

Overall, six of the seven aligners (all but TopHat2) were able to align > ~90% of the transcriptome using alignments that were at least 100 bases long. Bowtie2 (95.6% in end-to-end and 97.1% in local) and BWA (97.8%) had the three highest coverages and were almost able to cover the full transcriptome at this cutoff. In contrast, TopHat2 had the lowest coverage using this cutoff with only 74.1% transcripts found. Interestingly, both STAR and HISAT2 had slightly higher coverage for alignment lengths > 1,000 bases than the other five aligners which all converged into nearly the same values.

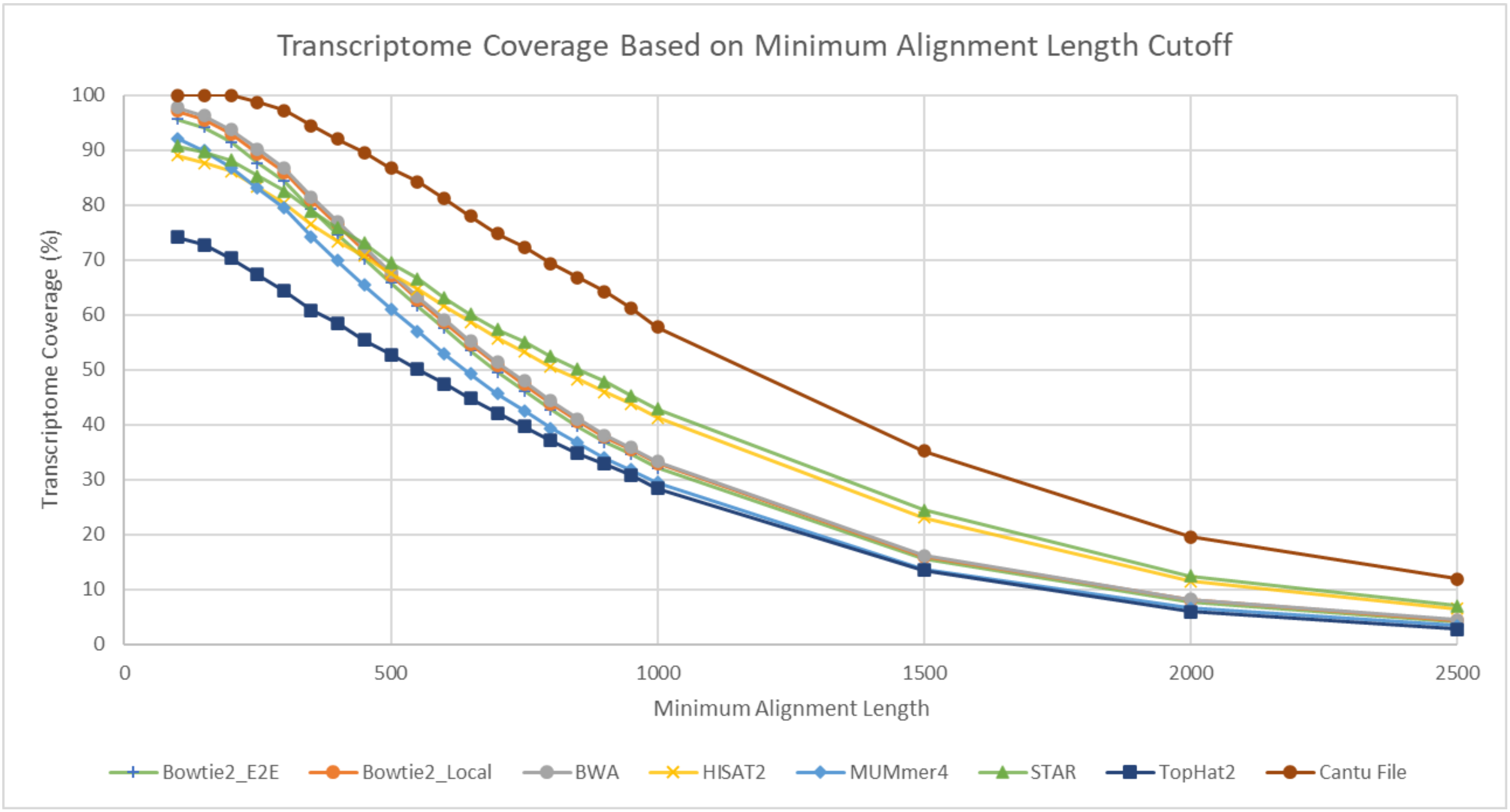


Figure 21: Transcriptome Coverage using a Minimum Alignment Length Cutoff from BLAST+ Results: This figure shows the transcriptome coverage for each aligner calculated from the BLAST+ alignment results. Coverage (y-axis) was calculated for varying alignment length cutoffs ranging from 100 to 2500 bases (x-axis) by dividing the number of BLAST+ reported alignments with length greater than or equal to the cutoff value. Each line represents a different aligner besides the "Cantu File" line, which is the theoretical maximum coverage and represents the length of transcripts greater than or equal to the cutoff value from the official *E. necator* transcriptome.

Checking for Gene Bias

After analyzing the number of genes that were mapped by each aligner, the next step was to examine those transcripts which were unmapped to determine if one aligner had difficulty aligning a particular category of genes. To do this, genes from the Cantu transcriptome that were not aligned by a certain aligner (did not have a hit from the BLAST+ analysis) were run through EggNOG-mapper in order to annotate these unmapped genes. EggNOG-mapper reports annotations from various databases, such as Kyoto Encyclopedia of Genes and Genomes and Gene Ontology, but also includes annotations from Clusters of Orthologous Groups of proteins (COG) (Tatusov *et al.*, 2000), which categorizes genes based on their function into 26 distinct categories (Table 5). To check for any bias in unmapped transcripts, the resulting COG categories were examined for overrepresentation within a given aligner. Overall, all aligners tested had similar distributions between COG categories with categories 'L' ("Replication, Recombination, and Repair") and 'S' ("Function Unknown") being overrepresented in each. The lowest number of unmapped reads was 106 from BWA with ~20% of these genes belonging to the "Replication, Recombination, and Repair" category and ~12% belonging to "Function Unknown". The highest number of unmapped reads belonged to TopHat2 with a total number of 1,570 unmapped genes resulting in ~12% belonging to "Replication, Recombination, and Repair" and ~29% belonging to "Function Unknown". Interestingly, as the number of unmapped reads increased between aligners, the percentage of genes belonging to "Replication, Recombination, and Repair" steadily decreased and the percentage of genes belonging to "Function Unknown" steadily increased (shown in Figure 22A-D; other aligners in Supplementary A1-A3). From these results, it was determined that no aligner had more trouble than another with aligning a particular category of genes as all aligners had the same categories represented. Although percentages differed between aligners, the increasing representation of the "Function Unknown" category of genes appears to be directly related to the number of unmapped genes.

