

RNA STRUCTURE ANALYSIS VIA THE RIGID BLOCK MODEL

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ABSTRACT OF THE DISSERTATION

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RNA structure is at the forefront of our understanding of the origin of life, and the mechanisms of life regulation and control. RNA plays a primordial role in some viruses. Our knowledge of the importance of RNA in cellular regulation is relatively new, and this knowledge, along with the detailed structural elucidation of the transcription machine, the ribosome, has propelled interest in understanding RNA to a level which starts to closely resemble that given to proteins and DNA.

In the process of progressively understanding the landscape of functionality of such a complex polymer as RNA, one practical task left to the structural chemist is to understand the details of how structure relates to large-scale polymer processes. With this in mind the fundamental problems which fuel the work described in this thesis are those of the conformations which RNA's assume in nature, and the aim to understand how RNA folds.

The RNA folding problem can be understood as a mechanical problem. Therefore efforts to determine its solution are not foreign to the use of statistical mechanical methods combined with detailed knowledge of atomic level structure. Such methodology is mainly used in this work in a long-term effort to understand the intrinsic structural features of RNA, and how they might relate to its folding.

As a thing among things, each thing is equally insignificant; as a world each one equally significant.

If I have been contemplating the stove, and then am told; but now all you know is the stove, my result does indeed sound trivial. For this represents the matter as if I had studied the stove as one among the many, many things in the world. But if I was contemplating the stove, it was my world, and everything else colorless by contrast with it ...

For it is equally possible to take the bare present image as the worthless momentary picture in the whole temporal world, and as the true world among shadows.

Ludwig Wittgenstein

As a molecule among molecules, each molecule is equally insignificant; as a world each one equally significant.

If I have been contemplating RNA, and then am told; but now all you know is RNA, my result does indeed sound trivial. For this represents the matter as if I had studied RNA as one among the many, many molecules in the world. But if I was contemplating RNA, it was my world, and everything else colorless by contrast with it ...

For it is equally possible to take the bare present image as the worthless momentary picture in the whole temporal world, and as the true world among shadows.

Anonymous Chemist

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Chapter 1

Introduction

RNA plays a primordial role in life, and perhaps also in the early history of its origins [1, 2, 3, 4]. In Biology RNA is a central player in the transcription and translation steps of what is known as its central dogma, i.e., DNA makes RNA (via transcription) and RNA makes protein (during translation). In the last decade of the twentieth century Fire and Mello [5] found that RNA also plays a role previously thought to be the job of proteins. That is, RNA can regulate translation using non-coding RNA's (ncRNA's). Another fundamental discovery about RNA came in 2000 with the elucidation at atomic level detail of a non-coding RNA, the ribosome [6, 7, 8].

Since its very beginnings, structural understanding of RNA has proven to be a very complex problem. It was not until 1956, three years after the famous Nature triad of papers by Watson and Crick, Wilkins, Stoke, and Wilson, and Franklin and Gosling [9, 10, 11] on the double-stranded structure of DNA, that Alex Rich and David Davies were able to produce double-stranded RNA from polyriboadenylic acid (poly-rA) and polyribouridylic acid (poly-rU) to produce a neatly difracting X-ray pattern typical of a double-helical structure. It was not until 1965 that Robert Holley was able to obtain the complete sequence of yeast Alanine tRNA, and also its secondary structure from cleavage of the whole structure into smaller frgments, and it was only in 1973, that the first complex, but small, tRNA structure, was solved at full atomic detail. Fifty seven years have passed since the description of the double-helical structure of DNA, but still RNA faces more challenges with the possibility of finding a whole new zoo of non-coding RNA structures [12], and the possibility of new engineered ones [13].

1.1 RNA chemistry

RNA is a poly-nucleotide chain, that is, a polymer whose monomeric unit is the nucleotide. The nucleotide unit is composed of three chemically distinct entities; base, sugar, and backbone. The bases can be of two types, purines (R), i.e. adenine (A) and guanine (G), and pyrimidines (Y), i.e. cytosine (C) and uracyl (U) as shown in Figure 1.1.

Heterocyclic bases can form a diversity of base-pairs through hydrogen bonding and can be classified in 28 classes as suggested by Saenger [16], and as seen in Figure 1.2. Nomenclatures which conform to Saenger's classes have been developed by Lee-Gutell [17], Leontis-Westhof [18], and Lemieux-Major [19].

The other non-covalent interactions which are common to the nucleotide bases are those of stacking through London dispersion forces and electrostatic interactions. It has been hypothesized that π electron interactions could also account for stacking, but very precise quantum calculations [20, 21] have show otherwise thus far.

Sugar, and backbone can adopt a variety of conformations constrained to the values of their torsion angles. In the case of the sugar these torsion angles are called puckers, perhaps in analogy to the gestures made by human lips. The prefered sugar pucker in RNA is called $C_{3'-endo}$, but in cases where an intercalated base is acommodated between two sequential bases, the sugar pucker changes to a non-prefered conformation called $C_{2'-endo}$. Standards to describe the conformations resulting from the specific sets of torsion angle values which sugars and backbone can attain have been developed and can be seen in textbooks [16], in the web [22], and in the IUPAC recommendations [15]. We refer the

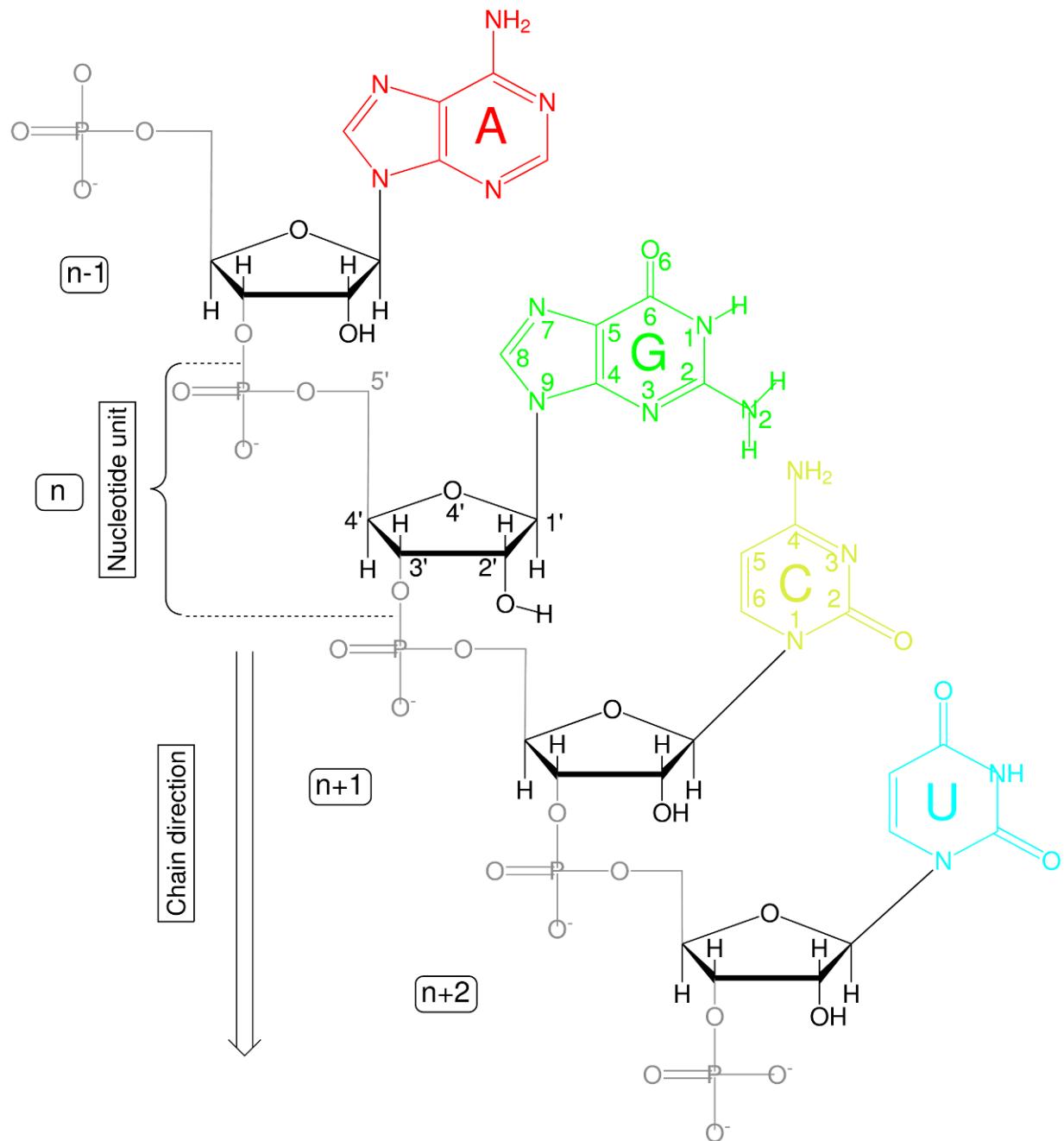


Figure 1.1: A single strand of RNA drawn in the 5' to 3' sense showing the main chemical entities which compose it; base, sugar, and backbone. The four bases (A, G, C, U) are colored according to the NDB (Nucleic Acid Database) convention [14], the backbone is colored gray, and the sugars black. The bases G, and C, and the furanose sugar are numbered according to the IUPAC rules [15]. This figure is a reproduction of Figure 2.1, in Wolfram Saenger's book [16].

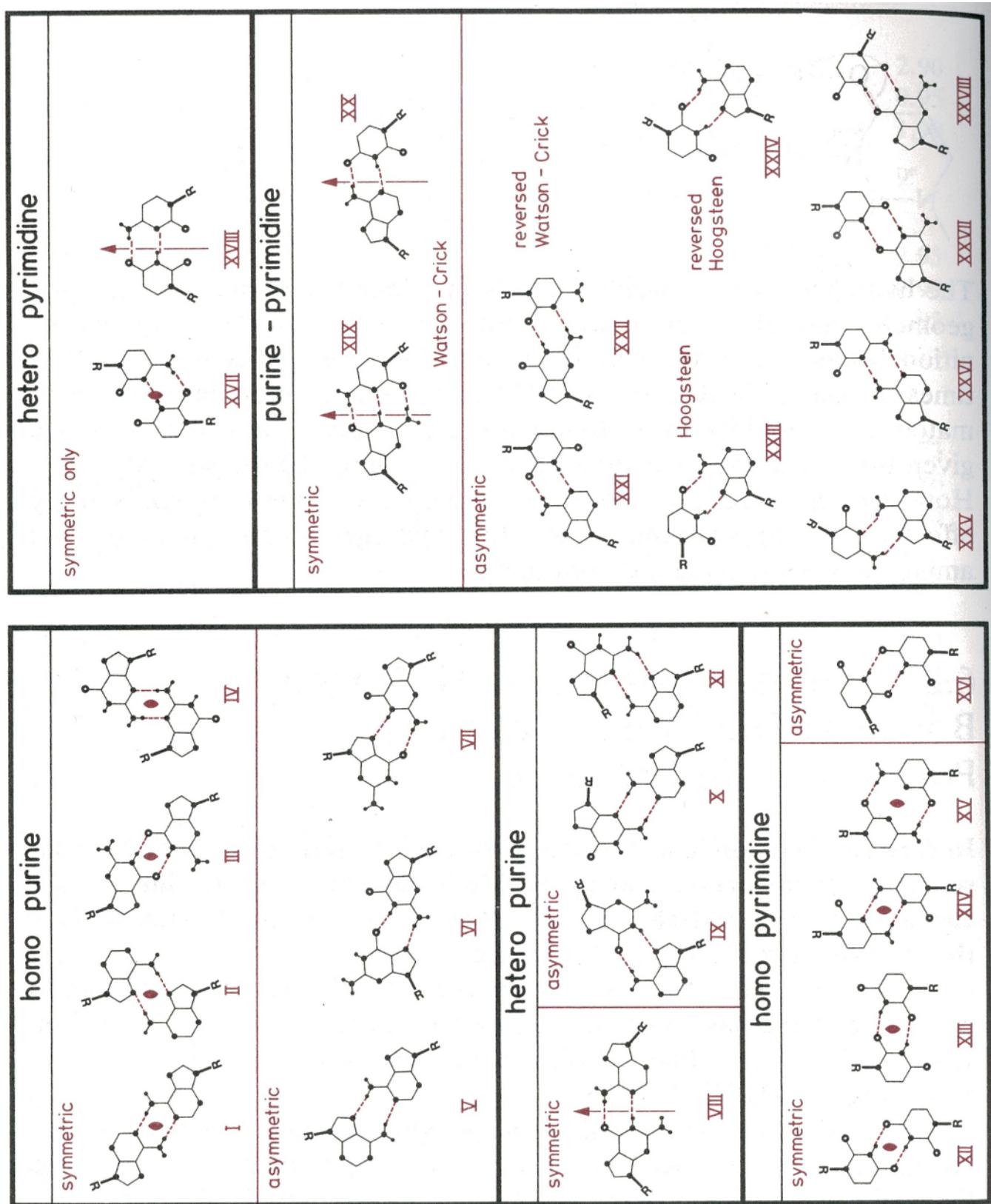


Figure 1.2: Saenger base-pairing classes, reproduced from his book, "Principles of Nucleic Acid Structure". [16].

