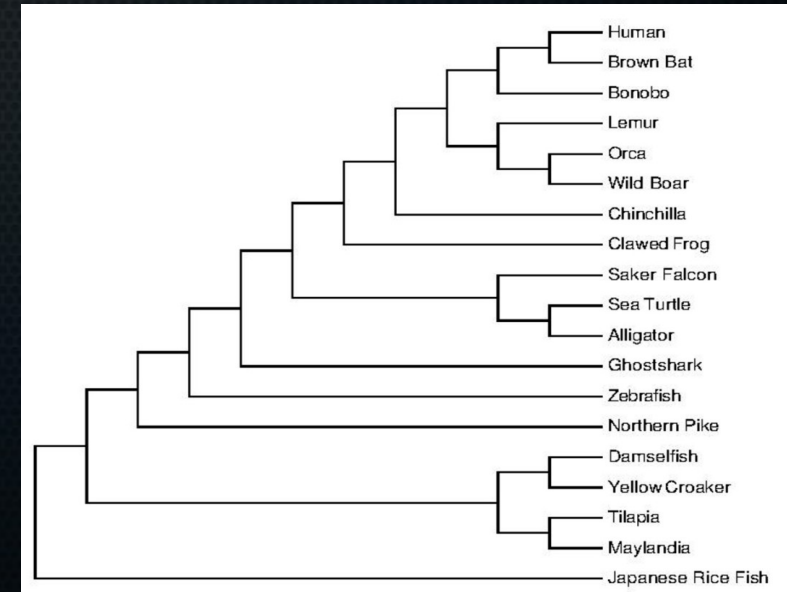


Clustering in practice: phylogeny

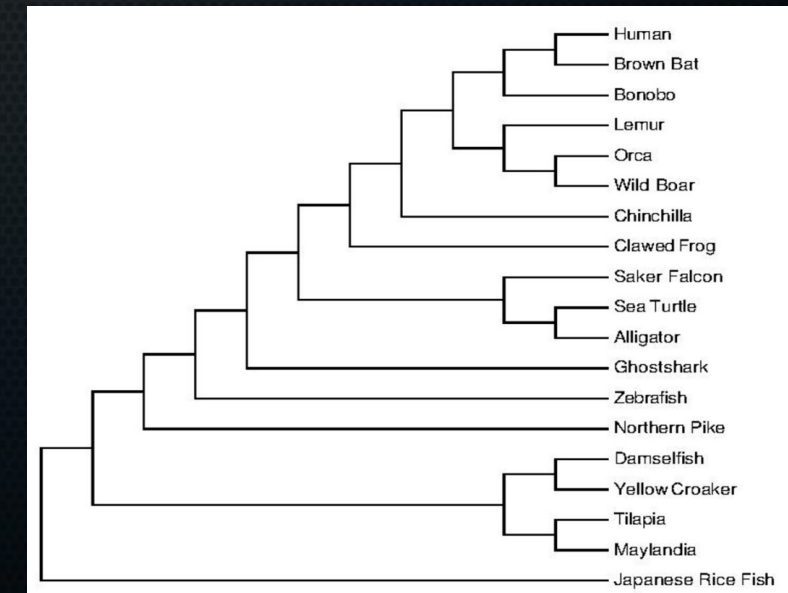
- A phylogeny is made based on a multiple-sequence alignment.



Source: https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree.pdf

Clustering in practice: phylogeny

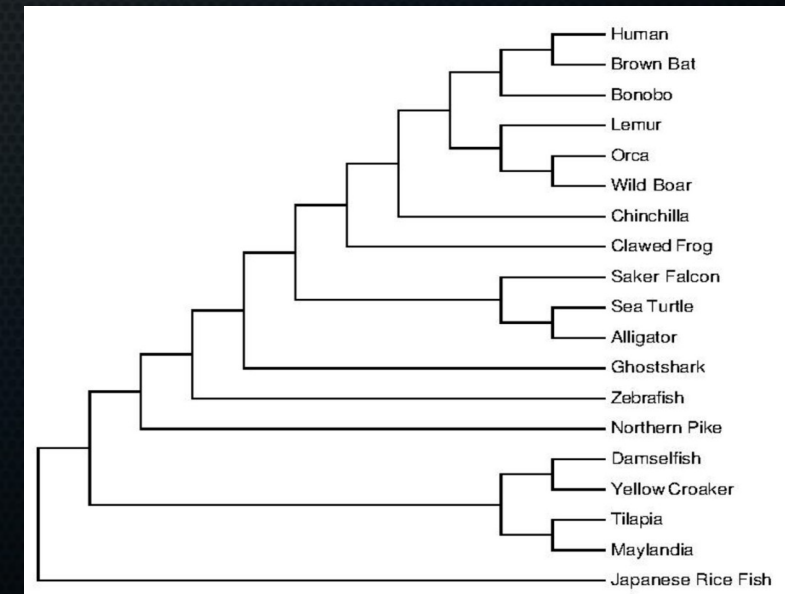
- Multiple-sequence alignment (MSA) is clustering of sequences according to similarity (and finding out which *parts* of the sequences are homologous/respond to each other to assess this similarity).
- Once we know *that* we can make our tree.



Source: https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree.pdf

Clustering in practice: phylogeny

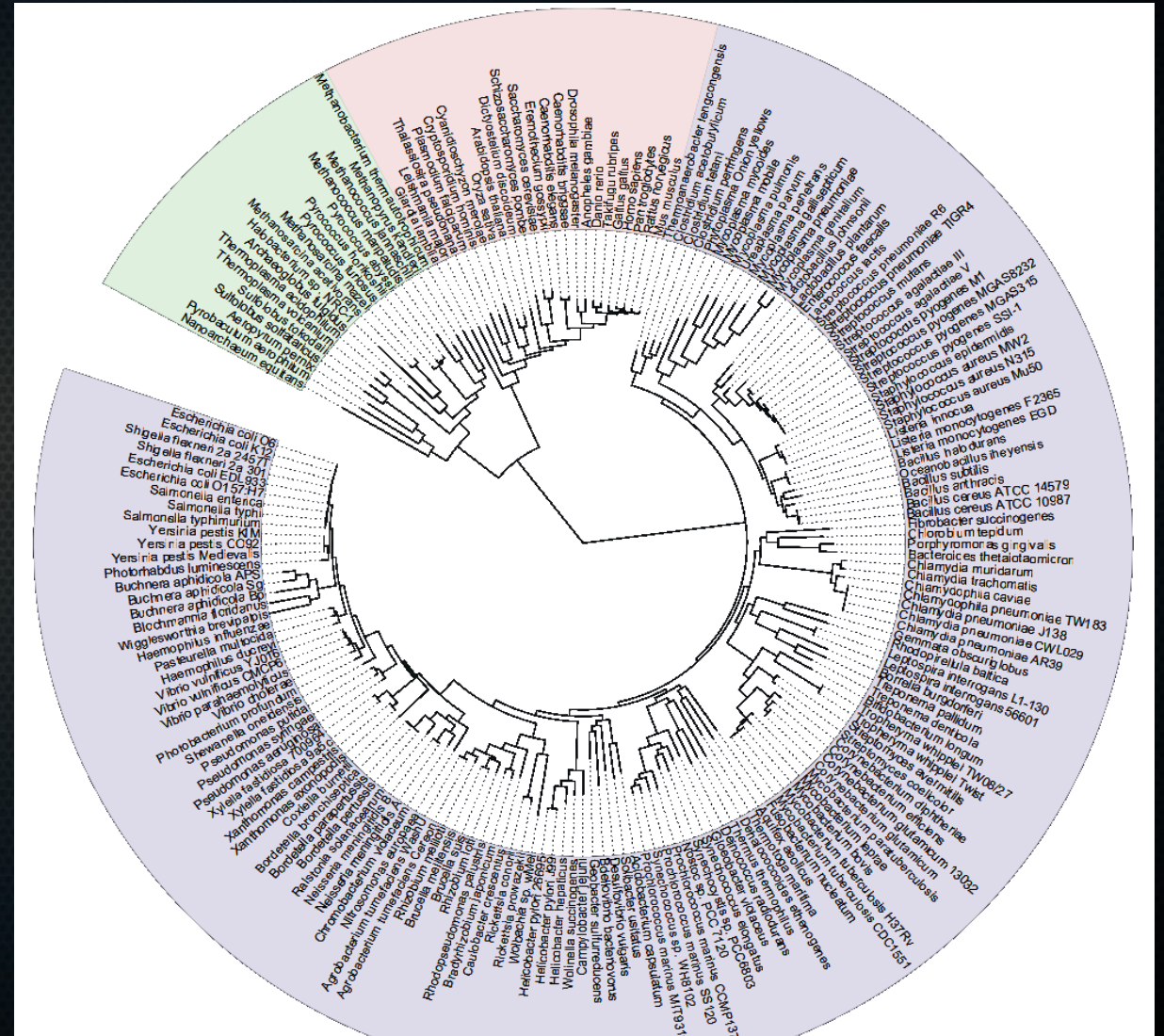
- Multiple-sequence alignment (MSA) is clustering of sequences according to similarity (and finding out which *parts* of the sequences are homologous/respond to each other to assess this similarity).
- Look into how it works, and some problems or caveats due to clusters and MSAs not being *the correct* clusters/MSAs



Source: https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree.pdf

Problems with phylogeny

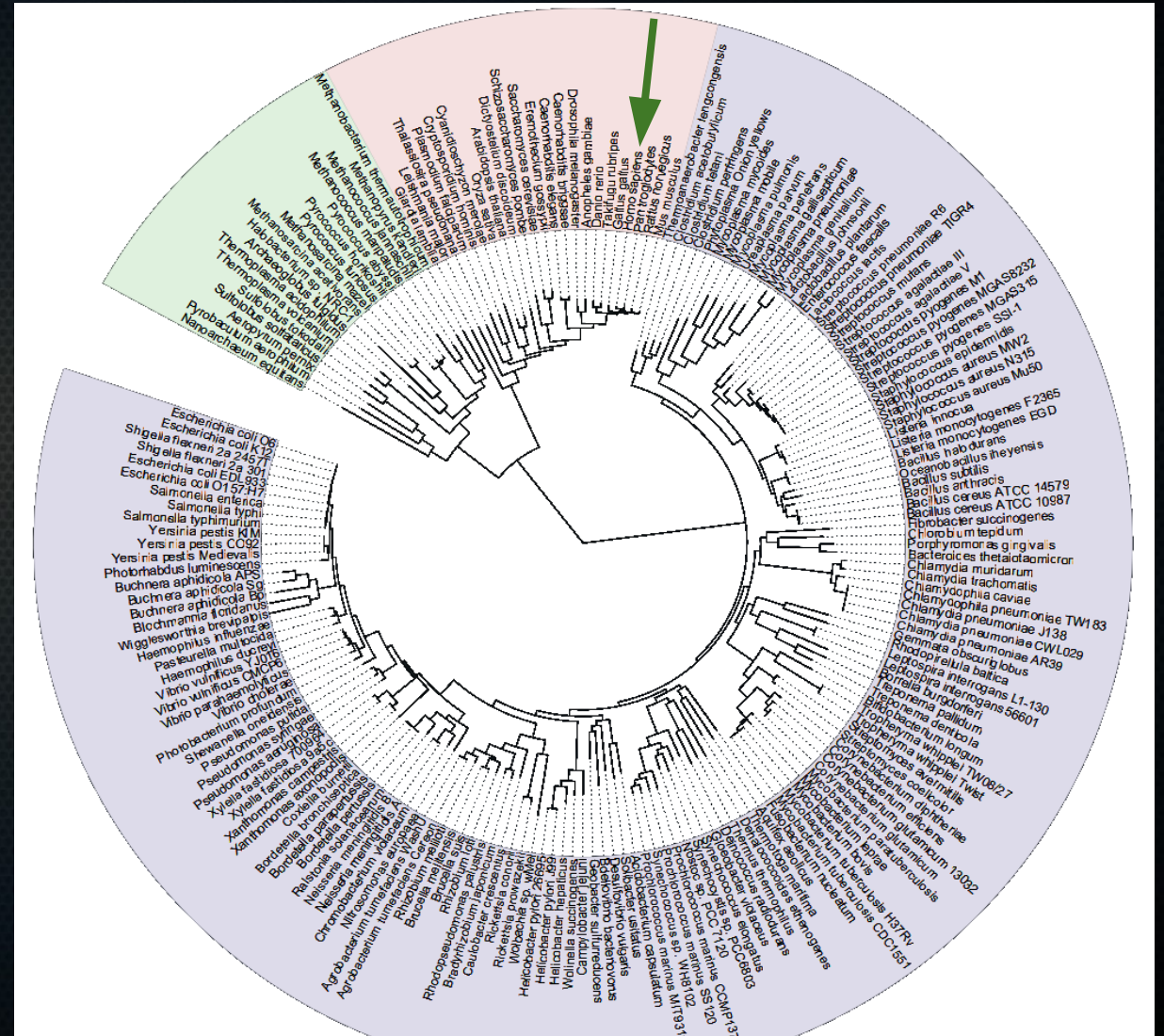
- What problems?



