Algorithms in Bioinformatics: A Practical Introduction

Multiple Sequence Alignment

Multiple Sequence Alignment

- Given k sequences $S = \{S_1, S_2, ..., S_k\}$.
- A multiple alignment of S is a set of k equal-length sequences $\{S'_1, S'_2, ..., S'_k\}$.
  - where S'_i is obtained by inserting gaps in to S_i.
- The multiple sequence alignment problem aims to
  - find a multiple alignment which optimize certain score.

Example: multiple alignment of 4 sequences

- S_1 = `ACG--GAGA`
- S_2 = `-CGTTGACA`
- S_3 = `AC-T-GA-A`
- S_4 = `CCGTTCAC-`

Applications of multiple sequence alignment

- Align the domains of proteins
- Align the same genes/proteins from multiple species
- Help predicting protein structure

Sum-of-Pair (SP) Score

- Consider the multiple alignment S' of S.
- SP-score($a_1, \ldots, a_k$) = $\Sigma_{1 \le i < j \le k}\ \delta(a_i, a_j)$
  - where a_i can be any character or a space.
- The SP-score of S' is
  - Σ_x SP-score($S'_1[x], \ldots, S'_k[x]$).

Example: multiple alignment of 4 sequences

- S_1 = `ACG--GAGA`
- S_2 = `-CGTTGACA`
- S_3 = `AC-T-GA-A`
- S_4 = `CCGTTCAC-`
- Assume score of
  - match and mismatch/insert/delete are 2 and -2, respectively.
- For position 1,
  - SP-score(A,-,A,C) = $2\delta(A,-) + 2\delta(A,C) + \delta(A,A) + \delta(C,-) = -8$
- SP-score= $-8+12+0+0–6+0+12–10+0 = 0$

Sum-of-Pair (SP) distance

- Equivalently, we have SP-dist.

- Consider the multiple alignment S' of S.
- SP-dist(a_1, ..., a_k) = $\Sigma_{1 \le i < j \le k}\ \delta(a_i, a_j)$
  - where a_i can be any character or a space.
- The SP-dist of S' is
  - Σ_x SP-dist($S'_1[x]$, ..., $S'_k[x]$).

Agenda

- Exact result
  - Dynamic Programming
- Approximation algorithm
  - Center star method
- Heuristics
  - ClustalW --- Progressive alignment
  - MUSCLE --- Iterative method

Dynamic Programming for aligning two sequences

- Recall that the optimal alignment for two sequences can be found as follows.
- Let $V(i_1, i_2)$ be the score of the optimal alignment between $S_1[1..i_1]$ and $S_2[1..i_2]$.

$$V(i_1,i_2) = \max \begin{cases} V(i_1-1, i_2-1) + \delta(S_1[i_1], S_2[i_2]) \\ V(i_1-1, i_2) + \delta(S_1[i_1], \_) \\ V(i_1, i_2-1) + \delta(\_, S_2[i_2]) \end{cases}$$

- The equation can be rephased as

$$V(i_1,i_2) = \max_{(b_1,b_2)\in\{0,1\}^2-\{(0,0)\}} \left\{ V(i_1-b_1, i_2-b_2) + \delta(S_1[i_1b_1], S_2[i_2b_2]) \right\}$$

Dynamic Programming for aligning k sequences (I)

- Let $V(i_1, i_2, \ldots, i_k)$ = the SP-score of the optimal alignment of $S_1[1..i_1], S_2[1..i_2], \ldots, S_k[1..i_k]$.

- Observation: The last column of the optimal alignment should be either $S_j[i_j]$ or '-'.
- Hence, the score for the last column should be SP-score$(S_1[b_1 i_1], S_2[b_2 i_2], \ldots, S_k[b_k i_k])$
  - For $(b_1, b_2, \ldots, b_k) \in \{0,1\}^k$.
  - (Assume that $S_j[0]$ = '-'.)

Dynamic programming for aligning k sequences (II)

- Based on the observation, we have
- $V(i_1, i_2, \ldots, i_k) = \max_{(b1, b2, \ldots, bk) \in \{0,1\}k}$
  $\{ V(i_1-b_1, \ldots, i_k-b_k) +$
  $\text{SP-score}(S_1[b_1 i_1], \ldots, S_k[b_k i_k]) \}$
- The SP-score of the optimal multiple alignment of $S=\{S_1, S_2, \ldots, S_k\}$ is
  - $V(n_1, n_2, \ldots, n_k)$
  - where n_i is the length of S_i.