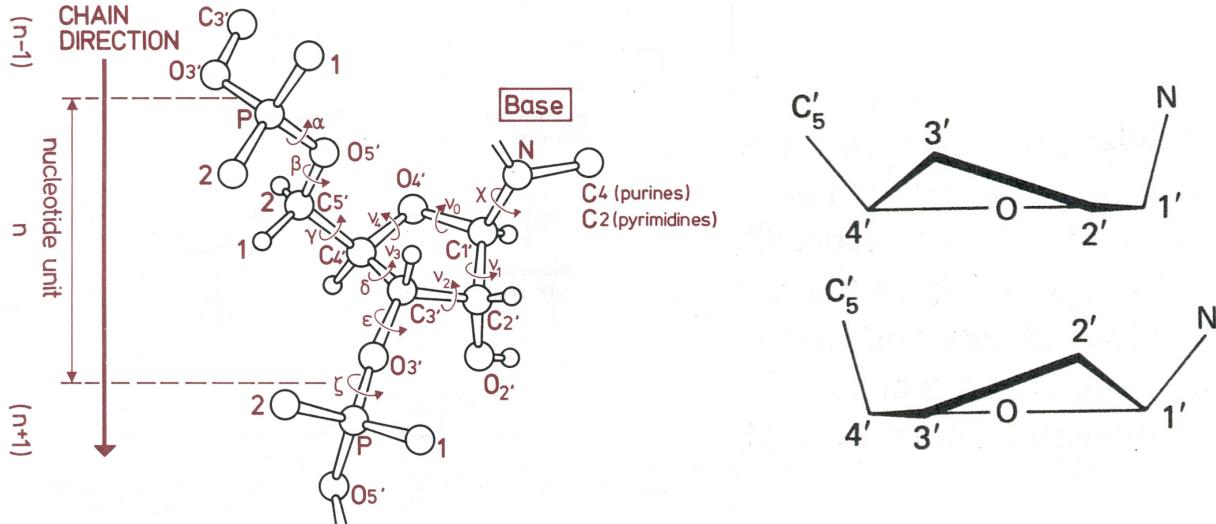


Figure 1.3: **Left:** Backbone and Sugar torsion angles. **Right:** The most common sugar pucker conformations in RNA, that is, C3'endo and C2'endo, reproduced from Wolfram Saenger's, "Principles of Nucleic Acid Structure". [16].

reader to these sources for a more detailed description, and limit ourselves to show in Figure 1.3 a brief description of these torsion angles.

1.2 Standard reference frame and local parameters

For the structural description of RNA the program 3DNA [23] has been used. In 3DNA there are three sets of symmetric local parameters:

1. Base-pair parameters,
2. Base (base-pair) step parameters,
3. Base (base-pair) local helical parameters.

Bases or base pairs are treated as "rigid bodies" using ideas from classical mechanics. The first two sets of parameters are based on Cartesian coordinates, whereas the third set of helical coordinates, resembles cylindrical coordinates and is based on Chasles's theorem [24].

1.2.1 Base-pair and base-step parameters

In 3DNA one starts with a Protein Data Bank (PDB) [25] file which is usually based on experimental informationⁱ and which can be downloaded from the NDB or PDB. This file contains the experimentally obtained Cartesian coordinates of the atoms. With this experimental data one performs a least-squares fit to a standard reference frame [26]. This can be done using the octave script at <http://rutchem.rutgers.edu/~{}esguerra/RNA/scripts.html> as a tutorial example. The coordinate origin which is embedded in the standard reference frame is kept and used for both base and base pairs. In the case of single unpaired bases, the program keeps the origin of one base of an ideal Watson-Crick pair. The definition of this frame is illustrated in Figure 1.4

ⁱThis is the most common case but the PDB file is sometimes the result of theoretical modeling.

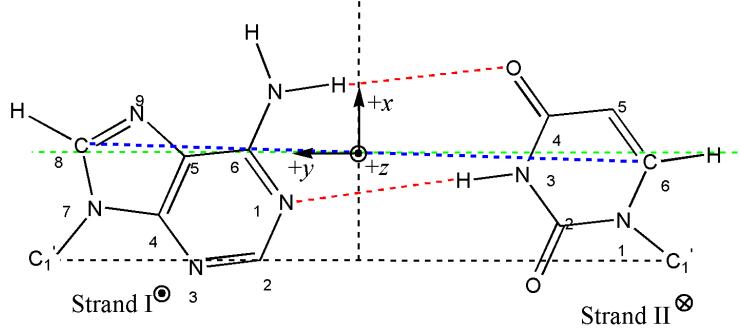


Figure 1.4: Standard reference frame of an A-T base-pair. The y -axis (dashed green line) is chosen to be parallel to the line connecting the $C1'$ of adenine and the $C1'$ of thymine associated in an ideal Watson-Crick base-pair. The x -axis is the perpendicular bisector of the $C1' - C1'$ line, and the origin is located at the intersection of the x -axis and the line connecting the $C8$ atom of adenine and the $C6$ atom of thymine. The z -axis is the cross product of the \hat{x} and \hat{y} unit vectors.

Once one has determined the coordinate origins for the two consecutive bases or base pairs comprising a step one defines a middle step triad (MST) [27]. This can be described by the following procedure:

1) Find the angle Γ between consecutive normals, *i.e.*, z -axis. Since these are unit vectors, the angle is defined by the dot product:

$$\Gamma = \cos^{-1}(\hat{z}_i \cdot \hat{z}_{i+1}) \quad (1.1)$$

2) Then find the vector which is perpendicular to the two normals (z -axis). This vector is obtained from the cross product of the consecutive z -axis (that is, the normal to the plane formed by the two vectors). This axis is called the roll-tilt axis and is normalized to form the unit vector \hat{r}_t ,

$$\hat{r}_t = \frac{\hat{z}_i \times \hat{z}_{i+1}}{|\hat{z}_i \times \hat{z}_{i+1}|} \quad (1.2)$$

3) To make consecutive z vectors coincide, one uses a linear homogeneous transformation $R(\theta)$ about the roll-tilt axis such that the original orientation matrices T_i and T_{i+1} are rotated by $\theta = \pm\Gamma/2$ to yield the transformed T'_i and T'_{i+1} orientation matrices.

$$T'_i = R_{rt}(\pm\Gamma/2)T_i \quad (1.3)$$

$$T'_{i+1} = R_{rt}(\mp\Gamma/2)T_{i+1} \quad (1.4)$$

The origin for the middle step triad is the average of the position vectors for the i and $i+1$ reference frames,

$$r_{MST} = \frac{(r_i + r_{i+1})}{2} \quad (1.5)$$

4) Again using the dot product one can find the angle between the transformed \hat{y}' vectors. This angle is equal to the magnitude of the Twist (Ω). The dot product of the \hat{z}_{MST} unit vector with the vector

resulting from the cross product of \hat{y}'_i and \hat{y}'_{i+1} gives the sign of Ω . Since the transformed x-y plane is orthogonal to \hat{z} then this applies in the same manner for x ,

$$\Omega = \cos^{-1}(\hat{y}'_i \cdot \hat{y}'_{i+1}) \quad (1.6)$$

$$(\hat{y}'_i \times \hat{y}'_{i+1}) \cdot \hat{z}_{MST} > 0, \quad \text{then } \Omega > 0 \quad (1.7)$$

$$(\hat{y}'_i \times \hat{y}'_{i+1}) \cdot \hat{z}_{MST} < 0, \quad \text{then } \Omega < 0 \quad (1.8)$$

5) With more scalar product one can find other angles, such as the phase angle ϕ ,

$$\phi = \cos^{-1}(\hat{r}t \cdot \hat{y}_{MST}) \quad (1.9)$$

$$(\hat{r}t \times \hat{y}_{MST}) \cdot \hat{z}_{MST} > 0, \quad \text{then } 180 \geq \phi \geq 0 \quad (1.10)$$

$$(\hat{r}t \times \hat{y}_{MST}) \cdot \hat{z}_{MST} < 0, \quad \text{then } -180 \leq \phi \leq 0 \quad (1.11)$$

6) The roll ρ and tilt τ angles, which are the remaining angular degrees of freedom for step parameters, are defined in terms of the bending angle and the phase angle:

$$\rho = \Gamma \cos(\phi) \quad (1.12)$$

$$\tau = \Gamma \sin(\phi) \quad (1.13)$$

Now to get the remaining three translational degrees of freedom for step parameters (D_x, D_y, D_z) one just needs to express the displacement vector in the middle step triad frame:

$$[D_x D_y D_z] = T_{MST}(r_{i+1} - r_i) \quad (1.14)$$

The procedure is completely analogous to compute the base-pair parameters. The opening ω , buckle κ , and propeller σ are the analogs of twist Ω , roll ρ , and tilt τ , and the middle step triad is called middle base triad MBT. The axis which are made to coincide are the y -axis and not the z -axis as in the base-pair step case [27].

The parameters obtained by this procedure are depicted graphically in Figure 1.5.

1.2.2 Local helical parameters

Local helical parameters are determined using Chasles's theorem, which states [24]:

“One can always transport a free rigid body from one position and orientation to another position and orientation by a single continuous motion along a unique axis of rotation.”