To further examine if a particular gene may have been overrepresented within these categories, the unmapped genes belonging to "Replication, Recombination, and Repair" and "Function Unknown" from the results of BWA were analyzed based on their function (given as "Description" from EggNOG-mapper). For BWA, 19 of the 22 genes belonging to "Replication, Recombination, and Repair" were determined to be transposons. For the "Function Unknown" category, no particular function was overrepresented.

Table 5: COG Categories: This table shows the categories from COG and their single letter representations.

A	RNA processing and modification
B	Chromatin structure and dynamics
C	Energy Production and conversion
D	Cell cycle control, cell division, chromosome partitioning
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
G	Carbohydrate transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
J	Translation, ribosomal structure, and biogenesis
K	Transcription
L	Replication, recombination, and repair
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
O	Post-translational modification, protein turnover, and chaperones
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis, transport, and catabolism
R	General function prediction only
S	Function unknown
T	Signal transduction mechanisms
U	Intracellular trafficking, secretion, and vesicular transport
V	Defense mechanisms
W	Extracellular structures
X	Mobilome: prophages, transposons
Y	Nuclear structure
Z	Cytoskeleton

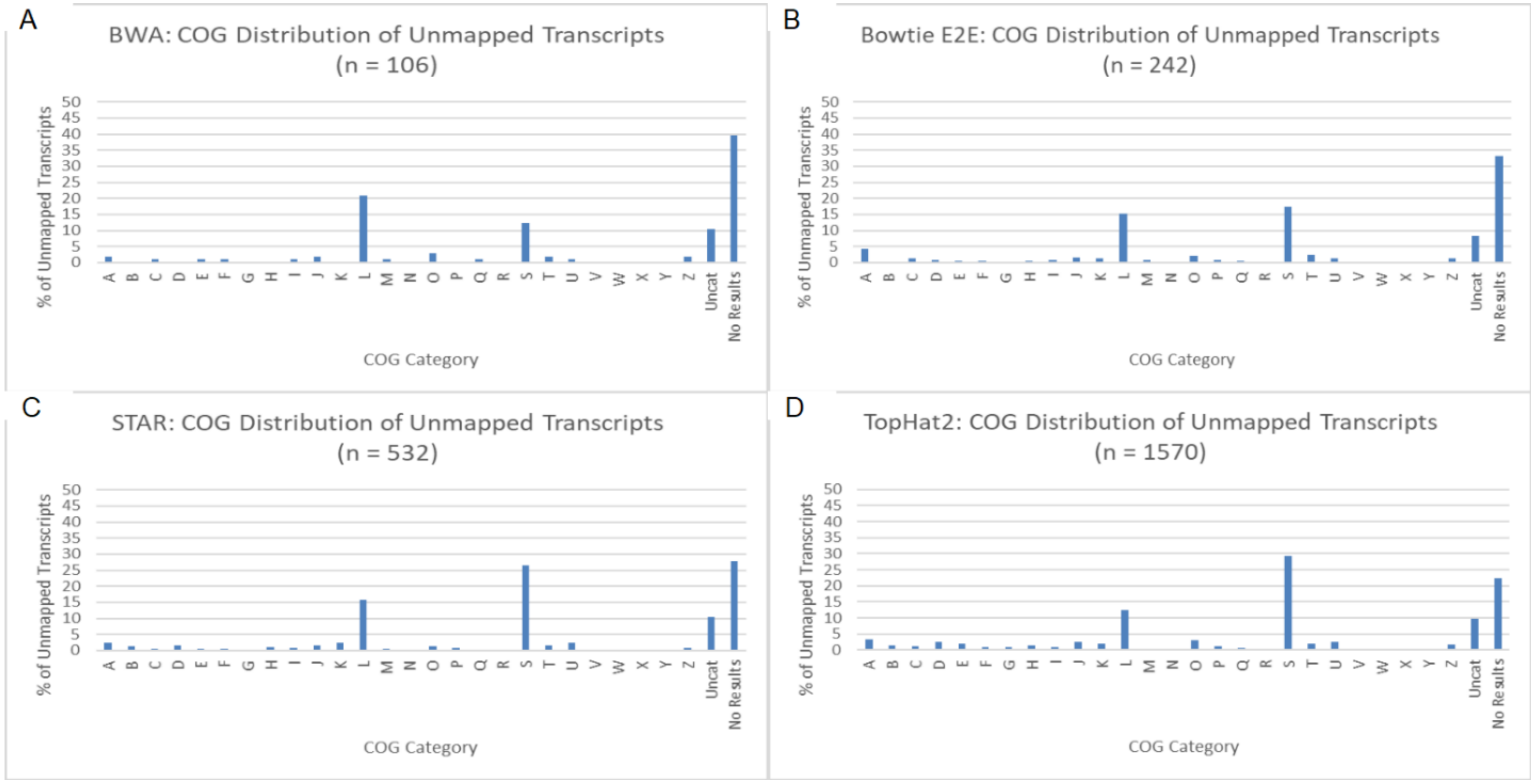


Figure 22: COG Category Distributions for Unmapped Transcripts: This figure shows the COG category distributions that are represented by the unmapped transcripts for four representative aligners. Figure A is for BWA, Figure B is for Bowtie in end-to-end mode, Figure C is for STAR, and Figure D is for TopHat2. The number of unmapped transcripts is listed as the value of 'n' in each figure's title and the value increases from Figure A (min value) to D (max value). The x-axis lists the single letter corresponding to each COG category (A-Z) along with "Uncat" (transcripts that had EggNOG-mapper results but no COG category) and "No Results" (transcripts that did not have any results from EggNOG-mapper). The y-axis represents the percentage of unmapped transcripts belonging to each category.