Dynamic Programming for aligning k sequences (III)

- By filling-in the dynamic programming table,
  - We compute $V(n_1, n_2, ..., n_k)$.
- By back-tracing,
  - We recover the multiple alignment.

Complexity

- Time:
  - The table V has $n_1 n_2 ... n_k$ entries.
  - Filling in one entry takes $2^k k^2$ time.
  - Total running time is $O(2^k k^2 \; n_1 n_2 ... n_k)$.
- Space:
  - $O(n_1 n_2 ... n_k)$ space to store the table V.
- Dynamic programming is expensive in both time and space. It is rarely used for aligning more than 3 or 4 sequences.

Center star method

- Computing optimal multiple alignment takes exponential time.
- Can we find a good approximation using polynomial time?
- We introduce Center star method, which minimizes Sum-of-Pair distance.

Idea

- Find a string S_c.
- Align all other strings with respect to S_c.
- Illustrate by an example:

```
S1: CCTGCTGCAG
S2: GATGTGCCG
S3: GATGTGCAG
S4: CCGCTAGCAG
S5: CCTGTAGG
```

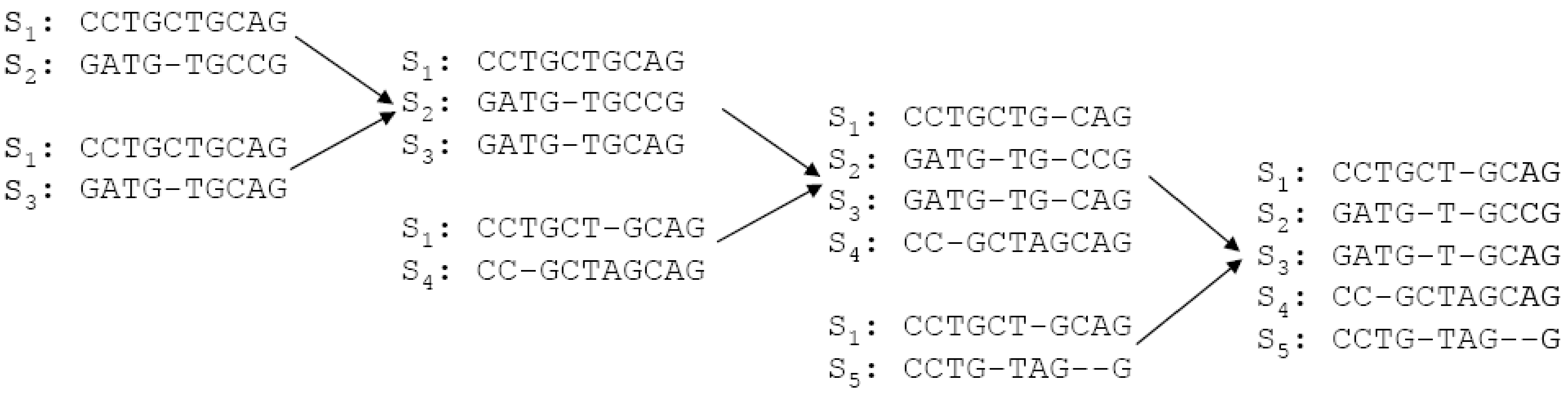
	S_1	S_2	S_3	S_4	S_5
S_1	0	4	3	2	4
S_2		0	1	6	5
S_3			0	5	5
S_4				0	4
S_5					0

$\Sigma_{i=1..k} D(S_1,S_i) = 13$
$\Sigma_{i=1..k} D(S_2,S_i) = 16$
$\Sigma_{i=1..k} D(S_3,S_i) = 14$
$\Sigma_{i=1..k} D(S_4,S_i) = 17$
$\Sigma_{i=1..k} D(S_5,S_i) = 18$

```
S1: CCTGCTGCAG
S2: GATG-TGCCG

S1: CCTGCTGCAG
S3: GATG-TGCAG

S1: CCTGCT-GCAG
S4: CC-GCTAGCAG

S1: CCTGCT-GCAG
S5: CCTG-TAG--G
```

```
S1: CCTGCT-GCAG
S2: GATG-T-GCCG
S3: GATG-T-GCAG
S4: CC-GCTAGCAG
S5: CCTG-TAG--G
```

