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New algorithmic and bioinformatic approaches for the analysis of data from high throughput sequencing

Evguenia Kopylova

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Algorithmes bio-informatiques pour l'analyse de données de séquençage à haut débit

*New algorithmic and bioinformatic approaches for the
analysis of data from high throughput sequencing*

THÈSE

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(spécialité informatique)

par

Evguenia KOPYLOVA

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Papers

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1. E. Kopylova, L. Noé and H. Touzet. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics*, 28(24):3211-7, 2012.

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3. E. Kopylova, L. Noé, M. Salson and H. Touzet. Universal read mapping of genomic and metagenomic data using SortMeDNA. (Submitted).

Introduction

A genome is a sequence of DNA nucleotides which encodes complex instructions to build and develop an organism, with a copy existing in every cell of the organism's body. Although an organism will commence with a genome inherited from its parents, throughout its lifetime, the genome will frequently experience changes, known as *mutations*, in the nucleotide sequence during replication and in response to the surrounding environment. These changes can manifest as single nucleotide polymorphisms (SNP) or through insertions or deletions of nucleotides in the genome. *Somatic* mutations occur in the non-reproductive cells of an organism and are not transmitted to the offspring. *Germ-line* mutations, on the other hand, occur in the gamete cells of an organism and are hereditary. If a mutation occurs in a protein coding region of the genome, it may alter the behavior of the protein and ordinarily cause harm to the organism. For example, a deletion of three nucleotides, namely the amino acid phenylalanine, in the human gene CFTR causes irregular folding of the synthesized protein and leads to a critical genetic disorder known as cystic fibrosis. Being able to discover, understand and cure such detrimental changes is one of the leading reasons for which biological sequence analysis is indispensable to humanity. On the contrary, sometimes mutations can lead to favorable changes, as for example the single nucleotide mutation which disables the production of the toxic hydrogen cyanide found in wild almonds, yielding to the sweet domesticated almonds popularly consumed today [Heppner, 1923].

Even more, the development of revolutionary sequencing technologies used to unveil the sequences of DNA molecules can now be applied to entire microbial communities in an exciting new field named *metagenomics*. One influential application of metagenomics is the diagnosis of pathogenic diseases, which can be used to detect candidate pathogens responsible for an infection, such as was shown in the discovery of bacteria *C. jejuni* causing acute diarrhea in an infected patient [Nakamura et al., 2008]. Another study in [Chan et al., 2013] focused on the stress responses of microbial communities in the McMurdo Dry Valleys of Antarctica in order to identify functional traits that motivate prosperity of these communities in the much threatened biome. In reality, applications of biological sequence analysis are innumerable, and new software and technologies are being developed every day to facilitate their studies.

The layout of nucleotides in a DNA molecule can be determined using sequencing technologies which exploit advanced techniques to read and record each nucleotide one letter at a time. The principal limitation to all of these methods is that the sequence cannot be read in one go, but must be randomly split into millions of overlapping fragments which are individually sequenced to produce *reads*. In the final step, the sequenced reads are either reassembled back into the original sequence, an approach known as *de novo* assembly, or aligned against an existing reference sequence, known as *read mapping*, using appropriate bioinformatic tools. With the arrival of next-generation sequencing technologies, gigabytes of DNA material can be sequenced in parallel within very short time periods [Qin et al., 2009]. Big data brings along big

challenges for the management and analysis of millions (to even billions) of short sequenced reads [Wandelt et al., 2012]. Moreover, different read lengths and sequencing error rates must be taken into account when developing bioinformatic tools applicable to data from multiple sequencing platforms. The objective of this thesis is to design and implement new algorithms for the analysis of data produced by (next-generation) high-throughput sequencing technologies (HTS). More precisely, we concentrate on algorithms for performing genomic sequence alignment, a common application in whole-genome resequencing, and also for aligning metatranscriptomic and metagenomic data, that is the RNA and DNA belonging to thousands of different species directly extracted from their environment.

Metatranscriptomics is the study of total RNA extracted from microorganisms in a community. Total RNA is a composition of many subgroups including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and other small non-coding RNA. Given that the former three RNA groups (rRNA, mRNA and tRNA) are chief participants in the synthesis of proteins, studying them can help to determine the activity and composition of the constituting species. The concentration and diversity of mRNA present in a sample can shed light on the actively expressed genes at the period of sampling. Changes in the environment, such as nutrient availability, temperature fluctuations and stress, can heavily influence normal gene expression. By tracking these changes, scientists can identify the cause and effect of threatened or thriving ecosystems and take measures to restore them. Another group of closely studied RNA is rRNA, which makes up to 90% of total RNA and is responsible for translating mRNA into protein. Ribosomal RNA is an ideal candidate for taxonomic analyses due to its presence in all cells and highly conserved structure. Depending on the question asked, it is often required that the sequenced reads from total RNA are sorted into their appropriate subgroups prior to further analysis. In this thesis, we introduce a new software tool called SortMeRNA to rapidly filter rRNA reads from metatranscriptomic data using a reference database of known rRNAs.

Another leading application of NGS is metagenomics, the study of total DNA retrieved from an environment. Whereas metatranscriptomic data renders information on the actively transcribed genes in a community, metagenomics provides the entire collection of genes and all other noncoding DNA within. The main goal of metagenomics is to obtain the complete genome sequences of all organisms in order to study their individual roles, whole-genome species diversity and symbiotic relationships of a community. Nowadays, metagenomic studies are popular in medicine, agriculture, biotechnology and environmental genomics. Global projects such as Tara Oceans or Tara Oceans Polar Circle endeavor on yearly expeditions to study marine ecosystems by collecting plankton from waters all over the world. An important subgroup of marine plankton, phytoplankton, is responsible for producing half of the total oxygen on earth, consequently changes to its population will directly affect all other organisms higher in the food chain hierarchy. In [Boyce et al., 2010], it has been reported that nearly 40% of surface phytoplankton in the northern hemisphere have vanished since 1950, and our understanding of how to replenish this loss is vital to sustain a healthy planet. MetaHIT [Ehrlich, 2011] is another metagenomic project focusing on the human intestinal microbiota and its connection to health and disease. The MetaHIT consortium hosts a broad database of publicly available draft genomes and partial sequences for the bacteria commonly found in the human intestinal tract. One of the greatest challenges in such large-scale projects is the ability to store, sort through and analyze the enormous amounts of sequenced data. In this thesis, we introduce a second software tool called SortMeDNA, which has been designed to align large-scale sequenced data against a database of known reference sequences with accuracy and speed.

Life on planet earth evolves at an accelerated pace as a result of our strong curiosity and innate

vivacity for growth and expansion. Understanding the biological building blocks of organisms is not only useful for modern day activities, but can be in the future applied to stimulating life on other planets. The Space Biosciences Division at NASA is actively exploring areas of research such as atmosphere revitalization, sources for biofuel [Buckwalter et al., 2013] and biological systems capable of self-producing natural resources. Given the hostile environments on other planets such as Mars, any signs of life would probably resemble single-celled microorganisms such as archaea known today [Morozova et al., 2007], therefore their current study through metatranscriptomics and metagenomics may one day offer advantages beyond our own world.

* * *

Contribution

The works of this thesis were supported by the ANR Project MAPPI (grant ANR-2010-COSI-004). Project MAPPI is a three-year collaboration between the teams LIAFA at the University of Paris-Diderot, IRISA in Rennes and the French National Sequencing Center (Genoscope) in Évry to develop bioinformatic tools for the analysis of metatranscriptomic and metagenomic data produced by the Tara Oceans expedition.

In this thesis we develop a new type of *approximate* seed allowing up to 1 error: The error can either be a mismatch or an indel, and its position in the seed is not predetermined. This unique feature gives the seed flexibility for different error types, such as indels in 454 reads, unpredictable error distribution, as readily observed with PacBio reads and capturing similarities between distant related species. Furthermore, we introduce an indexing data structure specifically tailored to perform fast queries in large texts using this approximate seed. We show the efficiency of our method with two developed software tools, SortMeRNA for filtering ribosomal RNA from metatranscriptomic data (see Chapter 3), and SortMeDNA for mapping reads from metagenomic and genomic sequences generated by second- and third-generation technologies (see Chapter ??).

Structure of this thesis

This thesis is organized into four main chapters.

- **Chapter 1** gives an introduction to sequence alignment, methods of traditional sequencing and the arrival of high-throughput sequencing technologies. Furthermore, new computational challenges for mapping gigabytes of high-throughput reads are discussed as well current algorithms and software aimed at solving them.
- **Chapter 2** presents the new approximate seed and the supporting data structures used to quickly search for short regions of similarity between two sequences.
- **Chapter 3** presents the software SortMeRNA which implements the techniques of Chapter 2 to quickly and accurately filter rRNA fragments produced by high-throughput sequencing technologies.

- **Chapter ??** presents the software SortMeDNA for read mapping which extends the algorithm of SortMeRNA to perform full alignments of high-throughput genomic or metagenomic sequences. SortMeDNA also applies statistical analysis to evaluate the significance of each alignment.
- **Conclusion** summarizes our findings and gives perspectives for future works.

Chapter 1

Background to DNA sequence analysis

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1.1 Overview

Biological sequence alignment is a method used to identify regions of similarity between organisms at the genomic, protein or structural level. Numerous applications include tracking species diversity and evolution, identifying new strains of bacteria or viruses, and predicting functional roles of organisms based on the similar genes they share. In genomic sequence alignment, the sequences used for comparison are derived from *deoxyribonucleic acid* (DNA) or *ribonucleic acid* (RNA) strands, which are long chains of nucleotides present in every cell of an organism. These chains are defined on the alphabet $\{A,C,G,T/U\}$ and comprise both coding regions such as protein coding genes, and noncoding regions such as repetitive DNA, telomeres, and noncoding RNA. The most recurring form of RNA is a single, linear strand of nucleotides, whereas for DNA, it is a double stranded helix whose two strands run in opposite directions of one another. In protein sequence alignment, the sequences used for comparison are long chains of *amino acids*, frequently defined on a 20 letter alphabet. An amino acid is encoded by 3 nucleotides, collectively known as a *codon*, and some amino acids are encoded by more than one codon (ex. codons UCU

and UCA both encode for amino acid Serine). *Sequence alignment* is the most commonly used method to compare two sequences by arranging them in a manner as to highlight their regions of similarity. Furthermore, secondary and tertiary structure information can complement sequence alignment to achieve a higher accuracy, such as reported for rRNA [Letsch et al., 2010] and protein molecules [Russell and Barton, 1992]. In this thesis, we focus exclusively on the sequence alignment between nucleotide sequences and apply our algorithms to DNA and RNA sequences generated by high-throughput sequencing technologies. In Section 1.2, we discuss the history and foundation of sequence alignment and demonstrate the fundamental algorithm behind most of today's alignment tools. In Section 1.3 we introduce Sanger sequencing, the pioneering 1st generation sequencing technology still used for small-scale DNA projects such as small bacterial or viral genomes [Yan et al., 2013] or hypervariable regions such as in mitochondrial DNA [Lemay et al., 2013]. We then go on to describe some of the leading 2nd and 3rd generation sequencing technologies which are vastly applied for large-scale studies such as eukaryotic genome resequencing [Nystedt et al., 2013] and environmental genomics [Hingamp et al., 2013].

To this day, all sequencing technologies depend upon bioinformatic tools to post-process their sequenced data. These two fields crisscross in a manner that one's success will often lead to the other's. Improvements in the data generated by sequencing technologies, such as longer read lengths and reduced error-rates, correspondingly improve the performance and accuracy of existing sequence alignment tools. On the other hand, new improvements in software tools, such as better algorithms for error handling and faster speeds, stimulate research for novel methods of sequencing. In any respect, both research areas are continually evolving to provide the most effective and low-cost approaches to whole-genome analysis. In Section 1.4, we describe the leading bioinformatic tools used for mapping data issued by high-throughput sequencing technologies.

1.2 Sequence alignment

1.2.1 Foundation

Intuitively, short and closely related sequences, such as the phrases `lalunaebella` and `laluneestbelle` can be easily aligned by hand. In fact, they can be aligned in any of the following forms shown in Figure 1.1, using either the entire sequences for alignment or only their selected regions.

Figure 1.1: Global (1-2) and local (3-4) alignments for strings `lalunaebella` and `laluneestbelle`.

target	LALUNAE---BELLA	LALUNAE--BELLA	LALUNEE	LALUNAE--BELL
	* *	* *	*	*
query	LALUN-EESTBELLE	LALUNEESTBELLE	LALUNAE	LALUNEESTBELL
	(1)	(2)	(3)	(4)

For any given alignment, one can associate mismatches (noted by `*`) to *substitutions* in biology, and gaps (noted by `-`) as *indel* (insertion and deletion) mutations. The regions where the sequences match exactly (noted by `|`) depict areas of high conservation. A *global alignment* will span the length of the entire target sequence (see Figure 1.1 (1),(2)), whereas a *local alignment* will only consider regions of high similarity between the sequences (see Figure 1.1 (3),(4)). If an alignment is found to be significant, it may represent a highly conserved region between two sequences which can help to compute their evolutionary distances or identify homologies based on,

for example, overlapping sequence motifs [Kumar and Filipinski, 2007]. Normally, for highly divergent sequences having undergone more complicated genome rearrangements, such as duplications and inversions of thousands of nucleotides [Lupski and Stankiewicz, 2005], local alignment will be a better choice as it will localize isolated regions of similarity and will not expect them to have the same order and orientation on the sequences. If however the order and orientation of similarity regions are important, for instance during the construction of phylogenetic trees using metabolic pathways [Ma et al., 2013], then global alignment would be better-suited for the task. In practice, local alignment is a more popular approach due to its wide range of applicability and the fact that relatively few highly similar complete genomes or sequences are available to examine in full-length.

Apart from the type of alignment one must consider, how do we distinguish the best, or most biologically significant alignment? Logically, the correct alignment would be the one that captures the true changes made throughout time for a pair of sequences sharing a common ancestor. However, this is often not possible for nucleotide sequences, since these changes are introduced over long periods (up to millions of years) and depend on many different environmental and hereditary factors. Instead, we opt to search for an *optimal alignment* which maximizes the number of similarities between two sequences, and applies an alignment scoring scheme to do so. The scoring scheme assigns nucleotide matches, mismatches and gaps in an alignment with reward and penalty values, respectively, and at the end of an alignment the values are summed to yield an overall score. The alignment with the highest score is considered to be the optimal alignment. For protein alignments, it is customary to use log-odds matrices such as PAM [Dayhoff and Schwartz, 1978] or BLOSUM [Henikoff and Henikoff, 1992] to set the scores for substitutions, whereas the gap open and gap extension penalties are left for the users to decide, although techniques such as inverse parametric sequence alignment [Kim and Kececioglu, 2007] can be used to set these parameters based on biologically correct reference alignments. To a much lesser degree, few works have been published on the assessment of optimal alignment parameters for nucleotide sequences. In Subsection 1.2.3, we describe the few experiments made (based on observed evolutionary patterns) to address this question and give a general guideline for choosing appropriate parameters.

1.2.2 Dynamic programming for sequence alignment

Dynamic programming is a method of dividing complex problems into several smaller subproblems and solving each one individually, then combining the results to give a complete (and optimal) solution to the original problem. Often, this method will reduce the computation from exponential to polynomial time in a problem involving recursion, by effectively reducing the number of repetitive calls made to reach the final solution. In bioinformatics, dynamic programming is the method of choice for finding an alignment between two sequences. The first *global* sequence alignment algorithm, known as the Needleman-Wunsch [Needleman and Wunsch, 1970] algorithm, was developed in 1970 for aligning two proteins in full-length, although it can be equally applied to nucleotide sequences. This dynamic programming algorithm guarantees to find the correct optimal alignment between two sequences of lengths n in $O(n^2)$ time. A modified version of the algorithm by Smith & Waterman [Smith and Waterman, 1981] was introduced a decade later to search for an optimal *local* alignment. The root idea behind both algorithms is to explore the space of all alignments without having to actually list them, as that would require exponential time, by progressively computing the maximum scoring alignment along the lengths of the two sequences.

Under a *linear gap penalty model*, the cost of introducing a gap is independent of any previous gaps and it can be computed using a linear function $\gamma(l) = -ld$ where l is the length of the gap and d is the penalty score. The setup to this algorithm is a 2-dimensional ($n \times m$) table representing all possible pairs of residues between the two sequences. For the Smith-Waterman algorithm, the entries in the alignment table M are computed using the rules listed in Figure 1.2.

Figure 1.2: Rules to construct the Smith-Waterman alignment table M of size $n \times m$ under a *linear gap penalty model*

$$(1) \quad M(i, 0) = 0, \forall i \in [0, m]$$

$$(2) \quad M(0, j) = 0, \forall j \in [0, n]$$

$$(3) \quad M(i, j) = \max \begin{cases} 0 & \\ M(i-1, j-1) + w(x_i, y_j) & \text{if match } x_i \text{ with } y_i \\ M(i-1, j) + d & \text{if insertion in } x \\ M(i, j-1) + d & \text{if insertion in } y \end{cases}$$

$$\forall i \in [1, m], \forall j \in [1, n], d < 0$$

The function $w(x_i, y_i)$ usually returns a positive score for a match if $x_i = y_i$ or a negative score for a substitution if $x_i \neq y_i$. The parameter d is the score for opening a gap (ex. introducing an insertion or deletion with respect to the query sequence). For a guide to choosing values for the alignment scores (match, mismatch, insertion, deletion), see subsection 1.2.3. In the following example, we will use the values match = 2, mismatch = -3, gap = -5 and go on to align the sequences,

$$\text{target} = \text{ATAGCCTTT} \quad \text{and} \quad \text{query} = \text{ATCGCCTT}. \quad (1.1)$$

To begin with the table construction, we place the target sequence on the horizontal axis of the table and the query sequence on the vertical axis, as illustrated in Figure 1.3.

The first row and first column entries are all set to 0, in order to simulate an alignment of all letters with a null character, and the remainder of the computation begins at the first empty cell in the top left corner of the table. The table values in Figure 1.3 are computed in the left to right, top to bottom manner beginning from $M(1, 1)$. At each new cell, the optimal score is updated using the previously computed values (see Figure 1.2 for the complete set of rules) and an arrow is placed from the current active cell to the cell used in the computation (the green arrow in Figure 1.3 signifies the value '1' was used to obtain the score, such that $M(4, 4) = M(3, 3) + 2 = 1 + 2 = 3$). Once the table has been filled in, the arrows are used to trace back a path for an alignment. The optimal local alignment begins from the highest computed value in the table, being the value 11 in Figure 1.4. To output the optimal alignment, we follow the arrows until we reach the end of the path. For our example, the optimal scoring path between the two sequences in our example is $11 \rightarrow 9 \rightarrow 7 \rightarrow 5 \rightarrow 3 \rightarrow 1 \rightarrow 4 \rightarrow 2$, which yields the local alignment shown in Figure 1.5.

Under an *affine gap penalty model*, the location of gaps relative to one another is considered since in biology gaps spanning multiple residues (multiple base insertions or deletions) are more likely to occur than as single residue mutations. A more fitting gap penalty for this property

Figure 1.3: Construction of Smith-Waterman table using dynamic programming

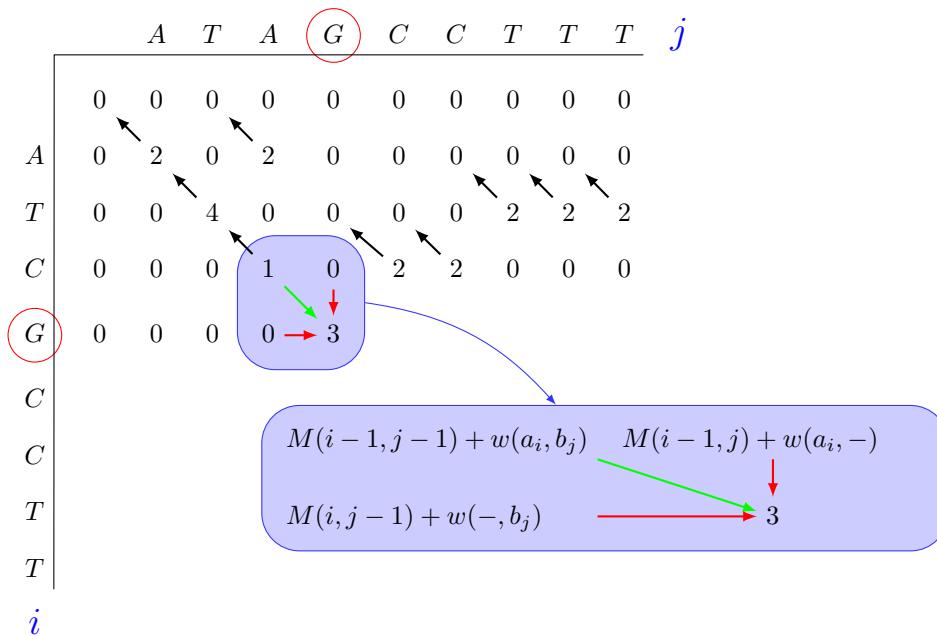
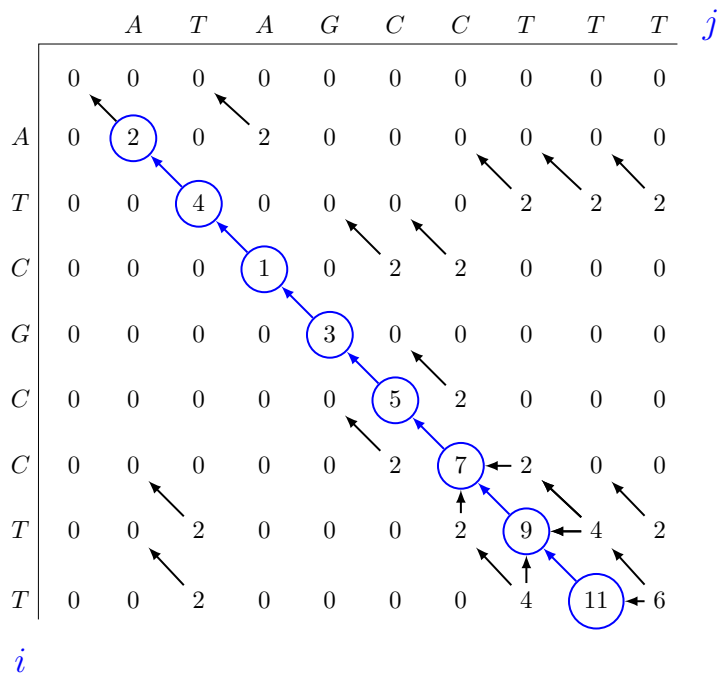


Figure 1.4: Optimal alignment trace back using Smith-Waterman table



follows the form $\gamma(l) = d + (l - 1)e$ for $l \geq 1$, where l is the length of the gap, d is the score for opening a gap and e is the score for extending a gap such that $d > e$. In this setup, additional gaps are penalized less than the initial ones. The recurrence relation shown in Figure 1.2 can be

Figure 1.5: Optimal local alignment for sequences ATAGCCTT and ATCGCCTT using the Smith-Waterman algorithm.

target	ATAGCCTT
	*
query	ATCGCCTT

modified to the one shown in Figure 1.6. Now, instead of keeping track only of the best score given that x_i is aligned with y_j , we must also keep track of two other tables: I_x and I_y . The table I_x keeps track of the best score given that x_i is aligned with a gap, and similarly the table I_y keeps track of the best score given that y_j is aligned with a gap. The recurrence relation in Figure 1.6 can be represented as a finite state automaton (FSA) shown in Figure 1.7. For every cell $M(i, j)$ the FSA is traversed to a new state (M, I_x or I_y) from the previous one depending on the next pair of residues (x_i, y_j) used for the alignment. Similar to the linear gap penalty model, the time required to align two sequences is $O(n^2)$. Ideally, the gap penalty model should follow a concave-like curve such that every new additional gap receives a slightly lower penalty than the previous one. However, the dynamic programming algorithm would require more time to align two sequences since for each new cell (x_i, y_j) we must consider every previous cell in the column *and* every previous cell in the row (extra $i + j + 1$ computations), and not only the one previous computation. Therefore, the affine gap penalty model is the most practiced in biological sequence alignment and can be implemented using the algorithm described in [Gotoh, 1982].

