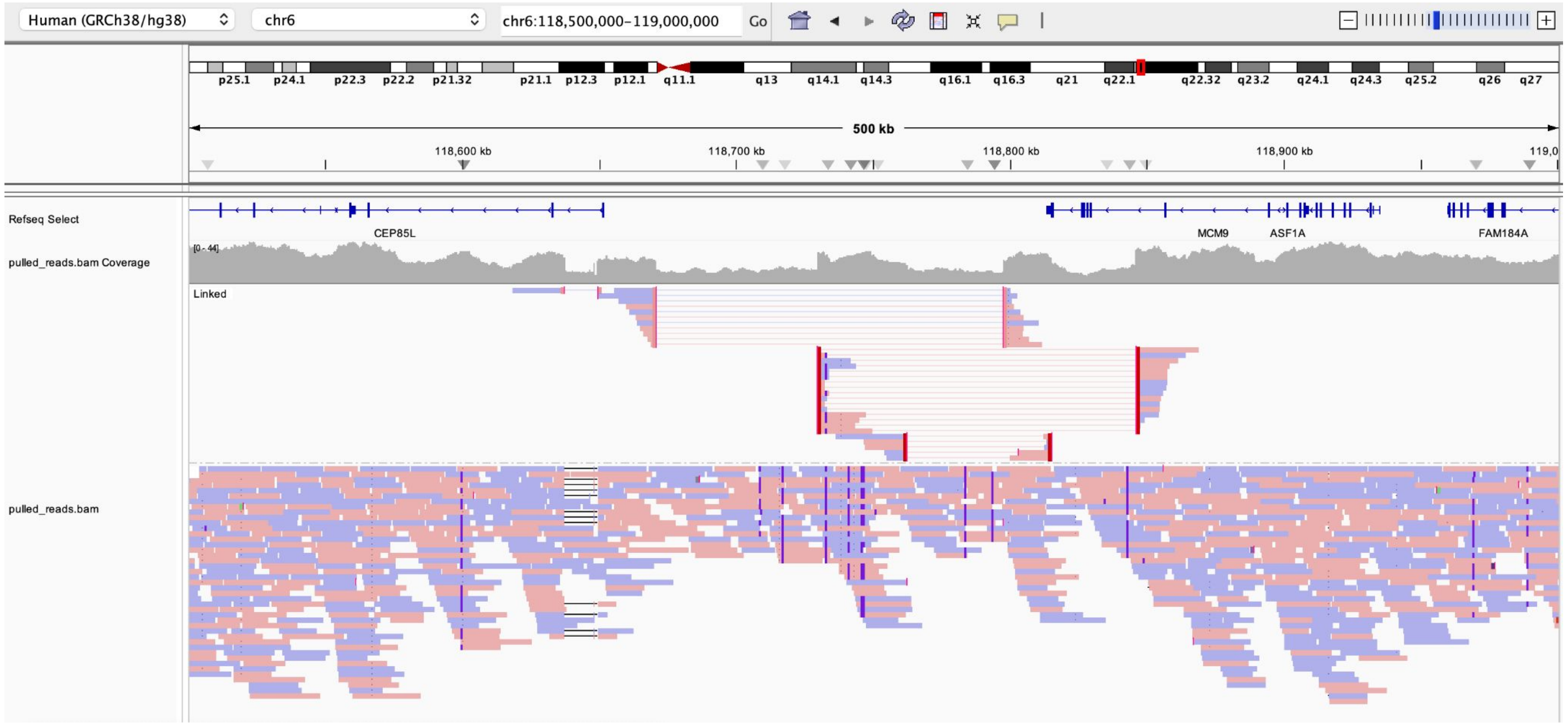
How to use long reads for de-novo assembly

Wolfram Höps
Radboudumc Nijmegen

Complex SVs can be hard to interpret from aligned reads

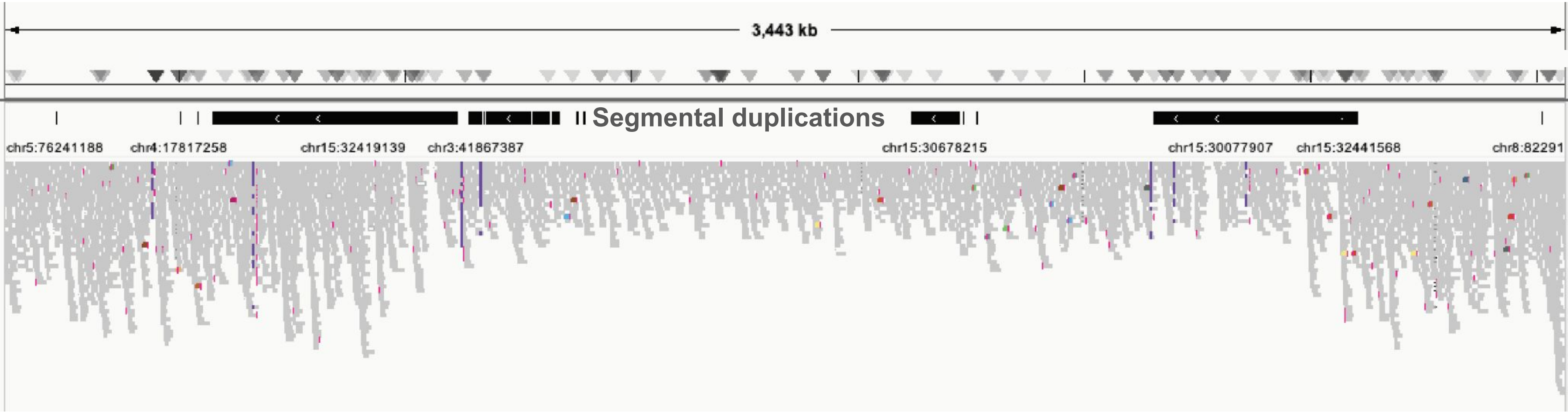


Human (GRCh38/hg38)
chr6
chr6:118,500,000-119,000,000
Go
p25.1 p24.1 p22.3 p22.2 p21.32 p21.1 p12.3 p12.1 q11.1 q13 q14.1 q14.3 q16.1 q16.3 q21 q22.1 q22.32 q23.2 q24.1 q24.3 q25.2 q26 q27
500 kb
118,600 kb
118,700 kb
118,800 kb
118,900 kb
119,0
Refseq Select
CEP85L
MCM9
ASF1A
FAM184A
pulled_reads.bam Coverage
[0 - 44]
Linked
pulled_reads.bam

Several Structural Variants are visible, but which genes are affected?

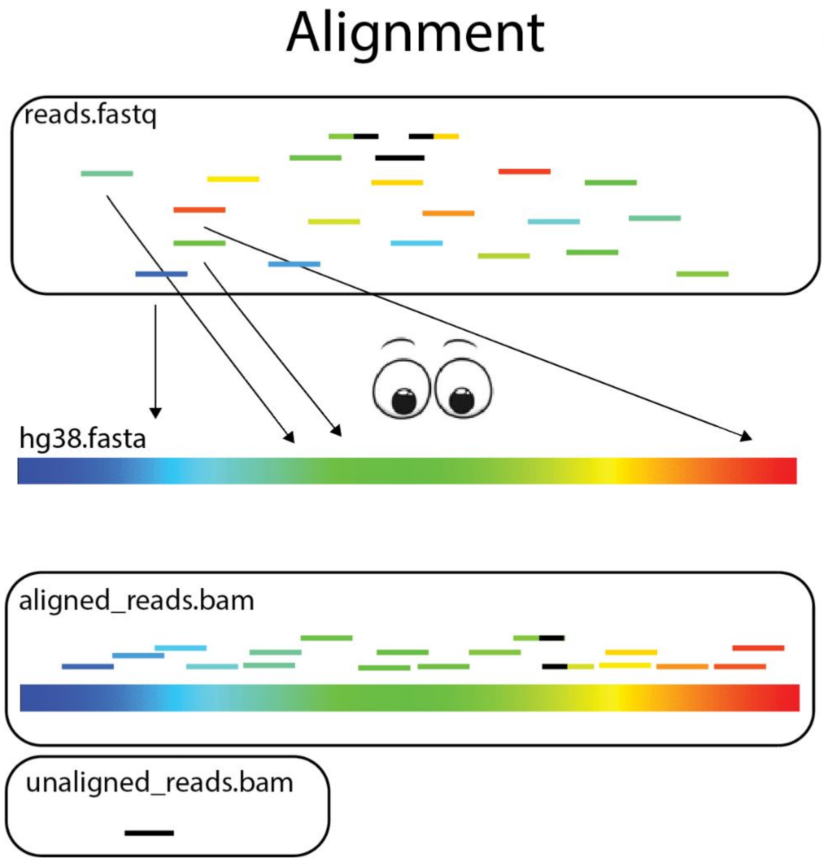
30-fold Hifi patient dataset: [Höps et al. 2025]

Complex loci can be hard to interpret from aligned reads

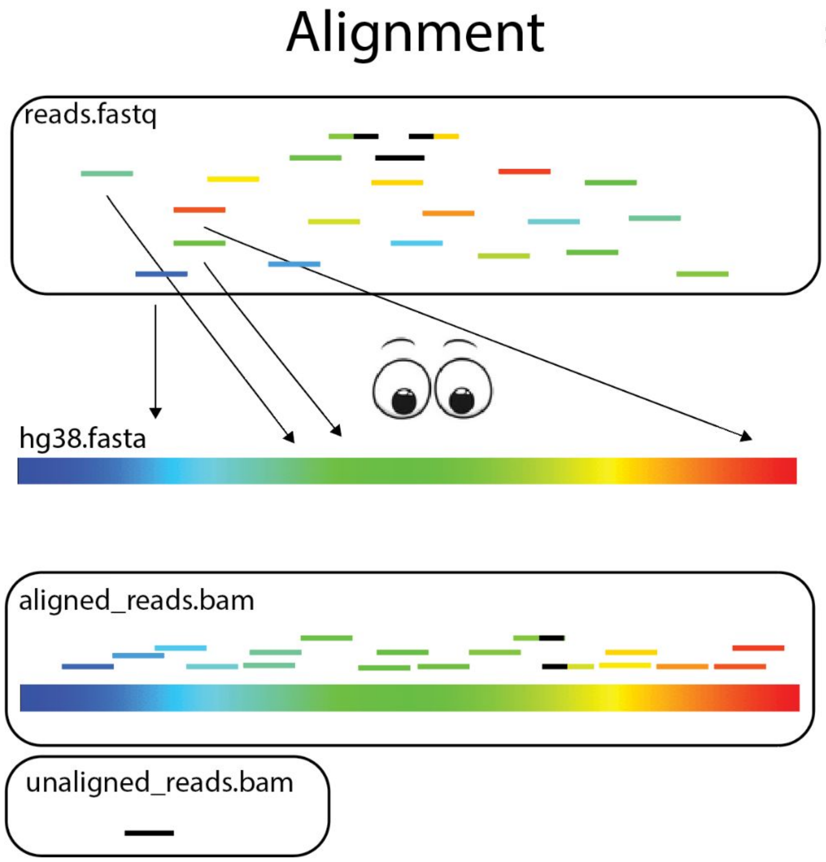


A multi-Mbp-sized deletion is visible, but where are the breakpoints?