Converting pair-wise alignment to multiple alignment



```
S1: CCTGCTGCAG
S2: GATG-TGCCG

S1: CCTGCTGCAG
S3: GATG-TGCAG
```

```
S1: CCTGCTGCAG
S2: GATG-TGCCG
S3: GATG-TGCAG

S1: CCTGCT-GCAG
S4: CC-GCTAGCAG
```

```
S1: CCTGCTG-CAG
S2: GATG-TG-CCG
S3: GATG-TG-CAG
S4: CC-GCTAGCAG

S1: CCTGCT-GCAG
S5: CCTG-TAG--G
```

```
S1: CCTGCT-GCAG
S2: GATG-T-GCCG
S3: GATG-T-GCAG
S4: CC-GCTAGCAG
S5: CCTG-TAG--G
```

Detail algorithm for center star method

```
Center_Star_Method
Require: A set $S$ of sequences
Ensure: A multiple alignment of $M$ with sum of pair distances at most twice
    that of the optimal alignment of $S$
1: Find $D(S_i, S_j)$ for all $i, j$.
2: Find the center sequence $S_c$ which minimizes $\sum_{i=1}^{k} D(S_c, S_i)$.
3: For every $S_i \in S - \{S_c\}$, choose an optimal alignment between $S_c$ and $S_i$.
4: Introduce spaces into $S_c$ so that the multiple alignment $\mathcal{M}$ satisfies the
    alignments found in Step 3.
```

```
S1: CCTGCTGCAG
S2: GATGTGCCG
S3: GATGTGCAG
S4: CCGCTAGCAG
S5: CCTGTAGG
```

	S_1	S_2	S_3	S_4	S_5
S_1	0	4	3	2	4
S_2		0	1	6	5
S_3			0	5	5
S_4				0	4
S_5					0

Step 1

$\Sigma_{i=1..k} D(S_1,S_i) = 13$

$\Sigma_{i=1..k} D(S_2,S_i) = 16$

$\Sigma_{i=1..k} D(S_3,S_i) = 14$

$\Sigma_{i=1..k} D(S_4,S_i) = 17$

$\Sigma_{i=1..k} D(S_5,S_i) = 18$

Step 2

```
S1: CCTGCTGCAG
S2: GATG-TGCCG

S1: CCTGCTGCAG
S3: GATG-TGCAG

S1: CCTGCT-GCAG
S4: CC-GCTAGCAG

S1: CCTGCT-GCAG
S5: CCTG-TAG--G
```

Step 3

```
S1: CCTGCT-GCAG
S2: GATG-T-GCCG
S3: GATG-T-GCAG
S4: CC-GCTAGCAG
S5: CCTG-TAG--G
```

Step 4

Running time of center star method

- Assume all k sequences are of length n.
  - Step 1 takes $O(k^2n^2)$ time.
  - Step 2 takes $O(k^2)$ time to find the center string S_c.
  - Step 3 takes $O(kn^2)$ time to compute the alignment between S_c and S_i for all i.
  - Step 4 introduces space into the multiple alignment, which takes $O(k^2n)$ time.
- In total, the running time is $O(k^2n^2)$.

S_1: CCTGCTGCAG
S_2: GATGTGCCG
S_3: GATGTGCAG
S_4: CCGCTAGCAG
S_5: CCTGTAGG

	S_1	S_2	S_3	S_4	S_5
S_1	0	4	3	2	4
S_2		0	1	6	5
S_3			0	5	5
S_4				0	4
S_5					0

Step 1
$O(k^2n^2)$

$\Sigma_{i=1..k} D(S_1,S_i) = 13$
$\Sigma_{i=1..k} D(S_2,S_i) = 16$
$\Sigma_{i=1..k} D(S_3,S_i) = 14$
$\Sigma_{i=1..k} D(S_4,S_i) = 17$
$\Sigma_{i=1..k} D(S_5,S_i) = 18$

Step 2
$O(k^2)$

S_1: CCTGCTGCAG
S_2: GATG-TGCCG

S_1: CCTGCTGCAG
S_3: GATG-TGCAG

S_1: CCTGCT-GCAG
S_4: CC-GCTAGCAG

S_1: CCTGCT-GCAG
S_5: CCTG-TAG--G

Step 3
$O(kn^2)$

S_1: CCTGCT-GCAG
S_2: GATG-T-GCCG
S_3: GATG-T-GCAG
S_4: CC-GCTAGCAG
S_5: CCTG-TAG--G

Step 4
$O(k^2n)$

Why center star method is good? (I)

