Strain-Level Comparative Genomics

Phylogenomics Crash Course
Multiple Genome Alignment & Visualization
(Bonus content: full-length 16S taxon abundance)

STAMPS – Day 7

July 25, 2024

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Agenda…

9:00am to 9:10am:	Intro/kickoff (Todd)
9:10pm to 9:35am:	Binning lecture (Mike)
9:35am to 9:55am:	Binning tutorial (Todd & Mike)
9:55am to 10:10pm:	We have genomes/genome bins, now what? (Todd)
10:10am to 10:35am:	Break (Group Photo at Lillie)
10:35am to 10:50am:	Phylogenetics + MSA lecture (Mike)
10:50am to 11:00am:	MSA game (Todd)
11:00am to 11:35am:	Parsnp/strain analysis lecture (Mike)
11:35am to 11:55am:	Parsnp tutorial (Mike & Todd)
11:55am to Noon:	Emu advertisement (Mike)

Quick Phylogenetics Refresher

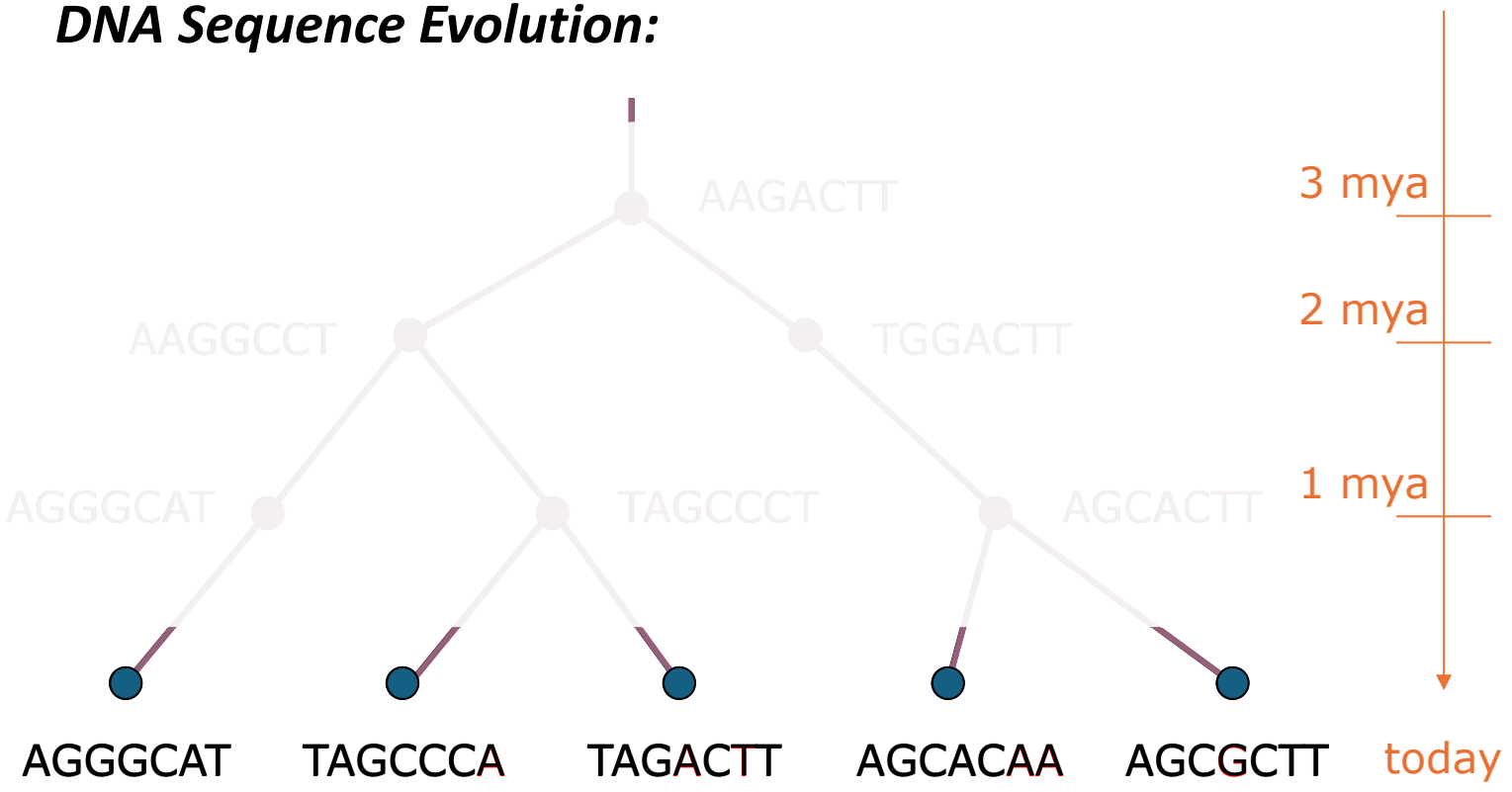
Multiple Sequence Alignment

Phylogeny Estimation

Population-level (“Species”) Tree Estimation

Brief Intro to Molecular Phylogenetics

DNA Sequence Evolution:



Notes:

- insertions and deletions also occur randomly on branches (not shown)
- In statistical terms, the extant sequences are the **observed data**, while the tree shape, tree topology and mutation rates are the **unknown model parameters** to be estimated.

Task is to estimate the tree shape from the extant sequences at the tree leaves...

Two Separate Problems (both NP-Hard):

1. Identify which groups of characters share a common ancestor. (Multiple Sequence Alignment)
2. Find the maximum likelihood tree and model parameters (ML Tree Estimation)

*mya = million years ago

*Sequence Animation Credit: Prof. Tandy Warnow

Multiple Sequence Alignment: Definition & Goal

Input: Sequences from different organisms (or different loci) that evolved from a common ancestor.

Goal: Align sequences so that all sets of positions having a common ancestor are grouped together.

- *Not the same as aligning short sequences (or reads) to a reference ("mapping").*
- *Not the same as **genome** alignment*
- *Typically done before creating a phylogenetic tree…*

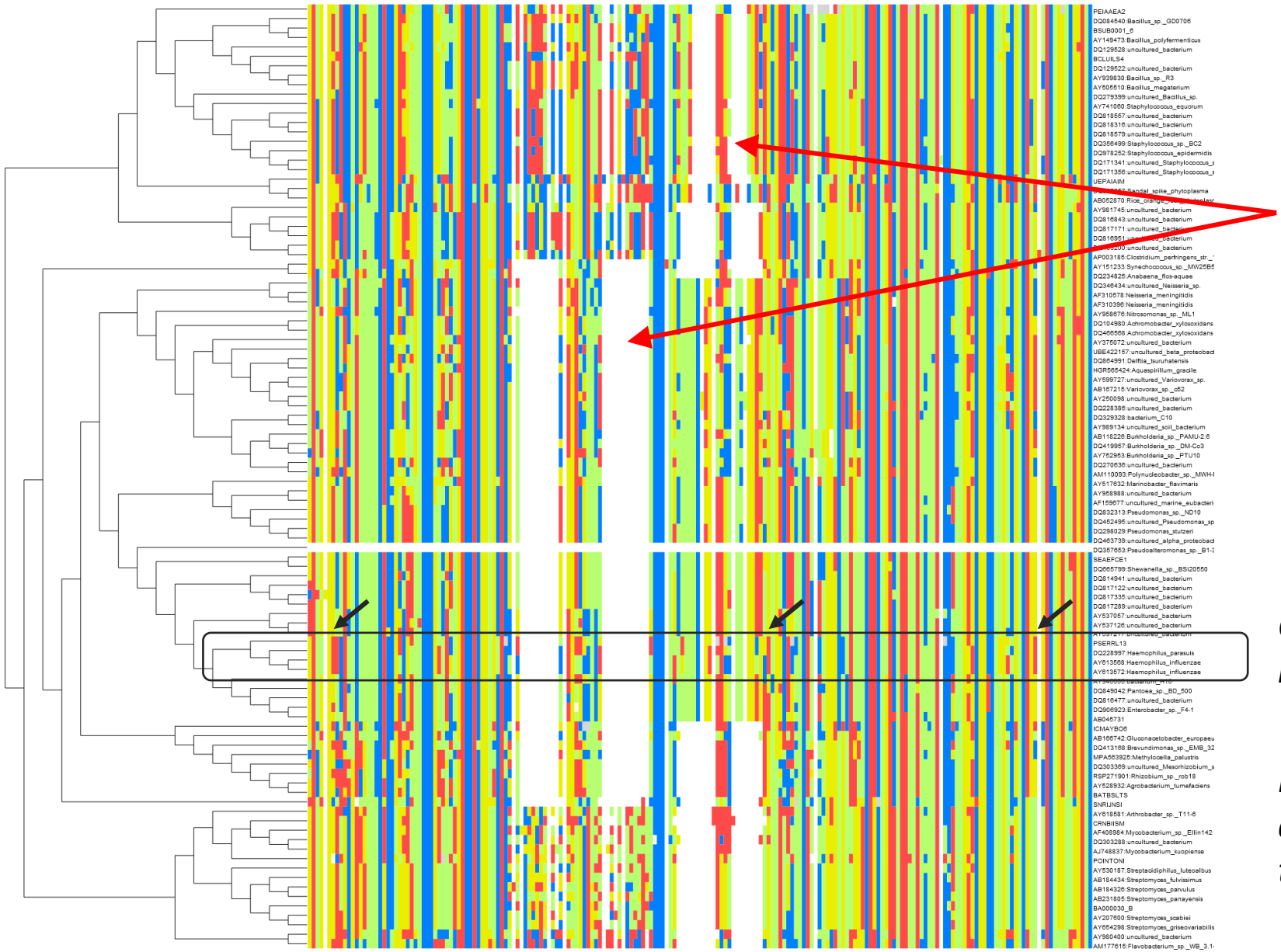
Tools:

- ***MAFFT***
- Muscle
- ***PASTA***
- ClustalW
- DiAlign
- BAli-Phy
- PRANK
- T-COFFEE
- …*et cetera*

Garbage Alignment → ***Garbage Tree***

Bold/Underlined = *My preferred methods*

Multiple Sequence Alignment (Example)



White indicates a “gap” in the alignment, a.k.a. an insertion or deletion (indel)

Conserved mutation patterns indicate evolutionary closeness.

Phylogeny estimation algorithms use this to build a tree…

Legend:

A	T
C	G

Data Properties Affecting Multiple Sequence Alignment

- Avg Sequence Similarity (rate of evolution)
- # of Sequences
- Presence of highly conserved regions
- Sequence Length heterogeneity
- Gap length/frequency
- Sequence fragmentation (*not the same as heterogeneity*)
- Avg Sequence Length

MSA Algorithms & Software (Partial List)

Tool	Use Case	Comments
MAFFT (Katoh et al., 2002)	Single Gene MSA (small N)	• Uses patterns of insertion/deletion to find optimal alignment • Generally pretty accurate in most conditions.
MUSCLE (Edgar, 2004)		• Progressive alignment. Suitable for relatively high overall sequence similarity.
CLUSTAL (Sievers et al., 2011)		• Ideal for protein alignments with structurally important sites.
PASTA (Mirarab et al., 2015)	Single Gene MSA (large N)	• Divide-and-conquer algorithm. Ideal for scaling alignment to large number of sequences (>1000)
HMMER (Eddy, 1998)	*Query sequence alignment to reference*	*• Represents reference alignment as HMM. Query sequence alignment performed using standard HMM algorithms.*

I tend to tell people:

- *Just run MAFFT for anything less than 500 sequences or so*
- *Muscle is fine too if the divergence is low…*
- *…or ClustalW for AA sequences with important structural sites*
- *Use PASTA for over 1k sequences or if avg. %-identity is very low (high rate of evolution).*

This simplistic advice is a STAMPS 2022 exclusive…

Final MSA Points (details in appendix)

- MSA accuracy can drop to 0% *fast*
  - *Especially as diversity & # sequences go up.*
- Bad MSA will lead to a Star-like tree
  - *i.e. no discernable relationships between sequences*
- MSA failure can take many forms (over/under alignment)

Tree Estimation Mechanics

- Given the MSA, how to estimate the Tree?
- Statistical Estimation:
  - Tree shape & branch length are ***parameters*** under a model of sequence evolution
  - Called GTR (generalized time reversible)
  - Each site (letter, residue, position, etc…) evolves I.I.D.
  - Mutation along a branch happens according to a continuous-time Markov process ("time" here = branch length).
  - Root is ***not identifiable***.
  - No indels under the model, MSA gaps treated as missing data.
- Under this model
  - Every tree shape/topology has a likelihood given the sequences (the "data")
  - **Maximum Likelihood Tree** will be "statistically consistent" (good)
    - I.e. given enough sites, the ML tree will be the correct one with $p \to 1$
    - Neighbor-joining, UPGMA, etc… are NOT statistically consistent (bad)
- Hence most good Tree estimators use an ML model (e.g. RAxML, FastTree, IQTree)

MGA & Visualization for Strain Analysis

Digging deep…

Introduction

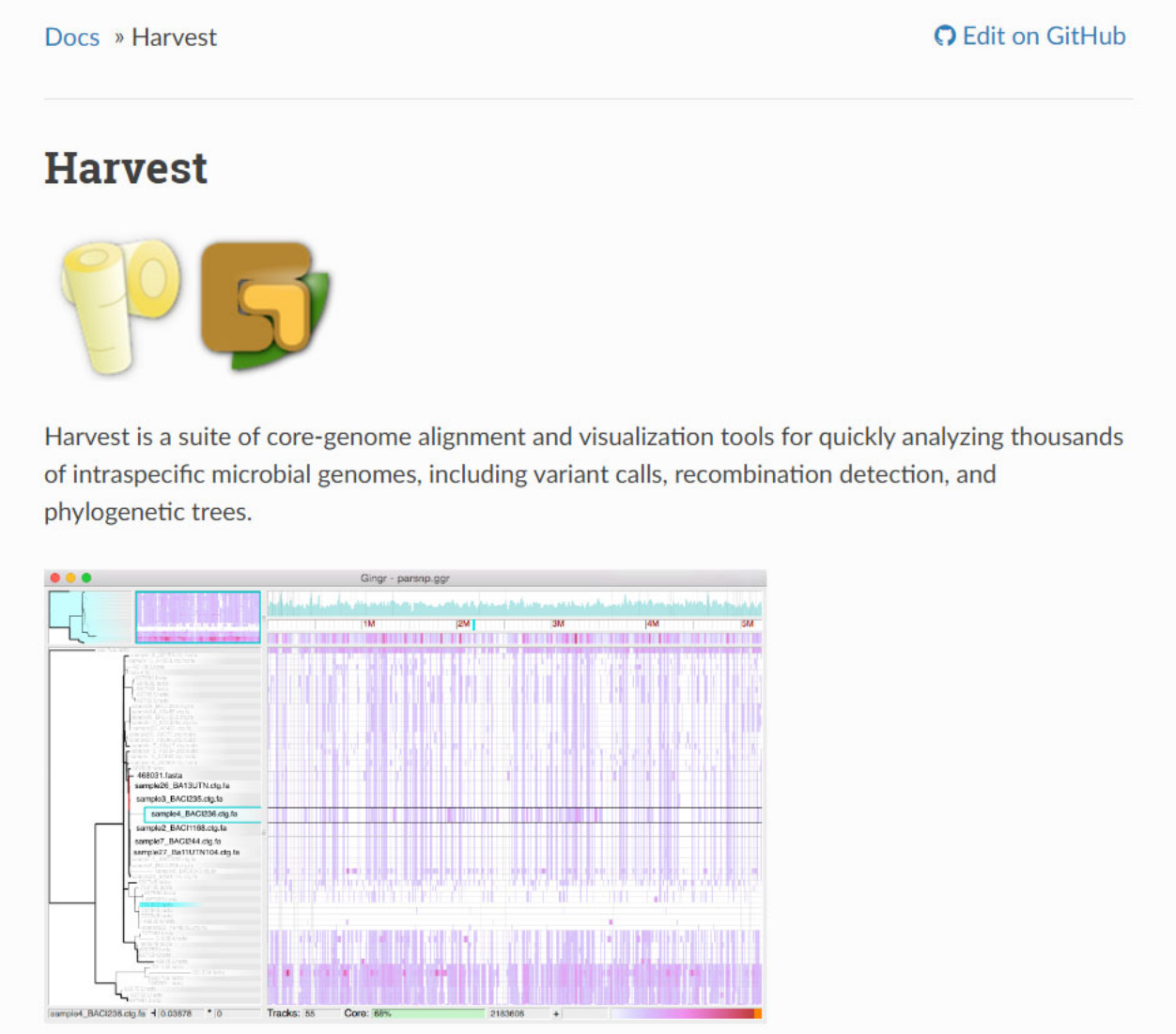
- What does “Strain” mean?
  - Particular SNP?
  - Multiple particular SNPs?
  - Presence/Absence of certain genes?
  - Phenotype?
  - *Other?*
- How do we compare strains?
  - Multiple Genome Alignment
  - Pangenome Analysis

Whole-Genome Alignment

- Idea: align specifically the *shared (“core”)* portion of several genomes.
- Use these aligned segments to identify phylogenetic relationships, etc…
- Visualize what exactly is similar and different…

Tools:

- Parsnp
- Mauve
- SibeliaZ
- (others…)



