
DTU

DTU Health Technology
Bioinformatics

Alignment

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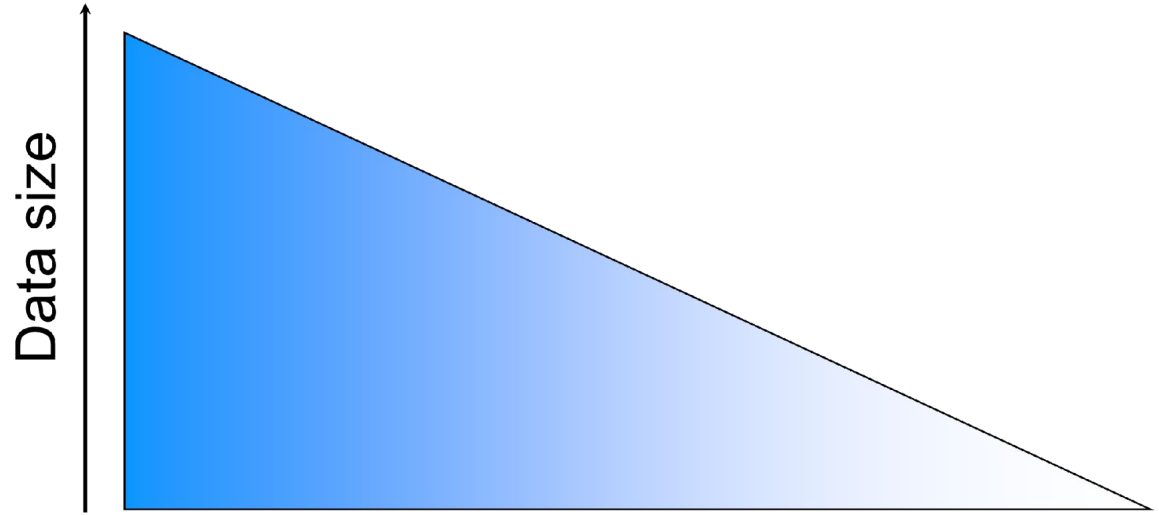
Menu

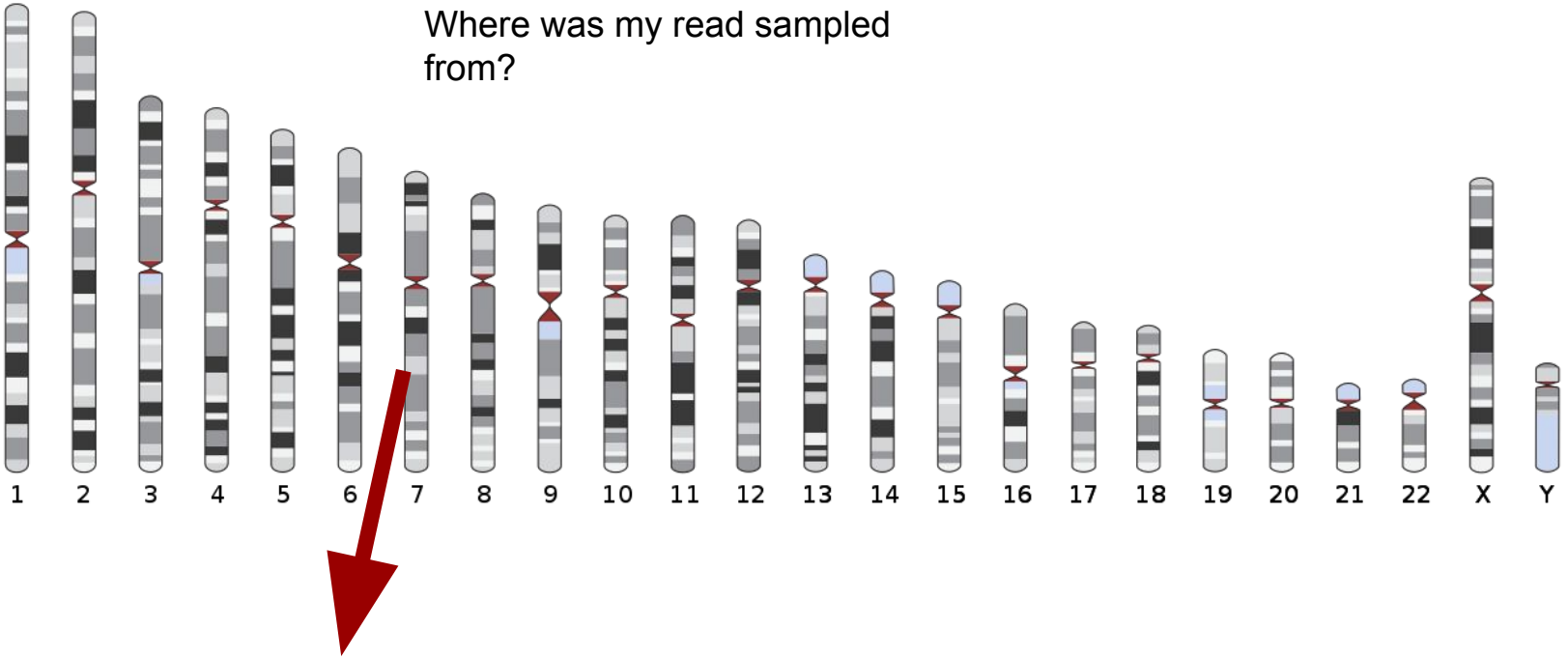
- Alignment approaches
- Burrows-Wheeler Transform
- More about coverage and depth
- Storing sequence alignments

Generalized NGS analysis



Question | Raw reads | Pre-processing | Assembly: Alignment / *de novo* | Application specific: Variant calling, count matrix, ... | Compare samples / methods | Answer?

Where was my read sampled from?



Alignment:

```
my read              GGGGCTGCCGCCCCATAGCA-GGACCTGGTCACAAGCAGC
                     ||||||||| |||||||||| |||||||||||| ||||||
chr7: 102,204,582    GGGGCTGCCCCCCCCATAGCAAGGACCTGGTCAC-AGCAGC
```

What is an alignment?

Alignment = story

```
seq1:   CAAGACTAACCTGAA
seq2:   CATGATAGCACTGCA
```

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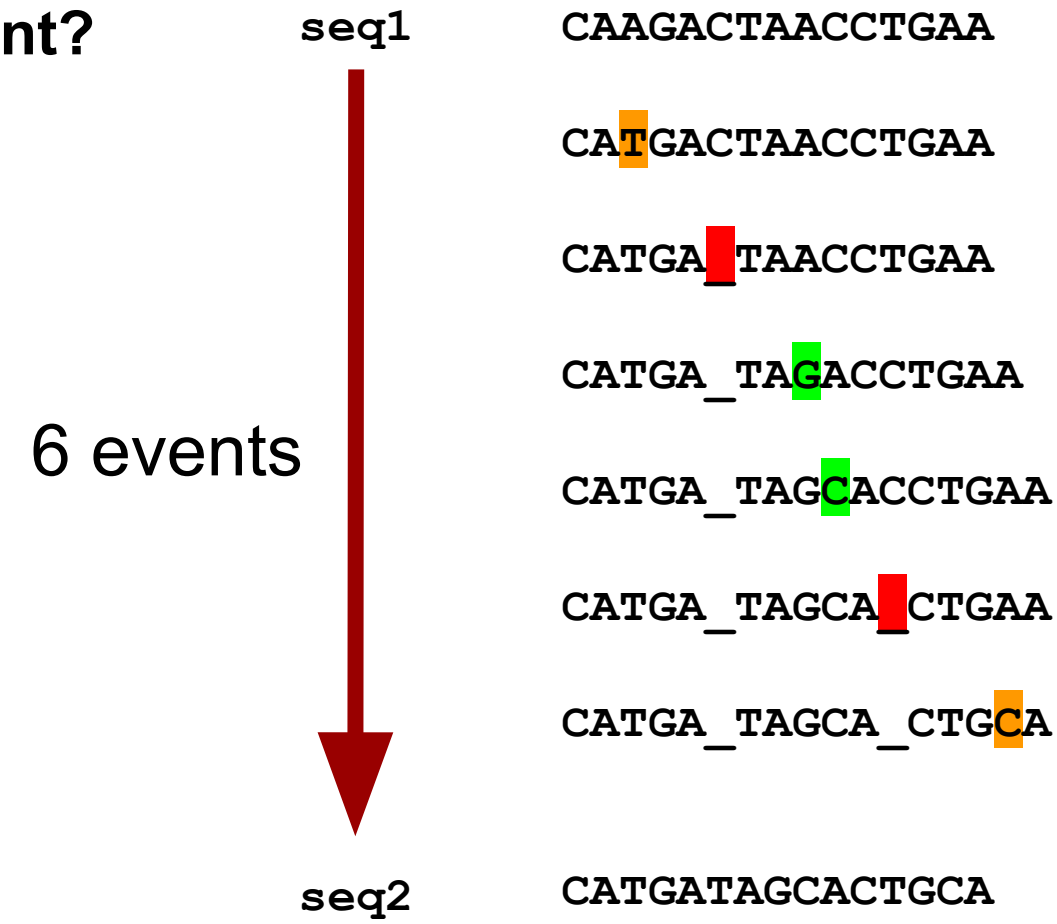


What is an alignment?

Alignment = story

seq1: **CAAGACTAACCTGAA**
seq2: **CATGATAGCACTGCA**

Needleman-Wunsch: best story?

- Dynamic programming
- Find the best path in the matrix

D		C	A	T	G	A	T	A	G	C	A	C	T	G	C	A
	0	-2	-4	-6	-8	-10	-12	-14	-16	-18	-20	-22	-24	-26	-28	-30
C	-2	1	-1	-3	-5	-7	-9	-11	-13	-15	-17	-19	-21	-23	-25	-27
A	-4	-1	2	0	-2	-4	-6	-8	-10	-12	-14	-16	-18	-20	-22	-24
A	-6	-3	0	1	-1	-1	-3	-5	-7	-9	-11	-13	-15	-17	-19	-21
G	-8	-5	-2	-1	2	0	-2	-4	-4	-6	-8	-10	-12	-14	-16	-18
A	-10	-7	-4	-3	0	3	1	-1	-3	-5	-5	-7	-9	-11	-13	-15
C	-12	-9	-6	-5	-2	1	2	0	-2	-2	-4	-4	-6	-8	-10	-12
T	-14	-11	-8	-5	-4	-1	2	1	-1	-3	-3	-5	-3	-5	-7	-9
A	-16	-13	-10	-7	-6	-3	0	3	1	-1	-2	-4	-5	-4	-6	-6
A	-18	-15	-12	-9	-8	-5	-2	1	2	0	0	-2	-4	-6	-5	-5
C	-20	-17	-14	-11	-10	-7	-4	-1	0	3	1	1	-1	-3	-5	-6
C	-22	-19	-16	-13	-12	-9	-6	-3	-2	1	2	2	0	-2	-2	-4
T	-24	-21	-18	-15	-14	-11	-8	-5	-4	-1	0	1	3	1	-1	-3
G	-26	-23	-20	-17	-14	-13	-10	-7	-4	-3	-2	-1	1	4	2	0
A	-28	-25	-22	-19	-16	-13	-12	-9	-6	-5	-2	-3	-1	2	3	3
A	-30	-27	-24	-21	-18	-15	-14	-11	-8	-7	-4	-3	-3	0	1	4

What is an alignment?

Alignment = story

```
seq1:   CAAGACTAACCTGAA
seq2:   CATGATAGCACTGCA
```


```
seq1   CAAGACTAACCTGAA
       CATGACTAACCTGAA
       CATGA_TAACCTGAA
       CATGA_TAGCCTGAA
       CATGA_TAGCACTGAA
       CATGA_TAGCACTGCA
seq2   CATGATAGCACTGCA
```

What is an alignment?

