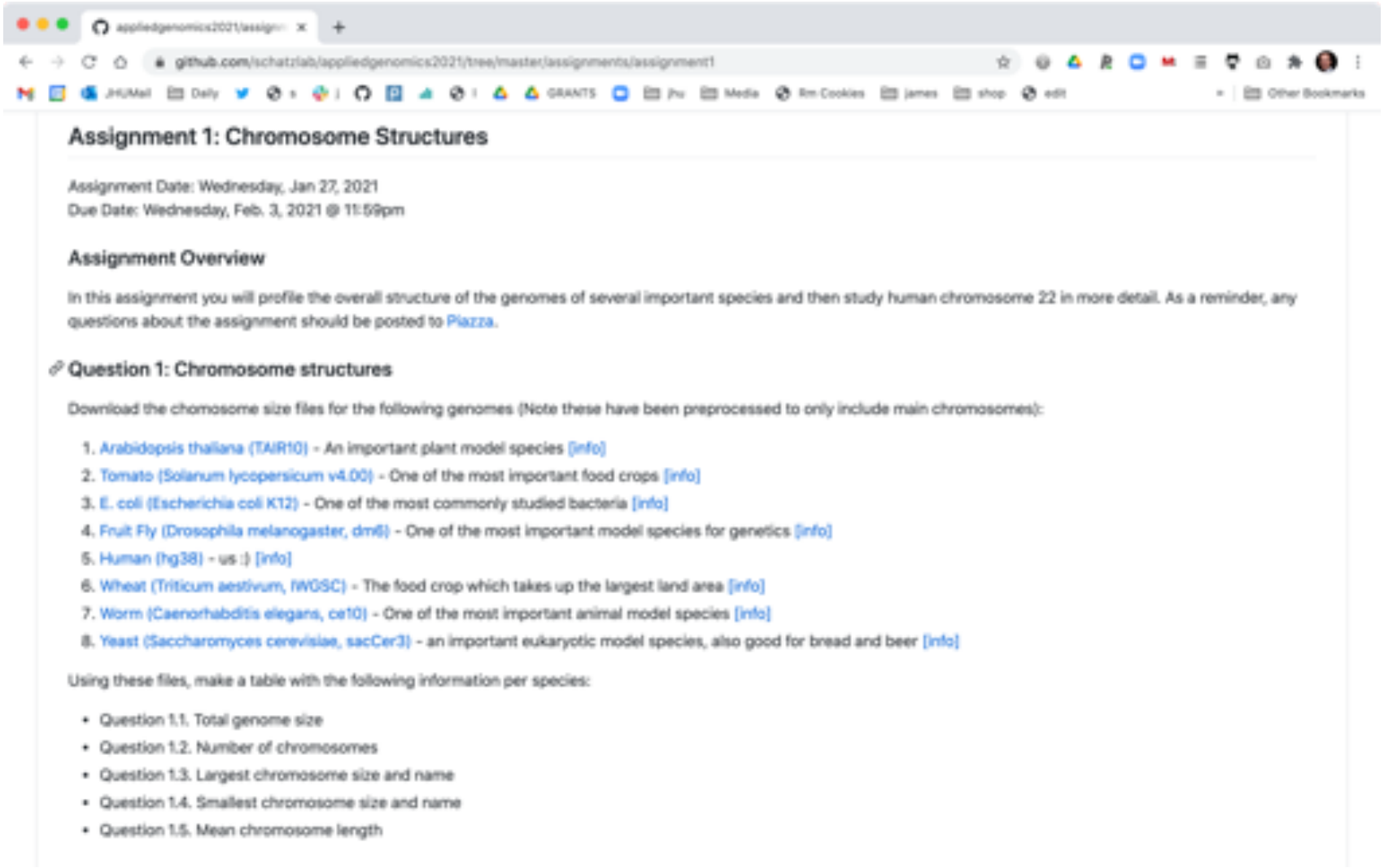
Genome Assembly

Michael Schatz

Feb 1, 2021

Lecture 3: Applied Comparative Genomics

Assignment 1: Chromosome Structures
Due Feb 3 @ 11:59pm



Assignment 1: Chromosome Structures

Assignment Date: Wednesday, Jan 27, 2021
Due Date: Wednesday, Feb. 3, 2021 @ 11:59pm

Assignment Overview

In this assignment you will profile the overall structure of the genomes of several important species and then study human chromosome 22 in more detail. As a reminder, any questions about the assignment should be posted to Piazza.

Question 1: Chromosome structures

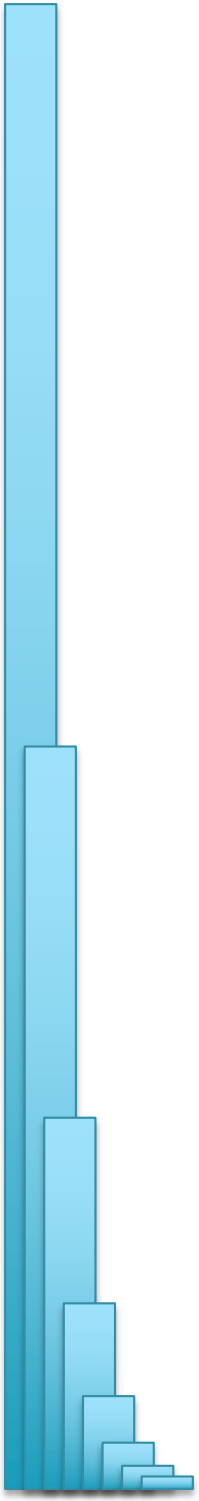
Download the chomosome size files for the following genomes (Note these have been preprocessed to only include main chromosomes):

1. Arabidopsis thaliana (TAIR10) - An important plant model species [info]
2. Tomato (Solanum lycopersicum v4.00) - One of the most important food crops [info]
3. E. coli (Escherichia coli K12) - One of the most commonly studied bacteria [info]
4. Fruit Fly (Drosophila melanogaster, dm6) - One of the most important model species for genetics [info]
5. Human (hg38) - us :) [info]
6. Wheat (Triticum aestivum, IWGSC) - The food crop which takes up the largest land area [info]
7. Worm (Caenorhabditis elegans, ce10) - One of the most important animal model species [info]
8. Yeast (Saccharomyces cerevisiae, sacCer3) - an important eukaryotic model species, also good for bread and beer [info]

Using these files, make a table with the following information per species:

- Question 1.1. Total genome size
- Question 1.2. Number of chromosomes
- Question 1.3. Largest chromosome size and name
- Question 1.4. Smallest chromosome size and name
- Question 1.5. Mean chromosome length

https://github.com/schatzlab/appliedgenomics2021

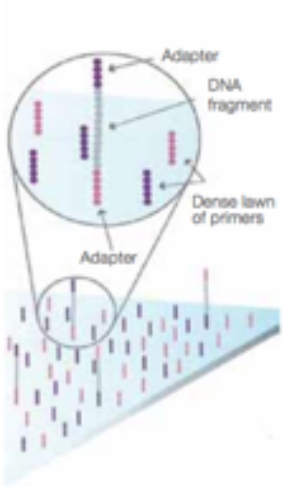


Part 1: Recap

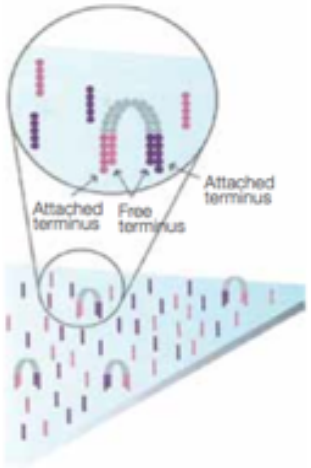
Second Generation Sequencing

Illumina HiSeq 2000
Sequencing by Synthesis

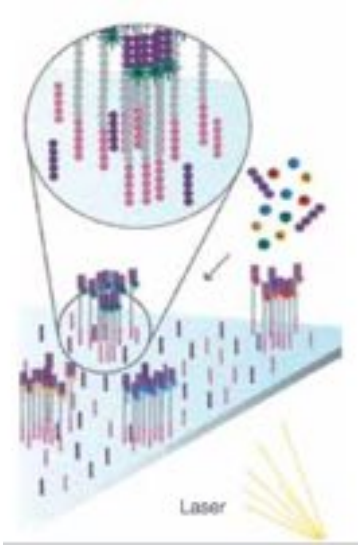
>60Gbp / day



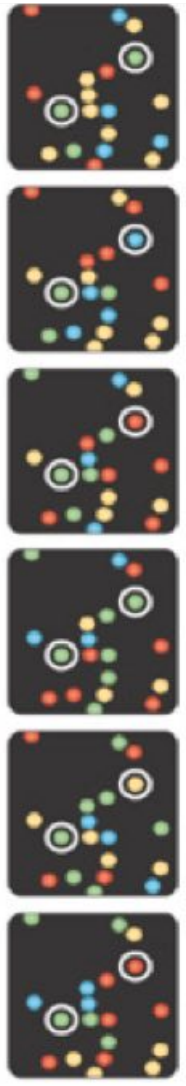
1. Attach



2. Amplify



3. Image

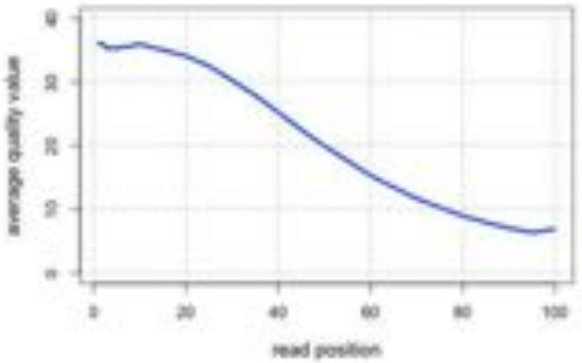


Metzker (2010) Nature Reviews Genetics 11:31-46
https://www.youtube.com/watch?v=fCd6B5HRaZ8