Figure 1.6: Rules to construct the Smith-Waterman alignment table M of size $n \times m$ under an *affine gap penalty model*

$$(1) M(i, 0) = 0, I_x(i, 0) = -\infty, I_y(i, 0) = -\infty \quad \forall i \in [0, m]$$

$$(2) M(0, j) = 0, I_x(0, j) = -\infty, I_y(0, j) = -\infty \quad \forall j \in [0, n]$$

$$(3) M(i, j) = \max \begin{cases} 0 \\ M(i-1, j-1) + w(x_i, y_j) & \text{if match } x_i \text{ with } y_i \\ I_x(i-1, j-1) + w(x_i, y_i) & \text{if insertion in } x \\ I_y(i-1, j-1) + w(x_i, y_i) & \text{if insertion in } y \end{cases}$$

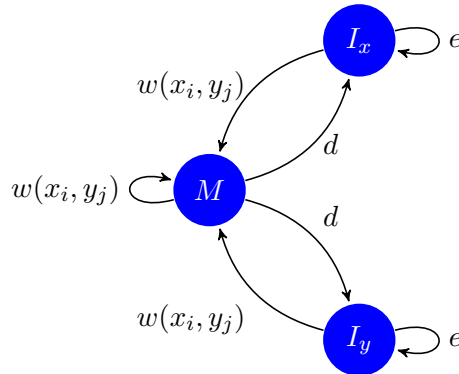
$$I_x(i, j) = \max \begin{cases} M(i-1, j) + d & \text{if open gap in } x \\ I_x(i-1, j) + e & \text{if extend gap in } x \end{cases}$$

$$I_y(i, j) = \max \begin{cases} M(i, j-1) + d & \text{if open gap in } y \\ I_y(i, j-1) + e & \text{if extend gap in } y \end{cases}$$

$$\forall i \in [1, m], \forall j \in [1, n], d < 0, e < 0$$

Although dynamic programming offers an important speedup over the naive recursion approach, it remains slow for aligning large eukaryotic genomes and when searching a large file of reads against a large database. To resolve this problem, significant efforts have been dedicated to improve the algorithm's performance using single instruction, multiple data (SIMD) parallelism. This accelerated approach has been reported to gain more than six times the speed over the traditional one [Farrar, 2007, Rognes, 2011] and has been implemented into many modern alignment software such as Novoalign (Novocraft), SHRiMP2 [David et al., 2011] and

Figure 1.7: A diagram of the relationships between the three states used for affine gap alignment [Durbin et al., 1998] (note $d < 0$ and $e < 0$)



Bowtie2 [Langmead and Salzberg, 2012]. Another acceleration used by all these tools is that none of them apply the dynamic programming algorithm to the entire search space of two sequences, but only to subregions showing potential to generate a high scoring alignment. In Section 1.4 we discuss the common heuristics used by many current read mapping tools and the efficacy of these approaches for different alignment applications.

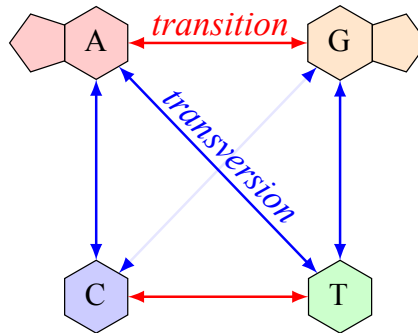
1.2.3 Choosing alignment parameters for nucleotide sequences

The Smith-Waterman sequence alignment parameters aim to maximize the score for true homologies of highly similar or distantly related species. Frequently, two different sets of parameters will produce two different results, especially if the query and reference data experience high rates of divergence. Since the start of the new millennium, very few investigations have been made into understanding the behavior of different alignment parameters for varying sets of data [Chiaromonte et al., 2002, Frith et al., 2010]. However, biological evolution suggests that substitution mutations occur more frequently than indels [Saitou and Ueda, 1994, Iengar, 2012], and thus should have a lower penalty in the scoring scheme. As a rule of thumb, a match should have a positive score and the mismatch, gap open and gap extend parameters should have negative scores such that the expected overall score for aligning two random sequences is negative. A simple model for a nucleotide scoring scheme is match=1, mismatch=-1, gap open=-2 and gap extend=-1. Interestingly, even though this model does not take into consideration any relationships between nucleotide bonds or mutation biases, it has been shown to work reasonably well on various data [Frith et al., 2010]. Indeed, more sophisticated models can be developed based on nucleotide compositions of known species to help increase accuracy of the results [States et al., 1991]. Certain types of models begin to consider biological and statistical properties of substitution mutations, as these are subject to certain biases in some species and do not come about as completely random events. For example, one of the species known to cause severe malaria in humans, the *Plasmodium falciparum*, has shown to experience a significant increase in mutations for two genes associated to sulfadoxine-pyrimethamine (drug) resistance [Ahmed et al., 2004].

Some of the commonly studied biases include CpG density, G+C content and transition/transversion ratios. A CpG site is a region of DNA where a C nucleotide is followed by a G nucleotide, or *vice versa*, along the same strand of DNA (this is different from a base-pair).

The CpG density shows a bias when the observed number of CpG dinucleotide sites does not correlate with the expected number (based on the G+C content), as witnessed in the subfamilies of HIV [Kypr et al., 1989] and the human genome [Sved and Bird, 1990]. A scoring scheme may be refined for acknowledging the presence of CpG dinucleotides by giving lower mismatch scores to more probable mutations of adjacent nucleotides, such as CpG→TpG (or complementary CpA), a common mutation caused by the methylation of cytosine and its subsequent deamination into thymine [Jabbari and Bernardi, 2004]. It has also been shown that mutations of CpG sites can affect the mutation rate of non-CpG DNA [Walser et al., 2008], a possible aftermath of incorrect DNA reparation of the deaminated nucleotide. However, the latter observation is largely specific to the sequence in question and would require strong knowledge of its evolutionary origins in order to be scored effectively. Another often recognized bias is related to the G+C (or A+T) content, which is simply the skewed percentage of nucleotides being either G or C in a genome. The G+C content can vary significantly between different genomes, such as the G+C rich actinobacteria or A+T rich firmicutes, and this characteristic can be incorporated into a scoring matrix by assigning higher scores to matches between less frequent residue pairs (eg. if G+C rich, assign higher scores to matches between A-A and T-T and lower scores to matches between G-G and C-C [Frith et al., 2010]). Lastly, the transition/transversion bias refers to the greater likelihood of seeing a mutation from a purine to a purine (A↔G), or a pyrimidine to a pyrimidine (C↔T) nucleotide, than from the purine to a pyrimidine or *vice versa*, the possibilities are illustrated in Figure 1.8. The expected ratio for a transition to transversion mutation is 1:2, however in vertebrate genomes transition mutations are more likely to occur than transversions (driven by methylation) [Zhao and Boerwinkle, 2002], thus a scoring matrix can be adjusted to consider this property by giving higher scores for a transition (i.e. A→G) mutation than for a transversion (i.e. A→T) mutation. However, it was also shown in [Yang and Yoder, 1999] that for a group of eutherian species the transition-transversion ratio approaches 1 for levels of divergence $\geq 20\%$ due to a saturation of transitions. Investigations into using *sequence specific* transition-transversion substitution matrices have been presented using the tool DNAlignTT [Agrawal and Huang, 2008], which reported comparable and sometimes significantly better results over the trivial match/mismatch scoring scheme in their preliminary results.

Essentially, the substitution scores of a scoring matrix are derived from log-odds ratios [Karlin and Altschul, 1990], which compare the probabilities of aligning two bases as a result of evolution versus as a result of a random alignment. The biases discussed in this section can be used to construct a simple 4×4 matrix, being the safest choice for data with unknown properties, or a 16×16 matrix if considering dinucleotide properties [Salama and Stekel, 2013]. For coding regions, an amino acid scoring matrix can be used which aligns amino acids rather than individual nucleotides. Along with an appropriate scoring scheme, it is important to choose an alignment cutoff score, a score which will capture enough biological homologies but also dismiss artificial alignments which happen to arise by chance. A good measure of alignment significance is the expectation value (E-value) which gives the expected number of alignments between two random sequences of lengths m and n having a certain alignment score. In the following Subsection 1.2.4, we will discuss the E-value and its application in our software SortMeDNA, further described in Chapter ??.

Figure 1.8: Transition and transversion mutations

1.2.4 Significance of alignment scores

The purchasing power of a dollar can define its relative value, so what then defines the relative value of a sequence alignment? Well, we can begin by asking how many random sequence alignments can statistically exist that achieve the same score as the homology at question. In [Gordon et al., 1986, Karlin and Altschul, 1990] it was proven that the distribution of the Smith-Waterman ungapped local alignment scores between two infinitely long random sequences (each successive character follows a Markovian model) follow the extreme value distribution type I, otherwise known as the Gumbel distribution. Moreover, the latter paper goes on to describe the expectation value (E-value), that is the number of random alignments expected to exist between two sequences of lengths m and n having a score S or greater. The equation for this calculation is given by,

$$E = Kmn e^{-\lambda S} \quad (1.2)$$

where K and λ are the Gumbel parameters, m and n are the lengths of the query and reference sequences, respectively, and S is their Smith-Waterman local alignment score. The logic behind Equation 1.2 follows the same principle as observing the longest run of heads (or tails) in a game of coin toss. During an alignment between two random sequences, we can consider a match between two letters as throwing a head and a mismatch as throwing a tail. Because of the randomness property, every subsequent comparison between two letters will yield a result independent from any previous one, synonymous to each new toss of an unbiased coin.

Although it has not been proven that an alignment involving gaps also follows the Gumbel distribution, it has been heavily conjectured to be the case through simulation analysis [Altschul and Gish, 1996]. The Gumbel parameters K and λ are the scaling factors and are computed using the background nucleotide frequencies of the reference sequence n and an alignment scoring matrix (including indel costs). The most straightforward approach to compute these values is to simulate many random sequences of lengths n and m , align them and fit the values K and λ onto the resulting score distribution [Waterman and Vingron, 1994]. However, this approach is inefficient since it requires thousands of random alignments to arrive at an accurate estimation. Various faster and on-the-fly methods have been developed to alleviate this time constraint by using the ‘islands method’ [Olsen et al., 1999], global alignment [Sheetlin et al., 2005] or importance sampling [Park et al., 2009] techniques.

The first, and still one of the few, local alignment tools that effectively applies this statistical measure for evaluating alignment score significance is BLAST [Altschul et al., 1990]. BLAST computes the E-value per query, meaning that the length m is for one query and n for the entire

reference database. It is also possible to compute the E-value for the entire search space, meaning the length m will represent all of query sequences and n , as in BLAST, will represent the entire reference database. When computing the E-value, it is also important to consider alignments carried out on the edges of either the query or reference sequences, where an alignment may run out of sequence before reaching the threshold score. To account for this boundary condition, we also compute the finite-size correction which adjusts the lengths of m and n by subtracting from them the minimal length l required to achieve a significant score. When computing the length correction per read, the relative entropy is used to take into account the nucleotide distributions for both the query and reference sequence. However, when computing it for the entire set of reads, the entropy of the reference sequence alone can be used. Although this approach is not theoretically optimal [Wang and Samudrala, 2006], it allows to compute the length correction one time and apply it to the global computation of the E-value for all reads. Below we outline the steps taken to compute the E-value for the entire search space, which apart from approximating the length correction using entropy (rather than relative entropy), closely follow those for BLASTN [Korf et al., 2003].

Let M and N define the total number of query and reference sequences in our search space, respectively. Then, we can represent the m and n values in Equation 1.2 as,

$$m = \sum_{i=1}^M m_i \quad n = \sum_{i=1}^N n_i \quad (1.3)$$

Considering the finite-size correction, we must additionally subtract this value l from every query and reference sequence,

$$m' = m - (l \times M) \quad n' = n - (l \times N) \quad (1.4)$$

The length correction l is given by,

$$l = \frac{\ln(Kmn)}{H} \quad (1.5)$$

If $l > m$ then set $m' = \frac{1}{k}$. The parameter H represents the relative Shannon entropy,

$$H = - \sum_{i=1}^4 \sum_{j=1}^i q_{ij} \ln \frac{q_{ij}}{p_i p_j} \quad (1.6)$$

for which q_{ij} are the pair-wise frequencies of the aligned region and p_i, p_j are the background frequencies of nucleotides in the reference database. However, since we would like to compute the E-value for all query sequences rather than per query, we assume that the pair-wise frequency probabilities are unknown or resemble the background frequencies of the reference database (see [Wang and Samudrala, 2006]). We use the Shannon entropy instead,

$$H = - \sum_{i=1}^4 p_i \ln p_i \quad (1.7)$$

Therefore, the E-value with length correction for m and n is,

$$E = Km'n'e^{-\lambda S} \quad (1.8)$$

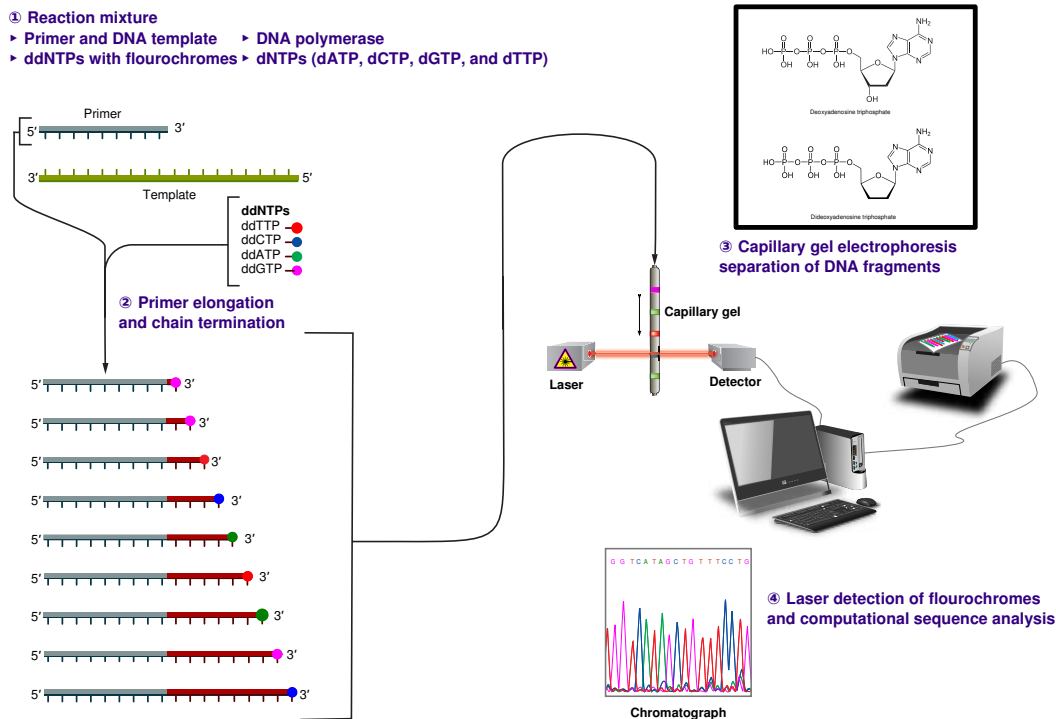
1.3 Next generation sequencing

1.3.1 1st generation Sanger sequencing

The first complete genome, the ϕ X174 virus, was sequenced in 1977 [Sanger et al., 1977] using a *chain termination technique* developed by Frederick Sanger. The original method required four reaction mixtures to be set up each containing single-stranded copies of a DNA template, DNA polymerase enzymes responsible for DNA replication, DNA primers (short DNA sequences complementary to the 5' end of the template sequence) and equal amounts of all four types of nucleotides. In addition, small amounts of *one type* of modified nucleotide are added to each reaction mixture. The modified nucleotides lack the hydroxyl group (in the sugar molecule) required for bonding of adjacent nucleotides during replication. Depending on the variant preferred, either the normal nucleotides, the modified nucleotides or the DNA primers are also radioactively labeled in order to see the DNA molecule in the final step.

The chain termination method begins with a single-stranded DNA template to which a primer is annealed at the 5' end (DNA can only be synthesized from the 5'→3' direction). As the DNA polymerase gradually incorporates normal nucleotides to form the complementary strand, the synthesis reaction proceeds until a modified nucleotide is integrated into the chain, terminating elongation and essentially exposing which of A,C,G or T nucleotides resides at that position. Therefore, in order to determine the position of every single nucleotide in a strand of DNA, this method must be applied to many copies of the same strand (in each reaction mixture) obtainable via plasmid vectors or the polymerase chain reaction (PCR). The final step of sequencing involves denaturing the elongated DNA fragments from their template strands and passing them through a gel electrophoresis setup (different lane for each reaction mixture), which separates the fragments according to length differing in size by 1 bp. Since each fragment is radioactively labeled (via primer, normal nucleotides or modified nucleotide), all that remains to do is read the radioactive bands from the start 5'→3' in the four lanes of gel to determine the complete sequence of the original DNA strand. In 1986, an automated variation to the Sanger method was described [Smith et al., 1986] where the modified nucleotides are fluorescently labeled with a different color to distinguish each type and added simultaneously into one reaction mixture. Afterwards, during electrophoresis a laser excites the atoms in the fluorescent labels to produce visible light which is translated by a computer into its corresponding nucleotide. Refer to Figure 1.9 for an illustration of the automated method. The main limitation to this approach of sequencing is that it can only be applied to short DNA sequences (~800 bp back then and ~2,645 bp today using Novex® precast gels by Life Technologies), in order to maintain a high resolution during gel electrophoresis for a pair of sequences differing in length by 1 bp. Therefore, even a genome as small as the one of the ϕ X174 virus had to be sequenced using a *whole-genome shotgun* approach, where the entire molecule was randomly sheared into smaller, possibly overlapping DNA fragments (~500 bp) to be individually sequenced using the chain termination method and finally reassembled back into the original genome using computational approaches [Staden, 1979].

Figure 1.9: Sanger sequencing using the chain termination method, automated version using fluorescently labeled nucleotides and computer reading



<http://commons.wikimedia.org/wiki/File:Sanger-sequencing.svg>

Milestones using Sanger sequencing

The first bacterial genome, *Haemophilus influenzae*, was entirely sequenced using the whole-genome shotgun sequencing approach and afterwards reassembled with the TIGR assembler [Fleischmann et al., 1995]. The sequencing of the first eukaryotic genome, the yeast *Saccharomyces cerevisiae*, was an international collaboration effort that commenced in 1989 and was completed seven years later [Goffeau et al., 1996]. Due to large computational resources required for assembling many small DNA fragments (and concerns of the authenticity of the results), the sequencing of *S. cerevisiae* was done in a hierarchical approach. Complete chromosomes were initially split up into ordered and slightly overlapping sections using BAC clones, and each section was then individually shotgunned into smaller fragments which were finally sequenced and reassembled back into the original parent sections. The Human Genome Project (HGP) was another international collaboration to sequence the first human genome using Sanger sequencing, beginning in 1990 and completing 13 years later with an estimated cost of \$2.7 billion. The same hierarchical shotgun sequencing approach (as for *S. cerevisiae*) was used to sequence the 3 billion base-pair genome [Consortium, 2001]. Although a remarkable accomplishment for mankind, at that rate and cost personalized genome sequencing was unrealistic for large-scale and commercial applications. During the same time period, another team took on the challenge to sequence the human genome using whole-genome shotgun sequencing [Venter et al., 2001], largely omitting the map-based (BAC clones) sequencing steps. Localized regional shotgun sequencing was also performed to improve resolution by first sorting reads to known BAC contigs (derived from the

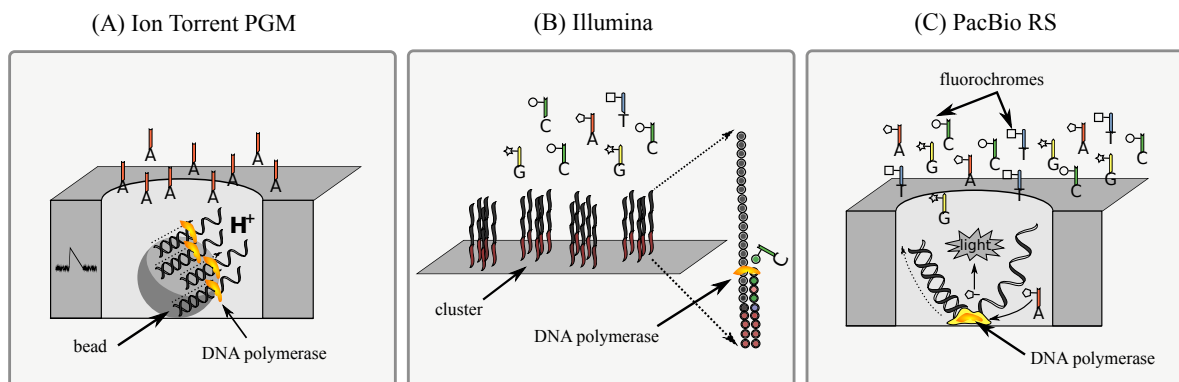
HGP) and then individually assembling them.

