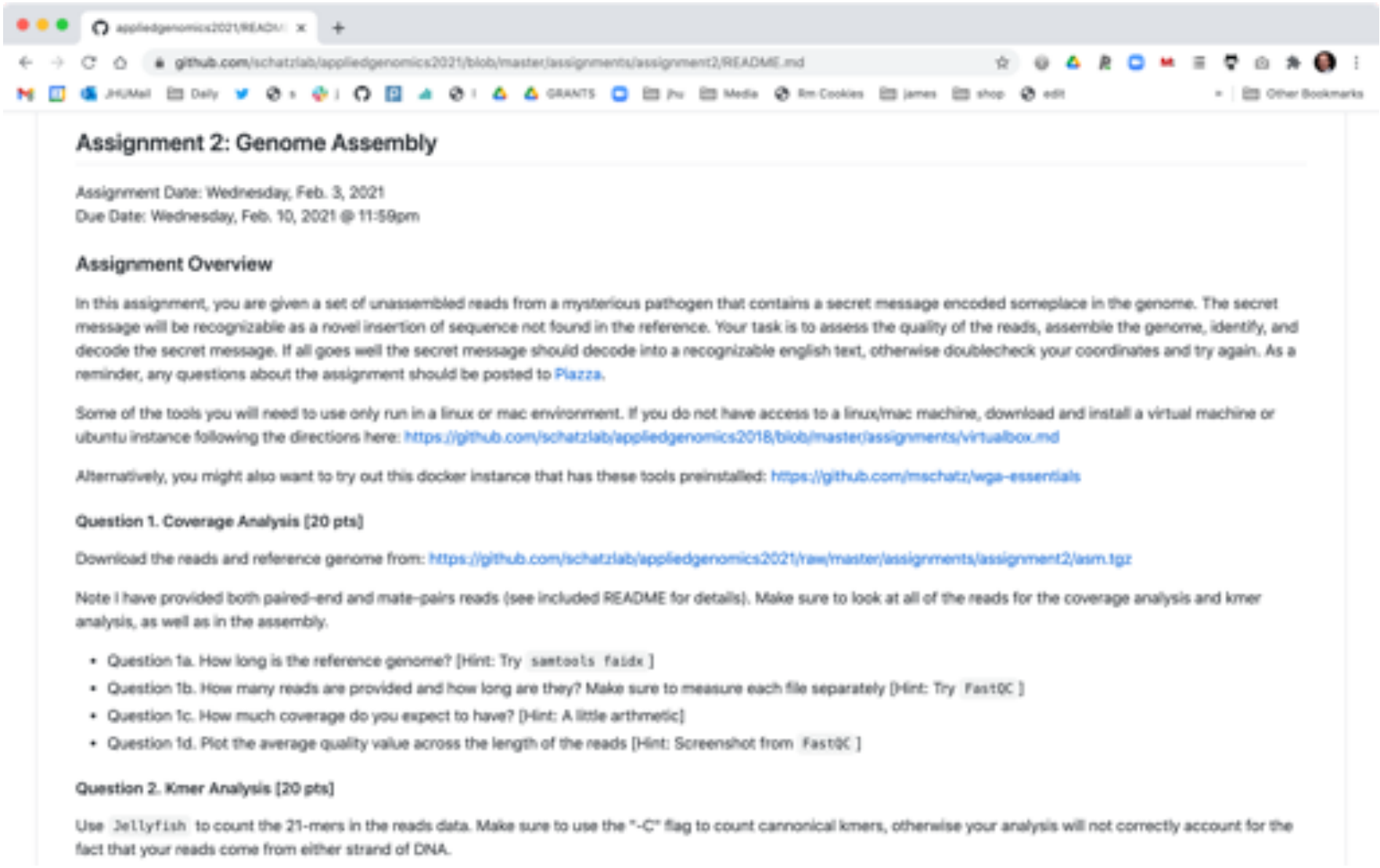
Assembly & Whole Genome Alignment

Michael Schatz

Feb 8, 2021

Lecture 5: Applied Comparative Genomics

Assignment 2: Genome Assembly
Due Feb 10 @ 11:59pm



Assignment 2: Genome Assembly

Assignment Date: Wednesday, Feb. 3, 2021
Due Date: Wednesday, Feb. 10, 2021 @ 11:59pm

Assignment Overview

In this assignment, you are given a set of unassembled reads from a mysterious pathogen that contains a secret message encoded someplace in the genome. The secret message will be recognizable as a novel insertion of sequence not found in the reference. Your task is to assess the quality of the reads, assemble the genome, identify, and decode the secret message. If all goes well the secret message should decode into a recognizable english text, otherwise doublecheck your coordinates and try again. As a reminder, any questions about the assignment should be posted to Piazza.

Some of the tools you will need to use only run in a linux or mac environment. If you do not have access to a linux/mac machine, download and install a virtual machine or ubuntu instance following the directions here: https://github.com/schatzlab/appliedgenomics2018/blob/master/assignments/virtualbox.md

Alternatively, you might also want to try out this docker instance that has these tools preinstalled: https://github.com/mschatz/wga-essentials

Question 1. Coverage Analysis [20 pts]

Download the reads and reference genome from: https://github.com/schatzlab/appliedgenomics2021/raw/master/assignments/assignment2/asm.tgz

Note I have provided both paired-end and mate-pairs reads (see included README for details). Make sure to look at all of the reads for the coverage analysis and kmer analysis, as well as in the assembly.

- Question 1a. How long is the reference genome? [Hint: Try `samtools faidx`]
- Question 1b. How many reads are provided and how long are they? Make sure to measure each file separately [Hint: Try `FastQC`]
- Question 1c. How much coverage do you expect to have? [Hint: A little arthmetic]
- Question 1d. Plot the average quality value across the length of the reads [Hint: Screenshot from `FastQC`]

Question 2. Kmer Analysis [20 pts]

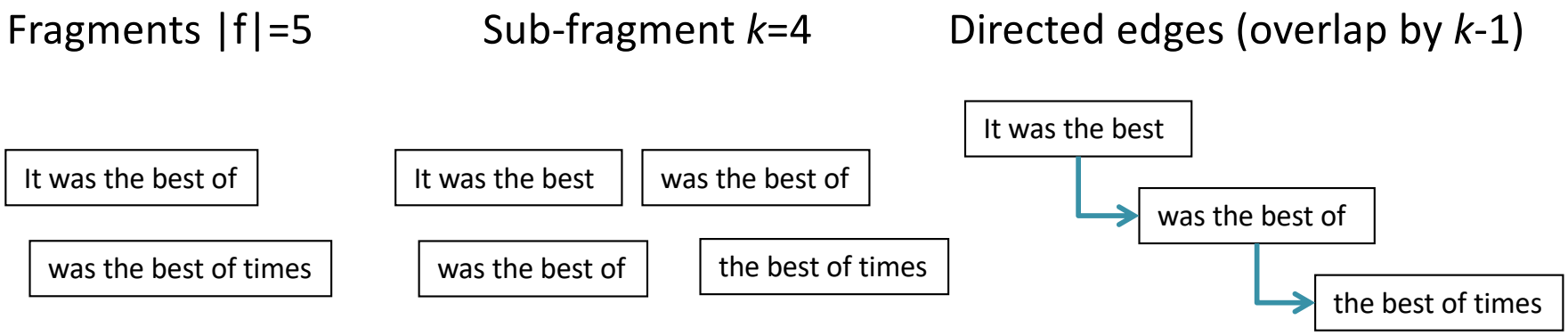
Use `Jellyfish` to count the 21-mers in the reads data. Make sure to use the "-C" flag to count cannonical kmers, otherwise your analysis will not correctly account for the fact that your reads come from either strand of DNA.

https://github.com/schatzlab/appliedgenomics2021

Recap

de Bruijn Graph Construction

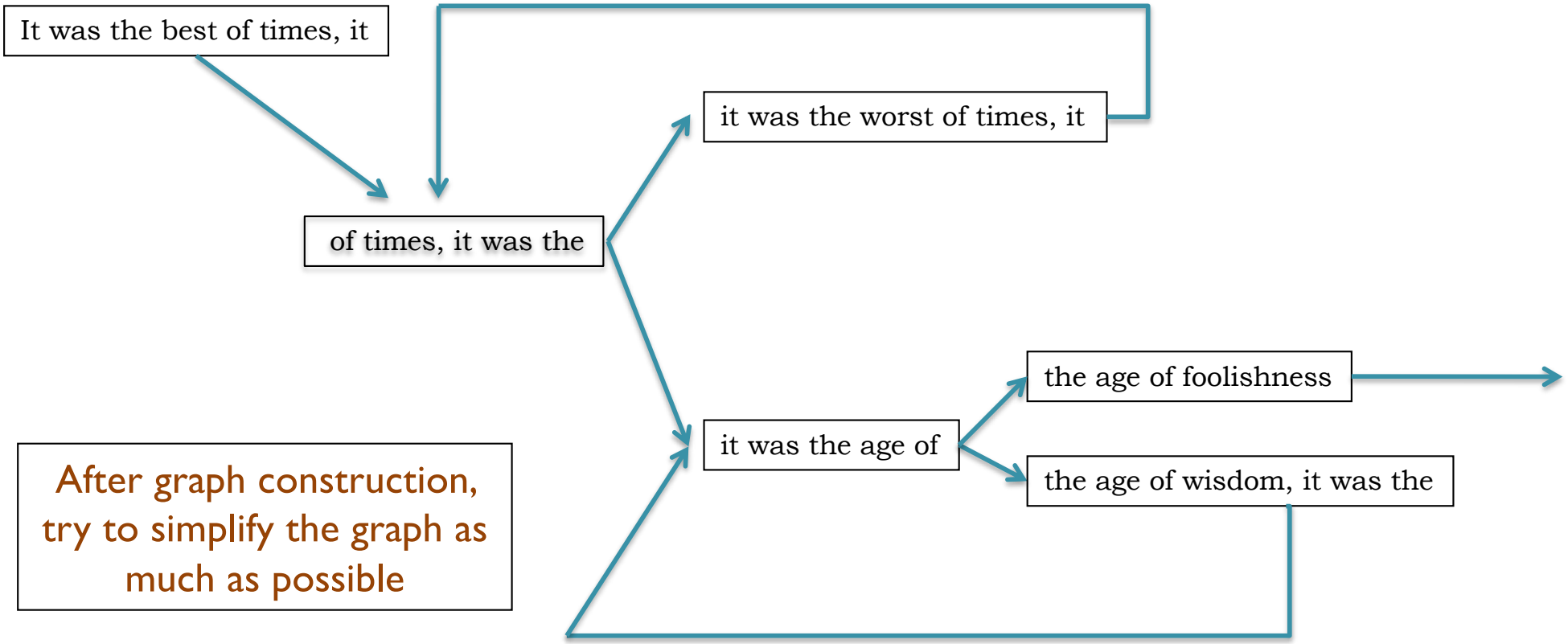
- $G_k = (V,E)$
  - V = Length-*k* sub-fragments
  - E = Directed edges between consecutive sub-fragments
    - Sub-fragments overlap by *k*-1 words



