Computational methods for RNA folding: Algorithms and Software

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What is RNA primary structure?

RNA primary structure is the sequence of ribonucleotides (bases), and can be written as $5' - r_1 r_2 \ldots r_n - 3'$, where n is the number of bases and each r_i is either A, C, G or U. Of course, bases are sometimes modified, either naturally or *in vitro*.

Nucleobase	Adenine	Guanine	Cytosine	Uracil
Nucleoside (NB + D-ribose)	Adenosine	Guanosine	Cytidine	Uridine
Nucleotide (NS+phosphate)	AMP, A_p	GMP, G_p	CMP, C_p	UMP, U_p

Ambiguous codes for RNA/DNA

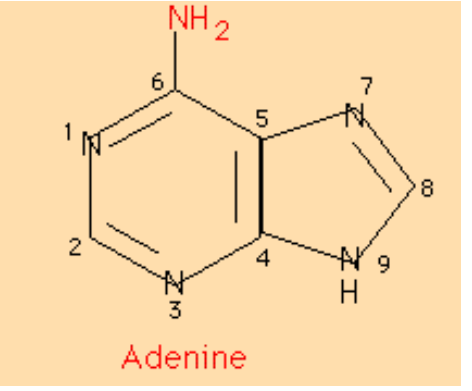
A,G R	C,U/T Y	A,U/T W	C,G S	A,C M	G,U/T K
C,G,U/T B	A,G,U/T D	A,C,U/T H	A,C,G V	A,C,G,U/T N	

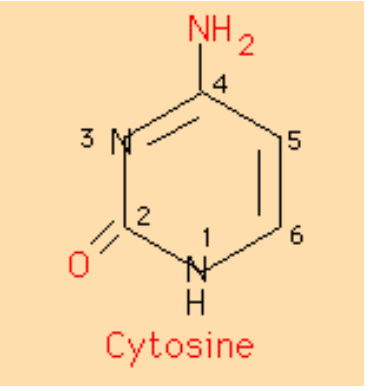
Remembering the Ambiguous Base Letters

How to remember the ambiguous codes?
The **NOT** cases use the next (available) letter.

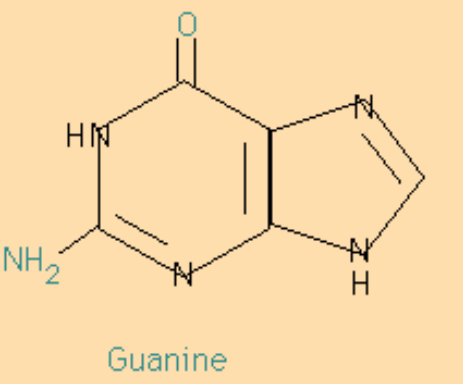
R: pu**R**ine
Y: p**Y**rimidine
M: a**M**ide
W: **W**eak
K: **K**eto
S: **S**trong
B: **NOT** A
D: **NOT** C
H: **NOT** G
V: **NOT** U/T
N: a**N**ything

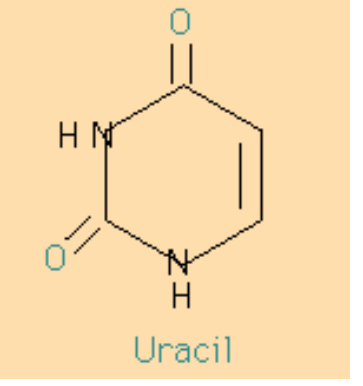
NH_3 (amino) opposite to the glycosidic bond.





C=O (keto) opposite to the glycosidic bond.





What is RNA secondary structure?

RNA secondary structure is the collection (set) of base pairs that form in 3D. The hydrogen bonds of base pairs and the stacking of adjacent base pairs are responsible for most of the thermodynamic stability of an RNA. The most common base pairs are Watson-Crick (W-C): C·G, G·C, A·U and U·A. Base pairs between G and U, G·U and U·G, are called wobble pairs. Other pairs are called non-canonical.

A base pair between r_i and r_j is denoted as $r_i \cdot r_j$ ($i < j$) or simply by $i \cdot j$ when the context is clear. A secondary structure is a collection, S, of base pairs that satisfy:

1. If $i \cdot j \in S$, then $j - i > 3$. The number 3 is called the minimum hairpin loop size. This "rule" is broken by the existence of tetra-loops.

2. If $i \cdot j, i' \cdot j' \in S$, then $j = j'$ if $i = i'$ and $i = i'$ if $j = j'$. This rule excludes base triples, and is violated in some structures.

3. If $i \cdot j, i' \cdot j' \in S$, then either $i < j < i' < j'$ ($i \cdot j$ precedes $i' \cdot j'$) or $i < i' < j' < j$ ($i \cdot j$ includes $i' \cdot j'$). Violations of this rule also occur and create "pseudoknots".

Anything can pair with anything else in twelve different ways (almost).

Recent work of Leontis and Westhof describe 12 distinct ways in which any two bases can pair.

- Some cannot occur.
- Some can be modeled but have not yet been observed in structures derived by X-ray crystalography or NMR methods.
- Many base pairs are structurally equivalent (isosteric).

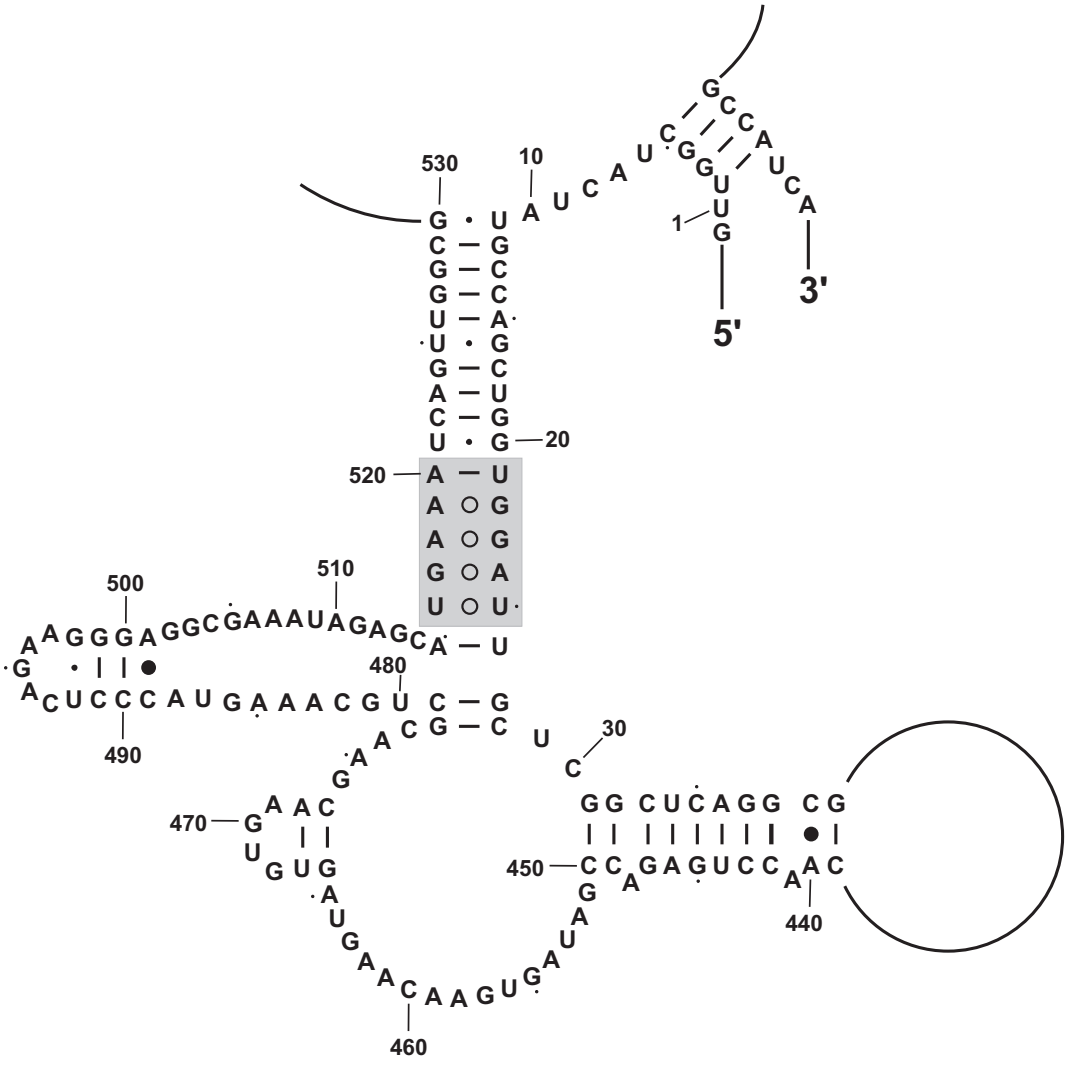
`http://www.bgsu.edu/departments/chem/RNA/pages/`

`PDB/index.html`

Non-canonical BPs Deduced by Comparative Sequence Analysis

The figure on the right displays five consecutive non-canonical BPs in a portion of a large-subunit rRNA.

Statistics were gathered from 5S rRNA, grpI introns, RNAse P RNA, SSU and LSU rRNA.



Nomenclature and Definitions

- Complete: Entire structures.
- Core: “Reliable” portions of rRNA structures deduced from an alignment with respect to *E. coli* rRNA.
- ext-: Base pairs as indicated.
- ext+: Base pairs added when found in small symmetric interior loops (up to 5×5).
- “$\mathrm{X_pY}$” refers to a di-nucleotide, not to a BP. Think $5' - \mathrm{XY} - 3'$.
- “XY” refers to a BP, canonical or not. Thus AU or GG are base pairs.
- “WX/YZ” refers to “tandem base pairs”, canonical or not. Think $\begin{array}{c} 5' - \mathrm{WY} - 3' \\ 3' - \mathrm{XZ} - 5' \end{array}$.
- “WX/YZ” is clearly equivalent to “ZY/XW”.
- $F(\mathrm{WX/YZ})$ is the ratio of the observed number of base pair stacks of type WX/YZ divided by the expected number. The expected number is based on observed frequencies of di-nucleotides.
- $F(\mathrm{WX/YZ})$ measures under or over represetation of tandem BPs.

The Numbers

Data	Sequences	Stacks	Base pairs	Nucleotides
Complete ext-	1900	337659	436327	1597471
Complete ext+	1900	345586	441117	1597471
core rRNA ext-	8058	1671686	2245790	10998639
core rRNA ext+	8058	1737666	2287177	10998639

The core data, though less reliable, especially for Eukaryote rRNA, was used because of the very large number of base pairs that could be analyzed.

Statistics on tandem base pairs - I

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
AA/AA	121	170	0.24	0.25	105	358	0.29	0.33
AA/AC	33	45	0.83	0.79	79	167	0.99	0.73
AA/AG	501	1283	0.64	0.86	412	13490	0.48	1.60
AA/AU	626	882	0.62	0.67	1746	7037	1.00	1.24
AA/CA	46	78	0.91	0.59	71	175	0.95	0.54
AA/CC	23	25	2.14	1.70	99	571	1.56	0.92
AA/CG	725	878	1.10	1.17	2883	3284	1.46	1.36
AA/CU	51	66	2.13	1.33	38	136	1.35	1.07
AA/GA	104	140	0.38	0.33	144	226	0.71	0.42
AA/GC	809	967	1.20	1.25	1627	5614	1.21	1.14
AA/GG	44	48	0.47	0.46	77	90	0.72	0.68
AA/GU	219	282	0.68	0.66	289	853	0.77	0.73
AA/UA	666	831	0.71	0.74	1316	2494	0.91	0.95
AA/UC	49	54	2.33	1.76	62	80	1.29	1.04
AA/UG	135	160	0.60	0.58	243	351	0.81	0.69
AA/UU	220	291	0.84	0.77	215	875	0.77	1.11
AC/AC	36	40	0.65	0.61	64	66	0.89	0.83

Statistics on tandem base pairs - II

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
AC/AG	36	103	0.53	0.56	121	235	0.80	0.50
AC/AU	337	518	0.77	0.73	1404	1794	1.22	0.94
AC/CA	32	34	0.68	0.68	120	128	1.23	1.16
AC/CC	31	33	1.39	1.21	80	85	1.50	1.29
AC/CG	1517	1529	1.83	1.79	10168	10372	1.95	1.87
AC/CU	17	18	1.31	1.17	126	129	1.60	1.49
AC/GA	43	59	0.66	0.66	149	491	0.95	0.94
AC/GC	693	764	1.14	1.12	2435	2827	1.49	1.48
AC/GG	46	49	0.70	0.64	163	165	0.88	0.83
AC/GU	240	260	0.80	0.81	1311	1361	1.15	1.10
AC/UA	618	636	1.08	1.07	700	796	1.36	1.28
AC/UC	62	62	1.66	1.60	28	29	1.44	1.32
AC/UG	177	190	0.76	0.75	1121	1123	1.18	1.12
AC/UU	55	84	0.94	0.94	91	94	1.27	1.16
AG/AC	40	43	0.57	0.53	33	116	0.98	0.57
AG/AG	394	549	0.50	0.44	2146	2719	0.62	0.54
AG/AU	575	708	0.64	0.69	2343	3132	0.75	0.76

Statistics on tandem base pairs - III

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
AG/CC	25	27	1.00	0.91	48	111	0.98	0.75
AG/CG	2803	3000	2.06	2.14	19452	26253	2.15	2.75
AG/CU	50	60	0.55	0.52	182	253	0.80	0.84
AG/GA	55	56	0.42	0.40	164	167	0.70	0.67
AG/GC	1804	2214	1.49	1.70	2188	8923	1.11	1.68
AG/GG	49	55	0.60	0.58	214	225	0.71	0.68
AG/GU	1351	1566	1.17	1.26	5687	8033	0.85	1.08
AG/UA	608	869	0.93	1.09	2566	6124	1.02	1.49
AG/UC	7	8	0.94	0.98	28	69	1.19	0.94
AG/UG	305	511	0.58	0.69	2303	6474	0.95	1.11
AG/UU	194	282	0.67	0.65	184	825	0.91	0.92
AU/AC	560	683	0.86	0.97	2440	2866	1.32	1.28
AU/AG	645	701	0.60	0.61	4551	10955	0.98	1.29
AU/CC	149	190	1.36	1.31	511	549	1.67	1.49
AU/CU	336	393	0.95	0.91	513	686	1.20	1.10
AU/GG	552	666	1.45	0.95	1444	1472	0.99	0.94

Statistics on tandem base pairs - IV

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
AU/UC	547	586	1.15	1.12	1791	1990	1.35	1.35
AU/UU	1447	1589	1.37	1.43	8180	8483	2.29	2.25
CA/AC	55	79	1.09	1.24	613	623	1.54	1.48
CA/AG	39	52	0.88	0.88	291	324	1.25	1.15
CA/AU	249	340	1.01	0.98	633	874	1.37	1.18
CA/CC	10	12	1.34	1.34	47	49	1.28	1.14
CA/CG	768	826	1.07	1.04	2293	2406	1.06	0.98
CA/CU	15	15	0.76	0.75	54	60	1.13	1.04
CA/GC	499	582	1.43	1.40	2216	2431	1.63	1.47
CA/GG	67	73	0.62	0.56	773	778	1.02	0.98
CA/GU	66	101	0.92	0.73	188	293	1.25	0.95
CA/UC	16	27	1.81	1.50	53	66	1.42	1.28
CA/UG	177	179	0.67	0.65	985	993	1.22	1.16
CA/UU	37	41	0.85	0.80	68	81	1.31	1.12
CC/AG	13	16	0.99	0.94	68	70	1.18	1.09
CC/AU	87	101	1.69	1.52	360	368	1.63	1.54

Statistics on tandem base pairs - V

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
CC/CC	9	11	0.70	0.30	46	47	0.65	0.56
CC/CG	187	202	0.91	0.89	1180	1252	1.28	1.09
CC/CU	12	14	2.69	1.64	44	45	1.56	1.39
CC/GC	207	219	0.77	0.76	1144	1616	1.22	0.97
CC/GG	35	36	0.44	0.42	680	687	0.97	0.92
CC/GU	57	58	0.93	0.93	153	156	1.20	1.11
CC/UC	13	15	1.52	1.41	30	31	1.06	0.99
CC/UG	86	87	0.92	0.90	880	885	1.27	1.22
CC/UU	48	95	0.86	0.96	170	174	1.25	1.17
CG/AG	723	764	0.86	0.87	5998	6049	1.03	0.99
CG/CU	428	644	0.91	1.15	1617	2154	1.14	1.27
CG/GG	403	505	0.62	0.55	3761	3772	0.82	0.79
CG/UC	390	633	1.23	1.28	1275	1848	1.31	1.23
CG/UU	1260	1326	1.78	1.80	3107	3870	1.51	1.75
CU/AG	35	37	1.84	1.76	108	168	1.09	0.97
CU/AU	307	339	0.95	0.93	1023	1143	1.51	1.38

Statistics on tandem base pairs - VI

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
CU/CG	492	564	1.10	1.04	2887	3060	1.19	1.10
CU/CU	60	63	0.93	0.89	489	489	1.07	1.03
CU/GG	24	26	0.68	0.60	203	229	1.12	0.96
CU/GU	105	109	1.27	1.21	204	225	1.18	1.15
CU/UC	6	6	1.11	1.08	80	80	1.14	1.10
CU/UG	124	126	0.82	0.80	944	950	1.35	1.29
CU/UU	46	67	0.69	0.64	362	366	1.02	0.98
GA/AG	738	966	0.70	0.73	1788	4959	0.63	0.77
GA/AU	2326	2641	1.94	1.96	9546	13561	1.55	2.04
GA/CG	1366	1401	1.30	1.28	15634	15920	2.05	1.97
GA/CU	17	19	1.16	1.06	73	124	1.31	0.89
GA/GG	335	343	1.79	1.68	134	159	0.59	0.53
GA/GU	260	277	0.65	0.63	1444	1704	0.85	0.78
GA/UG	183	189	0.61	0.59	1043	1069	0.89	0.83
GA/UU	55	64	0.57	0.53	245	653	0.91	0.98
GC/CU	398	429	1.09	1.07	3931	3947	1.48	1.42
GC/GG	471	513	0.60	0.61	3171	3175	0.71	0.68

Statistics on tandem base pairs - VII

WX/YZ	Complete				Core rRNA			
	Count		F(WX/YZ)		Count		F(WX/YZ)	
	-ext	+ext	-ext	+ext	-ext	+ext	-ext	+ext
GC/UU	1796	2179	2.42	2.69	15064	15462	3.31	3.15
GG/AU	354	377	0.72	0.73	2207	2211	1.06	1.01
GG/CU	21	24	1.08	0.87	94	116	0.85	0.69
GG/GG	10	14	0.29	0.29	91	93	0.41	0.38
GG/GU	101	115	0.44	0.42	255	386	0.58	0.50
GG/UG	68	76	0.47	0.44	380	385	0.69	0.65
GG/UU	46	49	0.52	0.50	119	194	0.87	0.60
GU/CU	63	64	0.68	0.66	429	431	1.13	1.08
GU/UU	238	256	0.60	0.61	1117	1168	0.92	0.87
UA/CU	320	358	1.45	1.34	2147	2166	2.26	2.14
UA/UU	969	1154	1.06	1.17	3784	3974	1.84	1.83
UC/CU	15	16	3.63	2.37	25	28	1.54	1.39
UC/GU	56	66	0.92	0.96	244	249	1.07	1.00
UC/UU	200	235	1.05	0.94	534	707	1.14	1.03
UG/UU	645	678	1.03	1.04	1830	1983	1.07	1.08
UU/UU	397	459	0.57	0.61	1418	1425	0.90	0.86

Non-canonical BP Count

Note: The W-C base pair counts are restricted to those occurring next to a non-canonical base pair. The numbers in the table are the base pair counts for complete and core BPs, ext- and ext+

The BP columns are ordered from least frequent to most.

BP	Complete	BP	Complete+	BP	Core	BP	Core+
CC	992	CC	1141	CC	5540	CC	6696
GG	2626	GG	2969	AA	9406	GG	14137
CU	4282	CU	5143	GG	13766	CU	22054
AA	4372	GU	5350	CU	19618	GU	29072
GU	4656	AA	6200	GU	21050	AC	31997
AC	6617	AC	7555	AC	28918	AA	35801
UU	7653	UU	8849	UU	36488	UU	40334
AU	12278	AU	14562	AU	49205	AU	72675
AG	15679	AG	19003	AG	79287	CG	124235
CG	17739	CG	20139	CG	102031	AG	133581

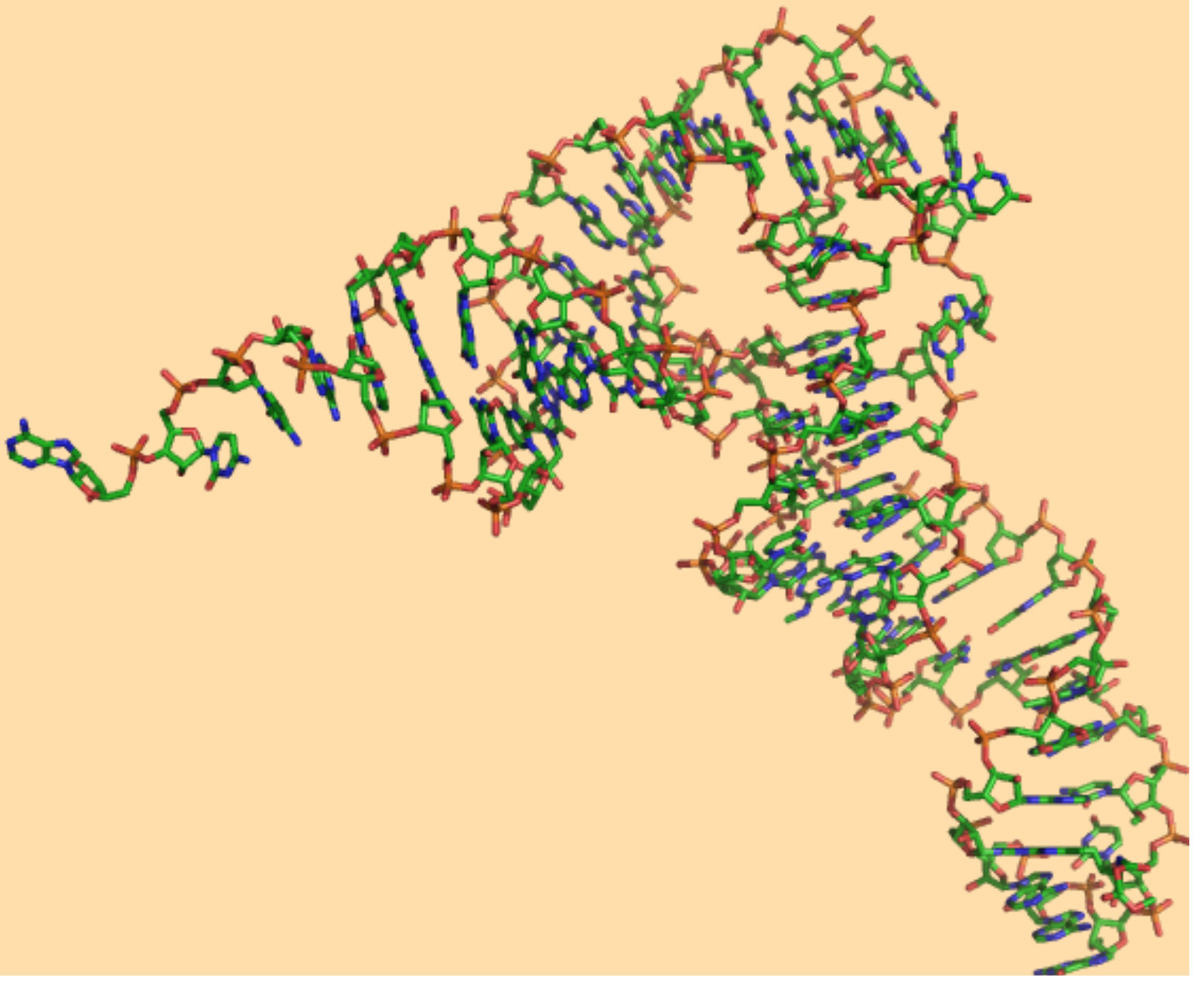
What is RNA tertiary structure?

Base triples and pseudoknots are best excluded from the definition of secondary structure. They comprise some of the interactions that characterize tertiary structure. Angles between pairs of adjacent helices are part of tertiary structure. Adding (sufficient) tertiary structure to secondary structure can lead to the modeling of atomic resolution structures.

A helix is a collection of two or more adjacent base pairs.

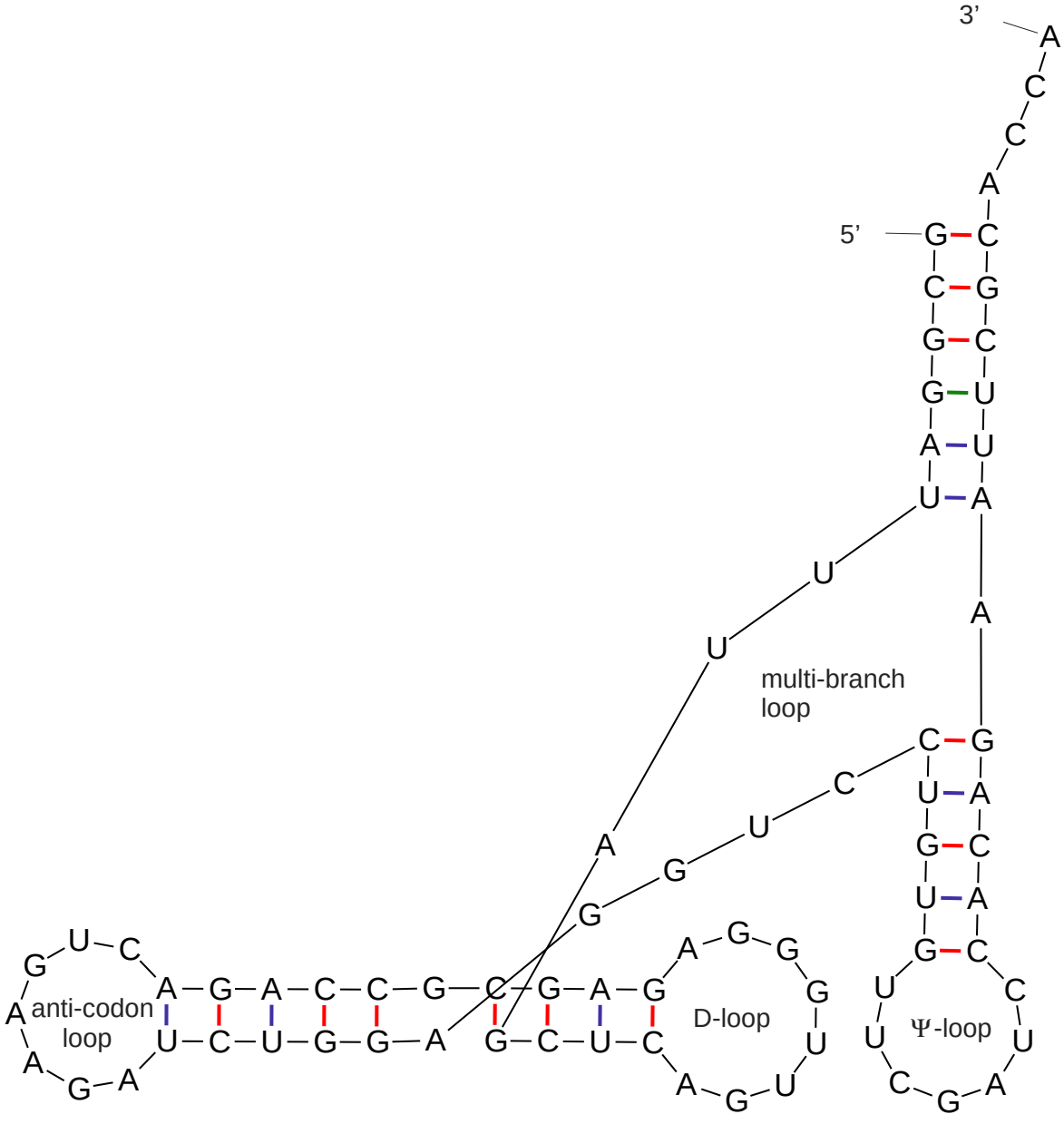
3D structure for *S. cerevisiae* Phe tRNA.

An atomic resolution model is complicated and full of details. What can secondary structure show?



A 3D-like secondary structure for *S. cerevisiae*

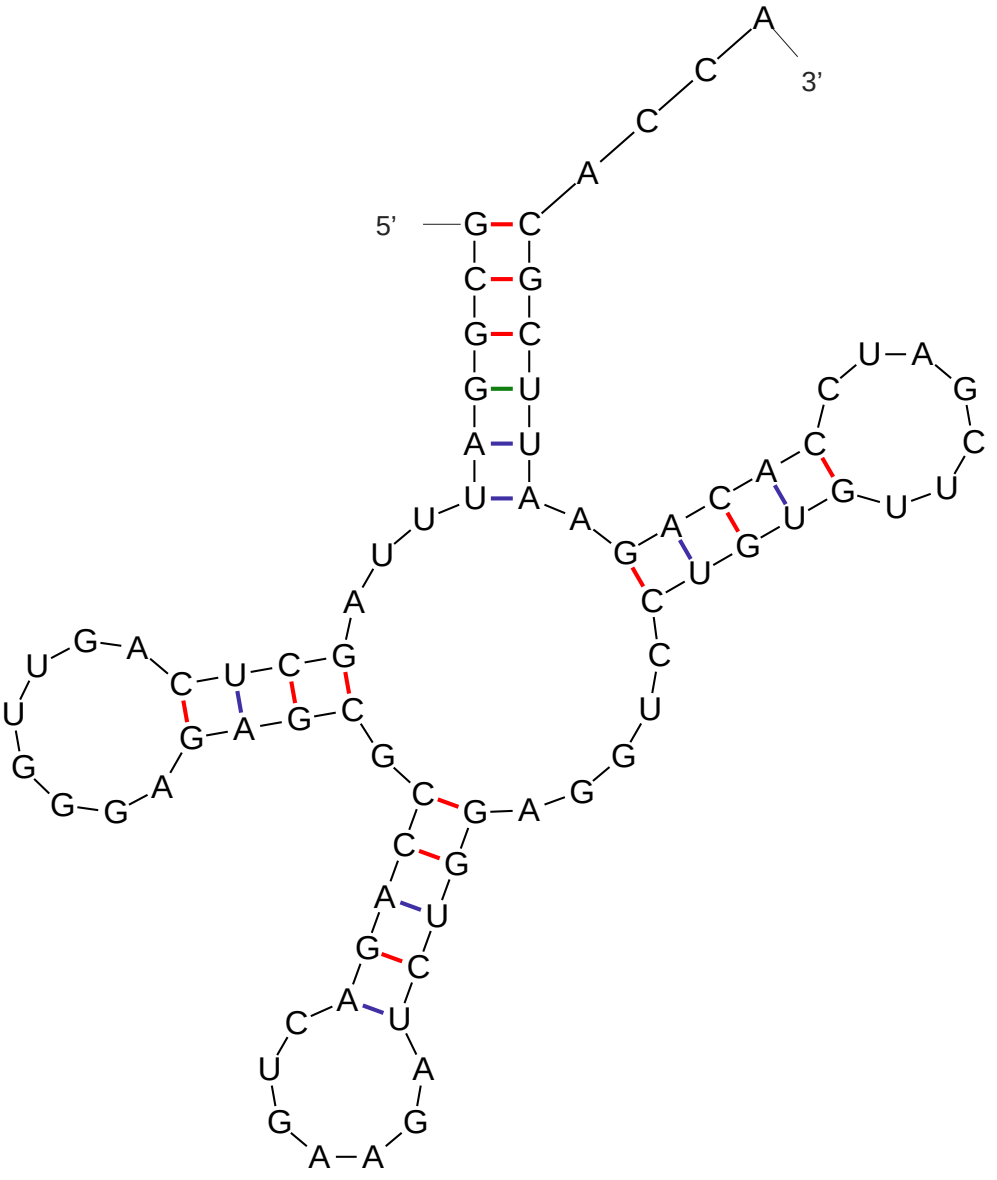
This image preserves some features of the 3D structure.



1TRA yeast Phe tRNA *(S. cerevisiae)*

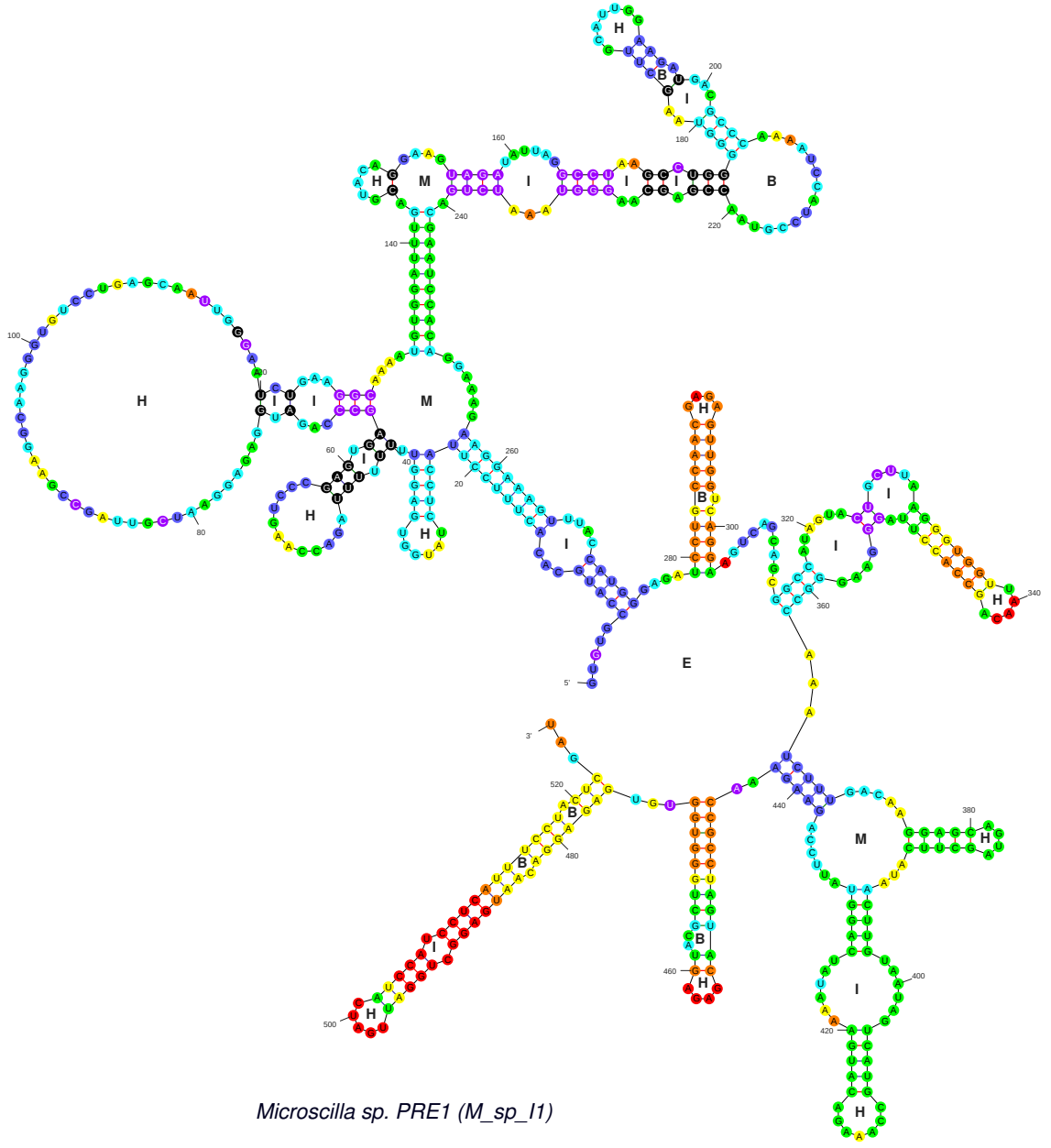
The “cloverleaf” secondary structure for *S. cerevisiae*

This traditional secondary structure representation for tRNA does not convey 3D structure information.



1TRA yeast Phe tRNA *(S. cerevisiae)*

Representations of RNA secondary structures.



Microscilla sp. PRE1 (M_sp_I1)

The traditional representation of secondary structure. Example is a group II intron from
Microscilla sp. PRE1
Source: Steve Zimmerly
`www.fp.ucalgary.ca/group2introns/species.htm`)

- E: exterior loop
- H: hairpin loop
- B: bulge loop
- I: interior loop
- M: multi-branch loop

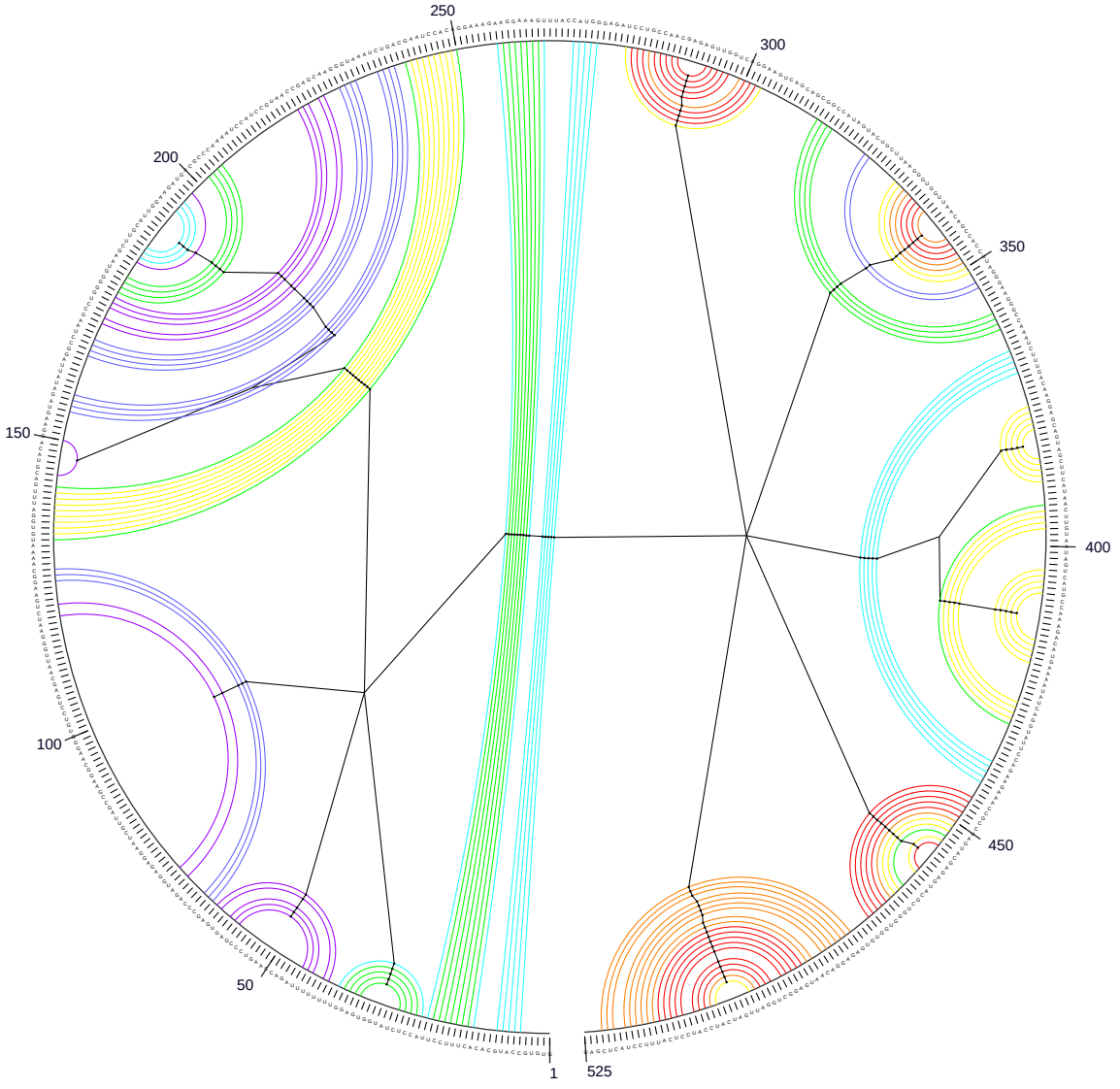
Colors depend on the probability of base pairs or the probability that a single-stranded base is single-stranded.

