ALIGNMENT

MY INTRODUCTION TO BIOLOGICAL SEQUENCE ALIGNMENT

About me

- Dr. Benjamin J. Buchfink, computer scientist and mathematician by training
- Started working on sequence alignment in 2012
- Developed the popular DIAMOND tool (12 citations/day) for protein alignment
- Mostly worked in academic research since then, at the University of Tübingen and the Max Planck institute for biology Tübingen
- Read more:
- Buchfink et al., Nature Methods 2014
- Buchfink et al., Nature Methods 2021
- Buchfink et al., Nature Methods 2026 (to appear)

Questions to the audience

1. Have you ever **run** a sequence alignment software?
2. Was it **directly** or as part of a pipeline?
3. Done **multiple** sequence alignment?
4. Know who/what **Smith-Waterman** is?
5. What is the difference between similarity and homology?
6. Why align? Variant calling, RNA-seq quantification, taxonomic classification, etc.
7. Slides adopted from Rayan Chikhi (last year)

Types of alignments

- Pairwise

meiotic recombination protein REC8 homolog [Lepidochelys kempii]

Sequence ID: XP_073164915.1 **Length:** 573 **Number of Matches:** 1

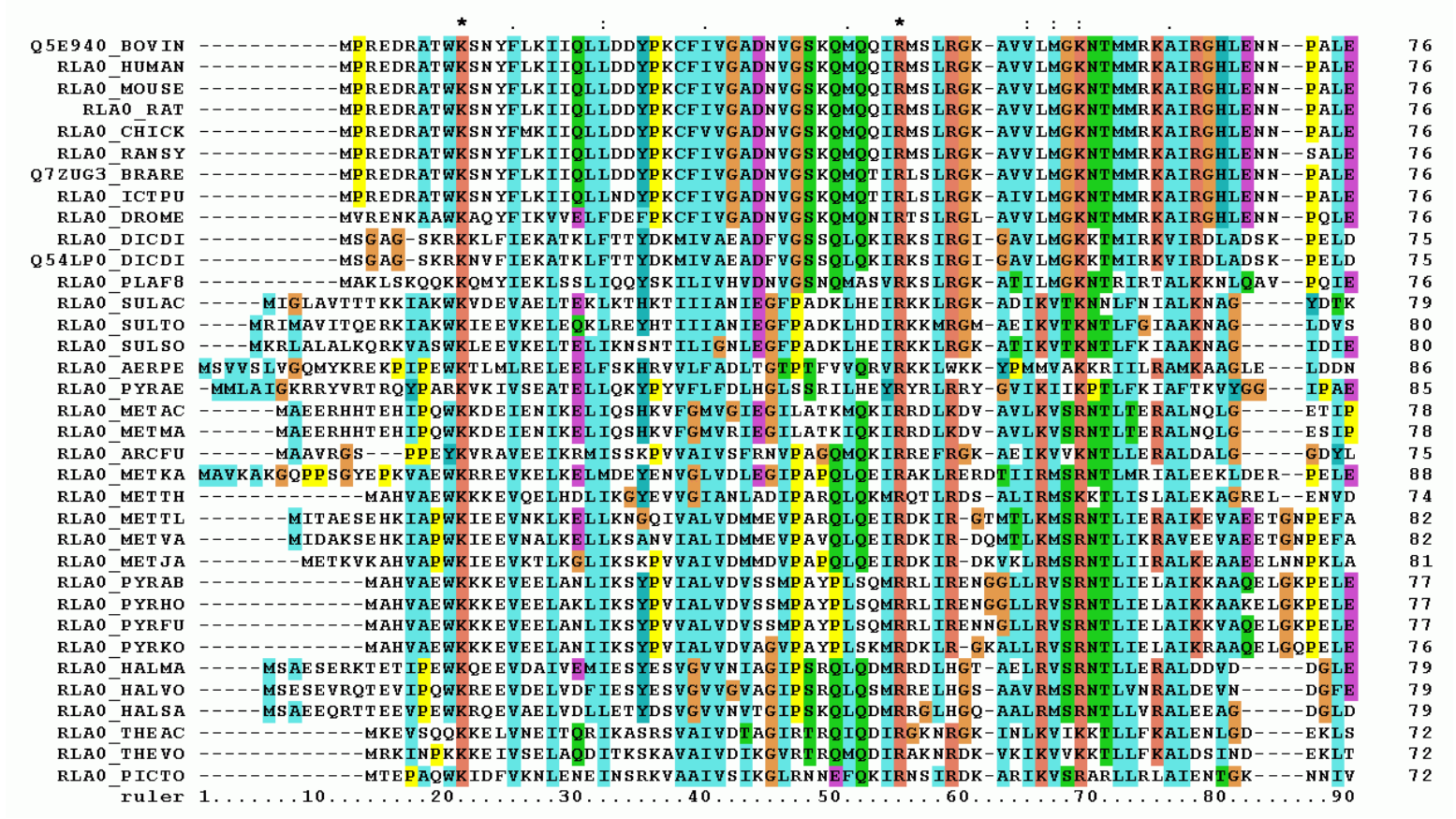
Range 1: 340 to 561 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
160 bits(405)	3e-47	Compositional matrix adjust.	115/241(48%)	145/241(60%)	22/241(9%)

```
Query  35   RRRLLFWDKETQISPEKFQEQ-LQTRAHCWECPMVQPPERTIRGPAELFRTPTLSGWLPP  93
            RR+L F D+ TQI  ++F++Q L  RA C    +V+   R  R PAEL R PT  GWLPP
Sbjct  340  RRQLRFLDEVTQIPRDQFKKQILDVRAQCRPLVVVEVAARQRRTPAELLRAPTY-GWLPP  398

Query  94   ELLGLWTHCAQPPPKALRRELPEEAAAEEERRKIEVPSEIEVPREALEPSVPLMVSLEIS  153
             L  LW HCA      + R +   A   EE RK E  SE+EV REALEPS+P+MVS EIS
Sbjct  399  ALHALWQHCA------ILRPVDYAALWAEEERKEEPVSELEVAREALEPSIPVMVSSEIS  452

Query  154  LEAAEEEKSRISLIPPEERWAWPEVEAPEAPALPVVPELPEVPMEMPLVLPPELELLSLE  213
            LE  EEE  R SL+ PEER   PE E     ALP+VPELPEV +E    LPP+ +L++LE
Sbjct  453  LETTEEEVPRPSLVTPEERRLVPEPEE----ALPIVPELPEVSLE----LPPDRDLITLE  504

Query  214  AVHRAVALELQANREPDFSSLVSPLSPRRMAARVFYLLLGECMHVCVCMWGR--DTETRG  271
             + R VA EL+   E DF SLV   + R +A+R+FYL    C+ +C   + +   TE  G
Sbjct  505  YIRRLVAAELEQVGEMDFRSLVPTTTSRGIASRIFYL----CLVLCGLQFLQLHQTEPYG  560

Query  272  P  272
            P
Sbjct  561  P  561
```

Types of alignments

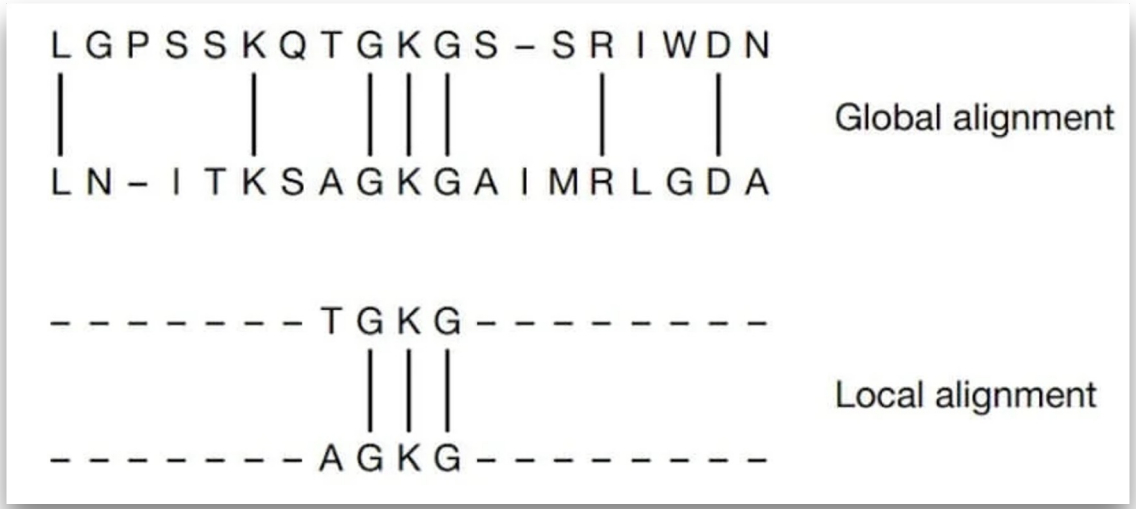
- Multiple



Terminology

- Query: sequence to align
- Reference (or target, subject): other sequence to align to
- Hit (or match or alignment): part of query aligned to part of reference
- Homology: shared ancestry
- Similarity, identity: mathematical ways to detect homology
- Letter (or residue or monomer): base pair or nucleotide or amino-acid
- CIGAR string (or edit transcript): series of edit operations to transform one sequence into the other

Global vs local



https://microbenotes.com/local-global-multiple-sequence-alignment/

Alignment is based on scoring

- What is a *good* alignment?
  One that minimizes a penalty (or **maximizes** a score).
- Example for DNA alignement:
- Match score +1 (M)
- Mismatch penalty -1 (X)
- Gap penalty -2
  Two kinds of gaps:
- Deletion from the reference (D)
- Insertion into the reference (I)

Needleman-Wunsch

- Make a dynamic programming matrix

reference

query

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2					
T	-4					
C	-6					
C	-8					

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1				
T	-4					
C	-6					
C	-8					

Each cell is the optimal alignment score of [query up to this row] vs [reference up to this column]

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1			
T	-4					
C	-6					
C	-8					

AG

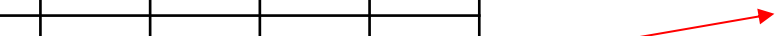
A-

MD

score: -1

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1			
T	-4	-1				
C	-6					
C	-8					



A-

AT

MI

score: -1

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1			
T	-4	-1	0			
C	-6					
C	-8					

Three possibilities:

MX -> score 0

MDI -> score -3

MID -> score -3

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1	-3	-5	-7
T	-4	-1	0			
C	-6	-3				
C	-8	-5				

M

MDDD

MX

MIII

Insight: each filled cell corresponds to the score of an optimal alignment of ref/query so far

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1	-3	-5	-7
T	-4	-1	0	?		
C	-6	-3				
C	-8	-5				

Three possibilities:

```
AGT-  or  AGT  or  AGT
A--T      A-T      AT-
```

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1	-3	-5	-7
T	-4	-1	0	0	-4	-6
C	-6	-3	-2	-1	1	-1
C	-8	-5	-4	-3	0	0

Needleman Wunsch

	-	A	G	T	C	A
-	0	-2	-4	-6	-8	-10
A	-2	1	-1	-3	-5	-7
T	-4	-1	0	0	-4	-6
C	-6	-3	-2	-1	1	-1
C	-8	-5	-4	-3	0	0

Then the alignment is
the CIGAR string at the bottom right cell.
It traces back to the top left cell:

MDMMX

AGTCA
A-TCC

Smith Waterman

	-	A	G	T	C	A
-	0	0	0	0	0	0
A	0	0	0	0	0	1
T	0	0	0	1	0	0
C	0	0	0	0	2	0
C	0	0	0	0	0	1

1. Cells cannot be negative
2. Find the highest scoring cell
3. Trace it back to a zero

Here: TC aligned to TC (.. how surprising)

$$H_{i,j} = \begin{cases} \max \begin{cases} H_{i-1,j-1} + P[q_i, d_j] \\ E_{i,j} \\ F_{i,j} \\ 0 \end{cases} & \begin{vmatrix} i > 0 \\ \cap \\ j > 0 \end{vmatrix} \\ 0 & \begin{vmatrix} i = 0 \\ \cup \\ j = 0 \end{vmatrix} \end{cases} \quad (1)$$

$$E_{i,j} = \begin{cases} \max \begin{cases} H_{i,j-1} - Q \\ E_{i,j-1} - R \end{cases} & |j > 0 \\ 0 & |j = 0 \end{cases} \quad (2)$$

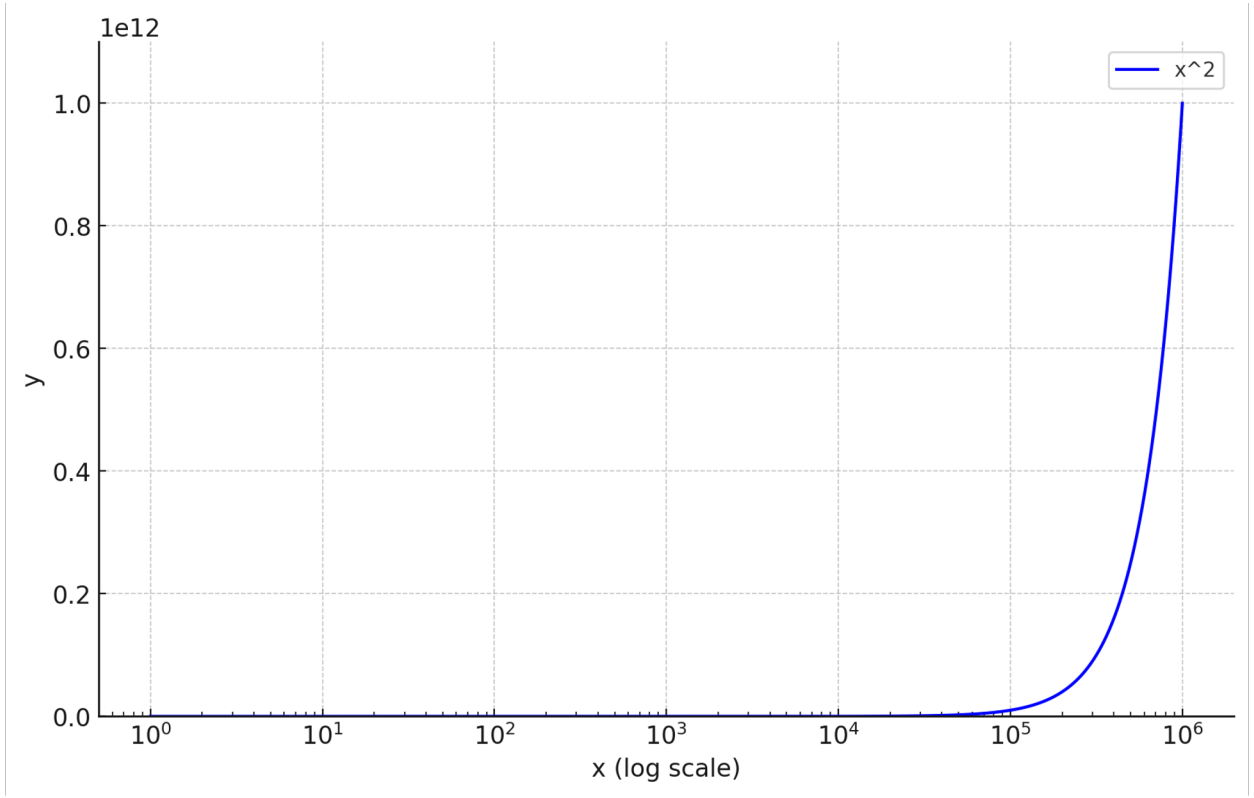
$$F_{i,j} = \begin{cases} \max \begin{cases} H_{i-1,j} - Q \\ F_{i-1,j} - R \end{cases} & |i > 0 \\ 0 & |i = 0 \end{cases} \quad (3)$$

$$S = \max_{1 \leqslant i \leqslant m \cap 1 \leqslant j \leqslant n} H_{i,j} \quad (4)$$

Why can’t we Smith Waterman everything?

It requires (n*m) operations, where n and m are the sequence lengths.

When n ~ m, it’s n^2 operations:



The BLAST algorithm

```
Query= d5gy3a_ a.102.1.0 (A:) automated matches {Klebsiella pneumoniae [TaxId: 573]}

>d1h12a_ a.102.1.2 (A:) Endo-1,4-beta-xylanase {Pseudoalteromonas haloplanktis [TaxId: 228]}
Length=404

 Score = 45.8 bits (107),  Expect = 1.3e-05
 Identities = 53/195 (27%), Positives = 84/195 (43%), Gaps = 37/195 (18%)

Query    7  YKARFMMPDGRIIDTANGNVSHTEGQGFAMLLAVANNDRPAFDKLWQWTDSTLRD----- 61
            YKA ++    + I+   G+   TEGQ + M  AV  N +  FD LW++  +  ++
Sbjct   59  YKAHYI----KAINPDEGDDIRTEGQSWGMTAAVMLNKQEEFDNLWRFAKAYQKNPDNHP 114

Query   62  --KSNGLFYW--RYNPVAPDPIADKNNASDGDTLIAWALLRAQKQWQDKRRYAIASDAIT 116
              K  G++ W  + N        D+  A DG+   A+ALL A  +W +   +DAIT
Sbjct  115  DAKKQGVYAWKLKLNQNGFVYKVDEGPAPDGEEYFAFALLNASARWGNSGEFNYYNDAIT 174

Query  117  A------SLLKYTVVTFAGRQVMLPGVKGFNLNDHLNLNPSYFIFPAWRAFAERTHLTA- 169
                   L++  ++ F+      P +   NL D     PSY I   +  FA      A
Sbjct  175  MLNTIKNKLMENQIIRFS------PYID--NLTD-----PSYHIPAFYDYFANNVTNQAD 221

Query  170  ---WRTLQTDGQALL 181
               WR + T  + LL
Sbjct  222  KNYWRQVATKSRTLL 236
```

The BLAST algorithm

1. Seed: Find two neighborhood 3-mer matches within a window of 40 on the same diagonal

```
Query= d5gy3a_ a.102.1.0 (A:) automated matches {Klebsiella pneumoniae [TaxId: 573]}

>d1h12a_ a.102.1.2 (A:) Endo-1,4-beta-xylanase {Pseudoalteromonas haloplanktis [TaxId: 228]}
Length=404

 Score = 45.8 bits (107),  Expect = 1.3e-05
 Identities = 53/195 (27%), Positives = 84/195 (43%), Gaps = 37/195 (18%)

Query    7  YKARFMMPDGRIIDTANGNVSHTEGQGFAMLLAVANNDRPAFDKLWQWTDSTLRD----- 61
            YKA ++    + I+   G+   TEGQ + M  AV  N +  FD LW++  +  ++
Sbjct   59  YKAHYI----KAINPDEGDDIRTEGQSWGMTAAVMLNKQEEFDNLWRFAKAYQKNPDNHP 114

Query   62  --KSNGLFYW--RYNPVAPDPIADKNNASDGDTLIAWALLRAQKQWQDKRRYAIASDAIT 116
              K  G++ W  + N        D+  A DG+   A+ALL A  +W +   +   +DAIT
Sbjct  115  DAKKQGVYAWKLKLNQNGFVYKVDEGPAPDGEEYFAFALLNASARWGNSGEFNYYNDAIT 174

Query  117  A------SLLKYTVVTFAGRQVMLPGVKGFNLNDHLNLNPSYFIFPAWRAFAERTHLTA- 169
                    L++  ++ F+      P +   NL D     PSY I   +  FA      A
Sbjct  175  MLNTIKNKLMENQIIRFS------PYID--NLTD-----PSYHIPAFYDYFANNVTNQAD 221

Query  170  ---WRTLQTDGQALL 181
               WR + T  + LL
Sbjct  222  KNYWRQVATKSRTLL 236
```

The BLAST algorithm

2. Extend using gapped X-drop

```
Query= d5gy3a_ a.102.1.0 (A:) automated matches {Klebsiella pneumoniae [TaxId: 573]}

>d1h12a_ a.102.1.2 (A:) Endo-1,4-beta-xylanase {Pseudoalteromonas haloplanktis [TaxId: 228]}
Length=404

 Score = 45.8 bits (107),  Expect = 1.3e-05
 Identities = 53/195 (27%), Positives = 84/195 (43%), Gaps = 37/195 (18%)

Query    7  YKARFMMPDGRIIDTANGNVSHTEGQGFAMLLAVANNDRPAFDKLWQWTDSTLRD----- 61
            YKA ++    + I+   G+   TEGQ + M  AV  N +  FD LW++  +  ++
Sbjct   59  YKAHYI----KAINPDEGDDIRTEGQSWGMTAAVMLNKQEEFDNLWRFAKAYQKNPDNHP 114

Query   62  --KSNGLFYW--RYNPVAPDPIADKNNASDGDTLIAWALLRAQKQWQDKRRYAIASDAIT 116
              K  G++ W  + N        D+  A DG+   A+ALL A  +W +   +  +DAIT
Sbjct  115  DAKKQGVYAWKLKLNQNGFVYKVDEGPAPDGEEYFAFALLNASARWGNSGEFNYYNDAIT 174

Query  117  A------SLLKYTVVTFAGRQVMLPGVKGFNLNDHLNLNPSYFIFPAWRAFAERTHLTA- 169
                   L++  ++ F+      P +   NL D     PSY I   +  FA      A
Sbjct  175  MLNTIKNKLMENQIIRFS------PYID--NLTD-----PSYHIPAFYDYFANNVTNQAD 221

Query  170  ---WRTLQTDGQALL 181
               WR + T  + LL
Sbjct  222  KNYWRQVATKSRTLL 236
```

Anything can align to anything

Two random DNA sequences:

```
ATTTTAGGGGGG-GAAGGTTG-

    ||||   | |   |    |

GCG--AGGGCCGTGTTGCCGGT
```

- Be careful of “coercing” alignments. Sometimes there is just no homology. Those alignments are meaningless.