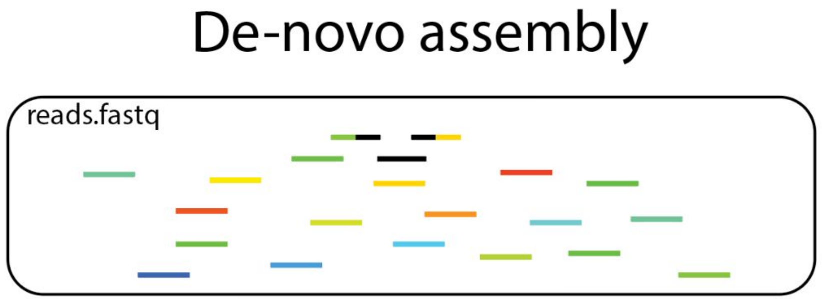
Read alignment != de-novo assembly



Read alignment != de-novo assembly

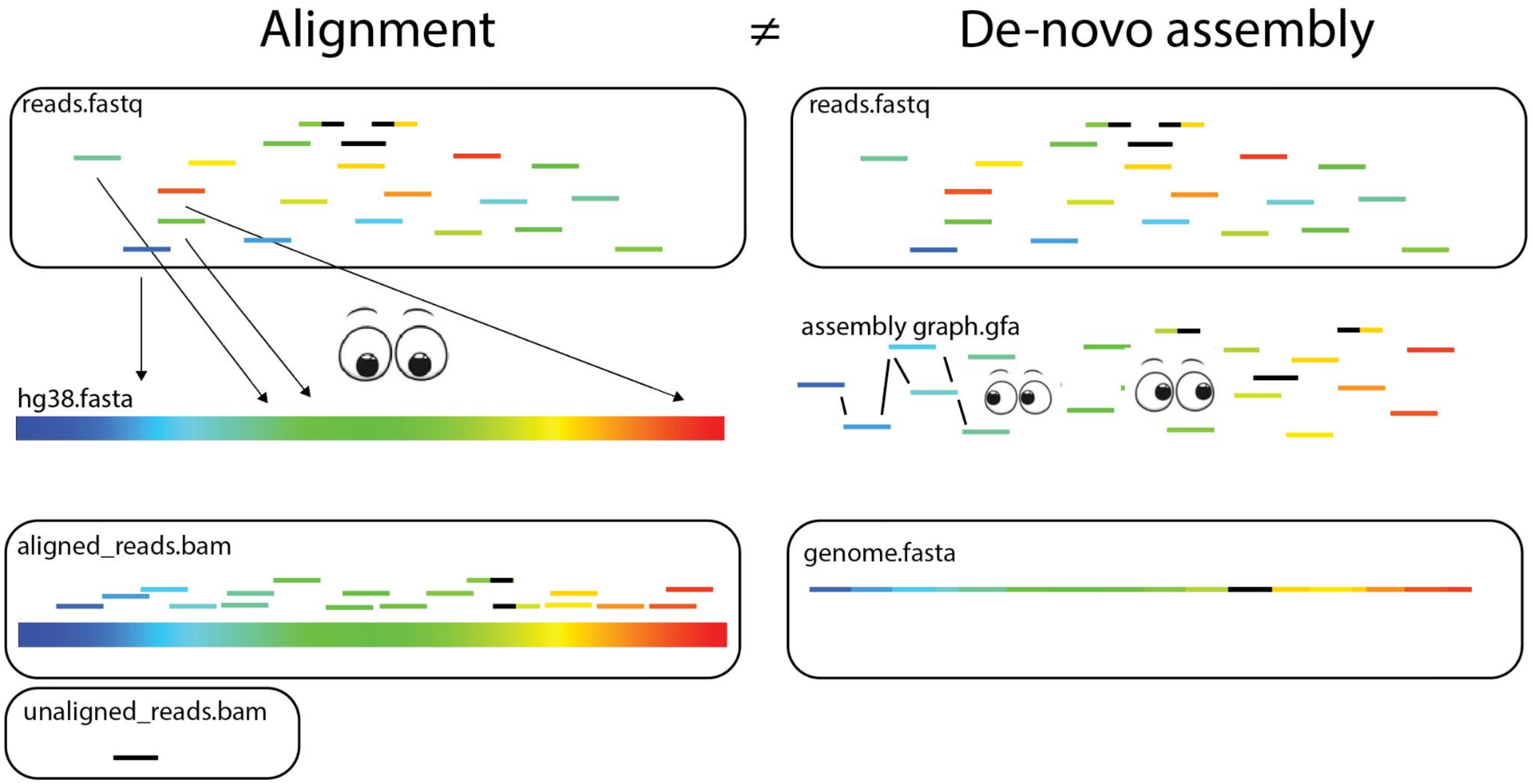


$\neq$



Own figure

Read alignment != de-novo assembly



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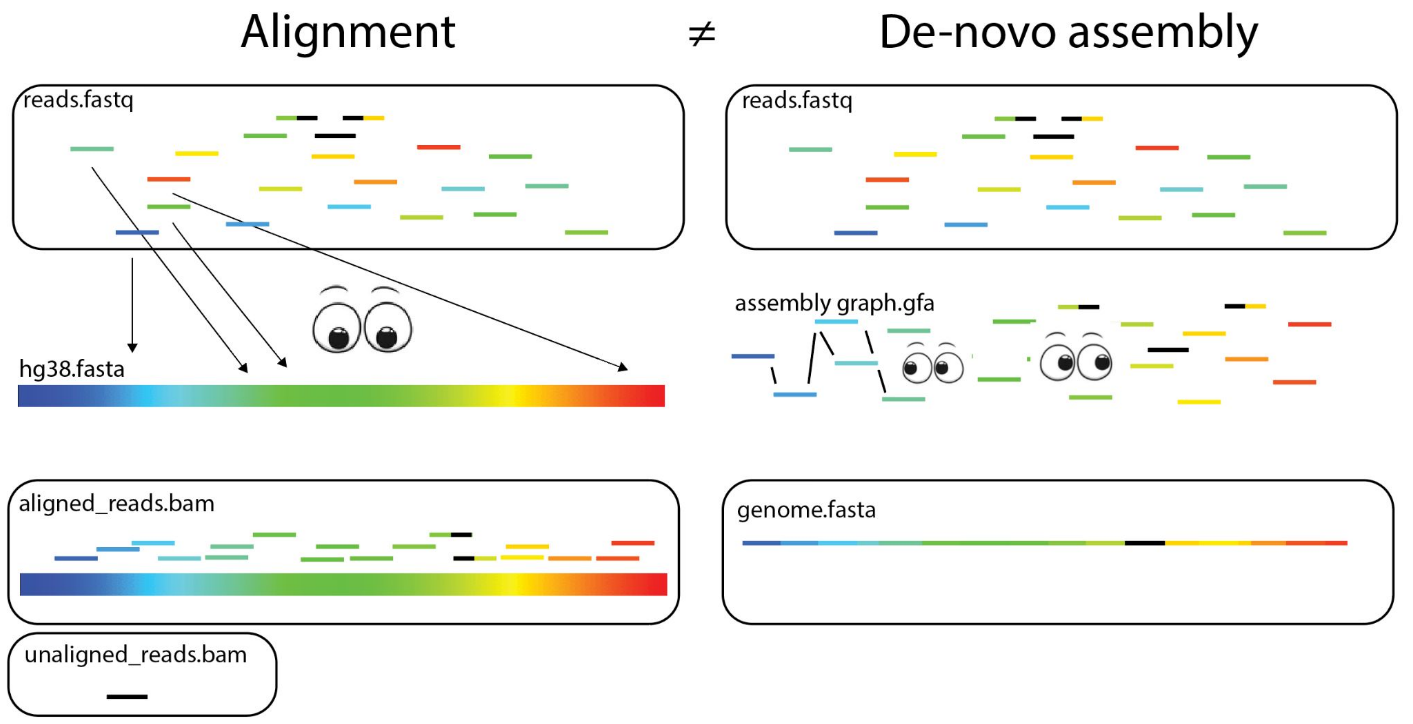
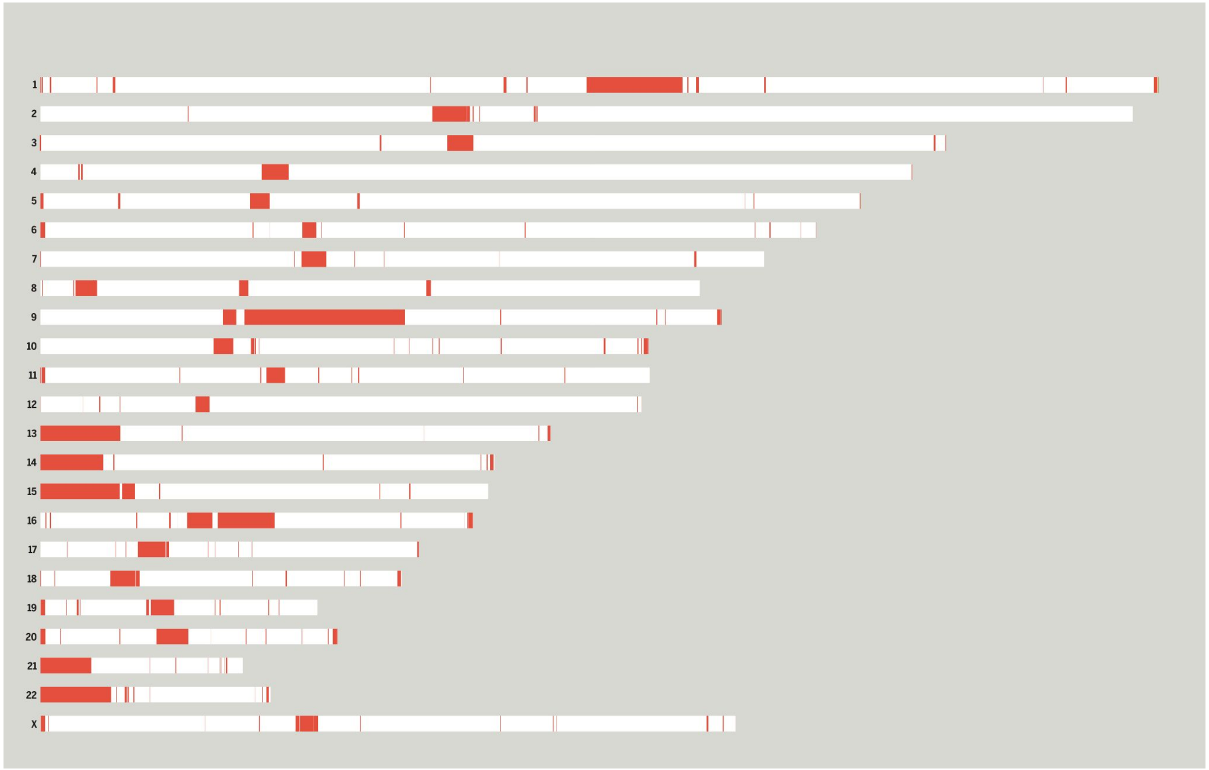


Requires Reference Genome
Short variant calling in non-complex regions
Computationally light (hours)
Automated, easy to use

No Reference required (though sometimes useful)
Long, complex variants in hard-to-map regions
Computationally heavy (hours - days)
Pitfalls, hard to use

Own figure

The telomere-to-telomere reference **assembly**

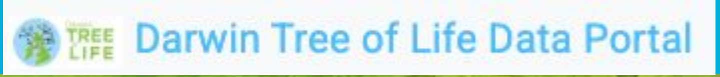


red: regions previously (hg38) unresolved

T2T consortium, 2021

Darwin Tree of Life Data Portal

Portal.darwintreeoflife.org



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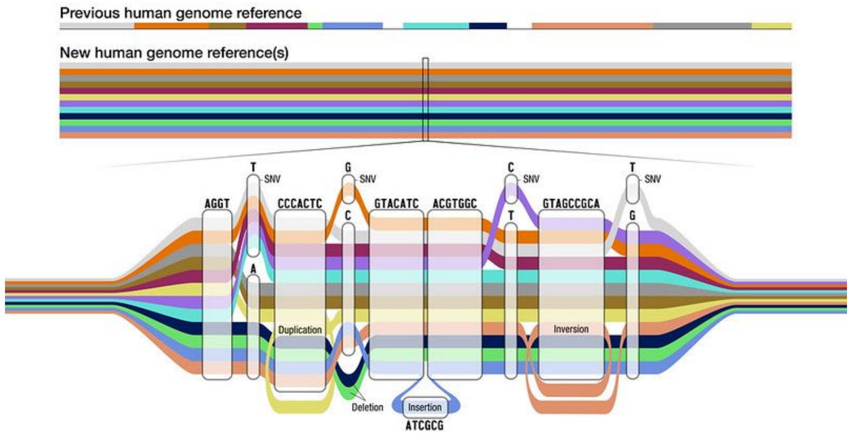
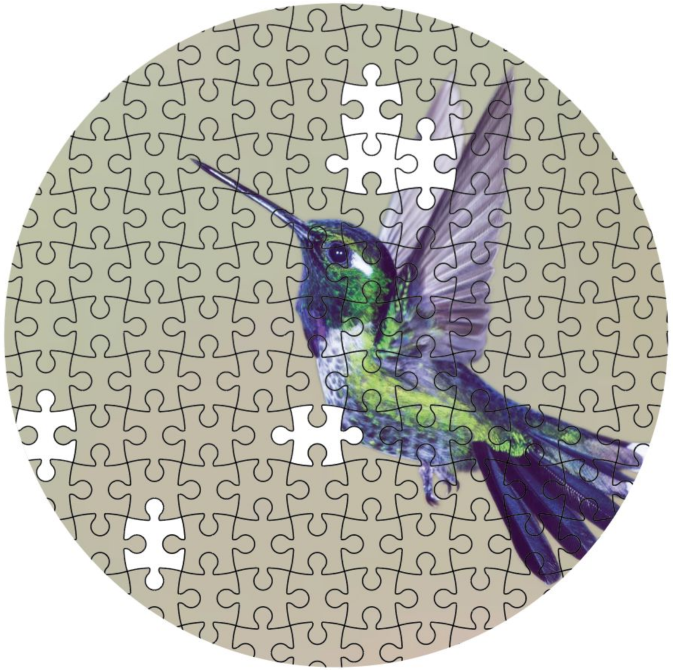


Human Pangenome Reference Consortium:
232 individuals fully assembled (-> 464 ‘genomes’)

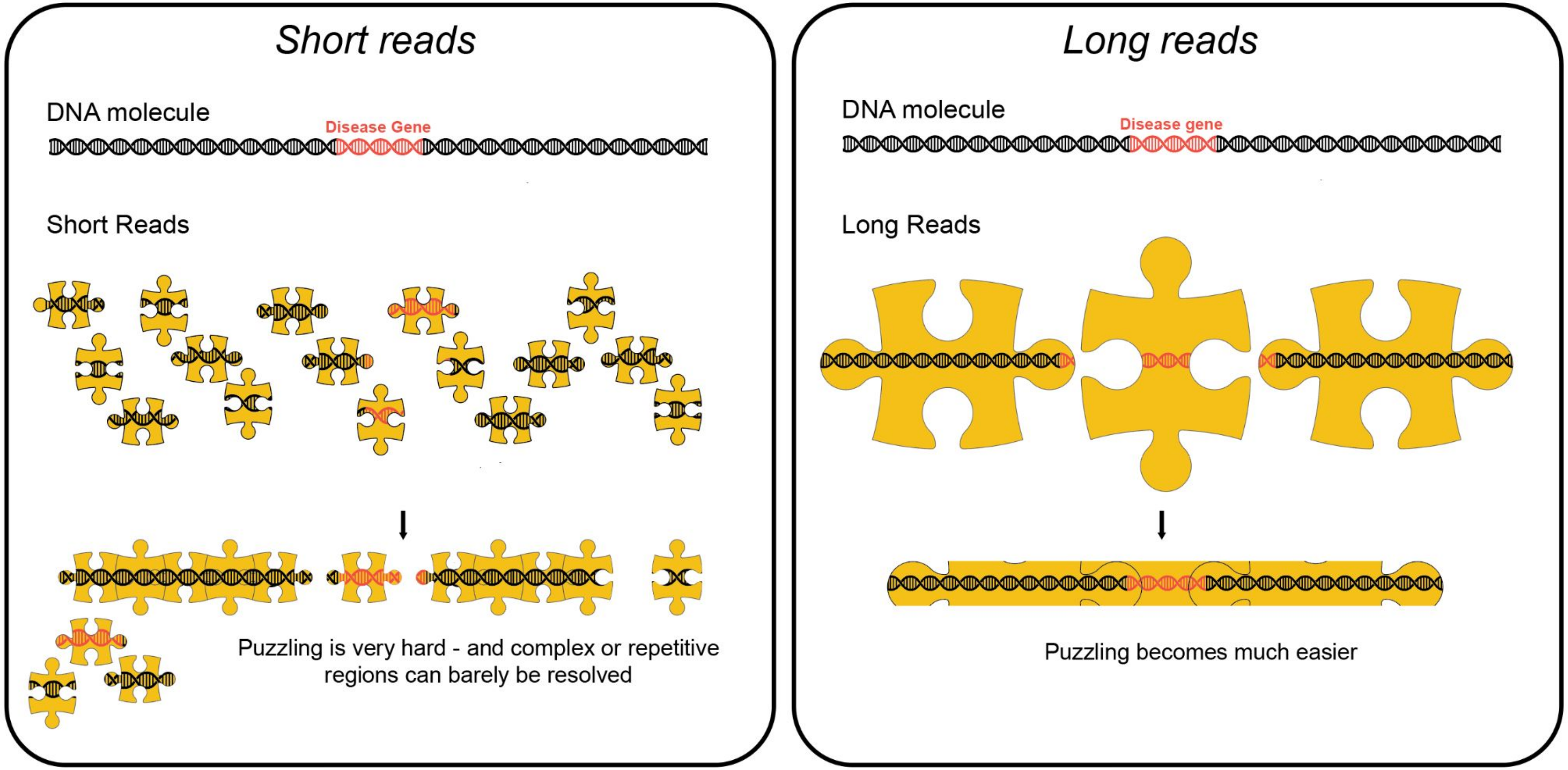
Liao et al. 2023

The assembly ‘hype’ is technology-driven



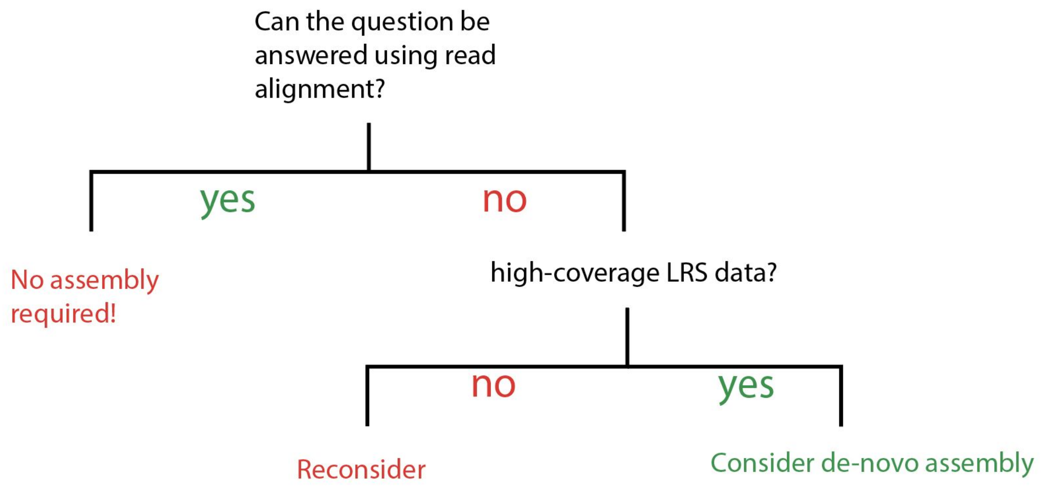
Own figure

The assembly ‘hype’ is technology-driven


Short reads
DNA molecule
Disease Gene
Short Reads
Puzzling is very hard - and complex or repetitive regions can barely be resolved
Long reads
DNA molecule
Disease gene
Long Reads
Puzzling becomes much easier

Own figure

De-novo assembly of whole human genomes or smaller loci is now feasible for well-equipped labs


Can the question be answered using read alignment?
yes
no
No assembly required!
high-coverage LRS data?
no
yes
Reconsider
Consider de-novo assembly

Typical applications in human genetics:

- resolve structural variations (SVs) longer than the read length
- resolve variants in or near unmappable or highly diverse genomic regions
- resolve complex genomic regions

Data requirements

Required

>15-fold accurate long reads per haplotype is needed.

