Multiple sequence alignments
similarity without sequence similarity

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angewandte Sequenzen
Sommersemester 2018

Bis jetzt

- Man hat eine Sequenz (Protein oder Nukleotid)
- Man will so viel wie möglich finden, um
  - Struktur vorherzusagen
  - Funktion vorherzusagen
- Jetzt Alignments, Evolution & Funktion

Multiple alignments

- mostly for proteins

- what does a set of sequences look like ?

haemoglobin as example

- summarise this data

```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALEKMFLSFPTTKTYFPHFDLSHGSAQVKGHG
 LSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGDYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTHVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEAWERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEAWERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSAADKTNVKAAWSKVGGHAGEYGAEALEMFLGFPTTKTYFPHFDLSHGSAQVKAHG
VLSAADKTNVKAAWSKVGGHAGEYGAEALEMFLGFPTTKTYFPHFDLSHGSAQVKAHG
VLSADDKANIKAAWGKIGGHGAEYGAEALERMFCSFPTTKTYFPHFDVSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEAFERMFLSFPTTKTYFPHFDLSHGSAQVKGQG
VLSPADKTNVKAAWGKVGAHAGEYGAEAFERMFLSFPTTKTYFPHFDLSHGSAQVKGQA
VLSAADKSNVKAAWGKVGGNAGAYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKSNVKATWDKIGSHAGEYGGEALERTFASFPTTKTYFPHFDLSPGSAQVKAHG
VLSPADKSNVKAAWGKVGGHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTGTYFPHFDLSHGSAQVKGHG
VLSSADKNNVKACWGKIGSHAGEYGAEALERTFCSFPTTKTYFPHFDLSHGSAQVQAHG
VLSAADKSNVKAAWGKVGGNAGAYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VLSANDKSNVKAAWGKVGNHAPEYGAEALERMFLSFPTTKTYFPHFDLSHGSSQVKAHG
VLSPADKSNVKAAWGKVGGHAGDYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
        ...        ...          ... ...
```

Conservation / variability

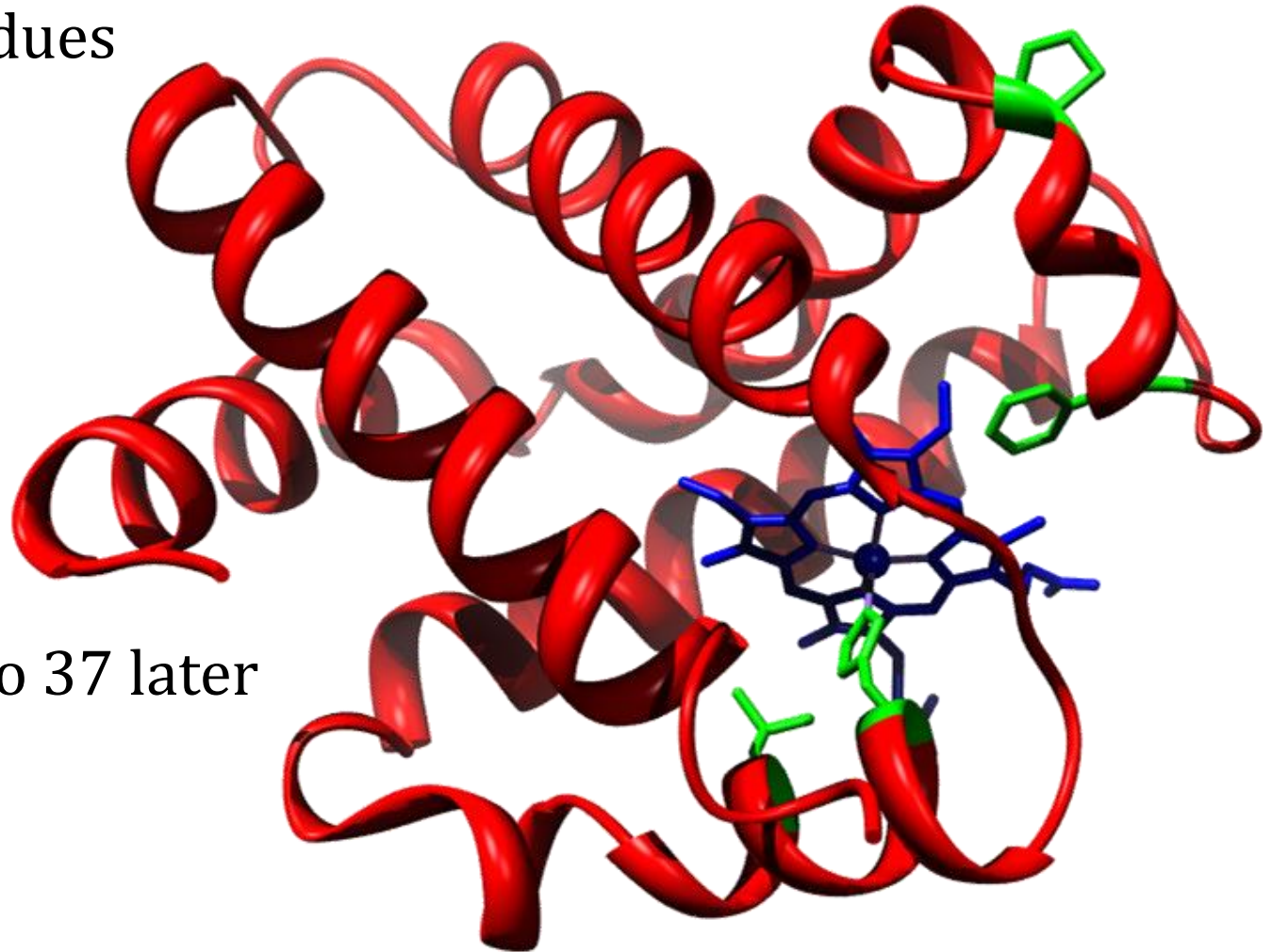Look at residues 37, 43, 83 and 87
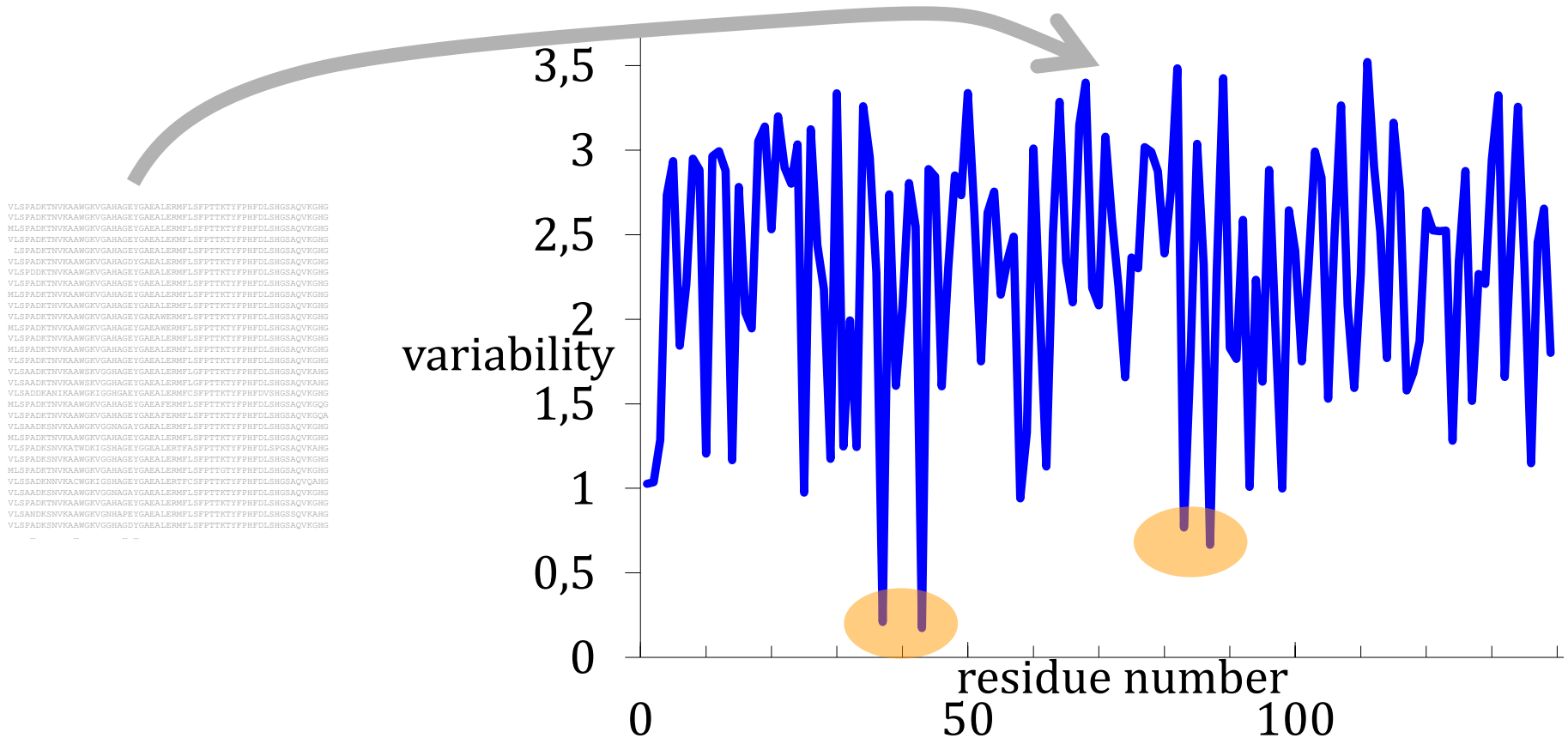
- how do we get these and what does it mean ?
- what does it mean for this protein ?