Source: Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic acids research, gkab301. Advance online publication. <https://doi.org/10.1093/nar/gkab301>

Problems with phylogeny

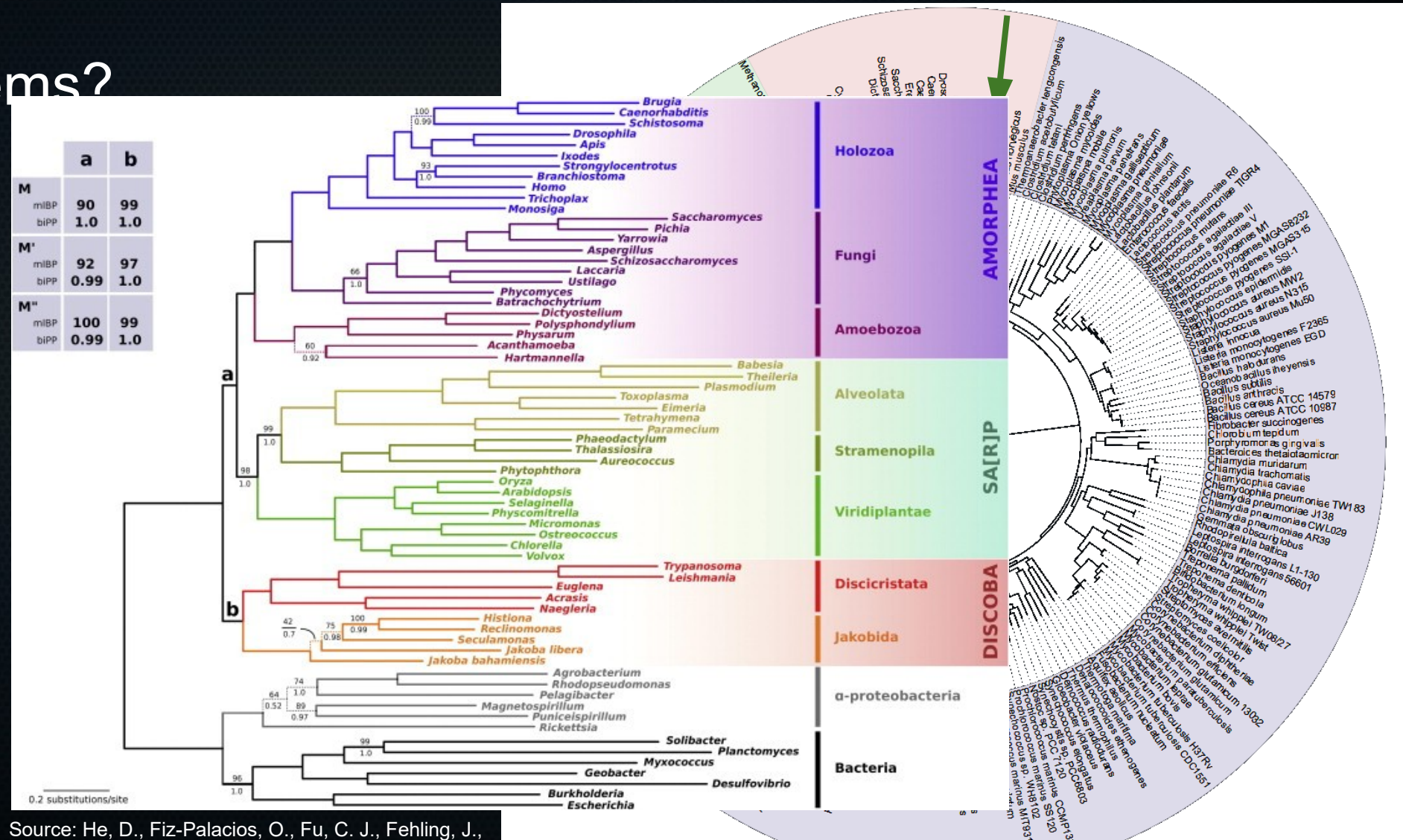
- What problems?



Source: Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic acids research, gkab301. Advance online publication. <https://doi.org/10.1093/nar/gkab301>

Problems with phylogeny

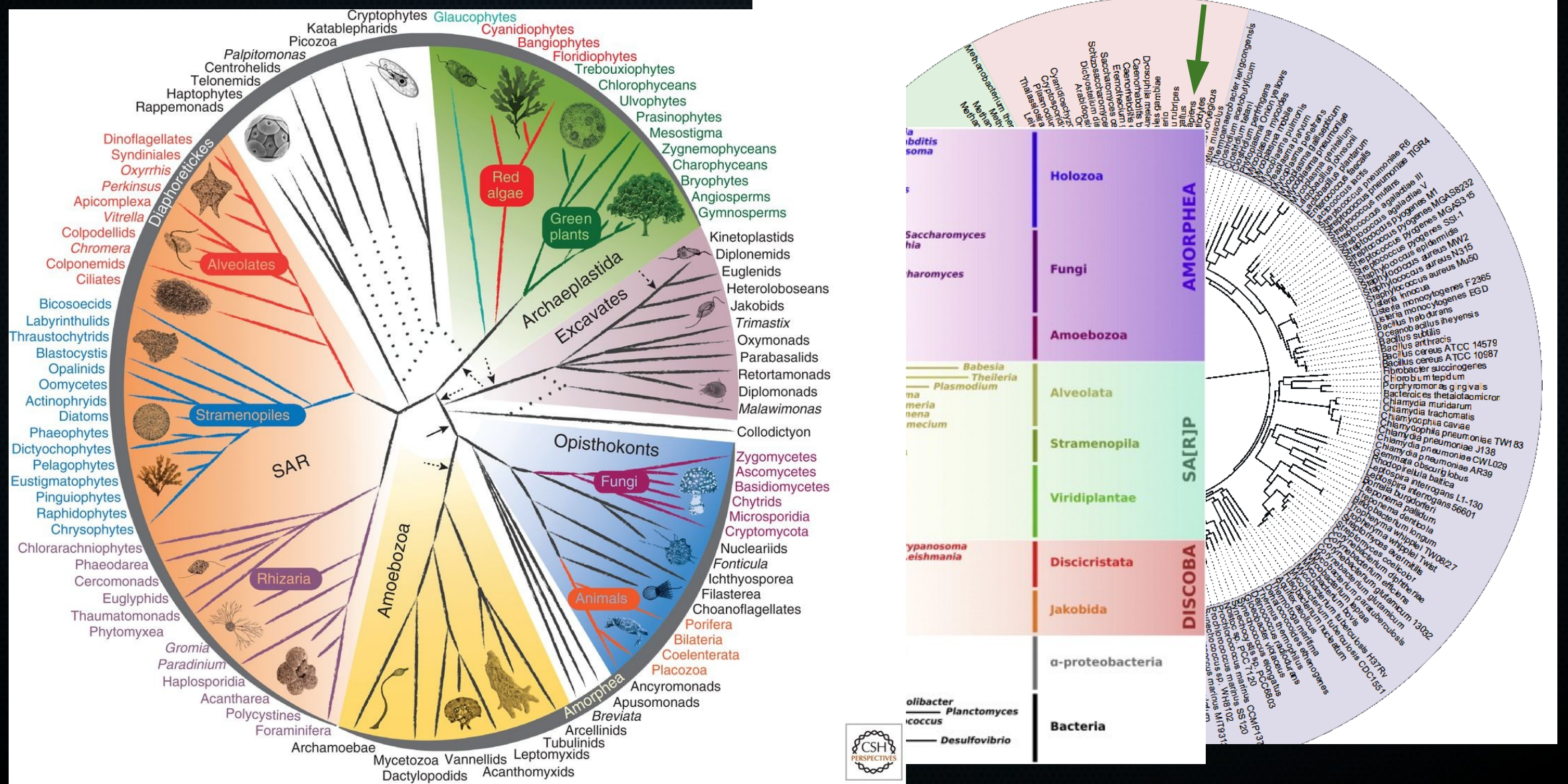
- What problems?



Source: He, D., Fiz-Palacios, O., Fu, C. J., Fehling, J., Tsai, C. C., & Baldauf, S. L. (2014). An alternative root for the eukaryote tree of life. *Current Biology*, 24(4), 465-470.

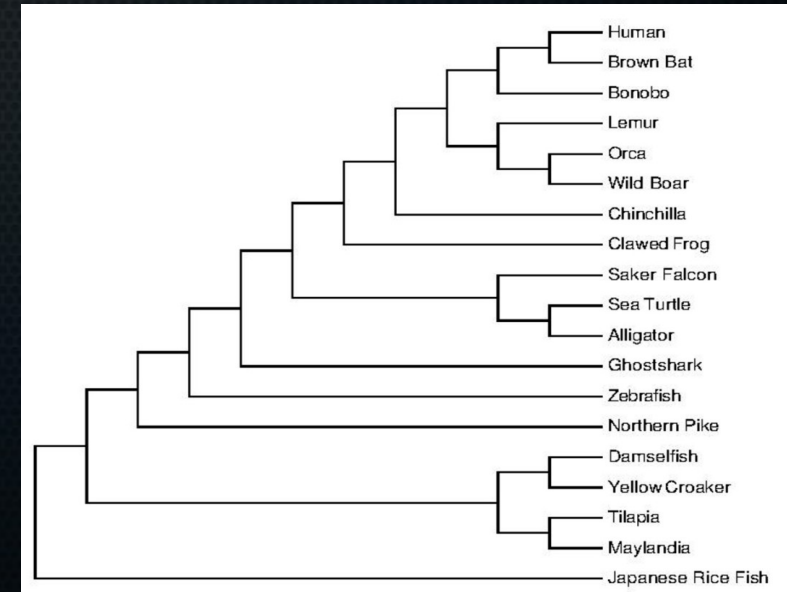
Problems with phylogeny

Source: Burki, F. (2014). The eukaryotic tree of life from a global phylogenomic perspective. Cold Spring Harbor perspectives in biology, 6(5), a016147.



Problems with phylogeny

- Pragmatically, these methods work extremely well and have yielded great insights.
- Under the hood, there are some caveats.



Source: https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree.pdf

Phylogenetic shaky ground

- Problem 1: How do we get a tree?

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

Phylogenetic shaky ground

- Problem 1: How do we get a tree?
→ Need to know correspondence between characters →
which letter in which sequence corresponds to which letter in
another sequence? → **alignment**

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

Phylogenetic shaky ground

- Problem 1: correspondence between characters.



