

Genomics for evolutionary inference

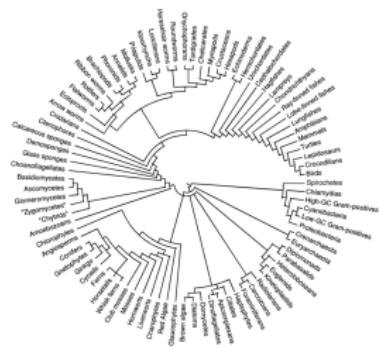
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How do you get from



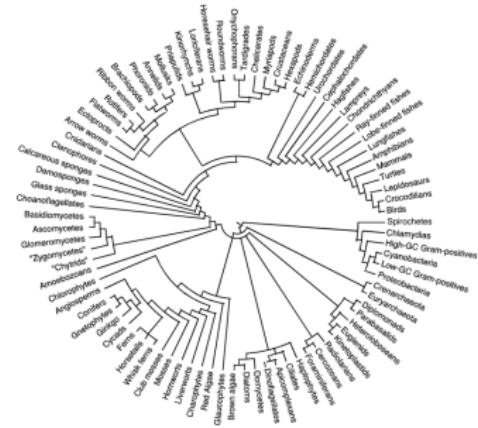
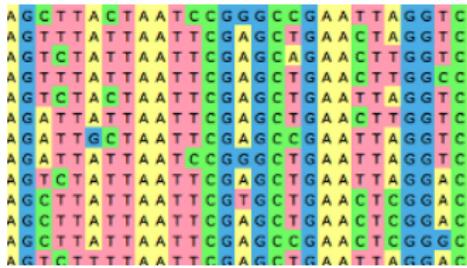
to



?

You've seen a lot about how to get from

to



I'm going to talk about going from



to



to

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A G C T T A C T C T A A T C C G G G C C G A A T T A G G T C
A G T T T A T T A A T T C A G C T A A T C A T G G C C
A C T C G T A T T A A T T G A G C A G A A A C T T G G C C
A G T T T A T T A A T T G A G C T G A G C T T G G C C
A G T C G T A C T A A T T G A G C T G A G C T T G G C C
A G A T T T A T T A A T T G A G C T G A A C T T G G C C
A G A T T T G C T A A T T G A G C C G C A A T T A G G T C
A G A T T T A T T A A T T C G G G G C C G T G A A T T A G G T C
A G T C G T A T T A A T T G A G C T G A C T T G G C C
A G G T T A T T A A T T G T G A G C T G A A T T A G G C C
A G G T T A T T A A T T G T G A G C T G A A T T A G G C C
A G G T T A T T A A T T G T G A G C T G A A T T A G G C C
A G G T T A T T A A T T G T G A G C T G A A T T A G G C C
A G G T T A T T A A T T G T G A G C T G A A T T A G G C C
A G G T C T T A T T A A T T G A G C T G A A T T A G G C C

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I'm going to talk about going from

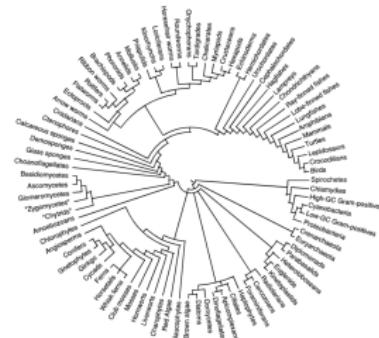


to

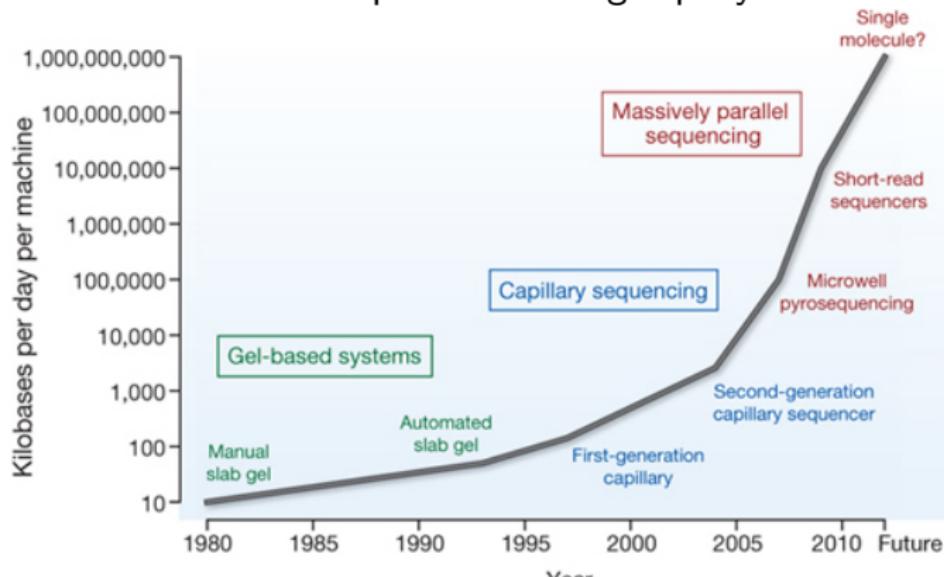


to

and how those choices can affect



The quantity of available sequence data for inferring evolutionary relationships is increasing rapidly



<http://genome.wellcome.ac.uk/>

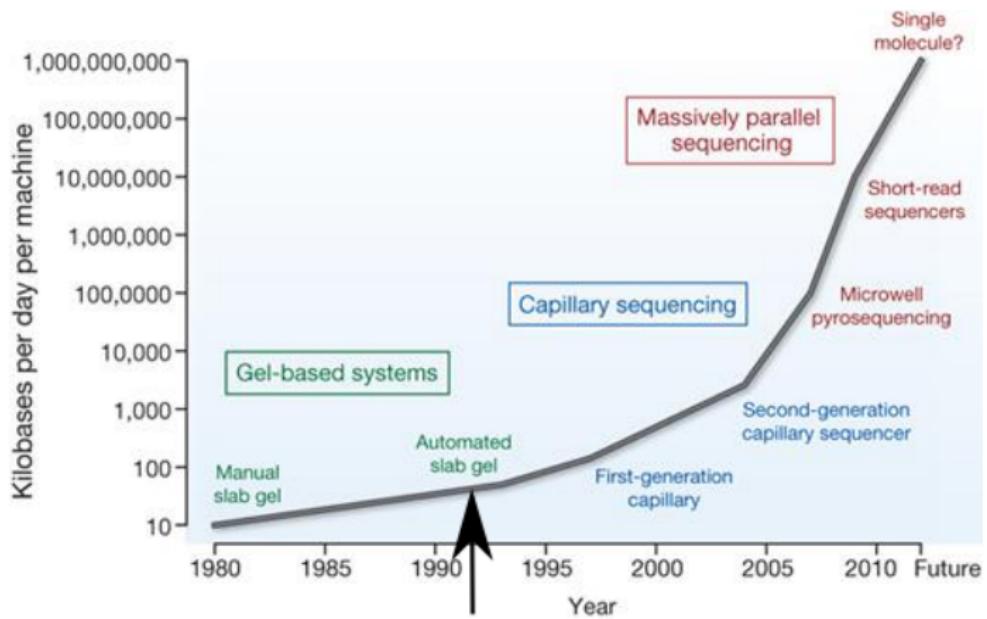
“With the advent of modern molecular biology, the ability to collect biological sequence data has out-paced the ability to adequately analyze these data”

– Jeff Thorne (Evolutionary biologist)

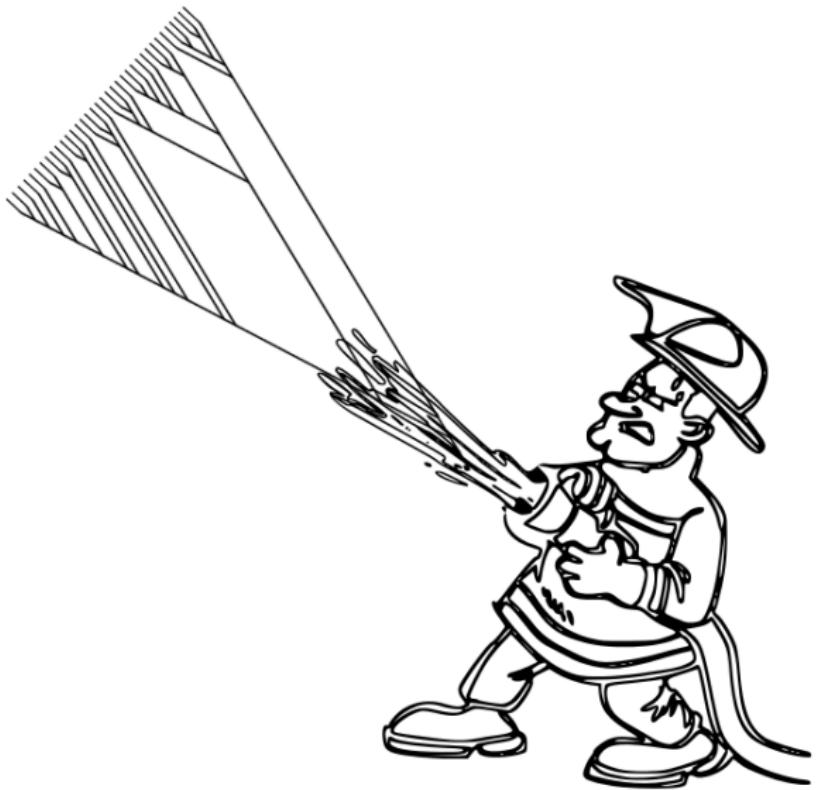
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Thorne et al., Journal of Molecular Evolution. **1991**



<http://genome.wellcome.ac.uk/>



There are a lot of choices to make!

Biological questions

- ▶ What do you want to know?
- ▶ What do you already know?

Biological questions

- ▶ What do you want to know?
- ▶ What do you already know?

Technical questions

- ▶ What data is right for our questions?
- ▶ Is a closely related reference genome available?
- ▶ How should we process and analyze our data?
- ▶ What biases may be affecting our inferences?

General approach

- Decide what to sequence ( to )
- Consensus sequence, alignment, locus selection
( to )
- Evolutionary analyses ( to )
- Success!

There are a lot of possible paths you can take!

/home/ejmctavish/Desktop/WH2019/FlowChart.png

What to sequence?



to



Different sequencing approaches enrich the samples for different components of the genome

Enrichment (smallest to largest proportion of genome)

- ▶ Directed PCR
- ▶ Targeted enrichment, Rad-tag etc
- ▶ Transcriptome
- ▶ Whole genome

Depending on your questions, any of these could be the best option!

Directed PCR

- ▶ Simple and cheap for a small number of genes
- ▶ Doesn't scale so well to many genes
- ▶ Doesn't sound fancy

Targeted enrichment (e.g. Ultra-conserved elements, probes for orthologous single copy genes, etc.)

- ▶ Use hybridization to enrich particular regions
- ▶ Works well even on degraded DNA
- ▶ Need to synthesize probes specific to each region
 - need data to get data!
- ▶ Data sets can be combined across projects if same probe set applied

Non-targeted enrichment (RAD-tag, ddRAD etc.)