BLAST's E-value

E-value = number of hits one can "expect" to see by chance on a database this size.

Always raise an eyebrow if your E-value is >= 0.01.

Common thresholds: < 0.01, or < 1e-5

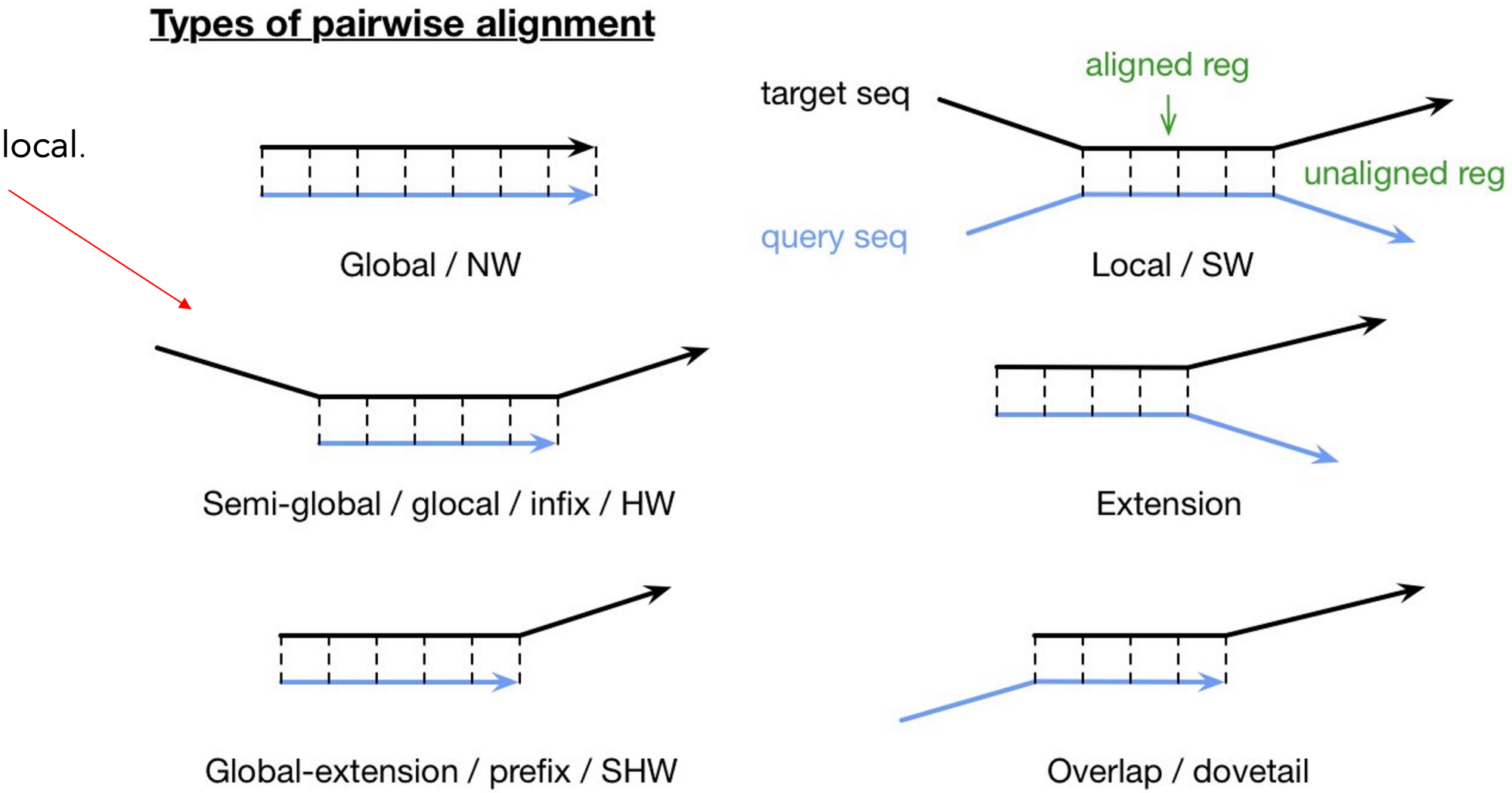
Short read mapping, in principle

```
ACAACTGTCTGCTTCAGGAGTTAAATCTTACA-GGATGA reference
ACAACTGTCTGCTT
read1
       TCTG-TTCAGGAGTT
       read2
        CTGCTTCAGGAGTT
        read3
               GGGAGTTAAATCTT
               read4
                 GAGTTAAAT

                 read5
```

Adapted from https://wikis.univ-lille.fr/bilille/_media/ngs2023_dnaseq_03_cours_dna_mapping.pdf

Wait.. is this local alignment or global alignment?

Neither. It's glocal.



https://twitter.com/lh3lh3/status/1488580381091770371

Why is it difficult? Need to find a home for every read

Problem: Half of the human genome is comprised of repeats

```
taaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaa
accctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccctaac
cctaacccaaccctaaccctaaccctaaccctaaccctaaccctaaccctaacccc
taaccctaaccctaaccctaaccctaacctaaccctaaccctaaccctaaccctaa
ccccccctaaccctaaccctaaccctaaccccctaaccctaaccctaaaccc
ccctaaaccctaaccctaaccctaaccctaaccctaaccccaaccccaac
cccaaccccaaccccaaccccaaccctaaccccctaaccctaaccctaacc
ctaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccccc
taaccctaaccctaaccctaaccctaaccctaaccctaaccctaaccct
aaccctaaccctaaccctcgcggtaccctcagccggcccgcccgcccggg
tctgacctgaggagaactgtgctccgccttcagagtaccaccgaaatctg
tgcagaggacaacgcagctccgccctcgcggtgctctccgggtctgtgct
gaggagaacgcaactccgccggcgcaggcgcagagaggcgcgccgcgccg
gcgcaggcgcagacacatgctagcgcgtcggggtggaggcgtggcgcagg
cgcagagaggcgcgccgcgccggcgcaggcgcagagacacatgctaccgc
gtccaggggtggaggcgtggcgcaggcgcagagaggcgcaccgcgccggc
gcaggcgcagagacacatgctagcgcgtccaggggtggaggcgtggcgca
ggcgcagagacgcaagcctacgggcgggggttgggggggcgtgtgttgca
ggagcaaagtcgcacggcgccgggctggggcgggggagggtggcgccgt
gcacgcgcagaaactcacgtcacggtggcgcggcgcagagacgggtagaa
```

(first bit of human chromosome 1)

Slide: A. Quinlan

Tools

- Bowtie2
- BWA-MEM
- Strobealign
- Minimap2
- …

Which one to choose? *Not going to answer that!*

Bowtie2, BWA-MEM: battle-tested, well-documented

minimap2: faster

Strobealign: ultra fast, newer

PacBio CLR / ONT:

- Minimap2
- Variants of minimap2 for ~ 2-5x speed gain (mm2-fast, BLEND, ..)

PacBio HiFi:

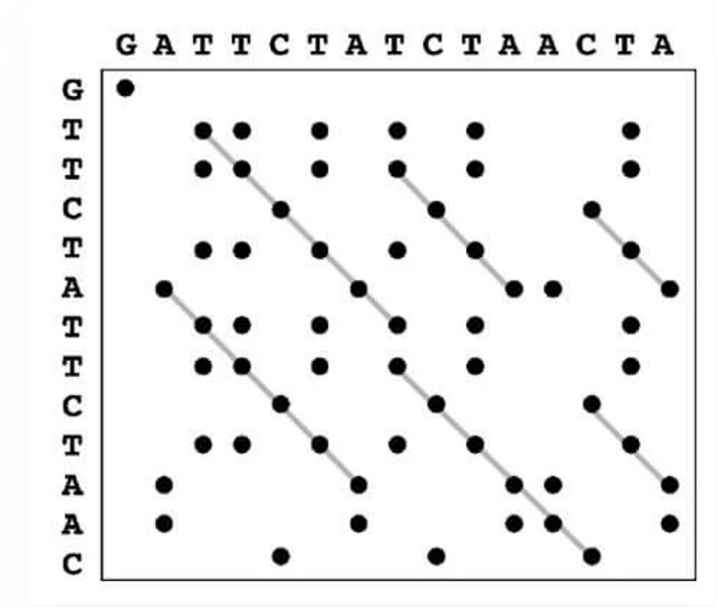
- Minimap2
- Winnowmap2 (better accuracy)
- Mapquik (30x faster mapping, but no alignment)

Tools (long vs long)

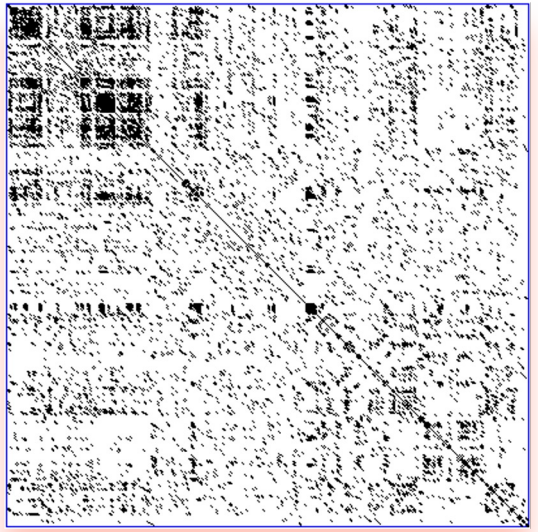
- BLAT
- Exonerate
- LASTZ
- MUMmer
- minimap2
- wfmash
- FASTGA

Dot plots

- Tools: LASTZ, D-Genies, yass, MUMmer, ModDotPlot



https://microbenotes.com/local-global-multiple-sequence-alignment/



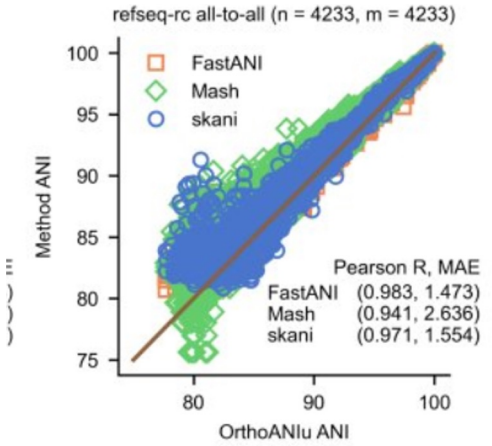
Wikipedia

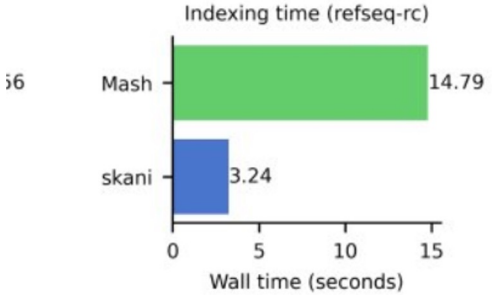
ANI (average nucleotide identity)

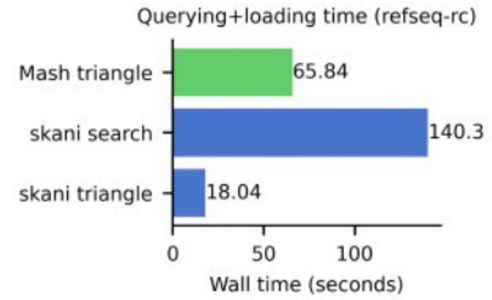
A strange identity metric, used to compare two bacterial genomes:

1. Extract many 1 Kbp fragments from query
2. ANI = mean identity of the reciprocal best hits

(from FastANI: https://www.nature.com/articles/s41467-018-07641-9)







How does Bowtie2 work?

Specializes in aligning Illumina reads to genomes.

1) Find seeds using FM-index, typically 20 nt length, up to 1 mismatch
2) Prioritizes seeds to further align
3) Extend seeds using SW-like algorithm

Minimizers

Minimap2 and strobealign use minimizers as seeds, then SW extension.

Minimizers: select only *some* k-mers as seeds

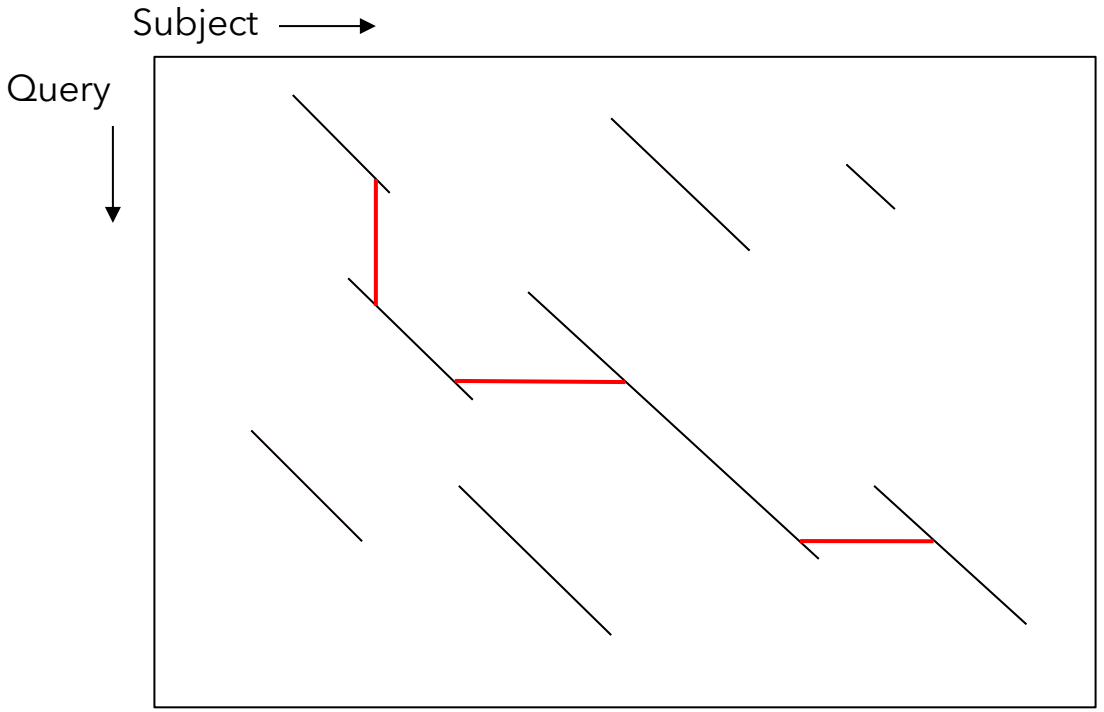
```
reference:     CTAAAAAGGTCA..
2nd window:     TAAAAAGG
                TAAAA
          seed:  AAAAA
                  AAAAG
                   AAAGG
```

all reference k-mers	Found at position(s)
AAAAA	10, 65, 147, …
AAAAC	80
….	
AAAGG	none
….	
TAAAA	49, 101

Which “some”? Slide a window over the reference, and pick the (lexicographically) smallest seed within that window. Do that for all windows

Chaining

DP on the ungapped extensions at seed hits



1. Provides estimate of alignment score to limit the search space.
2. Infer the trajectory of the best alignment to guide banded DP.

Paired reads

In some cases, Illumina sequencers output pairs of reads.

Aligners consider both reads jointly to improve precision. Need to specify:

- Orientation (forward-reverse is most common)
- Format: interleaved in one file, or two separate files

Mapping quality

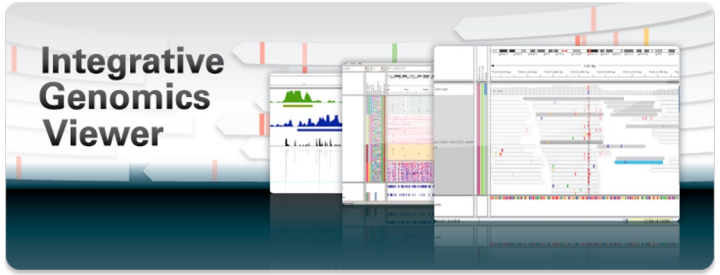
…is your best friend, to avoid errors downstreams.

Mapq: how confidently each read is mapped (in log probability).