- PacBio HiFi reads
- ONT (Ultra long)

Best case: both

Other data types can improve phasing and contiguity:

- Parental data
- Hi-C
- Strand-Seq
- Optical genome mapping

Technical background

Phased assembly of diploid genomes

Some commonly used assembly tools for human genomes:
Verkko [Rautainen et al. 2023], **Hifiasm** [Cheng et al. 2021], **Flye** [Kolmogorov et al. 2020]

Phased assembly of diploid genomes

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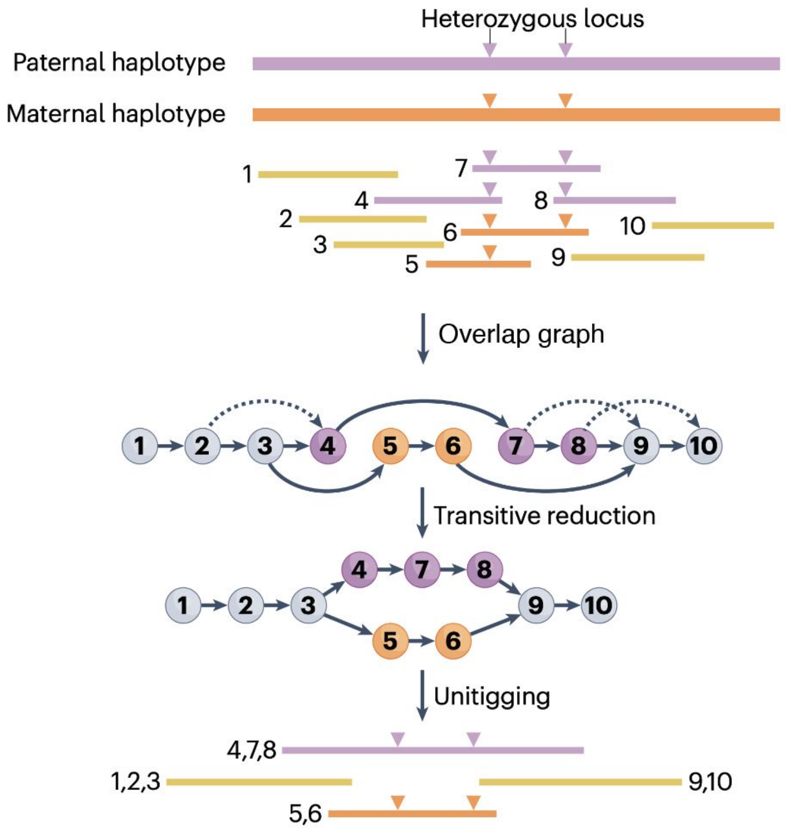
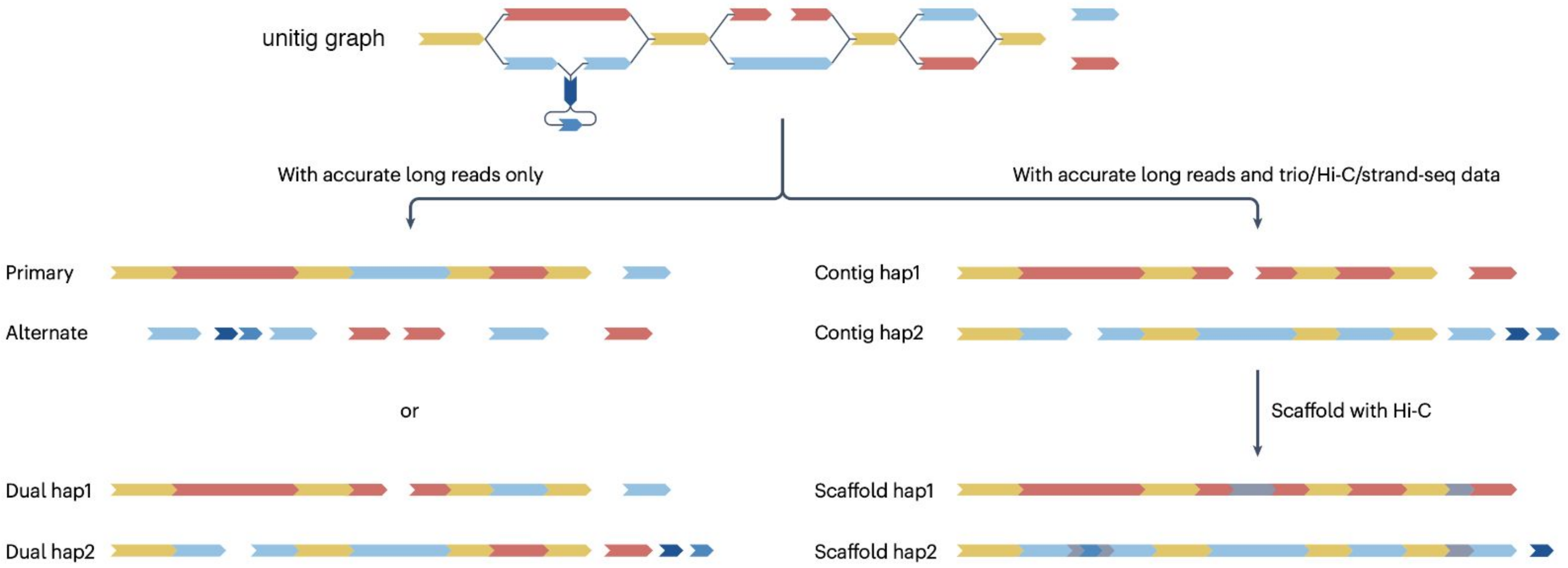


Hifiasm: assembly with overlap graphs
[adapted from Li & Durbin 2024]

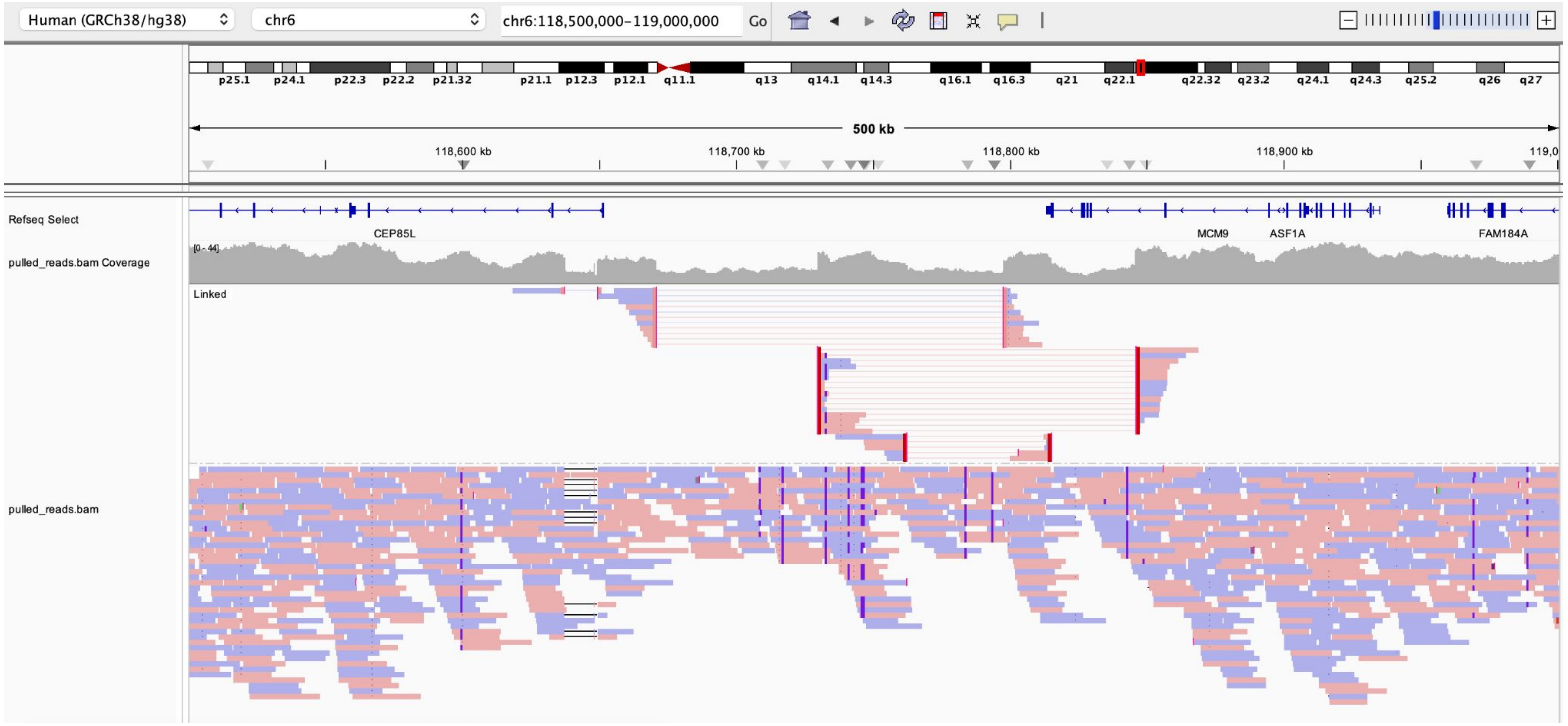
From unitigs to phased assembly



adapted from Li & Durbin 2024

Hands on: let’s assemble something

An example, HiFi-only assembly



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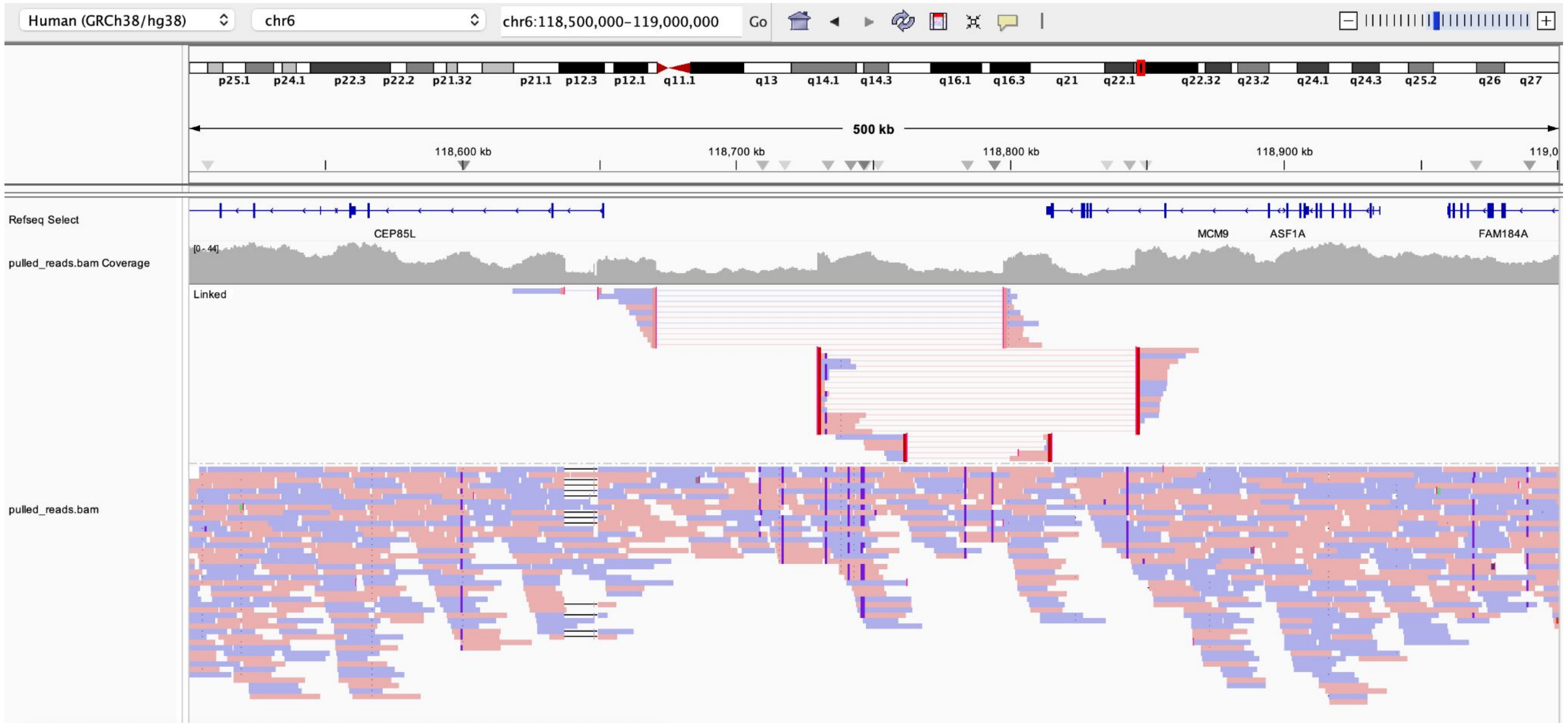
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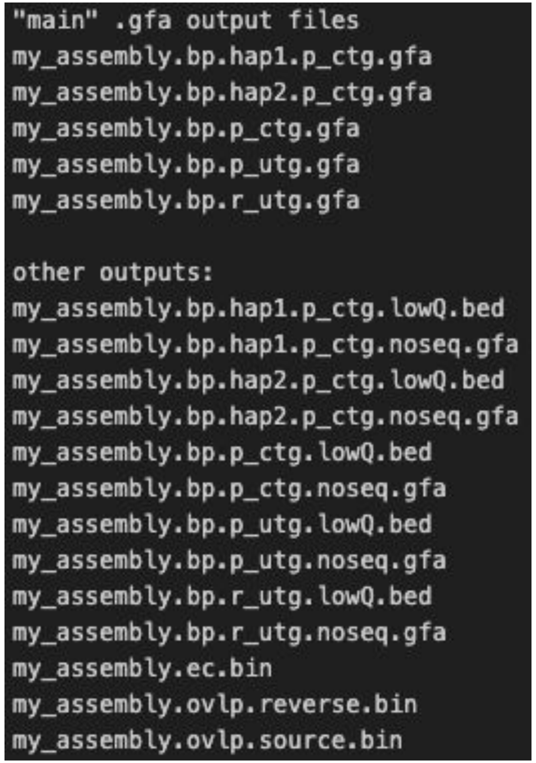
500 kb
118,600 kb
118,700 kb
118,800 kb
118,900 kb
Refseq Select
CEP85L
MCM9
ASF1A
FAM184A
pulled_reads.bam Coverage
[0 - 44]
Linked
pulled_reads.bam

```
samtools view sample.bam “chr6:117000000-120000000” -b > reads_region.bam
samtools fastq sample_local.bam > reads_region.fastq
hifiasm reads_region.fastq -o my_assembly  #<- here, we could include ONT, parental reads or  Hi-C
```

(all code on github!)