Color annotation of secondary structure.

The eight colors used to annotate probabilities. They emphasize the very high and very low probabilities.

0.9990 < Prob. <=1.0000
0.9900 < Prob. <=0.9990
0.9000 < Prob. <=0.9900
0.6500 < Prob. <=0.9000
0.3500 < Prob. <=0.6500
0.1000 < Prob. <=0.3500
0.0100 < Prob. <=0.1000
0.0000 < Prob. <=0.0100

Circle Plot & Tree Diagram



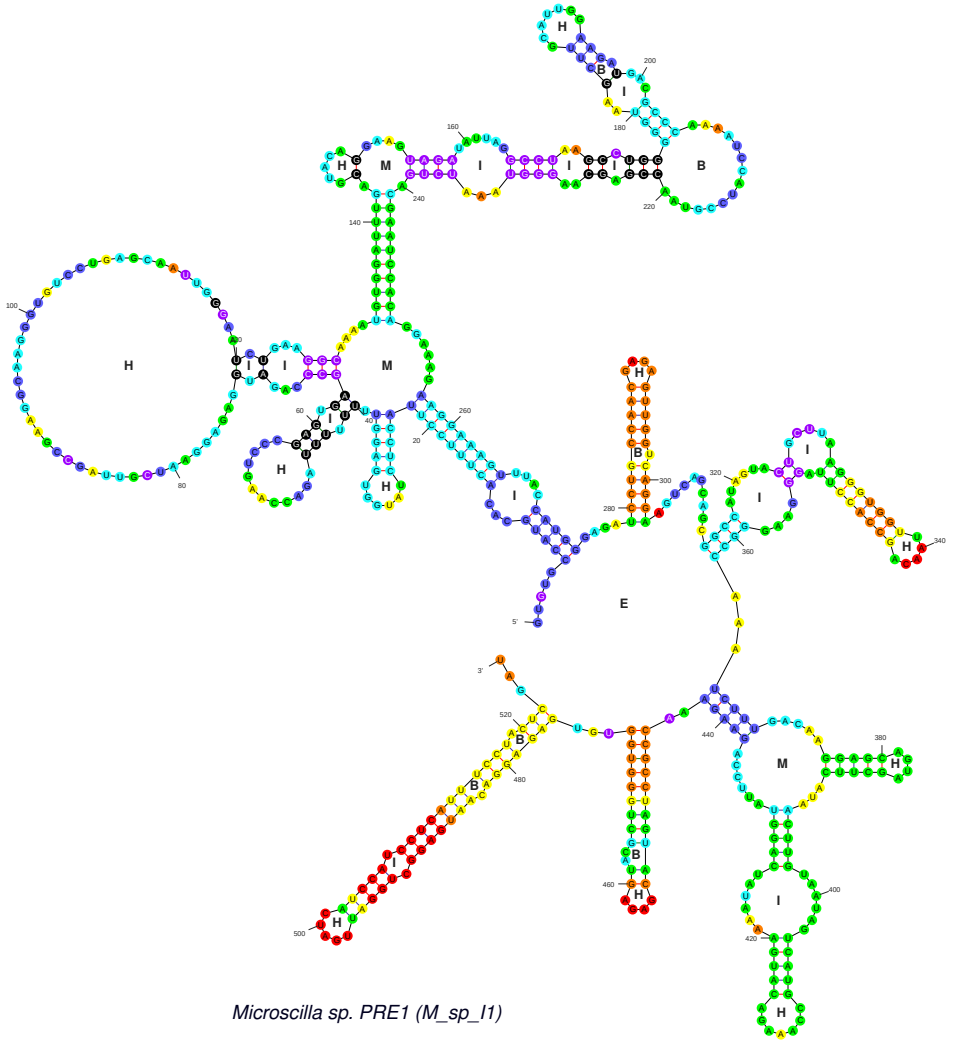
Microscilla sp. PRE1 (M_sp_l1)

Two plots in one.

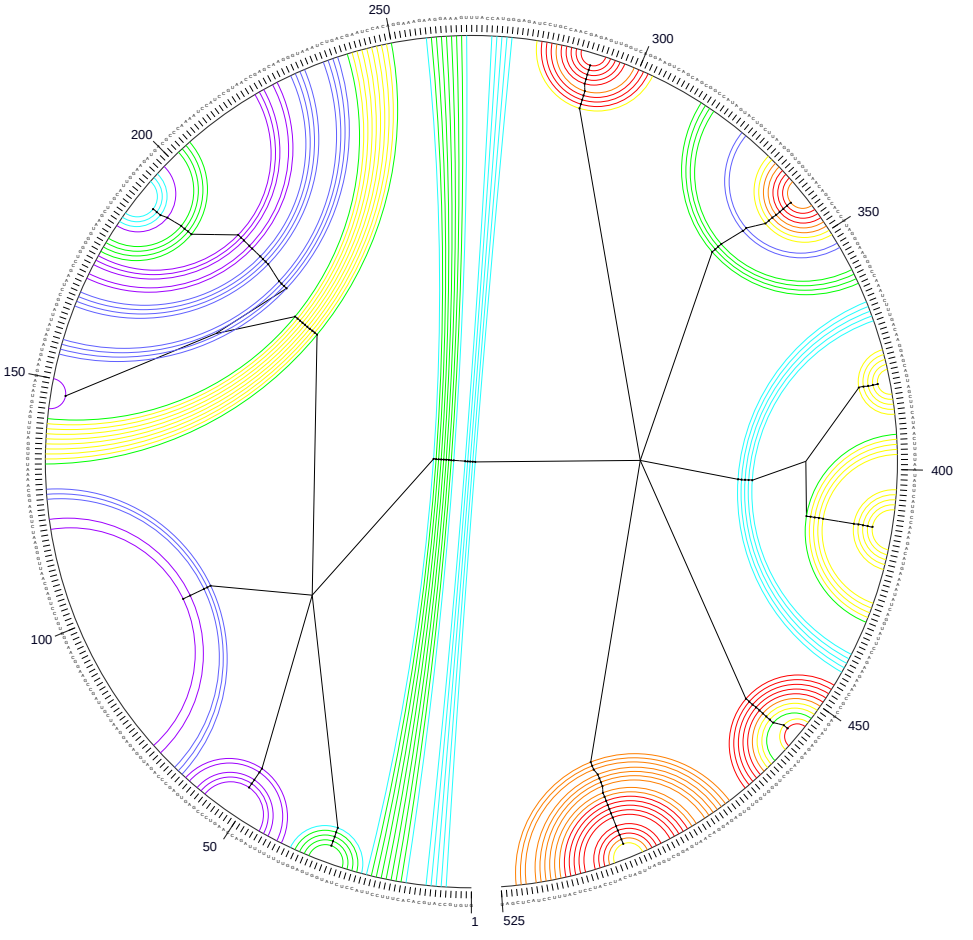
- Bases are drawn along the circumference of a circle
- Base pairs are circular arcs the intersect the circle at right angles.
- Black lines (edges) within the circle comprise a “tree representation” of the secondary structure.
  Every base pair and multi-branched loop is a node. Nodes connecting consecutive base pairs can be collapsed into a single “helix” node.

Colors depend on the probability of base pairs, as in the standard plot.

Side by side comparison - 1



Microscilla sp. PRE1 (M_sp_I1)



Microscilla sp. PRE1 (M_sp_I1)

Pseudoknot example

Two base pairs (BPs), $r_i \cdot r_j$ and $r_{i'} \cdot r_{j'}$, can be called "incompatible" if $i < i' < j < j'$. That is, they violate the third rule for RNA secondary structure.

A pseudoknot is created by two incompatible helices (stems). That is, every BP in one is incompatible with every BP in the other.

```
                A-C
3'- A-G-G-C-U /   U          <=== Example of a simple pseudoknot.
    U-C-C-G-A-G-G-G
     U        C-C-C - 5'
      C--U--C/
```

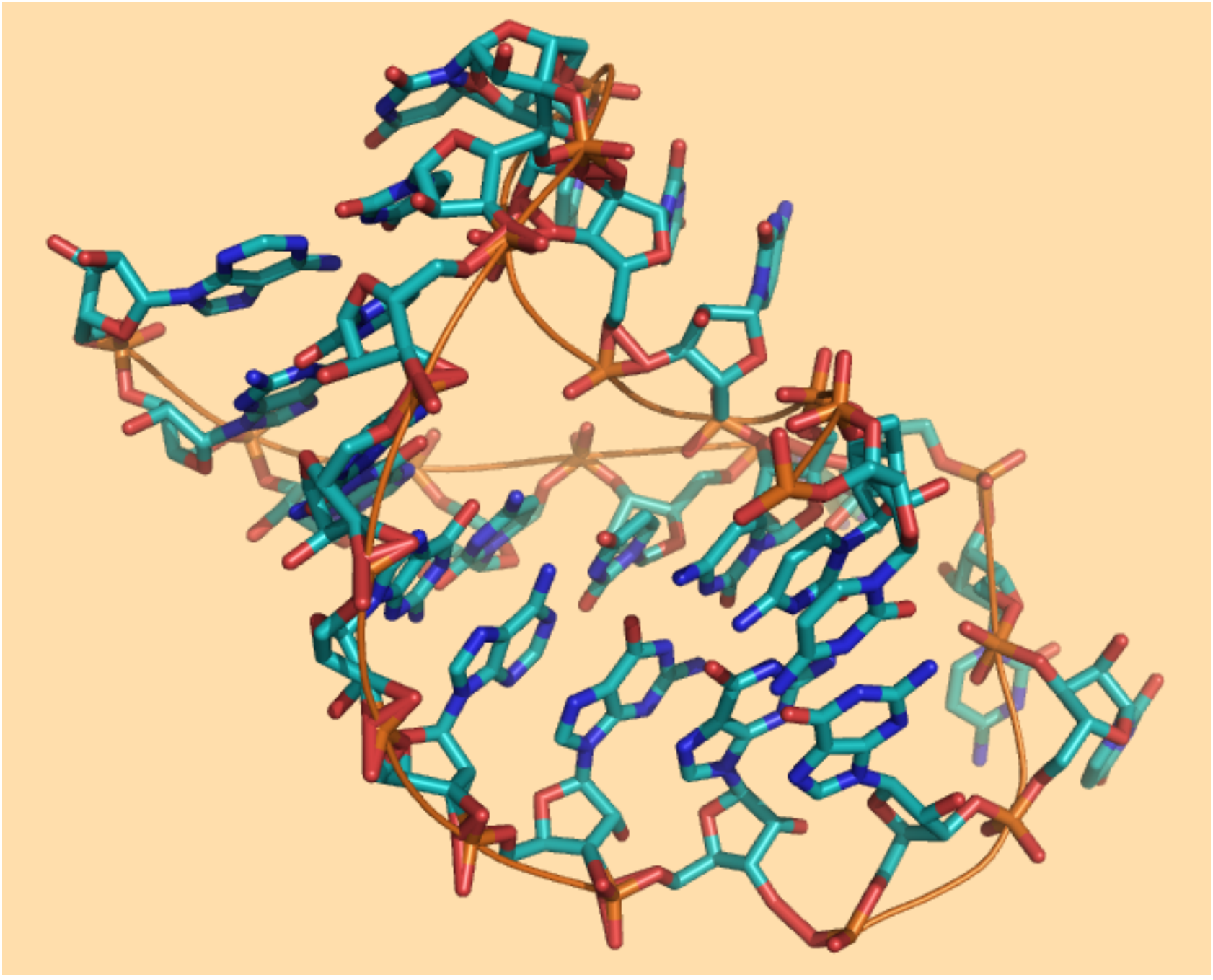
Stem 1: $\mathrm{C}_1 \cdot \mathrm{G}_{15}$, $\mathrm{C}_2 \cdot \mathrm{G}_{14}$, $\mathrm{C}_3 \cdot \mathrm{G}_{13}$,

is incompatible with

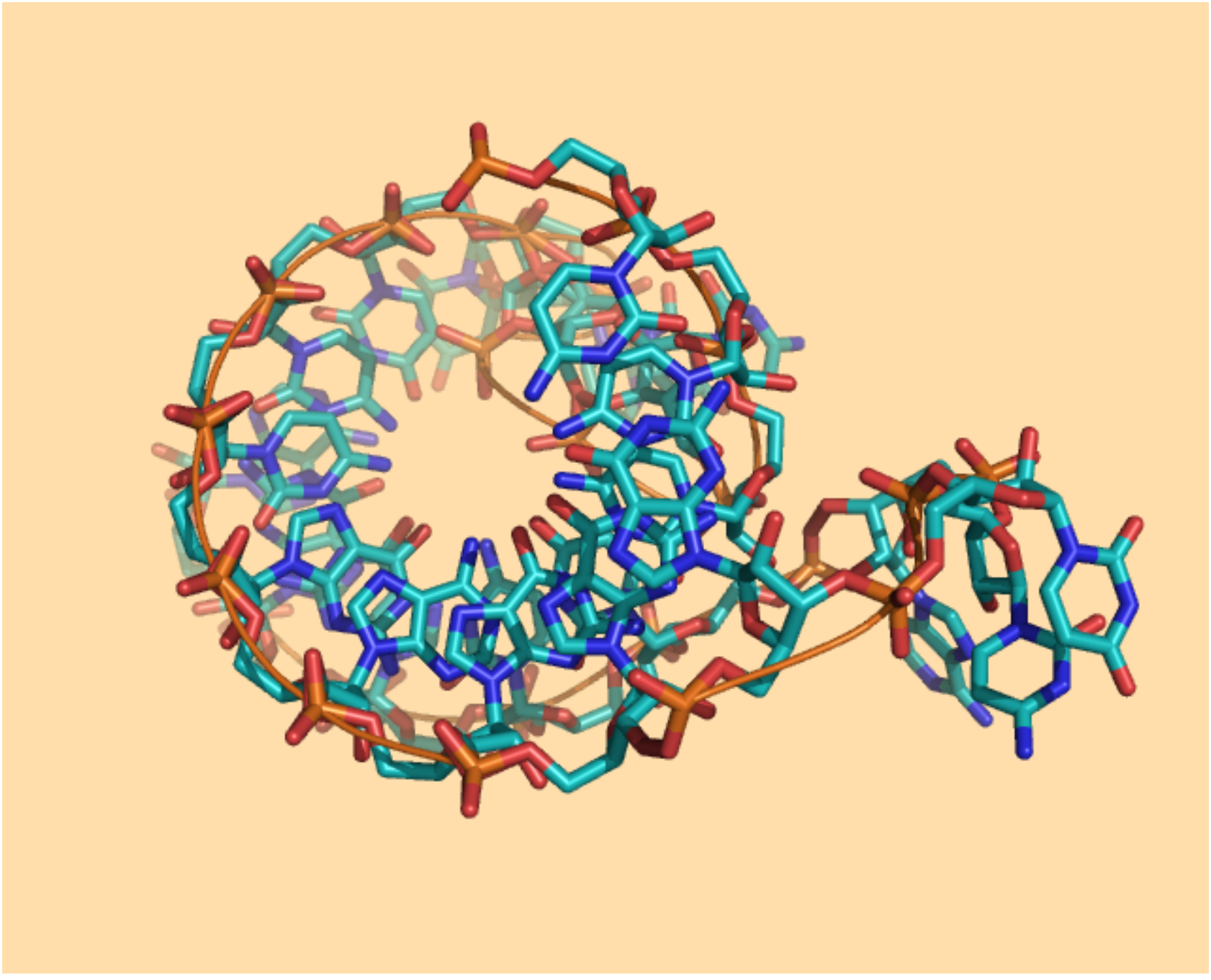
Stem 2: $\mathrm{U}_8 \cdot \mathrm{A}_{23}$, $\mathrm{C}_9 \cdot \mathrm{G}_{22}$, $\mathrm{C}_{10} \cdot \mathrm{G}_{21}$, $\mathrm{G}_{11} \cdot \mathrm{C}_{20}$, $\mathrm{A}_{12} \cdot \mathrm{U}_{19}$.

Is this possible? – Yes.

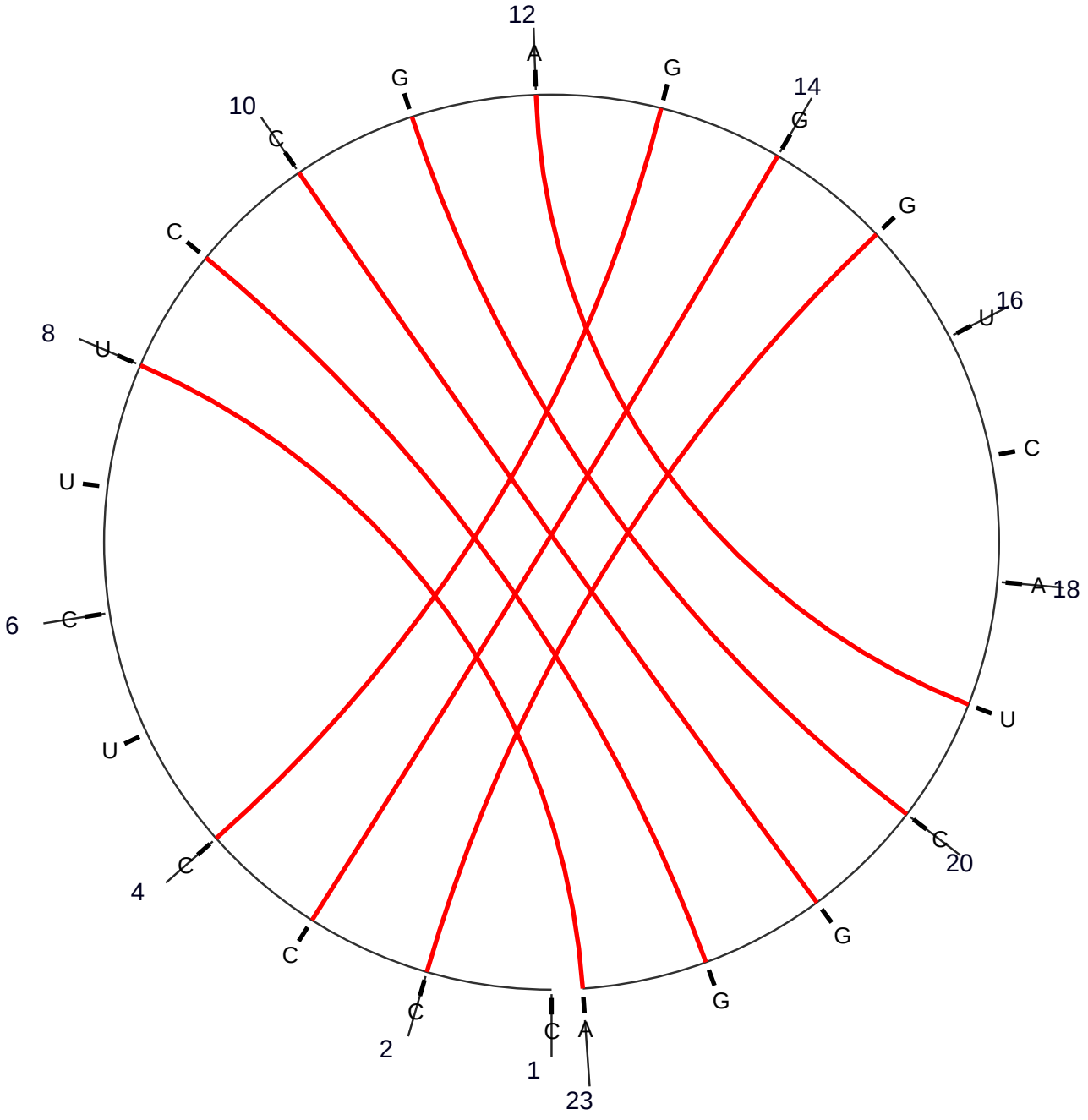
3D model of simple pseudoknot. Coordinates by F. Major



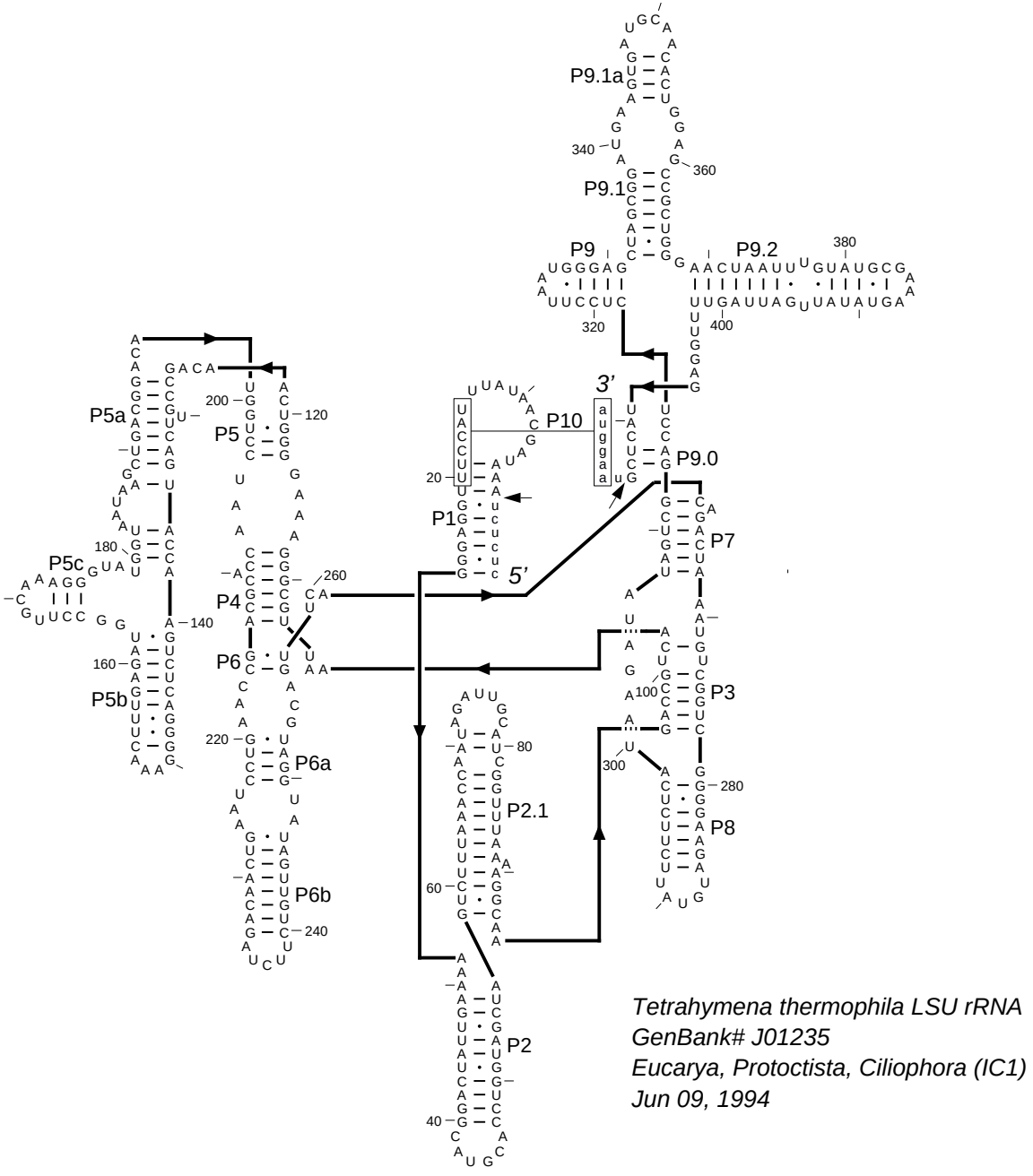
Same model - orientation shows coaxial stacking



In the circle plot, intersecting BP arcs indicate a pseudoknot

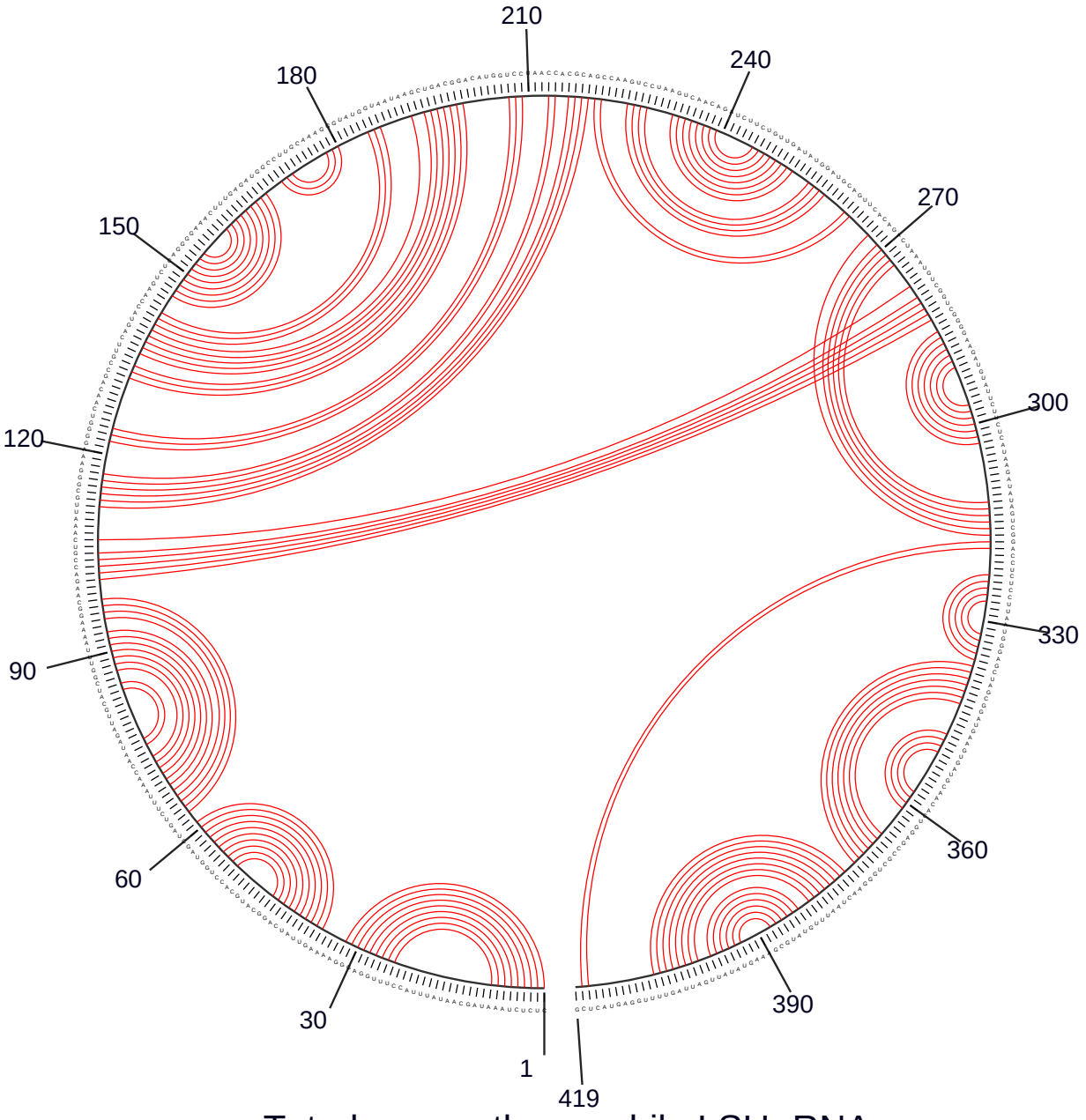


Can you find the pseudoknot?



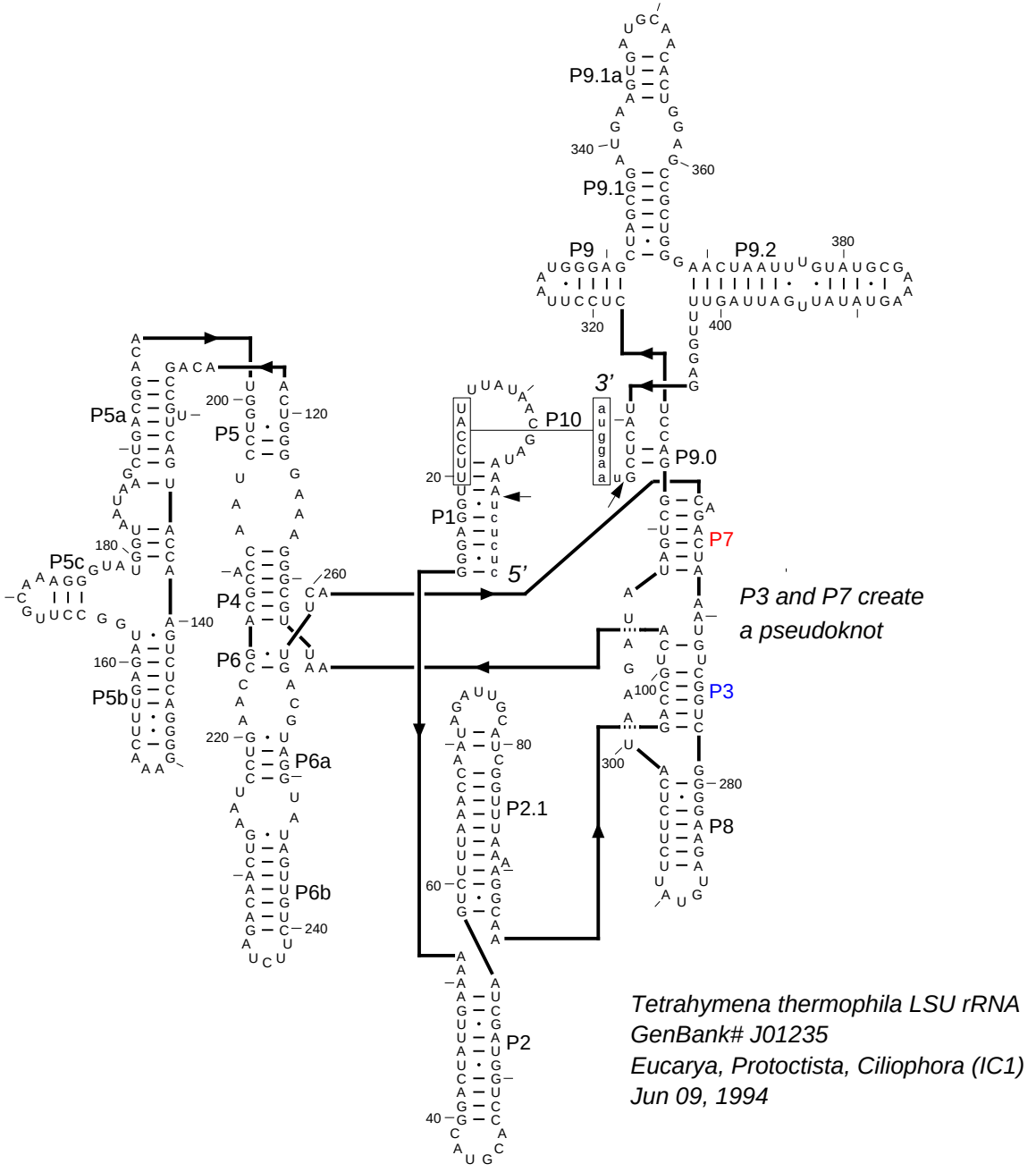
Tetrahymena thermophila LSU rRNA
GenBank# J01235
Eucarya, Protoctista, Ciliophora (IC1)
Jun 09, 1994

Now you can. P3 and P7 create a pseudoknot.

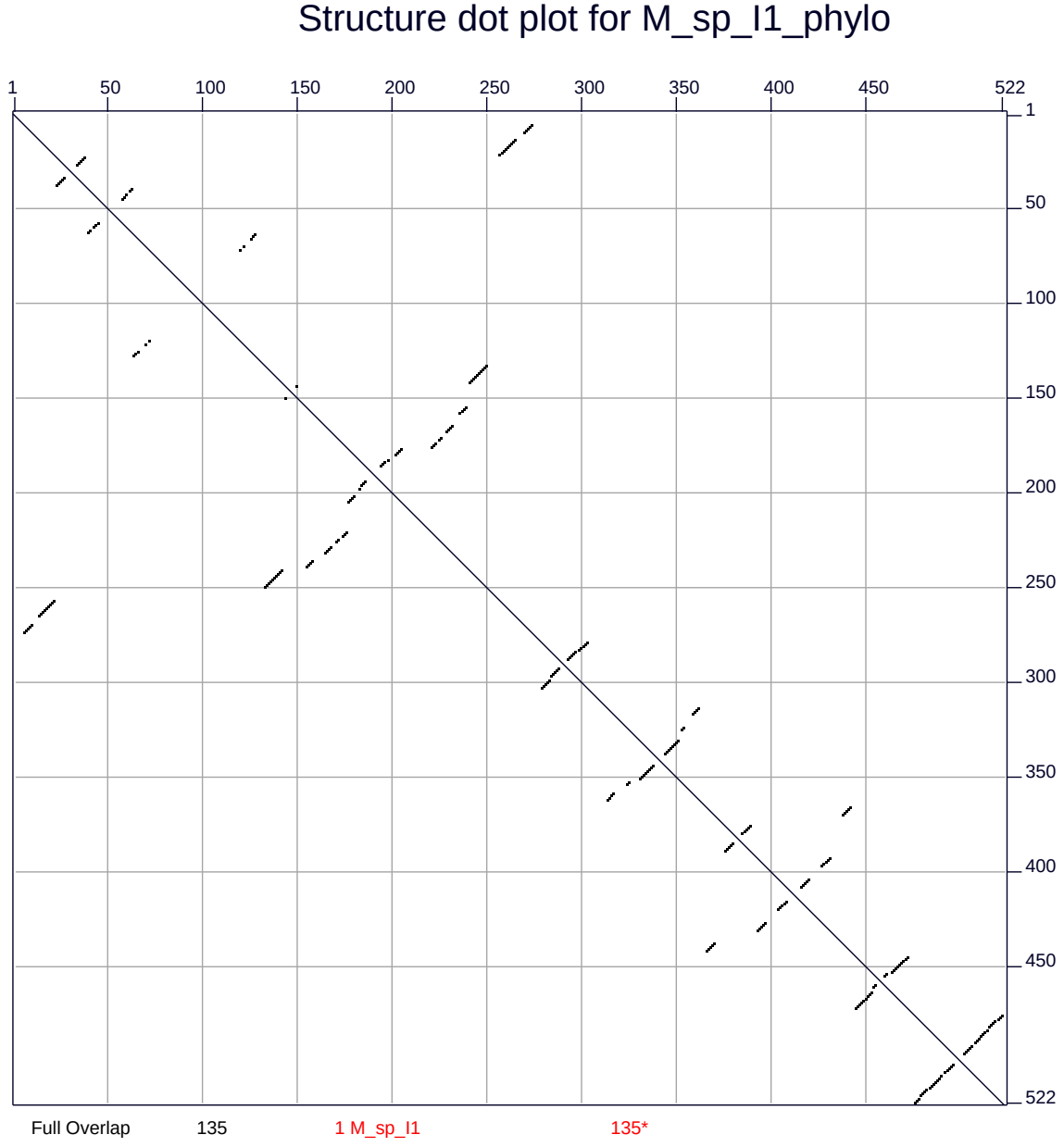


Tetrahymena thermophila LSU rRNA

Now you can see them in the original plot.



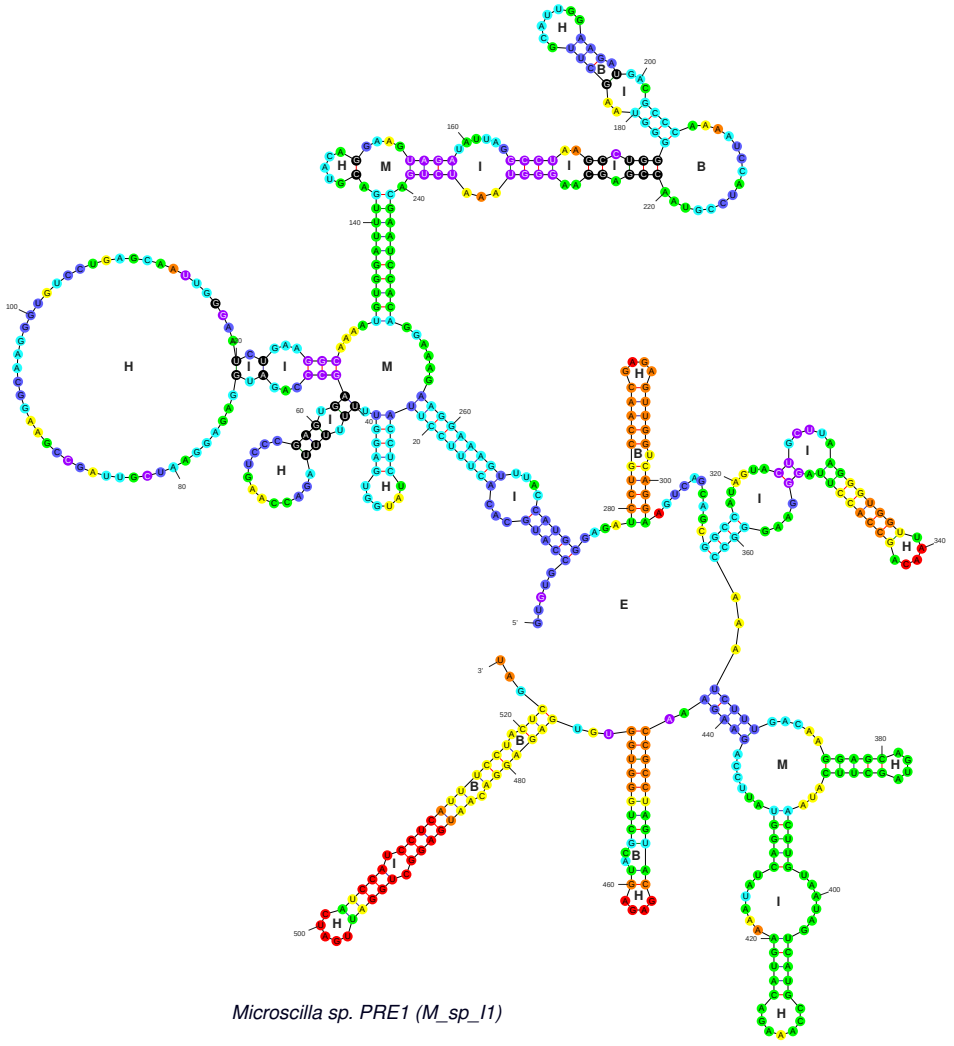
Dot plot



The most abstract representation of secondary structure.