For the three dimensional case of nucleic acid base steps what this means is that, instead of rotating around one reference-frame centered axis and then translating along another reference-frame centered axis, one rotates about and also translates along only one common axis, which is not reference-frame centered. This allows one to define the orientation of a helical axis (or unique rotational-translational axis) as a unit vector given by equation 2.15:

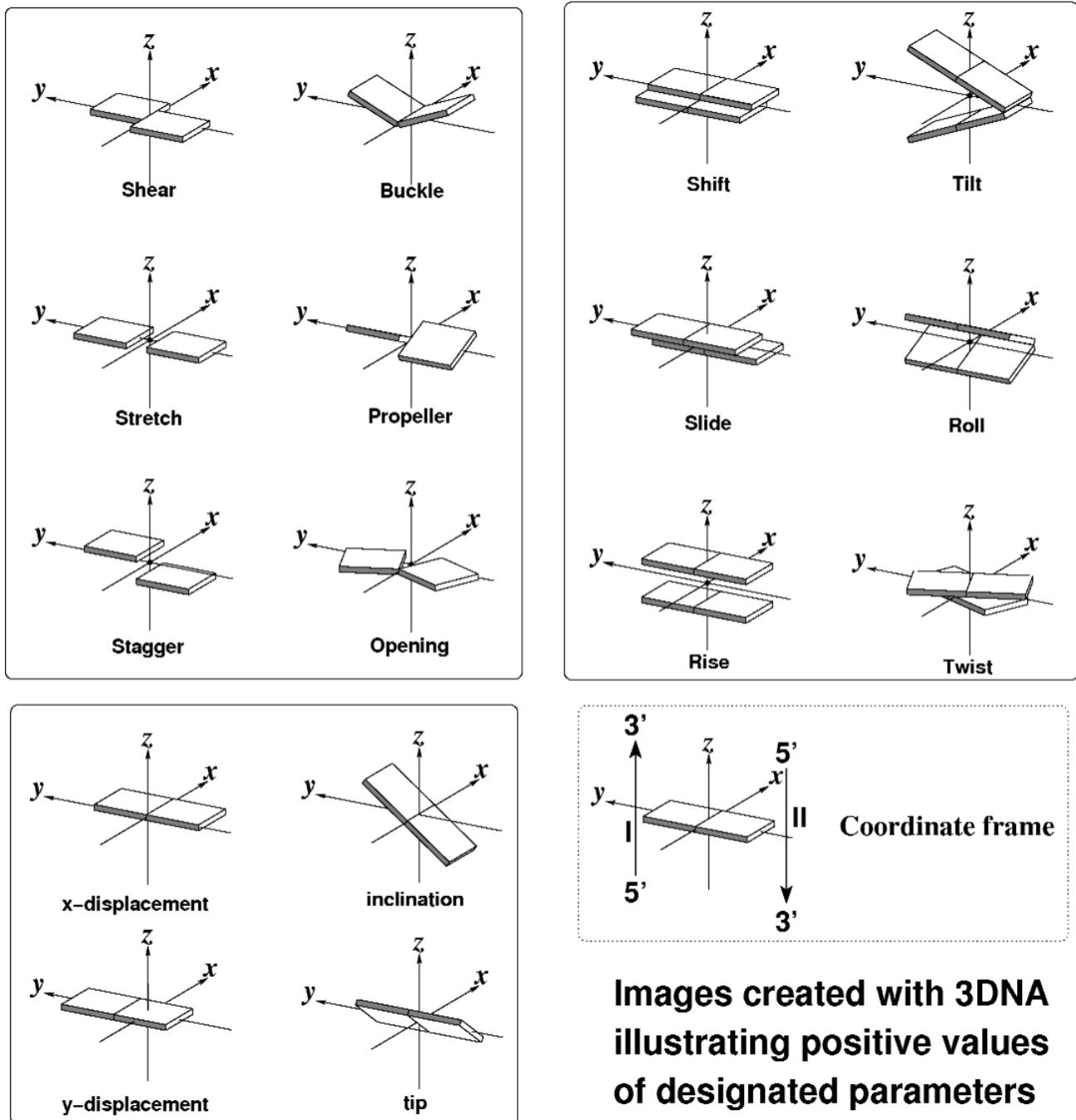


Figure 1.5: Illustration of base pair and base step parameters [23]

$$h = \begin{bmatrix} h_x \\ h_y \\ h_z \end{bmatrix} \quad (1.15)$$

where:

$$h_x = \frac{\tau}{\Omega_h}, \quad h_y = \frac{\rho}{\Omega_h}, \quad h_z = \frac{\Omega}{\Omega_h} \quad (1.16)$$

$$\Omega_h = \sqrt{\tau^2 + \rho^2 + \Omega^2} \quad (1.17)$$

The local helical axis can be defined alternatively [28] as a cross product:

$$h = (x_2 - x_1) \times (y_2 - y_1) \quad (1.18)$$

where the x and y refer to the reference frames on base pairs 1 and 2.

1.3 RNA folding

The first high-resolution X-ray structure of RNA larger than a dinucleotide was that of yeast tRNA^{Phe} at 3 Å in 1974 [29, 30, 31]. Thirty six years later there are two orders of magnitude more structural information about RNA [32], and new information from non-coding RNA's is expected [12]. This fact and the discovery of ribozymes [33, 34], which are catalytic RNA molecules, has renewed interest in solving the RNA folding problem, that is, starting from the primary sequence, finding in an automatedⁱⁱ way the native three-dimensional structure of an RNA molecule and the folding pathway that it follows. The RNA folding problem is usually seen as analogous to the protein folding problem, due both to the discovery of the enzymatic behavior of RNA [33, 34] and the complicated folding of large RNA molecules [38]. To take advantage of this analogy, a unified conceptual framework for describing RNA and protein folding, called the kinetic partitioning mechanism (KPM), has been developed by Thirumalai and Hyeon [39]. This and other methods are based on defining an adequate partition function for describing the correct conformational ensemble of folded, partially folded, and unfolded structures [40, 41, 42] of either protein or RNA.

1.4 Is RNA folding a hard or easy problem?

There are two trains of thought regarding the mechanism of RNA folding. One states that RNA folding is less complex than protein folding [43] because RNA is made up of a four letter alphabet of similar nucleotide units instead of a 20 letter alphabet of dissimilar amino acids. Therefore the number of possible sequential combinations is smaller. It is also well known that secondary and tertiary interactions can be separated in the case of RNA by the absence or presence of Mg²⁺ [44] (see Figure 1.6), and that the secondary structure motifs of RNA are more limited in number than those of protein, whereas secondary and tertiary elements are not as easily separable in proteins. The other point of view says

ⁱⁱThe term automated is used here to mean a theoretical model of tertiary folding, which could use experimental measures of secondary structure association in the same way that the traditional secondary structure folding model [35, 36] uses the Tinoco-Uhlenbeck dinucleotide postulate [37] to find total free energies.

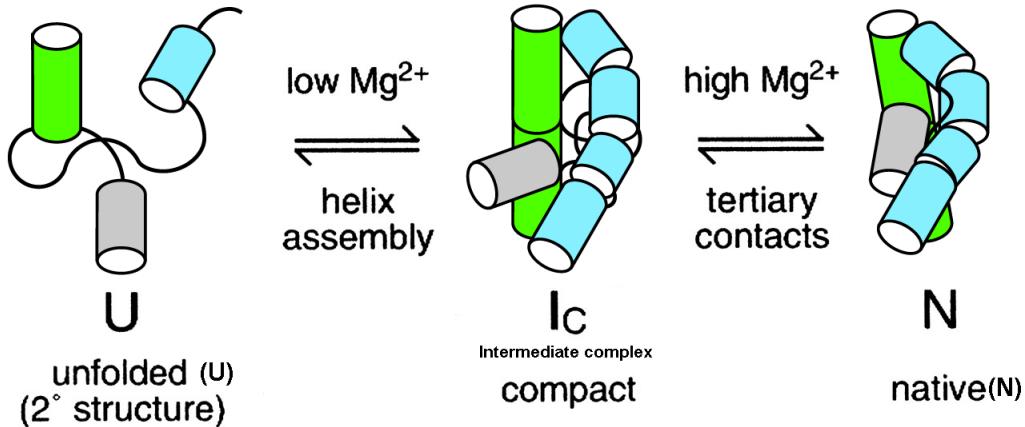


Figure 1.6: Separation of secondary and tertiary interaction in RNA [44]. Double helical secondary structure represented by individual cylinders and tertiary interactions by association of cylinders. Color coding stands for separate helical regions of RNA, and the connecting black strings represent single stranded loop structures.

that RNA folding can be at least as complex as protein folding [45, 46] since there is no such thing as hydrophobic burial of regions of RNA as in the case of proteins. Instead, the electrostatic problem stemming from a complex charged backbone must be dealt with in the case of RNA. For instance, the interactions of the RNA polyanionic backbone with water and cations [47] are not easily simulated with explicit solvent models like those used to treat proteins. The aforementioned interactions of RNA need to be modeled implicitly, and must aim to describe long dynamic processes of the order of seconds to minutes, in contrast to the typical time scales of tens of microseconds associated with protein folding.

Although secondary and tertiary structure can be separated experimentally, there have been few theoretical efforts to account for the folding of RNA from a random sequence of nucleotides into secondary structures and tertiary structures. What little is known has been investigated at low resolution. Stephen Harvey and associates have simulated the folding of yeast tRNA^{Phe}, [48] and the assembly of the 30S subunit of the ribosome [49] at various levels of detail, initially using only one pseudoatom per helical region, and later one pseudoatom per nucleotide. Recently François Major's group at Montreal has proposed a pipeline of two computer algorithms to study RNA structure [50]. One pipeline makes secondary structure predictions, and the other assembles 3D structures based on the best scoring secondary structures. By contrast, in the case of proteins many groups have simulated the transition from secondary to tertiary structure, including some calculations which account for the strong coupling of secondary and tertiary structure [51, 52, 53]. This type of work is often referred to as protein structural topology and there is no counterpart for RNA.

1.5 Experimental folding techniques

Traditionally RNA folding and unfolding have been followed calorimetrically and spectroscopically as a function of temperature and cation concentration [54, 55]. While this approach works well for studying two-state folders, *i.e.*, structures which populate only two states (native and melted), in general RNA's are not two-state folders. RNA seems to go through a rugged free energy landscape of conformations in the process of folding [56]. The experimental solution to this problem is offered by single-molecule techniques like fluorescence resonance energy transfer (FRET) and mechanical micromanipulation, in which the ends of RNA are attached to micron sized beads which are then pulled apart and monitored with a laser light trap [57, 58, 59, 60]. In the case of single-molecule force-induced unfolding, state transitions often occur under non-equilibrium conditions, thereby making it difficult to extract equilibrium

information from the data. Bustamante, Tinoco, and associates have shown that by using the Crooks fluctuation theorem [61], one can deal with such cases and extract RNA folding free energies from single-molecule experiments [62]. Recently an alternative solution to this problem has been proposed by Thirumalai and associates based on single-molecule force-quenching experiments, by using a so called de Genes "expanding sausage model" [63].

1.6 RNA simulations

Network and molecular mechanics-molecular dynamics (MM-MD) methods provide useful information relevant to the RNA folding-unfolding problem, especially for describing fluctuations away from the native conformation. Gaussian network models [64, 65, 66], which treat RNA at less than atomic detail, have been used to describe the motions of large RNA structures like the ribosome. Examples of the predicted normal modes of motion of the ribosome can be seen at: <http://ribosome.bb.iastate.edu/70SnK> mode. Using MM, Sanbonmatsu and coworkers obtained a static atomic model of the 70S ribosome structure through homology modeling [67]. Tung and associates used this structure for an all-atom MD simulation of the movement of tRNA into a fluctuating ribosome [68]. This type of simulation might be useful in a reverse-folding approach to the RNA folding problem. To the best of our knowledge, such calculations haven't as yet been done for RNA.

1.6.1 Local nucleotide interactions

The molecular interactions that rule RNA structures at the nucleic acid base level, *i.e.*, local level, are hydrogen bonding and stacking interactions. The former are related to base pairing and the latter, in most cases, to nucleotide steps. These interactions can be explored theoretically at various levels. At the highest level are ab-initio quantum mechanical calculations which are still too expensive for systems as large as hundreds of atoms. Such calculations, nevertheless, can tell a great deal about local electronic behavior. For example, Hobza and collaborators have found that the stacking interaction of free nucleotide bases is determined by dispersion attraction, short-range exchange repulsion, and electrostatic interaction. No specific $\pi - \pi$ interactions are found from electron correlated ab-initio calculations [20, 21]. This is why force field methods have been so successful in the study of nucleic acids, since the empirical potentials used in such studies mimic well the quantum mechanically obtained energy profiles [67, 69]. A currently debated ab-initio finding is whether small fluctuations in the configurations of neighboring base pairs (dimers) are iso-energetic or not. Recent calculations of Sponer and Hobza [70] seem to contradict their earlier work [69, 71], in which the stacking energies were reported to be relatively insensitive to dimer conformation. The new results use the so-called "coupled cluster singles doubles with triple electron excitations" CCSD(T) method, to account for electron correlation. Using this electron correlation energy correction, the stacking energy differences between dimer conformations turn out to be considerably higher than previously reported.