- Let M* be the optimal alignment.
- The SP-dist of M*

$$
\begin{aligned}
&= \sum_{1\le i<j\le k} d_{\mathcal{M}^*}(i,j) \\
&\ge \sum_{1\le i<j\le k} D(S_i,S_j) \\
&= \frac{1}{2}\sum_{i=1}^{k}\sum_{j=1}^{k} D(S_i,S_j) \\
&\ge \frac{1}{2}\sum_{i=1}^{k}\sum_{j=1}^{k} D(S_c,S_j) \\
&= \frac{k}{2}\sum_{j=1}^{k} D(S_c,S_j)
\end{aligned}
$$

Why center star method is good? (II)

- The SP-dist of M

$$
\begin{aligned}
&= \sum_{1\le i<j\le k} d_{\mathcal{M}}(i,j) \\
&= \frac{1}{2}\sum_{i=1}^{k}\sum_{j=1}^{k} d_{\mathcal{M}}(i,j) \\
&\le \frac{1}{2}\sum_{i=1}^{k}\sum_{j=1}^{k}[D(S_c,S_i)+D(S_c,S_j)] \\
&= \frac{k}{2}\sum_{i=1}^{k} D(S_c,S_i)+\frac{k}{2}\sum_{j=1}^{k} D(S_c,S_j) \\
&= k\sum_j D(S_c,S_j)
\end{aligned}
$$

- The SP-dist of M is at most twice of that of M* (the optimal alignment).

Progress alignment

- Progress alignment is first proposed by Feng and Doolittle (1987).

- It is a heuristics to get a good multiple alignment.
- Basic idea:
  - Align the two most closest sequences
  - Progressive align the most closest related sequences until all sequences are aligned.

- Examples of Progress alignment method include:
  - ClustalW, T-coffee, Probcons
- Probcons is currently the most accurate MSA algorithm.
- ClustalW is the most popular software.