- ▶ Select randomly distributed, but consistent, genome regions
- ▶ Comparable across closely related taxa, but not more distant taxa
- ▶ Each locus has very few variable sites (not good for generating gene trees)

Whole transcriptome

- ▶ Enriched for expressed protein coding genes
- ▶ Content will vary based on cell type, environment, etc.
- ▶ Provides expression level data

Whole genome sequencing

- ▶ Capture all the data
- ▶ In a phylogenetic context, currently only cost effective for small genomes
- ▶ Annotation is hard! Often need transcriptome to get genes
- ▶ Mapping or assembly can be slow

Need to put the pieces back together!



to

A G C T T A C T A A T T C C G G G C C G A A T T A G G T C
A G T T T A T T A A T T C G A G C T G A A C T T A G G T C
A G T C T A T T A A T T C G A G C A G A A C T T G G G T C
A G T T T A T T A A T T C G A G C T G A A C T T G G G C C
A G T C T A C T A A T T C G A G C T G A A A T T A G G T C
A G A T T A T T A A T T C G A G C T G A A C T T G G G T C
A G A T T G C T A A T T C G A G C C G A A T T A G G T C
A G A T T T A T T A A T T C G G G C T G A A A T T A G G T C
A G T C T A T T A A T T C G A G C T G A A A T T A G G A C
A G C T T A T T A A T T C G T G C T G A A C T C G G A C
A G C T T A T T A A T T C G A G C T G A A A C T C G G A C
A G C T T A T T A A T T C G A G C C G A A A C T C G G G C
A G T C T A T T T A A T T C G A G C T G A A A T T A G G A C

Genomic sequencing

- ▶ You have all the data! 
- ▶ You have to deal with all of the data. 

De novo assembly

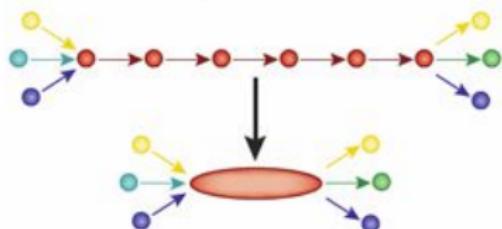
1. Fragment DNA and sequence



2. Find overlaps between reads

...AGCCTAGACCTACA**GGATGCGCGACACGT**
GGATGCGCGACACGTCGCATATCCGGT...

3. Assemble overlaps into contigs

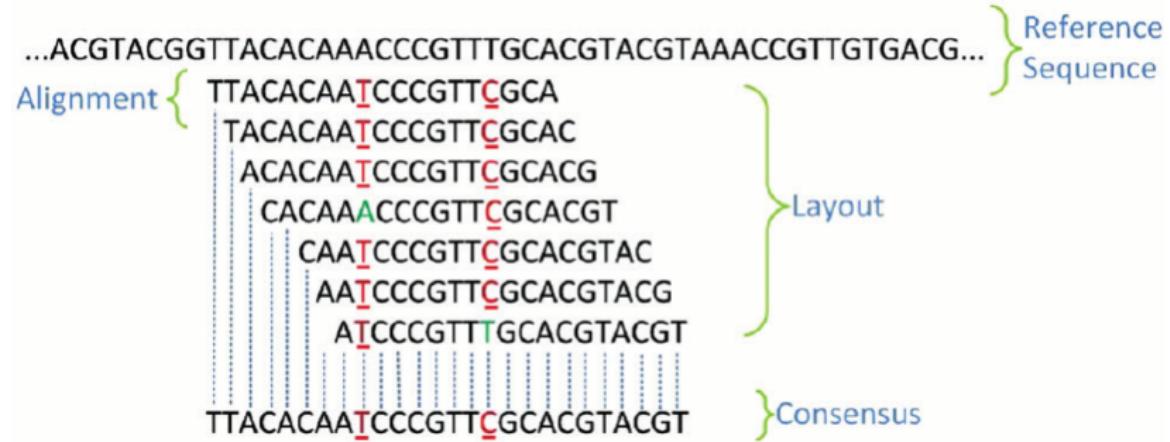


4. Assemble contigs into scaffolds



(Baker, 2012)

Mapping to a reference genome

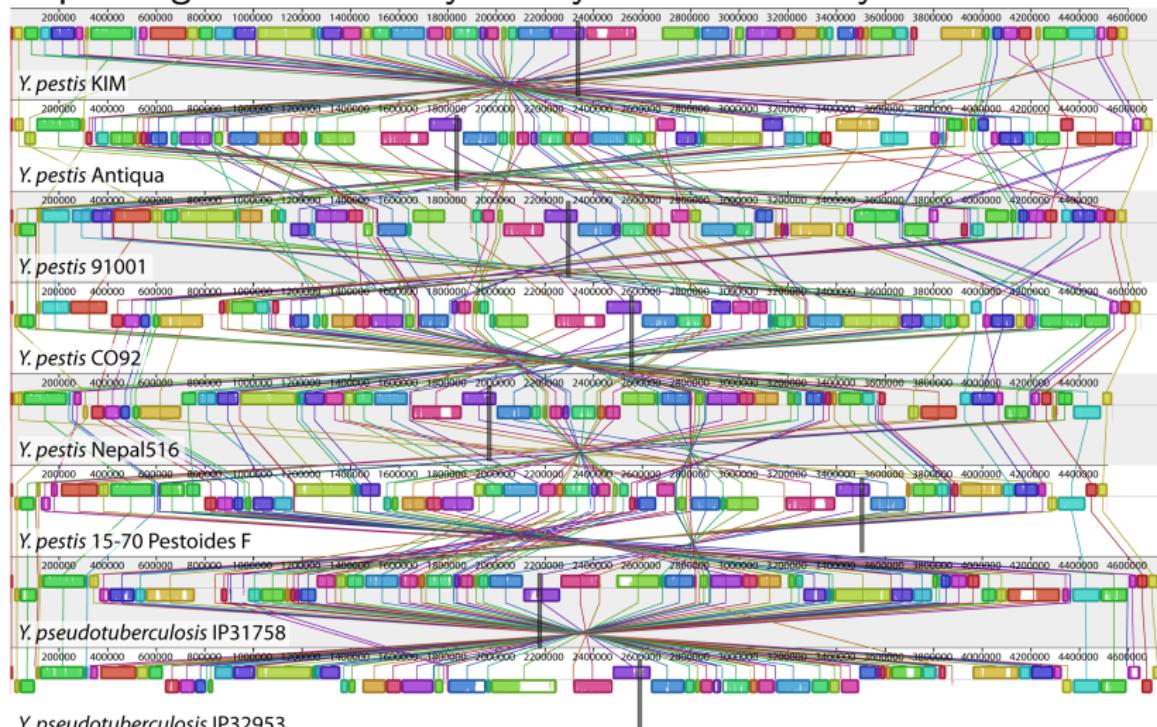


To make evolutionary statements, you need to align genomic regions across taxa.

Depending on evolutionary history this can be easy or hard!

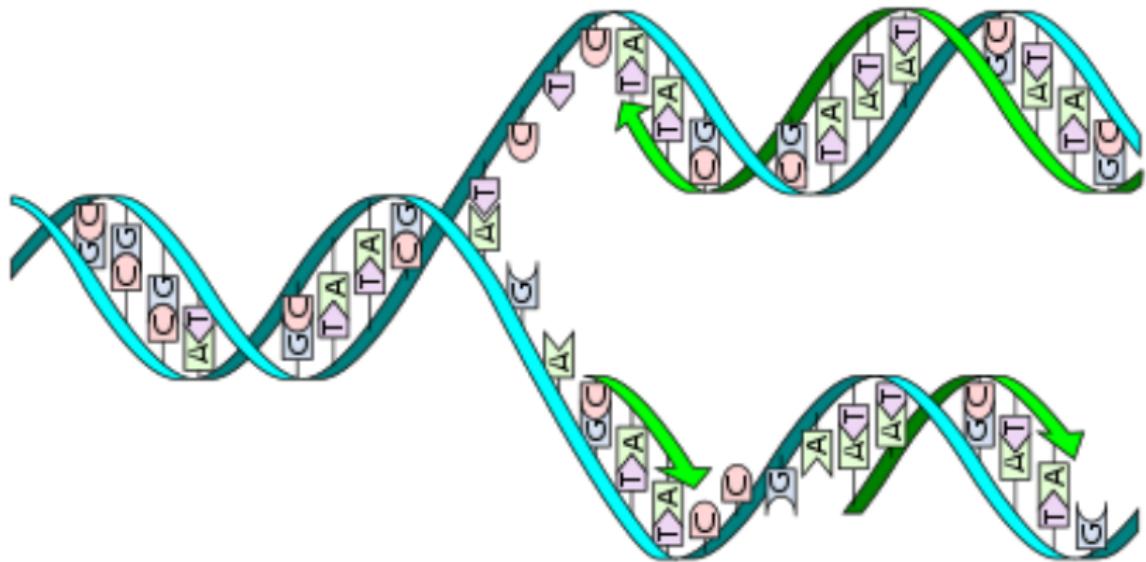
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(Darling et al., 2008)

An alignment is a statement of shared ancestry



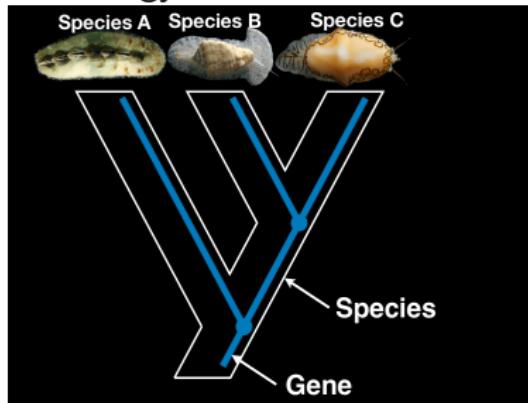
Gene tree (Locus tree)

The ancestry of a homologous region of the genome that has a single evolutionary history (no recombination)

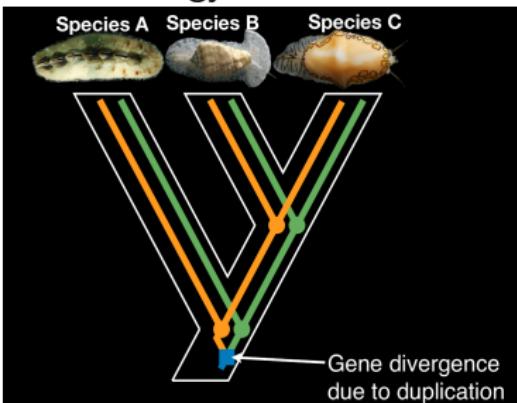
Enrichment methods focus our sequencing efforts on these regions

Gene duplication and loss

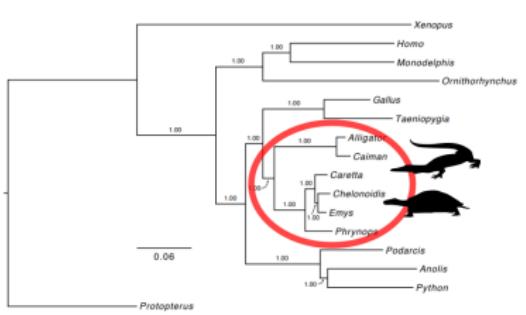
Orthology



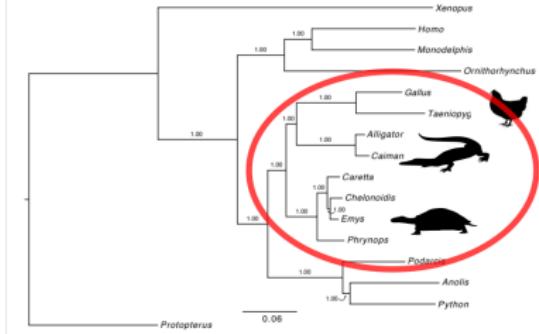
Paralogy



Inference of homology is not incorrect! But our current models are limited. If you treat paralogs as orthologs, you can make incorrect inferences. figure from Casey Dunn



A majority-rule consensus tree from Bayesian phylogenetic analysis of the concatenated dataset of Chiari et al.
248 nuclear genes



**Same analysis of the same dataset
after removal of the two genes
with evidence for paralogy.**

“investigation of genes with extreme support for turtle placement revealed unappreciated paralogy in a small proportion of alignments (<1%) that had an extraordinary influence on the inferred placement of turtles.”