– Overlaps between fragments are implicitly computed

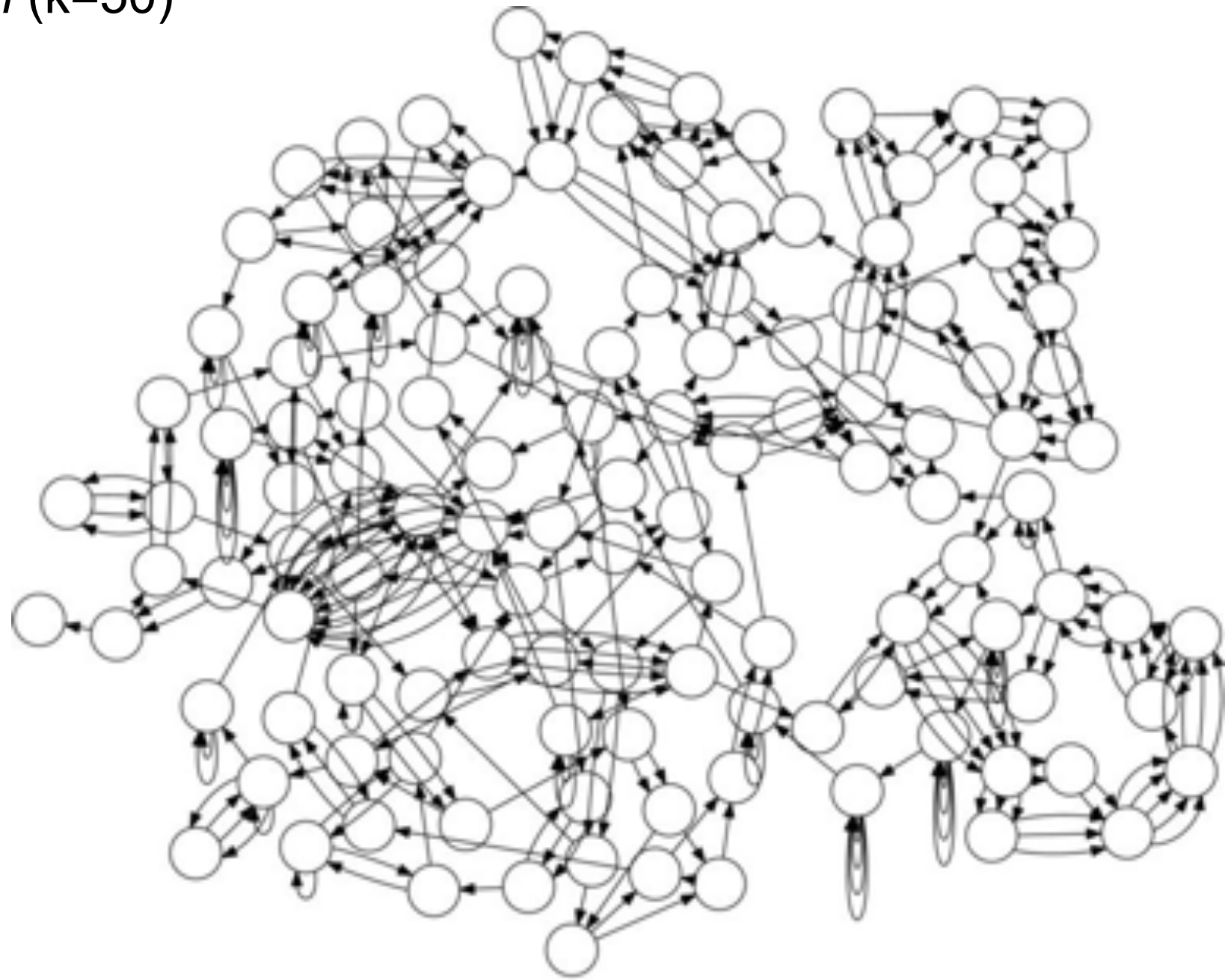
de Bruijn, 1946
Idury et al., 1995
Pevzner et al., 2001

de Bruijn Graph Assembly



After graph construction, try to simplify the graph as much as possible

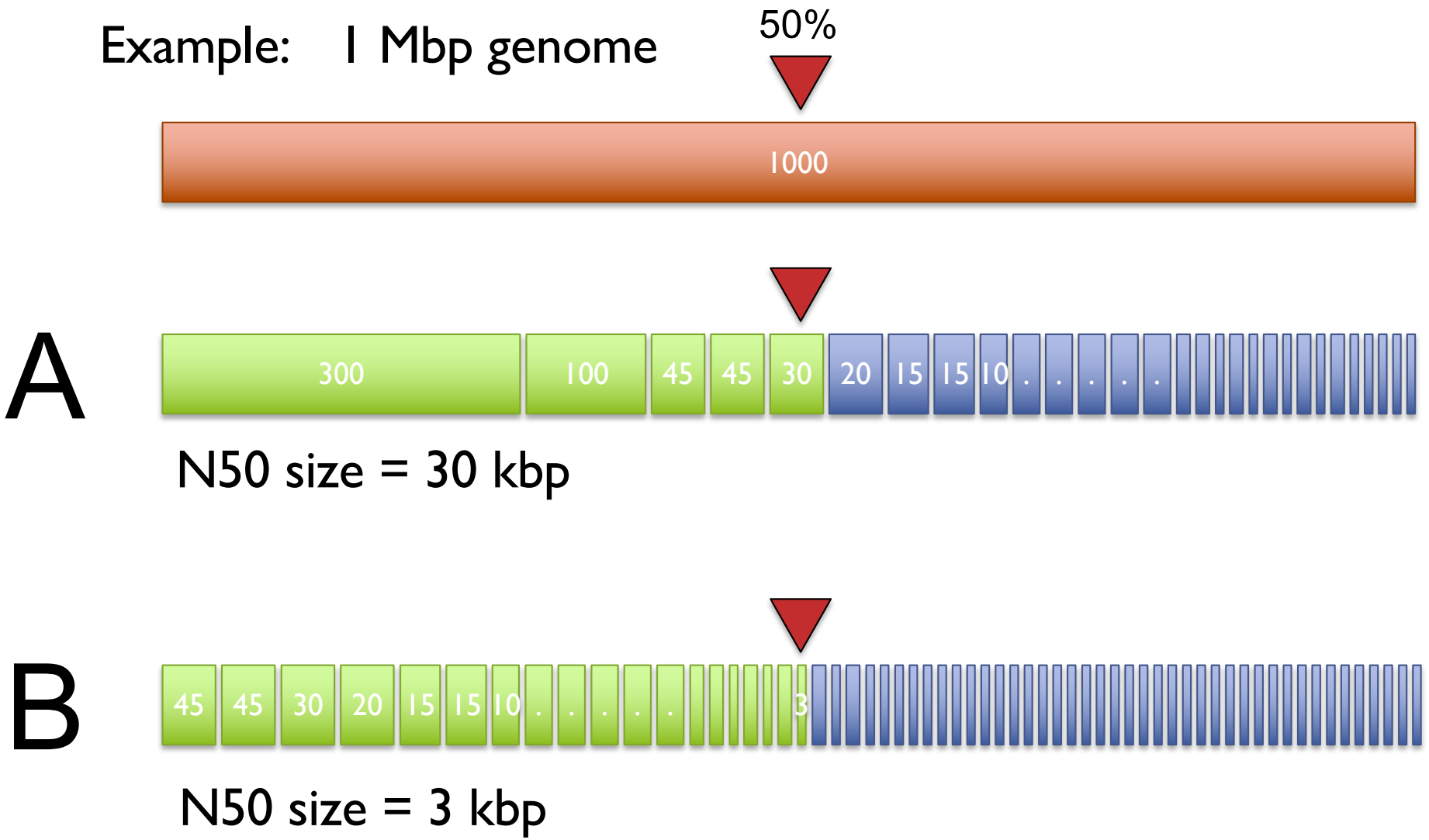
E. coli (k=50)



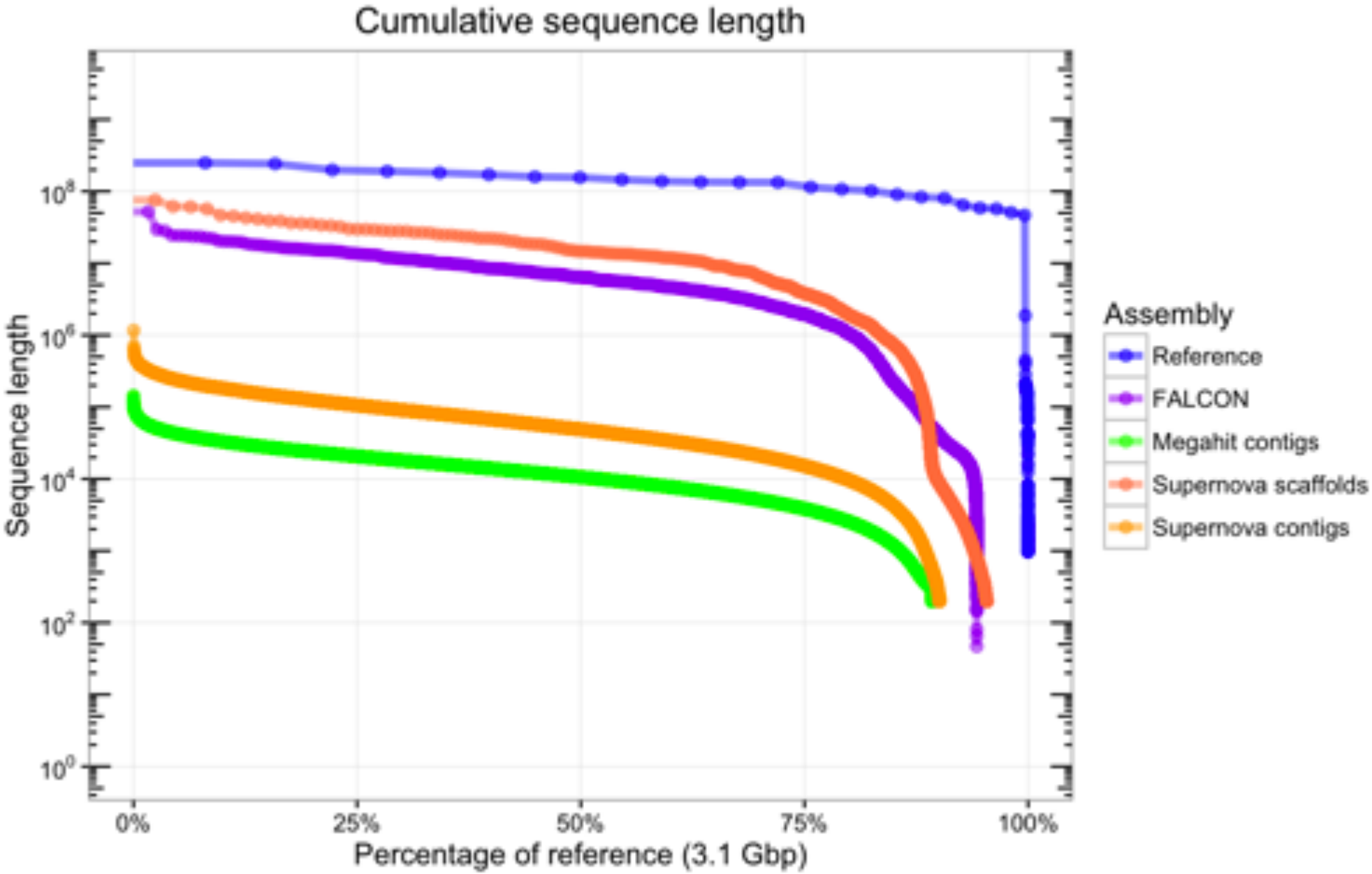
Reducing assembly complexity of microbial genomes with single-molecule sequencing
Koren et al (2013) Genome Biology. **14**:R101 https://doi.org/10.1186/gb-2013-14-9-r101

Contig N50

Def: 50% of the genome is in contigs as large as the N50 value



Contig Nchart



Contig N50

Def: 50% of the genome is in contigs as large as the N50 value

Better N50s improves the analysis in every dimension

- Better resolution of genes and flanking regulatory regions
- Better resolution of transposons and other complex sequences
- Better resolution of chromosome organization
- Better sequence for all downstream analysis

Just be careful of N50 inflation!

- *A very very very bad assembler in 1 line of bash:*
- *cat *.reads.fa > genome.fa*

N50 size = 3 kbp

Pop Quiz 1

Assemble these reads using a de Bruijn graph approach (k=3):

```
ATTA
GATT
TACA
TTAC
```

Pop Quiz 2

Assemble these reads using a de Bruijn graph approach (k=3):

```
ACGA
ACGT
ATAC
CGAC
CGTA
GACG
GTAT
TACG
```

Titus Brown
@ctitusbrown

Following

Wow, this could double as life philosophy, too!