Grab only highly-confident alignments: `samtools view -q 60 [file.bam]`

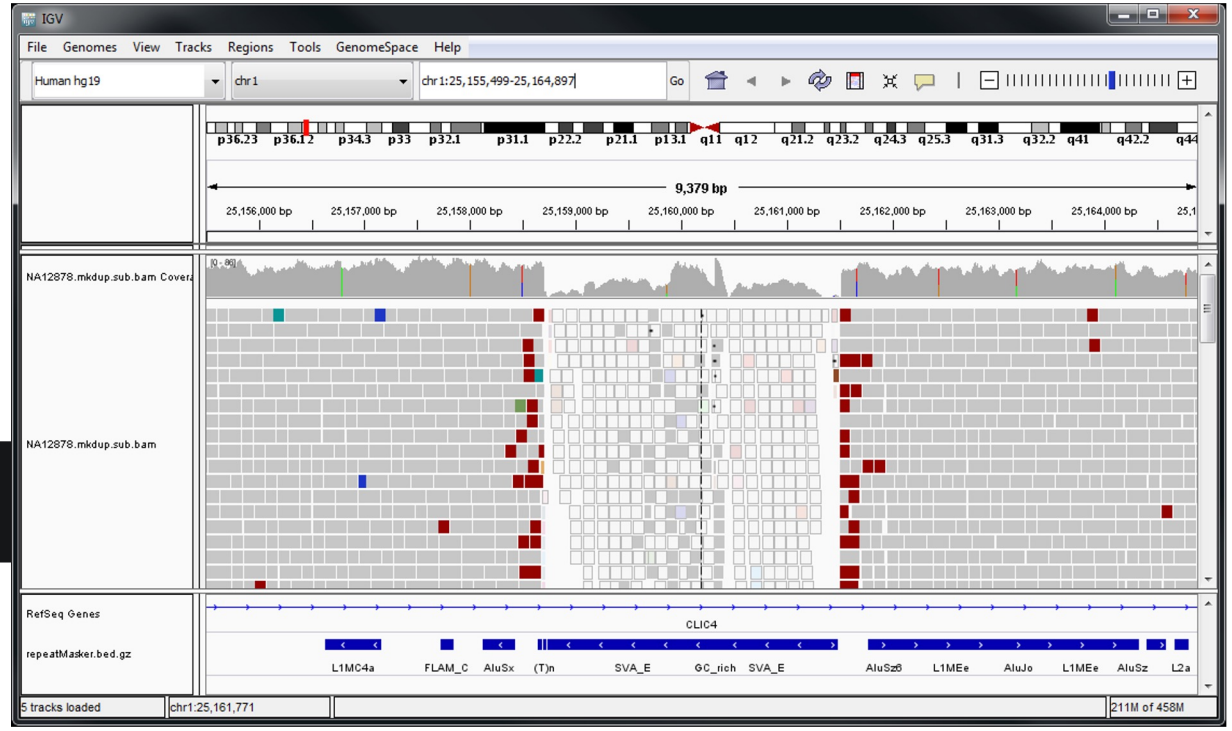
Grab all alignments except trash ones: `samtools view -q 1 [file.bam]`

Visualization of alignments



IGV
IGV desktop application

IGV Web App
IGV in a web browser



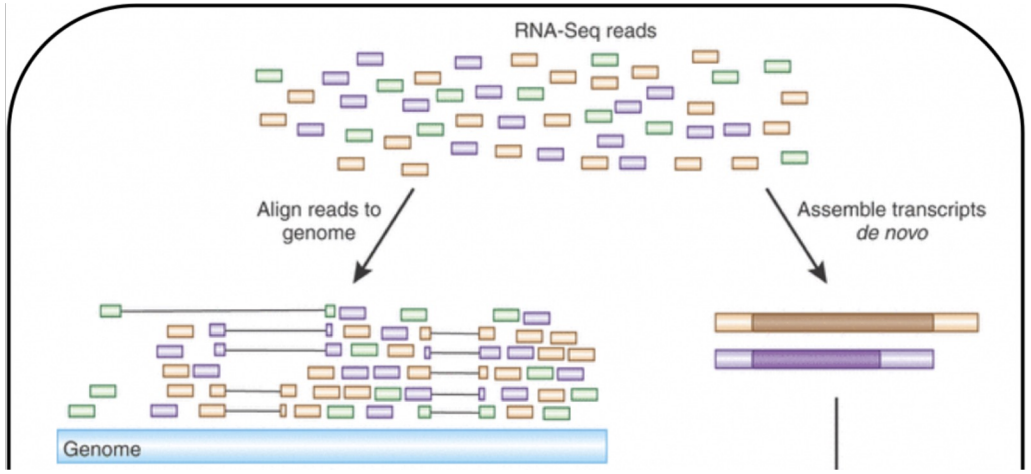
RNA

RNA read alignment is very similar to DNA, except:

- Split mapping (on genomes) due to splicing
- Ambiguity (on transcriptomes) due to many isoforms

Tools:

- Kallisto, Salmon
- STAR, HiSAT2



Proteins: What changes compared to pairwise DNA?

- Different alphabet, shorter sequences
- Some substitutions are more likely than others
- Distant homology search (high evolutionary distances)

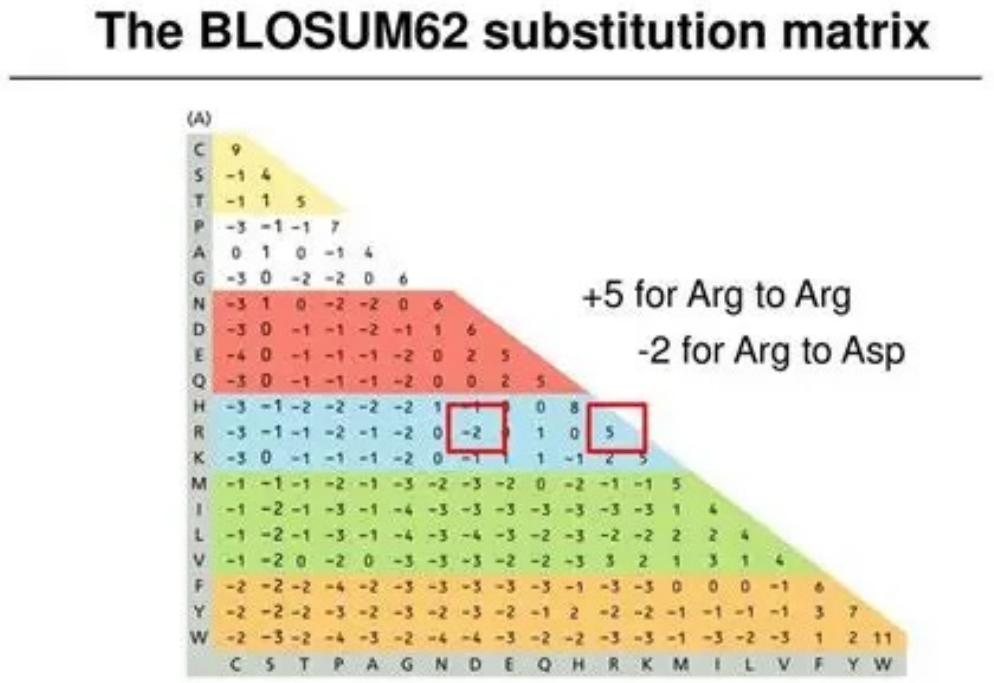


Fig 4.4

Slides: Mike Hallett, David Walsh

Algorithms

- To correctly interpret output from bioinformatics tools, you need to (at least roughly) understand its algorithm.
- Do not treat a tool as some magic black box!
- The tool cannot read your mind. Always adjust its parameters for your application.
- Risk of missing information, or reach wrong conclusions.
- Default settings are most likely not optimal for your application.

The BLAST algorithm

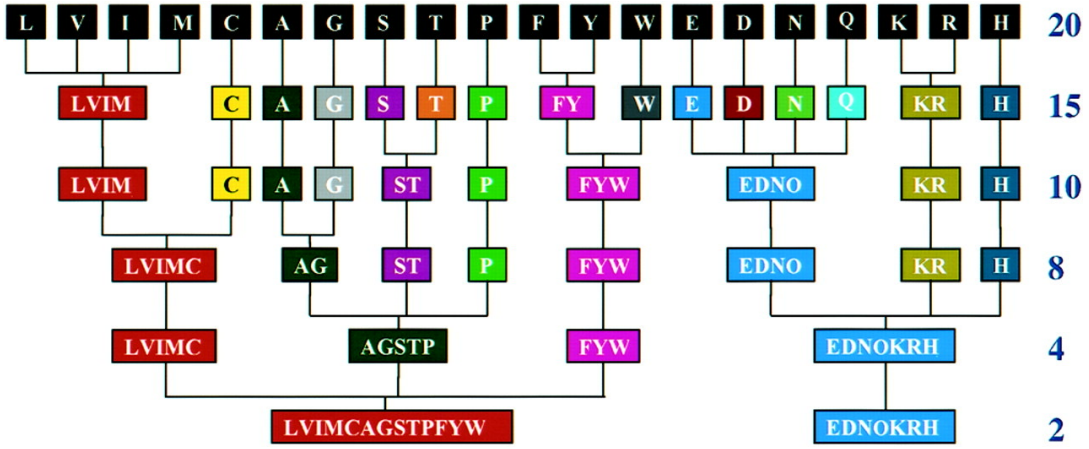
1. Seed: Find two neighborhood 3-mer matches within a window of 40 on the same diagonal

```
Query= d5gy3a_ a.102.1.0 (A:) automated matches {Klebsiella pneumoniae [TaxId: 573]}

>d1h12a_ a.102.1.2 (A:) Endo-1,4-beta-xylanase {Pseudoalteromonas haloplanktis [TaxId: 228]}
Length=404

 Score = 45.8 bits (107),  Expect = 1.3e-05
 Identities = 53/195 (27%), Positives = 84/195 (43%), Gaps = 37/195 (18%)

Query     7  YKARFMMPDGRIIDTANGNVSHTEGQGFAMLLAVANNDRPAFDKLWQWTDSTLRD----- 61
             YKA ++    + I+   G+   TEGQ + M  AV  N +  FD LW++  +  ++
Sbjct    59  YKAHYI----KAINPDEGDDIRTEGQSWGMTAAVMLNKQEEFDNLWRFAKAYQKNPDNHP 114

Query    62  --KSNGLFYW--RYNPVAPDPIADKNNASDGDTLIAWALLRAQKQWQDKRRYAIASDAIT 116
               K  G++ W  + N        D+  A DG+   A+ALL A  +W +   +  +DAIT
Sbjct   115  DAKKQGVYAWKLKLNQNGFVYKVDEGPAPDGEEYFAFALLNASARWGNSGEFNYYNDAIT 174

Query   117  A------SLLKYTVVTFAGRQVMLPGVKGFNLNDHLNLNPSYFIFPAWRAFAERTHLTA- 169
                    L++  ++ F+      P +   NL D     PSY I   +  FA      A
Sbjct   175  MLNTIKNKLMENQIIRFS------PYID--NLTD-----PSYHIPAFYDYFANNVTNQAD 221

Query   170  ---WRTLQTDGQALL 181
                WR + T  + LL
Sbjct   222  KNYWRQVATKSRTLL 236
```

Expected value of initial two-hit extensions between two random proteins of length 300: ~7.4

Alphabet reductions

Group amino acids based on substitution frequencies:
A [KR] [EDNQ] C G H [ILVM] [FYW] P [ST]



Murphy et al. Simplified amino acid alphabets for protein fold recognition and implications for folding. (2000)

Spaced seeds

Pattern of match and "don't care" positions to extract seeds from sequence:

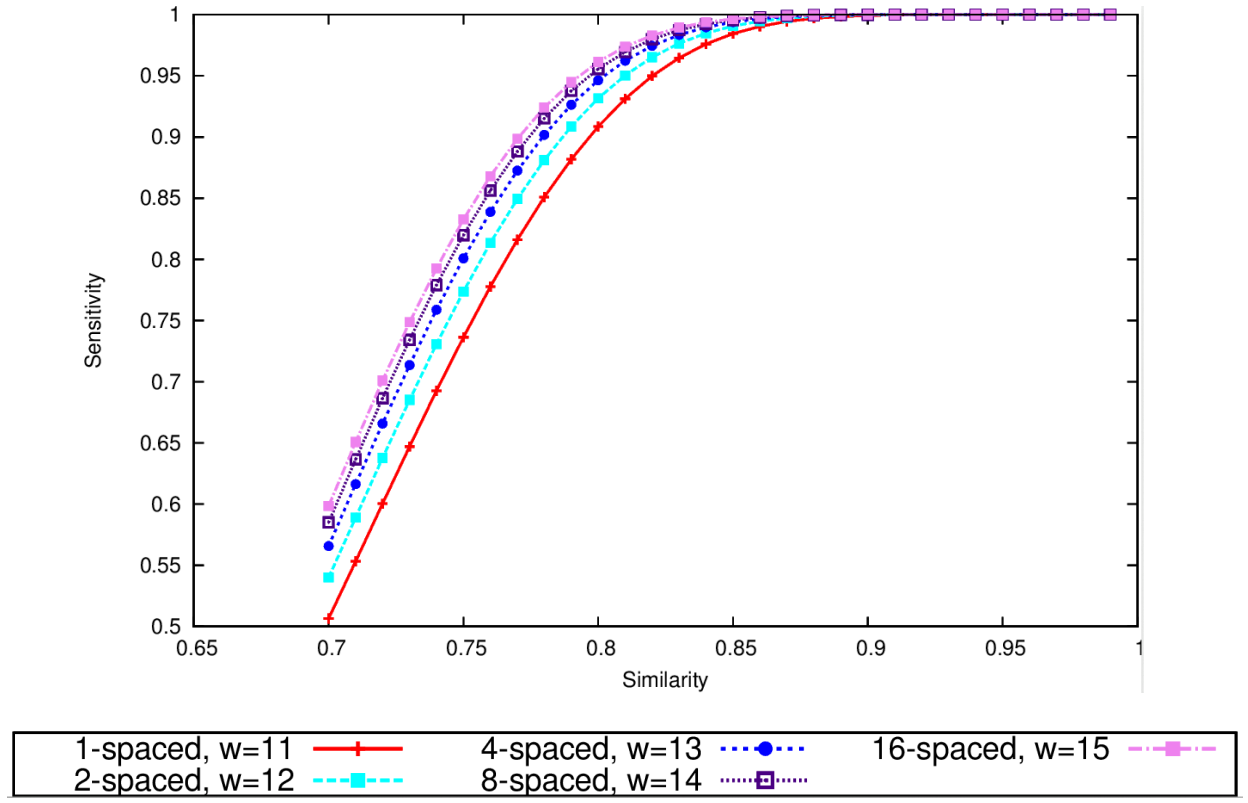
```
Reference        SLWAKKRTVDGQPKWLPLVAHLVDASNVSRMLFNQWLSD
Spaced Seed        1111011011110111
Query               FWAKKRTVNGQPLWLPLTQHLEDASNVSR
```

Choose set of seed shapes that complement each other:

```
1011110111          10101001101011
11001011111         1101001000101111
101110001111        1110010010010111
111010100111        1100110001100111
1111010010101       1111000010000111
1100011011011       1101010000011011
11011010001101      1110001010101001
11011101100001      1101001100010011
```

Multiple spaced seeds

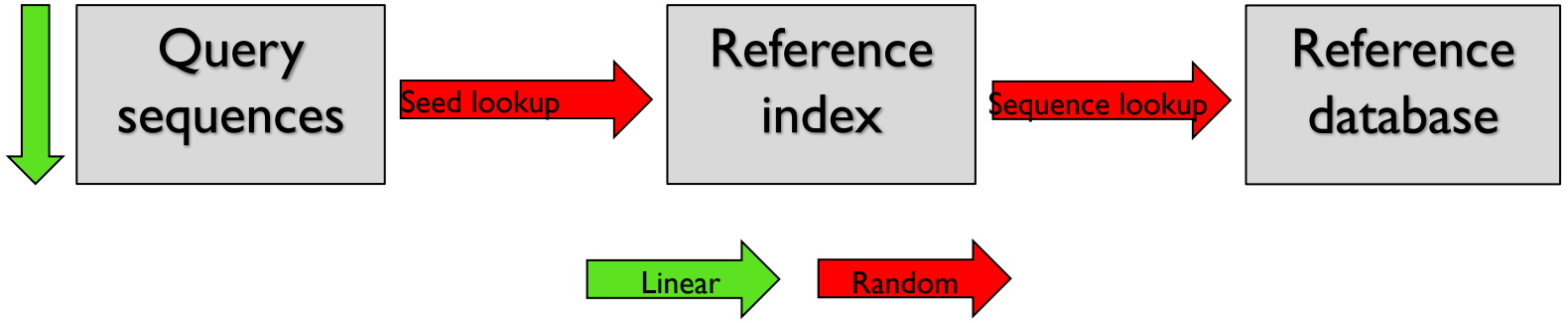
Sensitivity for homologous region of length 70 (Ilie et al., 2011)



Technical challenge: High memory consumption and many seed lookups

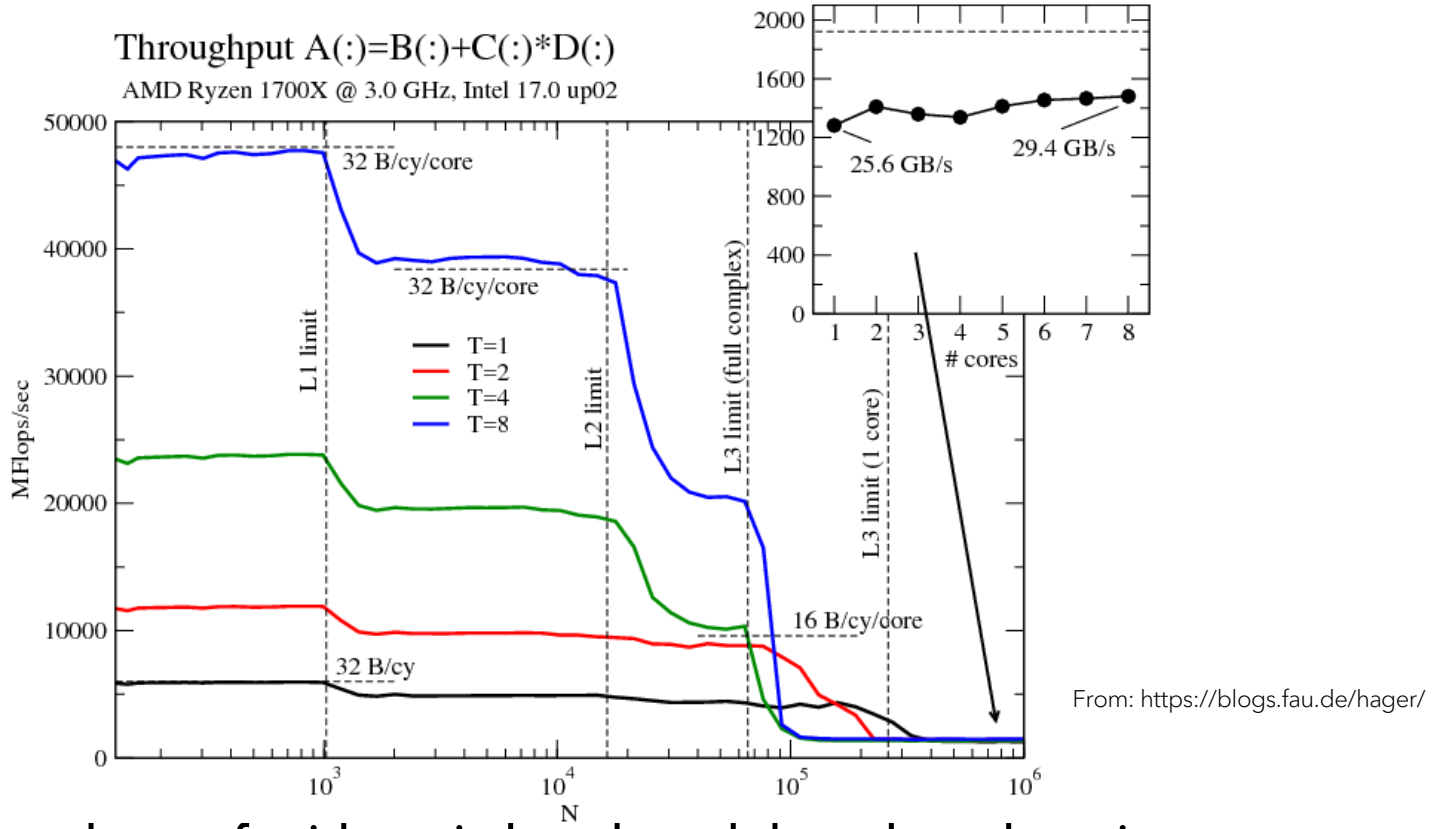
Index based alignment

Linear processing of the queries:



- Random memory access pattern to data structures that far exceed cache sizes.
- Bottleneck: latency of main memory.
- Compounded by using multiple spaced seeds.