1.3.2 2nd and 3rd generation sequencing

Although the Sanger method can produce average read lengths of 850 bp having 99% consensus accuracy, the throughput per run is less than 24 Kb (for a modern 24-capillary 3500xL Genetic Analyzer system) and can take up to 6 hours to prepare and run. The new millenium has brought forth many new state-of-the-art technologies which can sequence the entire human genome within two days under \$10,000 (rapid run mode using the Illumina HiSeq 2500 system), albeit often with significantly shorter read lengths and a higher error rate (at the time of writing).

Sequencing-by-synthesis is a technique used to sequence a single strand of DNA in real-time, by incorporating free nucleotides into a growing chain using DNA polymerase and immediately recording which nucleotide was added. In 1996, a sequencing-by-synthesis setup was used to sequence 15 bases of a 291 bp DNA template [Ronaghi et al., 1996] in real-time, setting a world record for this alternative approach to Sanger sequencing. Today, this cutting-edge principle is the foundation of all high-throughput sequencing technologies, such as 454 (Roche), Illumina (Solexa) and Ion Torrent (Life Technologies), adept for generating millions of high quality reads of lengths 100-1000 bp within several days. Similarly to Sanger sequencing, all sequencing-by-synthesis technologies employ DNA polymerase to gradually incorporate free nucleotides into a growing strand of complementary DNA. Per contra, the detection of incorporated nucleotides is performed using variant techniques other than the Sanger method and the entire sequencing process is massively parallelized. For 454, the method of detection is pyrosequencing [Ronaghi et al., 1998, Nyrén, 2007], for Ion Torrent it is the detection of hydrogen ions [Rothberg et al., 2011] and for Illumina it is nucleotide dye-termination [Bentley et al., 2008]. All of these techniques are performed over millions of amplified clones (derived via emulsion PCR using beads or clusters) for DNA fragments obtained via whole-genome shotgun sequencing, in order to provide a sufficient signal for detecting nucleotide incorporation. Moreover, the flow cells on which sequencing reactions take place, are optimized to permit massively parallel sequencing of millions of different DNA fragments simultaneously. The diagrams (A) and (B) in Figure 1.10 illustrate the general setup for Ion Torrent and Illumina platforms. In the following paragraphs we will give a short summary of each technique and highlight the steps most susceptible to *sequencing errors* and the factors limiting the production of long reads, as error types and read lengths vary between technologies (see Table 1.1) and are important to consider during the development of software for sequence analysis.

454 and Ion Torrent sequencing are based on detecting by-products of a synthesis reaction. During the synthesis of a DNA molecule, free nucleotides are incorporated in their natural triphosphate forms in cycles over a sequencing flow cell. Upon bonding of a free nucleotide to the next unpaired base in the template strand, two by-products, a pyrophosphate (two phosphate groups bonded together) and a positively charged hydrogen ion, are released. Each by-product is exclusively used by the 454 and Ion Torrent technologies, respectively, to detect the incorporation of a base. In the case of 454, the release of a high-energy pyrophosphate molecule triggers a series of catalytic reactions which ultimately end with an emission of visible light. The intensity of light emitted is proportional to the number of nucleotides incorporated into the DNA chain and can be detected by a charge-coupled device (CCD) camera. In the case of Ion Torrent, the release of a positively charged hydrogen ion decreases the pH of the solution (since $\text{pH} = -\log_{10}(H^+)$), and the change in ion concentration can be detected using a silicon substrate chip (see Figure 1.10(A)). In both cases, the addition of different types of nucleotides

Figure 1.10: Second and third generation sequencing technologies

Ion Torrent and PacBio images taken from http://en.wikipedia.org/wiki/File:From_second_to_fourth-generation_sequencing,_illustration_on_TAGGCT_template.svg, Illumina individually incorporated

is performed sequentially, in order to recognize which nucleotide was incorporated during the detection of a light signal or the change in ion concentration. One limitation to both methods is a higher error rate for detecting long homopolymers (a run of identical bases), which is caused by an over- or under-call of the signal for the incorporated nucleotides. These types of errors often manifest themselves as insertions or deletions of nucleotides and can sometimes lead to incorrect estimations of biodiversity for environmental studies [Fonseca et al., 2010]. Whether a nucleotide was incorporated or not, the flow cell must be thoroughly washed over to remove all unincorporated nucleotides before new enzymes and reagents can be added for the next cycle (this will ensure synchronized signals and reduce byproduct accumulation which is known to inhibit DNA polymerase [Mashayekhi and Ronaghi, 2007]).

Illumina sequencing uses fluorescently labeled nucleotides (different ‘color’ for each type) which work to serve two purposes: firstly, the label prohibits further elongation of the complementary strand such that a single nucleotide can be incorporated at each flow cycle (this avoids the over- or under-call of bases as in 454 and Ion Torrent) and secondly, its proper color allows for the identification of the incorporated nucleotide (here a miscall of a base can occur leading to a substitution error). Illumina’s labeled nucleotides have the property of having reversible terminators, meaning that when the incorporated nucleotide is recorded by a camera based on its color, an enzyme can cleave off the fluorescent label to reinstate synthesis. Because sequencing is carried out in a base-by-base manner, this technology produces far fewer errors at homopolymer and repetitive sequence regions than both 454 and Ion Torrent.

All of the three aforementioned sequencing approaches have limitations to the read lengths produced (see Table 1.1), some of these limiting factors attribute to nucleotide misincorporation by DNA polymerase or a reduced signal due to lost DNA fragments ($\sim 0.1\%$) washed away during each cycle [Mashayekhi and Ronaghi, 2007], as well as the complex management of massively parallelized setups. In terms of sequencing errors, it has been estimated that nowadays the Illumina technology has the lowest error rates in one round of sequencing at 0.1 errors per 100 bases, in comparison to 0.38 and 1.5 errors per 100 bases for 454 and Ion Torrent, respectively [Loman et al., 2012]. Error types occur mostly as substitutions for Illumina (frequently sequence-specific for GGC motifs and inverted repeats [Nakamura et al., 2011]) and homopolymer indels

for 454 and Ion Torrent.

PacBio single-molecule sequencing is a recent¹ technology capable of generating average read lengths of 4600 bp (possible up to 24Kb). This advanced technology uses fluorescent labels attached to the terminal phosphate group of free nucleotides (as opposed to the actual base) which are cleaved off by DNA polymerase during replication (see Figure 1.10(C)). The single strand of DNA to be sequenced is passed through a single DNA polymerase molecule attached to a *zero-mode waveguide* (ZMW) at the bottom of a chamber. The ZMW is a structure sensitive enough to detect the incorporation of single nucleotides [Levene et al., 2003]. During sequencing, all four types of nucleotides are introduced into the chamber simultaneously at equal amounts. When one of the four nucleotides is being incorporated into the chain, its distinct fluorescent label excites for several milliseconds and this illumination signal is recorded by the ZMW. Thusly, the entire strand of DNA can be sequenced continuously using a single strand of DNA without the need for PCR amplification as for other technologies (known to cause sequence-specific biased coverage [Ross et al., 2013]), or cyclic washing of flow cells. Although the error rates are much higher than for other technologies, nearly 15% (mostly indels), they appear to be independent of the sequencing context and can be resolved by multiple rounds of resequencing [Roberts et al., 2013]. Of all today's technologies, PacBio delivers the least invasive method for eavesdropping on natural DNA replication while delivering very long reads in a short time period (see Table 1.1). Small bacterial genomes are already being fully sequenced and assembled using this technology [Nicholsona et al., 2013, Khosravi et al., 2013], since the long reads can cover complete gene transcripts and low complexity regions.

1.4 Tools for high-throughput read mapping

The arrival of high-throughput sequencing technologies has introduced new problems for read mapping. Firstly, the low cost of sequencing has allowed many smaller laboratories worldwide to invest in personal machines and sequence their own data. However, not every laboratory has the sufficient computational resources capable of analyzing such large amount of throughput data. Secondly, all of today's technologies are susceptible to sequencing errors in the form of substitutions, insertions and deletions (see Section 1.3.2). Moreover, naturally occurring errors may exist in distant homologies and being able to consider them is an important characteristic for aligning divergent species.

In the context of speed, SSEARCH [Smith and Waterman, 1981, Pearson, 1991] is a tool which provides a modern implementation of the exhaustive Smith-Waterman sequence alignment, however to align 1000 Illumina reads of length 100 nt against human chromosome 21 takes about a half-hour (using one thread on an Intel Xeon CPU @ 2.67GHz). Given that a typical Illumina MiSeq machine can generate 15 million reads within 2 days (see Figure 1.1), using SSEARCH to map all of these reads against the same chromosome would take roughly 1 year. New heuristics have been developed to accelerate this search by first locating short regions of similarity called *seeds* between the query and reference and then extending the seeds into longer alignments using the Smith-Waterman algorithm (see Figure 1.11). In other words, a seed gives a clue for a potential alignment. To highlight the speedup gained with heuristic approaches in regard to the full dynamic programming algorithm, Bowtie2 [Langmead and Salzberg, 2012] takes less than a minute to index human chromosome 21 and only about 0.2 seconds to align the same set of 1000

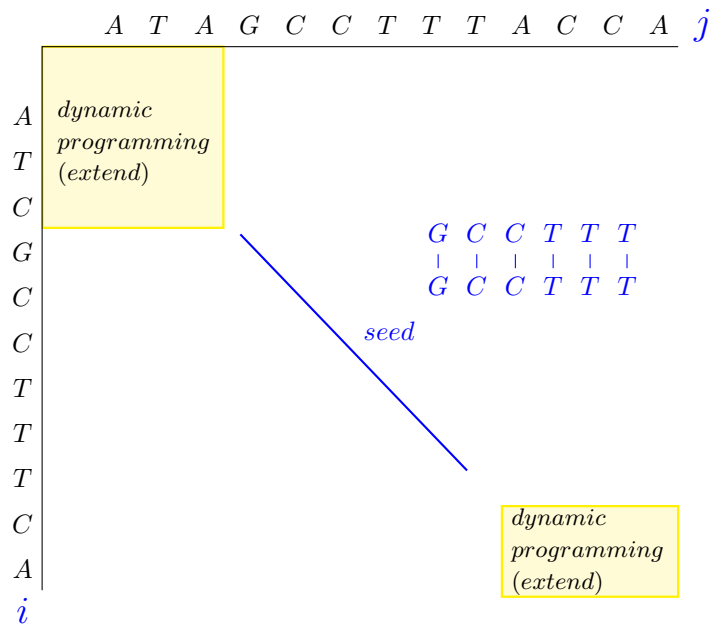
¹(11/04/2013, GlobeNewswire) Photo Release – Pacific Biosciences Launches the PacBio(R) RS II Sequencing System

Table 1.1: Specifications for various sequencing-by-synthesis technologies (taken from each company's official website 09/2013)

Platform	Read length (bp)	Reads per run	Run time	Base call accuracy	Application
<i>2nd generation</i>					
Illumina ¹					
MiSeq (paired-end)	250x2	15 million	39 hrs	99.9% for >75% of bases	Targeted, genome, metagenomics, transcriptomics, epigenetics
HiSeq 2500 (high output, paired-end)	100x2	6 billion	11 days	99.9% for >80% of bases	(same as MiSeq)
Roche/454 ²					
GS Junior	~400	~100,000	10 hrs	99% at first 400 bases	Targeted, genome, metagenomics, tran- scriptomics
GS FLX XL+	up to 1000 (average 700)	~1,000,000	23 hrs	99.997% consensus at 15x coverage	(same as GS Junior)
Ion Torrent ³					
Ion PGM (Ion 318 Chip v2)	200-400	4-5.5 million	4.4-7.3 hrs	99% expected	Exome, methylation, genome, transcrip- tomics
Ion Proton	up to 200	60-80 million	2-4 hrs	99% expected	(same as Ion PGM)
<i>3rd generation</i>					
Pacific BioScience ⁴					
PacBio RS II	up to 23,297 (average 4,606)	47,197	30 min - 2 hrs	99.999% consensus at 30x coverage	<i>de novo</i> assembly, targeted

¹www.illumina.com²www.454.com³www.lifetechnologies.com⁴www.pacificbiosciences.com

Figure 1.11: Seed-and-extend strategy to reduce the amount of search space examined by the dynamic programming algorithm for aligning sequences x and y



Illumina reads (or 50 minutes for the entire 15 million reads).

In the context of errors, many existing alignment tools have been optimized for genome resequencing ($\sim 99\%$ sequence similarity) and adopt an exact match seed model. However, for applications such as metagenomics or metatranscriptomics where the reference sequences may share $\sim 75\text{-}98\%$ similarity to the query, these tools are no longer sensitive enough.

Most of today's read mapping tools use a seed-and-extend approach coupled with a query and/or reference index to facilitate rapid alignment of HTS data or sequences to large-scale databases. In the following subsections we will describe the early techniques of using suffix trees and enhanced suffix arrays to align sequences using *exact contiguous seeds*, as well as the FM-index now used in many of the recent alignment tools for its near-linear time search and compressed structure [Chacón et al., 2013]. Furthermore, we will discuss the advantages of using hash tables for implementing *exact noncontiguous seeds*, that is seeds allowing mismatch errors, and the enhanced sensitivity achieved by these tools for aligning more distantly related species. Finally, we will outline our motivation for a new indexing structure implementing *approximate seeds* allowing mismatch and gap errors, for a further improvement in aligning sequences exhibiting high indel error rates or distant homologies.

1.4.1 Heuristic seed alignment

Typically there are three types of seeds that are continuously studied in pattern matching algorithms. The first type of seed we will call the *exact contiguous seed*, simply meaning the match must be contiguous and share no errors between the query and the reference sequence. All indexing data structures used today can easily support exact contiguous seeds, including the suffix tree, (enhanced) suffix array, the FM-index and hash tables which are all discussed in

the following subsections. Although exact matching algorithms are very fast, it has been shown in [Ma et al., 2002] that *exact noncontiguous seeds* or *spaced seeds* which allow mismatches at some predefined positions can improve sensitivity and help recognize distant homologies (provided that not too many indels are present). A spaced seed is defined by its size and weight. For example, a spaced seed of size 16 and weight 12 searches for k -mers (matches of length k) of length 16 but where only 12 of the predefined positions require to have an exact match. The most efficient indexing data structure by now which can support multiple *predefined* mismatches in the seed is based on hash tables. Suffix trees, the enhanced suffix array and the FM-index allow for mismatches also (not predefined to any positions) but the underlying backtracking algorithm can often render their search algorithms considerably slower, especially for a larger number of mismatches. However, sometimes even exact noncontiguous seeds are not sensitive enough to spot distant homologies (ex. environmental studies), or insertion/deletion errors specific to sequencing technologies such as 454, Ion Torrent and PacBio. In these contexts, *approximate seeds* allowing mismatch and indel errors anywhere in the seed would serve as an optimal choice (but at some computational cost) and the few tools today that explicitly implement them are based on some variant of the suffix tree.

1.4.2 Indexing using hash tables

A common practice in biological sequence alignment is to find all *exact* occurrences of a k -mer in a fixed text of length n , which can often be large and repetitive such as the human genome or a ribosomal RNA database. A naïve approach would take $O((n - k + 1)k)$ time by placing the k -mer at all possible positions $(n - k + 1)$ in the text n . The fastest alternative to this problem requires only constant $O(z)$ time where z is the number of occurrences of a k -mer, by indexing the text in a lookup table (or perfect hash table).

Shortly following the publication of the Smith-Waterman dynamic programming algorithm, a paper emerged introducing the idea of using a lookup table to efficiently index and search nucleotide sequences [Dumas and Ninio, 1982]. By translating each consecutive pair of overlapping nucleotides into a number (for example, AC = 15) and using it as a lookup index in a table² storing the original positions of each dinucleotide, the authors could quickly identify all positions of any dinucleotide within the original sequence. This method was later extended to exact overlapping k -mers [Wilbur and Lipman, 1983], being substrings of length k , and used to find regions of high similarity between two sequences prior to extending them to full alignments using dynamic programming. For example, by using a 2-bits-per-nucleotide encoding where A=00, C=01, G=10 and T=11, we can encode the string $x = \text{tatagata}$ to a binary number $x = 1100110010001100$ and subsequently use the decimal form $x = 52364$ as an index to a table. FASTA [Pearson and Lipman, 1988] and BLAST [Altschul et al., 1990] (BLASTN is the nucleotide version to which we will refer to from now on) are two established families of programs which index every query independently using contiguous k -mers and maintain an auxiliary lookup table for a list of their occurrences (for nucleotide sequences). Afterwards, a reference database is scanned for k -mers in common with the query using the lookup table and the best matching regions are extended into a full alignment. In this manner, only small subparts of the entire $O(n^2)$ search space are actually aligned, thus gaining a lot of time (see Figure 1.11). Although this heuristic method cannot guarantee to find all local alignments in contrast to the full dynamic programming algorithm, it has shown to be a good approximation in many applications

²The index was used to search the last occurrence of a dinucleotide in the primary lookup table, however a secondary table was also required to search for all preceding positions.

[Lipman and Pearson, 1985, Pearson and Lipman, 1988, Lam et al., 2008].

Since both FASTA and BLASTN localize their indexing to individual queries and the size of the k -mers is relatively small (smaller k -mers = more hits in the database), both of these tools are subject to long processing times for large data. For this reason, recently developed HTS alignment tools focus on indexing the full set of queries (MAQ [Li et al., 2008]), or the full set of reference sequences (Novoalign [Novocraft], GNUMAP [Clement et al., 2010] and SHRiMP2 [David et al., 2011]), or both at the same time (BWA-SW [Li and Durbin, 2009] but using trees). Although searching for each k -mer occurrence of a query in a reference sequence can be done in constant time using this structure, the memory requirement to index the human genome is $> 15\text{Gb}$ (for Novoalign, SHRiMP2 and BFAST [Homer et al., 2009]). BFAST applies a two-level hash system for long k -mers, where only the first $p < k$ nucleotides are hashed and the remaining parts are stored in a complementary bucket. During a lookup, the first p characters are used to index the hash table and then a binary search is performed on the tails in the complementary bucket, resulting in a lookup time slightly inferior to $O(1)$.

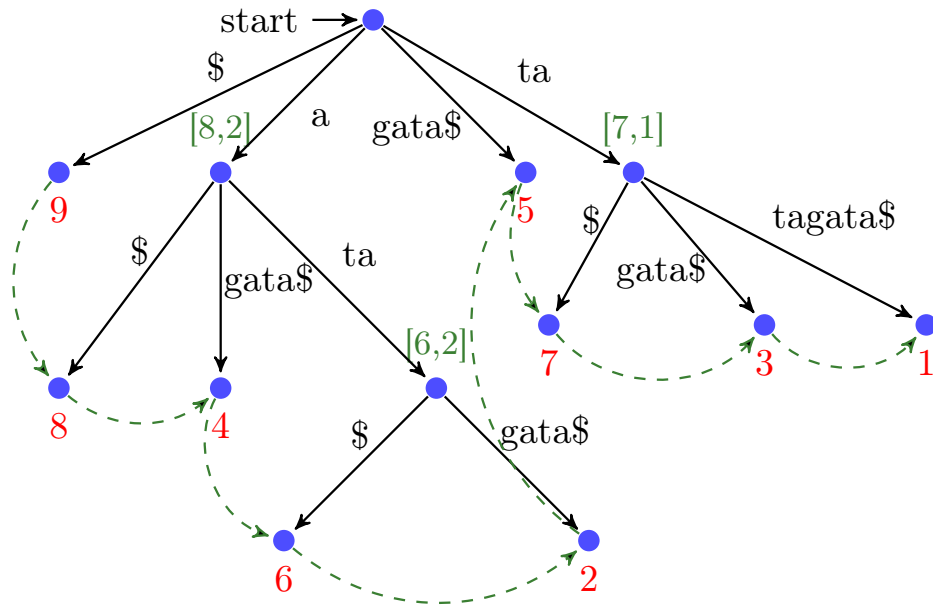
1.4.3 Indexing using suffix trees

An alternative to using a hash table to search for all k -mer occurrences is to use a *suffix tree*, which requires only $O(k + z)$ time (where z is the number of occurrences of the k -mer). One advantage of suffix trees over hash tables is that they can be used with a collection of strings of varying lengths and they also support approximate string matching [Navarro and Baeza-Yates, 2000, Russo et al., 2009]. Another advantage is that identical prefixes of suffixes are clustered into one path, thus greatly reducing the number of comparisons. Also, a tree traversal is rapid and can be performed in various modes to accommodate different applications [Gusfield, 1997]. The main drawback of a suffix tree is the large amount of memory it requires, with the most efficient implementation to date consuming 12-17 bytes per nucleotide [Kurtz, 1999, Kurtz et al., 2004]. For this reason, several more compact versions of the suffix tree have been developed, namely the enhanced suffix array and the suffix array-like FM-index. In the following subsections we will briefly describe these data structures and the software tools which implement them.

1.4.3.1 Suffix tree

Suffix trees have been used since the 1970's [Weiner, 1973, McCreight, 1976] to efficiently store all prefixes or suffixes of a string in a non-redundant manner. There exist both linear time methods for constructing a suffix tree [Ukkonen, 1995] on a constant-size alphabet (online-construction) and for any alphabet [Farach, 1997]. A suffix tree can represent all of the substrings of a string in an easily accessible format. For example, the suffix tree for the string $x = \text{tata}gata\$$ is shown in Figure 1.12. Every leaf node corresponds to a suffix and its label remarks the starting position of the suffix within the original string. Appending the special character '\$' to a string before constructing its suffix tree ensures that a preorder traversal yields the suffix nodes in lexicographical order, as '\$' is considered to be the lexicographically least character. To search a k -mer in the tree, one must begin at the root node (marked with 'start') and trace a path through the branches whose characters match exactly to the query. If all letters of a k -mer are exhausted during tree traversal at a non-leaf node j , then the number of occurrences of the k -mer in the string correspond to the number of terminal leaf nodes branching from j . To find all positions at which the k -mer occurs at, the suffix tree must also contain (a) a lexicographical linking between all leaf nodes (the dashed green arrows in Figure 1.12) (b) the paired information of the first and

Figure 1.12: Illustration of the suffix tree for the sequence $x = \text{tatagata}\$$. The leaf nodes hold the starting position (in color red) of each suffix in x . A preorder traversal of this suffix tree beginning from the root node (marked as start) yields all suffixes of x in lexicographical order, being $\{\$,a\$,agata\$,ata\$,atagata\$,gata\$,ta\$,tagata\$,tatagata\}$. To search for all occurrences of a string, we begin at the root node and follow the edges that match to the characters of our string. The string (or at least its prefix) exists in the tree if we exhaust all of the characters before or at a leaf node. For example, if we search the string $s = \text{ata}$, we will finish at the inner node marked with $[6,2]$. The green dashed path links together all leaf nodes in lexicographical order and the $[x,y]$ label at each inner node (except the root) gives the first and last position of a leaf node reachable from the current inner node. Both of these are optional as they are only useful for finding all of the locations at which s occurs (other methods exist too). To find all positions at which s occurs, we descend to the first lexicographically least leaf node and output its position (being 6). Then we follow the paths linking the leaf nodes and output their positions until we reach the last position (being 2).



last suffix position amongst all paths branching from j (the three green paired numbers $[x,y]$ in Figure 1.12). In this manner, all occurrences of a k -mer and its positions in the original string can be recuperated. Otherwise, if at some node l no more outgoing branches match to the remaining unmatched characters in a k -mer, then the k -mer does not exist in the string.