Single-strand and double-strand stacking free energies can be obtained calorimetrically [72]. One of the most popular methods used for obtaining such quantities is differential scanning calorimetry (DSC) [73]. These measurements show favorable dinucleotide stacking free energies as large as -3.6 kcal/mol for double-strand stacking. Experimentally, the magnitudes of these interactions are found to be sequence-dependent [54]. In fact, the stacking free energies for some sequencesⁱⁱⁱ are found to be negligible. Thus there may be no accountable stacking interaction at all for some sequences.

Besides taking into account the effects of stacking and hydrogen bonding, it is important to think

ⁱⁱⁱFree Energies for 5' unpaired nucleotides (e.g. UC/A UU/A) are quite small (*i.e.* < 0.4 kcal/mol) and are termed weakly stacking bases.[74, 75]

at the same time about the polyelectrolyte nature of the RNA backbone. Manning's counterion condensation theory [76, 77] provides a simple and quantitative picture of the interactions of a regular double-helical nucleic acid polyanion with its counterions, but it does not take into account the discrete nature of charge [54] or the folding of RNA. Poisson-Boltzmann theory offers a more detailed picture of the behavior of charged macroions in solution [78, 79].

The local conformational space of RNA has been studied using a large set of available RNA structures from the Nucleic Acid Database (NDB) [80]. The torsion angles of the nucleotide steps have been clustered using different techniques [81, 82]. The root-mean-square deviations (RMSD) of the distances between closely spaced atoms in the phosphates, sugars, and bases, have also been clustered [83]. The latter studies are aimed at finding the common nucleotide base steps and base-pair building blocks which have been given the name of RNA doublets. Recently, the RNA Ontology Consortium (ROC) has proposed a consensus set of RNA dinucleotide conformers integrating the work of various groups [84].

1.6.2 RNA secondary structure algorithms and the lack of tertiary ones

From secondary structure prediction algorithms like Zuker's *mfold* program [85], Hofacker's Vienna RNA package [36], or Mathews Dynaling software [86], one obtains a large ensemble of secondary structure graphs, i.e. 2D representations of the double-stranded helical stems, hairpin loops, bubbles formed by the constituent bases. These graphs can be analyzed with graph theory to produce a partition function describing a full arrangement of contacts for the total number of possible secondary structures, allowing the construction of a "relation of microscopic conformations to macroscopic properties" [87]. So far this type of model has not been generalized to take into account tertiary structural features, i.e., interhelical interactions of RNA. In the last two to three years a boom in prediction of small (≈ 200 nucleotides) RNA 3D structures has started. Basically three types of approaches are being followed. One is that of using a coarse-grained model, assigning a potential function to it, applying a minimization procedure, and then performing a molecular mechanics (MM) all-atom refinement [88, 89, 90]. Another starts from the predicted secondary structures, assumes that the helical regions adopt the canonical A-form structure, mechanically inserts residues as rigid bodies in the remaining non-helical regions, and finally carry out an MM optimization [91]. The third approach entails a pipeline between secondary structure prediction, and tertiary structure assembly is proposed. This pipeline uses as bridging concept between 2D and 3D structure, the graph theoretical definition of a minimum cycle basis, which for the case of nucleic acids has been renamed as Nucleic Cyclic Motifs (NCM) [50].

1.6.3 RNA overall fold

Whereas in the case of proteins one qualitatively describes the overall fold in terms of the arrangement of secondary structure motifs, i.e., using the helix-ribbon-coil images developed by Jane Richardson [92] (see Figure 1.7), there is still no comparable description of the overall fold of RNA. A ribbon representation of the sugar phosphate backbone (see Figure 1.8) helps to understand the folding of small RNA's, but in the case of the ribosome, a representation at such level of detail does not allow to make sense of such a large structure (close to 3000 nucleotides for the large subunit of the archaeal ribosome). In the past two years Holbrook [93] and Sykes [94] have proposed new representations for RNA based on helical region organization. Holbrook makes an analysis of continuous interhelical strands, so called, COINS, and Sykes makes an optimized projection of 3D helical axis to 2D images, which can later be annotated with, for example, hydroxyl radical footprinting results.

One can envision that a thorough investigation of the space of translational and rotational degrees of freedom of the helical regions of RNA could give clues as to how we might see an overall fold in RNA structures. To the best of our knowledge there is no comparable quantitative description of the folding of proteins.

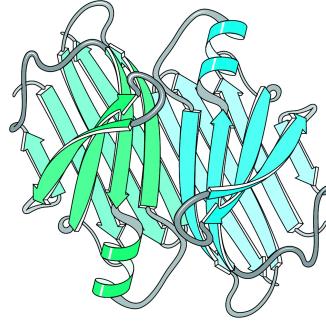


Figure 1.7: Ribbon-coil schematic illustrating the fold and intermolecular units of a dimer of prealbumin (PDB_ID:2pab), or transthyretin, taken from Richardson et al. [95]

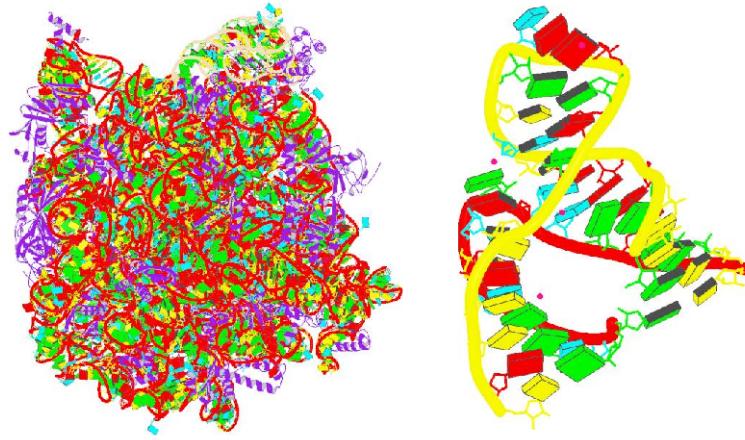


Figure 1.8: Images of the *Haloharcula marismortui*'s large ribosomal subunit NDB_ID:RR0033 (left) and the hammerhead ribozyme (right) NDB_ID:UR0029. The figures were taken directly from the NDB web pages, and show a 3DNA generated [96] ribbon representation of the phosphate backbone, and a block representation for the nucleotide bases. From the figures it's clear that, whereas the ribozyme fold can be clearly understood with this representation, the ribosome fold cannot.

In the case of proteins the SCOP (Structural Classification of Proteins) database [97], classifies proteins, among various qualitative descriptors, according to folds, which are recurrent arrangements of secondary structure, that is, a list of secondary structures with unique topological connections. The SCOR (Structural Classification of RNA) database [98, 99], aims to provide a similar classification to that obtained for proteins, but using RNA motifs instead. This classification focuses on the local folding of small pieces of RNA and cannot describe the overall fold. Local classification is also qualitative rather than quantitative.

1.6.4 RNA motifs

The term “RNA motif” is used in the literature to describe three different levels of RNA organization, namely, **RNA sequence** motifs, **RNA secondary structure** motifs, or **RNA 3D structure** motifs. Because these distinctions are not always clearly made the beginner may result in confused and frustrated bibliographical searches.

The lack of a unique definition of RNA motifs is yet another source of confusion in understanding RNA motifs is the lack of a unique definition. Three popular and somewhat recent definitions of RNA motifs include:

- “*a discrete sequence or combination of base juxtapositions found in naturally occurring RNA’s in unexpectedly high abundance.*”[100]
- “*conserved structural subunits that make up the secondary structures of RNAs.*”[101]
- “*ordered stacked arrays of non-Watson-Crick base pairs that form distinct folds on the phosphodiester backbones of RNA strands.*”[102]

The kind of RNA motifs addressed in this thesis are of the third type, that is, **RNA 3D structure** motifs which we henceforth term RNA motifs. From our point of view RNA motifs are to be understood as peculiar sets of geometrical (in the rigid block sense) arrangements in three-dimensional space.

Even though there is no unique definition, we can think of three practical tasks regarding RNA motifs. That is, given an RNA 3D structure automatically identify, describe, and find new motifs. For automatic identification of RNA motifs Pyle and collaborators have developed a software called AMIGOS. This software finds RNA motifs based on specific values of backbone virtual torsion angles η and θ [103, 104, 105] in a way which resembles a Ramachandran plot analysis. Lemieux and Major [106] provide the software MC-Fold, which implicitly finds RNA motifs based on an algorithm to determine so called nucleic cyclic motifs, which are just the minimal cycle basis of an RNA secondary structure interpreted as a mathematical graph. Leontis [107] and collaborators provide FR3D (read as FRED). F3RD is a matlab windows executable program which finds RNA motifs based on the isostericity matrices of base-pairs.

For description of RNA motifs Schlick and collaborators have used FR3D to localize RNA helical junctions of order four (i.e. four-way junctions) or higher, and performed a visual analysis to see if the helices in such junctions form coaxial stacks or not, and have classified them accordingly [108, 109]. As mentioned previously in the context of RNA folds, Holbrook, and Sykes, describe helical regions and display them in two-dimensional representations. Spomer's group has carried out the description of RNA motifs present in the ribosome using Molecular Dynamics (MD) methods implemented in the AMBER package. They have performed 25ns simulations of the Sarcin-Ricin Domain (SRD) of the ribosome [110], and also 80ns simulations of hydration of loop E in the 5S subunit of the ribosome [111].

The software programs which perform the task of identifying RNA motifs in RNA structures also have the ability to find new RNA motifs, as is the case for AMIGOS, MC-Fold, and FR3D.

1.7 Overview

Keeping always in mind the greater scope of the RNA folding problem, this thesis addresses various issues of RNA structural understanding using RNA crystallographic data from the Protein Data Bank (PDB). Such data has been analyzed statistically in terms of a rigorous rigid-body formalism. In Chapter 2 the consensus clustering technique is used to classify RNA base-step parameters of non-A-RNA conformations, and the resulting groups are localized and understood in the context of rRNA. Chapter 3 reconsiders previous work carried out by Dr. Yurong Xin at the Olson's lab, on classification of RNA base-pairs by resorting again to clustering analysis techniques, and database mining of the WWW available Base Pair Structures (BPS) database. In Chapter 4 we explore, using statistical analysis, the data available on RNA helical regions, and use this information to compute the persistence length of double-stranded RNA's and compare it to experimental results. In Chapter 5 we provide a new python software, pyRNAmotifs which interfaces with 3DNA to do a rigorous search of existing and perhaps new RNA motifs, and finally in Chapter 6 we propose the measurement and classification of RNA structures using a new graph theoretical index named folding index, based on a helical region "view" of RNA's, which is clearly concordant with the emerging necessity of new metrics beyond RMSD for structural understanding.

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Chapter 2

RNA Base Steps

The problem of classification of the space of conformations of RNA is not new, see for example, Olson 1972 [1], Saenger 1984 [2], and Gautheret 1993 [3]. This problem had only been addressed by a few researchers before the turn of the twenty first century, now this situation is changing rapidly. The reason for this fast change came in the year 2000, when a vast amount of RNA structural information became available due the elucidation of the structure of the 30S small ribosomal subunit of *Thermus thermophilus*, a bacterial ribosome [4, 5], and the 50S large ribosomal subunit of *Haloarcula marismortui*, an archaeal ribosome [6].