(Brown and Thomson, 2016) (Chiari et al., 2012)

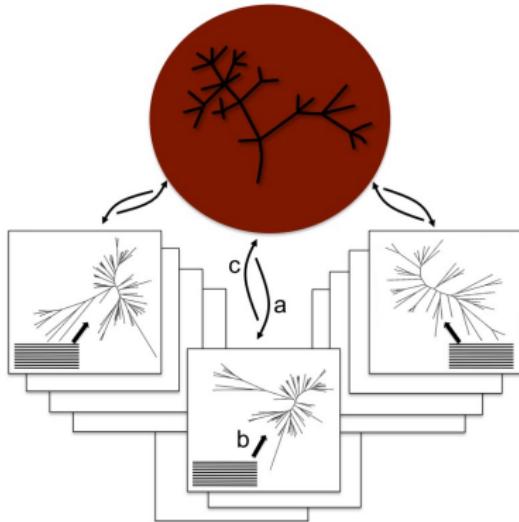
Challenge: The true (unknown) phylogenetic history is needed to assess orthology vs paralogy

"We used to be so blissful, back in the day. We just had data for one gene."

Paul Lewis (this morning)

Integrated approaches can jointly estimate gene trees and species trees

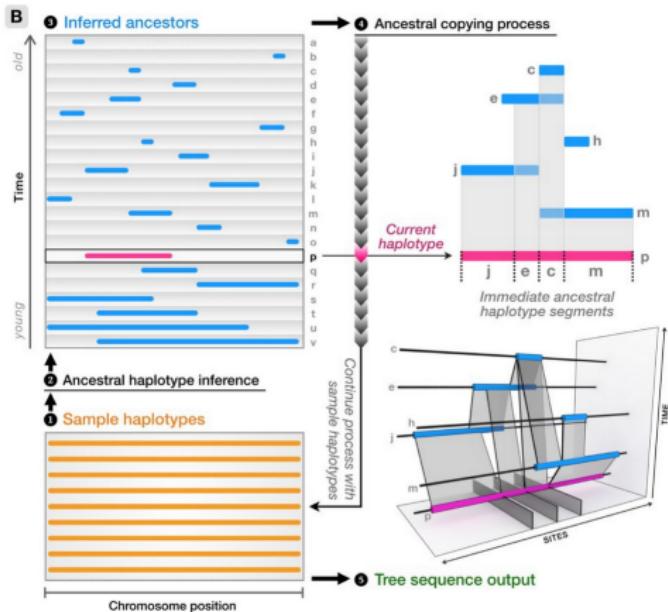
$$L(T, S, N | A) = \prod_{G_i \in \mathcal{G}} L(G_i)$$



(Boussau et al., 2013) But are computationally expensive.

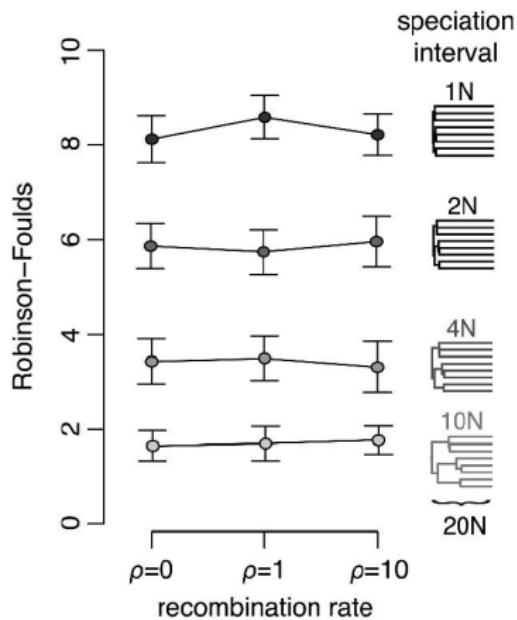
As we densely sample genomes and taxa, the size of a ‘locus’ or ‘un-recombined region’ will get smaller.

Jointly estimate recombination and ancestries along genomes



Currently only applied within humans! (Kelleher et al., 2018)

Species tree methods are robust to intra-locus recombination (based on analyses of simulated data)



Robinson Foulds (RF)
distance: the symmetric
difference between trees -
the number of branches in
tree 1 and not in tree 2 +
the number of branches in
tree 2 and not in tree 1.

(Lanier and Knowles, 2012)

Do you need a whole genome to answer your questions?

Do you need a whole genome to answer your questions?

For phylogenetic and population genetic questions, not necessarily!

Most phylogenetic methods cannot directly handle whole genome data, but from whole genome sequencing you can get homologous loci, as well as a bunch of other stuff!

How do the choices we make in



to



to

A grid of DNA sequence data represented by colored boxes. The columns are labeled with the four bases: A (green), T (red), C (blue), and G (yellow). The rows show different sequence contexts, such as AT, TA, CT, CG, AG, GC, etc. The sequence reads from top to bottom as follows:

AG	CT	T	A	C	T	A	A	T	C	G	G	G	C	G	A	A	T	T	A	G	G	T	C				
A	G	T	T	T	A	T	T	C	G	A	G	C	A	G	A	C	T	G	A	A	C	T	A	G	G	T	C
A	G	T	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	T	C				
A	G	T	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	C	C				
A	G	T	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	T	C				
A	G	A	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	T	C				
A	G	A	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	T	C				
A	G	C	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	A	C				
A	G	C	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	A	C				
A	G	C	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	G	C				
A	G	C	T	T	A	T	T	C	G	A	G	C	T	G	A	A	C	T	T	G	G	G	A				

How do the choices we make in



to



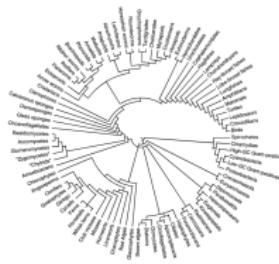
to

affect

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A G C T T A C T A T T C C G G G C C G A A T T A T T A G G T C
A T T T A T T A T T C C G A C T G A A C T A T T A G G T C
A G T C T A T T A A T T C C G A C G A A C T A T T G G G C
A T T T A T T A A T T C C G A C T G A A C T A T T G G G C
A T C T A C T A A T T C C G A C T G A A C T A T T A G G T C
A G A T T T A T T A A T T C C G A C G A A C T A T T G G G C
A G A T T T C C T A T T C C G A C G C G A A C T A T T A G G T C
A G A T T T A T T A A T T C C G G G C C G A A T T A T T A G G T C
A G T C T A T T A A T T C C G A C T G A A C T A T T A G G T C
A G C T T A T T A A T T C C G T G A A C T C C G G A C
A G C T T A T T A A T T C C G A C T G A A C T C C G G A C
A G C T T A T T A A T T C C G A C T G A A C T C C G G A C

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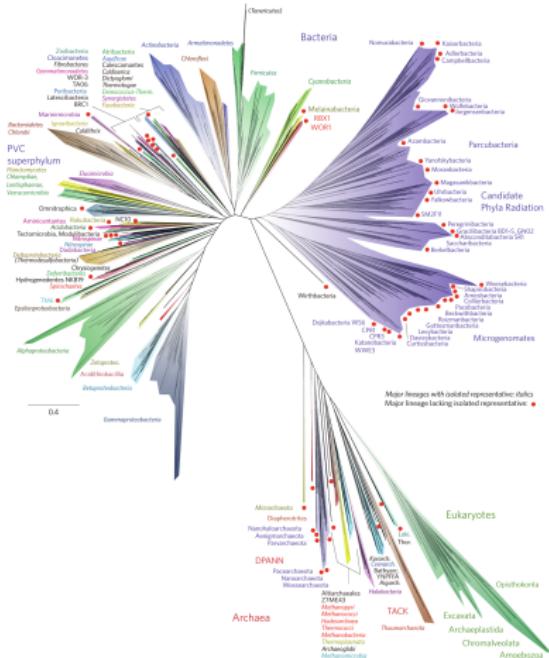
Ascertainment bias

A bias in parameter estimation or testing caused by non-random sampling of the data.
(also sometimes overlapping with 'selection bias' or 'acquisition bias')

Ascertainment bias is ubiquitous!

- Surveying volunteers
- Studying undergraduates
- Sampling across 'species'
- Discarding rare outliers

Sampling across the tree of life



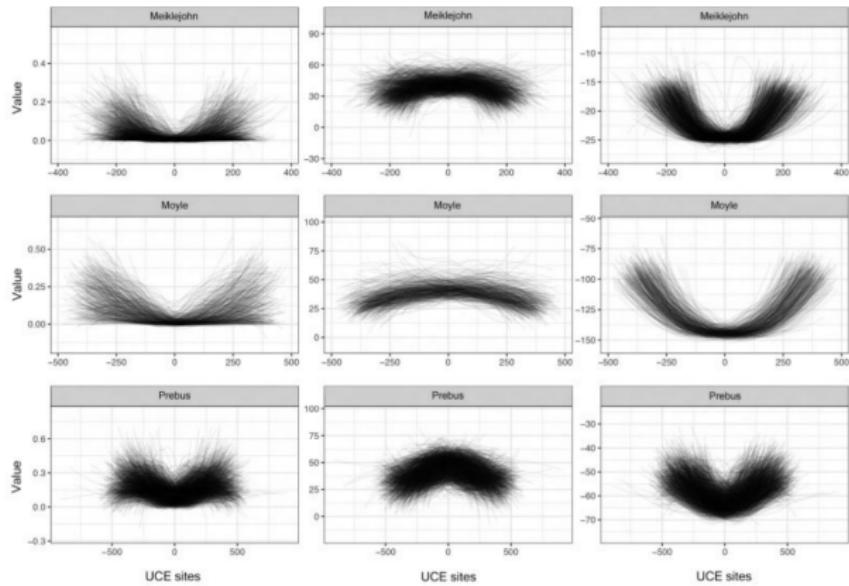
(Hug et al., 2016)

Ascertainment bias in genomic data

It is important to consider what models of evolution are appropriate for your data types

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Entropy (rate proxy), GC content, Multinomial likelihood



Extreme rate heterogeneity in Ultra Conserved Elements, can be handled with appropriate partitioning
(Tagliacollo and Lanfear, 2018)

Analyzing only variable sites (e.g. Single Nucleotide Polymorphism (SNP) analyses)

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'Something Definitely Happened Once!'

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Instead of 'Anything Can Happen Now'
'Something Definitely Happened Once!'

This affects our ability to estimate branch lengths using likelihood
Intuitively, will increase inferred branch lengths
can also affect tree topology

Short Tree

AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA
AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA
AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA
AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA
AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA

The sequence logo displays the consensus sequence AAGTATACACATTATCGAAATCAAAAGAAAATTTCAAAAAATCTATAGA. The letters are color-coded: A (green), T (yellow), C (blue), and G (red). The height of each letter at a position indicates its frequency of occurrence at that position across the aligned sequences.

Short Tree

AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA
AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA
AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA
AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA
AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA
AAGTATACACATTATCGAACTAAAAAGAAAATTTCCTAAAAATCTATAGA

Long Tree

CAGCAGGGTTTACCTTGCAAGGGCAAAACAGTCCACCACTTCCTGGCAC
CACCAAGATTTTACATGCAAGGGCAAAACAGTCCACCACTTCATGAACAC
CAGCAGGGTTTACCTTGCAAGGGAGCCATTTCCTTCACTTCAGGGAAC
CAGCAGGGTTTACCTTGCAAGGGAAAAACAGTTTACCATTCCTGGAAC
CAGCAGGGTTTACCTTGCAAGGGAAAAACAAATATAACACTTGGTAATAC

How surprised should we be to see no invariant sites?

Very surprising, unless branches are very long

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Very surprising, unless branches are very long
but only if we looked for them!