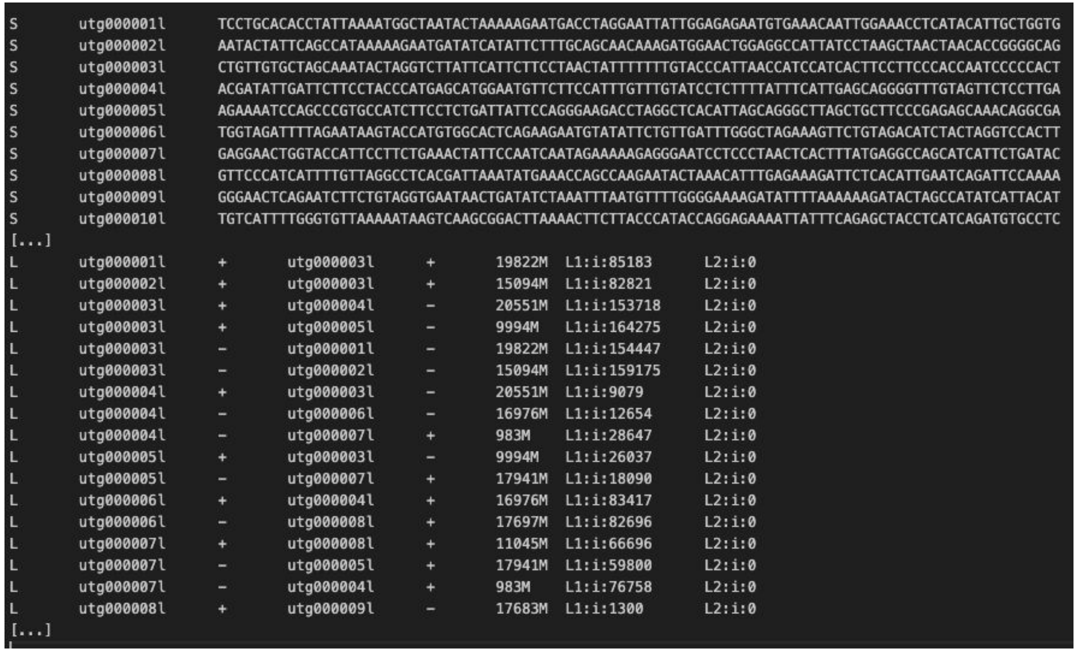
gfa: graphical fragment assembly files

hifiasm outputs:



```
"main" .gfa output files
my_assembly.bp.hap1.p_ctg.gfa
my_assembly.bp.hap2.p_ctg.gfa
my_assembly.bp.p_ctg.gfa
my_assembly.bp.p_utg.gfa
my_assembly.bp.r_utg.gfa

other outputs:
my_assembly.bp.hap1.p_ctg.lowQ.bed
my_assembly.bp.hap1.p_ctg.noseq.gfa
my_assembly.bp.hap2.p_ctg.lowQ.bed
my_assembly.bp.hap2.p_ctg.noseq.gfa
my_assembly.bp.p_ctg.lowQ.bed
my_assembly.bp.p_ctg.noseq.gfa
my_assembly.bp.p_utg.lowQ.bed
my_assembly.bp.p_utg.noseq.gfa
my_assembly.bp.r_utg.lowQ.bed
my_assembly.bp.r_utg.noseq.gfa
my_assembly.ec.bin
my_assembly.ovlp.reverse.bin
my_assembly.ovlp.source.bin
```

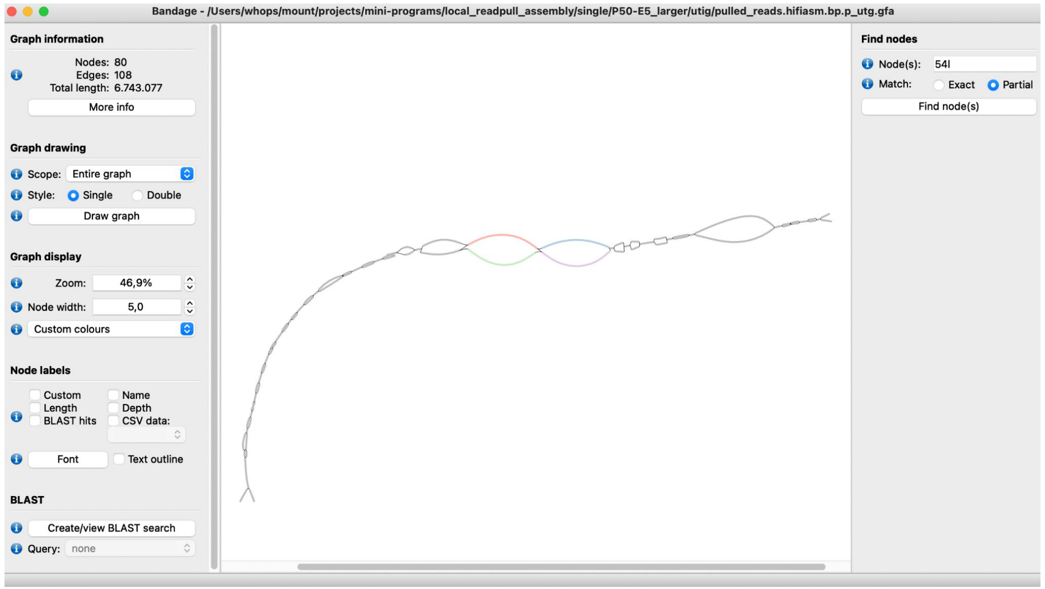


```
S       utg000001l      TCCTGCACACCTATTAAAATGGCTAATACTAAAAAGAATGACCTAGGAATTATTGGAGAGAATGTGAAACAATTGGAAACCTCATACATTGCTGGTG
S       utg000002l      AATACTATTCAGCCATAAAAAGAATGATATCATATTCTTTGCAGCAACAAAGATGGAACTGGAGGCCATTATCCTAAGCTAACTAACACCGGGGCAG
S       utg000003l      CTGTTGTGCTAGCAAATACTAGGTCTTATTCATTCTTCCTAACTATTTTTTTGTACCCATTAACCATCCATCACTTCCTTCCCACCAATCCCCCACT
S       utg000004l      ACGATATTGATTCTTCCTACCCATGAGCATGGAATGTTCTTCCATTTGTTTGTATCCTCTTTTATTTCATTGAGCAGGGGTTTGTAGTTCTCCTTGA
S       utg000005l      AGAAAATCCAGCCCGTGCCATCTTCCTCTGATTATTCCAGGGAAGACCTAGGCTCACATTAGCAGGGCTTAGCTGCTTCCCGAGAGCAAACAGGCGA
S       utg000006l      TGGTAGATTTTAGAATAAGTACCATGTGGCACTCAGAAGAATGTATATTCTGTTGATTTGGGCTAGAAAGTTCTGTAGACATCTACTAGGTCCACTT
S       utg000007l      GAGGAACTGGTACCATTCCTTCTGAAACTATTCCAATCAATAGAAAAAGAGGGAATCCTCCCTAACTCACTTTATGAGGCCAGCATCATTCTGATAC
S       utg000008l      GTTCCCATCATTTTGTTAGGCCTCACGATTAAATATGAAACCAGCCAAGAATACTAAACATTTGAGAAAGATTCTCACATTGAATCAGATTCCAAAA
S       utg000009l      GGGAACTCAGAATCTTCTGTAGGTGAATAACTGATATCTAAATTTAATGTTTTGGGGAAAAGATATTTTAAAAAAGATACTAGCCATATCATTACAT
S       utg000010l      TGTCATTTTGGGTGTTAAAAATAAGTCAAGCGGACTTAAAACTTCTTACCCATACCAGGAGAAAATTATTTCAGAGCTACCTCATCAGATGTGCCTC
[...]
L       utg000001l      +       utg000003l      +       19822M  L1:i:85183      L2:i:0
L       utg000002l      +       utg000003l      +       15094M  L1:i:82821      L2:i:0
L       utg000003l      +       utg000004l      -       20551M  L1:i:153718     L2:i:0
L       utg000003l      +       utg000005l      -       9994M   L1:i:164275     L2:i:0
L       utg000003l      -       utg000001l      -       19822M  L1:i:154447     L2:i:0
L       utg000003l      -       utg000002l      -       15094M  L1:i:159175     L2:i:0
L       utg000004l      +       utg000003l      -       20551M  L1:i:9079       L2:i:0
L       utg000004l      -       utg000006l      -       16976M  L1:i:12654      L2:i:0
L       utg000004l      -       utg000007l      +       983M    L1:i:28647      L2:i:0
L       utg000005l      +       utg000003l      -       9994M   L1:i:26037      L2:i:0
L       utg000005l      -       utg000007l      +       17941M  L1:i:18090      L2:i:0
L       utg000006l      +       utg000004l      +       16976M  L1:i:83417      L2:i:0
L       utg000006l      -       utg000008l      +       17697M  L1:i:82696      L2:i:0
L       utg000007l      +       utg000008l      +       11045M  L1:i:66696      L2:i:0
L       utg000007l      -       utg000005l      +       17941M  L1:i:59800      L2:i:0
L       utg000007l      -       utg000004l      +       983M    L1:i:76758      L2:i:0
L       utg000008l      +       utg000009l      -       17683M  L1:i:1300       L2:i:0
[...]
```

S: Segment / Sequence
L: Link

Assembly graph visualization with *Bandage*

bandage -> load graph…. -> my_assembly.p_utig.gfa



Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/single/P50-E5_larger/utig/pulled_reads.hifiasm.bp.p_utg.gfa

Graph information

Nodes: 80
Edges: 108
Total length: 6.743.077

More info

Graph drawing

Scope: Entire graph

Style: [✓ Single] Double

Draw graph

Graph display

Zoom: 46,9%

Node width: 5,0

Custom colours

Node labels

Custom, Name, Length, Depth, BLAST hits, CSV data:

Font, Text outline

BLAST

Create/view BLAST search

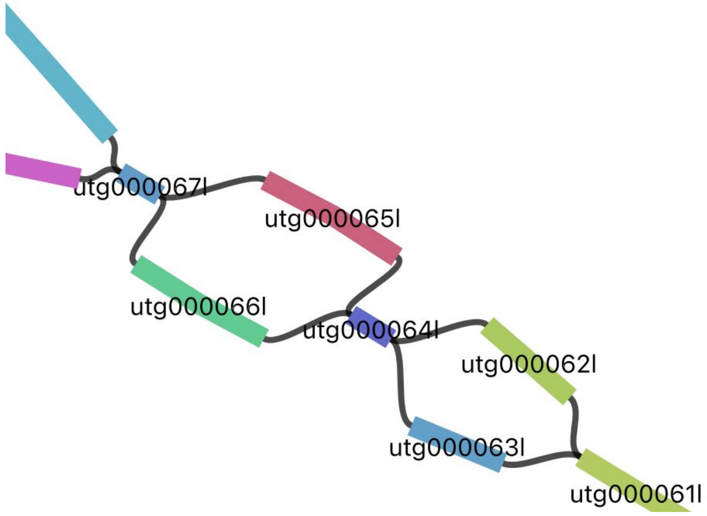
Query: none

Find nodes

Node(s): 54l

Match: Exact [✓ Partial]

Find node(s)

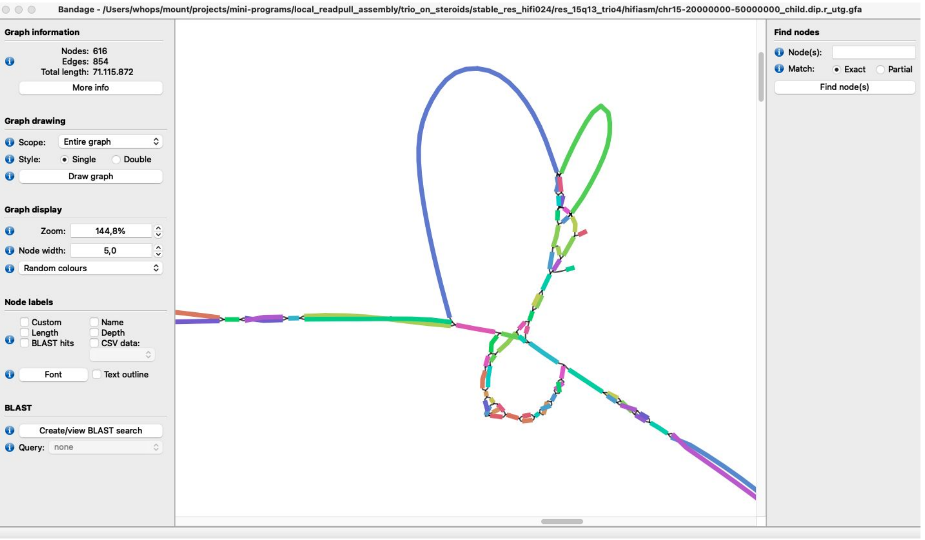


nodes: Segments (“utigs”)
connections: Links

Excursion: interpreting assembly (unitig / contig) graphs

Excursion: Understanding assembly graphs (.gfa)

HiFi reads; 5Mbp repeat-rich region


Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/trio_on_steroids/stable_res_hifi024/res_15q13_trio4/hifiasm/chr15-20000000-50000000_child.dip.r_utg.gfa

Graph information

Nodes: 616
Edges: 854
Total length: 71.115.872

Graph drawing

Scope: Entire graph
Style: [✓ Single] Double

Graph display

Zoom: 144,8%
Node width: 5,0
Random colours

Node labels

Custom, Length, BLAST hits, Name, Depth, CSV data:

BLAST

Query: none

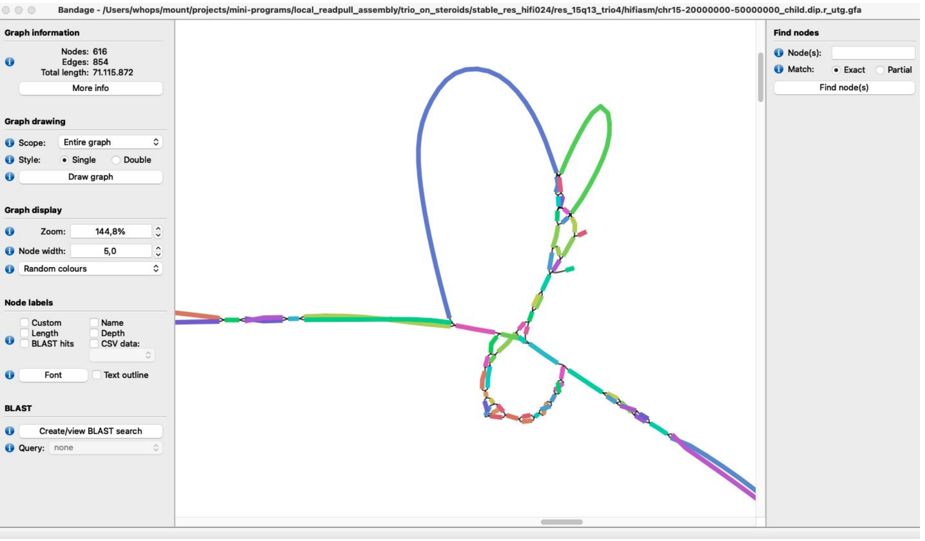
Find nodes

Node(s):
Match: [✓ Exact] Partial

Adverse signs:
- branching / tangles ⭕
- 'tips': loose ends ⭕

Excursion: Understanding assembly graphs (.gfa)

HiFi reads; 5Mbp repeat-rich region



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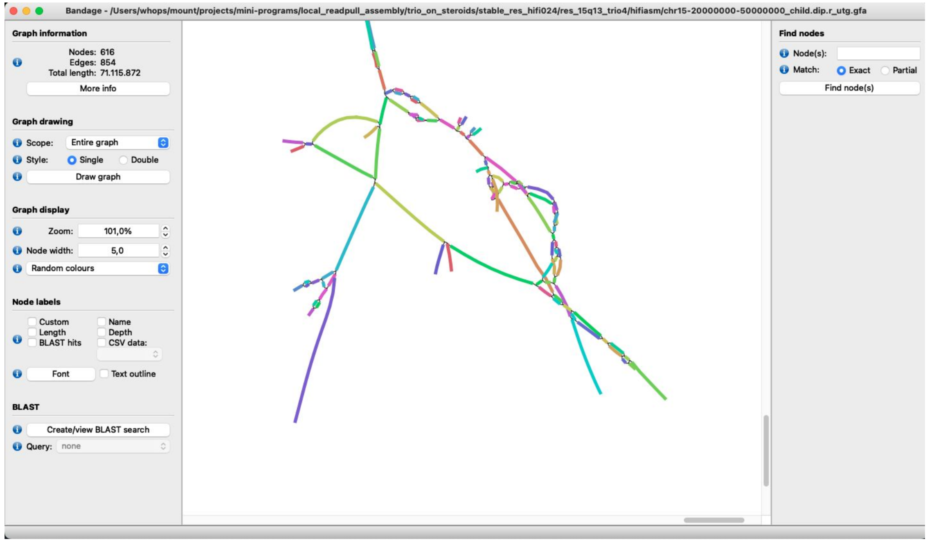
HiFi reads; (peri) centromeric region on chr15