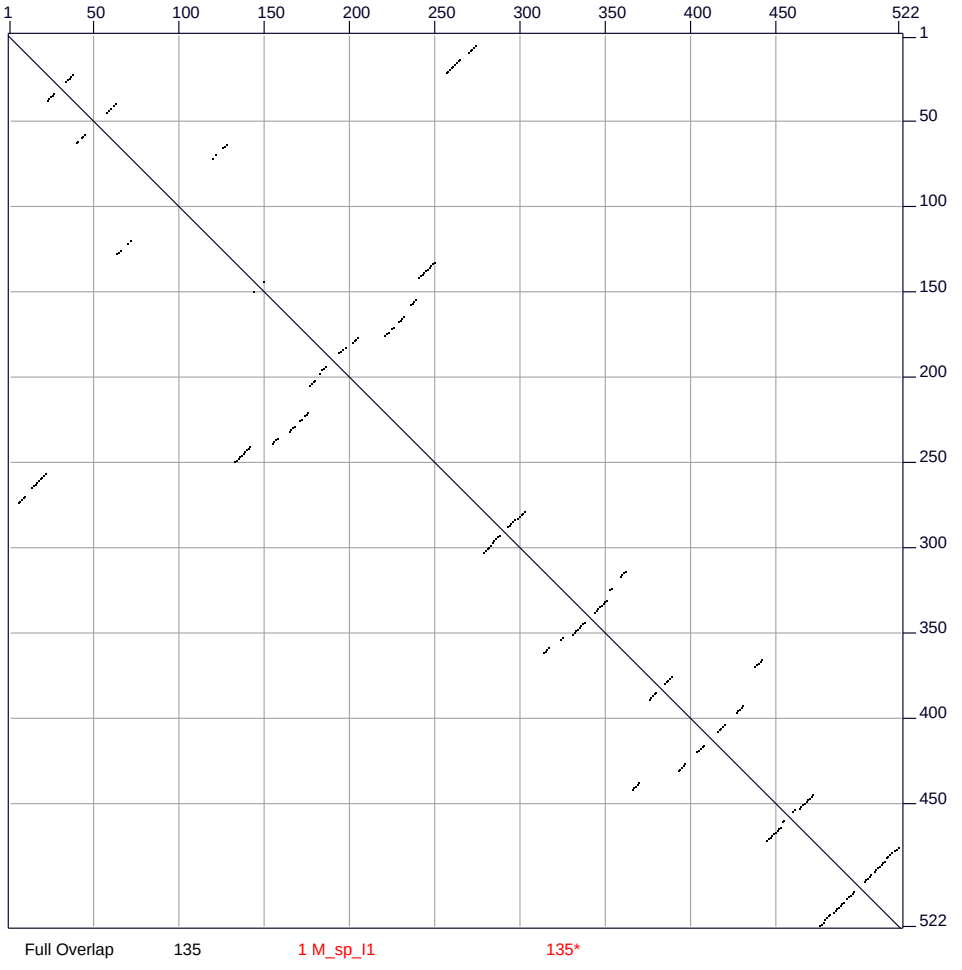
- Bases are not drawn.
- Base pairs are dots (filled in circles, squares, diamonds or other shapes).
  Row is $5'$ base & Column is $3'$ base. A dot in row i and column j represents the base pair $r_i \cdot r_j$ in the RNA whose sequence is $r_1 r_2 \ldots r_n$.
- Helices and hairpin loops are easy to detect.
- Bulge and interior loops are a bit harder to detect.
- Multi-branch loop detection is not easy.

Side by side comparison - 2



Microscilla sp. PRE1 (M_sp_I1)

Structure dot plot for M_sp_I1_phylo



Prediction of RNA secondary structure

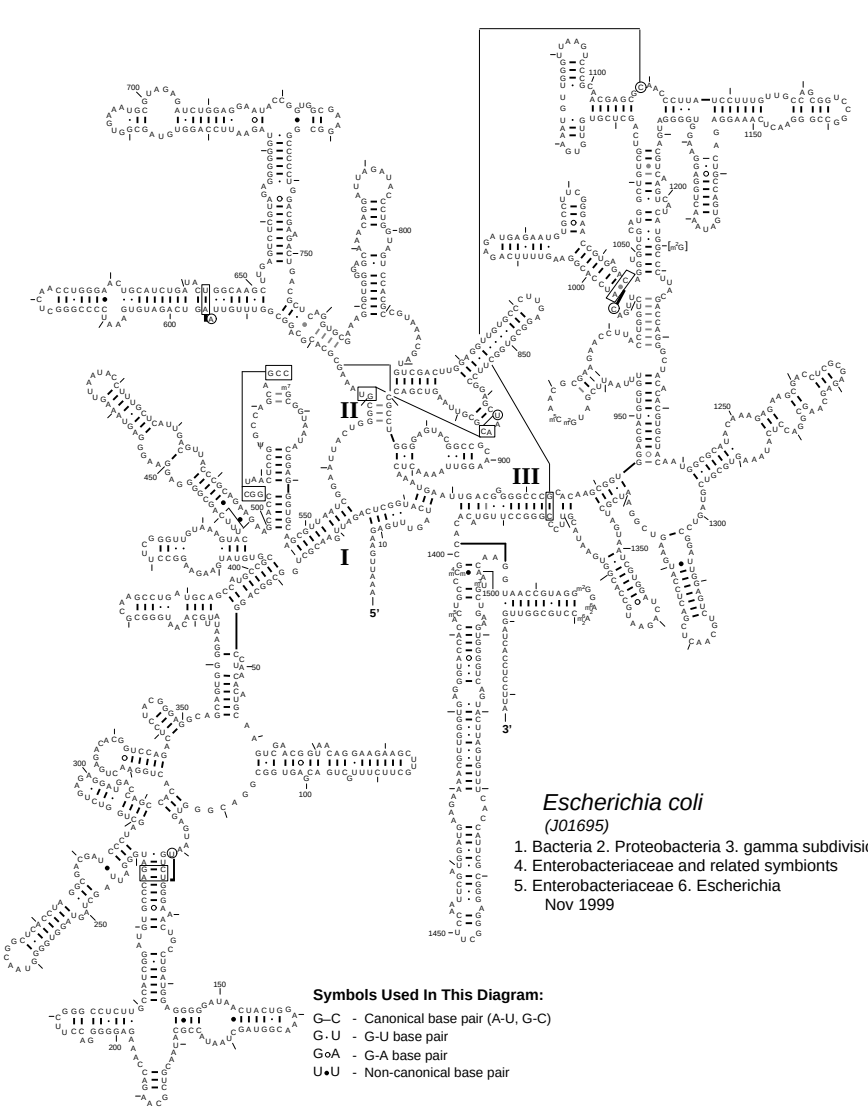
What are the common methods?

- Comparative, or phylogenetic methods.
  - Considered the “gold standard”.
  - Labor intensive
  - Requires numerous homologous sequences that can be well aligned.
- Free energy minimization methods
  - Works on single sequences.
  - Fast, cheap and easy to perform.
  - Unreliable in general.
  - Cannot predict pseudoknots. (Some time consuming exceptions exist.)

Comparative methodology

- A “golden rule” in biology: Structure is conserved more than sequence.
- This principle can be used to predict RNA secondary structure.
- It is used together with site directed mutagenesis to confirm the existence of specific base pairs.
- It can be used, for example, to design non-virulent strains of an RNA virus by interrupting significant secondary structure.

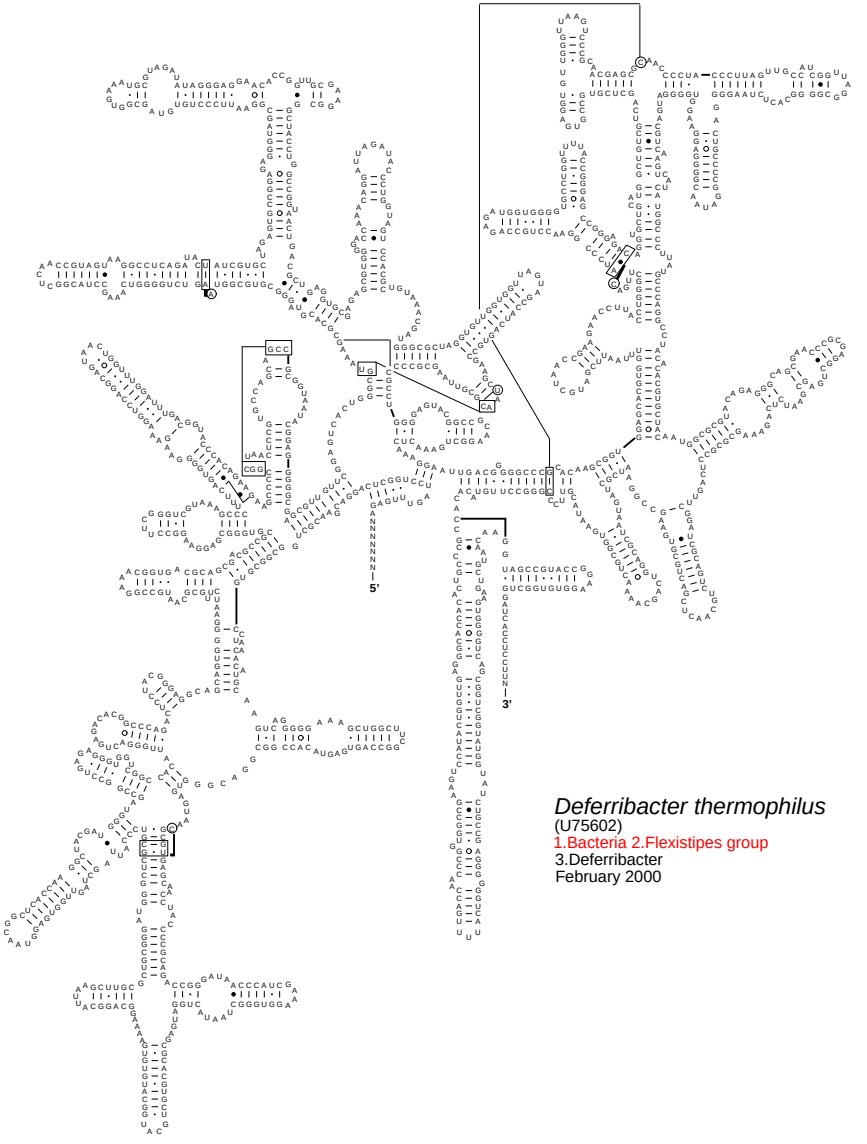
SSU rRNA: *Escherichia coli versus Deferribacter thermophilus*



Escherichia coli
(J01695)
1. Bacteria 2. Proteobacteria 3. gamma subdivision
4. Enterobacteriaceae and related symbionts
5. Enterobacteriaceae 6. Escherichia
Nov 1999

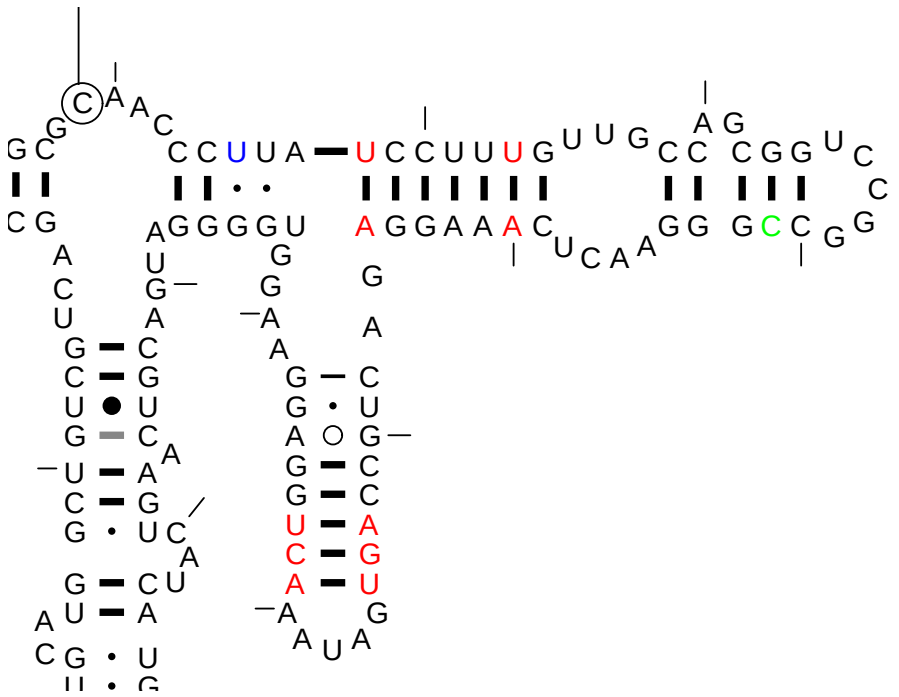
Symbols Used In This Diagram:

G–C - Canonical base pair (A-U, G-C)
G·U - G-U base pair
G∘A - G-A base pair
U•U - Non-canonical base pair

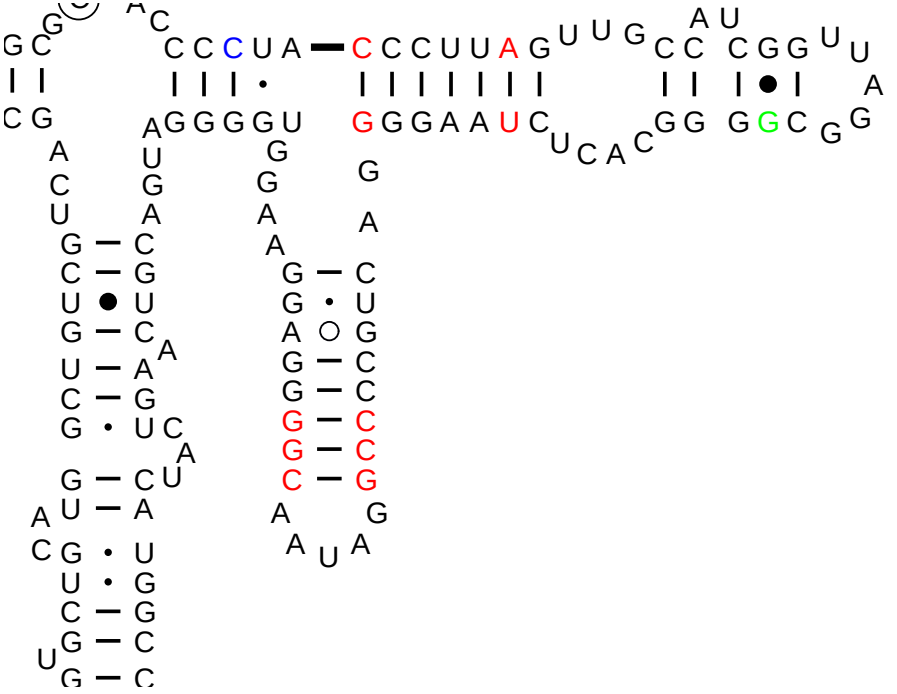


Deferribacter thermophilus
(U75602)
1.Bacteria 2.Flexistipes group
3.Deferribacter
February 2000

Secondary structure comparison between two 16S rRNAs.



Escherichia coli



Deferribacter thermophilus

Compare a small domain in one with the corresponding domain in the other.

- BP is conserved – Both bases unchanged.
- BP is conserved – Both bases change (compensatory change)
- BP is conserved – One base changes (W-C $\leftrightarrow$ wobble pair)
- BP not conserved – One base changes (W-C $\leftrightarrow$ non-canonical pair)

Need an alignment of homologous sequences

Given m RNA sequences, $R_1, R_2, \ldots, R_m$. The i^{th} sequence has length n_i. After alignment, they all have a common length, n. They can be written as

$$\begin{array}{lcccccc}
R_1 & = & r_1(1), & r_1(2), & r_1(3), & \ldots, & r_1(n), \\
R_2 & = & r_2(1), & r_2(2), & r_2(3), & \ldots, & r_2(n), \\
R_3 & = & r_3(1), & r_3(2), & r_3(3), & \ldots, & r_3(n), \\
\vdots & & \vdots & \vdots & \vdots & \vdots & \vdots \\
R_m & = & r_m(1), & r_m(2), & r_m(3), & \ldots, & r_m(n).
\end{array}$$

$R_k(i)$ is the i^{th} "base" in the k^{th} sequence. It is A, C, G, U or "-". The last symbol stands for an inserted gap.

Constructing a "correct" alignment is usually slow and difficult work. Many methods have been developed to automate this procedure.

"Correct" refers to computing an alignment that captures the evolution of these RNAs.

Some History: Quick-n-Dirty

Copied from the Gutell Lab web site:

`http://www.rna.ccbb.utexas.edu/CAR/1D/Paradigms/Visual`

Red-dot Green-dot: 1978–1981; attributed to Carl Woese

- Strict covariation.
- Can detect W-C and wobble pairs only (consecutive, anti-parallel & nested)
- Provides a comparative proof for a helix. Two or more compensatory base changes within a helix.
- Less similar sequences provide more supporting changes.
- Aligns sequences to maximize primary structure similarity.
- Analyzes just two sequences at one time.
- Identifies secondary interactions only.

Red-dot, green-dot: Gutell tRNA example

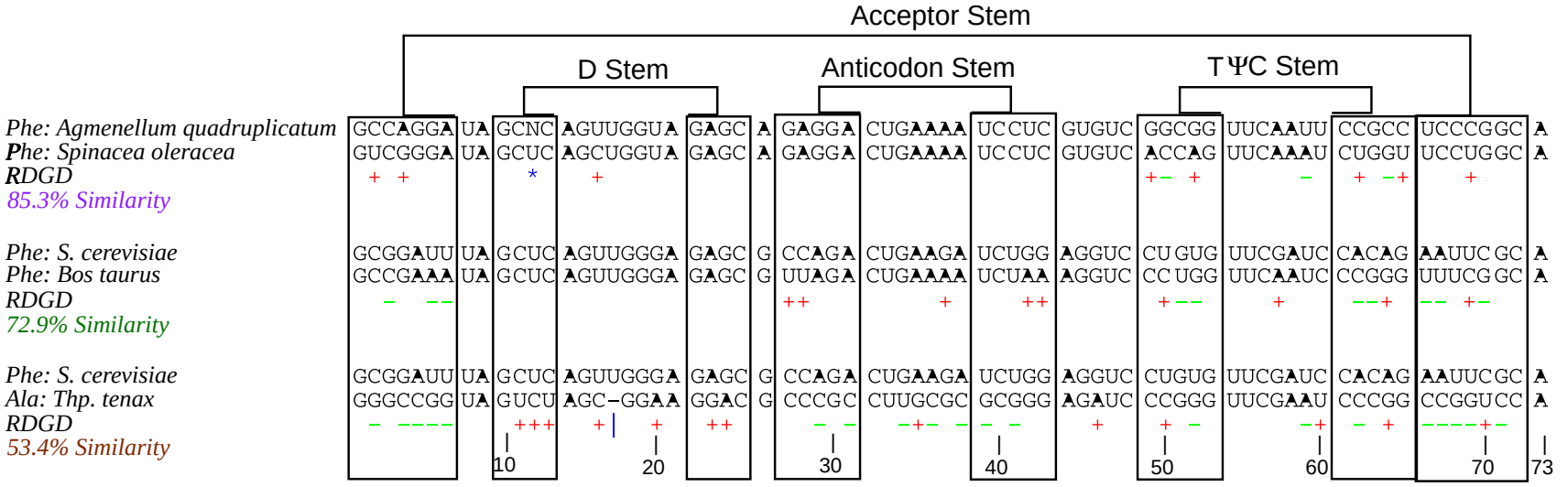


Figure 1. Reddot-greendot examples from tRNA. Symbols used: +: transition; -: transversion; |: deletion; *: ambiguous nucleotide. Experimentally verified helices from the secondary structure are boxed and connected with black lines. Nucleotide position numbers refer to the S.cerevisiae Phe reference sequence. Sequence names are shown as *amino acid:organism*.

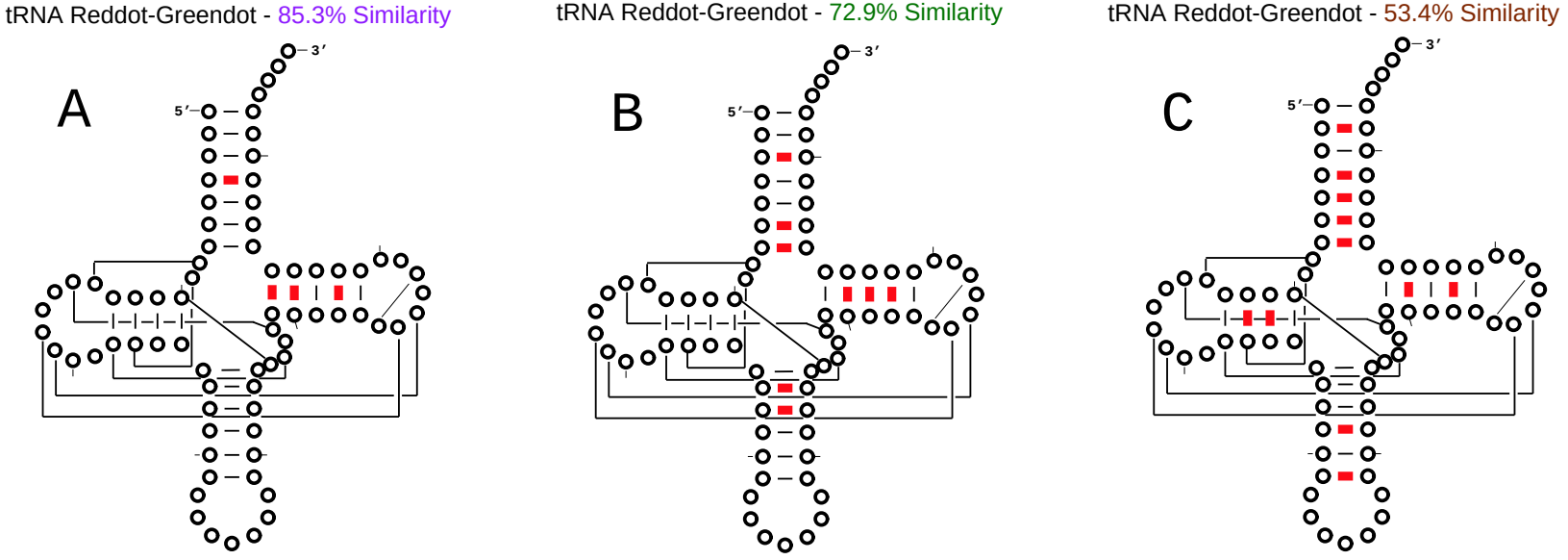
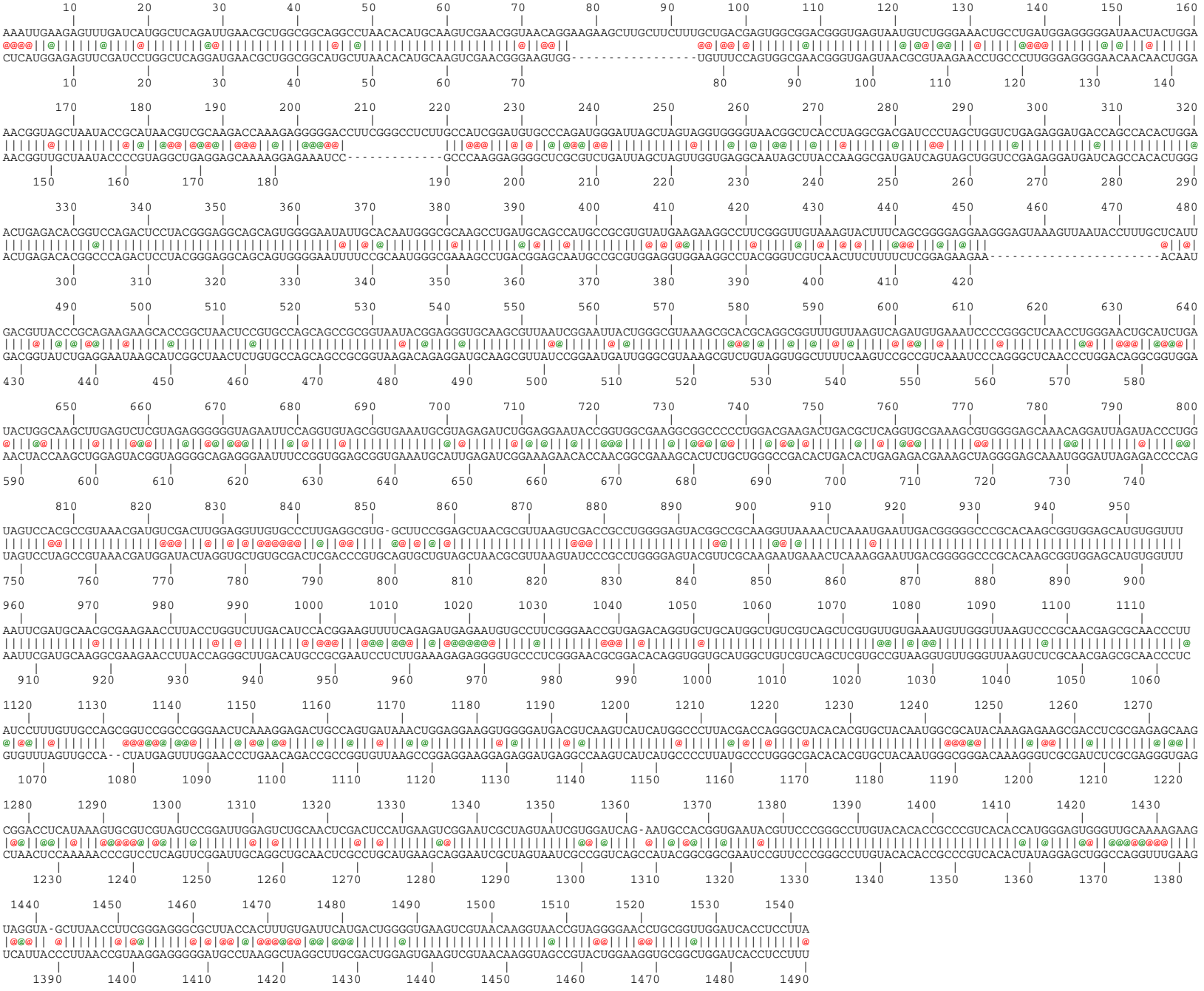
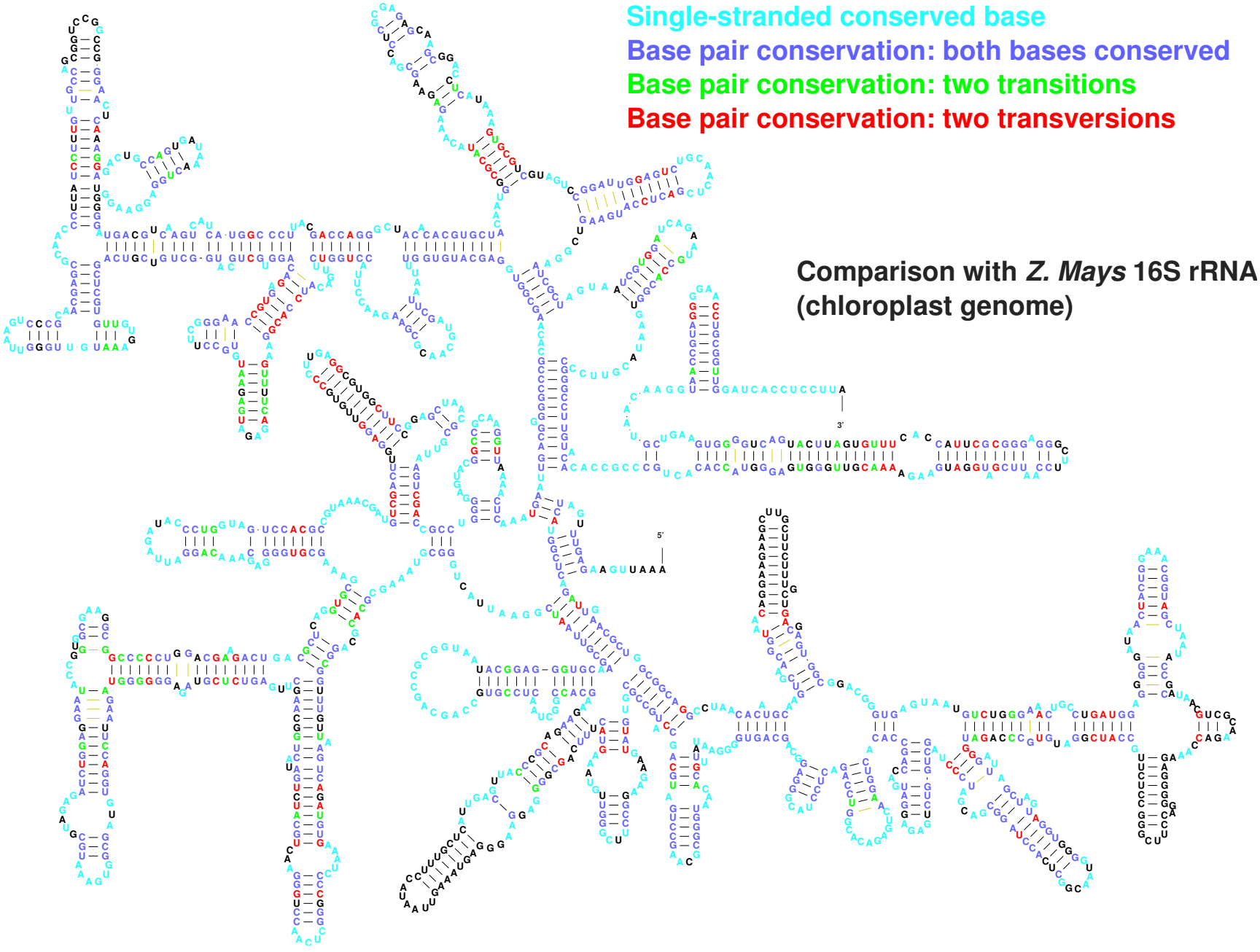


Figure 2. Results of the reddot-greendot analysis shown on tRNA secondary structure diagrams. Base pairs which are predicted with the method are shown with red tick marks. **A**. Sequences with 85.3% similarity. **B**. 72.9% similarity. **C**. 53.4% similarity.

Green dot Red dot: *E. coli vs. Z. mays 16S rRNA.*



Annotated secondary structure plot for *E. coli* 16S rRNA.



Modern approach. A no-brainer alignment.

The twenty 5S rRNAs shown below all have the same length. Alignment is simple. No gaps are introduced.

```
GCUUACGGCCAUACCAGCCUGAAUACGCCCGAUCUCGUCCGAUCUCGGAAGCUAAGCAGGGUCGGGCCUGGUUAGUACUUGGAUGGGAGACCGCCUGGGAAUACCAGGUGCUGUAAGCUU
GCUUACGGCCAUACCACCCUGAGCACGCCCGAUCUCGUCCGAUCUCGGAAGCUAAGCAGGGUCGGGCCUGGUUAGUACUUGGAUGGGAGACUGCCUGGGAAUACCAGGUGUUGUAAGCUU
GCUUACGGCCACACCAACCUGAGCAAGCCCGAUCUCGUCUGAUCUCGGAAGCCAAGCAGGGUUGGGCCUGGUUAGUACUUGGAUGGGAGACUGCCUGGGAAUACCAGGUGUUGUAAGCUU
GCCUACGACCAUACCACCAUGAGUAUACCGGUUCUCGUCCGAUCACCGGAGUCAAGCAUGGUCGGGCCGGGUCAGUACCUGGAUGGGUGACCGCCUGGGAACACCUGGUGUUGUAGGCCU
GCCUACGACCAUACCACCAUGAAUACACCGGUUCUCGUCCGAUCACCGAAGUUAAGCAUGGUCAGGCCGGGUCAGUACCUGGAGUGGUGACCGCCUGGGAACACCCGGUGUUGUAGGCCU
GUCUACGACCAUACCACAAUGAACACACCGGUUCUCGUCCGAUCACCGAAGUUAAGCAUUGUCGGGCCAGGAUAGUACCUGGAUGGGGGACCGCCUGGGAACGCCUGGUGUCGUAGACUU
GUCUACGGCCAUACCACCGGGAAAAAACCGGUUCUCGUCCGAUCACCGAAGUCAAGCCCGGUAGGGCCAGGUUAGUACUUGGAUGGGUGACCGCCUGGGAAUACCUGGUGCUGUAGACUU
GCCUGCGGCCAUACCACGUUGAAUGCACCGGUUCCCAUCUGAACACCGAAGUUAAGCAACGUCGGGCCAGCUUAGUACCUGGAUGGGUGACCGCCUGGGAAUCGCUGGUGCUGCAGGCUU
GCCUACGGCCAUACCACGUUGAAAACACCGGUUCUCGUCUGAUCACCGAAGUUAAGCAACGUAGGGCCUGCCCAGUACUUGGAUGGGUGACCGCCUGGGAACAGCAGGUGUUGUAGGCUU
GCCUAGGACCAUAUCACGUUGAAUGCACCGGUUCUCGUCCGAUCACCGAAGUUAAGCAACGUCGAGCCCGGUUAGUACUUGGAUGGGUGACCGCCUGGGAAUACCGGGUGUUCUAGGCCU
GGCAACGACCAUACCACGUUGAAUACACCAGUUCUCGUCCGAUCACUGAAGUUAAGCAACGUCGGGCGUAGUUAGUACUUGGAUGGGUGACCGCUUGGGAACACUACGUGCCGUUGGCAU
GCCAACGUCCAUACCAUGUUGAAUACACCGGUUCUCGUCCGAUCACCGAAGUCAAGCAACAUCGGGCGUGGUCAGUACUUGGAUGGGUGACCGCCUGGGAACACCACGUGAUGUUGGCUU
GAUAGCGUCCAUACCACACUGAAAACACCGGUUCUCGUCCGAUCACCGCAGUUAAGCAGUGUCGGGCCCAGUUAGUACUUGGAUGGGUGACCGCCUGGGAAUACUGGGUGUCGCUACCUU
ACCAACGGCCAUACCACGUUGAAAGUACCCAGUCUCGUCAGAUCCUGGAAGUCACACAACGUCGGGCCCGGUCAGUACUUGGAUGGGUGACCGCCUGGGAACACCGGGUGCUGUUGGCAU
GUCGACGCUCAUACUAGGUUGGGUCCACCCGAUCUCGUUCGAUCUCGGCAGUUAAACAACCUUAGGCCUCGUUAGUACUUGAAUGCGUGAGCGUCUGGGAAUACGAGGUGGUGUCGACUU
GUUGUCGGCCAUACUAAGGUGAAAACACCGGAUCCCAUUCGAACUCCGAAGUUAAGCGCCUUAAGGCUGGGUUAGUACUAAGGUGGGGGACCGCUUGGGAAGUCCCAGUGUCGACAACCU
GUUGGUGGCCAUACUAAGCCUAAAGCACCGGAUCCCAUUCGAACUCCGAAGUUAAGCGGCUUAAGGCGAGGUUAGUACUAAGGUGGGGGACCGCUUGGGAAGUCCUCGUGUUGACAACCC
GUUAUCGGCCAUACUAAGCCAAAAGCACCGGAUCCCAUUCGAACUCCGAAGUUAAGCGGCUUAAGGCAUGGUUAGUACUAAGGUGGGGGACCGCUUGGGAAGCCCAUGUGCUGAUAGCUU
GCUAUCGGCCAUACUAAGCCAAAUGCACCGGAUCCCAUCCGAACUCCGAAGUUAAGCGGUUUAAGGCCUGUUAAGUACUGAGGUGGGGGACCACUCGGGAACUUCAGGUGCUGAUAGCUU
GUUGUCGGCCAUACUAUGCCUAACGCACCAGAUCCCAUCCGAACUCUGAAGUUAAGCGGCAUAAGGCGAGGUUAGUACUUGGGUGGGGGACCGCCAGGGAAGCCCUCGUGCUGACAGCUA
```

Then what? How to find BPs conserved by compensatory mutations?