> **Michael Schatz** @mike_schatz
> Replying to @ZaminIqbal @nomad421 and 4 others
> Yep, very easy to find *a* path, very hard to find *the* path

11:40 AM - 22 Jan 2018

4 Retweets **17** Likes

2 4 17

Outline

1. ***Assembly theory***
   - Assembly by analogy

2. ***Practical Issues***
   - Coverage, read length, errors, and repeats

3. ***Next-next-gen Assembly***
   - Canu: recommended for PacBio/ONT project

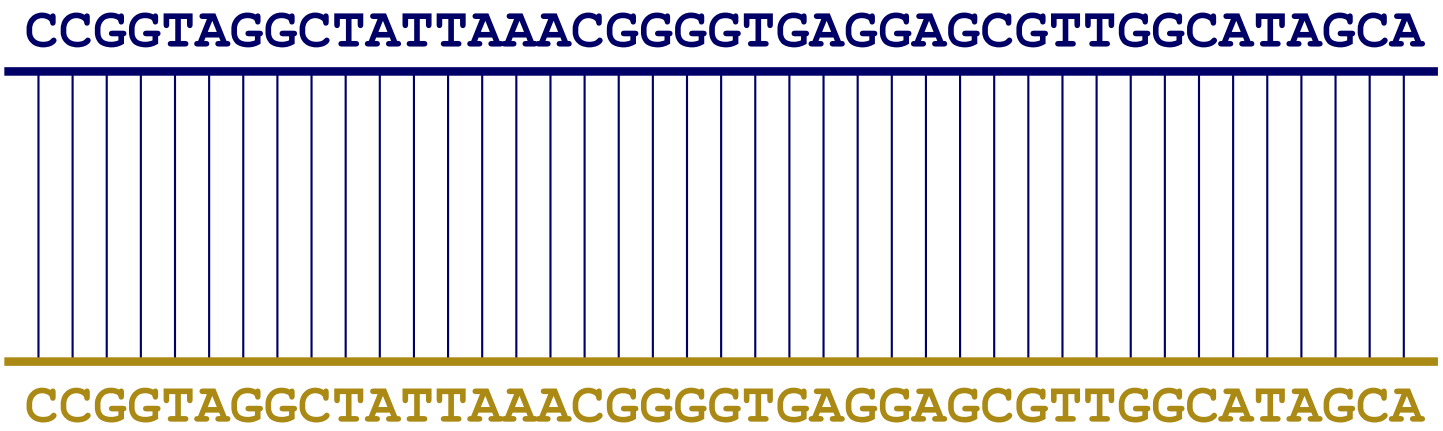
4. ***Whole Genome Alignment***
   - MUMmer recommended

Whole Genome Alignment with MUMmer

Slides Courtesy of Adam M. Phillippy
NHGRI

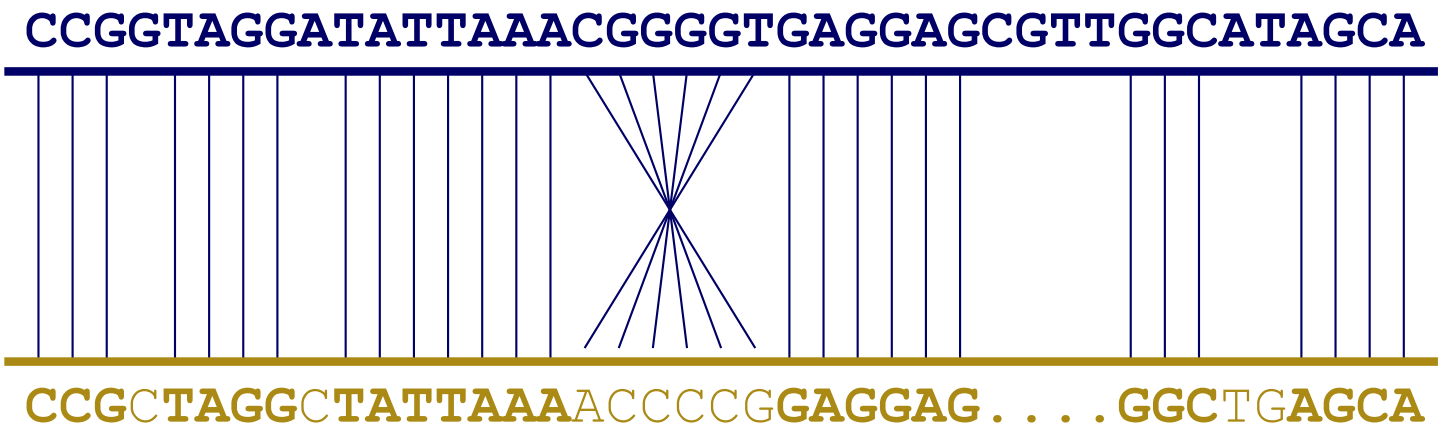
Goal of WGA

- For two genomes, *A* and *B*, find a mapping from each position in *A* to its corresponding position in *B*



Not so fast...

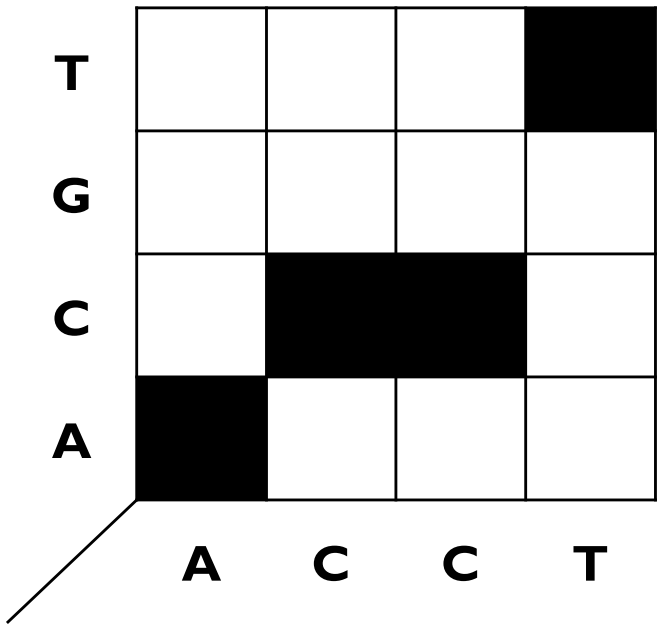
- Genome *A* may have insertions, deletions, translocations, inversions, duplications or SNPs with respect to *B* (sometimes all of the above)



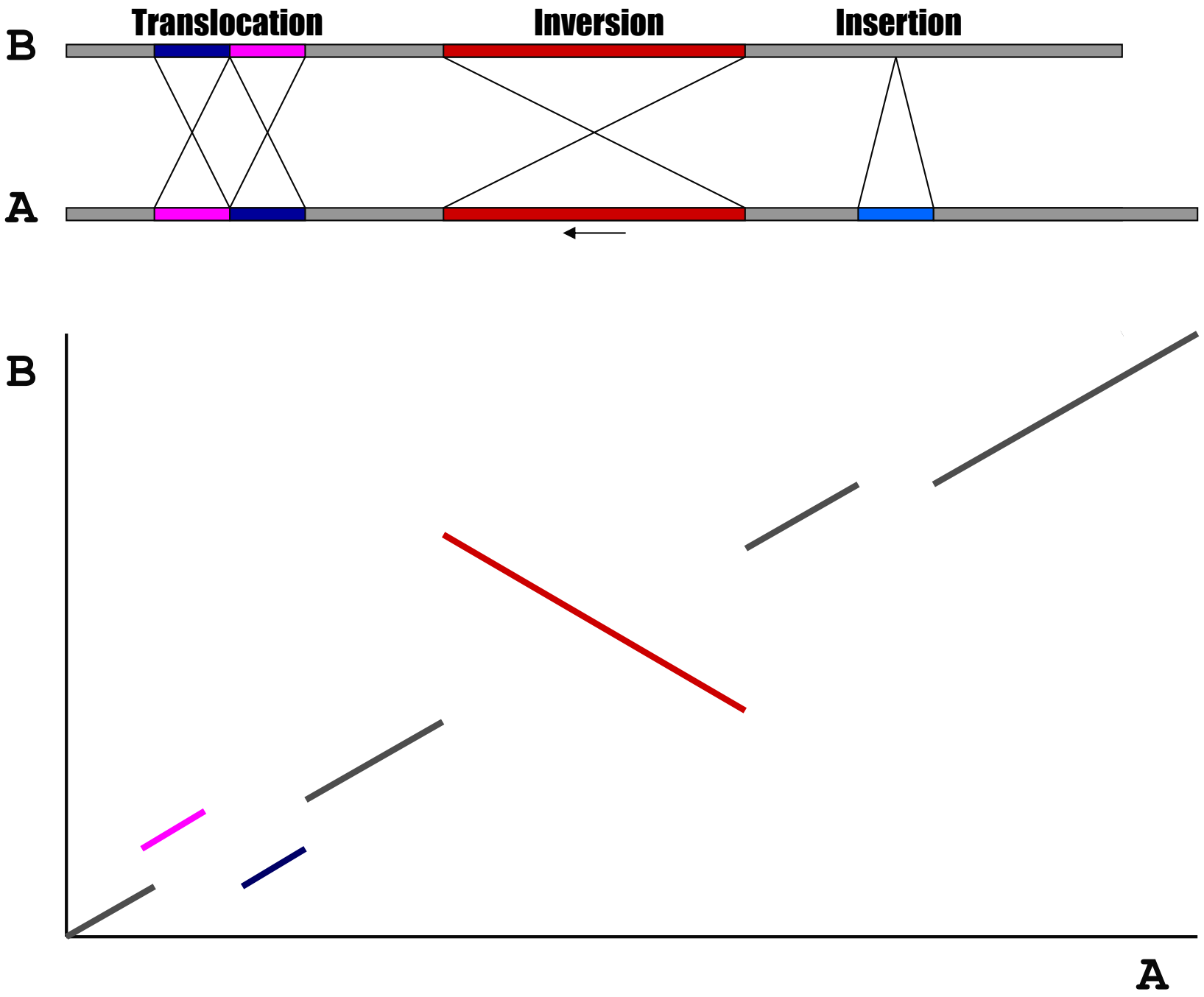
WGA visualization

- How can we visualize *whole* genome alignments?

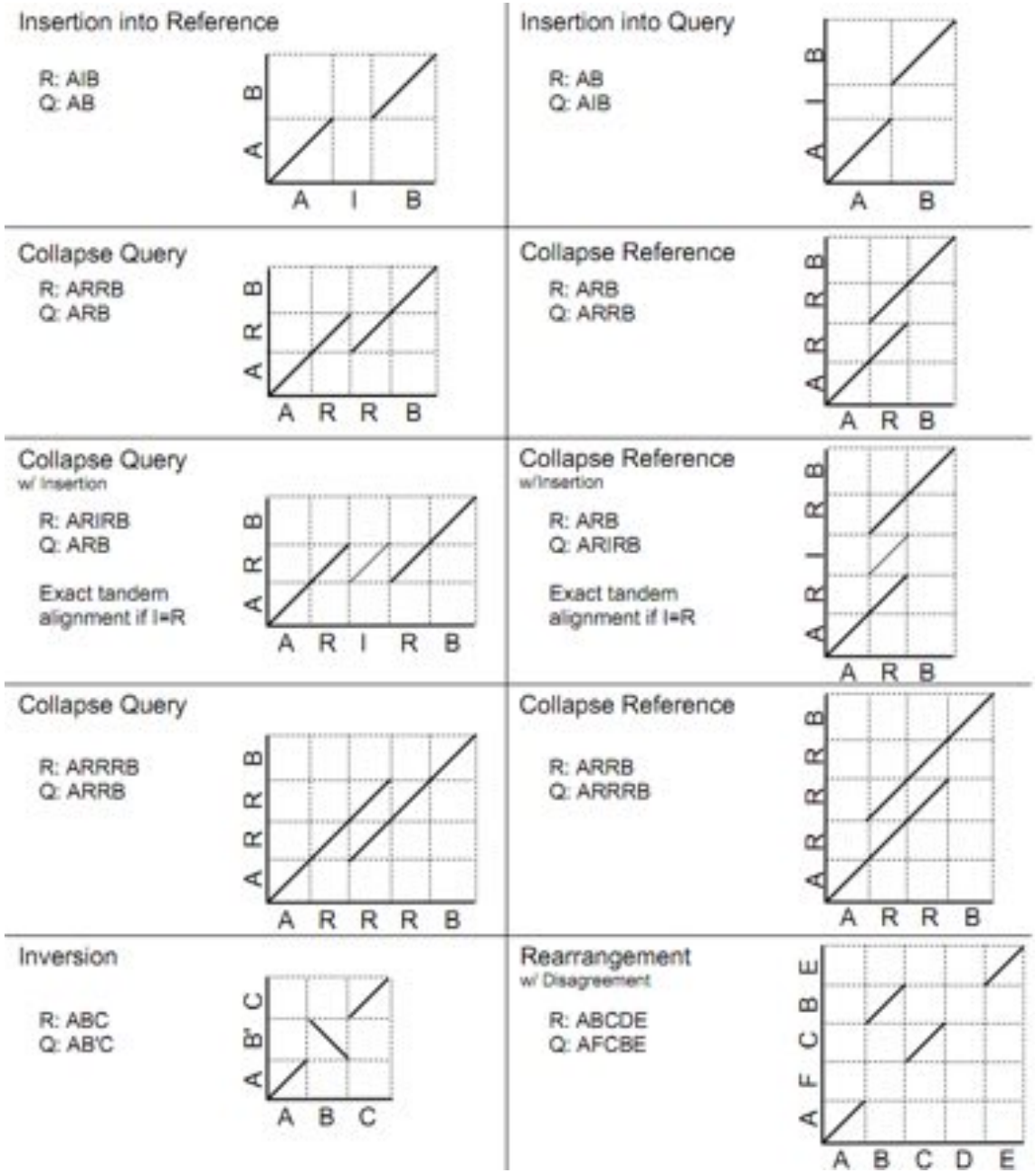
- With an alignment dot plot
  - *N* x *M* matrix
    - Let *i* = position in genome *A*
    - Let *j* = position in genome *B*
    - Fill cell *(i,j)* if A_i shows similarity to B_j



  - A perfect alignment between *A* and *B* would completely fill the positive diagonal