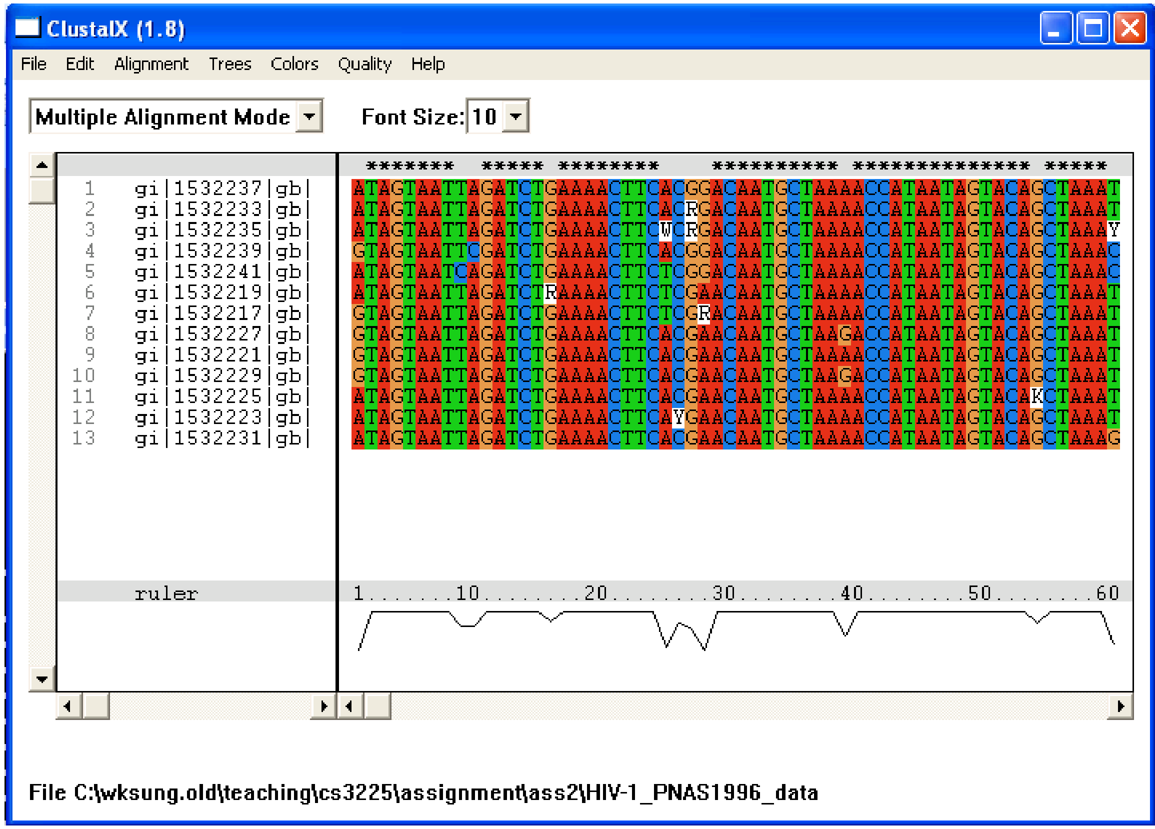
Basic algorithm

1. Computing pairwise distance scores for all pairs of sequences
2. Generate the guide tree which ensures similar sequences are nearer in the tree
3. Aligning the sequences one by one according to the guide tree

ClustalW

- A popular progressive alignment method to globally align a set of sequences.

- Input: a set of sequences
- Output: the multiple alignment of these sequences



```
                              ******* ***** ******** ********** ************** *****
 1  gi|1532237|gb|   ATAGTAATTAGATCTGAAAACTTCACGGACAATGCTAAAACCATAATAGTACAGCTAAAT
 2  gi|1532233|gb|   ATAGTAATTAGATCTGAAAACTTCACRGACAATGCTAAAACCATAATAGTACAGCTAAAT
 3  gi|1532235|gb|   ATAGTAATTAGATCTGAAAACTTCWCRGACAATGCTAAAACCATAATAGTACAGCTAAAY
 4  gi|1532239|gb|   GTAGTAATTCGATCTGAAAACTTCACGGACAATGCTAAAACCATAATAGTACAGCTAAAC
 5  gi|1532241|gb|   ATAGTAATCAGATCTGAAAACTTCTCGGACAATGCTAAAACCATAATAGTACAGCTAAAC
 6  gi|1532219|gb|   ATAGTAATTAGATCTRAAAACTTCTCGAACAATGCTAAAACCATAATAGTACAGCTAAAT
 7  gi|1532217|gb|   GTAGTAATTAGATCTGAAAACTTCTCGRACAATGCTAAAACCATAATAGTACAGCTAAAT
 8  gi|1532227|gb|   GTAGTAATTAGATCTGAAAACTTCACGAACAATGCTAAGACCATAATAGTACAGCTAAAT
 9  gi|1532221|gb|   GTAGTAATTAGATCTGAAAACTTCACGAACAATGCTAAAACCATAATAGTACAGCTAAAT
10  gi|1532229|gb|   GTAGTAATTAGATCTGAAAACTTCACGAACAATGCTAAGACCATAATAGTACAGCTAAAT
11  gi|1532225|gb|   ATAGTAATTAGATCTGAAAACTTCACGAACAATGCTAAAACCATAATAGTACAKCTAAAT
12  gi|1532223|gb|   ATAGTAATTAGATCTGAAAACTTCAYGAACAATGCTAAAACCATAATAGTACAGCTAAAT
13  gi|1532231|gb|   ATAGTAATTAGATCTGAAAACTTCACGAACAATGCTAAAACCATAATAGTACAGCTAAAG

    ruler            1.......10........20........30........40........50........60
```

File C:\wksung.old\teaching\cs3225\assignment\ass2\HIV-1_PNAS1996_data

Step 1: pairwise distance scores

- Example: For S_1 and S_2, the global alignment is
  - S_1=P-PGVKSDCAS
  - S_2=PADGVK-DCAS
- There are 9 non-gap positions and 8 match positions.
- The distance is 1 – 8/9 = 0.111