Whole Genome Alignment: Quick How-To with Parsnp

- Get *assembled* genomes from individual organisms
  - Isolates are nice, MAGs will do
  - Contigs are fine for this, doesn't have to be complete
  - Helps to have at least 1 high-quality, annotated reference genome
  - Useful to run QUAST to QC the assembly
- Run Parsnp:

```
contig_repo=./parsnp_contigs
parsnp_out=./parsnp_output_13
ref_genbank=./ref_assembly_GCF_008121495/Ref_ATCC_29149.gbff

parsnp -g $ref_genbank -d $contig_repo -p 15 -o $parsnp_out
```

Annotated Reference Genome (.gbff format)

Folder with 1 fasta file for each assembly (containing all contigs) *...OR...*
File with a newline-separated list of assembly fasta files (full

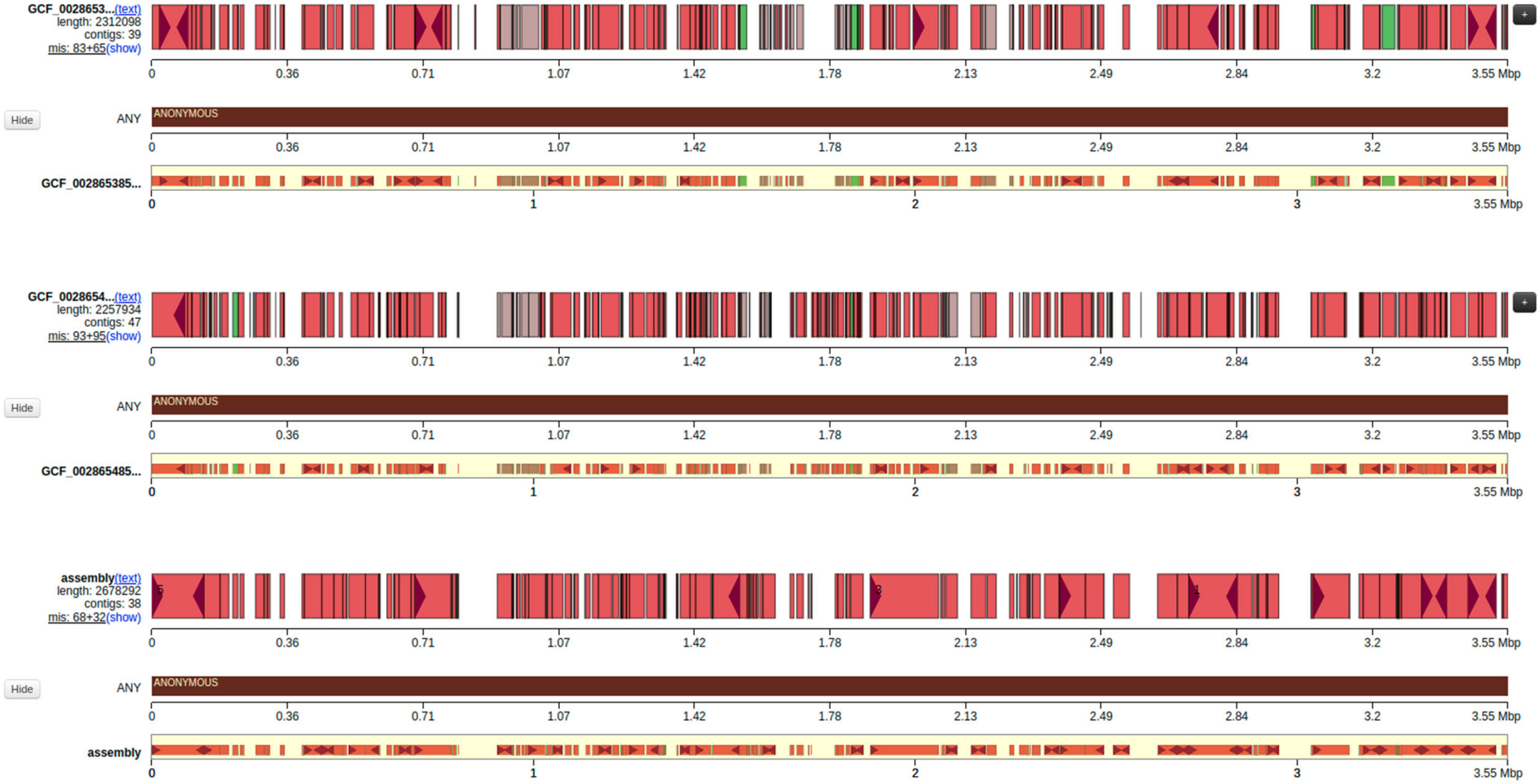
 # processors

Output folder

Interlude: QC-ing an Assembly with QUAST

What can we learn?

- Assembly Quality Issues?
- Issues with Reference?



Notes:

The top two assemblies are SPAdes assemblies done by the original authors of the R. Gnavus paper (citation later).

The bottom is a Unicycler assembly from the same reads.

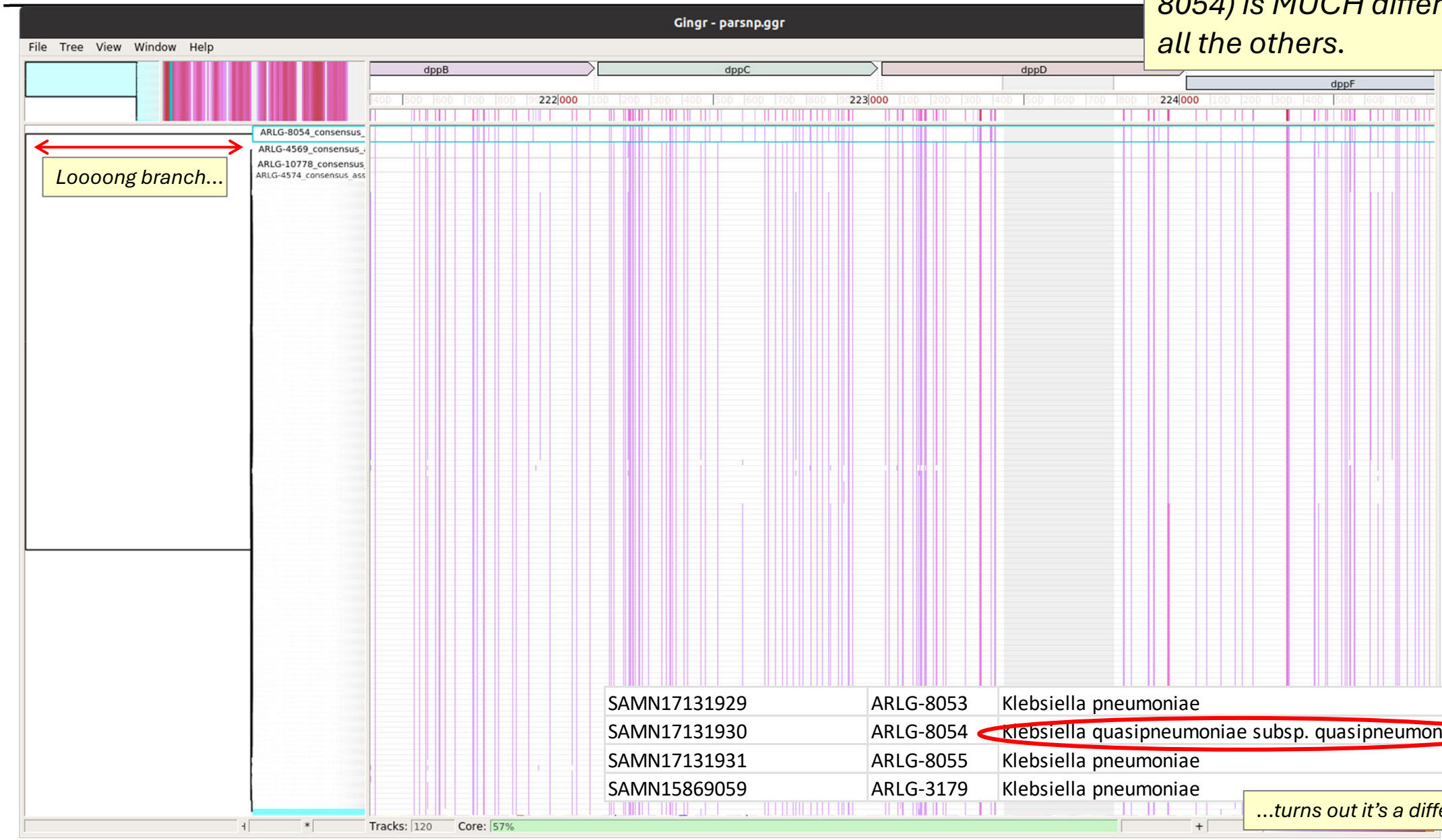
Case Study #1: *Klebsiella pneumoniae*[1]

- 119 Carbapenam-resistant *Kp* isolates (95 from Houston Hospitals)
- Hybrid short/long-read assemblies
  - so they *should* be high-quality
- Focus of study was to show spread of two separate “clonal groups”

[1]Shropshire, et al. (2022), DOI: 10.1128/mbio.00497-22

Case Study #1: Observations…

Observation #1:
One of these genomes (ARLG-8054) is MUCH different from all the others.



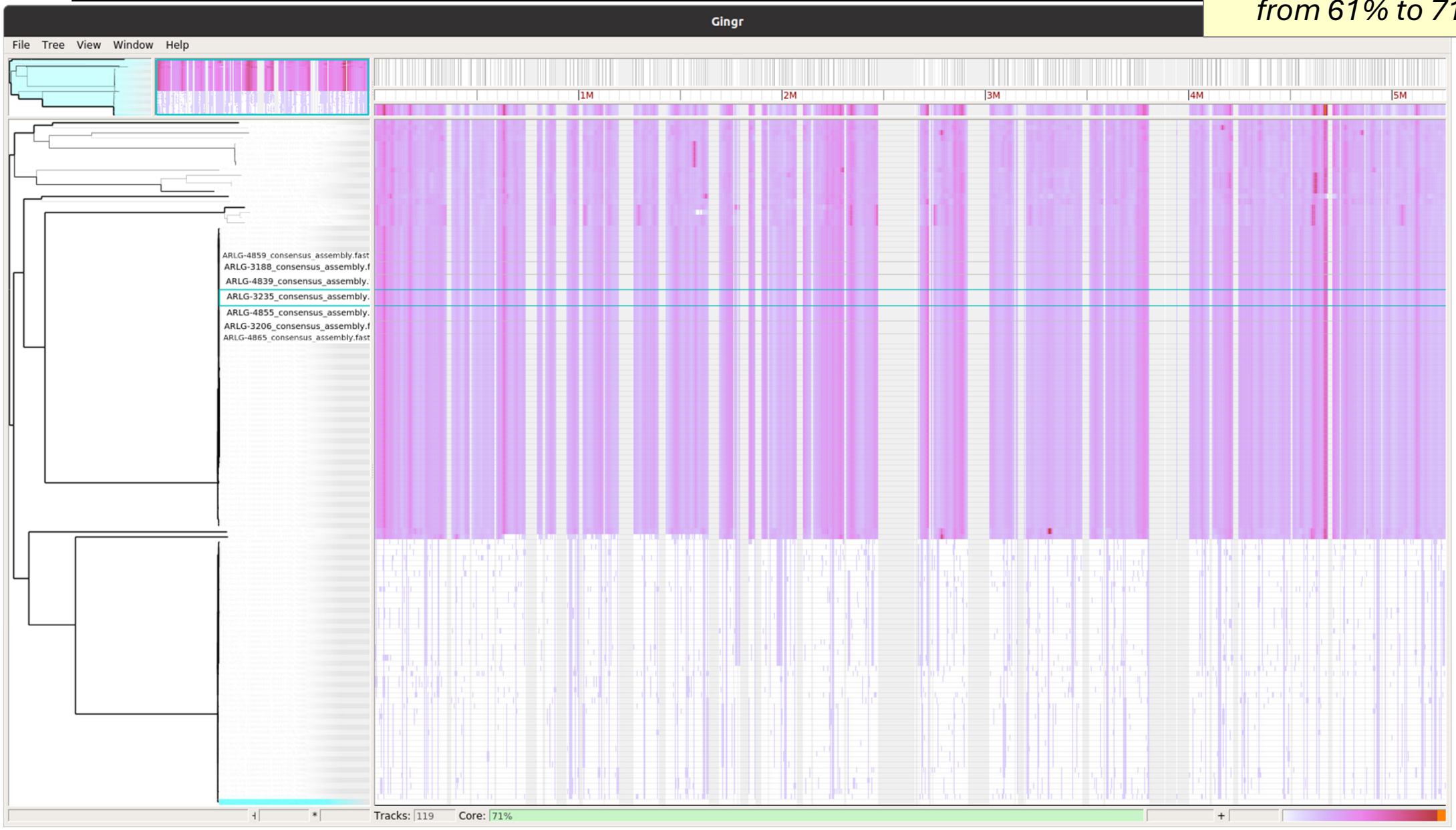
SAMN17131929	ARLG-8053	Klebsiella pneumoniae	TX
SAMN17131930	ARLG-8054	Klebsiella quasipneumoniae subsp. quasipneumoniae	TX
SAMN17131931	ARLG-8055	Klebsiella pneumoniae	TX
SAMN15869059	ARLG-3179	Klebsiella pneumoniae	MI

…turns out it's a different species

Case Study #1: Excluding ARLG-8054

***Observation #2*:**

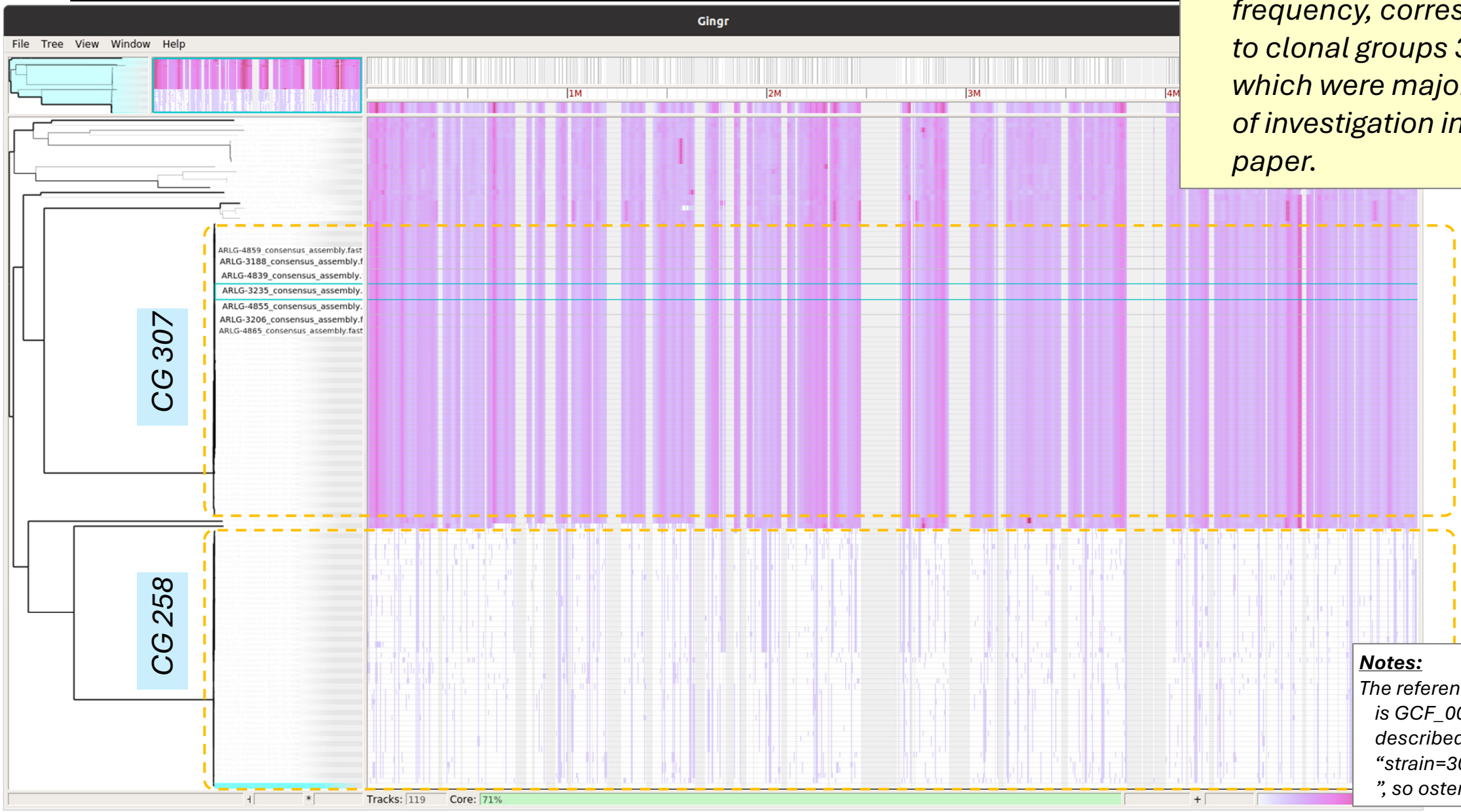
- *Without 8054, core % goes from 61% to 71%*



Case Study #1: Excluding ARLG-8054

Observation #3:

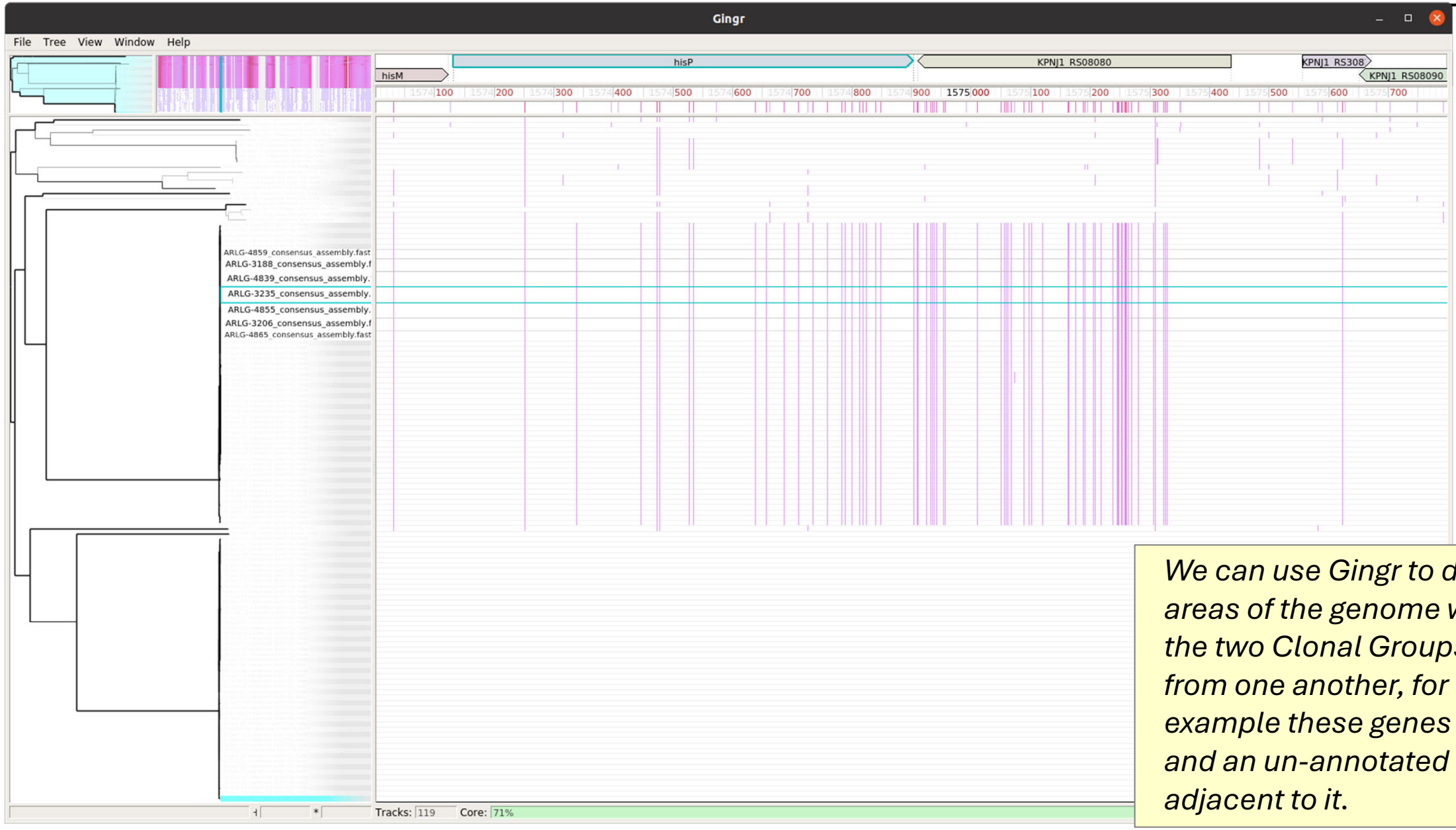
- *2 Clades with highest frequency, corresponding to clonal groups 307, 258 which were major targets of investigation in this paper.*



Notes:

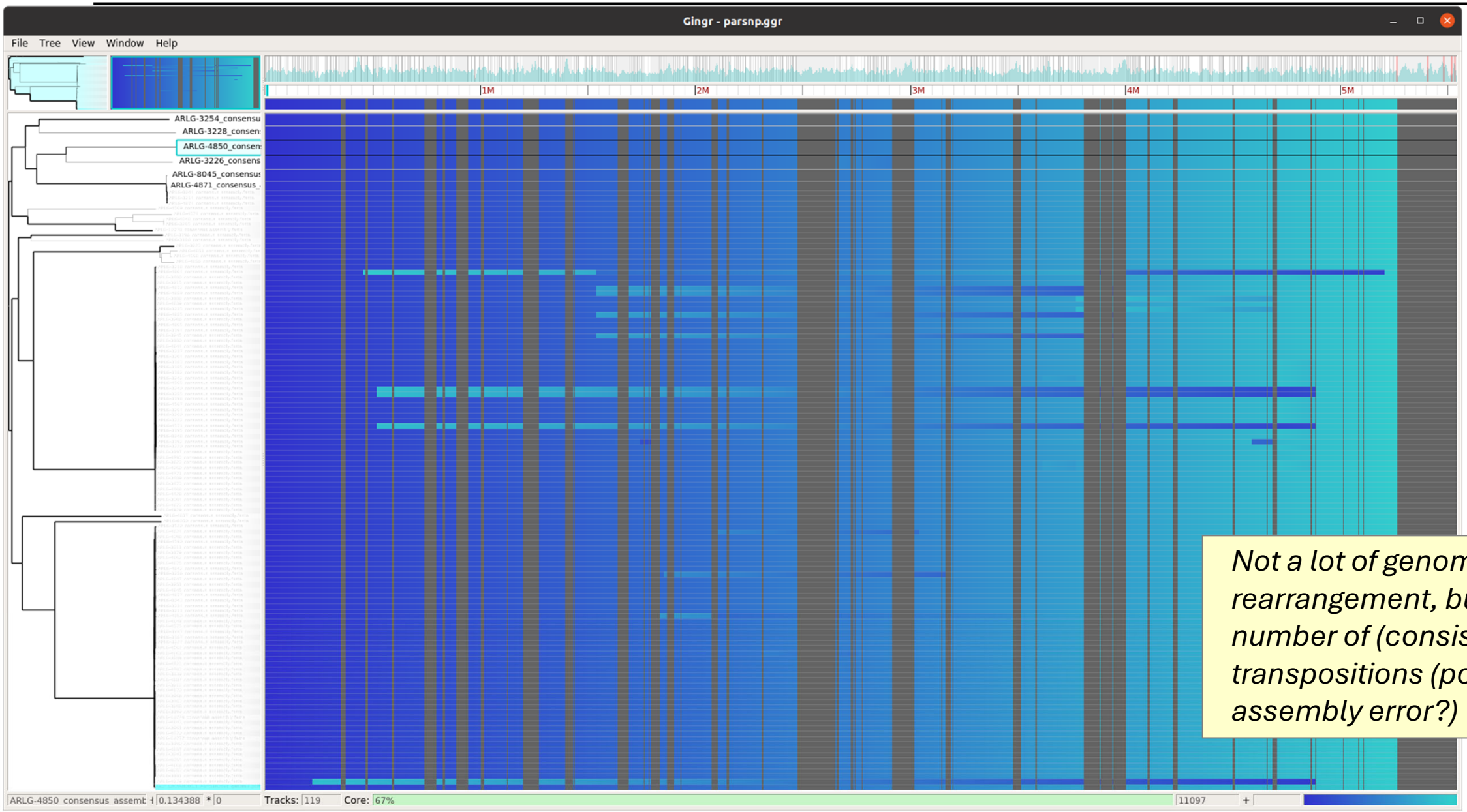
The reference genome here is GCF_000598005.1, described as "strain=30660/NJST258_1", so ostensibly ST258.

Case Study #1: Digging In…



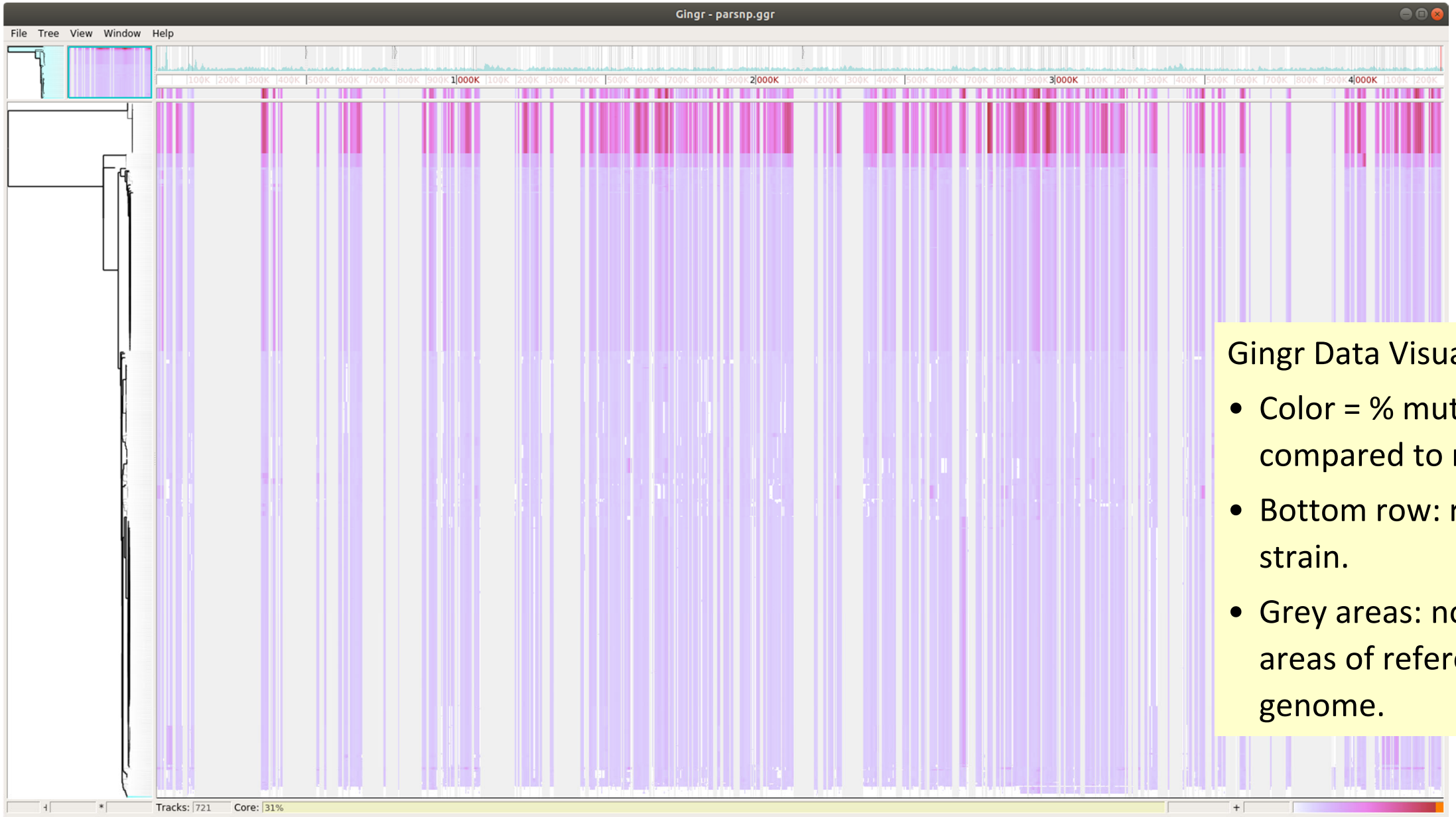
We can use Gingr to dig into areas of the genome where the two Clonal Groups differ from one another, for example these genes "hisP" and an un-annotated one adjacent to it.

Case Study #1: Synteny Comparison



Not a lot of genome rearrangement, but a small number of (consistent) large transpositions (possible assembly error?)

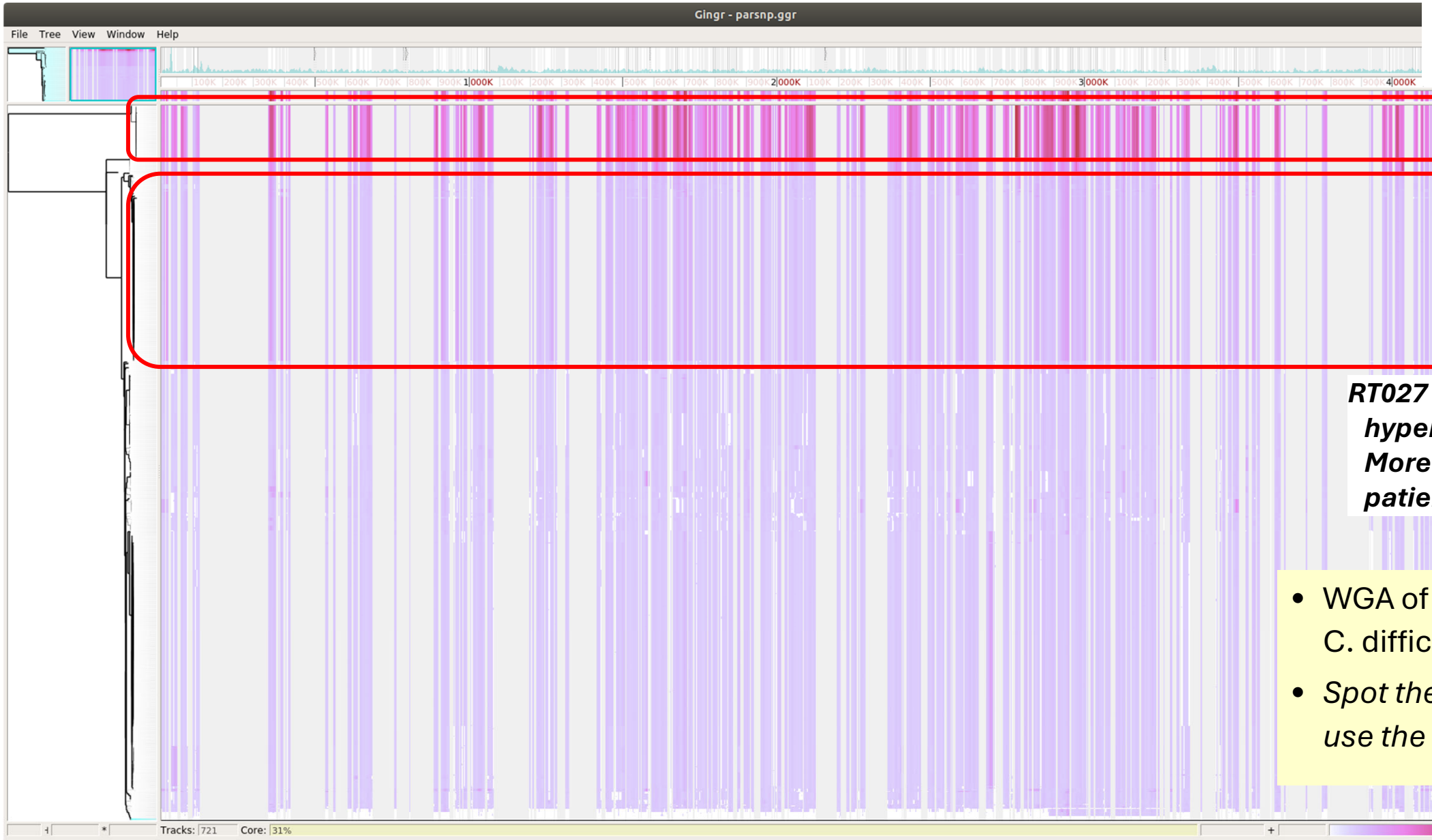
Case-Study #2: *C. difficile* Genomes



Gingr Data Visualization:

- Color = % mutation compared to reference
- Bottom row: reference strain.
- Grey areas: non “core” areas of reference genome.