Discussion

The field of bioinformatics is constantly flooded with new tools claiming to be the best at what they do. Often trying to search for a tool to do the required job yields more questions than answers by giving the researcher too many options to choose from. The aim of this project was to ease that burden in terms of searching for which genome aligner performs the best. By using a consistent dataset and running the aligners on their default settings, performance for each aligner was determined by the algorithms built into the tools themselves. If the algorithms were independent of overall performance, then all genome aligners should generate the same results given the same dataset. As this study has shown, this was not the case as aligners such as TopHat2 struggled to keep up with many of the other aligners like STAR and BWA. Aligners differed in the number of reads that were successfully mapped, overall runtimes, efficiency of using multiple cores for parallel computing, and transcriptome coverage completeness.

Using Default Settings

There are two major reasons for why default settings were used in this project. The first reason being that there is an assumption being made that the developers for each aligner thoroughly tested there product and assigned default values for which the aligner performs best on most datasets. Another reason involves who is actually going to be using these types of tools out in the field. A researcher who is using a genome aligner is most likely not familiar enough with the function of each of the variables and how much of an impact changing one of these values will have on the output. Further, a majority of the time when using a tool such as a genome aligner the tool will always first be run using its default settings. If the results are sufficient enough, the researcher will move on with their work and have only ever run the tool with its default settings. If more work needs to be done at the alignment step, then extra care is taken to change variables as needed to get the intended results. Since running on the default settings is often used as an initial test of an aligner's overall performance, it is important for the tool to perform well using these settings.

Correlations Between Alignment Rate & Runtime per Read

One interesting finding during this analysis was the correlation between alignment rates and runtime per read that was discovered in a majority of the aligners. The initial hypothesis was that normalizing the runtimes by the number of reads in each sample would remove any correlation. Theoretically, a sample with 1,000 reads should take roughly 10x's longer to process than a sample with 100 reads. By removing this variable, one would assume that a sample that had a longer runtime would not necessarily result in a higher alignment rate and therefore no correlation should be found between alignment rate and runtime as seen in aligners like HISAT2 and TopHat2. However, most of the aligners tested resulted in a positive correlation between these two variables. Overall, Bowtie2 (in local and end-to-end mode) had a strong positive correlation and BWA and MUMmer4 had slightly positive correlations. The reason for a positive correlation between these two variables may simply come down to how an aligner stores an alignment. Prior to reporting all alignments for a given sample, an aligner must create a data structure to store all current alignments. Most often, an aligner has some order as to where an alignment is placed within the structure and may not simply attach the next alignment to the end of the queue as would occur in an array. As the data structure grows more complex by adding more alignments, it gets computationally more intense to traverse and add an additional alignment to its designated place in the structure. In a simple example, if one was to be given a random number and told to place that number in its correct position, it is much easier (and faster) to find this number's correct position in a list of 10 numbers than a list of 100 numbers.

Assuming that these lists of numbers are now the data structure filled with already aligned reads, finding the correct location for the next aligned read becomes more difficult and therefore requires more time as the alignment rate increases.

Additionally, STAR resulted in a negative correlation between runtime and alignment rate. This means that the longer it took to run, the lower the number of successfully mapped reads would be and ultimately letting the aligner run for a long period of time would only result in worse alignment rates. Although this seems inexplicable, if this correlation was defined in terms of the percentage of unmapped reads, a better solution presents itself. A sample with a low alignment rate inherently has a large percentage of unmapped reads. Relating this back to the initial negative correlation yields the idea that a large percentage of unmapped reads results in a longer runtime, meaning that STAR may spend longer time than other aligners at attempting to align unmappable reads. Although this seems like a poor quality for an aligner to have, it may give STAR an advantage when trying to align transcripts that are often misaligned by other aligners either due to the reads being of low quality or highly variable such as having a higher SNP rate than others. This idea is further reinforced by the fact that STAR accounts for higher variability by allowing for a greater number of mismatches in resulting alignments than other aligners. Whereas other aligners negatively weight a mismatch when calculating an alignments overall score (see the “Mismatch” column in Table 1), STAR allows for a maximum of 10 mismatched bases by default for a given alignment. In contrast, TopHat2 only allows for a maximum of 2 mismatches before terminating an alignment. This higher mismatch tolerance would allow for STAR to align more highly variable transcripts, but ultimately hinder its runtime when aligning reads in a less variable specie.

Explanation of Speedup Curves

When examining speedup curves, a tool is always judged based on how much the curve itself differs from the line y = x. This is because a linear line represents a tool running X times faster on X number of cores and therefore has an ideal linear speedup. Although ideal, linear speedup is often rare as simply adding to the number of cores being used does not guarantee that a process will run faster. Instead, the code itself must be structured accordingly to accommodate using more cores. Often there are large portions of code which can only be run in serial (on 1 corre). This results in a tool beginning with using only 1 core, then a point is reached in the process where work can be divided among multiple cores, followed by the tool normally finishing in serial. Therefore, speedup depends mainly on the runtime and complexity of these portions of code that must be run in serial. If the serial portions are time consuming as they appear to be in TopHat2 and STAR, using a large number of cores will not necessarily result in faster times, resulting in a logarithmic curve. If the code is well-structured and ready for parallelization, such as Bowtie2 in local mode, BWA, HISAT2, and MUMmer4, an ideal linear speedup is achieved.

In other cases, such as Bowtie2 in end-to-end mode, super-linear speedup is found which results in a tool performing > X times faster on X cores. Super-linear speedup is most often achieved due to parallel computing taking advantage of an increased cache of memory outside of the constantly available random access memory (RAM) (Ristov *et al.*, 2016). For example, say that a computer has 5 available processing cores that can each hold 1 MB of data resulting in a total cache of 5 MB. Next, assume that the data being used requires 4 MB to run to completion. If only one core was being used, the machine only has access to 1 MB of the data at a time. The other 3 MB of data are stored in RAM. When the first 1 MB of data has been processed, the next

1 MB must be transferred between RAM and the central processing unit (CPU). If all 5 cores were being utilized, all 5 MB of data could be processed at once without the use of RAM and therefore the additional step of transferring data between RAM and CPU would not be required (data must still be transferred between CPU and memory cache but RAM is not required). This increase in available cached memory by using multiple cores is often the most logical reason for super-linear speedup.