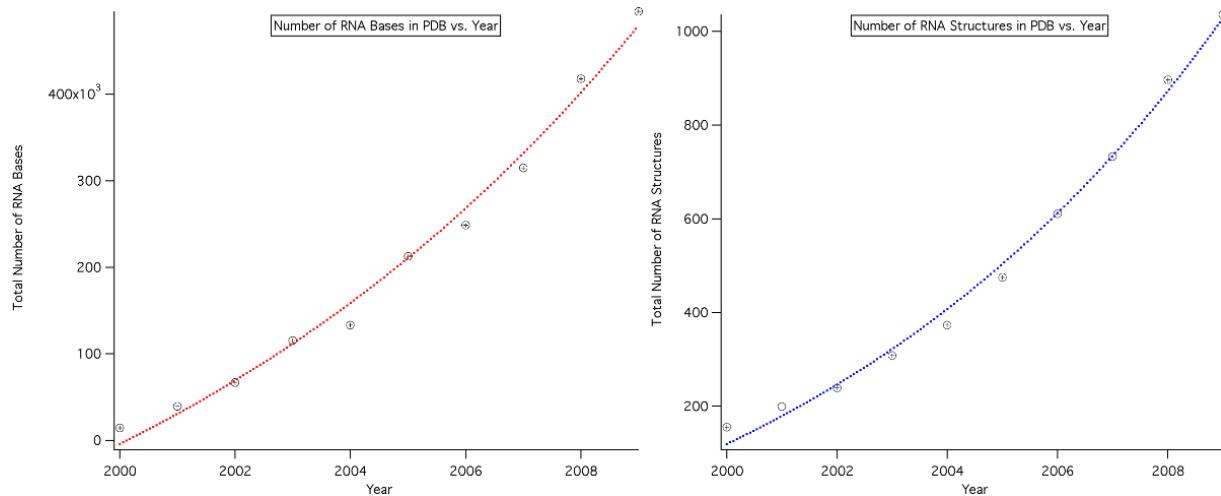


Figure 2.1: **Left:** Total number of RNA bases added to the PDB database between 2000 and 2010 (Exponential fit line in blue). **Right:** Total number of RNA structures solved yearly by X-Ray crystallography between 2000 and 2010 (Exponential fit line in red).

Between 1978 and 2000 a total of 116 RNA structures with resolution greater than 3.5\AA , and comprising around 5500 nucleotide bases are found in the Protein Data Bank (PDB), and between 2000 and today a total of 931 RNA structures comprising 491158 nucleotide bases are found. That is, the increase in information due to the solution of large RNA structures is about two orders of magnitude as pointed out by Noller [7]. Looking at the growth of RNA structural information from 2000 until today, it is clear that both the total number of RNA structures deposited to the PDB, and the total number of nucleotide bases in these structures, is growing in an exponential way (as can be seen by the exponential fits in Figure 2.1). It's important to note that such growth comes mainly from ribosomal structures which contain 88 percent of all RNA bases in the PDB. So, even though structural interest in RNA is growing since ribosomal structures became available in 2000, and several Nobel prizes have been awarded for work in this field, along with the exciting possibilities of deciphering large RNA [8] structures other than the ribosome, still the growth of the RNA structural field is far from that of proteins if weighed by the growth in diversity of RNA structural information in the past decade. At the present time if we look at the distribution of RNA sizes counted by number of bases, as can be seen in Figure 2.2 it's clear

that there are great patches where there are no RNA structures whatsoever, roughly between 600 and 1400 bases and between 1800 and 2700 bases. The area of non-coding RNA's holds great promise for finding structured RNA's in such length ranges as has recently been suggested by Breaker [8]. A representative example of the characteristic ranges of RNA structures available to date in the PDB can be seen in Table 2 for structures larger than 300 bases. An interesting comparison between the total number of structures of RNA, protein, dna, and nucleic acid plus protein, available at the PDB from the seventies until today can be seen in Supplement Figure S2.

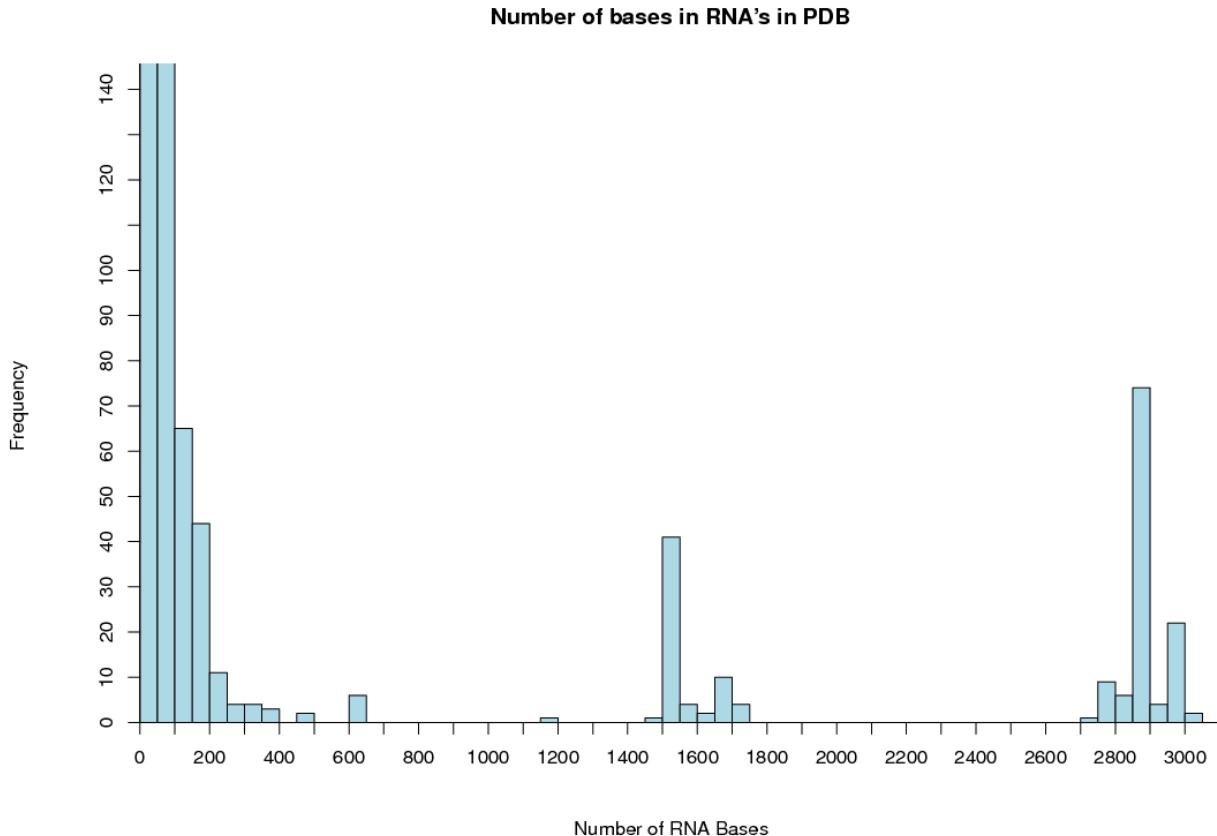


Figure 2.2: Frequency of nucleotide bases in RNA molecules found in the PDB classified by the size of RNA molecules. We define the size as the total number of nucleotide bases present per molecule.

The analysis of RNA conformational information contained in RNA structural data can be divided into three main perspectives: an atom based perspective; a bond based perspective; and a third, as yet unexplored to our knowledge, rigid-body based perspective. In the atom based perspective, either direct comparison of backbone atom positions is made [9], or a comparison of distances between a reduced set of atoms taken from the nucleotide backbone, sugar, and base [10]. The bond based perspective is divided into three main categories; the first considers the consecutive covalent bonds in the RNA backbone and the glycosidic bond between the sugar and base, that is, six backbone torsion angles and one glycosidic torsion angle [9, 11, 12, 13, 14]; or alternatively the pseudo-bonds between consecutive P and C4' atoms and the resulting pseudo-torsion angles η and θ [1, 15, 16, 17]¹. The third category considers the networks of horizontal hydrogen bonding patterns coming from a definition of interacting edge boundaries in the nucleotide bases [19, 20, 21]. In this chapter we study the rigid body based perspective using clustering analysis.

¹Previously the pseudotorsion angles η and θ were given the names ω_{ν} , and $\omega_{\nu\nu}$.[18]

PDBID	Structure Name	Phylogenetic Group	Number of bases	Year
1l8v	Mutant of P4-P6 Domain of Group I Intron	Eukaryote	314	2002
3igi	Group II Intron	Bacteria	395	2009
1fg0	Central Loop in Domain V of 23S rRNA	Archaea	499	2000
2nz4	GlmS Ribozyme	Eukaryote	604	2006
1xmq	30S rRNA	Bacteria	1522	2004
1ffk	50S rRNA Subunit	Archaea	2828	2000

Table 2.1: Some large RNA structures (>300 bases) elucidated in the last decade.

2.1 Consensus Clustering of Single Stranded Base Step Parameters

To our knowledge there has been no classification of rigid-body base-step parameters for RNA structures available from the PDB. It is important to note here that in crystal structures, RNA bases are determined more accurately than backbone torsion angles, as has been shown by Richardson and collaborators from analysis of van der Waals steric clashes. This can be seen more clearly in Figure 2.3, reproduced from Richardson's work [11], where the red and orange dots in the backbone atoms region denote steric clashes and the green and yellow dots in the base atoms region denote very good agreement with expected van der Waals distances.

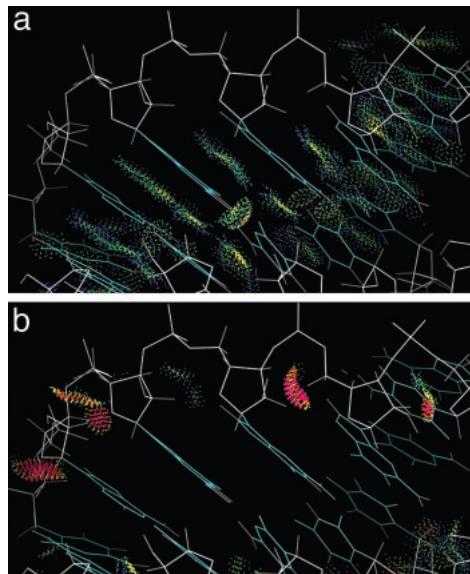


Figure 2.3: Figure taken from Richardson et al. [11] where the blue and green dots in a) mean very accurate van der Waals distances, and in b) the red and orange dots mean steric clashes, that is, distances outside the acceptable van der Waals range.

2.1.1 Combining Fourier Averaging Results and Clustering Analysis

Using the coordinate files of 20 rRNA structures provided by Schneider et al.[13] we used standard clustering analysis (CA) techniques (see Appendix A) to classify a set of non-ARNA base-steps using, rather than the more common torsion angles space, the base-step parameters space, that is, three

translational parameters (Shift D_x , Slide D_y , Rise D_z), and three rotational parameters (Tilt τ , Roll ρ , Twist ω), which we describe with the hexaparametric vector ν :

$$\nu = (D_x, D_y, D_z, \tau, \rho, \omega) \quad (2.1)$$

The results illustrated in the dendrogram shown in Figure 2.4 and whose corresponding structures are shown in Figures 2.5 S1 were obtained by performing clustering analysis and consensus clustering on 20 structures provided by Schneider et al. [13]. These twenty structures were obtained by Schneider applying a Fourier averaging technique, and lexicographical clustering, to torsion angles of 23S rRNA. The methodology we used follows that used by others to recover the periodic table classification from multidimensional property vectors for elements [22, 23].

In Figures 2.5, and S1 we see that Group I contains structure 1 with base-plane normals pointing in opposite directions, Group II includes extended conformations with neighboring bases roughly parallel but not stacked and is formed by structures 15, 16, 10, 14, Group III also contains extended conformations with bases perpendicular to one another and is formed by structures 8, 9, 17, Group IV 18, 19, 20, 13, 11, 12, 5, 3, 6, 7, 2, 4 contains four major subgroups: (a) structures 2, 4 which are unstacked with bases neither parallel nor perpendicular; (b) structures 18, 19, 20 which closely relate to A-RNA; (c) structures 11, 12, 13 which are unstacked and have parallel bases; and (d) structures 3, 5, 6, 7 which are also unstacked and have parallel bases. We also see in Group IV that the conformers in subgroups IV (c) and IV (d) are closely related, and that the dimers in these two subgroups are more closely related to those in subgroup IV (b) than to those in subgroup IV (a).