The memory hierarchy

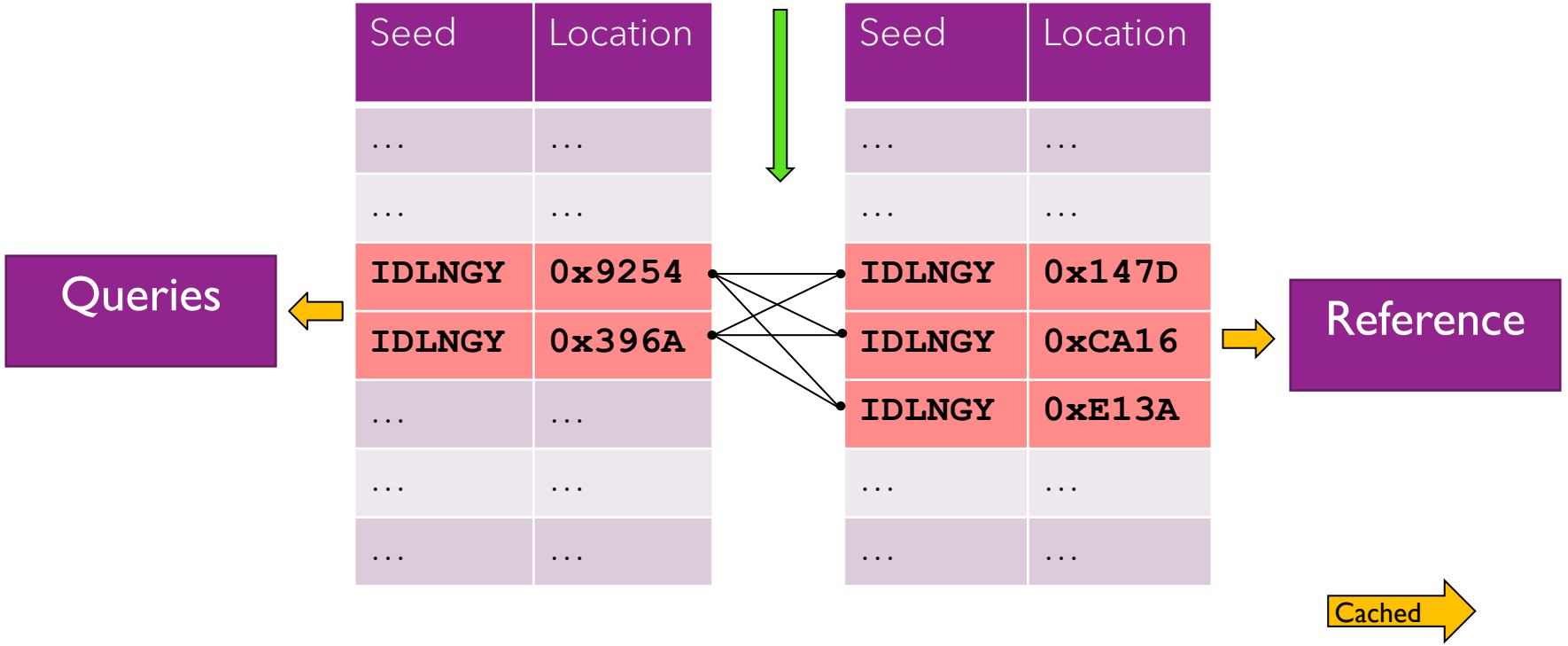


From: https://blogs.fau.de/hager/

Throughput of arithmetic benchmark based on data size

Double indexing

Sort-merge join technique with on-the-fly computed double indexes



Compare operations for each seed are streamed through the CPU efficiently by cache-aware partitioning.

Example run (nr database, 514 million sequence)

>tr|A0A0D6XPW3|A0A0D6XPW3_9STAP MerR family transcriptional regulator OS=Staphylococcus microti OX=569857 GN=tipA PE=4 SV=1

```
MTTYAIGDIAKMFDISTRTLQYYDEKGVLPPAYVDHNNYRVYTEHEVEKLQLIVLMKTLG
MQLKEIQALLTAEGTLDTVRLLLDQKSKALEQTIQKQQAQYQQIQAMQRMINETSQSSIT
KLQEMDRMLMKNEHLKPLRVKSLGLASIGTLSLWSGIWVGTKLGTPTYPLLGLGTSVGIA
YYLTHDYYHQVKYMCPNCQHVFVPTMRQFILARHTPSTRHLTCPSCEFTGYCVEVYEPH
```

Mode	Shapes	Seed hits	Alignments	Ratio
Default	2x10	1,083,599	10,524	100
Sensitive	16x8	248,372,076	49,204	5,000
Very-sensitive	14x7	1,055,031,248	51,464	20,000
Ultra-sensitive	64x7	4,687,907,463	51,684	90,000

Hamming distance filter

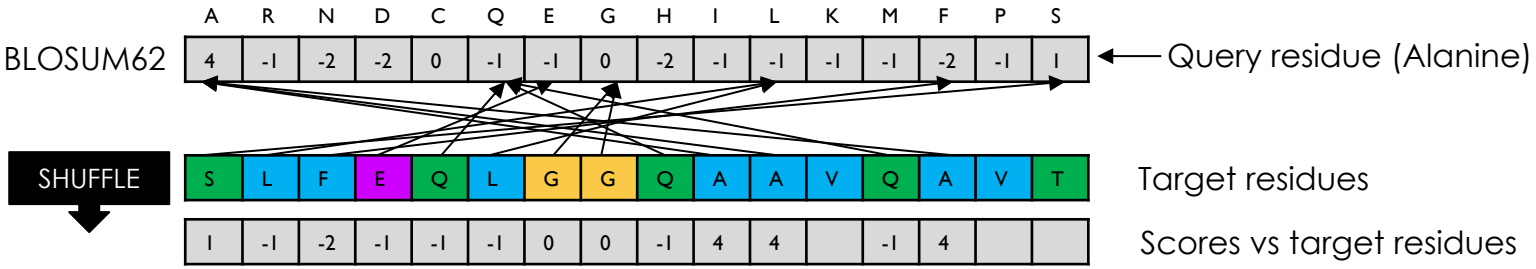
```
KKLNHPCIIKIKNFFDAEDYYAIVLELMEGHELFDKVVGNKRLKEATC
   + P  + +   F   D  + +L+LM G++L   +  +    E
STGDCPFAVCMSYAFHTPDKLSFILDLMNGNDLHYHLSQHGVFSENDM
```

- Compute the hamming distance of 2 16-letter strings:

```
_popcnt32(_mm_movemask_epi8(_mm_cmpeq_epi8(x, y))
```

- Throughput: 16 ps/Letter, compared to 170 ps/Letter for ungapped extension with BLOSUM62 scores.
- Preload all query and subject windows into linear buffers.
- Use loop tiling to optimise cache use.
- Filtration: ~92%

Ungapped extension filter




```
s1,…,s16 ← 16x16 byte subject sequences
t1,…,t16 ← transpose(s1,…,s16)
for i in [0,16[
        match_scores ← BLOSUM62[query[i], ti]
        score += match_scores
        max_score ← max(max_score, score)
```

Filtration: ~83%

Data-parallel Smith Waterman

- Technical challenges: Intra-register data dependencies, loading match scores into registers.
- SWIPE: Inter-sequence SIMD parallelization
- Does not easily afford banded DP.

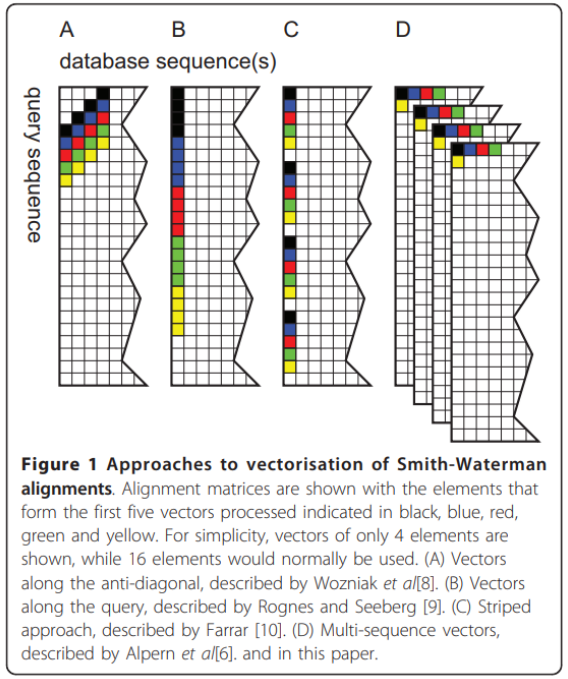


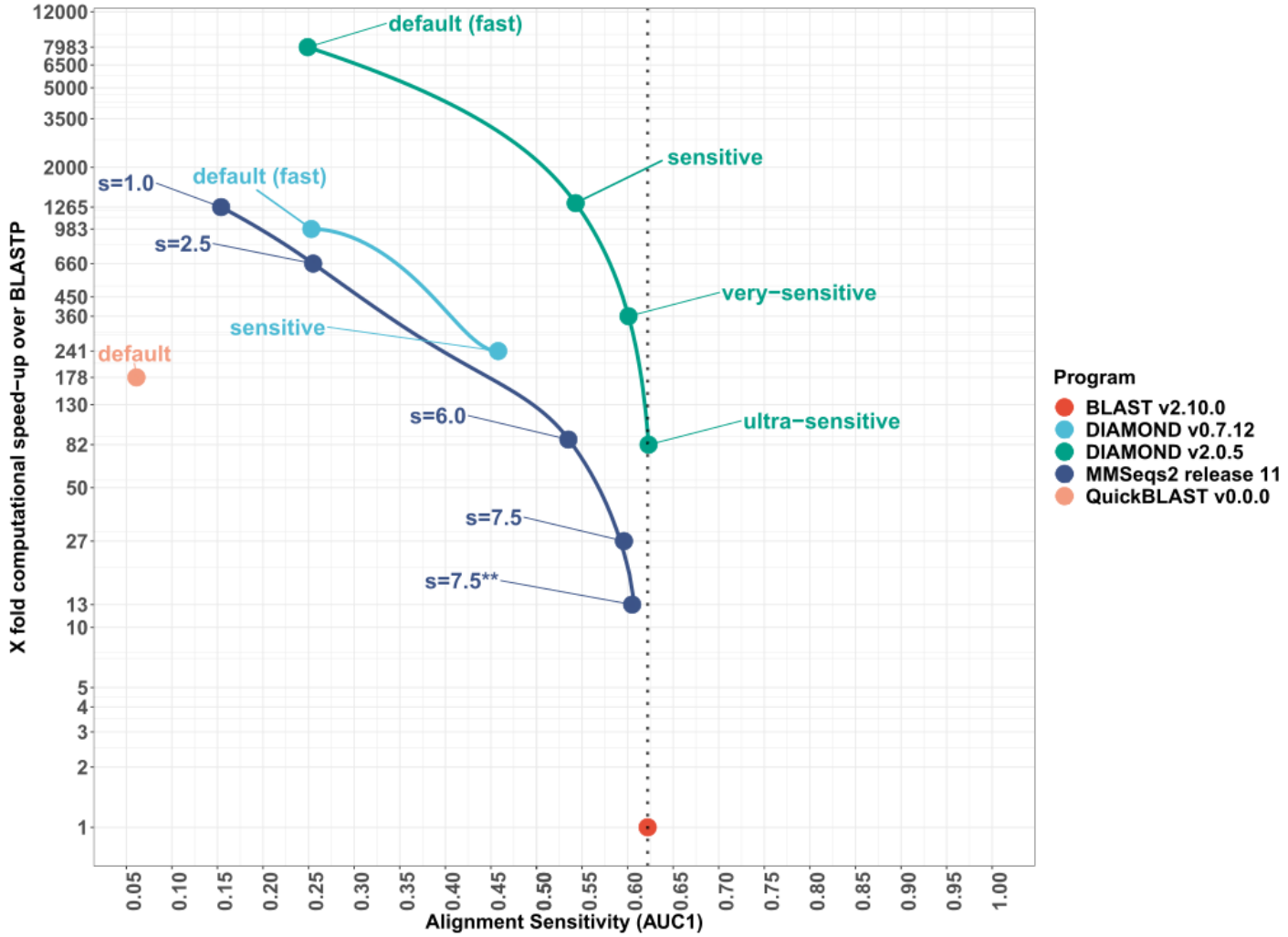
Figure 1 Approaches to vectorisation of Smith-Waterman alignments. Alignment matrices are shown with the elements that form the first five vectors processed indicated in black, blue, red, green and yellow. For simplicity, vectors of only 4 elements are shown, while 16 elements would normally be used. (A) Vectors along the anti-diagonal, described by Wozniak *et al*[8]. (B) Vectors along the query, described by Rognes and Seeberg [9]. (C) Striped approach, described by Farrar [10]. (D) Multi-sequence vectors, described by Alpern *et al*[6]. and in this paper.

From: Rognes, Faster Smith-Waterman database searches with inter-sequence SIMD parallelization (2011)

Benchmark

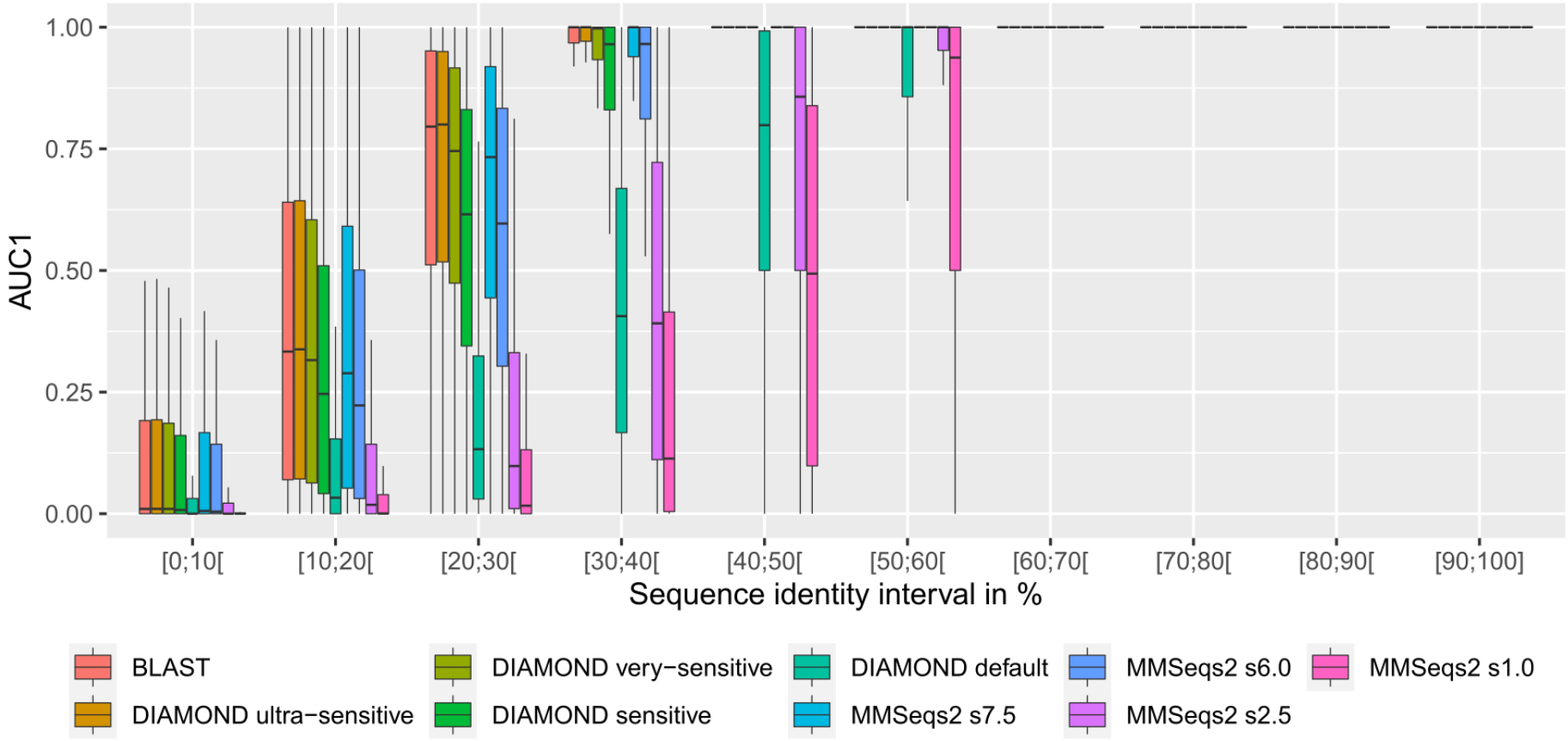
- Annotate a query file and sequence database using SCOP domains.
- SCOP (Structural Classification of Proteins): Proteins are classified into classes, folds, superfamilies, and families. Contains ~2,000 superfamilies and ~4,500 families.
- For each query, compute AUC1: fraction of sequences from the same family covered until the first false positive.
- Alignments to targets annotated with a different SCOP fold are false positives; shuffle non-annotated ranges.
- In our case: 1.7 million queries vs. 8 million targets.

DIAMOND (2021)



From: Buchfink et al., Nature Methods (2021)

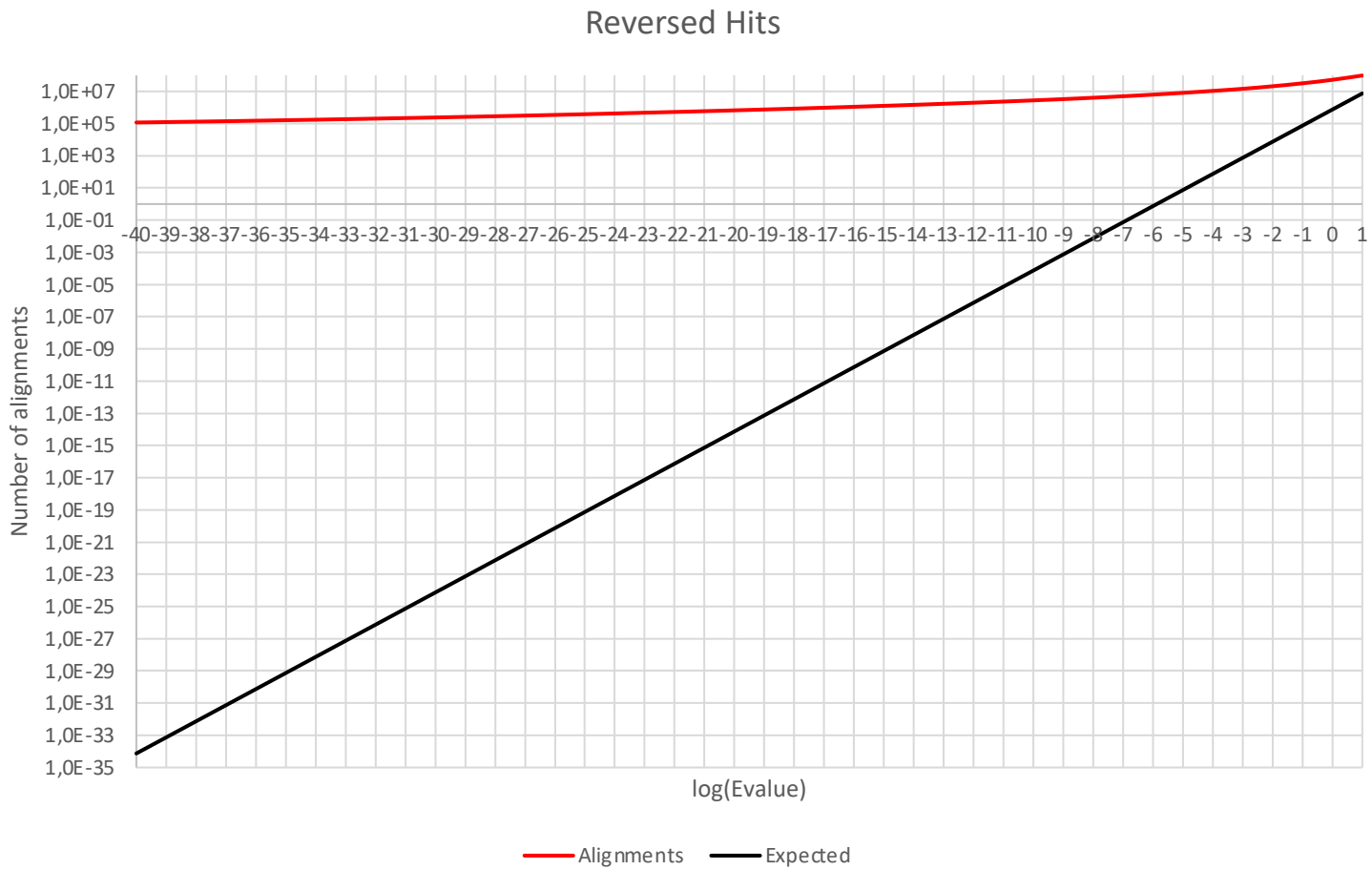
Sensitivity by sequence identity



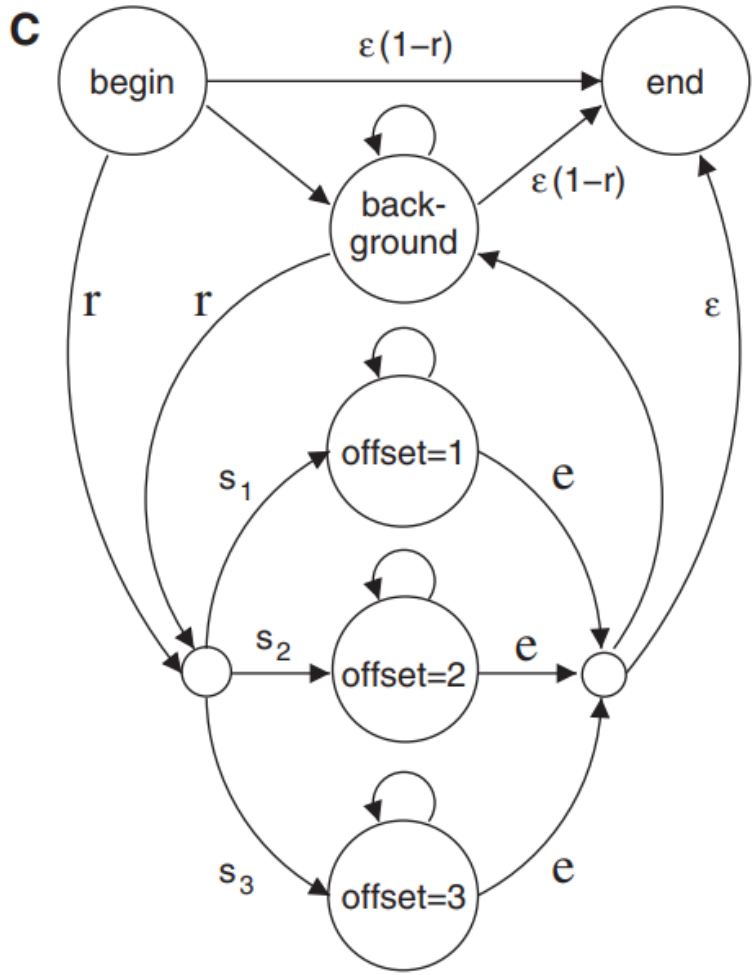
From: Buchfink et al., Nature Methods (2021)