Alignment = story

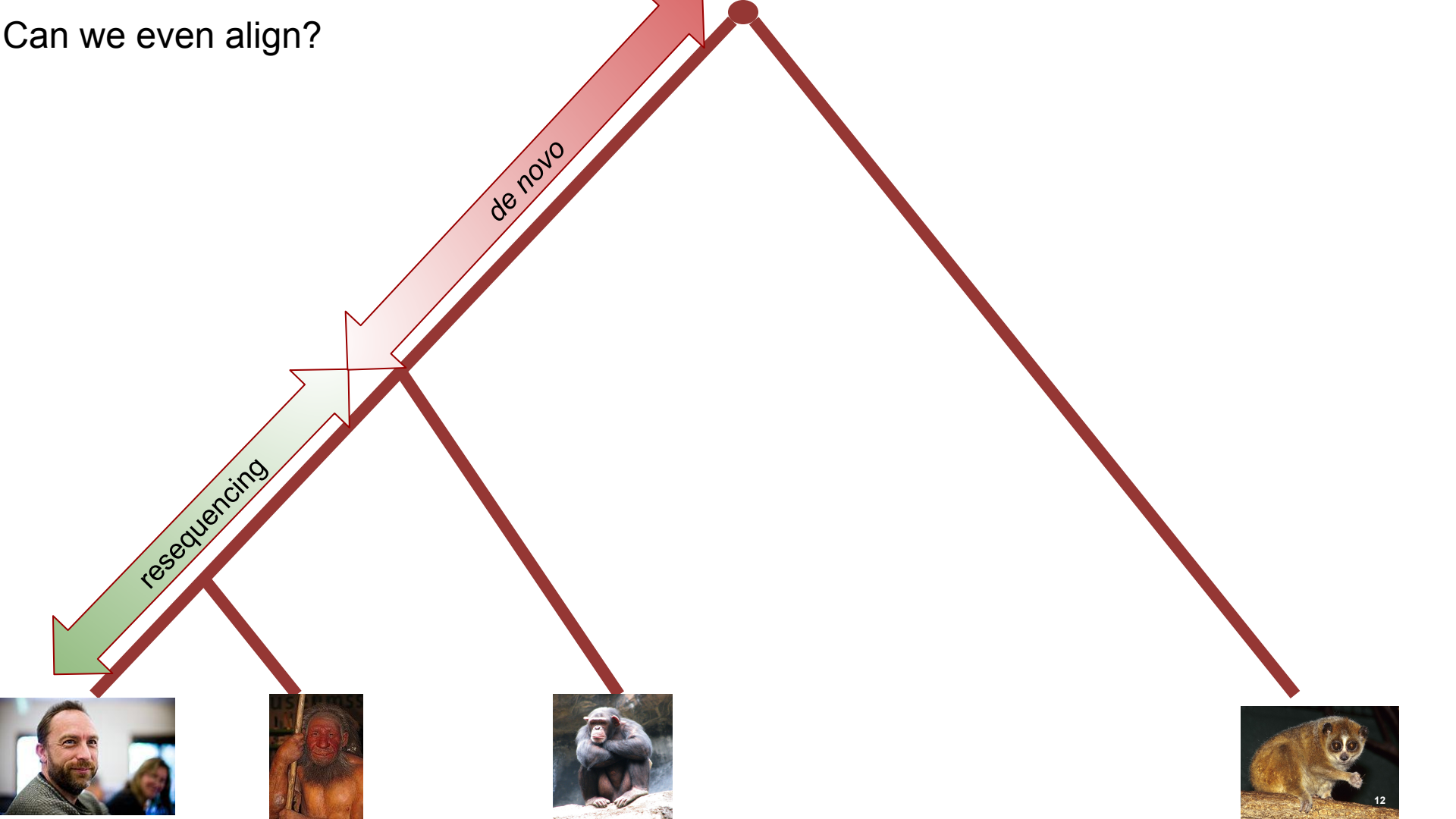
- 2 sequences can have a lot of alignments
- Not every alignment is equally likely
- Important to quantify the likelihood of seeing that alignment
- Be skeptical when you hear: “This is the alignment!”

What is an alignment?

Types of alignment:

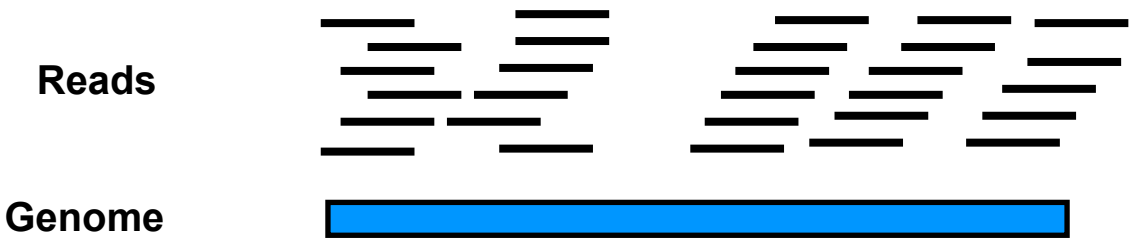
- “Short” read alignment
- Whole sequence alignment
- Whole genome/chromosome alignment
- Multiple sequence alignment

Can we even align?



Alignment/Mapping

- Assemble your reads by aligning them to a closely related reference genome
- High sequence similarity between individuals makes this possible



Sounds easy?

- Some pitfalls:
  - Divergence between sample and reference genome
  - Repeats in the genome
  - Recombination and re-arrangements
  - Poor reference genome quality
  - Read errors
  - Regions not in the ref. genome
  - Surprise sample

Simplest solution

- Exact string matches!
- Calling “grep” on a read of 150bp on a 3B base genome takes only 1 second!
  - … but aligning 20B reads from a NovaSeq would take 20B seconds so **634 years**
- We need bespoke tools
- What about mutations, sequencing errors etc?

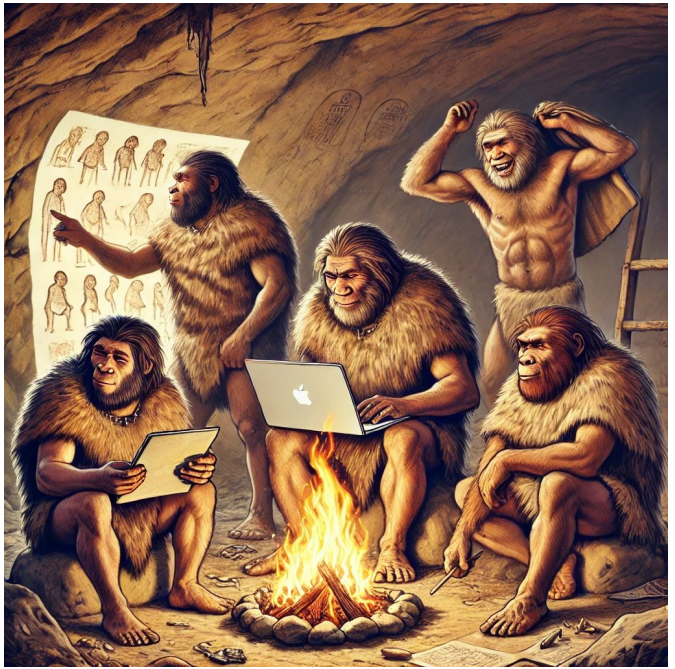
```
Reference:  ...ACGTGCGGACGCTGAACGTGA...
                       ||| |||||| ||
Read:                  GCGCACGCTGTAC
```

A simpler solution?

- Allow imperfect matches

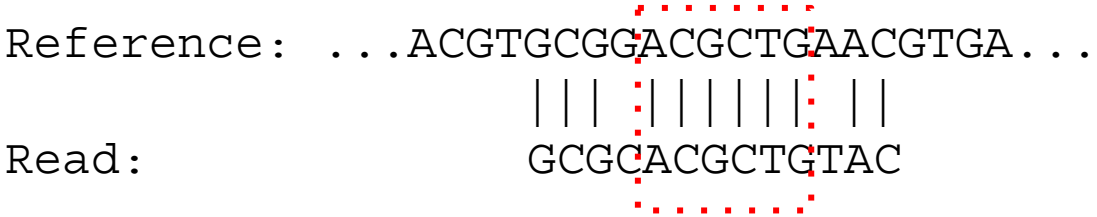
```
Reference:  ...ACGTGCGGACGCTGAACGTGA...
                       ||| |||||| ||
Read:                  GCGCACGCTGTAC
```

- Use the Smith-Waterman algorithm to find the best match
- Aligning a read took 2.5 hours so aligning 20B reads from a NovaSeq would take **0.5M years**



Smart solution

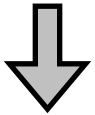
- Any “reasonable” match will have perfect stretches:



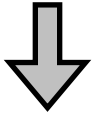
- Some alignments won’t have such stretches, we will lose them...
- Maybe we can index the strings in the human genome as a data structure

Smart solution

1. Use algorithm to quickly find *possible* matches

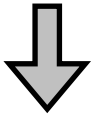


Drastically reduced search space

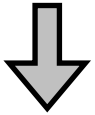


2. Allow us to perform slow/precise alignment for possible matches (Smith-Waterman)

3.2Gb



X possible matches

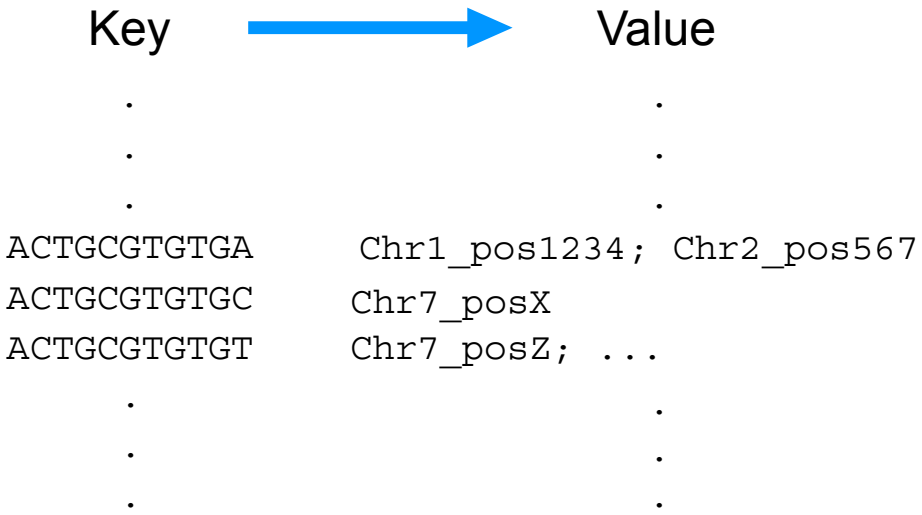


1 best match

Hash based algorithms

Lookups in hashes are *fast!*

1. Index the reference using *k*-mers.
2. Search reads vs. hash *k*-mers
3. Perform alignment of entire read around seed
4. Report best alignment



Also known as *Seed and extend*

Spaced seeds

- Key/*k*-mer is called a seed

```
11111111111
```

L = 11, 11 matches

- Smarter: Spaced seeds (only care about ‘1’ in seed, ‘0’ = wildcards)
  - Higher sensitivity
  - One can use several seeds

```
1110100101001101111
```

L = 18, 11 matches

Multiple seeds & drawbacks

- One could require multiple short seeds
  - Instead of extending around each seed, extend around positions with several seed matches

- Drawbacks of hash-based approaches:
  - Lots(!) of RAM to keep index in memory (hg ~48Gb!)

Burrows-Wheeler Transform

- Hash based aligners require lots of memory and are only reasonable fast
- Can we make it better/faster?
- Burrows Wheeler Transformation (BWT)
- BWT was originally created for compression

BWT: Create index

Genome

Marks end-of-string, lexicographically smallest

T = AGGAGC$

1. Create all possible shifts of the string

(move first base to end)

0 AGGAGC$

BWT: Create index

Genome

Marks end-of-string, lexicographically smallest

```
T = AGGAGC$
```

1. Create all possible shifts of the string

(move first base to end)

```
0  AGGAGC$
1  GGAGC$A
```

BWT: Create index

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1. Create all possible shifts of the string

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0  AGGAGC$
1  GGAGC$A
2  GAGC$AG
```

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1  GGAGC$A
2  GAGC$AG
3  AGC$AGG
```

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1. Create all possible shifts of the string

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0  AGGAGC$
1  GGAGC$A
2  GAGC$AG
3  AGC$AGG
4  GC$AGGA
5  C$AGGAG
6  $AGGAGC
```

BWT: Create index

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0	AGGAGC$
1	GGAGC$A
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5	C$AGGAG
6	$AGGAGC

2. Sort the strings lexicographically to create BWT matrix and Suffix Array

$	A	G	G	A	G	C
A	G	C	$	A	G	G
A	G	G	A	G	C	$
C	$	A	G	G	A	G
G	A	G	C	$	A	G
G	C	$	A	G	G	A
G	G	A	G	C	$	A

BWT matrix

BWT: Create index

Genome

Marks end-of-string, lexicographically smallest

T = AGGAGC$

BWT(T) = CG$GGAA

$	A	G	G	A	G	C
A	G	C	$	A	G	G
A	G	G	A	G	C	$
C	$	A	G	G	A	G
G	A	G	C	$	A	G
G	C	$	A	G	G	A
G	G	A	G	C	$	A

BWT matrix

BWT: Create index

Genome

Marks end-of-string, lexicographically smallest

```
T = AGGAGC$
```

- Reversible
- BTW(T) is easier to compress than T due to repeated characters tend to cluster ex:

```
BWT(T)= CG$GGAA
```

```
Ringeren_I_Ringe_ringer_ringere_end_ringeren_ringer_i_Ringsted$
$d__ _nIiernerdenrgtrr_gggggnnnnnnn_RrrrRrReeeiiiiiieeeee____gs
```

- try bzip2

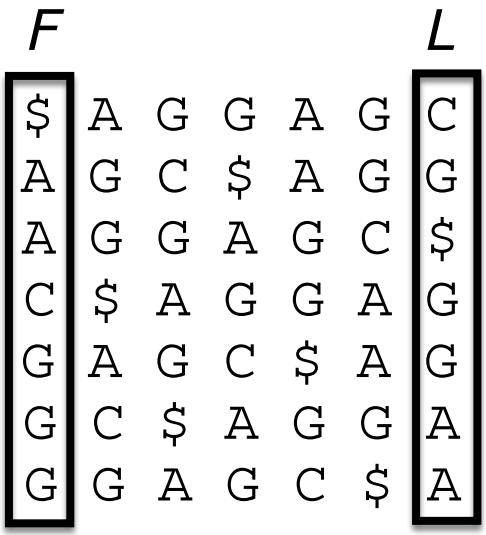
BWT: *T*-rank

T = AGGAGC$

T-ranking:

# of times the base occurred previously in T

A_0 G_0 G_1 A_1 G_2 C_0 $



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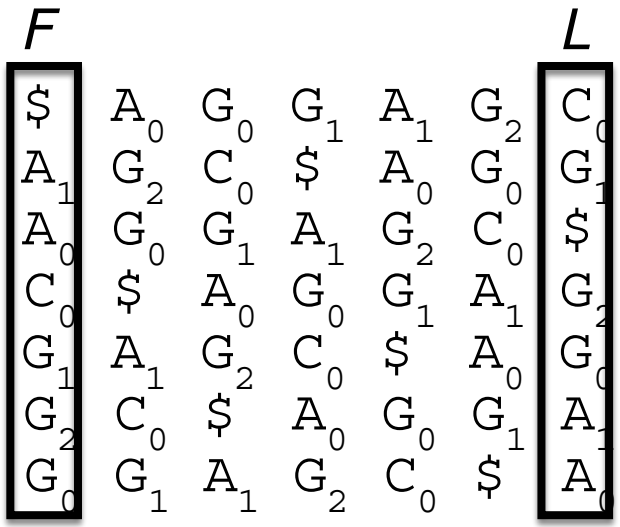

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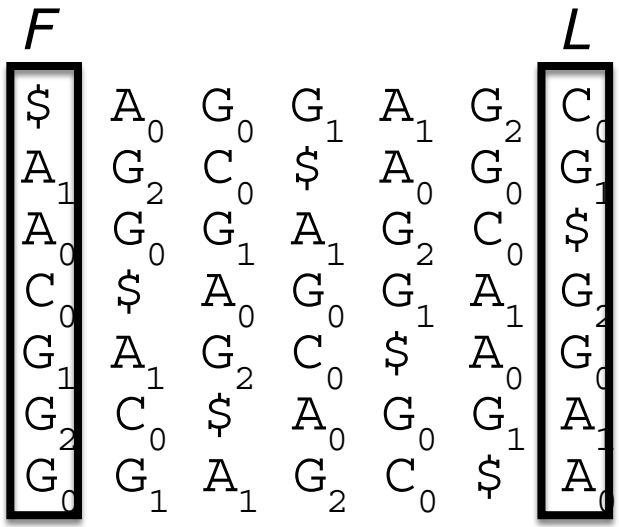


Notice that individual base-rank is the same in *F* and *L*

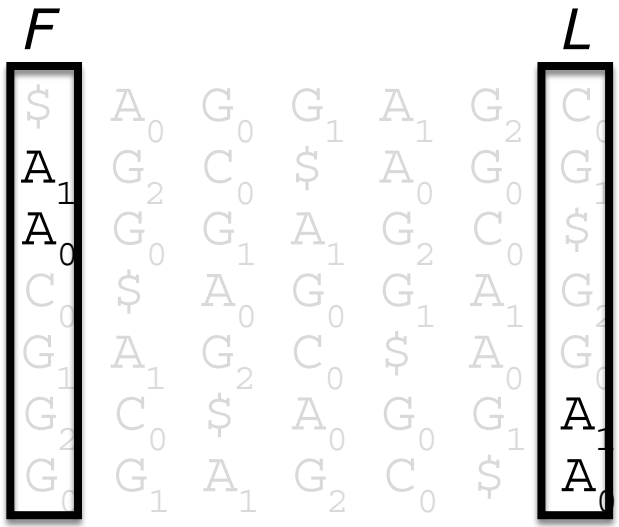
➡ Rank will always be the same in *F* and *L*

BWT: *T*-rank

T = AGGAGC$

T-ranking: # of times the base occurred previously in T

A_0 G_0 G_1 A_1 G_2 C_0 $



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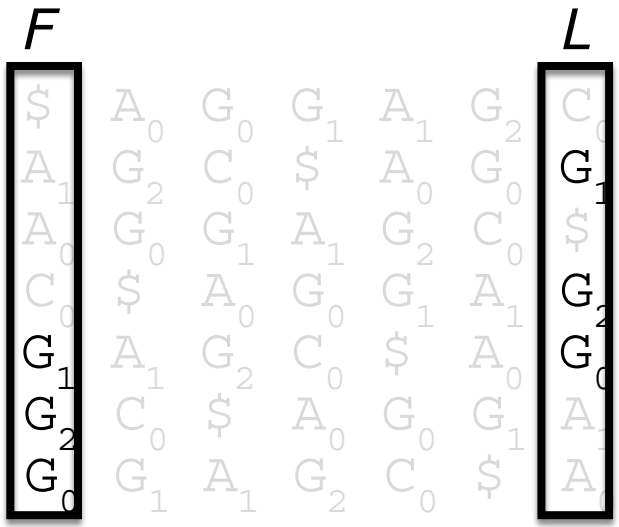

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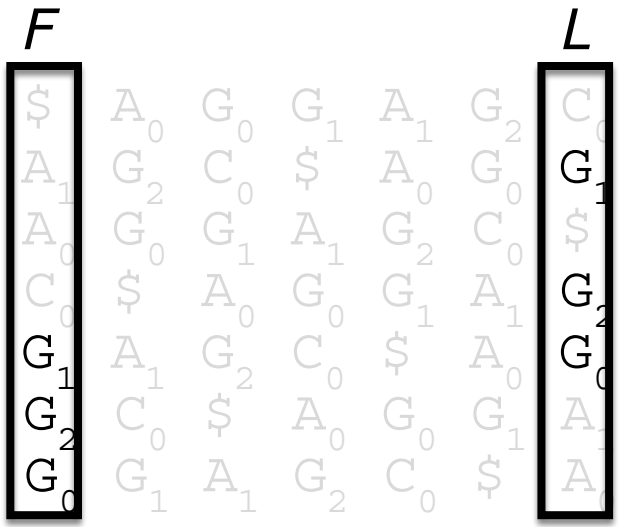
➡ Rank will always be the same in *F* and *L*

BWT: *T*-rank

Why does this generalize?



BWT: *T*-rank

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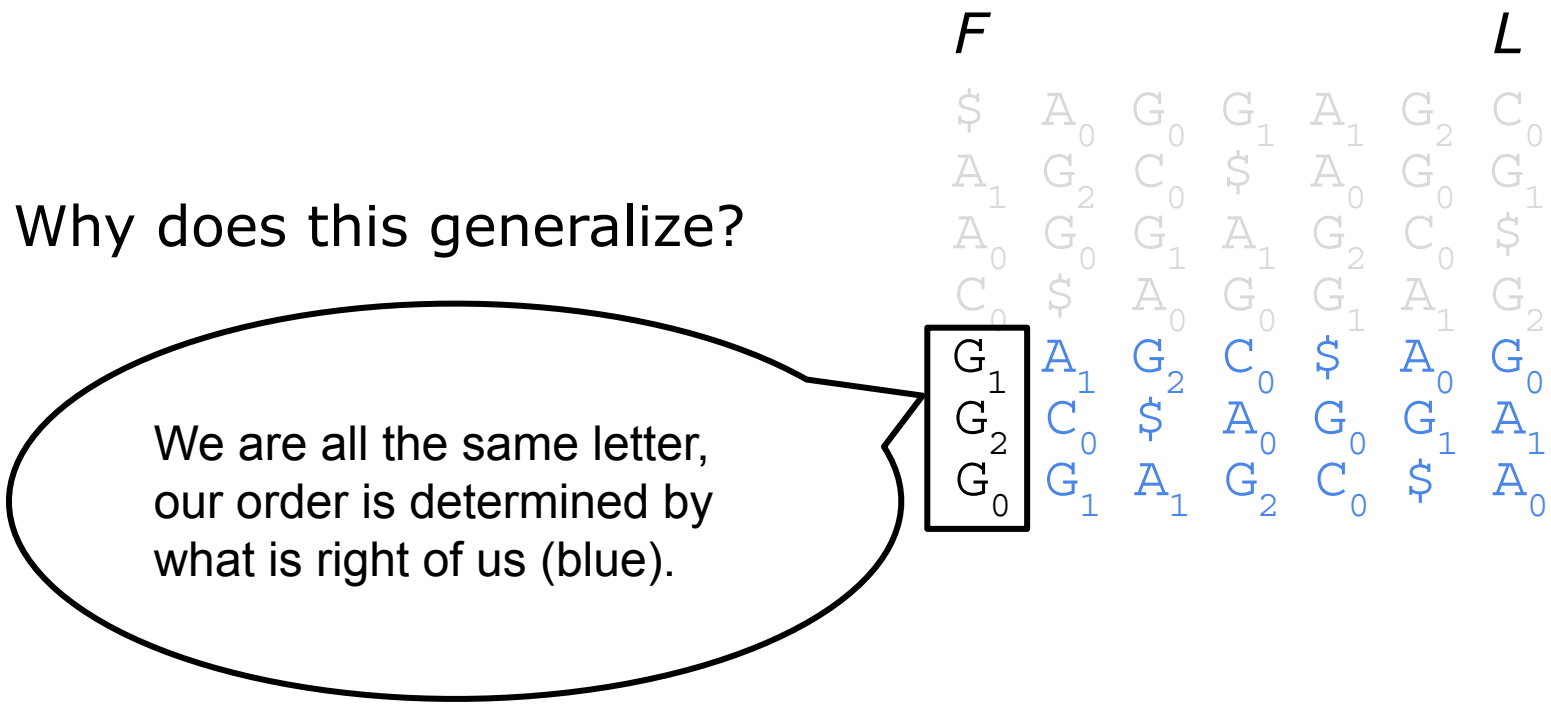
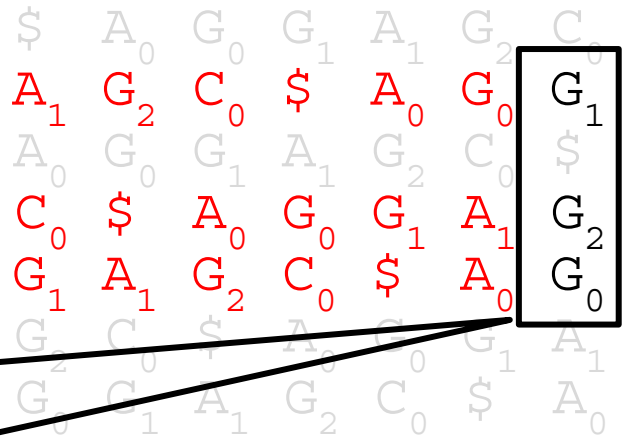


BWT: *T*-rank

Why does this generalize?

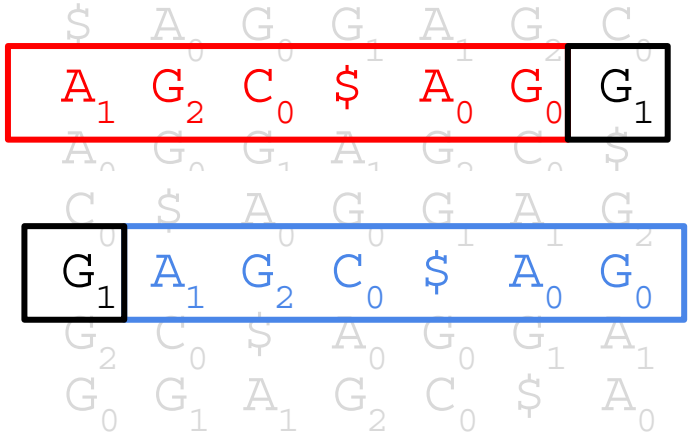


We are all the same letter, our order is determined by what is left of us (red).

BWT: *T*-rank

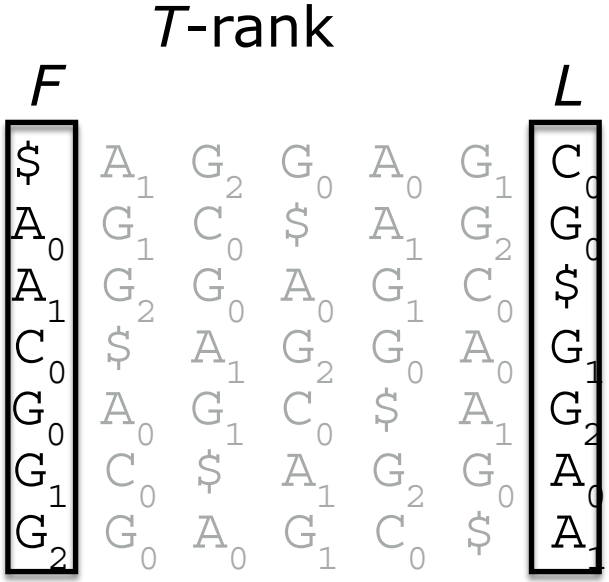
Why does this generalize?

- The string left (red) of the G1 and right (blue) of G1 are identical
- They are sorted
- Therefore the order is the same



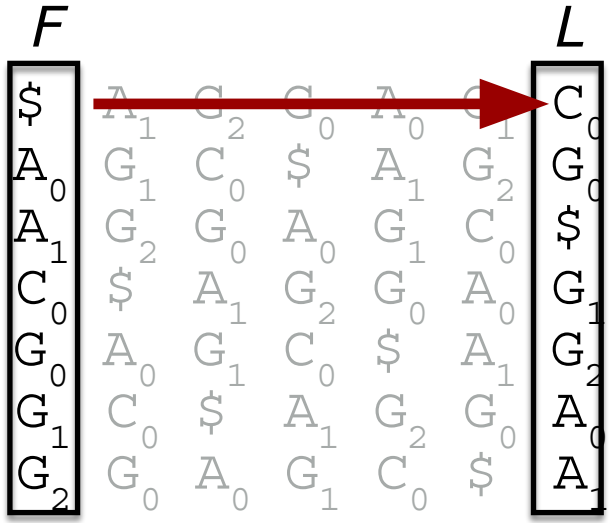
BWT: *T*-rank

How to reverse the BTW back the original string *T* ?



BWT is reversible

LF-mapping: LF can be used to recreate the original genome



C_0

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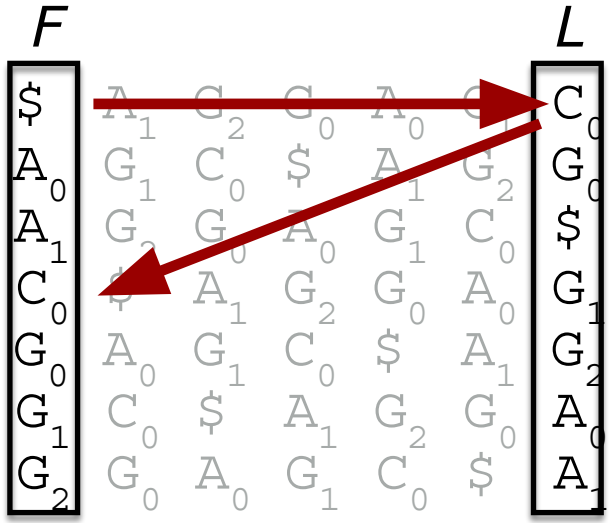
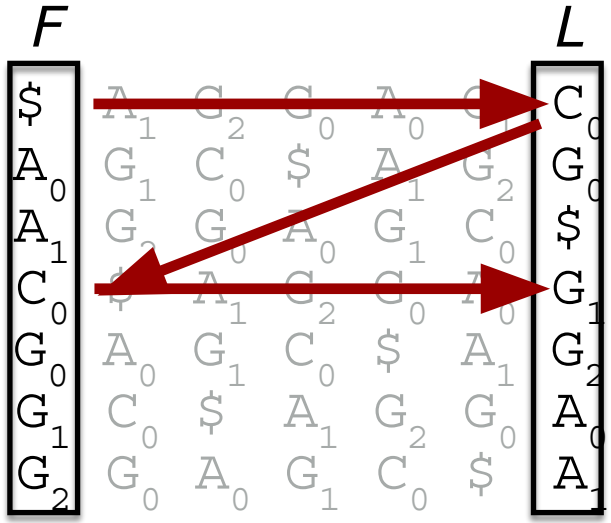


C_0

BWT is reversible

LF-mapping: LF can be used to recreate the original genome

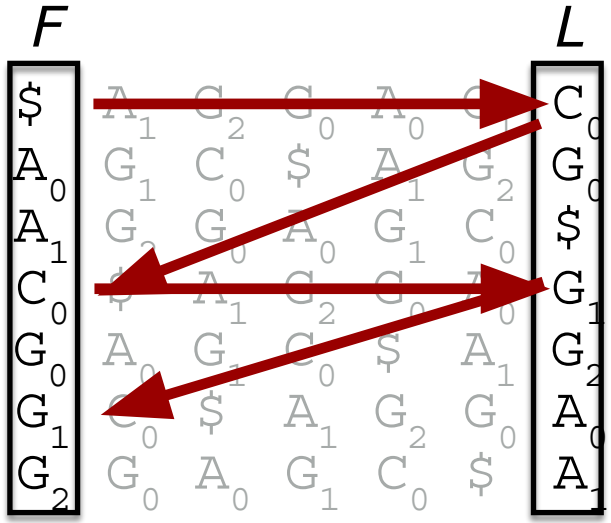
C_0G_1



BWT is reversible

LF-mapping: LF can be used to recreate the original genome

C_0G_1



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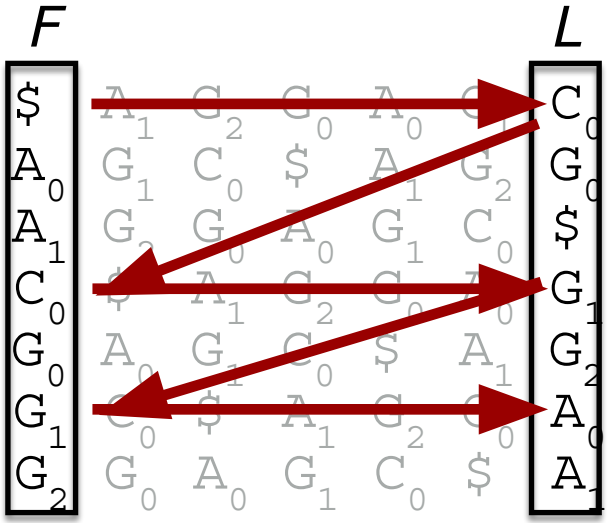
LF-mapping: LF can be used to recreate the original genome

$C_0G_1A_0$



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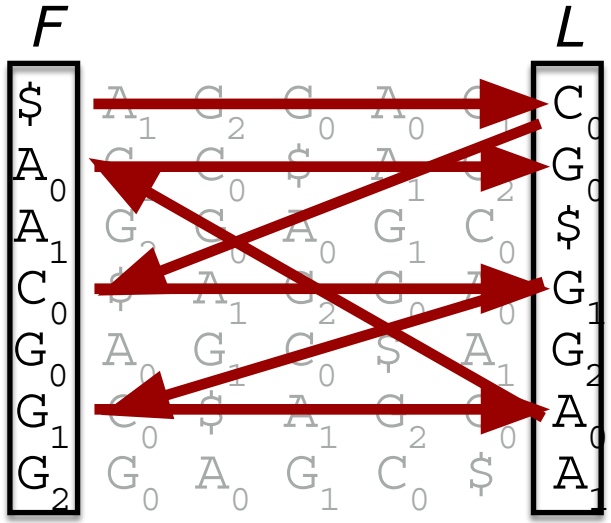
LF-mapping: LF can be used to recreate the original genome

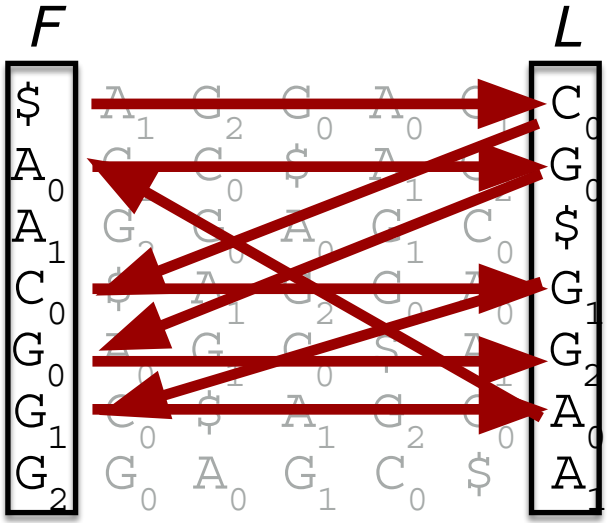
$C_0G_1A_0G_0$



BWT is reversible

LF-mapping: LF can be used to recreate the original genome

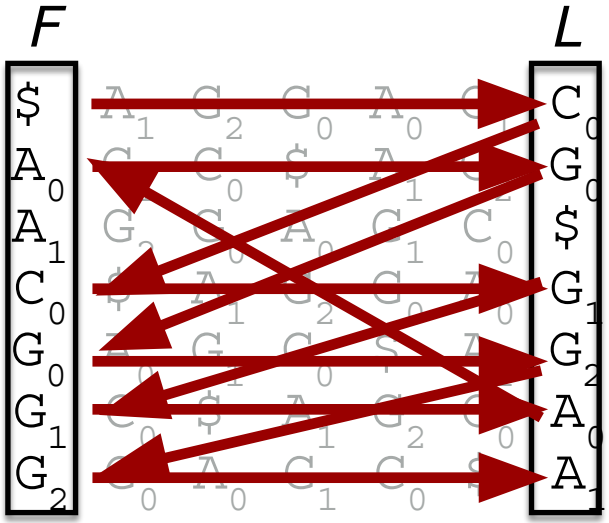
$C_0G_1A_0G_0G_2$



BWT is reversible

LF-mapping: LF can be used to recreate the original genome

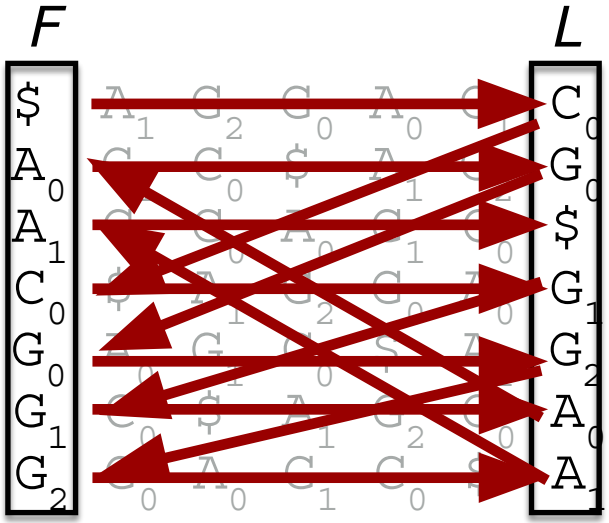
$C_0G_1A_0G_0G_2A_1$



BWT is reversible

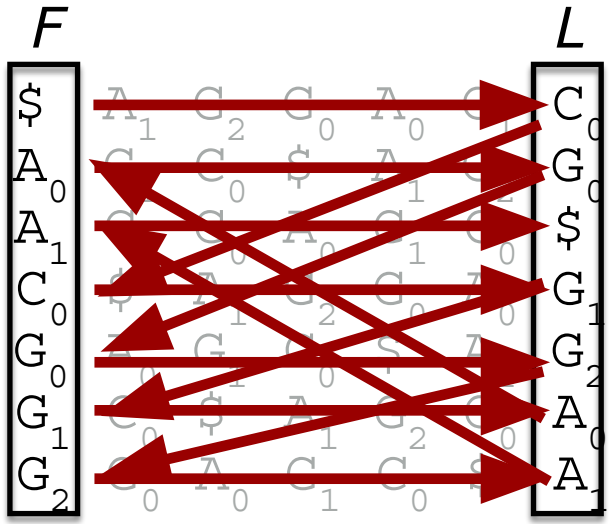