Illumina Quality

QV	p_{error}
40	1/10000
30	1/1000
20	1/100
10	1/10

$$Q_{\text{sanger}} = -10 \log_{10} p$$

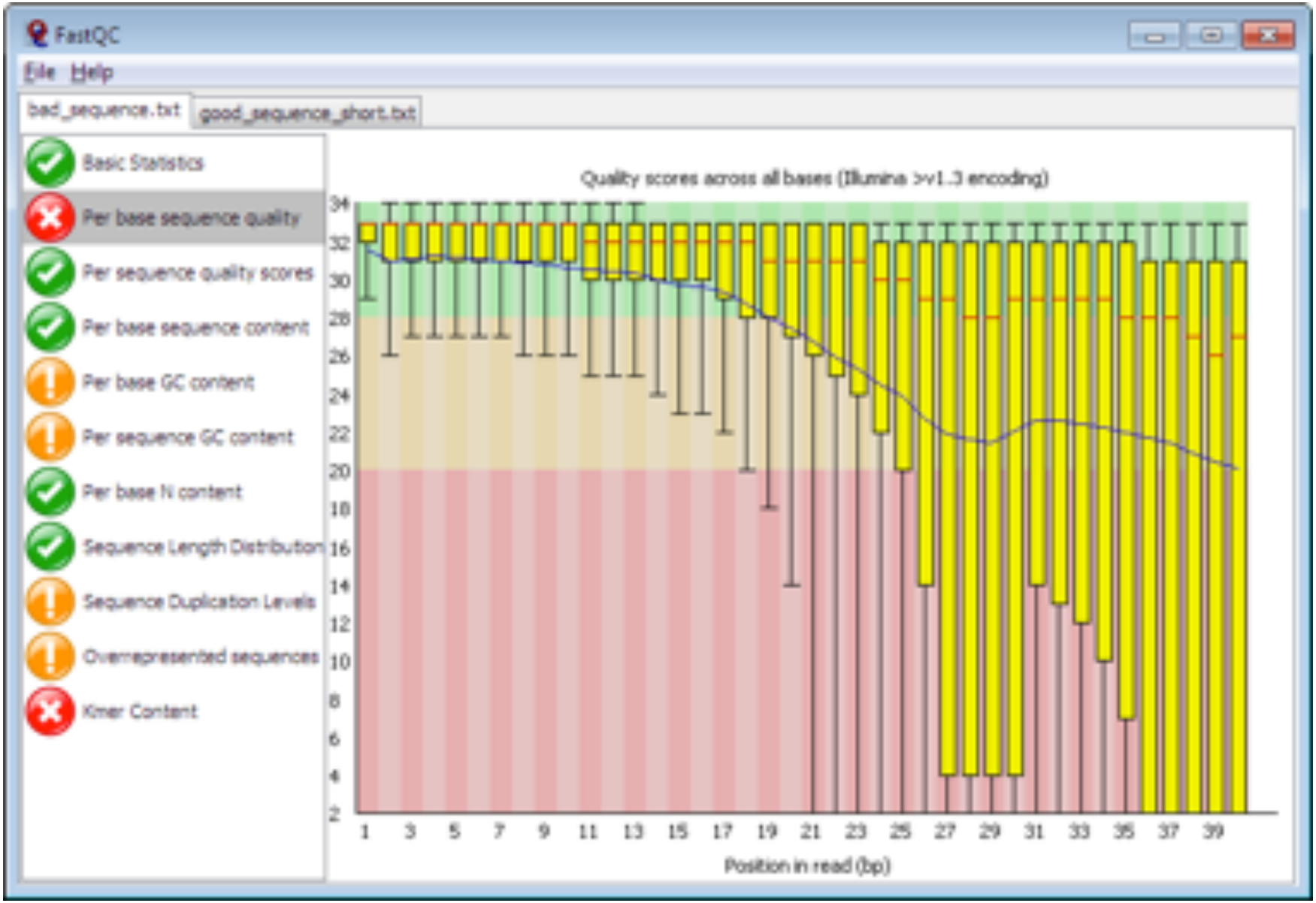



```
  SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
  ........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.....................
  ....................................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII.....................
  ....................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ.....................
  LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL.....................................................
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                         |    |        |                              |                     |
 33                        59   64       73                            104                   126

 S - Sanger        Phred+33,  raw reads typically (0, 40)
 X - Solexa        Solexa+64, raw reads typically (-5, 40)
 I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
 J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
     with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
     (Note: See discussion above).
 L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

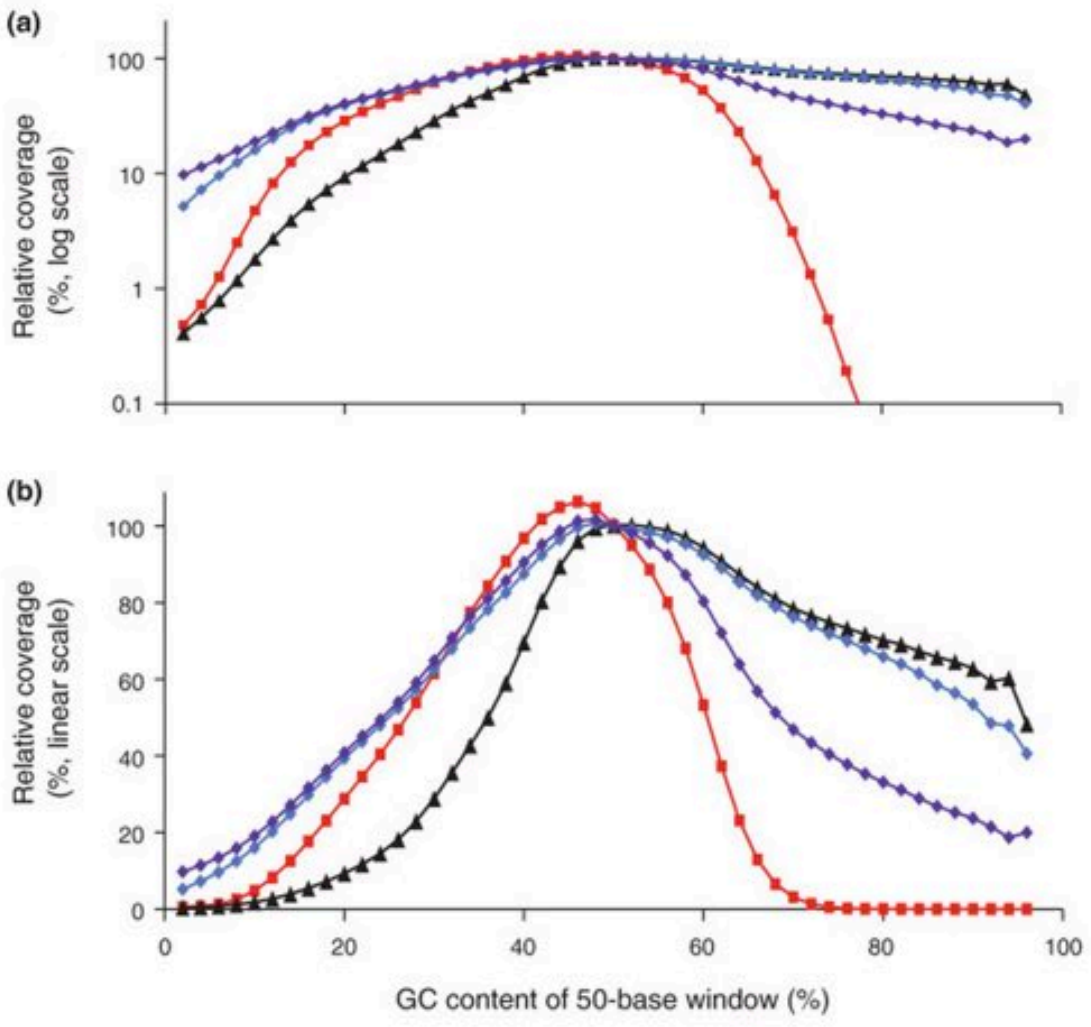
http://en.wikipedia.org/wiki/FASTQ_format

FASTQC: Is my data any good?



http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Beware of GC Biases



Illumina sequencing does not produce uniform coverage over the genome

- Coverage of extremely high or extremely low GC content will have reduced coverage in Illumina sequencing
- Biases primarily introduced during PCR; lower temperatures, slower heating, and fewer rounds minimize biases
- This makes it very difficult to identify variants (SNPs, CNVs, etc) in certain regions of the genome

Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries.
Aird et al. (2011) *Genome Biology.* 12:R18.

Question?

We would love to generate
longer and longer reads with this technology

What can we do?

Illumina Hacking

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Genome analysis

Advance Access publication May 22, 2013

Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data

Inanc Birol[1,2,3,*], Anthony Raymond[1], Shaun D. Jackman[1], Stephen Pleasance[1], Robin Coope[1], Greg A. Taylor[1], Macaire Man Saint Yuen[4], Christopher I. Keeling[4], Dana Brand[1], Benjamin P. Vandervalk[1], Heather Kirk[1], Pawan Pandoh[1], Richard A. Moore[1], Yongjun Zhao[1], Andrew J. Mungall[1], Barry Jaquish[5], Alvin Yanchuk[6], Carol Ritland[4,6], Brian Boyle[7], Jean Bousquet[7,8], Kermit Ritland[6], John MacKay[7,8], Jörg Bohlmann[4,6], Steven J.M. Jones[1,2,9]

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Associate Editor: Michael Brudno

ABSTRACT

White spruce (*Picea glauca*) is a dominant conifer of the boreal forests of North America, and providing genomics resources for this commercially valuable tree will help improve forest management and conservation efforts. Sequencing and assembling the large and highly repetitive spruce genome though pushes the boundaries of the current technology. Here, we describe a whole-genome shotgun sequencing strategy using two Illumina sequencing platforms and an assembly approach using the ABySS software. We report a 20.8 giga base pairs draft genome in 4.9 million scaffolds, with a scaffold N50 of 20 356 bp. We demonstrate how recent improvements in the sequencing technology, especially increasing read lengths and paired end reads from longer fragments have a major impact on the assembly contiguity. We also note that scalable bioinformatics tools are instrumental in providing rapid draft assemblies.

Availability: The *Picea glauca* genome sequencing and assembly data are available through NCBI (Accession#: ALWZ01000000000 PID: PRJNA83435). http://www.ncbi.nlm.nih.gov/bioproject/83435.

Contact: ibirol@bcgsc.ca

Supplementary information: Supplementary data are available at *Bioinformatics* online.

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1 INTRODUCTION

The assembly of short reads to develop genomic resources for non-model species remains an active area of development (Schatz *et al.*, 2012). The feasibility of the approach and its scalability to large genomes was demonstrated b (Simpson *et al.*, 2009) using human and was later used to assemble the SOAPdenovo tool (Li *et al.*, 2010). high quality results, as demonstrate *et al.*, 2012; Ladner *et al.*, 2013; Rib been successfully applied numerous ti omes (Chan *et al.*, 2011; Chu *et al.*, 2 2011; Godel *et al.*, 2012; Swart *et al.*,

Estimated at 20 giga base pairs (Gi cing and assembly of the genome of t the pine (*Pinaceae*) family present uni generation end, those challenges inclu whole-genome shotgun sequencing da ing reduced representation resources tude of the problem. On the bioin massive sequencing datasets is extrem ing cycles, memory usage, storage req programming implementations on co

We addressed the data representati and sequencing multiple whole-genom HiSeq 2000 and MiSeq sequencers (CA, USA). Compared with localized as building and sequencing fosmi approach of isolating ~10 kb DNA s sequencing fragments in high through CA, USA), a shotgun only sequencing sequence data effectively covering th that can be an order of magnitude le especially substantial when sequencin

In this work, we demonstrate that at this scale remains viable and pro

*To whom correspondence should be addressed.

assemble the spruce genome, we used the ABySS algorithm (Simpson *et al.*, 2009), which captures a representation of read-to-read overlaps by a distributed de Bruijn graph and uses parallel computations to build the target genome. The modular nature of the tool allowed us to execute a large number of tests to tune the message passing interface for a successful execution, train the assembly parameters for an optimal assembly and quantify the utility of long reads for large genome assemblies. To the best of our knowledge, the ABySS algorithm is unique in its ability to enable genome assemblies of this scale using whole-genome shotgun sequencing data.

2 METHODS

2.1 Sample collection

Apical shoot tissues were collected in April 2006 from a single white spruce (*Picea glauca*, genotype PG29) tree at the Kalamalka Research Station of the British Columbia Ministry of Forests and Ranges, Vernon, British Columbia, Canada. Genomic DNA was extracted from 60 gm tissue by BioS&T (http://www.biost.com), Montreal, QC, Canada) using an organelle exclusion method yielding 300 µg of high quality purified nuclear DNA.

2.2 Library preparation and sequencing

DNA quality was assessed by spectrophotometry and gel electrophoresis before library construction. DNA was sheared for 45 s using an E210 sonicator (Covaris) and then analysed on 8% PAGE gels. The 200–300 bp (for libraries with 250 bp insert size) or 450–550 bp (for libraries with 500 bp insert size) DNA size fractions were excised and eluted from the gel slices overnight at 4°C in 300 µl of elution buffer [5:1 (vol/vol) LoTE buffer [3 mM Tris–HCl (pH 7.5), 0.2 mM EDTA]/7.5 M ammonium acetate] and was purified using a Spin-X Filter Tube (Fisher Scientific) and ethanol precipitation. Genome libraries were prepared using a modified paired-end tag (PET) protocol supplied by Illumina Inc. This involved DNA end repair and formation of 3′ adenosine overhangs using the Klenow fragment of DNA polymerase I (3′–5′ exonuclease minus) and ligation to Illumina PE adapters (with 5′ overhangs). Adapter-ligated products were purified on QiAquick spin columns (Qiagen) and amplified using Phusion DNA polymerase (NEB) and 10 PCR cycles with the PE primer 1.0 and 2.0 (Illumina). PCR products of the desired size range were purified from adapter ligation artifacts using 8% PAGE gels. DNA quality was assessed and quantified using an Agilent DNA 1000 series II assay (Agilent) and Nanodrop 7500 spectrophotometer (Nanodrop). DNA was subsequently diluted to 8 nM. The final concentration was confirmed using a Quant-iT dsDNA HS assay kit and Qubit fluorometer (Invitrogen).

The mate pair (MPET, a.k.a. jumping) libraries were constructed using 4 µg of genomic DNA with the Illumina Nextera Mate-Pair library construction protocol and reagent (FC-132-1001). The genomic DNA sample was simultaneously fragmented and tagged with a biotin containing mate pair junction adapter, which left a short sequence gap in the tagmented DNA. The gap was filled by a strand displacement reaction using a polymerase to ensure that all fragments were flush and ready for circularization. After an AMPure Bead cleanup, size selection was done on a 0.6% agarose gel to excise 6–9 kb and 9–13 kb fractions, which were purified using a Zymoclean Large Fragment DNA Recovery Kit. The fragments were circularized by ligation, followed by a digestion to remove any linear molecules and left circularized DNA for shearing. The sheared DNA fragments that contain the biotinylated junction adapter (mate pair fragments) were purified by means of binding to streptavidin magnetic beads, and the unwanted unbiotinylated molecules were washed away. The DNA fragments were then end repaired and A-tailed following the protocol and ligated to indexed TruSeq adapters. The final library was enriched by a 10-cycle PCR and purified by AMPure bead clean-up. Library quality and size were assessed by Agilent DNA 1000 series II assay and KAPA Library Quantification protocol. The two fractions were pooled for sequencing paired end 100 bp using Illumina HiSeq2000.

The construction of the 12 kb mate pair libraries was achieved by a hybrid 454/Illumina procedure. Briefly, 50 µg of genomic DNA was fragmented for 20 cycles at speed code 12 using a Hydroshear (Digilab, Marlborough, MA) equipped with a large assembly module. The fragmented DNA was loaded on a 1% agarose gel, and fragments of 9–18 kb were extracted. Biotinylated circularization adapters from the Titanium Paired-end Adaptor set (454 Life Sciences/Roche, Branford, CT) were added to ends of the gel-extracted fragments. After recombination of the ends was performed with Cre recombinase (New England Biolabs, Ipswich, MA), and linear molecules remaining after digestion were removed with Plasmid Safe (Epicentre, Madison, WI), the molecules were fragmented using GS Rapid Library Nebulization (454 Life Sciences/Roche, Branford, CT), and fragment end-repair followed by A-tailing was performed with the GS Rapid Library preparation kit (454 Life Sciences/Roche, Branford, CT). TruSeq Adaptors (Illumina, Inc., Hayward, CA) were ligated to the repaired/A-tailed ends. Biotinylated fragments were enriched using Streptavidin-coupled Dynabeads (Life Technologies, Grand Island, NY) and amplified by PCR using Illumina TruSeq primers.

Random bacterial artificial chromosome (BAC) sequencing was performed using DNA from the same genotype on 454 Titanium with 6 kb paired-end libraries at the PlateForme Génomiques of the Institute for Systems and Integrative Biology (Université Laval, Quebec City, QC). A single paired-end library was prepared on a pool of 15 BACs (equimolar concentrations) as described earlier in the text with the following modifications: 15 µg of DNA was fragmented using a Hydroshear with a standard assembly module at speed code 18, 6–10 kb fragments were extracted from the gel, and GS-FLX library adaptors were ligated to the repaired/A-tailed fragments. GS-FLX sequencing using the titanium chemistry was performed according to manufacturer's instructions (454 Life Sciences/Roche, Branford, CT). Sanger sequencing method was used to obtain BAC sequencing data as previously described (Hamberger *et al.*, 2011; Keeling *et al.*, 2010).

2.3 MiSeq modification

In sequencing the spruce genome, we generated longer read lengths by modifying the MiSeq platform. The MiSeq uses a clamshell style cartridge (Supplementary Fig. S1A) to hold reagent tubes in an array accessed by the MiSeq's sippers. Most of the reagents are used in length independent steps such as denaturation and cluster generation. The three reagents, the Scan, Cleavage and Incorporation mix, are consumed at each cycle. Although the MiSeq allows any read length specified in the control software, the reagent cartridge cannot be changed during the run without stopping it. Increasing the read length therefore requires increasing the quantity of the length-dependent reagents in the cartridge. This led to the solution of combining the length-dependent reagents of two kits into one.

A tool was designed that opens the snap-hook latches holding the cartridge together (Supplementary Figs S1B and S2), giving access to the reagent tubes, yet allowing the cartridge to be put back on without damage to its components (Supplementary Figs S3–S5). The 40 ml, the stock length-dependent reagent containers allow a maximum of ~650 cycles in total. To maximize the potential of the kit approach, a new reagent tray with 70 ml wells was designed and placed in a modified clamshell base.

Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data

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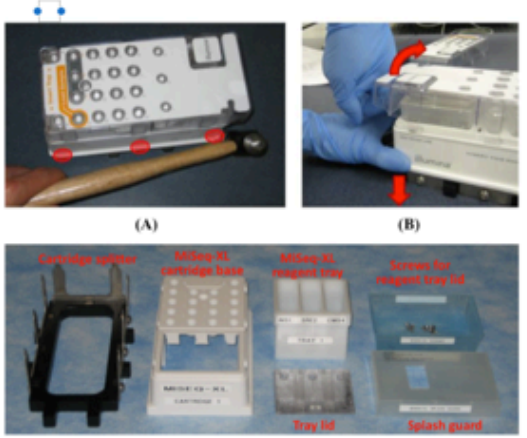


Figure S1. Modification of the MiSeq cartridge. MiSeq reagent cartridge was modified to allow for longer read lengths. **(A, B)** Opening of the clamshell style cartridge. **(C)** Contents of the modified cartridge. This was initially used to combine two PE150 kits for PE300 runs. When Illumina introduced the P250 kit, the same apparatus was used to enable PE500 runs.

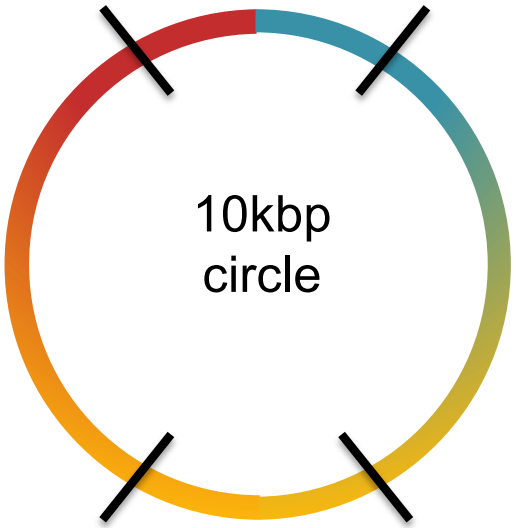
Paired-end and Mate-pairs

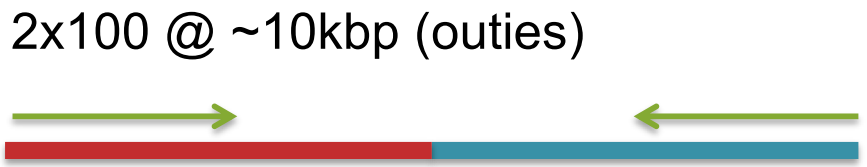
Paired-end sequencing

- 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





FASTQ Files

reads.1.fq

reads.2.fq