Conserved residues

Proximity to haem group

- green residues

- more on pro 37 later

Beliefs in multiple sequence alignments

Similar proteins found in many organisms
* where they are conserved - connected with function
* variation reflects evolution (phylogeny)

How many homologues might you have ?
* many
  * some DNA replication proteins – almost every form of life
  * profilin – cell mobility – bacteria, mammals, plants
  * ..
* few
  * exotic viral proteins
  * messengers exclusively in human biochemistry
  * ...

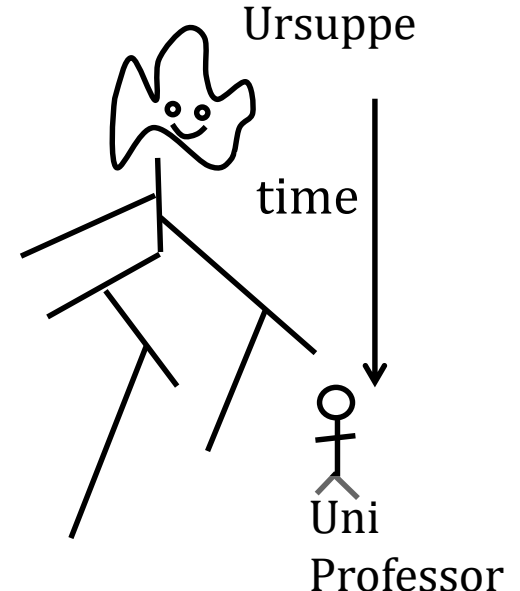# Trees / Phylogeny

Multiple sequence alignments are fun
- conservation, function…
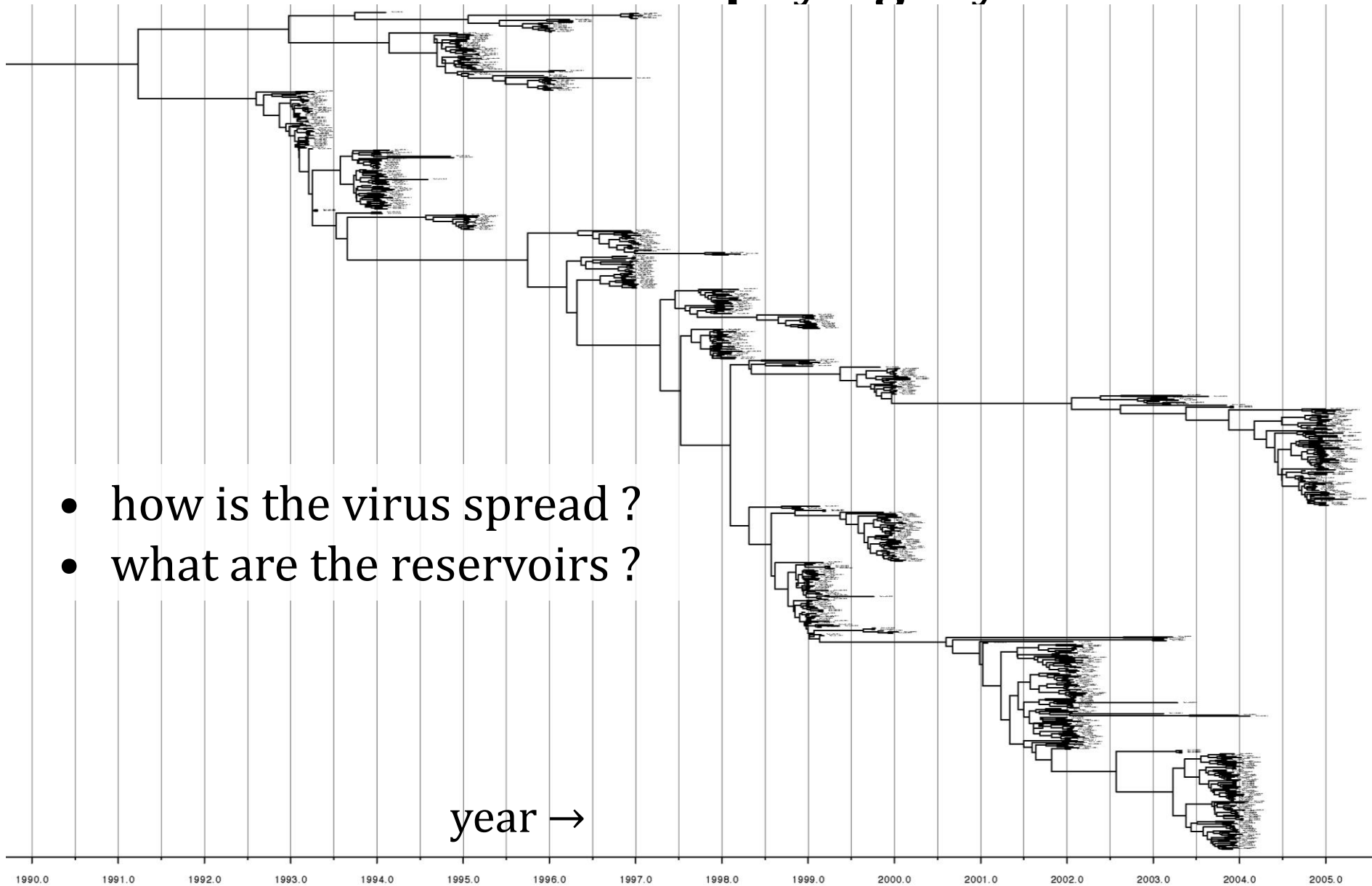
What next ? Phylogeny - making trees
- Need multiple sequence alignments to make trees
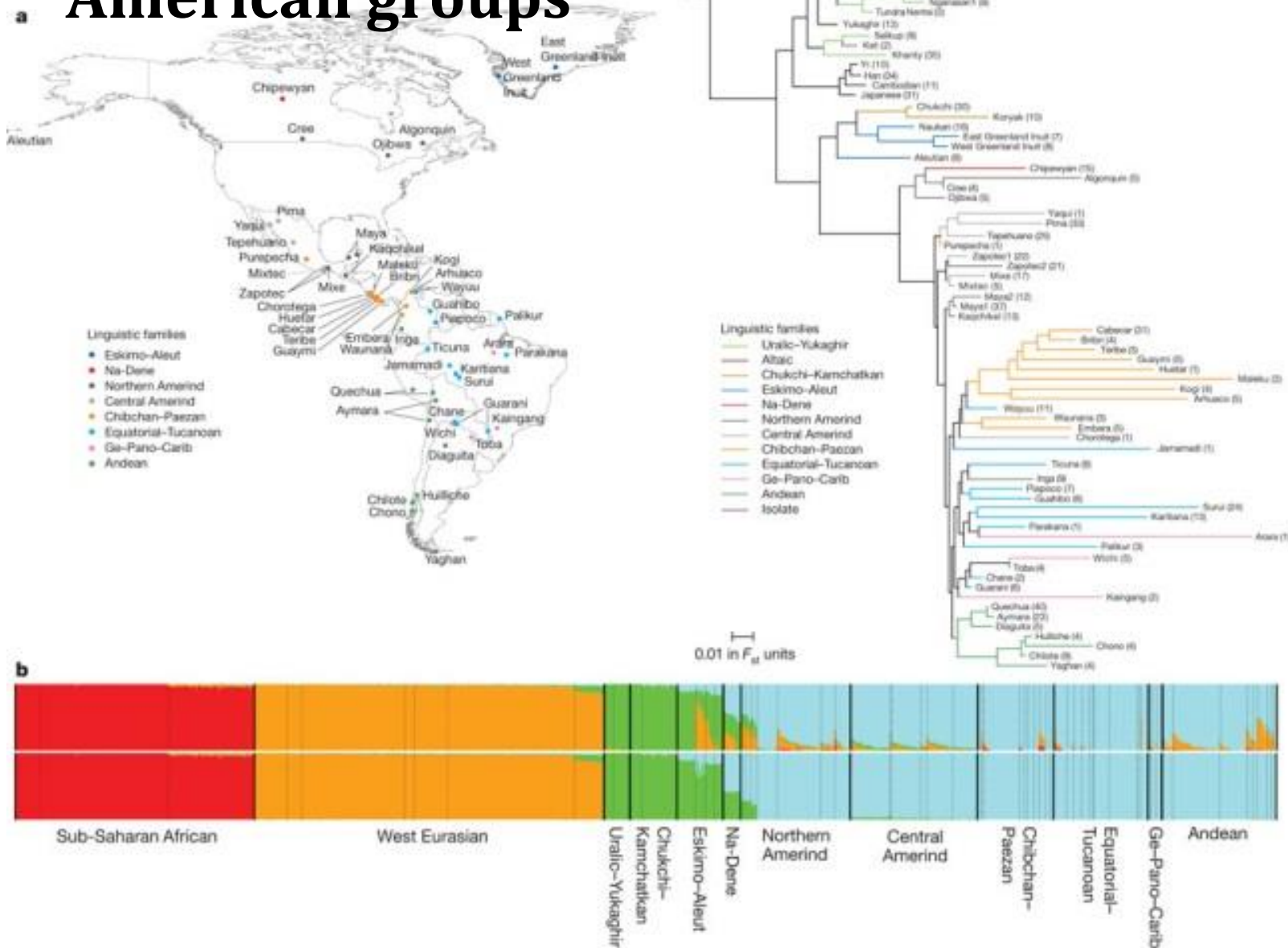
Do you just want the tree of life ?
- who killed the bananas ?
- where does influenza come from ?
- lassa, swine flu, ebola
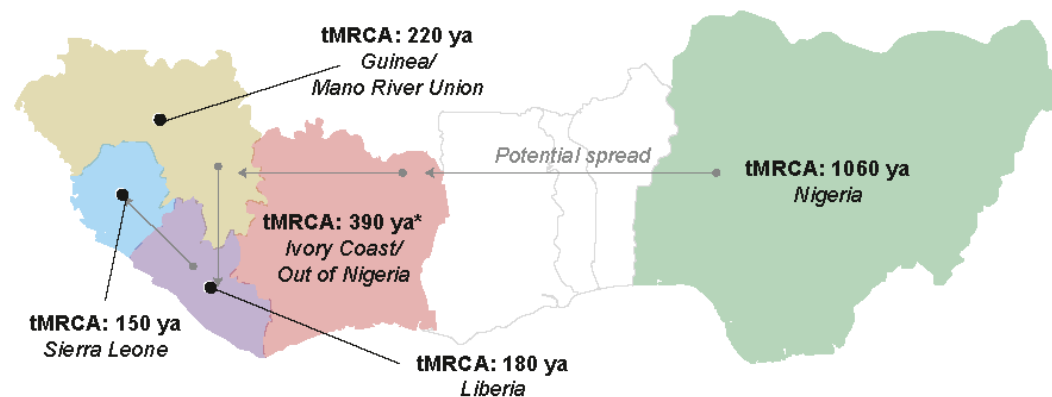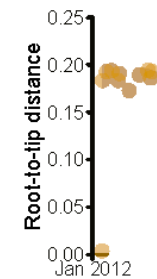- who killed the ladies ?