To account for the representation of the groups obtained by clustering in the 23S subunit of the ribosome we have computed the root-mean-square deviation (RMSD) between the average step parameters of the structures composing each group, and the step-parameters of the 2753 steps present in 23S. That is, for each group we have obtained a set of RMSD values which have been plotted as histograms as shown in Figures 2.6, and 2.7. The results are also summarized in Table 2.2, where we can see that they only constitute 31% of the total amount of steps in the 23S subunit of the ribosome. We used a cutoff of 10 Åⁱⁱ to select the structures which belong to each group, based on visual analysis of superimposed reconstructed structures. For example, for Group I; if we reconstruct the ribosomal steps with an RMSD of 10 Å or less, we get the figure shown in the left panel of Figure 2.8. But if we reconstruct with the set of structures with an RMSD of 15 Å or less we start getting structures, which after being superimposed based on the reference frames of the first base are clearly not related to that group, as can be seen in the right panel of Figure 2.8.

We have also noticed that the starting structures kindly provided to us by Dr. Berman, have a large rise in the case of A-RNA, that is, a value of 4.39 Å, which is larger than the 3.30 Å value obtained for the "classical" A-RNA structure from Arnott and collaborators [24]. This might have a significant effect on the amount of structures which can be grouped under the A-RNA like group.

Because of not getting a good representation of the total diversity of base-steps in the 23S subunit of the ribosome, we have opted to perform an analysis based fully on base-step parameters. We believe that the reason for such poor representation is due to the mixing of Fourier averaging for backbones, and the base-step perspective.

2.1.2 Selection of a Clustering Methodology

In order to analyze our dataset of base-step parameters we have decided to use clustering analysis methods. Clustering analysis methods can be broadly classified in two main categories, that is, they can be partitional or hierarchical. In either case the main problem one faces for classification purposes

ⁱⁱWe retain the traditional unit of Angstroms to refers to our RMSD's, but it is important to note that since we are not referring to an all-atom model such unit does not have a direct physical meaning.

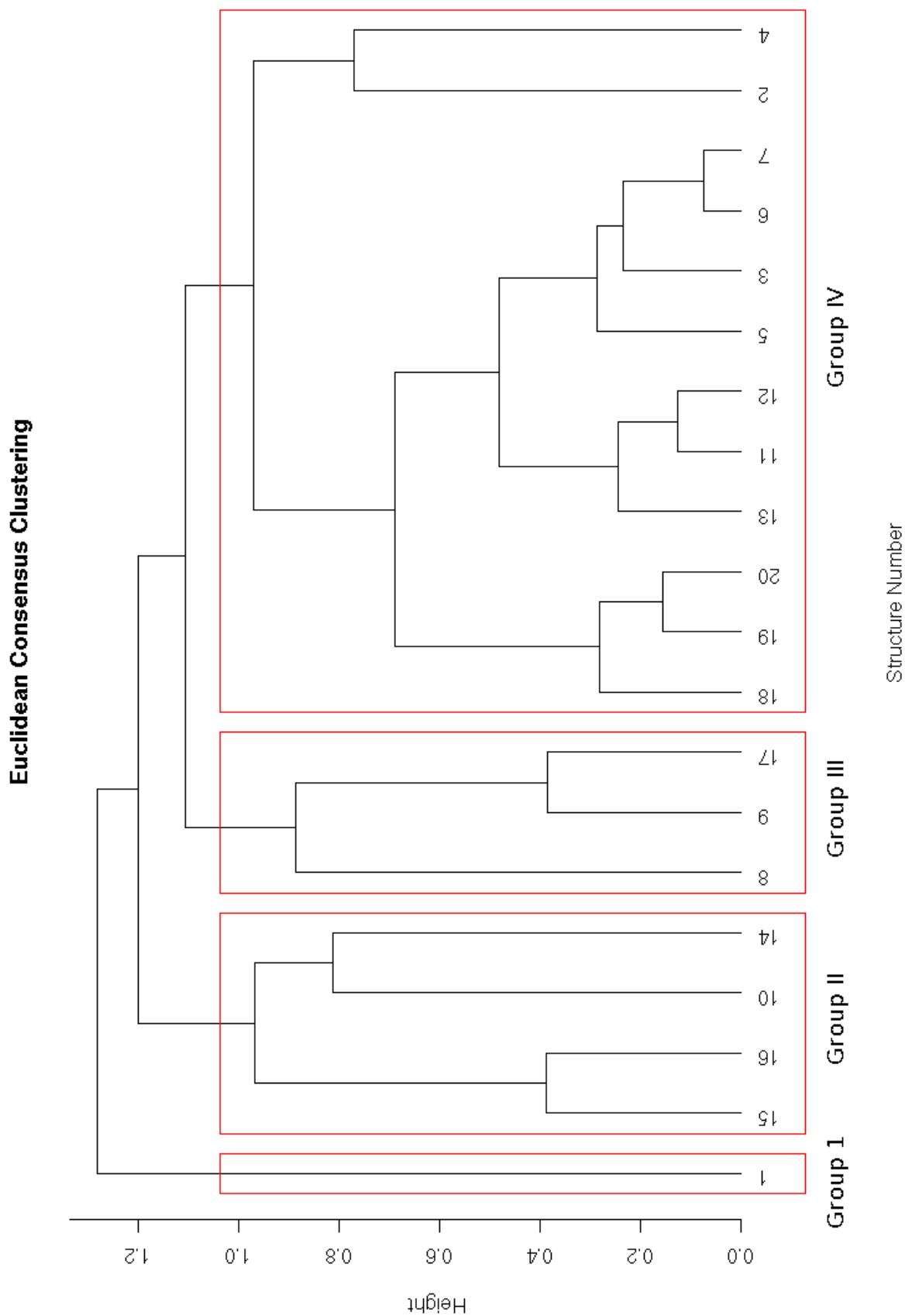


Figure 2.4: Dendrogram showing the results of consensus clustering of 20 non-A-type rRNA dinucleotides according to their hexadimensional base-step parameter vectors.

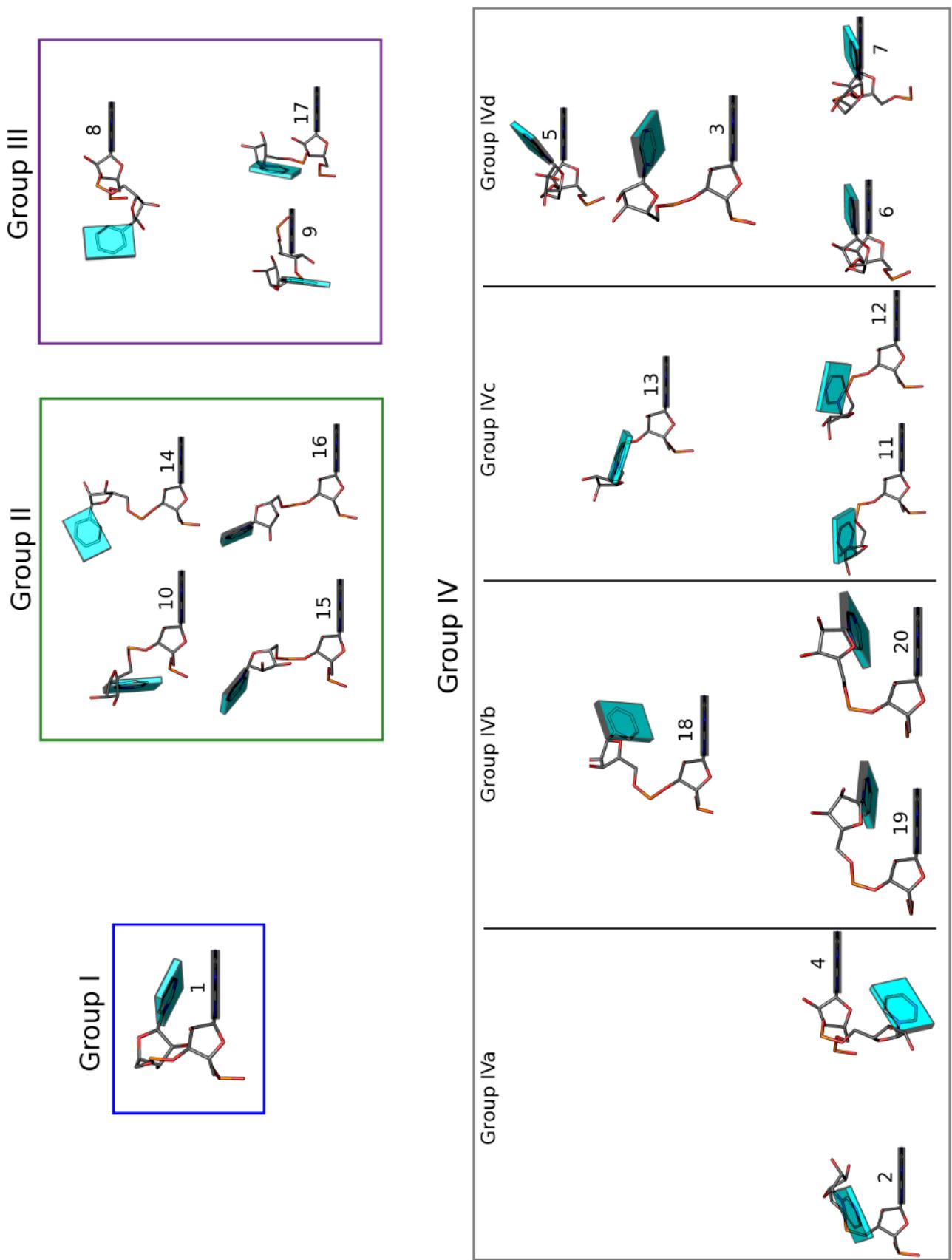


Figure 2.5: RNA dinucleotide structures organized by clusters obtained from consensus clustering of their hexadimensional base-step parameter vectors. The structures have been centered on the reference frame of the first step, that is, the adenine base, and the minor groove face of the rigid block parameter associated to adenine is facing the viewer.