OASIS [Meek et al., 2003] and MUMmer [Kurtz et al., 2004] are two sequence alignment tools that index the reference database using a suffix tree. Both of these tools require 12 to 17 bytes per nucleotide and are practical for whole-genome alignment of smaller bacterial genomes, as the resulting index can reside comfortably in a low-memory machine (>36 Gb required for the human genome) and maintain better cache locality. Another compromise to using a suffix tree-based aligner is that the index is often rebuilt for each new run, which can become time consuming for aligning large collections of high throughput reads. In a different application called RISOTTO [Pisanti et al., 2006], suffix trees were also used to quickly extract repeated patterns (functional elements in genetic sequences or entire genes) from a text allowing mismatch errors.

1.4.3.2 Suffix array

A *suffix array* (SA) is a sorted array of all suffices' starting positions in a string. It was first introduced in [Gonnet et al., 1992, Manber and Myers, 1993] as a space-efficient alternative to a suffix tree for finding all occurrences of a pattern of length k in a text of length n in $O(k \log(n))$ time (using a simple binary search algorithm). The slower search time (compared to querying a suffix tree) is compensated by a three- to five-fold decrease in memory, requiring only 4 bytes per nucleotide in its basic form. An indirect approach for constructing a suffix array is to simply traverse a suffix tree using preorder traversal and record the values stored at each leaf node. For example, a preorder traversal of the tree in Figure 1.12 will yield the suffix array $\{9,8,4,6,2,5,7,3,1\}$ shown in Figure 1.13. However, more direct and lightweight methods to construct the suffix array have been introduced since [Puglisi et al., 2007].

1.4.3.3 Enhanced suffix array

In [Abouelhoda et al., 2004] it was shown that every suffix tree algorithm can be replaced with an equivalent algorithm based on an *enhanced suffix array*, a data structure consisting of a suffix array and additional tables which help to navigate it. The ESA was primarily invented to be a space efficient alternative to the suffix tree, although this depends on the memory requirements of additional tables. Using an enhanced suffix array, the time to search a pattern of length k in a text of length n can be reduced back down to $O(k + z)$, equivalent to a suffix tree. One of the routinely used auxiliary tables is the *Longest Common Prefix array* which stores the lengths of the longest common prefixes between successive suffices of a suffix array, or $LCP[i] = \text{lcp}(x[\text{SA}[i-1]..n], x[\text{SA}[i]..n])$ for $1 < i \leq n$. For example, the LCP for position $i = 5$ in the string $x = \text{tatagata\$}$ in Figure 1.13 is computed between the suffices 'ata\\$' and 'atagata\$', such that $LCP[5] = \text{lcp}(\text{ata\$}, \text{atagata\$}) = 3$. Both tools Vmatch [Abouelhoda et al., 2004] and Segemehl [Hoffmann et al., 2009] use enhanced suffix arrays rather than suffix trees to align short sequences, where Vmatch reports using ~ 7 bytes per nucleotide and Segemehl ~ 13 bytes per nucleotide. The difference in the memory requirements is due to the fact that Segemehl supports non-exact read mapping using a conceptual suffix-interval tree and thus their enhanced suffix array data structure is slightly more complex. In the same (2004) paper, it was shown that the enhanced suffix array can be combined together with another data structure called the *Burrows-Wheeler Transform* (BWT) [Burrows and Wheeler, 1994] to efficiently locate repetitive regions in a sequence. The BWT is a reversible rearrangement of characters in a string with the property of grouping together identical characters to facilitate more efficient text compression, such as done in the tool bzip2. Figure 1.14 shows how to obtain the BWT of the string $x = \text{tatagata\$}$. In the following subsection we will describe the latest FM-index data structure which strategically combines the BWT and the suffix array to provide fast query searches with the added benefit of a compressed index.

1.4.3.4 FM-index

The FM-index is a compressed suffix array-like data structure which is based on the Burrows-Wheeler Transform and stems roots from traditional suffix trees and enhanced suffix arrays. In [Ferragina and Manzini, 2000], it was shown that the suffix array implicitly resides within the BWT and a new data structure called the FM-index can be used to perform fast searches on a compressed index. The equivalence property between the suffix array and BWT can be intu-

Figure 1.13: Illustration of the enhanced suffix array (ESA) for the sequence $x = \text{tatagata\$}$. The ESA consists of a suffix array (SA) and additional tables such as the longest-common-prefix (LCP) or Burrows-Wheeler-Transform (BWT). The SA is a list of positions of all suffices of x in lexicographical order. A preorder traversal of the suffix tree in Figure 1.12 will yield the same list as given in the SA[i] column. The LCP array stores the length of the longest common prefix between a pair of consecutive suffices in the suffix array. For example, $\text{LCP}[5] = 3$ because $x[\text{SA}[5]..9] = \text{atagata\$}$ and $x[\text{SA}[4]..9] = \text{ata\$}$. The construction of the BWT array is given in Figure 1.14. The BWT is frequently used to quicken navigation of the suffix array.

i	SA[i]	LCP[i]	BWT[i]	$x[\text{SA}[i]..9]$
1	9	0	a	\$
2	8	0	t	a\$
3	4	1	t	agata\$
4	6	1	g	ata\$
5	2	3	t	atagata\$
6	5	0	a	gata\$
7	7	0	a	ta\$
8	3	2	a	tagata\$
9	1	2		tatagata\$

itively seen by looking at Step 2 of Figure 1.14, where the permuted strings of $x = \text{tatagata\$}$ contain within them all suffices of the suffix array in $x[\text{SA}[i]..9]$ of Figure 1.13. By using two auxiliary tables supporting last-to-first BWT column mapping (see [Ferragina and Manzini, 2000]) along with the BWT, a binary search algorithm can be performed to quickly find all locations of a pattern of length k in the index of length n in $O(k + z \log^\epsilon n)$ time (where z is the number of occurrences as before and $\epsilon > 0$ is chosen at the time the FM-index is built). In most implementations, the FM-index requires only 0.5-2 bytes per nucleotide [Li and Homer, 2010] which makes it the smallest indexing data structure currently employed for performing exact pattern matching (and approximate with performance compromises, see Section 1.4.1) in biological sequence analysis. Almost all recent HTS alignment tools use the FM-index including Bowtie [Langmead et al., 2009], BWA-SW [Li and Durbin, 2009], SOAP2 [Li et al., 2009], Bowtie2 and GEM [Marco-Sola et al., 2012]

Figure 1.14: Construction of the Burrows-Wheeler Transform for the sequence $x = \text{tatagata}\$$. In bioinformatics, the BWT is often simultaneously used for text compression and indexing. To construct the BWT of x , first all rotations of x are determined (Step 1). Next, the rotations are sorted lexicographically (Step 2) and the last character of each rotation is taken to construct the BWT (Step 3). The resulting string $BWT = \text{attgtaa}\$$ groups together runs of similar characters which can be easier compressed using run-length encoding. Moreover, the BWT can be reversed into the original string x using a simple inverse transformation algorithm (time to compute same as sorting in Step 2).

Step 1: generate all rotations	Step 2: sort sequences lexicographically	Step 3: print last character of every sorted sequence
tatagata\$	\$tatagata	
atagata\$t	a\$tatagat	
tagata\$ta	agata\$tat	
agata\$stat	ata\$tatag	
gata\$tata	atagata\$t	
ata\$statag	gata\$tata	attgtaa\$
ta\$stataga	ta\$stataga	
a\$statagat	tagata\$ta	
\$tatagata	tatagata\$	

Chapter 2

New approximate seeding technique and supporting data structures

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In this chapter we will describe a novel approximate seeding technique that can detect whether two strings W and V have an edit distance $d(W, V) \leq 1$. The *edit distance* $d(W, V)$ measures the amount of difference between two strings. It is defined as the minimum number of edit operations needed to transform one string into the other – with allowable edit operations being *insertion*, *deletion* or *substitution* of a single character. Figure 2.1 shows various examples of strings W and V with edit distance $d(W, V) = 1$.

W = A-CCTGA	CTAGGATAA	GACACATT
	*	
V = ATCCTGA	CTAGCATAA	GACAC-TT

Figure 2.1: Example of edit distance $d = 1$ for various strings.

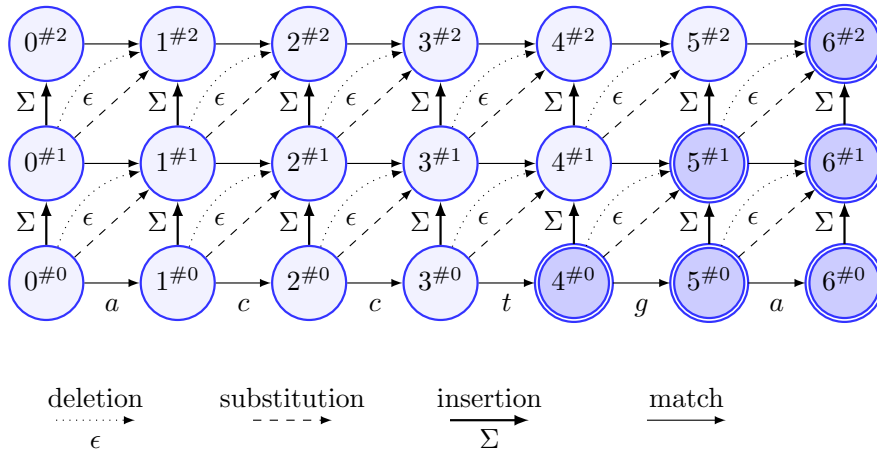
We apply this seeding technique in *approximate pattern matching* for querying a string in a large text allowing up to 1 error of any type. Given a query W and a reference text R , we want to find all occurrences of $R[i, j]$ such that $d(W, R[i, j]) \leq 1$.

In Section 2.1 we describe the technicalities of our novel seeding method using the universal Levenshtein automaton for $d = 1$. In Section 2.2.1 we go on to describe the indexing data structures used together with the universal Levenshtein automaton to find k -mers allowing up to 1 error in a reference text.

2.1 The Levenshtein automaton

The classical nondeterministic Levenshtein automaton for a pattern p and a number of errors d recognizes the set of strings which are at most edit distance d to p (see Figure 2.2). This automaton is not suitable for large-scale computations because of the presence of multiple active states (enhanced by epsilon transitions) which represent all feasible scenarios of alignments between two strings. Epsilon transitions allow for states to be activated without consuming any input character. In the Levenshtein automaton their purpose is to consider possible deletions of characters in both strings. For example, prior to searching the query $q = ctga$ in the automaton for the pattern $p = acctga$ of Figure 2.2, the states $0\#0, 1\#1$ and $2\#2$ will be activated before the first letter $q[0] = c$ is input into the automaton. In this manner, active state $2\#2$ considers two possible deletions in q prior to evaluating a state transition for input letter c (which would result in a match from $2\#2 \rightarrow 3\#2$).

Figure 2.2: The nondeterministic Levenshtein automaton for $p = acctga$ and $d = 2$. The $s\#e$ notation for each state corresponds to s number of characters read in the pattern p and e number of errors recorded. The initial state is $0\#0$ and the six final states are $4\#0, 5\#0, 5\#1, 6\#0, 6\#1$ and $6\#2$. Each non-final state has three outgoing arcs, one for each type of edit operation.



A common solution to deal with the multiple active states is to transform the nondeterministic automaton into an equivalent deterministic one using classical powerset construction [Hopcroft et al., 2004], which will construct a new deterministic state for every possible set of nondeterministic active states. However, the resulting automaton may be exponential in the length of p and it will continue to be limited to representing strings of that defined length. In [Schulz and Mihov, 2002] and [Mihov and Schulz, 2004] a universal Levenshtein automaton was introduced based upon insightful observations of the classical one. The term *universal* conveys its one-time construction and independency of p , thus it can be applied to any pair of strings of arbitrary lengths. The intuition arises from the symmetry of the nondeterministic automaton, which applies the same set of transition rules to every new input character and each new set of active states is a subset of a known bounded superset. Formally, these bounded supersets are called *symbolic triangular areas* and defined in Definition 1.

Definition 1. [Mihov and Schulz, 2004] Let p denote a pattern of length ρ and $A(p, d)$ the nondeterministic Levenshtein automaton for error d . The **triangular area of a state** λ of $A(p, d)$ consists of all states β of $A(p, d)$ that can be reached from λ using a (potentially empty)

sequence of u upward transitions and, in addition, $h \leq u$ horizontal or reverse (i.e., leftward) horizontal transitions. Let $0 \leq i \leq \rho$. By **triangular area** i , we mean the triangular area of state i^0 . For $j = 1, \dots, d$, by **triangular area** $\rho + j$, we mean the triangular area of state ρ^j .

To highlight the idea behind this principle, let us search any query in the generic nondeterministic automaton for $\rho = 7$ and $d = 2$ illustrated in Figure 2.3 (meaning the same Levenshtein automaton for $d = 2$ can be applied to any pattern p of length $\rho = 7$). After reading the 2^{nd} character in our query, the set of all possible active states is bounded by the yellow symbolic triangular area (A). By reading the 3^{rd} character, the symbolic triangular area will shift one position to the right. The semantics of the Levenshtein automaton transitions enforce that the horizontal span of all reachable states is $\leq 2d + 1$. For example for $d = 2$, the base of the triangular area will span 5 states (see Figure 2.3). The new set of active states for each triangular area only depends on the previous set of active states and the new transitions taken by reading any character. As long as the number of characters read is $< \rho - d$, the new subset of active states will always contain *non-accepting* (I states). Beginning at the $(\rho - d)^{th}$ character, the triangular area will also contain *accepting* states (M states), see Figure 2.4. Together, these general observations have led to the formulation of a universal Levenshtein automaton. In full generality, the size of the automaton is exponential in a function of d . More specifically, the total number of deterministic states is classified by $O((d + 1)2^{4d - \log_2 \sqrt{2d+1}})$ [Mitankin, 2005]. Therefore, the automaton grows quickly for higher number of errors, where for $d = 1$ there are only 14 states and for $d = 2$ or $d = 3$ there are already 90 or 602 states respectively.

A set of characteristic vectors symbolizing the homology of p and a word W serve as the alphabet to the automaton and must be precomputed using Definition 2.

Definition 2. [Mihov and Schulz, 2004] The **characteristic vector** $\vec{\chi}(w, V)$ of a symbol $w \in \Sigma$ in a word $V = v_1 \dots v_n \in \Sigma^*$ is the bitvector of length n where the i th bit is set to 1 iff $w = v_i$.

The length of the bitvectors is $\leq (2d + 1) + 1$, where $2d + 1$ is the longest span of reachable states in the nondeterministic Levenshtein automaton and the additional sum of 1 is for the last bit to identify the transition between non-accepting and accepting states.

Let $d = 1$, the input word $W = acaga$ and the pattern $p = \$actaga$ (d number of \$ characters are added to the prefix to standardize the length of bitvectors obtained for the initial characters), then $\chi_1(\underline{a}, \$act) = 0\underline{1}00$, $\chi_2(\underline{c}, acta) = 0\underline{1}00$, $\chi_3(\underline{a}, ctag) = 00\underline{1}0$, $\chi_4(\underline{g}, taga) = 00\underline{1}0$, $\chi_5(\underline{a}, aga) = \underline{1}0\underline{1}$ are the computed characteristic bitvectors. It follows that $\{\chi_1, \dots, \chi_5\}$ is the *characteristic bitvector array* carrying the similarity information of W and p .

Beginning from χ_1 to $\chi_{|W|}$, the bitvectors are sequentially passed into the universal Levenshtein automaton and serve as the new alphabet (rather than the letters themselves). Each bitvector leads to a transition between states in constant time (provided that the bitvector fits into a computer word) corresponding to the number of errors encountered thus far. If some χ_i reaches a failure state, greater than d errors exist between W and p , and the strings are rejected. The automaton only recognizes two strings if the input of the last bitvector $\chi_{|W|}$ leads to a final state. An example of bitvector computations and automaton traversal for the two words $p = acctga$ and $W = atcctga$ is shown in Figure 2.5.

The mathematics behind constructing this automaton are well described in [Schulz and Mihov, 2002, Mihov and Schulz, 2004], however here we also give a simple example of how the universal property can be identified using a classical non-deterministic Levenshtein automaton. Figure 2.6 illustrates a sequence of snapshots for the active states of

a non-deterministic Levenshtein automaton for $d = 1$ for the word $W = acgt$ and the input pattern $p = agt$ as p is introduced into the automaton character by character. The universal Levenshtein automaton determinizes the active states in the yellow triangle, and the changes of transitions are carried out using characteristic bitvectors (in red) rather than individual letters of the input pattern.

2.1.1 Application in biological sequence alignment

The ability of the universal Levenshtein automaton to quickly recognize two strings with edit distance $\leq d$ is an extremely valuable property for developing new approximate seeds allowing indel errors. We chose to work with $d = 1$ as the corresponding automaton is very small and the search time is up to 20x faster than for $d = 2$ and 60x faster than for $d = 3$ [Mihov and Schulz, 2004]. However, higher error automata may be useful for more specialized applications such as tracking dinucleotide mutations or identifying variations in protein coding regions. Our approach to using a universal seed with $d = 1$ is to search for short k -mer matches for $k \in [8, 26]$ between a read and a database of reference sequences allowing up to 1 error. Since the input to the universal Levenshtein automaton is a series of characteristic bitvectors computed from two strings (rather than the strings themselves), we had to determine a clever technique to quickly build these bitvectors between any k -mer on the read and those found in the reference database. For this, we devised the *dynamic bitvector table* which is illustrated in Figure 2.7.

Going back to the problem of finding all *approximate* k -mer occurrences in a text (see Section 1.4.1), we can precompute a bitvector table of size $4k$ (for DNA text) and use it to retrieve any combination of characteristic bitvectors for all 4^{k+1} possible matches (a match is defined to have ≤ 1 error to the k -mer) in the text *without* actually ever seeing the text. During the search for a k -mer in a text, we can quickly retrieve the correct bitvector corresponding to a character being observed in the text without needing to recompute the bitvector for every new candidate. Initially, the dynamic bitvector table is computed for the first k -mer on the read where this k -mer is equivalent to the word V in Definition 2 and each input symbol $w \in \{a, c, g, t\}$ belongs to a k -mer in the reference text (see Figure 2.7). Thusly, in order to search for all k -mers on the read in the reference text, the bitvector table can be shifted by one position to the right on the read and updated using bitwise operations to remove the first character of the preceding k -mer and add a new character to the suffix. This operation can be seen in Figure 2.8.

By continuously shifting the bitvector table along the read, a new characteristic bitvector table can be quickly precomputed for each k -mer using the previous one and used to search the reference text. As discussed in Section 1.4, naïvely searching for a query k -mer in a large text by comparing each k -mer against all possible candidates is extremely inefficient, thus we have also developed a new lossless text indexing data structure for rapidly listing all k -mer matches. This reference index is constructed one time and may be reused for any set of reads. The following subsections describe this indexing data structure and its main utilization in searching for seeds.

2.2 Indexing with the Burst trie

In Section 1.4.3 we have seen that tree-like index data structures, such as the suffix tree, are suitable for approximate seeds. However, suffix trees require large memory resources and more suitable data structures exist for searching large collections of *identical length* strings. Here, we propose to use an alternative data structure, namely the Burst trie [Heinz et al., 2002]. Further-

Figure 2.3: Symbolic triangular area for non-accepting states of type I . The yellow symbolic triangle (A) represents the set of all *possible* active states after the 2nd letter x_2 of an input word was read by the automaton. Similarly, after reading the 3rd letter x_3 , the triangular area shifts one position to the right. The new triangular area (B) will encompass all new states reached by transitions from triangular area (A). Since neither (A) nor (B) contain a final state of type M , they fall under the same name of “symbolic triangular area for non-accepting states of type I ”.

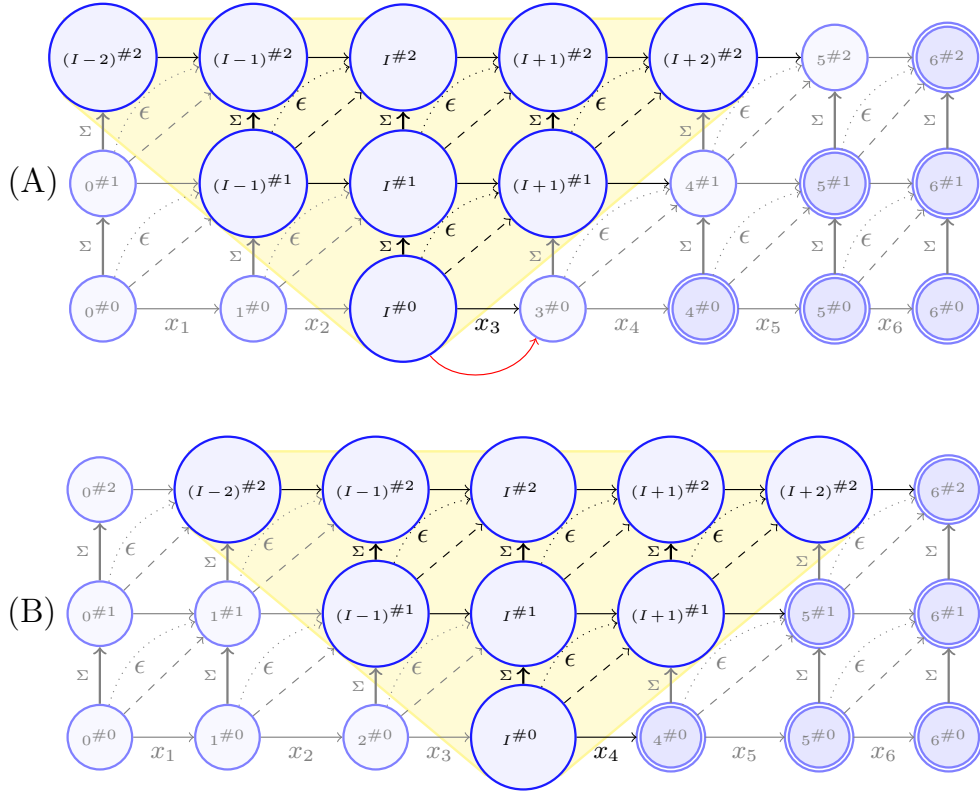


Figure 2.4: Symbolic triangular area for accepting states of type M . The yellow symbolic triangle represents all *possible* active states after the 4th letter x_4 was read by the automaton. This triangular area encompasses three of the six final non-deterministic states $\{(M-2)\#0, (M-1)\#1$ and $M\#2\}$ and is known as the *accepting-state* triangular area. All deterministic states derived from this area will form *accepting states* in the universal Levenshtein automaton for $d = 2$.