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Can correct by applying Lewis (2001) model for analysis of only variable sites implemented inference software

Based on correction for problem of not counting un-observed restriction sites in (Felsenstein, 1992)

“it is possible in many cases to correct the ascertainment bias relatively easily, if reliable information is available regarding the details of the ascertainment scheme.” (Nielsen, 2004)

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This information is not always available. Bias can be driven by the true, evolutionary history you are attempting to estimate!

Despite the large volume of data in genomic studies,
ascertainment bias is still an issue

Despite **because of** the large volume of data in genomic studies, ascertainment bias is still an issue

Two case studies:
Phylogenetics of *Penstemon*
Tracing gonorrhea outbreaks

Phylogenetics of Penstemon using RADseq data

Question: How often have transitions between hummingbird and bee pollination occurred in *Penstemon*?



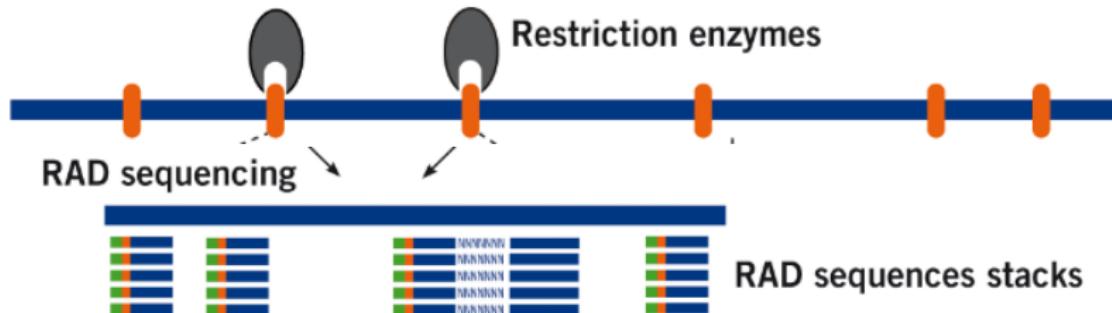
Data:

- ▶ Restriction site-associated DNA sequencing (RADSeq)
- ▶ 83 species, two samples per species
- ▶ No closely related reference genome

RADseq

Uses restriction enzymes to fragment DNA

Targets sequencing to the same regions across taxa

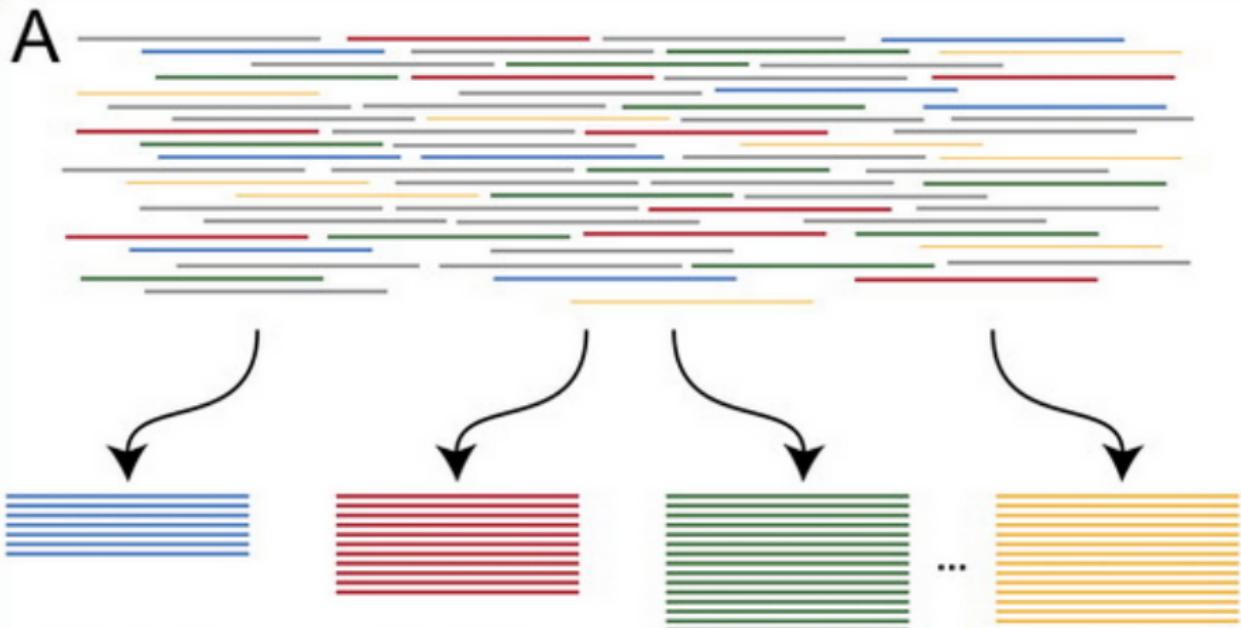


In comparison: Shotgun Sequencing



(figures from floragenex.com)

In the absence of a reference genome, you need to cluster reads
A 'cluster' is an inference of homology



Clustered using Stacks (Catchen et al., 2011)

Several factors can cause drop-out of alleles in RAD-seq data (i.e. not observing homologous alleles)

- Mutations at restriction digest sites
- Clustering parameters exclude homologous regions
- Low coverage

There have been many conflicting studies on the importance of missing data in phylogenetic analyses,
broadly, as long as missing data is random, it shouldn't be very
problematic, but phylogenetically-biased missing data is likely to be.
(Roure et al., 2013; Lemmon et al., 2009)

Missing data in RADseq can mislead inference

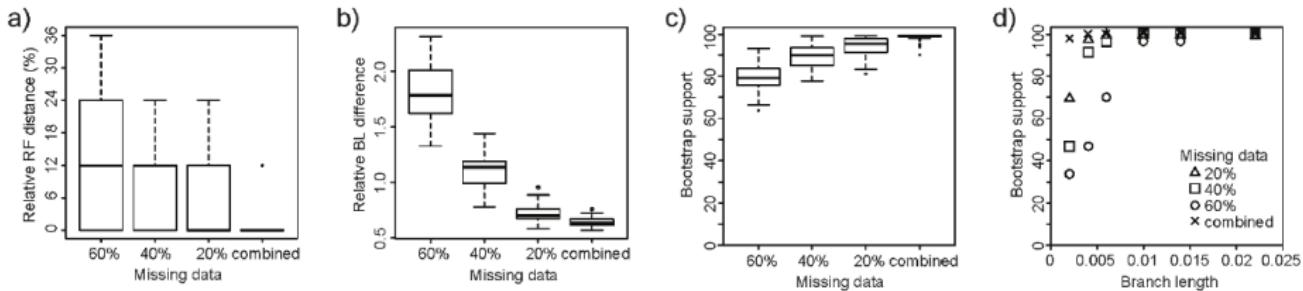


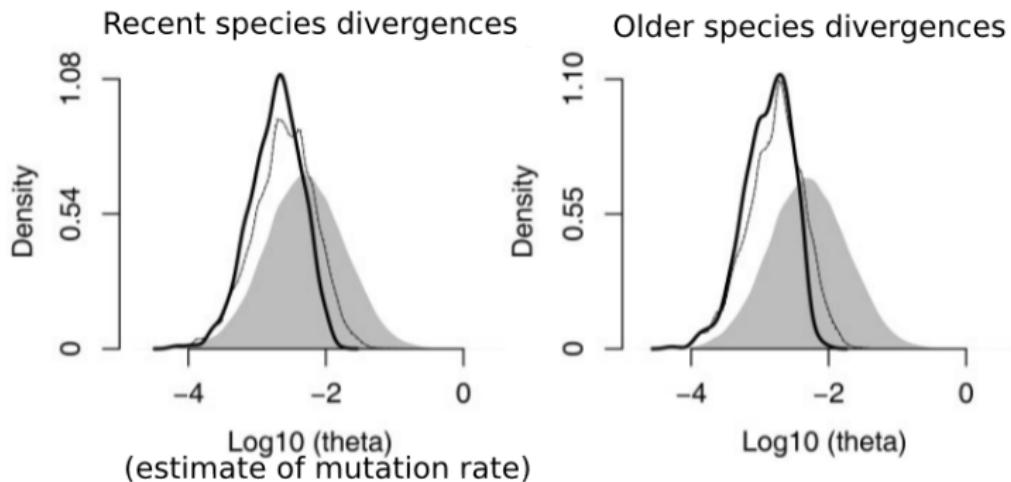
Figure 4: Properties of simulated RAD loci with different amounts of missing data. Loci that contain more missing data tend to result in discordant topologies (a), increased branch length errors (b), and lower bootstrap support (c). Loci that contain less missing data provide higher bootstrap support for shorter branches (d).

(Leaché et al., 2015)

But excluding sites with high levels of missing data doesn't solve the problem.

But excluding sites with high levels of missing data doesn't solve the problem.

It biases rate estimation downwards by preferentially removing high rate loci



Gray shading is simulated rates, dashed line is shift due to loss of RAD sites, black line is shift due to loss of cut sites, black line shift due to loss of cut sites + post sequencing processing.

(Huang and Knowles, 2014)

Advice?

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“Given that the data matrix reflects complex interactions between aspects of library construction and processing with the divergence history itself, our results also suggest that general rules-of-thumb are unlikely.”

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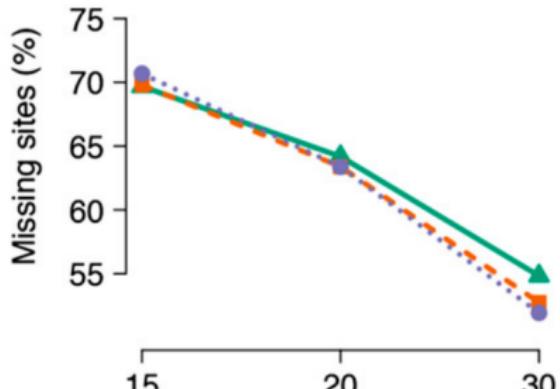


Tradeoffs:

- ▶ Decreasing similarity cutoff captures more loci shared across the tree, at risk of incorrect homology
- ▶ Decreasing taxon representation threshold allows you to capture more loci, but representing fewer individuals