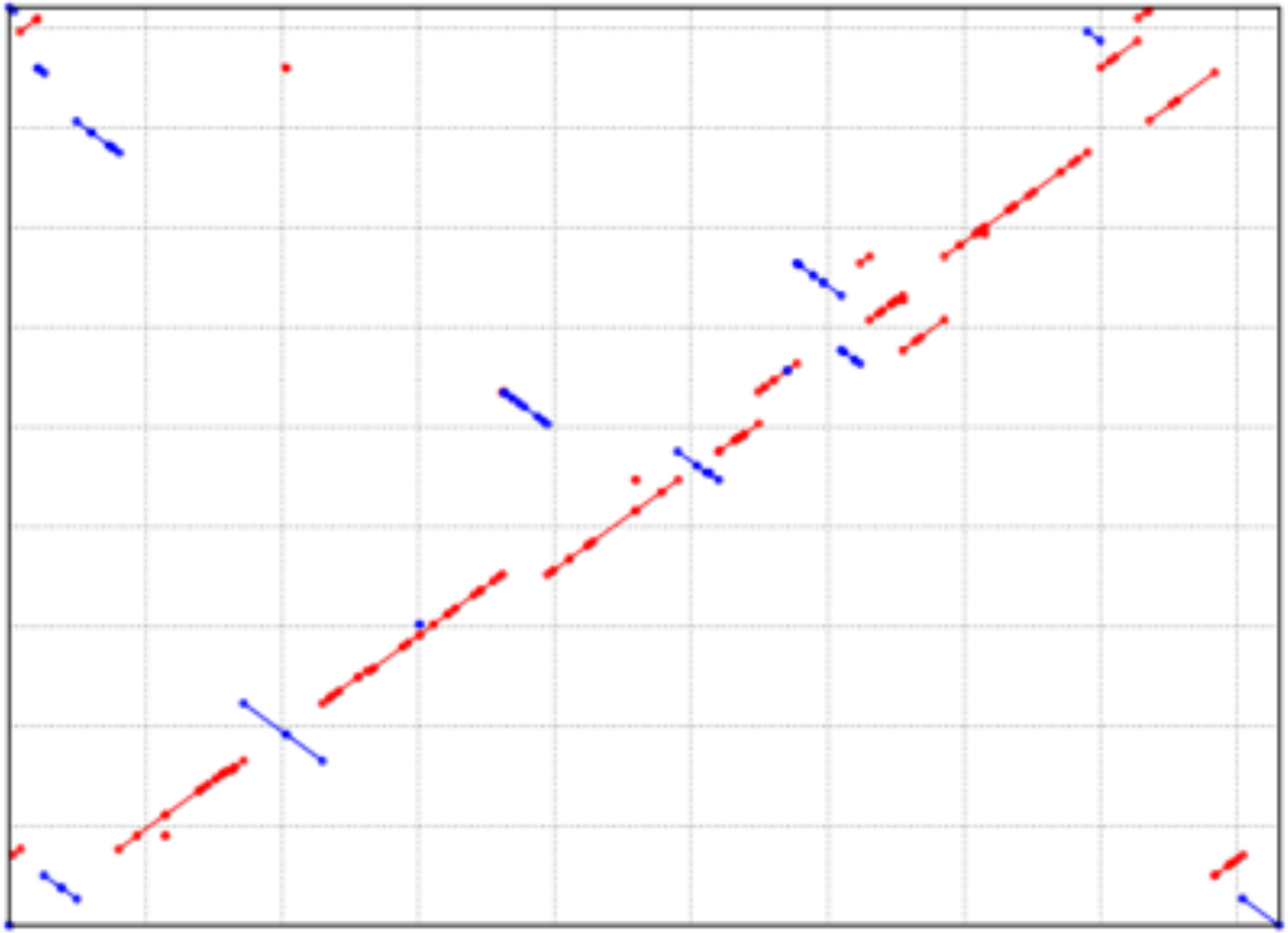

Translocation
Inversion
Insertion
B
A
B
A

SV Types



- Different structural variation types / misassemblies will be apparent by their pattern of breakpoints
- Most breakpoints will be at or near repeats
- Things quickly get complicated in real genomes

http://mummer.sf.net/manual/AlignmentTypes.pdf



Alignment of 2 strains of *Y. pestis*

http://mummer.sourceforge.net/manual/

Assignment 2: Genome Assembly
Due Wednesday Feb 10 @ 11:59pm

1. ***Setup Docker/Ubuntu***
2. ***Initialize Tools***
3. ***Download Reference Genome & Reads***
4. ***Decode the secret message***
    1. *Estimate coverage, check read quality*
    2. *Check kmer distribution*
    3. *Assemble the reads with spades*
    4. *Align to reference with MUMmer*
    5. *Extract foreign sequence*
    6. *dna-encode.pl -d*

https://github.com/schatzlab/appliedgenomics2021/blob/master/assignments/assignment2/README.md

Find and decode

```
nucmer -maxmatch ref.fasta \
   default/ASSEMBLIES/test/final.contigs.fasta
   -maxmatch     Find maximal exact matches (MEMs) without repeat filtering
   -p refctg     Set the output prefix for delta file

mummerplot --layout --png out.delta
   --layout      Sort the alignments along the diagonal
   --png         Create a png of the results

show-coords -rclo out.delta
   -r            Sort alignments by reference position
   -c            Show percent coverage
   -l            Show sequence lengths
   -o            Annotate each alignment with BEGIN/END/CONTAINS

samtools faidx default/ASSEMBLIES/test/final.contigs.fasta
   Index the fasta file

samtools faidx default/ASSEMBLIES/test/final.contigs.fasta \
    contig_XXX:YYY-ZZZ | ./dna-encode -d
```

See manual at http://mummer.sourceforge.net/manual

Outline

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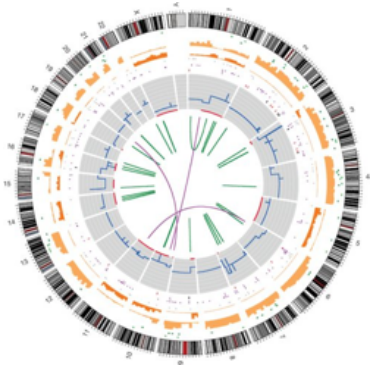
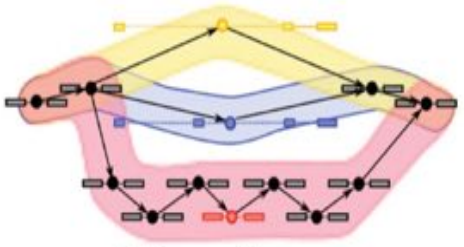
Assembly Applications

- Novel genomes

- Metagenomes

- Sequencing assays
  - Structural variations
  - Transcript assembly
  - …





Why are genomes hard to assemble?

1. ***Biological***:
   - (Very) High ploidy, heterozygosity, repeat content

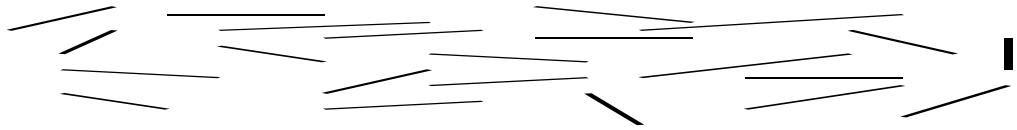
2. ***Sequencing***:
   - (Very) large genomes, imperfect sequencing

3. ***Computational***:
   - (Very) Large genomes, complex structure

4. ***Accuracy***:
   - (Very) Hard to assess correctness

Assembling a Genome

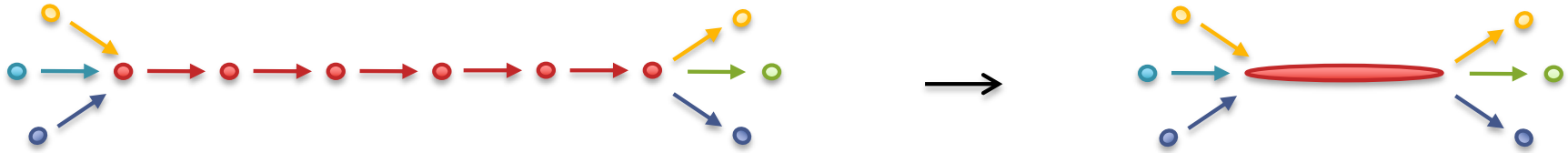
1. Shear & Sequence DNA



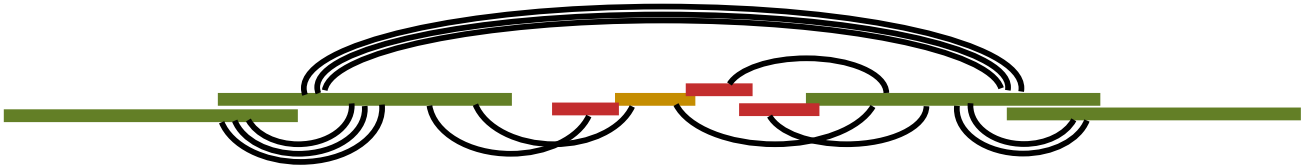
2. Construct assembly graph from reads (de Bruijn / overlap graph)

...AGCCTAGGGATGCGCGACACGT
GGATGCGCGACACGTCGCATATCCGGTTTGGTCAACCTCGGACGGAC
CAACCTCGGACGGACCTCAGCGAA...

3. Simplify assembly graph

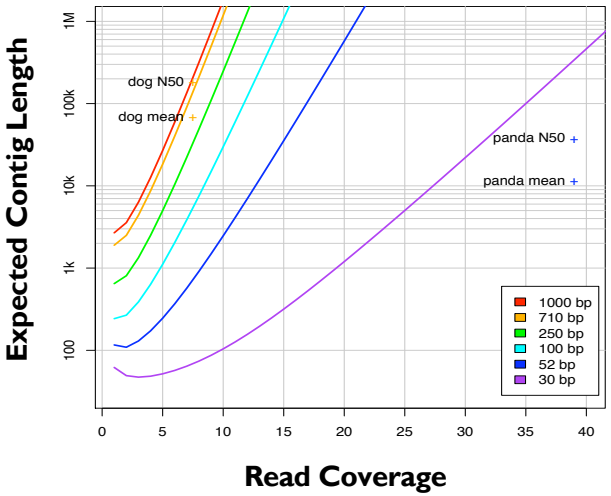


4. Detangle graph with long reads, mates, and other links



Ingredients for a good assembly

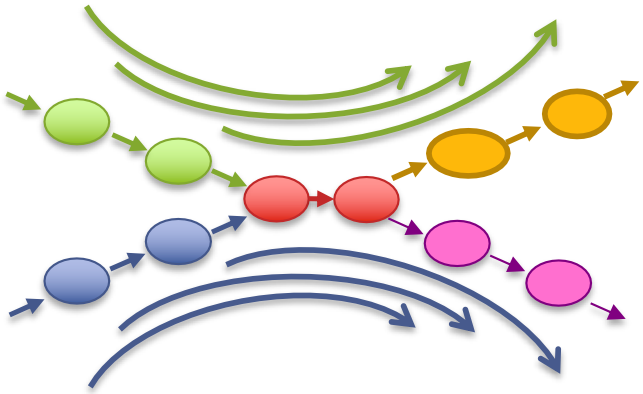
Coverage



High coverage is required

- Oversample the genome to ensure every base is sequenced with long overlaps between reads
- Biased coverage will also fragment assembly

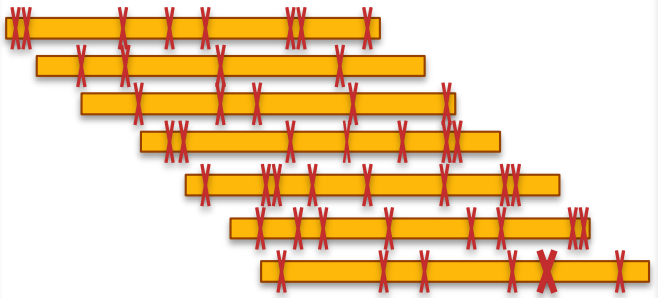
Read Length



Reads & mates must be longer than the repeats

- Short reads will have ***false overlaps*** forming hairball assembly graphs
- With long enough reads, assemble entire chromosomes into contigs

Quality



Errors obscure overlaps

- Reads are assembled by finding kmers shared in pair of reads
- High error rate requires very short seeds, increasing complexity and forming assembly hairballs

Current challenges in *de novo* plant genome sequencing and assembly
Schatz MC, Witkowski, McCombie, WR (2012) *Genome Biology*. 12:243