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Excursion: Understanding assembly graphs (.gfa)

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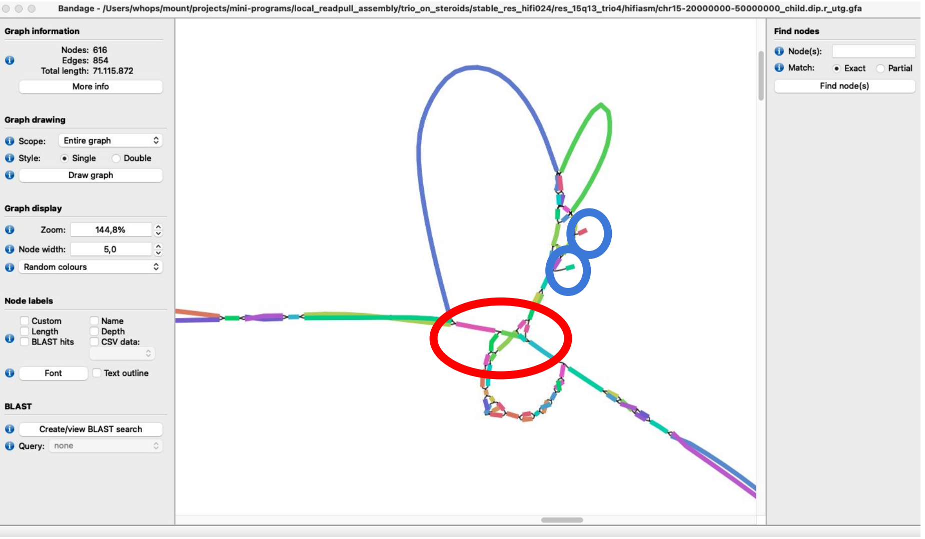

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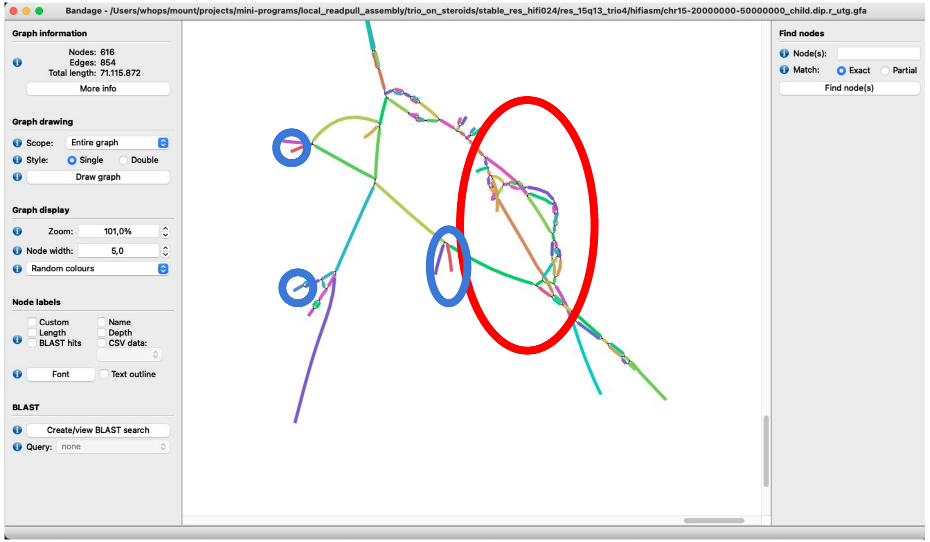
HiFi reads; (peri) centromeric region on chr15



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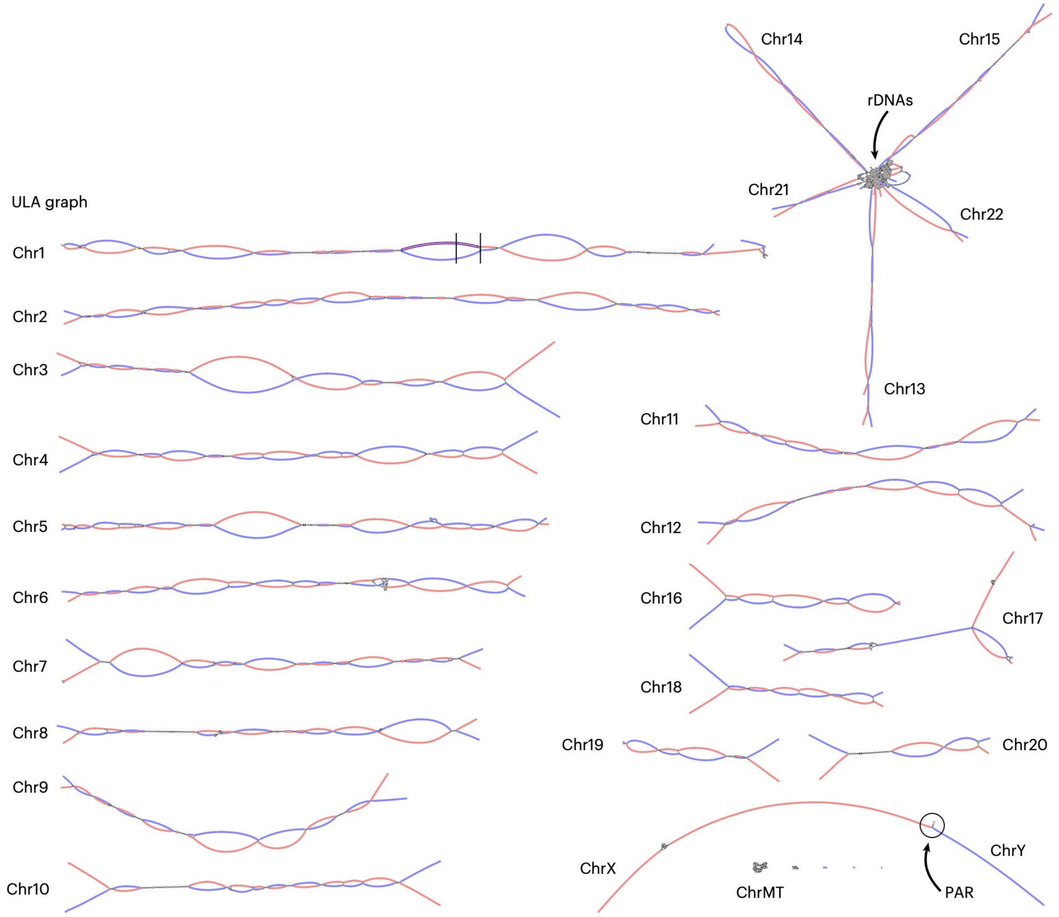
Excursion: Verkko assembly with >100X Hifi + ~80X ONT UL

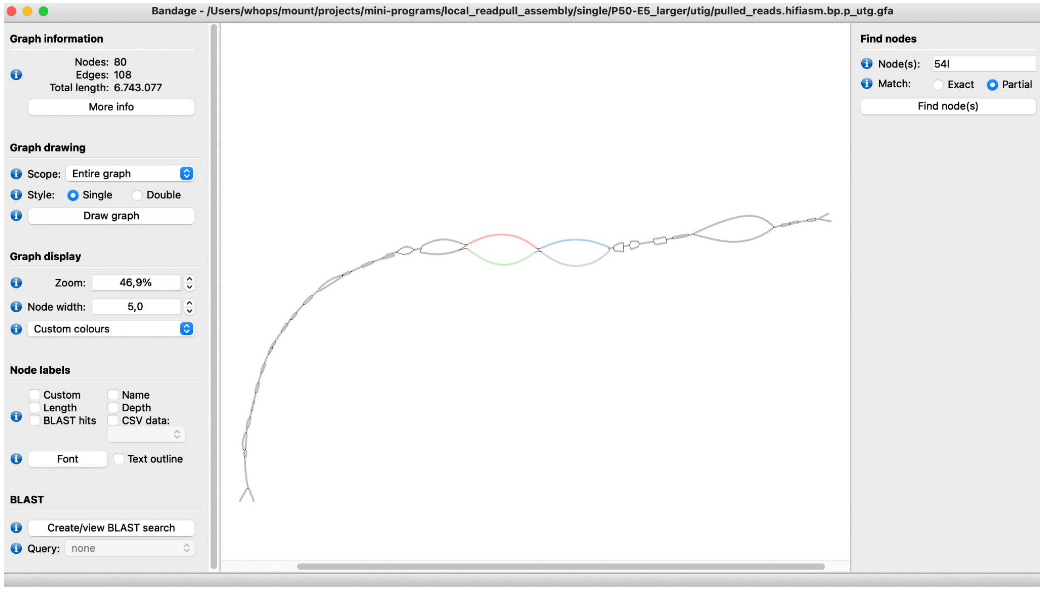


adapted from Rautiainen et al. 2023

Excursion over

Assembly graph visualization with *Bandage*

bandage -> load graph…. -> my_assembly.p_utig.gfa



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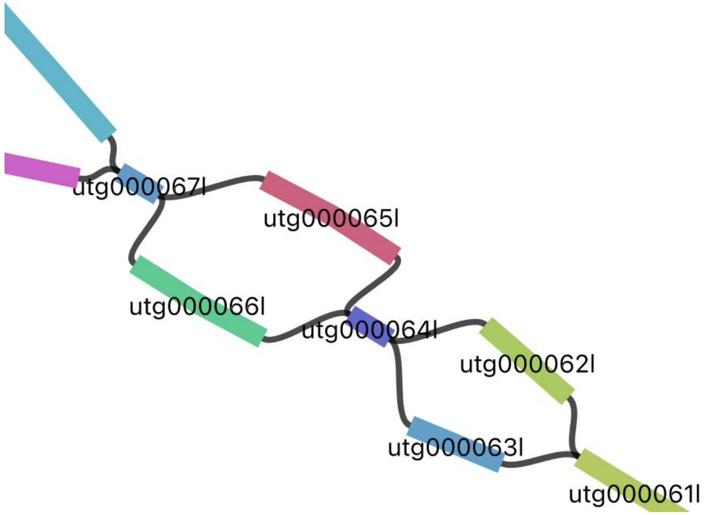

nodes: Segments ("utigs")
connections: Links

Assembly graph visualization with *Bandage*

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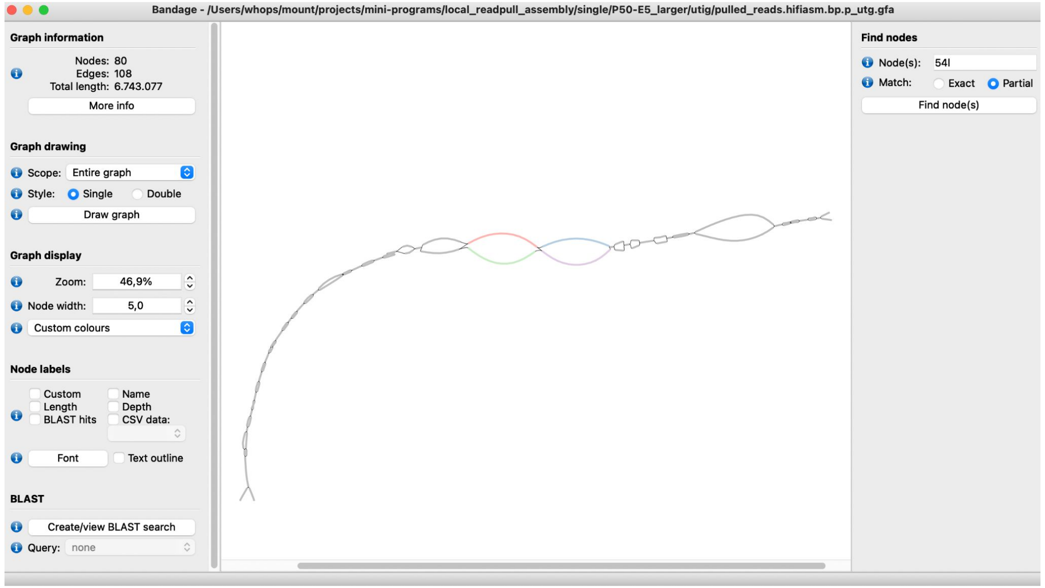

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Custom colours

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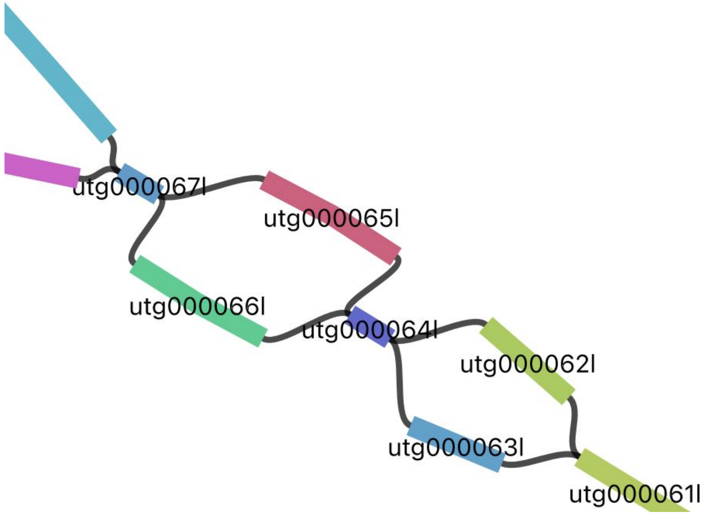
Find nodes

Node(s): 54l
Match: Exact [✓ Partial]