LF-mapping: LF can be used to recreate the original genome

$C_0G_1A_0G_0G_2A_1\$$



BWT is reversible

LF-mapping: LF can be used to recreate the original genome



$C_0G_1A_0G_0G_2A_1\$$

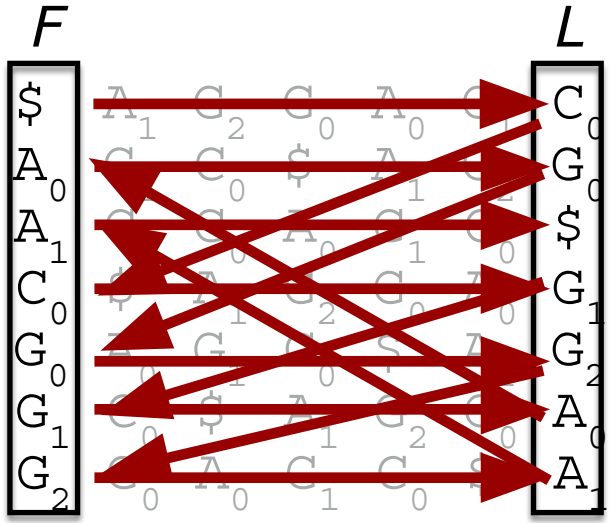
Reversed:

$A_1G_2G_0A_0G_1C_0$

T = AGGAGC$

BWT is reversible

LF-mapping: LF can be used to recreate the original genome



$C_0G_1A_0G_0G_2A_1\$$

Reversed:

$A_1G_2G_0A_0G_1C_0$

T = AGGAGC$

F can be represented = 2x A, 1x C, 3x G
we need |Σ| integers

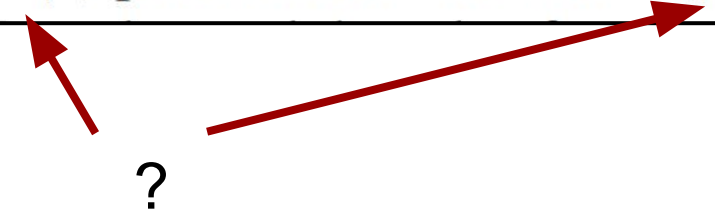
We therefore only need to store *L*

Why are we talking about BTW for alignments?

- In 1994, Michael Burrows and David Wheeler created the Burrows-Wheeler Transform (BWT)
  - A reversible transformation of the genome
- Full-text index in Minute space (FM) index
  - *Paolo Ferragina, and Giovanni Manzini. "Opportunistic data structures with applications." Foundations of Computer Science, 2000. Proceedings. 41st Annual Symposium on. IEEE, 2000.*
  - Implementations: BWA, bowtie and SOAP2
  - How fast? First an intro to “big O” notation

Brief intro to “Big O” notation

The CALCULATED procedure in Figure 3 gives a better, though not optimal, bound. It is conceptually equivalent to the one described in Figure 4, which is simpler to understand. We use the BWT of the reverse (not complemented) reference sequence to test if a substring of W is also a substring of X. Note that to do this test with BWT string B alone would make CALCULATED an $O(|W|^2)$ procedure, rather than $O(|W|)$ as is described in Figure 3.

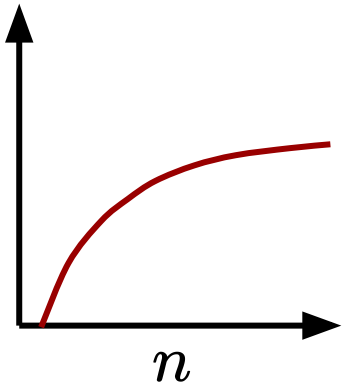


from: Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009 Jul 15;25(14):1754-60.

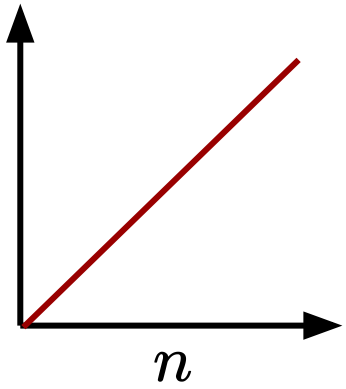
Brief intro to “Big O” notation

- If I have n sequences, how does the amount of time required by the program increase?

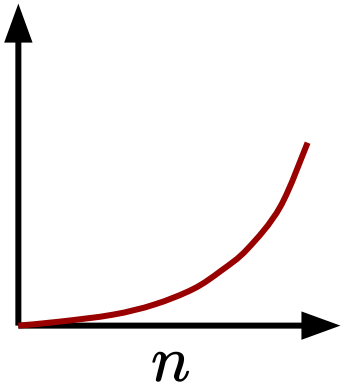
$log(n)$



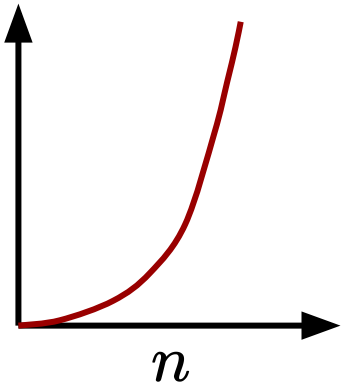
n



n^2



2^n



Brief intro to “Big O” notation

- If I have n sequences, how does the amount of time required by the program increase?

Number of steps

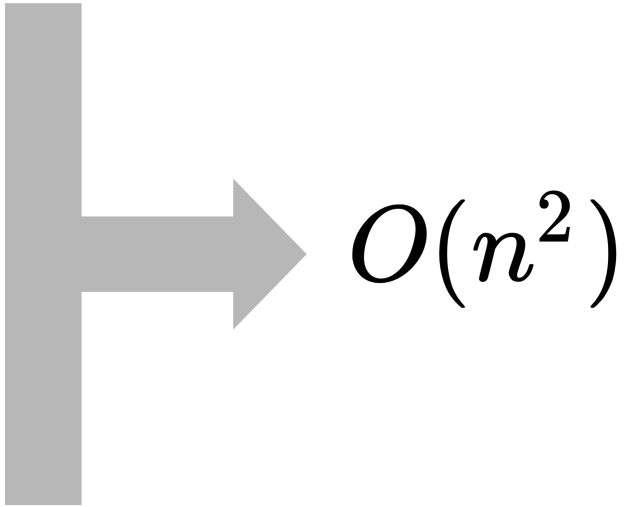
$3n^2 + 6n + 1$

$25n^2 + 5n + 4$

$9n^2$

$1000n^2$

$n^2 - 9$



$O(n^2)$

BWT for alignment

- The FM index uses $O(|L|)$ memory
- Can be searched in $O(|pattern|)$ steps
- Entire FM-index is 1.5Gb for human genome
- FM-index
  - We also need certain other data structures that we did not cover

- Human genome can be effectively indexed and searched using 3Gb RAM!

Implementation in BWA

- Burrows Wheeler Aligner (BWA) can use:
  - bwa aln: First ~30nt of read as seed
    - Extend around positions with seed match
    - For short reads
  - bwa mem: Multiple short seeds across the read
    - Extend around positions with several seed matches
    - For longer reads

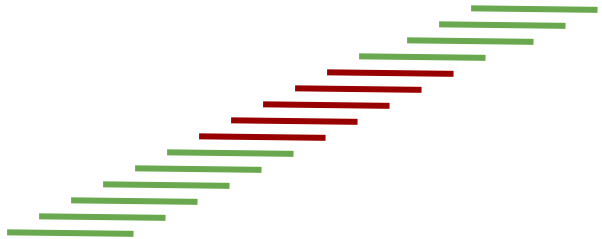
Hash methods making a come back?

Let’s go back to our example:

```
my read              GGGGCTGCCGCCCCATAG
                     ||||||||| ||||||||
chr7: 102,204,582    GGGGCTGCCCCCCCCATAG
```

Hash methods making a come back?

match with k=4



match
mismatch

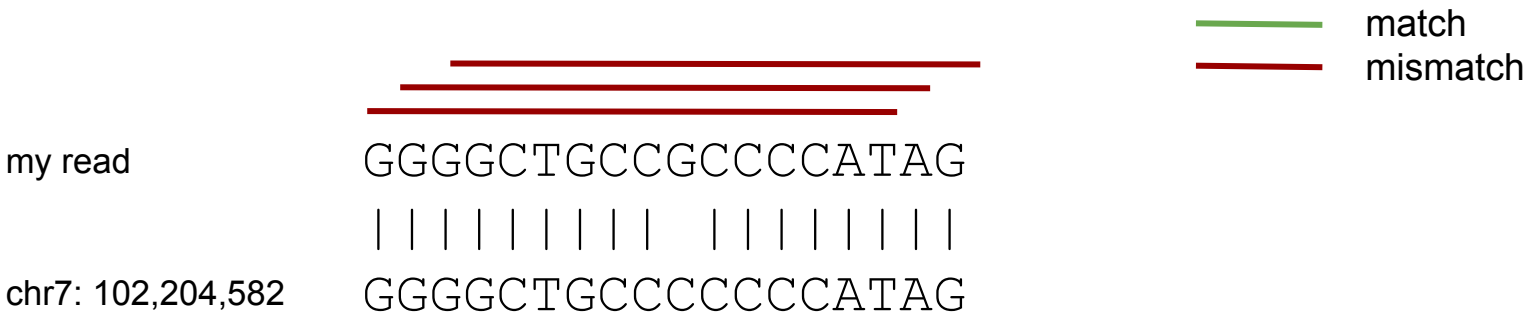
my read GGGGCTGCCGCCCCATAG

chr7: 102,204,582 GGGGCTGCCCCCCCATAG

But wait, 4^4 is 256, that is too little!
Everything will match, poor specificity

Hash methods making a come back?

match with k=16

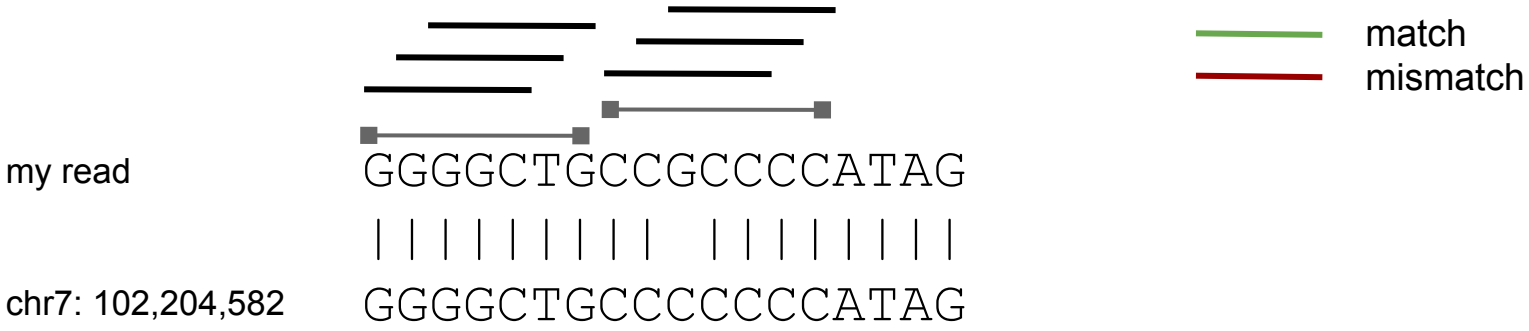


Nothing matches! poor sensitivity

4^{16} = 4,294,967,296

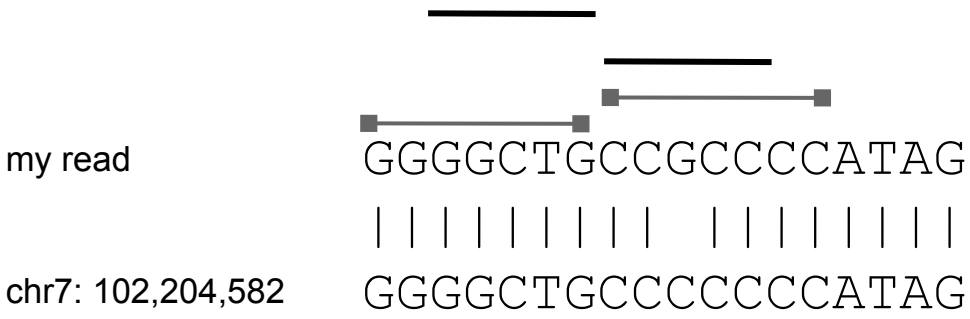
Hash methods making a come back?

Mimimizer with w=7 k=5



Hash methods making a come back?

Mimimizer with w=7 k=5

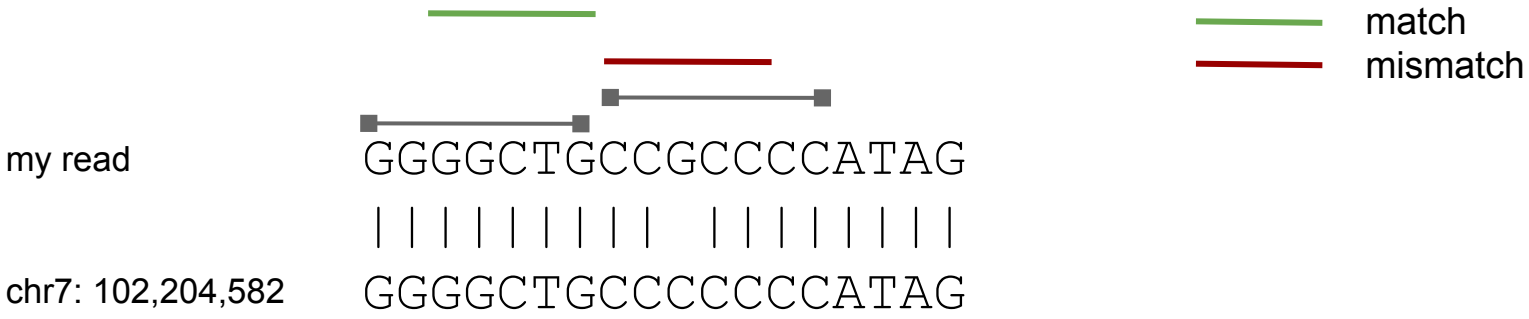


match
mismatch

Pick the kmer with lowest lexicographical value

Hash methods making a come back?

Mimimizer with w=7 k=5



- Balance between short and long kmers
- Sensitive+specific

Great for long reads!

JOURNAL ARTICLE

Minimap2: pairwise alignment for nucleotide sequences FREE

Heng Li

Bioinformatics, Volume 34, Issue 18, September 2018, Pages 3094–3100,
https://doi.org/10.1093/bioinformatics/bty191
Published: 10 May 2018 **Article history** ▾

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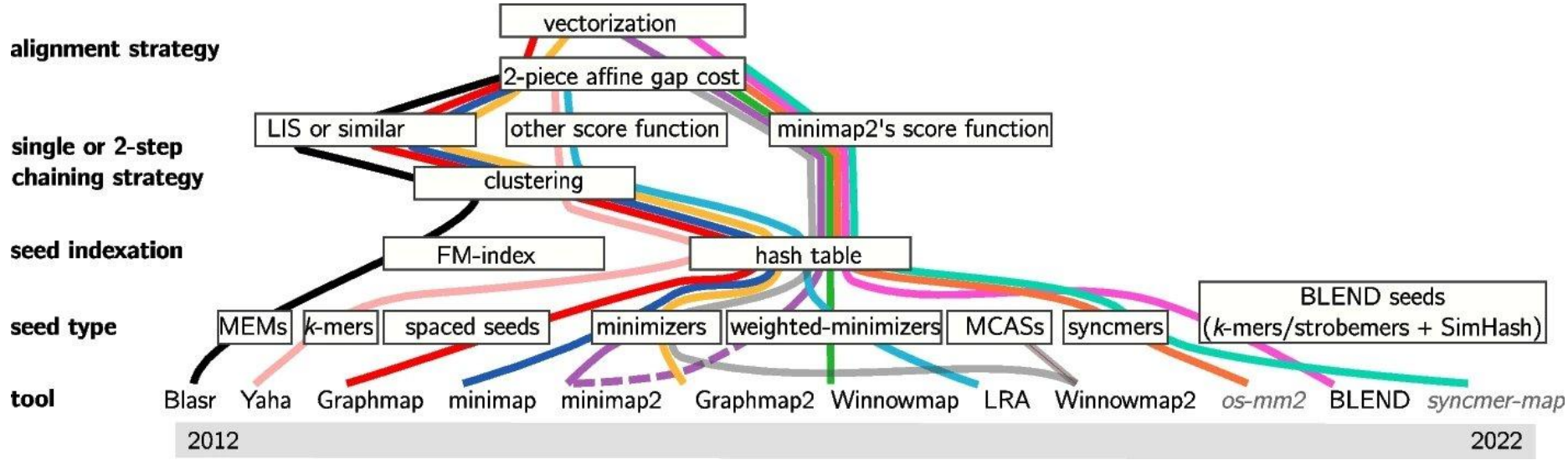
Abstract

Motivation

Recent advances in sequencing technologies promise ultra-long reads of ~100 kb in average, full-length mRNA or cDNA reads in high throughput and genomic contigs over 100 Mb in length. Existing alignment programs are unable or inefficient to process such data at scale, which presses for the development of new alignment algorithms.

Results

Minimap2 is a general-purpose alignment program to map DNA or long mRNA sequences against a large reference database. It works with accurate short reads of ≥100 bp in length, ≥1 kb genomic reads at error rate ~15%, full-length noisy Direct RNA or cDNA reads and assembly contigs or closely related full chromosomes of hundreds of megabases in length. Minimap2 does split-read alignment, employs concave gap cost for long insertions and deletions and introduces new heuristics to reduce spurious alignments. It is 3–4 times as fast as mainstream short-read mappers at comparable accuracy, and is ≥30 times faster than long-read genomic or cDNA mappers at higher accuracy, surpassing most aligners specialized in one type of alignment.



Review | Open access | Published: 01 June 2023

A survey of mapping algorithms in the long-reads era

Kristoffer Sahlin, Thomas Baudeau, Bastien Cazaux & Camille Marchet

Genome Biology **24**, Article number: 133 (2023) | Cite this article

Abstract