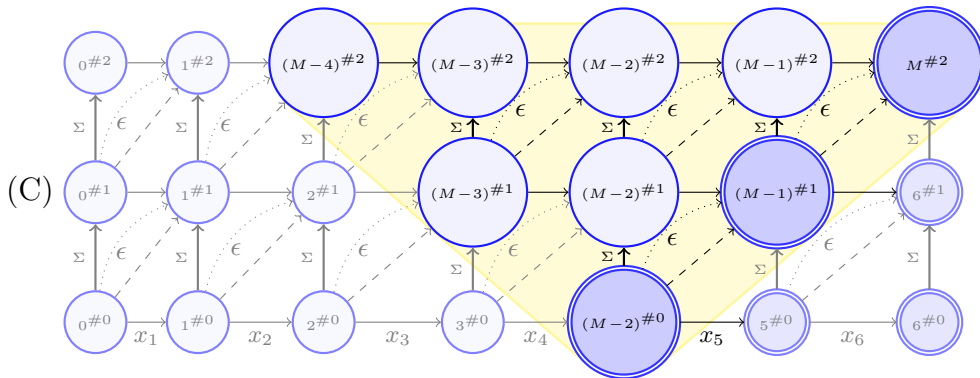


Figure 2.5: Conceptual example of the universal deterministic Levenshtein automaton for $d = 1$ (not all of the transitions are shown). To see whether the word $W = \text{atcctga}$ and the pattern $p = \text{acctga}$ have Levenshtein distance ≤ 1 , we first compute the set of characteristic bitvectors representing them using Definition 2. The resulting characteristic bitvector array is $\chi = \{0100, 0001, 1100, 1000, 100, 10, 1\}$. In the universal Levenshtein automaton, the I states are the deterministic non-accepting states of the symbolic triangular area shown in Figure 2.3. Similarly, the M states are the deterministic accepting states of the symbolic triangular area shown in Figure 2.4. The transitions between states are made using one bitvector from the characteristic bitvector array. For each transition, the x character in the bitvector is a joker and will accept both a ‘0’ or a ‘1’ in its corresponding position. Moreover, if the joker character is found inside brackets, i.e. (x) , then it is not obligatory to exist in the bitvector. For example, the transition labeled as $x1x(x)$ will accept the bitvectors 0101, 1100 and 111 (amongst other possibilities). However, it will not accept the bitvectors 1001, 1000 and 11 (amongst other possibilities). Beginning from the initial state (labeled ‘start’), each bitvector in χ is input to the automaton in order and the transitions are followed accordingly. If the last bitvector transition leads to an M state, then W and p match with $d \leq 1$. Otherwise if a null state is reached (meaning there does not exist a transition from the current state corresponding the next bitvector) or an I state is reached using the last bitvector in χ , then W and p do not match with $d \leq 1$.

Step	Precomputed bitvectors	State transitions
(a)	$\chi_1(a, \$acc) = 0100$	$\{I\#0\} \xrightarrow{0100} \{I\#0\}$
(b)	$\chi_2(t, acct) = 0001$	$\{I\#0\} \xrightarrow{0001} \{(I-1)\#1, I\#1\}$
(c)	$\chi_3(c, cctg) = 1100$	$\{(I-1)\#1, I\#1\} \xrightarrow{1100} \{(I-1)\#1, I\#1\}$
(d)	$\chi_4(c, ctga) = 1000$	$\{(I-1)\#1, I\#1\} \xrightarrow{1000} \{(I-1)\#1\}$
(e)	$\chi_5(t, tga) = 100$	$\{(I-1)\#1\} \xrightarrow{100} \{(I-1)\#1\}$
(f)	$\chi_6(g, ga) = 10$	$\{(I-1)\#1\} \xrightarrow{10} \{(I-1)\#1\}$
(g)	$\chi_8(a, a) = 1$	$\{(I-1)\#1\} \xrightarrow{1} \{M\#1\}$

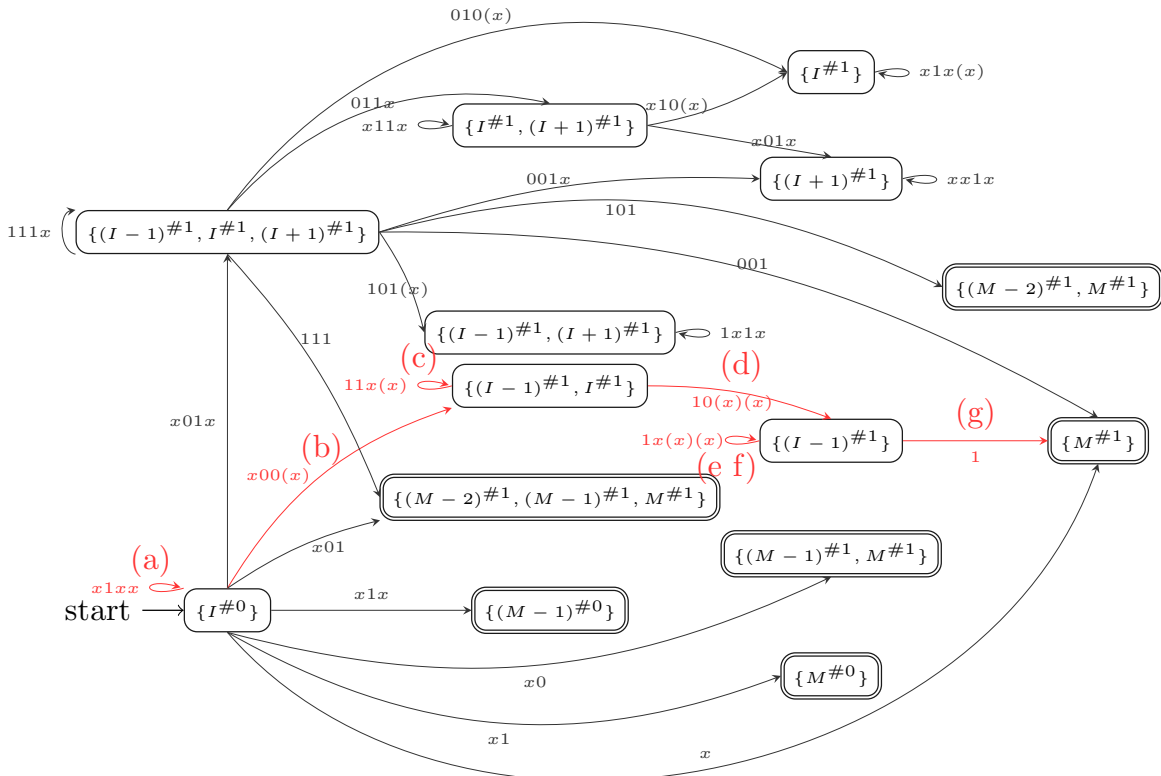


Figure 2.7: The precomputed bitvector table for pattern $p = \$actaga$ covering all possibilities of q for $d = 1$. The first bit in each entry of column $i = 0$ represents the $\$$ symbol and is always set to ‘0’. If the query $q = actag$ was being searched, then the highlighted set of bitvectors 0100, 0100, 0010, 0010, 101 would be passed to the universal Levenshtein automaton.

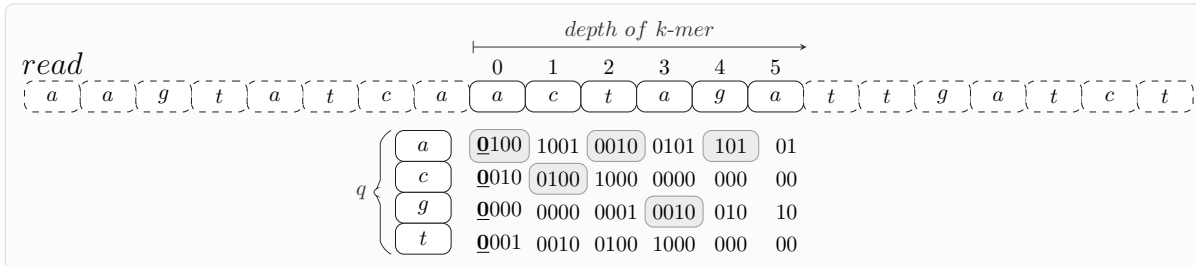
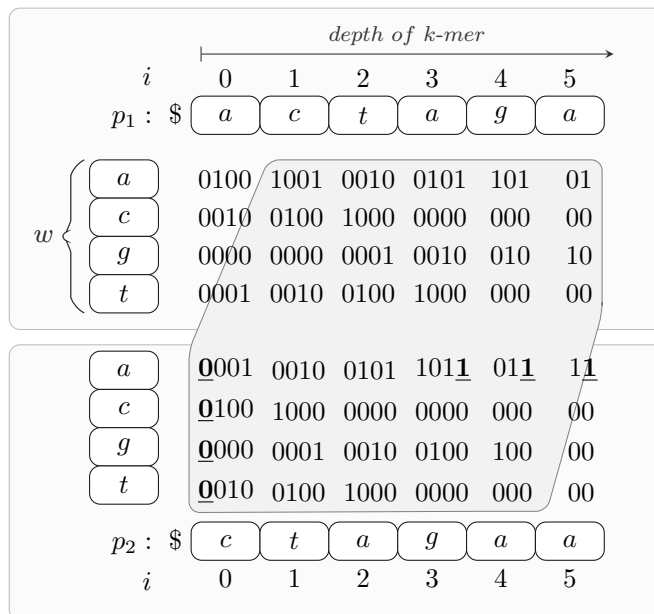


Figure 2.8: The modification of the bitvector table from pattern $p_1 = \$actaga$ to $p_2 = \$ctagaa$ for $d = 1$. Columns 0-2 of p_2 are equal to columns 1-3 of p_1 , except for column 0, where the most significant bit (MSB) of every bitvector represents the symbol $\$$ and is set to ‘0’. Column 3 of p_2 equals to column 4 of p_1 with an additional bit appended. The appended bit is set to ‘1’ in the bitvector corresponding to the newly appended character, otherwise it is set to 0. Column 4 of p_2 is equal to column 3 of p_2 , although the MSB is not considered. The same rule applies to column 5 of p_2 , where the two MSBs of the column 3 bitvectors are not considered.



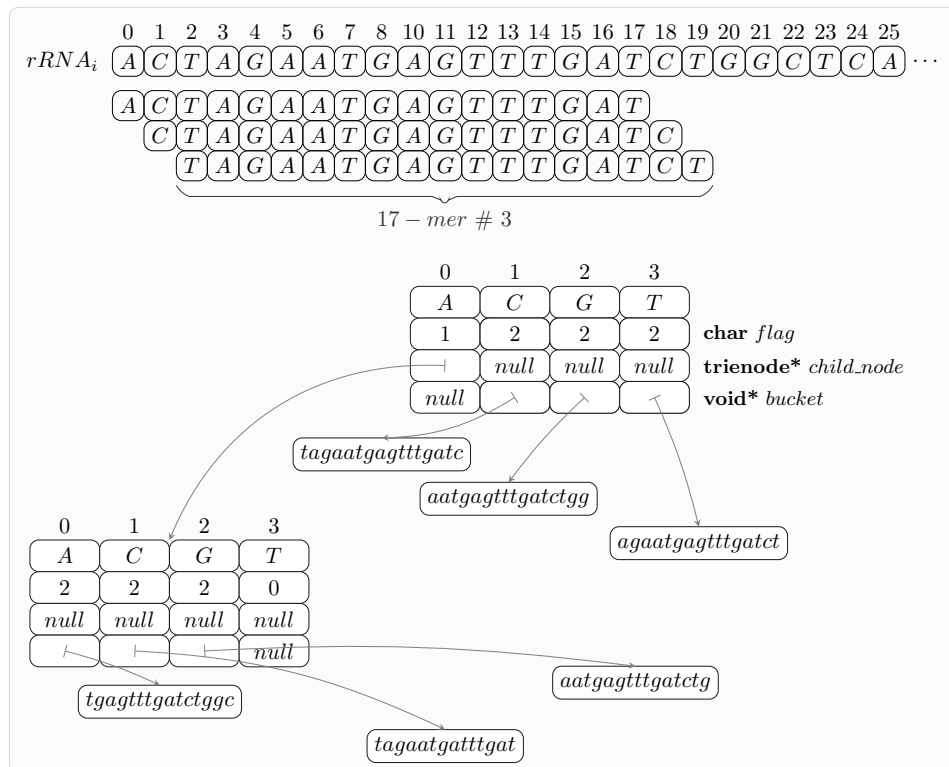
more, we explain how to optimize this data structure for searching words of length k allowing up to one error.

2.2.1 The Burst trie

The Burst trie is a fast and versatile data structure which effectively stores large numbers of strings such as an rRNA database or large collections of genomic sequences. Given a sequence $x = ab$, the Burst trie can store the prefix a as a link of trie nodes and the suffix b as an array of characters appended to the last trie node: this extension to the last node is called a “bucket” (see

Figure 2.9). Normally, subtrees become more sparse in the depth of a trie and representing them as reduced “buckets” of contiguous memory preserves space and boosts cache-efficiency. When the number of sequences sharing a common prefix a reaches a fixed threshold, the appended bucket of suffixes bursts to form a new trie node and smaller sub-buckets. To optimize memory access during subtree traversal, the threshold size of a bucket should be less than the lower level cache. A systematic use of this trie can be observed in the fastest sorting algorithm for large sets of strings, the Burstsrt [Sinha and Zobel, 2004].

Figure 2.9: Let $k = 16$, the Burst trie below is constructed on the first six 17-mers of a reference sequence. The ‘char flag’ describes whether a pointer is set to a trie node ‘1’, a bucket ‘2’ or neither ‘0’. Additional information on the origin of the 17-mer directly follows each element, as shown in the dashed bucket.



2.2.2 Improvement: Lookup table & mini-burst tries

We use an additional optimization to improve access into the Burst trie. Since we consider at most one error between the window and the database, we have this simple property: For every two words of length $\sim k$ such that the edit distance between them is bounded by 1, there exists a common substring of length $\frac{k}{2}$ which is either a prefix or a suffix of the two words. We apply this property to construct a lookup table storing all $\frac{k}{2}$ -mers existing in the reference database. Note that for $k \in [8, 26]$, transposing the nucleotide alphabet onto a binary equivalent, such that $\{a, c, g, t\} = \{00, 01, 10, 11\}$, we can represent each $\frac{k}{2}$ -mer in k bits which maps to a unique integer value. Upon completion of the forward and reverse Burst tries, a scan of each trie is performed to record the existence of all $\frac{k}{2}$ -mers and, if present, associated pointers to the trie node representing the immediate letter following the prefix. The precomputed lookup table

quickly determines whether an exact match of the prefix or suffix exists in the Burst tries and furthermore it provides us with direct access to the remaining part of the word in the Burst trie.

2.2.3 Implementation

Following a similar method of an array-structured trie as described in [Sinha et al., 2006], our Burst trie is assembled exactly on the nucleotide alphabet $\{a, c, g, t\}$. As illustrated in Figure 2.9, the trie stores every unique $(k + 1)$ -mer substring in a reference database, since we look at seeds of length k with at most 1 error between any two words (note that the extra nucleotide for a reference $(k + 1)$ -mer is to account for a possible insertion or deletion of a nucleotide in the query k -mer). The information on whether the $(k + 1)$ -mer belongs to a forward strand, the reverse-complement or both (*strand*), and its origin (*hashid*) follows each entry in a bucket (not shown in Figure 2.9). When the exact location of the $(k + 1)$ -mer needs to be found in the reference database, the *hashid* value serves as an index in a complementary table storing this information. For databases containing highly conserved sequences, such as the 16 rRNA for which nearly one-quarter of the positions are 99 – 100% conserved [Cannone et al., 2002, Mears et al., 2002], this data structure moderates the size of the tree since many identical or closely similar substrings are shared between sequences.

In our initial implementation of the index we constructed one Burst trie on all possible unique $(k + 1)$ -mers in the reference database and used a $\frac{k}{2}$ -mer lookup table to quickly access different nodes of the Burst trie. However, this approach can be further improved by “cutting off” the trie nodes from the root of the Burst trie to depth k and replacing them by a lookup table. Then, every entry in the lookup table is connected to a separate (mini) burst trie, which represent the children nodes (below depth k) of the original full trie. In the following subsections we will describe how such a data structure can be easily computed directly, without the prior need to explicitly construct a full Burst trie, to both reduce the memory requirements and speed up the search by optimizing calls to the local cache.

Index construction for the reference database

To find all occurrences of a k -mer in a reference database with an edit distance of at most 1, we apply the pigeonhole principle to divide the k -mer into two equal parts, such that at least one part will match with 0 error. This property allows us to take advantage of the following arborescent data structure setup:

1. build a $(\frac{k}{2})$ -mer exact match lookup table (see Figure 2.10(2a))
2. connect each entry in the lookup table to a *mini-burst trie* storing the remaining $\frac{k}{2} + 1$ characters and allowing search up to one error (see Figure 2.10(2b/c))

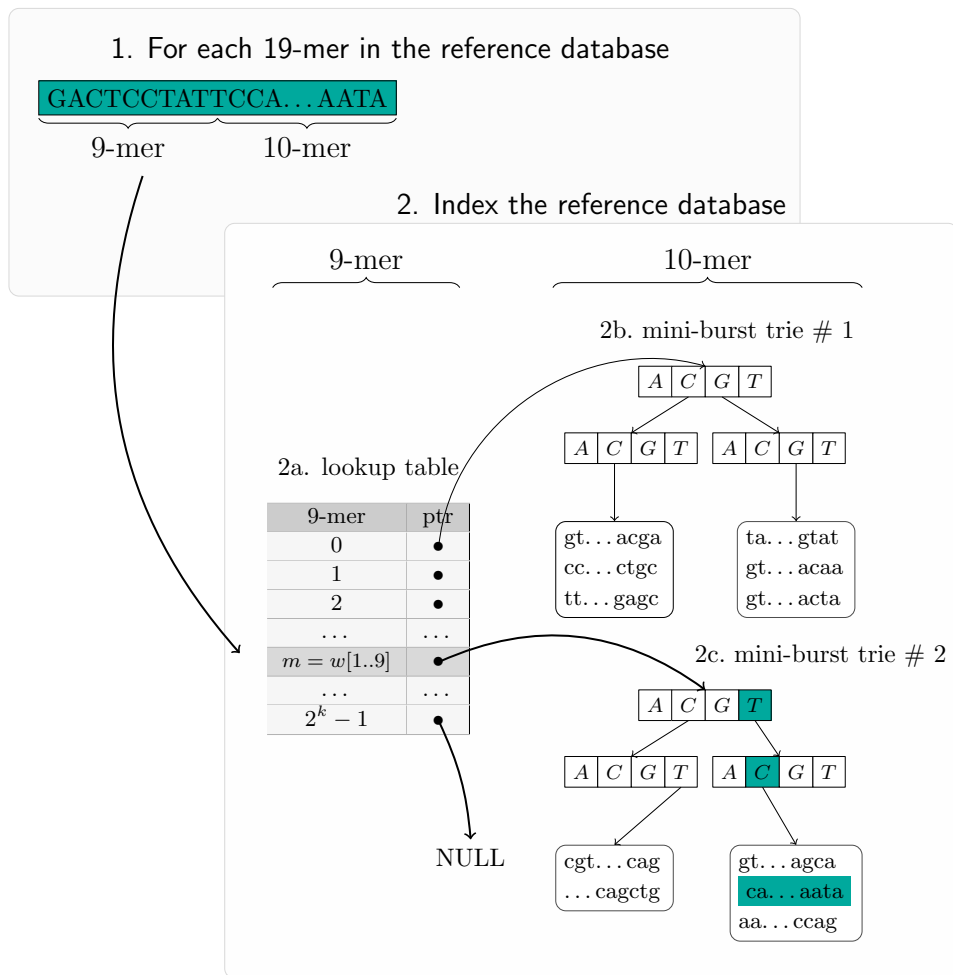
The arborescent index was inspired by the burst trie data structure, but with a twist to index the prefixes of strings with a lookup table and the suffixes in a collection of branching (mini) burst tries. The burst trie data structure was chosen due to its relatively low memory footprint and fast access to each entry.

The $(\frac{k}{2})$ -mer lookup table and the $(\frac{k}{2} + 1)$ -mer mini-burst tries work hand-in-hand to store all unique $(k + 1)$ -mers of the reference database, and a separate list of positions gives occurrences for all unique $(k + 1)$ -mers. The $(\frac{k}{2})$ -mer lookup table is accessed by converting a $(\frac{k}{2})$ -mer into

a unique decimal value using 2 bits per nucleotide encoding, where $A = 00$, $C = 01$, $G = 10$ and $T = 11$. For example the lookup index i for the string $x = ACTAGTATT$ would be $i = 000111001011001111 = 29391$.

This arborescent data structure was designed to optimally enumerate all $(k+1)$ -mer sequences which match to a k -mer query sequence allowing up to 1 error, note that the extra nucleotide for a reference $(k+1)$ -mer is to account for a possible insertion or deletion of a nucleotide in the query k -mer. The original positions of all $(k+1)$ -mers in the reference index are stored in a separate inverted list of positions. The index for this list is computed using the CHM minimal perfect hash function (CMPH library [Reis et al., 2012]) and stored next to each $(\frac{k}{2} + 1)$ -mer entry in the mini-burst trie leaves. In future implementations, the size of this auxiliary positions list can possibly be reduced in size by using a pairing function [Lisi, 2007].