Coverage Statistics

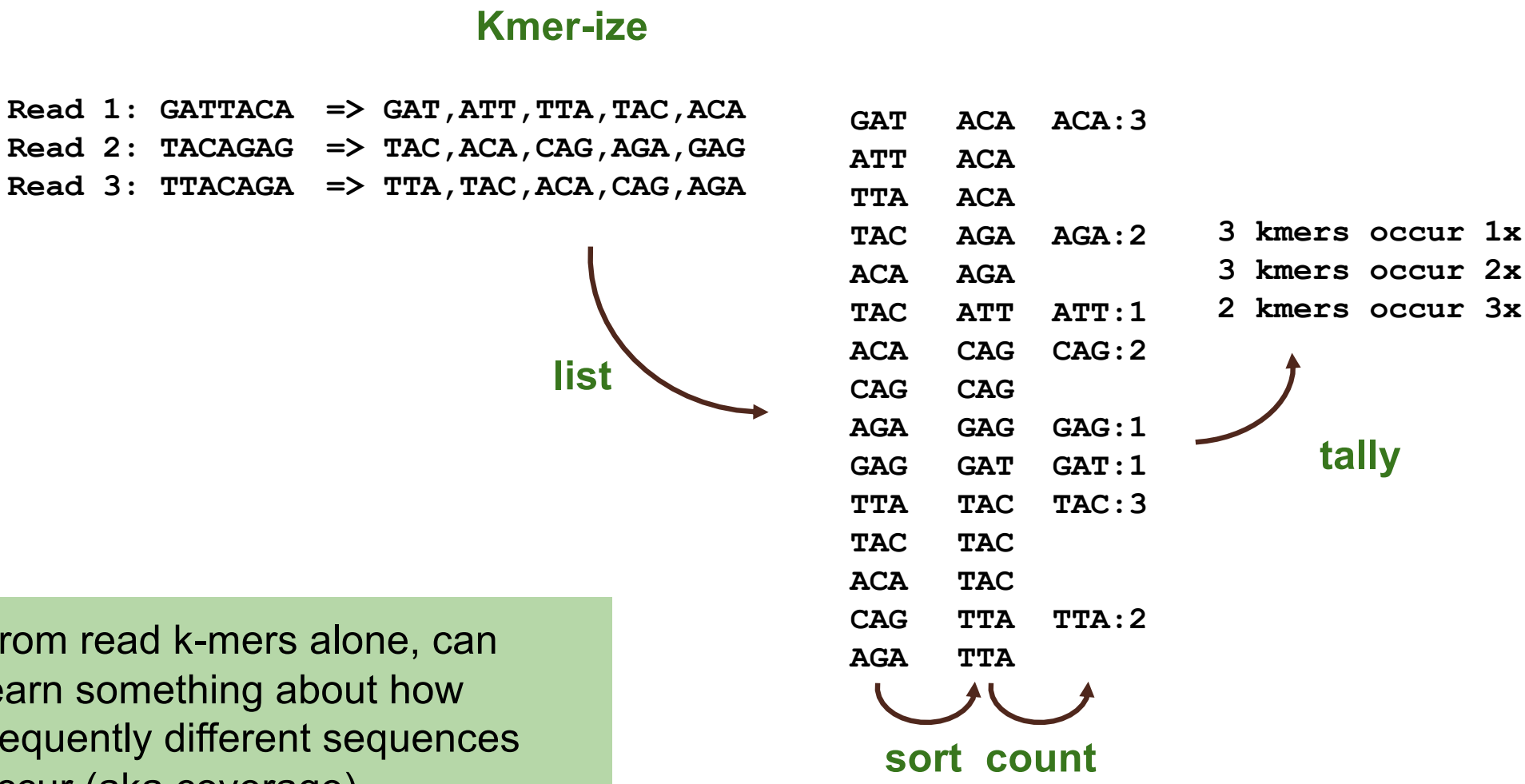
$$\text{sequencing\_coverage} = \frac{\text{total\_bases\_sequenced}}{\text{genome\_size}}$$

$$\text{genome\_size} = \frac{\text{total\_bases\_sequenced}}{\text{sequencing\_coverage}}$$

$$\text{genome\_size} = \frac{100\text{Gb}}{50\text{x}} = 2\text{Gb}$$

But how can you figure out
the coverage without a genome?

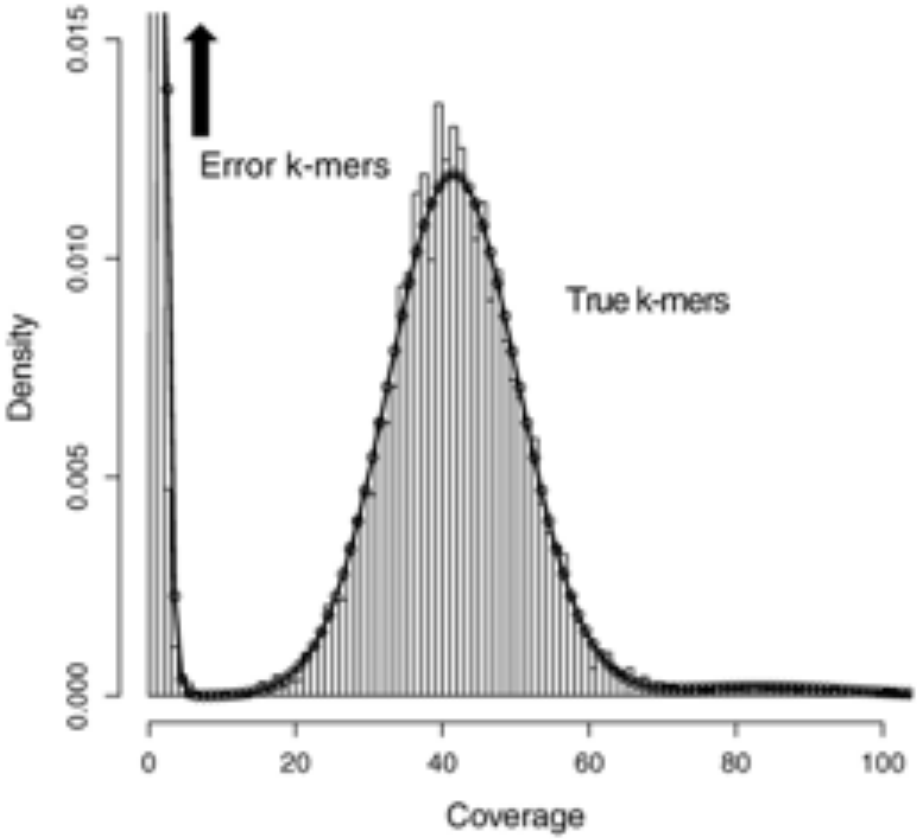
K-mer counting



From read k-mers alone, can learn something about how frequently different sequences occur (aka coverage)

Fast to compute even over huge datasets

K-mer counting in real genomes



- The tally of k-mer counts in real genomes reveals the coverage distribution.
- Here we sequenced 120Gb of reads from a female human (haploid human genome size is 3Gb), and indeed we see a clear peak centered at 40x coverage
- There are also many kmers that only occur <5 times. These are from errors in the reads
- There are also kmers that occur many times (>>70 times). These are repeats in the genome

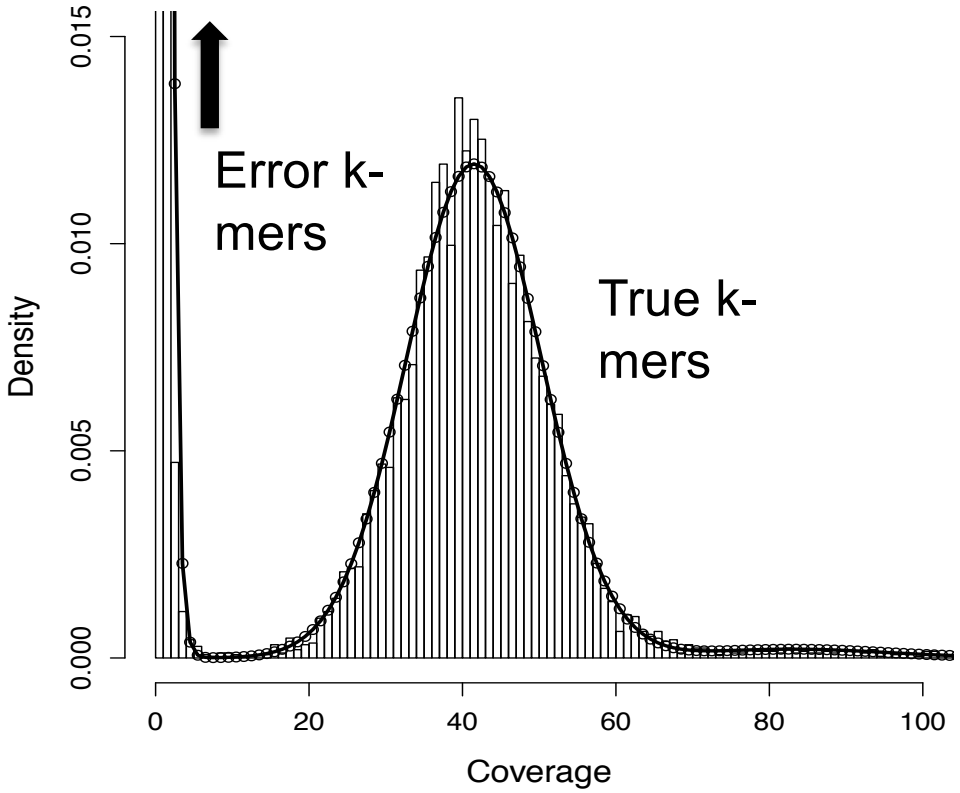
Error Correction with Quake

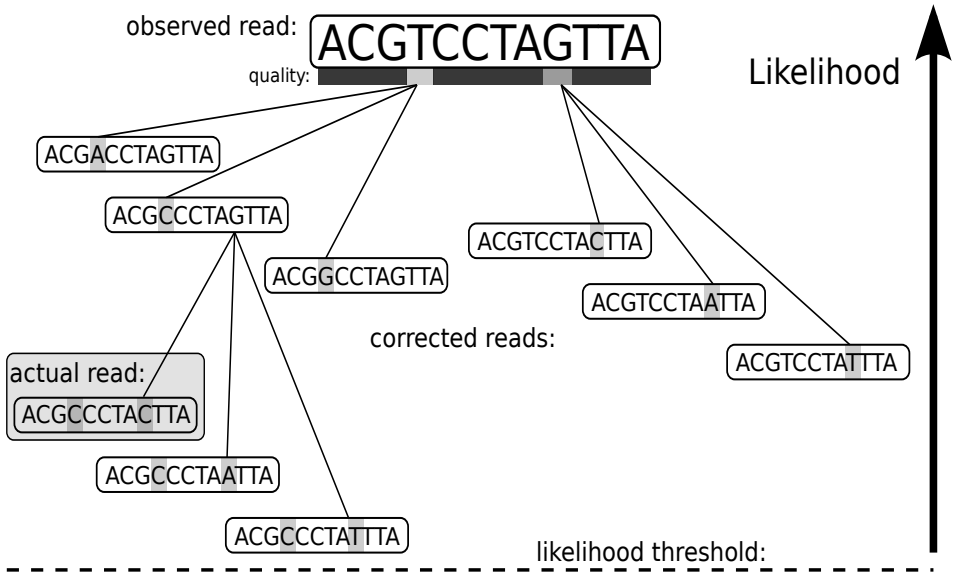
1. Count all "Q-mers" in reads

- Fit coverage distribution to mixture model of errors and regular coverage
- Automatically determines threshold for trusted k-mers

2. Correction Algorithm

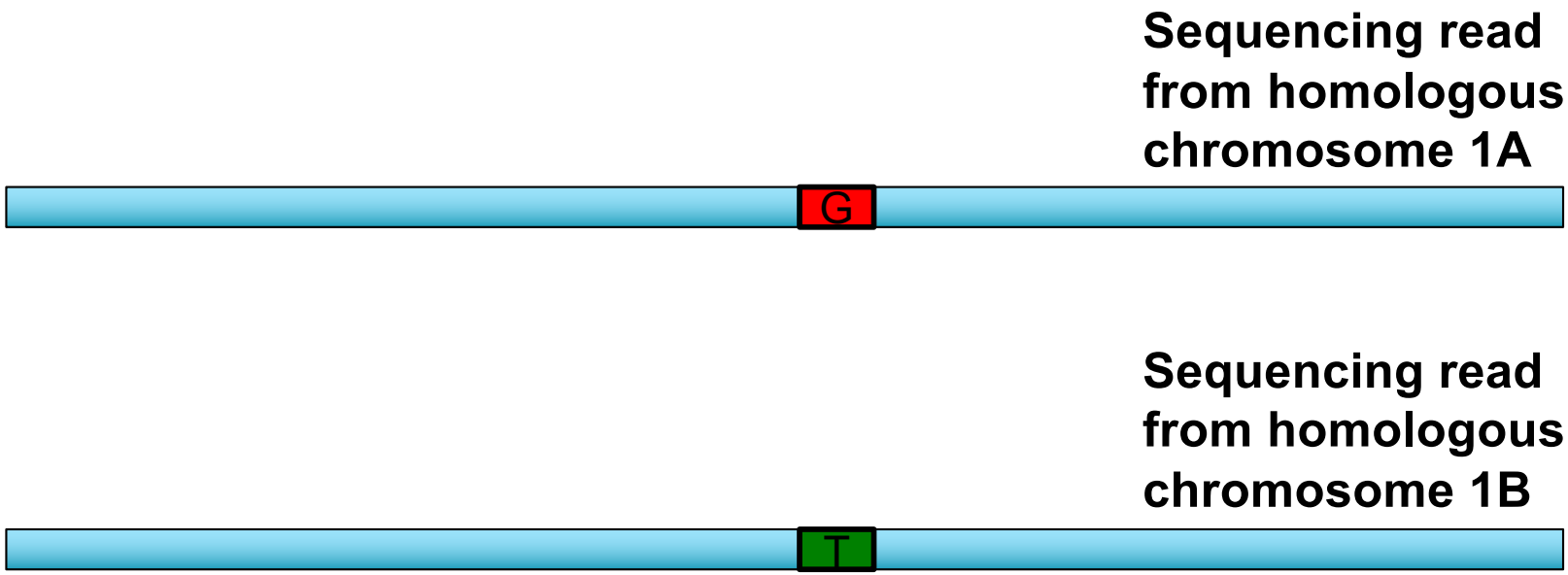
- Considers editing erroneous kmers into trusted kmers in decreasing likelihood
- Includes quality values, nucleotide/nucleotide substitution rate