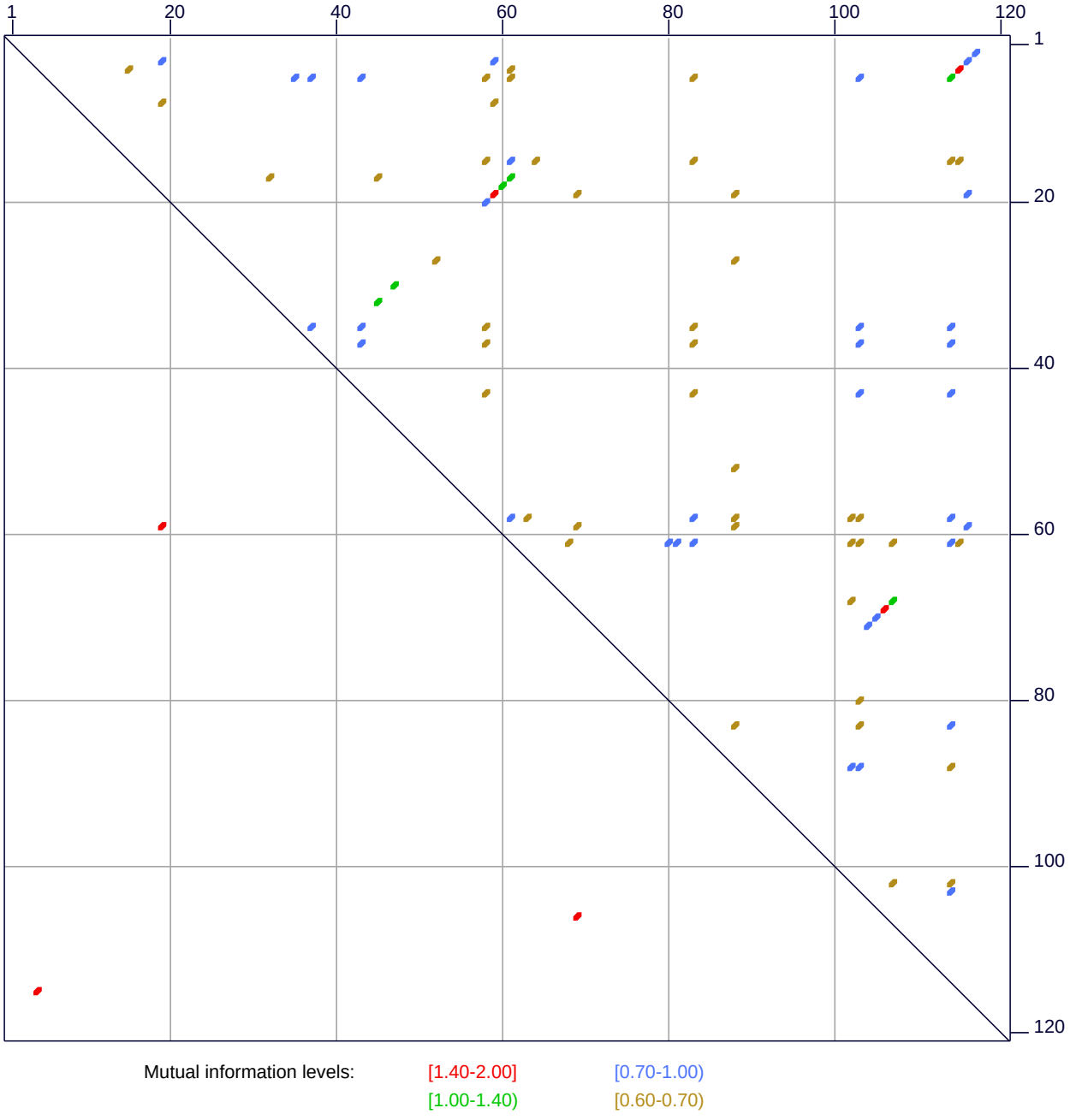
Mutual information content

- $M(i,j) =$ "mutual information" between columns i and j. It measures the "degree of correlation".
- A large $M(i,j)$ suggests that $r_k(i) \cdot r_k(j)$ exists for all (or most) k between 1 and m.
- Base pairs that are 100% conserved yield **no** mutual information.
- $M(i,j)$ is the "relative entropy" between a pair of probability distributions. If $f_{i,j}(B_1,B_2)$ is the observed frequency of the base pair, $B_1 \cdot B_2$, in columns i and j, and if $f_i(B)$ is the observed frequency of B in column i, then

$$M(i,j) = \sum_{B_1,B_2 \in \{A,C,G,U\}} f_{i,j}(B_1,B_2) \log_2 \frac{f_{i,j}(B_1,B_2)}{f_i(B_1) f_j(B_2)}.$$

Comment: The sum of $f_{i,j}(B_1,B_2)$ over all pairs and the sum of $f_i(B)$ over all bases may be < 1, since gaps are ignored.

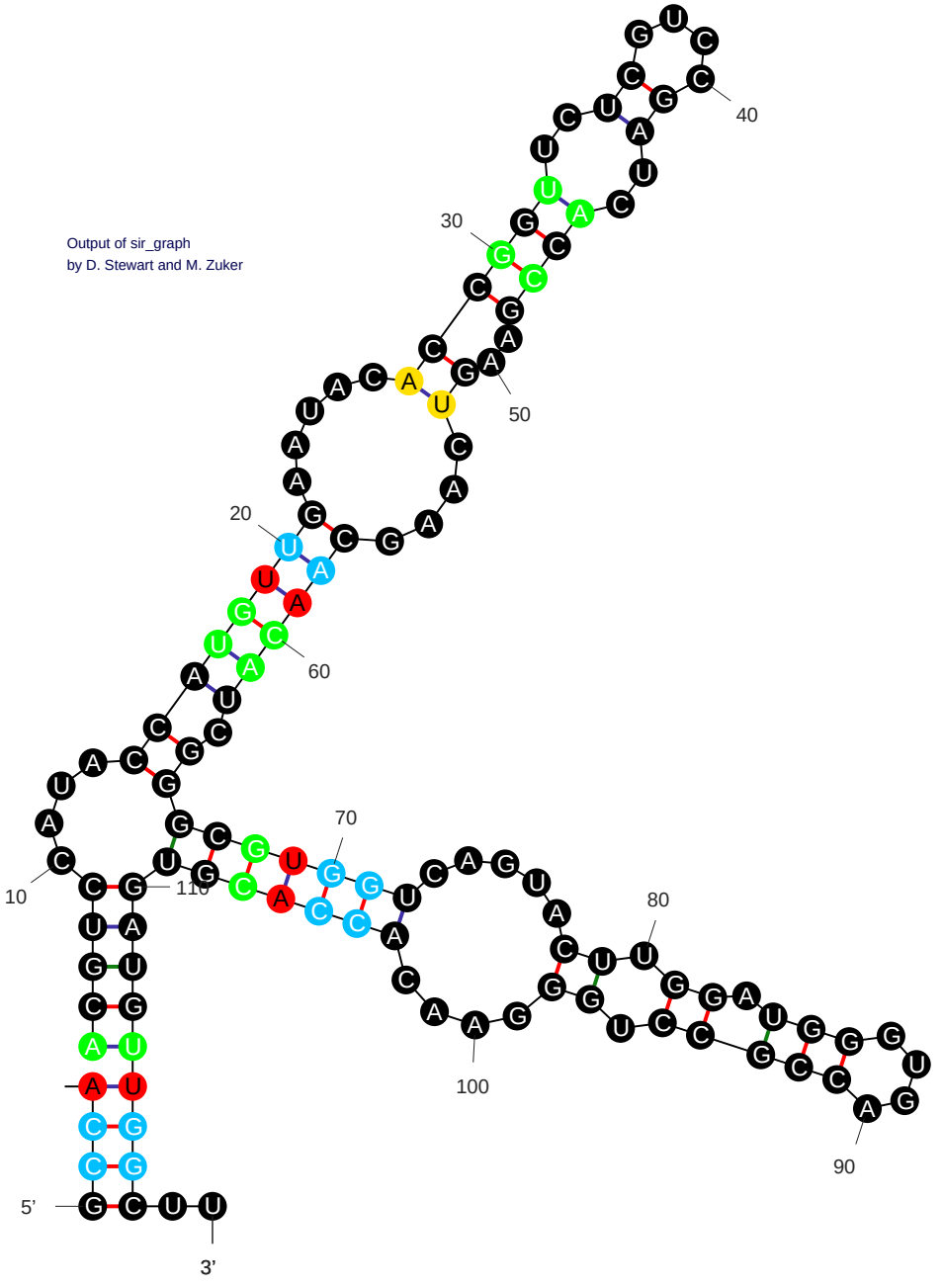
Mutual information plot for 20 Eukaryote 5S rRNAs



Twenty sequences perfectly aligned

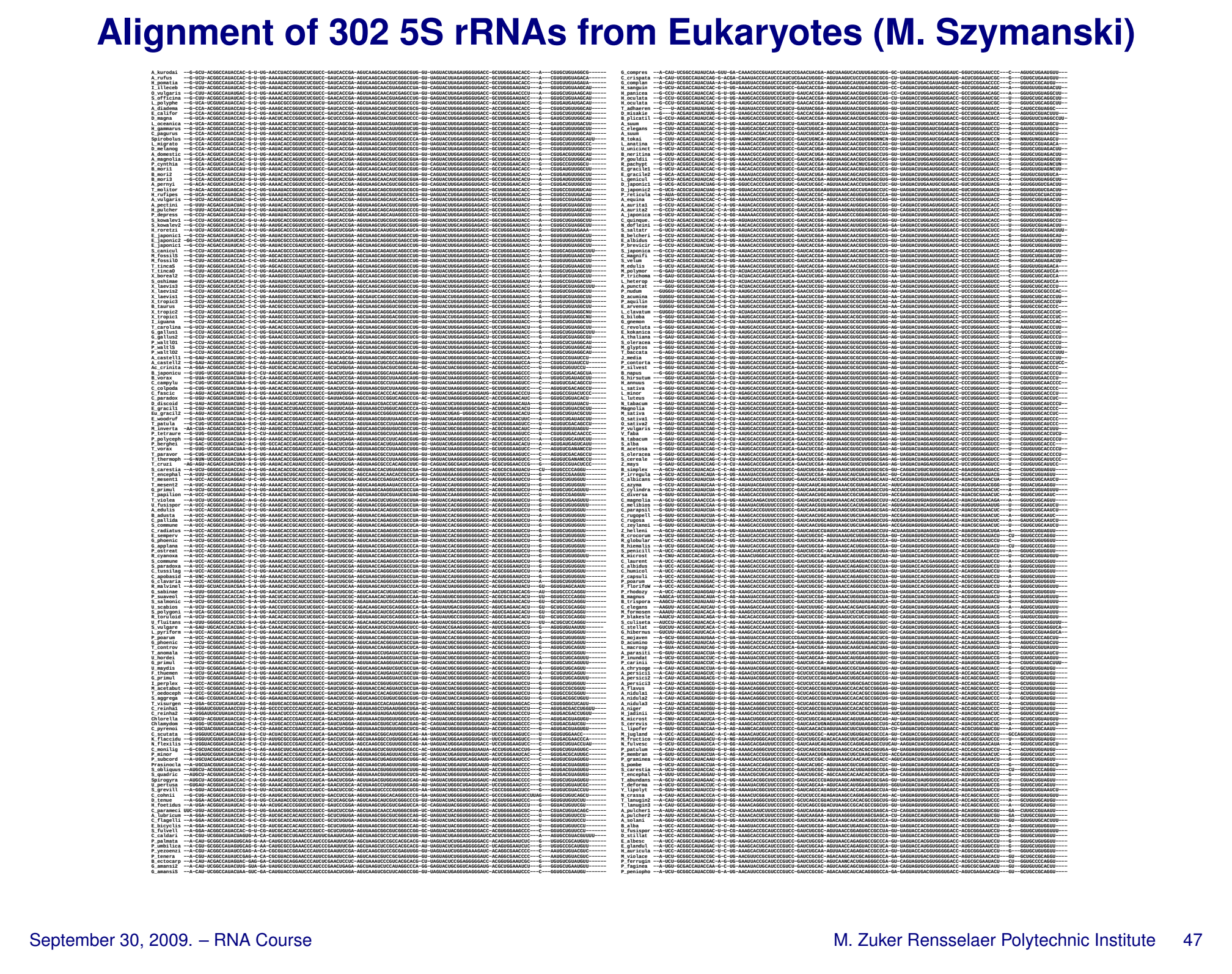
- Only (4+4+2+4=14) out of 39 (40 with non-canonical $U \cdot U$) base pairs are identified (35%)
- There is a fair amount of “noise”.
- 100% conserved base pairs not shown. They greatly add to the “noise”, but can be useful to “fill in” or extend stems (helices). Total of 86 base pairs in plot.

Comparative model for *Bombyx mori* 5S rRNA

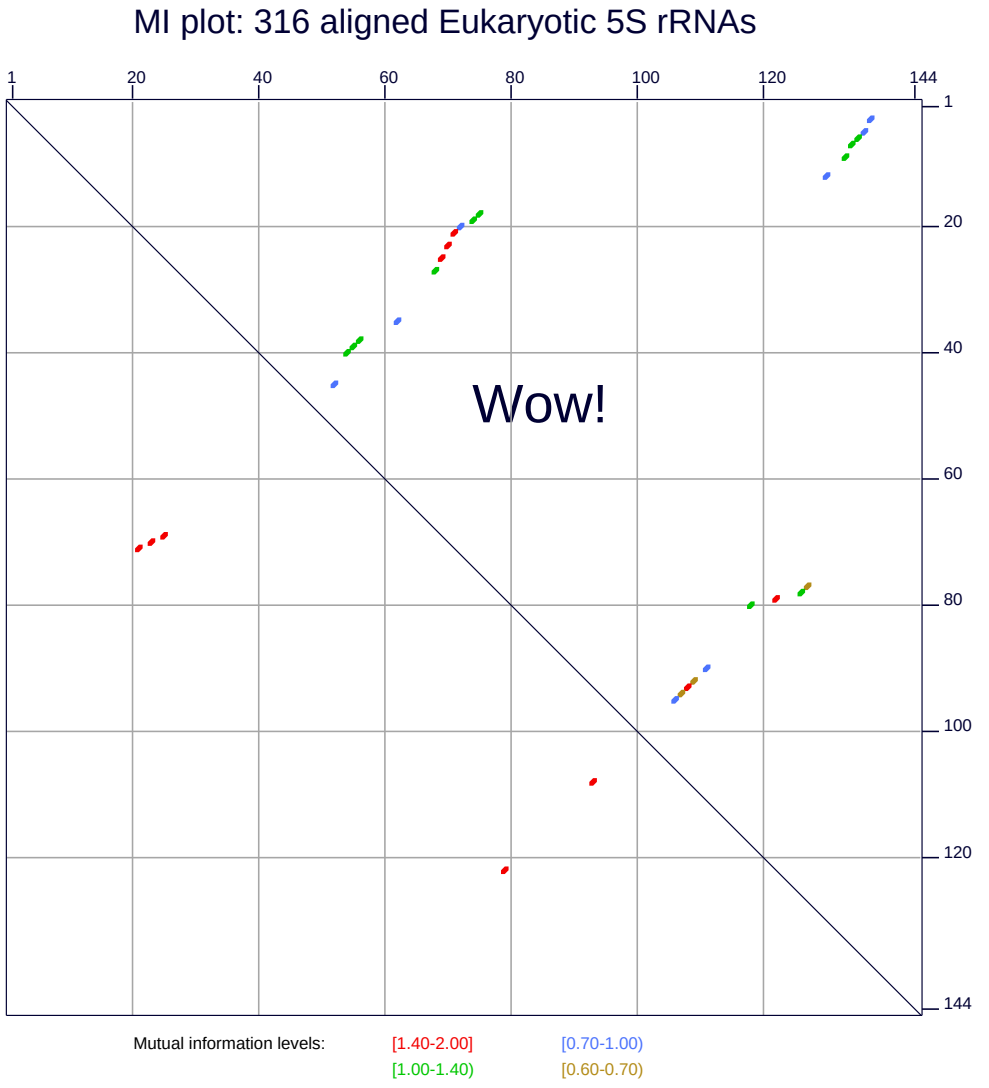


BPs with MI ≥ 0.6 are annotated in color. Its free energy can be used to compare with the minimum energy folding.

Alignment of 302 5S rRNAs from Eukaryotes (M. Szymanski)

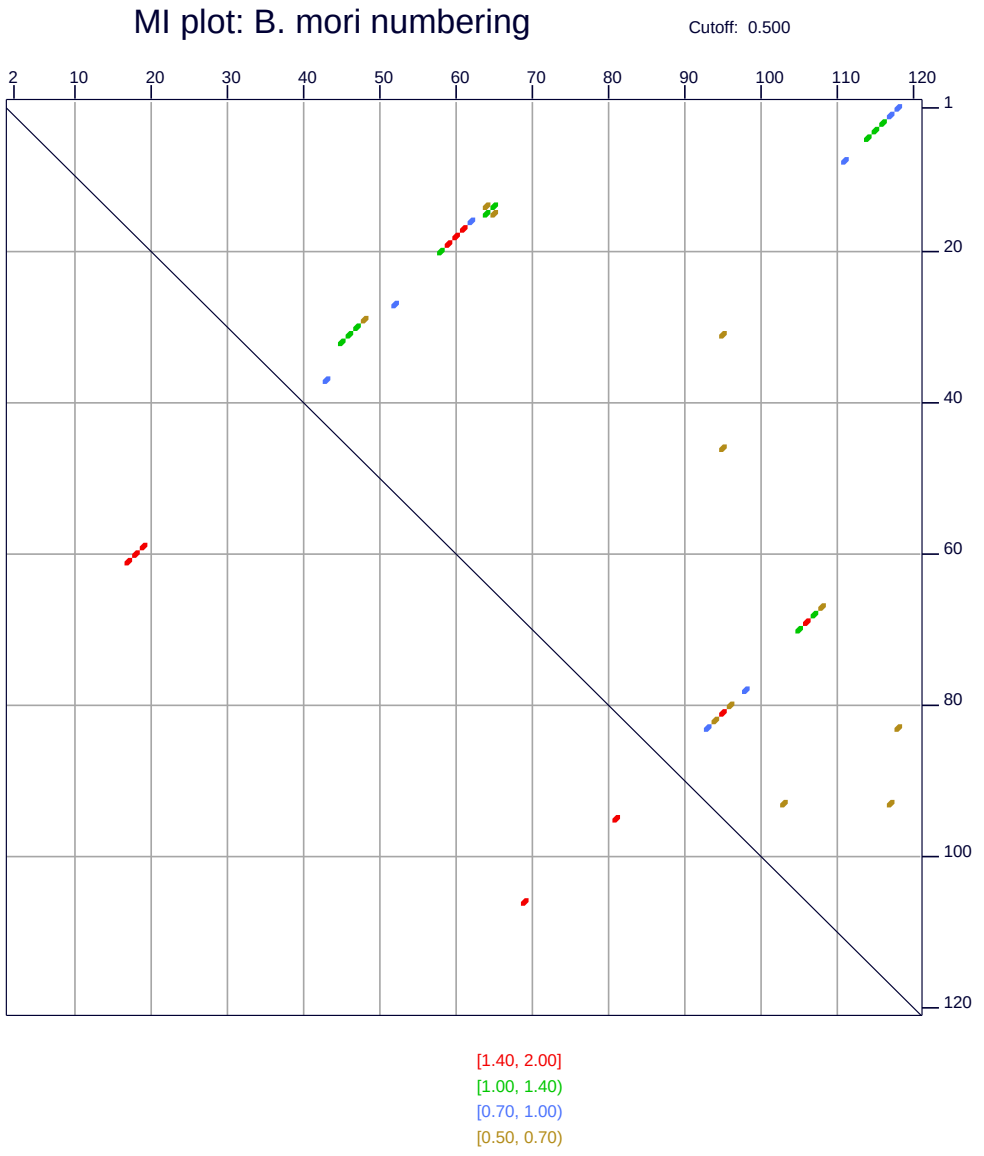


“Low noise” MI plot



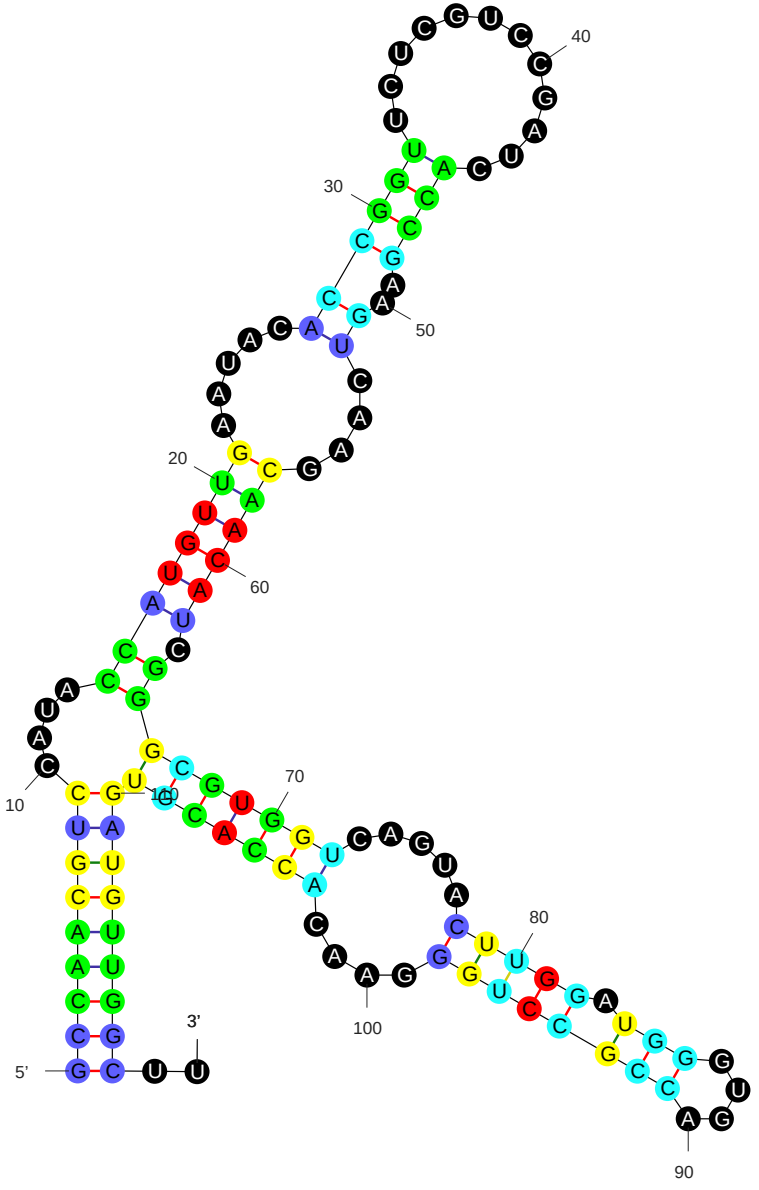
- Most BPs are detected.
- The irregularities in stems is a consequence of embedded gaps in the alignment.
- Alignment BPs must be converted when a particular sequence is extracted and “degapped”.

“Low noise” MI plot - *Bombix mori* numbering



Helices appear properly when numbering with respect to a single sequence.

Comparative model for *Bombyx mori* 5S rRNA - Update



BPs with MI ≥ 0.5 are annotated in color. All base pairs are supported by comparative data.

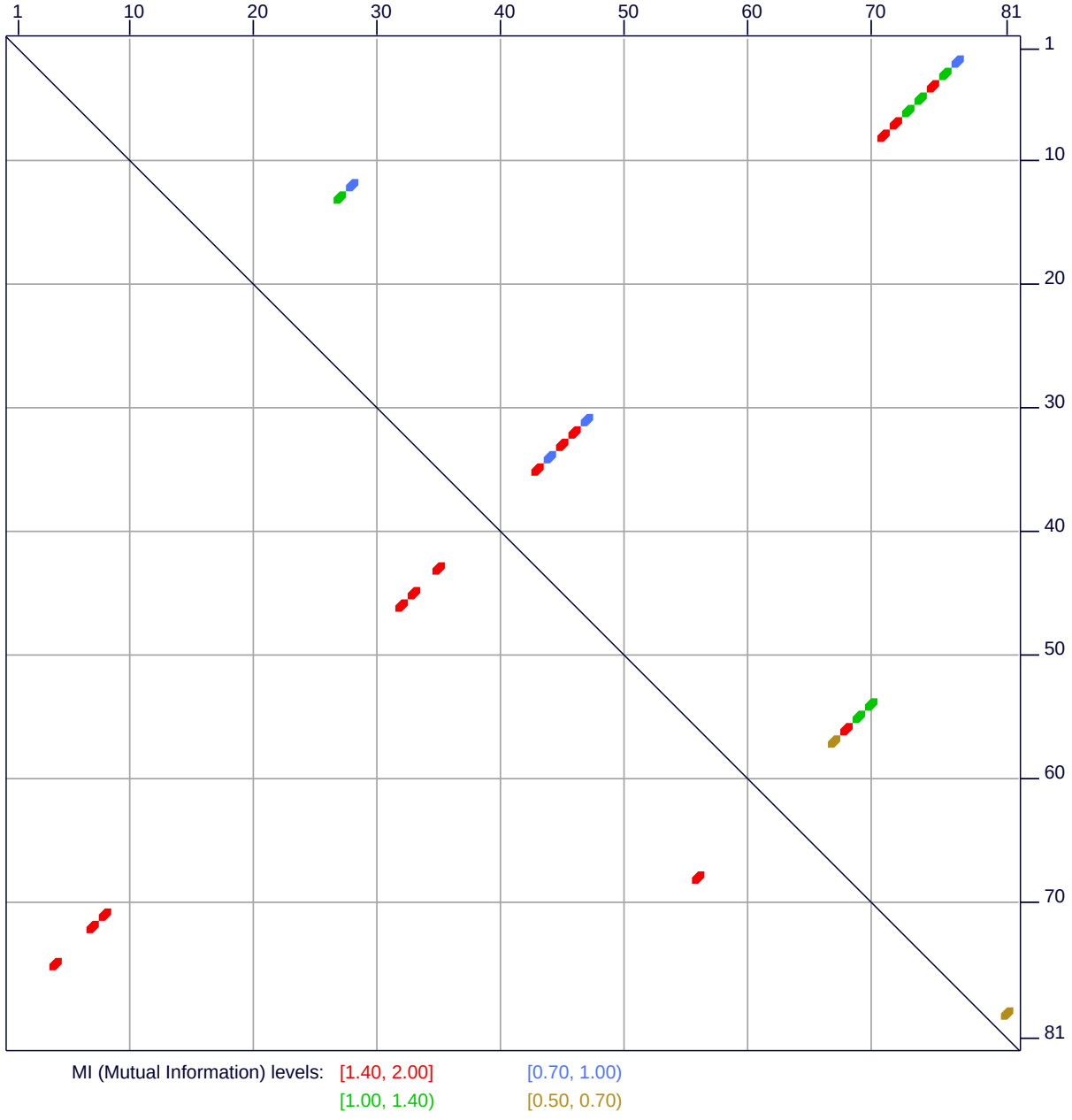
Transfer RNA – tRNA

- A huge number are known.
- Secondary structure deduced from perhaps 12 sequences in 1969 (Michael Levitt)
- For this presentation, 654 aligned tRNAs were selected from Sprinzl's database

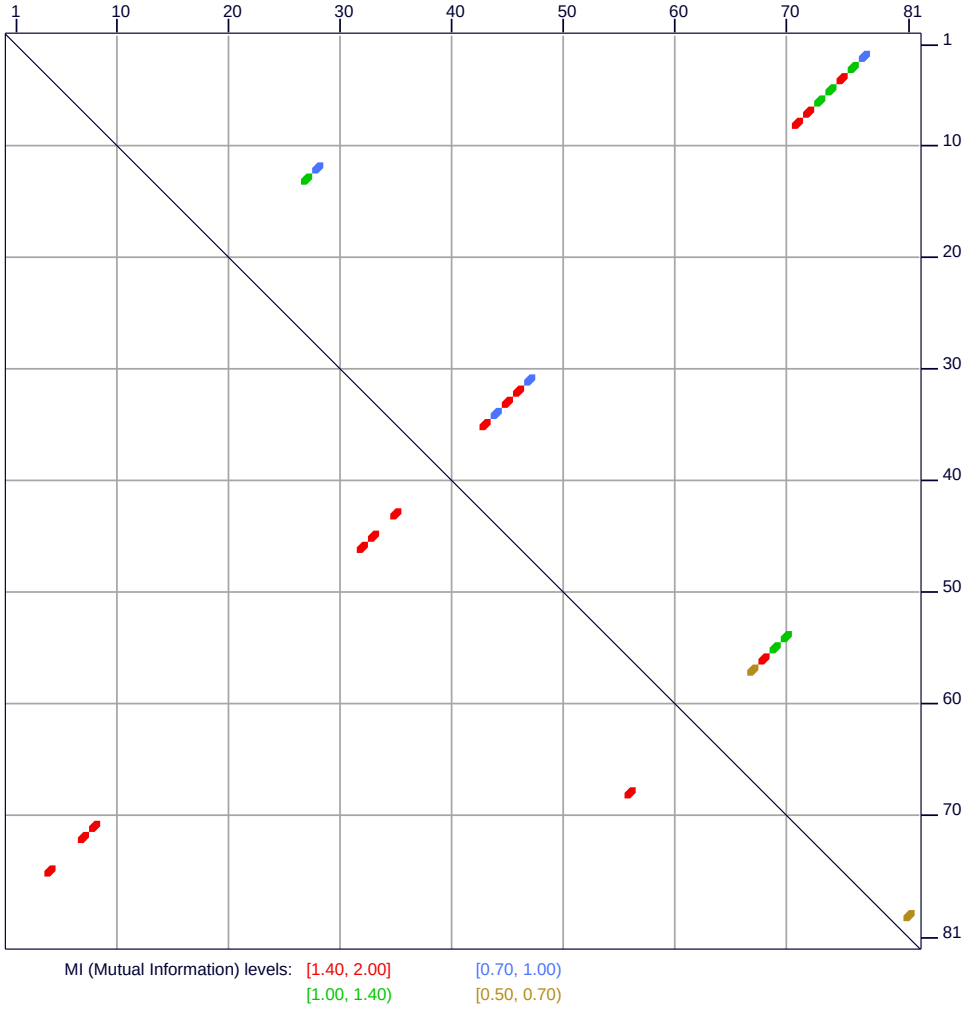
Sample entry:

```
DA0380 TGC HALOBACTERIUM CUT. ARCHAE
-GGGCCCATAGCTCAGT--GGT--AGAGTGCCTCCTTTGCAAGGAGGAT-17more-GCCCTGGGTTCGAATCCCAGTGGGTCCA---
 ==*====  *===           ===* =====       =====             =====       =========*==
 A stem   Dstem           D   aC           aC                TPsiC       TPsiC Astem
```

MI plot for 654 aligned tRNAs (Sprintzl, 1993)

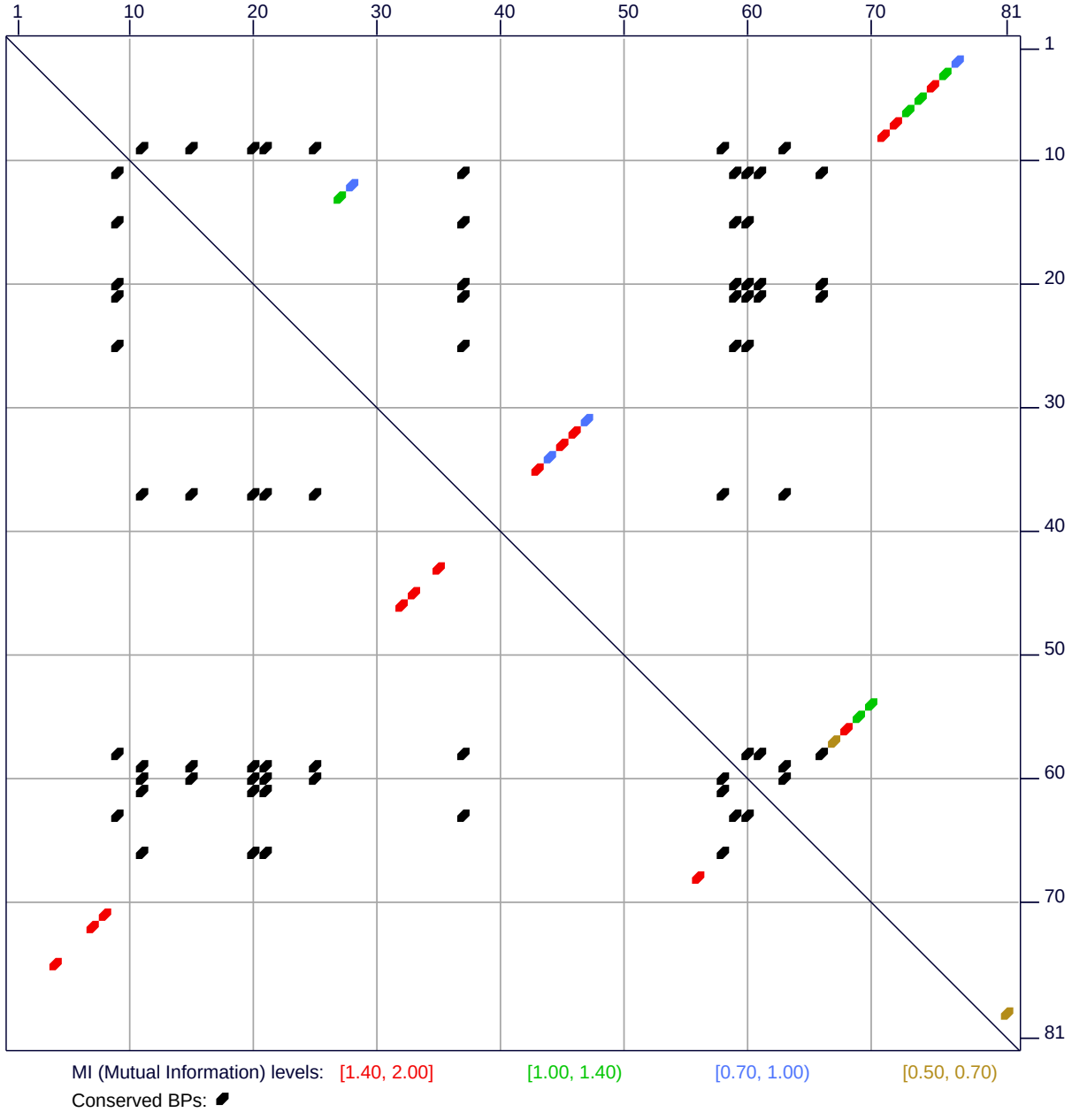


MI plot for 654 aligned tRNAs (Sprintzl, 1993)

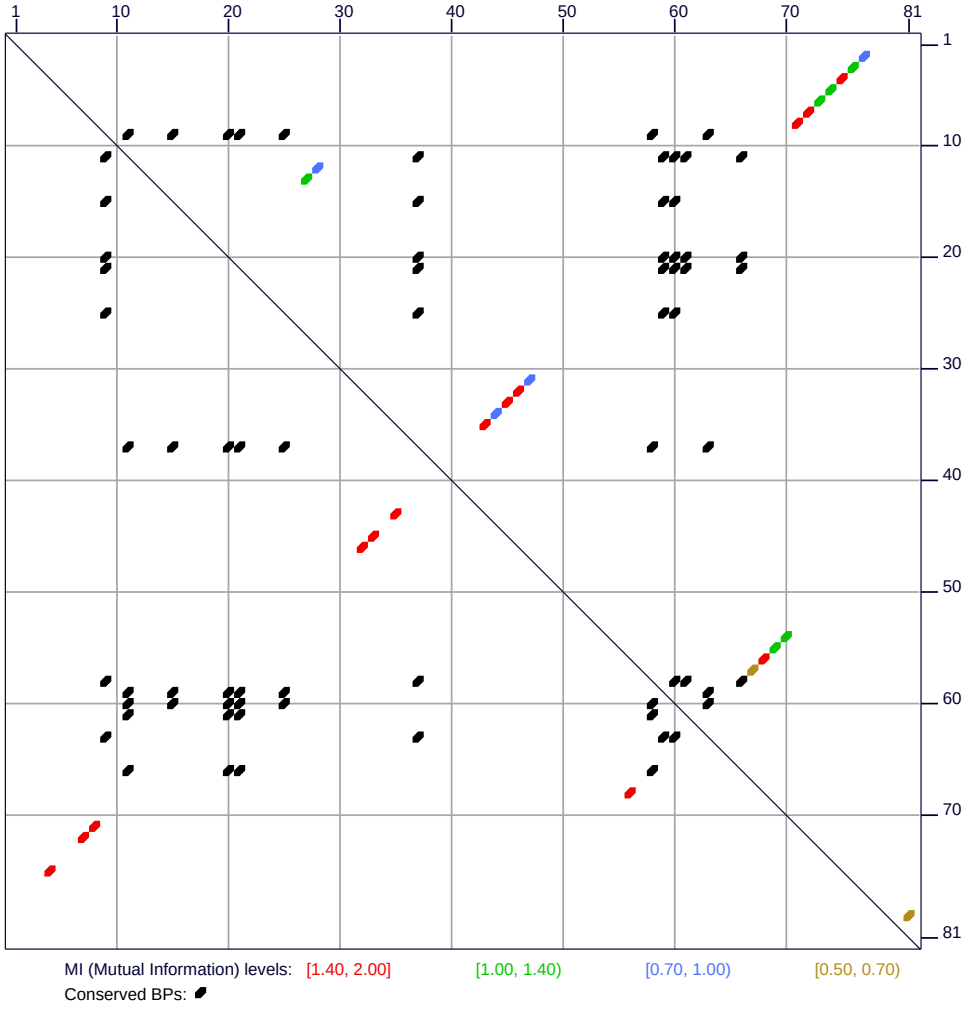


With 654 sequences, Secondary structure is very well determined using MI. The quality of the alignment is critical.

MI plot + conserved BPs for 654 aligned tRNAs (Sprintzl, 1993)

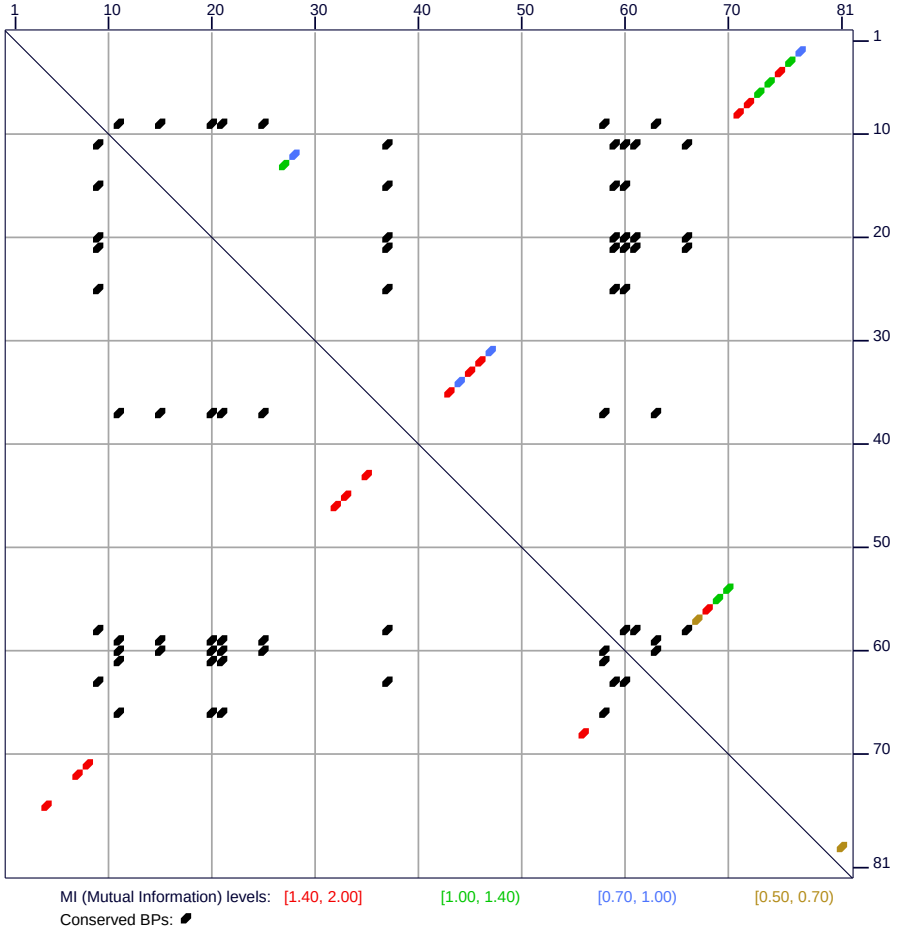


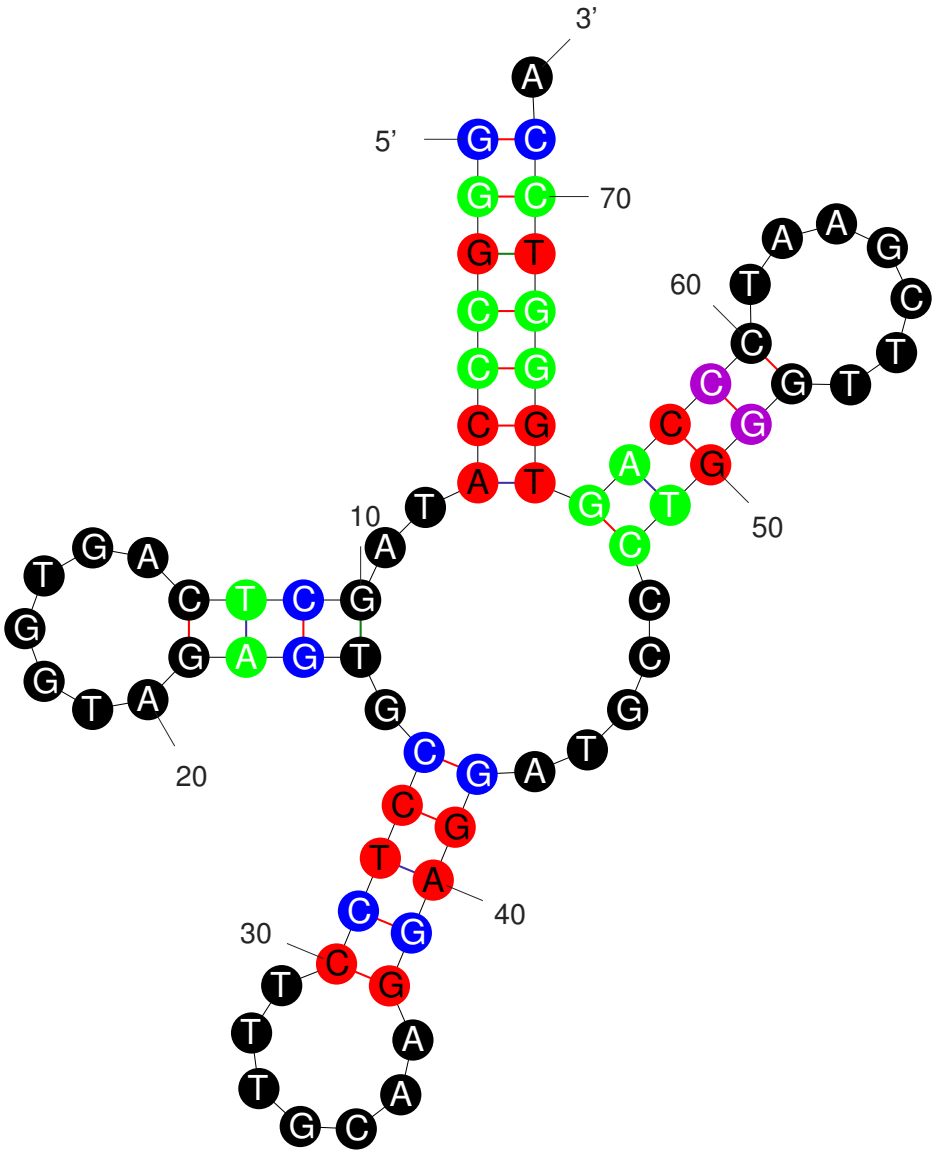
MI plot + conserved BPs for 654 aligned tRNAs (Sprintzl, 1993)



Plotting conserved BPs adds noise. Only one extra BP is discovered. Is it worth it?