Figure 2.10: Lookup table and mini-burst trie index



Due to the dynamic nature of index construction (self-adjusting tree composed of trie nodes and bucket nodes), one cannot precisely calculate the size of the index before it is built. However, from multiple simulations on varying data, the estimated size has shown to be inferior to

$100 \times (\text{length of reference sequence})$ bytes. To keep the index of a practical size, it can be divided into multiple subparts. During the mapping of reads, each index subpart can be sequentially loaded and processed in memory, always maintaining the memory for the index below its designated threshold. Although the optimal mapping time is achieved if the index can be constructed in one part, the overhead time with processing multiple subparts is not impractical. Thus, the index fragmentation feature nonetheless allows convenient utilization of this data structure.

2.2.4 Searching for matching seeds in the reference index

The search for a k -mer seed in the indexed database is performed in two steps. Firstly, the k -mer is split into two equal parts and the first part is directly searched in the $(\frac{k}{2})$ -mer lookup table (with 0 errors). Then, if the $(\frac{k}{2})$ -mer exists, a pointer redirects the remaining of the search to a corresponding mini-burst trie (allowing up to 1 error). Here, a parallel traversal is performed between the second part of the k -mer against all entries in the mini-burst trie using the universal Levenshtein automaton for edit distance $d = 1$. At every depth of the mini-burst trie, we assume that the symbol q in $\chi_i(q, V)$ appears as one of $\{a, c, g, t\}$ with equal probability. Following a pre-order path, the traversal of the Burst trie begins at the root node. Through knowledge of the nucleotide letter and the depth of the node being visited, the coinciding bitvector is accessed in the precomputed bitvector table, indifferent to whether the node is a trie node or a character in the bucket. Subsequently, the bitvector is passed to the universal Levenshtein automaton which decides whether to continue traversal of the current subtree or backtrack to the first branching point with a non-failure Levenshtein state and recommence traversal of a new subtree. In this manner, a complete traversal of the mini-burst trie remains unlikely as backtracking occurs each time the edit distance between the pattern and a traversed branch exceeds k . To further speed up Burst trie traversal for every k -mer, a ‘backwards dictionary’ approach as described in [Mihov and Schulz, 2004] was implemented. This means that we build mini-burst tries for the forward and reverse sense of $(k + 1)$ -mer in the reference database so that it can be traversed quickly from both ends.

2.3 Extending seeds into longer matches using the LIS

The Smith-Waterman dynamic programming algorithm is the most precise method to score a homology between two sequences and is used in the majority of short-read alignment tools after the initial seeding step. Here we describe our method to use the collection of matching seeds accumulated during mini-burst trie traversal to isolate longer homologous regions between the read and a reference sequence prior to performing localized Smith-Waterman alignment. We begin by binning the seeds to their corresponding reference sequences by using the $(k + 1)$ -mer list of positions to link each matching seed to the original location on a reference sequence as illustrated in Figure 2.11. Next, we isolate regions of length equal to the read on each reference sequence containing a threshold number of matching seeds (by default 2). The next step involves computing variant of the *longest increasing subsequence* (LIS) on the seeds’ positions on the read relative to a region on the reference sequence, as shown in Steps 3-4 of Figure 2.11. The LIS is the longest subsequence of elements in a sequence where the elements in the subsequence are in increasing (sorted) order. For example for the given the sequence of integers $\{7, 1, 9, 6, 42, 23, 40, 26\}$ one possible LIS is $\{1, 6, 23, 40\}$. By first sorting the (position on reference - position on read) k -mer positions using the ‘position on reference’ as a key and then computing the LIS on the reads’

positions, we try to reassemble our collection of k -mers into a longer contiguous region common to both sequences. Finally, if the LIS is composed of a threshold number of seeds (by default 2), we proceed to the final step of Smith-Waterman alignment beginning near the LIS.

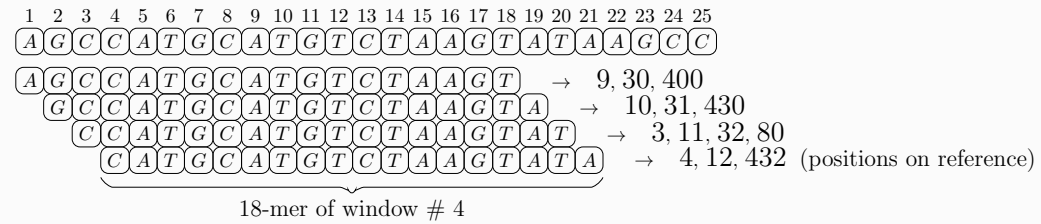
2.4 Conclusion

In this chapter we introduced a novel approximate seeding technique along with the complementary machinery optimized for its efficiency. We described a new index data structure never used before in short-read sequence alignment which can accommodate seeds allowing up to 1 error of any type. Moreover, the error is not restricted to any predefined position in the seed which gives it flexibility for unpredictable error distribution. The tradeoff for maintaining such seeds is the size of the new index data structure which is able to accommodate searches with insertions and deletions, requiring more space than the BWT. However, due to the malleability of the data structures used, the index can be divided into multiple subparts without significant effects on its performance.

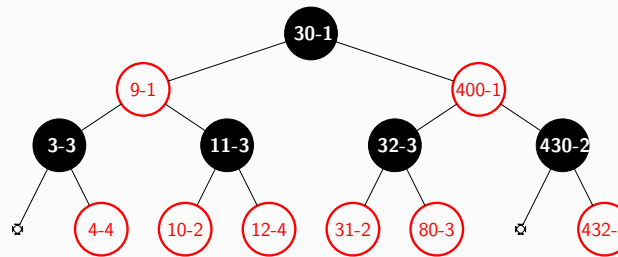
* * *

Figure 2.11: How to extend multiple k -mer matches into a longer homologous region using the longest increasing subsequence of k -mer positions

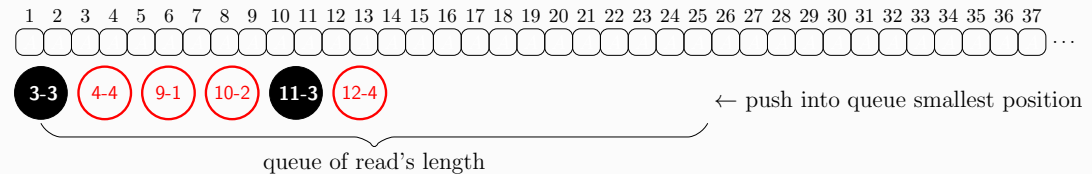
Step 1. For each k -mer on the read, collect the list of positions where it appears on the reference



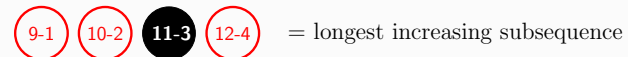
Step 2. Sort all lists by position on the reference using quicksort, or a self-balancing tree, or a min-heap array (the key is the position on reference, the value is the position on the read)



Step 3. Using a double-ended queue of the length of the read, push into the queue the smallest reference positions fitting within the range of read's length



Step 4. If the number of elements in the queue is greater than threshold r , find the **longest-increasing-subsequence** of the corresponding read k -mer positions



Step 5. Apply **Smith-Waterman** alignment starting near the first position of the LIS



Chapter 3

SortMeRNA: a filter for metatranscriptomic data

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In this chapter, we present a first example for an application of the approximate seeding framework introduced in Chapter 2. We designed an efficient filter to rapidly sort through millions of reads generated by metatranscriptomic sequencing projects and identify the ribosomal RNA fragments within. This classification step is a prerequisite to any further bioinformatic analysis.

The method is implemented in a software called SortMeRNA, that was released in October 2012 and published in the journal *Bioinformatics* [Kopylova et al., 2012]. It is now used in production by Genoscope (French National Center for Sequencing) to process data from Tara Oceans. It has also been integrated in two published computational pipelines [Leimena et al., 2013, Krohn-Molt et al., 2013] and received excellent feedback from multiple research laboratories worldwide³.

This chapter is organized as follows. In Section 3.1, we present the biological context of metatranscriptome sequencing projects and the computational challenges posed by this new sort

³Umeå University (Sweden), Leibniz Institute DSMZ (Germany), NGS department of Campus Science Support Facilities GmbH (Austria), Oxford Centre for Integrative Systems Biology (Great Britain), Laboratoire d'Ecologie Alpine (Grenoble), PRABI (Lyon), Wageningen University (Netherlands), SciLifeLab (Stockholm), SCELSE (Singapore), ..

of data. In Section 3.2, we describe the algorithm behind SortMeRNA. In Section 3.3, we provide performance results on simulated and real data, with a comprehensive comparison with other software programs. In Section 3.4, we conclude the chapter with a discussion on the overall results.

3.1 Application context: metatranscriptomics analysis

The transcriptome of an organism consists of the set of total RNA, which regularly varies and harmonizes with external environmental conditions and the *metatranscriptome* is an ensemble of all RNA molecules found within a microbial community. Metatranscriptomic profiling via next-generation sequencing provides an authentic representation of species richness within a community at the time of the sampling. It becomes particularly important for samples which cannot be cultivated outside their native environment.

The initial challenge of metatranscriptomic sequenced data analysis is to sort apart the RNA fragments based on their biological significance. Phylogenetic structure of a community is primarily established using the 16S and 18S ribosomal RNA (rRNA) genes, which remain highly conserved among different species of bacteria, archaea and eukarya [Janda and Abbott, 2007]. Other inquiries can be made regarding the functionality of a community by studying the messenger RNA (mRNA) of the actively transcribed genes. Thus, it is of primary interest to sort out the rRNA from the mRNA in the total RNA for answering the most basic questions regarding the species composition, gene regulation and protein information of a metatranscriptome. Subject to prokaryotic or eukaryotic cells, the rRNA content can represent up to 80-85% of total RNA and the protein coding mRNA as little as 1-6% [Sorek and Cossart, 2010]. If one wants to focus exclusively on mRNA, there exist prior-to-sequencing methods to help isolate and enrich mRNA from the total RNA. These methods focus on the depletion of rRNA with technologies such as subtractive hybridization (Life Technologies), exonuclease digestion [Boissinot et al., 2007] and the duplex-specific nuclease treatment (DSN) [Yi et al., 2011]. However, even selective removal of rRNA genes does not guarantee definite depletion, and some rRNA genes can still remain in the metatranscriptomic sample. Alternatively, if the goal is to sort apart RNA without any loss of information, in the case of studying rRNA for species identification, non-invasive methods for separating RNA are also preferred. So there is a need for computational tools able to efficiently catalog families of rRNA sequences in metatranscriptomic datasets.

3.2 SortMeRNA

The goal of SortMeRNA is to solve the following problem: Given a large set of metatranscriptomic reads, how to rapidly isolate the reads belonging to rRNA sequences. Multiple software have been recently developed to address this issue. All of them take advantage of the fact that rRNA sequences are homologs and that the primary structure is well conserved within a biological domain (archaea, bacteria or eukarya). Their major difference is the method used to compare reads against a database of rRNA sequences. The first set of programs, Meta-RNA [Huang et al., 2009], SSU-ALIGN [Nawrocki et al., 2009] and rRNASelector [Lee et al., 2011] share a common approach to represent a rRNA database as a probabilistic model. Both Meta-RNA and rRNASelector use prebuilt Hidden Markov Models (HMM) and consequently sort reads against the database with the HMMER3 package [Eddy, 2011] whereas SSU-ALIGN uses Covariance Models to sup-

port secondary structure information. Alternative tools such as BLASTN [Altschul et al., 1990], used in numerous home-made workflows, and riboPicker [Schmieder et al., 2012] exploit a variation of the seed-and-extend strategy. The tool riboPicker implements a modified version of the Burrows-Wheeler Aligner [Li and Durbin, 2009]. In this context however, reads should be compared against large rRNA databases to achieve a good sensitivity level. In all cases, computational time is still an issue to handle large collections of reads.

3.2.1 Principle of the algorithm

SortMeRNA uses a seeding strategy, just like BLASTN and riboPicker. The novelty is that it combines two main fundamentals to achieve a high sensitivity and fast speed. First, it uses approximate seeds, instead of exact seeds as riboPicker does. We will see in Section 3.3 that it allows us to override some limitations of riboPicker on the sensitivity. Second, the rRNA database is stored in a text index. This allows us to take advantage of redundancy between homolog sequences, as HMMs do, and to build a compressed model of all rRNA sequences. This yields a significant speed up in the computational time, compared to BLASTN.

The algorithm is a straightforward application of the approximate seed framework presented in Chapter 2. Let k be the size of the seed.

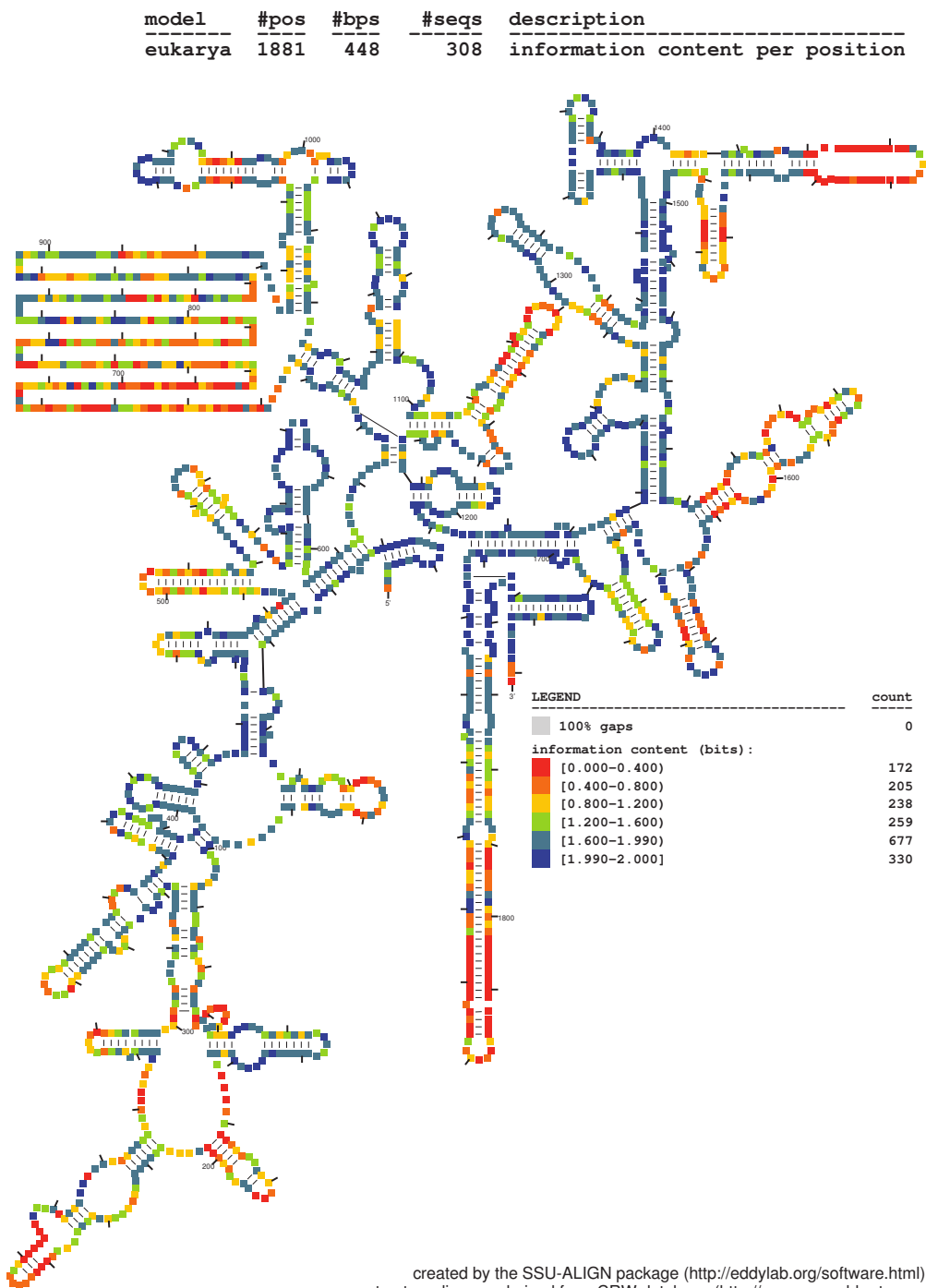
1. The reference database is composed of a collection of rRNA sequences that are indexed in the look up table and the mini burst tries. A multiple sequence alignment of an rRNA database can clearly define areas of high nucleotide conservation and emphasize the evolutionary origins shared between organisms. Figure 3.1 shows such an example of different levels of conservation. Since we are interested in well-conserved regions of the rRNA sequences, before a k -mer is traversed in the mini-burst trie its prefix or suffix must exist a threshold β^4 number of times in the lookup table. This notion enforces that a read matches closely to one region in a database rather than multiple scattered ones leading to a false alignment.
2. We scan each read with a sliding window of length k (the size of the seed), position by position, and count the number of windows present in the reference database, with up to one error. This is done with the universal Levenshtein automaton.

SortMeRNA does not implement an extension phase. The accepted reads are those which have more than a threshold ratio r of windows with a match in the database. This threshold is proportional to the length of the read.

The performances of the algorithm heavily depend on two parameters: The size k of the sliding window, and the minimal proportion r of accepted windows in a read. To find a robust choice for k and r , we ran the algorithm for several values of k and r on different rRNA databases and for several sets of reads. We discuss the choice for parameter settings in the remaining of this section.

⁴The algorithm to compute β ascertains that each read (in the set of reads provided by the user) has at least ratio r seeds, of which at least one $\frac{k}{2}$ -mer has an occurrence greater than β in the rRNA database

Figure 3.1: 18S rRNA secondary structure diagram showing the high conservation (blue areas) of nucleotides in the primary structure. This diagram was generated by SSU-ALIGN for a multiple alignment of 308 eukaryotic 18S rRNA.



alignment file: CRW_plus_mix/CRW_plus_mix.eukarya.stk

page 2

3.2.2 Parameter setting

To estimate best values for k and r , we purposely designed four rRNA databases differing in terms of similarity and the rRNA subunit. We also generated several sets of simulated reads, that should be classified either as *rRNA reads*, or as *non-rRNA reads*, and for which the classification results are known.

3.2.2.1 Construction of rRNA databases

We use the SILVA databases [Pruesse et al., 2007] that administer a comprehensive set of quality checked rRNAs, including a software tool ARB [Ludwig et al., 2004] to aid with phylogenetic analyses of the data via graphical representation. From SILVA, we purposely designed four databases with distinctive features: Small 16S and large 23S subunit, varying identity percentage and from distinct phylogeny tree subparts.

Set 1 : 80% identity 16S rRNA bacteria & archaea (2262 sequences)

Set 2 : 80% identity 16S rRNA bacteria & archaea+truncated phylogeny tree (2187 sequences)

Set 3 : 95% identity 23S rRNA bacteria & archaea (1969 sequences)

Set 4 : 95% identity 23S rRNA bacteria & archaea+truncated phylogeny tree (1906 sequences)

Each database was constructed by applying the ARB package and UCLUST [Edgar, 2010] to the small 16S and large 23S subunit databases from SILVA. The procedure is as follows (see also Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5 for each Set),

1. use the ARB package to extract the phylogeny trees of 16S rRNA and 23S rRNA bacteria & archaea databases in fasta and xml formats,
2. for Set 2 and Set 4, remove a branch in the phylogenetic tree to induce missing species (see Figures 3.3 and 3.5)
3. remove all occurrences of rRNA other than 16S or 23S using description information in the xml format
4. remove long sequences (1600 for 16S rRNA and 5000 for 23S rRNA) and those having > 1% of ambiguous N's,
5. apply UCLUST on the filtered set of rRNAs to create representative 16S and 23S rRNA databases with identity $x\%$.

The identity percentage x refers to the definition of clusters used by UCLUST: Each cluster is defined by a representative sequence, and each sequence in a cluster matches the representative sequence according to the identity threshold. Finally, each database is constituted by the set of representative sequences. By construction, every pair of sequences in the representative database has an identity percentage lower than the threshold x .