Case-Study #2: *C. difficile* Genomes

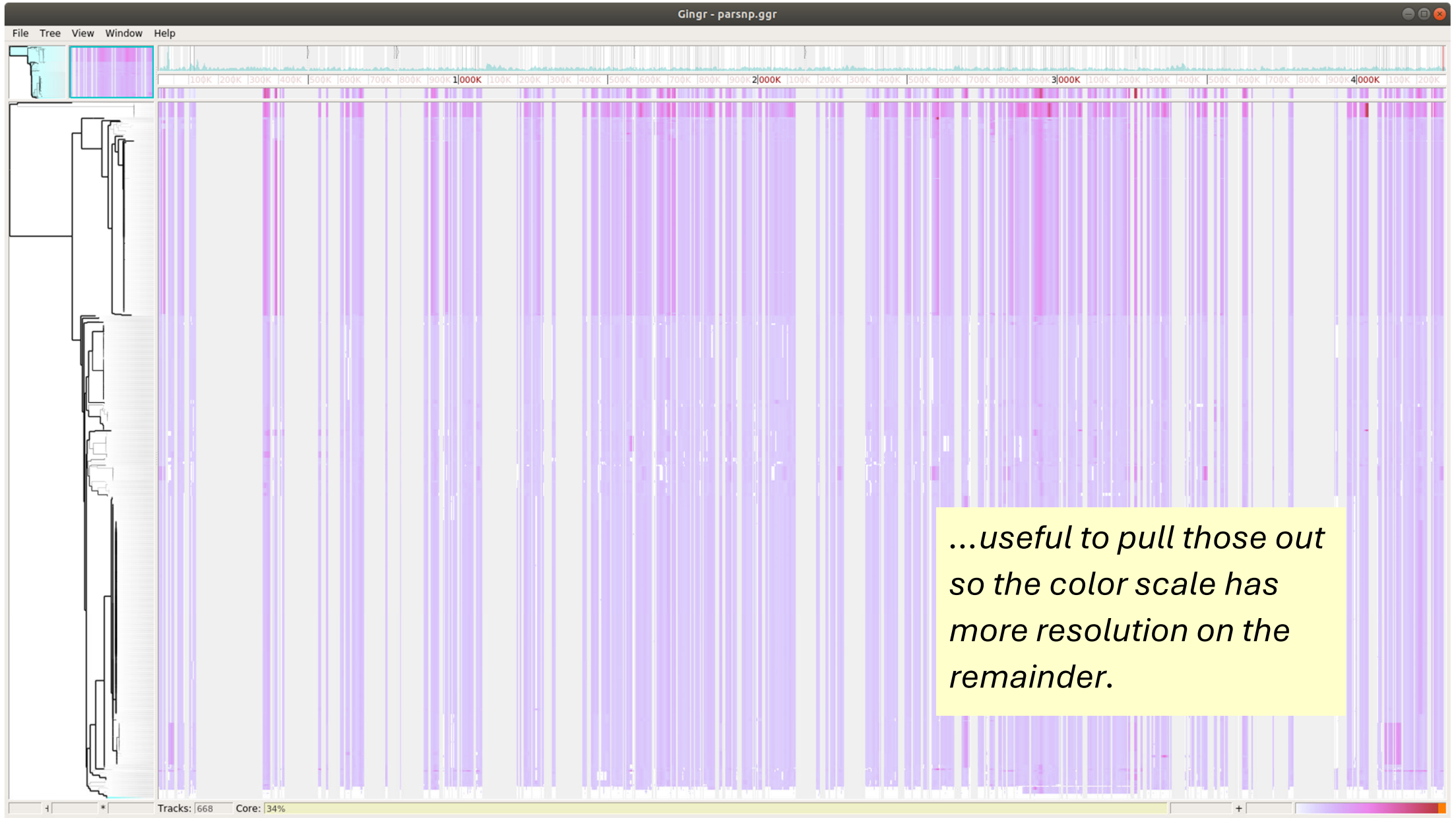


RT078 – Originated in animal host, crossed over

RT027 – Known hypervirulent strain. More recurrent, nastier patient outcomes.

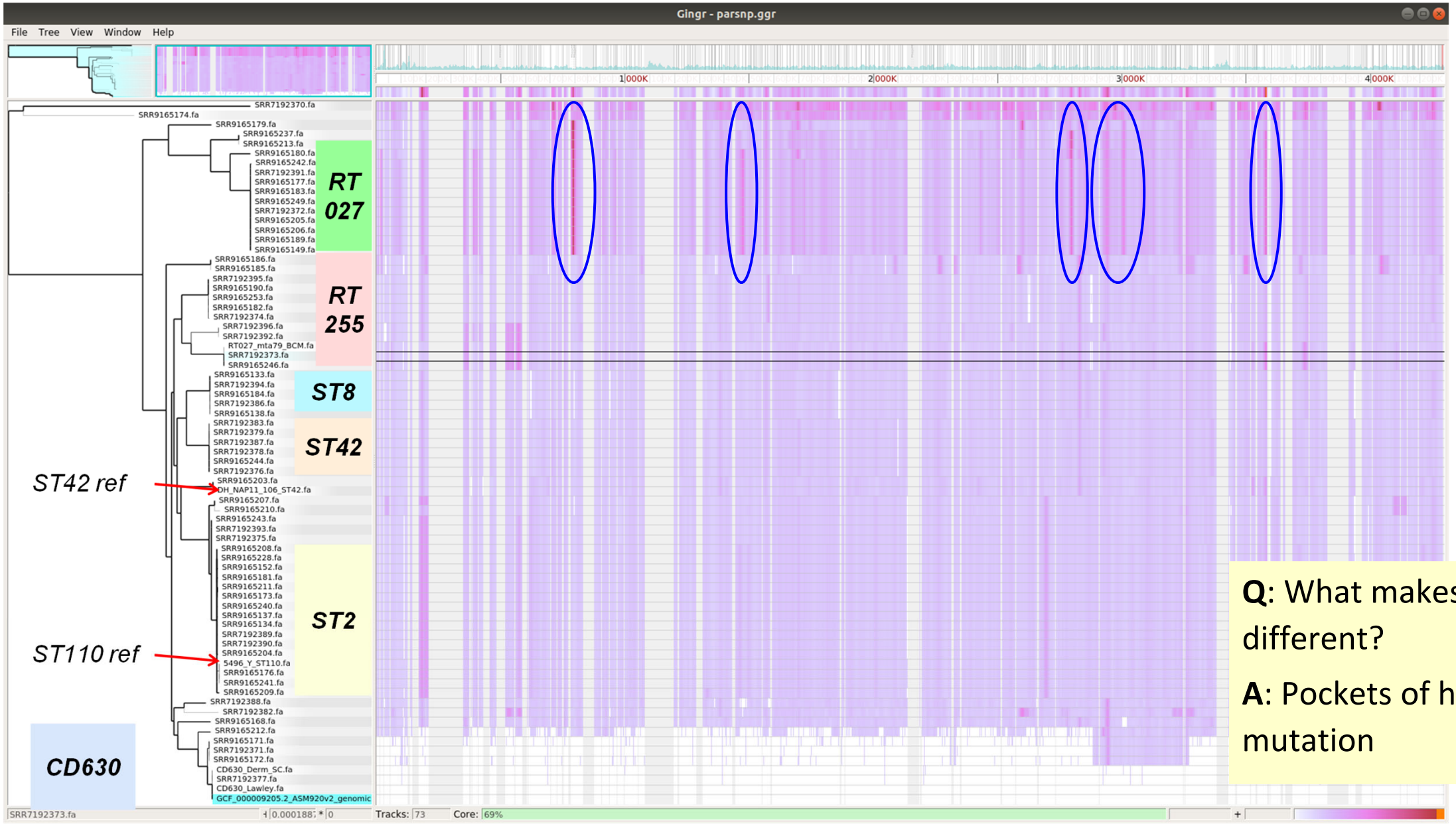
- WGA of 720 assembled C. difficile genomes
- *Spot the strains… (hint: use the tree)*

Case-Study #2: *C. difficile* Genomes (excluding RT078 samples)



Case-Study #2

Subset of Genomes w/ST annotation



Q: What makes RT027 different?

A: Pockets of heavy mutation

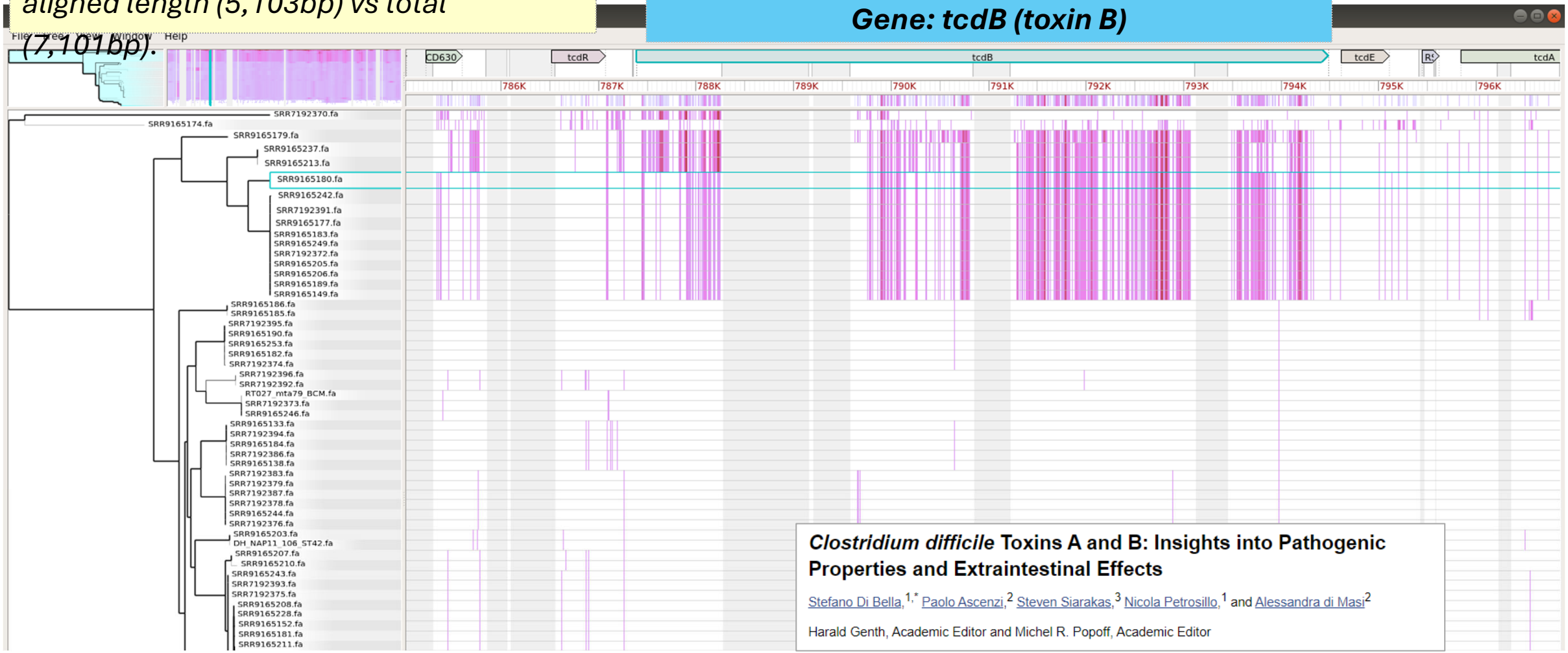
Case-Study #2

Digging Deeper…

Note: *not all of the tcdB gene was aligned by Parsnp, so this table represents the aligned length (5,103bp) vs total (7,101bp).*

- *This particular region is precisely the coding locus for Toxin B.*
- *RT027 carries a variant tcdB gene with altered function that contributes to its virulence.*

Gene: tcdB (toxin B)



***Clostridium difficile* Toxins A and B: Insights into Pathogenic Properties and Extraintestinal Effects**

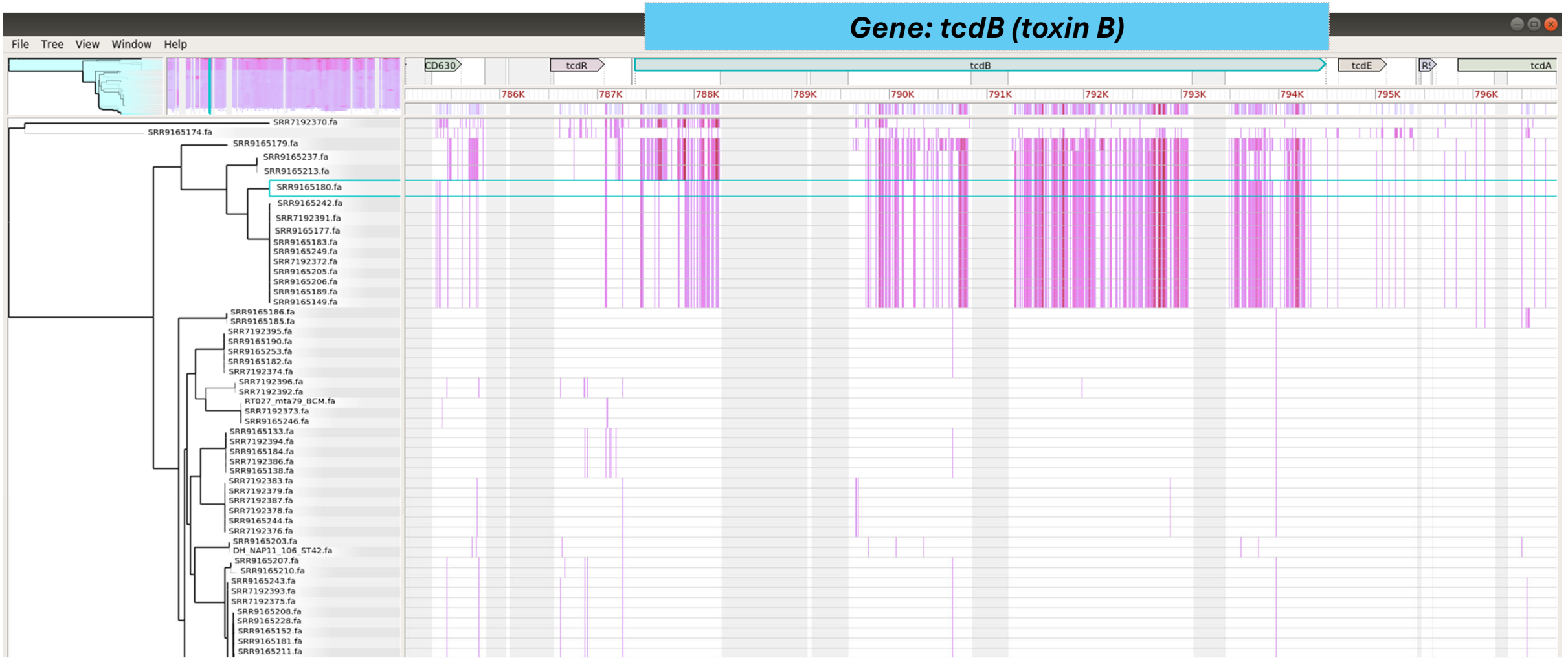
Stefano Di Bella,[1,*] Paolo Ascenzi,[2] Steven Siarakas,[3] Nicola Petrosillo,[1] and Alessandra di Masi[2]

Harald Genth, Academic Editor and Michel R. Popoff, Academic Editor

Case-Study #2

Remark...

Gene-level phylogenetic signal largely matches up with whole-genome phylogeny...

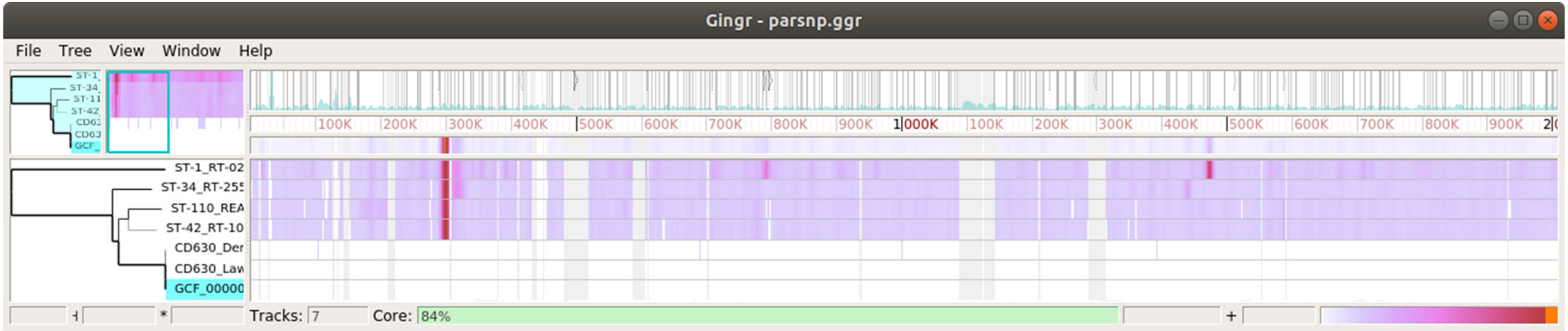


Case-Study #2

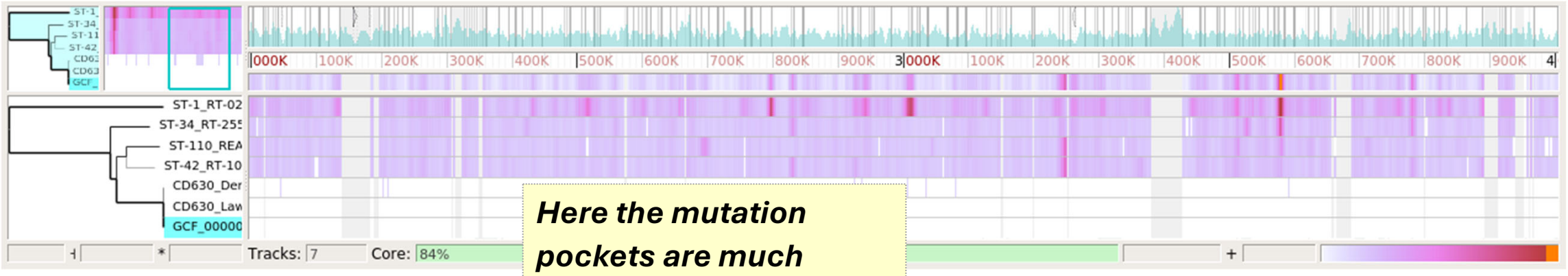
Comparing Reference Genomes for Some Strains

***Note**: RT027 is in the top row. CD630 is a lab strain used as a common reference.*

Segment 1 (positions 0-2mbp)