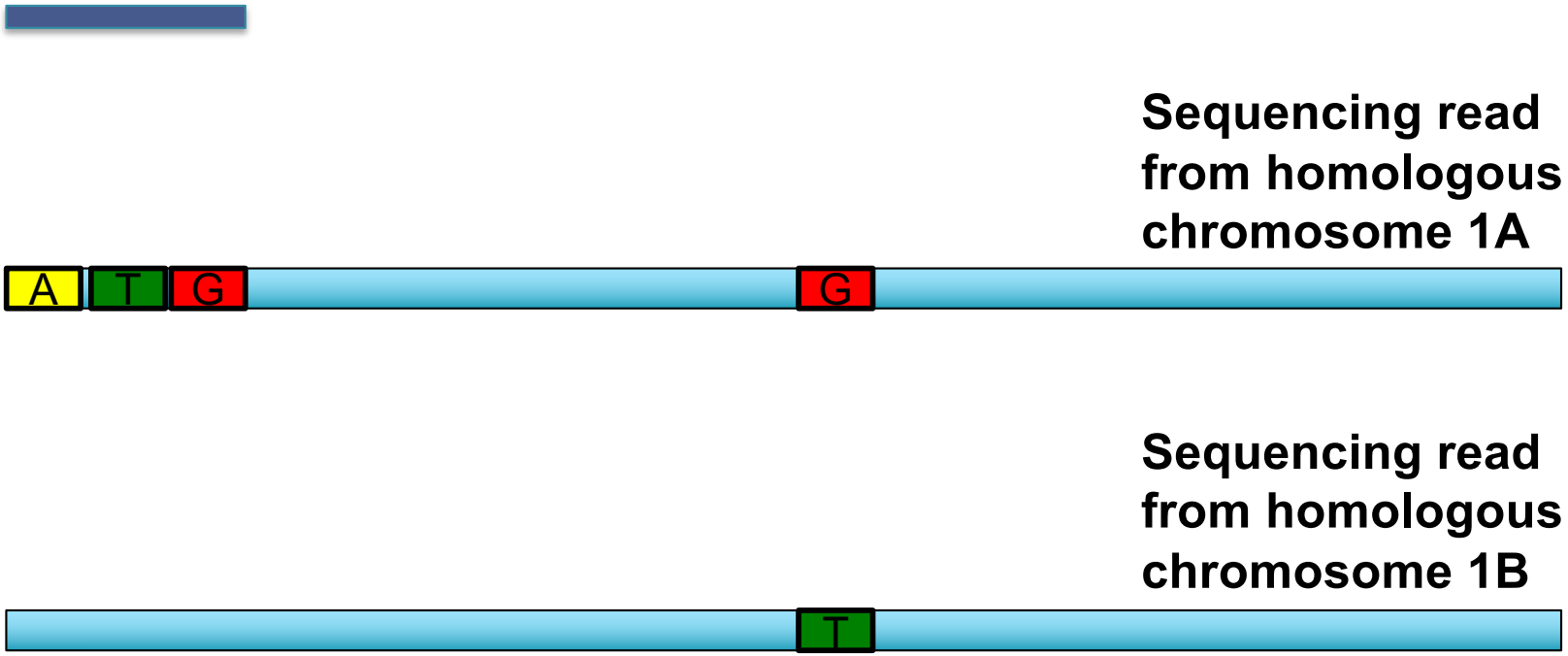


Quake: quality-aware detection and correction of sequencing reads.
Kelley, DR, Schatz, MC, Salzberg SL (2010) *Genome Biology*. 11:R116