Ursuppe

time

Uni
Professor

Influenza virus phylogeny

- how is the virus spread ?
- what are the reservoirs ?

year →

1990.0   1991.0   1992.0   1993.0   1994.0   1995.0   1996.0   1997.0   1998.0   1999.0   2000.0   2001.0   2002.0   2003.0   2004.0   2005.0

Rambaut, A., .. Holmes, C. The genomic.. influenza A virus, Nature 452, 1-6, 2008

52 Native American groups

Reich, D., …Ruiz-Linares, A., Nature, 488, 370 (2012), Reconstructing Native American .. History

lassa virus

Andersen, KG... Sabeti, PC, Cell, 162, 738-750 (2015), "Clinical sequencing .. lassa virus"
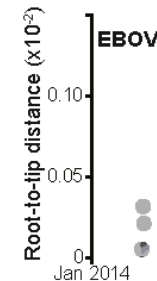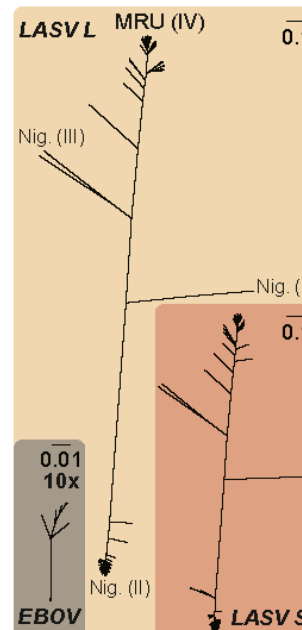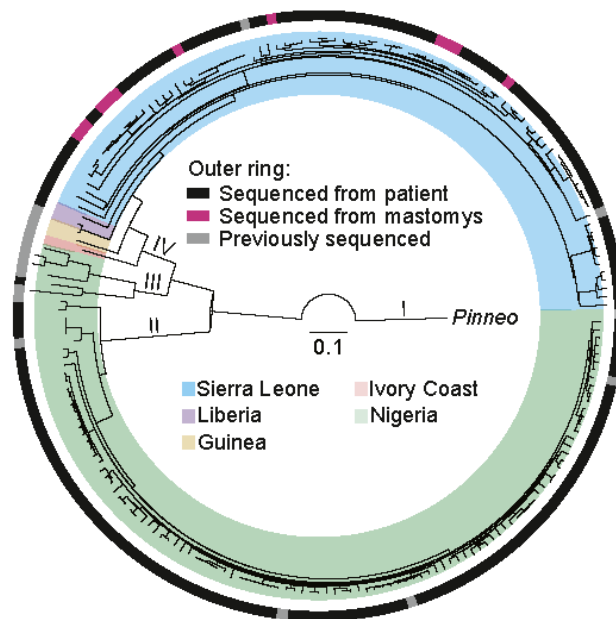
Ebola

How did the virus spread ?

Did national borders help ?

Was there just one jump from animals to people ?

Locations of lineages

How did the virus spread ?

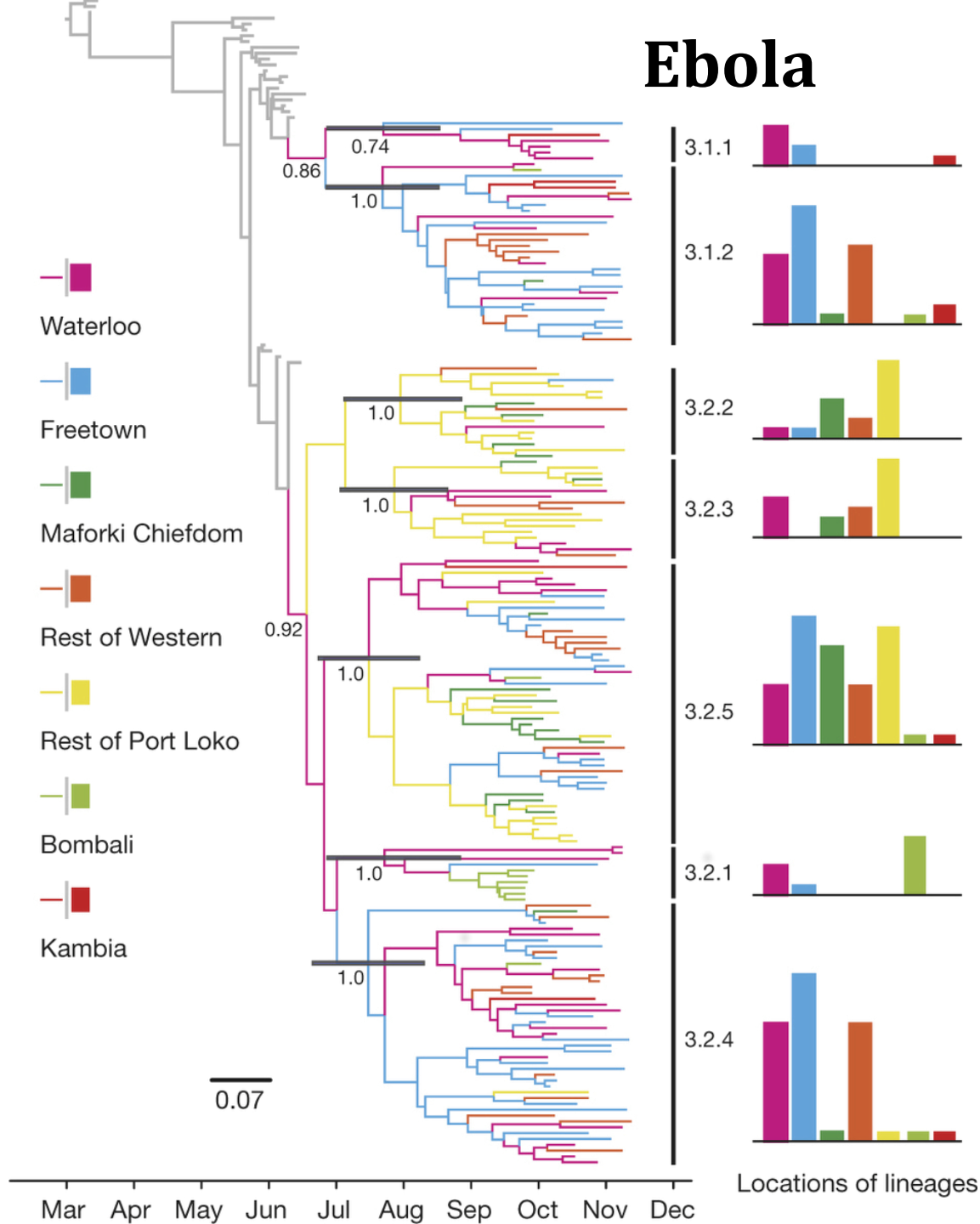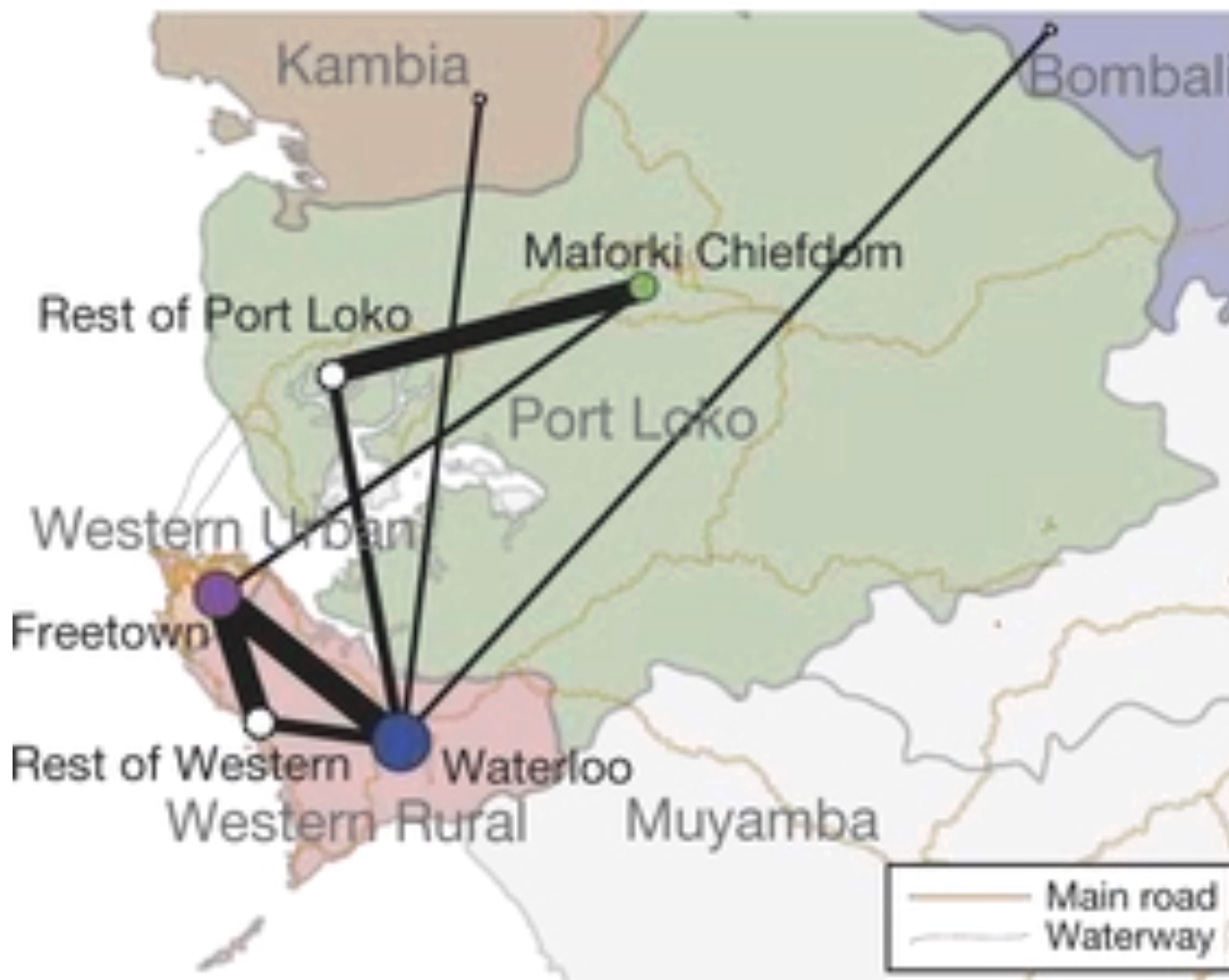Thickness of lines – closeness of sequences