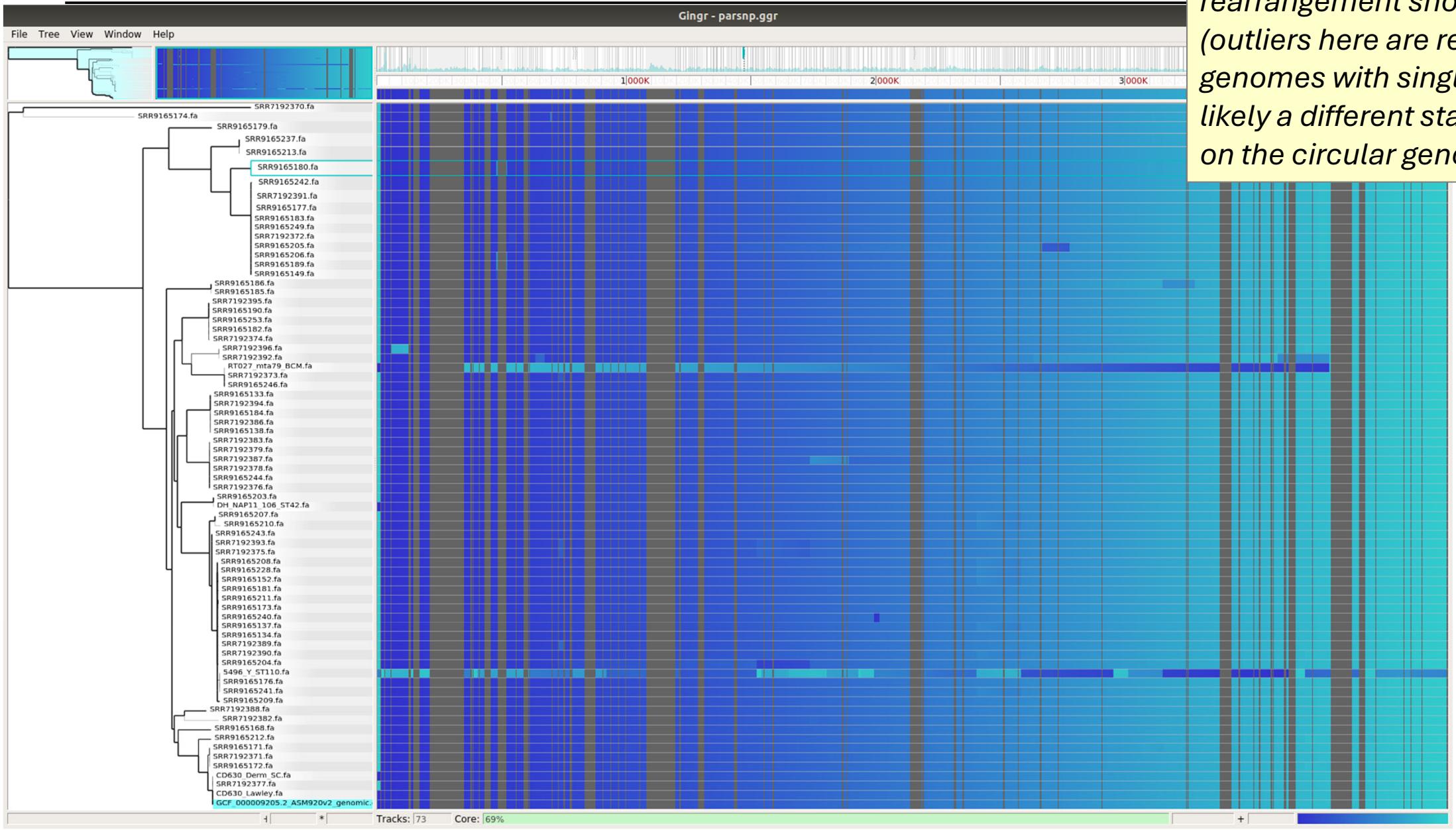
Segment 2 (positions 2-4mbp)



Here the mutation pockets are much clearer.

Case-Study #2

Synteny Comparison: *C. difficile* Isolates



For C. diff, even across a huge number of isolates, very little rearrangement shows up (outliers here are reference genomes with single contig, likely a different starting point on the circular genome.)

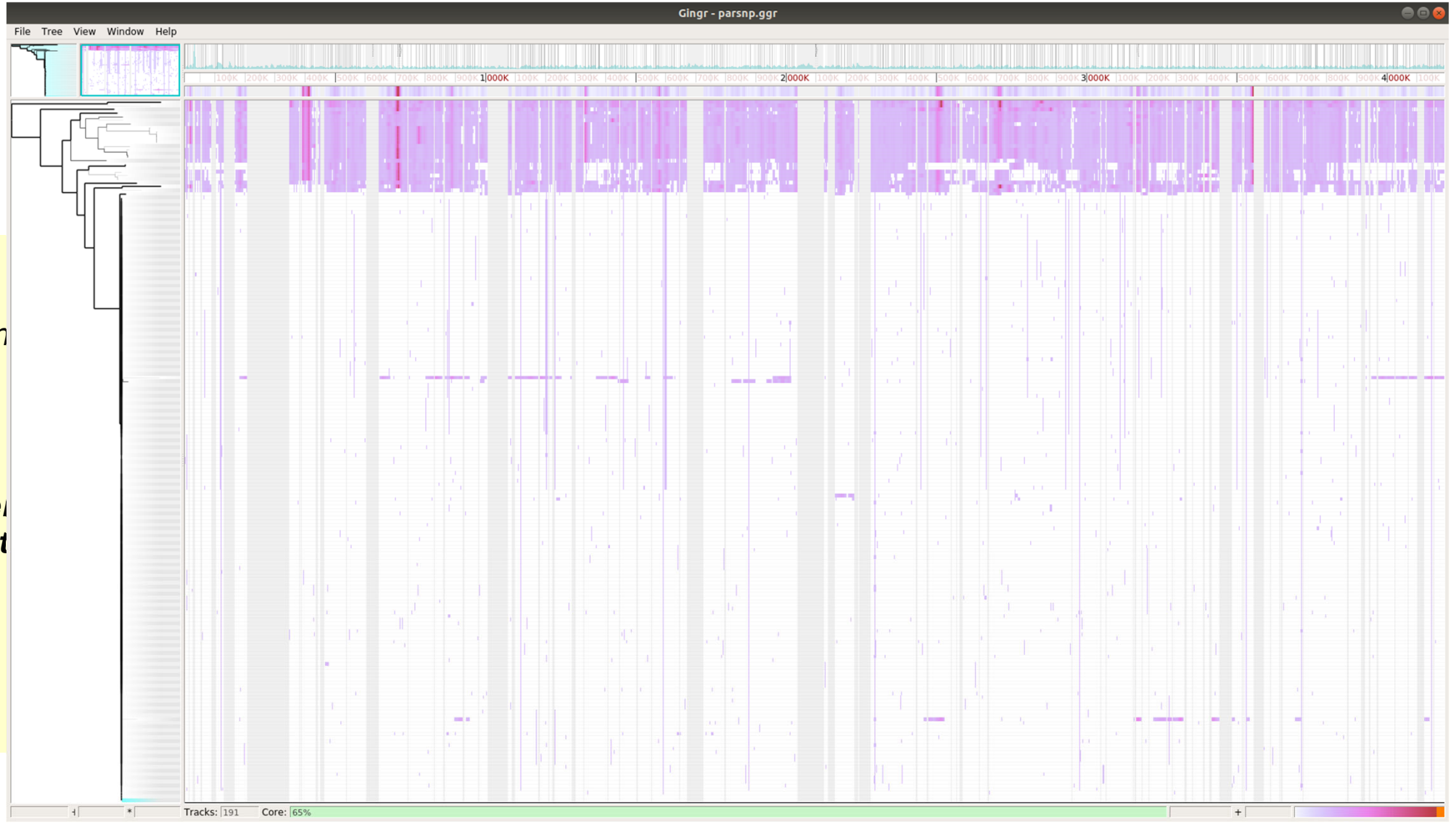
Case-Study #2

Alignment of RT027 isolates (and near relatives) to RT027 ref.

Q: *Does the RT027 Reference match the genomes from the clin*

A: *...Yes*

- ***Very little to see, ve high match level wit all RT027 isolates except 3.***



Case Study #3: *R. gnavus* Isolates from IBD Patients

14 Genomes:

- Reference: ATCC 29149 (RefSeq GCF_008121495)
- ATCC 29149 *de novo* assembly (by me)
- ATCC 35913 (GenBank GCA_900036035)
- 12 Genomes from Hall et al. (2017) (table at right)

RJX1118*	Stool from infant treated with antibiotics
RJX1119*	Stool from infant treated with antibiotics
RJX1120*	Biopsy from IBD patient
RJX1121*	Biopsy from IBD patient
RJX1122*	Biopsy from IBD patient
RJX1123*	Biopsy from IBD patient
RJX1124*	Biopsy from IBD patient
RJX1125*	Biopsy from IBD patient
RJX1126*	Biopsy from IBD patient
RJX1127*	Biopsy from IBD patient
RJX1128*	Stool from IBD patient

Case-Study #3

R. gnavus Isolates from IBD Patients

Game 1 : Spot the 2 Genomes from Infant Stool (non-IBD)

Case-Study #3

R. gnavus Isolates from IBD Patients

Game 1 : Spot the 2 Genomes from Infant Stool (non-IBD)

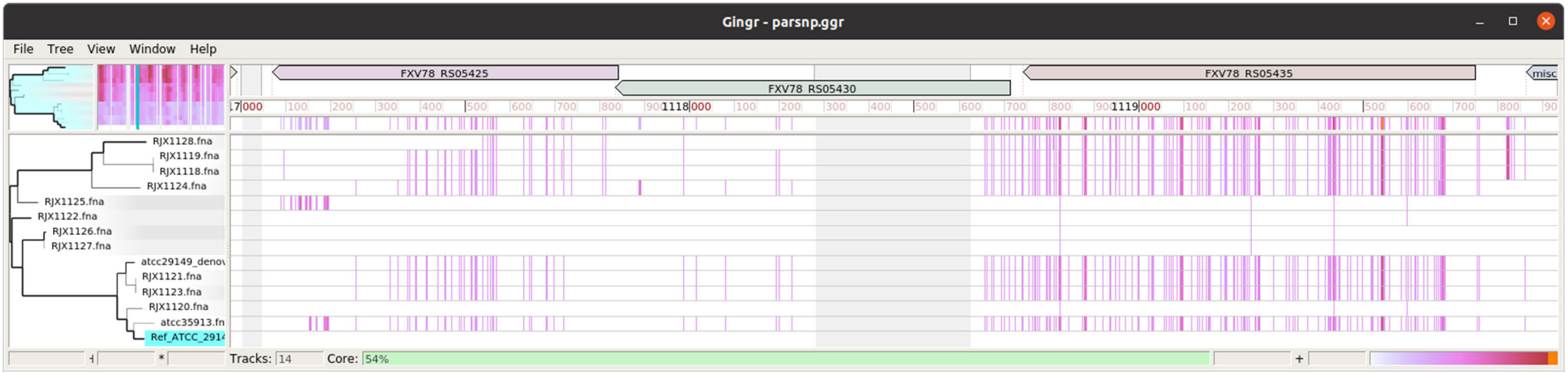
Game 2 : Spot the 2nd ATCC 29149 genome (supposedly the same as the reference)

Case-Study #3

R. gnavus strain-level phylogenetic signal is a mes

Depending on the operon, the phylogenetic appearance is completely different
⇒ ***Highly plastic genome***

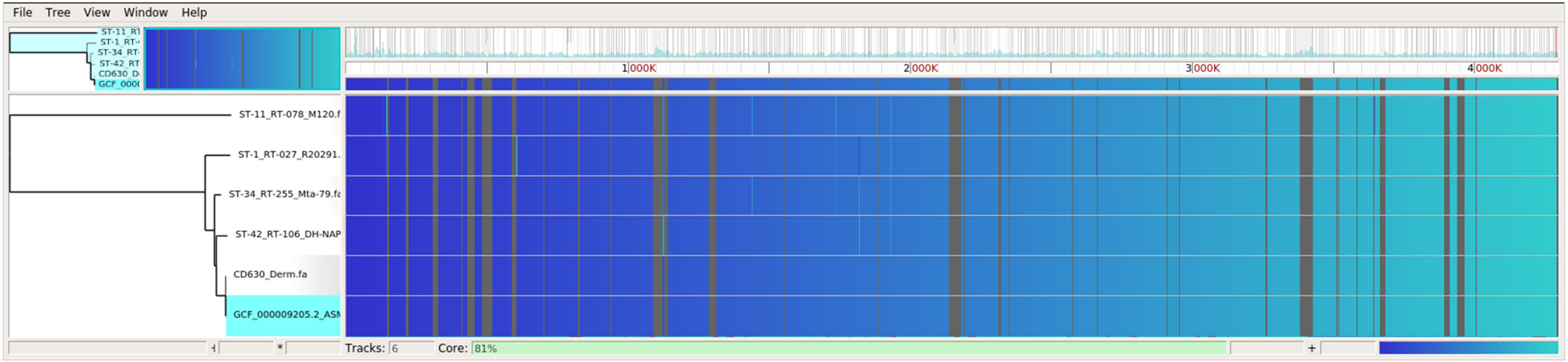
This does NOT show genome rearrangements (synteny)

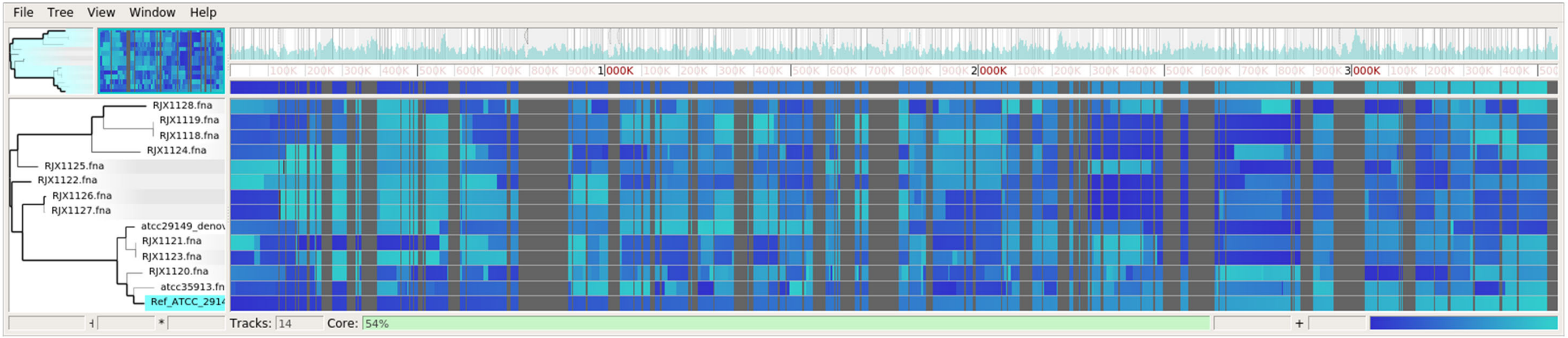


Case-Study #3

Synteny Comparison: *R. gnavus* & *C. difficile*

These two organisms have very different types of genome plasticity.





Conclusions

- Whole-genome alignment will give a detailed comparison specifically of the *core* genome
  - Maybe also auxiliary genes (*pan*-genome)
- Visualization can get you up close and personal with the data
  - (This statement applies to almost everything, not just genomes)
- Strains can differ from one another in weird ways.
  - Selective mutation at points of interest
  - Gene gain/loss depending on environment
  - Genome-wide phylogenetic signal vs. Locus-specific signal
  - Etc…?