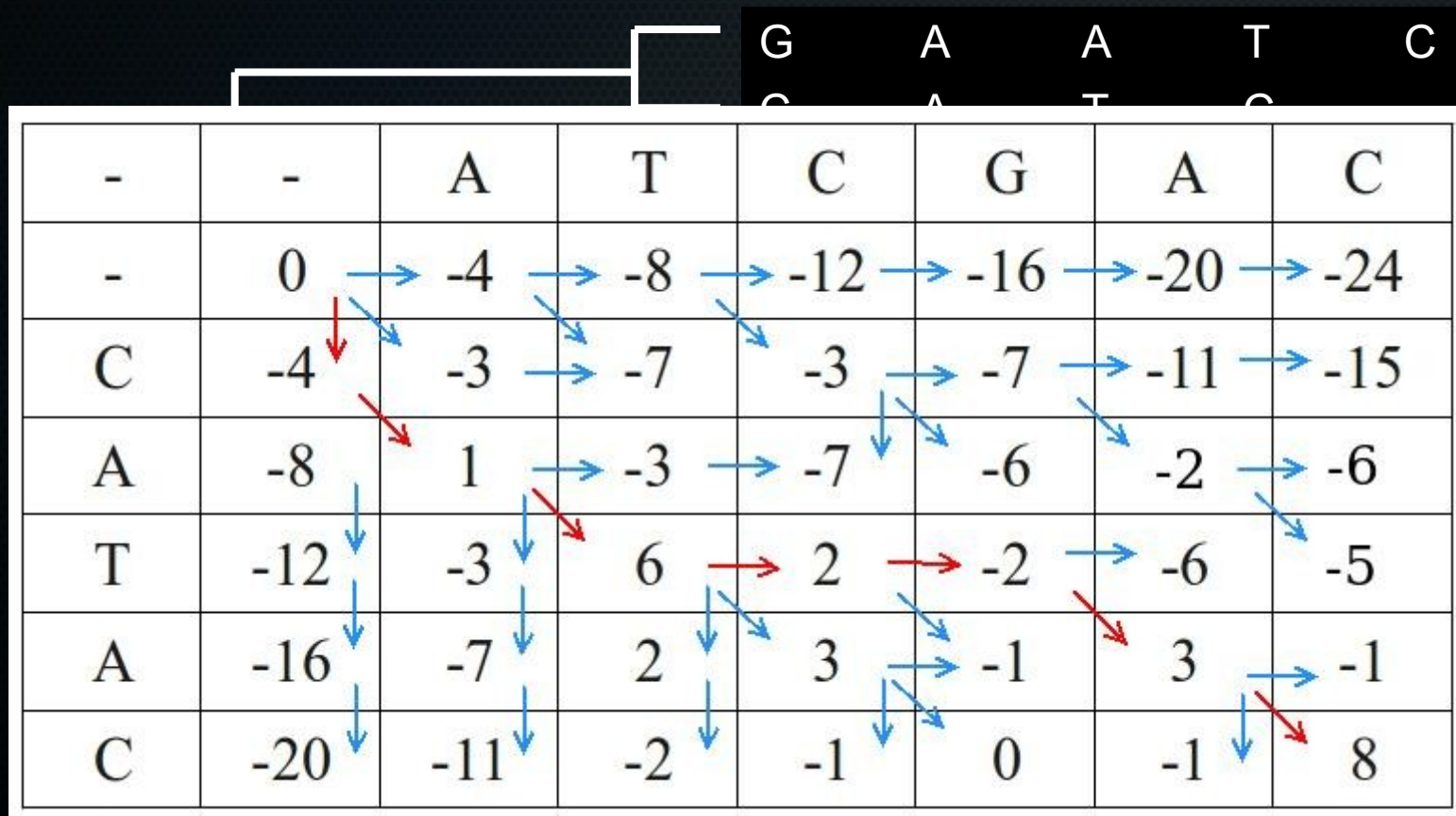
Phylogenetic shaky ground

- Problem 1: correspondence between characters



Phylogenetic shaky ground

- Problem 1: correspondence between characters



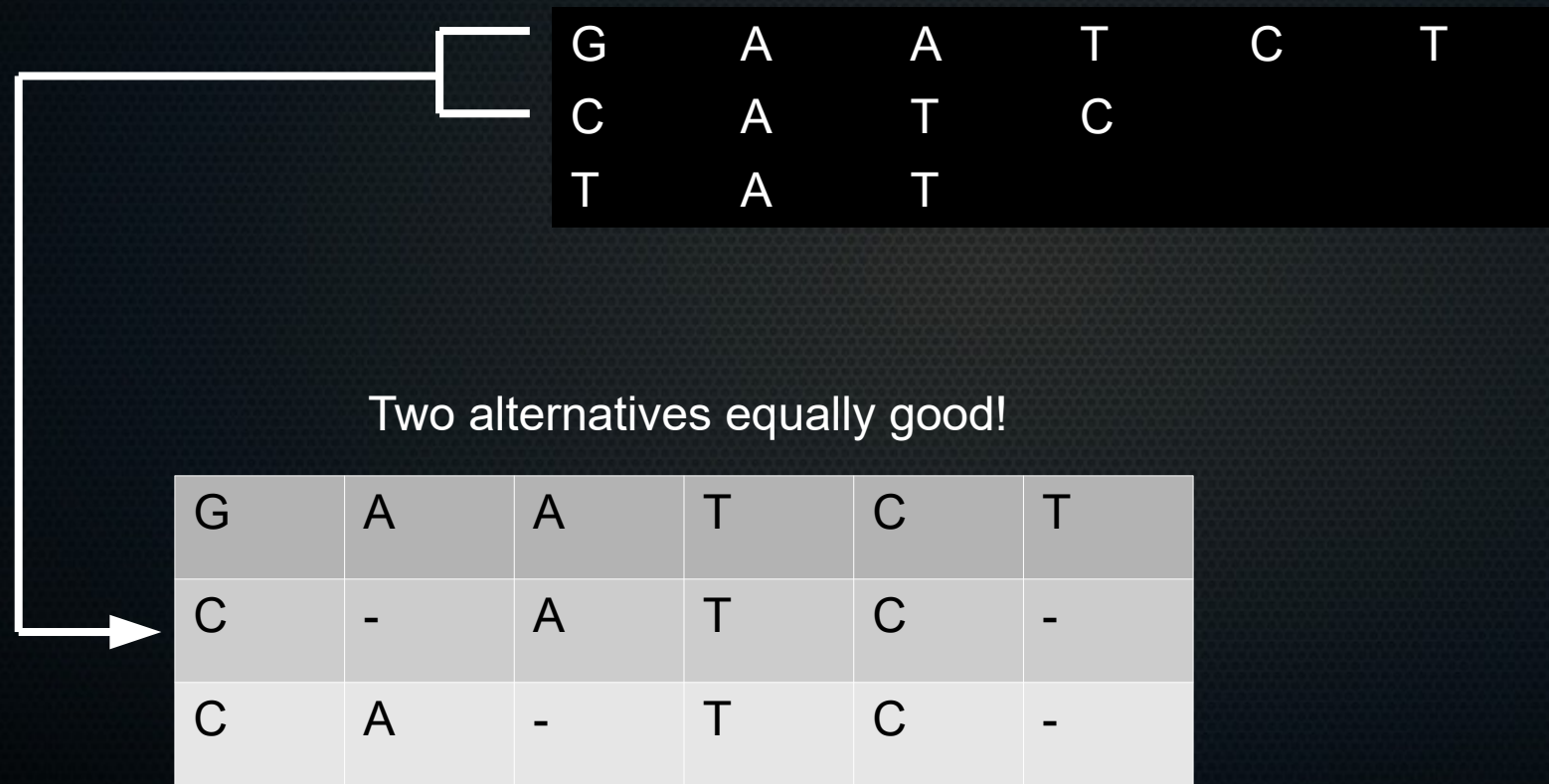
		G	A	A	T	C	T
-	-	A	T	C	G	A	C
-	0	-4	-8	-12	-16	-20	-24
C	-4	-3	-7	-3	-7	-11	-15
A	-8	1	-3	-7	-6	-2	-6
T	-12	-3	6	2	-2	-6	-5
A	-16	-7	2	3	-1	3	-1
C	-20	-11	-2	-1	0	-1	8

Every gap gives a gap penalty

Aligned via dynamic programming (Needleman Wunsch-like).