Staphylococcus aureus

countries
- United Kingdom
- Ireland
- Germany
- Czech Republic
- New Zealand
- Nigeria
- Sudan
- Portugal
- Australia
- Hungary
- Sweden
- Denmark
- Singapore
- Namibia

fluoroquinolone resistance

methicillin resistance
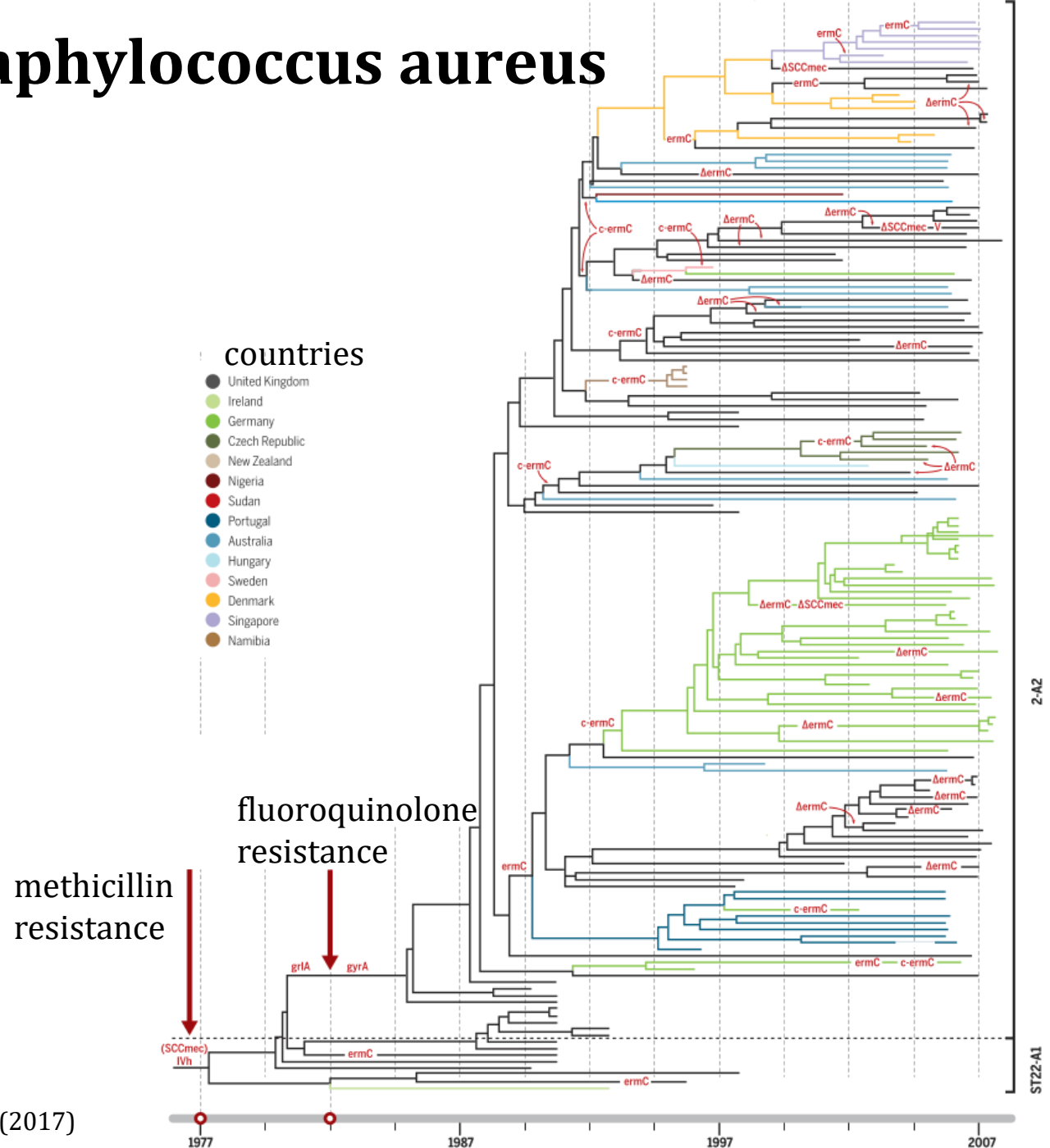
Baker, S., Thomson, N., Weill, F-X, Holt, K.E. (2017)
Science 360, 733-738

1000 acts of sex

"the defendant intended to inflict "great bodily harm"…
Between 1999 and 2004, he engaged in more than 1000 oral,
vaginal, and anal acts of unprotected sex with his female
partners"

black: normal population
colours: associated with
Herr WA04

Scaduto, D.I., Brown, JM, Haaland, WC, Zwicki, DJ, Hillis, DM, Metzker, ML, (2010) Proc Natl Acad Sci USA, 107, 21242
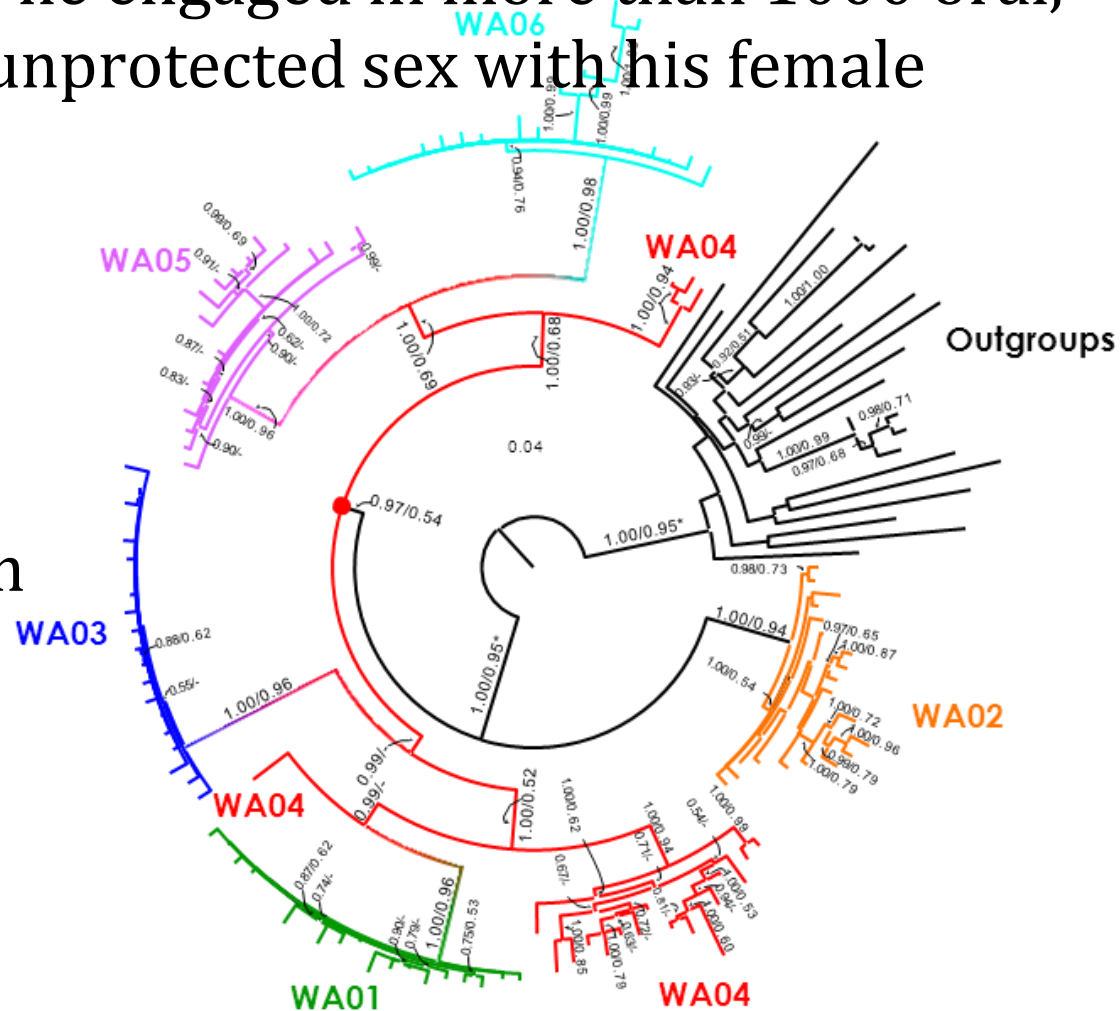
The plan