Sample Outliers

Across all aligners tested, 46 of the 48 samples appeared to cluster well together in terms of their runtime and alignment rate. Interestingly, the same two samples, A06 and D11, were listed as outliers for nearly every aligner with most aligners finding lower alignment rates for these samples compared to the others. As the 48 samples of *E. necator* were all from geographically distinct locations, one could assume that these two samples were from the same region and perhaps genetically distinct from the reference genome used. Although both samples came from the southern region of the United States, sample A06 came from the more central United States in Purdy, Missouri whereas sample D11 came from the eastern United States in Mocksville, North Carolina (see Supplementary S4). Additionally, these samples were not the only samples used in this project from these respective areas. There were 2 other samples from the state of Missouri and 7 other samples from North Carolina. It was also suspected that these samples may have had two of the smallest number of reads after quality control and were therefore at a disadvantage initially prior to alignment. However, both samples had nearly the same number of reads (A06 had 741,883 and D11 had 737,552) with these counts being nearly equivalent to the average read count of 741,219. Aside from being technical outliers, there appears to be no biological reason why these samples continuously had lower alignment rates than the others.

Transposon Bias

After checking for a bias in the type of genes which were unable to be aligned, it was discovered that a large portion of these genes were transposons. Although it may seem that an aligner's struggle with aligning transposons comes from the gene's function, it most likely has to do with overall sequence of the gene and has little to do with function if at all. Transposable elements have caused trouble for researchers trying to align them in the past due to their sequences being made up of large sections of repeatable elements. Aligners often struggle with aligning repeatable sequences as these reads often are aligned to multiple locations in the genome. Some aligners will weight an alignment negatively if a read is assigned to multiple locations and therefore may not report the resulting alignment to the user. Other aligners, such as most of the aligners tested in this project, report a large number of potential alignments and will list separately in their output that "X number of reads were aligned to >1 location". However, even listing multiple alignments does not seem to be enough to aid in the alignment of transposons. Teissandier *et al.* discovered that using paired end data (single end was used in this project) results in a better mapping rate of transposons as well as having aligners report multiple hits for every read aligned (Teissandier *et al.*, 2019). In other words, it is not uncommon for a study such as this to identify a large number of unmapped transposons.

"Best" Aligner

To determine which aligner performed the "best", a few clarifications must first be made. First, aligners behave differently when using different datasets. This could be due to biological differences such as GC content and proportion of repeated elements, which was discussed previously as being troublesome for aligners. Another difference guaranteed to make an impact is a more technical issue with the overall quality of the dataset being used. The dataset used in this project had very poor quality and attempts to clean up the reads saw roughly 80% of reads removed. It is possible for a much cleaner dataset having much different results then the ones portrayed here. Therefore, the results of this project can be seen as which aligner performed best on this dataset for *E. necator*. Second, with fine tuning of the parameters, it is possible for other aligners to outperform one another. This project worked under the assumption that the creators of each tool allowed for the default settings to result in the best quality results for an aligner. Additionally, RNA-seq based aligners, such as HISAT2, STAR, and TopHat2, all were run without the use of an annotated transcriptome. It is speculated that running these aligners with an annotation file results in higher mapping rates, however, to keep all aligners on the same level, it was decided against for this project.

Deciding on which aligner performed the best came down to which tool achieved the best balance of the two major categories: speed and accuracy. Analyzing the results as is made deciding on which aligner was the "best" difficult. In contrast, the worst aligner on this dataset was TopHat2, which had the longest runtimes and the lowest alignment rate by a large margin. To better illustrate the major differences between all aligners tested, a figure was generated (which excluded TopHat2 to show a greater difference between individual aligners) to directly compare the average runtime per read with the average alignment rate for a given aligner (Figure 23). The first conclusion drawn from this figure was that Bowtie2 in local mode and BWA had a near tie for the highest alignment rate, however BWA's lesser runtime results in the tool being preferable. Similar reasoning was applied to determining STAR being a better tool to use over MUMmer4 and HISAT2 over Bowtie2 in end-to-end mode. When comparing BWA to STAR, both aligners had similar runtimes, however BWA had on average a higher alignment rate. This narrowed the field of aligners down to two: BWA with high alignment rate and HISAT2 with a fast runtime. To decide which of these two variables is more important, the BLAST+ results for transcriptome coverage come into play (Figure 21). Although BWA had ~20% higher alignment rates on average, HISAT2 not only achieved approximately the same coverage of the transcriptome but also generated a higher proportion of larger alignments. This result suggests that having a higher alignment rate may not be as important than a faster runtime. For this reason, it was determined that HISAT2 was the best performing aligner on this dataset.