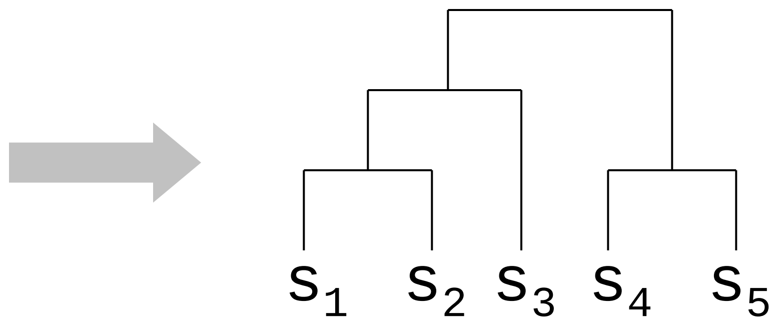
S_1: PPGVKSDCAS
S_2: PADGVKDCAS
S_3: PPDGKSDS
S_4: GADGKDCCS
S_5: GADGKDCAS

	S_1	S_2	S_3	S_4	S_5
S_1	0	0.111	0.25	0.555	0.444
S_2		0	0.375	0.222	0.111
S_3			0	0.5	0.5
S_4				0	0.111
S_5					0

Step 2: generate guide tree

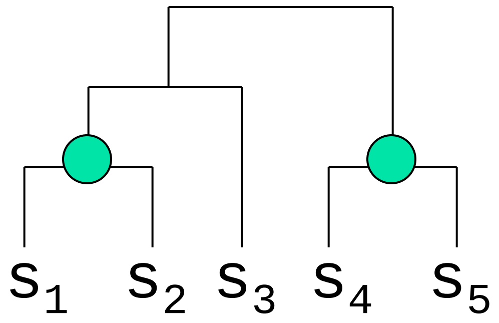
- By neighbor-joining, generate the guide tree.

	S_1	S_2	S_3	S_4	S_5
S_1	0	0.111	0.25	0.555	0.444
S_2		0	0.375	0.222	0.111
S_3			0	0.5	0.5
S_4				0	0.111
S_5					0



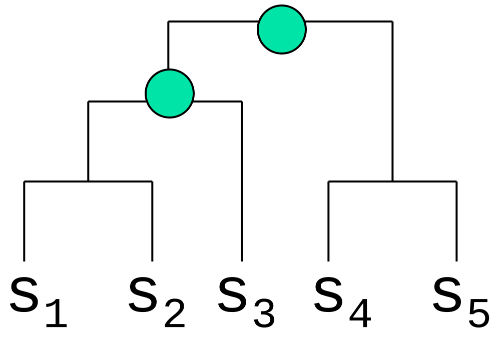
Step 3: align the sequences according to the guide tree (I)

- Aligning S1 and S2, we get
  - S_1=P-PGVKSDCAS
  - S_2=PADGVK-DCAS
- Aligning S4 and S5, we get
  - S_4=GADGKDCCS
  - S_5=GADGKDCAS



Step 3: align the sequences according to the guide tree (II)

- Aligning (S1, S2) with S3, we get
  - S_1=P-PGVKSDCAS
  - S_2=PADGVK-DCAS
  - S_3=PPDG-KSD--S
- Aligning (S1, S2, S3) with (S4, S5), we get
  - S_1=P-PGVKSDCAS
  - S_2=PADGVK-DCAS
  - S_3=PPDG-KSD--S
  - S_4=GADG-K-DCCS
  - S_5=GADG-K-DCAS



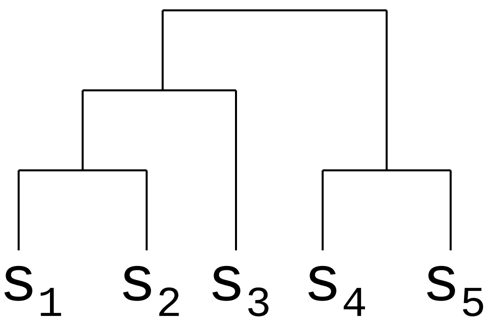
$\Rightarrow$

```
S1:  P-PGVKSDCAS
S2:  PADGVK-DCAS
S3:  PPDG-KSD--S
S4:  GADG-K-DCCS
S5:  GADG-K-DCAS
```

Summary

```
S1: PPGVKSDCAS
S2: PADGVKDCAS
S3: PPDGKSDS
S4: GADGKDCCS
S5: GADGKDCAS
```

	S_1	S_2	S_3	S_4	S_5
S_1	0	0.111	0.25	0.555	0.444
S_2		0	0.375	0.222	0.111
S_3			0	0.5	0.5
S_4				0	0.111
S_5					0



```
S1: P-PGVKSDCAS
S2: PADGVK-DCAS
S3: PPDG-KSD--S
S4: GADG-K-DCCS
S5: GADG-K-DCAS
```

Detail of Profile-Profile alignment (I)

- Given two aligned sets of sequences A_1 and A_2.
- Example:
  - A_1 is a length-11 alignment of S_1, S_2, S_3
    - S_1=`P-PGVKSDCAS`
    - S_2=`PADGVK-DCAS`
    - S_3=`PPDG-KSD--S`
  - A_2 is a length-9 alignment of S_4, S_5
    - S_4=`GADGKDCCS`
    - S_5=`GADGKDCAS`
- Similar to the sequence alignment,
  - the profile-profile alignment introduces gaps to A_1 and A_2 so that both of them have the same length.