It has been over a decade since the first publication of a method dedicated entirely to mapping long-reads. The distinctive characteristics of long reads resulted in methods moving from the seed-and-extend framework used for short reads to a seed-and-chain framework due to the seed abundance in each read. The main novelties are based on alternative seed constructs or chaining formulations. Dozens of tools now exist, whose heuristics have evolved considerably. We provide an overview of the methods used in long-read mappers. Since they are driven by implementation-specific parameters, we develop an original visualization tool to understand the parameter settings (http://bcazaux.polytech-lille.net/Minimap2/).

Intro to mapping quality

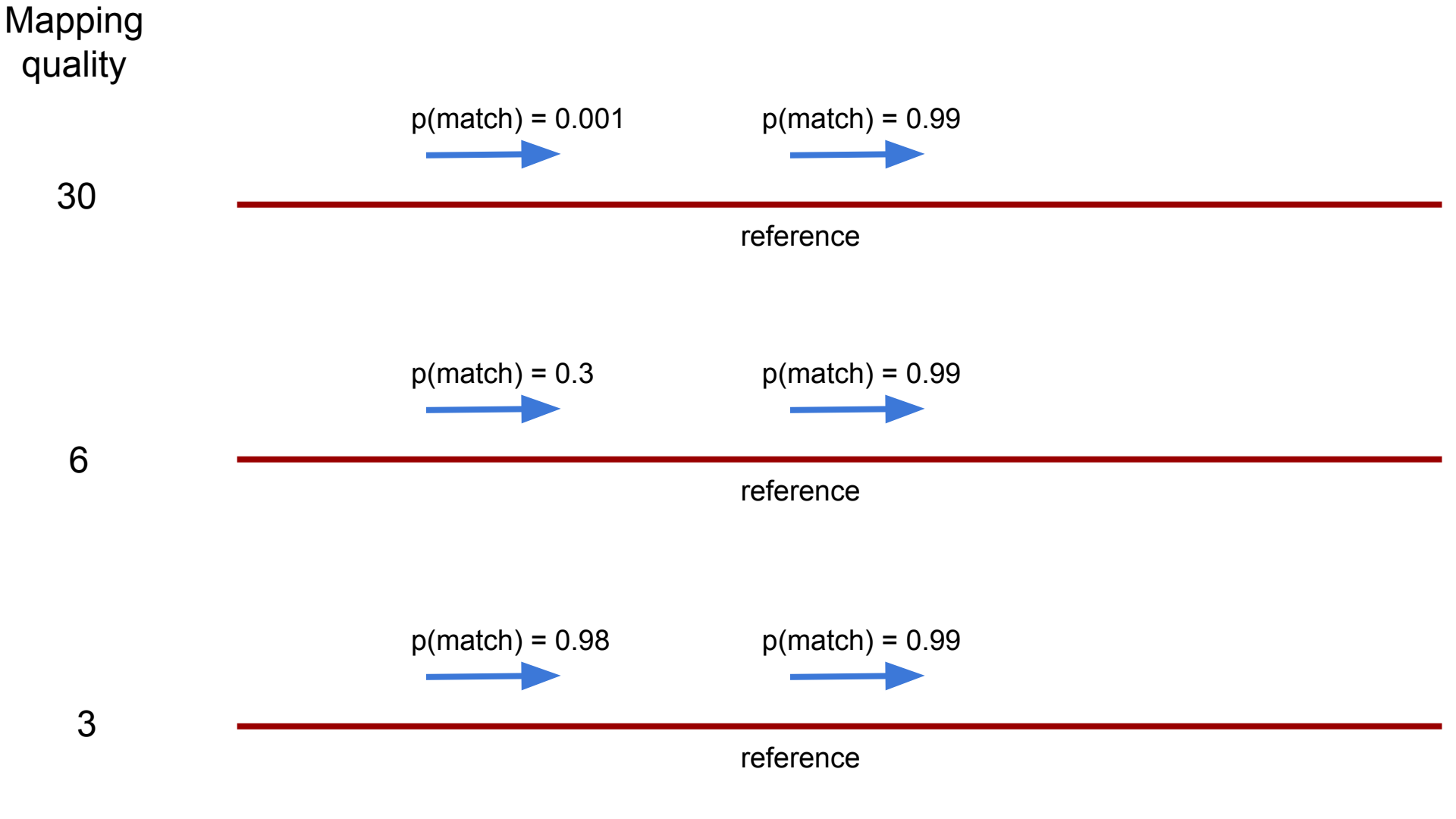
What happens when a sequence has multiple hits to the genome?

Depends on the aligner, Burrows-Wheeler Aligner (BWA) does the following:

- Assign to the genomic location with the best score
- Use other matches to compute the probability of mismapping on a log scale:

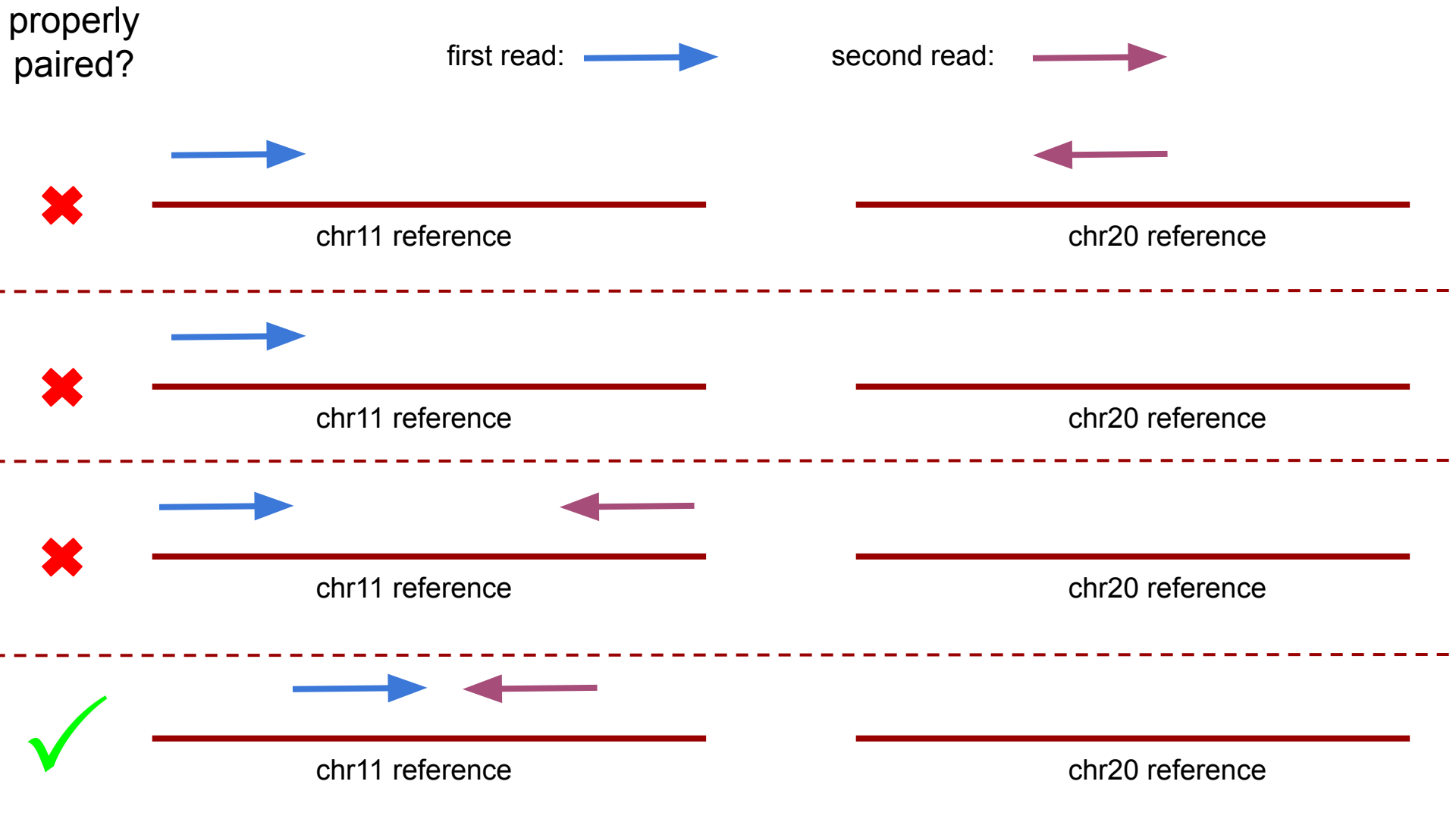
$$MAPQ = -10 \log (P[\text{mismapping}])$$

e.g. MAPQ 30 = P[mismapping] = 1/1000


Mapping quality
p(match) = 0.001
p(match) = 0.99
30
reference
p(match) = 0.3
p(match) = 0.99
6
reference
p(match) = 0.98
p(match) = 0.99
3
reference

Intro to “proper pairs” vs “unpaired”

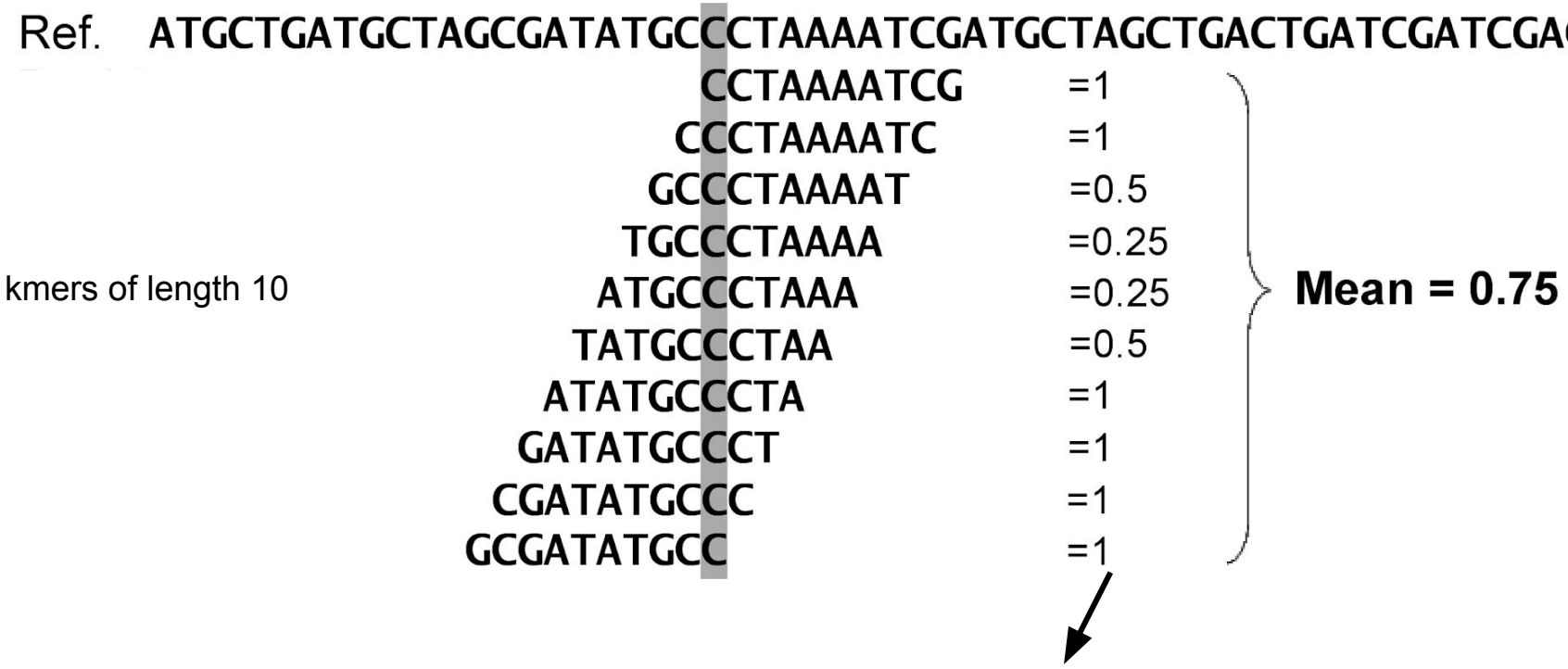
- Some aligners add an extra flag to indicate that 2 paired reads were found:
  - on the same chromosome
  - facing each other (one + strand, the other - strand)
  - within a “reasonable” distance


properly paired?
first read:
second read:
chr11 reference
chr20 reference
chr11 reference
chr20 reference
chr11 reference
chr20 reference
chr11 reference
chr20 reference

mapping quality vs mappability

- Mapping quality is often (poorly) approximated for speed
- Use another technique to avoid spurious mappings: genomic mappability
- Mapping quality is per read
- Mappability is for a genomic region

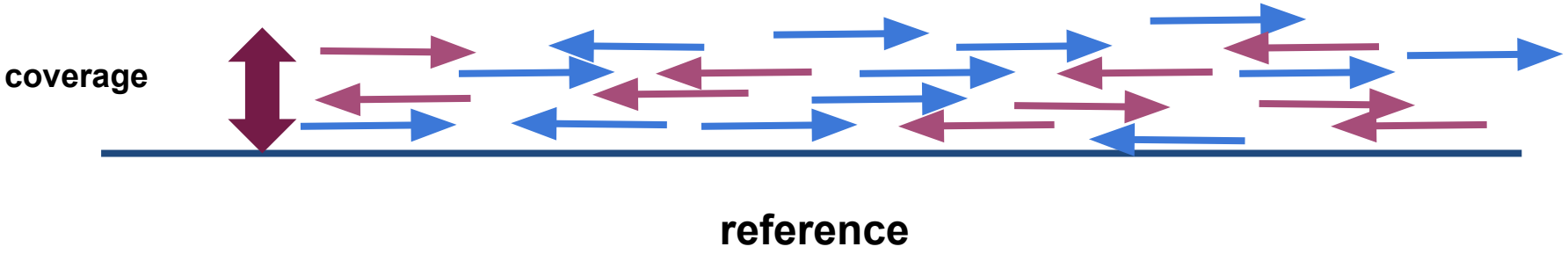
Mappability



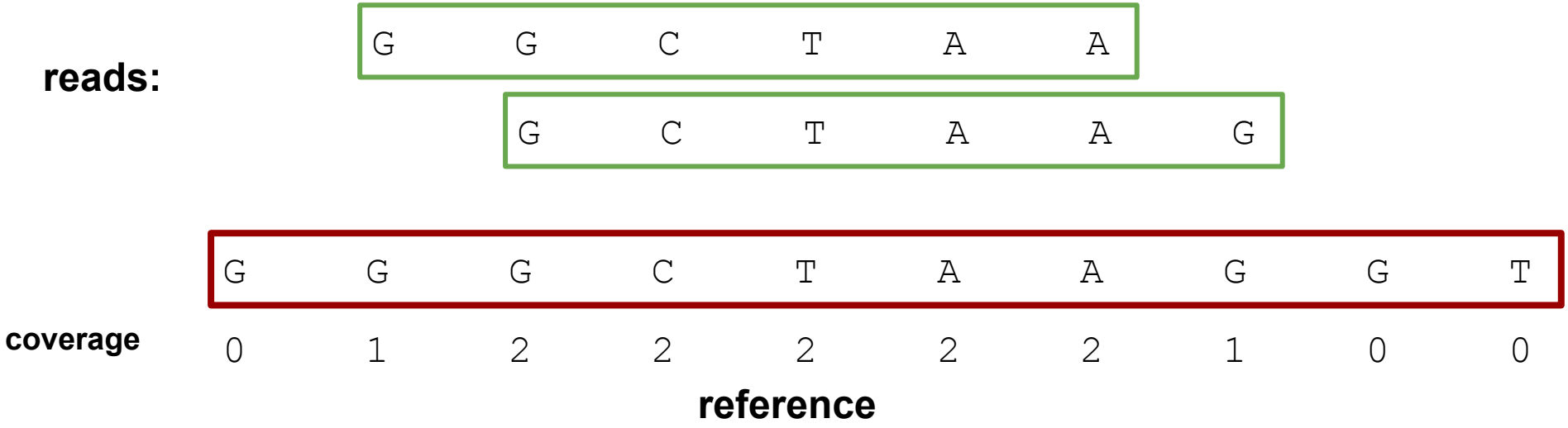
mappability score 1 = unique <1=not unique

Coverage

- Coverage/depth is how many times that your data covers the genome (on average)

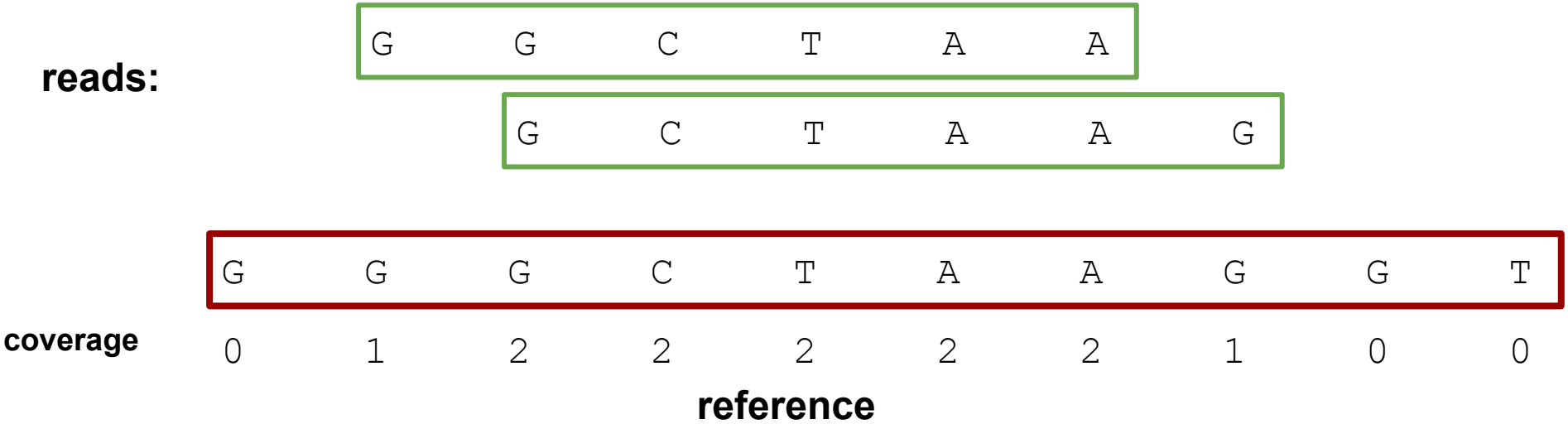


Depth of Coverage



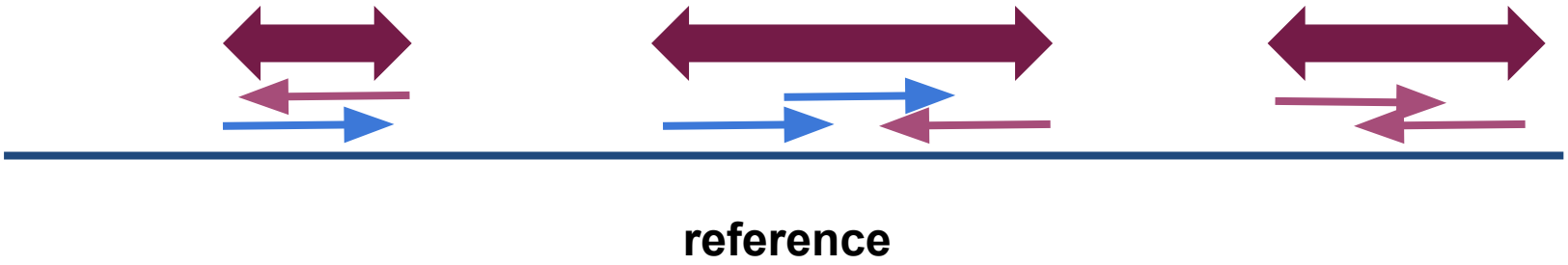
Depth of Coverage

average: (0+1+2+2+2+2+2+1+0+0)/10 = 1.2X



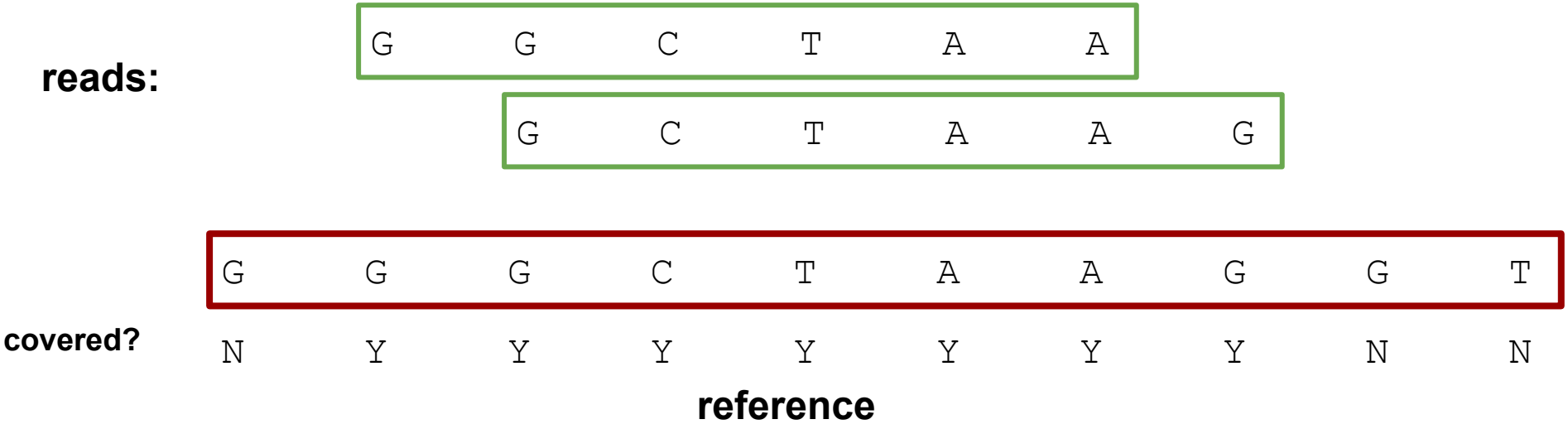
Breadth of coverage

- breadth of coverage: fraction of the genome covered (1X or more)



Breadth of Coverage

average: 7 sites with Y / 10 = 70%



Coverage

- Coverage/depth is how many times that your data covers the genome (on average)

- Example:
  - N: Number of reads: 5M
  - L: Read length: 100
  - G: Genome size: 5Mbp
  - C = 5M*100/5M = 100X
  - On average there are 100 reads covering each position in the genome

$$C = N \times \frac{L}{G}$$

SAM/BAM format

- Sequence Alignment / Map format
- BAM = Binary SAM and zipped - always convert to BAM
- Two sections
  - Header: All lines start with “@”
  - Alignments: All other lines