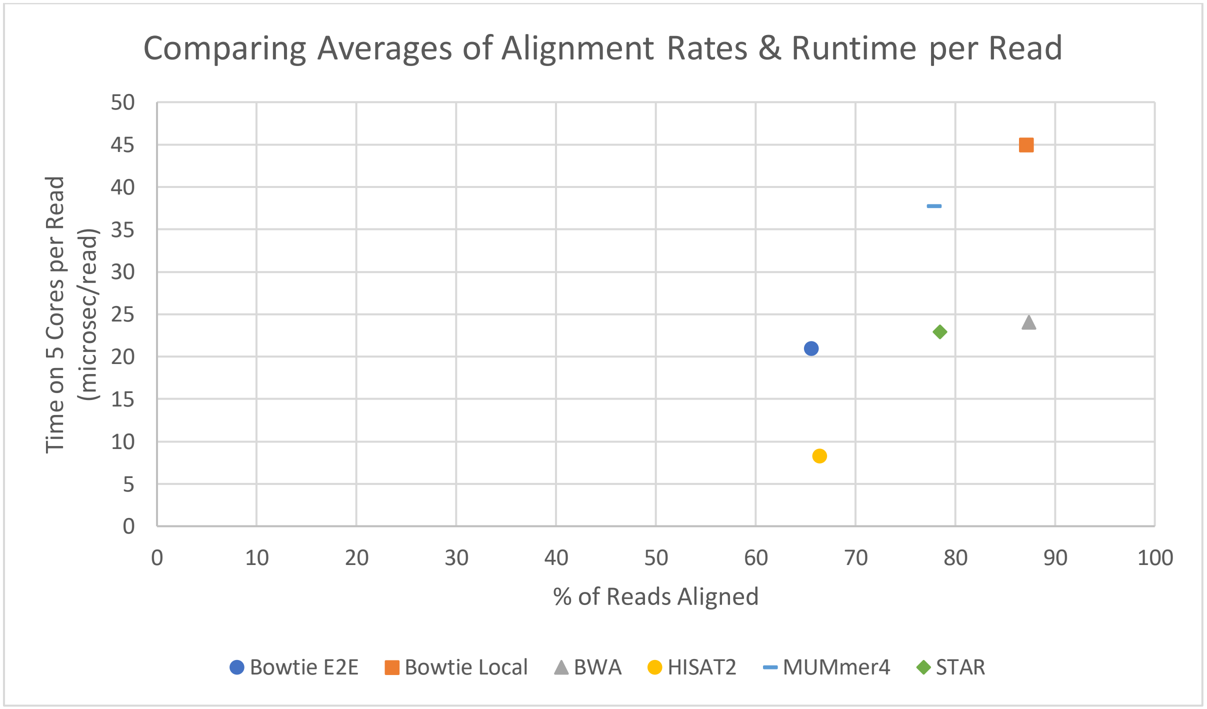


Figure 23: Average Alignment Rate vs Average Runtime per Read: This figure illustrates the comparison of averages for runtime per read on 5 cores (y-axis) and alignment rate (x-axis) for all aligners (except TopHat2).

Comparing with Other Studies

To determine if the results found in this study were simply in outlier, the results from other similar benchmarking studies were surveyed. Although a crucial step in the pipeline for RNA-seq analysis, there have not been many studies performed which analyze the performance of the aligners themselves as many studies choose to compare tools used downstream when performing differential expression. Of the few studies found, no particular study used the same exact set of aligners, however most of them contained at least two of the tools which allowed for some comparative conclusions to the project performed here. For example, many of the studies found that STAR almost always outperformed TopHat2 in terms of higher alignment rates as well as reporting less mismatches in the overall alignments (Engström *et al.*, 2013; Teng *et al.*, 2016). Additionally, TopHat2 was found to perform better (higher alignment rates) when using an annotation file (Engström *et al.*, 2013). Similar to this project, one study found that HISAT2 outperformed both STAR and BWA in regards to runtime by a factor of 1.7 and 3.4 respectively (Lachmann *et al*., 2020). This same study also found that although BWA recorded the highest gene counts (similar to the higher alignment rates reported here) it was determined during differential expression analysis that BWA recorded the lowest number of enriched biological terms (Lachmann *et al*., 2020). This finding confirms the conclusion for the project outlined in this paper that having a high alignment rate does not necessarily guarantee better results as found by the similar transcriptome coverages for all aligners.

Future Work

To officially determine which aligner is the best, a wide variety of datasets must be used consisting of datasets for various organisms, datasets ranging from low to high quality, etc. Projects similar to this one could be performed by comparing an aligners ability to process data of different quality and from other organisms to determine if the results found for aligning average quality reads for *E. necator* (as performed here) are comparable. It is possible that an aligner such as TopHat2 only performs well with extremely high-quality data, which would drastically increase its alignment rate but also makes the tool impractical to use as extremely high-quality data is expensive to produce. Also, STAR, TopHat2, and HISAT2 were theoretically run at a disadvantage to keep them on the same level as the other aligners tested that were unable to accept annotation files. All three of these aligners are specifically designed to align RNA-seq reads to a reference genome with added help from an annotated transcriptome for the same species. Assuming that running these aligners with a transcriptome attached would increase alignment rates, it may result in an aligner such as STAR surpassing BWA in terms of average alignment rate. Additionally, it may increase HISAT2's alignment rate, further separating it from the other aligners. Another area for future work comes from the fact that tools for bioinformatics are constantly being updated, so it is inevitable that an aligner such as HISAT3 or STAR2 should eventually release with the authors claiming that this new tool/version is the "best" aligner available. Adding the results from that new aligner to a project such as this would truly determine how much better the aligner is against its competition.

Works Cited

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Appendix A: Supplementary Figures & Tables