Repeat masking

Alignment of 740,000 queries against the NR database (220 million REVERSED proteins) using BLAST 2.10.0:



tantan masking



(Frith, 2016)

Examples of reversed alignments

```
Query= VUS24436.1 hypothetical protein SB6410_00906 [Klebsiella grimontii]

Length=1828

>\RSK04010.1 hypothetical protein D8809_02240 [Streptococcus gordonii]
Length=1896

 Score = 1144 bits (2958),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 537/1621 (33%), Positives = 1361/1621 (84%), Gaps = 20/1621 (1%)

Query  10    SESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSE  69
             +++LS++E L+E+ +LS++E L+++ +LS++E L+++ +LS++E L+++++L E++ L+E
Sbjct  262   TDALSDAEVLAETLALSDAEVLADTLALSDAEVLADTLALSDAEVLADTDALVEADVLAE  321

Query  70    SESLSESESLSESESLSESESLS----------LSESESLSESESLSESESLSESESLSE  119
             +E+LS++E L+++ +LS+++ L+          L+++ +LS+++ L+++ +L ++E L++
Sbjct  322   TEALSDAEVLADTLALSDADVLADTLALSDADVLADTLALSDADVLADTLALVDAEVLAD  381

Query  120   SESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSE  179
             +++L E++ L+++ +L E+E L+++ +L ++E L+++++LS++E L+++ +LS+++ L+E
Sbjct  382   TDALVEADVLADTLALVEAEVLADTLALVDAEVLADTDALSDAEVLADTLALSDADVLAE  441

Query  180   SESLSESESLSESESLSESESLSESESLSESESLSASESLSESESLSESESLSESESLSE  239
             +++L +++ L+++E+LS+++ L+++ +LS+++ L+ S++LS++E L+++ +LS+++ L+E
Sbjct  442   TDALVDADVLADTEALSDADVLADTLALSDADVLADSDALSDAEVLADTLALSDADVLAE  501

Query  240   SESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSE  299
             +E+LS+ E L+++ +LS+ E L+E+ +LS++E L+E+E+LS++E L++S++LS++E L++
Sbjct  502   TEALSDDEVLADTLALSDDEVLAETLALSDAEVLAETEALSDAEVLADSDALSDAEVLAD  561

                                    • • •

Query  1490  SESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSL  1549
             +E L+++ +LS+++ L+++++LS++E L+++++LS+++ L+++E+L +++ L+E+ + S
Sbjct  1762  AEVLADTLALSDADVLADTDALSDAEVLADTDALSDADVLADTEALVDADVLAETLAFSD  1821

Query  1550  SESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSESESLSE  1609
             +E L+++ +LS++E L+++++LS+++ L+++++LS++E L+++++LS+++ L+++E+L +
Sbjct  1822  AEVLADTLALSDAEVLADTDALSDADVLADTDALSDAEVLADTDALSDADVLADTEALVD  1881

Query  1610  S  1610
             +
Sbjct  1882  A  1882
```

Examples of reversed alignments

```
Query= BAG82822.1 polyprotein [Enterovirus A71]

Length=2438

>\WP_124134815.1 hypothetical protein [Campylobacter hepaticus]
Length=113

 Score = 185 bits (470),  Expect = 1e-50, Method: Composition-based stats.
 Identities = 89/96 (93%), Positives = 90/96 (94%), Gaps = 2/96 (2%)

Query  84   HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHK  143
            DFFKSAMPEGYVQERTI FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL HK
Sbjct  20   QDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNIL-HK  78

Query  144  LEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSV  179
            LEYNYNSH VYI ADKQKNGIK NFKIRHNIEDGS+
Sbjct  79   LEYNYNSH-VYITADKQKNGIKANFKIRHNIEDGSM  113
```

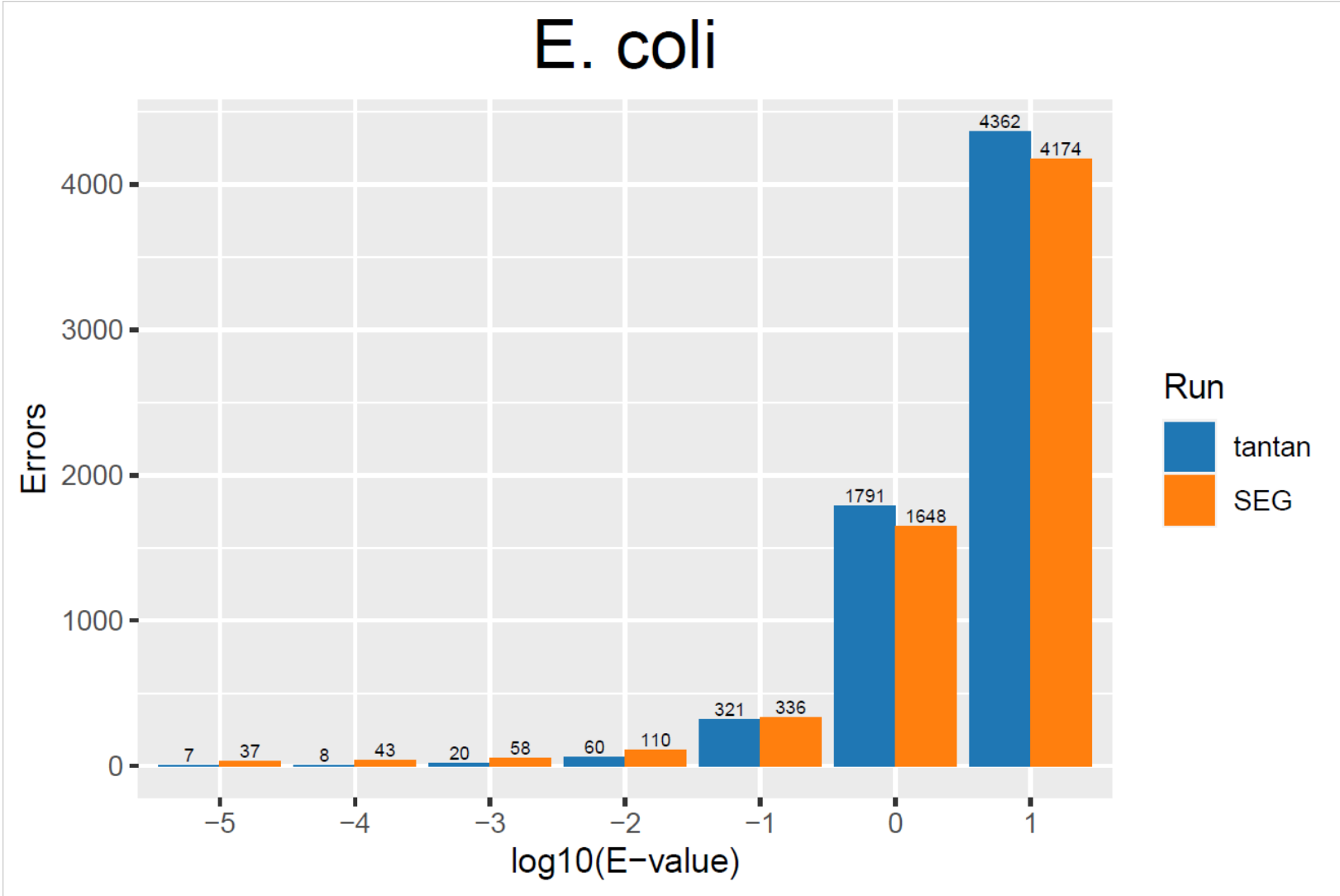
Examples of reversed alignments

```
Query= XP_018736024.1 DNA-binding transcription factor YAP1 [Sugiyamaella
lignohabitans]

Length=561

>\XP_027639320.1 plectin [Falco peregrinus]
Length=6846

 Score = 64.7 bits (156),  Expect = 7e-07, Method: Composition-based stats.
 Identities = 43/146 (29%), Positives = 71/146 (49%), Gaps = 6/146 (4%)

Query  66    ETPSPNNTFSLPEKRKIASPDRENSEEPVQVKSEANTESDNIKSKAAKADASSTVSSSKV  125
             E  +    FSL + +  A   R+  E+ VQ++ E   E+  ++ +A +A        +
Sbjct  5209  ELQATKEAFSLRKSQLEAEASRQAVEQAVQIQREKEVEAQRMRREAEEAQLRLKELDAVA  5268

Query  126   AAAAAAAAKKAGRKPEEKEPENKRKAQNRAAQRAFRE------RKERHLKELEDRVLQLE  179
                   AA ++A  K + +E   ++K      QR  RE      RK+R   E   R+ QLE
Sbjct  5269  KQKERAADREAQVKRKLEEEAERKKQSEEKVQRRLREAEEQAQRKQREADEARARLEQLE  5328

Query  180   NEATATNDENNFLRLQVERLQEELKK  205
              EA+A   E N L+L++ R++EE+KK
Sbjct  5329  TEASARQKETNELQLRIVRIEEEVKK  5354
```

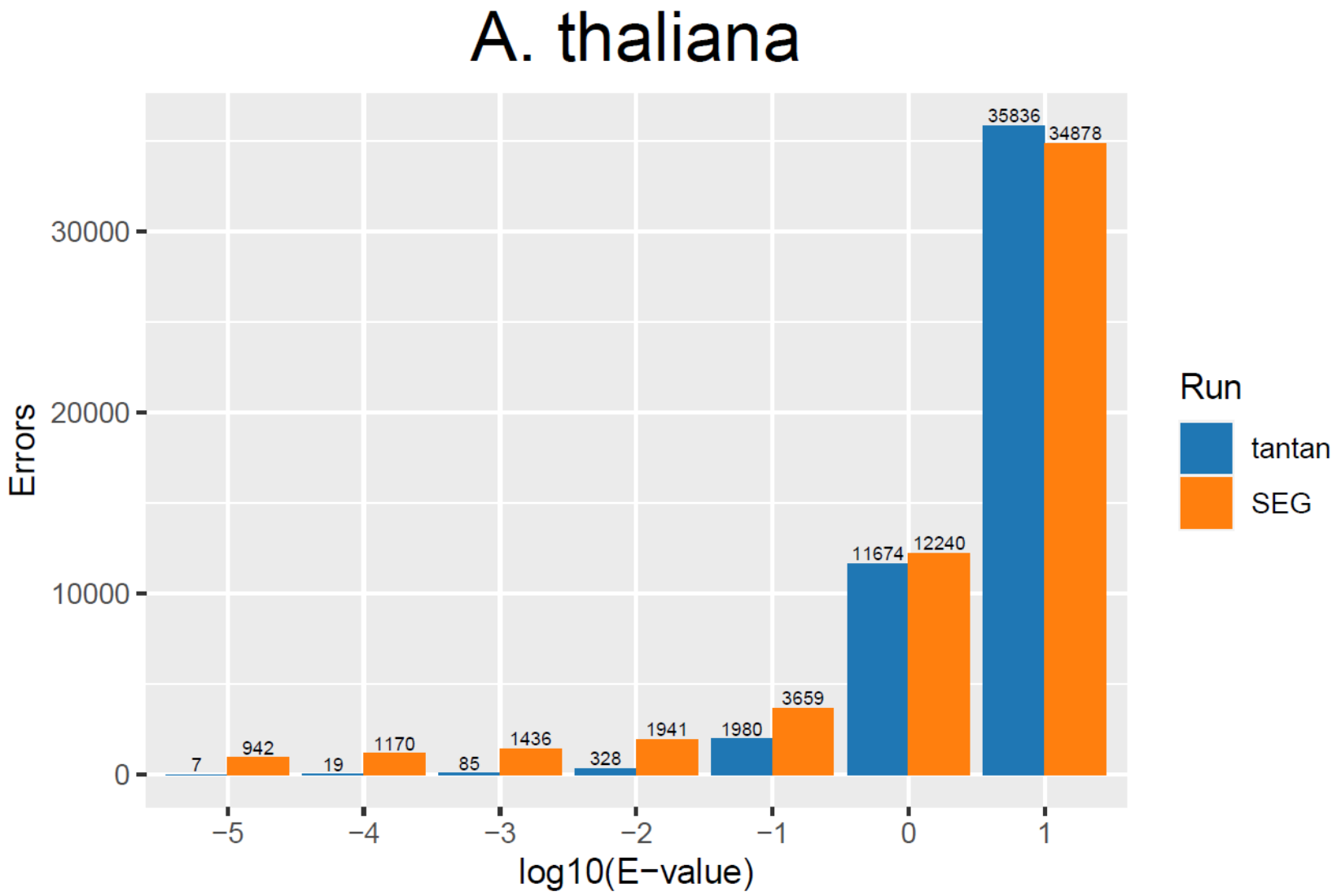
Error benchmark

- Database: UniRef50 processed by DecoyPyrat (Wright, 2016).
- Query: E. coli, A. thaliana (TAIR10) reference assemblies
- Diamond in very-sensitive mode