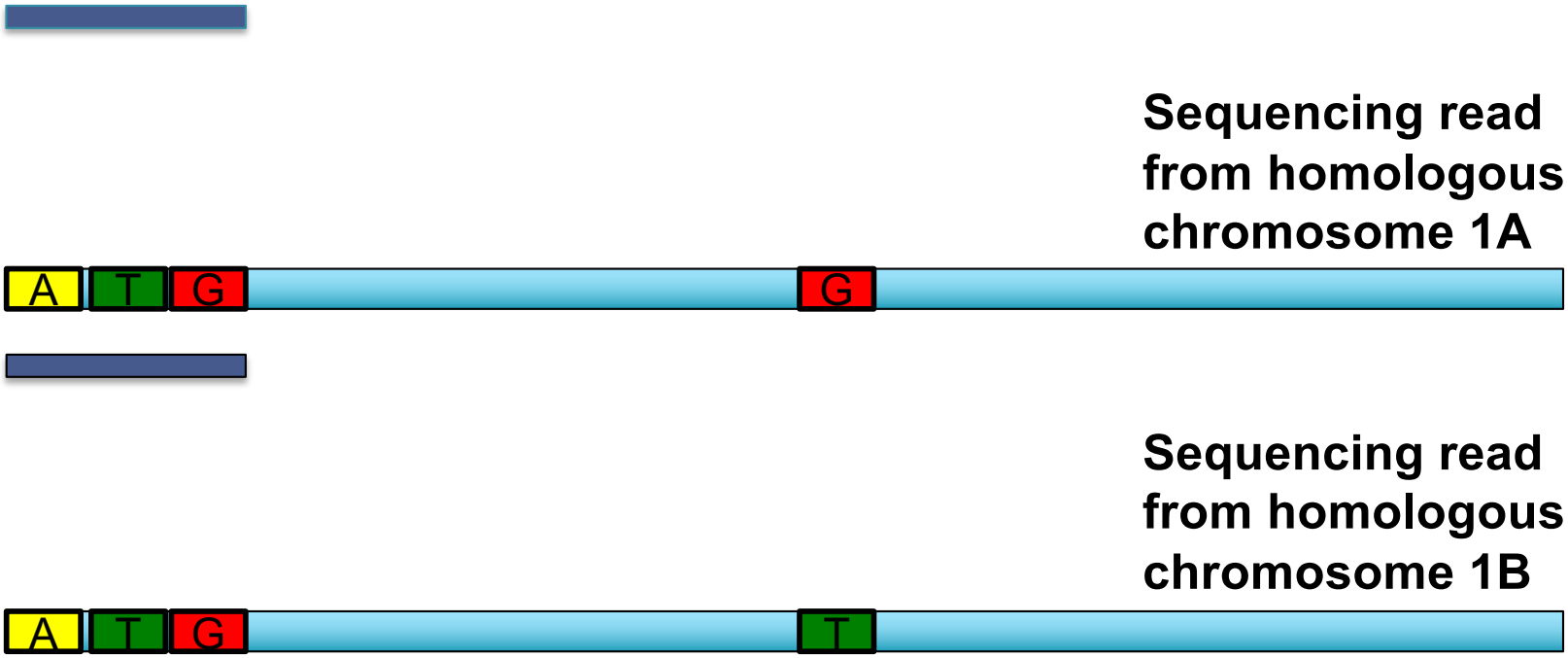
K-mer counting in heterozygous genomes



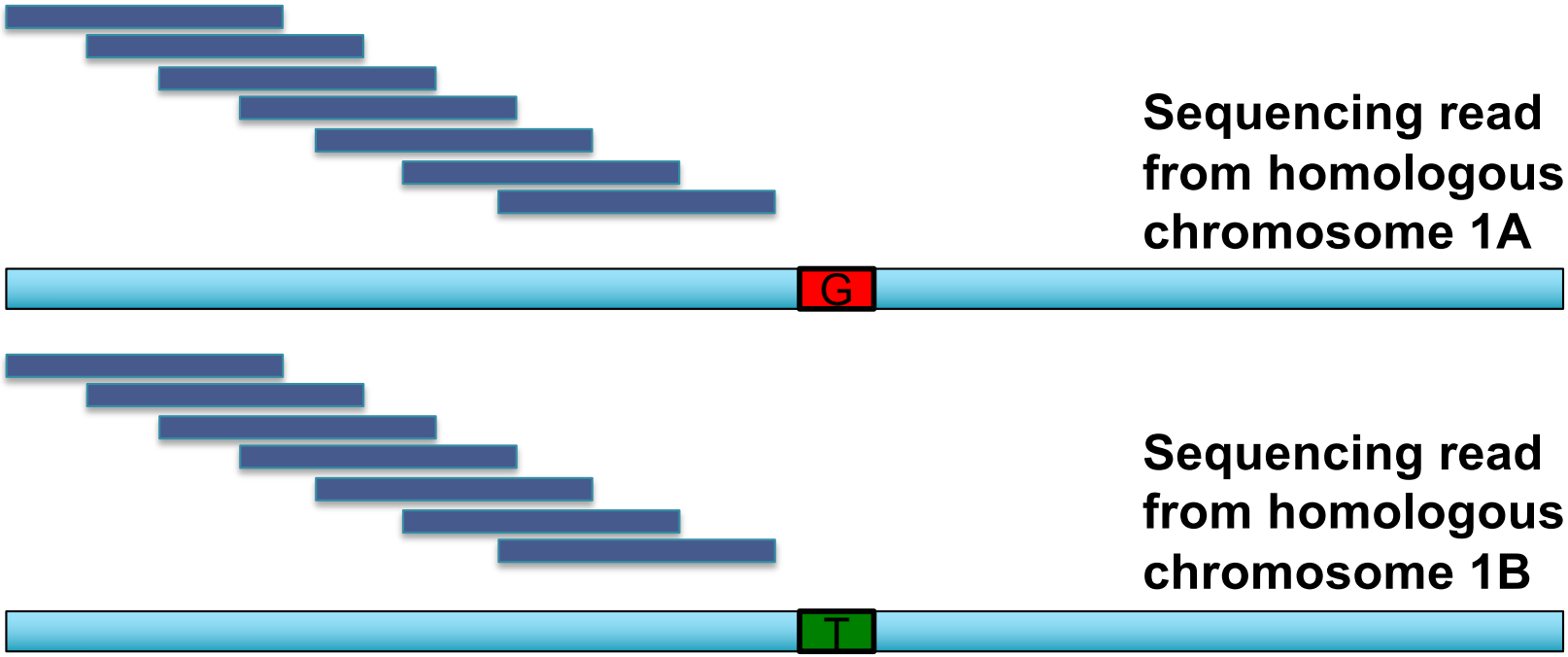
K-mer counting in heterozygous genomes



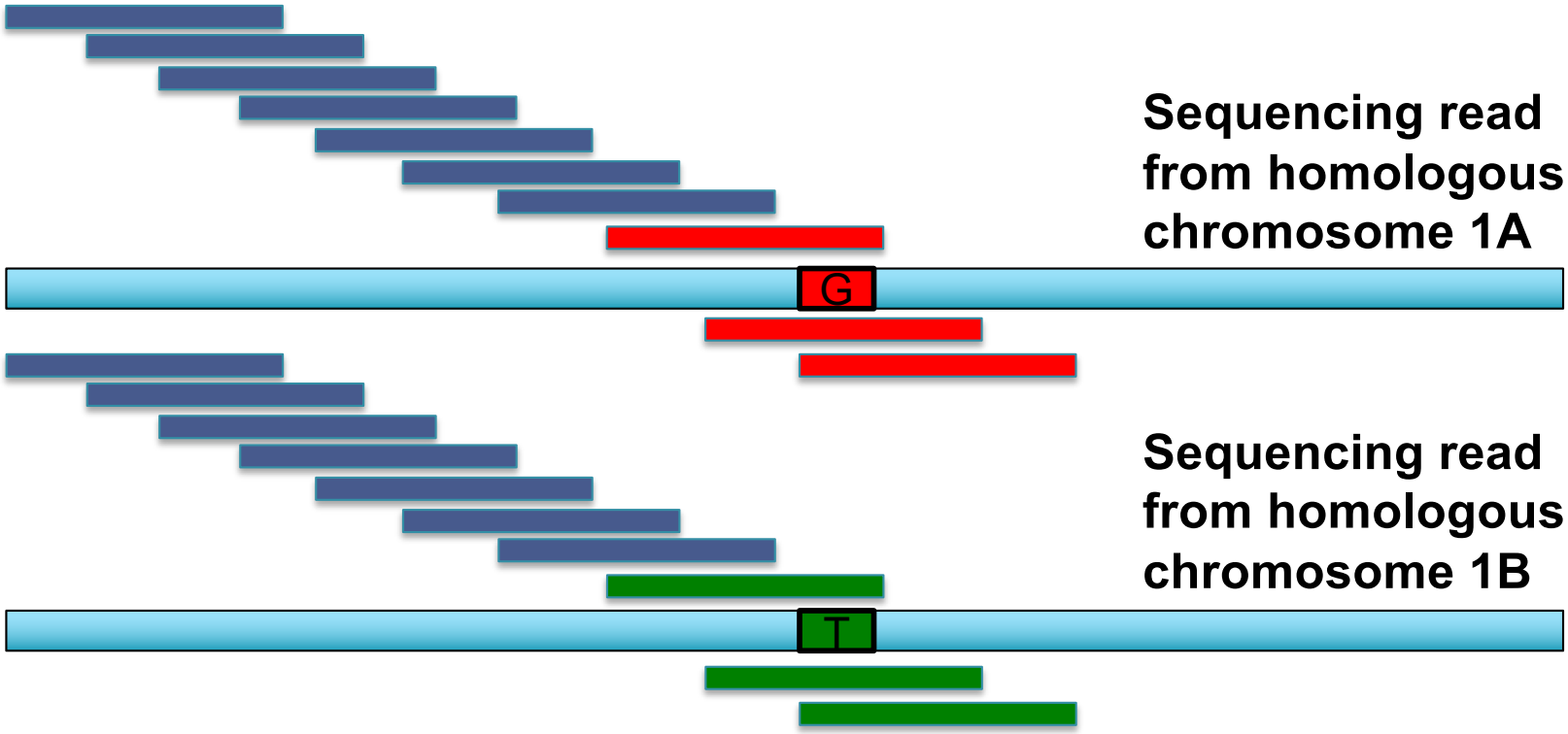
K-mer counting in heterozygous genomes



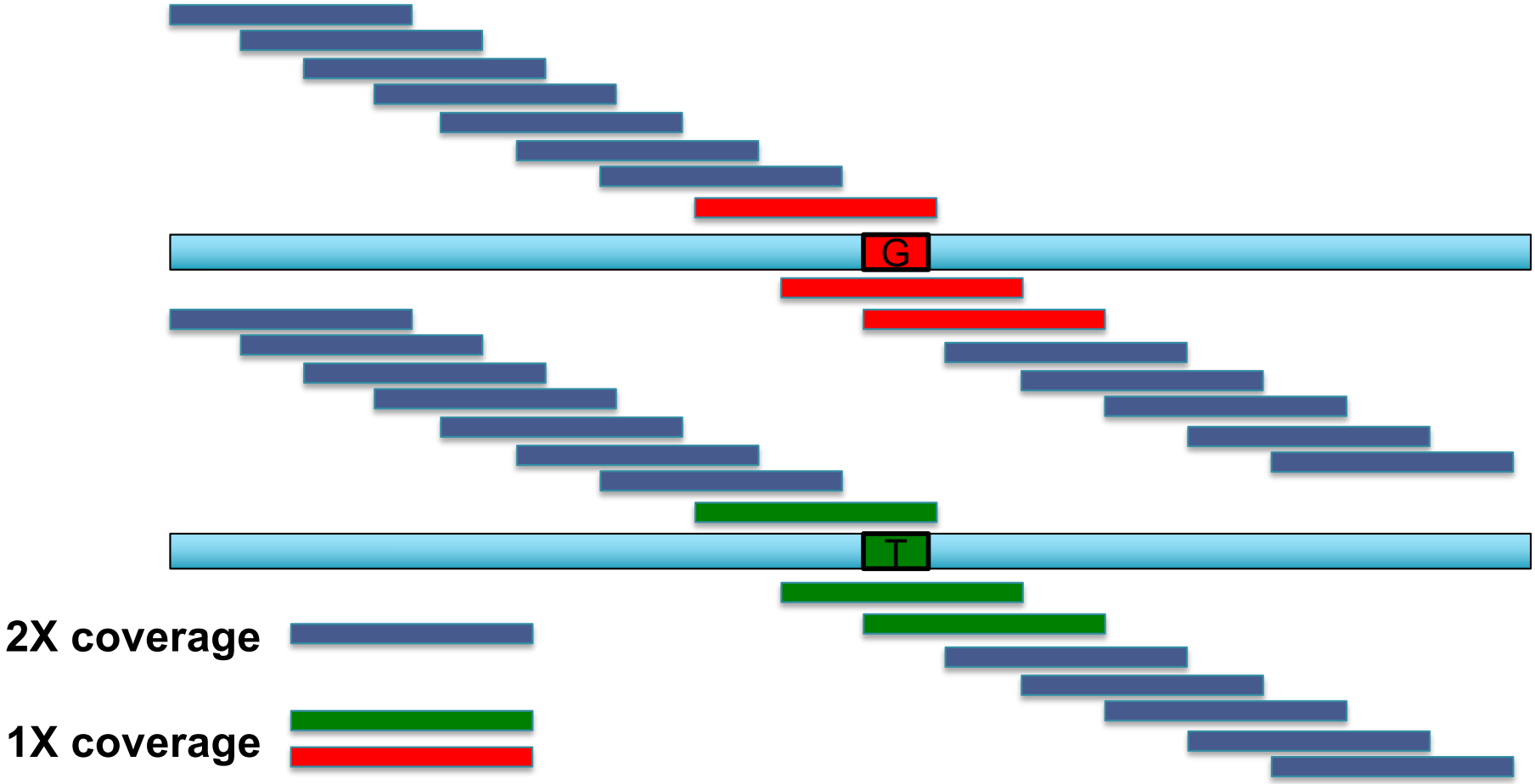
K-mer counting in heterozygous genomes



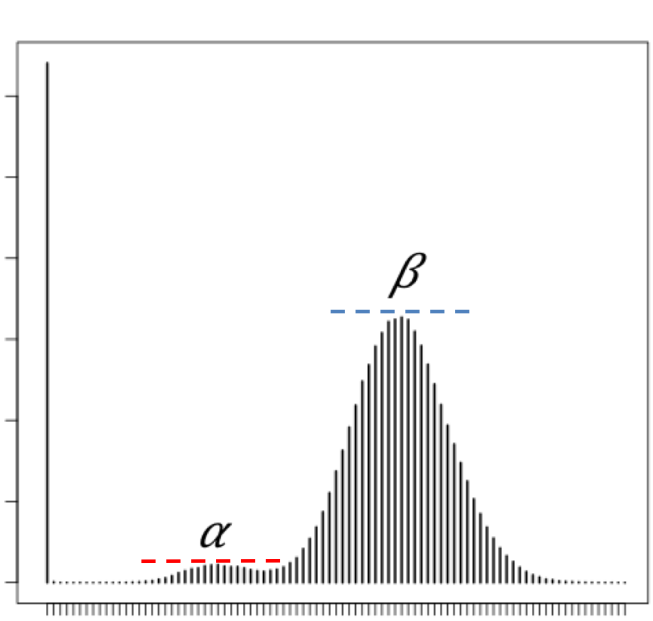
K-mer counting in heterozygous genomes



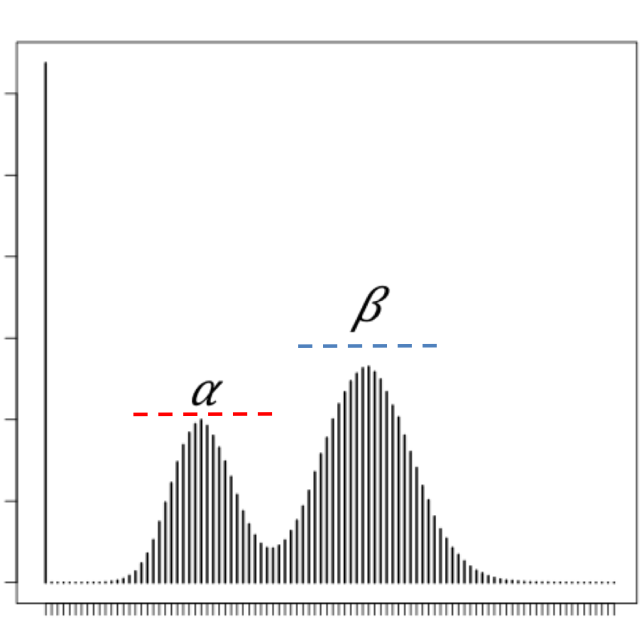
K-mer counting in heterozygous genomes



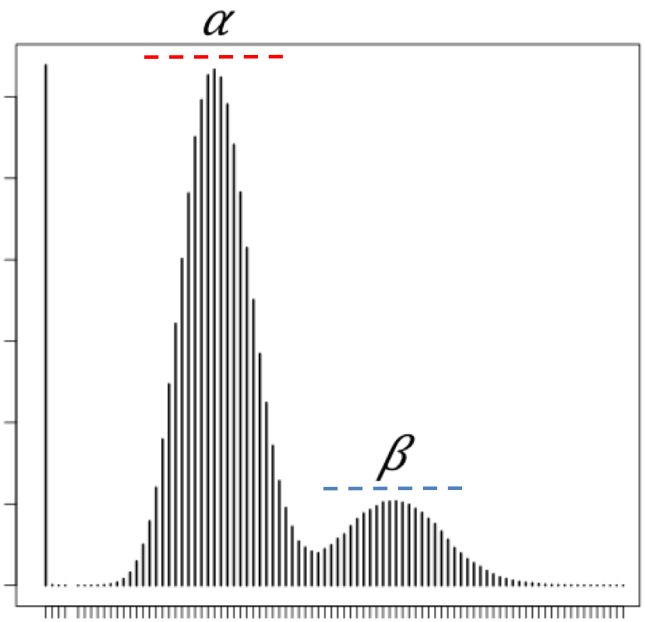
Heterozygous Kmer Profiles



0.1% heterozygosity



1% heterozygosity



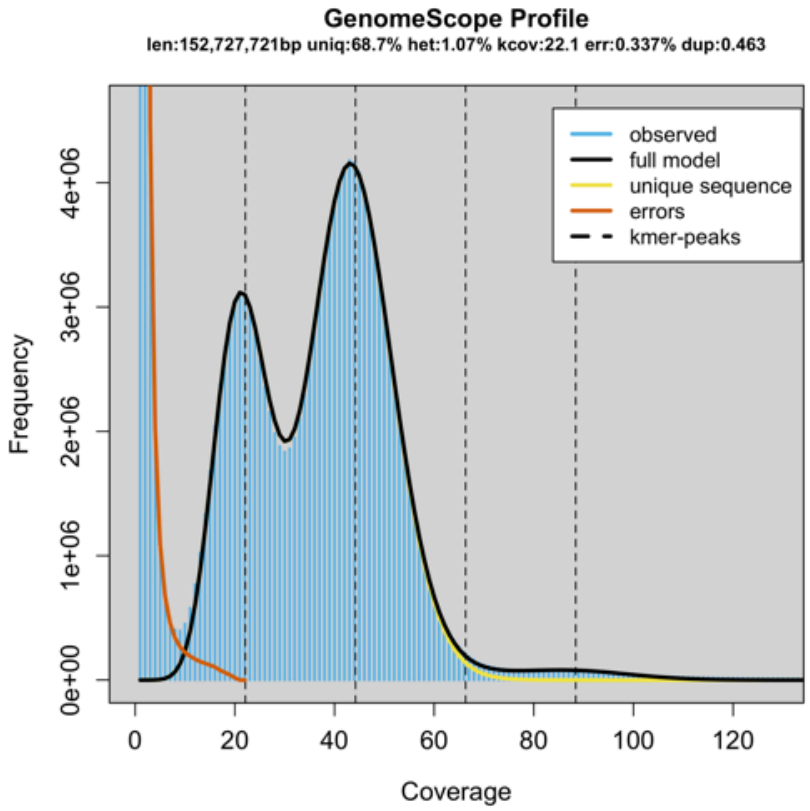
5% heterozygosity

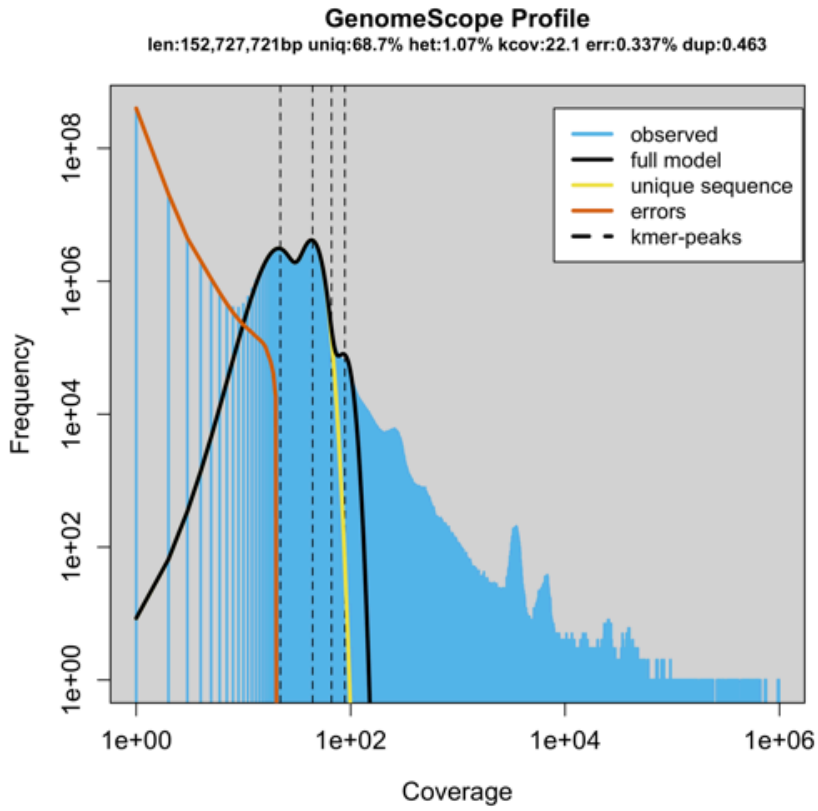
- ***Heterozygosity creates a characteristic "double-peak" in the Kmer profile***
  - Second peak at twice k-mer coverage as the first: heterozygous kmers average 50x coverage, homozygous kmers average 100x coverage

- ***Relative heights of the peaks is directly proportional to the heterozygosity rate***
  - The peaks are balanced at around 1.25% because each heterozygous SNP creates 2*k heterozygous kmers (typically k = 21)

GenomeScope: Fast genome analysis from short reads

http://genomescope.org





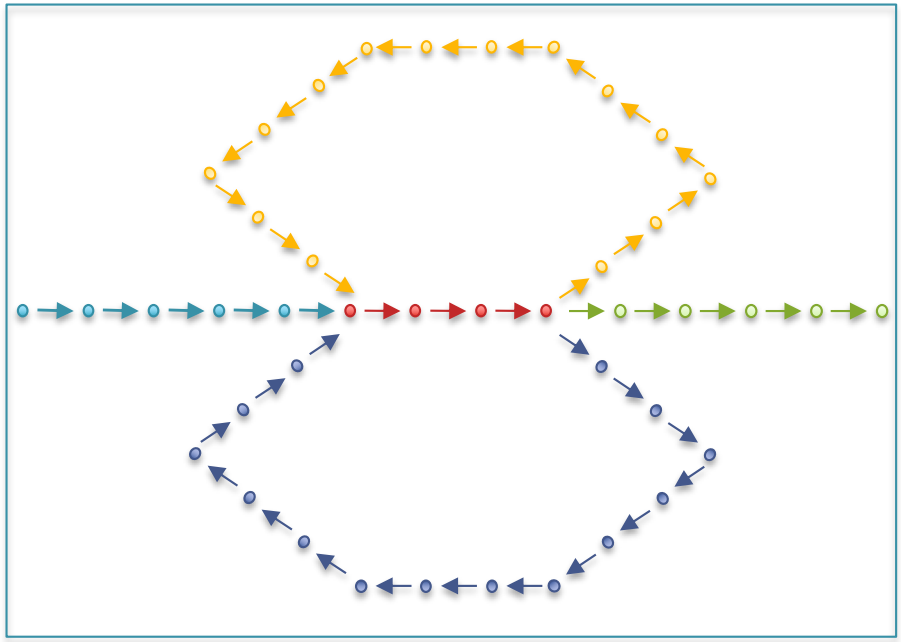
- Theoretical model agrees well with published results:
  - Rate of heterozygosity is higher than reported by other approaches but likely correct.
  - Genome size of plants inflated by organelle sequences (exclude very high freq. kmers)

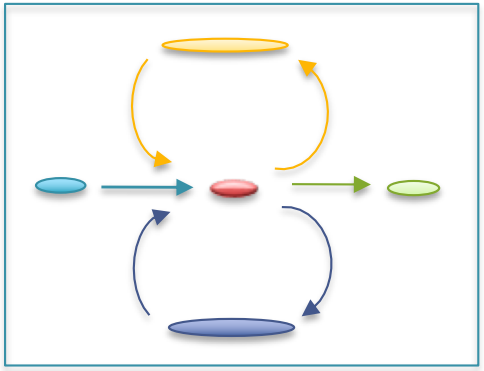
Vurture, GW*, Sedlazeck FJ*, et al. (2017) *Bioinformatics*
Ranallo-Benavidez, TR. *et al.* (2019) *bioRxiv*

Errors

Unitigging / Unipathing

- After simplification and correction, compress graph down to its non-branching initial contigs
  - Aka "unitigs", "unipaths"





Why do contigs end?

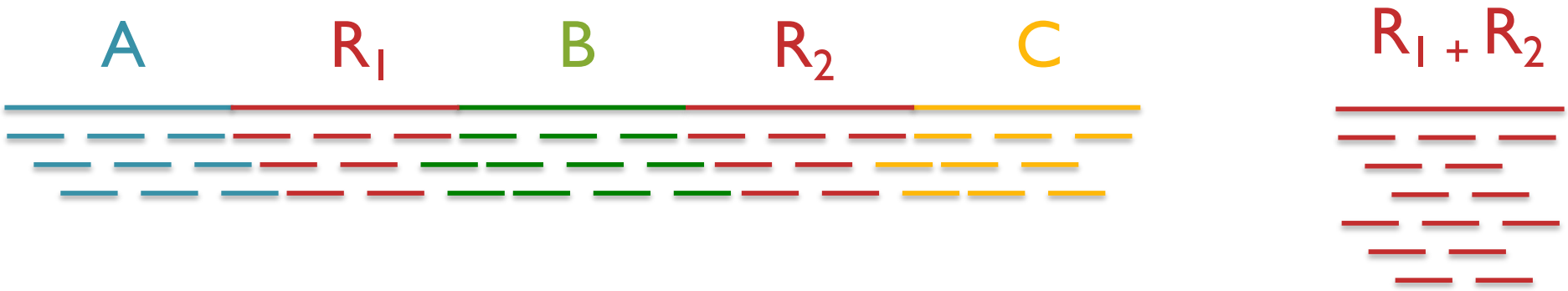
(1) End of chromosome! ☺, (2) lack of coverage, (3) errors,
(4) heterozygosity and (5) repeats

Repetitive regions

Repeat Type	Definition / Example	Prevalence
Low-complexity DNA / Microsatellites	$(b_1b_2\ldots b_k)^N$ where $1 \leq k \leq 6$ CACACACACACACACACACA	2%
SINEs (Short Interspersed Nuclear Elements)	*Alu* sequence (~280 bp) Mariner elements (~80 bp)	13%
LINEs (Long Interspersed Nuclear Elements)	~500 – 5,000 bp	21%
LTR (long terminal repeat) retrotransposons	Ty1-copia, Ty3-gypsy, Pao-BEL (~100 – 5,000 bp)	8%
Other DNA transposons		3%
Gene families & segmental duplications		4%