3.2.2.2 Simulated reads

We generated simulated reads to be able to estimate the selectivity and the sensitivity of SortMeRNA with varying parameter values. For that, we applied MetaSim [Richter et al., 2008] using provided error models: Roche 454 and Illumina. MetaSim's maximum length error model for the

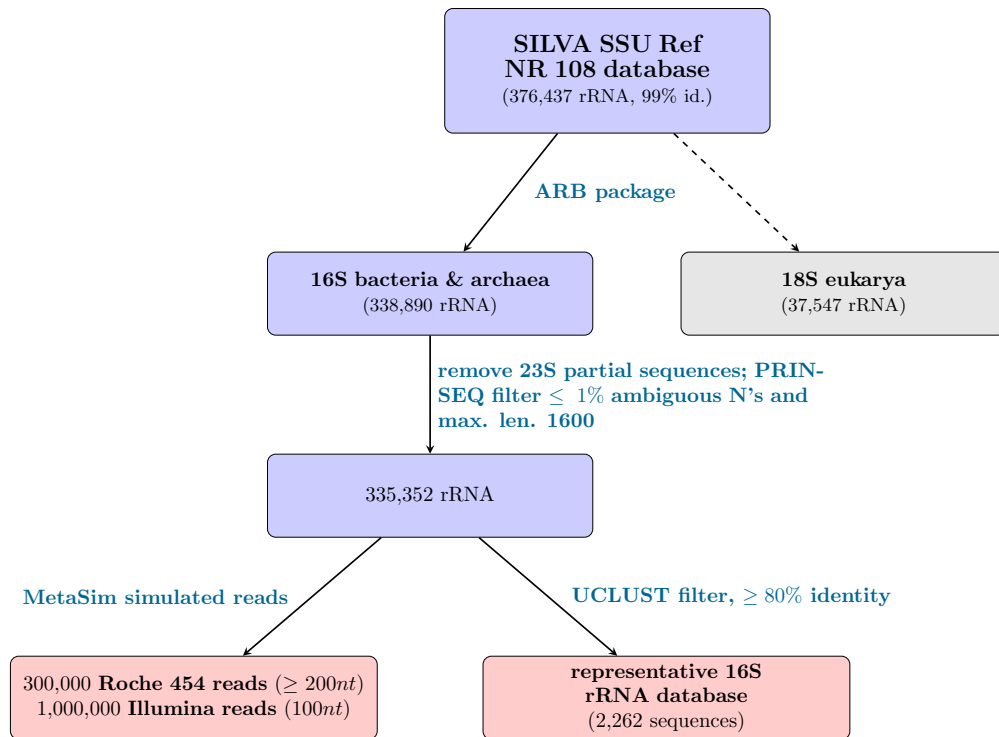
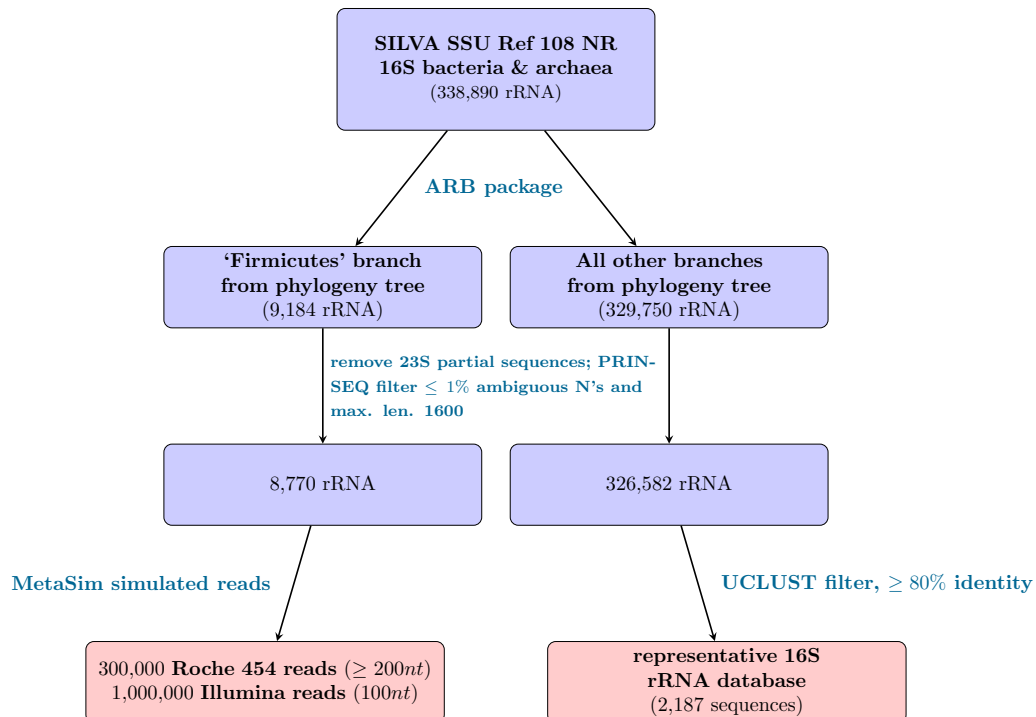
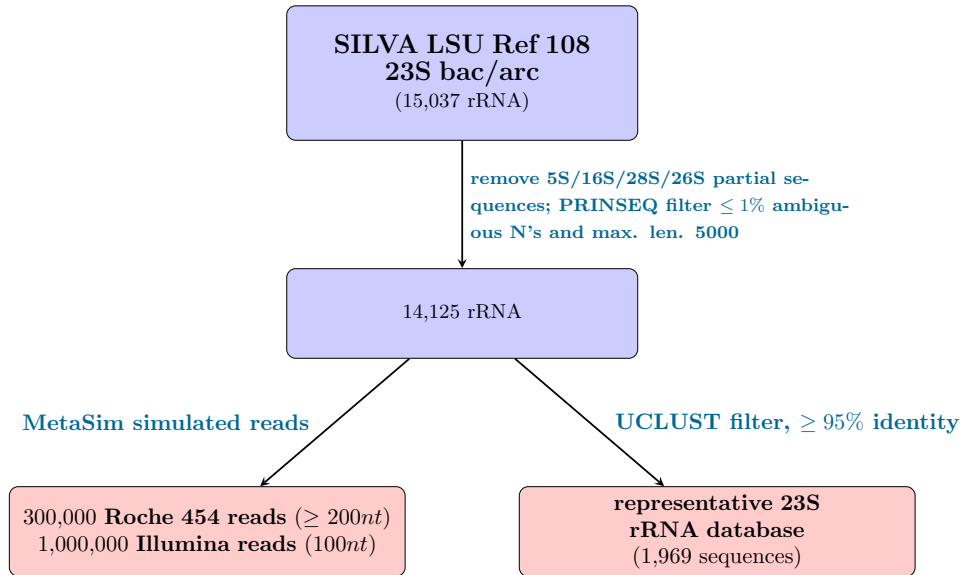
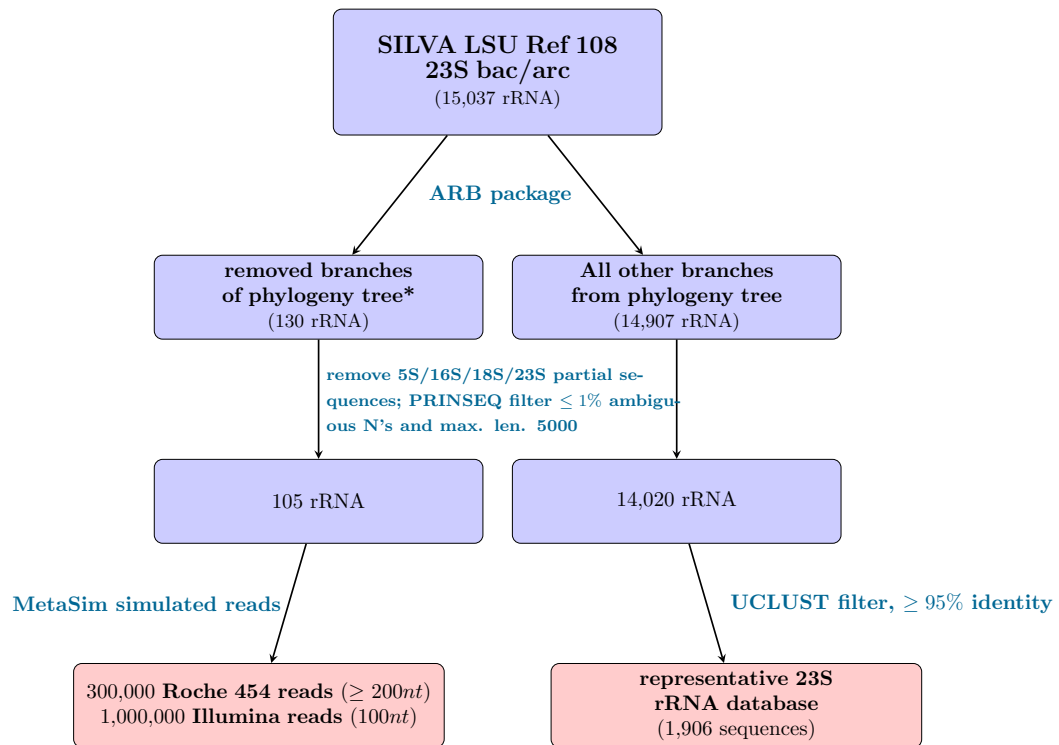
Figure 3.2: Construction of Set 1 – 16S rRNA database with 80% identity**Figure 3.3:** Construction of Set 2 – 16S rRNA database with 80% identity + truncated phylo. tree

Figure 3.4: Construction of Set 3 – 23S rRNA database with 95% identity**Figure 3.5:** Construction of Set 4 – 23S rRNA database with 95% identity + truncated phylogenetic tree. Section of phylogenetic tree: 36 Planctomycetes, 14 Fibrobacteres, 44 Verrucomicrobia, 21 Chloroflexi_1, 6 Candidate division TM7, and 9 Lentisphaerae.

Illumina technology is 80nt, in order to adapt this model for 100nt the last probability value of the 80th position was extended by 20nt. In practice, MetaSim had simulated Roche 454 reads with 2.8-3% sequencing error rate of which approximately 79% were insertions and 21% deletions. Additionally, for Illumina reads, the sequencing error rate was 1.2% of which 100% were substitutions.

Generation of Roche 454 and Illumina rRNA reads. We started from sequences of the SILVA database that have not been already selected in the representative sets (Set 1 to Set 4).

1. apply MetaSim on the filtered set of rRNAs of SILVA not belonging to the representative databases to create 300,000 Roche 454 reads of $\geq 200nt$ and 1,000,000 Illumina reads of 100nt.

In the MetaSim simulator settings, the parameters ‘Mean’ (mean length of clone) and ‘Second Parameter’ (standard deviation of clone length) as defined in the user manual, were set to 1000 and 100 respectively. The default values are 2000 and 200, however since many rRNA sequences are shorter than 2000 nt, we reduced this value to 1000 and the standard deviation to 100.

2. filter out ‘circular’ reads produced by MetaSim, approximately 10% of the reads.
3. filter out reads shorter than 200nt (only for Roche 454) and reads with $> 1\%$ of ambiguous character N (both Roche 454 & Illumina).

Generation of Roche 454 and Illumina non-rRNA reads We started from the NCBI complete bacterial genomes: <ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/>

1. find all locations of rRNAs on the complete NCBI bacterial genomes in the Genbank file format,
2. mask the locations of rRNA in the Fasta file format by a contiguous sequence of N’s (+150nt to cover misannotation)
3. run MetaSim on the NCBI complete bacterial genomes with masked rRNAs to create 1,000,000 Roche 454 reads of $\geq 200nt$ and 1,000,000 Illumina reads of 100nt,
4. filter out ‘circular’ reads produced by MetaSim, approximately 10% of the reads.

In the MetaSim simulator settings, the parameters ‘Mean’ (mean length of clone) and ‘Second Parameter’ (standard deviation of clone length) as defined in the user manual, were set to 2000 and 200 respectively. These default values work well since the average length of a bacterial genome exceeds 2000 nt.

5. filter out reads shorter than 200nt (only for Roche 454) and reads with $> 1\%$ of ambiguous character N (both Roche 454 & Illumina).

Note that some rRNA genes are not annotated and therefore missed during the masking process. This explains that there may be some rRNA fragments in the generated reads, which will impact the selectivity for all programs.

3.2.2.3 Choice of values for k and r

To estimate robust values for k and r , we ran SortMeRNA on Set 1 to Set 4 and varied the parameters $k \in [14, 16, 18, 20]$ and $r \in [0.05, 0.10, 0.15, \dots, 0.95]$. The acceptance rule is as follows. Let ℓ be the length of a read, and ϵ the number of accepted windows for this read. A read is classified as rRNA if the number of accepted windows divided by the number of total full windows on a read is greater or equal to r ,

$$\frac{\epsilon}{\ell - k + 1} \geq r.$$

The results for Roche 454 reads are demonstrated in Figure 3.6 and those for Illumina reads in Figure 3.7. The main conclusion is that $k = 18$, $r = 0.15$ for Roche 454 reads and $k = 18$, $r = 0.25$ for Illumina reads give best sensitivity/selectivity balance for all rRNA databases. Moreover, as shown in the Matthews correlation coefficient tables, varying r within short ranges does not significantly affect the results. We use these values as default settings in all subsequent analyses of Section 3.3.

3.2.3 Availability

SortMeRNA is written in C++ and freely distributed under the GPL license as a stand-alone version or as a Galaxy wrapper. Galaxy is an open, web-based platform that provides users with a graphical workflow management system, and emphasizes accessibility, reproducibility and transparency [Goecks et al., 2010]. Both distributions, including the user manual with installation instructions, can be downloaded from <http://bioinfo.lifl.fr/RNA/sortmerna/>.

The software uses OpenMP functions to parallelize filtering of the reads. The input criteria are a fasta/fastq file of letter space reads produced by Roche 454 or Illumina technologies, and a fasta file of rRNA sequences. There are eight rRNA databases included in the software package covering the small (16S/18S), large (23S/28S) and 5/5.8S ribosomal subunit rRNAs, which were all derived from the SILVA and RFAM databases. Additionally, the user can work with their own RNA databases.

SortMeRNA supports multi-threading and has been tested on Linux (Ubuntu, Fedora, CentOS and Debian) and Mac OS 10.6.8 systems. For compilation, a g++ compiler version 4.3 or higher is required.

3.3 Performance results

The performance of SortMeRNA was measured in terms of sensitivity, selectivity and real-data analysis compared to the previously mentioned software SSU-ALIGN, Meta-RNA, rRNASelector, riboPicker and BLASTN.

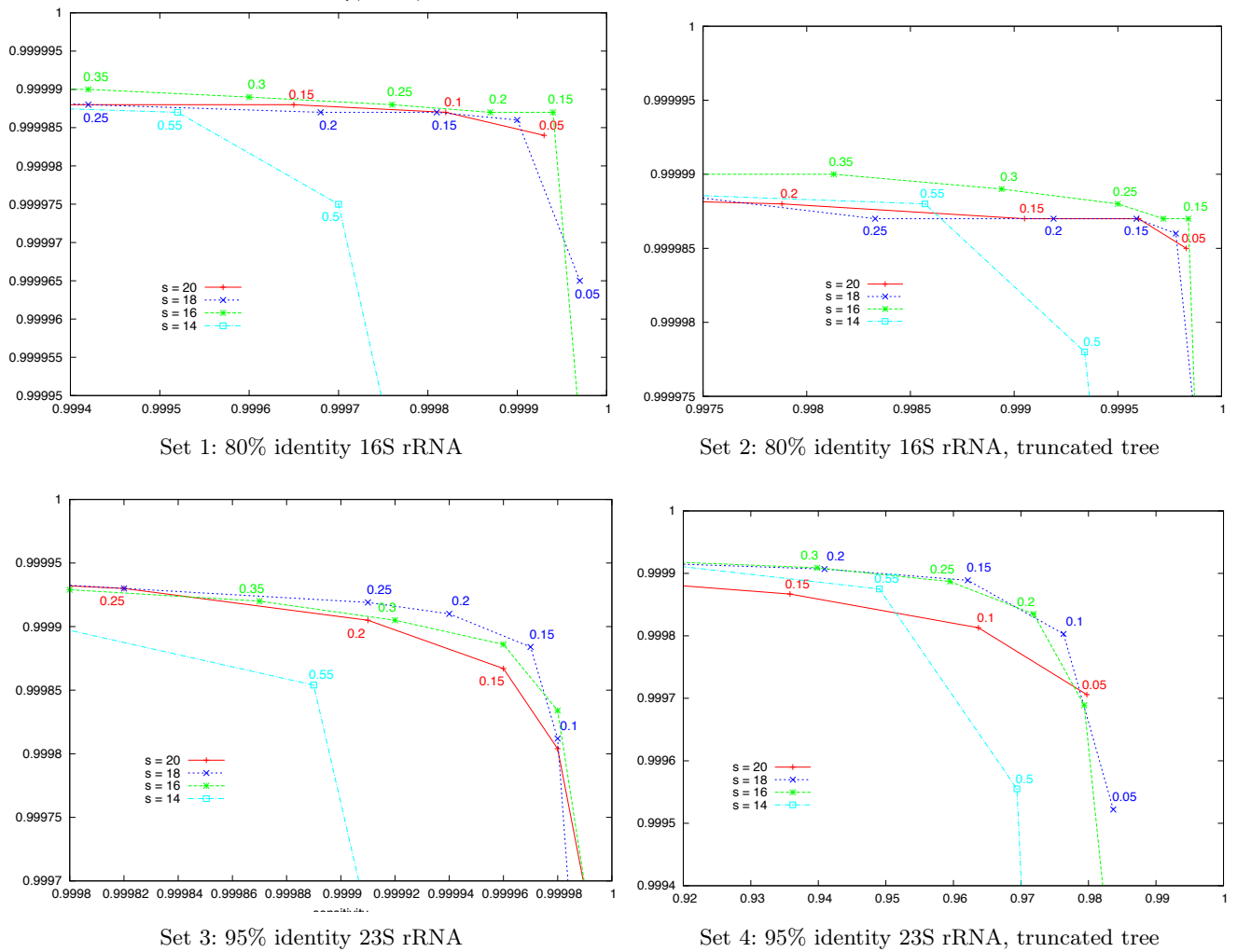
For evaluating all software on an equal basis, another two databases were created: One for 16S rRNA, and one for 23S rRNA.

Set 5 : 85% identity 16S rRNA bacteria & archaea (7659 rRNA), see Figure 3.8

Set 6 : 98% identity 23S rRNA bacteria & archaea (2811 rRNA), see Figure 3.9

The 16S rRNA database was used by SortMeRNA, riboPicker, BLASTN and SSU-ALIGN in Test 1 and Test 3, and the 23S rRNA database by SortMeRNA, riboPicker and BLASTN in

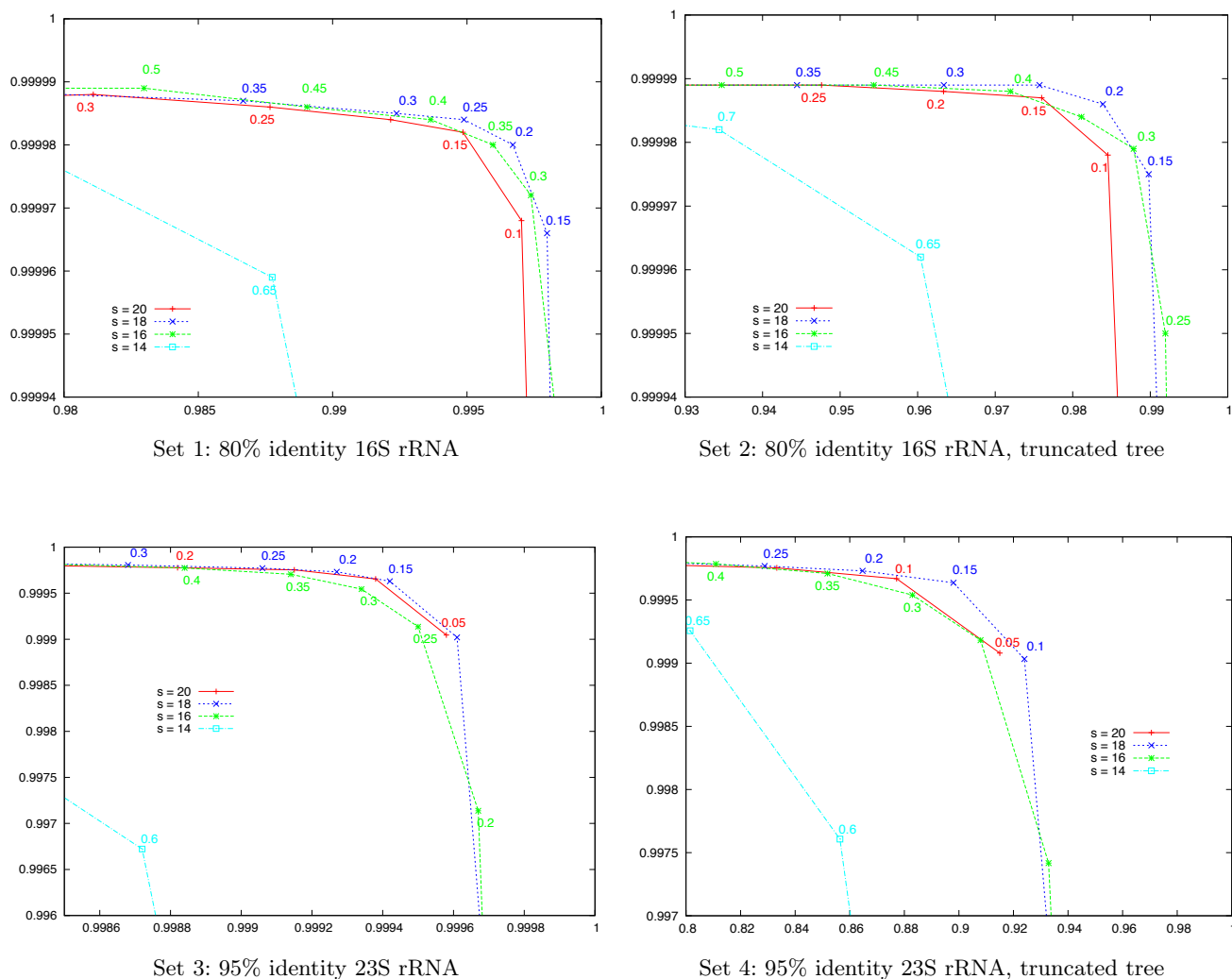
Figure 3.6: SortMeRNA results by varying parameters k (length of the sliding window) and r (ratio of accepted windows) on Set 1 to Set 4 for **Roche 454** simulated reads. For each graph, the horizontal axis is for the sensitivity, and the vertical axis is for the selectivity. Each curve corresponds to a different value for k : $k = 14, 16, 18, 20$. Each dot on a curve corresponds to a different value for r . Below: Matthews correlation coefficients for $k = 18$ and various values of r .



Matthews correlation coefficient

r	0.05	0.1	0.15	0.2	0.25	0.3	0.35
Set 1	0.9999	0.9999	0.99985	0.99976	0.99959	0.99933	0.99895
Set 2	0.99987	0.99982	0.99971	0.99945	0.99889	0.99773	0.99536
Set 3	0.99895	0.99958	0.99973	0.99977	0.99977	0.99973	0.99970
Set 4	0.98834	0.98417	0.97512	0.96131	0.93811	0.89803	0.84026

Figure 3.7: SortMeRNA results by varying parameters k (length of the sliding window) and r (ratio of accepted windows) on Set 1 to Set 4 for **ILLUMINA** simulated reads. For each graph, the horizontal axis is for the sensitivity, and the vertical axis is for the selectivity. Each curve corresponds to a different value for k : $s = 14, 16, 18, 20$. Each dot on a curve corresponds to a different value for r . Below: Matthews correlation coefficients for $k = 18$ and various values of r .



Matthews correlation coefficient

r	0.05	0.1	0.15	0.2	0.25	0.3	0.35
Set 1	0.99598	0.99856	0.99795	0.99670	0.99489	0.99240	0.98674
Set 2	0.99366	0.99395	0.98982	0.98401	0.97600	0.96401	0.94588
Set 3	0.99306	0.99863	0.99905	0.99901	0.99883	0.99849	0.99792
Set 4	0.94040	0.92567	0.90233	0.87242	0.84096	0.80262	0.75228

Test 2 and Test 4. SSU-ALIGN was written for aligning small ribosomal subunits and does not provide models for 23S rRNA. The tool riboPicker was also tested with a more comprehensive database made available from their website: All 16S and 23S rRNA sequences taken from SILVA, RDP-II, Greengenes, NCBI archaeal and bacterial genomes, and HMP (3,232,371 16S and 19,602 23S unique sequences). The results for this larger database are indicated by riboPicker* in the subsequent tables and figures. For Meta-RNA and rRNASelector, we used the HMMs provided with the software.