```
@SEQ_ID
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
+
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65
```

@Identifier
Sequence
+Separator
Quality Values
…

http://en.wikipedia.org/wiki/FASTQ_format

Illumina Sequencing Summary

Advantages:

- Best throughput, accuracy and read length for any 2nd gen. sequencer
- Fast & robust library preparation

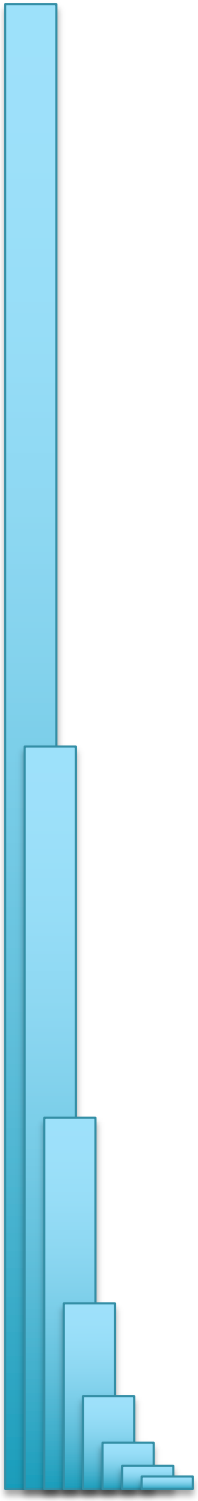
Disadvantages:

- Inherent limits to read length (practically, 150bp)
- Some runs are error prone
- Requires amplification, sequences a population of molecules

Illumina HiSeq
~3 billion paired 100bp reads
~600Gb, $10K, 8 days
(or “rapid run” ~90Gb in 1-2 days)

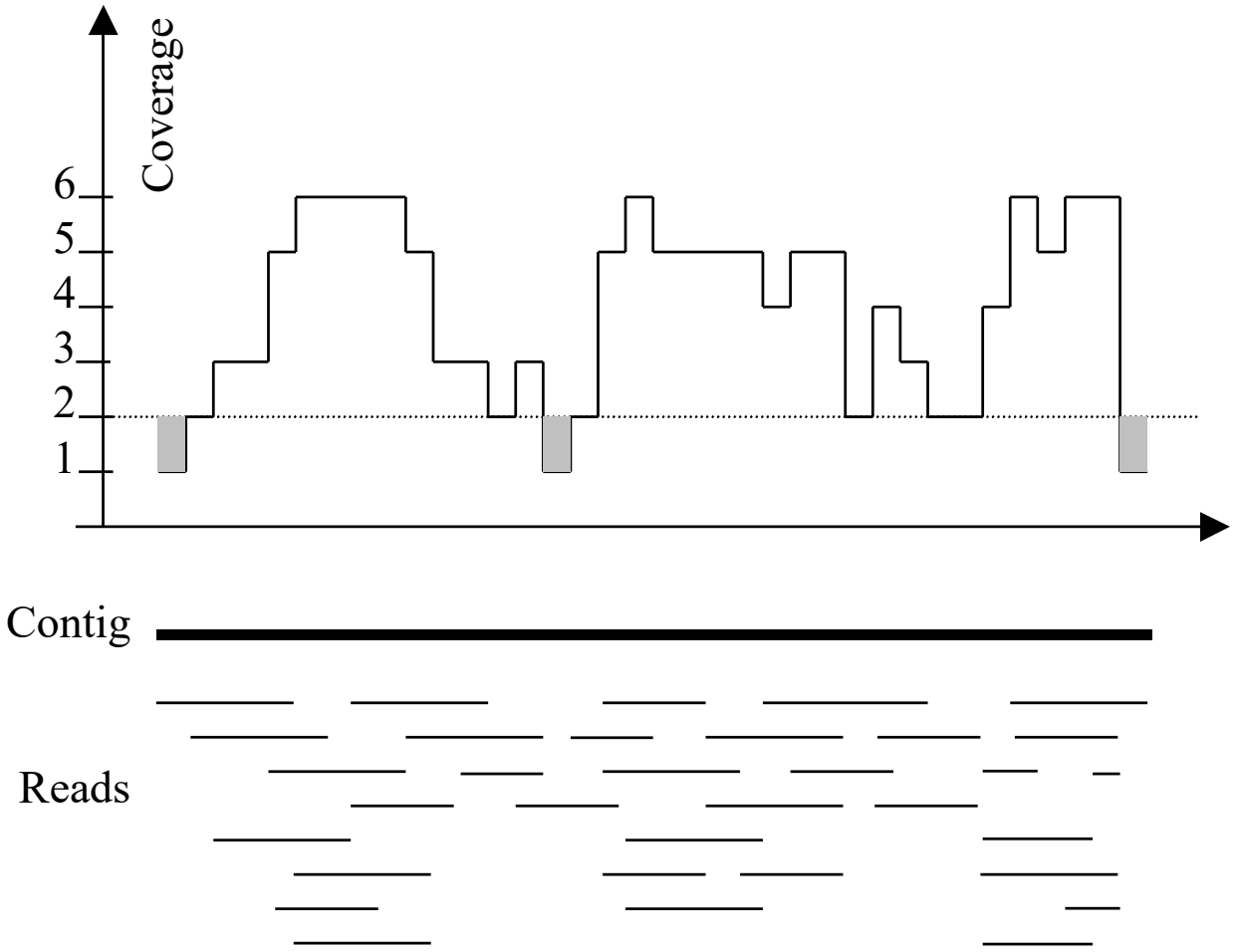
Illumina X Ten / NovaSeq
~6 billion paired 150bp reads
1.8Tb, <3 days, ~1000 / genome($$)
(or “rapid run” ~90Gb in 1-2 days)

Illumina NextSeq
One human genome in **<30 hours**



Part 2: Coverage

Typical sequencing coverage

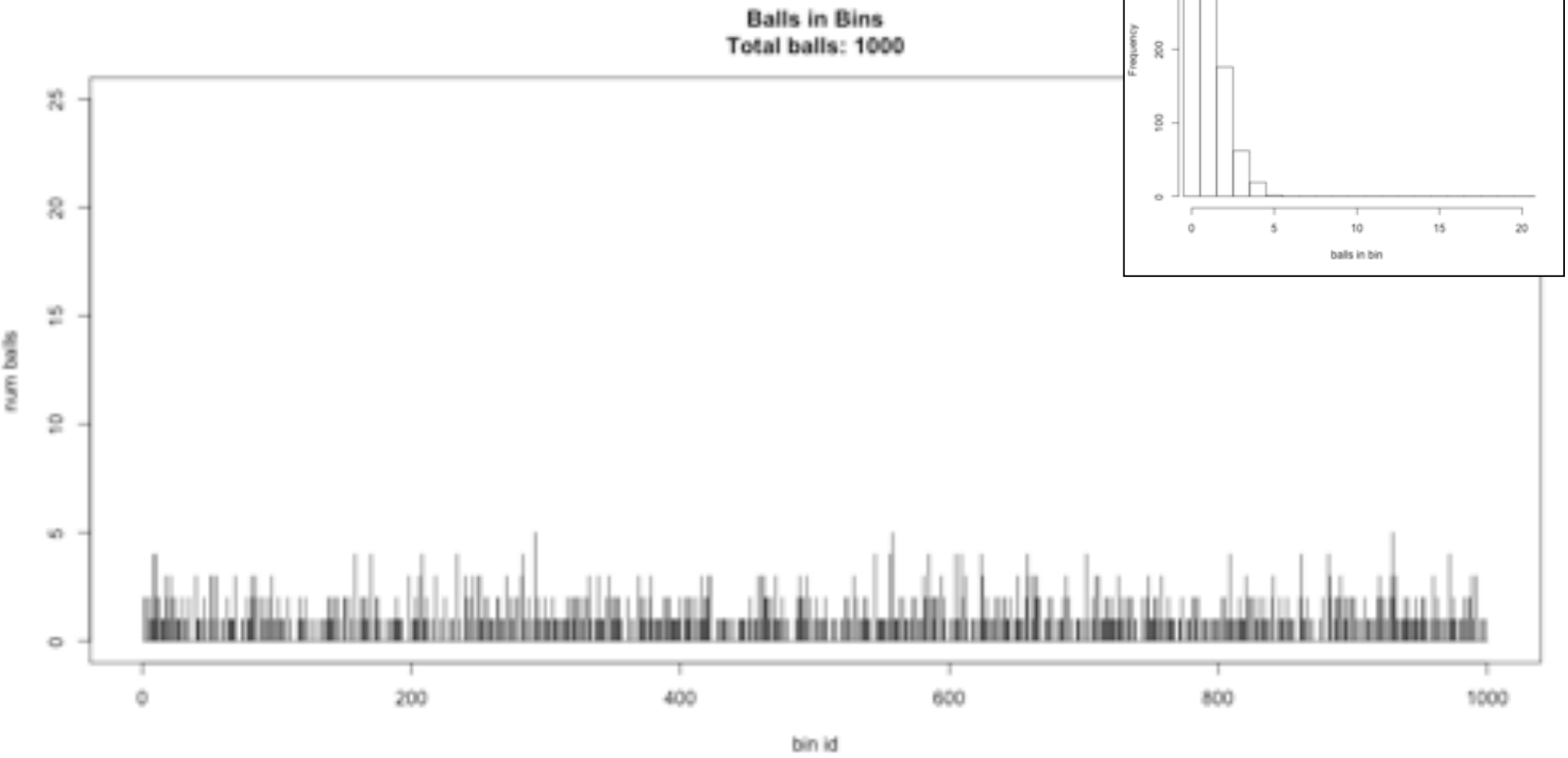


Imagine raindrops on a sidewalk

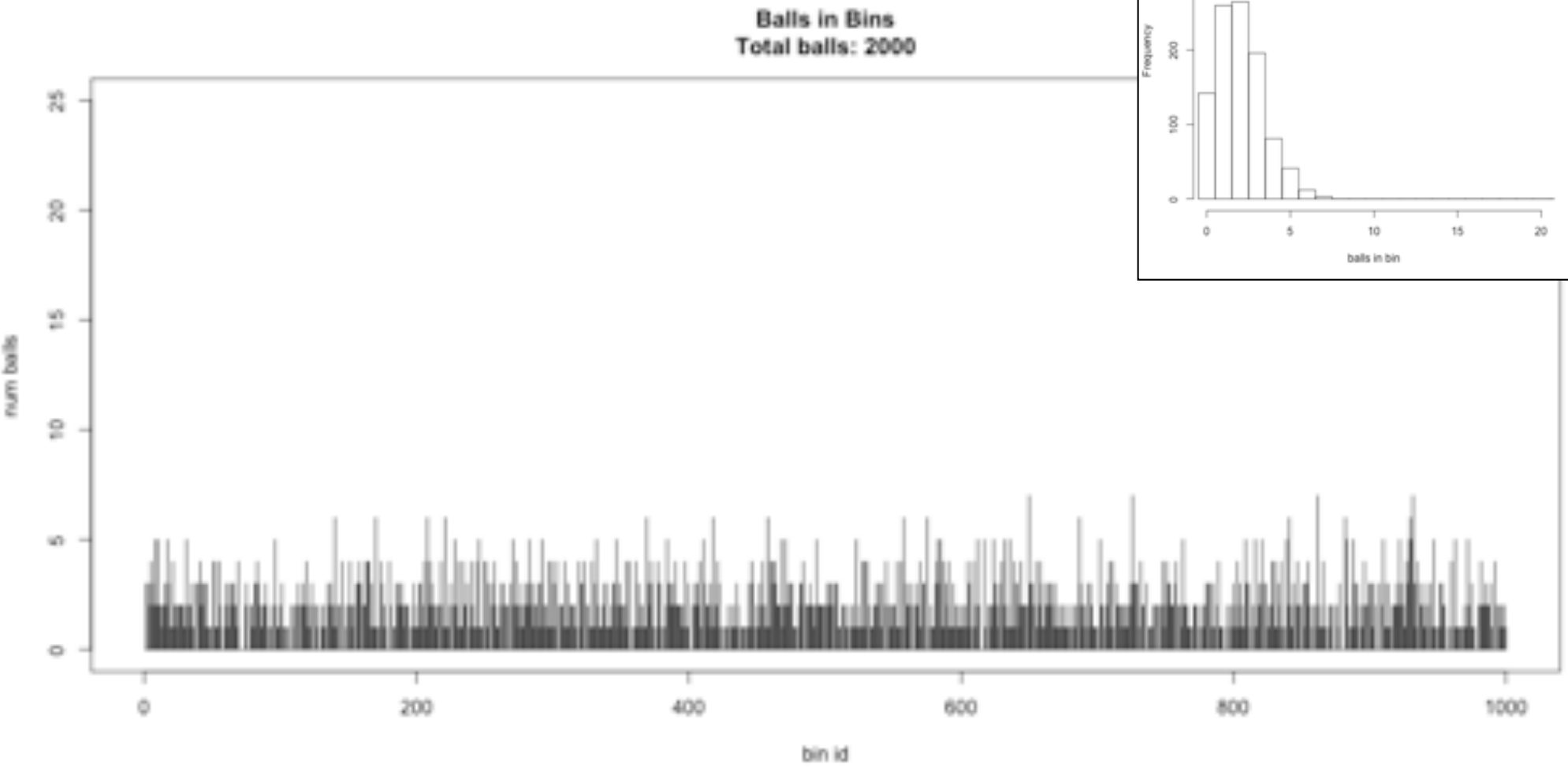
We want to cover the entire sidewalk but each drop costs $1

If the genome is 10 Mbp, should we sequence 100k 100bp reads?

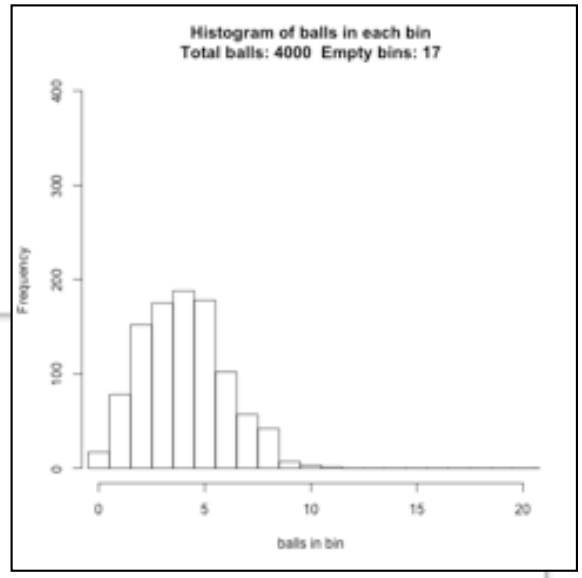
1x sequencing



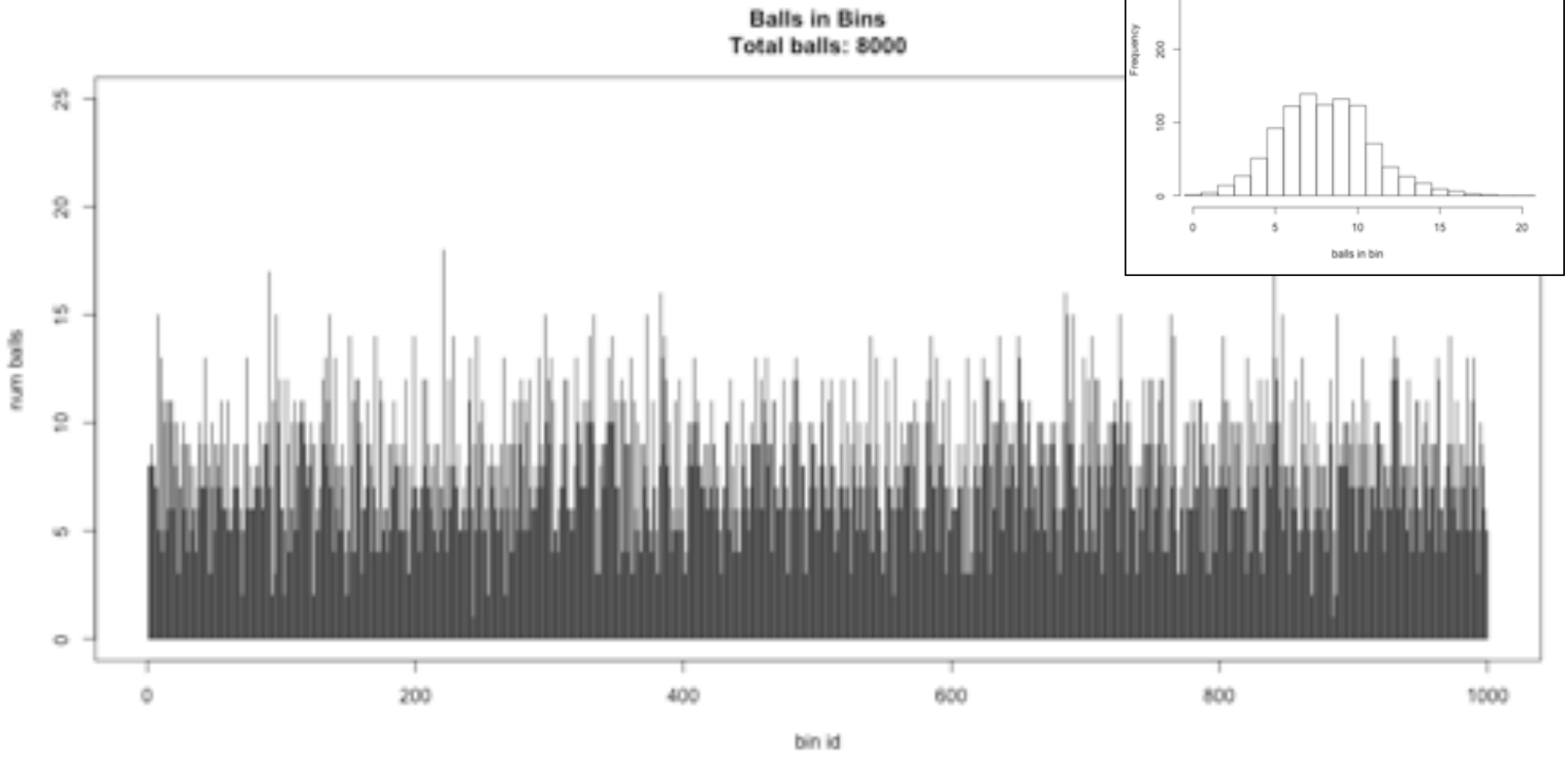
2x sequencing



4x sequencing



8x sequencing



Poisson Distribution

The probability of a given number of events occurring in a fixed interval of time and/or space if these events occur with a known average rate and independently of the time since the last event.

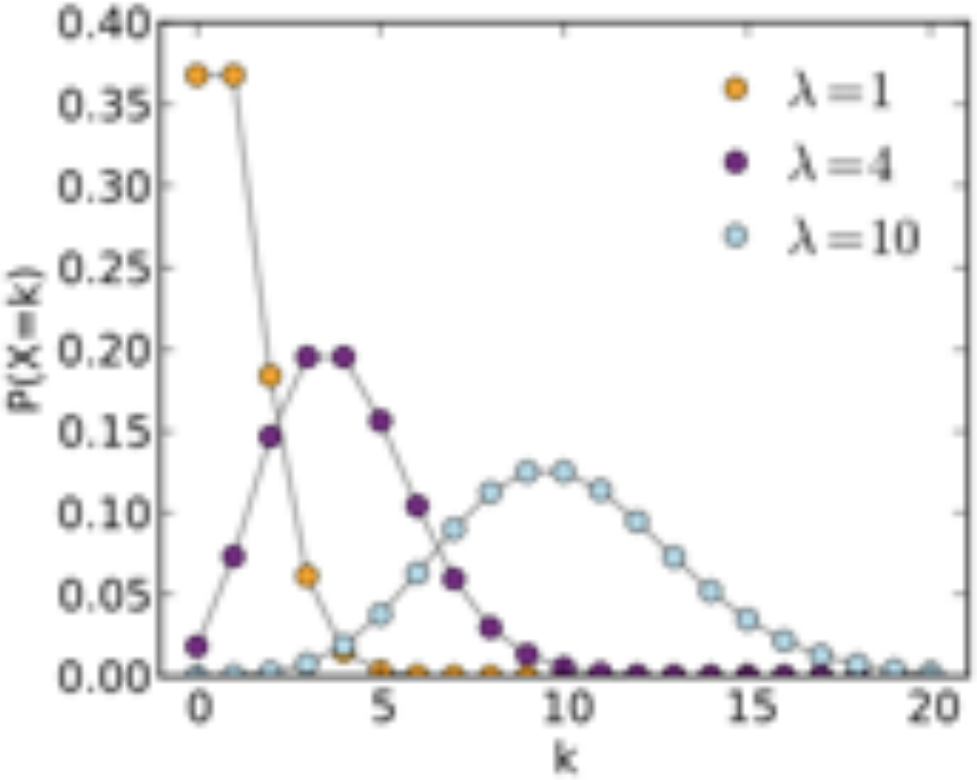
$$P(k) = \frac{\lambda^k}{k!} e^{-\lambda}$$

Formulation comes from the limit of the binomial equation

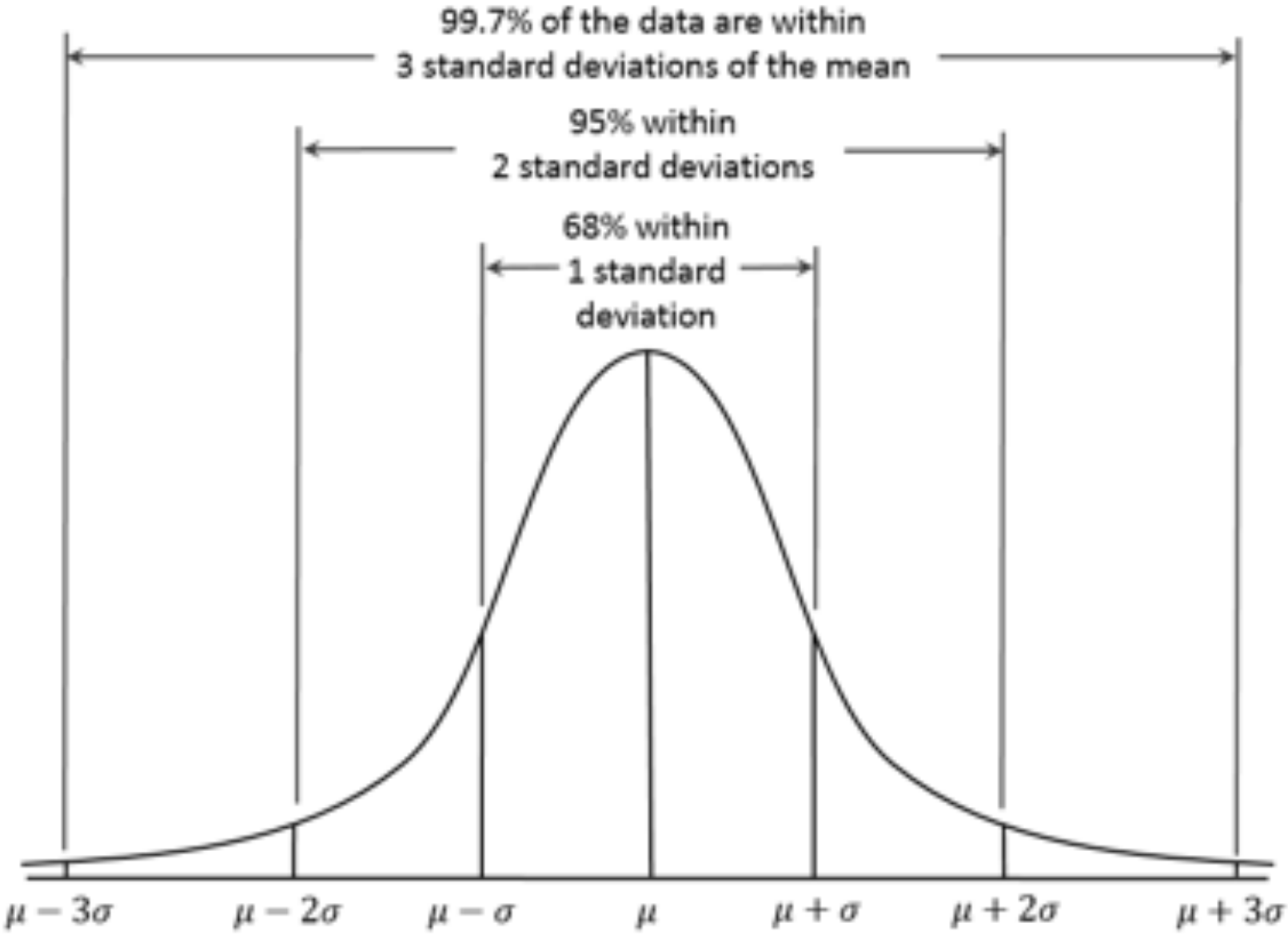
Resembles a normal distribution, but over the positive values, and with only a single parameter.

Key properties:

- ***The standard deviation is the square root of the mean.***
- ***For mean > 5, well approximated by a normal distribution***



Normal Approximation

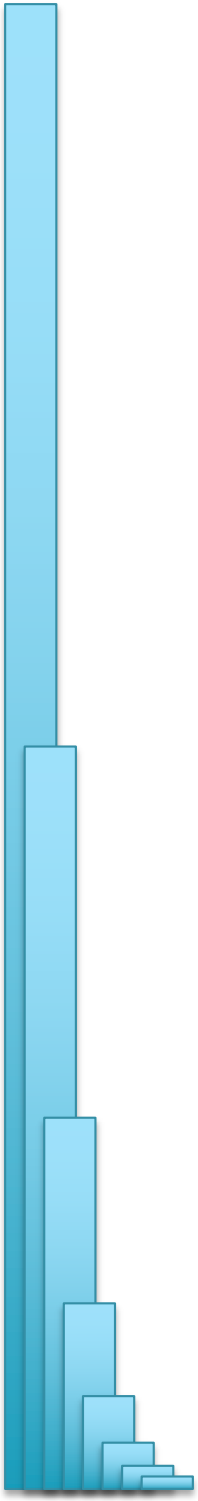