MI plot & tRNA-TGC *Halobacterium cutirubrum*





Halobacterium cutirubrum tRNA-TGC

Energy Minimization

- How is energy assigned? Answer: “nearest-neighbor” energy rules are used.
- A stem with n BPs is broken into $n-1$ “BP stacks”. Energy ΔG is assigned to the “BP stacks”, but takes into account hydrogen bonds and stacking. These energies are negative “favorable”.
- Mismatched BPs at the ends of stems also contribute to stability.
- The loops are destabilizing.

δG for BP stacks

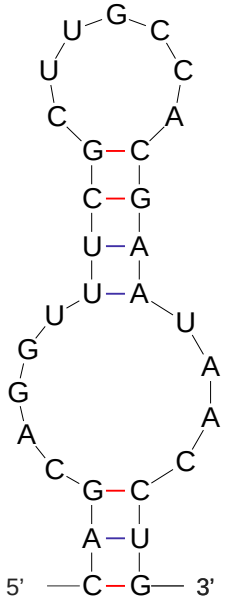
NN (nearest neighbor) free energies for RNA at 37°.
Doug Turner's group at the University of Rochester.

$$\delta G\begin{pmatrix} 5'-\mathrm{CGAGTATTCGG}-3' \\ 3'-\mathrm{GCTCATAAGCC}-5' \end{pmatrix} = \delta G\begin{pmatrix} 5'-\mathrm{CG}-3' \\ 3'-\mathrm{GC}-5' \end{pmatrix} +$$
$$\delta G\begin{pmatrix} 5'-\mathrm{GA}-3' \\ 3'-\mathrm{CT}-5' \end{pmatrix} + \delta G\begin{pmatrix} 5'-\mathrm{AG}-3' \\ 3'-\mathrm{TC}-5' \end{pmatrix} + \delta G\begin{pmatrix} 5'-\mathrm{GT}-3' \\ 3'-\mathrm{CA}-5' \end{pmatrix} +$$
$$\delta G\begin{pmatrix} 5'-\mathrm{TA}-3' \\ 3'-\mathrm{AT}-5' \end{pmatrix} + \delta G\begin{pmatrix} 5'-\mathrm{AT}-3' \\ 3'-\mathrm{TA}-5' \end{pmatrix} + \delta G\begin{pmatrix} 5'-\mathrm{TT}-3' \\ 3'-\mathrm{AA}-5' \end{pmatrix} +$$
$$\delta G\begin{pmatrix} 5'-\mathrm{CG}-3' \\ 3'-\mathrm{GC}-5' \end{pmatrix} + \delta G\begin{pmatrix} 5'-\mathrm{GG}-3' \\ 3'-\mathrm{CC}-5' \end{pmatrix}$$

- Don't sum "scores" as in sequence alignment.
- Consider two BPs at a time.
- Consecutive δG's are not independent.

Other stacking free energies

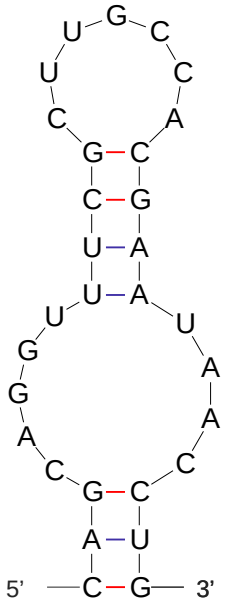
δG for mismatched pairs and dangling



In the example structure on the left:

- Stacking of the $\mathrm{C}_{13} \cdot \mathrm{A}_{19}$ mismatch stabilizes the H-loop.
- Stacking of the $\mathrm{C}_4 \cdot \mathrm{C}_{27}$ mismatch and the $\mathrm{U}_8 \cdot \mathrm{U}_{24}$ mismatch stabilizes the I-loop.
- These negative (favorable) energies are added to the unfavorable (positive) energies of the H-loop or the I-loop. **They are really associated with the adjacent stem. The energy assignment to the loop is done for algorithmic reasons**.
- Stacking of single bases at the end of stems is also considered (not shown).

Entropic terms for loops: δG is unfavorable



In the same example structure on the left:

- Both the H-loop and the I-loop have penalty energies that grow logarithmically with loop size (number of single-stranded bases).
- $\delta G \approx 1.75RT \ln(l)$, for loop size l.
- In addition, there is an I-loop asymmetry penalty. The asymmetry of the I-loop in the example is $1 = |5 - 4|$. This is the difference between the number of single-stranded bases on each side of the loop.

Energy methods for single sequences - Multi-structure dot plots

The energy dot plot (EDP)

- The minimum free energy (mfe) of a folding, ΔG_{mfe}, can be computed.
- Let $\delta G \geq 0$ be a free energy increment.
- All secondary structures with free energies between ΔG_{mfe} and $\Delta G_{\mathrm{mfe}} + \delta G$ are superimposed in a single plot.
- Different colors are used for different values of δG.
- For $\delta G_1 < \delta G_2$, with colors c_1 and c_2, respectively, c_1 is "on top" and obscures c_2.

The probability dot plot (PDP)

- All base pairs with probabilities above some cutoff are plotted as "dots".
- The area of the dots is (usually) proportional to the probability.
- The dots are colored to indicate a probability range.

Algorithms - Free energy minimization

- Simplified model that assigns energies to base pairs and ignores loop instabilities.
- For RNA sequence, $R = r_1 r_2 \ldots r_n$, let $e(i,j)$ be the free energy of pairing r_i with r_j. This can be $+\infty$ when a base pair is impossible.
- If $\mathbf{S}$ is a secondary structure on R, then the free energy of $\mathbf{S}$ is denoted by $\Delta G(\mathbf{S})$ and is defined as

$$\Delta G(\mathbf{S}) = \sum_{r_i \cdot r_j \in \mathbf{S}} e(i,j).$$

- Reasonable values of e at 37 °C are -3, -2 and -1 kcal/mol for GC, AU and GU base pairs, respectively.
- The goal is to compute $\Delta G_{\text{mfe}}(R) = \min_{\mathbf{S} \in \mathcal{S}} \Delta G(S)$, where "mfe" refers to minimum free energy and $\mathcal{S}$ is the collection of all secondary structures.
- If $1 \leq i \leq j \leq n$, let $E(i,j) = \Delta G_{\text{mfe}}(R_{i,j})$, where $R_{i,j} = r_i \ldots r_j$.
- $E(i,j) = 0$ if $j - i < 4$, since no structure can form in such a case.
- Otherwise,

$$E(i,j) = \min \left\{ E(i+1,j), E(i,j-1), e(i,j) + E(i+1,j-1), \min_{k=i+1}^{j-1} \left(E(i,k) + E(k+1,j) \right) \right\}$$

Algorithms - Free energy minimization - II

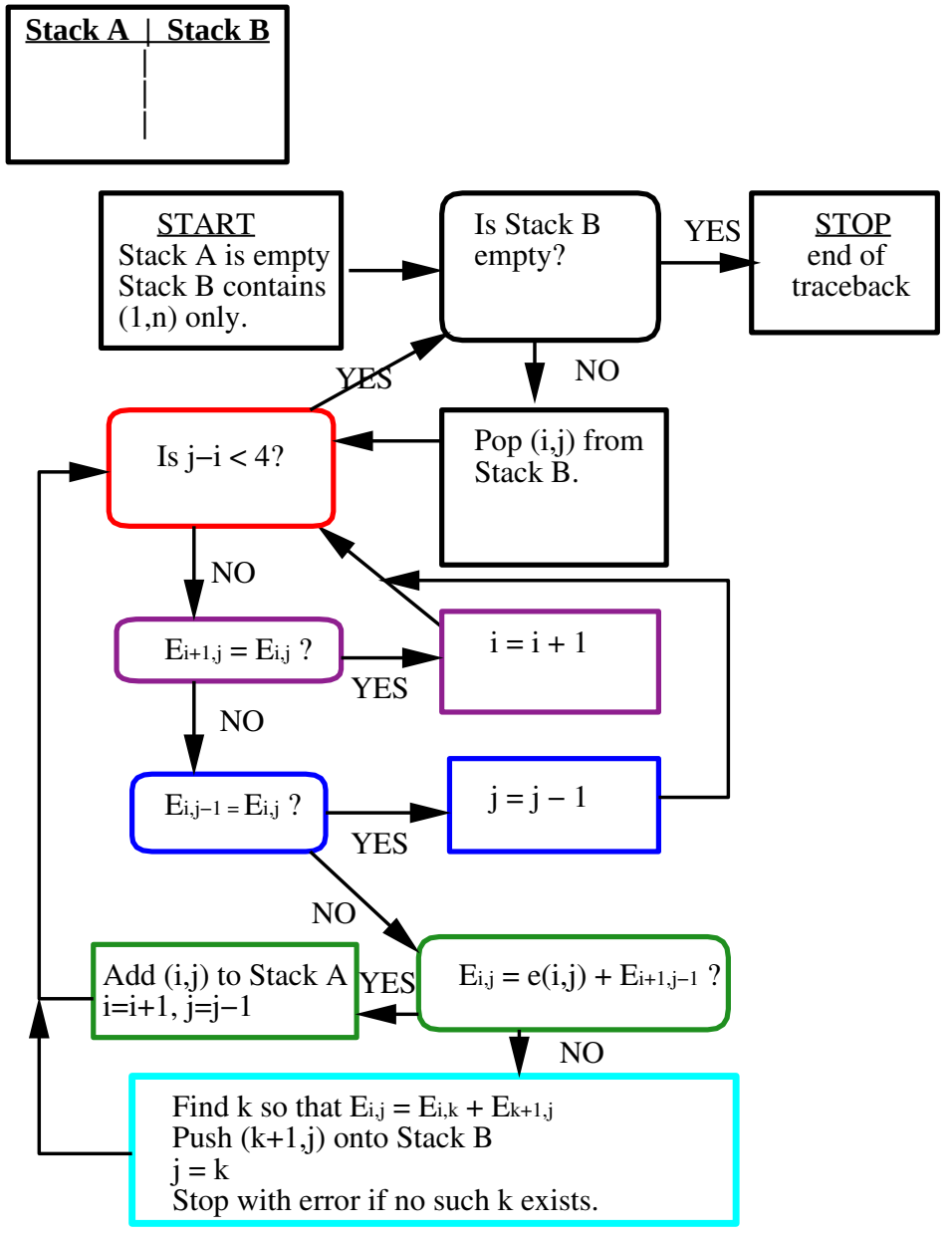
In (color coded) words:

- Fragments of length ≤ 4 have 0 folding energy, since they cannot fold. Otherwise,
- r_i is unpaired, or
- r_j is unpaired, or
- r_i and r_j pair with each other, or
- r_i and r_j both pair, but **not** with each other. In this case, r_i pairs with r_{k_1} and r_j pairs with r_{k_2}, where $i < k_1 < k_2 < j$. The k in the recursion can be any integer satisfying $k_1 \leq k < k_2$.

The above equations describe the "fill algorithm".

- $E(i,j)$ is computed recursively for ever larger sequence fragments.
- $\Delta G(R) = E(1,n)$, but what structure(s) has (have) this free energy?
- The "traceback algorithm" computes a structure.

Algorithms - Free energy minimization - Traceback - I



An illustrated traceback chart for RNA folding using base pair dependent energy rules.

Algorithms - Free energy minimization - Traceback - II

- Start: Set $i = 1$ and $j = n$. Put i and j on to the "traceback stack".
- Recursion:

1. If the traceback stack is empty, the traceback terminates. Otherwise, take i and j from the traceback stack.
2. If $E_{i+1,j} = E_{i,j}$, then i is not paired.
   (a) If $j - i > 3$, set $i = i + 1$ and continue with 2.
   (b) If $j - i \leq 3$, continue with 1.
   Otherwise, continue with 3.
3. If $E_{i,j-1} = E_{i,j}$, then j is not paired.
   (a) If $j - i > 3$, set $j = j - 1$ and continue with 3.
   (b) If $j - i \leq 3$, continue with 1.
   Otherwise, continue with 4.
4. If $E_{i,j} = e(i,j) + E_{i+1,j-1}$, then r_i pairs with r_j. Add $i.j$ to the list of base pairs, set $i = i + 1$ and $j = j - 1$ and continue with 2. Otherwise, continue with 5.
5. If $E_{i,j} = E_{i,k} + E_{k+1,j}$, for some $k \in (i,j)$, put the fragment $k+1 \ldots j$ on to the traceback stack (i is $k+1$ and j is the current j) and deal with $i \ldots k$ by setting $j = k$ and continue with 2. (Note that some k must exist if this point is reached.)

Fill and traceback example.

Given the sequence $R =$ GCAGCACCCAAAGGGAAUAUGGGAUACGCGUA.

The base pair folding energies are -3, -2 and -1 for GC (CG), AU (UA) and GU (UG) base pairs, respectively. $E(i, j)$ appears in row i and column j of the triangular array below.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32		
G	C	A	G	C	A	C	C	C	A	A	A	G	G	G	A	A	U	A	U	G	G	G	A	U	A	C	G	C	G	U	A		
0	0	0	0	-3	-3	-3	-3	-6	-6	-6	-6	-6	-9	-12	-12	-12	-14	-14	-14	-16	-17	-17	-17	-18	-18	-20	-21	-24	-24	-25	-25	G	1
	0	0	0	0	0	0	-3	-3	-3	-3	-3	-6	-9	-9	-9	-9	-11	-11	-13	-16	-17	-17	-17	-17	-17	-20	-21	-22	-24	-24	-24	C	2
		0	0	0	0	0	-3	-3	-3	-3	-3	-6	-6	-9	-9	-9	-11	-11	-13	-14	-14	-14	-14	-16	-16	-18	-18	-21	-21	-23	-23	A	3
			0	0	0	0	-3	-3	-3	-3	-3	-6	-6	-9	-9	-9	-11	-11	-12	-14	-14	-14	-14	-15	-15	-18	-18	-21	-21	-22	-22	G	4
				0	0	0	0	0	0	0	0	-3	-6	-9	-9	-9	-11	-11	-11	-14	-14	-14	-14	-15	-15	-17	-18	-20	-21	-21	-21	C	5
					0	0	0	0	0	0	0	-3	-6	-9	-9	-9	-11	-11	-11	-11	-11	-13	-13	-15	-15	-15	-18	-18	-18	-20	-20	A	6
						0	0	0	0	0	0	-3	-6	-9	-9	-9	-9	-9	-11	-11	-11	-13	-13	-13	-15	-15	-18	-18	-18	-18	-20	C	7
							0	0	0	0	0	-3	-6	-6	-6	-6	-6	-6	-8	-8	-10	-10	-10	-10	-12	-15	-15	-15	-18	-18	-18	C	8
								0	0	0	0	-3	-3	-3	-3	-3	-4	-4	-5	-7	-7	-7	-7	-8	-9	-12	-12	-15	-15	-15	-15	C	9
									0	0	0	0	0	0	0	0	-2	-2	-4	-4	-4	-4	-4	-6	-6	-9	-9	-12	-12	-14	-14	A	10
										0	0	0	0	0	0	0	-2	-2	-4	-4	-4	-4	-4	-6	-6	-9	-9	-12	-12	-14	-14	A	11
											0	0	0	0	0	0	-2	-2	-3	-3	-3	-3	-4	-6	-6	-9	-9	-12	-12	-14	-14	A	12
												0	0	0	0	0	-1	-1	-2	-2	-2	-2	-3	-5	-6	-9	-9	-12	-12	-13	-13	G	13
													0	0	0	0	-1	-1	-2	-2	-2	-2	-3	-5	-6	-9	-9	-12	-12	-12	-12	G	14
														0	0	0	0	0	-2	-2	-2	-2	-2	-4	-6	-9	-9	-9	-9	-9	-11	G	15
															0	0	0	0	-2	-2	-2	-2	-2	-4	-6	-6	-6	-8	-8	-9	-11	A	16
																0	0	0	0	0	-1	-1	-2	-4	-6	-6	-6	-6	-7	-9	-11	A	17
																	0	0	0	0	-1	-1	-2	-4	-6	-6	-6	-6	-7	-9	-11	U	18
																		0	0	0	0	0	-2	-4	-4	-4	-4	-6	-7	-9	-9	A	19
																			0	0	0	0	-2	-2	-3	-3	-4	-6	-7	-7	-9	U	20
																				0	0	0	0	-1	-1	-3	-3	-6	-6	-7	-7	G	21
																					0	0	0	0	0	-3	-3	-6	-6	-6	-6	G	22
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																							0	0	0	0	0	0	-1	-3	-4	A	24
																								0	0	0	0	0	-1	-2	-4	U	25
																									0	0	0	0	0	-2	-2	A	26
																										0	0	0	0	0	0	C	27
																											0	0	0	0	0	G	28
																												0	0	0	0	C	29
																													0	0	0	G	30
																														0	0	U	31
																															0	A	32

Fill & traceback example: A traceback route

5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
C	A	C	C	C	A	A	A	G	G	G	A	A	U	A	U	G	G	G	A	U	A	C	G	C	G	U	A	
-3	-3	-3	-3	-6	-6	-6	-6	-6	-9	-12	-12	-12	-14	-14	-14	-16	-17	-17	-17	-18	-18	-20	-21	-24	-24	-25	-25	G 1
0	0	0	-3	-3	-3	-3	-3	-6	-9	-9	-9	-9	-11	-11	-13	-16	-17	-17	-17	-17	-17	-20	-21	-22	-24	-24	-24	C 2
0	0	0	-3	-3	-3	-3	-3	-6	-6	-9	-9	-9	-11	-11	-13	-14	-14	-14	-14	-16	-16	-18	-18	-21	-21	-23	-23	A 3
0	0	0	-3	-3	-3	-3	-3	-6	-6	-9	-9	-9	-11	-11	-12	-14	-14	-14	-14	-15	-15	-18	-18	-21	-21	-22	-22	G 4
0	0	0	0	0	0	0	0	-3	-6	-9	-9	-9	-11	-11	-11	-14	-14	-14	-14	-15	-15	-17	-18	-20	-21	-21	-21	C 5
	0	0	0	0	0	0	0	-3	-6	-9	-9	-9	-11	-11	-11	-11	-11	-13	-13	-15	-15	-15	-18	-18	-18	-20	-20	A 6
		0	0	0	0	0	0	-3	-6	-9	-9	-9	-9	-9	-11	-11	-11	-13	-13	-13	-15	-15	-18	-18	-18	-18	-20	C 7
			0	0	0	0	0	-3	-6	-6	-6	-6	-6	-6	-8	-8	-10	-10	-10	-10	-12	-15	-15	-15	-18	-18	-18	C 8
				0	0	0	0	-3	-3	-3	-3	-3	-4	-4	-5	-7	-7	-7	-7	-8	-9	-12	-12	-15	-15	-15	-15	C 9
					0	0	0	0	0	0	0	0	-2	-2	-4	-4	-4	-4	-4	-6	-6	-9	-9	-12	-12	-14	-14	A 10
						0	0	0	0	0	0	0	-2	-2	-4	-4	-4	-4	-4	-6	-6	-9	-9	-12	-12	-14	-14	A 11
							0	0	0	0	0	0	-2	-2	-3	-3	-3	-3	-4	-6	-6	-9	-9	-12	-12	-14	-14	A 12
								0	0	0	0	0	-1	-1	-2	-2	-2	-2	-3	-5	-6	-9	-9	-12	-12	-13	-13	G 13
									0	0	0	0	-1	-1	-2	-2	-2	-2	-3	-5	-6	-9	-9	-12	-12	-12	-12	G 14
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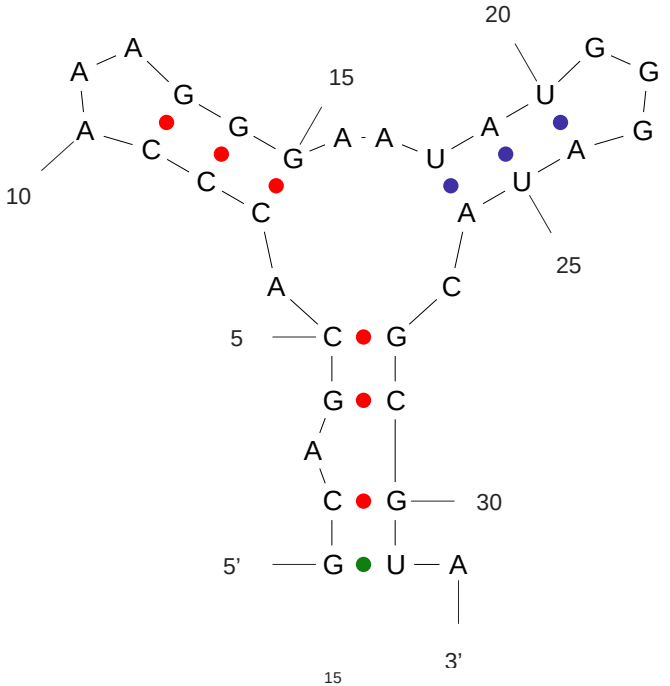
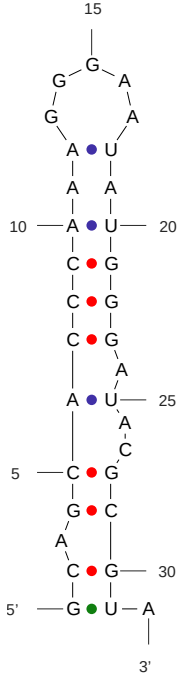
											0	0	0	0	-2	-2	-2	-2	-2	-4	-6	-6	-6	-8	-8	-9	-11	A 16
												0	0	0	0	0	-1	-1	-2	-4	-6	-6	-6	-6	-7	-9	-11	A 17
													0	0	0	0	-1	-1	-2	-4	-6	-6	-6	-6	-7	-9	-11	U 18
														0	0	0	0	0	-2	-4	-4	-4	-4	-6	-7	-9	-9	A 19
															0	0	0	0	-2	-2	-3	-3	-4	-6	-7	-7	-9	U 20
																0	0	0	0	-1	-1	-3	-3	-6	-6	-7	-7	G 21
																	0	0	0	0	0	-3	-3	-6	-6	-6	-6	G 22
																		0	0	0	0	-3	-3	-3	-3	-3	-4	G 23
																			0	0	0	0	0	0	-1	-3	-4	A 24
																				0	0	0	0	0	-1	-2	-4	U 25
																					0	0	0	0	0	-2	-2	A 26
																						0	0	0	0	0	0	C 27
																							0	0	0	0	0	G 28
																								0	0	0	0	C 29
																									0	0	0	G 30
																										0	0	U 31
																											0	A 32

Resulting Structure(s)





The structure on the left corresponds to the traceback route depicted in red on the previous page.

This alternative structure is also mfe. It would be found by following the "-15"s to position (6,25).

Instability of Predictions

Problems in folding prediction became evident as soon a minimum free energy folding predictions became available. How was this detected?

1. Folding a slightly different homologous sequence could result in a totally different predicted secondary structure.

2. Small changes in the free energy parameters could dramatically alter folding prediction.

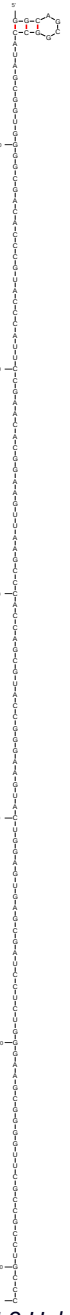
3. Adding folding constraints would change predicted sturctures, but in many cases the mfe increased very little.

4. Attempts to model equilibrium folding of elongating RNA molecules failed to give satisfactory results.

On the next twelve pages, mfe foldings are predicted for the same 5S rRNA on the segments $5' - r_1 \ldots r_N - 3'$, for $N = 10, 20, \ldots 110, 123$. Base pairs in previous foldings are not conserved in general.

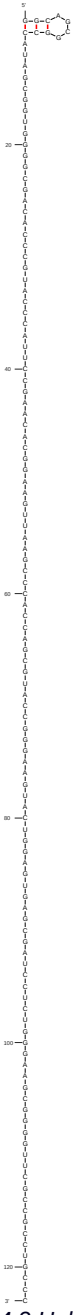
Thus, it became important to predict sub-optimal foldings as well as mfe foldings.

Folding *Halobacterium saccharovorum 5S rRNA*: 1–10



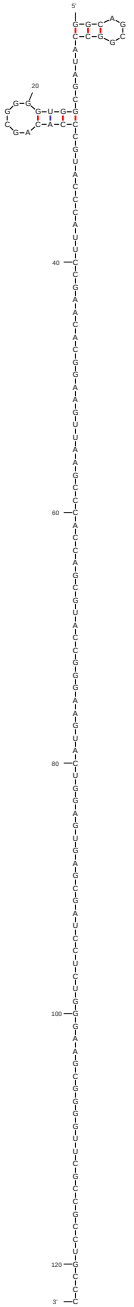
dG = -4.2 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–20



dG = -4.2 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–30

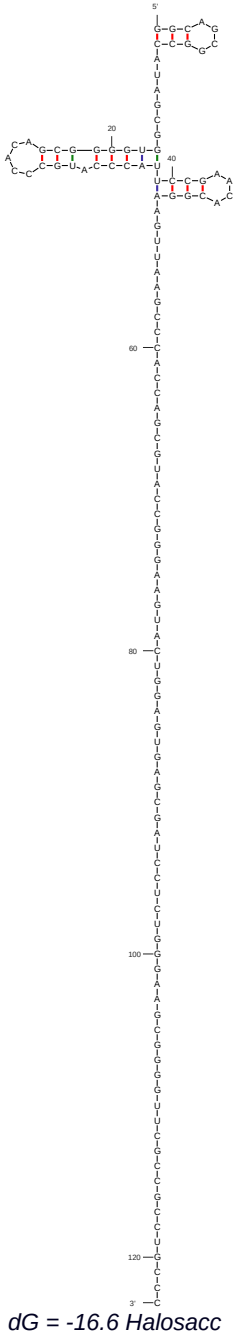


dG = -9.9 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–40

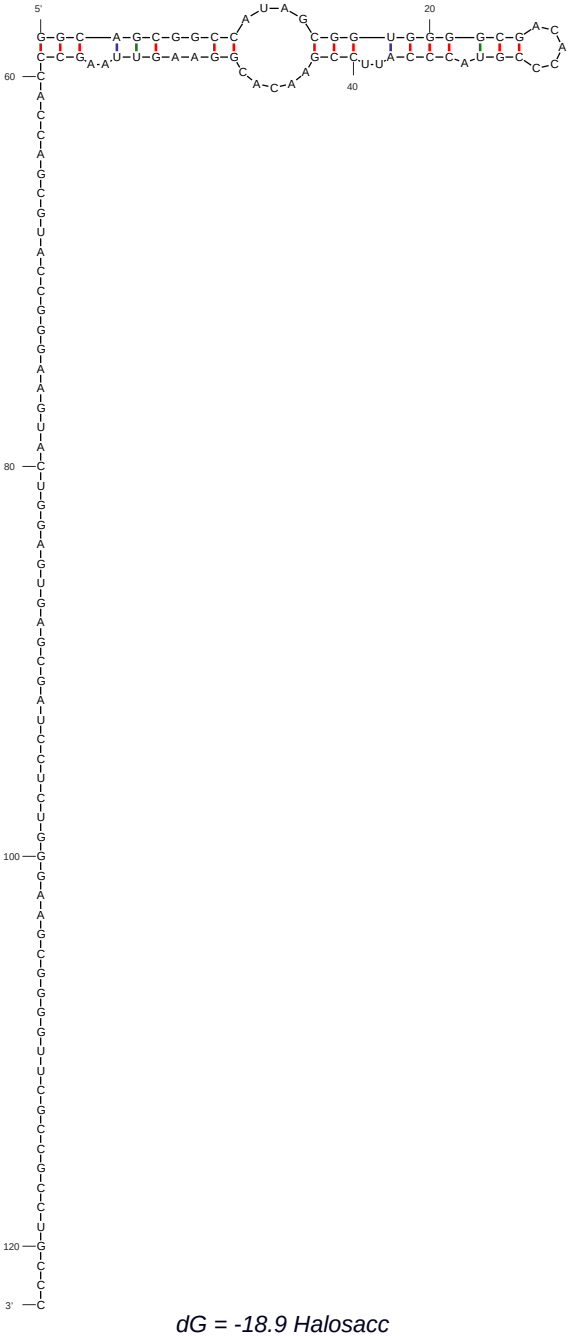
dG = -13.4 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–50



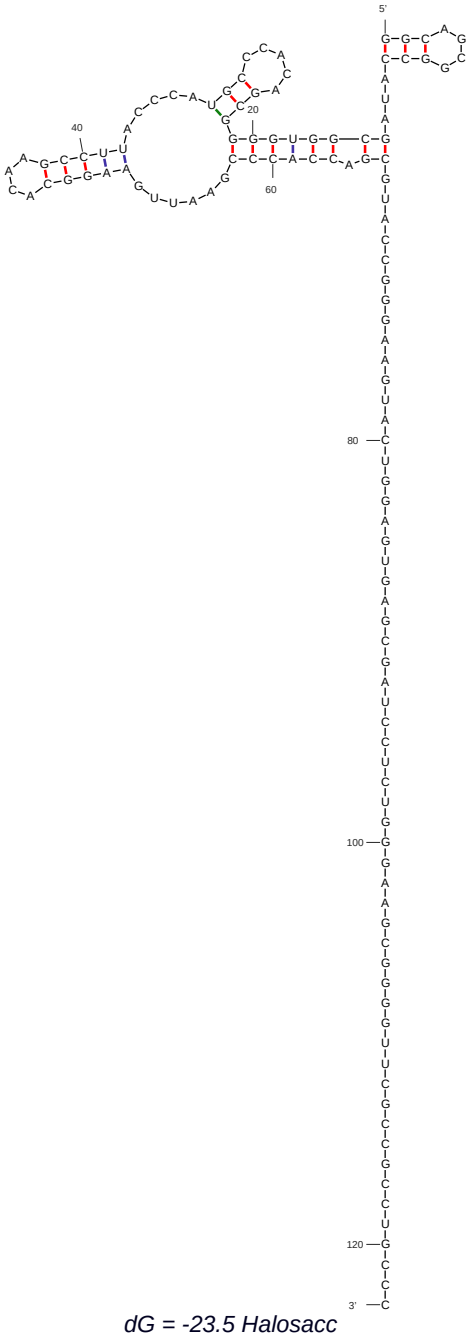
dG = -16.6 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–60



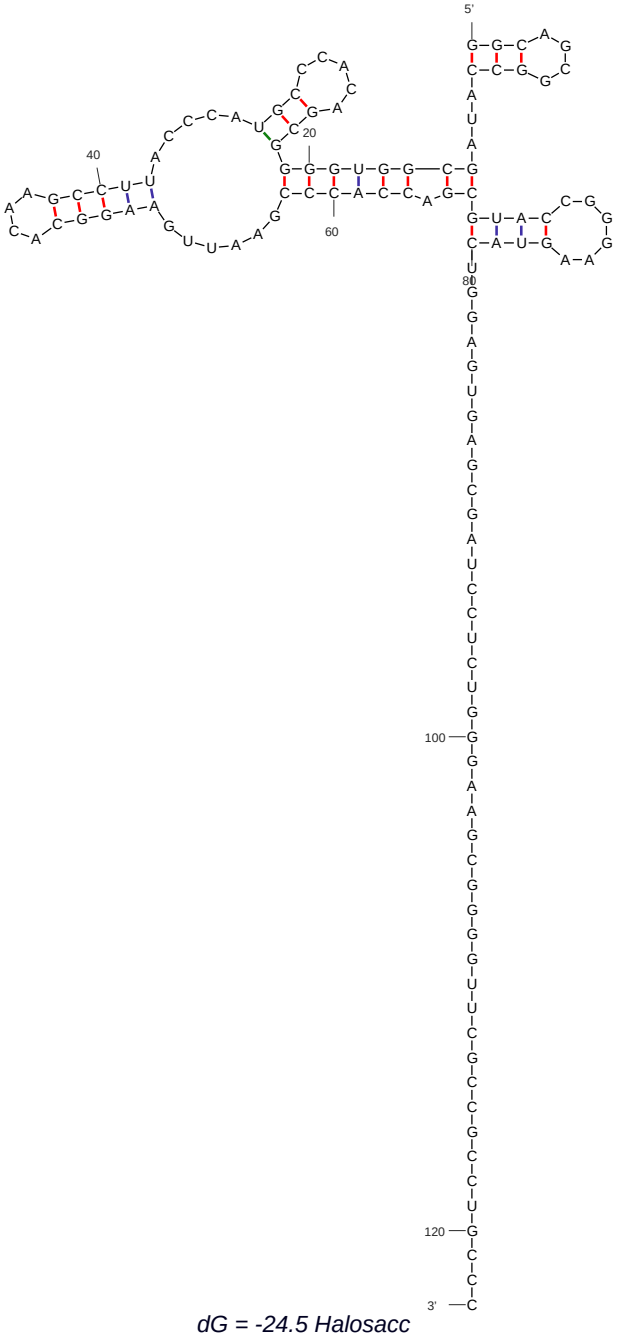
dG = -18.9 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–70



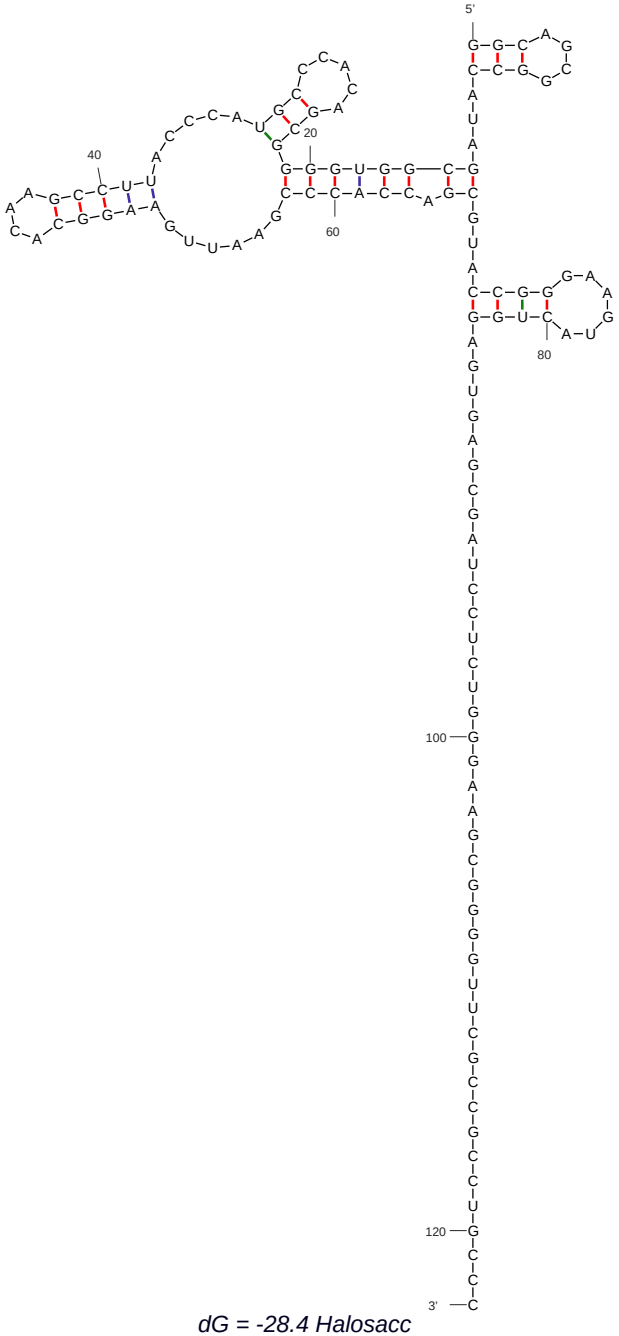
dG = -23.5 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–80