- optimise and alignment and tree simultaneously

Many sequences - rigorous alignment

- two sequence alignment
  - optimal path through $n \times m$ matrix
- three sequence alignment
  - optimal path through $n \times m \times p$ matrix
- four sequence alignment
  - ...
- m sequence alignment of n residues.... $O(n^m)$

Excuse to use lots of approximations

- no guarantee of perfect answer

Reasonable starting point

- begin with pairs of proteins

Scoring schemes

$$S_{a,b} = \sum_{i=1}^{N_{res}} \text{match}(s_{a,i}, s_{b,i})$$

In pairwise problem

```
VLSPADKSNVKAGWGQVGAHAGDYGAEAIERMYLSFPSTKTYFPHTDISHGSAQVKGHG
MLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
```

- Sum over match()
  N_{res} is sequence length
- $\text{match}(s_{a,i}, s_{b,i})$ is the match/mismatch score of sequence a and b at position i
- invent a distance between two sequences like

$$d_{a,b} = \frac{1}{S_{a,b}}$$

- distance measure..
  which sequences are most dissimilar to each other

Scoring schemes for a multiple alignment

In the best alignment

```
1 VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
2 VITP-EQSNVKAAWGKVGAHAGEYGAEALEQMFLSYPTTKTYFP-FDLSHGSAQIKGHG
3 MLSPGDKTQVQAGFGRVGAHAG--GAEALDRMFLSFPTTKSFFPYFELTHGSAQVKGHG
4 VLSPAEKTNIKAAWGKVGAHAGEYGAEALEKMF-SYPSTKTYFPHFDISHATAQ-KGHG
5 -VTPGDKTNLQAGW-KIGAHAGEYGAEALDRMFLSFPTTK-YFPHYNLSHGSAQVKGHG
6 VLSPAEKTNVKAAWGRVGAHAGDYGAEALERMFLSFPSTQTYFPHFDLS-GSAQVQAHA
7 VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
```

- 1 is aligned to 2, 3, ..
- 2 to 3,4, ...

- then I move 5 and 2 & 5 and 3 – messes up 2 and 3

Mission: for N_{seq} sequences
- $S_{a,b}$: alignment score sequences a and b

$$score = \sum_{b \neq a}^{N_{seq}} \sum_{a=1}^{N_{seq}} S_{a,b}$$

- not quite possible
  - this method is just an approximation

Aligning average sequences

```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VITPAEKTNVKAAWGKVGAHAGEYGAEALEQMFLSYPTTKTYFPHFDLSHGSAQIKGHG
```

and

```
IITPGDKTNVKAAFGKVGAHGGEYGAEALDRMFISFPSTKTYYPHFDLSHASAQVKAHG
VITPAEQTNIKGAWGQIGAHAGDYAADALEQMFLSYPTSKTYFPYFDLTHGSAQIKGHG
VITPAEKTQVKAAWGKVGGHAGEYGAEAIEQMFLTYPTTQTYFPHFELSHGTAQIKGHG
```

At each position

- use some kind of average in scoring
- if a column has 2×D and 1×E score
  - score as 2/3 D + 1/3 E
- later.. call the average of S1 and S2: av(S1, S2)

Summarise ingredients

- pairwise scores + distances
- ability to align little groups of sequences

Progressive alignments

Guide tree / progressive / neighbour joining method

Steps
- build a distance matrix
- build a guide tree
- build up overall alignment in pieces
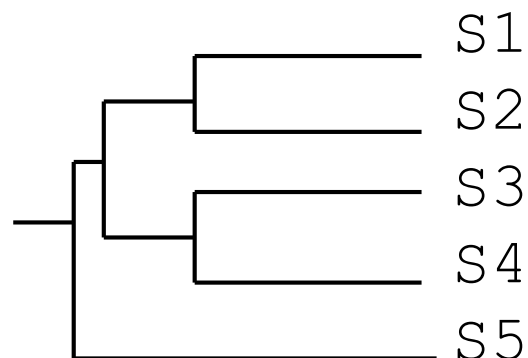
Progressive alignment - tree

S1	ATCTCGAGA
S2	ATCCGAGA
S3	ATGTCGACGA
S4	ATGTCGACAGA
S5	ATTCAACGA

Compute pairwise alignments, calculate the distance matrix

	S1	S2	S3	S4	S5
S1	–				
S2	.11	–			
S3	.20	.30	–		
S4	.27	.36	.09	–	
S5	.30	.33	.23	.27	–

calculate guide tree

S1
S2
S3
S4
S5

Multiple alignment from guide tree

- gaps at early stages remain

Problems..

  - S1/S2 and S3/S4 good
  - no guarantee of S1/S4 or S2/S3

- av(S1,S2) is average of S1 and S2

```
align S1 with S2
S1      ATCTCGAGA
S2      ATC-CGAGA

align S3 with S4
S3      ATGTCGAC-GA
S4      ATGTCGACAGA

align av(S1,S2) with av(S3,S4)
S1      ATCTCGA--GA
S2      ATC-CGA--GA
S3      ATGTCGAC-GA
S4      ATGTCGACAGA

align av(S1,S2,S3,S4) with S5
S1      ATCTCGA--GA
S2      ATC-CGA--GA
S3      ATGTCGAC-GA
S4      ATGTCGACAGA
S5      AT-TCAAC-GA
```
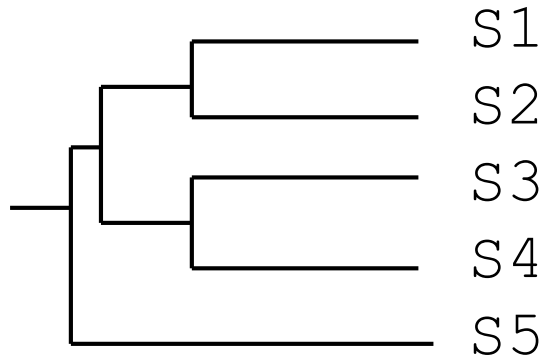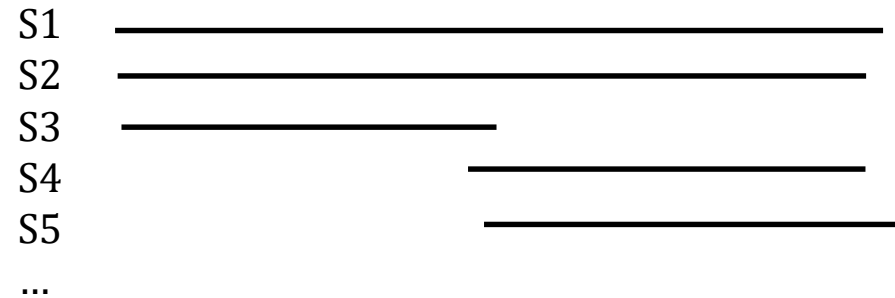
Problems and variations

	S1	S2	S3	S4	S5
S1	–				
S2	.11	–			
S3	.20	.30	–		
S4	.27	.36	.09	–	
S5	.30	.33	.23	.27	–

What order should we join ?

- pairs are easy (S1+S2) and (S3+S4)
- which next ?

Real breakdown

S1 and S2 are multi-domain proteins

- S3 is not really related to S4 or S5
- distance matrix elements are rubbish

Given an alignment

How reliable / believable ?

- set of very related proteins (an enzyme from 100 mammals)
  - no problem
- diverse proteins (an enzyme from bacteria to man)
  - lots of little errors
- can break completely (domain example)

Is the tree a "phylogeny" ? A reflection of evolution ?

- more later

Measuring conservation / entropy

Entropy

- how much disorder do I have ? $S = -k_b \sum_{i=1}^{N_{states}} p_i \ln p_i$
- in how many states may I find the system ?

Our question

- look at a column – how much disorder is there ?

```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VITP-EQSNVKAAWGKVGAHAGEYGAEAIEQMFLSYPTTKTYFP-FDLSHGSAQIKGHG
MLSPGDKTQVQAGFGRVGAHAG--GAEAVDRMFLSFPTTKSFFPYFELTHGSAQVKGHG
VLSPAEKTNIKAAWGKVGAHAGEYGAEAAEKMF-SYPSTKTYFPHFDISHATAQ-KGHG
-VTPGDKTNLQAGW-KIGAHAGEYGAEALDRMFLSFPTTK-YFPHYNLSHGSAQVKGHG
VLSPAEKTNVKAAWGRVGAHAGDYGAEAGERMFLSFPSTQTYFPHFDLS-GSAQVQAHA
VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
```

           no   much
        disorder   disorder

Calculate an "entropy" for each column

Entropy

- forget k_b (Boltzmann – just scaling)