Can estimate Poisson distribution as a normal distribution when $\lambda > 10$

Pop Quiz!

I want to sequence a 10Mbp genome to 24x coverage.
How many 120bp reads do I need?

I want to sequence a 10Mbp genome so that
>97.5% of the genome has at least 24x coverage.
How many 120bp reads do I need?



Part 3: De novo genome assembly

Outline

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

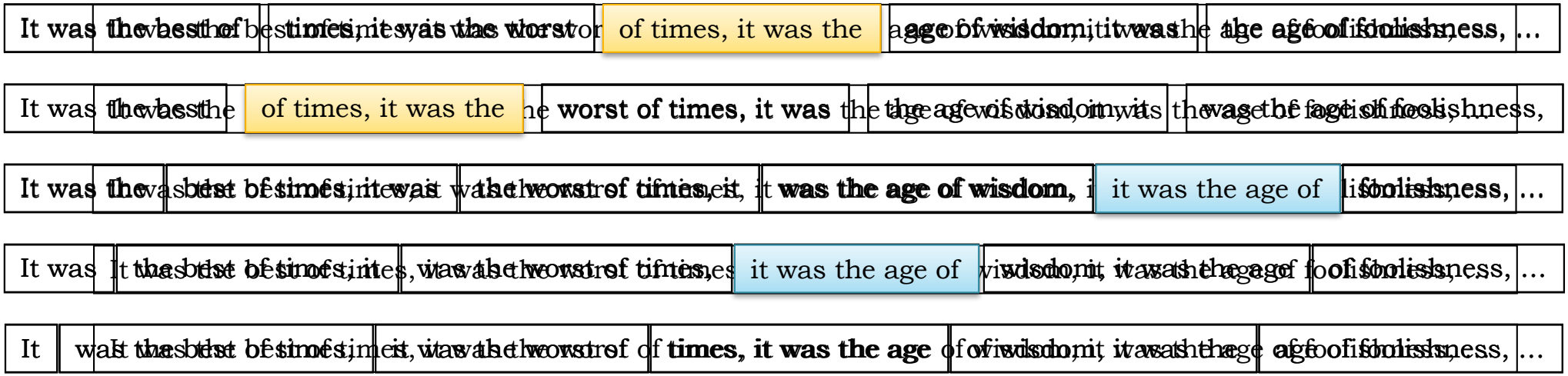
2. ***Practical Issues***
   - Coverage, read length, errors, and repeats

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

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

Shredded Book Reconstruction

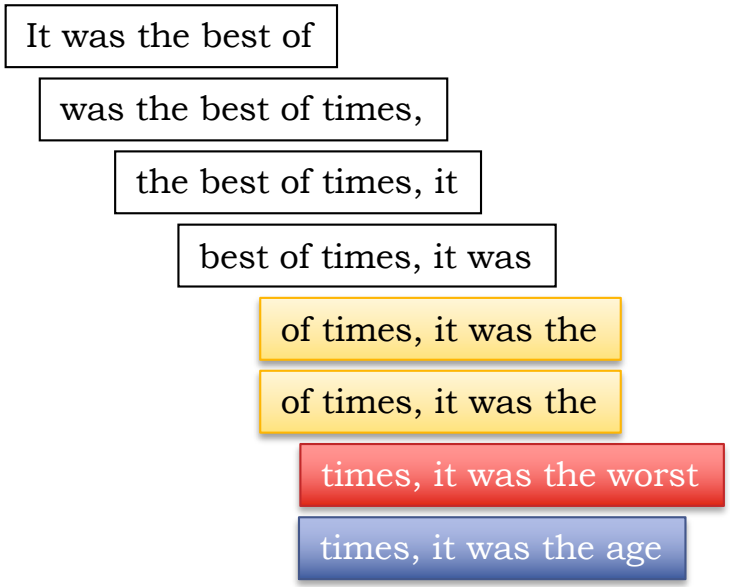
- Dickens accidentally shreds the first printing of A Tale of Two Cities
  - Text printed on 5 long spools



- How can he reconstruct the text?
  - 5 copies x 138, 656 words / 5 words per fragment = 138k fragments
  - The short fragments from every copy are mixed together
  - Some fragments are identical

Greedy Reconstruction

It was the best of
age of wisdom, it was
best of times, it was
it was the age of
it was the age of
it was the worst of
of times, it was the
of times, it was the
of wisdom, it was the
the age of wisdom, it
the best of times, it
the worst of times, it
times, it was the age
times, it was the worst
was the age of wisdom,
was the age of foolishness,
was the best of times,
was the worst of times,
wisdom, it was the age
worst of times, it was



The repeated sequence make the correct reconstruction ambiguous

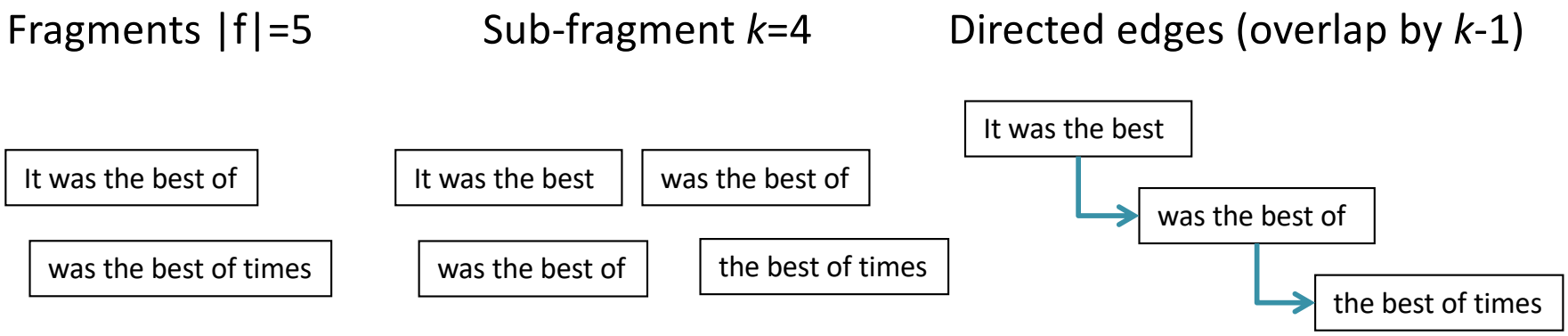
- It was the best of times, it was the [worst/age]

Model the assembly problem as a graph problem

How long will it take to compute the overlaps?

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

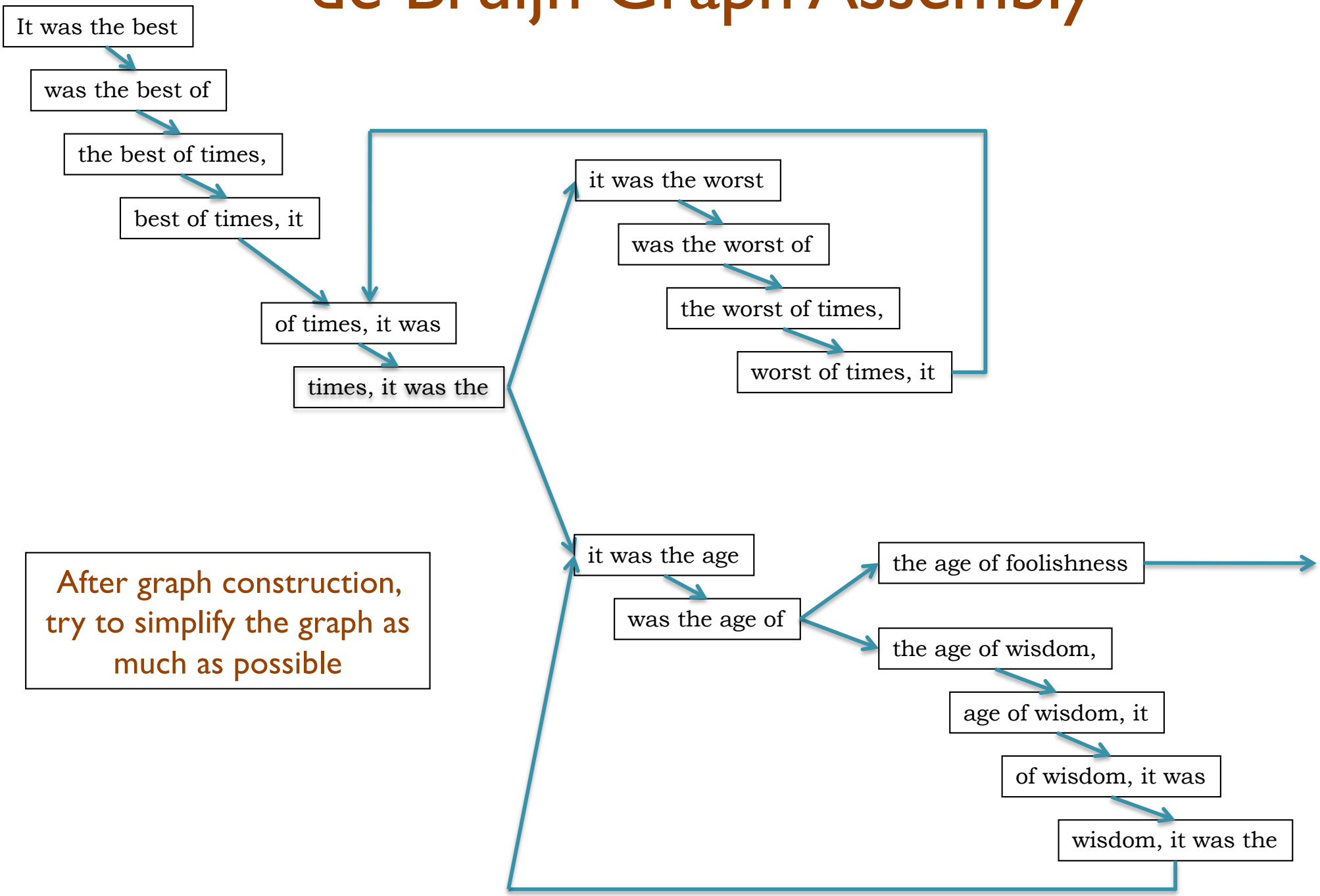


– Overlaps between fragments are implicitly computed

How to pronounce:
https://forvo.com/word/de_bruijn/

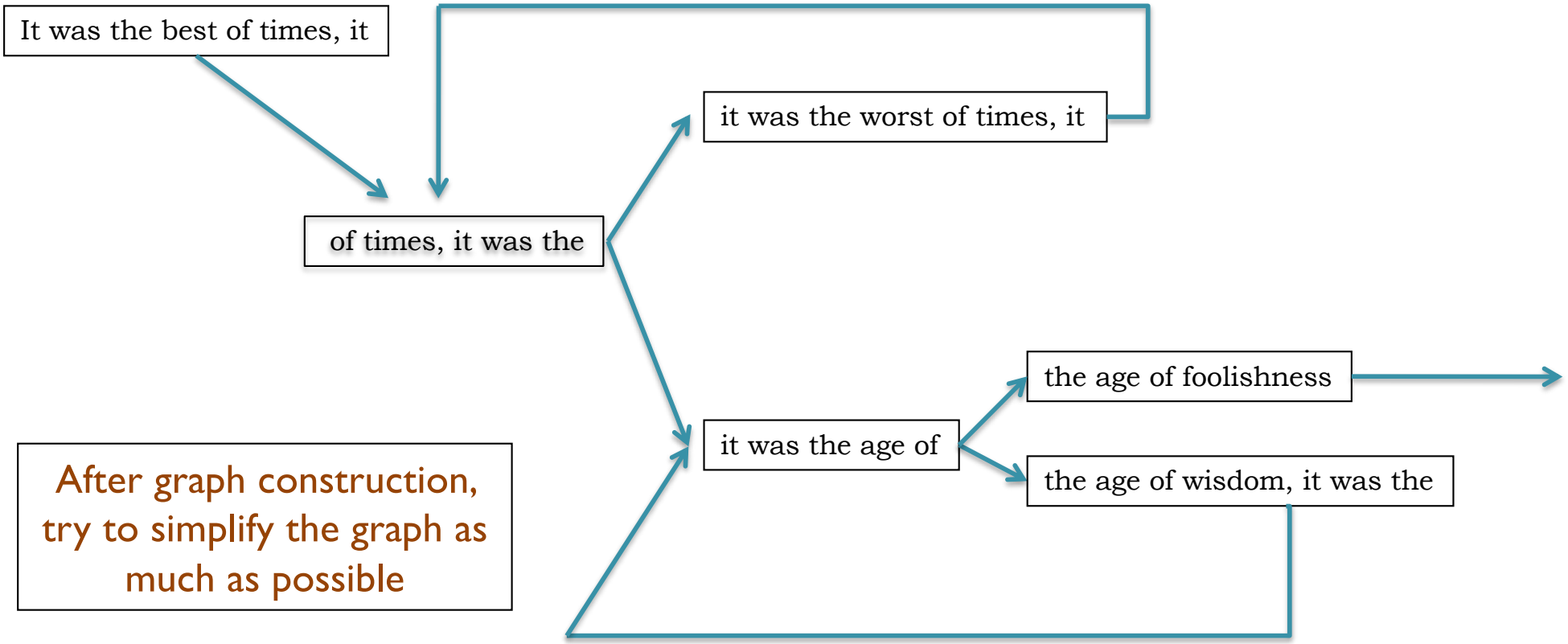
de Bruijn, 1946
Idury et al., 1995
Pevzner et al., 2001

de Bruijn Graph Assembly