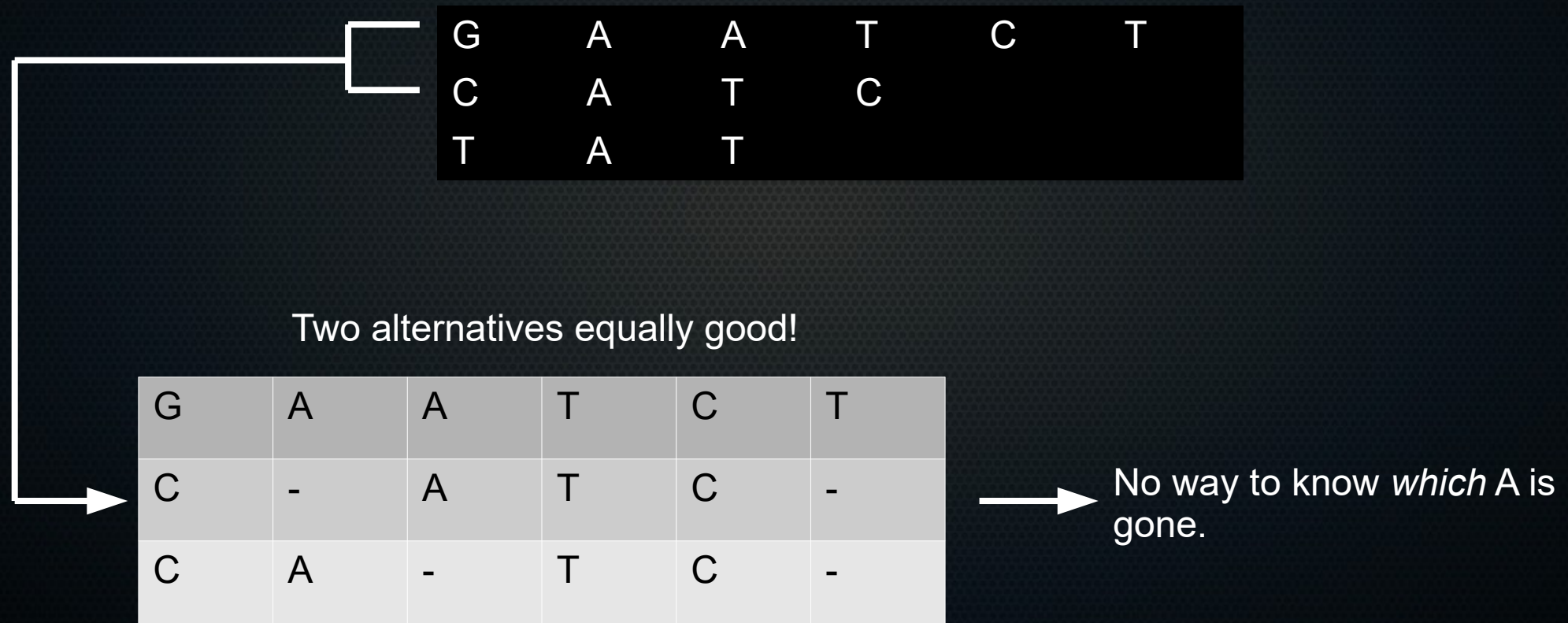
Phylogenetic shaky ground

- Problem 1: correspondence between characters



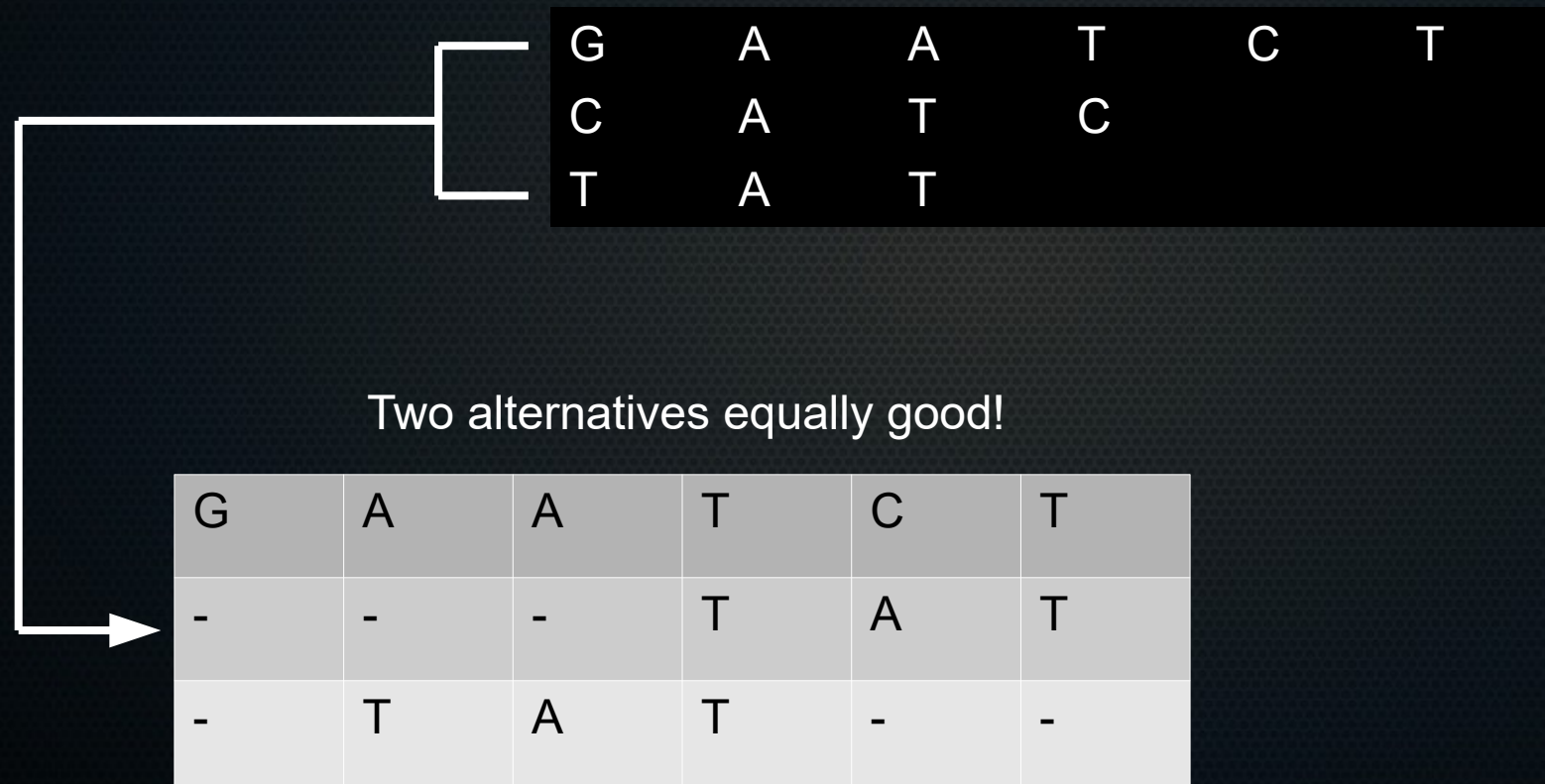
Phylogenetic shaky ground

- Problem 1: correspondence between characters



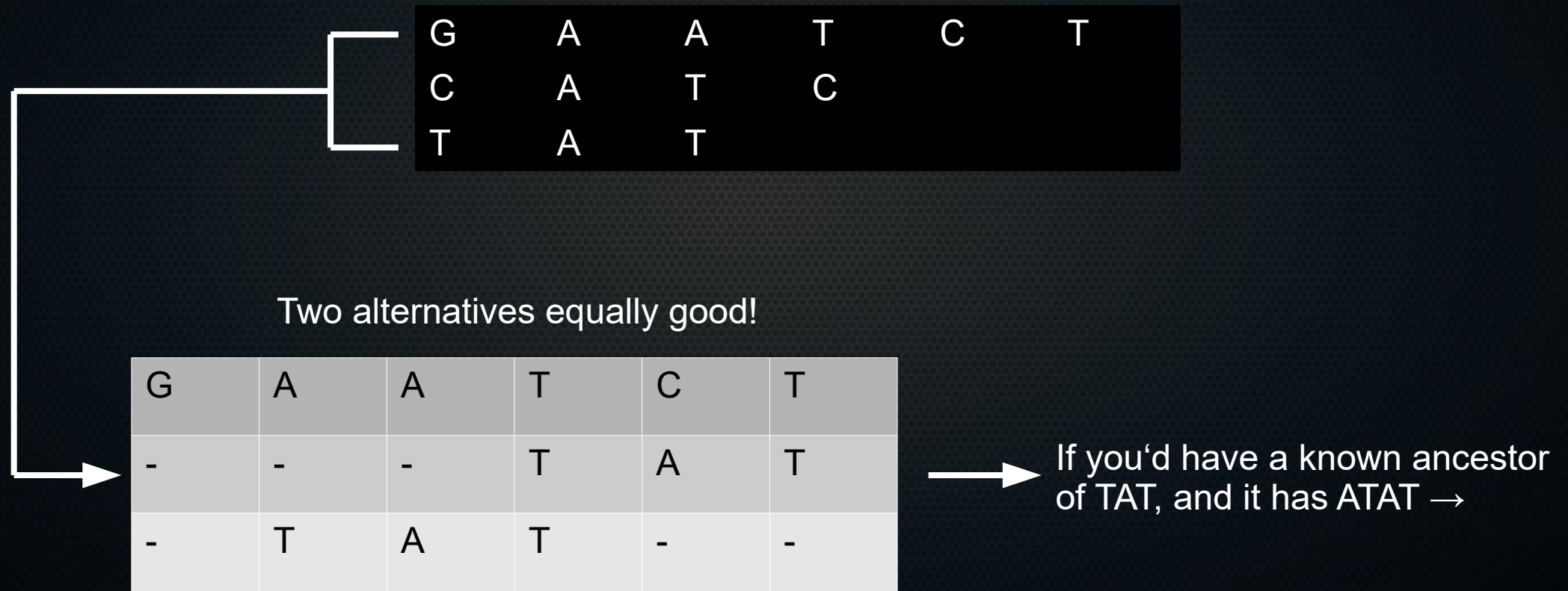
Phylogenetic shaky ground

- Problem 1: correspondence between characters



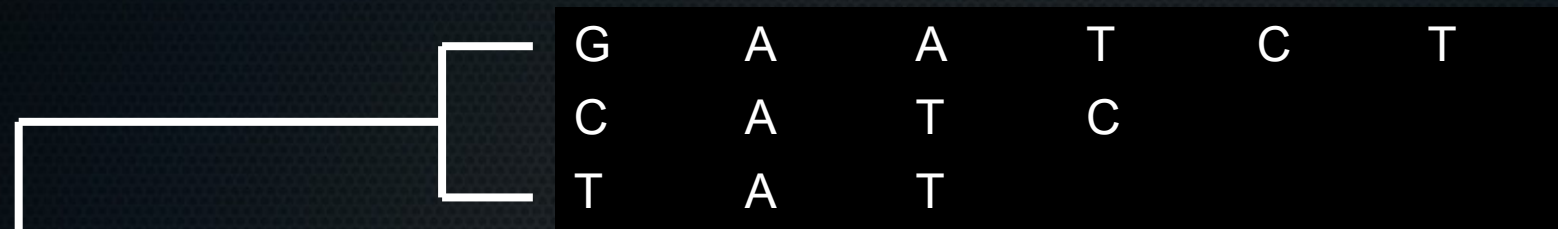
Phylogenetic shaky ground

- Problem 1: correspondence between characters



Phylogenetic shaky ground

- Problem 1: correspondence between characters



Two alternatives equally good!

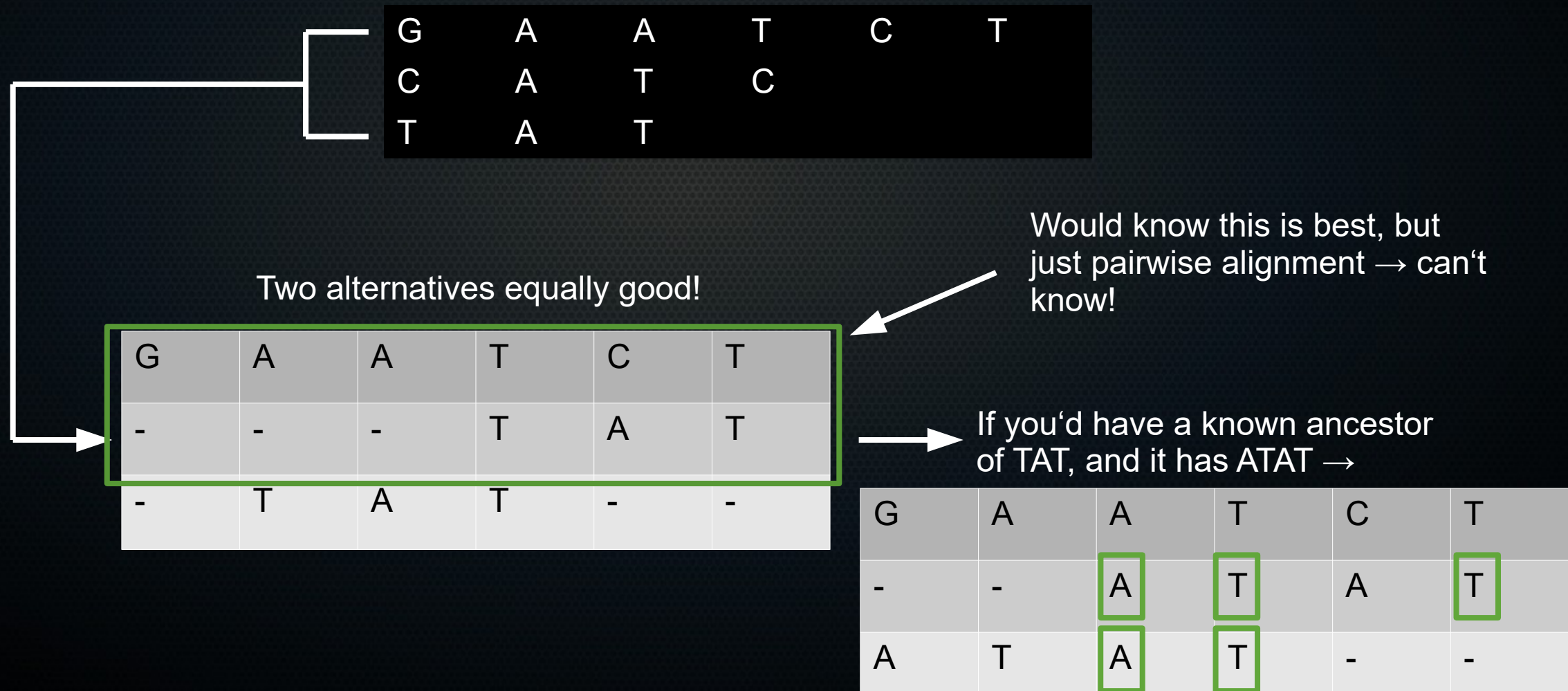
G	A	A	T	C	T
-	-	-	T	A	T
-	T	A	T	-	-

→ If you'd have a known ancestor of TAT, and it has ATAT →

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

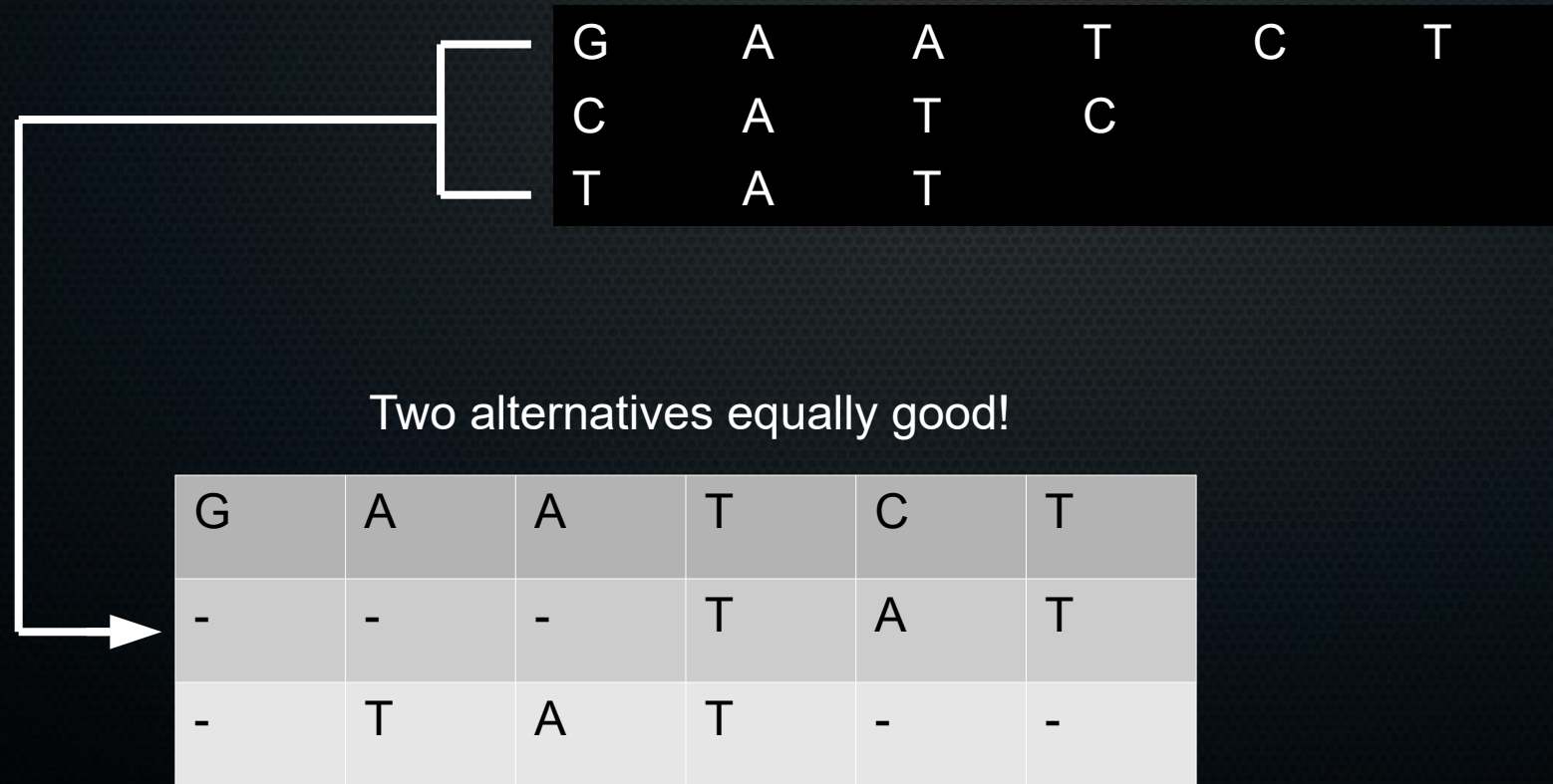
Phylogenetic shaky ground

- Problem 1: correspondence between characters



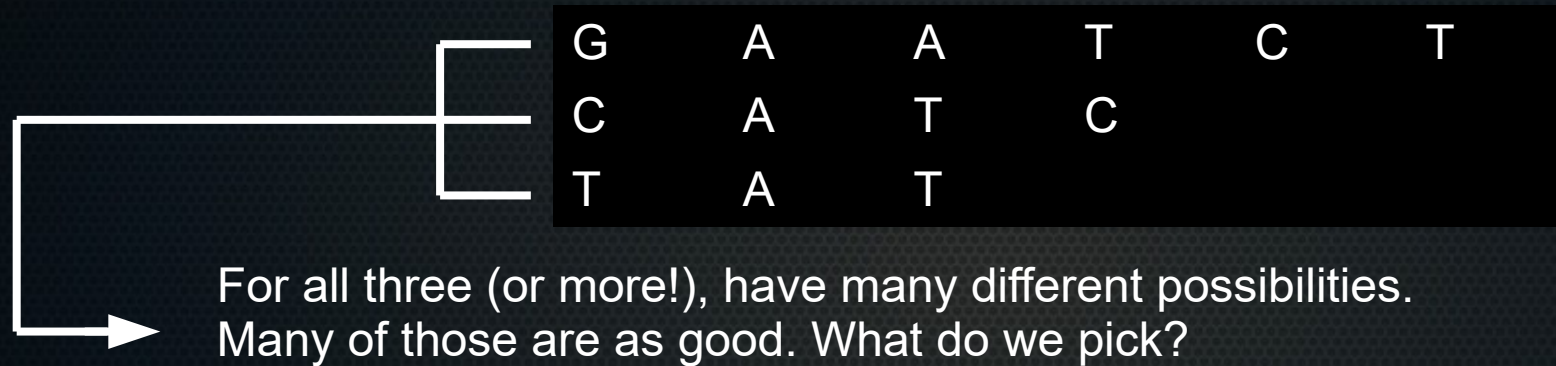
Phylogenetic shaky ground

- Problem 1: correspondence between characters
So: can get best alignment between two sequences, but can already be non-unique. → in practice one is picked at random.