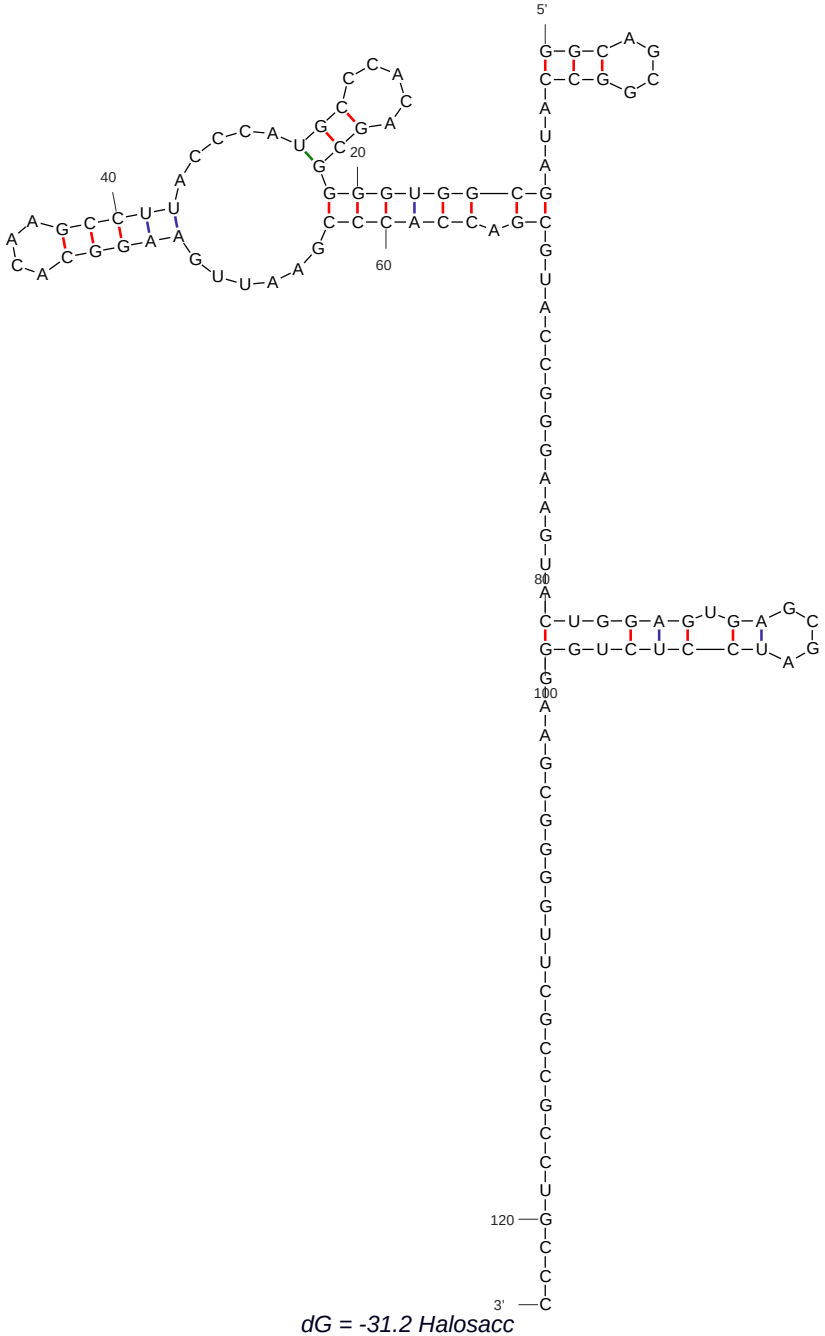
dG = -24.5 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–90



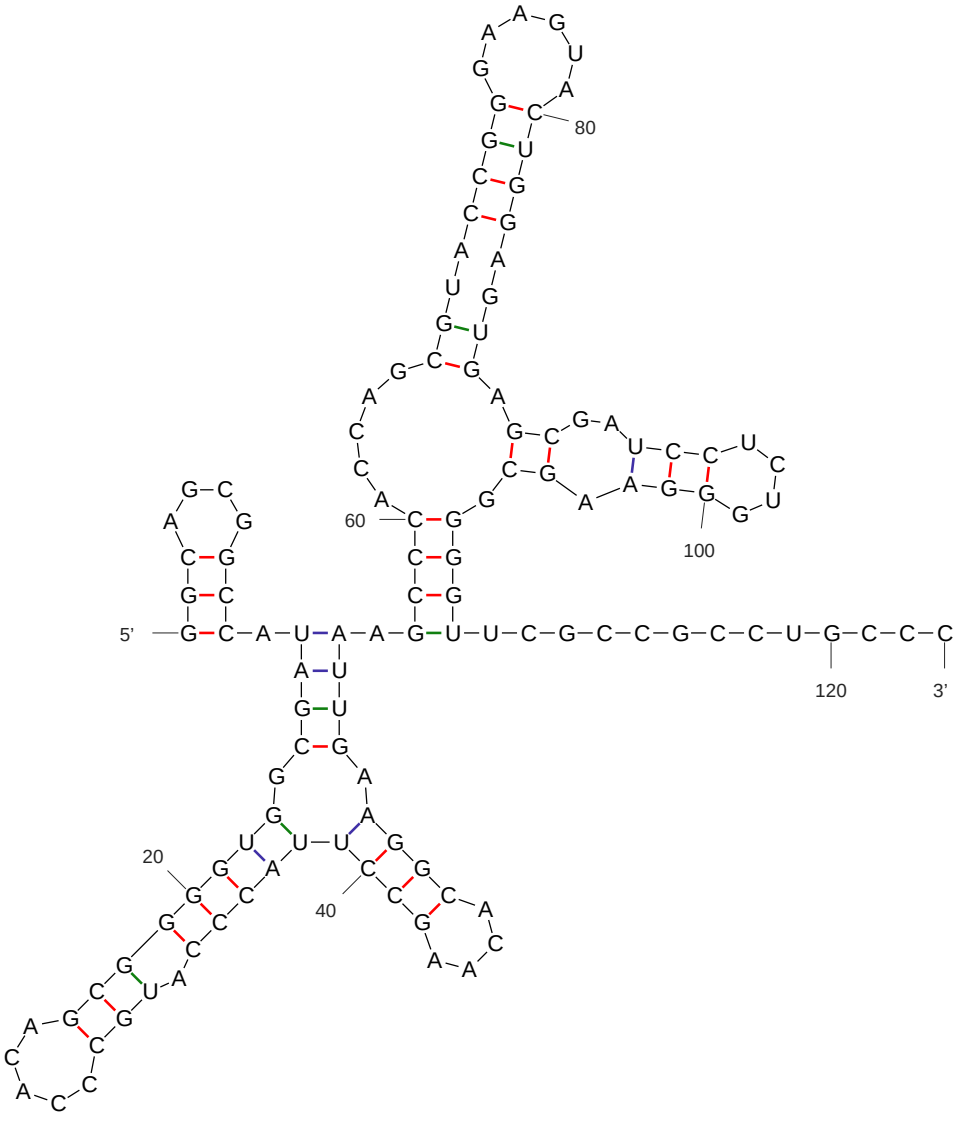
dG = -28.4 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–100



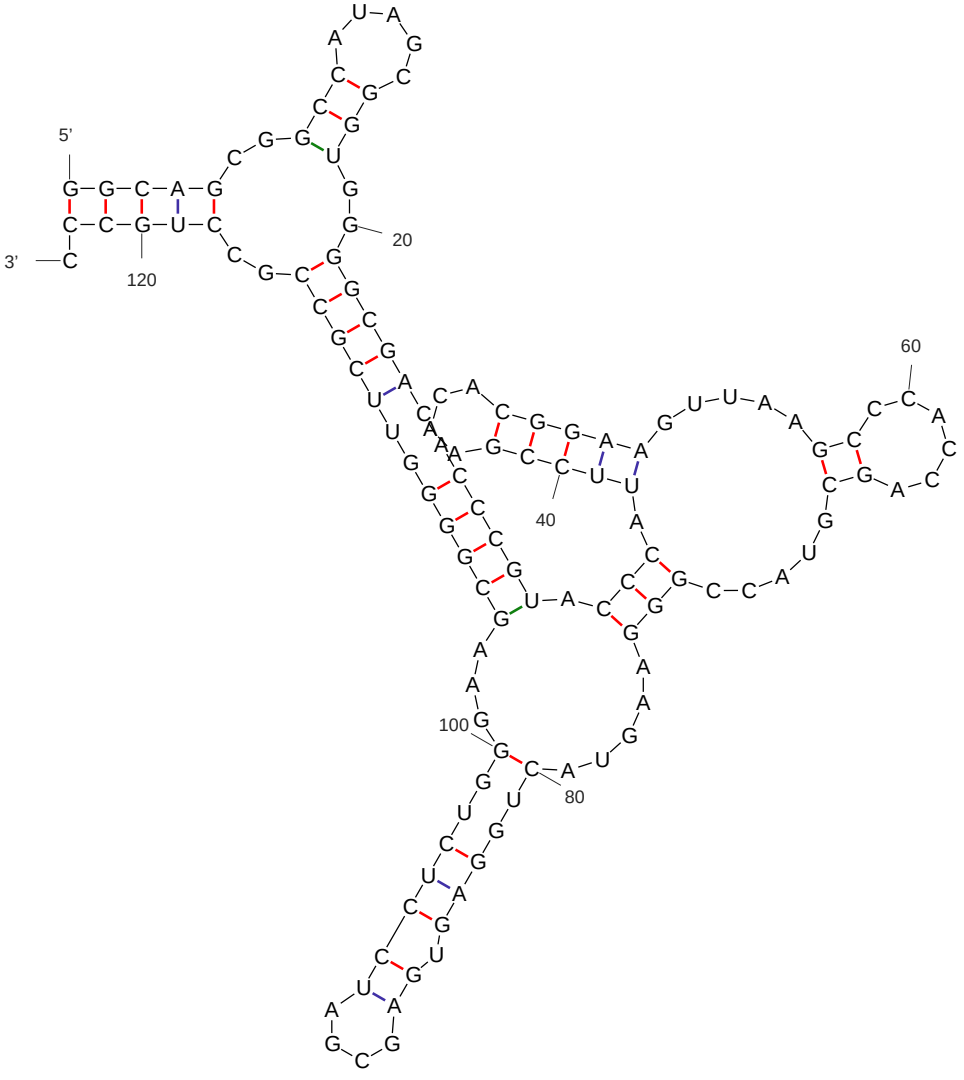
dG = -31.2 Halosacc

Folding *Halobacterium saccharovorum 5S rRNA*: 1–110



dG = -34.8 Halosacc

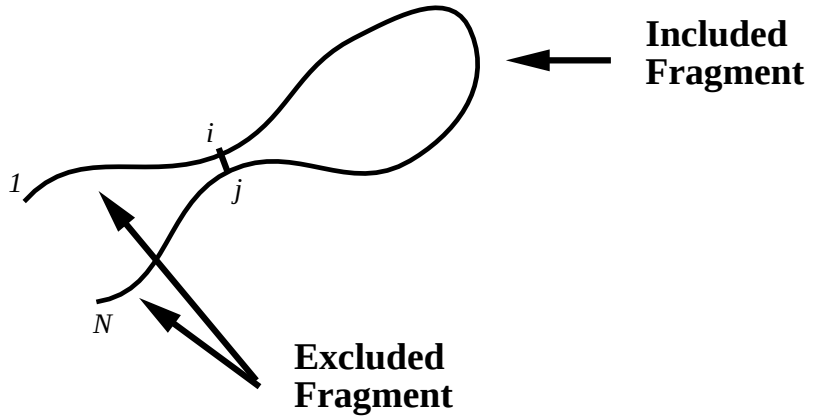
Folding *Halobacterium saccharovorum 5S rRNA*: 1–123



dG = -47.6 Halosacc

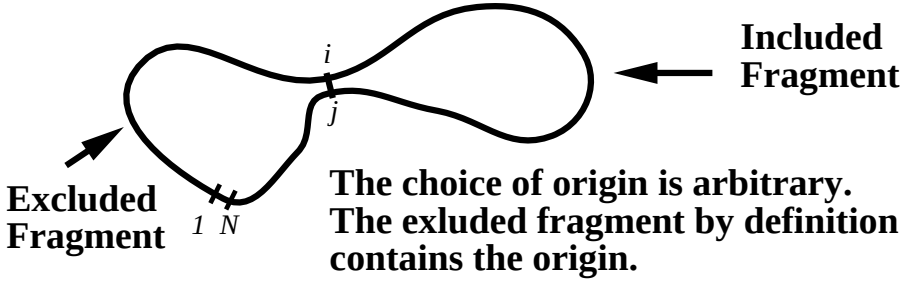
The suboptimal algorithm: Circularize the RNA

Apparent Lack of Symmetry:



For any *i,j* pair, *V(i,j)* gives the best folding energy for the included region only.

Solution: "Circularize" the sequence.



The choice of origin is arbitrary. The exluded fragment by definition contains the origin.

Define $V(i,j) = e(i,j) + E(i+1, j-1)$. That is, $V(i,j)$ is the mfe of all foldings on $R_{i,j}$ where r_i and r_j pair. Call $R_{i,j}$ an "included" fragment of R.

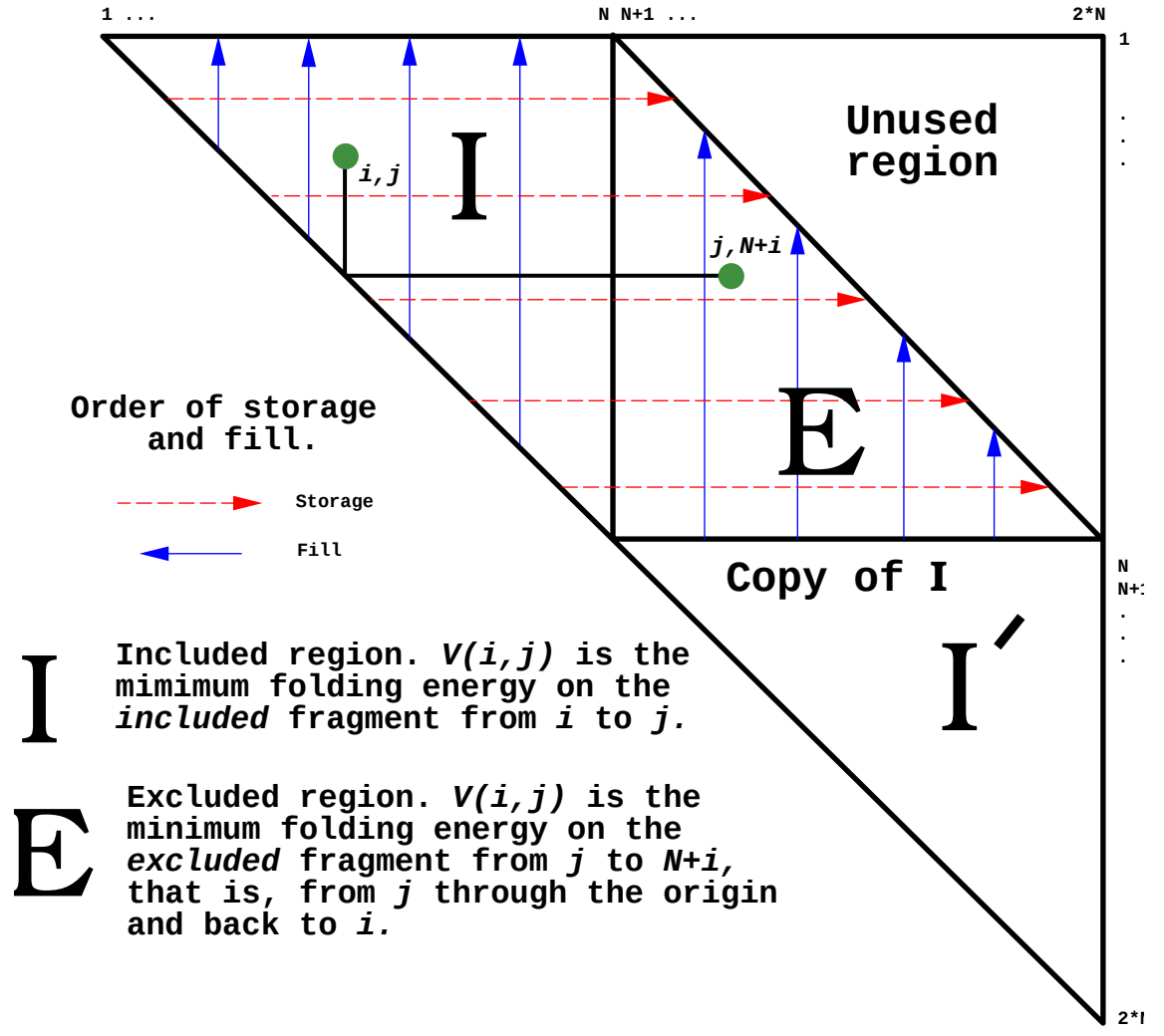
Circularize the RNA (some RNAs, such as viroids, are naturally circular.) For $i < j$, let $E(j,i)$ and $V(j,i)$ be defined for the "excluded" fragment, $R_{j,i} = r_j r_{j+1} \ldots r_n r_1 \ldots r_i$. Then $V(i,j) + V(j,i) - e(i,j)$ is the mfe over all foldings containing the base pair, $r_i \cdot r_j$.

Q: How to compute E and V for excluded fragments?

The suboptimal algorithm: Folding $\mod n$

Solution:

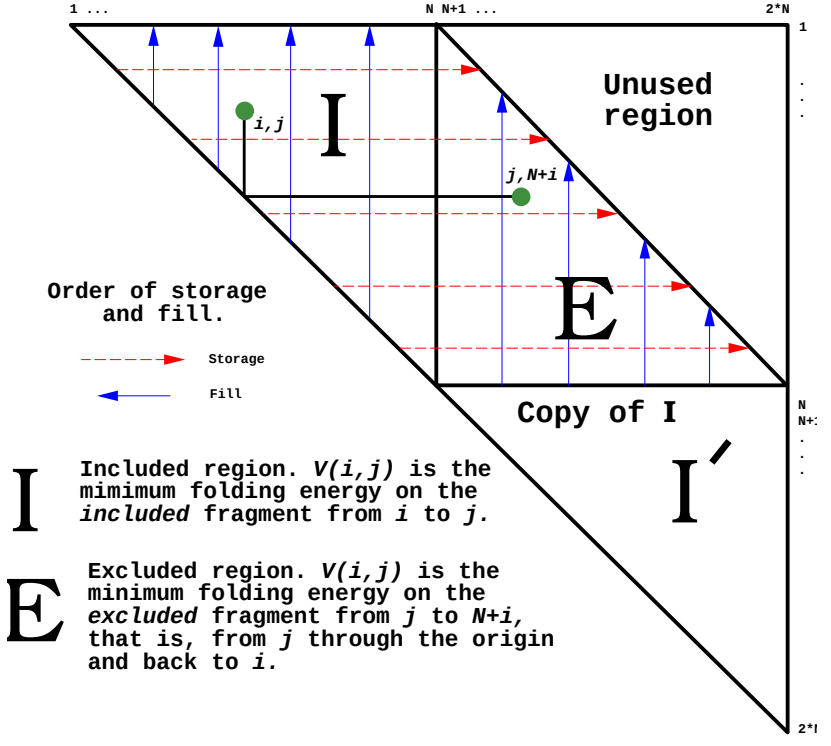
- Double the sequence.
- Fold modulo n.
- For $n < k \leq 2n$, $r_k = r_{k-n}$.
- For $i < j$, $R_{j,i+n}$ corresponds to $R_{j,i}$.



Apply the regular fill algorithm to the doubled sequence.

The suboptimal algorithm: Practical details

- I′ region, $n < i \leq j \leq 2n$, need not be defined or filled, since $E(i,j) = E(i-n, j-n)$ and $V(i,j) = V(i-n, j-n)$.
- The region defined by $j - i \geq n$ is not required (unused).
- Only 1/2 of the doubled array need be defined in computer memory.
- The folding rules are slightly altered for fragments containing the origin. For example, the base pair, $r_1 \cdot r_n$ can exist, implying that $r_n \cdot r_{n+1}$ exists, even though n and $n+1$ are adjacent.



The suboptimal algorithm: Results

- For base pair energy rules, the mfe on any folding containing the base pair, $r_i \cdot r_j$, is

$$\delta G_{i,j} = V(i,j) + V(j,i+n) - e(i,j).$$

  For loop energy rules, $\delta G_{i,j} = V(i,j) + V(j,i+n)$.

- A structure containing $r_i \cdot r_j$ with free energy $\delta G_{i,j}$ may be computed by computing and combining two tracebacks; one for $R_{i,j}$ beginning with $r_i \cdot r_j$ and one for $R_{j,i+n}$, starting with $r_j \cdot r_{i+n}$.

The Equilibrium Partition Function: Definition

The definition of the partition function for all secondary structures on R is

$$Z(R) = \sum_{\mathbf{S} \in \mathcal{S}} e^{-\frac{\Delta G(\mathbf{S})}{RT}}.$$

This is a weighted counting of all structures. Note that the lower the free energy, the higher the weighting. According to statistical mechanical theory, this Boltzmann weighting gives the probability density for every folding. That is, the probability of any particular folding, $\mathbf{S}$, is given by $\exp(-\Delta G(\mathbf{S})/RT)/Z$.

The number of secondary structures grows roughly as 1.8^n, but the computation can be performed in reasonable time using a recursion similar to the original dynamic programming algorithm for computing an optimal folding. We need to introduce new terms.

Algorithm for base pair dependent rules.

AUXILIARY PARTITION FUNCTIONS:

$Q(i,j) = Z(R_{i,j})$

$Q'(i,j)$: *restricted*, must contain $r_i \cdot r_j$ base pair

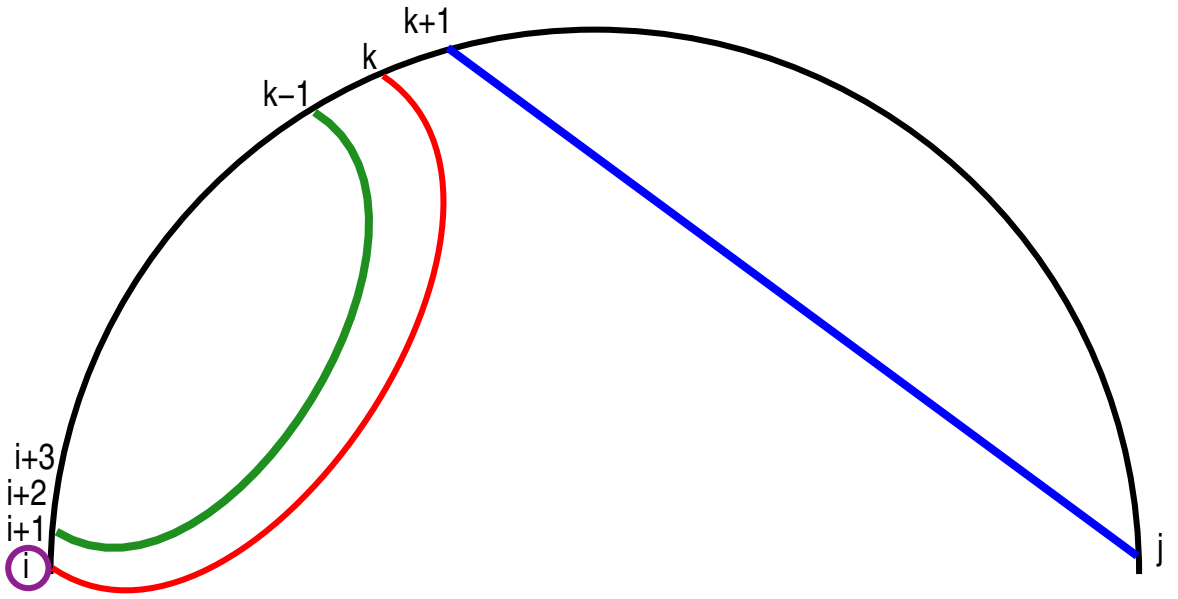
That is, we consider partition functions for every fragment of the original sequence. The Q's are required for loop dependent energy rules, but not for the simpler base pair energy rules. Simple recursions exist for base pair dependent energy rules.

$$
\begin{aligned}
Q(i,j) &= Q'(i,j) = 1, \qquad \text{for} \quad j-i<4, \quad \text{otherwise} \\
Q(i,j) &= Q(i+1,j) + \sum_{k=i+4}^{j} Q'(i,k)Q(k+1,j) \quad \text{and} \\
Q'(i,j) &= \exp\left(-\tfrac{e(i,j)}{RT}\right) Q(i+1,j-1),
\end{aligned}
$$

where $Q(j+1,j)$ is defined to be 1 for convenience.

Algorithm in picture form.

Partition function − base pair dependent rules



$$Q_{ij} = Q_{i+1,j} + \sum_{k=i+4}^{j} exp(-e(i,k)/RT)\, Q_{i+1,k-1}\, Q_{k+1,j}$$

Cases:

1. i is unpaired
2. i pairs with a base k, where $Q_{j+1,j} = 1$

The figure on the left illustrates the recursion step for computing the partition function for base pair energy rules. For each summand, the contribution is broken into 3 components, as shown by the colors.

Partition functions: Computing probabilities

When the recursion is finished, we know the probability, $P(\mathbf{S})$, of any structure $\mathbf{S}$. That is:

$$P(\mathbf{S}) = \frac{e^{-\frac{\Delta G(\mathbf{S})}{RT}}}{Q(1,n)}$$

As with the suboptimal algorithm, the partition function algorithm may also be run on the **doubled sequence**.

For $1 \leq i \leq j \leq n$, $Q(i,j)$ and $Q'(i,j)$ are partition functions for the included fragment, $R_{i,j}$ and $Q(j,i+n)$ & $Q'(j,i+n)$ are partition functions for the excluded fragment, $R_{j,i+n}$.

The probability of the base pair $r_i \cdot r_j$ is given by

$$P(r_i \cdot r_j) = \frac{Q'(i,j)Q'(j,i+n)}{e^{-\frac{e(i,j)}{RT}}Q(1,n)}.$$

The exponential term in the denominator corrects for the fact that both $Q'(i,j)$ and $Q'(j,i+n)$ count the energy contribution of the base pair $r_i \cdot r_j$. This term is absent when base pair energy rules are used.

Partition functions: Stochastic traceback

Ratios of partition functions are conditional probabilities.

For any fragment, $R_{i,j}$ of R,

- $P_{i,j}(i) = \dfrac{Q(i+1,j)}{Q(i,j)}$ is the probability that r_i is single-stranded.
- $P_{i,j}(k) = \dfrac{Q'(i,k)Q(k+1,j)}{Q(i,j)}$ is the probability that r_i pairs with r_k, for $i < k \leq j$.
- These probabilities are **conditional**.
- That is, they are conditioned on the premise that no base pair of the form $r_h \cdot r_l$ exists for $h < i$ and $i \leq l \leq j$ or for $i \leq h \leq j$ and $l > j$.

Partition functions: Stochastic traceback algorithm.

Algorithm flowchart:

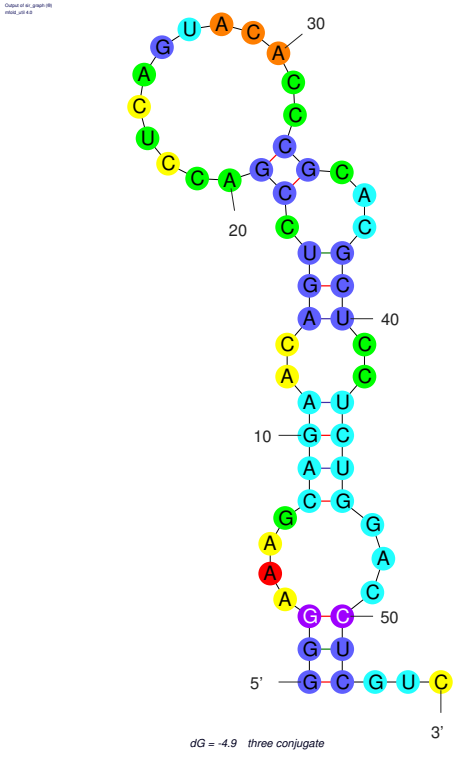
- Initial conditions: The list of base pairs and the "traceback stack" are both empty.
- Start: Set $i = 1$ and $j = n$. Put i and j on to the "traceback stack".
- Recursion:

1. If the traceback stack is empty, the traceback terminates. Otherwise, take i and j from the traceback stack.
2. Choose a random number, x, between 0 and 1 (uniform distribution). If $x \leq P_{i,j}(i)$, continue with 3; otherwise, continue with 4
3. r_i will be unpaired.
   (a) If $j - i > 3$, set $i = i + 1$ and continue with 2.
   (b) If $j - i \leq 3$, continue with 1.
4. Find the unique k, $i < k \leq j$ such that $\sum_{h=i}^{k-1} P_{i,j}(h) < x$ and $\sum_{h=i}^{k} P_{i,j}(h) \geq x$.
   (a) Add $r_i \cdot r_k$ to the list of base pairs.
   (b) If $j - k \geq 3$, place $k + 1$ and j on to the traceback stack.
   (c) Set $i = i + 1$, $j = k - 1$. Continue with 2 if $j - i > 3$; otherwise, continue with 1

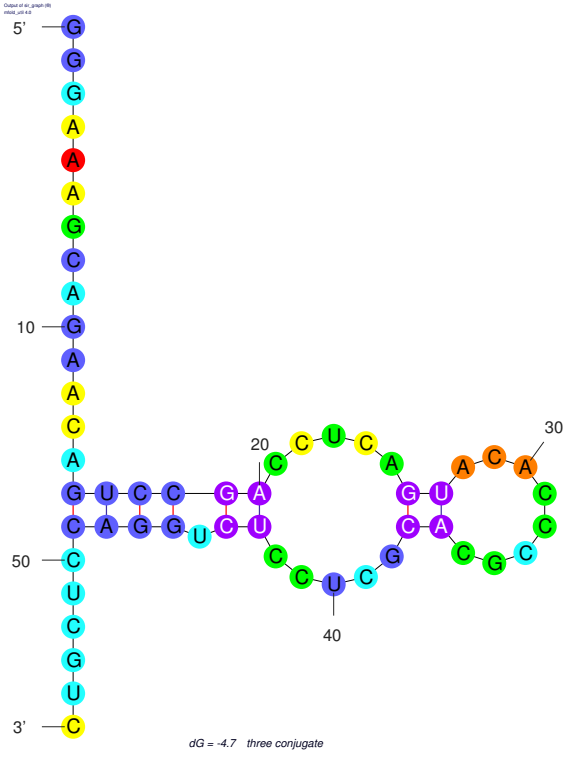
The folding problem is ill-conditioned in general

As bad as it gets.

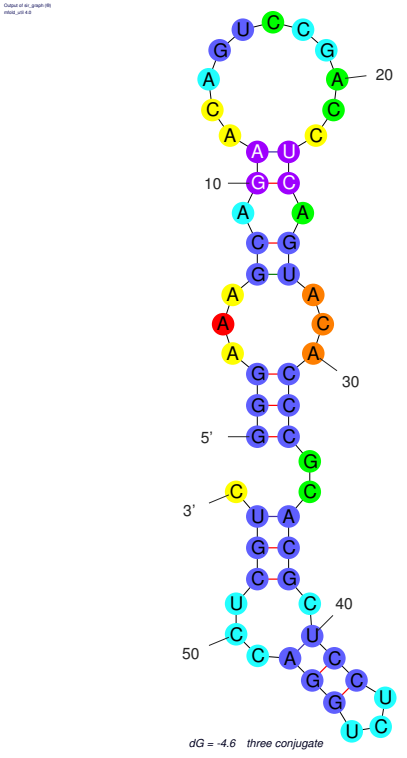
Three *conjugate*[1] foldings of a short sequence. From left to right, $\Delta G = -4.9$ kcal/mol (mfe folding), $\Delta G = -4.7$ and $\Delta G = -4.6$!



Folding 1



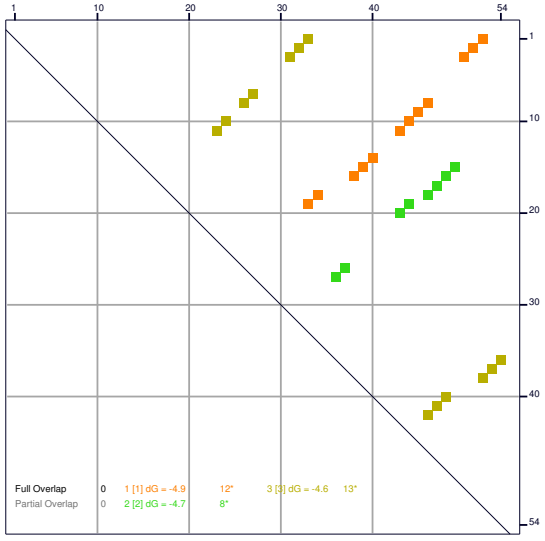
Folding 2

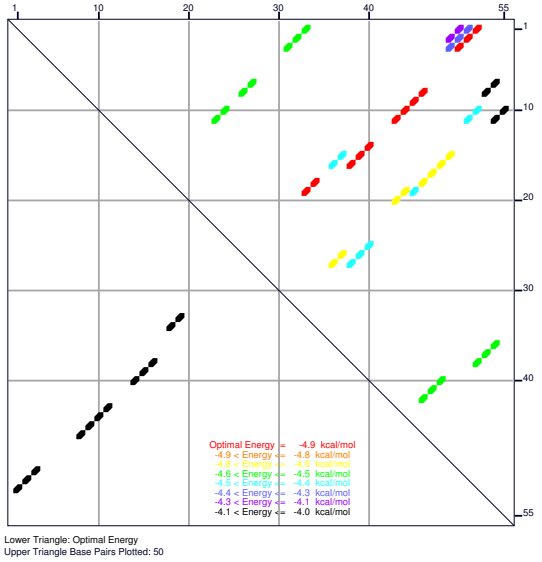


Folding 3

[1] Two foldings are called conjugate if they share no base pairs in common.

Other views of the “three conjugate” foldings example



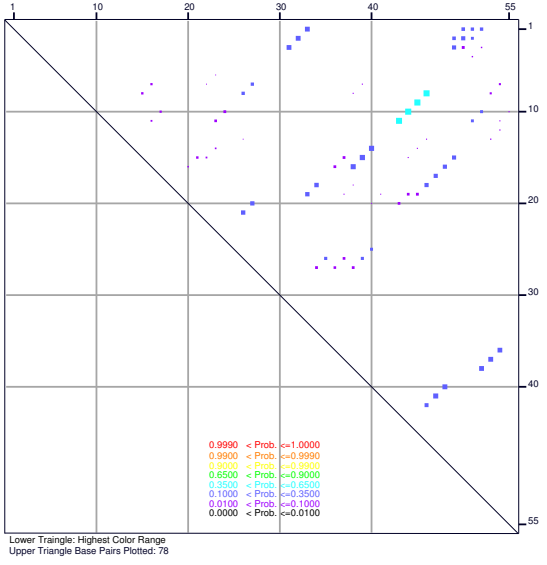


Clockwise from above left:

1. Structure dot plot. Superposition of three conjugate foldings.

2. EDP (energy dot plot). Superposition of **all possible** foldings within 0.3 kcal/mol from mfe.

3. PDP (probability dot plot) A plot of all base pairs with probabilities ≥ 0.01. The maximum probability is 0.44 (poor).



Heresy: Statistics on one Datum

For a single secondary structure, predicted or otherwise:

- Not all base pairs are created equal.
- The EDP and PDP can be used to distinguish.
- In a secondary structure plot:
  1. color base pairs according to the probability that they form.
  2. color single-stranded bases according to the probability that they are **not** paired.
- Higher probability base pairs are more likely to be correct.

The *consensus* structure.

The consensus structure contains all base pairs whose probabilities are $> 50\%$, and no others. (Ye Ding & Chip Lawrence call this the “centroid”.)

Quiz:

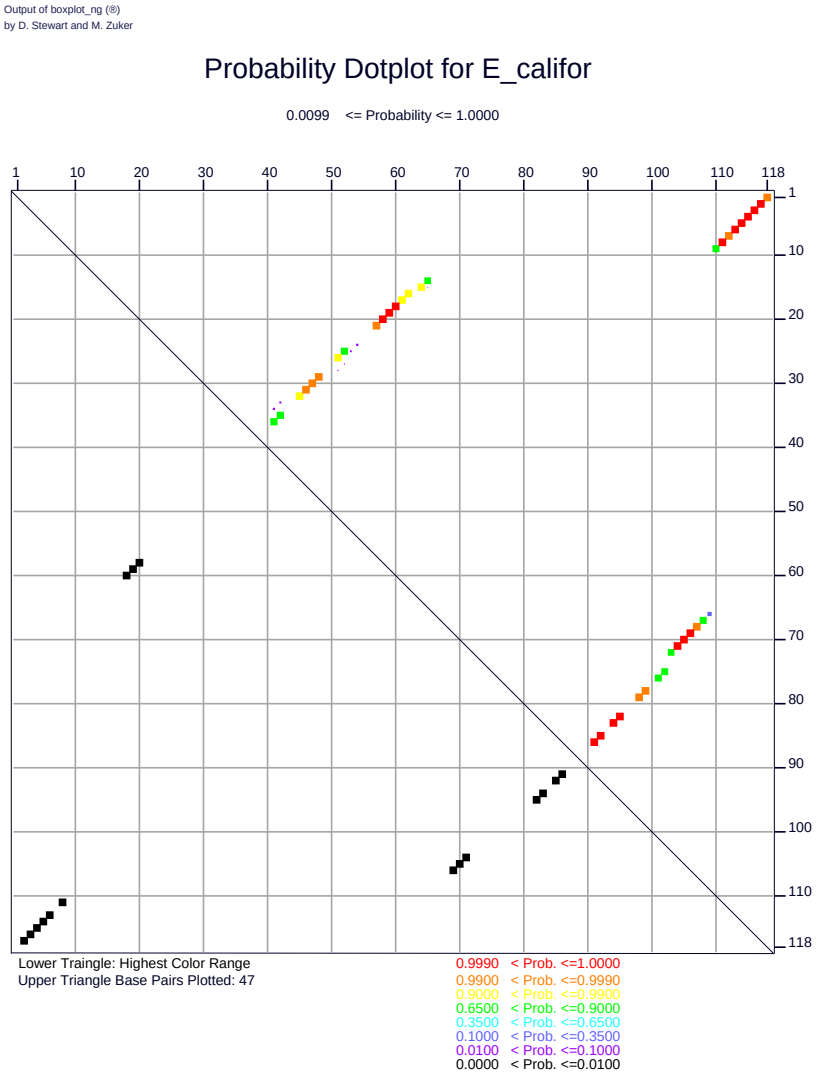
- Prove that the consensus structure is valid (no base triples). [20 points]
- Prove that the consensus structure contains no pseudoknots. [40 points]

Good *versus* Bad RNAs

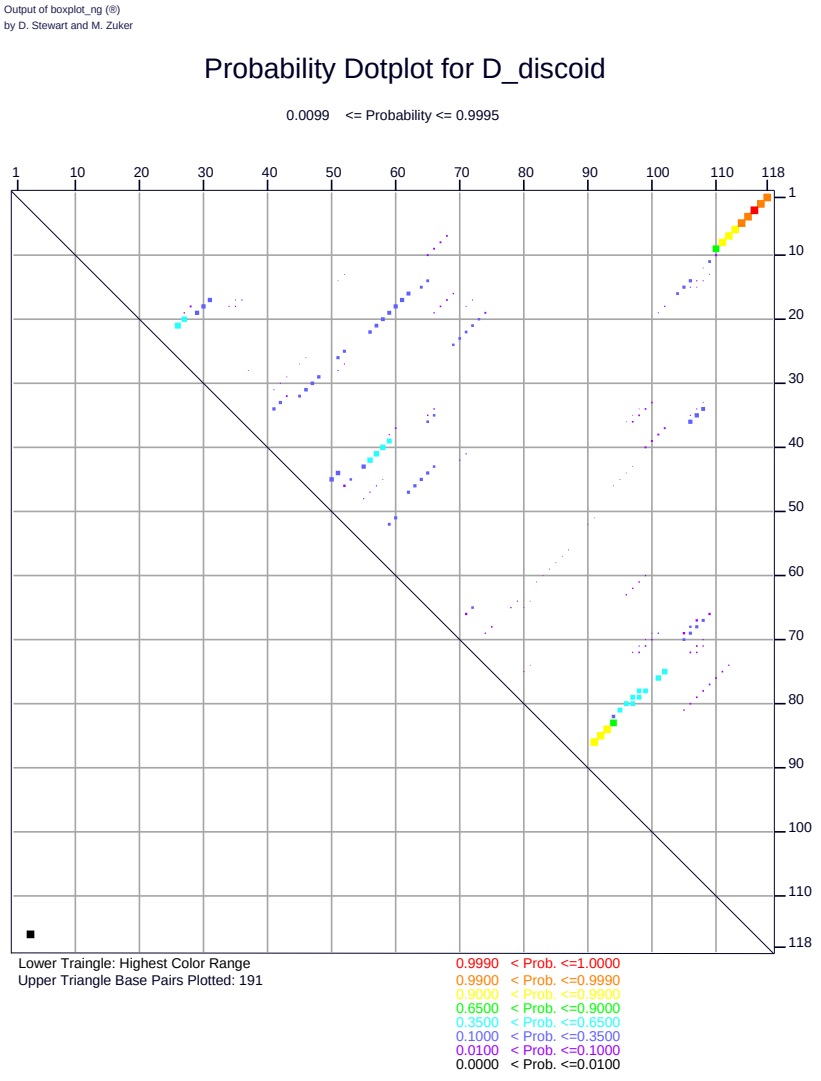
- A good RNA has an “uncluttered” EDP or PDP. Base pairs in a mfe folding are more likely to be correct. High probability base pairs are “usually” correct, but a good RNA has a lot more high probability base pairs than a bad RNA.
- A bad RNA has a “cluttered” EDP or PDP. A mfe folding has fewer correct base pairs. Even though high probability base pairs are likely to be correct, there are relatively few of them.