```
@SQ   SN:mito_ref     LN:16569
@PG   ID:bwa     PN:bwa     VN:0.7.17  CL:bwa samse human_MT.fa test_cut.sai input.fq.gz
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

The header section

```
@SQ   SN:mito_ref      LN:16569
@PG   ID:bwa      PN:bwa      VN:0.7.17  CL:bwa samse human_MT.fa test_cut.sai input_fq.gz
```

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

The alignment section

```
@SQ   SN:mito_ref      LN:16569
@PG   ID:bwa     PN:bwa     VN:0.7.17  CL:bwa samse human_MT.fa test_cut.sai input_fq.gz
```

Contains information like:

- What command line was used to generate this SAM/BAM file?
- What does the reference genomes look like? (chromosome names+length)

```
@SQ   SN:mito_ref     LN:16569
@PG   ID:bwa     PN:bwa     VN:0.7.17  CL:bwa samse human_MT.fa test_cut.sai input.fq.gz
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

```
HN3BX:8:2115:3985:442  16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:315  0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:133  16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:134  16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

Read IDs (should be unique)

```
HN3BX:8:2115:3985:4426  16  mito_ref 5265   37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157  0   mito_ref 9373    0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335  16  mito_ref 7241   15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342  16  mito_ref 14440   0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237  0   mito_ref 1994    0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

flag (https://broadinstitute.github.io/picard/explain-flags.html)

ex:

```
 0 = single-end read, mapped, read mapping to + strand
16 = single-end read, mapped, read mapping to - strand
```

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

name of chromosome (ex: chr1, chr2 ...

```
HN3BX:8:2115:3985:4426 16 mito_re  5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_re  9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_re  7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_re  14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_re  1004   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

coordinate on chromosome

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

mapping quality

ex:

MQ = prob of correct mapping

37 = 99.98%

15 = 96.84%

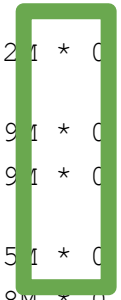
0 = 00.00%

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

CIGAR

Number of operations:
matches (**M**)
insertions (**I**)
deletions (**D**)

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```



chromosome of the other pair (not used here)

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

position on the chromosome of the other pair (not used here)

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 TTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 ATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 TACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 TACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC  AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

length between pairs (not used here)

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

sequence of the reads

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

quality scores of the reads

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

optional flags, tell us:

- how many mismatches in alignment
- total number of matches
- read group

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

multiplexing: to which read group does the read belong to?