Detail of Profile-Profile Alignment (II)

- To determine the alignment, we need a scoring function PSP(A_1[i], A_2[j]).
- In clustalW, the score is defined as follows.
  - $PSP(A_1[i],A_2[j]) = \Sigma_{x,y}\, g_x^i\, g_y^j\, \delta(x,y)$

  where g_x^i is the observed frequency of amino acid x in column i.
- This is a natural scoring for maximizing the SP-score.

- Our aim is to find an alignment between A_1 and A_2 to maximizes the PSP score.

Example

- $A_1[1..11]$ is the alignment of S_1, S_2, S_3
  - S_1=P-PGVKSDCAS
  - S_2=PADGVK-DCAS
  - S_3=PPDG-KSD--S
- $A_2[1..9]$ is the alignment of S_4, S_5
  - S_4=GADGKDCCS
  - S_5=GADGKDCAS

- $PSP(A_1[3],A_2[3]) = 1x2x\delta(P,D)+2x2x\delta(D,D)$
- $PSP(A_1[9],A_2[8]) = 2\delta(C,C)+2\delta(C,A)+\delta(-,C)+\delta(-,A)$

Dynamic Programming

- Let $V(i,j)$ = the score of the best alignment between $A_1[1..i]$ and $A_2[1..j]$.
- We have $V(i,j)$ = maximum of
  - $V(i-1,j-1)+PSP(A_1[i],A_2[j])$
  - $V(i-1,j)+PSP(A_1[i],-)$
  - $V(i,j-1)+PSP(-,A_2[j])$

- By fill-in the dynamic programming table, we can find the optimal alignment.
- Time complexity: $O(k_1n_1+k_2n_2+n_1n_2)$ time.

Example

- By profile-profile alignment, we have
  - S_1=`P-PGVKSDCAS`
  - S_2=`PADGVK-DCAS`
  - S_3=`PPDG-KSD--S`
  - S_4=`GADG-K-DCCS`
  - S_5=`GADG-K-DCAS`

Complexity

- Step 1 performs k^2 global alignments, which takes $O(k^2n^2)$ time.
- Step 2 performs neighbor-joining, which takes $O(k^3)$ time.
- Step 3 performs at most k profile-profile alignments, each takes $O(kn+n^2)$ time. Thus, Step 3 takes $O(k^2n+kn^2)$ time.

- Hence, ClustalW takes $O(k^2n^2+k^3)$ time.

Limitation of progressive alignment method

- Progressive alignment method will not realign the sequence
  - Hence, the final alignment is bad if we have a poor initial alignment.
- Progressive alignment method does not guaranteed to converge to the global optimal.

Iterative method

- To reduce the error in progress alignment, iterative methods are introduced.

- Iterative methods are also heuristics.
- Basic idea:
  - Generate an initial multiple alignment based on methods like progress alignment.
  - Iteratively improve the multiple alignment.

- Examples of iterative method include:
  - PRRP, MAFFT, MUSCLE
- We discuss the detail of MUSCLE.

Multiple sequence comparison by log-expectation (MUSCLE)

- Idea 1:
  - Try to construct a draft multiple alignment as fast as possible; then, MUSCLE improves the alignment.
- Idea 2:
  - Introduce the log-expectation score for profile-profile alignment

Profile-profile alignment

- For clustalW, we use the PSP score
  - $PSP(A_1[i],A_2[j]) = \Sigma_{x,y}\ g_x^i\ g_y^j\ \delta(x,y)$

  where g_x^i is the observed frequency of amino acid x in column i.
- PSP score may favor more gaps. So, we use the log-expectation (LE) score.
  - $LE(A_1[i],A_2[j]) =$