Approach

Investigate a range of parameters

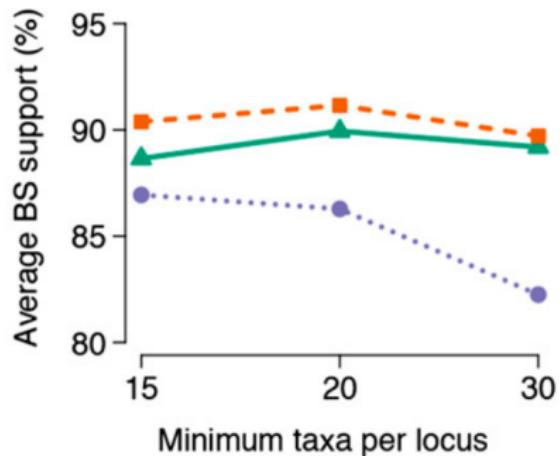


▲ $W_{\text{clust}} = 0.80$

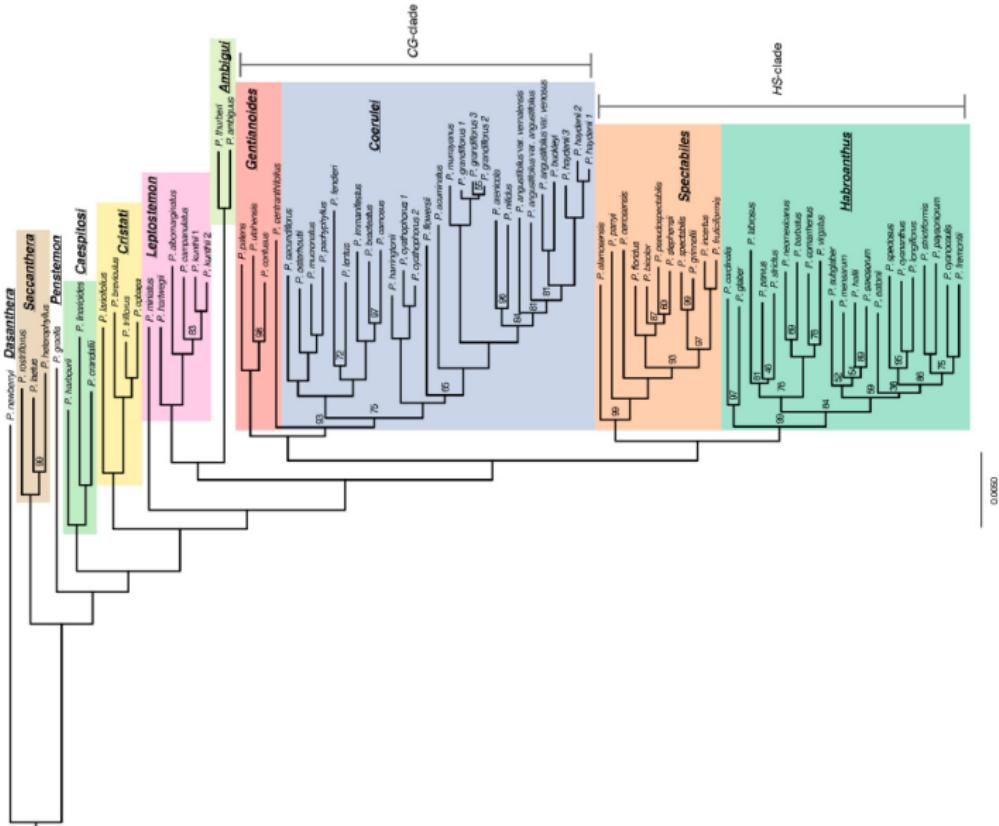
■ $W_{\text{clust}} = 0.90$

● $W_{\text{clust}} = 0.95$

(Wessinger et al., 2016)



Missing data is phylogenetically biased

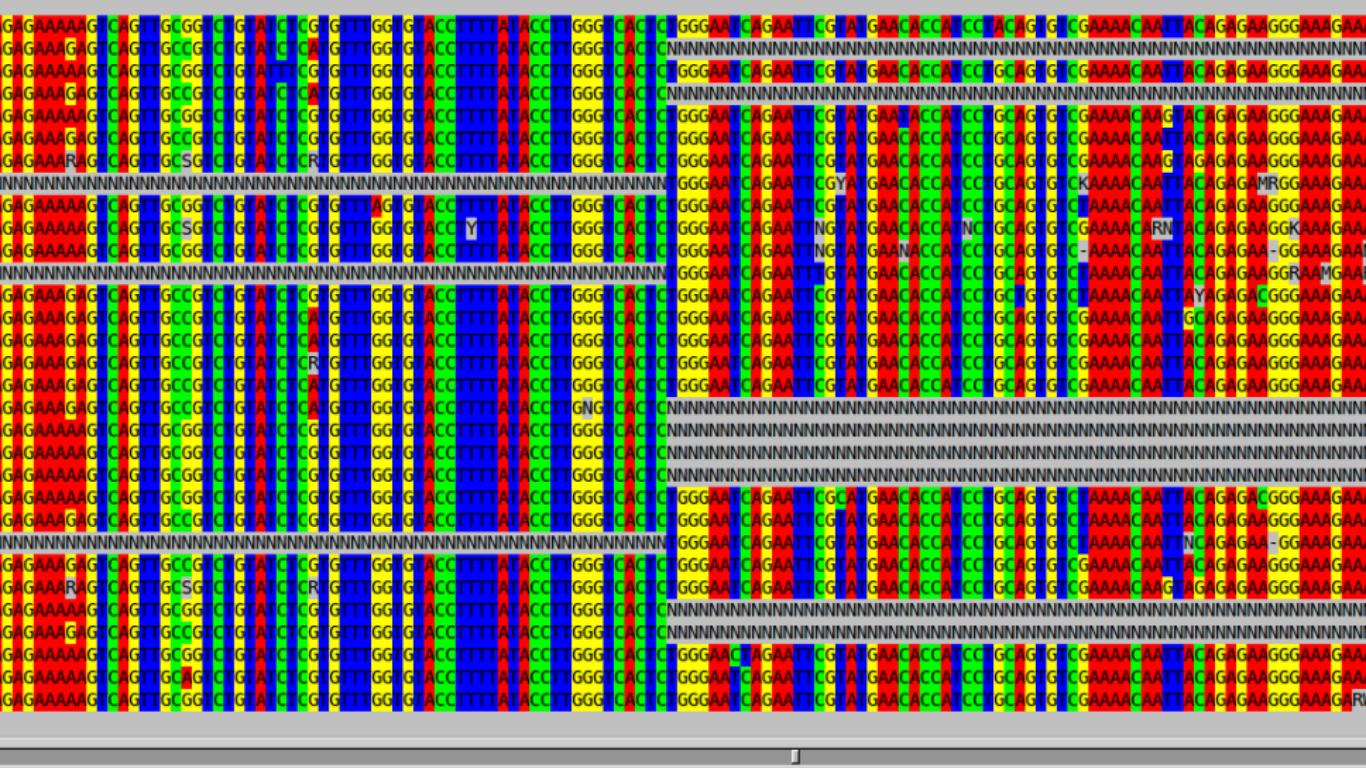


Across full dataset, many loci are only found in one of the major clades

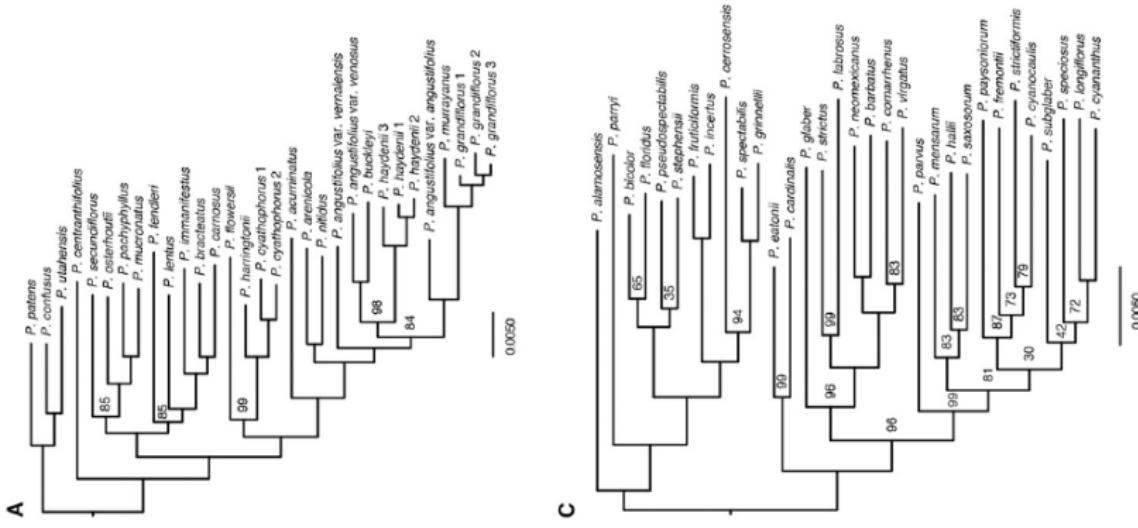
ees Search: Goto: Help

Variation within clades is better captured by dividing the data set and clustering separately

Trees | Search: | Goto: | Help |



Build (and report!) multiple trees using different filtering parameters



Trees from separate clade analyses (Wessinger et al., 2016)

Summary:

Bias:

- ▶ Clustering parameters drive non-random missing data

Potential effect on inference:

- ▶ No topological resolution
- ▶ Tip branch lengths are shortened
- ▶ Non-homologous regions align

Mitigation:

- ▶ Estimate relationships under a range of filtering parameters

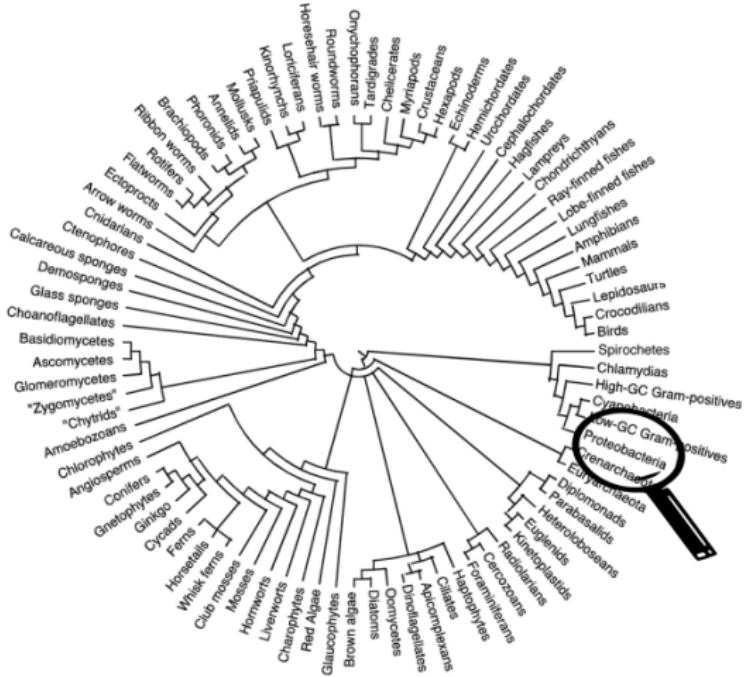
Conclusions:

Branch lengths and bootstrap support differ across filtering parameters

Different data sets may be appropriate at different phylogenetic scales

Evolutionary inferences about pollinator shifts need to be robust to this uncertainty

Case study - tracing gonorrhea outbreaks



Rapid phylogenetic updating to trace gonorrhea outbreaks



Collaboration with
Jack Cartee , Dr. Jeanine Abrams-McLean , and Jasper Toscani
Field (PhD student, UC Merced)

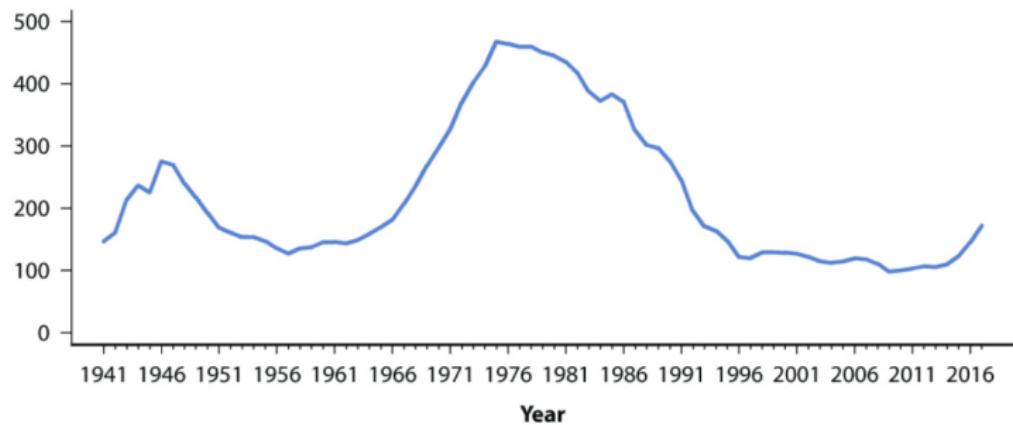
Neisseria gonorrhoeae

- Gram-negative, diplococci bacteria
- Responsible for the sexually transmitted infection known as gonorrhea
- One of two pathogenic *Neisseria* species known to infect humans
- WHO estimated 78 million new cases among adults worldwide in 2012