We have a protein

- 20 possible states.. use log base 20

$$S = -\sum_{i=1}^{N_{states}} p_i \log_{20} p_i$$

If a residue is always conserved ? $p_i = 1$ or $p_i = 0$

$$S = \log_{20} 1 = 0 \quad \text{(no entropy)}$$

What if all residues are equally likely ? $p_i = \frac{1}{20}$

$$S = -\sum_{i=1}^{20} \frac{1}{20} \log_{20} \frac{1}{20} = -20 \cdot \frac{1}{20} \log_{20} \frac{1}{20} = -20 \cdot \frac{1}{20}(-1)$$

$$= 1$$

- my toy alignment…

Entropy

- First column is boring
- Second

$$p_\text{D} = {}^5/_7$$

$$p_\text{E} = {}^1/_7$$

$$p_\text{N} = {}^1/_7$$

```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VITP-EQSNVKAAWGKVGAHAGEYGAEAIEQMFLSYPTTKTYFP-FDLSHGSAQIKGHG
MLSPGDKTQVQAGFGRVGAHAG--GAEAVDRMFLSFPTTKSFFPYFELTHGSAQVKGHG
VLSPAEKTNIKAAWGKVGAHAGEYGAEAAEKMF-SYPSTKTYFPHFDISHATAQ-KGHG
-VTPGDKTNLQAGW-KIGAHAGEYGAEALDRMFLSFPTTK-YFPHYNLSHGSAQVKGHG
VLSPAEKTNVKAAWGRVGAHAGDYGAEAGERMFLSFPSTQTYFPHFDIS-GSAQVQAHA
VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDISHGSAQVKGHG
```

$$S = -\left(\frac{5}{7}\log_{20}\frac{5}{7} + \frac{1}{7}\log_{20}\frac{1}{7} + \frac{1}{7}\log_{20}\frac{1}{7}\right)$$

$$\approx 0.27$$

Entropy from DNA

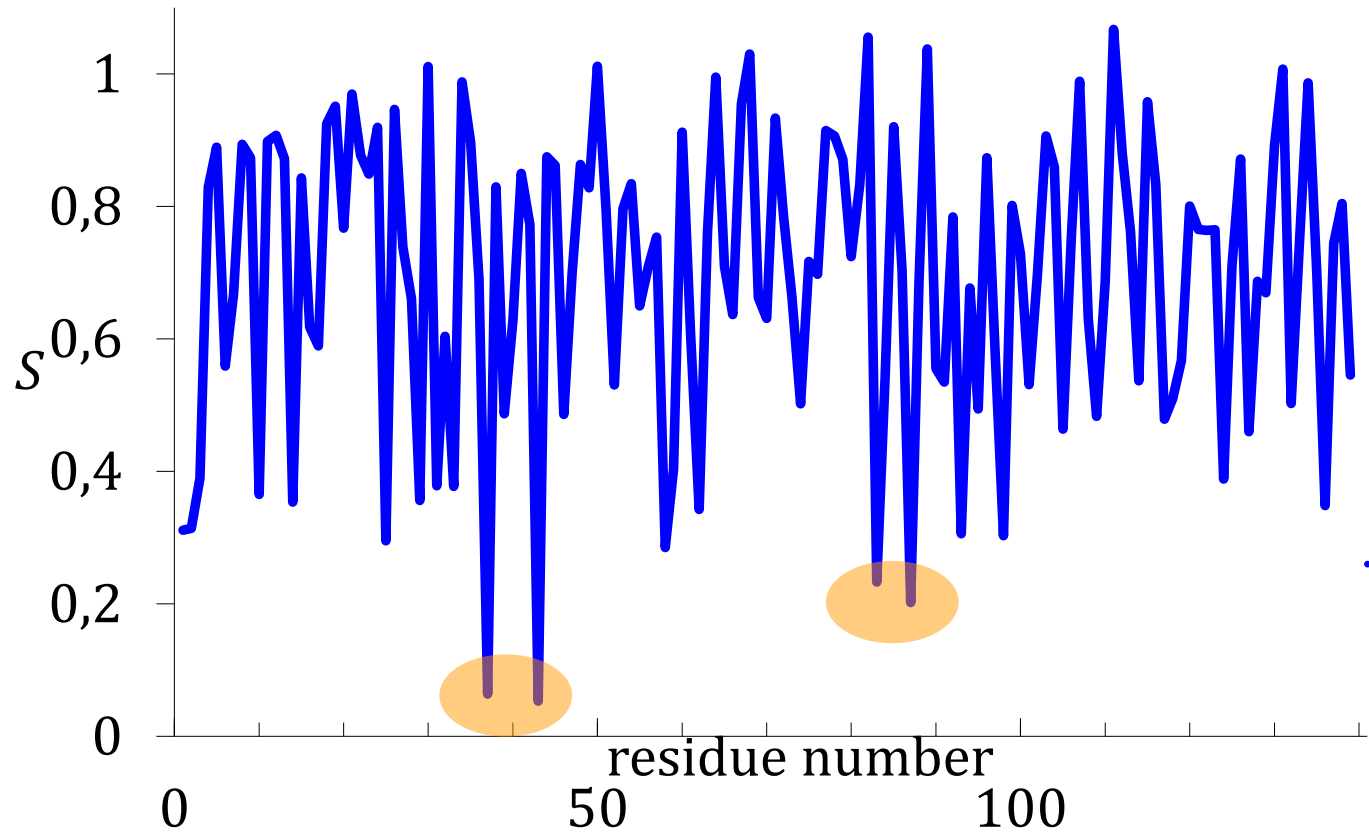Exactly as for proteins (use $p_i \log_4 p_i$)

max possible entropy

$$S = -4\left(\frac{1}{4}\log_4\frac{1}{4}\right)$$

$$= -4\left(\frac{1}{4}\cdot(-1)\right)$$

$$= 1$$

example from start of this topic

Haemoglobin conservation

Look at residues 37, 43, 83 and 87

4 residues (maybe more) stand out as conserved
- why ?

Conserved residues in haemoglobin
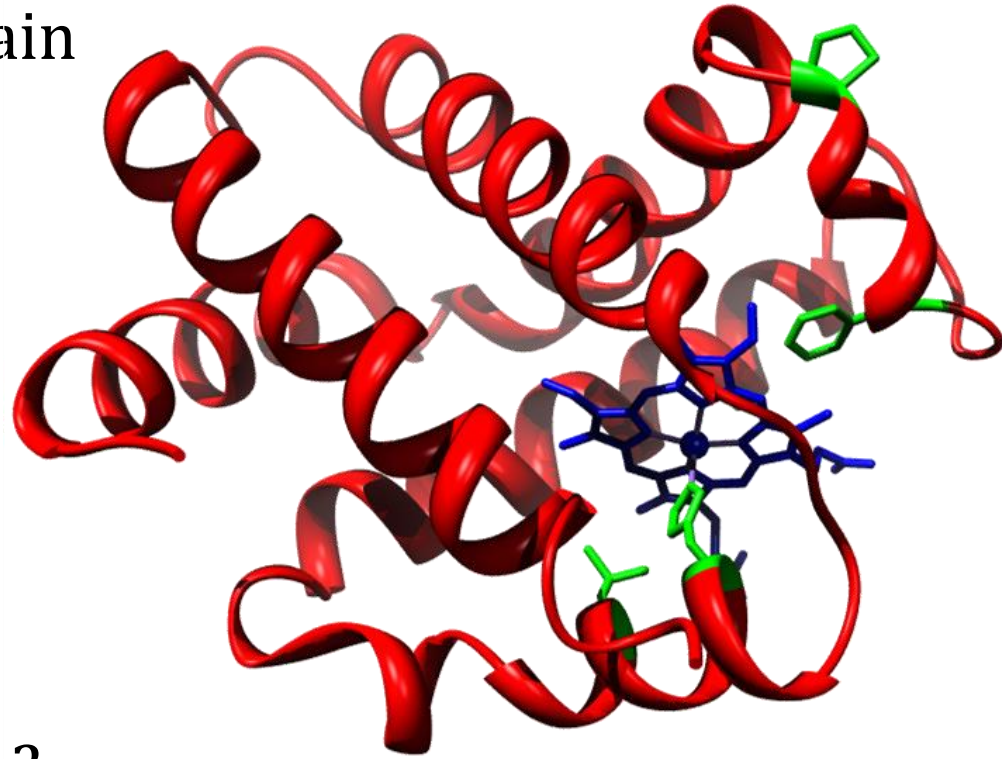
3 of the sites are easy to explain
- interact with haem group

Look at fourth site
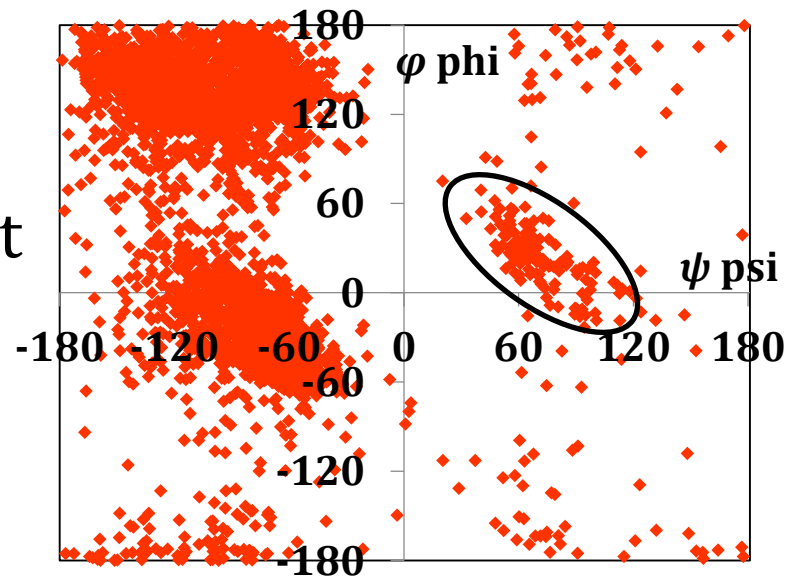- proline
- end of a helix

What is special about proline ?
- no H-bond donor
- here – if it mutates, haemoglobin does not fold

Conservation for structure

Some residues have very special structural roles

- proline – not an H-bond donor
  - often end of a helix
- glycine – can visit part of $\varphi\ \psi$ plot
  - found in some turns

Are all gly residues so important ?

- NO – they occur in many places sometimes in turns

Are all pro residues very conserved ? No

Conservation for function

In a serine protease
- always a "catalytic serine"
- can it mutate ? Not often

In haemoglobin – residues necessary for binding haem
- can they mutate ? rarely
- changes properties of haemoglobin (bad news)

Dogma
- residues in active site will be more conserved than other sites

Important summary

Conservation may reflect

- important function
- structural role

Mutagenesis / chemistry

- what residue may I change to allow binding to a solid substrate ? (for biosensor/immobilized enzyme ?)
- try error prone PCR to select for new enzyme activity – which sites might I start with (active site) ?