Phylogenetic shaky ground

- What about three sequences?



Phylogenetic shaky ground

- What about *many more* sequences?

Source: <https://2017-lapaz-assembly.readthedocs.io/en/latest/qiime1.html>

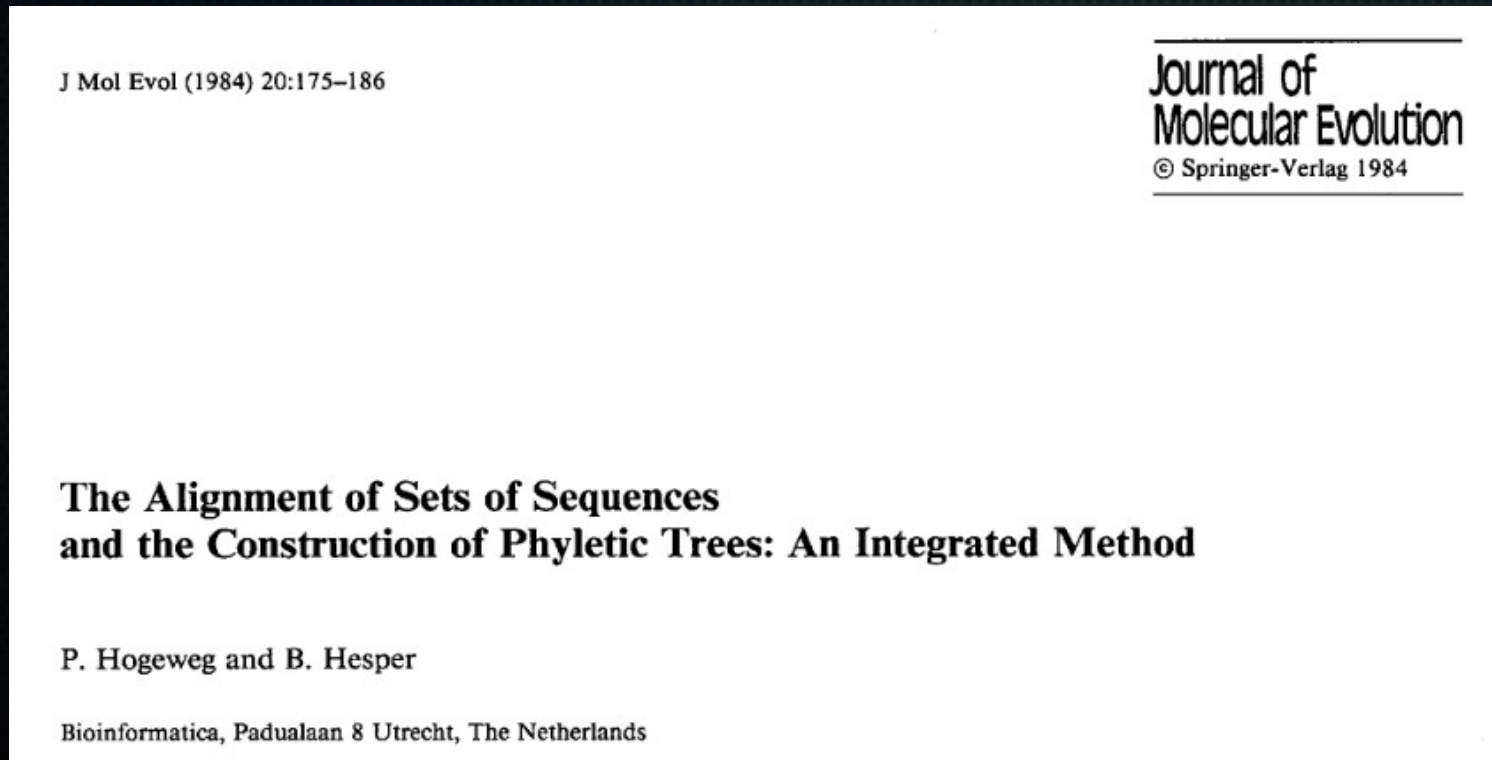
```
>C12.06102014.R2.D01.TCACGGGAGTTG_1 HWI-M03127:41:ACE13:1:2107:7445:12380
GACGTAGGGAGCGAGCGTTCTCCGGATTTACTGGGCGTAAAGGGTCCGCAGGCGGGTGTTTAAGTTTCACCTGACAGCCTCCGGCTTAACCGGGGGAGGTGGTGGAAACTGGAG
ACCTTGAGACATCGAGAGGCAGAGGGAATTCGTGTGGAGCAGTGAAATGCGTAGAGATGCGGAAGAACACCAGAGGCGAAGGCGCTCTGCTGGCGATGCTCTGACGCTCAGGGA
CGAAAGCCAGGGGAGCAAACAGG
>C12.06102014.R2.D01.TCACGGGAGTTG_2 HWI-M03127:41:ACE13:1:1111:28215:12905
TACCAGCTCCCCGAGTGGTCGGGACGATTATTGGGCCTAAAGCATCCGTAGCCGGCTTCACAGGTCTCTTGTTAAATCCAACGGCTCAACCGTTGGACTGCAGGGGATACCATGG
AGCTAGGGGGCGGGAGAGGCGGACGGTACTCCATGGGTAGGGGTAAATCCGTTGATCCATGGAAGACCACCAGTGGCGAAGGCGGTCCGCTAGAACGCGCCCGACGGTGAGGGA
TGAAAGCTGGGGGAGCGAACCGG
>C12.06102014.R2.D01.TCACGGGAGTTG_3 HWI-M03127:41:ACE13:1:2109:17440:18773
TACCAGCTCCCCGAGTGGTCGGGACGATTATTGGGCCTAAAGCATCCGTAGCCGGCTTCACAGGTCTCTTGTTAAATCCAACGGCTCAACCGTTGGACTGCAGGGGATACCATGG
AGCTAGGGGGCGGGAGAGGCGGACGGTACTCCATGGGTAGGGGTAAATCCCTTTGATCCATGGAAGAGCACCAGTGGCGAAGGCGGTCCGCTAGAACGCGCCCGACGGTGAGGGA
TGAAAGCTGGGGGAGCGGACCGG
>C12.06102014.R2.D01.TCACGGGAGTTG_4 HWI-M03127:41:ACE13:1:1106:5583:21341
TACCAGCTCCCCGAGTGGTCGGGACGATTATTGGGCCTAAAGCATCCGTAGCCGGCTTCACAGGTCTCTTGTTAAATCCAACGGCTCAACCGTTGGACTGCAGGGGATACCATGG
AGCTAGGGGGCGGGAGAGGCGGACGGTACTCCATGGGTAGGGGTAAATCCCTTTGATCCATGGAAGACCACCAGTGGCGAAGGCGGTCCGCTAGAACGCGCCCGACGGTGAGGGA
TGAGAGCTGGGGGAGCGAACCGG
>C12.06102014.R2.D01.TCACGGGAGTTG_5 HWI-M03127:41:ACE13:1:2106:15029:18423
TACCAGCTCCCCGAGTGGTCGGGACGATTATTGGGCCTAAAGCATCCGTAGCCGGCTTCACAGGTCTCTTGTTAAATCCAACGGCTCAACCGTTGGACTGCAGGGGATACCATGG
```


Phylogenetic shaky ground

- What about *many more* sequences?
- Becomes impossible to try all possibilities, cannot use Dynamic Programming (Needleman-Wunsch-like) approach for more than 3 or 4 sequences. So we use heuristics to get something.

Progressive tree alignment

- Idea: we can do pairwise alignment well



Progressive tree alignment

- Idea: we can do pairwise alignment well
- So: do pairwise alignment on all sequences, get their pairwise distances (non-agreeing bases and gaps), cluster them hierarchically.

Progressive tree alignment

- Idea: we can do pairwise alignment well
- So: do pairwise alignment on all sequences, get their pairwise distances (non-agreeing bases and gaps), cluster them hierarchically.
- Then, to get a multiple sequence alignment: align the two closest sequences, add the third closest to that, fourth closest, etc.
Note: this means that you continuously make a pairwise alignment, where the already aligned sequences are taken as one.

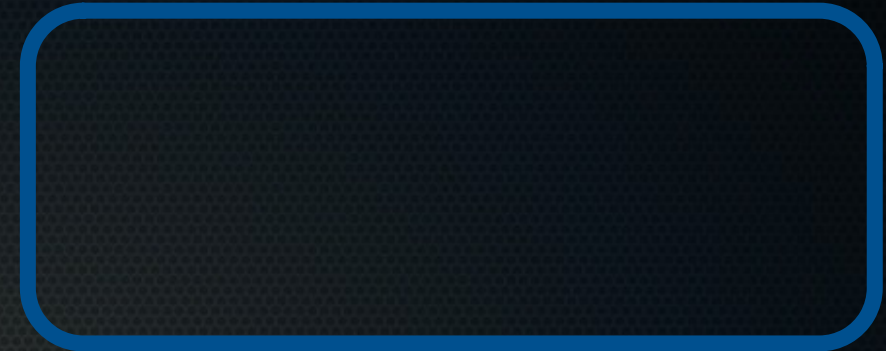
Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

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Pairwise alignment
(Needleman-Wunsch)



Progressive tree alignment

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4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)
↓
Example
(used EMBOSS Needle)

```
#=====
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 13
# Identity:      7/13 (53.8%)
# Similarity:    7/13 (53.8%)
# Gaps:          6/13 (46.2%)
# Score: 13.5
#
#=====
EMBOSS_001      1 -ATG--CTGTTTA      10
                  ||| |  |||
EMBOSS_001      1 AATGGCC---TTA      10
```

Progressive tree alignment

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#=====
EMBOSS_001      1 -ATG--CTGTTTA      10
                  ||| | |||
EMBOSS_001      1 AATGGCC---TTA      10
```

	1	2	3	4
1	50	13.5		
2	-	50		
3	-	-	50	
4	-	-	-	50

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)
↓
Example
(used EMBOSS Needle)

Note that real similarity is not measured by what letters match exactly, but by substitution matrices (based on many aligned sequences and substitution rates in those sequences), think BLOSUM62 and PAM for proteins.