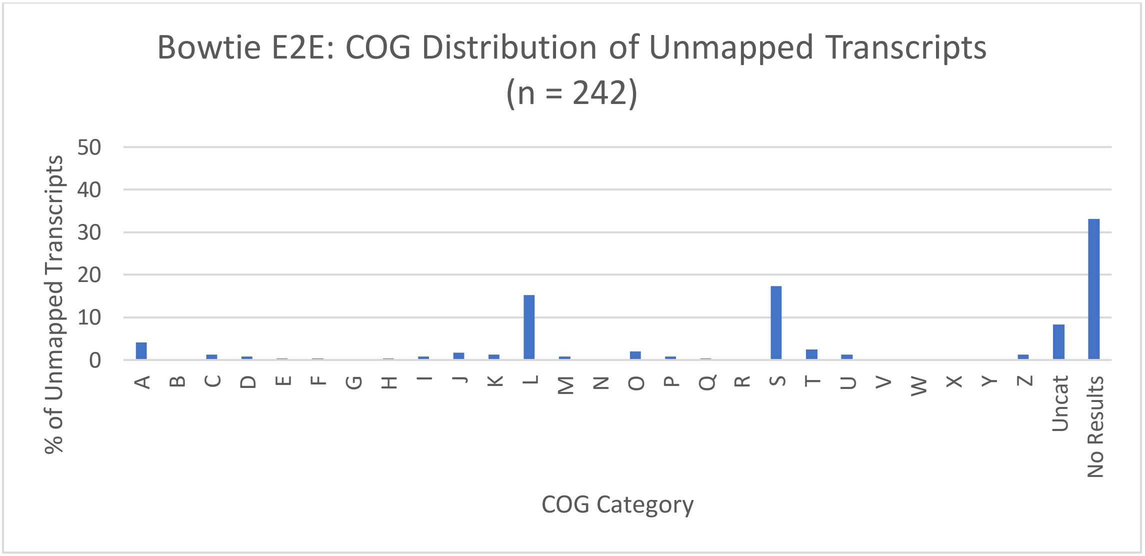

Figure S1: COG Category Distribution for Unmapped Transcripts for Bowtie2 in End-to-End Mode: This figure shows the COG category distributions that are represented by the unmapped transcripts for Bowtie2 in end-to-end mode. The number of unmapped transcripts is listed as the value of 'n' in the title. The x-axis lists the single letter corresponding to each COG category (A-Z) along with "Uncat" (transcripts that had EggNOG-mapper results but no COG category) and "No Results" (transcripts that did not have any results from EggNOG-mapper). The y-axis represents the percentage of unmapped transcripts belonging to each category.

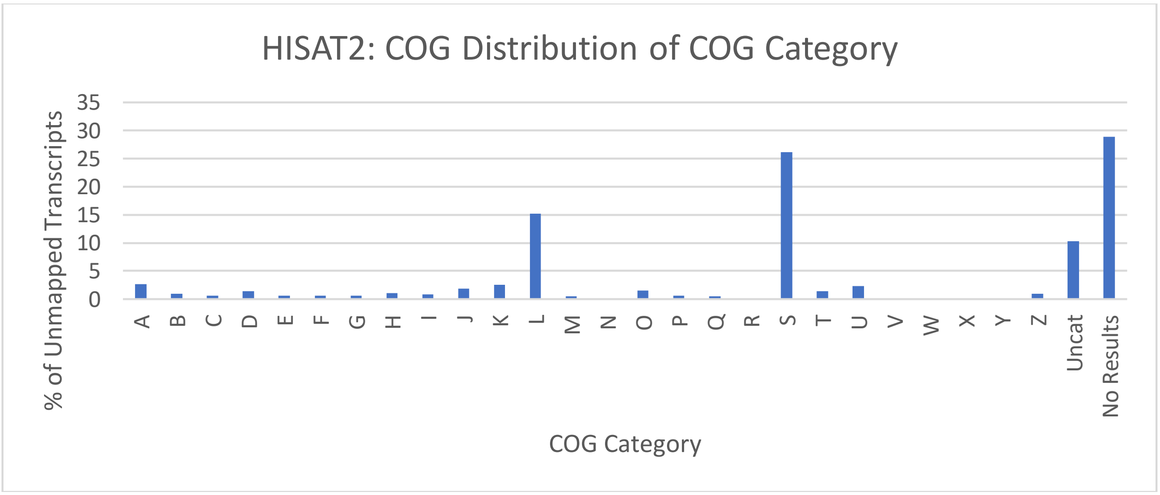

Figure S2: COG Category Distribution for Unmapped Transcripts for HISAT2: This figure shows the COG category distributions that are represented by the unmapped transcripts for HISAT2. The number of unmapped transcripts is listed as the value of 'n' in the title. The x-axis lists the single letter corresponding to each COG category (A-Z) along with "Uncat" (transcripts that had EggNOG-mapper results but no COG category) and "No Results" (transcripts that did not have any results from EggNOG-mapper). The y-axis represents the percentage of unmapped transcripts belonging to each category.

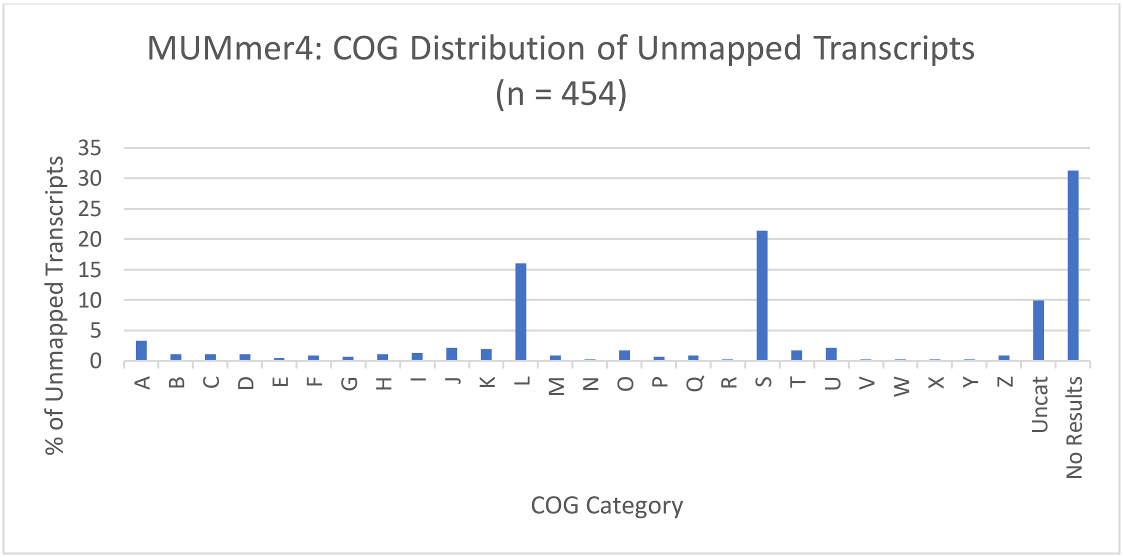

Figure S3: COG Category Distribution for Unmapped Transcripts for MUMmer4: This figure shows the COG category distributions that are represented by the unmapped transcripts for MUMmer4. The number of unmapped transcripts is listed as the value of 'n' in the title. The x-axis lists the single letter corresponding to each COG category (A-Z) along with "Uncat" (transcripts that had EggNOG-mapper results but no COG category) and "No Results" (transcripts that did not have any results from EggNOG-mapper). The y-axis represents the percentage of unmapped transcripts belonging to each category.