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

de Bruijn Graph Assembly



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

The full tale

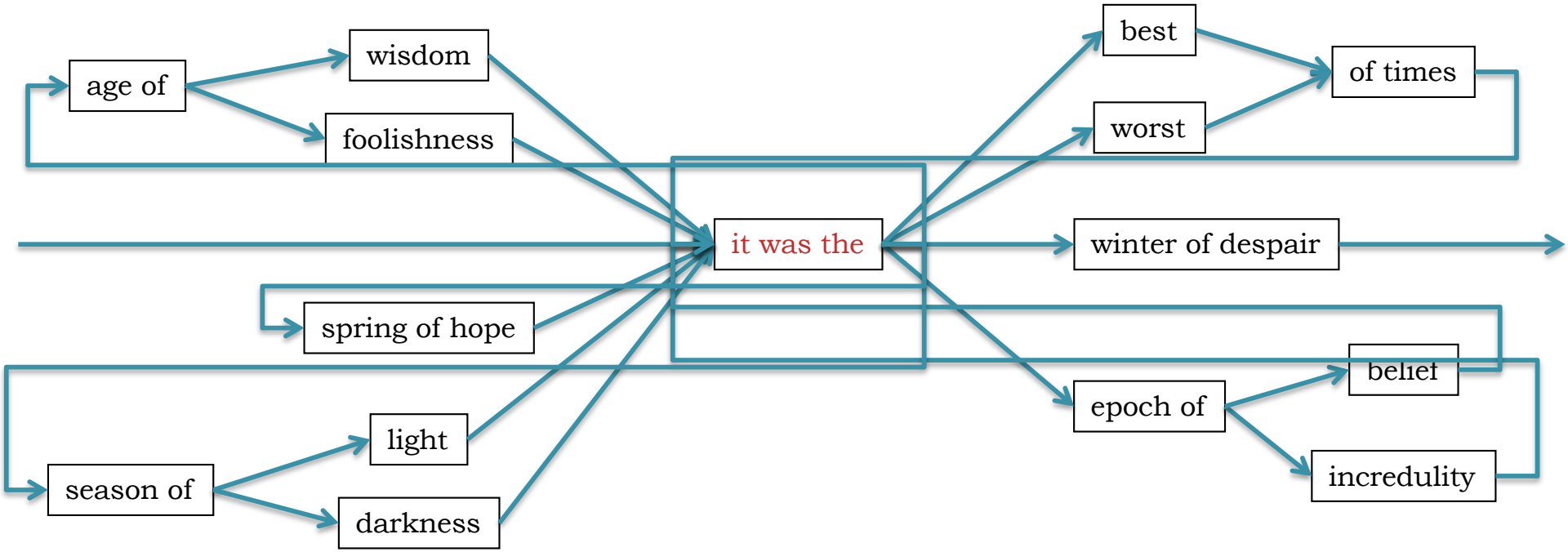
… it was the best of times it was the worst of times …

… it was the age of wisdom it was the age of foolishness …

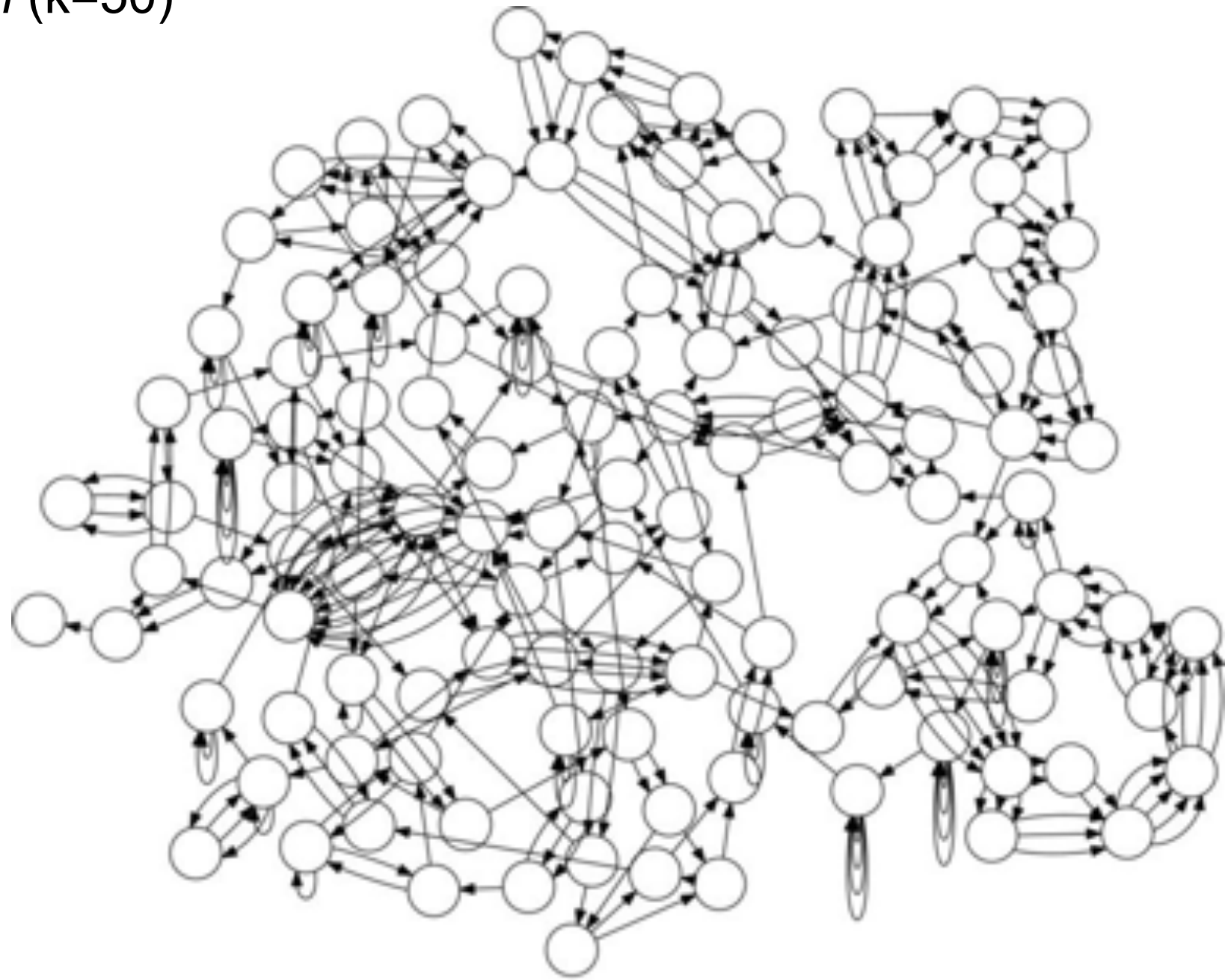
… it was the epoch of belief it was the epoch of incredulity …

… it was the season of light it was the season of darkness …

… it was the spring of hope it was the winder of despair …

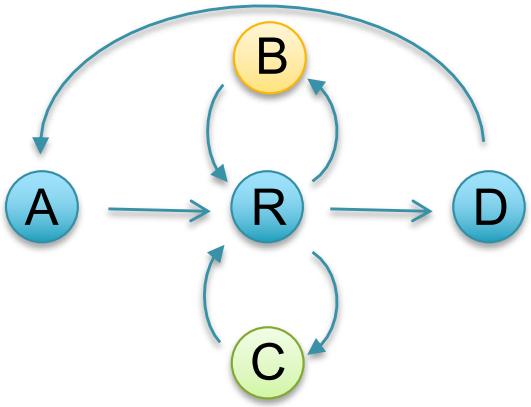


E. coli (k=50)



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

Counting Eulerian Cycles



ARBRCRD
or
ARCRBRD

Generally an exponential number of compatible sequences

– Value computed by application of the BEST theorem (Hutchinson, 1975)

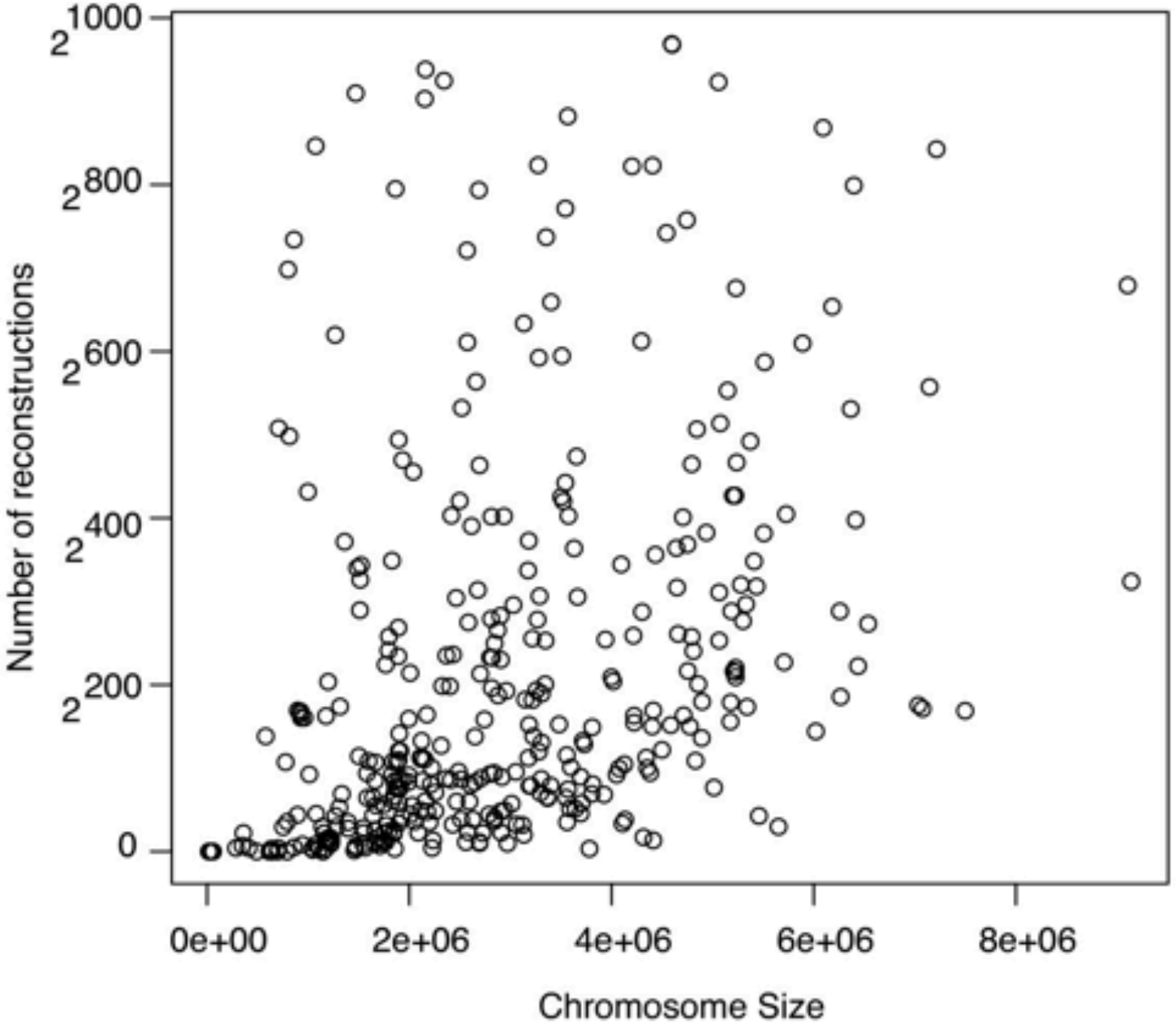
$$\mathcal{W}(G,t) = (\det L)\left\{\prod_{u\in V}(r_u-1)!\right\}\left\{\prod_{(u,v)\in E} a_{uv}!\right\}^{-1}$$

L = *n x n* matrix with r_u-a_{uu} along the diagonal and -a_{uv} in entry uv

$r_u = d^+(u)+1$ if $u=t$, or $d^+(u)$ otherwise

a_{uv} = multiplicity of edge from *u* to *v*

Assembly Complexity of Prokaryotic Genomes using Short Reads.
Kingsford C, Schatz MC, Pop M (2010) *BMC Bioinformatics*.



Assembly Complexity of Prokaryotic Genomes using Short Reads.
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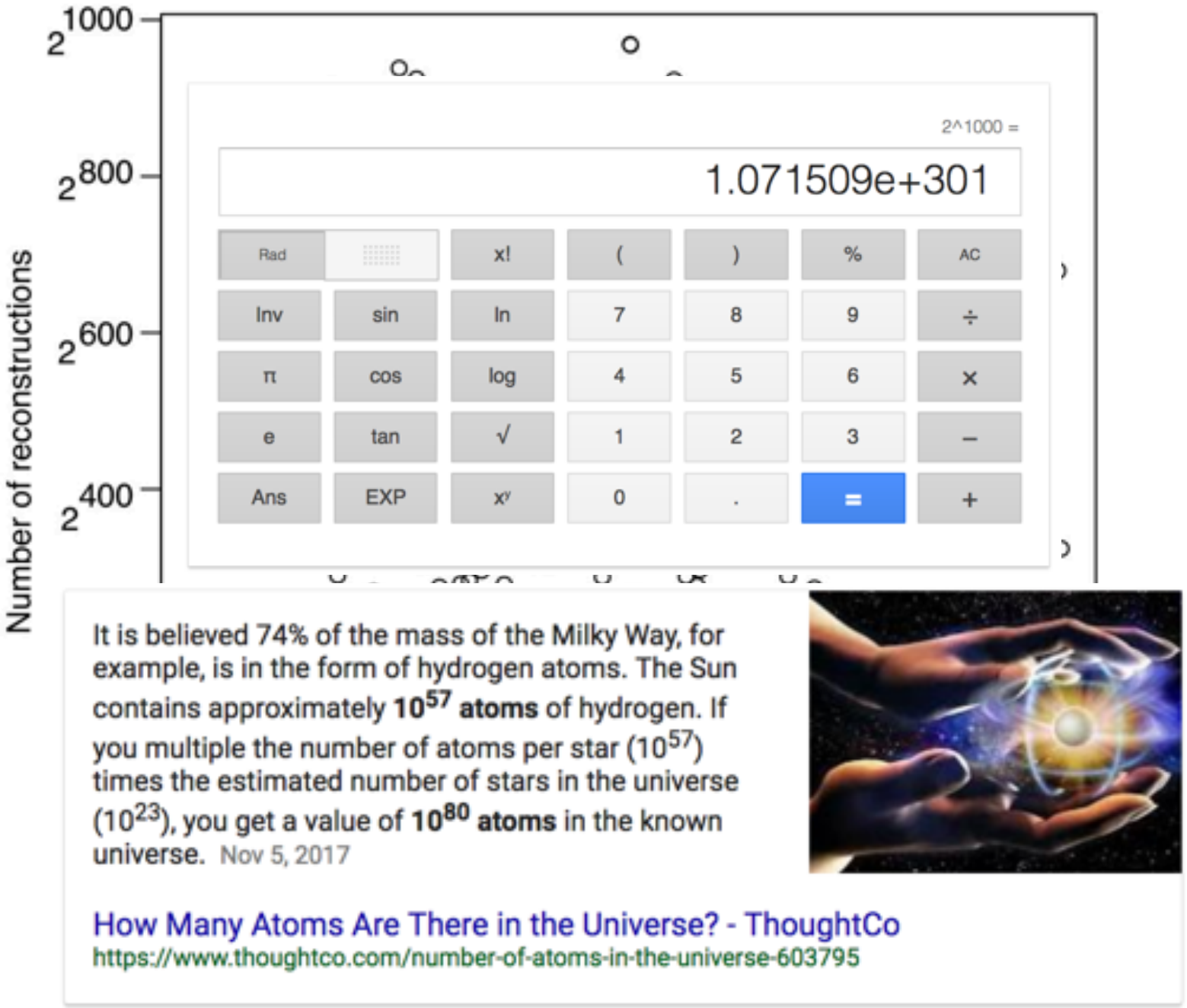

Assembly Complexity of Prokaryotic Genomes using Short Reads.
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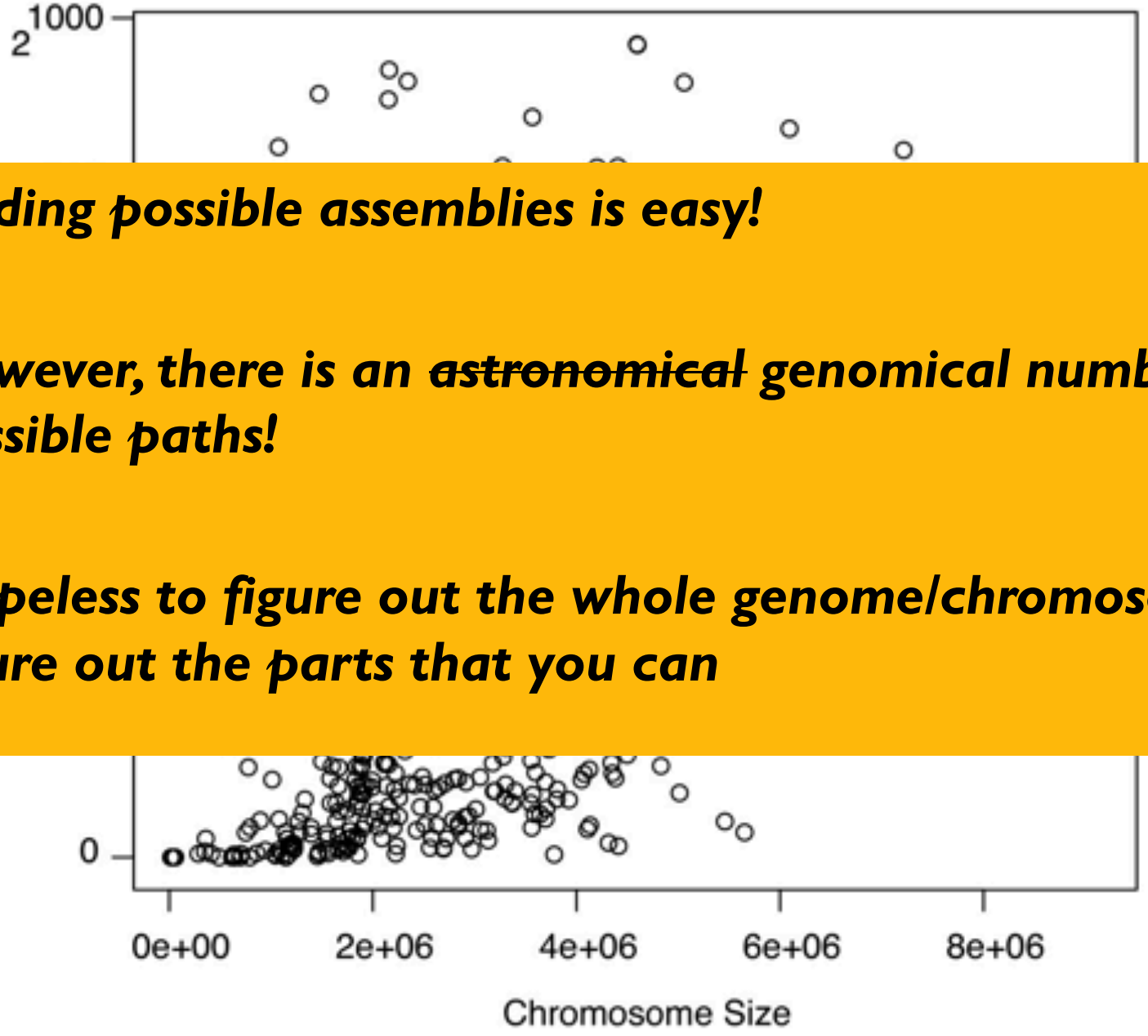
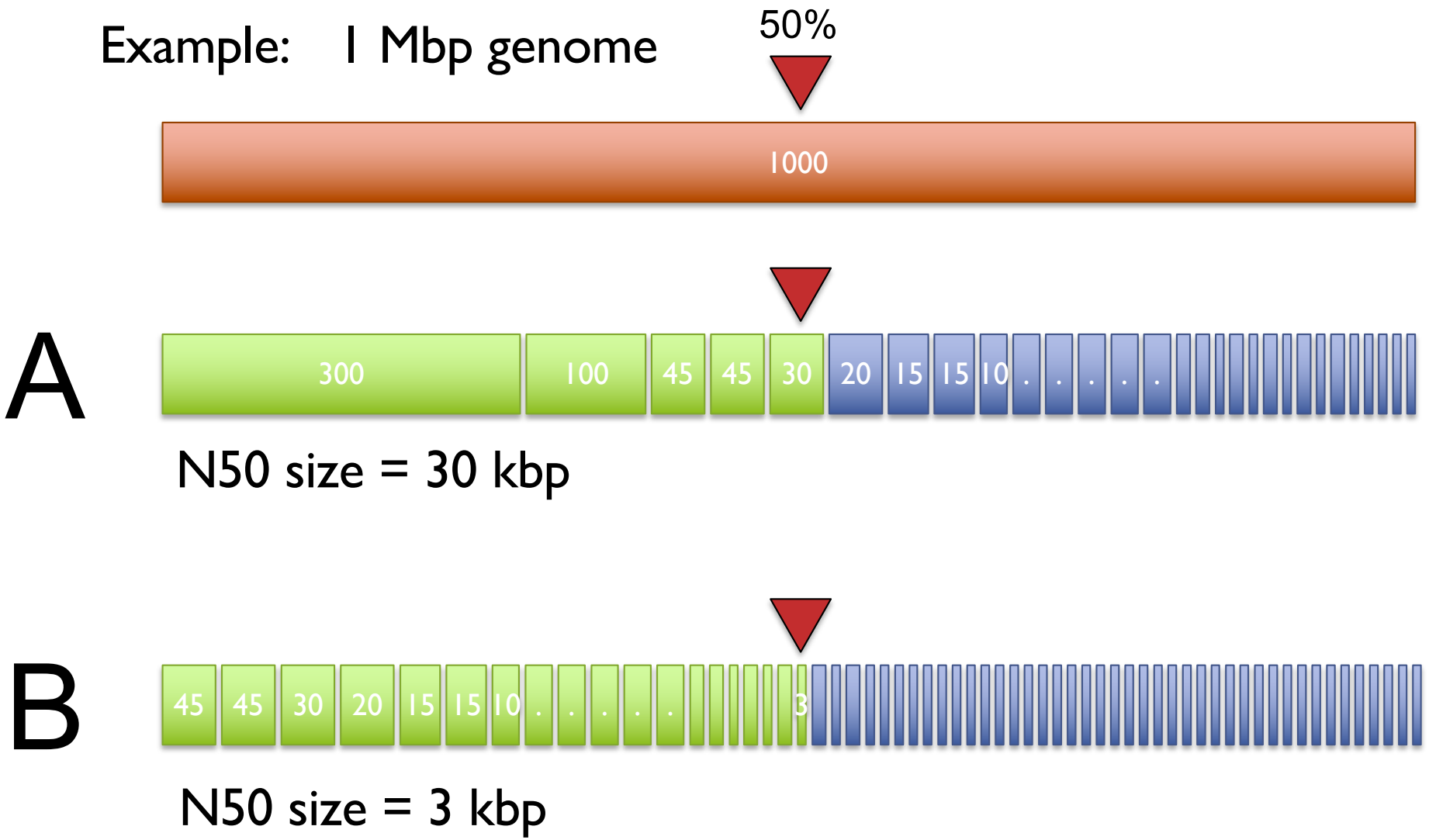


- ***Finding possible assemblies is easy!***
- ***However, there is an ~~astronomical~~ genomical number of possible paths!***
- ***Hopeless to figure out the whole genome/chromosome, figure out the parts that you can***

Assembly Complexity of Prokaryotic Genomes using Short Reads.
Kingsford C, Schatz MC, Pop M (2010) *BMC Bioinformatics*.

Contig N50

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