```
#=====
#
# Aligned_sequences: 2
# 1: EMBOSS_001
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# Matrix: EDNAFULL
# Gap_penalty: 10.0
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```

	1	2	3	4
1	50	13.5		
2	-	50		
3	-	-	50	
4	-	-	-	50

Progressive tree alignment

1 ...ATGCTGTTTA...
 2 ...AATGGCCTTA...
 3 ...CCCCCCCCCCC...
 4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)

Example
(used EMBOSS Needle)

	A	T	G	C	S	W	R	Y	K	M	B	V	H	D	N
A	5	-4	-4	-4	-4	1	1	-4	-4	1	-4	-1	-1	-1	-2
T	-4	5	-4	-4	-4	1	-4	1	1	-4	-1	-4	-1	-1	-2
G	-4	-4	5	-4	1	-4	1	-4	1	-4	-1	-1	-4	-1	-2
C	-4	-4	-4	5	1	-4	-4	1	-4	1	-1	-1	-1	-4	-2
S	-4	-4	1	1	-1	-4	-2	-2	-2	-2	-1	-1	-3	-3	-1
W	1	1	-4	-4	-4	-1	-2	-2	-2	-2	-3	-3	-1	-1	-1
R	1	-4	1	-4	-2	-2	-1	-4	-2	-2	-3	-1	-3	-1	-1
Y	-4	1	-4	1	-2	-2	-4	-1	-2	-2	-1	-3	-1	-3	-1
K	-4	1	1	-4	-2	-2	-2	-2	-1	-4	-1	-3	-3	-1	-1
M	1	-4	-4	1	-2	-2	-2	-2	-4	-1	-3	-1	-1	-3	-1
B	-4	-1	-1	-1	-1	-3	-3	-1	-1	-3	-1	-2	-2	-2	-1
V	-1	-4	-1	-1	-1	-3	-1	-3	-3	-1	-2	-1	-2	-2	-1
H	-1	-1	-4	-1	-3	-1	-3	-1	-3	-1	-2	-2	-1	-2	-1
D	-1	-1	-1	-4	-3	-1	-1	-3	-1	-3	-2	-2	-2	-1	-1
N	-2	-2	-2	-2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

	Symbol	Meaning	Mnemonic
DNA Bases	G	Guanine	<u>G</u> uanine
	T	Thymine	<u>T</u> hymine
	A	Adenine	<u>A</u> denine
	C	Cytosine	<u>C</u> ytosine
Ambiguity Characters	R	G + A	pu <u>R</u> ine
	Y	T + C	p <u>Y</u> rimidine
	S	G + C	<u>S</u> trong interactions (3 H bonds)
	W	T + A	<u>W</u> eak interactions (2 H bonds)
	K	G + T	<u>K</u> eto
	M	A + C	a <u>M</u> ino
	D	G + T + A	Not-C (<u>D</u> follows C in alphabet)
	H	T + A + C	Not-G (<u>H</u> follows G)
	B	G + T + C	Not-A (<u>B</u> follows A)
	V	G + A + C	Not-T or U (<u>V</u> follows U)
	N	G + A + T + C	a <u>N</u> y

Source: <http://rosalind.info/glossary/iupac-notation/>

Source: <http://rosalind.info/glossary/dnafull/>

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)

	1	2	3	4
1	50	13.5	0	1
2	-	50	0	3
3	-	-	50	16
4	-	-	-	50

Similarity

Progressive tree alignment

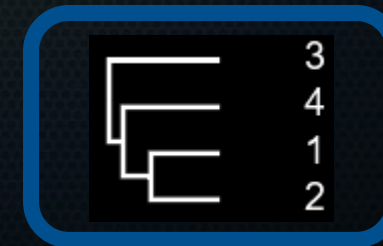
1 ...ATGCTGTTTA...
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(Needleman-Wunsch)

	1	2	3	4
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2	-	50	0	3
3	-	-	50	16
4	-	-	-	50

Similarity

Make a guide tree



Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)

	1	2	3	4
1	50	13.5	0	1
2	-	50	0	3
3	-	-	50	16
4	-	-	-	50

Similarity

Make a guide tree



Guide tree does not correspond
with pairwise distance matrix,
what gives!?

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)

	1	2	3	4
1	50	13.5	0	1
2	-	50	0	3
3	-	-	50	16
4	-	-	-	50

Similarity

Make a guide tree



Used Clustal Omega, which calculates distances slightly differently (optimised for large alignment + with different IUB DNA matrix where every match scores 1.9 and every mismatch is 0*)

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Needleman-Wunsch)

	1	2	3	4
1	50	13.5	0	1
2	-	50	0	3
3	-	-	50	16
4	-	-	-	50

Our guide tree
would look like this.



Similarity


Make a guide tree



Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Clustal Omega)



	1	2	3	4
1	50	?	?	?
2	-	50	?	?
3	-	-	50	?
4	-	-	-	50

Similarity

Make a guide tree
(with Clustal Omega
distances)



Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Clustal Omega)

	1	2	3	4
1	50	?	?	?
2	-	50	?	?
3	-	-	50	?
4	-	-	-	50

Similarity

Make a guide tree
(with Clustal Omega
distances)



Make a MSA by aligning
along this guide tree

1 -ATGCTGTTTA 10
2 AATGGCCTTA- 10
*** **

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCCAC...

Pairwise alignment
(Clustal Omega)

	1	2	3	4
1	50	?	?	?
2	-	50	?	?
3	-	-	50	?
4	-	-	-	50

Similarity

Make a guide tree
(with Clustal Omega
distances)



Make a MSA by aligning
along this guide tree

4 --GGGGGCCCAC 10
1 -ATGCTGTTTA- 10
2 AATGGCCTTA-- 10
★

Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Clustal Omega)

	1	2	3	4
1	50	?	?	?
2	-	50	?	?
3	-	-	50	?
4	-	-	-	50

Similarity

Make a guide tree
(with Clustal Omega
distances)

3 --CCCCCCCCC 10
4 --GGGGGCCAC 10
1 -ATGCTGTTA- 10
2 AATGGCCTTA-- 10

Make a MSA by aligning
along this guide tree



Progressive tree alignment

1 ...ATGCTGTTTA...
2 ...AATGGCCTTA...
3 ...CCCCCCCCCCC...
4 ...GGGGGCCAC...

Pairwise alignment
(Clustal Omega)

	1	2	3	4
1	50	?	?	?
2	-	50	?	?
3	-	-	50	?
4	-	-	-	50

Can now:

- calculate distances between aligned sequences
- choose a joining criterion (here: neighbour-linking)
- make an (unrooted) phylogenetic tree!

	3	0.35556
	4	0.24444
	1	0.23611
	2	0.20833

Similarity

Make a guide tree
(with Clustal Omega
distances)

Make a MSA by aligning
along this guide tree

3 --CCCCCCCCC 10
 4 --GGGGGCCAC 10
 1 -ATGCTGTTA- 10
 2 AATGGCCTTA-- 10



Progressive tree alignment

- Caveat: this introduces a feedback loop and doesn't ensure that we get the best tree!

Progressive tree alignment

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- We say: we want to align these sequences, and then cluster them to make a tree.
- We cannot align all the sequences together: incalculable.

Progressive tree alignment

- Caveat: this introduces a feedback loop and doesn't ensure that we get the best tree!
- We say: we want to align these sequences, and then cluster them to make a tree.
- We cannot align all the sequences together: incalculable.
- So what we do: pairwise alignments, score how well the sequence pairs align (distance/similarity), make a guide tree based on that, use it to align all together, then make a tree. This does *not* guarantee the *best* alignment of all sequences (global alignment).

Progressive tree alignment

- Caveat: this introduces a feedback loop and doesn't ensure that we get the best tree!
- We say: we want to align these sequences, and then cluster them to make a tree.
- We cannot align all the sequences together: incalculable.
- So what we do: pairwise alignments, score how well the sequence pairs align (distance/similarity), make a guide tree based on that, use it to align all together, then make a tree. This does *not* guarantee the *best* alignment of all sequences (global alignment).
- So we need a non-optimal/non-true tree to align the sequences, to make our tree. Do you see the strange recursion there?

Summary so far:

- Want to cluster nucleotide sequences based on evolutionary relatedness (phylogeny, phylogenetic tree)
- Need to align them all to calculate distances
- Can't do that directly



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- With the MSA in hand, we can calculate all distances, cluster hierarchically, and make a phylogenetic tree

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 - What about aligning thousands or hundreds of thousands of sequences? Does progressive alignment work fine?

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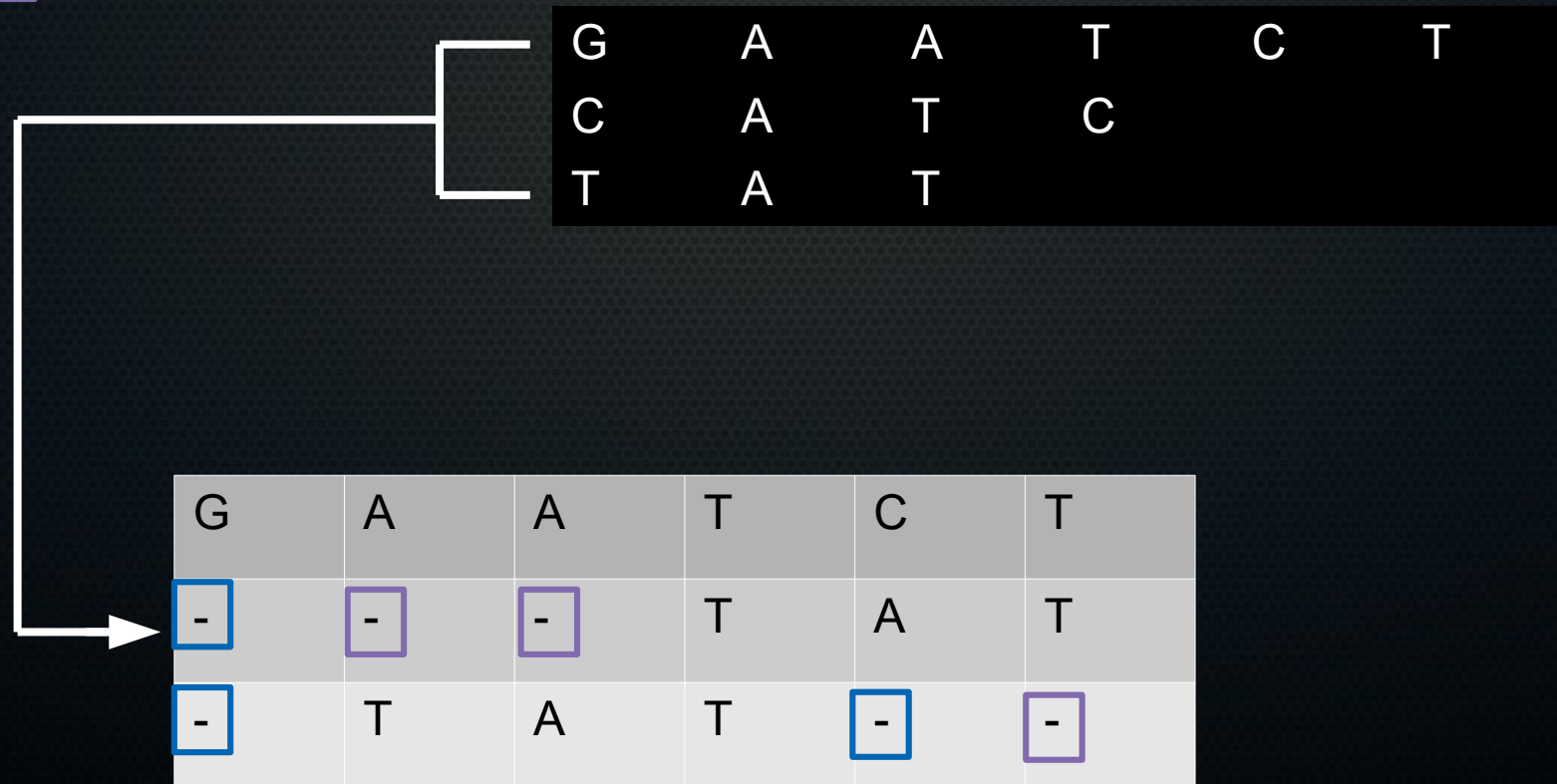
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Gaps and evolution

- Actually have separate scores for **opening** a gap (introducing a mismatch/SNP) and **extending** a gap.