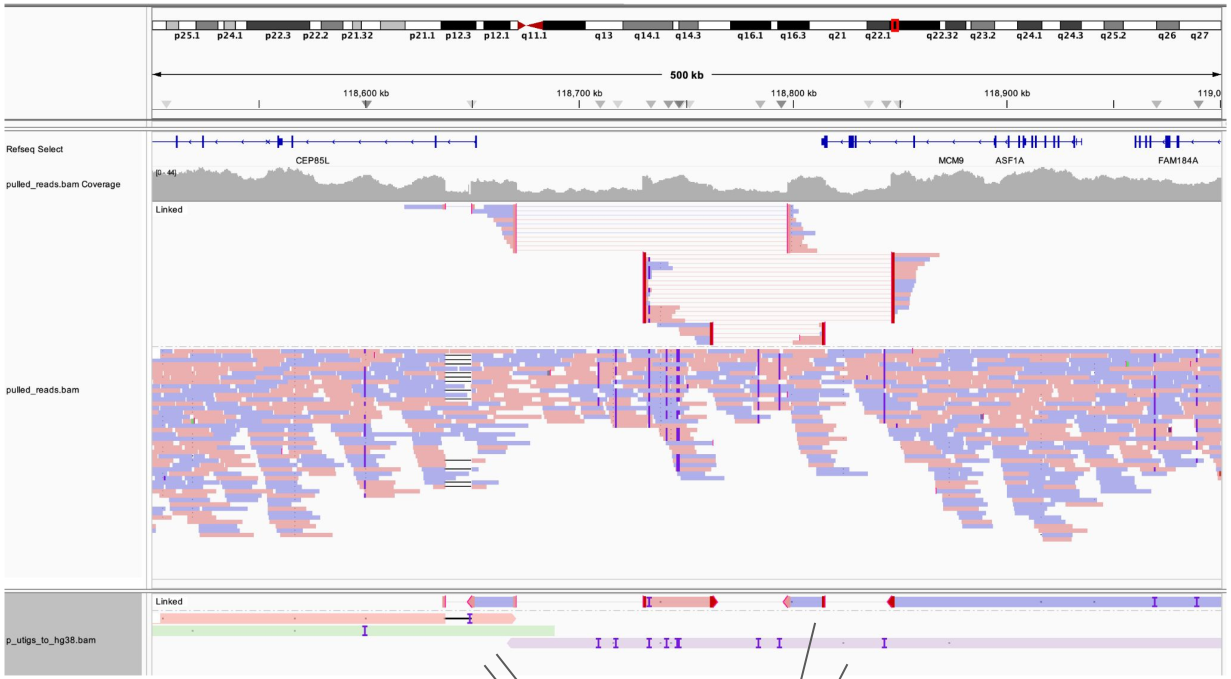


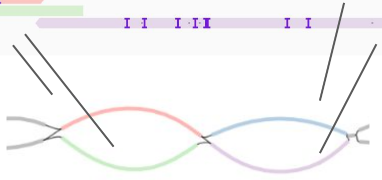
nodes: Segments ("utigs")
connections: Links

no tips or tangles

manipulating gfa files - mapping segments back to the reference

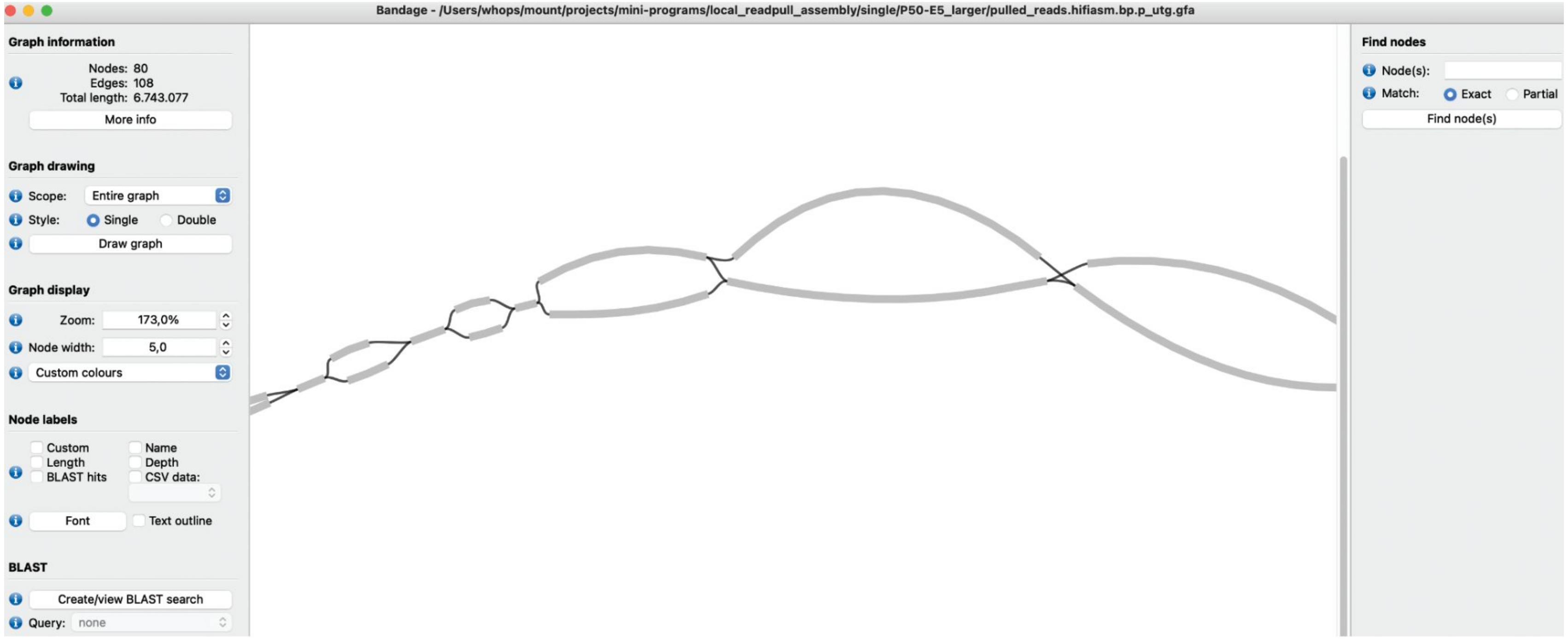
```
gfatools gfa2fa unitigs.gfa > unitigs.fa
minimap2 -x asm5 -a hg38.fa unitigs.fa > aligned_utigs.sam
```





unitigs.gfa

From unitigs to contigs: How to connect the sequences?



Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/single/P50-E5_larger/pulled_reads.hifiasm.bp.p_utg.gfa

Graph information

Nodes: 80
Edges: 108
Total length: 6.743.077

Graph drawing

Scope: Entire graph
Style: [✓ Single] Double

Graph display

Zoom: 173,0%
Node width: 5,0
Custom colours

Node labels

BLAST

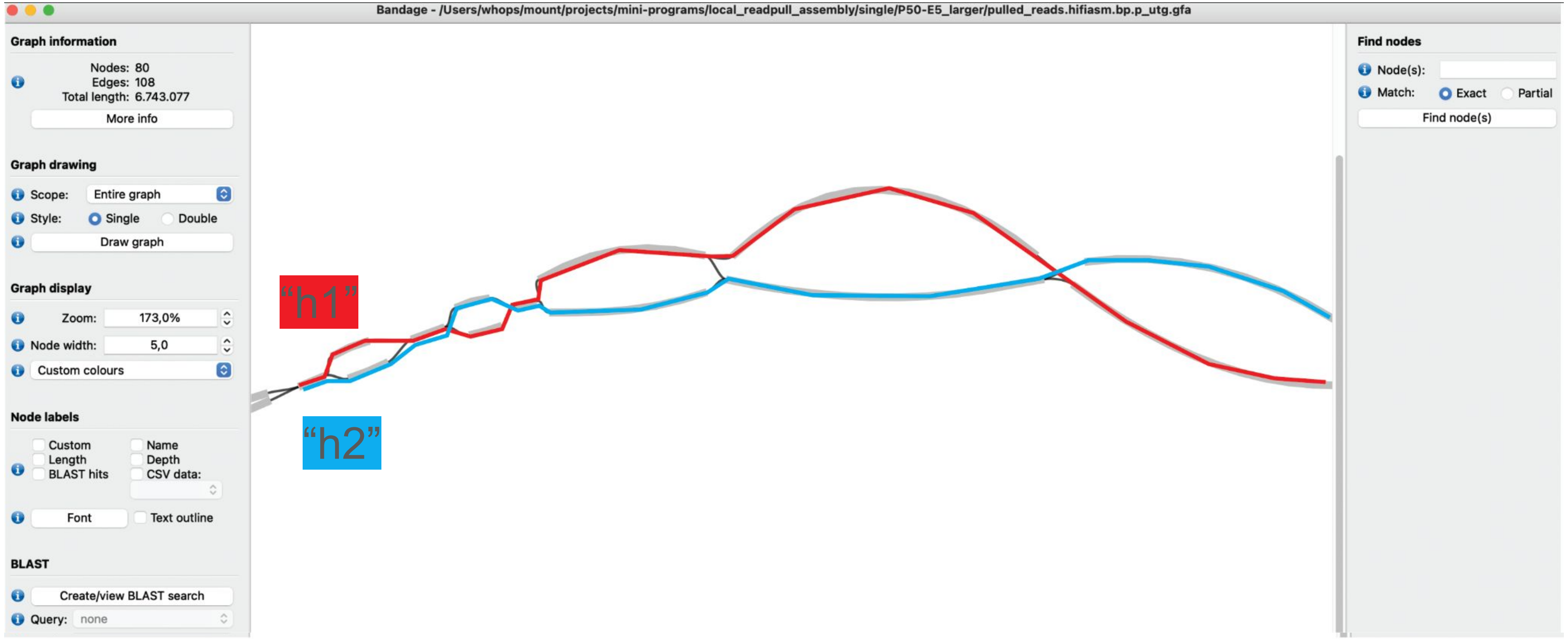
Query: none

Find nodes

Match: [✓ Exact] Partial

Additional data types (Parent info, Hi-C, ONT UL) will greatly improve this step

From unitigs to contigs: How to connect the sequences?


Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/single/P50-E5_larger/pulled_reads.hifiasm.bp.p_utg.gfa

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Node labels

Custom, Length, BLAST hits, Name, Depth, CSV data:

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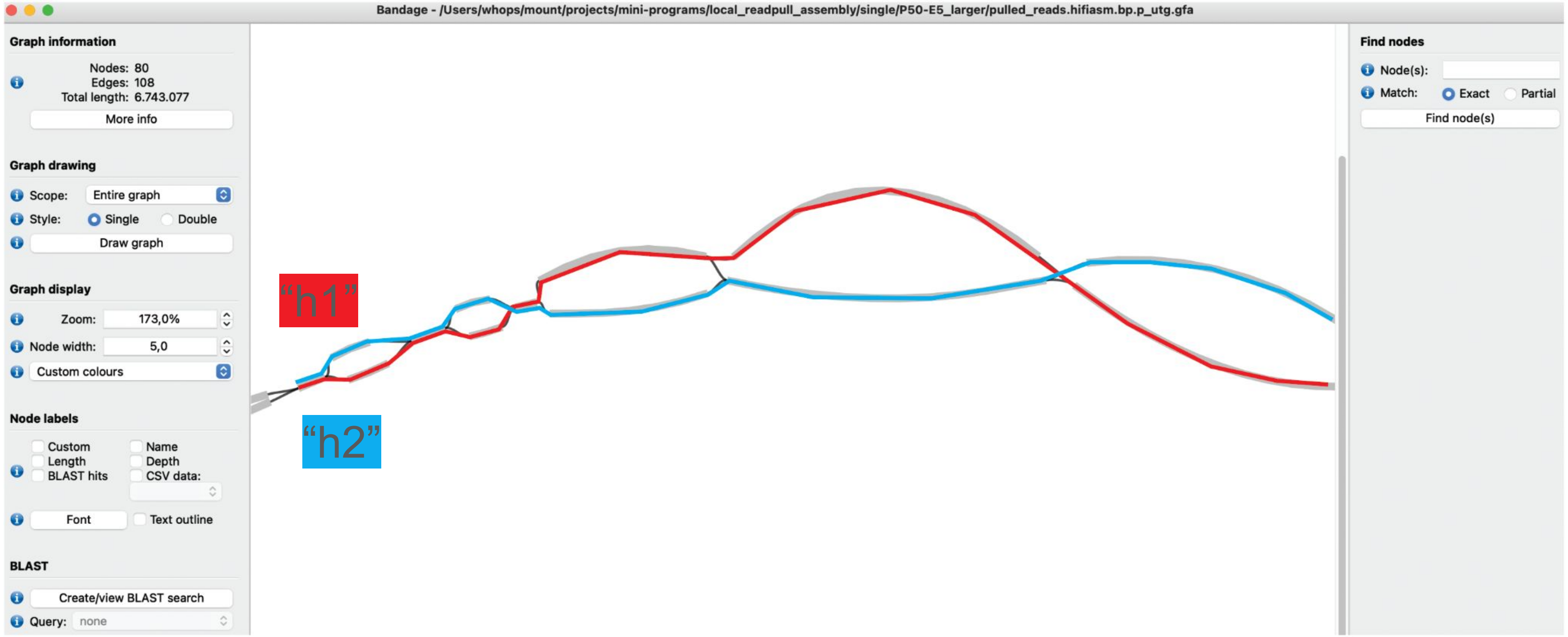
Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/single/P50-E5_larger/pulled_reads.hifiasm.bp.p_utg.gfa

Graph information

Nodes: 80
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More info

Graph drawing

Scope: Entire graph
Style: [✓ Single] Double

Draw graph

Graph display

Zoom: 173,0%
Node width: 5,0
Custom colours

Node labels

Custom, Length, BLAST hits, Name, Depth, CSV data:

Font, Text outline

BLAST

Create/view BLAST search
Query: none

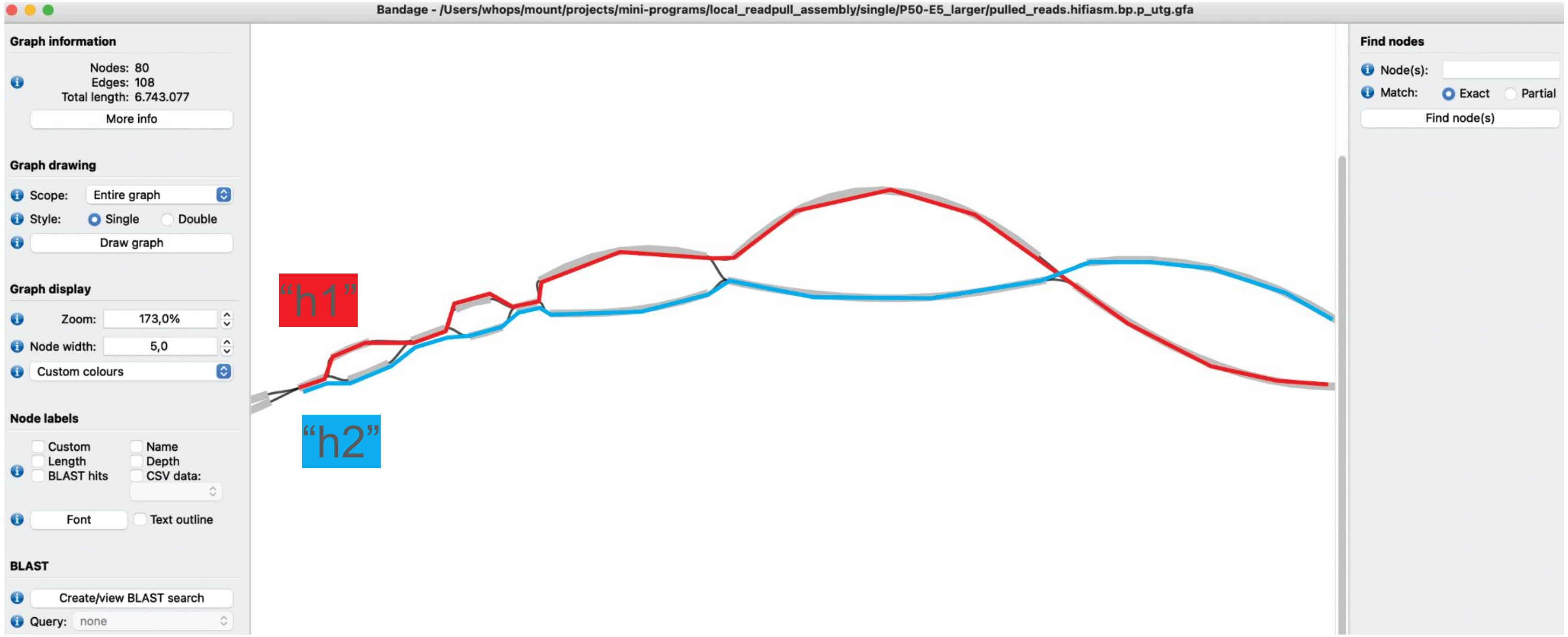
Find nodes

Node(s):
Match: [✓ Exact] Partial

Find node(s)

Additional data types (Parent info, Hi-C, ONT UL) will greatly improve this step

From unitigs to contigs: How to connect the sequences?