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

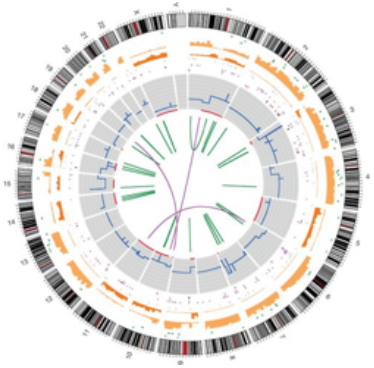
4. Whole Genome Alignment
   - MUMmer recommended

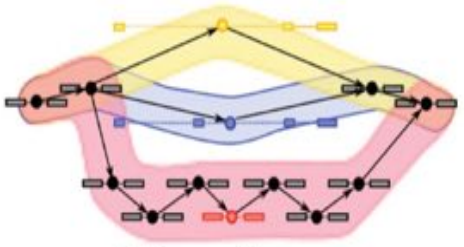
Assembly Applications

- Novel genomes

- Metagenomes

- Sequencing assays
  - Structural variations
  - Transcript assembly
  - …





Why are genomes hard to assemble?

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

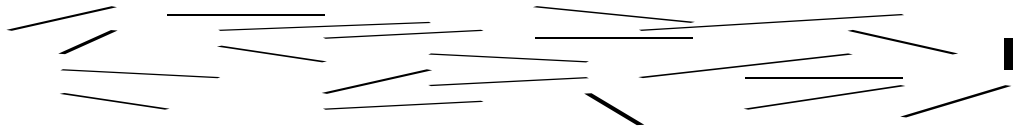
2. ***Sequencing***:
   - (Very) large genomes, imperfect sequencing

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

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

Assembling a Genome

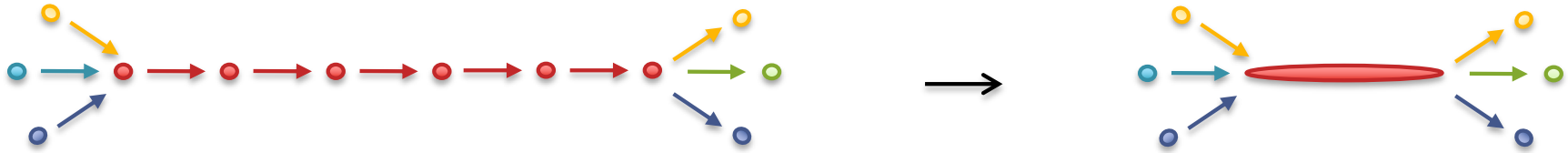
1. Shear & Sequence DNA



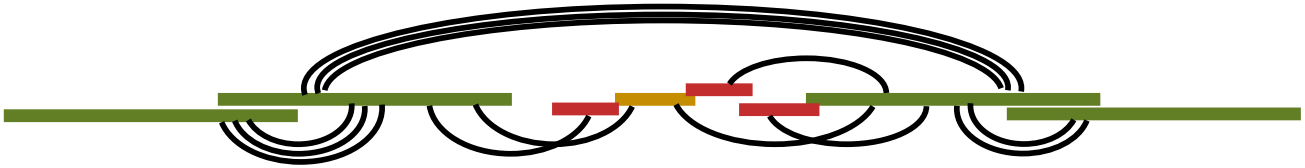
2. Construct assembly graph from reads (de Bruijn / overlap graph)

...AGCCTAGGGATGCGCGACACGT
GGATGCGCGACACGTCGCATATCCGGTTTGGTCAACCTCGGACGGAC
CAACCTCGGACGGACCTCAGCGAA...

3. Simplify assembly graph

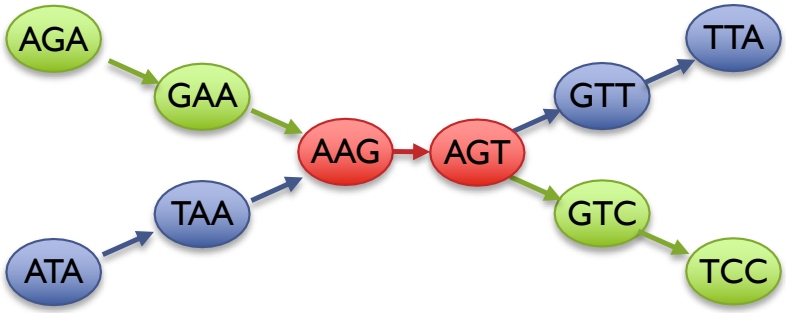


4. Detangle graph with long reads, mates, and other links



Two Paradigms for Assembly

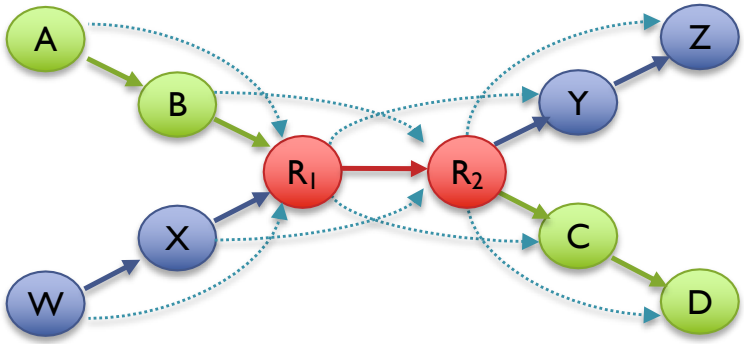
de Bruijn Graph



Short read assemblers

- Repeats depends on word length
- Read coherency, placements lost
- Robust to high coverage

Overlap Graph



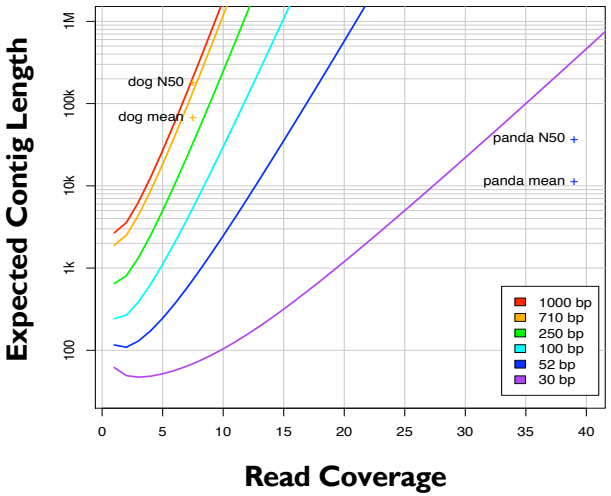
Long read assemblers

- Repeats depends on read length
- Read coherency, placements kept
- Tangled by high coverage

Assembly of Large Genomes using Second Generation Sequencing
Schatz MC, Delcher AL, Salzberg SL (2010) *Genome Research*. 20:1165-1173.

Ingredients for a good assembly

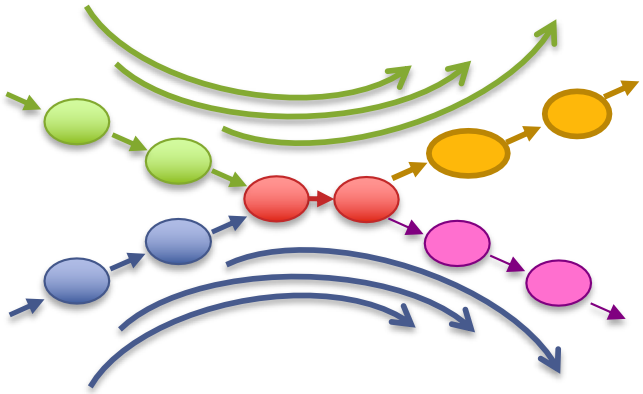
Coverage



High coverage is required

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

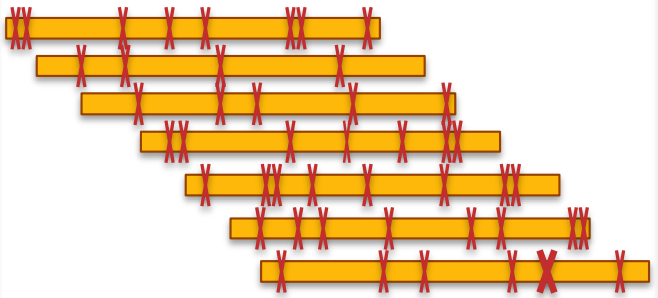
Read Length



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

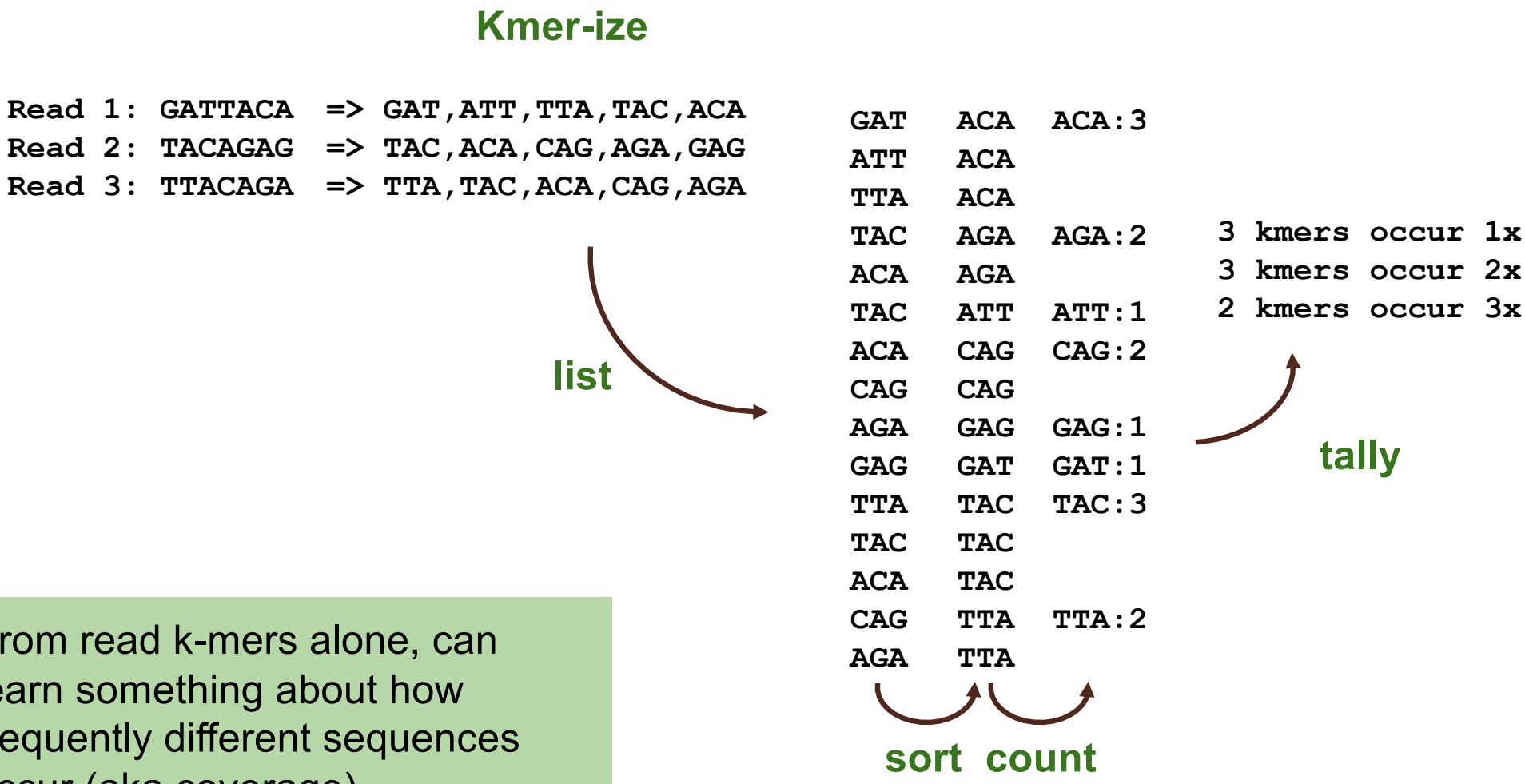
$$\text{sequencing\_coverage} = \frac{\text{total\_bases\_sequenced}}{\text{genome\_size}}$$

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