Table S4: Sample Locations: This table shows the geographic location where each sample originated (Cadle-Davidson, personal communication).

Sample ID	**Location**
A01	St. James, MO
A02	Comanche, TX
A03	Hurdle Mills, NC
A04	Chile
A05	Blue Ridge, NC
A06	Purdy, MO
A07	Chile
A08	Panther Creek Falls, GA
A09	Blood Mountain, GA
A10	Watkins Glen, NY
A11	Fredonia, NY
A12	Chile
B01	Lockport, NY
B02	Riverhead, NY
B03	Highland, NY
B04	Lockport, NY
B05	Watkins Glen, NY
B06	East Brunswick, NJ
B07	Chile
B08	Lansing, NY
B09	Dresden, NY

B10	DeSoto Falls, GA
B11	Watkins Glen, NY
B12	Waverly, MO
C01	Tryon, NC
C02	Winchester, VA
C03	Burdett, NY
C04	Chile
C05	Asheville, NC
C06	Panther Creek Falls, GA
C07	Chile
C08	Winchester, VA
C09	Burdett, NY
C10	Emporia, KS
C11	Pittsboro, NC
C12	Burdett, NY
D01	Chile
D02	Blairsville, GA
D03	Hurdle Mills, NC
D04	Chile
D05	Athens,NY
D06	Highland, NY
D07	Winchester, VA
D08	Lawrence, KS
D09	Blood Mountain, GA
D10	Winchester, VA
D11	Mocksville, NC
D12	Geneva, NY

Appendix B: Supplemental Code

B1: preprocess_bowtie2.sh:

```
#!/bin/bash
#Running Bowtie2 Pre-Processing

time /usr/local/bin/bowtie2-2.3.5.1-linux-x86_64/bowtie2-build e-necator.scaffolds.c.NCBI.fasta
e_necator
```

B2: run_bowtie2.sh:

```
#!/bin/bash
#Running Bowtie2 End-to-End or Local

#Create empty files for Times and Accuracy
echo > Alignments/Bowtie_E2E/1_Core/Times.txt
echo > Alignments/Bowtie_E2E/1_Core/Accuracy.txt

#Iterate through sample files
for file in $(ls trimmed_seqs/); do
        echo "Starting $file"
        result=$(echo ${file} | cut -c 7-9)

        echo ${result} >> Alignments/Bowtie_E2E/1_Core/Times.txt
        echo ${result} >> Alignments/Bowtie_E2E/1_Core/Accuracy.txt

        #Runs Bowtie2 in End-to-End and stores alignments in sample.sam
        #Moves time results from stdout to Time file
        #Moves Bowtie2 output from stdout to Accuracy file
        { time /usr/local/bin/bowtie2-2.3.5.1-linux-x86_64/bowtie2 -x e_necator
trimmed_seqs/$file -S Alignments/Bowtie_E2E/1_Core/$result.sam -p 1 2>>
Alignments/Bowtie_E2E/1_Core/Accuracy.txt ; } 2>>
Alignments/Bowtie_E2E/1_Core/Times.txt

        #Runs Bowtie2 in Local
        #{ time /usr/local/bin/bowtie2-2.3.5.1-linux-x86_64/bowtie2 -x e_necator
trimmed_seqs/$file -S Alignments/Bowtie_Local/1_Core/$result.sam -p 1 --local 2>>
Alignments/Bowtie_Local/1_Core/Accuracy.txt ; } 2>>
Alignments/Bowtie_Local/1_Core/Times.txt

        echo >> Alignments/Bowtie_E2E/1_Core/Times.txt
        echo >> Alignments/Bowtie_E2E/1_Core/Accuracy.txt

        echo "Finished $file"
done;
```

B3: preprocess_bwa.sh:

```
#!/bin/bash
#Running BWA Pre-Processing

time /usr/local/bin/bwa index -p e_necator e-necator.scaffolds.c.NCBI.fasta
```

B4: run_bwa.sh:

```
#!/bin/bash
#Running BWA

#Create empty files for Times
echo > Alignments/BWA/1_Core/Times.txt

#Iterate through sample files
for file in $(ls trimmed_seqs/); do
        echo "Starting $file"
        result=$(echo ${file} | cut -c 7-9)

        echo ${result} >> Alignments/BWA/1_Core/Times.txt
        echo ${result} >> Alignments/BWA/1_Core/Accuracy.txt

        #Runs BWA and stores alignments in sample.sam
        #Moves time results from stdout to Time file
        #Moves BWA output from stdout to tmp.txt
        { time /usr/local/bin/bwa mem -t 1 e_necator trimmed_seqs/$file >
Alignments/BWA/1_Core/$result.sam 2>> tmp.txt ; } 2>>Alignments/BWA/1_Core/Times.txt

        echo >> Alignments/BWA/1_Core/Times.txt

        echo "Finished $file"

done;
```

B5: preprocess_hisat2.sh:

```
#!/bin/bash
#Running HISAT2 Pre-Processing

time /usr/local/bin/hisat2/hisat2-build e-necator.scaffolds.c.NCBI.fasta e_necator
```

B6: run_hisat2.sh:

```
#!/bin/bash
#Running HISAT2

#Create empty files for Times and Accuracy
echo > Alignments/HISAT2/1_Core/Times.txt
echo > Alignments/HISAT2/1_Core/Accuracy.txt

#Iterate through sample files
for file in $(ls trimmed_seqs/); do
        echo "Starting $file"
        result=$(echo ${file} | cut -c 7-9)

        echo ${result} >> Alignments/HISAT2/1_Core/Times.txt
        echo ${result} >> Alignments/HISAT2/1_Core/Accuracy.txt

        #Runs HISAT2 and stores alignments in sample.sam
        #Moves time results from stdout to Time file
        #Moves BWA output from stdout to Accuracy file
        { time /usr/local/bin/hisat2/hisat2 -x e_necator trimmed_seqs/$file -S
Alignments/HISAT2/1_Core/$result.sam -p 1 2>> Alignments/HISAT2/1_Core/Accuracy.txt ; }
2>> Alignments/HISAT2/1_Core/Times.txt

        echo >> Alignments/HISAT2/1_Core/Times.txt
        echo >> Alignments/HISAT2/1_Core/Accuracy.txt

        echo "Finished $file"

done;
```