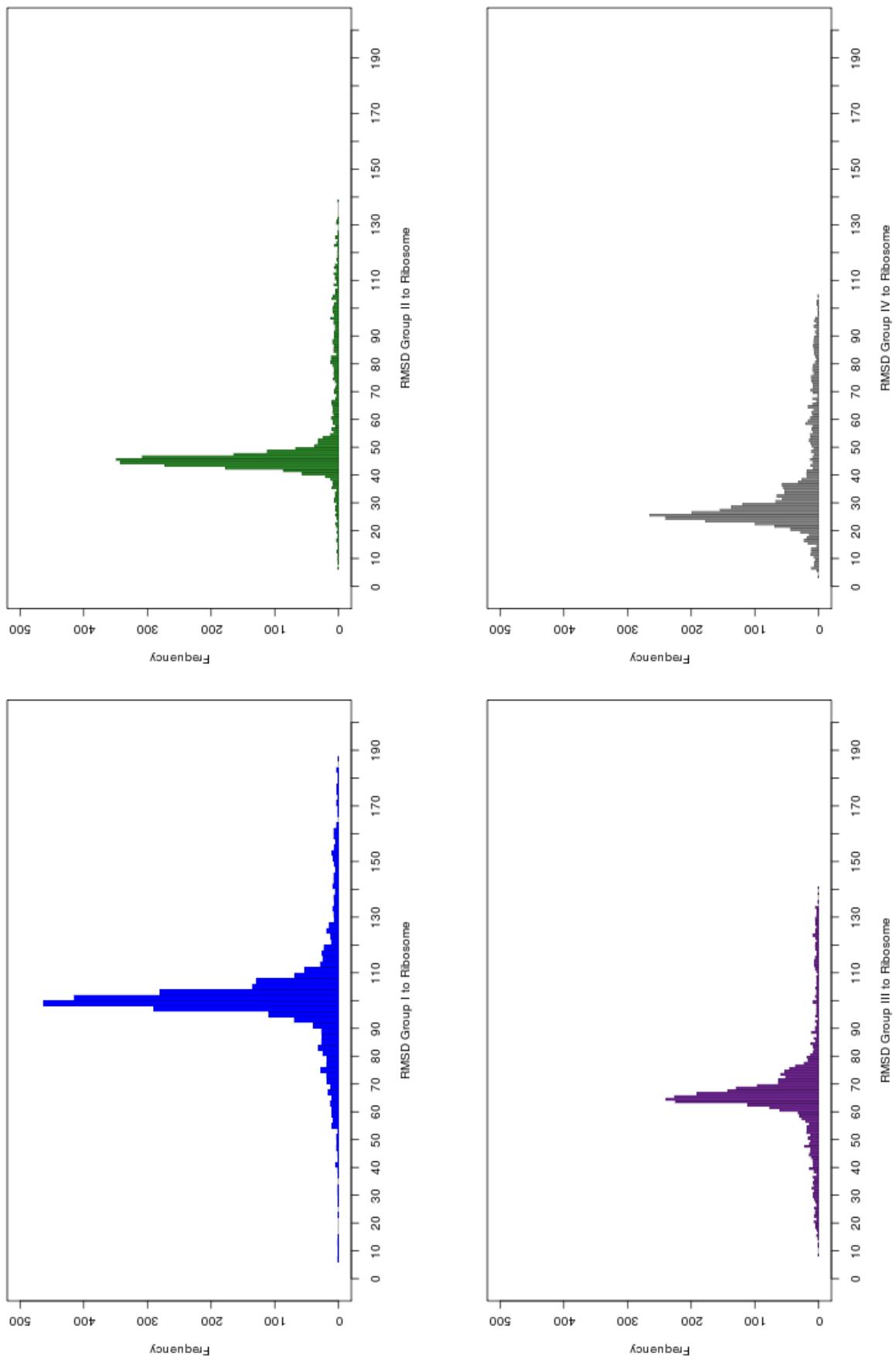


Figure 2.6: Root mean square deviation of the main four groups show in Figure 2.5. The color of the histograms is the same as that of the boxes surrounding the structures of Figure 2.5

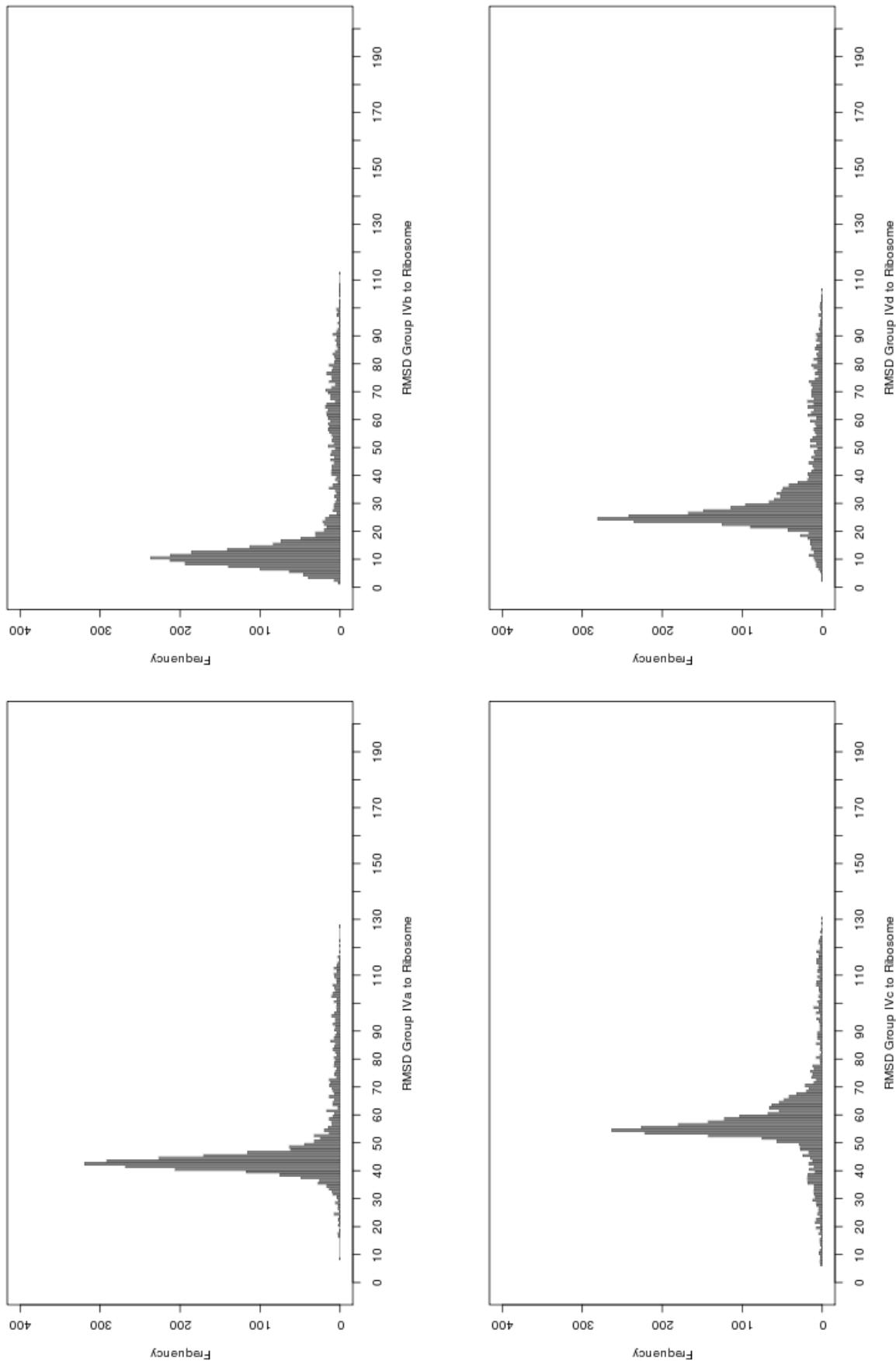


Figure 2.7: Root mean square deviation histograms for the subgroups present in group IV. Since subgroup IVb is composed of A-RNA like conformations we see in the upper left histogram that the highest proportion of small RMSD values belongs to this group.

Group	Percentage	Number of Base-Steps
I	0.11	3
II	0.18	5
III	0.04	1
IVa	0.36	1
IVb	29.31	807
IVc	0.33	9
IVd	1.27	35
Total	31.28	861

Table 2.2: Number of base-steps with RMSD values less than or equal to 10 Å between the reference base-step vectors from the four groups of non-A-type RNA dinucleotide conformations and all base-step vectors found in the 23S strand of *Haloarcula marismortui*. The percentage is calculated with respect to a total of 2753 base-steps present in the 23S chain of the 50S subunit of the ribosome.

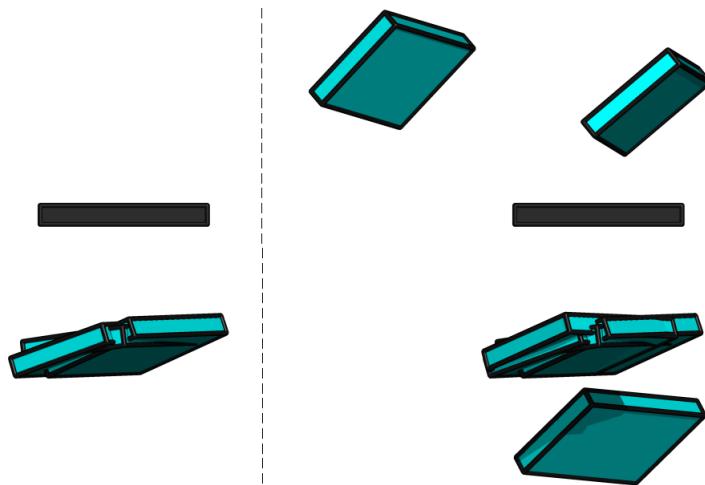


Figure 2.8: **Left:** With an RMSD cutoff of less than or equal to 10 Å, we identify three steps from the 23S subunit. **Right:** With an RMSD less than or equal to 15 Å we get a total of seven structures, where we clearly see that three of them are farther from the original Group I main structure of Figure 2.5

is that of deciding which is the optimal number of hierarchies or partitions that the analyzed data is split into. To obtain a criteria for an optimal number of clusters, and also to decide which method might be better for our dataset, we have used two types of cluster validation techniques. They are known as internal measures and stability measures. Full detail on the definition of such measures are provided in appendix A. To perform the validation analysis mentioned above we used a cluster validation package implemented in the R [25] statistical analysis program called `c1Valid` [26].

In Figures 2.9 and 2.10 we present the results for internal and stability validation results exploring the same dataset of base-step parameters for the 23S subunit of the ribosome that we've used before. In the clustering analysis literature it's customary to use the variable k to define the number of clusters and we will use variable k in that sense in what follows.

Our analysis computed the validation scores for a number of clusters ranging from $k = 2$, up to $k = 80$ clusters, and evaluated hierarchical methods (hierarchical, diana), and partitional methods (kmeans, pam, som, sota). The connectivity measure must be minimized, and the average silhouette width (silhouette) and dunn index must be maximized. With this in mind, we see that the method labeled as hierarchicalⁱⁱⁱ performs better in connectivity and dunn index for the whole range, and it is also the best performer in silhouette from $k = 12$ onwards.

In the case of the stability measures it is important to note here that mainly these measures are well suited for highly correlated data sets, therefore they are not very indicative for our data set, which has correlation mainly between the shift and twist parameters, but not in general. We include such measures for completeness. In the case of the stability measurements in all cases they are better the smaller their values are. We have quantified three measures, that is, the average proportion of non-overlap (APN), the average distance (AD), and the average distance between means (ADM), again the details of such measures are given in the Appendix A. As seen in Figure 2.10 the method with the best stability measures is sota for the case of APN, and ADM almost in all the range until it reaches a number of cluster of around 70. For the AD measure the best performers are pam and sota in the whole range. Notice that the hierarchical method performs similarly to other methods, and that in general, apart from the APN measure and the sota method, all methods have a similar behaviour due to the fact that our data set is not highly correlated, that is, it cannot be split into say, two, three, or four, principal components.

In all cases we also see that the best overall number of clusters is two, which is not surprising since we haven't filtered out A-RNA structures from our data set, leaving two main groups; that of A-RNA type base-steps, and those which are not A-RNA like.

We focus our attention in the group of structures which are not so closely related to A-RNA. Therefore we have filtered them out based on Figure 2.11, and end out with a data set of 797 (about 29% of the total number of steps) base-step parameters whose values are greater than an RMSD of 18 Å. These RMSD values have been computed between the base-step parameters of 23S RNA and the standard base-step parameter values derived from Arnott and collaborators [24] work. Such values are provided in table 2.1.2.

With the filtered dataset, which we will refer to as the non-ARNA dataset, we have performed a cluster validation analysis using only the hierarchical methods, that is, the agglomerative clustering method (labeled hierarchical in Figure ??), and the divisive clustering method (labeled as diana in Figure ??). In Figure ?? we have plotted the results of the internal validation scores and we can see that the connectivity and silhouette measures still give us an optimal number of cluster of two, whereas the dunn index tells us that the optimal number of clusters is 64. We find it interesting that for the case of the dunn index we see that this measure becomes constant at values of 17, 30, 56, and 64.