Drug design example

- target is an essential protein (basic metabolism, DNA synthesis, protein synthesis..)
- is there some set of sequence features common to pathogen, different to mammalian protein ?

Evolution – do not trust conservation

Imagine: two possible systems for some important enzyme
1. active site fits to essential biochemistry
   - any mutation – you lose
   - active site residues are conserved in a conservation plot
2. maybe enzyme is not absolutely perfect
   - some mutations kill you
   - some mutations OK
   - site does not appear perfectly conserved

Where would you evolve to ?

   1. very fragile
   2. likely to survive mutations

Resistance to mutations…
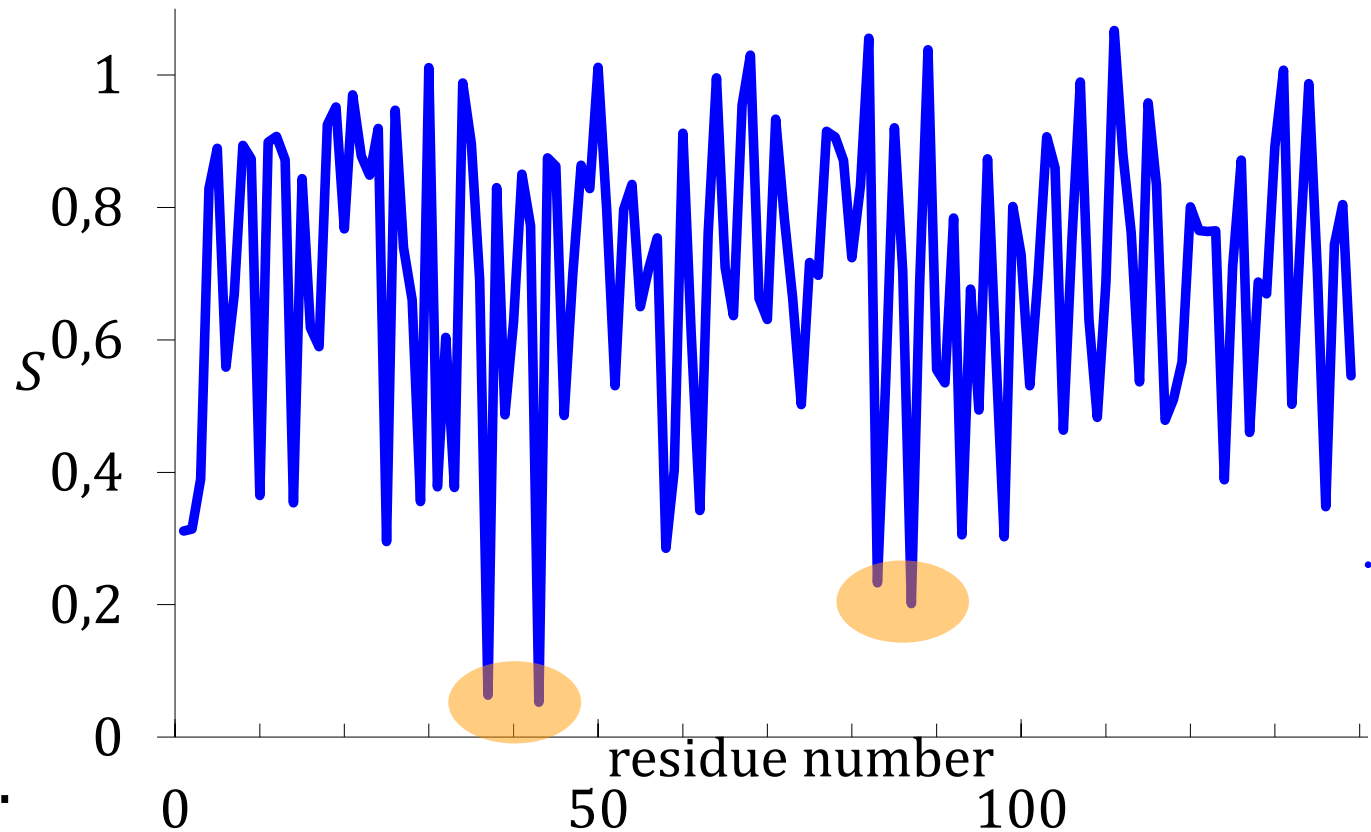
Tolerance of mutations

Boring answer
- some amino acids are similar to each other

Better answer – if your protein can tolerate mutations
- your genes have a better chance of being passed on
- will be selected for
- it is a Darwinian trait

Conservation – how meaningful ?

Earlier Folien…

- values from 0 to 1
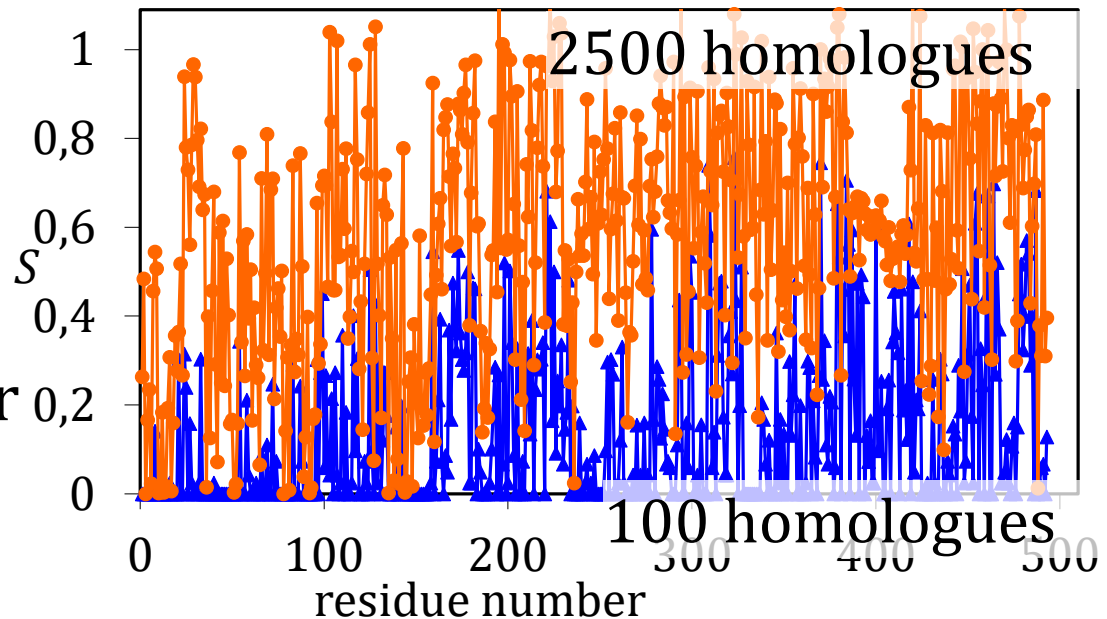
What if I used more homologues ?

Conservation – how meaningful ?

Example sequence (DNA gyrase)

- find 100 close homologues (mostly > 80% similarity)
  – calculate conservation

- find 2500 close homologues (mostly > 50 % similarity)
  calculate conservation

Fewer sequences

- lots of conserved sites

- you can get the answer you want

Consequences - summarise

Significance of conservation

You read in a paper – residue 37 is conserved
- how many sequences did they look at ?
  - 10 ? bad, 100 better, 1000 better

- choosing the number of sequences lets you manipulate results

- statistically
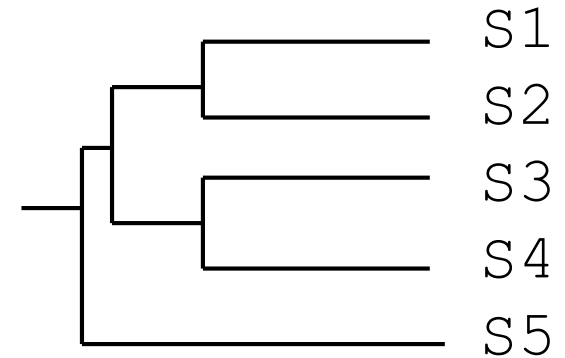 - have you sampled enough sequences ?

Phylogeny / Evolution

The trees in text books are almost never perfect

One rarely knows the correct history

Problems..

Previously we had a "guide tree"

- did (S1,S2) and (S3,S4) share an ancestor but not S5 ?
- branch lengths do not reflect evolutionary time
- there may be other similar trees which could be evolutionary paths

S1
S2
S3
S4
S5

Evolutionary time

Compare two DNA sequences see

      1 mutation (represents time t)

      2 mutations (time $2t$)

      3 mutations (time $3t$)…

No !