    $(1-f_i^G)(1-f_j^G)\log(\Sigma_{x,y}\ f_i^x f_j^y\ p_{xy}/(p_x p_y))$

  f_i^G is the proportion of gaps in A_1
  f_i^x is the proportion of amino acid x in A_1
  p_x is the background proportion of amino acid x
  p_{xy} is the probability that x aligns with y
  Note: $p_{xy}/(p_x p_y) = e^{\delta(x,y)}$

3 Stages of MUSCLE

1. Draft progressive
   - Generate an initial alignment based on some progressive alignment method
2. Improved progressive
   - Based on the alignment generated, compute a more accurate pairwise distance
   - An improved multiple alignment is generated by using a progressive alignment method
3. Refinement
   - An optional tree-based iteration step is included to further improve the alignment.

Stage 1: Draft progressive

- The steps are similar to ClustalW.

1. Pairwise distance matrix
   - To improve efficiency, we first compute the q-mer similarity, which is the fraction F of q-mers shared by two sequences. Then, the distance is 1-F.
2. Build guide tree
   - Instead of using neighbor joining, we use UPGMA, which is more efficient.
3. Profile-profile alignment
   - When performing profile-profile alignment, we uses log-expectation score.

Complexity of Stage 1

- Step 1 performs k^2 q-mer distance computation, which takes $O(k^2n)$ time.
- Step 2 performs UPGMA, which takes $O(k^2)$ time.
- Step 3 performs at most k profile-profile alignments, each takes $O(kn+n^2)$ time. Thus, Step 3 takes $O(k^2n+kn^2)$ time.

- Hence, Stage 1 takes $O(k^2n+kn^2)$ time.

Stage 2: Improved progressive

- The steps are similar to ClustalW.

1. Pairwise distance matrix
   - We first find the fraction D of identical bases shared by two aligned sequences. Then, the distance is $-\log_e(1-D-D^2/5)$.
2. Build guide tree
   - The guide tree is built using UPGMA.
3. Profile-profile alignment
   - When performing profile-profile alignment, we uses log-expectation score.
   - Only perform re-alignment when there are changes relative to the original guide tree.

Complexity of Stage 2

- Step 1 performs k^2 distance computation, which takes $O(k^2n)$ time.
- Step 2 performs UPGMA, which takes $O(k^2)$ time.
- Step 3 performs at most k profile-profile alignments, each takes $O(kn+n^2)$ time. Thus, Step 3 takes $O(k^2n+kn^2)$ time.

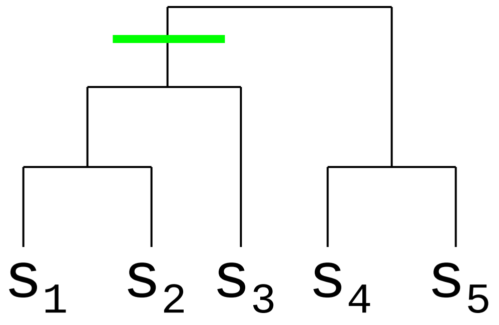
- Hence, Stage 2 takes $O(k^2n+kn^2)$ time.

Stage 3: Refinement

- This stage is optional. It refines the multiple sequence alignment to maximizes the SP-score.

A. Visit the edges e in decreasing distance from the root,
   1. Partition the alignment into two sets by deleting the edge e from the guide tree.
   2. The two sets are realigned using profile-profile alignment.
   3. Compute the SP-score for the new alignment.
   4. If the SP-score is improved, we keep the new alignment.

B. Iterate Step A until there is no improvement in SP-score or a user defined maximum number of iterations.



Complexity of Stage 3

- Step A.1 takes $O(1)$ time.
- Step A.2 takes $O(kn+n^2)$ time.
- Step A.3 takes $O(k^2n)$ time.

- Step A iterates k times. So, Step A takes $O(k^3n+kn^2)$ time.
- Suppose we perform x refinements. This stage takes $O(xk^3n+xkn^2)$ time.

Total running time of MUSCLE

- Stage 1: $O(k^2n+kn^2)$ time
- Stage 2: $O(k^2n+kn^2)$ time
- Stage 3: $O(xk^3n+xkn^2)$ time

- Total time: $O(xk^3n +xkn^2)$ time.

- Assuming $x=O(1)$, we have
  - Runing time: $O(k^3n+kn^2)$ time.

- Note: The time complexity we got is a bit different from MUSCLE analysis since MUSCLE assumes the length of the alignment is $(k+n)$ instead of n.

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