Special Thanks To:

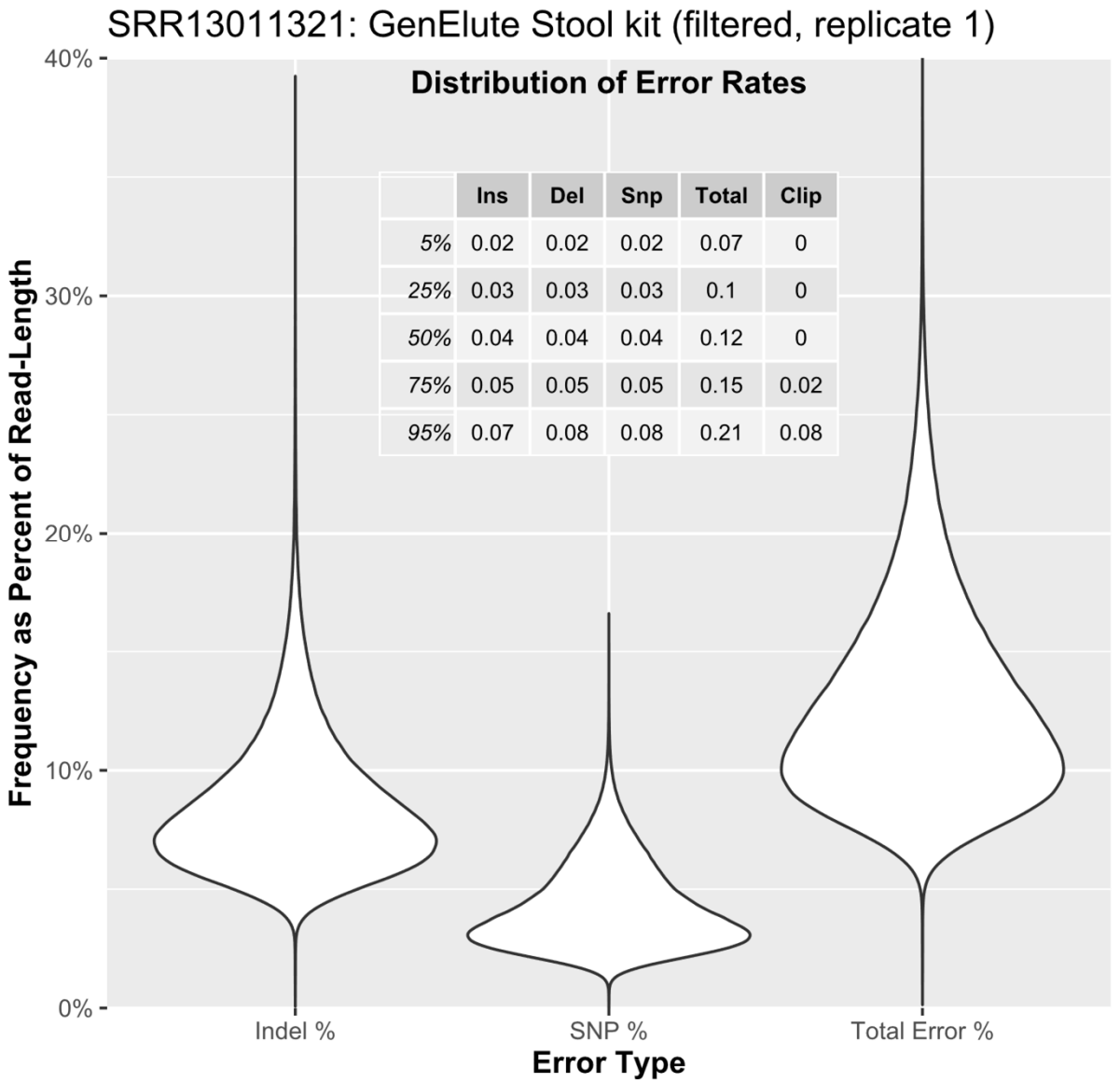
- The Treangen Lab (Rice)
  - Todd Treangen
  - Bryce Killie
  - Kristen Curry
  - Nick Sapoval
  - Yunxi Liu
  - Yilei Fu
  - Advait Balaji
- The Savidge Lab (Baylor College of Medicine)
  - Qinglong Wu
  - Charlie Seto
- Taylor Reiter (for the *R. gnavus* idea)

Bonus Topic: Taxon Abundance with Full-Length 16S ONT Reads

Not the HiFi ones, the noisy stuff…

ONT reads still have high error

- Data from Mann, et al. (2023)[1]
  - 16S amplicons with 27F/1492R primers
  - Multiple extraction protocols
    - *Only GenElute shown at left*[2]
    - *Others have nearly identical error rates*
  - Zymo standard, sequenced on MinION
  - R9.4.1 chemistry
  - Base-called with Guppy 3.2.4
  - Reads filtered with NanoFilt
- Median total error rate: 12%
  - 90% of reads between 7-21% error as percent of read-length
  - *Not* including soft-clipping, which remains in some reads even post-filtering.



	Ins	Del	Snp	Total	Clip
5%	0.02	0.02	0.02	0.07	0
25%	0.03	0.03	0.03	0.1	0
50%	0.04	0.04	0.04	0.12	0
75%	0.05	0.05	0.05	0.15	0.02
95%	0.07	0.08	0.08	0.21	0.08

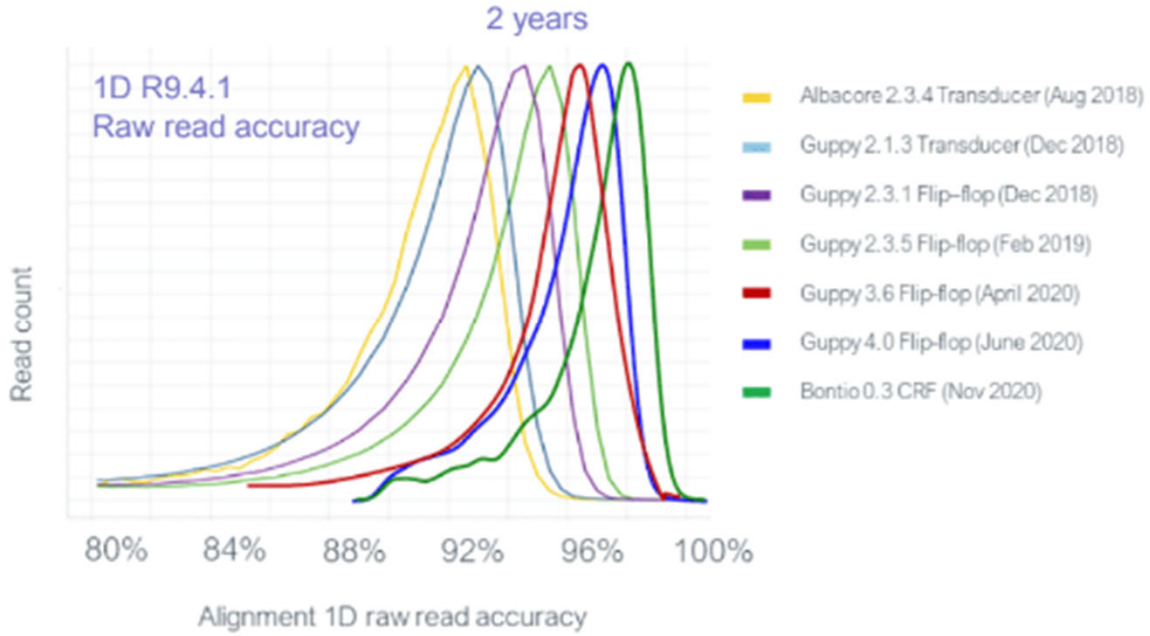
[1]*DOI: 10.1016/j.dib.2021.107036*2

[2]*As a sidenote, one of the kits was the QIAamp DNA Microbiome Kit which appeared to **really** mess up the profile of microbes recovered somehow.*

Errors coming down, but still material

- R10 vs. R9 chemistry:
  - Zhang, et al (2023)[3] reports error rates on Zymo Standard
  - Qiagen DNeasy PowersoilPro, PromethION, Guppy v6,
  - R9.4.1 and R10 chemistry
  - *(Data is non-public)*
- Error Rates[4]:
  - 9% total error with Guppy v6 vs. 11-12% ca. 2020

Chem.	Samp.	SNP	Ins.	Del.	Total
R9.4.1	S1	3.1%	1.5%	2.6%	7.2%
	S2	3.5%	1.5%	2.6%	7.6%
	Zymo	4.5%	1.6%	2.8%	8.8%
	All	3.6%	1.5%	2.7%	7.9%
R10.4.1	S1	1.5%	0.4%	0.6%	2.4%
	S2	1.8%	0.4%	0.6%	2.9%
	Zymo	2.9%	0.6%	0.7%	4.2%
	All	2.1%	0.5%	0.6%	3.2%



Basecalling has been improving, but true error rates still have a ways to go…

Image credit: Clive Brown via @nanopore on ~~Twitter~~X

[3]*DOI: 10.1128/aem.00605-23*

[4]*This is a subset of Table 2 from Zhang, et al. Zymo is the Zymo Standard D6305, S1/S2 are other samples*

16S Taxonomic Profiling: Read-Length vs. Error-Rate

- Relative Abundance from 16S Amplicons:
  - First 16S taxon identification was accomplished by the ancient Romans *citation needed*
- Illumina Reads:
  - ***Short reads*** ⟹ limited power to discriminate at species level (at least without extra effort)
  - ***Low error*** ⟹ classification generally accurate
  - ∗ *Recall/Sensitivity problem*
- Nanopore Reads:
  - ***Full length 16S*** ⟹ Species-level classification
  - ***High error rate*** ⟹ Cross confusion common with near relatives
  - ∗ *Precision/Specificity problem*

› Microbiome. 2020 May 15;8(1):65. doi: 10.1186/s40168-020-00841-w.

Construction of habitat-specific training sets to achieve species-level assignment in 16S rRNA gene datasets

Isabel F Escapa [1 2 3], Yanmei Huang [1 2], Tsute Chen [1 2], Maoxuan Lin [1], Alexis Kokaras [1], Floyd E Dewhirst [1 2], Katherine P Lemon [4 5 6 7]

Affiliations + expand

PMID: 32414415 PMCID: PMC7291764 DOI: 10.1186/s40168-020-00841-w

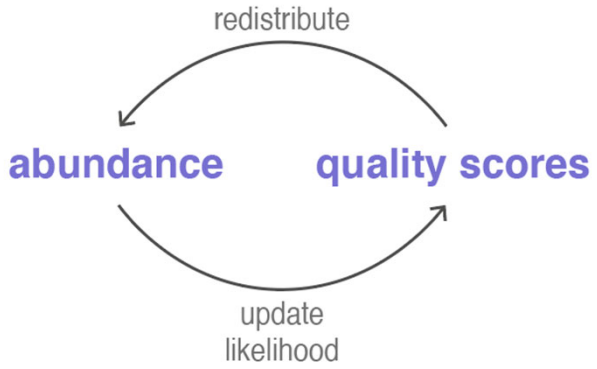
Free PMC article

This is one of the few references that attempts species-level classification based on 16S sequencing using Illumina sequences…

…does it by designing domain-specific database.

Emu: Species-level Taxon ID for full-length 16S reads

The EM algorithm corrects taxon assignment errors by considering prior likelihood that read was mis-assigned to a close relative.



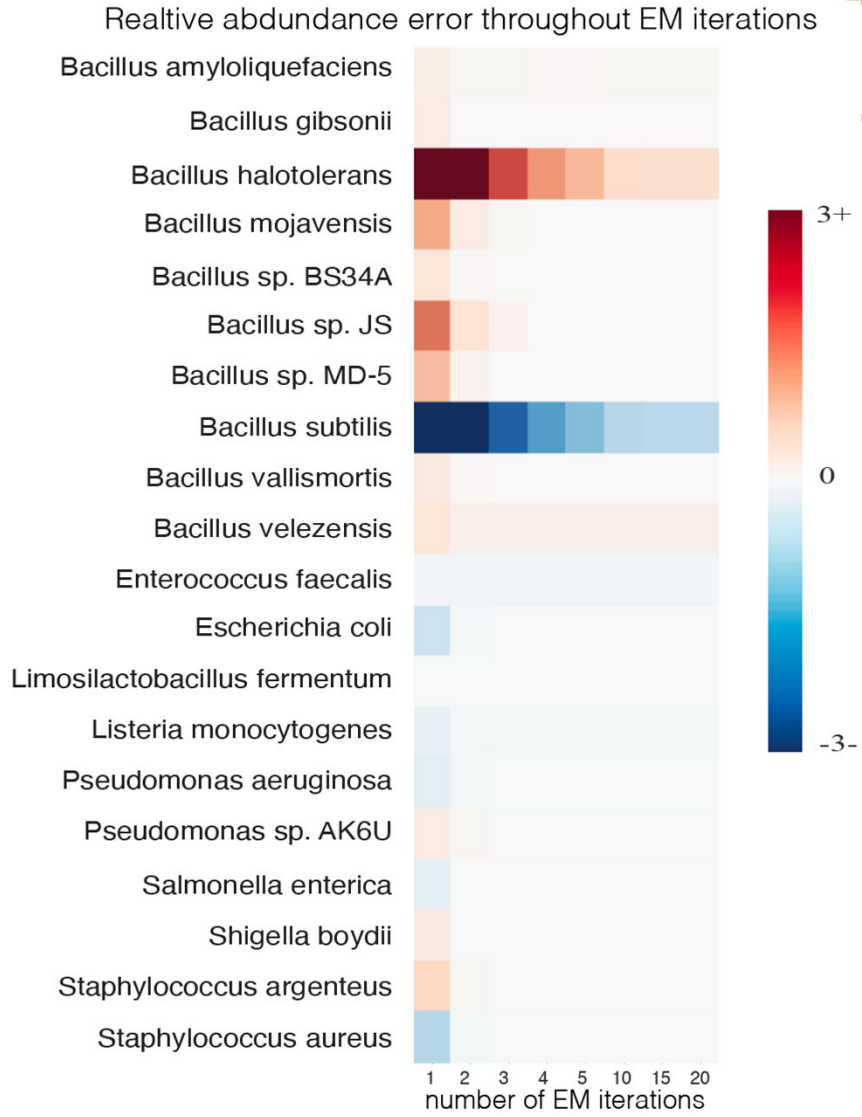
Prob. that read r comes from taxon t.

Likelihood of seeing read r when actual sequence is taxon t.
(Entirely driven by ONT error rates.)

Likelihood function:

$$P(t|r) = \frac{L(r|t)\pi(t)}{\sum_{t \in T} L(r|t)\pi(t)}$$

Prior probability of the read coming from taxon t (i.e. current estimate of abundance profile)



In collaboration with: Alexander Dilthey and Alona Tyshaieva, Institute of Medical Microbiology and Hospital Hygiene, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Slide Credit: Kristen Curry

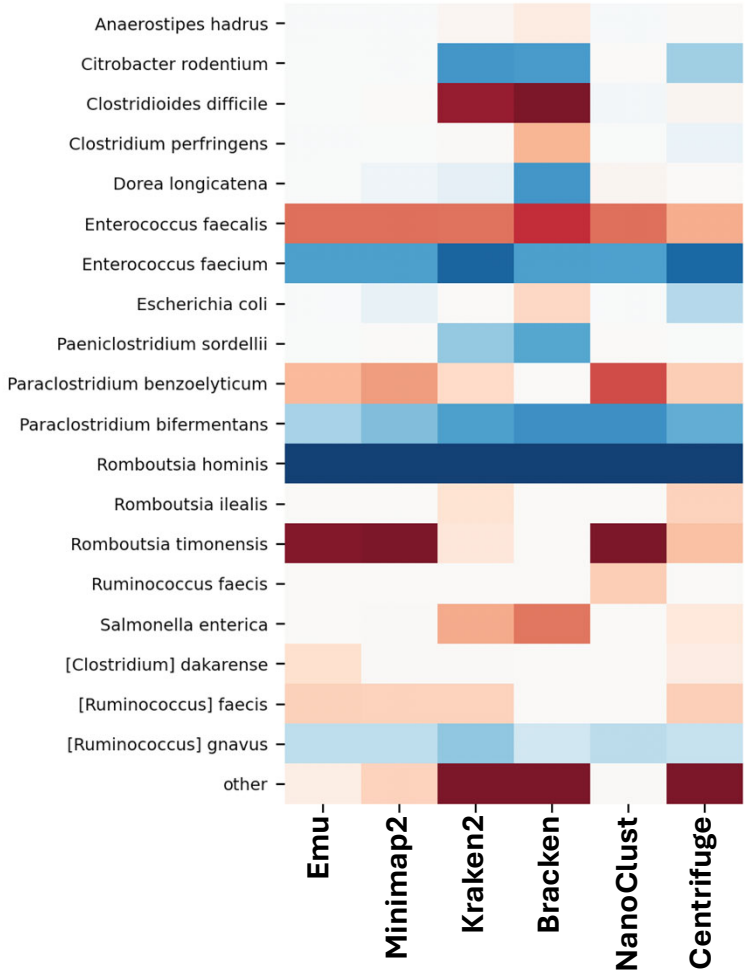
Example Results: Species-Level Relative Abundance

Synthetic Gut Community

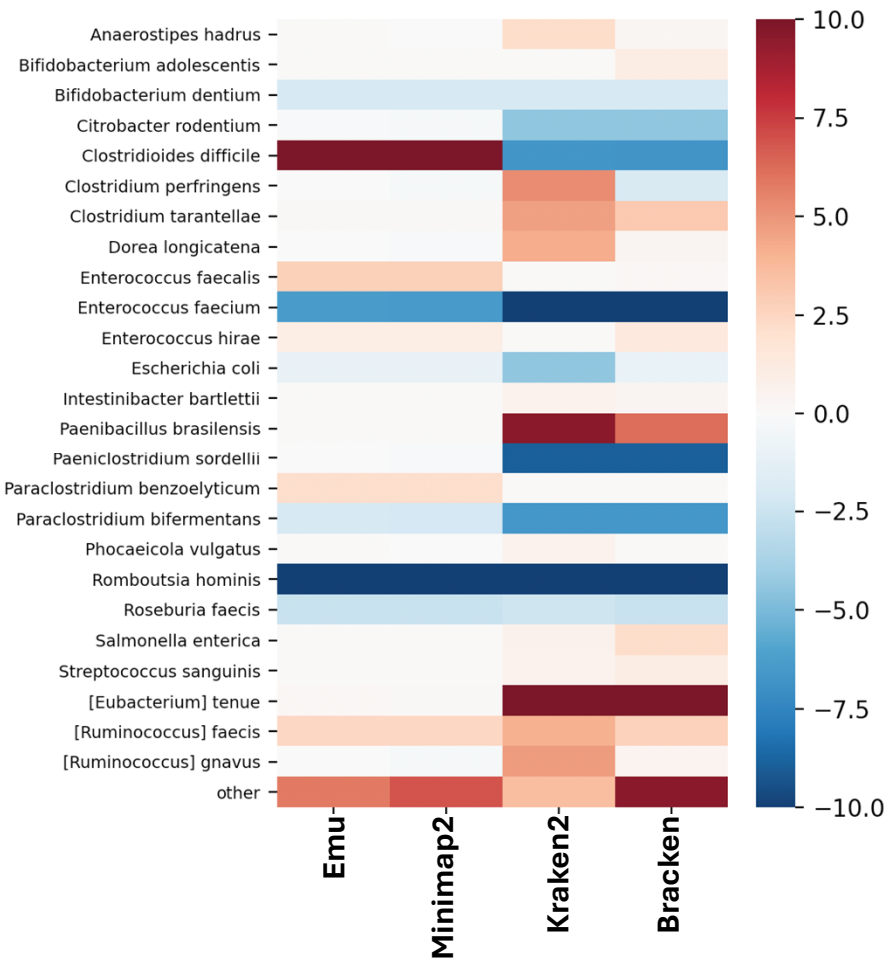
Approximate Ground Truth Abundance

Strain	CFU	Pct
Bifidobacterium dentum ATCC 27678	20000	18.9
Entercocccus faecium S66643	20000	18.9
Citribacter rodentium ATCC 51459	13000	12.3
Gemmiger formicilis ATCC 27749	10000	9.4
Escherichia coli MG1655	10000	9.4
Clostridium perfringens MT676	9000	8.5
Romboutsia hominis FRIFI	7000	6.6
Clostridium leptum ATCC 29065	5000	4.7
Clostridium scindens ATCC 35704	4000	3.7
Ruminococcus gnavus ATCC 29149	3000	2.8
Bacteroides vulgatus PC510	2000	1.9
Clostridium bartlettii DSM 16795	600	0.5
Clostridum innocuum ATCC 14501	440	0.4
Clostridium bifermentans ERIN_30100	300	0.2
Clostridium sordellii ATCC 9714	240	0.2
Bacteroides thetaiotaomicron VPI -5482	200	0.19
Eubacterium hadrum DSM 3319	200	0.19
Clostridium difficile 630	200	0.19
Dorea longicatena DSM 13814	100	0.095
Roseburia faecis DSM 16840 (M72)	4	0.004