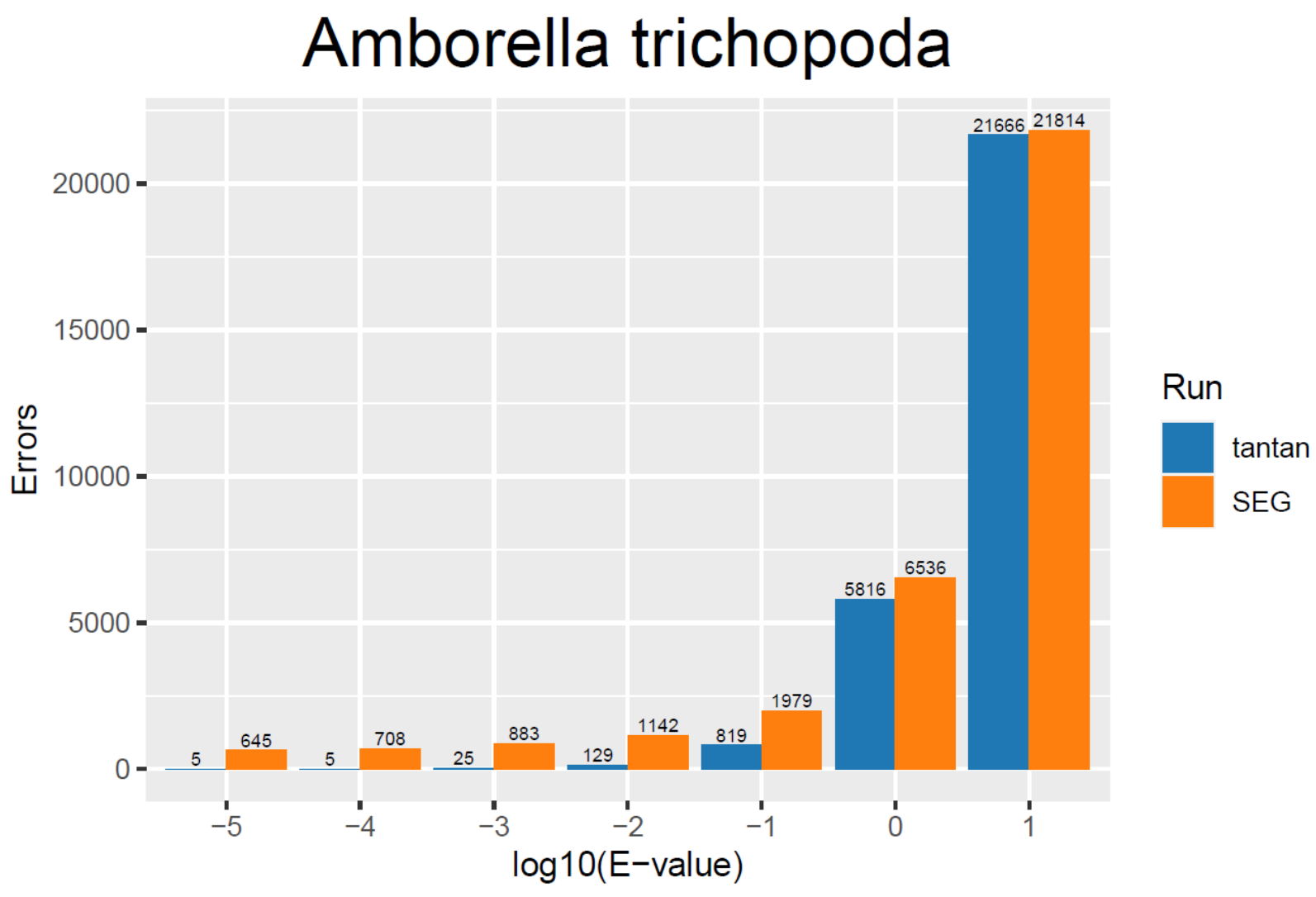
Queries with errors



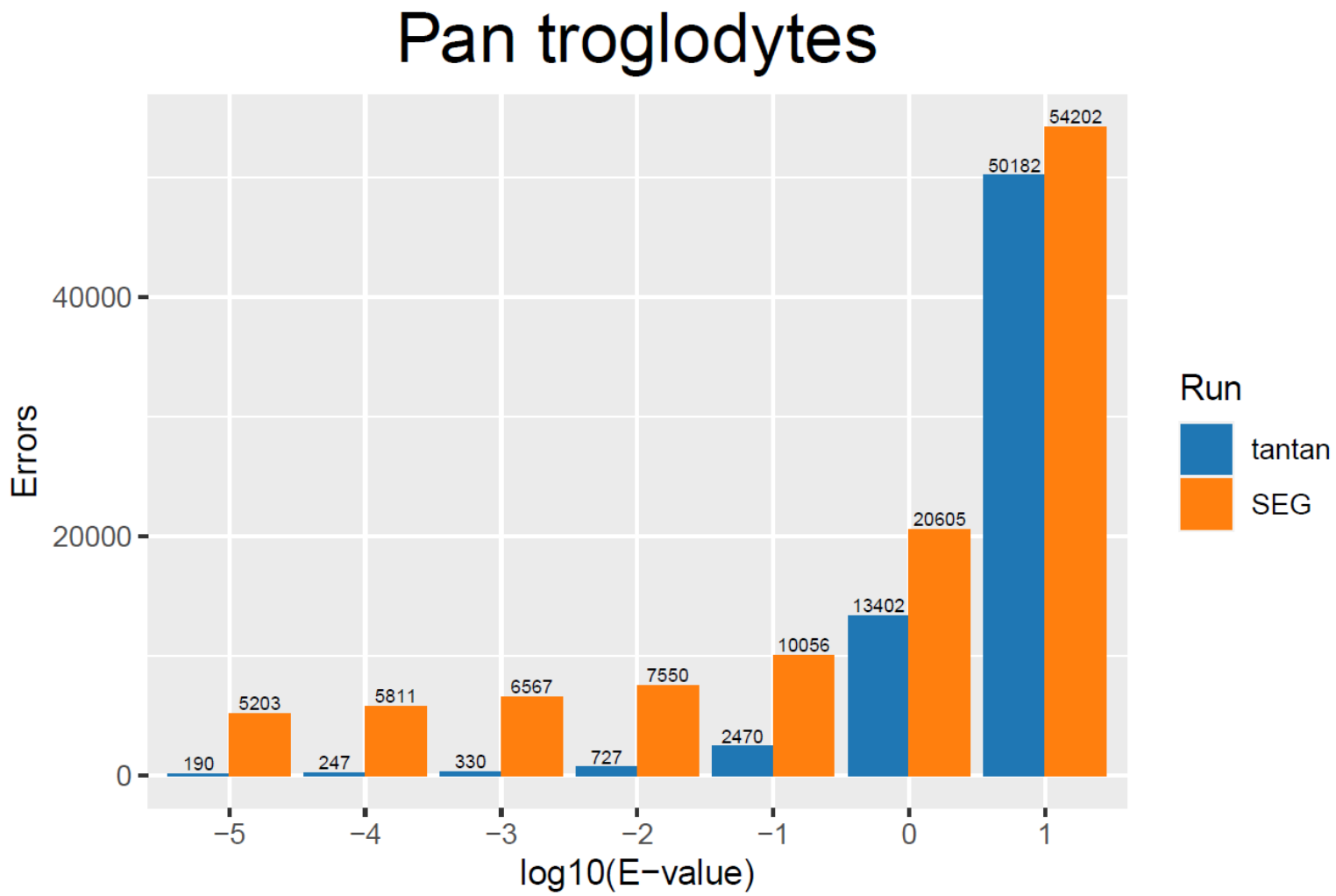
Queries with errors



Queries with errors



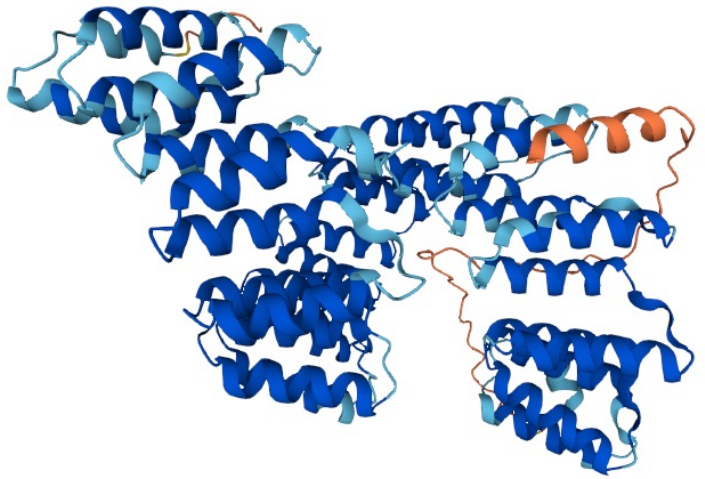
Queries with errors



Examples

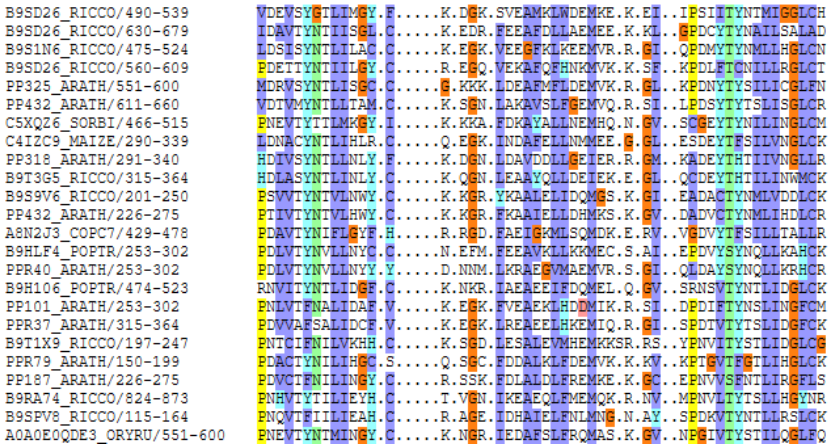
Pentatricopeptide repeat-containing protein At1g12775, mitochondrial

MVRMMIRRLSSQASRFVQPRLLETGTLRIALINCPNELLFCCERGFSTFS
DRNLSYRDKLSSGLVGIKADDAVDLFRDMIQSRPLPTVIDFNRLFSAIAK
TKQYELVLALCKQMESKGIAHSIYTLSIMINCFCRCRKLSYAFSTMGKIM
KLGYEPDTVIFNTLLnglclecrvsealelvdrmvemghkptlitlntlv
nglclngkvsdavvlidrmvetgfqpnevtygpvlnvmcksgqtalamel
lrkmeernikldavkysiiidglckdgsldnafnlfnemeikgfkadiit
yntliggfcnagrwddgakllrdmikrkispnvvtfsvlidsfvkegklr
eadqllkemmqrgiapntitynslidgfckenrleeaiqmvdlmiskgcd
pdimtfnilingyckanriddglelfremslrgviantvtyntlvqgfcq
sgklevakklfqemvsrrvrpdivsykilldglcdngelekaleifgkie
kskmeldigiymiiihgmcnaskvddawdlfcslplkgvkldaraynimi
selcrkdslskadilfrkmteeghapdeltynilirahlgdddattaael
ieemkssgfpadVSTVKMVINMLSSGELDKSFLDMLSTTRASLK



PFAM: PPR repeat family

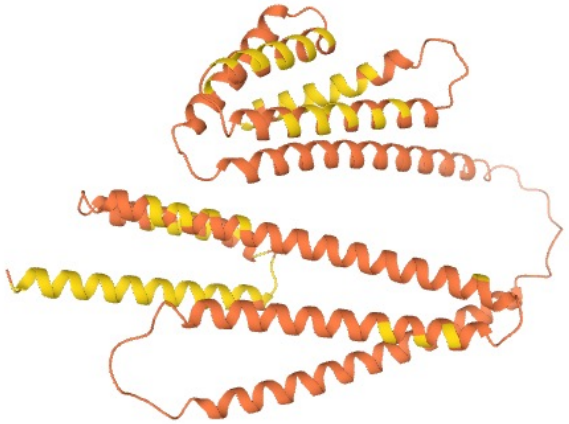
This repeat has no known function. It is about 35 amino acids long and is found in up to 18 copies in some proteins. The family appears to be greatly expanded in plants and fungi.



Examples

Late embryogenesis abundant domain-containing protein

MMERRRTALVLFVVVVVLTWQEGVLGKWLESTAKEKTGSWAGWVSDKITT
GFGTKKEETGIYQKSKDEARKAAQAAENYAYDKANyvkdsaydnagyakd
faenkaeyakdfaydkagdakdmayekaghakdfaydkagnakdmayeta
gyakdfaydkggyakdvaydkagnakdmayekagnakdmayekaehikdf
tydkVGSAygsaqsmmdsgydkagdakdmayekaGIVkdMAydkagdakd
vayekagiakdmaydkagnakdmaydkvGSAYGSAQkakdsgyekageak
dyaykkagnakdiayekaqdakdfaydkaGYGydkagdvirmatdksgea
yegakeksksakdTAGEAMDDSIDYMkdkshnakdgatrgfeeamekvge
kygvakestkyayetakkkaSQVAGEIRDRYAEL



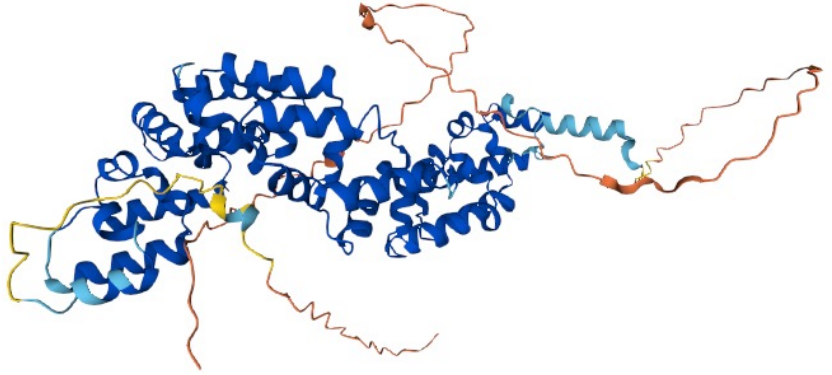
PFAM: Late embryogenesis abundant protein

Different types of LEA proteins are expressed at different stages of late embryogenesis in higher plant seed embryos and under conditions of dehydration stress. The function of these proteins is unknown.

Examples

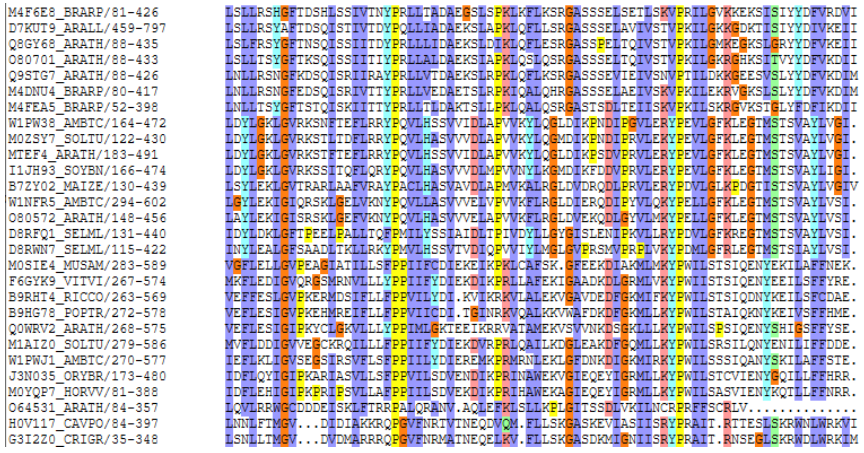
Transcription termination factor MTERF4, chloroplastic

MKIRFCNGFTKPGFLLVHFEPPSFFAVRSRSLSDSTYGNLCNHKKRPGTG
IGLTVQCAIANRRFSSRSLDSPRRERSSRssssssgrdrdrdkdkGRDSKS
LYSRPSLLDMNKEKAANRAKVYEFLRGIGIVPDELDGLELPVTADVMKER
VEFLHKLGLTIEDINNYPLVLGCSVKKNMVPVLDYLGKLGVRKSTFTEFL
Rrypqvlhssvvidlapvvkylqgldikpsdvprvlerypevlgfklegt
mstsvaylvgigvarreiggiltrypeilgmrvariikplveylevlgip
rlaaarliekrphilgfelddtvkpnvqilqdfnvretslpsiiaqypei
igidLKPKLDTQRKLLCSAIHLNPEDLGSLIERMPQFVSLSESPMLKHID
FLTKCGFSIDQTREMVIGCPQVLALNLGIMKLSFEYFQKEMKRPLQDLVD
FPAFFTYGLESTVKPRHKKIIKKGIKCSLAWMLNCSDEKFEQRMSYDTID
IEEVETDPSSFDMNTLMQPEREeesdseyeeeeddddEEFA



PFAM: mTERF

This family contains one sequence of known function Human mitochondrial transcription termination factor (mTERF) the rest of the family consists of hypothetical proteins none of which have any functional information. mTERF is a multizipper protein possessing three putative leucine zippers one of which is bipartite. The protein binds DNA as a monomer. The leucine zippers are not implicated in a dimerisation role as in other leucine zippers [1].



Rost, 1999

Twilight zone of protein sequence alignments

Burkhard Rost[1,2,3]

[1]EMBL, 69 012 Heidelberg, [2]LION Bioscience AG, Im Neuenheimer Feld 517, 69 120 Heidelberg, Germany and [3]Columbia University, Department of Biochemistry and Molecular Biophysics, 650 West 168 Street, New York, NY 10032, USA

Sequence alignments unambiguously distinguish between protein pairs of similar and non-similar structure when the pairwise sequence identity is high (>40% for long alignments). The signal gets blurred in the twilight zone of 20–35% sequence identity. Here, more than a million sequence alignments were analysed between protein pairs of known structures to re-define a line distinguishing between true and false positives for low levels of similarity. Four results stood out. (i) The transition from the safe zone of sequence alignment into the twilight zone is described by an explosion of false negatives. More than 95% of all pairs detected in the twilight zone had different structures. More precisely, above a cut-off roughly corresponding to 30% sequence identity, 90% of the pairs were homologous; below 25% less than 10% were. (ii) Whether or not sequence homology implied structural identity depended crucially on the alignment length. For example, if 10 residues were similar in an alignment of length 16 (>60%), structural similarity could not be inferred. (iii) The 'more similar than identical' rule (discarding all pairs for which percentage similarity was lower than percentage identity) reduced false positives significantly. (iv) Using intermediate sequences for finding links between more distant families was almost as successful: pairs were predicted to be homologous when the respective sequence families had proteins in common. All findings are applicable to automatic database searches.

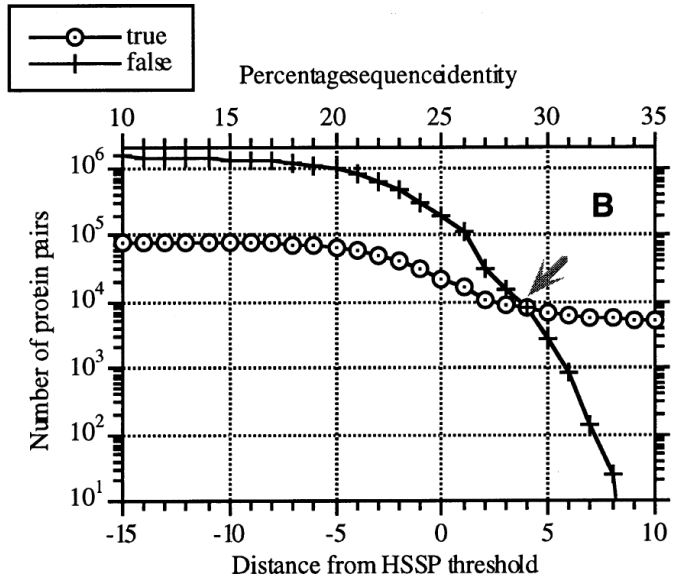


Fig. 2. Explosion of structurally dissimilar pairs in the twilight zone.

Distance to HSSP threshold

The HSSP-curve was originally defined by (Sander and Schneider, 1991):

$$p^I(n) = n + \begin{cases} 290.15 \cdot L^{-0.562}, & \text{for } L < 80 \\ 25 \quad , & \text{doe } L \geqslant 80 \end{cases} \qquad (1)$$

where L gave the number of residues aligned between two proteins; p^I the cut-off percentage of identical residues over the L aligned residues; and n described the distance in percentage points from the curve ($n = 0$ corresponds to the original HSSP-curve; $n = 5$ to the official HSSP database releases; curve plotted in Figure 3). Once Schneider and Sander

Rost, 1999

```
Query= A0A011

Length=281

>A0A023GPI5
Length=231

 Score = 22.7 bits (47),  Expect = 95.7
 Identities = 31/124 (25%), Positives = 39/124 (31%), Gaps = 25/124 (20%)

Query  109  VRGADALLLPALLGSGDDYFVWKSFLETLAAFPGRIPREEWPELLLTVALT-------FG 161
            VRGA   +  AL  + D+  ++  F+ T     G   R E P  L             FG
Sbjct   39  VRGATLAMPGALDRADDETLIYPLFMAT-----GWFTRSELPRRLALAGAPKARILPPFG 93

Query  162  EDPRTGDLLGTVPVSTASTEEID----RYLHVARAFGFHMVYLYSRNEHVPPEVVRHFRK 217
             DP    L   +    A T+       R L  A   G             P E  R
Sbjct   94  SDPGLPALCLALIAQAAETQGWPLAGTRLLVAAHGSG---------RSRAPSEAARRIAA 144

Query  218  GLGP 221
            GL P
Sbjct  145  GLAP 148
```

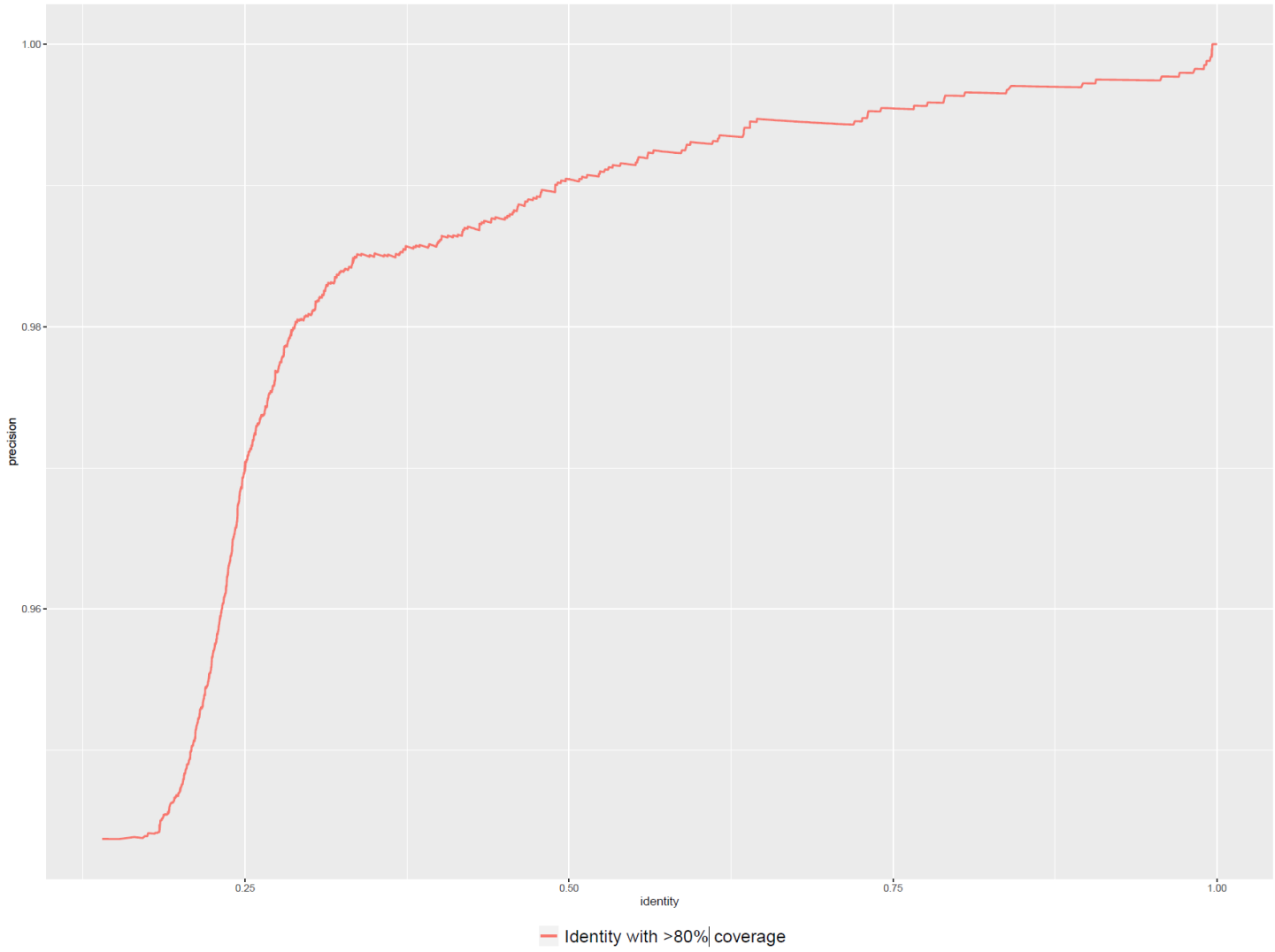
CATH annotation of PDB sequences

187,170 structures in the PDB, 128,000 with CATH fold annotation, corresponding to 35,713 primary sequences from UniProt.
14,987 of these sequences are annotated with CATH domains over >70% of their range.