- Over 50% of mammalian genomes are repetitive
  - Large plant genomes tend to be even worse
  - Wheat: 16 Gbp; Pine: 24 Gbp

Repeats and Coverage Statistics



- If *n* reads are a uniform random sample of the genome of length *G*, we expect $k=n\Delta/G$ reads to start in a region of length Δ.
  - If we see many more reads than k (if the arrival rate is > A) , it is likely to be a collapsed repeat

$$\Pr(X-copy)=\binom{n}{k}\left(\frac{X\Delta}{G}\right)^{k}\left(\frac{G-X\Delta}{G}\right)^{n-k}$$

$$A(\Delta,k)=\ln\left(\frac{\Pr(1-copy)}{\Pr(2-copy)}\right)=\ln\left(\frac{\frac{(\Delta n/G)^{k}}{k!}e^{\frac{-\Delta n}{G}}}{\frac{(2\Delta n/G)^{k}}{k!}e^{\frac{-2\Delta n}{G}}}\right)=\frac{n\Delta}{G}-k\ln 2$$

The fragment assembly string graph

Myers, EW (2005) Bioinformatics. 21(suppl 2): ii79-85.

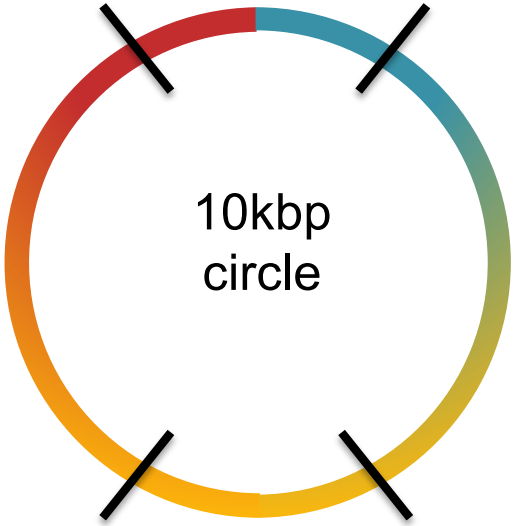
Paired-end and Mate-pairs

Paired-end sequencing

- Read one end of the molecule, flip, and read the other end
- Generate pair of reads separated by up to 500bp with inward orientation

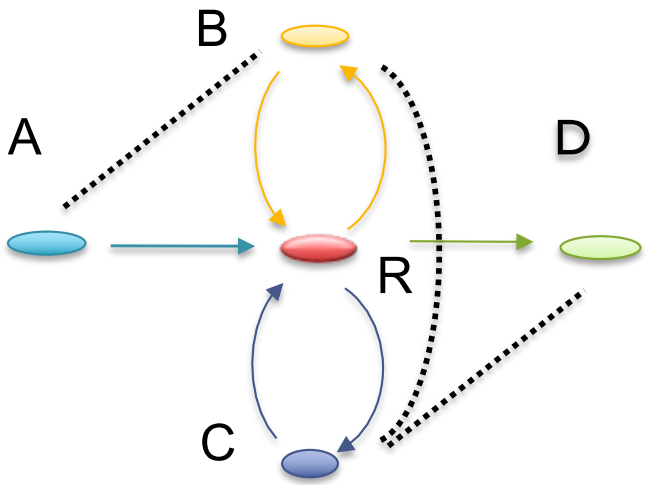
Mate-pair sequencing

- Circularize long molecules (1-10kbp), shear into fragments, & sequence
- Mate failures create short paired-end reads

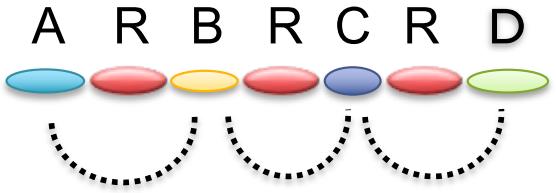


Scaffolding

- Initial contigs (*aka* unipaths, unitigs) terminate at
  - *Coverage gaps*: especially extreme GC
  - *Conflicts*: errors, repeat boundaries



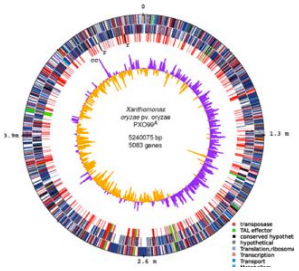
- Use mate-pairs to resolve correct order through assembly graph
  - Place sequence to satisfy the mate constraints
  - Mates through repeat nodes are tangled



- Final scaffold may have internal gaps called sequencing gaps
  - We know the order, orientation, and spacing, but just not the bases. Fill with Ns instead

Why do scaffolds end?

Assembly Summary



Assembly quality depends on

1. ***Coverage***: low coverage is mathematically hopeless
2. ***Repeat composition***: high repeat content is challenging
3. ***Read length***: longer reads help resolve repeats
4. ***Error rate***: errors reduce coverage, obscure true overlaps

- Assembly is a hierarchical, starting from individual reads, build high confidence contigs/unitigs, incorporate the mates to build scaffolds
  - Extensive error correction is the key to getting the best assembly possible from a given data set

- Watch out for collapsed repeats & other misassemblies
  - Globally/Locally reassemble data from scratch with better parameters & stitch the 2 assemblies together