Example: A good RNA *versus* a bad RNA.

E. californium 5S rRNA

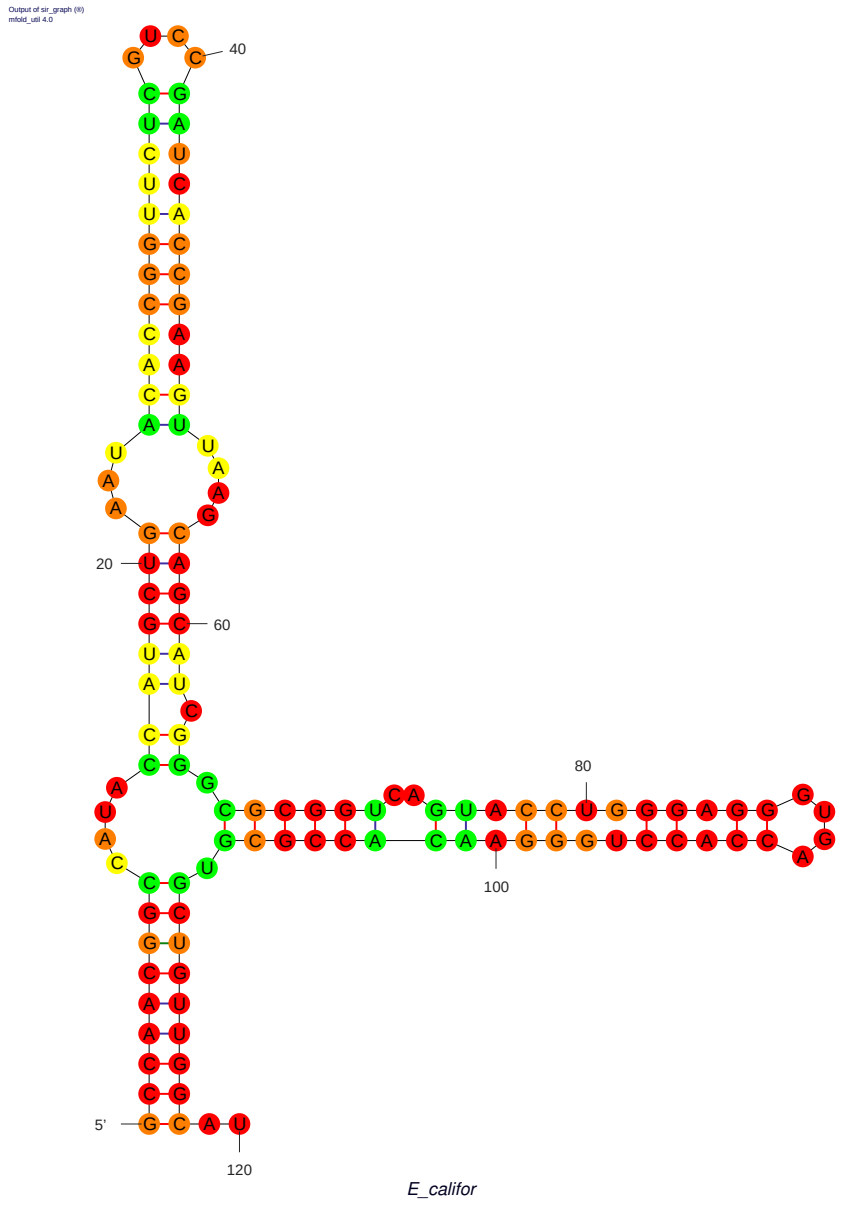


D. discoidium 5S rRNA

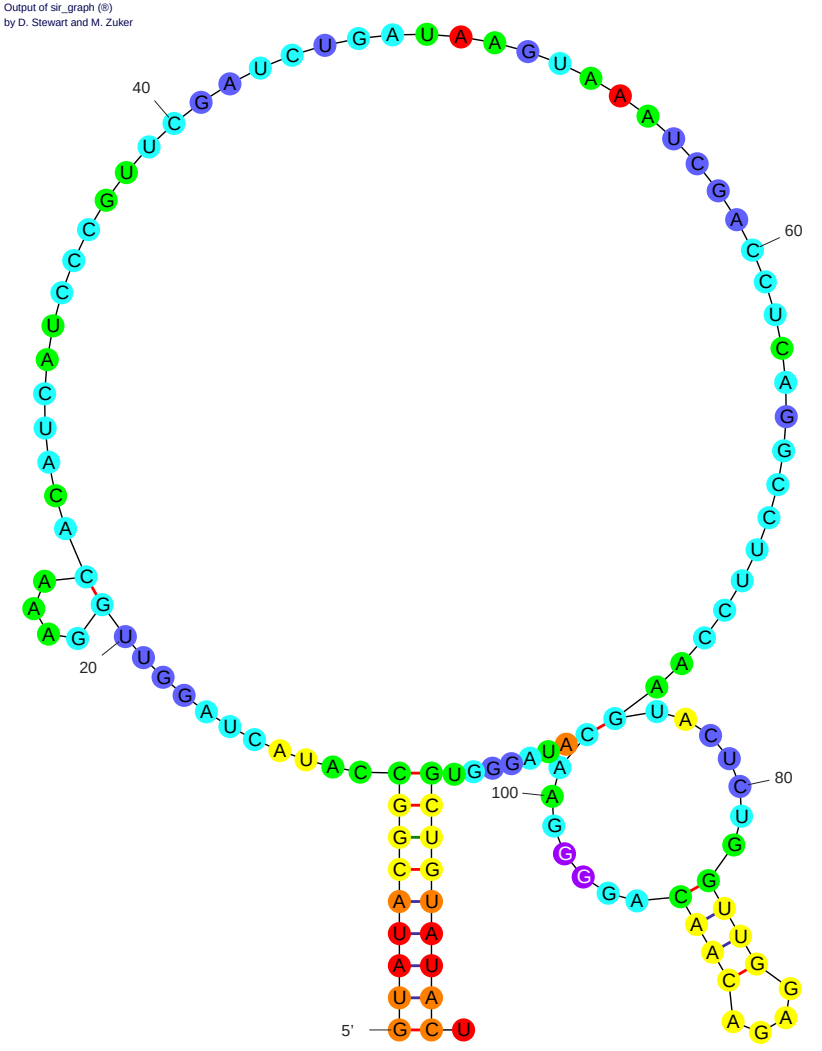


"Uncluttered" PDP *versus* a "cluttered" one.

Consensus structures for the same pair of 5S rRNAs



E_califor



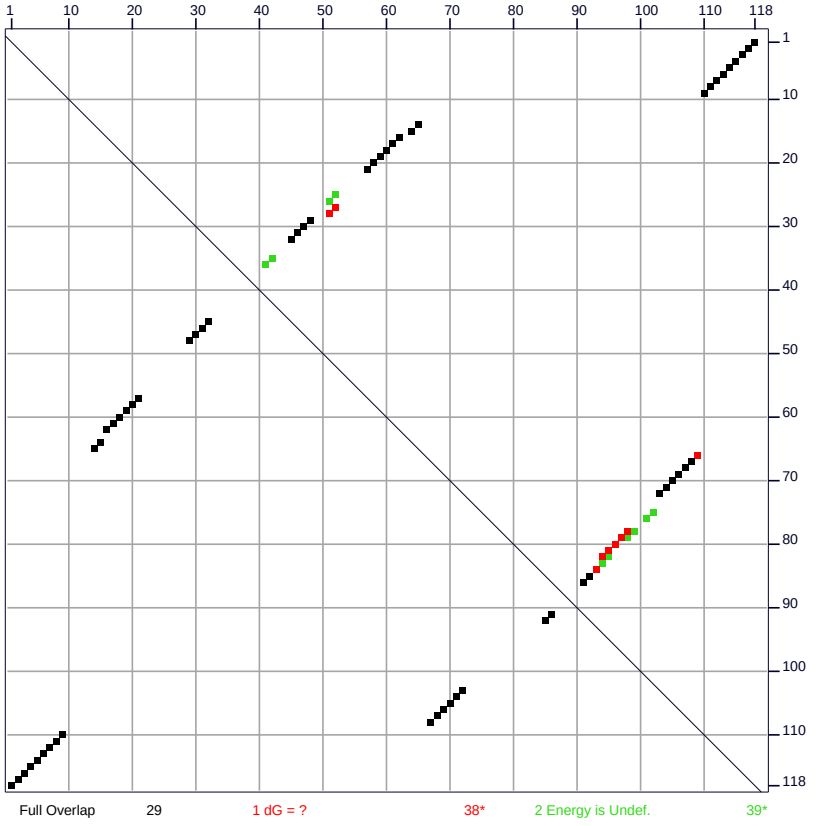
D_discoid Consensus

Not much predicted with confidence *versus* a lot predicted with high confidence. In this case, the second folding is a very good prediction.

The numbers

Output of ct_boxplot (®)
by D. Stewart and M. Zuker

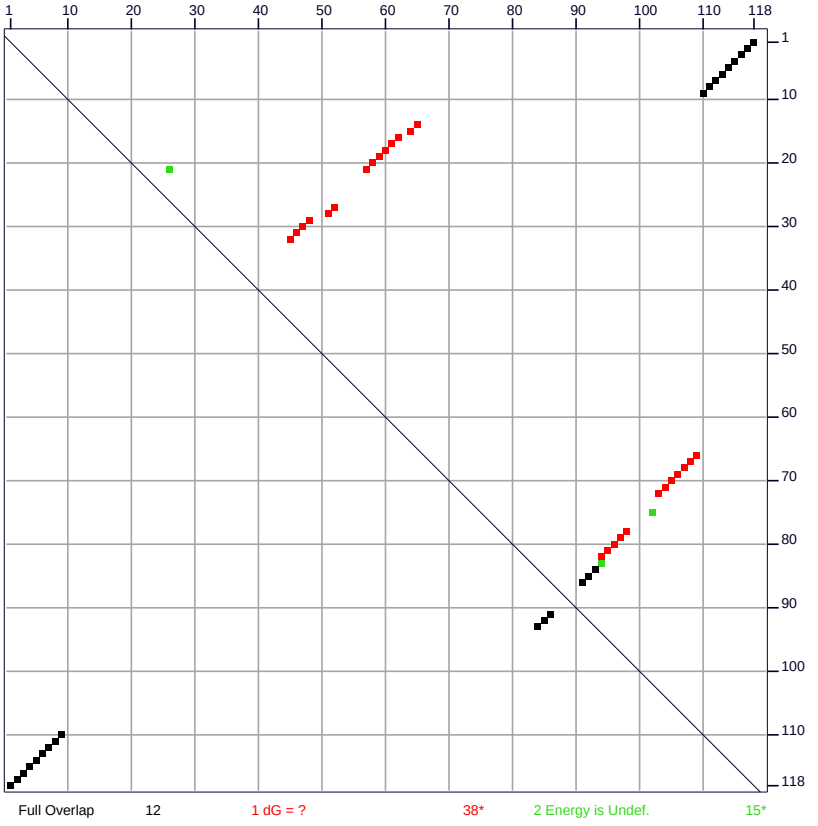
Structure dot plot for E_califor



*Counts for each structure include overlap dots.

Output of ct_boxplot (®)
by D. Stewart and M. Zuker

Structure dot plot for D_discoid



*Counts for each structure include overlap dots.

Entropy: Quantifying "good" and "bad"

If $P = \{p_i\}_{i=1}^{\infty}$ is a countable probability distribution (may be finite), then

$$\sum_{i=1}^{\infty} p_i = 1.$$

The (Shannon) entropy of P is defined by

$$\mathfrak{S}(P) = -\sum_{i=1}^{\infty} p_i \log(p_i).$$

(Treat $0\ln(0)$ as 0.) For infinite distributions, the entropy might be $+\infty$. We do not consider infinite or continuous distributions here. The base for the logarithm is not specified and may be chosen arbitrarily to scale the entropy. For example, base 2 gives the entropy in "bits".

When there are n "outcomes", $1 \leq i \leq n$ and the maximum entropy distribution sets $p_i = 1/n$, so that $\mathfrak{S}(P) = -\sum_{i=1}^{n} p_i \log(p_i) = \log(n)$.

If $\mathcal{S}(R)$ is the set of all foldings of $r_1 r_2 \ldots r_n$, then the maximum entropy loss to select a single folding is given by making all foldings equally likely, and so:

$\mathfrak{S}(\mathcal{S}(R)) \sim Cn - 1.5\ln(n)$ as $n \to \infty$, for some constant C. What is C?

The expected number of foldings

Suppose that an RNA sequence is totally random, with equal probabilities for A, C, G and U. Then the probability that two different bases can pair is $\frac{3}{8}$. This represents $1/16$ for A·U, U·A, C·G, G·C, G·U and U·G. In general, call this probability p.

Many years ago, I showed that the expected number of foldings, T_n, on a random RNA sequence of length n is given by

$$T_n \sim \frac{(1+4\sqrt{p})^{\frac{1}{4}}}{2\sqrt{\pi}p^{\frac{3}{4}}} n^{-\frac{3}{2}} \left(\frac{1+\sqrt{1+4\sqrt{p}}}{2} \right)^{2n+2},$$

as $n \to \infty$.

This implies a maximum entropy of $2\log\left(\frac{1+\sqrt{1+4\sqrt{p}}}{2} \right)$ per base. This reduces to $2\log(1+\sqrt{1+4\sqrt{p}}) - 2$ bits/base.

For $p = 3/8$, the maximum entropy is 1.029 per base. For $p = 1/4$, it is 0.900 bits per base.

The entropy of the Boltzmann distribution of all foldings.

The partition function for $\mathcal{S}(R)$ is defined by

$$Z = \sum_{\mathbf{S} \in \mathcal{S}(R)} \exp\left(-\frac{\Delta G(\mathbf{S})}{RT}\right)$$

The probability of structure $\mathbf{S}$ is $\frac{e^{-\frac{\Delta G(\mathbf{S})}{RT}}}{Z}$

The log probability is $-\frac{\Delta G(\mathbf{S})}{RT} - \ln Z$, so

$$\mathfrak{S}(\mathcal{S}(R)) = \frac{\overline{\Delta G(\mathbf{S})} - \Delta G_{\text{ens}}}{RT}.$$

ΔG_{ens} is computed directly by standard algorithms and $\overline{\Delta G(\mathbf{S})}$ can be estimated by averaging the free energies of a stochastic sample of foldings. Replacing RT with $RT\ln(2)$ expresses the entropy in bits.

I have chosen entropy per base to be the overall measure of "well-definedness" for RNA folding.

Entropy of random RNA

For each of 302 Eukaryote 5S rRNAs, 100 random sequences were generated with the same length and dinucleotide distribution. For each of these, the entropy of the Boltzmann distribution was computed with a sample of 100 foldings. GC content in the random sequences was $55.57 \pm 3.79\%$.

The entropy was 0.226 ± 0.007 bits/base.

The entropies of 100 randomized versions of the RNase P RNA from *Desulfovibrio desulfuricans* (length 360) gave similar results, as did foldings of the first 150 bases of these sequences.

Good and bad revisited

- Good refers to low entropy RNAs, for which many base pairs are predicted with high probability.
- Bad refers to high entropy RNAs, for which few base pairs are predicted with high probability.
- Problems: As is usual in biology, there are exceptions to each "rule".

1. In "good" or "bad" RNAs (especially for "good"), one expects that base pairs with high probability should be correct. Some high probability base pairs are not correct and some correct base pairs have very low probabilities.
2. In homologous RNAs that are similar enough to be readily aligned, some high probability base pairs in one may be low probability base pairs in the other, and *vice versa*.

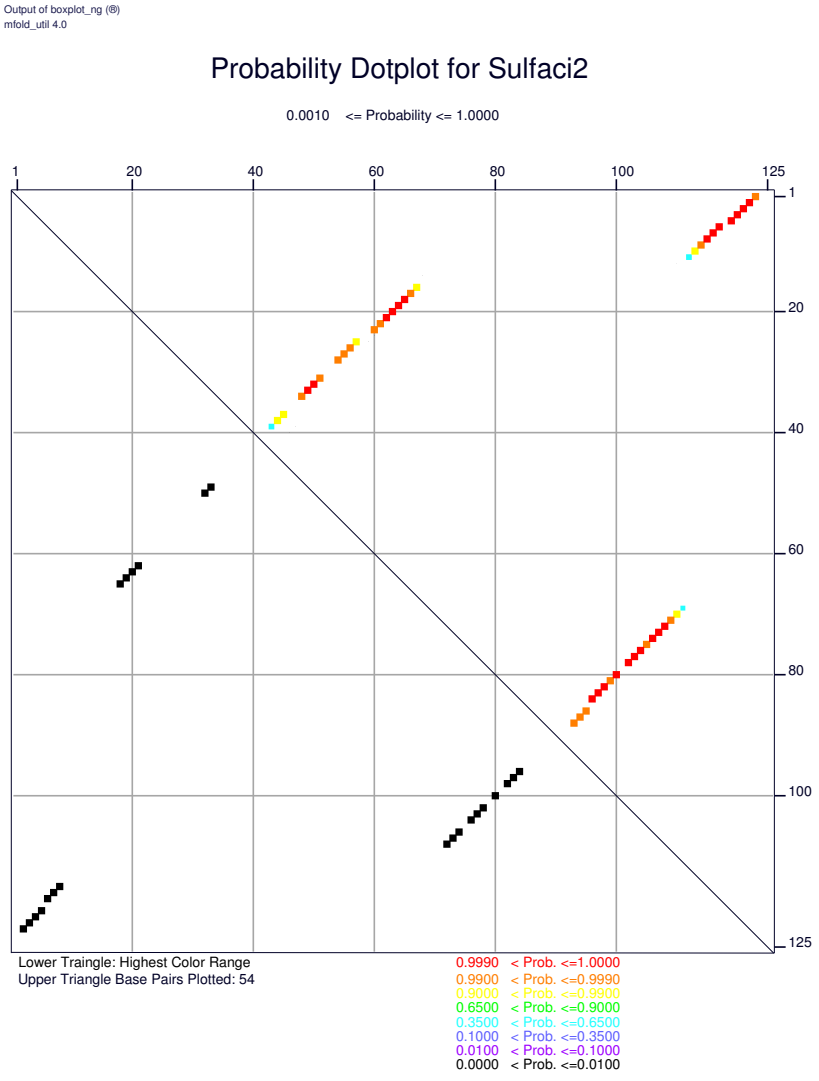
Table of computations

Type	Number	$\min \mathfrak{S}$	$\overline{\mathfrak{S}}$	$\max \mathfrak{S}$	r GC content	$r\ \delta G_{\mathrm{mfe}}$
5S rRNA Eubacteria	439	0.123 *Thermus sp1*	0.223	0.311 *Planctomyces limnophilus*	-0.308	0.500
5S rRNA Archaea	57	0.063 *Sulfolobus faci2*	0.165	0.246 *Halobacterium saccharovorum*	-0.386	0.768
5S rRNA Eukaryota	302	0.106 *Lineus geniculatus*	0.182	0.264 *Spirogyra sp*	-0.119	0.543
group I introns	81	0.161 *Staurastrum sp.* Chl (M753) SSU rRNA	0.212	0.307 *Zea mays* Chl tRNA Leu	-0.431	0.362
group II introns	51	0.177 *Trichodesmium* Chl *erythraeum* (sp I1)	0.217	0.258 *Sinorhizobium meliloti* (I1)	-0.770	0.597
RNase P Eubacteria	179	0.161 *Desulfovibrio desulfuricans*	0.205	0.263 *Campylobacter jejuni*	-0.573	0.762
RNase P Archaea	36	0.162 *Aeropyrum pernix*	0.213	0.278 *Methanococcus maripaludus*	-0.745	0.693
tRNA	803	0.090 *Bacillus subtillus* GAT	0.201	0.308 (Chl) *Euglena gracilis* CAT	-0.207	0.443

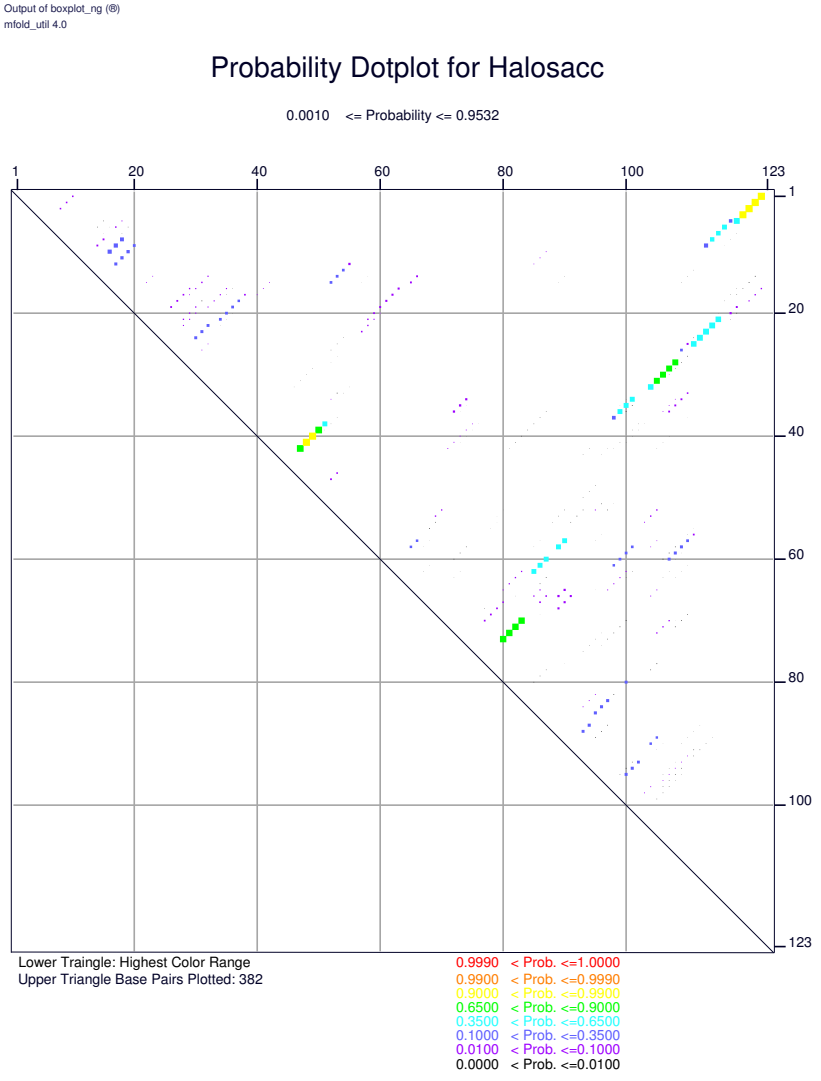
Minimum, average and maximum entropies, in bits/base, for different types of RNA. r is the correlation of entropy with GC content or minimum free energy.

Best *versus* worst. 5S rRNA from Archaea. I

Sulfolobus faci2

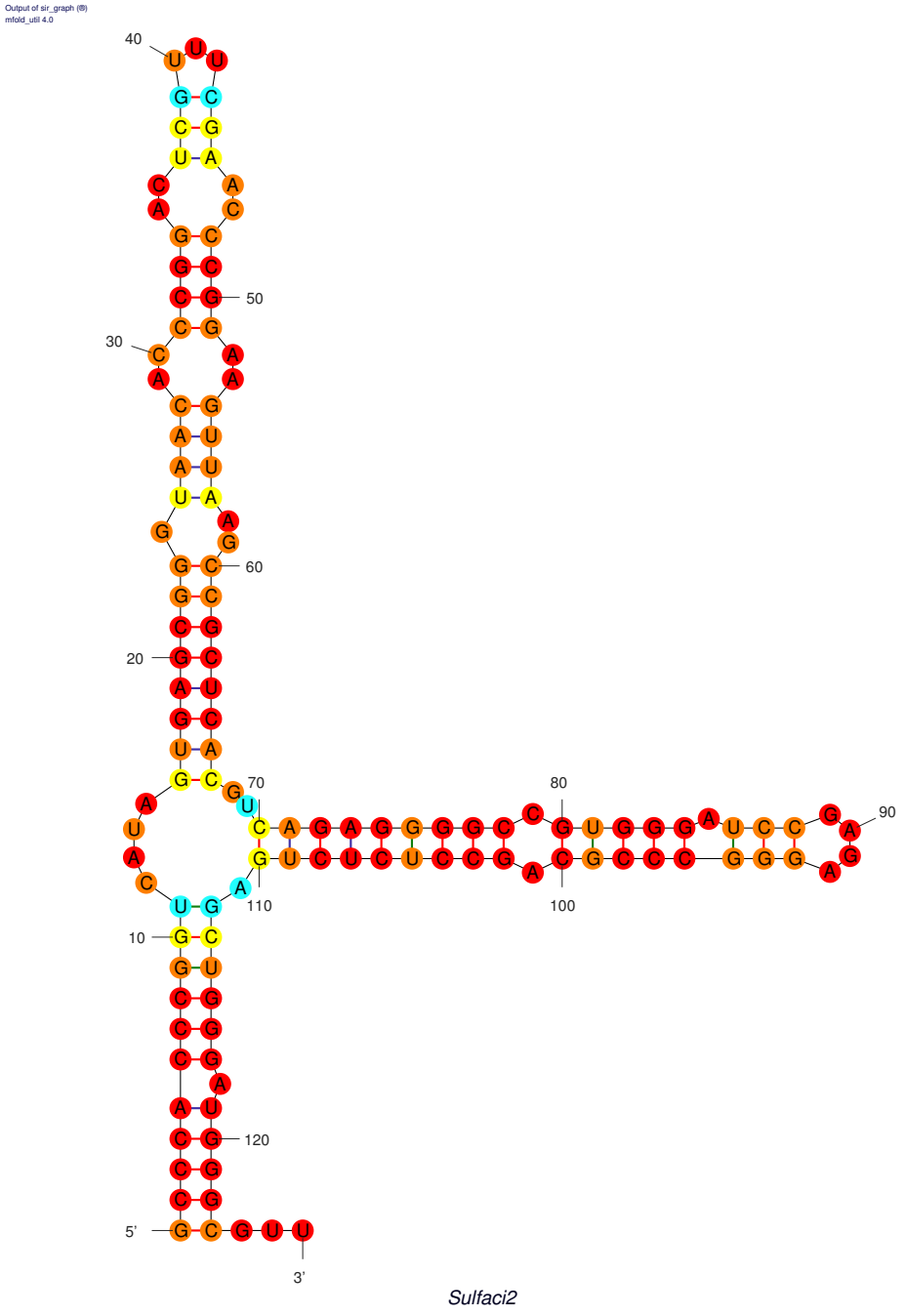


Halobacterium saccharovorum

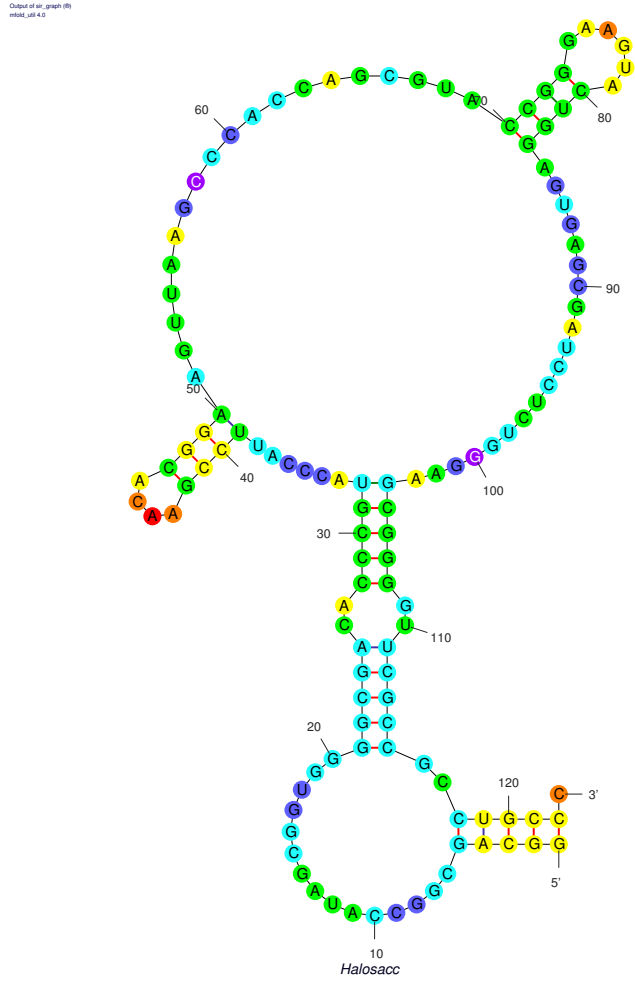


Best *versus* worst. 5S rRNA from Archaea. II

Sulfolobus faci2

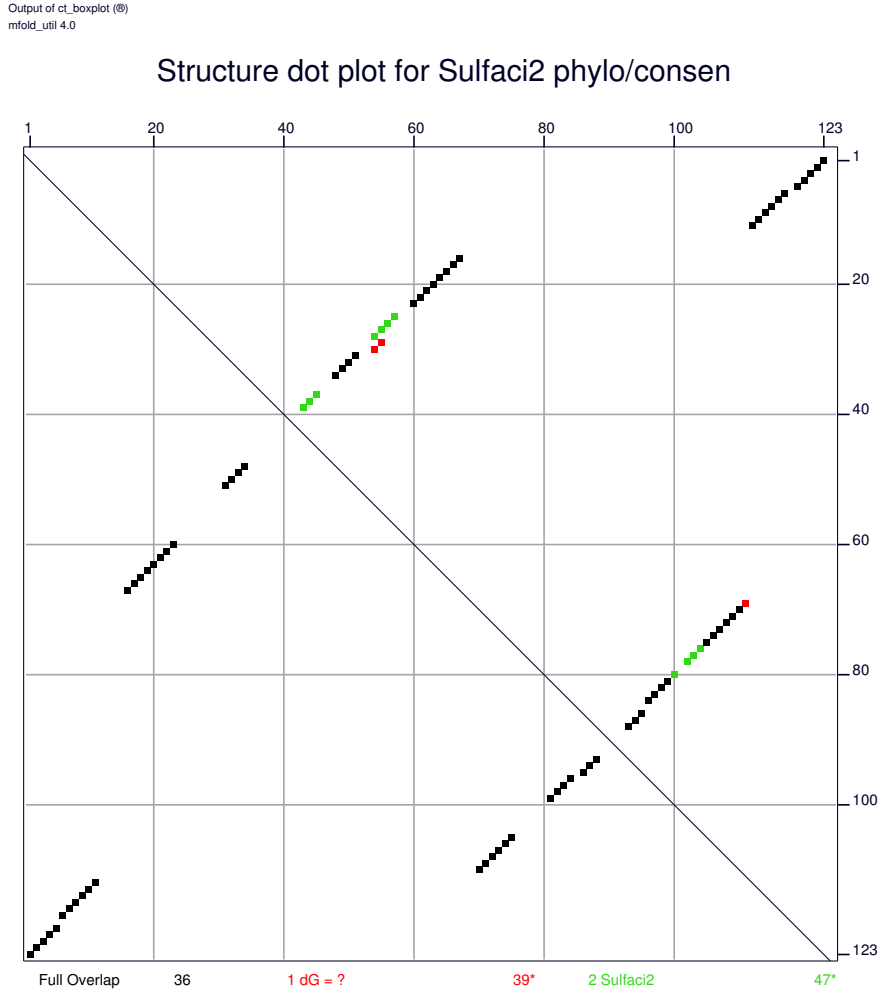


Halobacterium saccharovorum



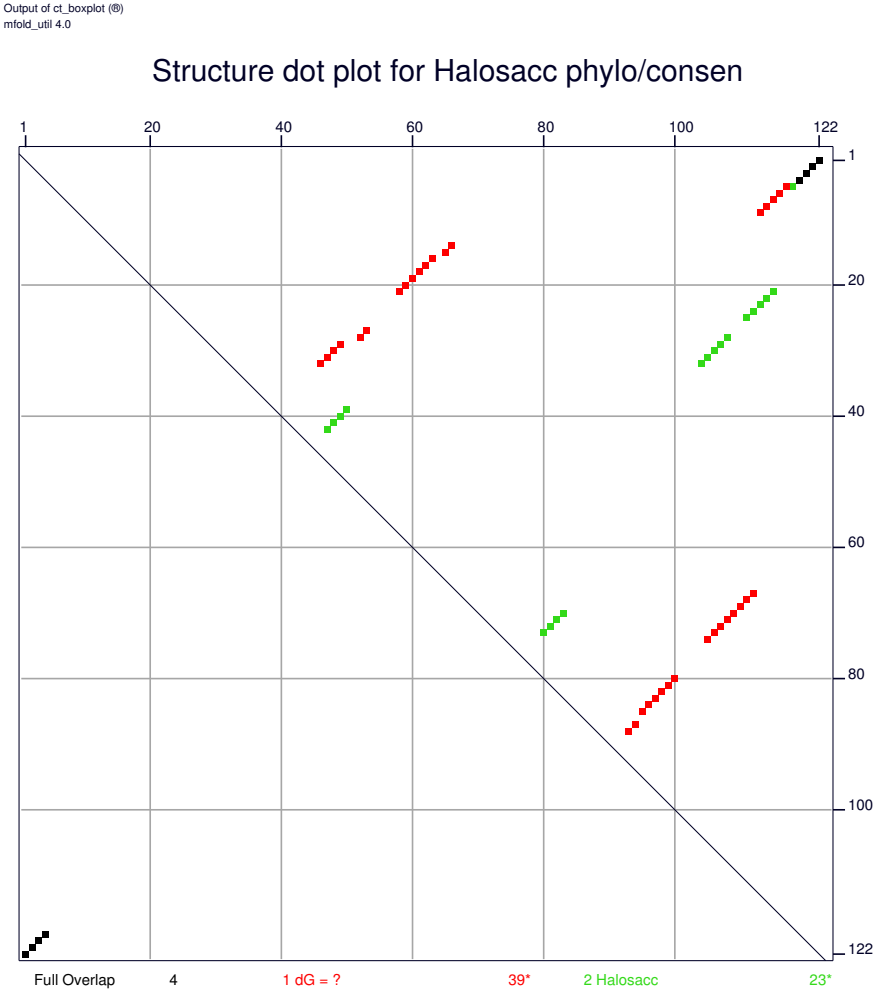
Best *versus* worst. 5S rRNA from Archaea. III

Sulfolobus faci2



*Counts for each structure include overlap dots.