↓ ↓

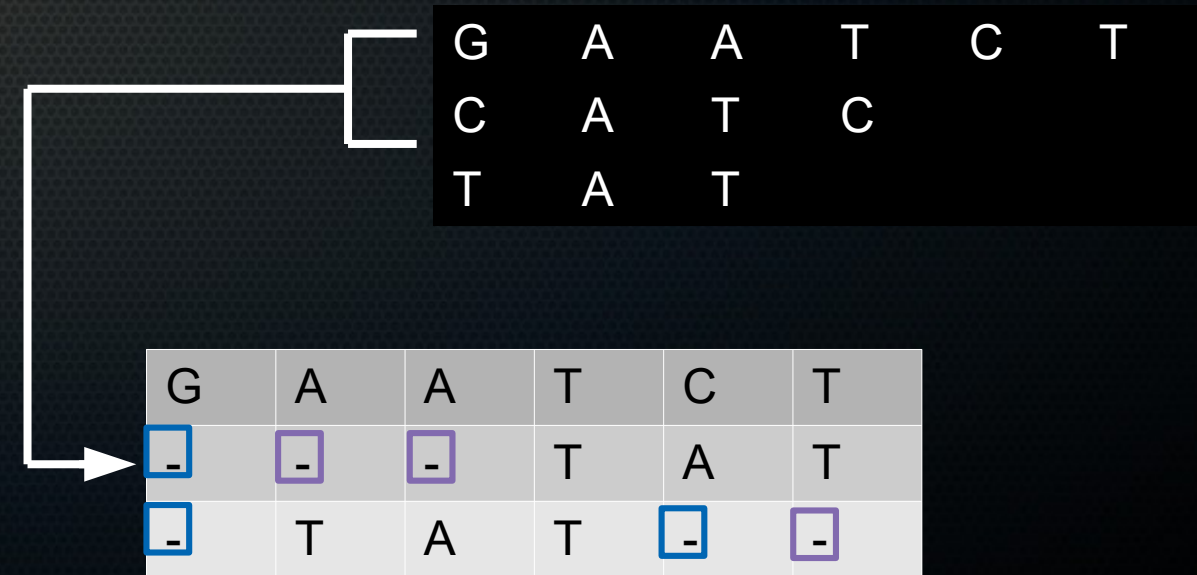
3	--CCCCCCCCC	10
4	--GGGGCCCAC	10
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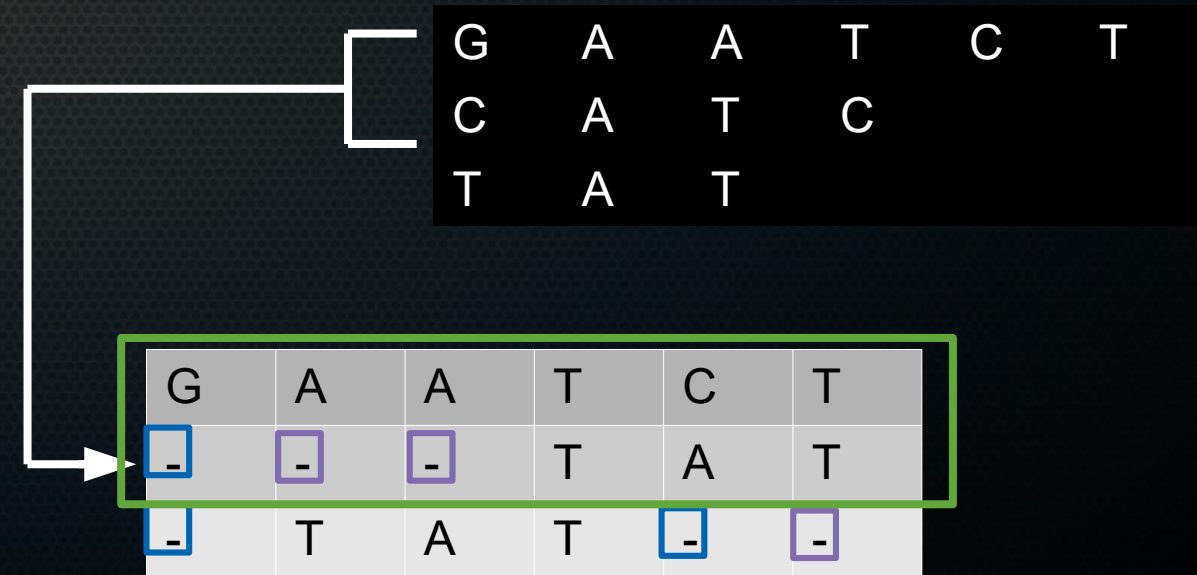
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- But: SNPs and small indels are *far* from the sole mediators of evolution!


We also have huge deletions, whole genome duplications, or large chromosomal rearrangements. That is just *one* evolutionary event, however our gap extension penalty will not favour an alignment that shows these!



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→ Our idea is that the tree shows evolutionary relatedness, but given how it works, it does that best for small changes only.

Gaps and evolution


- What about clustering nucleotide sequences for a different reason than phylogeny?



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- For example: align homologs of a protein that catalyses lipid flipping (flippase) → want to know if they are functional in all ancestors and in which organisms the gene lost function.



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- Note also that for proteins, the functional unit of evolution is the amino acid. Hence might be better to align proteins first, then codon triplets coding for them in DNA



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(Kapli, P., Yang, Z., & Telford, M. J. (2020). Phylogenetic tree building in the genomic age. Nature Reviews Genetics, 21(7), 428-444.)

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Progressive alignment of many sequences

- Still a problem:
Calculating all pairwise distances between 100.000 or more sequences requires N^2 calculations. Infeasible.

Progressive alignment of many sequences

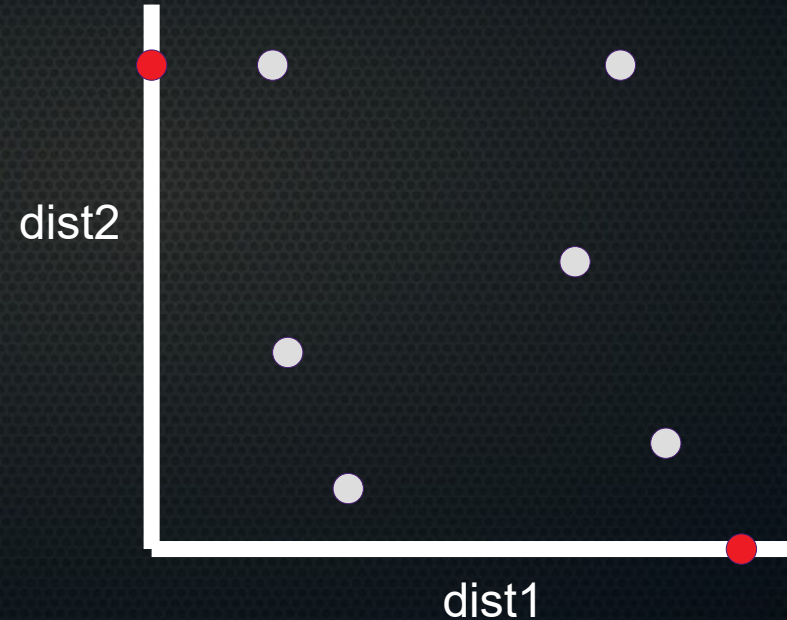
- Still a problem:
Calculating all pairwise distances between 100.000 or more sequences requires N^2 calculations. Infeasible.
- Solution: take $\log_2(100.000)$ *seed sequences*. Calculate distance of each sequence to each seed sequence (by pairwise alignment).

Progressive alignment of many sequences

- Now you can treat each sequence as a vector of these distances:

$$\begin{bmatrix} \text{dist}(\text{thisSeq}, \text{seedSeq}_1) \\ \text{dist}(\text{thisSeq}, \text{seedSeq}_2) \\ \text{dist}(\text{thisSeq}, \text{seedSeq}_3) \\ \dots \\ \text{dist}(\text{thisSeq}, \text{seedSeq}_n) \end{bmatrix}$$

Illustration of 8 sequences, 2 of which are seed sequences

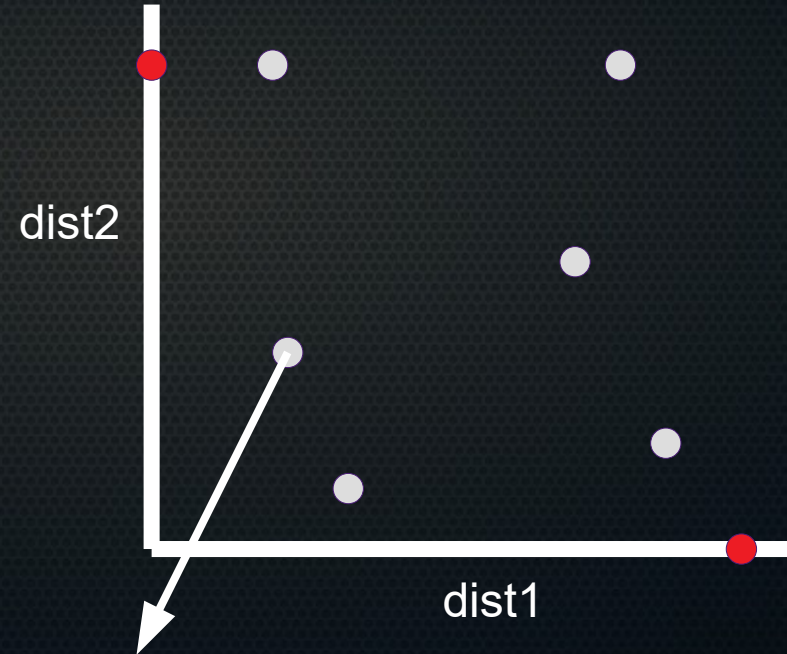


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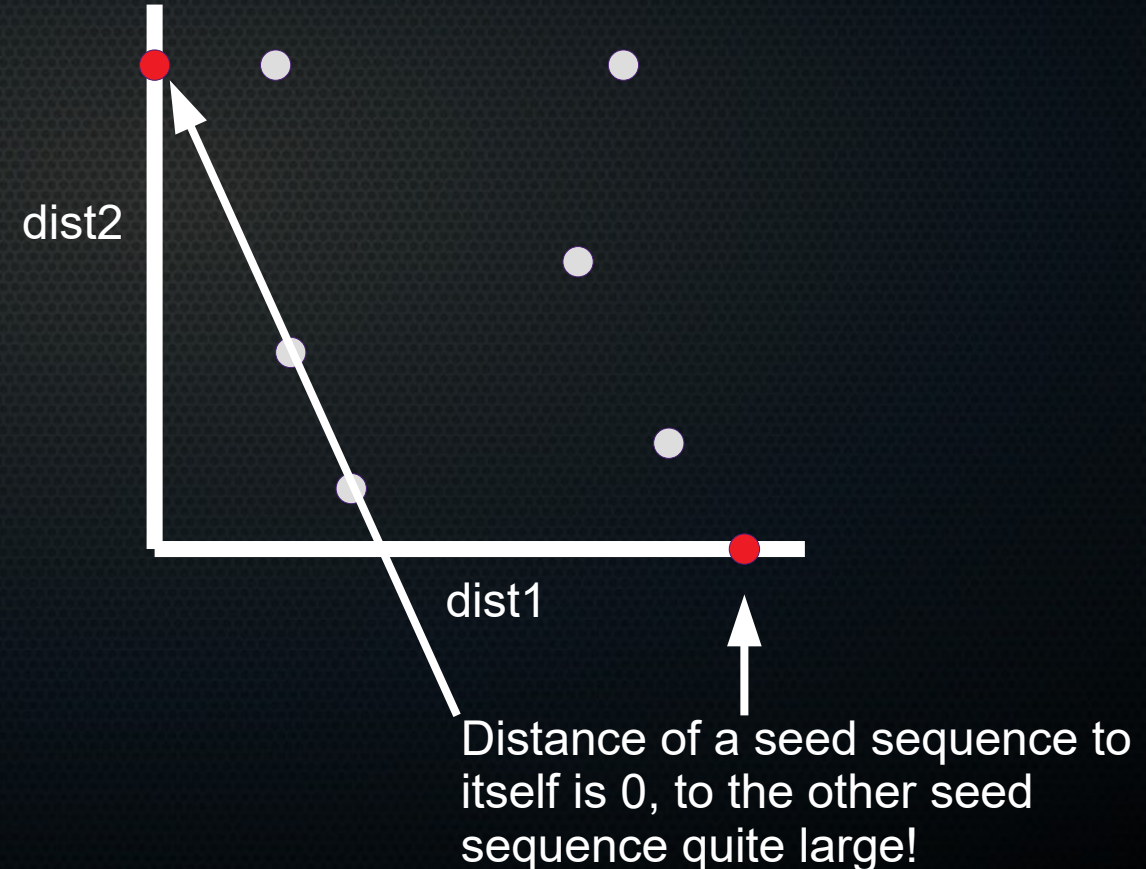
$$\begin{bmatrix} \text{dist}(\text{thisSeq}, \text{seedSeq}_1) \\ \text{dist}(\text{thisSeq}, \text{seedSeq}_2) \end{bmatrix} = \begin{bmatrix} \text{dist}_1 \\ \text{dist}_2 \end{bmatrix}$$

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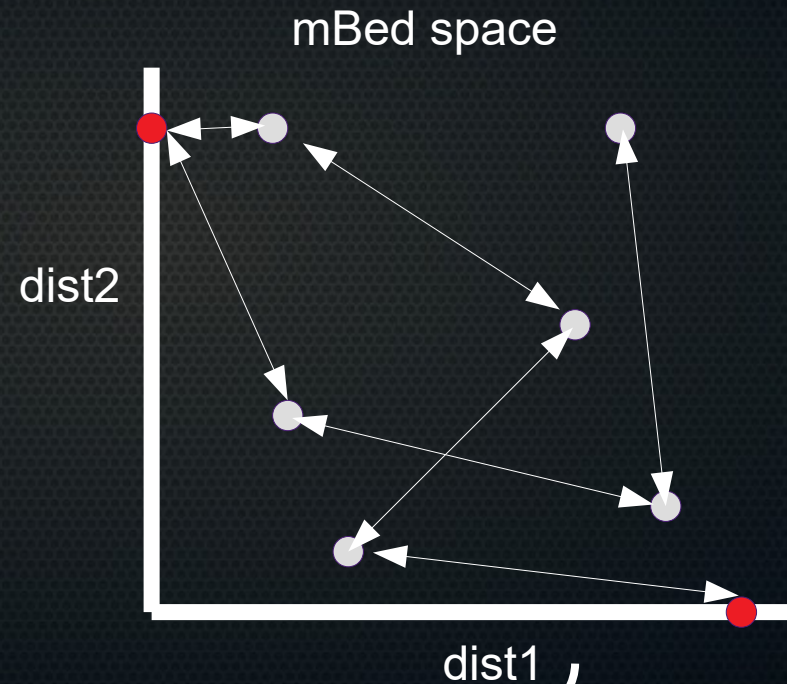
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Can easily calculate distances between
vectors (linear algebra is fast!)