B7: preprocess_mummer.sh:

```
#!/bin/bash
#Running pre-processing for MUMmer4

time /usr/local/bin/mummer4/bin/nucmer --save=e_necator e_necator.fa
```

B8: run_mummer.sh:

```
#!/bin/bash
#Run MUMmer4

#Create empty file for Times
echo > Alignments/mummer/1_Core/Times.txt

#Iterate through sample files
for file in $(ls trimmed_seqs/); do
	echo "Starting $file"
	result=$(echo ${file} | cut -c 7-9)

	echo ${result} >> Alignments/mummer/1_Core/Times.txt

	#Runs MUMmer4 and stores alignments in sample.sam
	#Moves time results from stdout to Time file
	{ time /usr/local/bin/mummer4/bin/nucmer --load=e_necator --sam-
long=Alignments/mummer/1_Core/${result}.sam --threads=1 e-necator.scaffolds.c.NCBI.fasta
trimmed_seqs/${file} ; } 2>> Alignments/mummer/1_Core/Times.txt

	echo >> Alignments/mummer/1_Core/Times.txt

	echo "Finished $file"

done;
```

B9: preprocess_STAR.sh:

```
#!/bin/bash
#Run pre-processing for STAR

time /usr/local/bin/STAR/STAR --runMode genomeGenerate --genomeDir
/home/ryan/STAR_Genome --genomeFastaFiles e-necator.scaffolds.c.NCBI.fasta
```

B10: run_STAR.sh:

```
#!/bin/bash
#Run STAR

#Create empty files for Times
echo >> Alignments/STAR/1_Core/Times.txt

#Iterate through sample files
for file in $(ls trimmed_seqs/); do
        echo "Starting $file"
        result=$(echo ${file} | cut -c 7-9)

        echo ${result} >> Alignments/STAR/1_Core/Times.txt

        #Runs STAR and stores alignments in sample.sam
        #Moves time results from stdout to Time file
        #Moves STAR output from stdout to tmp file
        { time /usr/local/bin/STAR/STAR --genomeDir /home/ryan/Thesis/STAR_Genome/ --
runThreadN 1 --readFilesIn trimmed_seqs/$file --outFileNamePrefix
Alignments/STAR/1_Core/${result}_ 2>> Alignments/STAR/1_Core/tmp.txt ; } 2>>
Alignments/STAR/1_Core/Times.txt

        #Changes output SAM name to sample.sam
        mv Alignments/STAR/1_Core/${result}_Aligned.out.sam
Alignments/STAR/1_Core/${result}.sam

        echo >> Alignments/STAR/1_Core/Times.txt

        echo "Finished $file"

done;
```

B11: run_tophat.sh:

```
#!/bin/bash
#Running TopHat2

export PATH=/usr/local/bin/bowtie2-2.3.5.1-linux-x86_64:$PATH
export BOWTIE_INDEXES=/home/ryan/Thesis

#Creates empty files for Times and Accuracy
echo > Alignments/Tophat/1_Core/Times.txt
echo > Alignments/Tophat/1_Core/Accuracy.txt

#Iterates through sample files
for file in $(ls tmp_seqs/); do
	echo "Starting $file"
	result=$(echo ${file} | cut -c 7-9)

	echo ${result} >> Alignments/Tophat/1_Core/Times.txt
	echo ${result} >> Alignments/Tophat/1_Core/Accuracy.txt

	#Runs TopHat2 and stores alignments in sample.sam
	#Moves time results from stdout to Time file
	#Moves TopHat2 output from stdout to Accuracy file
	{ time /usr/local/bin/tophat2/tophat -o Alignments/Tophat/1_Core/${result} -p 1 e_necator
tmp_seqs/$file 2>> Alignments/Tophat/1_Core/Accuracy.txt ; } 2>>
Alignments/Tophat/1_Core/Times.txt

	echo >> Alignments/Tophat/1_Core_Anno/Times.txt
	echo >> Alignments/Tophat/1_Core_Anno/Accuracy.txt

	echo "Finished $file"

done;
```

B12: fix_mummer_sam.sh:

```
#!/bin/bash
#Add correct header to MUMmer SAM files

#Creates empty file for Accuracy
echo > Accuracy.txt

#Creates empty directory for BAM and fixed SAM
mkdir Alignments/mummer/BAM
mkdir Alignments/mummer/SAM_2.0

#Iterates through bad SAM files
for file in $(ls Alignments/mummer/SAM/); do
	echo "Starting $file"
	result=$(echo ${file} | cut -c 1-3)

	echo ${result} >> Accuracy.txt

	#Adds correct header to MUMmer4 SAM files
	grep '@HD' Alignments/mummer/SAM/${file} > tmp_${result}.sam
	grep '@SQ' BWA_A01.sam >> tmp_${result}.sam
	grep '@PG' Alignments/mummer/SAM/${file} >> tmp_${result}.sam
	grep -v '@HD' Alignments/mummer/SAM/${file} > tmp.txt
	grep -v '@PG' tmp.txt >> tmp_${result}.sam

	mv tmp_${result}.sam Alignments/mummer/SAM_2.0/${result}.sam

done;
```

B13: SAMtoFA.sh:

```
#!/bin/bash
#Converts SAM to FASTA file

#Iterate through SAM files
for file in $(ls Alignments/BWA/SAM); do
        echo "Starting $file"
        result=$(echo ${file} | cut -c 1-3)

        #Converts SAM to BAM file
        samtools view -S -b Alignments/BWA/SAM/$file > Alignments/BWA/BAM/${result}.bam

        #Sorts the BAM file
        samtools sort Alignments/BWA/BAM/${result}.bam -o Alignments/BWA/BAM/tmp.bam

        mv Alignments/BWA/BAM/tmp.bam Alignments/BWA/BAM/${result}.bam

        #Converts BAM to FASTA file
        samtools fasta Alignments/BWA/BAM/${result}.bam >
Alignments/BWA/FASTA/${result}.fa

        echo "Finished $file"
done;
```