Gonorrhea – Rates of Reported Cases by Year, United States, 1941–2017

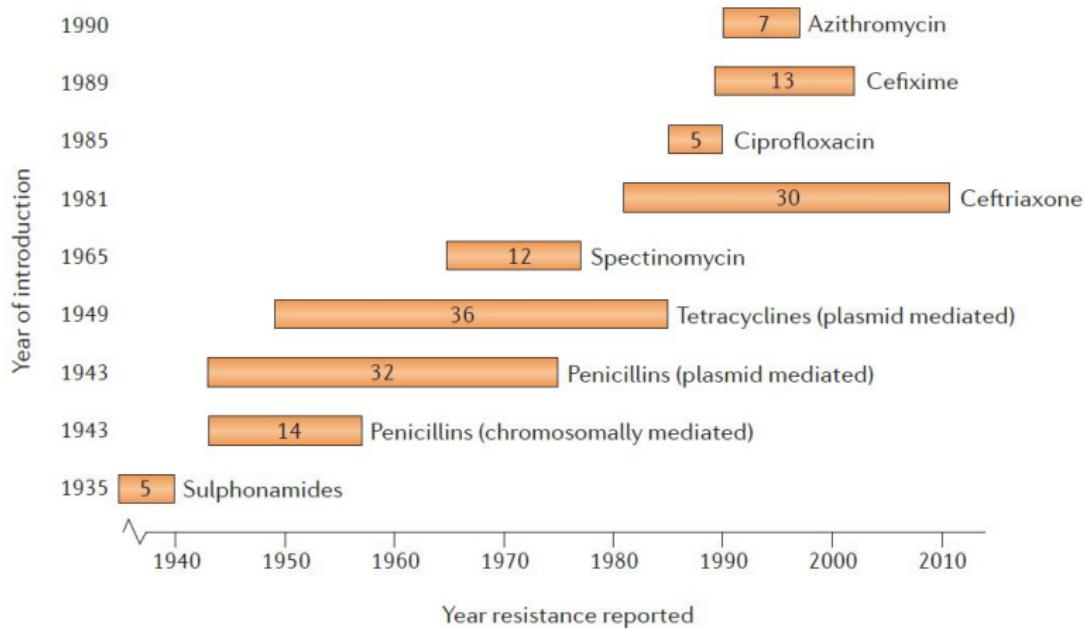
Rate (per 100,000 population)



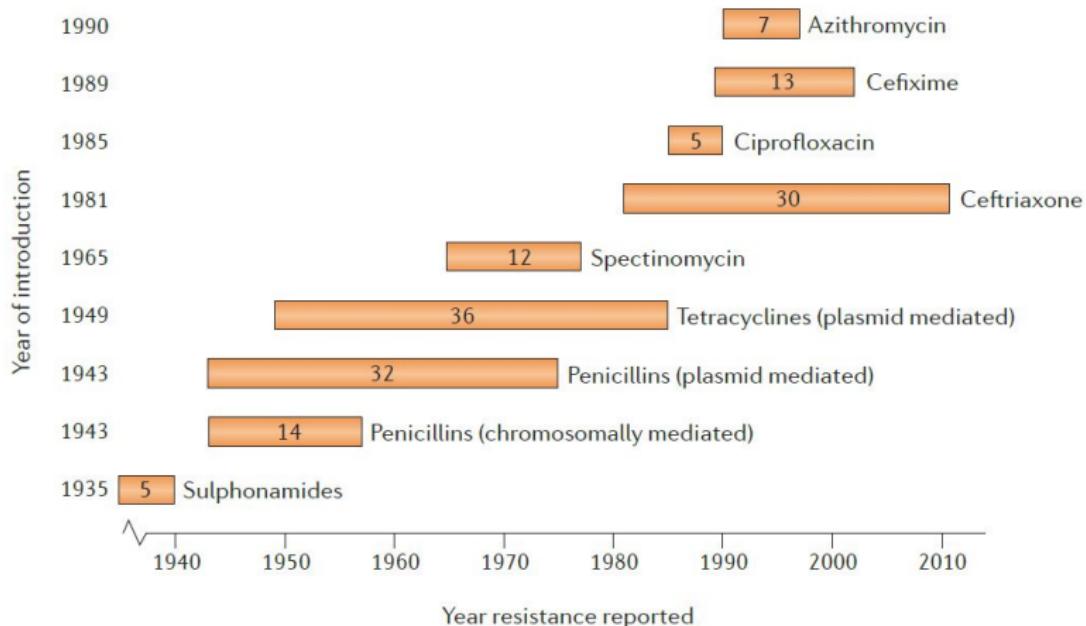
<https://www.cdc.gov/std/gonorrhea/stats.htm>

Recent increase in rates of gonorrhea infections

Neisseria gonorrhoeae has progressively developed resistance to each single dose antibiotic.



Neisseria gonorrhoeae has progressively developed resistance to each single dose antibiotic.

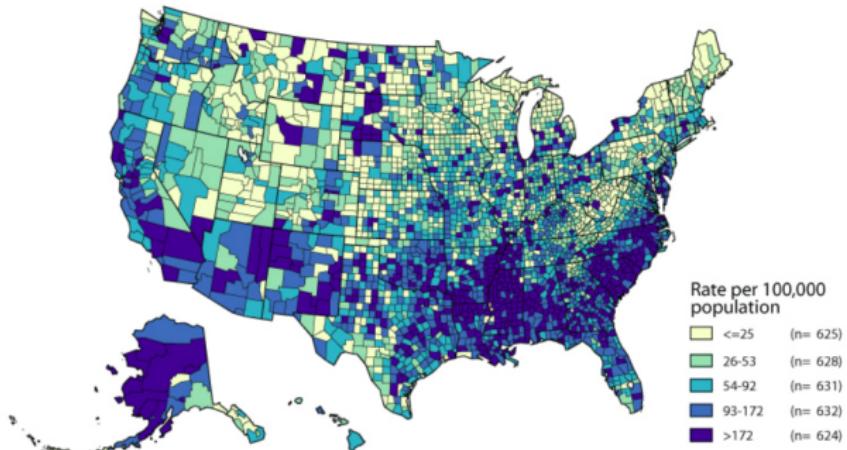


Only remaining recommended treatment option is dual therapy with a ceftriaxone plus azithromycin

"It is widely recognised that few antimicrobials remain effective in the treatment of *Neisseria gonorrhoeae* infection and that gonorrhoea could become untreatable in the future."
(Chisholm et al. Sex Transm Infect 2015)

To track and control outbreaks, the CDC is tracing evolutionary history of gonorrhea, across the US and globally.

Gonorrhea – Rates of Reported Cases by County, United States, 2017

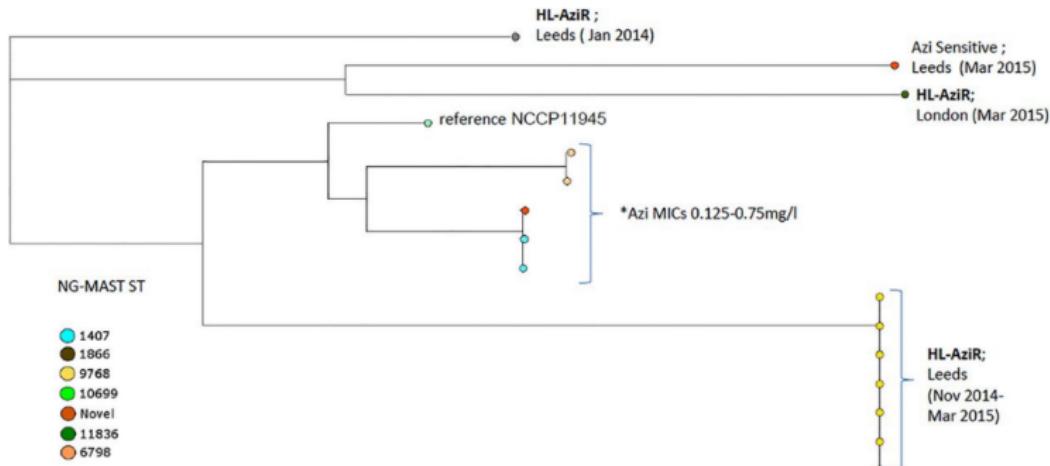


<https://www.cdc.gov/std/stats17/fignatpro.htm#gon>

Approach:

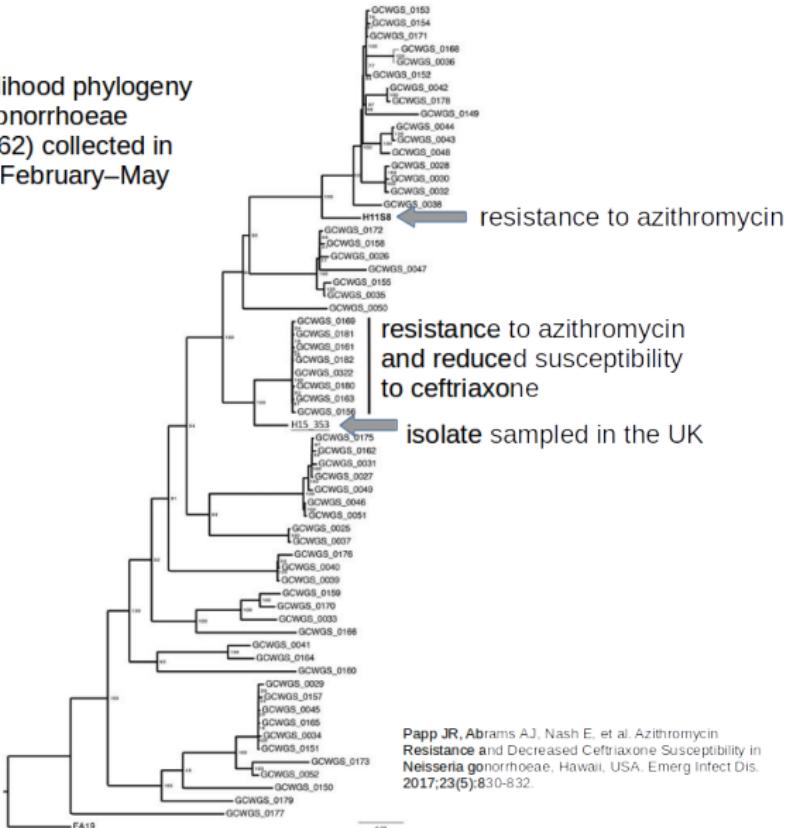
- ▶ Whole genomic sequencing of *Neisseria gonorrhoea* isolates - up to thousands of lineages
- ▶ Phylogenetic inference to track geographic spread and horizontal gene transfer of resistance genes

Combining geographic and evolutionary information can trace transmission, and transfer of resistance alleles across lineages



Stephanie A Chisholm et al. Sex Transm Infect doi:10.1136/sextrans-2015-052312

Maximum-likelihood phylogeny
of *Neisseria gonorrhoeae*
samples (N = 62) collected in
Hawaii during February–May
2016



Challenges:

- ▶ Thousands of samples; new isolates sequenced every day
- ▶ Speed from sampling → phylogeny important
- ▶ Need to rely on phylogenies for public health action (requires high confidence)
- ▶ Often very little nucleotide variability, but horizontal gene transfer is common.

Potential issues:

- ▶ Sequencing error
- ▶ Effect of choice of reference genome

Sequencing error

Potentially problematic when real variable sites are rare

Sequencing errors are likely to be singletons

Will overestimate tip branch lengths

Kuhner and McGill (2014) developed a correction for sequencing error in maximum likelihood phylogenetic inference.