All tests were performed on an Intel(R) Xeon(R) CPU W3520 2.67GHz machine with L1 cache size of 32 KB, L2 cache size of 256 KB and L3 cache size of 8192 KB. Since riboPicker and SSU-ALIGN do not provide a direct option for multi-threading, all tests were carried out using one thread.

3.3.1 Test 1: simulated 16S rRNA reads

Sensitivity for 16S rRNA. 300,000 Roche 454 and 1,000,000 Illumina 16S rRNA reads were simulated in the same manner as described in Section 3.2.2.2. The performance results can be viewed in Table 3.1. All software programs except riboPicker and SSU-ALIGN have a sensitivity level higher than 97%, and even higher than 99% for BLASTN and SortMeRNA. The sensitivity for riboPicker is very low (56%) because BWA-SW works well with error rates 2%-3% for 100-200nt reads, and loses sensitivity for new species. As expected, the sensitivity increases with a larger database (indicated riboPicker*). Considering the computation time, SortMeRNA runs in less than 2 minutes, or 72x faster than the next fastest tool with proportionate sensitivity (Meta-RNA). Note also that BLASTN executes at a very slow speed (several hours), because reads should be compared against all of sequences in the representative database.

Selectivity for 16S rRNA. 1,000,000 Roche 454 and 1,000,000 Illumina non-16S rRNA reads were simulated in the same manner as described in Section 3.2.2.2. The performance results can be viewed in Table 3.2. All programs have a selectivity level higher than 99.98%. The number of false positives for the HMM-based programs remains comparable to SortMeRNA for both Illumina and Roche 454 reads. The difference in the simulated data results between Meta-RNA and rRNASelector can be attributed to the number of bacteria vs. archaea rRNA sequences used in the construction of the HMMs, as well as additional parameter settings in rRNASelector. riboPicker* and BLASTN show the lowest selectivity. Concerning the running time, the order of the fastest programs is rRNASelector, Meta-RNA and SortMeRNA. Both rRNASelector and Meta-RNA use the HMMER3 package, which applies a pre-filter to quickly reject sequences which would score very low in the HMM. This acceleration heuristic gives these programs a competitive advantage on the artificial dataset for selectivity where all of the sequences are negative.

3.3.2 Test 2: simulated 23S rRNA reads

Similarly, we generated rRNA and non-rRNA reads for 23S rRNAs using the same protocol, as described in Section 3.2.2.2. Results are analogous as those of Test 1 in terms of accuracy and running time. They can be found in Table 3.3 and Table 3.4.

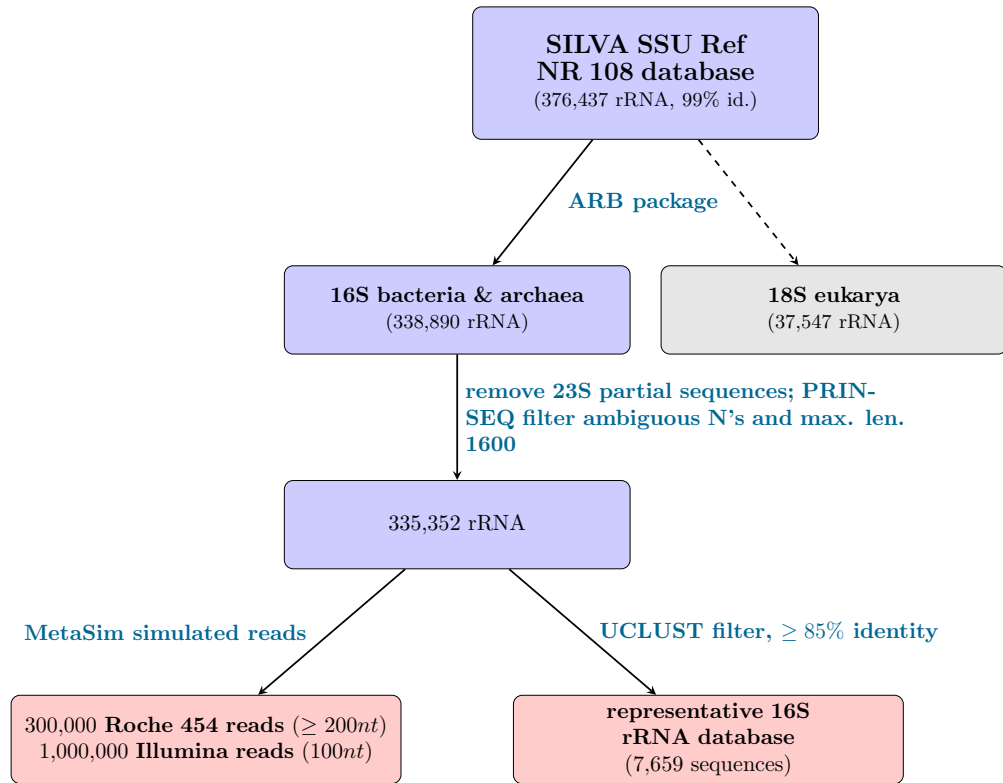
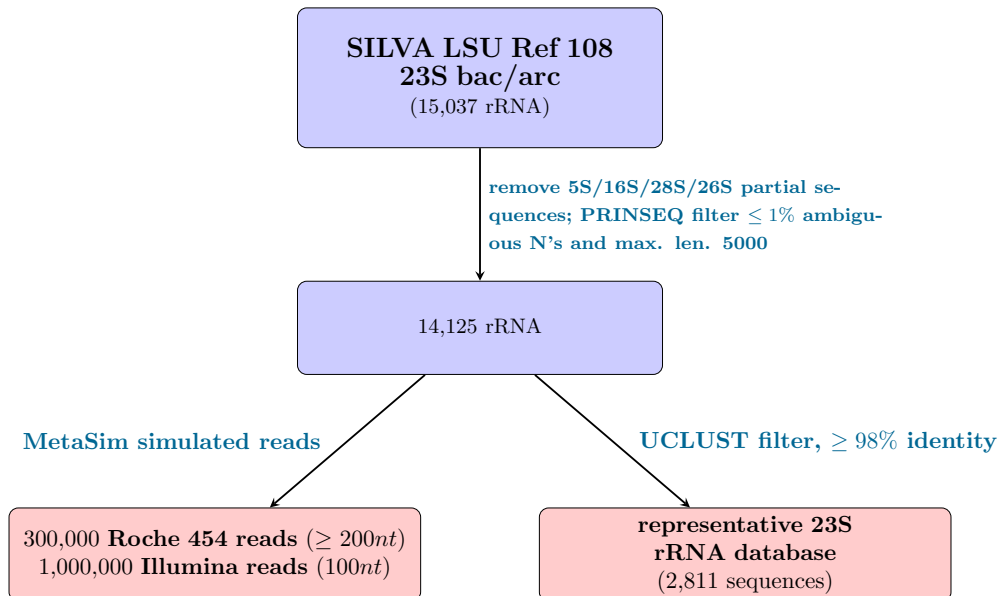
Figure 3.8: Construction of Set 5 – representative 16S rRNA database with 85% identity**Figure 3.9:** Construction of Set 6 – representative 23S rRNA database with 98% identity

Table 3.1: TEST 1, SENSITIVITY. 1,000,000 of MetaSim simulated Illumina (100nt) and 300,000 Roche 454 ($\geq 200nt$) rRNA reads against a representative 16S rRNA database of 7,659 sequences.

	Illumina				Roche 454			
	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)
<i>SortMeRNA</i>	998615	0:02	8.5	99.861	299979	0:02	6.3	99.993
<i>riboPicker</i>	558607	0:19	6.8	55.860	123024	0:19	5.6	41.008
<i>riboPicker</i> *	999941	6:33	35.3	99.994	299999	9:00	34	99.999
<i>BLASTN</i>	995322	23:52	3.0	99.532	299978	18:35	1.4	99.992
<i>Meta-RNA</i>	983332	2:00	33.3	98.333	299980	1:57	12.9	99.993
<i>rRNASelector</i>	974118	1:47	17.4	97.411	299976	2:00	7	99.992
<i>SSU-ALIGN</i>	971221	6:49	0.1	97.122	299902	5:50	0.1	99.967

Table 3.2: TEST 1, SELECTIVITY. 1,000,000 of MetaSim simulated Illumina (100nt) and 1,000,000 Roche 454 ($\geq 200nt$) non-rRNA reads against a representative 16S rRNA database of 7,659 sequences.

	Illumina				Roche 454			
	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)
<i>SortMeRNA</i>	17	0:02	7.6	99.9983	13	0:04	10.2	99.9987
<i>riboPicker</i>	7	0:10	6.7	99.9993	3	0:30	16.8	99.9997
<i>riboPicker</i> *	158	0:57	35.1	99.9842	53	2:43	45.2	99.9947
<i>BLASTN</i>	33	0:14	0.3	99.9967	33	0:16	0.3	99.9967
<i>Meta-RNA</i>	11	0:02	0.1	99.9989	11	0:04	0.2	99.9989
<i>rRNASelector</i>	10	0:01	0.1	99.9990	11	0:03	0.2	99.9989
<i>SSU-ALIGN</i>	8	3:51	0.1	99.9992	11	10:30	0.1	99.9989

Table 3.3: TEST 2, SENSITIVITY. 1,000,000 of MetaSim simulated Illumina (100nt) and 300,000 Roche 454 ($\geq 200nt$) rRNA reads against a representative 98% identity 23S rRNA database of 2,811 sequences.

	Illumina				Roche 454			
	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)
<i>SortMeRNA</i>	999909	0:01	7.2	99.909	300000	0:01	4.7	100
<i>riboPicker</i>	659494	0:19	6.7	65.949	213989	0:21	5.6	71.329
<i>riboPicker</i> *	986917	1:26	8.4	98.691	296584	1:46	7.3	98.861
<i>BLASTN</i>	999549	15:10	2.7	99.954	299999	11:25	1.3	99.999
<i>Meta-RNA</i>	936314	4:25	31.8	93.631	298918	4:29	13.1	99.639
<i>rRNASelector</i>	908344	4:7	16.4	90.834	298733	4:37	7.2	99.577

* Searching through all 23S rRNA databases provided by SILVA (only 23S), NCBI archaeal and bacterial genomes, and HMP.

Table 3.4: TEST 2, SELECTIVITY. 1,000,000 of MetaSim simulated Illumina (100nt) and 300,000 Roche 454 ($\geq 200nt$) **non-rRNA** reads against a representative 98% identity 23S rRNA database of 2,811 sequences

	Illumina				Roche 454			
	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)	rRNA	run time (hrs:min)	memory (%)	sensitivity (%)
<i>SortMeRNA</i>	243 ⁺	0:01	6.3	99.9757	112 ⁺	0:03	8.9	99.9888
<i>riboPicker</i>	39 ⁺	0:10	6.6	99.9961	24 ⁺	0:32	16.7	99.9976
<i>riboPicker</i> *	103	0:30	8.2	99.9897	54	1:32	18.3	99.9946
<i>BLASTN</i>	310 ⁺	0:26	0.3	99.9690	571 ⁺	0:26	0.2	99.9429
<i>Meta-RNA</i>	36	0:03	0.1	99.9964	29	0:06	0.3	99.9971
<i>rRNASelector</i>	34	0:02	0.1	99.9966	29	0:06	0.3	99.9971

* Searching through all 23S rRNA databases provided by SILVA (only 23S), NCBI archaeal and bacterial genomes, and HMP. ⁺ SortMeRNA, riboPicker and BLASTN use the same database (Set 6). riboPicker* searches through a database with 19,602 23S rRNA sequences, and both Meta-RNA and rRNASelector use prebuilt HMM models. For SortMeRNA, 82% of the 243 Illumina reads and 100% of the 112 Roche 454 reads are in common with the 310 and 571 reads classified by BLASTN. For riboPicker, 97% of the 39 Illumina reads and 100% of the 24 Roche 454 reads are in common with the 310 and 571 reads classified by BLASTN. The majority of these reads map to mRNA. Further investigation showed that due to misannotation, the database for Set 6 was contaminated with several mRNA, and hence the classified reads were correctly spotted in the database.

3.3.3 Test 3: photosynthetic microbial community

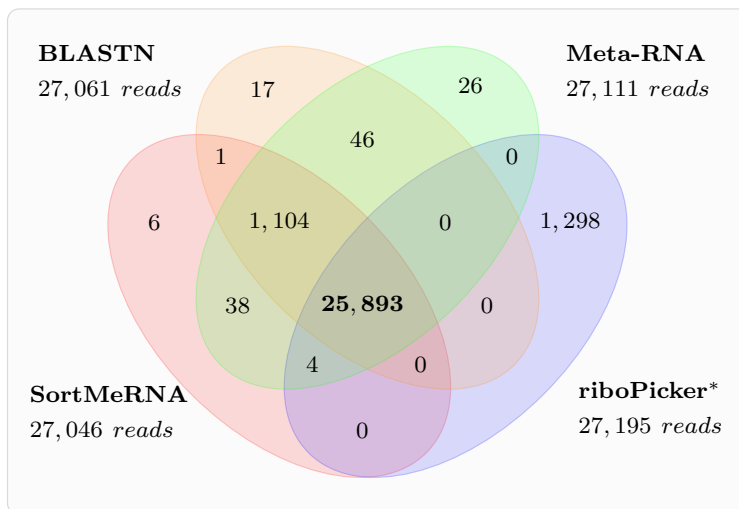
The metatranscriptomic dataset SRR106861 of a photosynthetic microbial community from 454 sequencing was downloaded from the NCBI Sequence Read Archive. We filtered this read set to remove any bias caused by shorter reads ($<200nt$), as well as low-quality reads which have more than 1% of the ambiguous character N. The reason for eliminating shorter reads is that riboPicker, Meta-RNA, rRNASelector and SSU-ALIGN require longer reads to achieve a higher sensitivity. In general, this length is suggested to be $\geq 200nt$ for Roche 454 reads.

The results can be viewed in Table 3.5, and the overlap of the results between tools in a Venn diagram displayed in Figure 3.10. The results obtained with SortMeRNA adhere to the accuracy of the HMM-based programs and are computed in a fraction of the time. riboPicker finds only a subpart of all potential rRNAs, which confirms its low sensitivity for small databases. The majority of 16S rRNA reads found only by riboPicker* (1,298) map to mRNA.

Table 3.5: TEST 3: Runtime for the SRR106861 metatranscriptome of 105,873 reads against a 16S rRNA database of 7,659 sequences.

	rRNA	run time (hrs:min)	memory (%)
<i>SortMeRNA</i>	27046	< 0:01	4.8
<i>riboPicker</i>	11389	0:04	2.3
<i>riboPicker</i> *	27195	0:39	30.8
<i>BLASTN</i>	27061	1:29	0.6
<i>Meta-RNA</i>	27111	0:11	1.8
<i>rRNASelector</i>	27085	0:11	0.8

Figure 3.10: TEST 3: Venn diagram for reads classified as 16S rRNA by BLASTN, Meta-RNA, SortMeRNA and riboPicker* in the SRR106861 metatranscriptome.



3.3.4 Test 4: tidal salt marsh creek

Similarly, we retrieved the dataset SRR013513 of a tidal salt marsh creek from 454 sequencing from the NCBI Sequence Read Archive. We removed low quality and short reads using the same cleaning procedure as in Test 3.

Results are available in Table 3.6 and Figure 3.11. Approximately 99% of the excess reads of Meta-RNA (12,112) and rRNASelector likewise map to 28S, along with 83% of the (624) reads found only by BLASTN and Meta-RNA. The (537) reads found only by BLASTN map to 16S rRNA, ncRNA and mRNA.

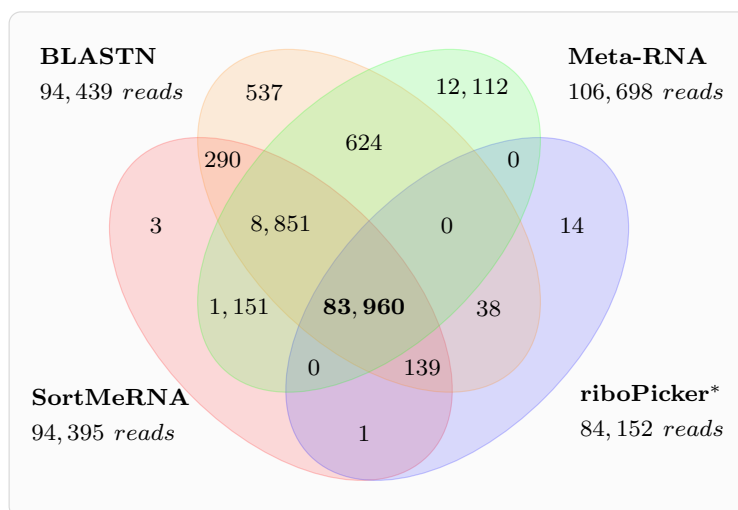
Table 3.6: TEST 4: Runtime for the SRR013513 metatranscriptome of 207,368 reads against a 23S rRNA database of 2,811 sequences.

	rRNA	run time (hrs:min)	memory (%)
<i>SortMeRNA</i>	94395	< 0:01	3.8
<i>riboPicker</i>	71937	0:10	3.9
<i>riboPicker</i> *	84152	0:36	5.5
<i>BLASTN</i>	94439	3:42	0.9
<i>Meta-RNA</i>	106698	1:33	4.8
<i>rRNASelector</i>	107900	1:36	3

3.4 Discussion

SortMeRNA has shown to be a rapid and efficient filter which can sort a large set of metatranscriptomic reads with high accuracy comparable to the HMM-based programs and a significantly lower running time. SortMeRNA implements our approximate seeds and this important charac-

Figure 3.11: TEST 4: Venn diagram for reads classified as 23S rRNA by BLASTN, Meta-RNA, SortMeRNA and riboPicker* in the SRR013513 metatranscriptome.



teristic renders the algorithm robust to errors of different types of sequencers while providing the ability to discover new rRNA sequences from unknown species. The method used by the algorithm is universal and flexible. The database can be constructed on any family of sequences that show some conservation at the nucleic acid level, which can be useful for identifying other families, such as transfer RNAs, or sequencing adapters. Moreover, the algorithm does not require a multiple sequence alignment file to build the database, as HMM-based programs do, and this is an advantage when sequences are hard to align or only partial sequences are available. Another advantage of SortMeRNA is the small number of parameter settings required by the program, most of which are precomputed using statistical analysis of the rRNA database.

In [Kopylova et al., 2013], we discuss in more detail how to use SortMeRNA in a global strategy for analyzing metatranscriptomic data: How to clean the data prior to the classification, how to map and assemble the filtered reads to proceed to the reconstruction of mRNA transcripts for functional analyses and phylogenetic classification of a community using the ribosomal RNA.

Conclusion

In this thesis, we have introduced a novel seeding technique for approximate seeds and the supporting indexing data structures used to quickly locate short regions of similarity between two sequences. In Chapter 3 we presented SortMeRNA, an efficient filter implementing approximate seeds to quickly remove ribosomal fragments from metatranscriptomic data. Nowadays, SortMeRNA is the fastest algorithm to filter ribosomal RNA with an accuracy comparable to the probabilistic HMM tools. In Chapter ?? we presented SortMeDNA, a read mapping tool that can align genomic and metagenomic data generated by second- and third-generation sequencing. SortMeDNA applies statistical analysis to verify the significance of an alignment since when mapping reads against distantly divergent species the chances of seeing random alignments reaching a threshold score increase.

Advancements in DNA sequencing techniques will one day provide technologies that skip the whole-genome shotgunning step and sequence complete genomes in one go. This trend is already visible with the 4,000-20,000 bp reads produced by PacBio, and entire DNA strand sequencing for the Lambda phage genome of 48,000 bp (both strands) by GridION [GridION, 2012]. However, although short read mapping tools may no longer be necessary, the algorithms behind them can prove to be invaluable for other sequence analysis applications. Genetic sequence databases such as GenBank [Benson et al., 2005], RefSeq [Pruitt et al., 2009] and Ensembl [Flicek et al., 2013] are growing at an exponential rate, with GenBank storing over 150 billion nucleotide bases in August, 2013 (see GenBank release notes for release 197.0). What is more, the databases are composed of a variety of sequences including whole genomes, metagenomic sequences, ribosomal, chloroplast, mitochondrial and uncultured sequences. Sorting through and analyzing all of this data can only be done by extremely efficient software and large computational resources. Finding DNA sequence variations [Cheng et al., 2013], making genetic diagnoses of diseases, studying microbial pathogens responsible for crop losses [Newton et al., 2010] and plant biology, as well as understanding the vast network and interdependency of environmental communities [Fierera et al., 2012] are just a few possibilities of where new bioinformatic analysis tools can evolve. In the meantime, as high-throughput short read technologies still dominate the market, new software tools capable of quickly and accurately analyzing their data are essential.

Both of our tools offer a good balance between speed and sensitivity, which is important for the analysis of high-throughput data for new applications in metatranscriptomics and metagenomics. The compromise to this feature is the much larger index required for implementing an approximate seed search, especially for large and repetitive eukaryotic genomes. Since SortMeRNA works with highly conserved ribosomal sequences, it requires a small representative database to achieve a very high sensitivity. By using either the 8,000 16S rRNA representative sequences provided with the SortMeRNA distribution or the 400,000 16S rRNA sequences available in the original SILVA databases, the differences in the number of reads classified by SortMeRNA will

be very small. SortMeDNA on the other hand is capable of managing the amount of memory loaded in RAM by internally splitting the index into multiple subparts. Investigations into reducing the size of the index are important to consider for future improvements of both tools. Perhaps a compressed version of the burst trie can be devised, or a completely different data structure similar to an enhanced suffix array or the BWT can be adopted. Compressed full-text indices such as the BWT or the compressed suffix array may deliver the most promising algorithms for future generation software as they are able to simultaneously compress and index a text [Navarro and Mäkinen, 2007]. It was also shown in [Russo et al., 2009] that it is possible to perform approximate string matching (up to $d = 6$) using compressed indices such as the Lempel-Ziv and suffix array self indices.

A possible extension to the seed model could be adding weights to the transitions of the universal Levenshtein automaton. Currently, seeds having a mismatch error or an indel error are scored equally, however as in the Smith-Waterman scoring matrix, options giving different weights to mismatches and indels can influence the results (for example fewer false positives by filtering out low probability matches). Furthermore, having a weighted automaton could also be exploited for seeds allowing 2 or even 3 errors. Although these latter options are not yet available in our tools, they could prove to be useful than the 1-error model in studies involving many low-complexity sequences or to discover more distant species.

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