Bandage - /Users/whops/mount/projects/mini-programs/local_readpull_assembly/single/P50-E5_larger/pulled_reads.hifiasm.bp.p_utg.gfa

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Node width: 5,0
Custom colours

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Text outline

BLAST

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Find nodes

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Additional data types (Parent info, Hi-C, ONT UL) will greatly improve this step

… and this is what asm_hap1 and asm_hap2 in our case are

gfatools gfa2fa unitigs.gfa > unitigs.fa
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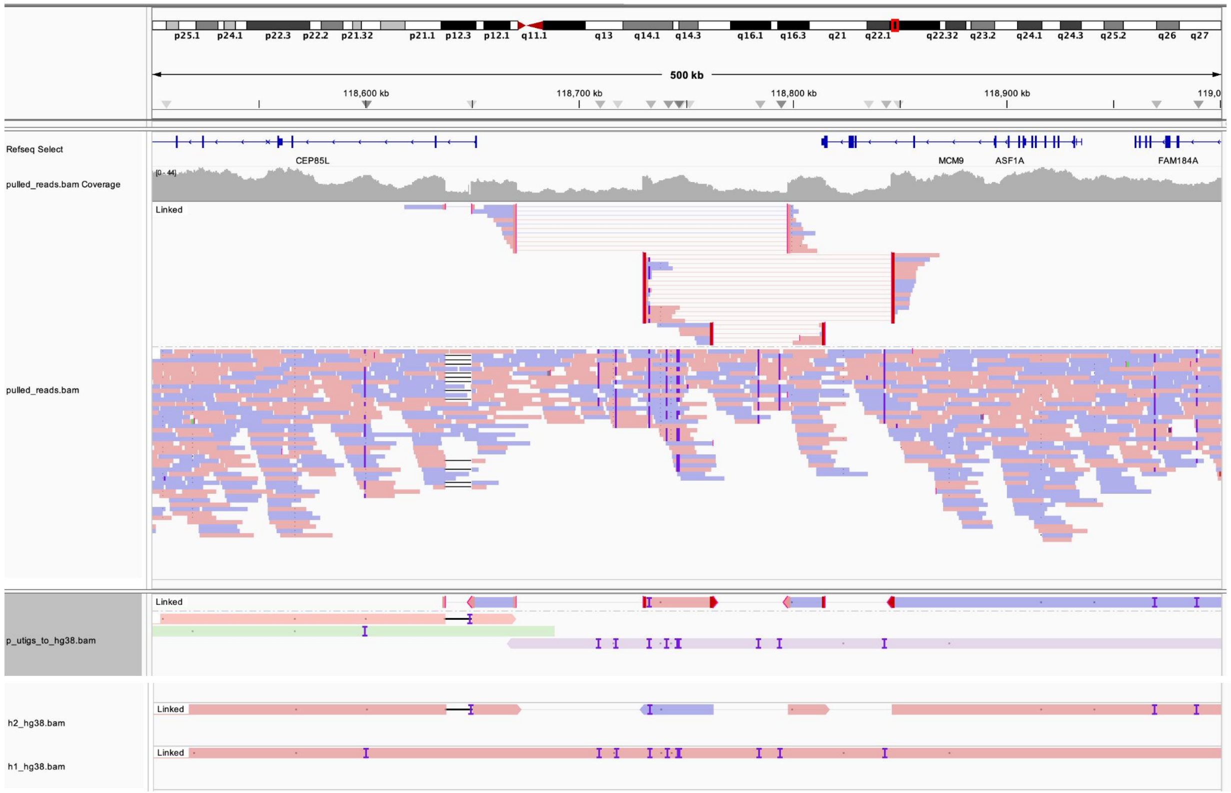

Assembly assessment

(1) Contiguity and Correctness with *Quast*
(2) k-mer based evaluation with *Merqury*
(3) Completeness with *BUSCO*

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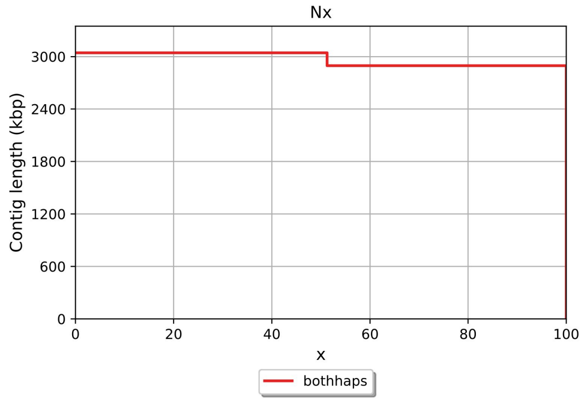

quast.py <(cat asm_hap1.fa asm_hap2.fa)

Report

	bothhaps
# contigs (>= 0 bp)	2
# contigs (>= 1000 bp)	2
# contigs (>= 5000 bp)	2
# contigs (>= 10000 bp)	2
# contigs (>= 25000 bp)	2
# contigs (>= 50000 bp)	2
Total length (>= 0 bp)	5939444
Total length (>= 1000 bp)	5939444
Total length (>= 5000 bp)	5939444
Total length (>= 10000 bp)	5939444
Total length (>= 25000 bp)	5939444
Total length (>= 50000 bp)	5939444
# contigs	2
Largest contig	3044186
Total length	5939444
GC (%)	38.20
N50	3044186
N75	2895258
L50	1
L75	2
# N's per 100 kbp	0.00

Gurevich et al. 2013

Assembly assessment

(1) Contiguity and Correctness with *Quast*
(2) k-mer based evaluation with *Merqury*
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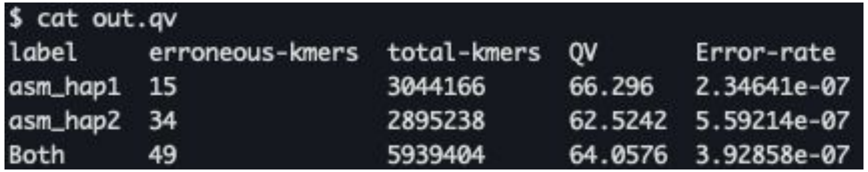


```
$ cat out.qv
label      erroneous-kmers   total-kmers   QV        Error-rate
asm_hap1   15                3044166       66.296    2.34641e-07
asm_hap2   34                2895238       62.5242   5.59214e-07
Both       49                5939404       64.0576   3.92858e-07
```

```
meryl count k=21 ../../../pulled_reads.fastq.gz output meryl_read_counts
$MERQURY/merqury.sh meryl_read_counts asm_hap1.fa asm_hap2.fa
```

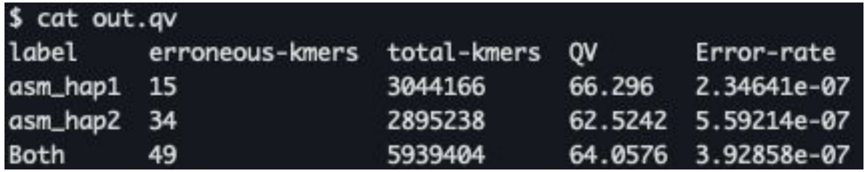
Rhie et al. 2020

Assembly assessment

(1) Contiguity and Correctness with *Quast*

(2) k-mer based evaluation with *Merqury*

(3) Completeness with *BUSCO*



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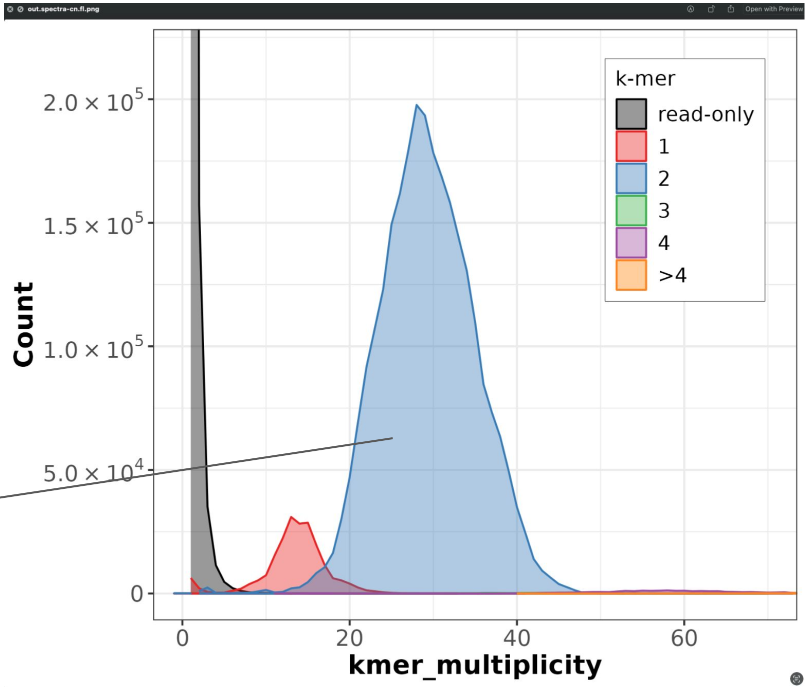


“k-mers which appear twice on our assembly:
usually appear ~30X in the raw reads”
-> homozygous peak

meryl count k=21 ../../../pulled_reads.fastq.gz output meryl_read_counts
$MERQURY/merqury.sh meryl_read_counts asm_hap1.fa asm_hap2.fa

Rhie et al. 2020

Completeness: how many expected genes are Complete, Duplicated, Fragmented, or Missing?

(1) Contiguity and Correctness with *Quast*
(2) k-mer based evaluation with *Merqury*
(3) Gene completeness with *BUSCO (or its reimplementation 'compleasm')*

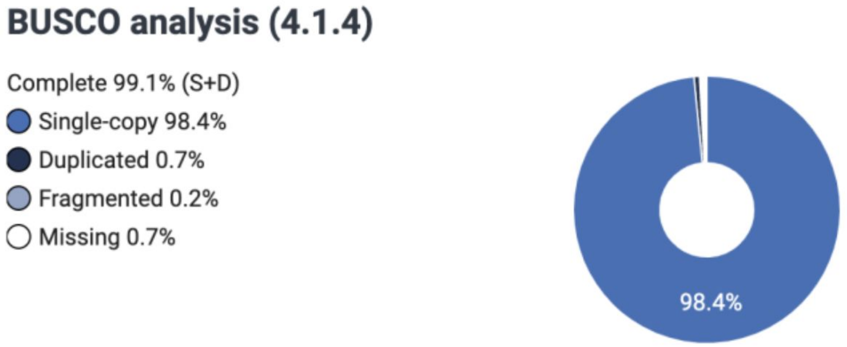
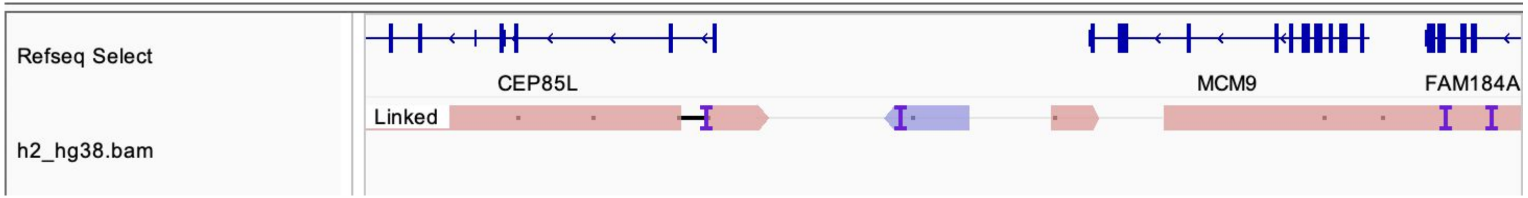
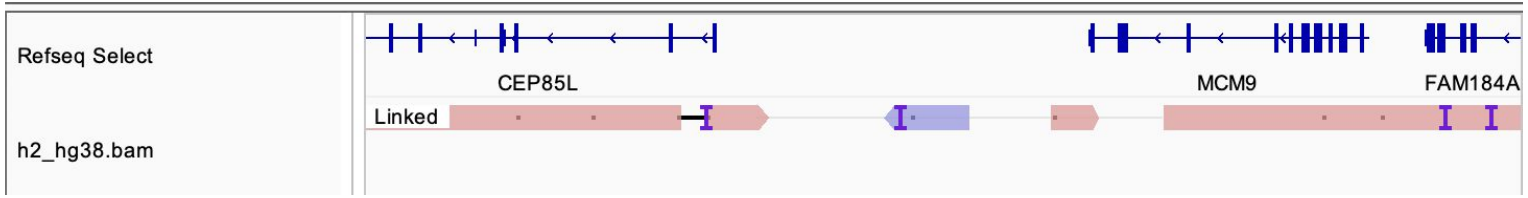


Figure: Genome institute at Washington University School of Medicine
BUSCO: Simão et al. 2015

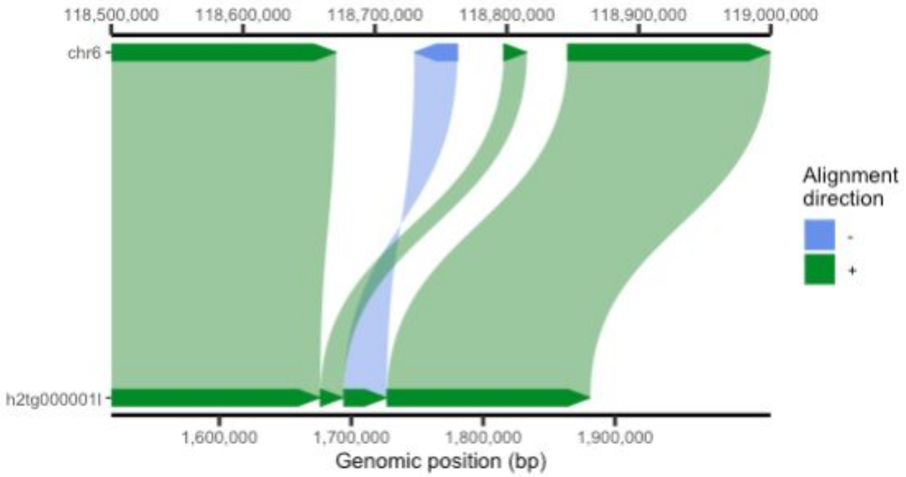
Complex regions require new visualizations



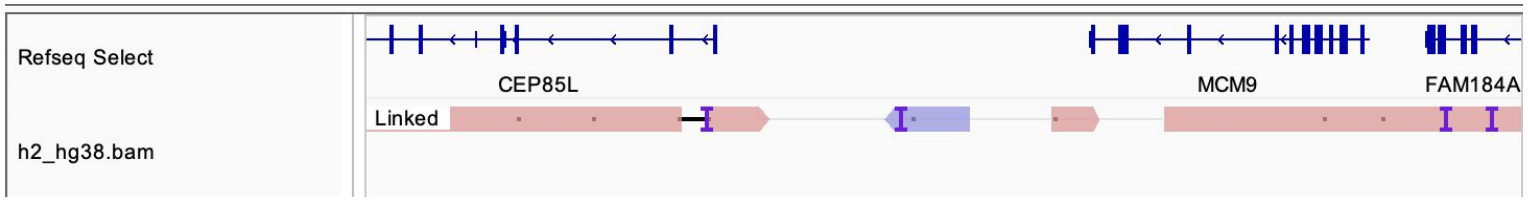
Complex regions require new visualizations



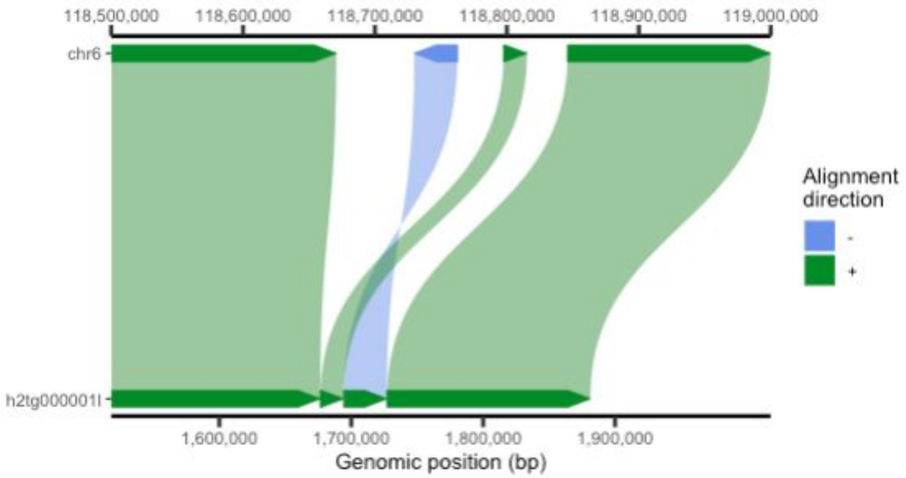
Miropeats plot [*SVbyEye*; Porubsky et al. 2025]