Progressive alignment of many sequences

- In essence we thus assume that the seed sequences are sufficiently dissimilar that calculating distances to them is informative enough to approximate a full distance matrix.

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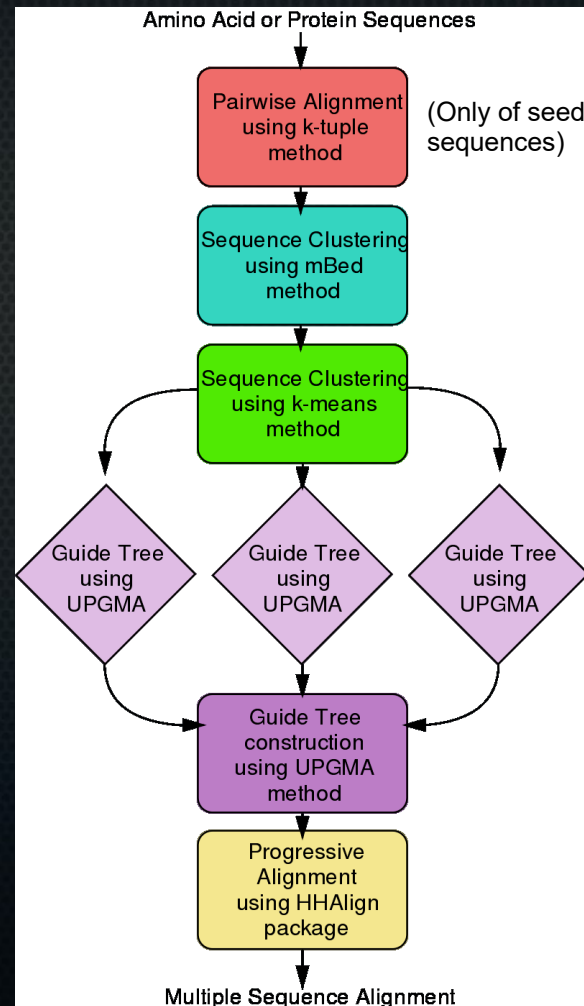
- In essence we thus assume that the seed sequences are sufficiently dissimilar that calculating distances to them is informative enough to approximate a full distance matrix.
- Checks are in place to make sure of this
(no duplicates, sample stratified by sequence length)

(Blackshields, G., Sievers, F., Shi, W., Wilm, A., & Higgins, D. G. (2010). Sequence embedding for fast construction of guide trees for multiple sequence alignment. *Algorithms for Molecular Biology*, 5(1), 1-11.).

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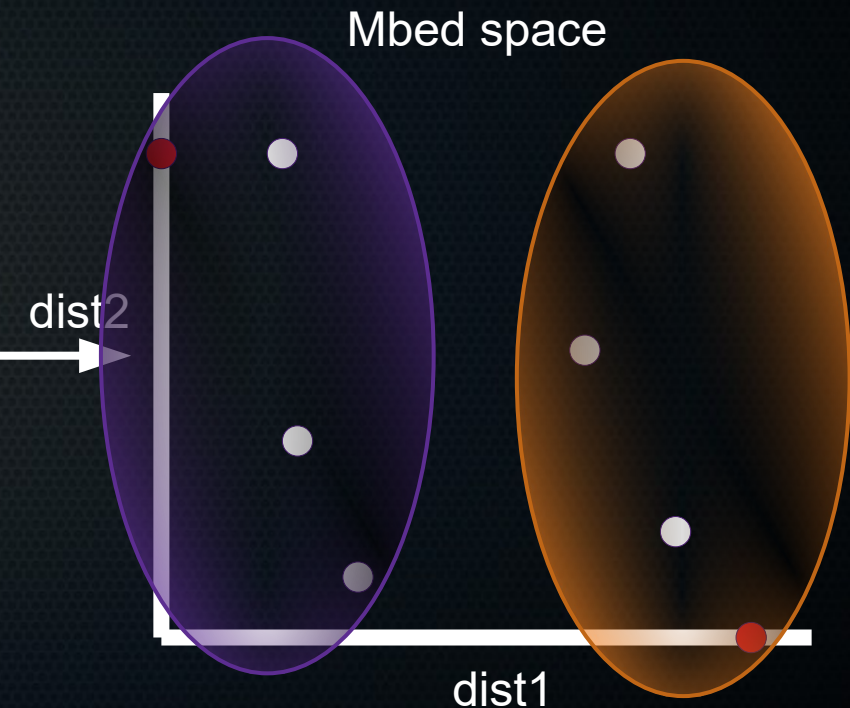
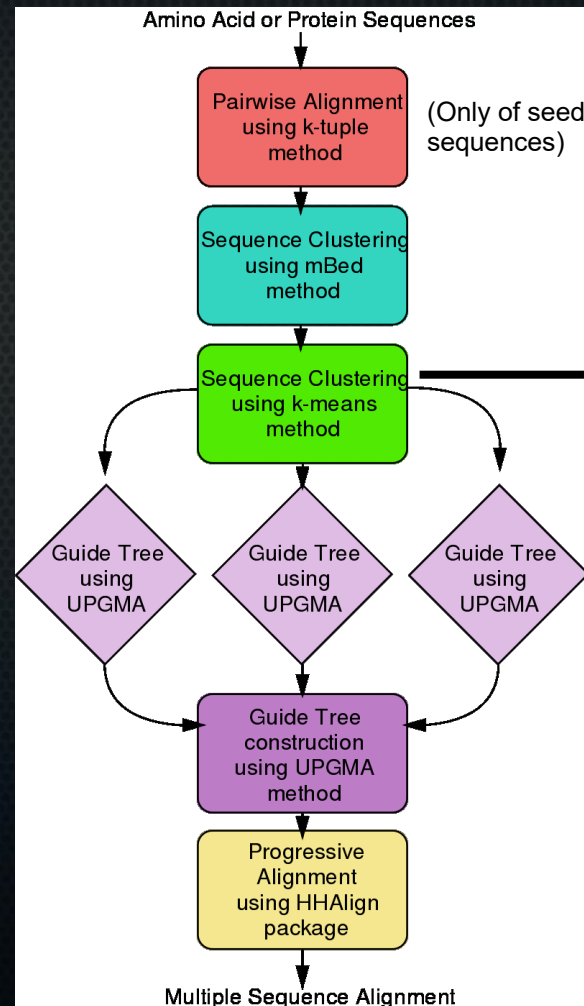
- Actually aligners use more short-cuts and tricks for large alignments.



Source:
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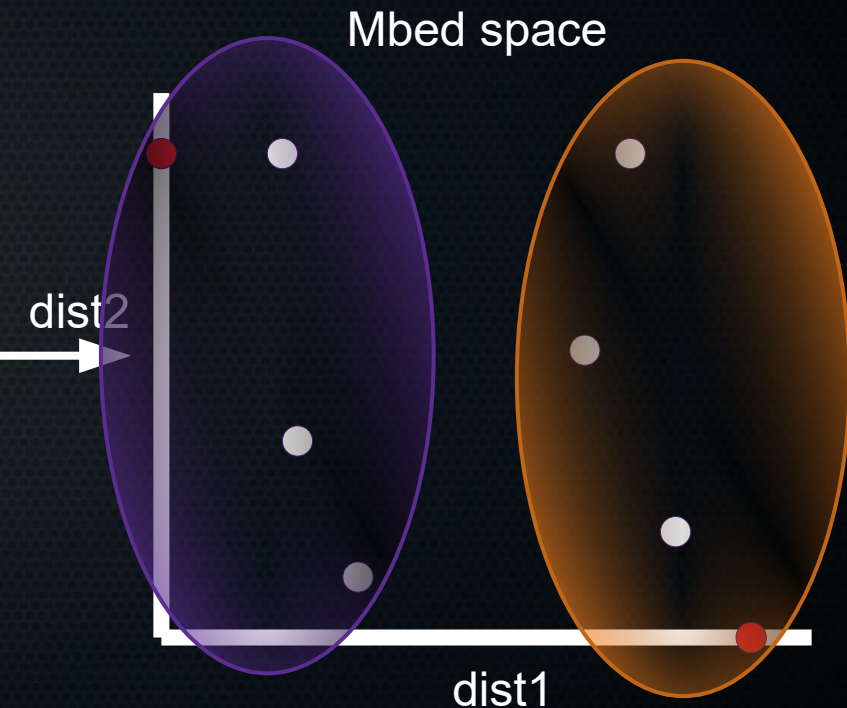
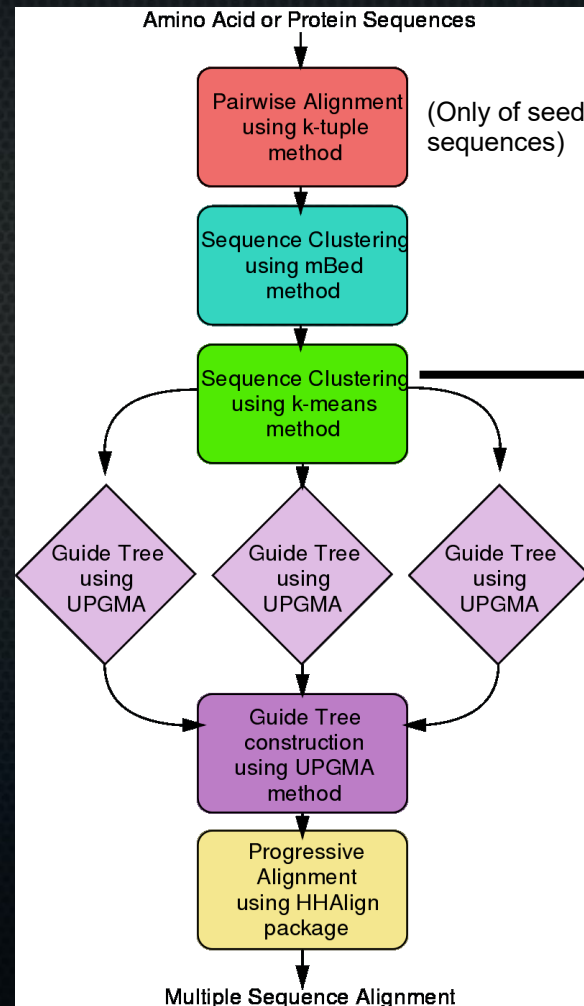
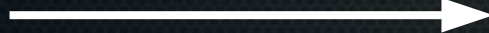
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Progressive alignment of many sequences

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Make separate guide trees per cluster for large datasets, connect them later

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So are we done?

- Well, no:
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 - separate opening and extending costs.
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 - ~~What about aligning thousands or hundreds of thousands of sequences? Does progressive alignment work fine?~~
 - No, need to use embedding to reduce distance calculations
 - What about current best practice?

Current best practice

- Two parts:
 - How do we get the MSA?
 - How do we make the tree once we have the MSA?

Current best practice: making the MSA

- Progressive alignment used often (Clustal, Muscle)
- Alternative: consistency-based methods (T-Coffee, ProbCons)
 - Do pairwise alignment, but also keep track of not-quite optimal pairwise alignment
 - When making the total MSA, look back at these not-quite optimal pairwise alignments, and try to maximise total score of all sequences.

Current best practice: making the MSA

- Progressive alignment used often (Clustal, Muscle)
- Alternative: consistency-based methods (T-Coffee, ProbCons)
- Alternative 2: statistical/Bayesian approaches (Bali-Phy, StatAlign)
 - assume complete evolutionary models (i.e. how often deletions occur, base changes, etc.), and fit tree and MSA simultaneously → most sound, but computationally very expensive.

Current best practice

- Two parts:
 - ~~How do we get the MSA?~~
 - How do we make the tree once we have the MSA?

Current best practice: making the tree

- Most-used: Bayesian (MrBayes, RevBayes) and Maximum Likelihood (IQ-TREE, RAxML) approaches.
→ won't go into this here, but safe to say that there's a lot more sophistication when going from MSA to phylogenetic tree!

Current best practice

- Two parts:
 - ~~How do we get the MSA?~~
 - ~~How do we make the tree once we have the MSA?~~

So are we done?

- Well, yes!