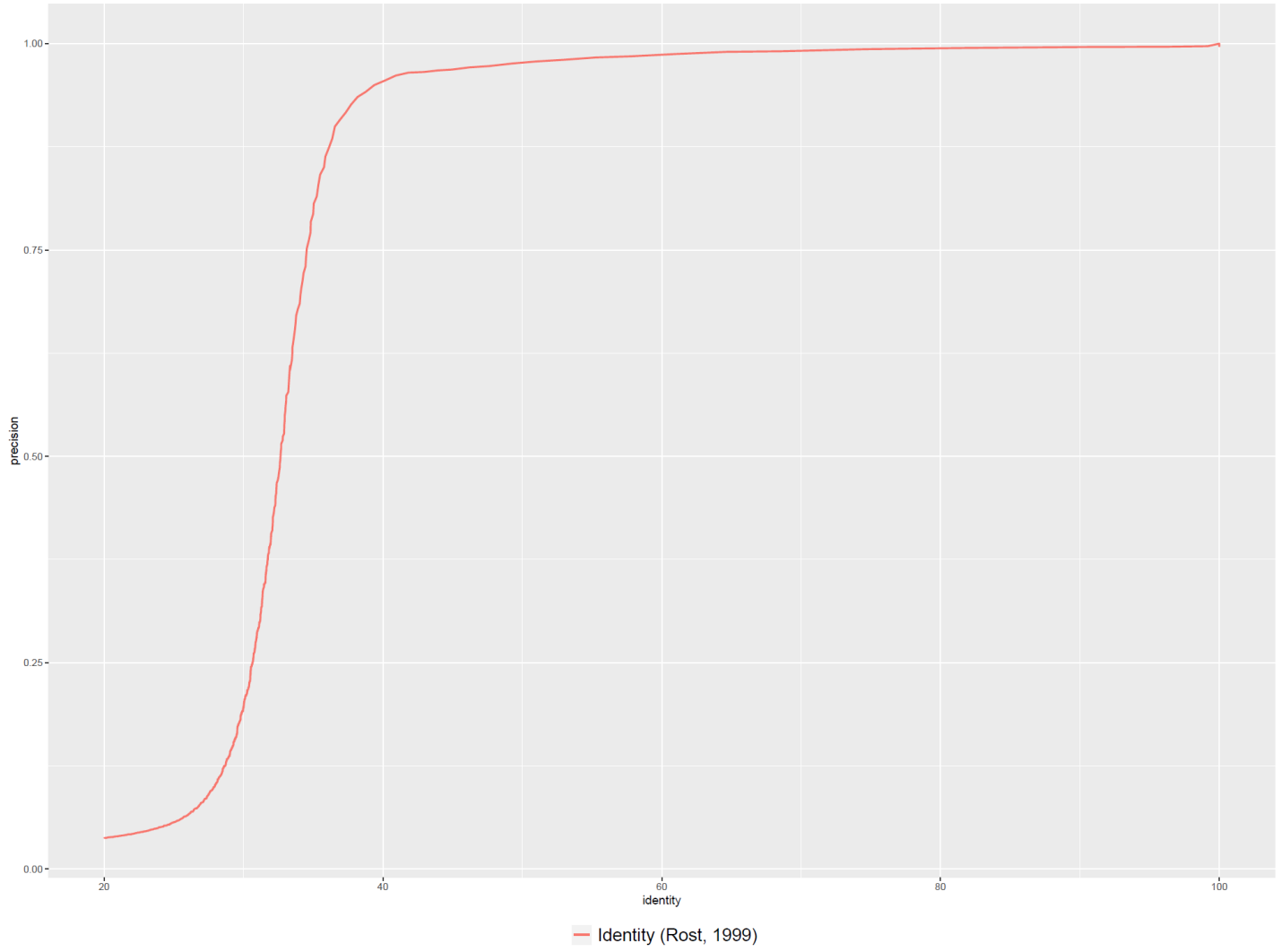
Clustering at 25% identity results in a query set of 3,138 sequences.

Positives := Sequence pairs with identical CATH domain architecture

CATH annotation of PDB sequences



Rost, 1999


1.00
0.75
0.50
0.25
0.00
precision
20
40
60
80
100
identity
Identity (Rost, 1999)

Vector instructions

Count the matching amino acids:

…ITVTAGATEALYAAITALVRNGDEVICFDPSYDSYAPAIALSGGIVKR…
…VVITPGSSGGFLLAFTALFDSGDRVGIGAPGYPSYRQILRALGLVPVD…

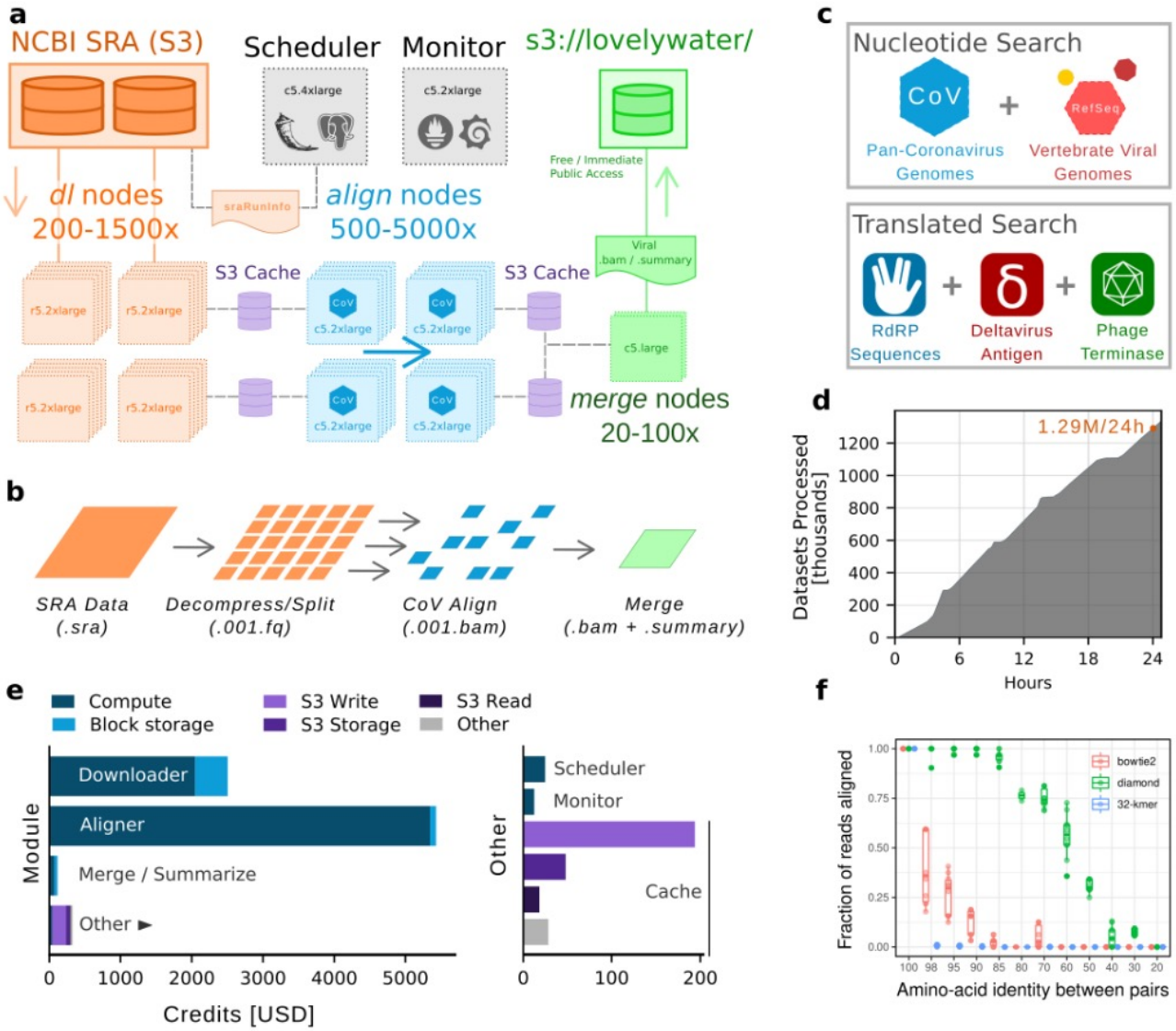
Naïve solution (~15ns):

```
int n = 0;
for(int i = 0; i < 48; ++i)
          if(a[i] == b[i]) ++n;
```

Using AVX2 instructions (~0.58ns)

```
_popcnt32(_mm256_movemask_epi8(_mm256_cmpeq_epi8(a, b)))
```

Project Serratus (http://serratus.io) (Edgar et al., Nature 2021)



Project Serratus (http://serratus.io)

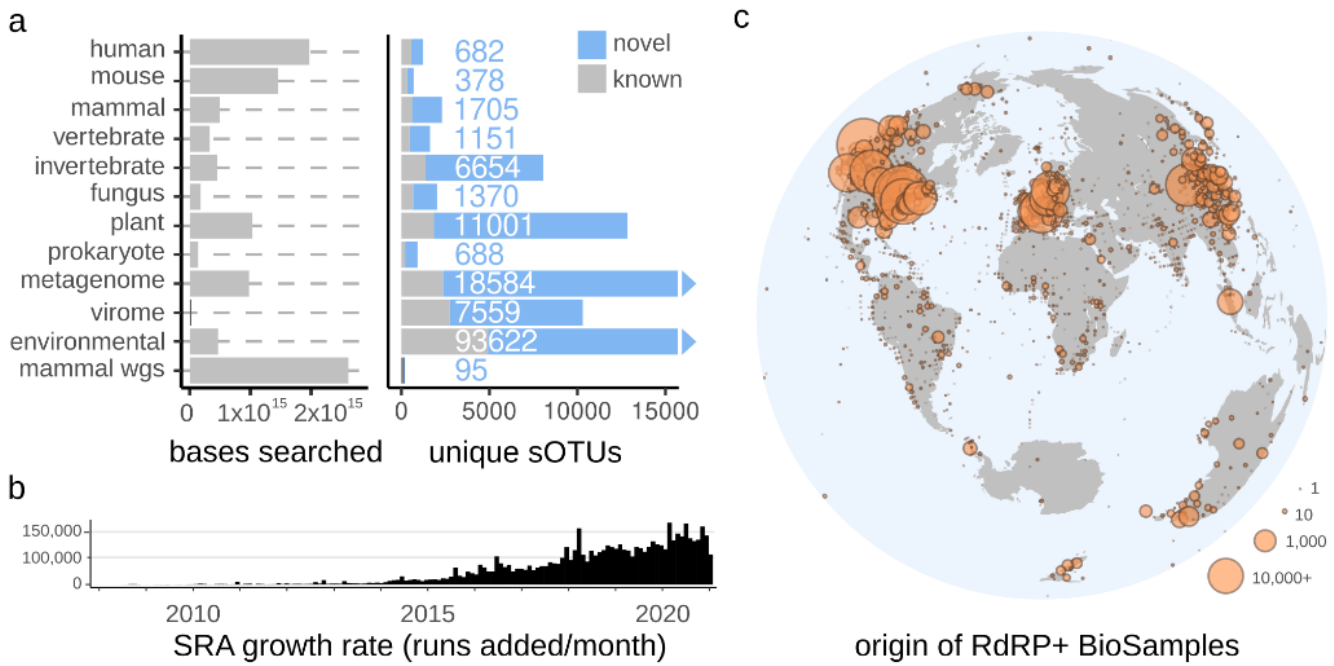


Figure 1: **Searching the planetary virome**

a Total bases searched from the 5,686,715 SRA sequencing runs analysed in the viral RdRP search grouped by sample taxonomy, where available (see Extended Figures 1 and 3, and Extended Table 1). 8,871/15,016 (59%) of known RdRP species-like operational taxonomic units (sOTUs) were observed in the SRA, and 131,957 unique and novel RdRP sOTUs were identified (see Extended Figure 2). sOTUs identified in multiple taxonomic groups are counted in each group separately, numbers shown indicate the number of novel sOTUs in each group. **b** Release dates of the runs included in the analysis reflecting the growth rate of available data. **c** Sample locations for 635,656 RdRP-containing contigs (27.8% of samples lacked geographic metadata). The high density of RdRP seen in North America, Western Europe and Eastern Asia reflects the substantial acquisition bias for samples originating from these regions. Interactive map is available at `https://serratus.io/geo`.

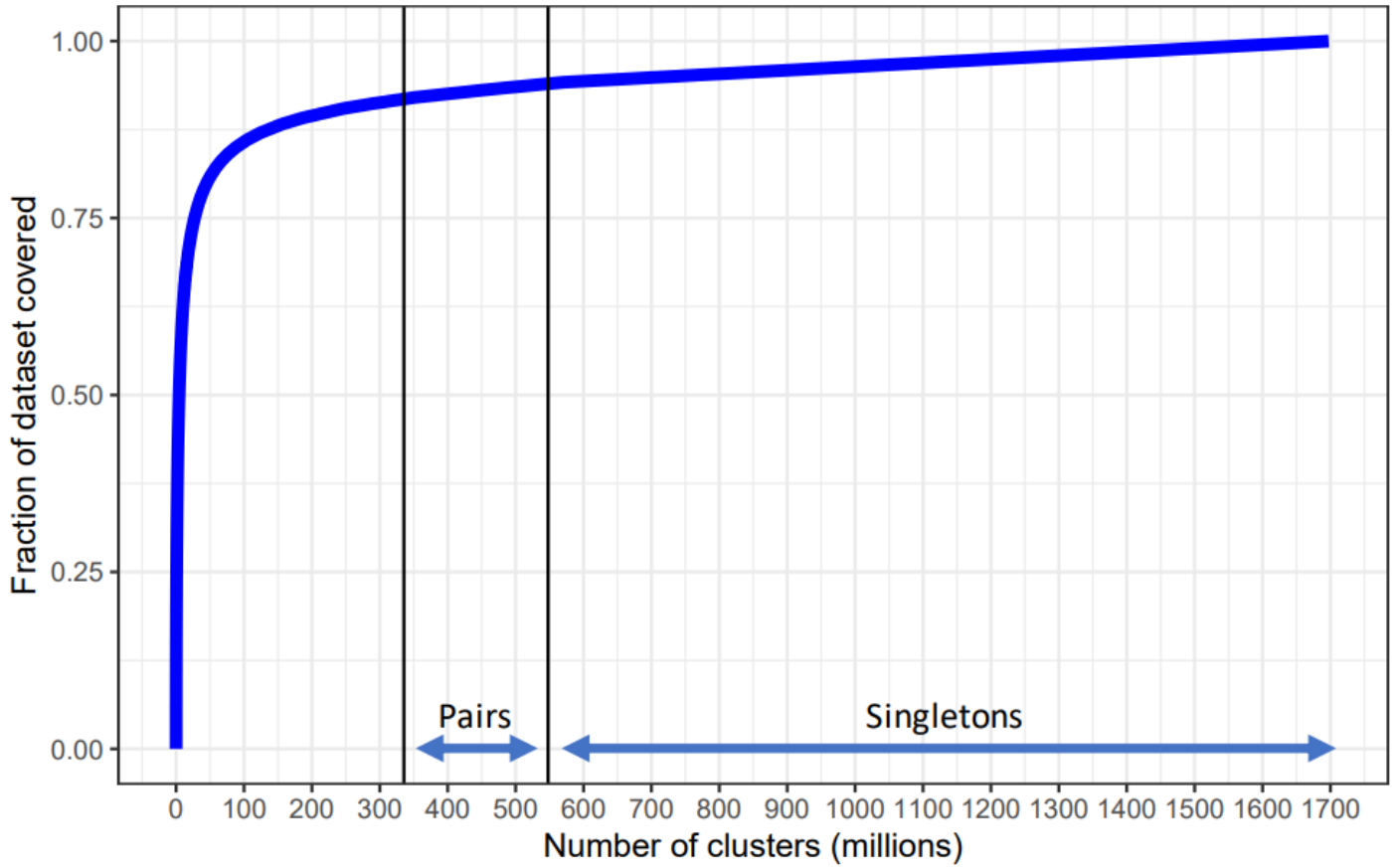
DIAMOND DeepClust

Database name	Number of sequences	Download date/release
IMG Environmental Aquatic Metagenomes	6,232,188,951	March 2022
IMG Environmental Non-Aquatic Metagenomes	6,851,118,226	March 2022
IMG Host-Associated Metagenomes	1,905,657,909	March 2022
IMG Engineered Metagenomes	801,972,930	March 2022
SRC	2,022,891,389	March 2022
MGnify	1,977,479,951	2022_05
metaclust	1,757,323,526	March 2022
NCBI NR	465,406,186	April 2022
AGNOSTOS	427,306,945	Ver 5
MERC	292,137,902	March 2022
MetaEuk	12,111,301	2019_11
SMAGs	10,207,435	v1
TOPAZ	8,405,914	v1
GPD	7,581,807	April 2022
NovelFams	4,587,583	March 2022
MGV	11,837,198	v1.0
Total	**22,788,215,153**	

	Alphafold2 BFD	DIAMOND run
Input sequences	2,423,213,294	22,788,215,153
Deduplicated sequences	2,423,213,294	19,387,935,704
Clusters	345,159,030	1,697,446,279
Singleton clusters		1,149,664,058
Clusters ≥3 members	61,083,719	335,433,899