How to reconstruct the alignment? it's complicated...

```
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

```
Ref+:      9373 AATGATGGC 9381
                |||||||| |
Qry+:         1 AATGATGAC 9
```

```
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0                       AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

sort by coordinate

```
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT        FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
```

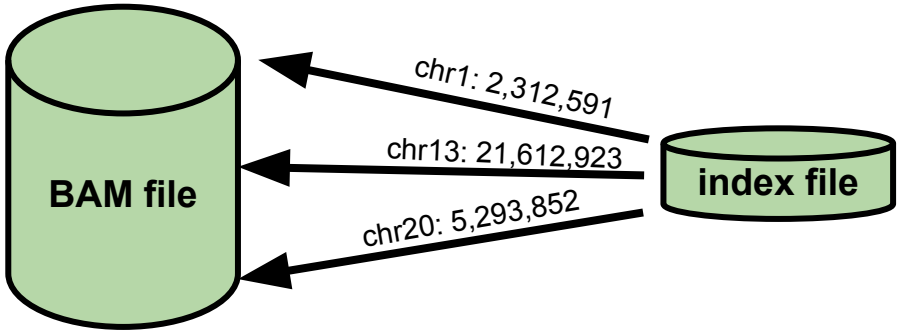
```
HN3BX:8:1102:2678:3157 0  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT       FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
HN3BX:8:1222:8146:4237 0  mito_ref 1994   0                      AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
```

sort by read name

```
HN3BX:8:1102:2678:3157 (  mito_ref 9373   0  9M * 0 0 AATGATGAC    AAFFFJJJJ    NM:i:1 MD:Z:7G1 RG:Z:sample1
HN3BX:8:1217:1588:1335 16 mito_ref 7241  15  9M * 0 0 ATACACCAC    JJJJFFFAA    NM:i:0 MD:Z:9   RG:Z:sample2
XG:i:0
HN3BX:8:1222:8146:4237 (  mito_ref 1994   0  8M * 0 0 AACCTACC     AAFFFJJJ     NM:i:0 MD:Z:8   RG:Z:sample1
HN3BX:8:2115:3985:4426 16 mito_ref 5265  37 12M * 0 0 ATTATCGAAGAA JJJJJAFFFAAA NM:i:0 MD:Z:12  RG:Z:sample1
XG:i:0
HN3BX:8:2216:6248:1342 16 mito_ref 14440  0  5M * 0 0 ATACT       FFFAA        NM:i:0 MD:Z:5   RG:Z:sample1
```

BAM indexing

- I want all reads mapping to chrX
- Go through the entire BAM file until we reach chrX
- Better idea: keep a small file telling you where chromosome/coord start:

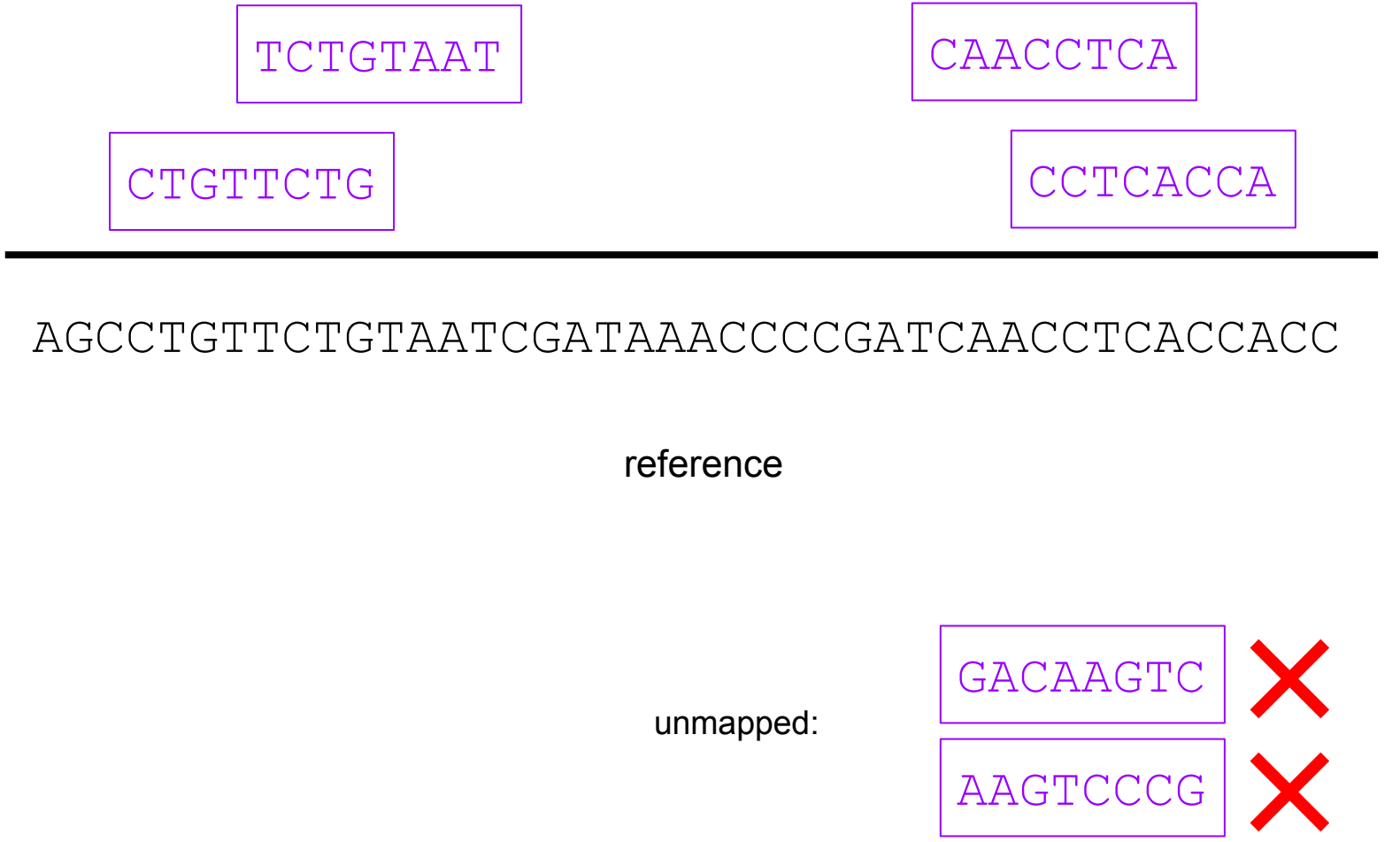


- Need a BAM file sorted by coordinate
- Is usually a .bai or .csi file

BAM vs CRAM

- Idea: why store the original reads? You only the differences to a reference
- Can be 30-60% smaller
- Good for long term storage
- BAM can be converted to CRAM
- Can be indexed

Brief note about pangenomes graphs


TCTGTAAT
CAACCTCA
CTGTTCTG
CCTCACCA
AGCCTGTTCTGTAATCGATAAACCCCGATCAACCTCACCACC
reference
unmapped:
GACAAGTC
AAGTCCCG

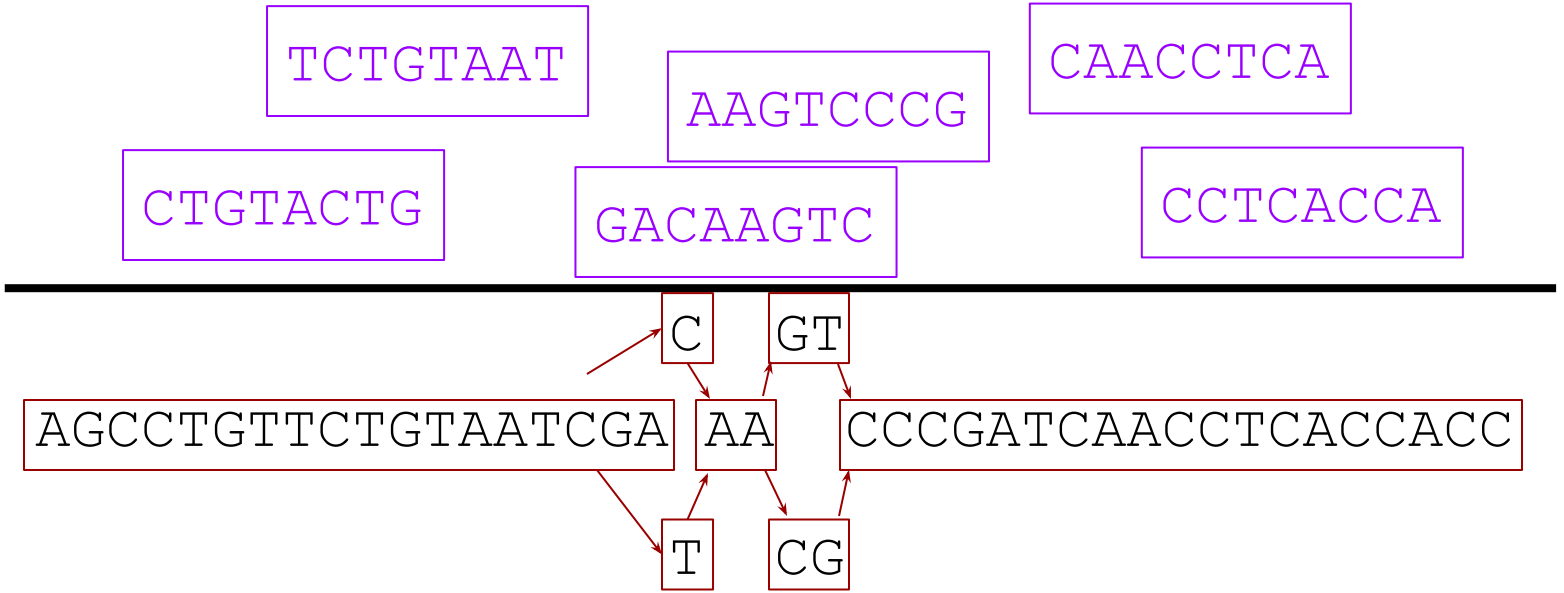
another
sample

AGCCTGTTCTGTAATCGACAAGTCCCGATCAACCTCACCACC

|||||||||||||||||||| || ||||||||||||||||||||

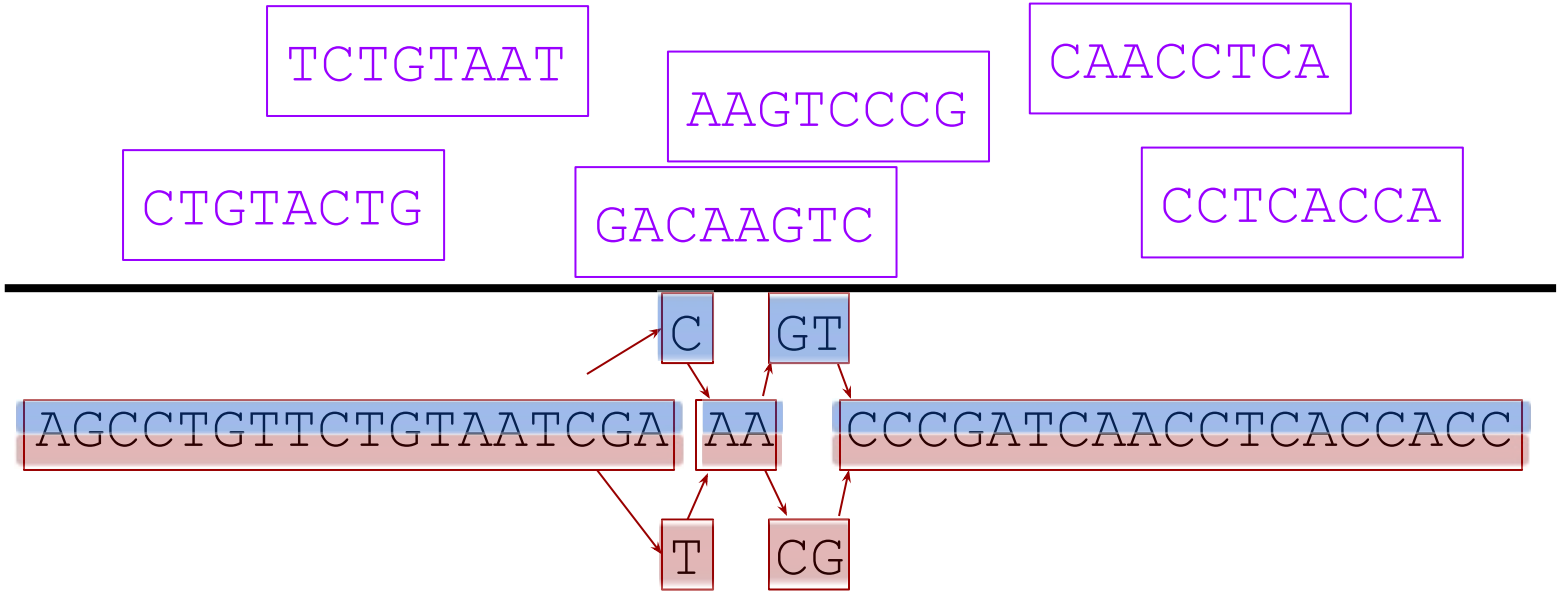
AGCCTGTTCTGTAATCGATAAACCCCGATCAACCTCACCACC

reference



references

unmapped:



references

Paths:

unmapped:

Exercise time!