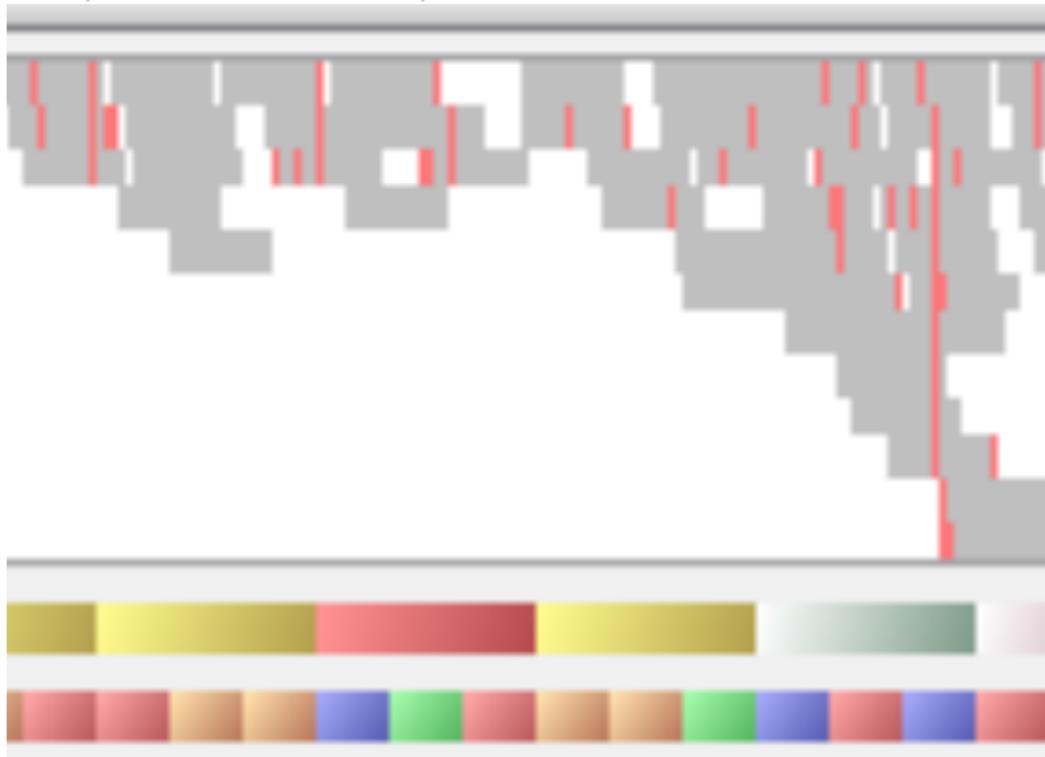
Uses a constant expected error per site

Currently, coverage and error information from sequence reads are discarded following  to 

We have information on confidence in individual base calls, but don't use it



Could use a “genotype likelihood”, capturing coverage and read quality (Nielsen et al., 2011)

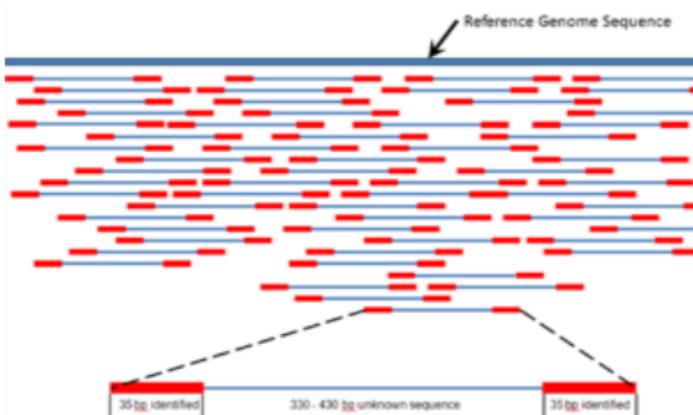


Not currently implemented in phylogenetic likelihood models

At high coverage, effect of sequencing error is likely low, but newer long read data have higher error rates!

Effect of reference choice

Reference based mapping of short reads can speed up generating a consensus sequence.



BUT: Reference choice can affect evolutionary inference

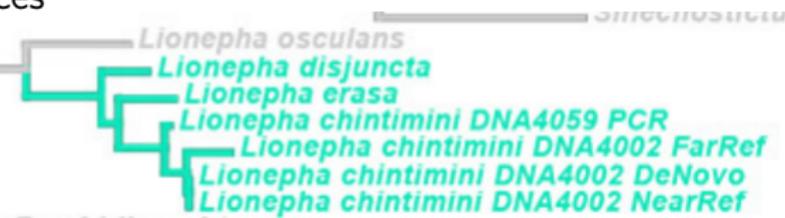
BUT: Reference choice can affect evolutionary inference

In humans, in highly polymorphic regions variant calling is biased toward the reference base (Brandt et al., 2015)

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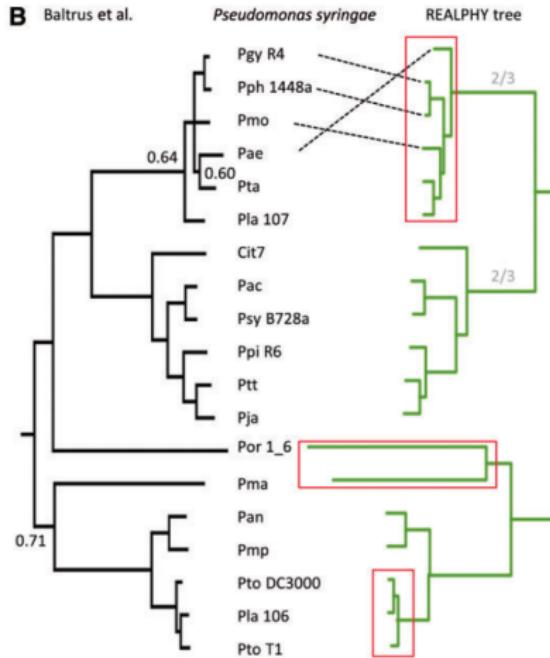
In fragmented DNA samples from beetles, branch lengths change based on reference choices



(Kanda et al., 2015)

A reference mapping based approach will discard information about structural variants not found in the reference

Reference choice can affect topology



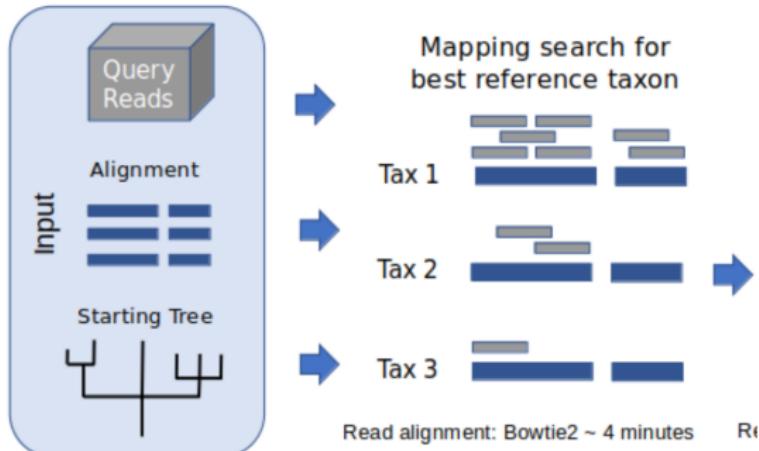
Mapping sequencing reads to reference genomes requires similarity cutoffs that generate biased missing data (Bertels et al., 2014)

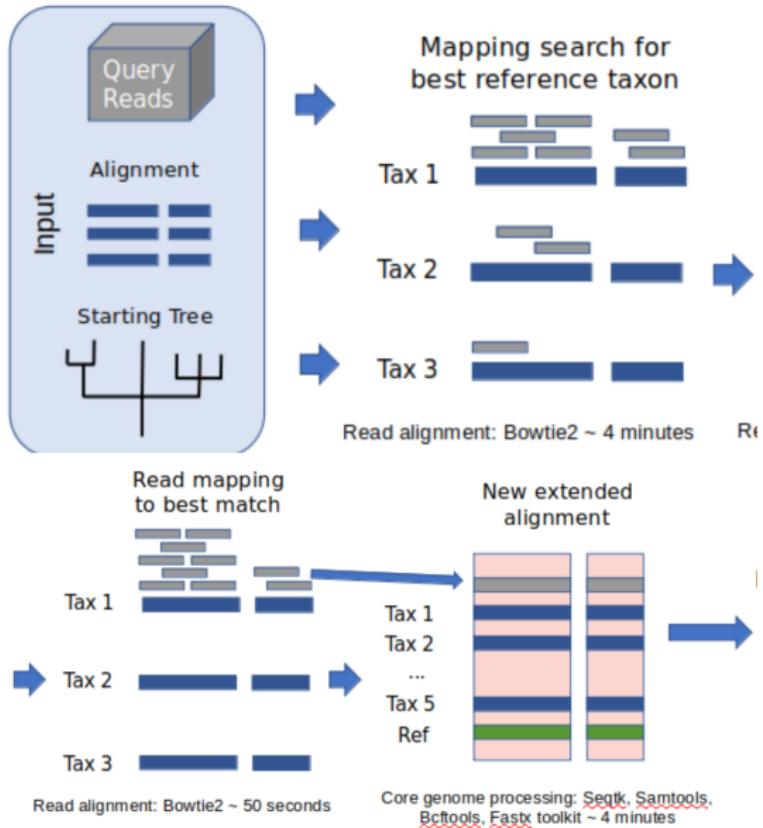
Problem: The true (unknown) phylogenetic history will affect how reads map across the genome.

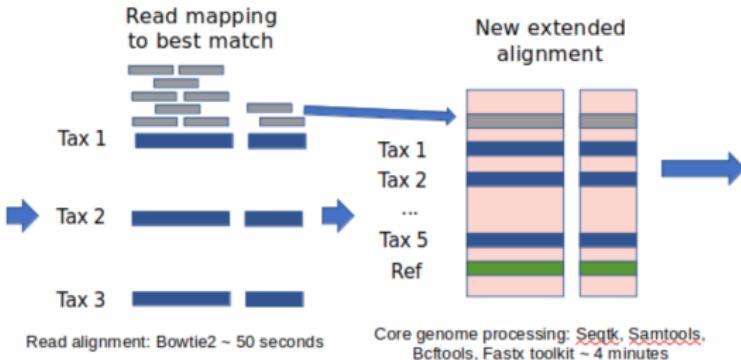
How can we efficiently estimate phylogenies from genomic data, without being biased by the evolutionary history itself?

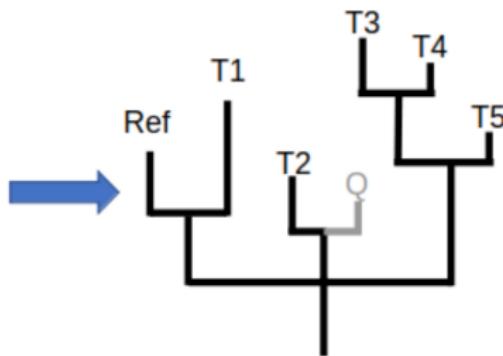
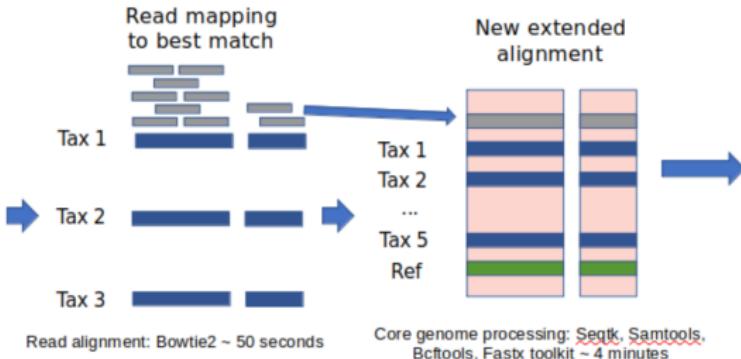
How can we efficiently estimate phylogenies from genomic data, without being biased by the evolutionary history itself?

Take advantage of prior inferences.