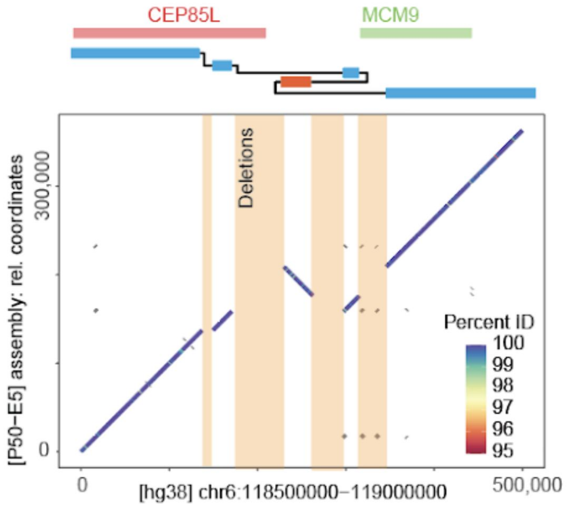
Complex regions require new visualizations



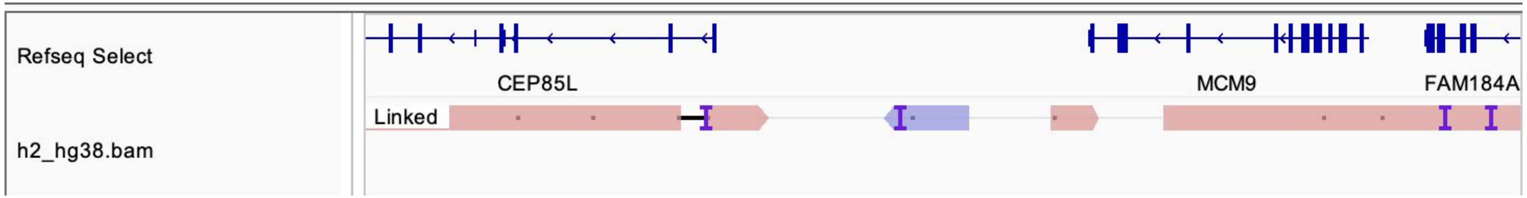
Miropeats plot [*SVbyEye*; Porubsky et al. 2025]



Dotplot [*NAHRWhals*; Höps et al. 2023]



Complex regions require new visualizations



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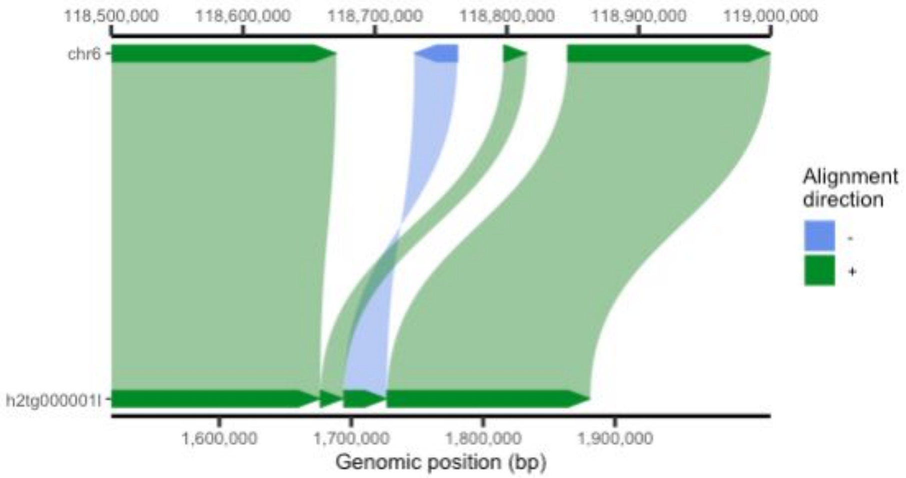
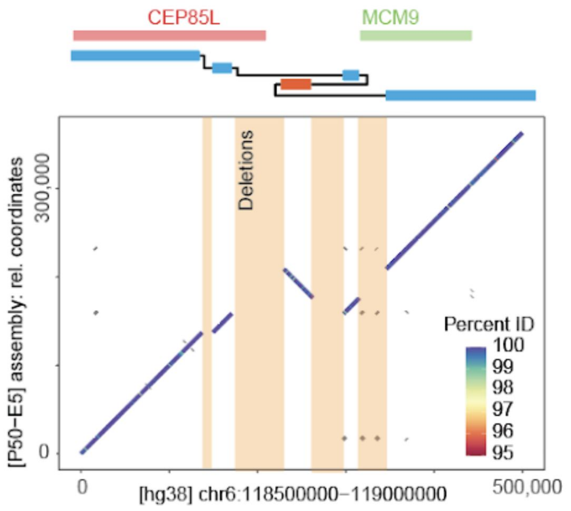
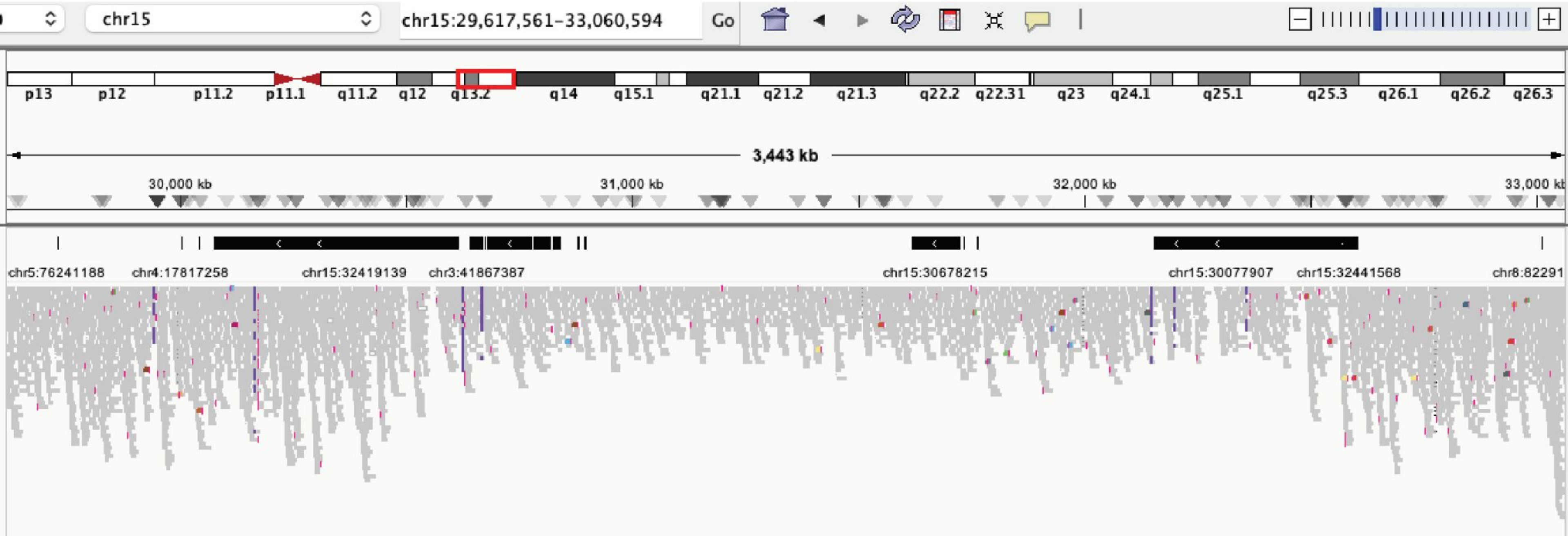


Dotplot [*NAHRWhals*; Höps et al. 2023]



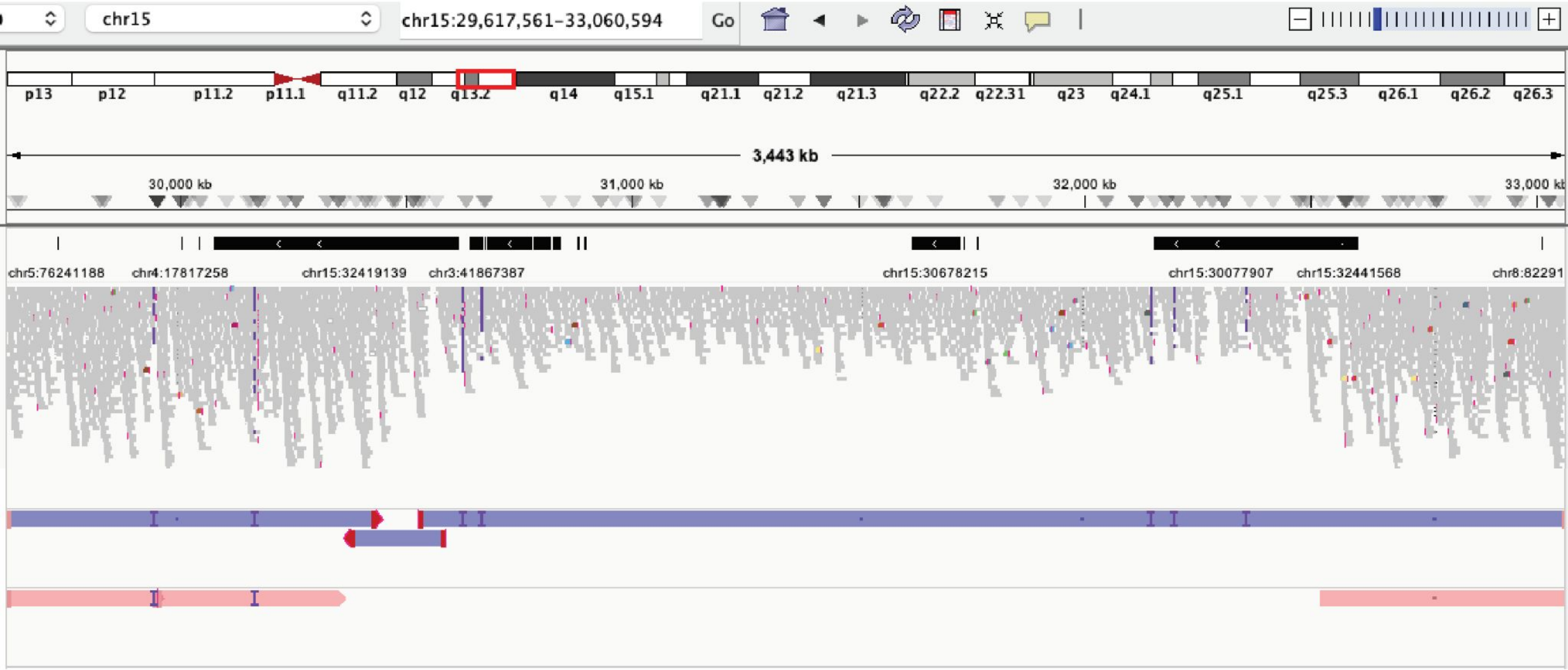
SV calling tools: e.g. *Dipcall, Smartie-sv, SVIM-asm, PAV.* [See e.g. review: Liu et al. 2024]

Example: Long duplications can obscure breakpoints



Deletion is visible, breakpoints are not.

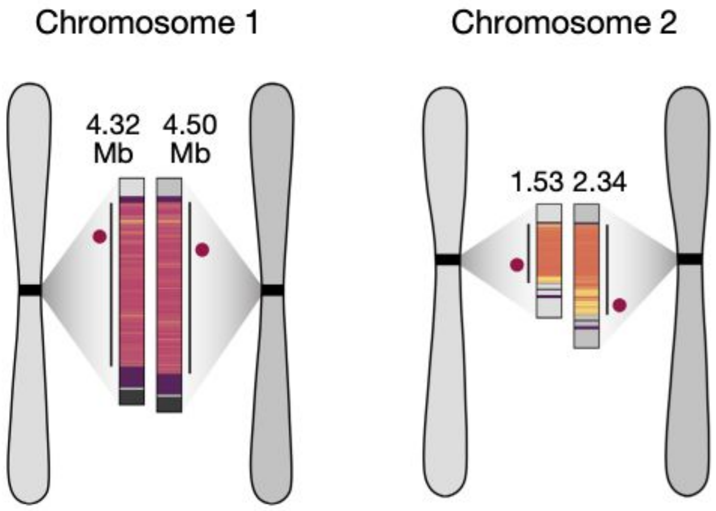
Example: Long duplications can obscure breakpoints



assembly_h1 and _h2: mapped ‘back’ to hg38 to reveal more detail

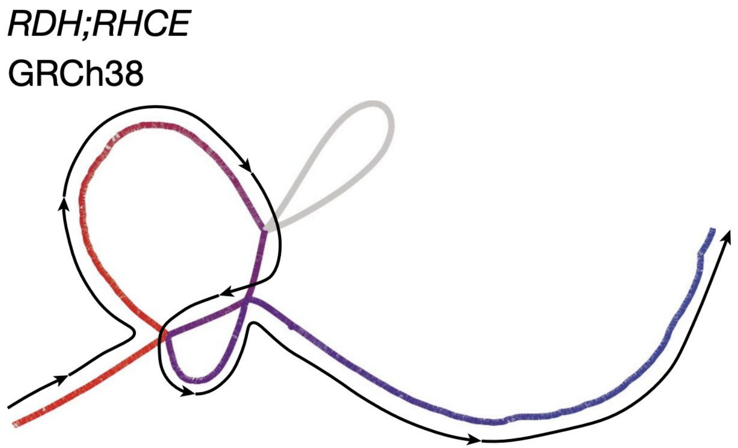
Beyond: assemblies unlock new biology

The most complex genomic loci



[Logsdon et al. 2024]

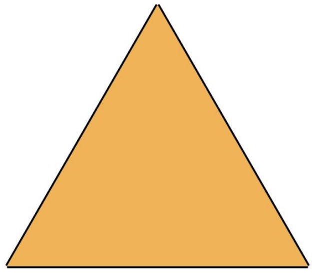
Pan-genome graphs to understand complex variation



[Liao et al. 2023]

Take-homes

Research question
Are assemblies necessary?
Of what contig length + quality?



Available data
Are assemblies of desired quality possible?

Expertise or Time
Are assemblies feasible?

Assemblies are only as good as the data - and always require expertise, time and careful QC.

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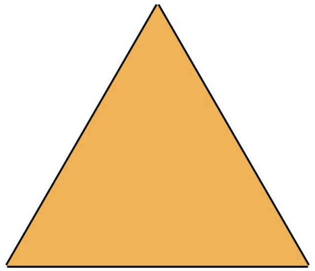

Available data
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Expertise or Time
Are assemblies feasible?

Assemblies are only as good as the data - and always require expertise, time and careful QC.

It's worth it: De-novo assembly can answer questions that standard read-mapping can't.

Materials & Further reading

Genome assembly algorithms
[1] Heng Li & Richard Durbin 2025: Genome assembly in the telomere-to-telomere era; Nat. reviews genetics
[2] Rautiainen et al. 2023: Telomere-to-telomere assembly of diploid chromosomes with Verkko
[3] Kolmogorov et al. 2019: Assembly of long, error-prone reads using repeat graphs

Assembly-based variant calling
[4] Olson et al. 2023: Variant calling and benchmarking in an era of complete human genome sequences
[5] Ebler et al. 2022: Pangenome-based genome inference allows efficient and accurate genotyping across a wide spectrum of variant classes
[6] Liu et al. 2024: Tradeoffs in alignment and assembly-based methods for structural variant detection with long-read sequencing data

Tutorials
Assembly step by step (highly recommend!):
https://training.galaxyproject.org/training-material/topics/assembly/tutorials/vgp_genome_assembly/tutorial.html
Assembly QC: (Quast, BUSCO, Mercury, Chromeister):
https://training.galaxyproject.org/training-material/topics/assembly/tutorials/assembly-quality-control/tutorial.html

https://github.com/WHops/ESHG2025_assemby_workshop