Nanopore

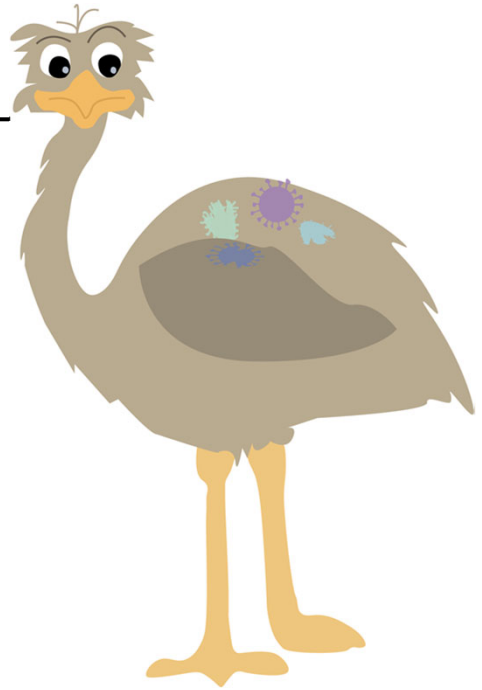


Illumina



...note that Emu does best here but it was also the only purpose-built 16S ONT method at the time (2021)

Emu Conclusions



- **Download:** https://github.com/treangenlab/emu
- Purpose-built for full-length, high-error 16S amplicon reads
  - I.e. ONT reads, especially R9 chemistry, even with old base-caller
  - Was the first algorithm *really* for this
    - Some newer ones now but we haven't evaluated
- Advantages (big):
  - Relative abundance accuracy (★ ★ ★)
  - Lower false positives (★ ★ ★ ★)
- Disadvantages (no big deal):
  - Some extra run time vs. just minimap2
  - Abundance below some minimum (e.g. 0.5%) is set to 0,
    - So not ideal for estimating very-low abundance members
- Extension to shotgun data via Lemur/Magnet[5] (pre-printed)

[5]*Image Credit: DALL-E*

Thank You

Please don’t hesitate to follow up with me after STAMPS if I can help or go through any of this material again.

Appendix

Multiple Sequence Alignment: Additional Slides

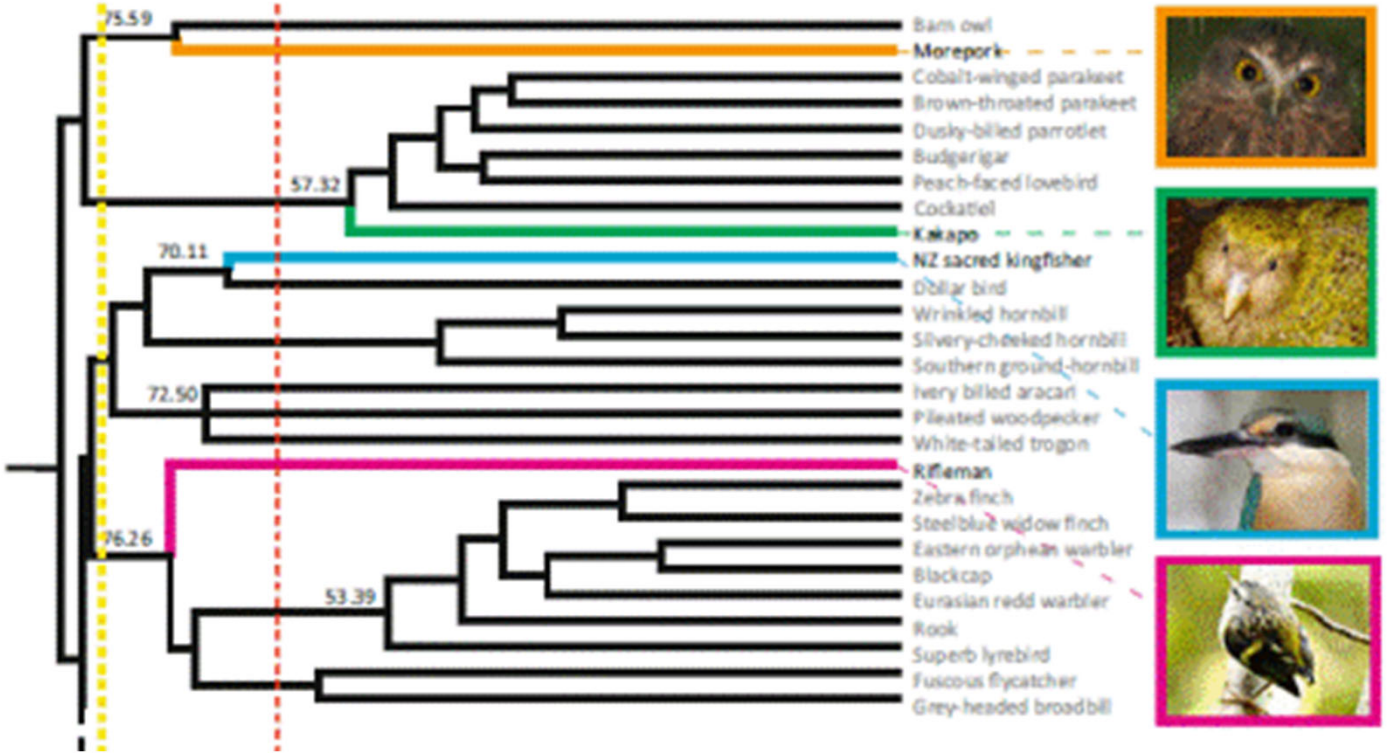
Contents

- MSA Extras
  - Star Trees, Failure Modes, Accuracy Dropoff
- Tree Estimation Extras
  - GTR details, Phylogenetic Placement
- Species Tree Estimation
- Gingr:
  - Quick How-to & Example

What Happens If the Alignment is Garbage?

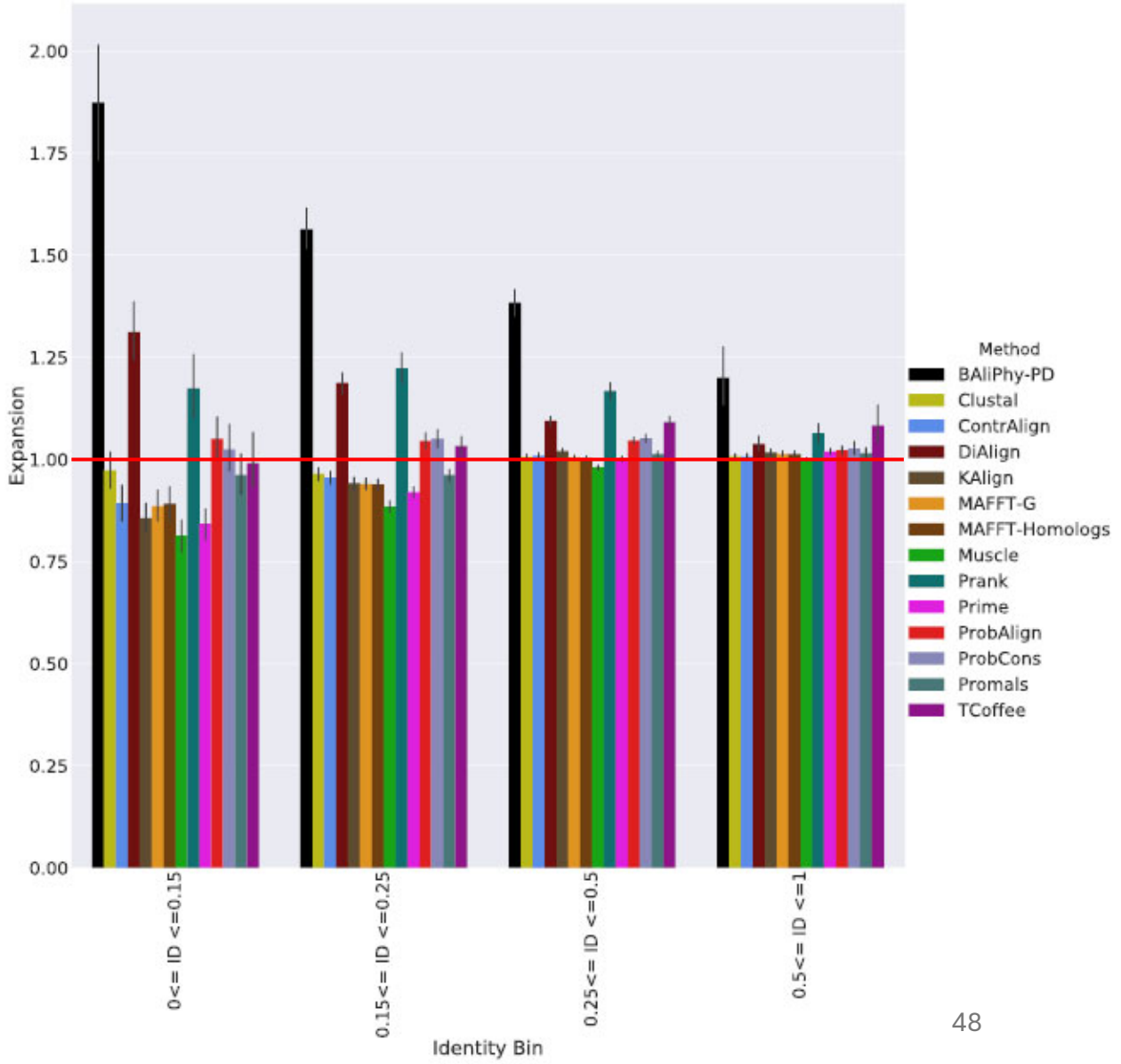
A poor alignment will give a tree with ***very deep* branches**

- I.e. long branches above leaves.
  - A "Star"-like tree
- "Nothing in this tree is systematically related to anything else in any significant capacity."
  - *Could be because relationships were nuked by bad alignment*
- Of course, star-like trees *can* be real! (e.g. birds)



Failure Modes: Under/Over-Alignment

- Most of the commonly used alignment methods will tend to *over*-align.
- *…better than aligning just the right amount incorrectly!*
- *…but can lead to some weird internal branches…*



Large N, Low %ANI ⟶ Very Hard Alignment

It is far easier than widely appreciated to get an alignment with ≈0% accuracy.

BE CAREFUL OUT THERE!

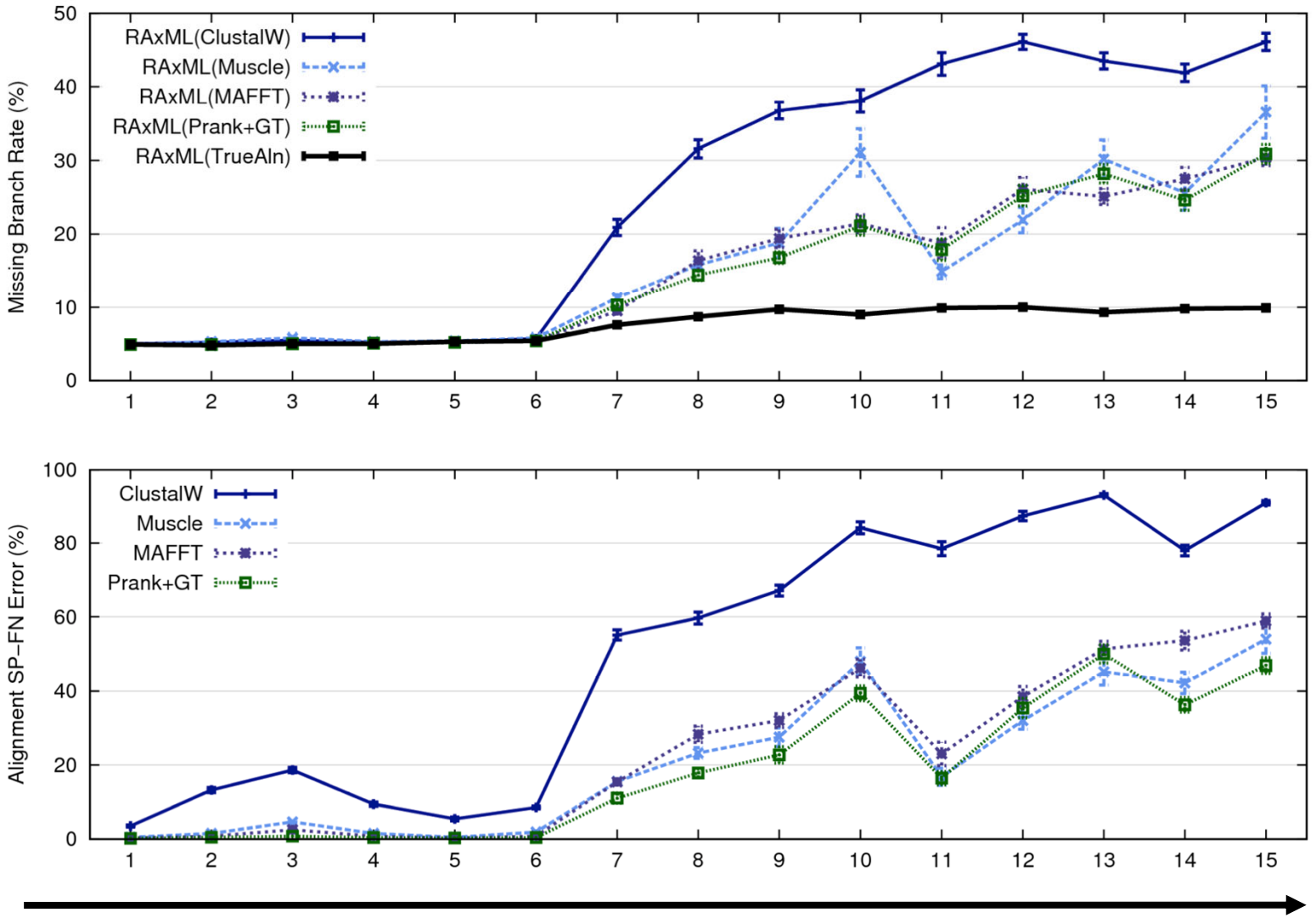
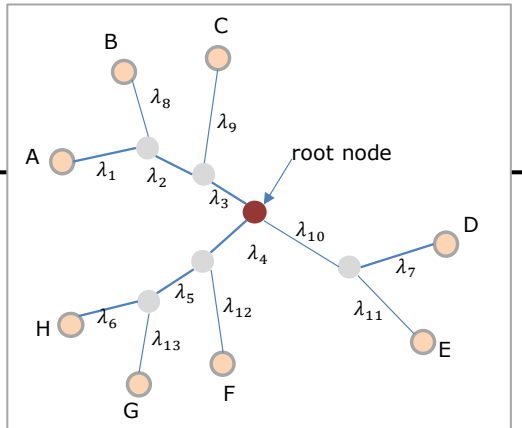


Image Credit: Tandy Warnow.