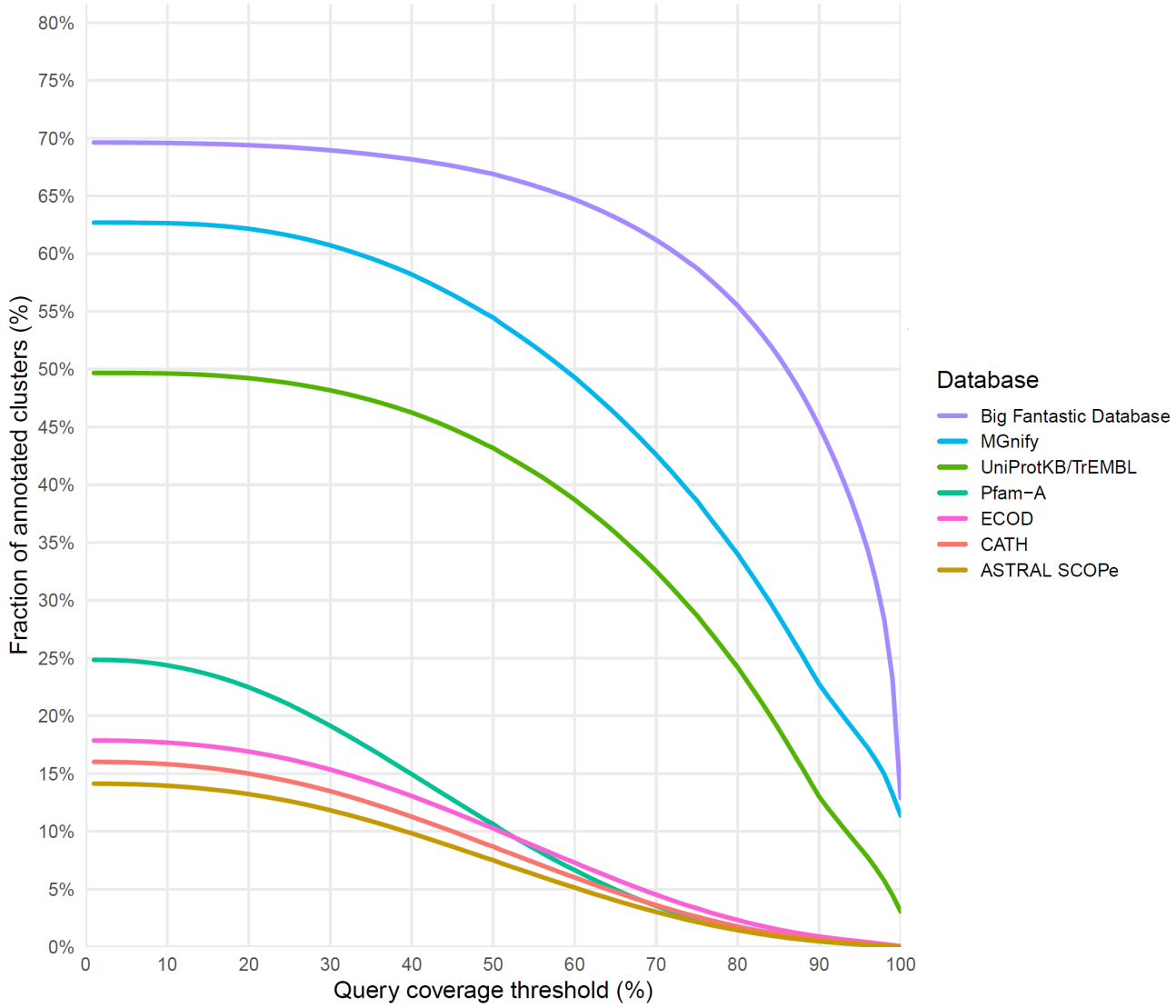
Time: ~250,000 CPU hours

DIAMOND DeepClust

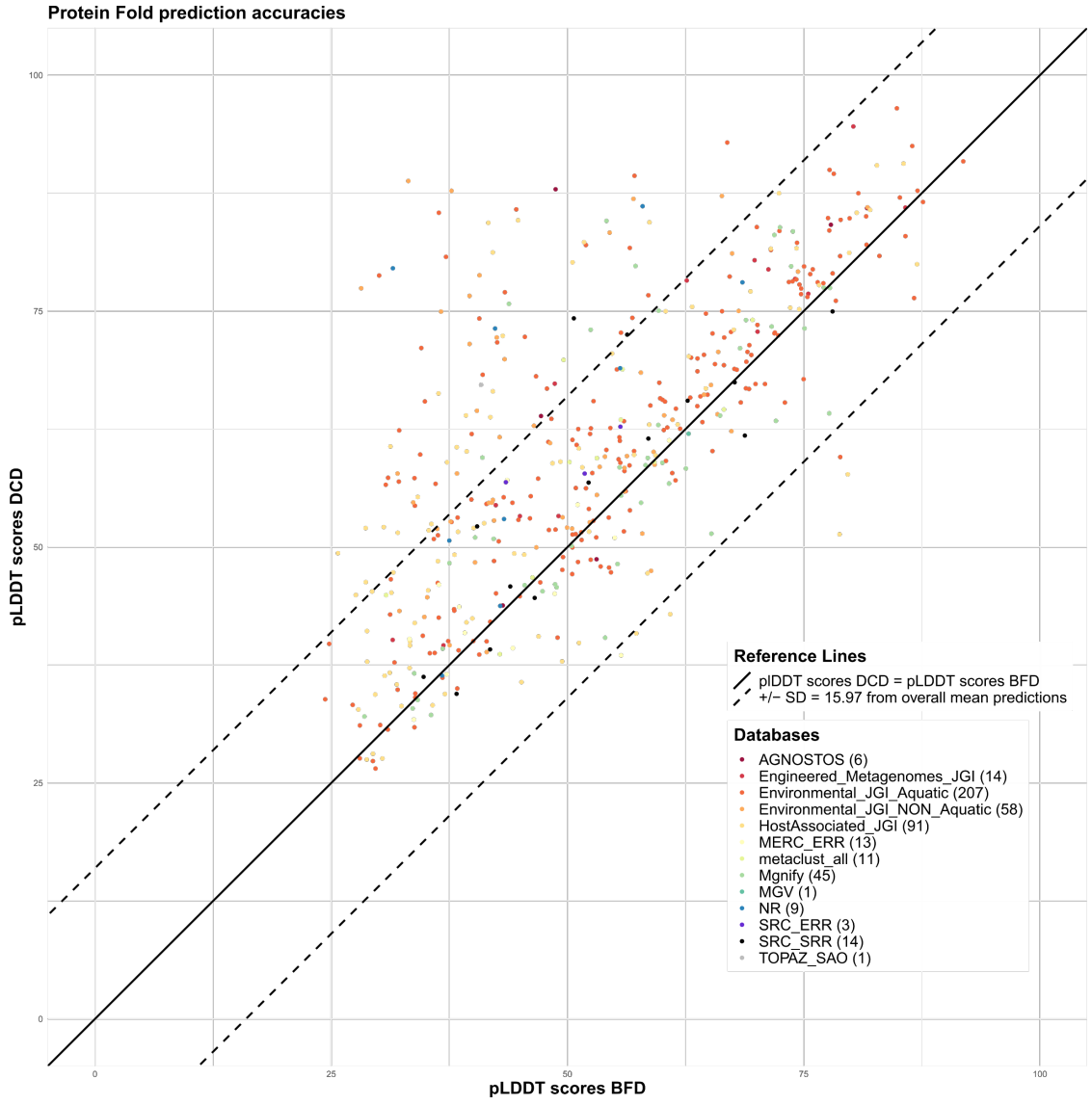


Supplementary fig. 10 | Cluster representation for the experimental study. Shown is the number of clusters generated with DIAMOND DeepClust against the fraction of the 19.4 billion input proteins that are covered by the clustering. The vertical lines indicate the start of clusters of size two and one (from left to right). The result illustrates that ~335 million representatives can capture 92% (~17.48 billion sequences) of the protein universe that comprises 19 billion sequences.

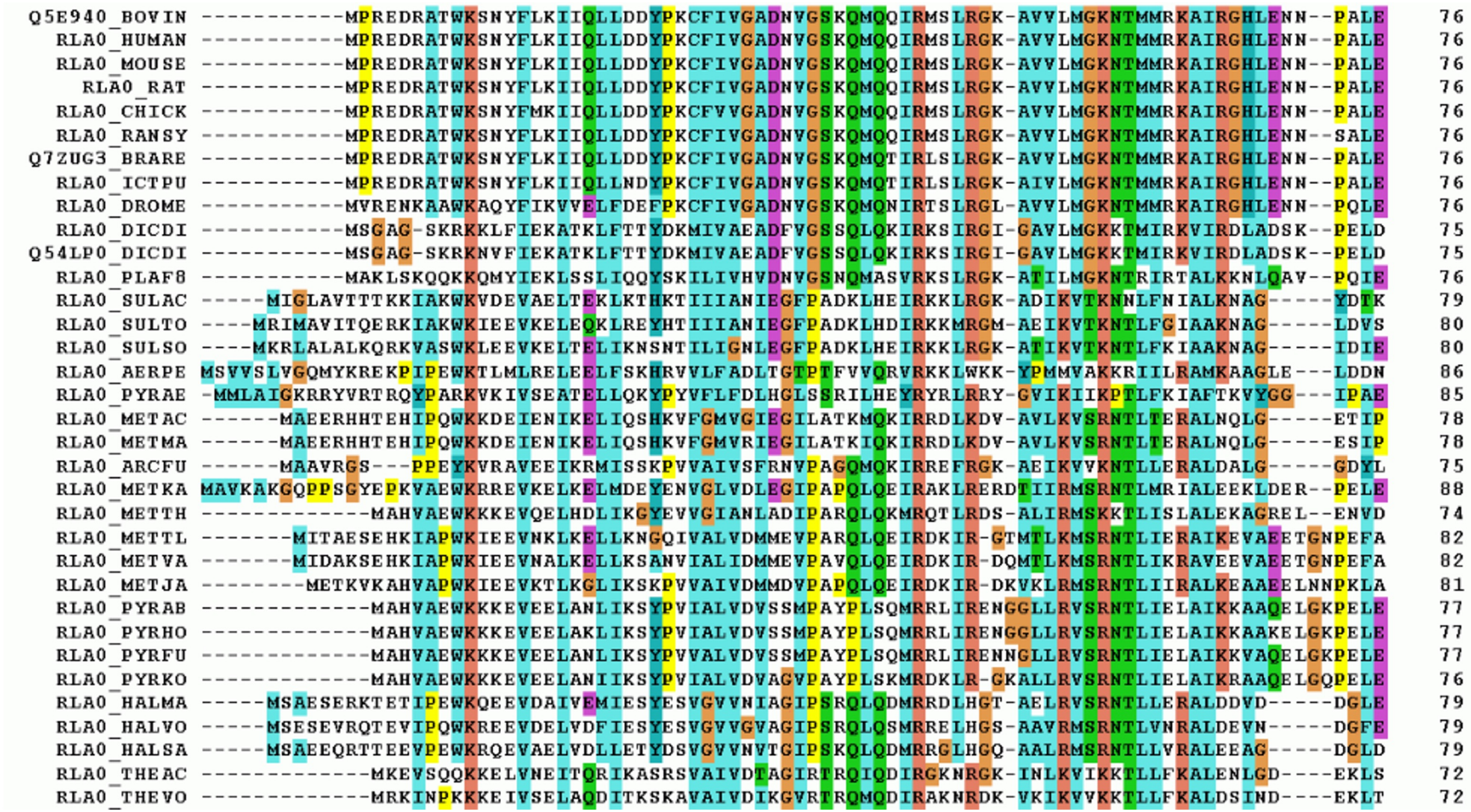
DIAMOND DeepClust



DIAMOND DeepClust



Multiple Sequence Alignment

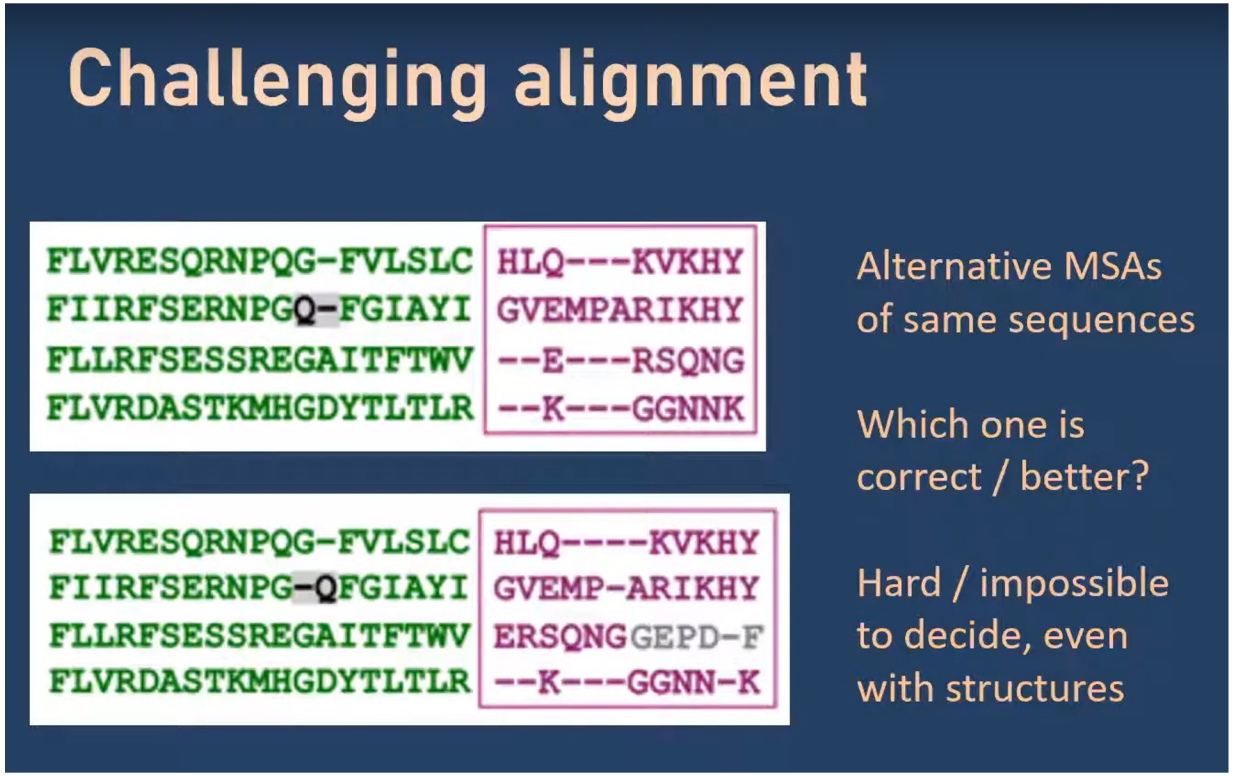


Multiple Sequence Alignment

- Comparative genomics
- Phylogeny
- Protein structure prediction
- RNA structure and function
- …

Multiple Sequence Alignment

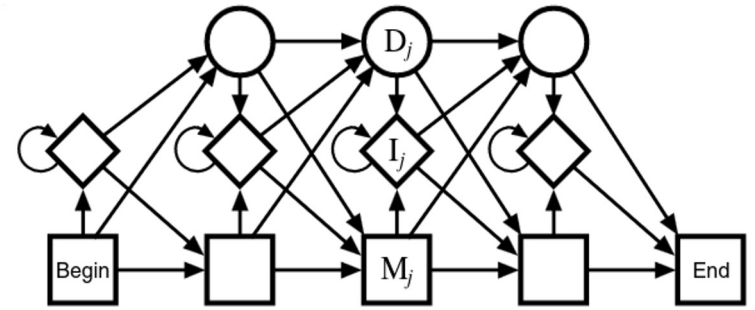
- MUSCLE
- ClustalW
- T-Coffee
- MAFFT



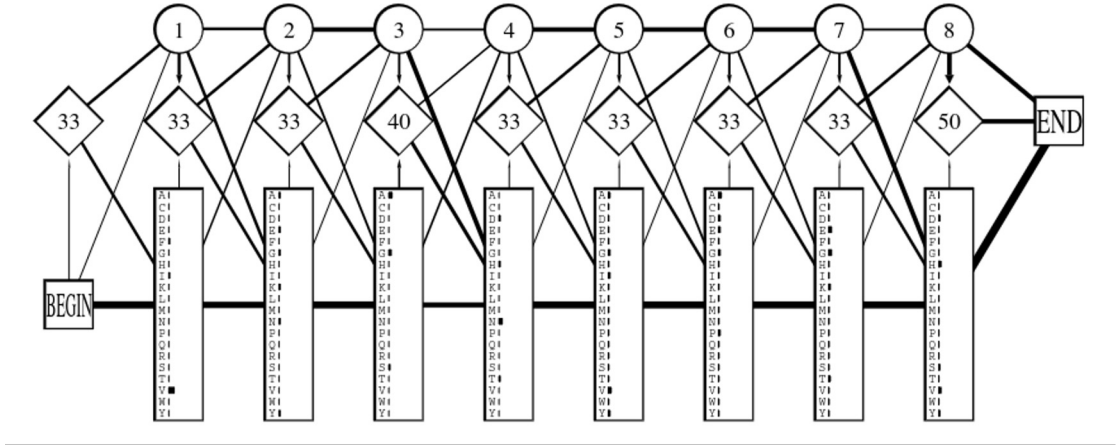
https://www.youtube.com/watch?v=2HmjHStpu7I

HMM profiles

Tools: HMMER, HHblits



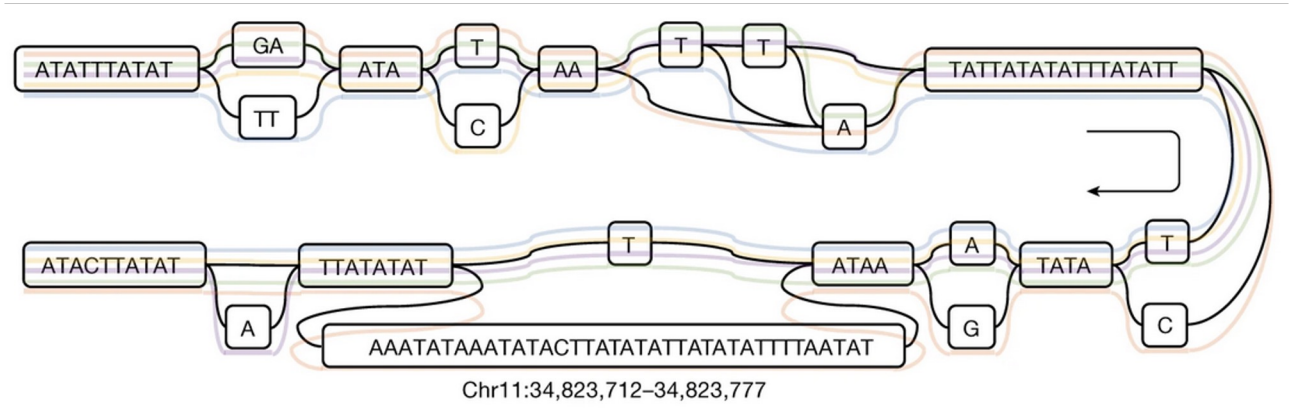

```
HBA_HUMAN    ...VGA--HAGEY...
HBB_HUMAN    ...V----NVDEV...
MYG_PHYCA    ...VEA--DVAGH...
GLB3_CHITP   ...VKG------D...
GLB5_PETMA   ...VYS--TYETS...
LGB2_LUPLU   ...FNA--NIPKH...
GLB1_GLYDI   ...IAGADNGAGV...
                ***  *****
```



http://www.mcb111.org/w06/durbin_book.pdf

Multiple DNA

- Wayyy longer sequences
- Duplications, inversions, and translocations wreak linearity
- Tools: SibeliaZ, Cactus
- State of the art: human genome graphs, look for pangenomics papers.



AI

- More and more important.
- Main issue in biology: overfitting, limited training data
- Very easy to game any given benchmark.
- Something looks good on paper does not mean it will perform in the real world.
- Be careful and critical.

Und es rief der Herr von Sachsen,
der von Bayern, der vom Rhein:
"Graf im Bart, Ihr seid der Reichste!
Euer Land trägt Edelstein!„
Lied der Württemberger, Justinus Kerner (1818)

Thank you!

- Contact me: buchfink@gmail.com
- https://github.com/bbuchfink/diamond
- Join my discord