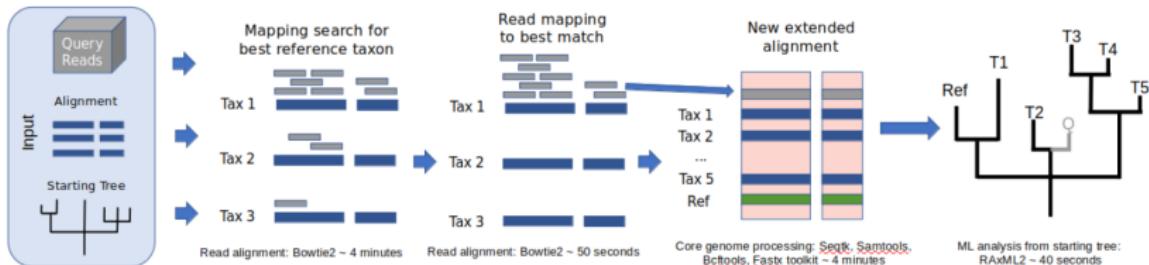




ntools,
es

ML analysis from starting tree:
RAxML2 ~ 40 seconds

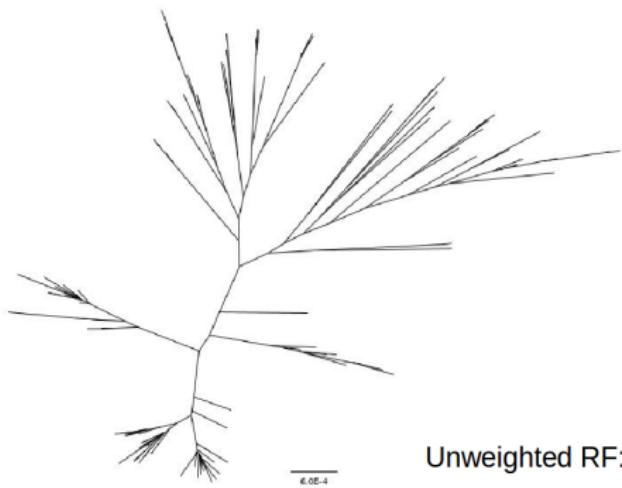
Phylogenetically informed phylogenomic updating approach:



- ▶ Assembles only homologous regions of interest
- ▶ Uses phylogenetic structure to select multiple references to generate consensus sequence
- ▶ Tree search speed up due to starting tree

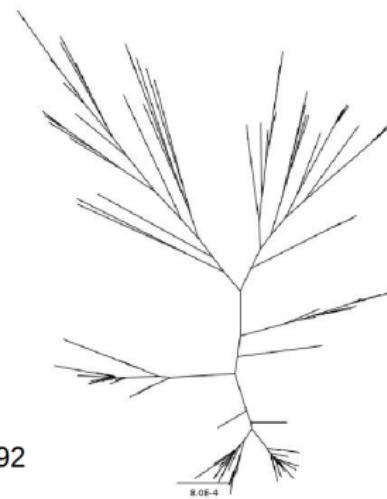
github.com/mctavishlab/phycorder

Tree from traditional method



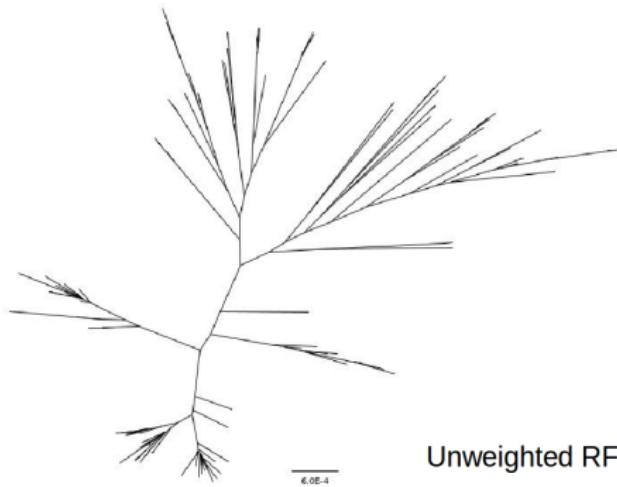
Unweighted RF: 92

Updated tree

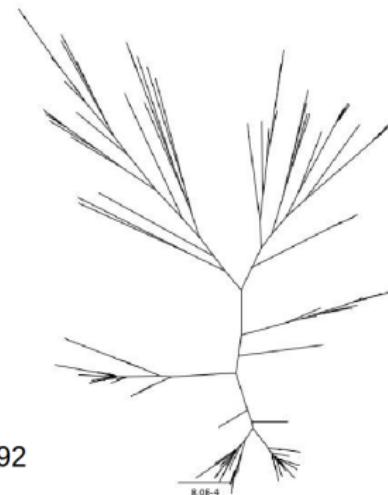


Results:

Ok... 🤔 the tree is different! but is it better or worse?



Unweighted RF: 92



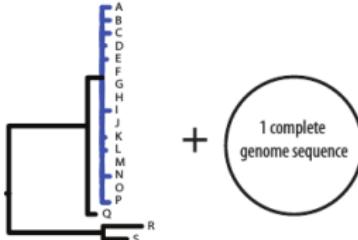
Testing the approach using simulations:

TreeToReads

Takes into account:

- Phylogeny and model of evolution
- Insertions and deletions
- Distribution of mutations across the genome
- Read coverage
- Sequencing error profiles (observed or estimated)

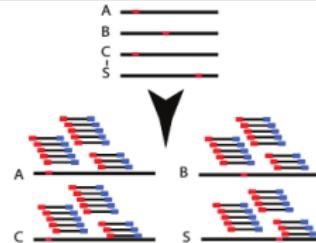
Generates short read data with which to test assembly, alignment and inference pipelines.



Input: 1) Tree file (newick)
2) Complete genome (fasta)

TreeToReads

Simulate mutations across
taxa according to defined
set of parameters



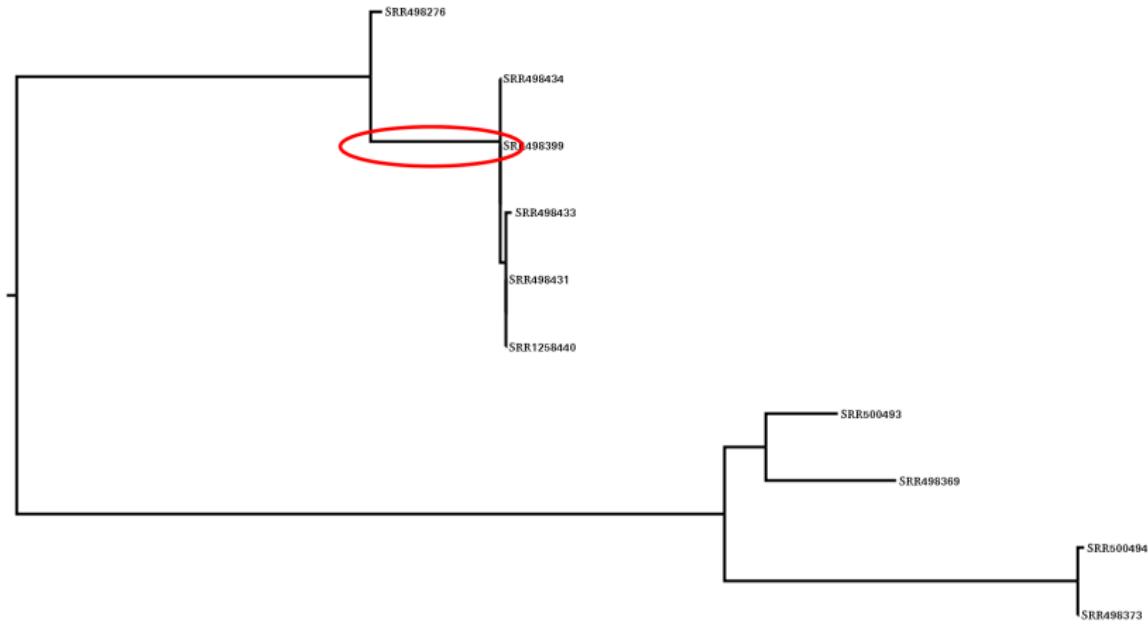
Output: set of raw reads (fastq)

Input genome for simulation is a tip on simulated tree
Can test alignment to other empirically observed genomes
(McTavish et al., 2017)

github.com/snacktavish/treetoreads

Other new approaches for generating reads from phylogenies:
NGSphy (Escalona et al., 2018), *Jackalope* (R package) (Nell,
2019)

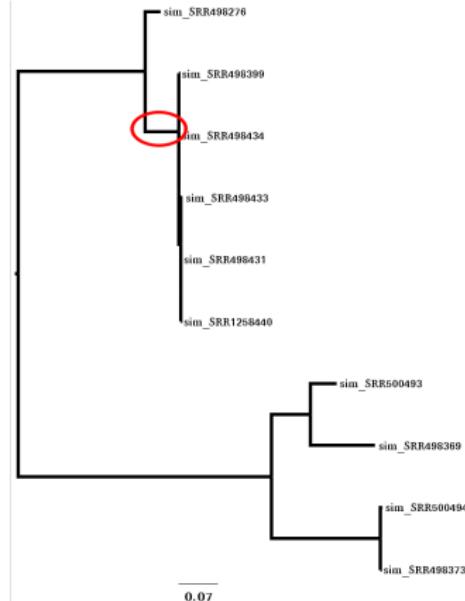
Take observed outbreak tree



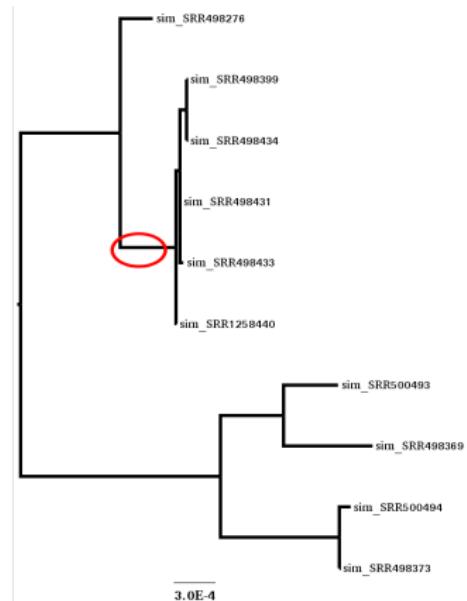
Simulate reads using empirical parameters

Infer trees from reads using two different reference genomes.

Reference within outbreak
reference



Distant (1% sequence divergence)



Simulation summary

- In this example, even distant reference genome did not affect parameter of interest (monophyly of outbreak), although it did affect branch lengths
- Effects of read mapping parameters and reference genome choice are likely to be idiosyncratic
- By using empirical estimates for evolutionary model, can investigate effects on parameters of interest
- Currently applying this approach to test gonorrhea phylogenetic updating procedure

Summary

Bias: Sequencing error, reference choice

Effect on inference:

Sequencing error can increase terminal branch lengths relative to internal branches

Not mapping reads on lineages more distant from reference genome will decrease those branch lengths

Mitigation: Use multiple reference genomes, simulation based tests to assess accuracy

Conclusions:

When a closely related reference is available, alternatives worsen inference

At high (around 40x) coverage all mutations are confidently recovered

Even at lower coverage (around 5x) high confidence in monophyly of outbreak clade

Big picture

- ▶ All data sets are biased, genome scale data is no exception
- ▶ Careful project planning helps
- ▶ Interrogate potential biases in data sets

What to do?

- What data will answer **your** questions?
- Are there existing data you want to be able integrate with?
- Consider in which direction biases are likely to sway results
- Use the most an appropriate available model for your data
- Re-sample your data to test if your key conclusions are robust to choices
- Simulation approaches to test if parameters of interest are affected by sampling and ascertainment schemes

Questions?

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