Generalized Time Reversible (GTR) Model



Example model tree $\mathcal{T}$ with 8 leaves

- A probability model for sequence evolution.
  - Characters drawn randomly at the root, then follows a CTMC on each branch in sequence.
- Parameters:
  - Binary Tree $\mathcal{T} = (T, \Lambda, \rho)$ with n leaves, topology T, branch lengths $\Lambda = (\lambda_1, \ldots, \lambda_{2n-3})$ and root node ρ (actually a nuisance parameter).
  - $\boldsymbol{\pi} = [\pi_A \quad \pi_C \quad \pi_G \quad \pi_T]$ is the probability vector of each character state at the root.
  - Transition rate matrix:

$$Q = \begin{bmatrix} - & a\pi_C & b\pi_G & c\pi_T \\ a\pi_A & - & d\pi_G & e\pi_T \\ b\pi_A & d\pi_C & - & f\pi_T \\ c\pi_A & e\pi_C & f\pi_G & - \end{bmatrix}$$

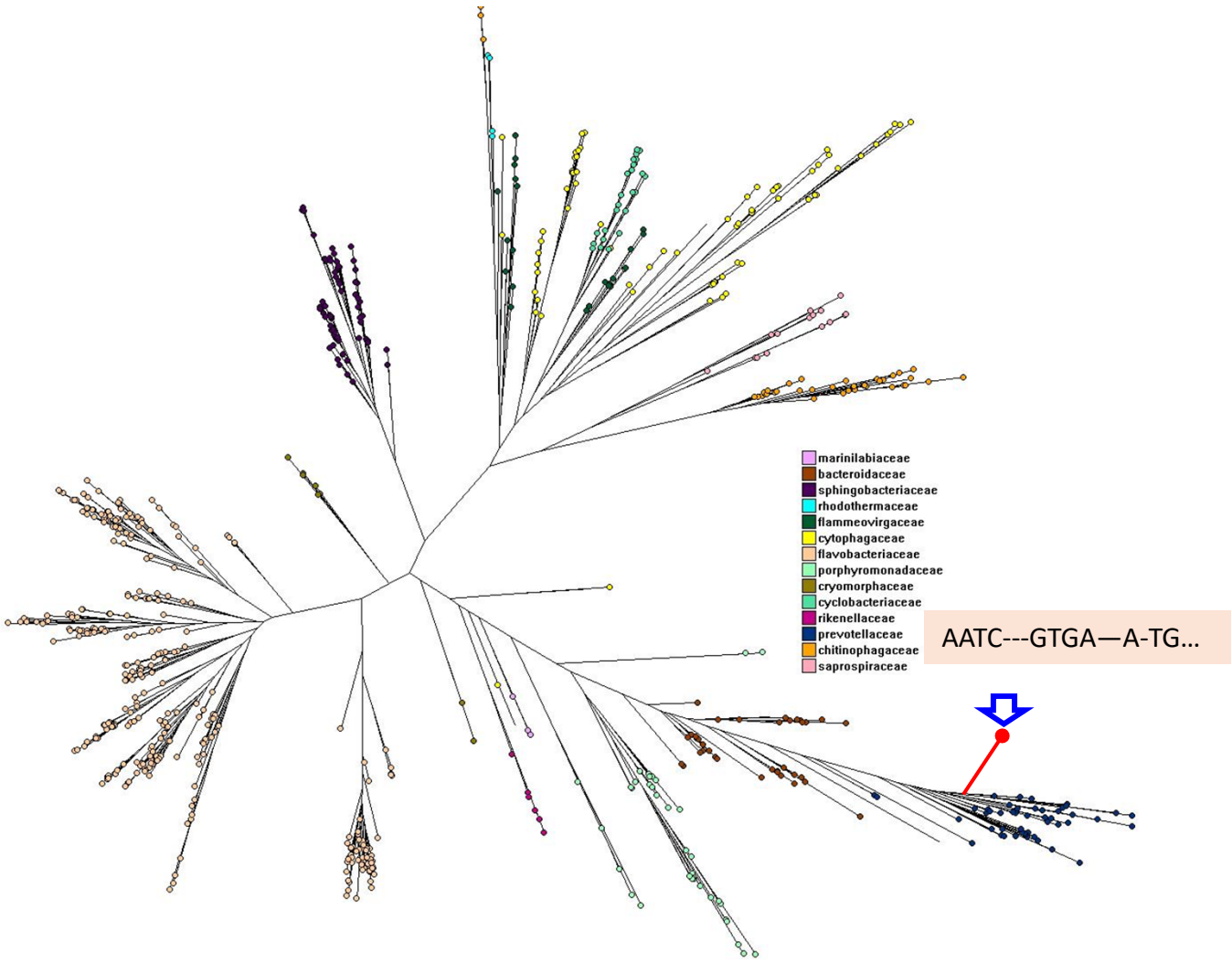
  where the values on the diagonal are set so the row sums to 0.

- Notes:
  - Assumes all sites (columns) evolve *i.i.d.*
  - No indels.
  - Given sequences generated according to the GTR, ML estimation of $(T, \Lambda, \boldsymbol{\pi}, Q)$ *is* statistically consistent.
  - Model is equivalent for any choice of root location.
  - In practice, gaps in the alignment are treated as missing data.
  - RAxML[1] and FastTree[2] are two off-the-shelf programs for ML phylogeny estimation.
  - ML topology estimation is NP-Hard.
  - **Branch lengths are denominated in *expected mutations per site.***

[1]https://cme.h-its.org/exelixis/web/software/raxml/index.html
[2]http://www.microbesonline.org/fasttree/

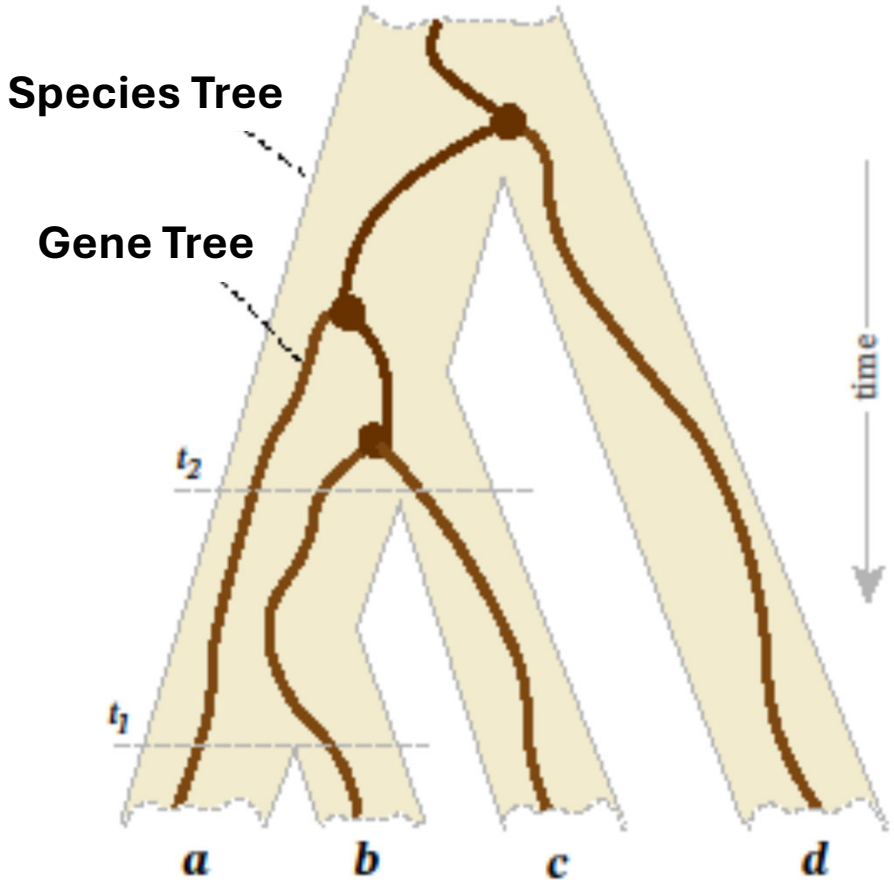
Related Problem: Phylogenetic Placement

- **Problem:** consider a fixed "reference" group of aligned sequences and a phylogeny T.
  - given a new sequence q find the best ***ne branch*** in $\boldsymbol{T}$ with q at the leaf (via ML).
- New branch includes:
  1. **Attachment Point**
     - *Identifies the closest relative in the data.*
  2. **New Branch Length ("pendant length")**
     - *Distance from its closest relative.*
     - *A measure of **novelty** relative to the original sequences*



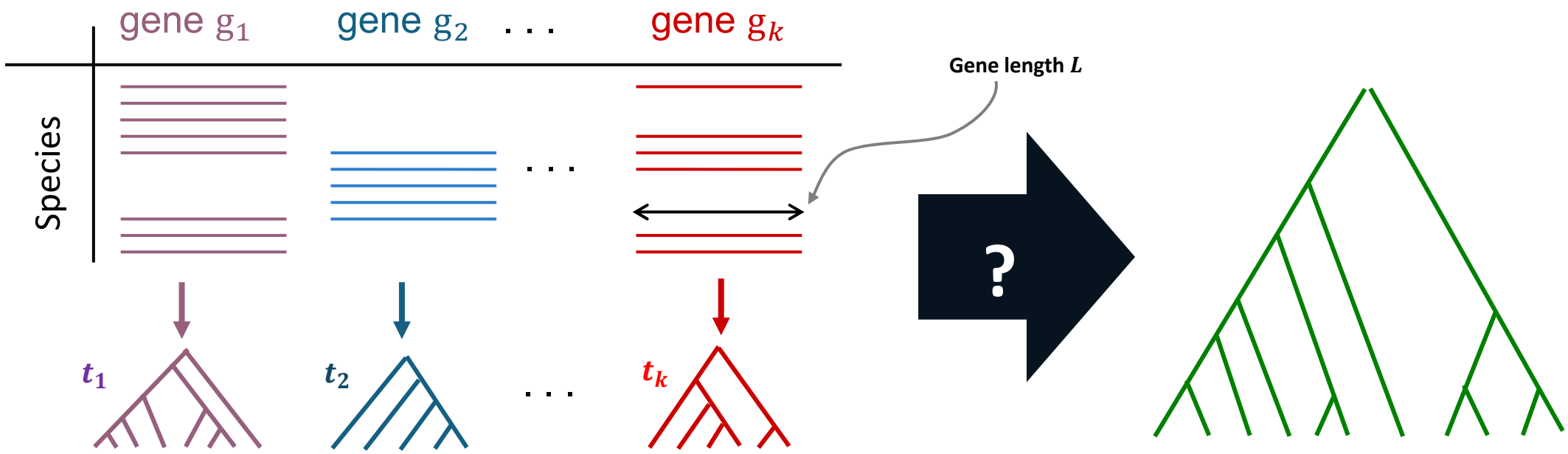
Species Trees: The Multi-Species Coalescent

- Genes evolve within a *population*
  - So gene branching events can happen without full population speciation
  - Can give rise to genes with a different tree shape than the species



Species Tree Estimation Problems

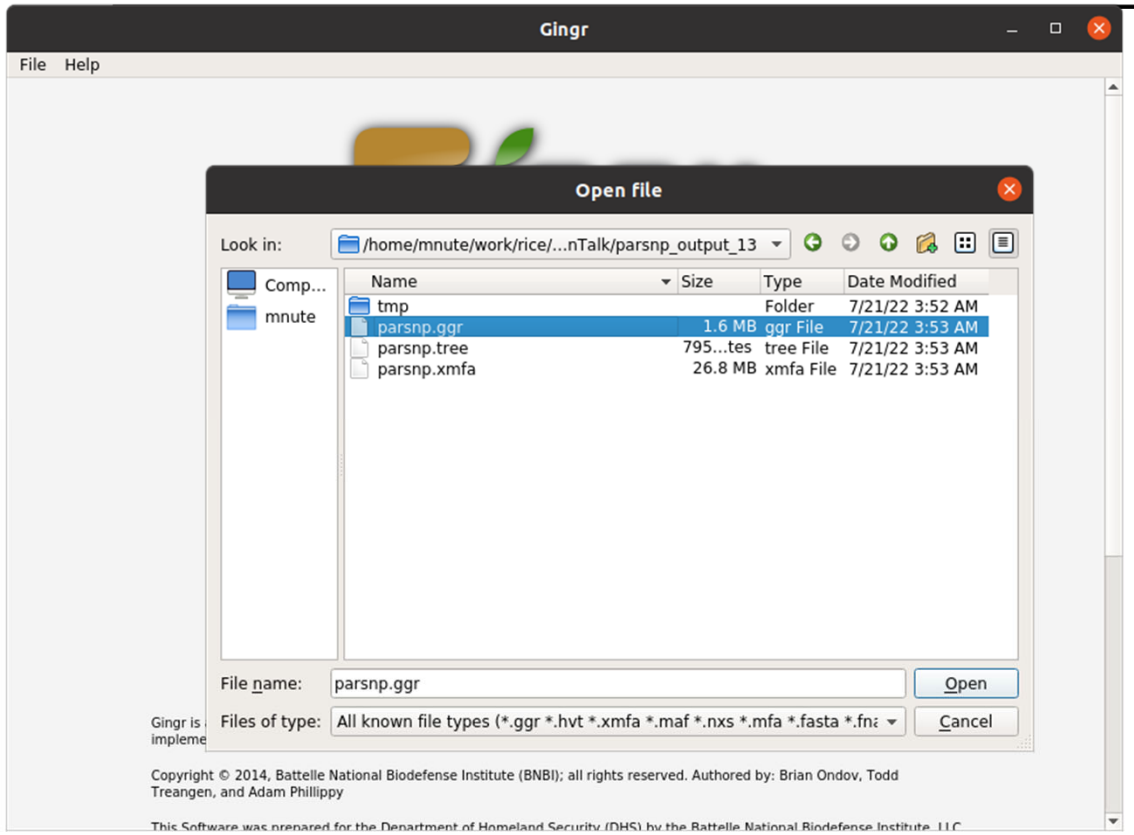
Problem: given multiple different genes generated from a single population species tree according to the multi-species coalescent (MSC), how do we estimate the population phylogeny for the species? And when is our estimate consistent?



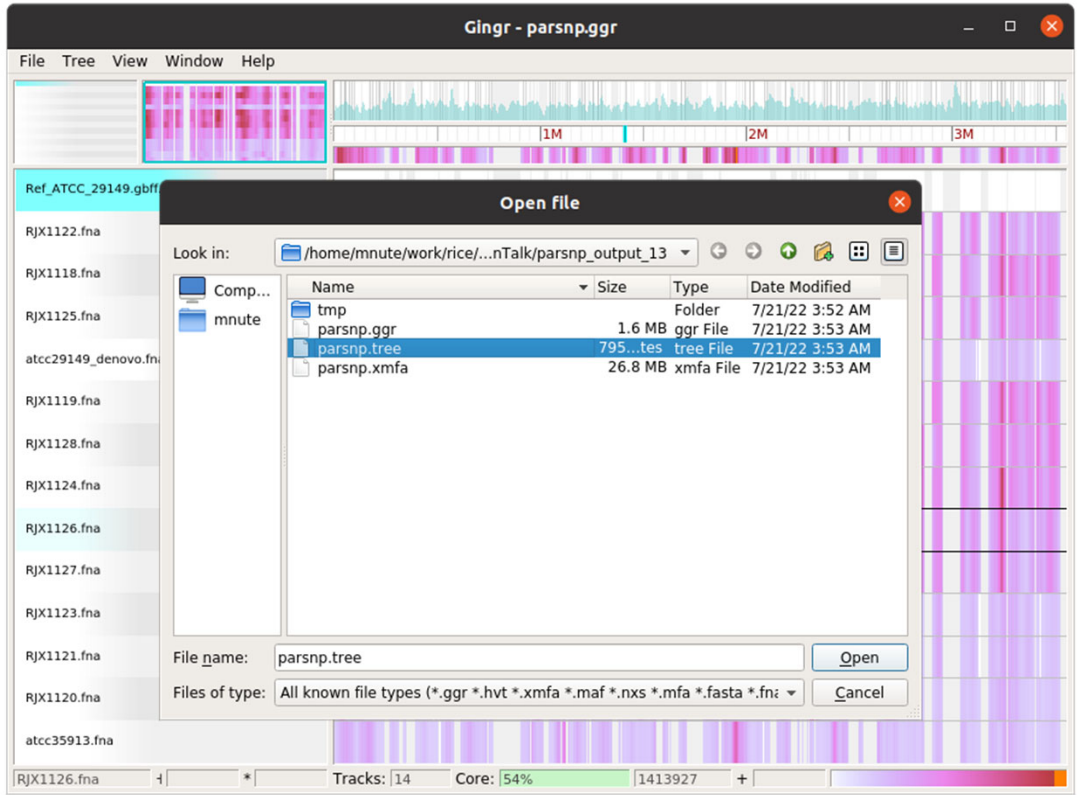
Species Tree Estimation Options

- What can we do to estimate a species tree when all the individual genes have their own topology?
  - Concatenation: join the individual gene-alignments together, in order, and estimate a tree under GTR.
    - Advantages: simple, RAxML can handle long sequences,
    - Disadvantages: ***not*** statistically consistent.
      - Not always a problem in practice though unless there is a lot of heterogeneity in gene trees.
      - In other words, species tree has many *relatively* recent speciation events.
  - Quartet methods: estimate the tree separately for every gene, then use a software specifically to combine the gene trees
    - E.g. ASTRAL
    - **IS** statistically consistent
    - Not quite as simple of a pipeline.
- My advice: use ASTRAL
  - *…but I know that most people don't bother.*

Appendix: Quick How-to with Gingr (1 of 2)



*1.) Open the *.ggr file created in the parsnp output folder.*



*2.) Once it is open, go back to the "Open" dialogue and open the *.tree file in the same folder.*

Appendix: Quick How-to with Gingr (2 of 2)

3.) This will give you the standard Gingr view. Other options to re-root the tree or to switch to Synteny view are available under the "Tree" and "View" menus.