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

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

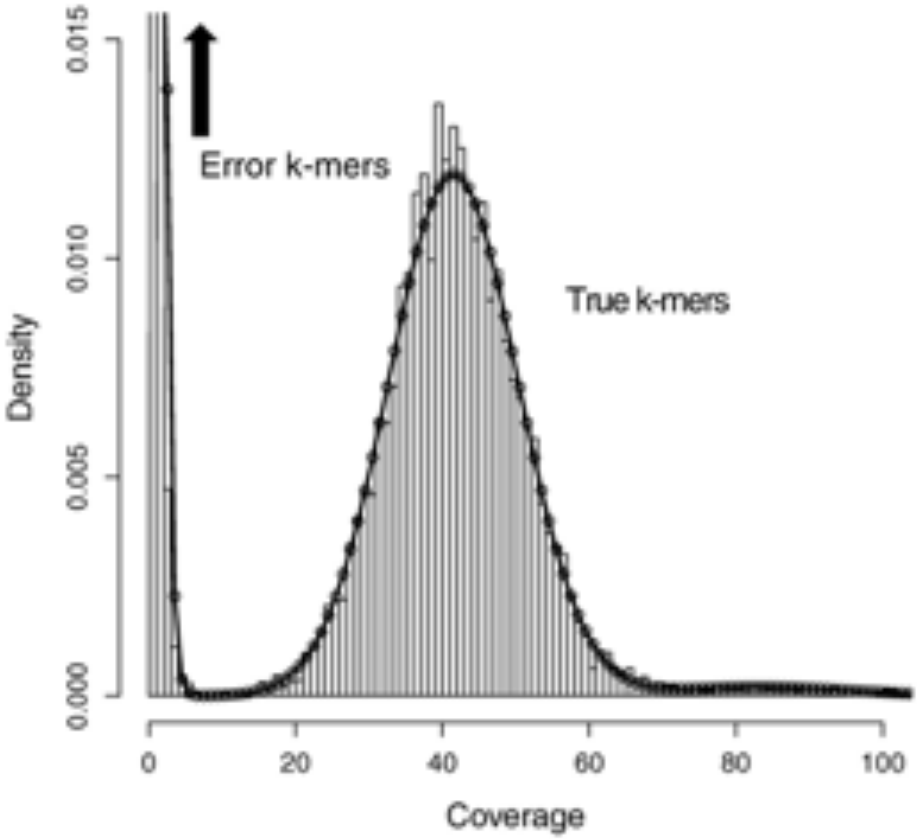
K-mer counting



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

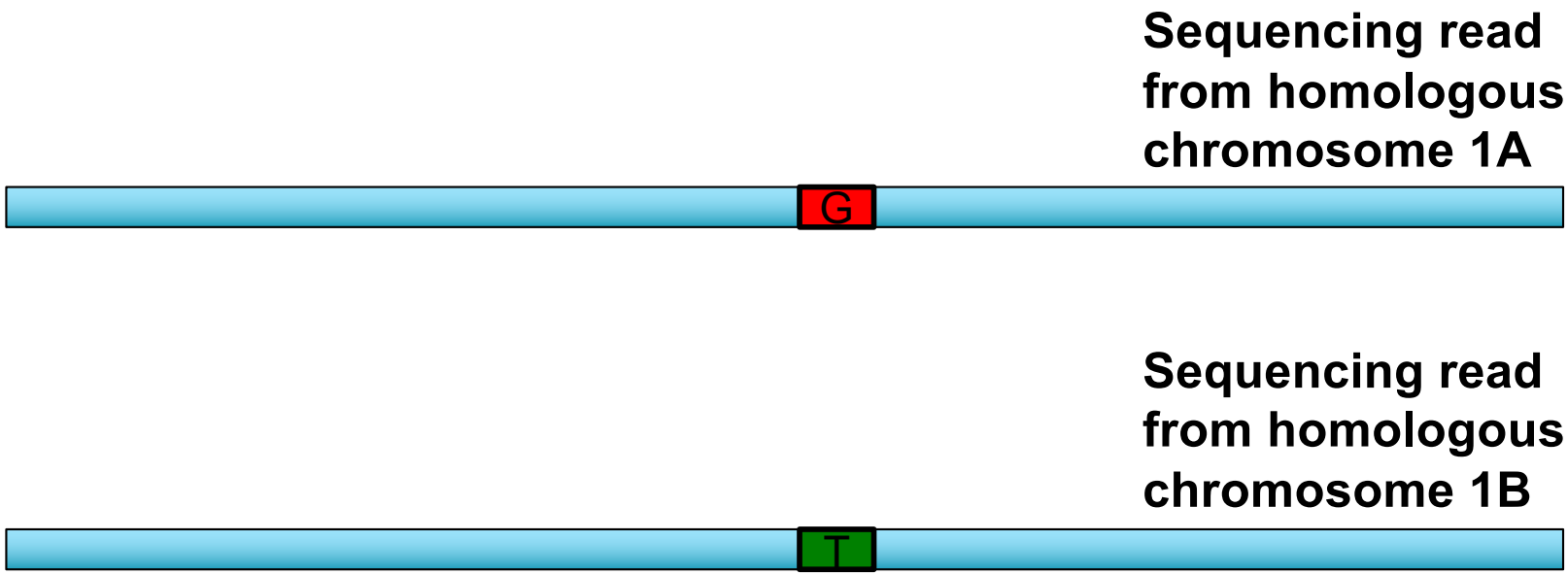
Fast to compute even over huge datasets

K-mer counting in real genomes

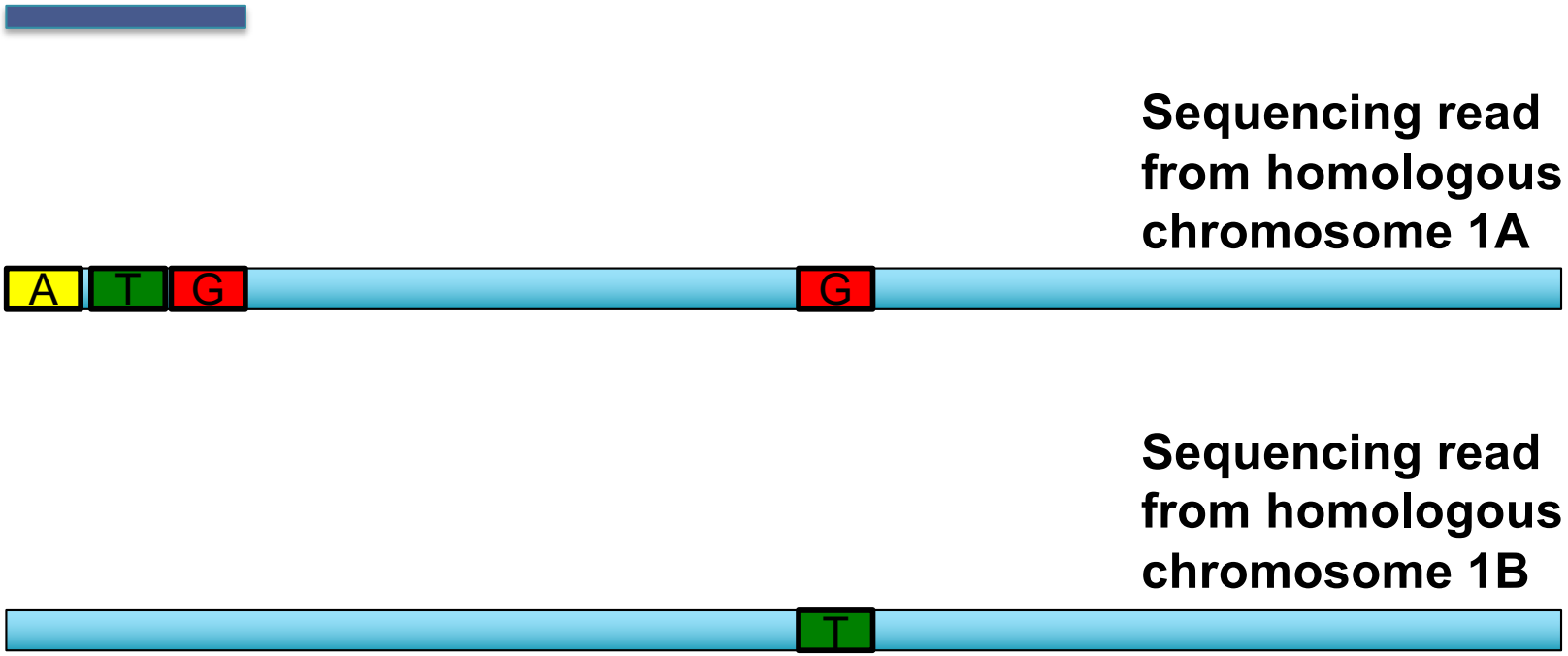


- The tally of k-mer counts in real genomes reveals the coverage distribution.
- Here we sequenced 120Gb of reads from a female human (haploid human genome size is 3Gb), and indeed we see a clear peak centered at 40x coverage
- There are also many kmers that only occur <5 times. These are from errors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

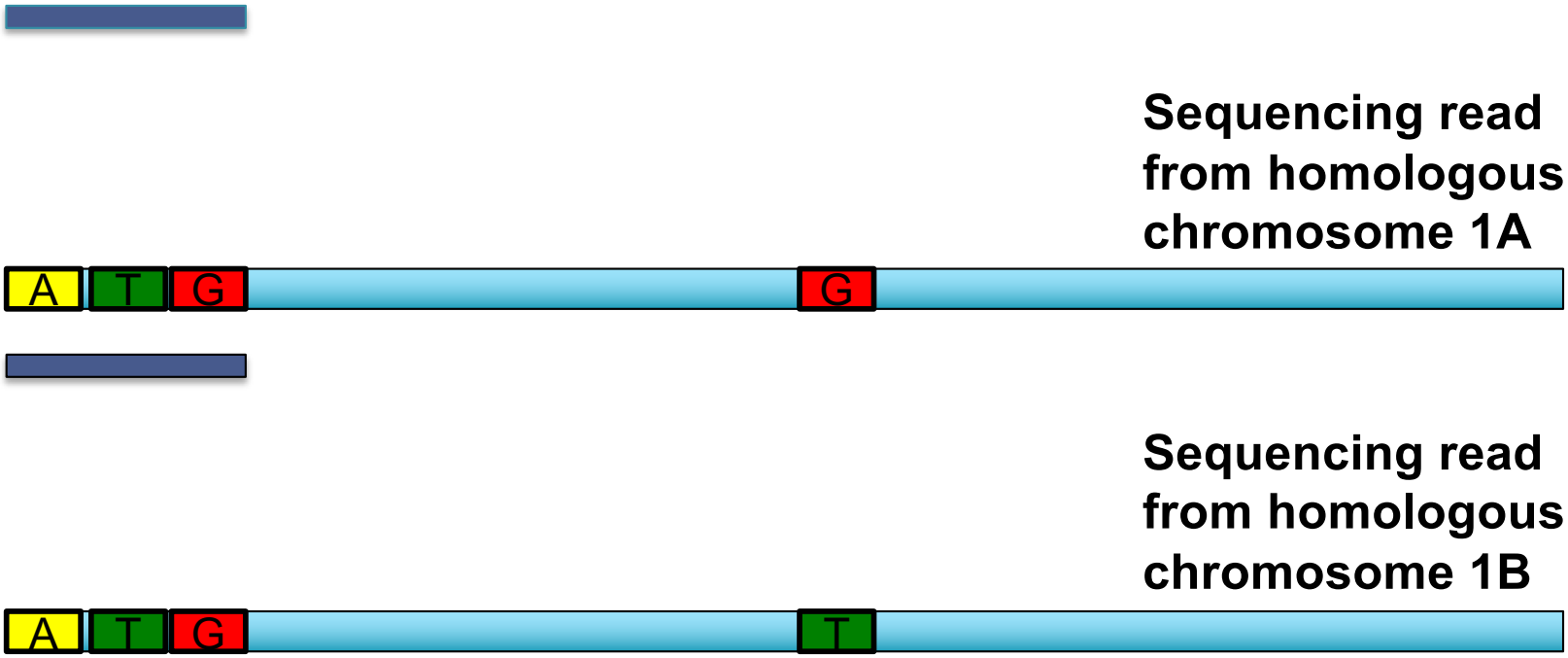
K-mer counting in heterozygous genomes



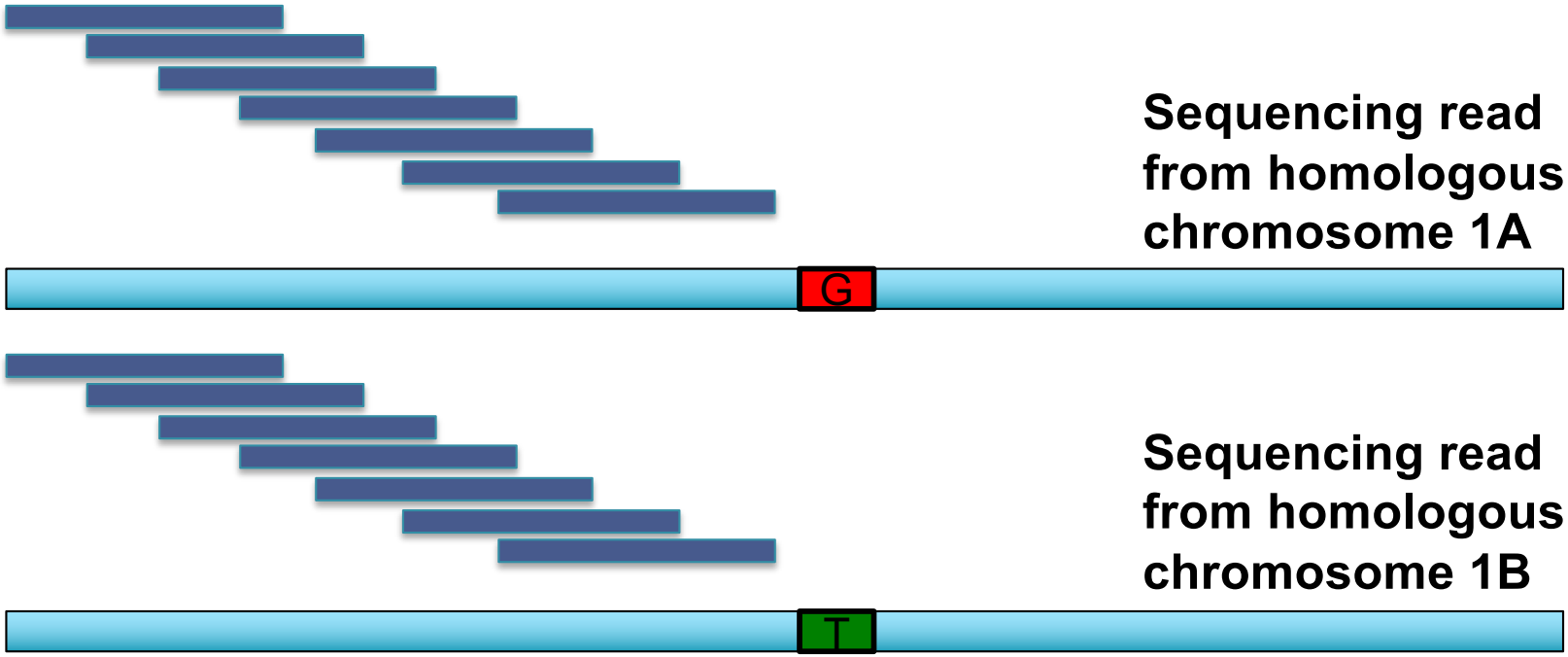
K-mer counting in heterozygous genomes



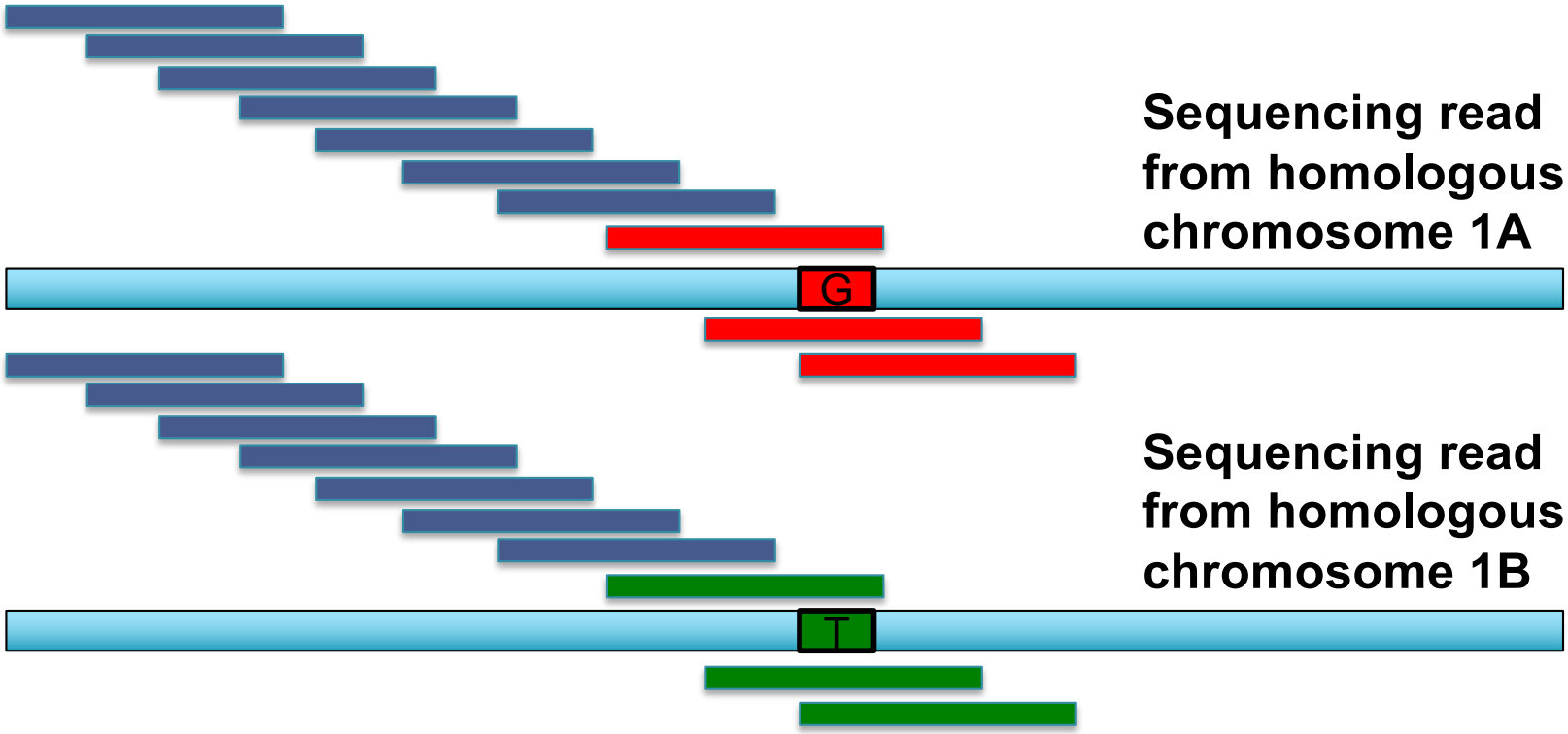
K-mer counting in heterozygous genomes



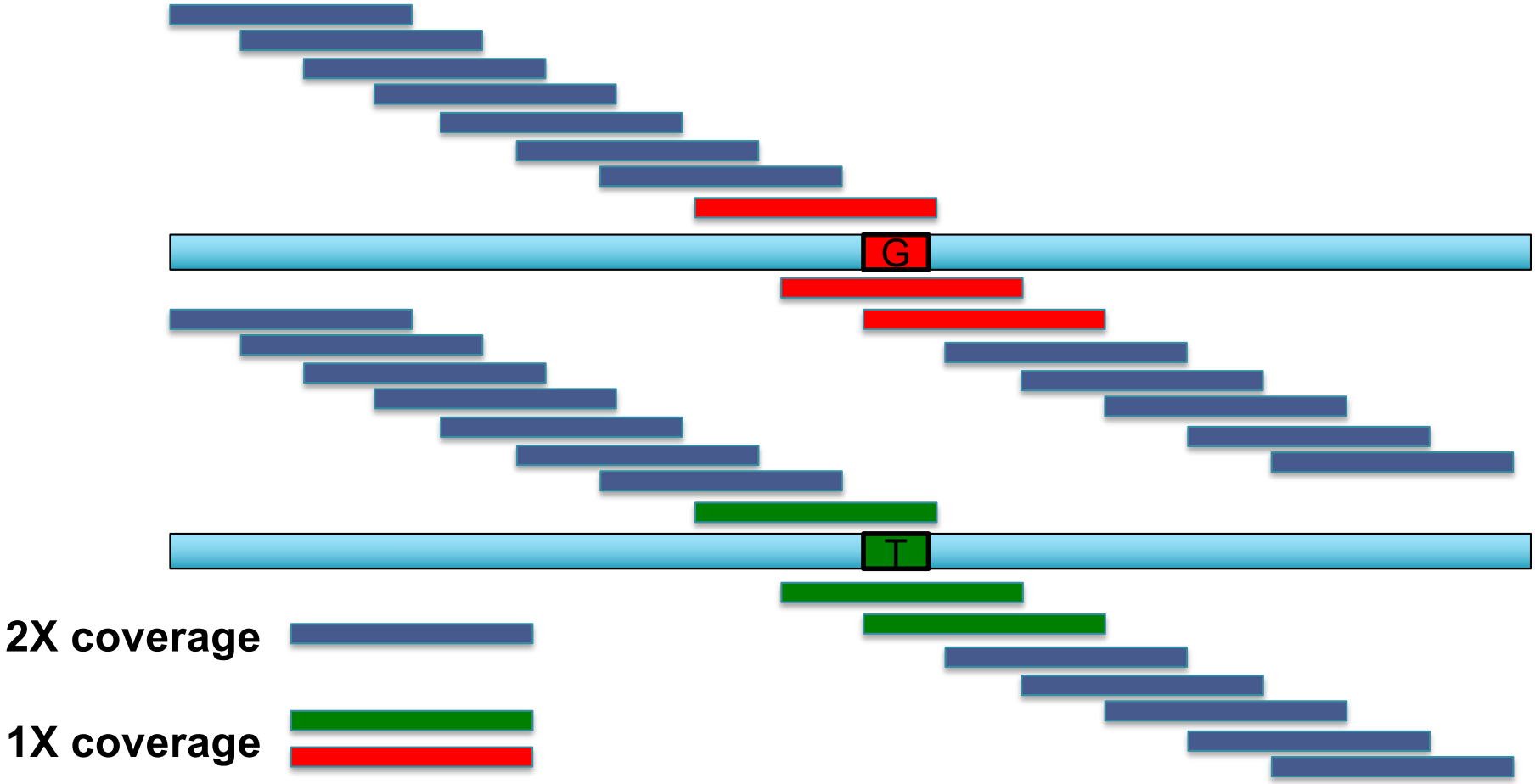
K-mer counting in heterozygous genomes



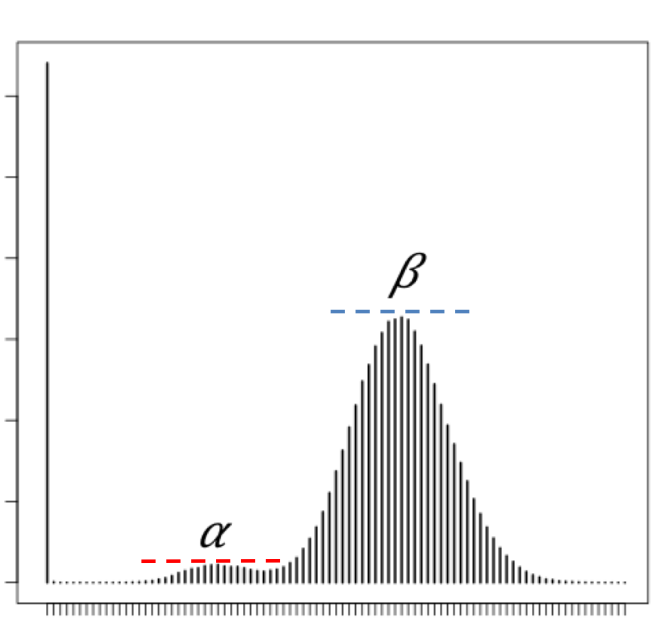
K-mer counting in heterozygous genomes



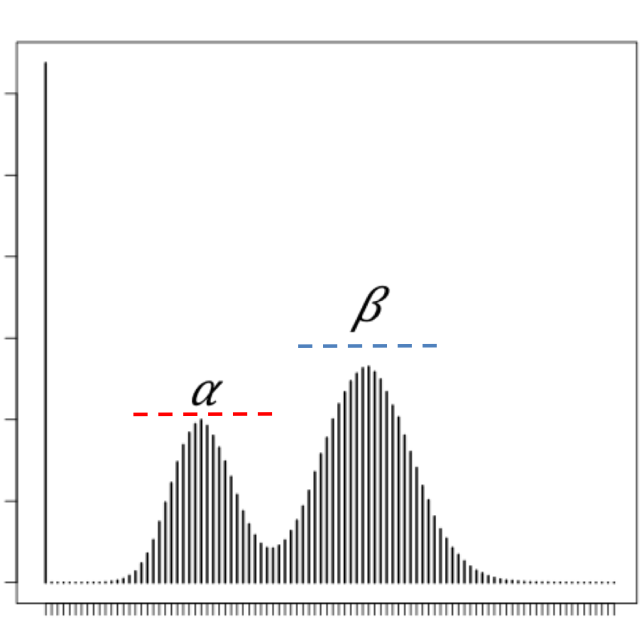
K-mer counting in heterozygous genomes



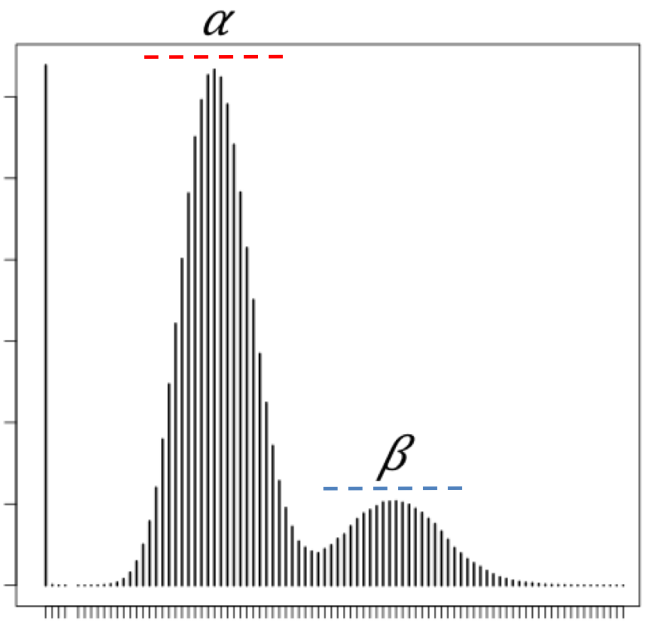
Heterozygous Kmer Profiles



0.1% heterozygosity



1% heterozygosity



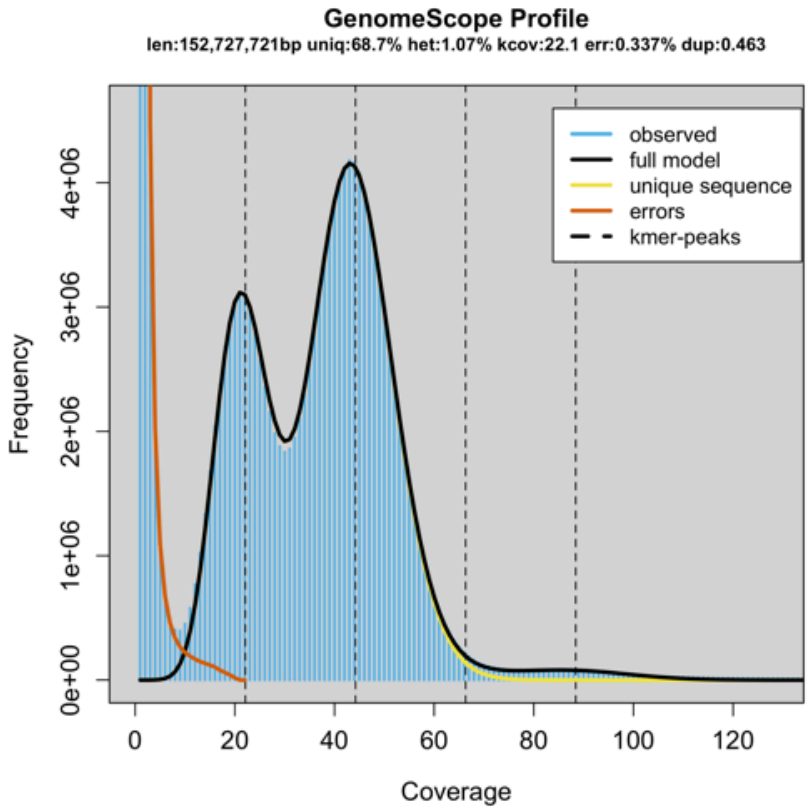
5% heterozygosity

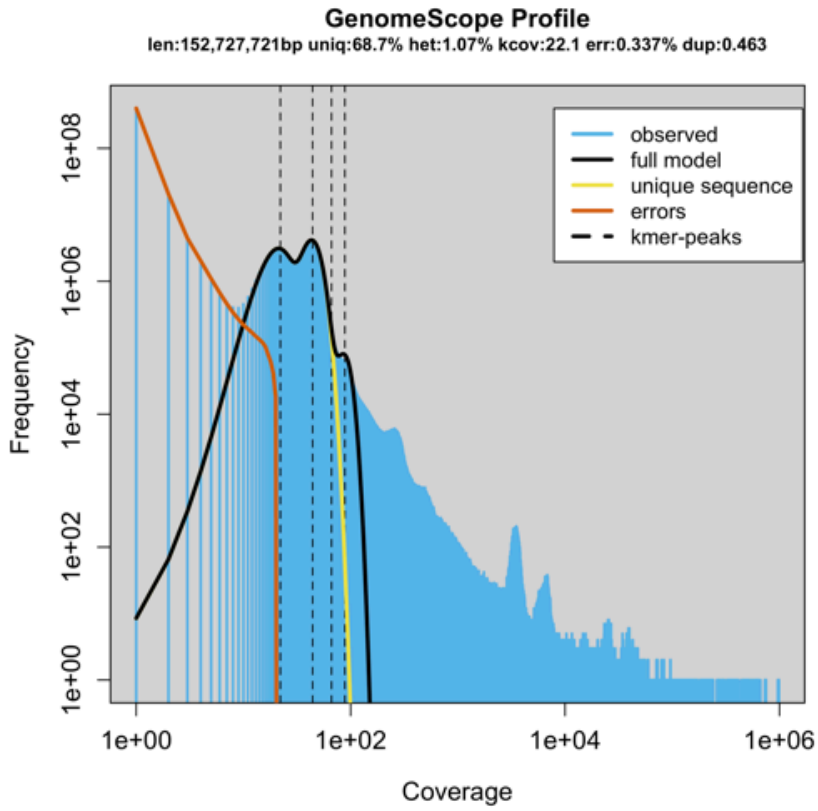
- ***Heterozygosity creates a characteristic "double-peak" in the Kmer profile***
  - Second peak at twice k-mer coverage as the first: heterozygous kmers average 50x coverage, homozygous kmers average 100x coverage

- ***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.* (2020) *Nature Communication*

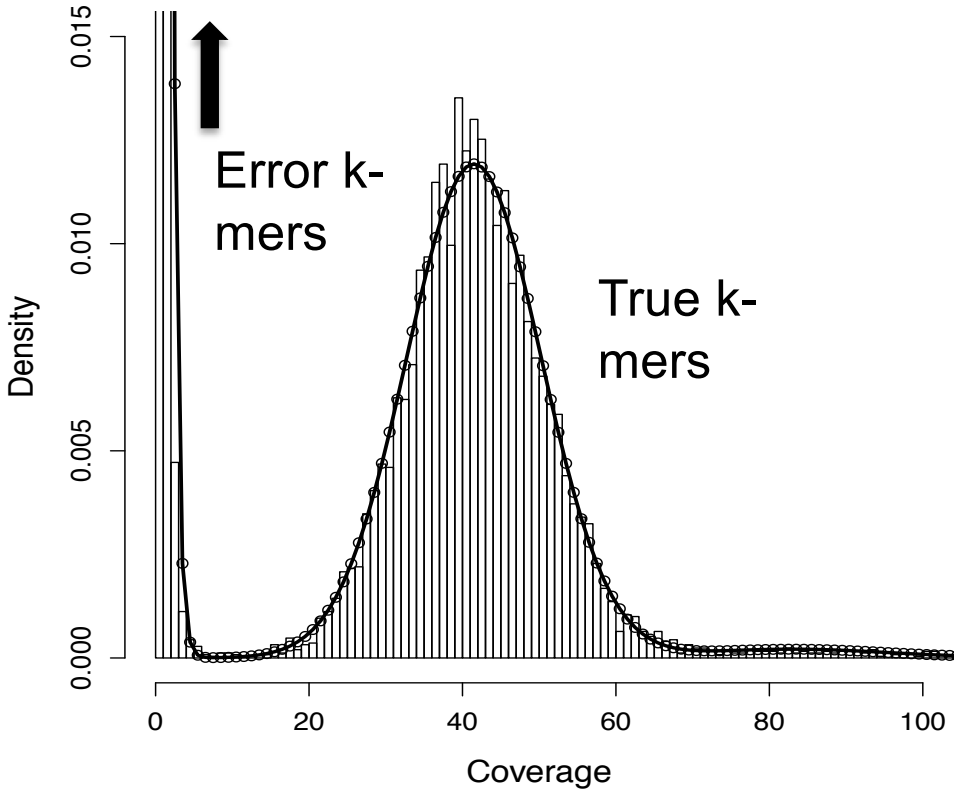
Error Correction with Quake

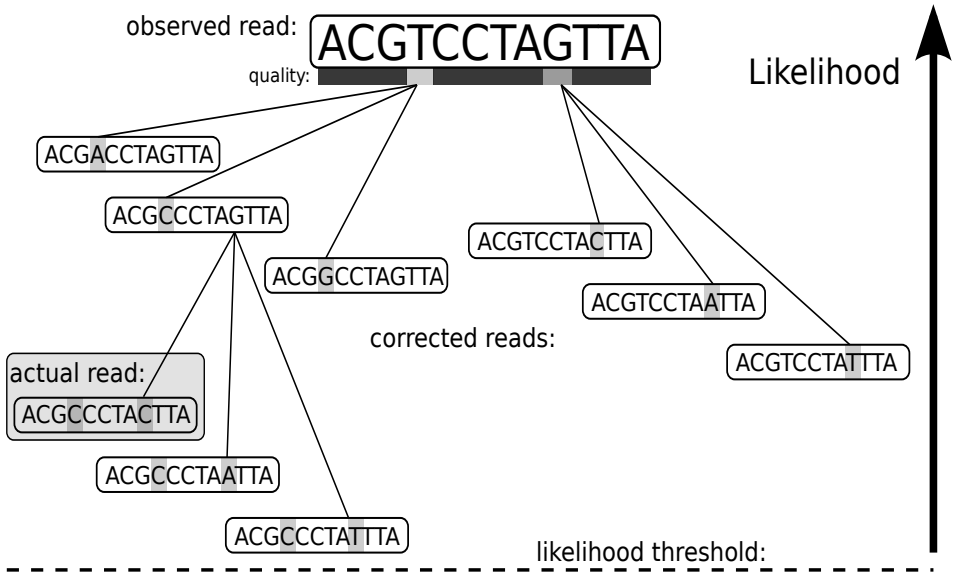
1. Count all "Q-mers" in reads

- 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



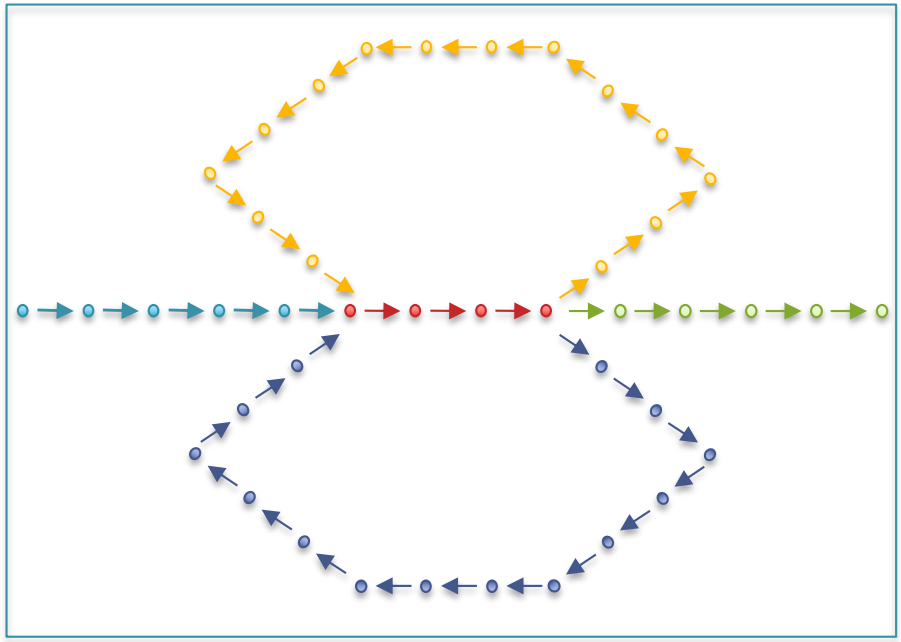


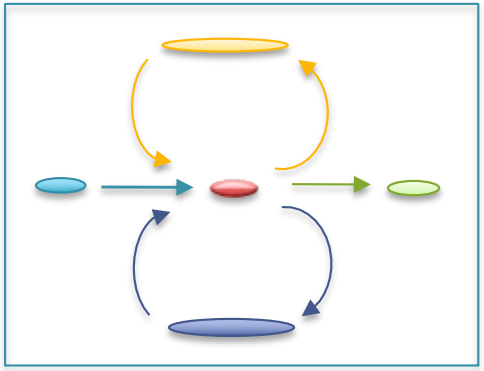