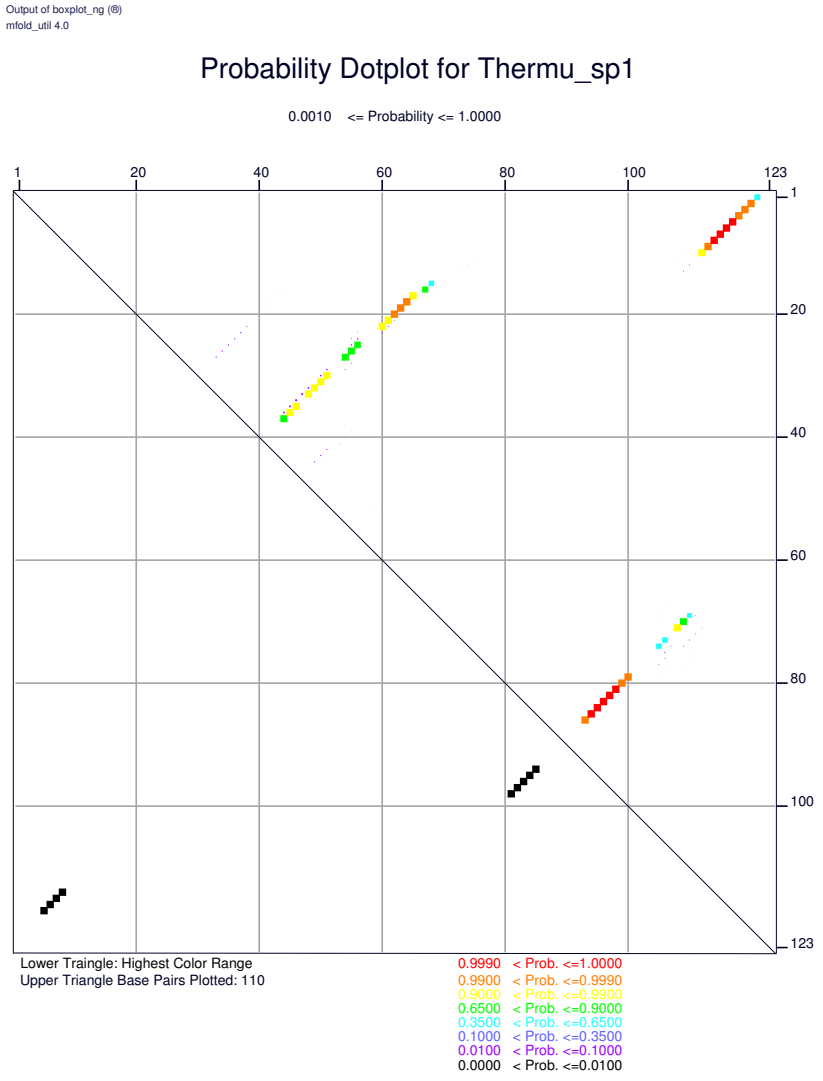
Halobacterium saccharovorum



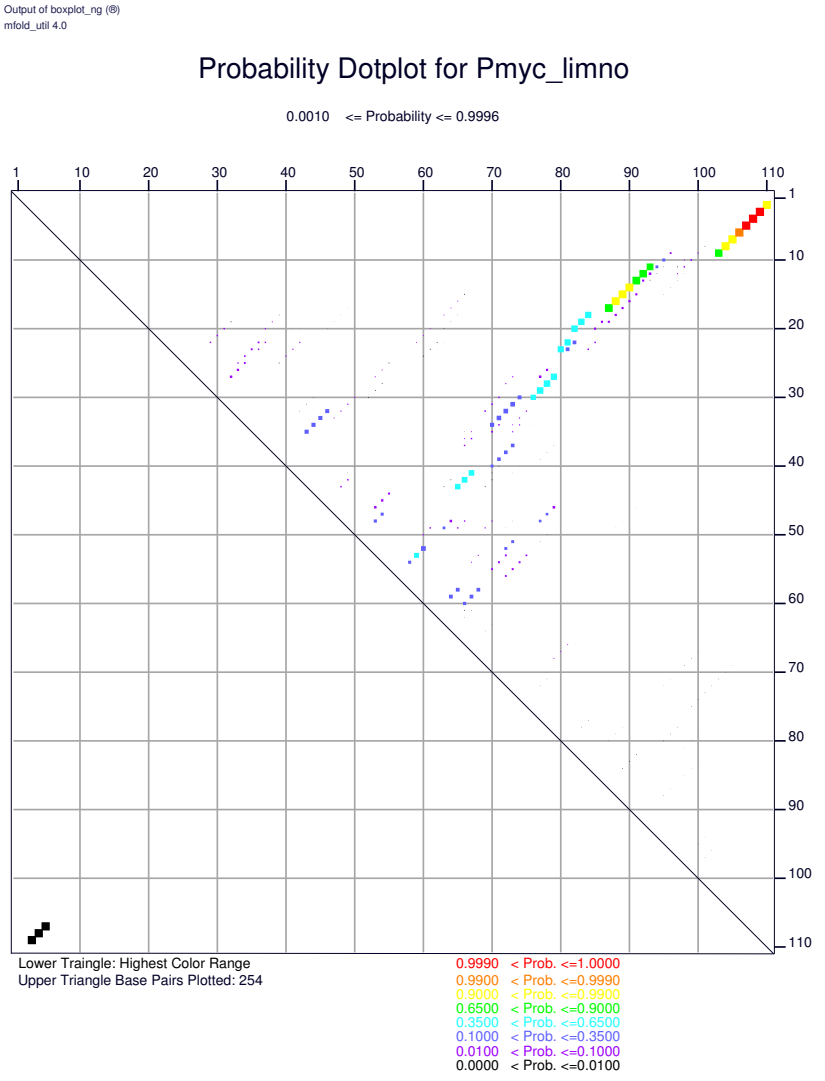
*Counts for each structure include overlap dots.

Best *versus* worst. 5S rRNA from Eubacteria. I

Thermus sp1

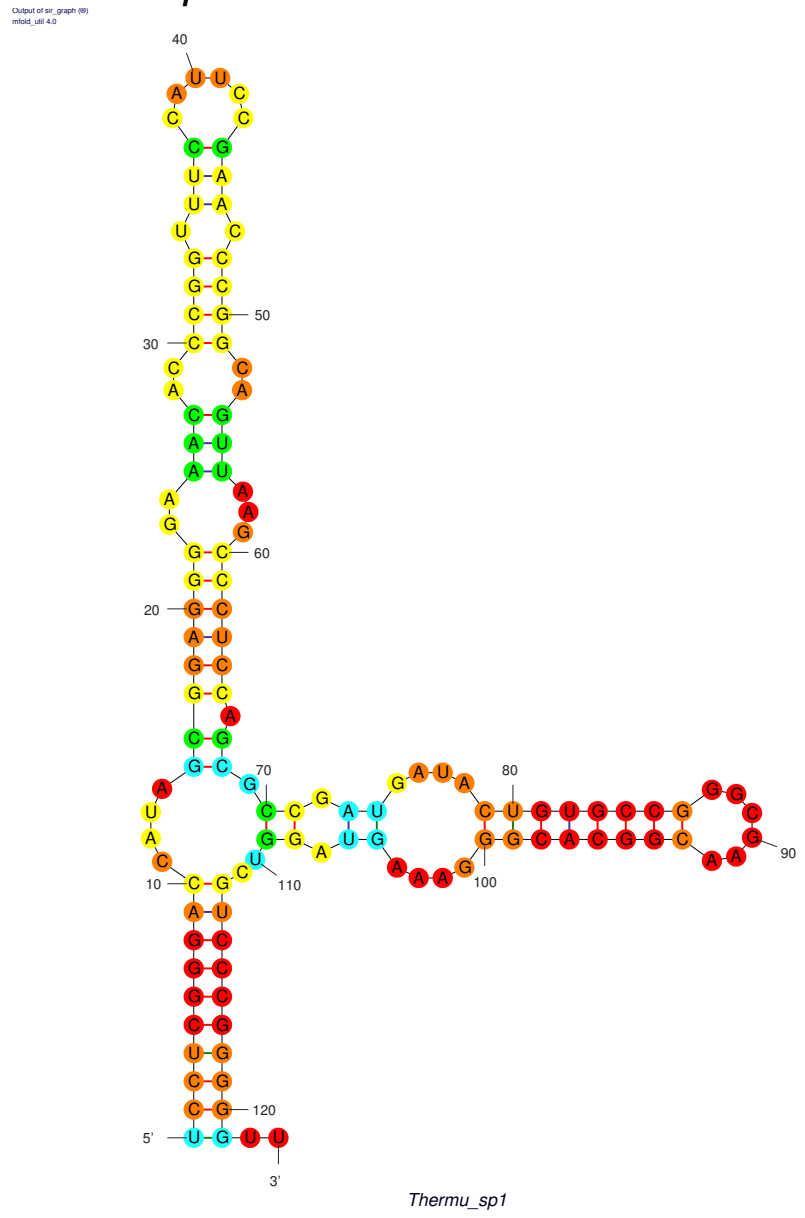


Planctomyces limnophilus

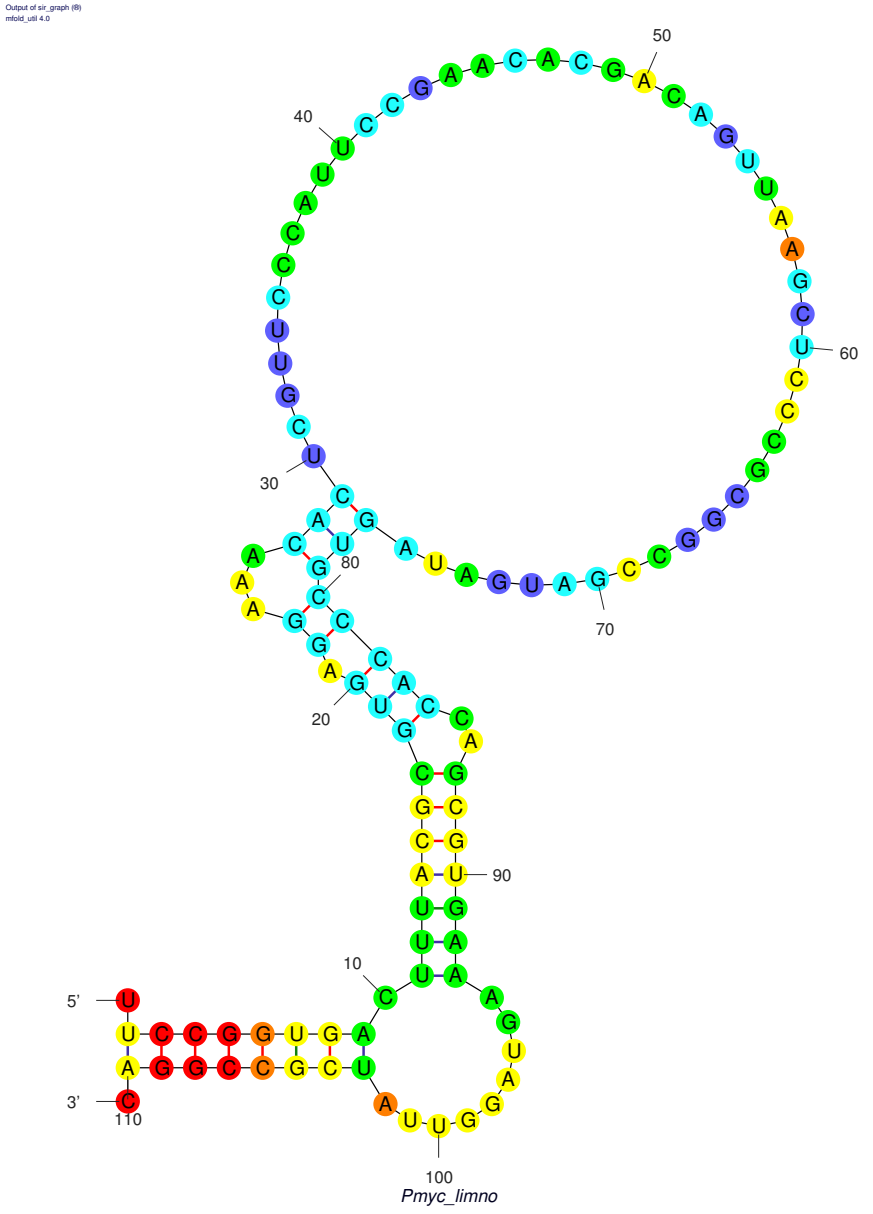


Best *versus* worst. 5S rRNA from Eubacteria. II

Thermus sp1

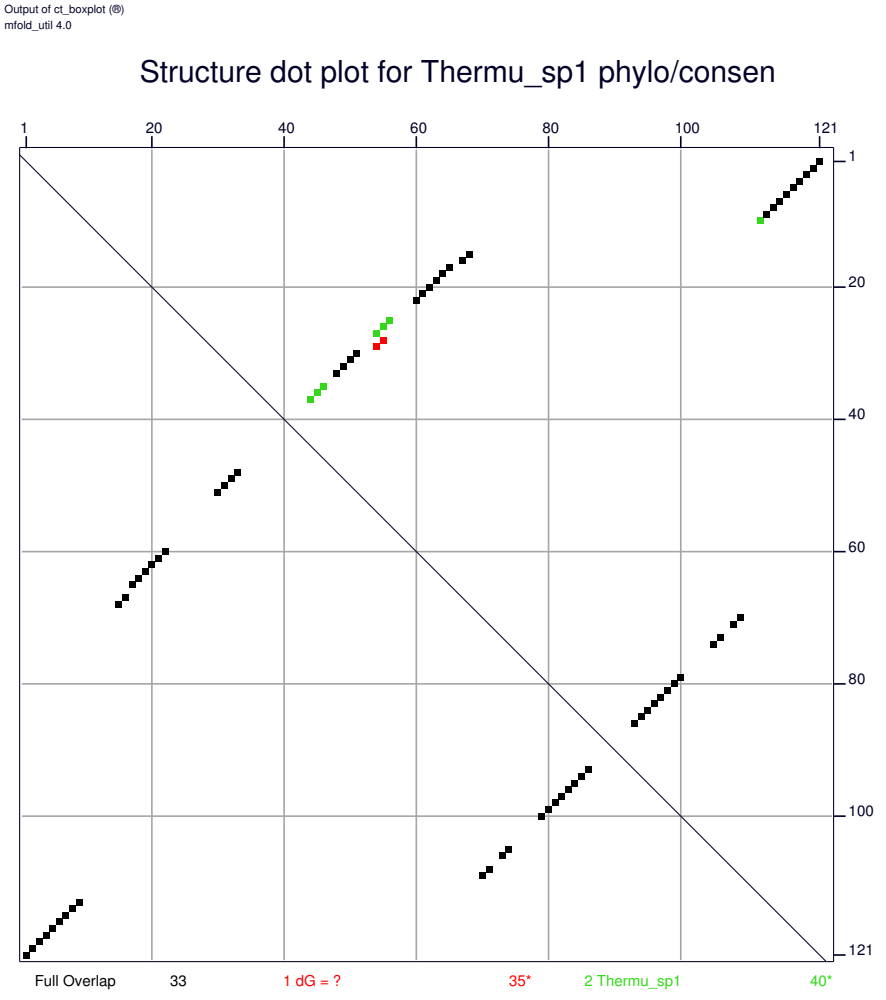


Planctomyces limnophilus



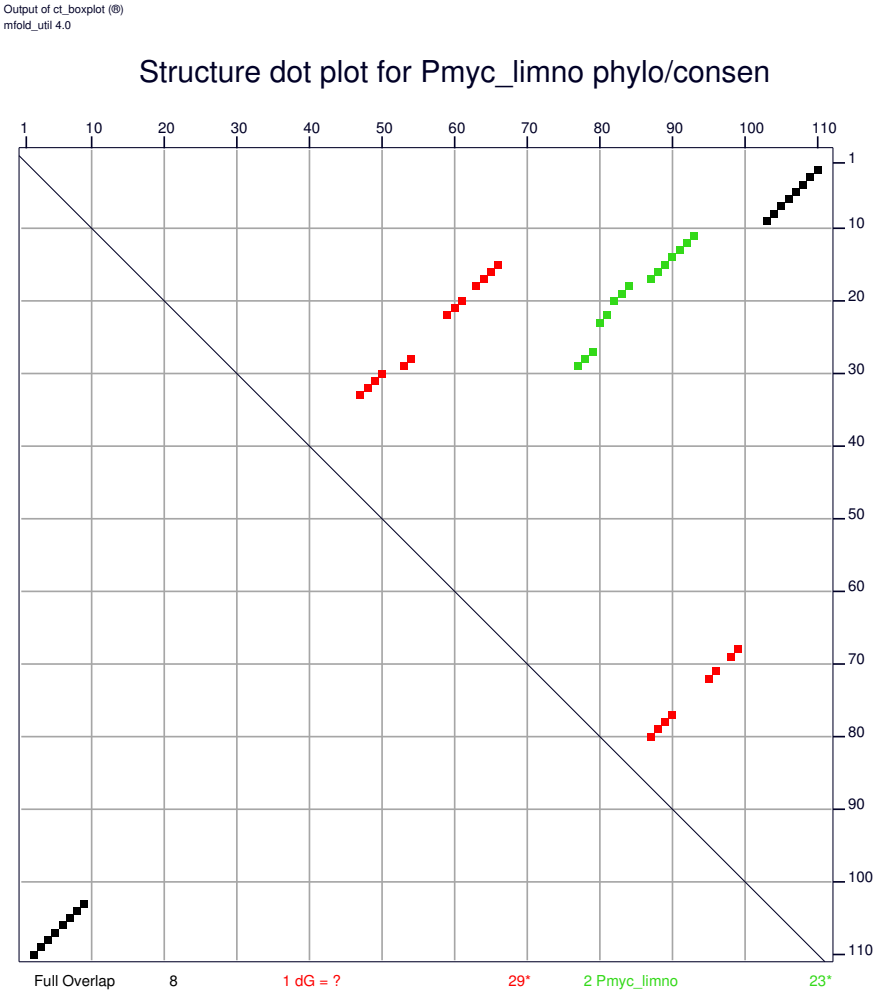
Best *versus* worst. 5S rRNA from Eubacteria. III

Thermus sp1



*Counts for each structure include overlap dots.

Planctomyces limnophilus



*Counts for each structure include overlap dots.

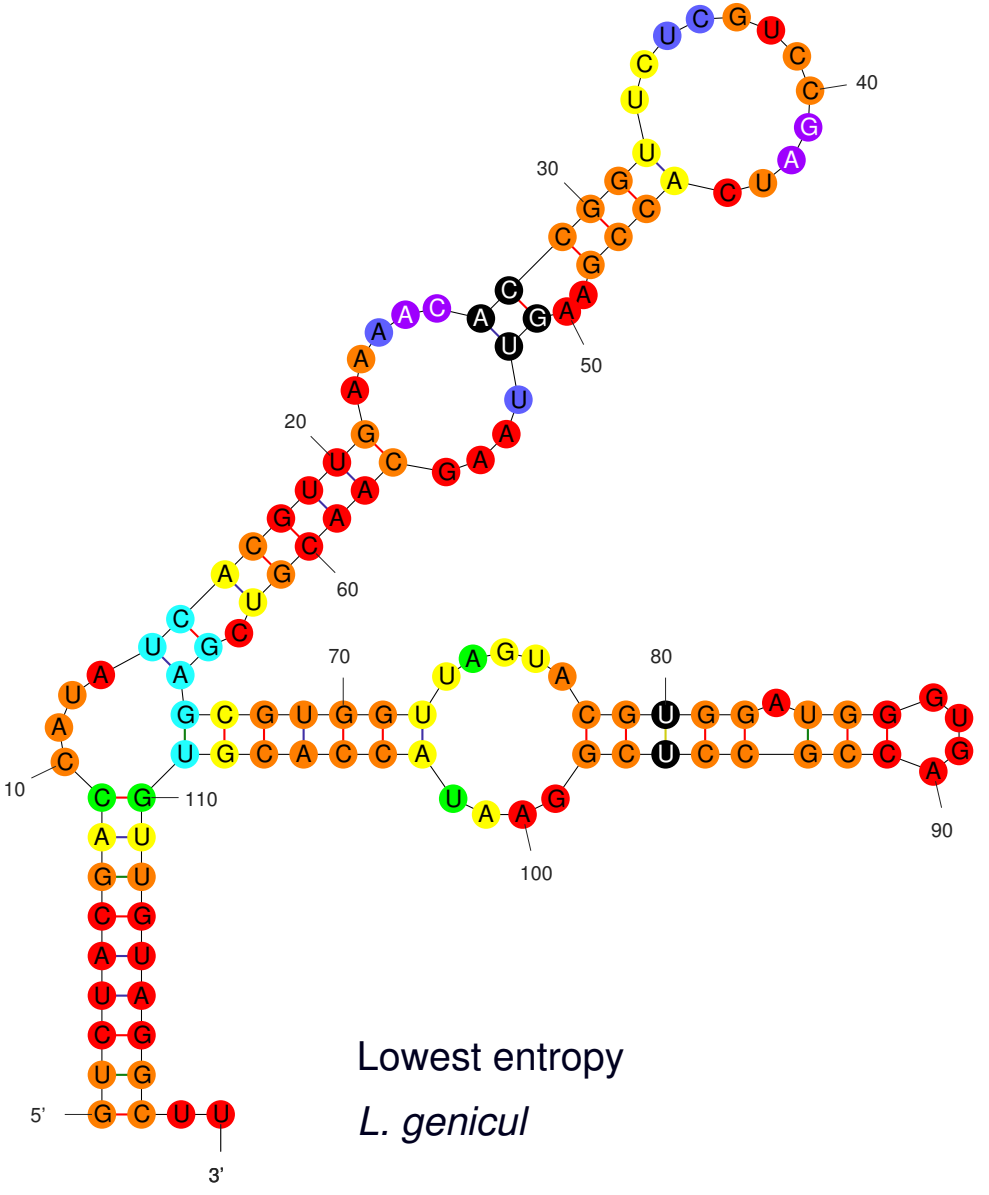
Problems I

The tandem base pairs in black have probabilities of roughly $1/2$ of 1 percent. They occur in the phylogenetic models for 5S rRNA and seem correct in terms of alignment and covariation.

This motif strongly contradicts the energy rules. What is happening here?

The table below shows good conservation evidence for the base pair $A_{27} \cdot U_{52}$. MI = 0.85 bits.

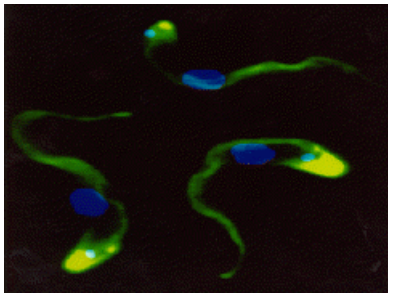
Number	.	A	C	G	U	Total
A	0	0	0	0	246	246
C	0	0	0	0	0	0
G	0	0	40	0	0	40
U	0	16	0	0	0	16
.	0	0	0	0	0	0
Total	0	16	40	0	246	302



Lowest entropy

L. genicul

Trypanosomes



Copied from the Matthews Lab web site:
www.biology.ed.ac.uk/research/groups/kmatthews/

African trypanosomes are parasites, spread between mammals by tsetseflies. They are responsible for epidemics of sleeping sickness in sub-Saharan Africa. This disease is always fatal unless treated and is the second biggest killer behind HIV in parts of Africa.

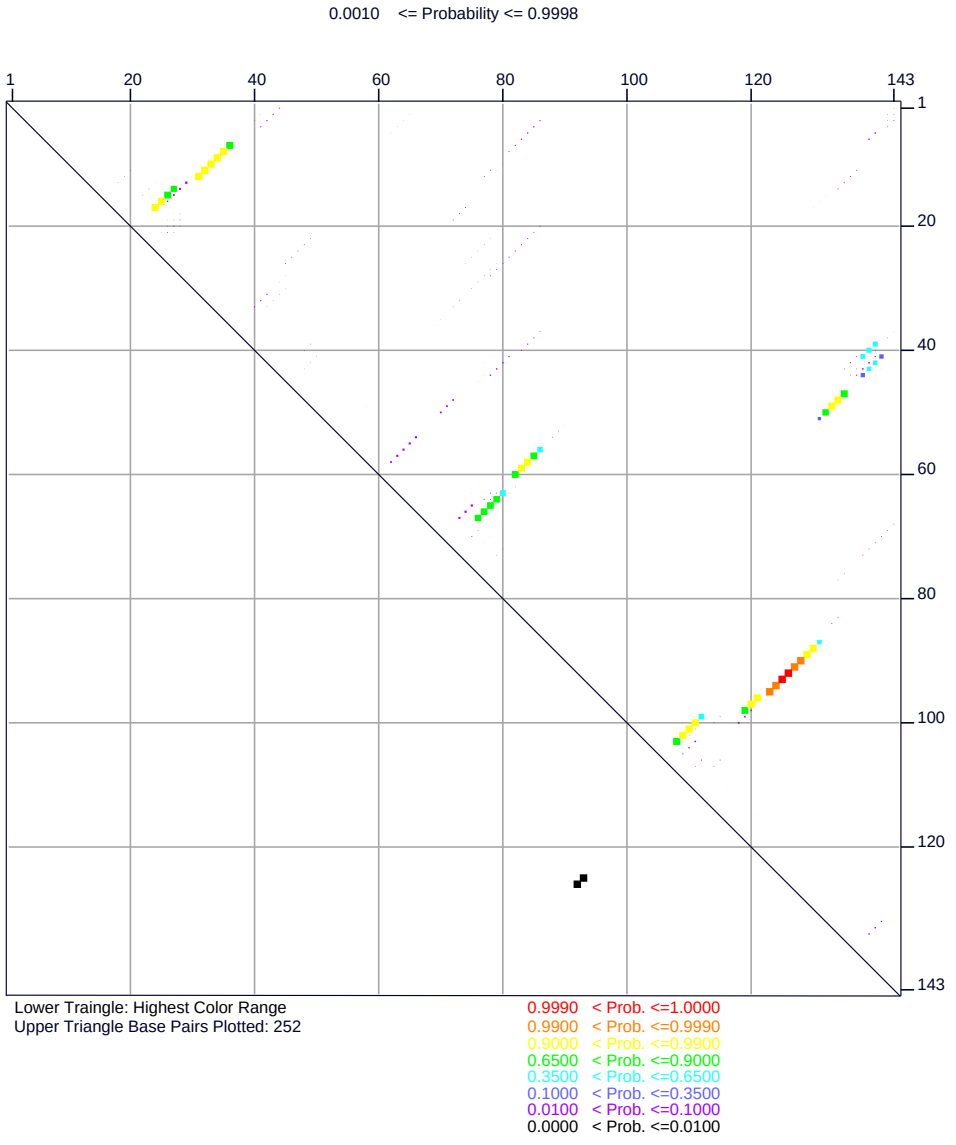
Trypanosomes are very ancient and interesting organisms: they are among the earliest branching eukaryotes. Fundamental discoveries have been made in these parasites which have had major impact on what we know about all cells. They also are unique in many respects, an example of the “differently evolved”.

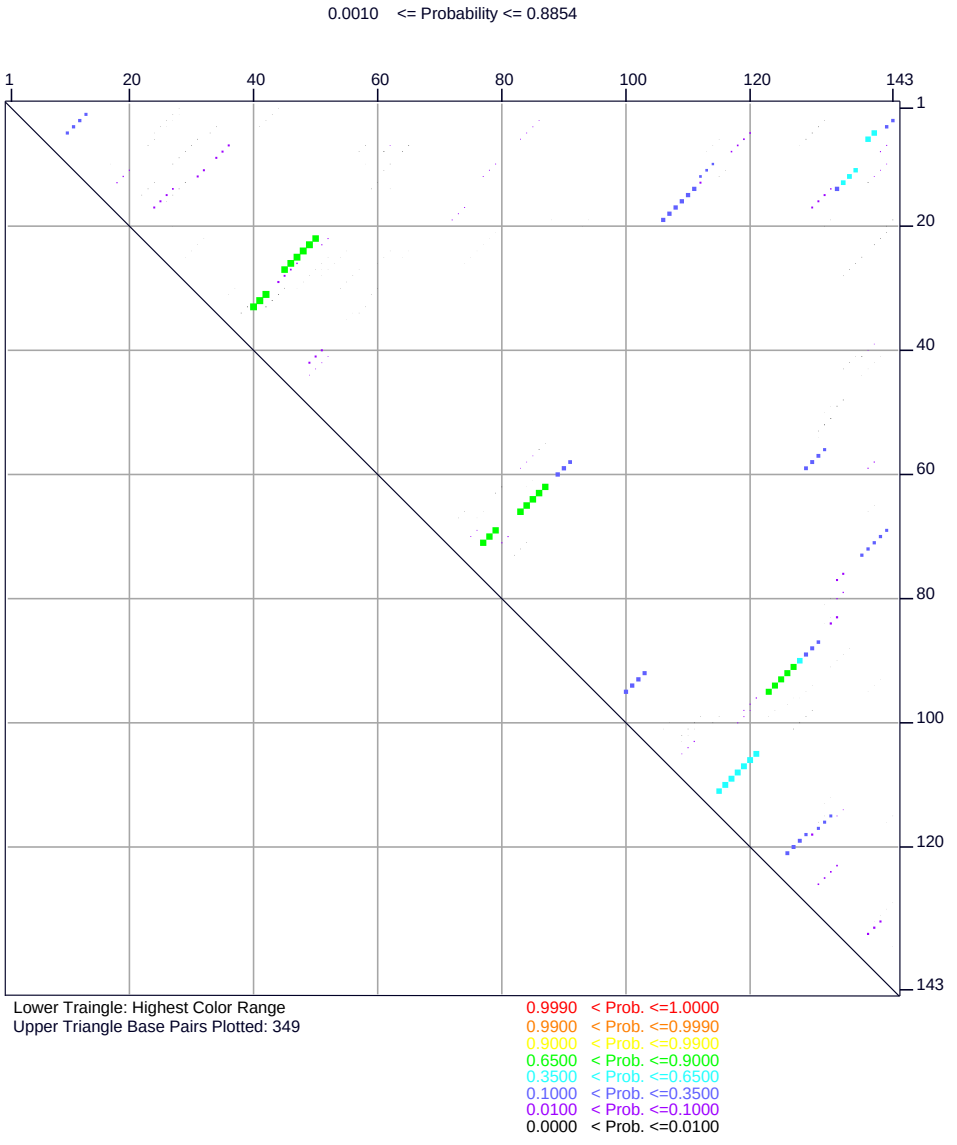
Life cycle: Different forms in insect and mammalian hosts.

Tyrpanosomes are ugly (nasty) protists. Their biology is very odd (RNA editing). It is not surprising that they are “ugly” in terms of RNA folding.

Problems II - The Ugly

The U3 RNAs from *L. collosoma* and *T. brucei* are problematic. Their entropy is high, but the problem is that they contradict each other.





Amastin mRNA $3'$-UTRs

Amastin surface proteins belong to a large family of developmentally regulated proteins comprising up to 45 members that have recently been discovered in the genus *Leishmania* and are highly similar to the amastin proteins in *Trypanosoma cruzi*.

Pfam Family: Amastin (PF07344) Summary: Amastin surface glycoprotein

This family contains the eukaryotic surface glycoprotein amastin (approximately 180 residues long). In Trypanosoma cruzi, amastin is particularly abundant during the amastigote stage.

Reference: Teixeira SM, Russell DG, Kirchhoff LV, Donelson JE; , *J Biol Chem* 1994;**269**:20509-20516.: A differentially expressed gene family encoding "amastin," a surface protein of *Trypanosoma cruzi* amastigotes. PUBMED:8051148

Amastin species tree

```
# Species tree for PF07344
# Generated from Pfam version 22.0
   |
   +--Eukaryota (58)
      +--Euglenozoa (58)
         +--Kinetoplastida (58)
            +--Trypanosomatidae (58)
               +--Trypanosoma brucei (2)
               +--Leishmania mexicana (1)
               +--Leishmania amazonensis (1)
               +--Leishmania (1)
               |  +--Leishmania braziliensis (1)
               +--Leishmania major (39)
               +--Trypanosoma (13)
               |  +--Trypanosoma cruzi (13)
               +--Leishmania donovani infantum (1)
```

Goal: Look for a common RNA structure in the $3'$-UTRs of 60 amastin mRNAs that would be involved in expression control.

Very ugly – Amastin mRNA $3'$-UTRs

Facts:

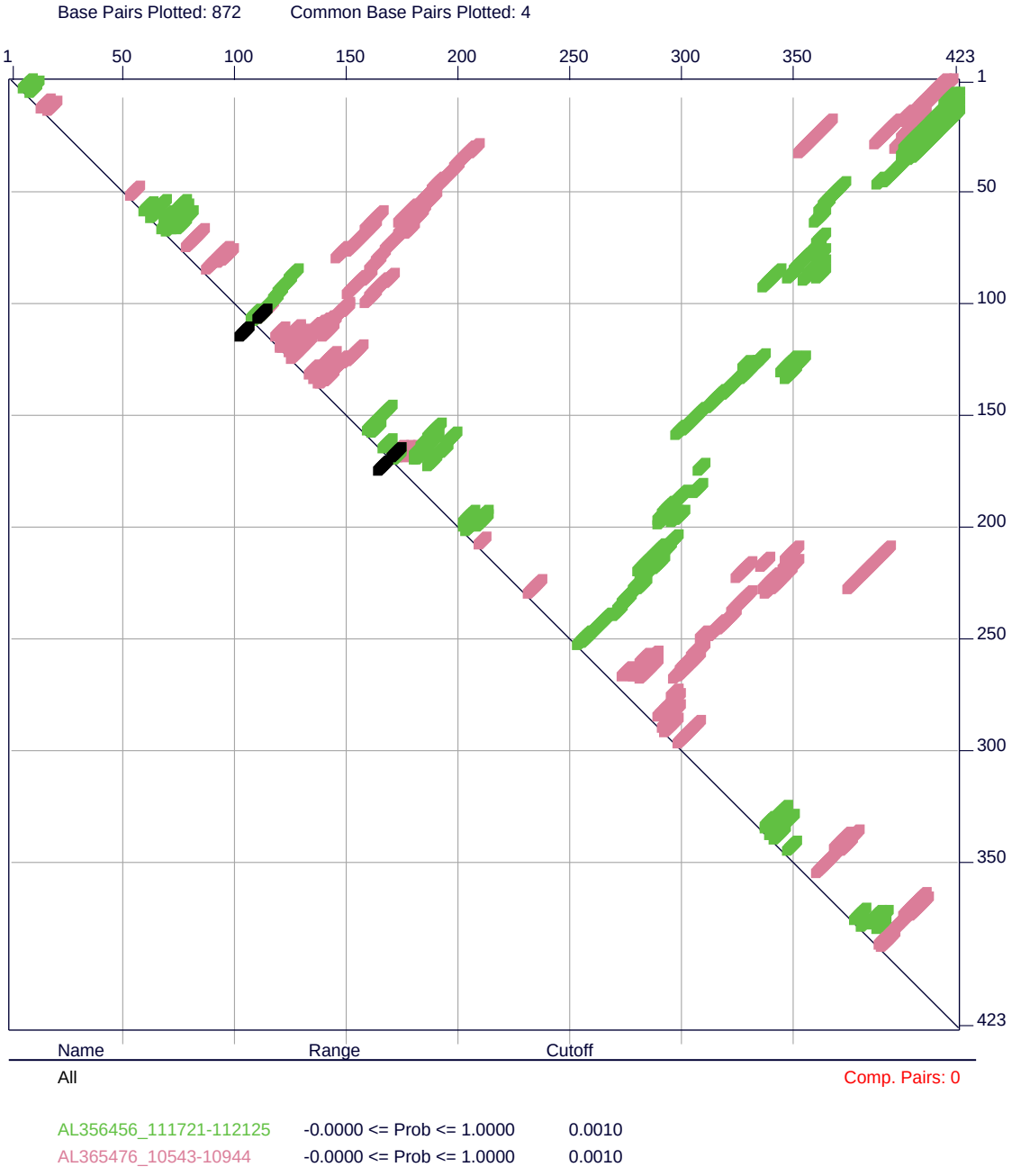
- The 60 UTRs cannot be aligned in any meaningful way.
- As with the Trypanosome U3 RNAs, the PDPs are very dissimilar.
- Worse, some of the UTRs have very low entropy, and still contradict one another.

Right: Alignment of the two lowest entropy amastin UTRs.

```
CLUSTAL W (1.83) multiple sequence alignment

AL356456_111721-112125      CG-AUCCGCUCAGACGCCCAUGCGCAUACACACCAUGCCACGGCAUACCG
AL365476_10543-10944        CGUGUGUGCGCAGAGCAUGCGGCGUCUCCUGCGUCCGACCCGGUGGGCUG
                            **  *  ** ****       ***  * *       * * ***    * *

AL356456_111721-112125      CGGCCUGAAGCGACUGAGAGC--GAAGCGUGGCAUGCGAGCAGAGCGCAG
AL365476_10543-10944        AUAUCAUGAGGGCACGGGUGCCGGAAGCAUGGCAUACGGCCGGGUUGCAC
                                *   ** *   * * **  ***** ****** **  * *   ***

AL356456_111721-112125      GGUCUCGGAG-GGCCCUGGCCUGCGGCUGGCCAACUGCCUCCCUGAGCUC
AL365476_10543-10944        GGGCUCAGAAAGGCUCUCACCGGAGGCUGGCGGACUUGUUCCUCGAGCUC
                            ** *** **  *** **  ** * *******  ***   ***  ******

AL356456_111721-112125      AUGUGGCACGCACCUGGGUCACC--------CCCUGCUGCCC-GCGCGAC
AL365476_10543-10944        AUCCGGCACGCGCCUGGACCACCACCACCACCUCUGUUACCCCGCAGGGC
                            **  ******* *****  ****        * *** * *** **  * *

AL356456_111721-112125      AGACGUCACCGCCACCGCACCGGAGCAGGGCUCUG-GGCGCUGCCCGCCU
AL365476_10543-10944        GGACGCCACCGCCACCGCCCUCGAGCAGAGCCCUGUGACGCCGACCGC--
                             **** ************ *  ****** ** *** * *** * ****

AL356456_111721-112125      GGAUCAGGGCGUGCAGUCCACCCAGGCCCGCGUGGUGCCGGCGGGGCAGC
AL365476_10543-10944        ---GCAGCACGUACCG------GAGGAAGGGAUGG-GCAGGUGCUGUUGG
                                ***  *** * *       ***   *  *** ** ** *  *  *

AL356456_111721-112125      ACACAUGGGAAGCGGGGACGGCGGAAGGGCAGGACGGGCUCGUCCUAGGC
AL365476_10543-10944        GCGCGUGGCG---CGGUUCGGCGGCAGGGC------GGCUCGUUCUCGGC
                             * * ***      **  ****** *****      ******* ** ***

AL356456_111721-112125      GGCCACGA--UGCAGCCGUGGUUCAACAGAAG--UAGAUGAGCGGUGGUU
AL365476_10543-10944        GACCAGGAGAUGUCGCCGCCGUGCGGCCAGAGGGUGGGGGGCGCUGUGGUU
                            * *** **  **  **** ** *  *   **  * *  * ** ******

AL356456_111721-112125      GUGUGUGUGUGUGUGUGUGUG
AL365476_10543-10944        GCGGCGGCGUGUAUACGCUGUUG
                            * *   * **** *  *    **
```

Complot: Dot plot superposition using alignment

As ugly as it can be. **NO common base pairs with probabilities** ≥ 0.001**!**, with the exception of four nonsense pairs (in black). This occurs despite the fact that **both** *Leishmania major* $3'$-UTRs have significantly low entropy.



Rfam - Database of aligned RNAs with consensus structure

The Rfam database, at
`www.sanger.ac.uk/Software/Rfam/`,
and at `rfam.janelia.org/`
is a significant and growing web resource.

- Currently[2], 1371 families. Each family has a *seed* file as well as a full alignment file.
- The purpose of the database is to use families to search genomic data for similar sequences.
- Many entries contain little or no mutual information to support consensus structures annotated in the alignment files.

A total of 14654 sequences from the 607 Rfam seed files (Version 8.1, October 2007) were processed.

[2] Version 9.1, December 2008

Some remarks on Rfam

The use of single sequence entropy calculations on Rfam RNAs might identify families for which the alignment needs to be improved, or families that might better be split into two (or more) groups.

Low entropy foldings might be useful in generating an initial folding for a family that could then be refined as needed using the other members together with a multiple sequence alignment.

This method fails badly with the amastin $3'$-UTRs. It works in other cases.

Acknowledgments

- NIGMS: Algorithm and software development support.
- Doug Turner, Dave Mathews *et al.* RNA energy parameters and algorithm development
- HHMI, Sean Eddy: Sabbatical support.
- Darin Stewart, Alex Yu, Nick Markham: mfold_util display software
- Nick Markham: UNAFold software
- Rfam consortium (Sean Eddy, Alex Bateman *et al.*): Rfam database and amastins
- Jim Brown: RNAse P RNA database
- Robin Gutell: group I intron database
- Sprinzl, Szymanski, Zimmerly already mentioned
- many others