After some evolution

      `A` →`C` → `G`   two events (although looks like `A`→`G`)

      `A` →`C` → `G` →`C` → `A`   looks like zero mutations

If I have infinite time

- all bases / residues equally likely
- p_{mut} =3/4 = 0.75 (DNA) or p_{mut}=19/20 (protein)

Mutation probability

- time units are arbitrary
- how would I estimate time ? (for DNA)

- $t \propto -\ln\left(1 - \frac{4}{3}p_{mut}\right)$

- p_{mut} ? count $\frac{n_{mut}}{n_{res}}$

- work in relative time

$$p_{mut} = \frac{3}{4}\left(1 - e^{-ct}\right)$$
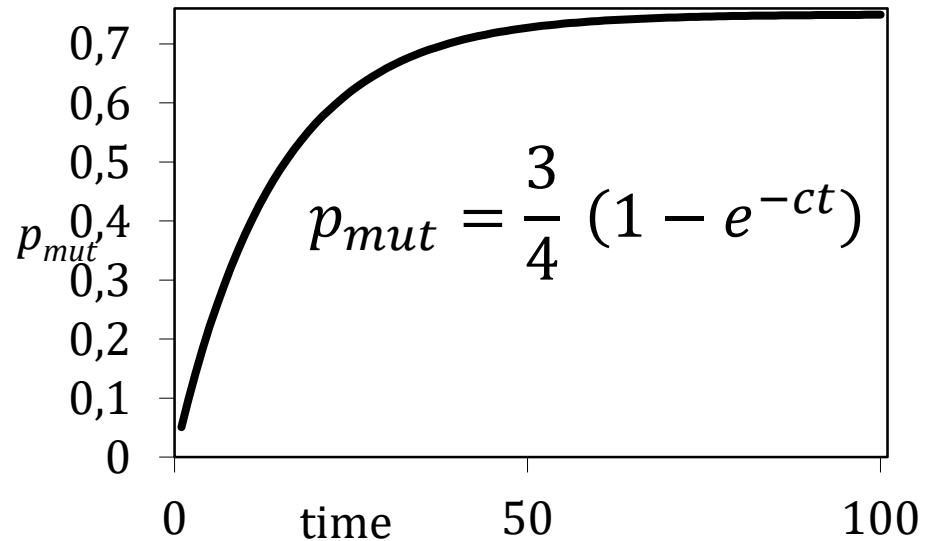
For short times, p_{mut} changes fast
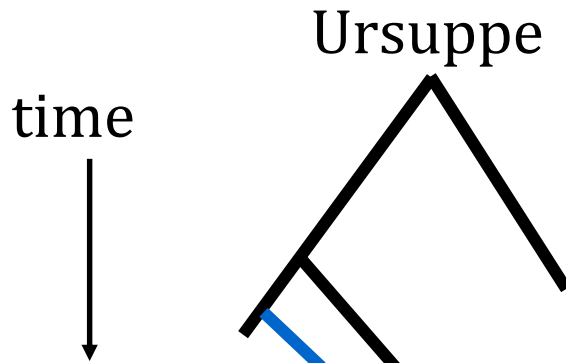- for small t, distances will be more reliable
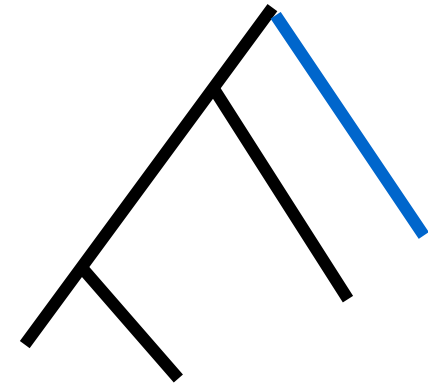 - as will be alignments
Is this enough for phylogeny ?
- what about reliability ?

Problems in phylogeny

- not all sites mutate equally quickly
- not all species mutate equally quickly

Ursuppe

time

but blue species (protein) mutates quickly

blue appears to have branched off earlier

Backwards mutations ?

- not really a problem (Klausurerfahrung)

Problems estimating time

1. mutation rates vary wildly
    - changing environments – pH, temperature,..

2. imagine time t is such that $p_{mut}= 0.25$
    - we have random events
    - sometimes you see 23% mutation, sometimes 28%

- time estimates will never be accurate
- maybe we cannot find the correct tree
    - can we roughly estimate reliability ?

Reliability

Think of first
alignment

```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VITP-EQSNVKAAWGKVGAHAGEYGAEAIEQMFLSYPTTKTYFP-FDLSHGSAQIKGHG
MLSPGDKTQVQAGFGRVGAHAG--GAEAVDRMFLSFPTTKSFFPYFELTHGSAQVKGHG
VLSPAEKTNIKAAWGKVGAHAGEYGAEAAEKMF-SYPSTKTYFPHFDISHATAQ-KGHG
-VTPGDKTNLQAGW-KIGAHAGEYGAEALDRMFLSFPTTK-YFPHYNLSHGSAQVKGHG
VLSPAEKTNVKAAWGRVGAHAGDYGAEAGERMFLSFPSTQTYFPHFDLS-GSAQVQAHA
VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
```

What would happen if you deleted a column ?

- if the data is robust /reliable
  - not much
- if the tree is very fragile /sensitive
  - tree will change

better…

Reliability

Repeat 10^2 to 10^3 times

- delete 5 to 10 % of columns
- copy random columns so as to have original size
- recalculate tree

How often did you see each branch ?


```
VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
VITP-EQSNVKAAWGKVGAHAGEYGAEAIEQMFLSYPTTKTYFP-FDLSHGSAQIKGHG
MLSPGDKTQVQAGFGRVGAHAG--GAEAVDRMFLSFPTTKSFFPYFELTHGSAQVKGHG
VLSPAEKTNIKAAWGKVGAHAGEYGAEAAEKMF-SYPSTKTYFPHFDISHATAQ-KGHG
-VTPGDKTNLQAGW-KIGAHAGEYGAEALDRMFLSFPTTK-YFPHYNLSHGSAQVKGHG
VLSPAEKTNVKAAWGRVGAHAGDYGAEAGERMFLSFPSTQTYFPHFDLS-GSAQVQAHA
VLSPDDKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG
```
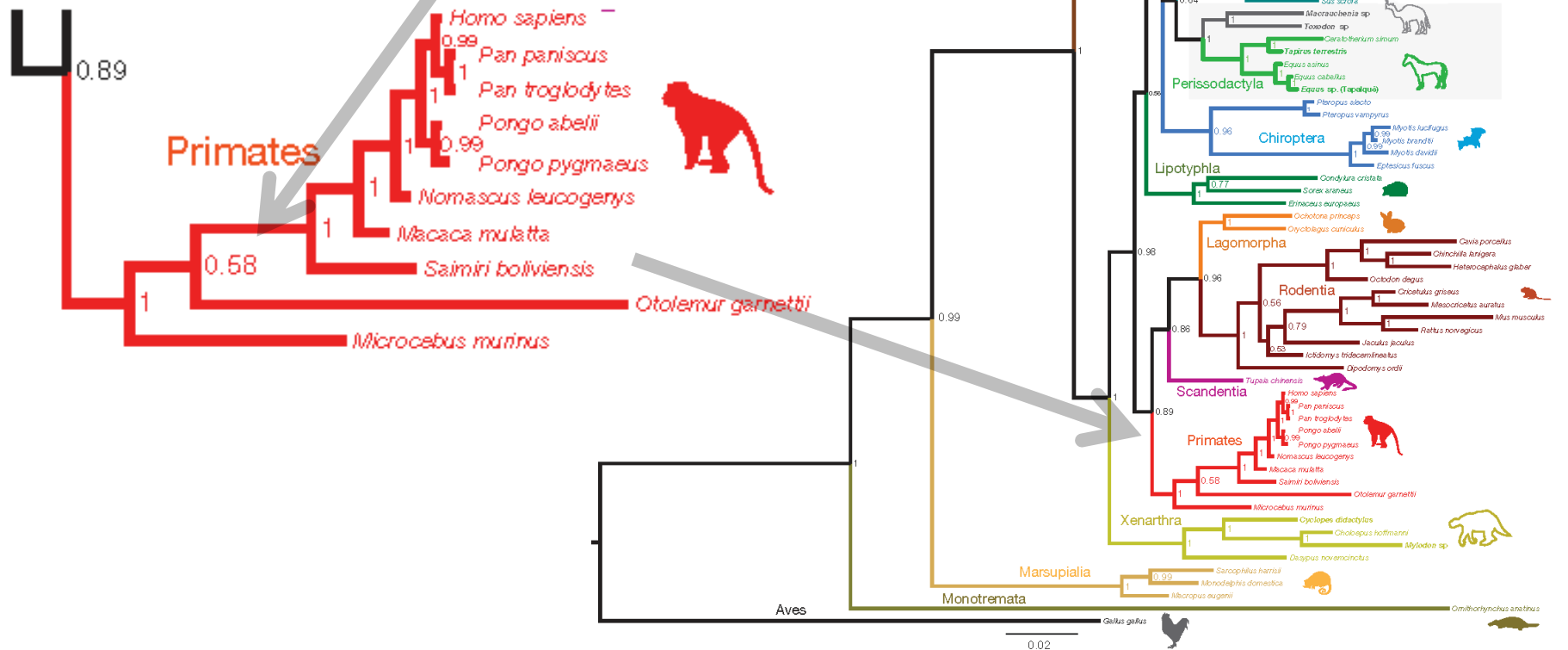
Monster example

- generate 1000 trees
- for each sub-tree
  - see how often it is present

- example from nature

Monster calculation

Took a long time

- look at this number

Welker, F., ... MacPhee, D.E. Nature, 522, 81-84 (2015) Ancient proteins resolve the evolutionary ...

DNA or protein sequences ?

The issues

- regulatory regions, RNA genes
- synonymous mutations (common – only seen in DNA)
- non-synonymous mutations (amino acid changes)
  - more information D⇌E, I⇌L⇌V, ..

Alignment reliability

- proteins
  - uses codon structure (implicitly)
  - better, amino acid similarity, I⇌L⇌V is not bad
- DNA
  - less information

DNA or protein sequences ?

	protein	DNA	time
synonymous changes	not seen	yes	short
a.a. changes	yes	yes	longer
a.a. similarity	accounted for	not seen	
frame shifts	not seen	yes	
non-coding regions	not helpful	yes	

Very short time or not protein-coding
- use DNA

Longer time and coding for protein
- use proteins

Summary

- multiple sequence alignment – conservation
  - find important residues (function or structure)
  - can quantify conservation
- relations between most similar proteins are most reliable
- best tree is never found
  - too difficult algorithmically
  - lots of errors – evolution is a random process
- rough idea of reliability
- quick tree – possible for 1000s of sequences
- more complicated methods – phylogeny in Biologie courses