Quake: quality-aware detection and correction of sequencing reads.
Kelley, DR, Schatz, MC, Salzberg SL (2010) *Genome Biology*. 11:R116

Errors

Unitigging / Unipathing

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

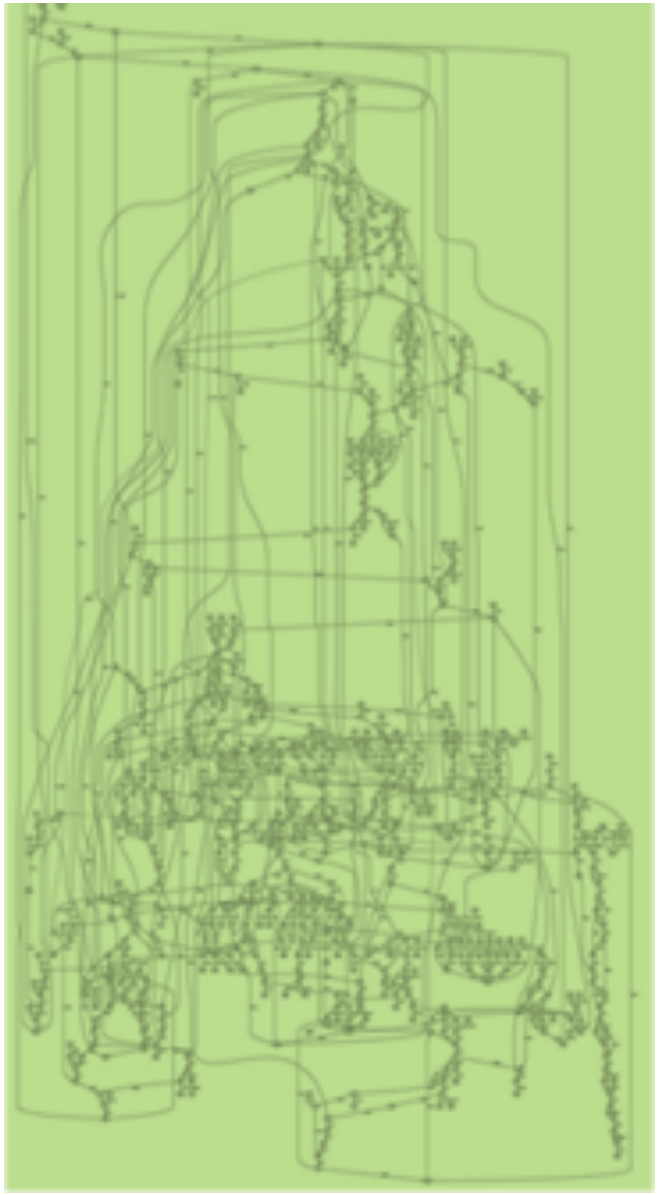




Why do contigs end?

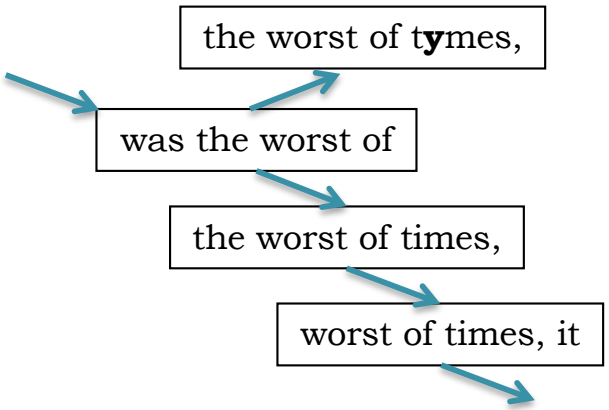
(1) End of chromosome! ☺, (2) lack of coverage, (3) errors,
(4) heterozygosity and (5) repeats

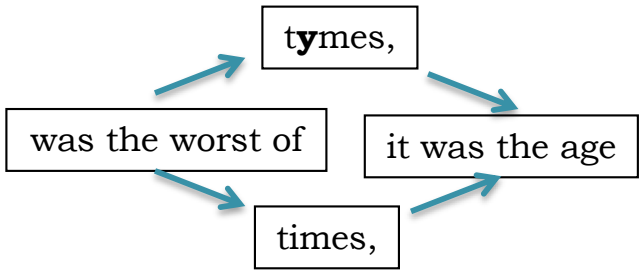
Errors in the graph



(Chaisson, 2009)

Clip Tips	Pop Bubbles
was the worst of times,	was the worst of times,
was the worst of t**y**mes,	was the worst of t**y**mes,
the worst of times, it	times, it was the age
	t**y**mes, it was the age



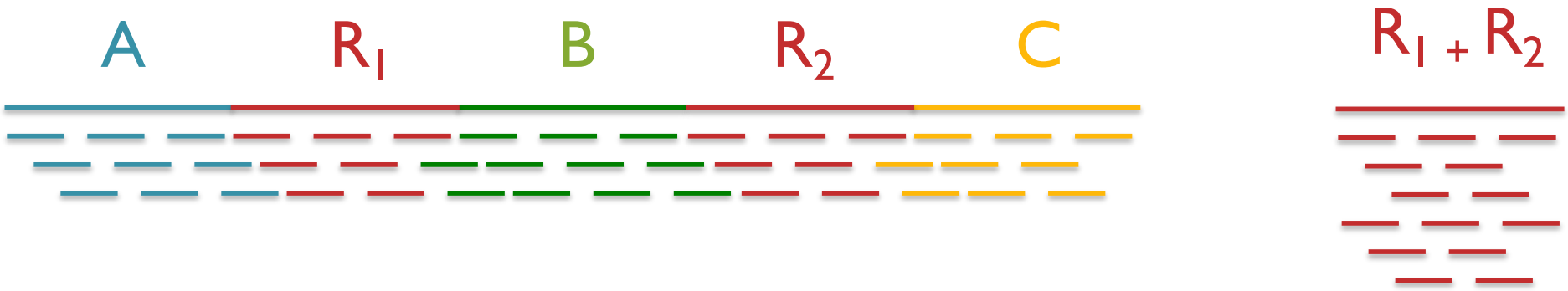


Repetitive regions

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.

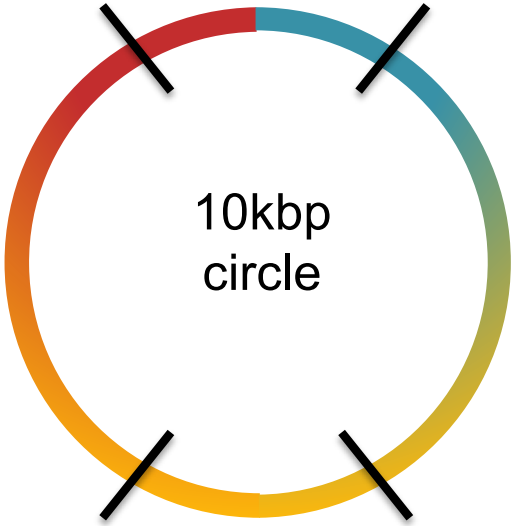
Paired-end and Mate-pairs

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

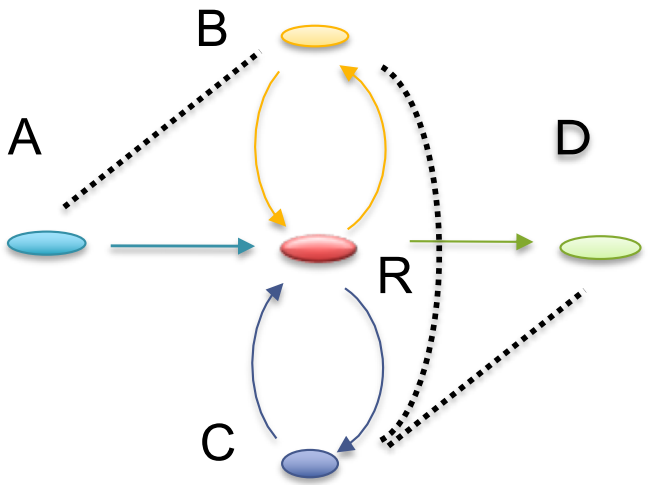
Mate-pair sequencing

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

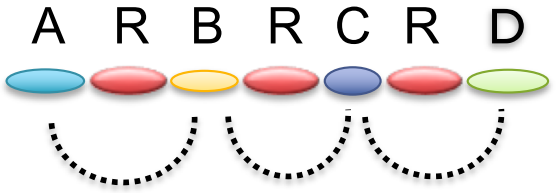


Scaffolding

- 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?

Assemblathon Results

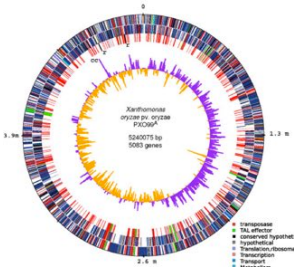
ID	Overall	CPNG50	SPNG50	Struct.	CC50	Subs.	Copy. Num.	Cov. Tot.	Cov. CDS
BGI	36	★					☆	★	☆
Broad	37	☆	★	★	★				
WTSI-S	46		★	☆	★	★			
CSHL	52	★							☆
BCCGSC	53							☆	★
DOEJGI	56		☆	★	☆	★			
RHUL	58								

- SOAPdenovo and ALLPATHS came out neck-and-neck followed closely behind by SGA, Celera Assembler, ABySS
- My recommendation for "typical" short read assembly is to use ALLPATHS or Spades

Assemblathon 1: A competitive assessment of de novo short read assembly methods

Earl et al. (2011) Genome Research. 21: 2224-2241

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

Next Steps

1. Reflect on the magic and power of DNA ☺
2. Check out the course webpage
3. Register on Piazza
4. Work on Assignment 1
    1. Set up Linux, set up Virtual Machine
    2. Set up Dropbox for yourself!
    3. Get comfortable on the command line