ⁱⁱⁱThe hierarchical label refers precisely to the agglomerative (bottom-up) technique, the euclidean metric, and the average method

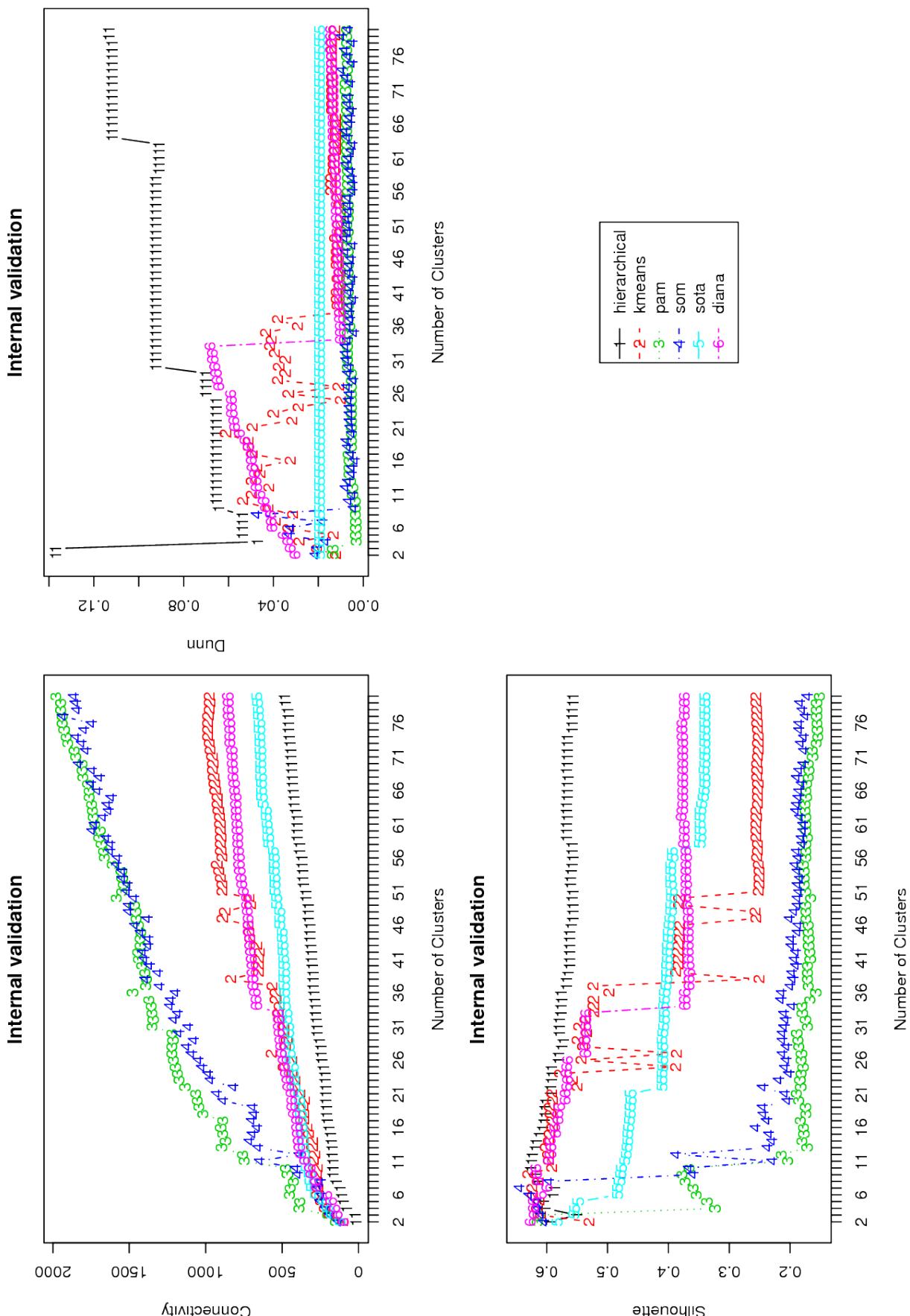


Figure 2.9: Cluster validity scores for internal measures. Notice how the agglomerative hierarchical method, labeled as 1 and in black color, behaves better for the whole range of connectivity and Dunn

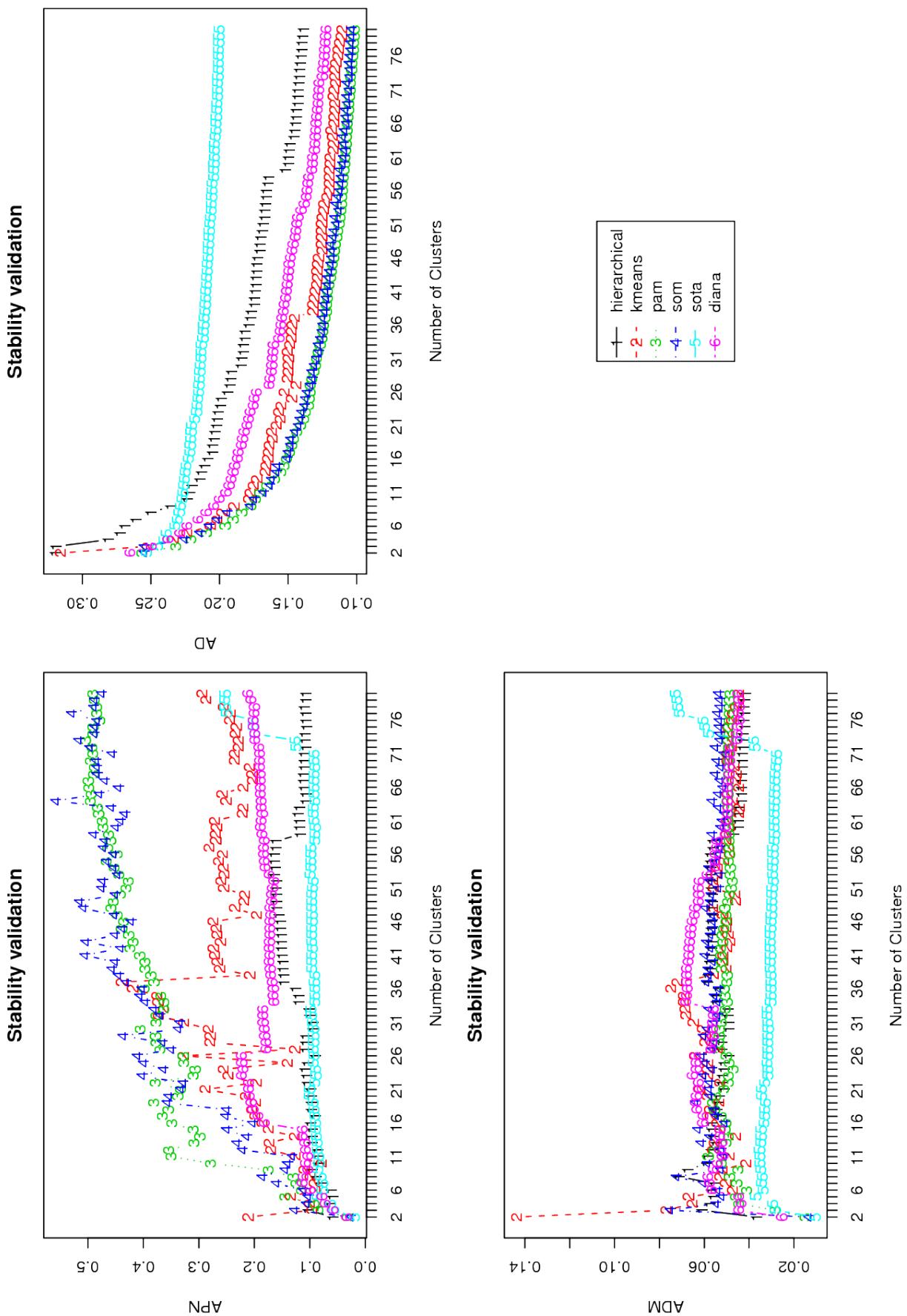


Figure 2.10: Cluster validity scores for stability measures.

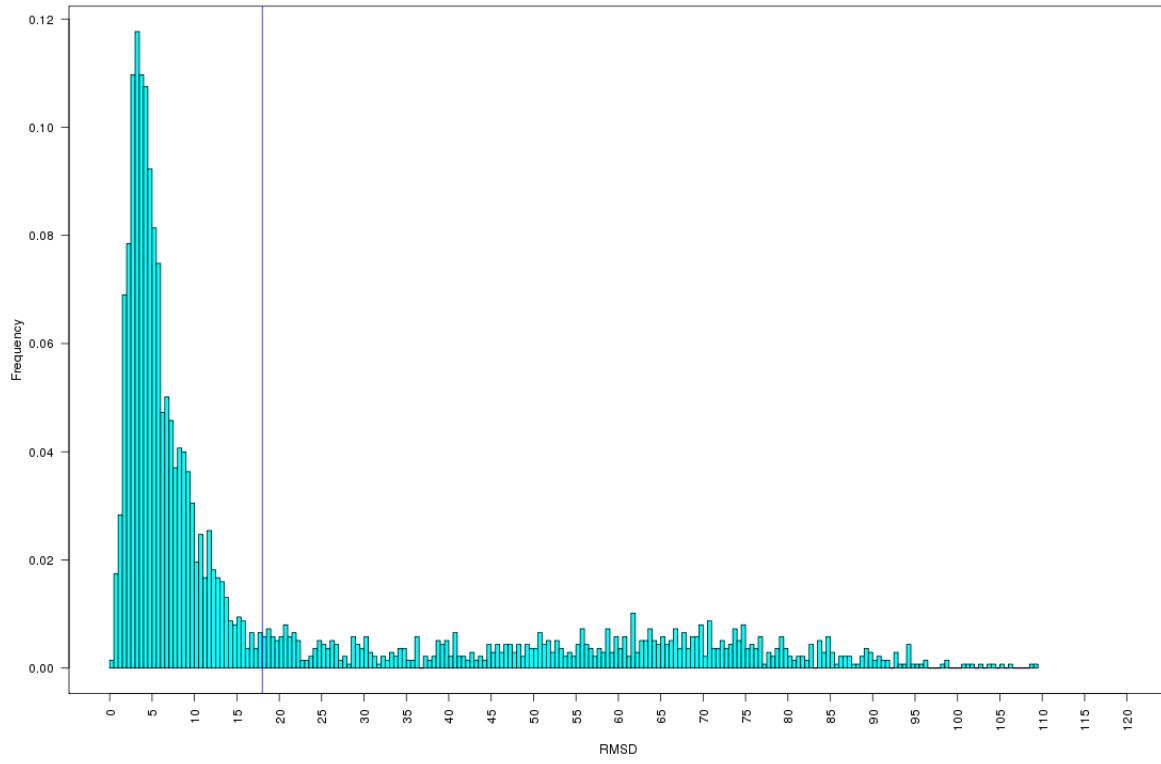


Figure 2.11: RMSD values between base-step parameters of the 23S subunit of ribosomal RNA and the standard base-step parameters derived from Arnott and collaborators [24] work.

Structure Name	Shift (D_x)	Slide (D_y)	Rise (D_z)	Tilt (τ)	Roll (ρ)	Twist (Ω)	Reference
A-DNA	0.36	-1.39	3.29	2.46	12.50	30.19	
B-DNA	0.44	0.47	3.33	4.63	1.77	35.67	
A-RNA	-0.08	-1.48	3.30	-0.43	8.64	31.57	Arnott
A'-RNA	0.05	-1.88	3.39	-0.12	5.43	29.52	Arnott
All-RNA	1.01	-2.52	3.33	2.94	9.75	25.12	Schneider

Table 2.3: Base step parameters for common DNA and RNA conformations. The base-step parameters are computed for a single-stranded base-step rather than a double-stranded base-pair step.

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Appendix A

Clustering Analysis (CA)

A.1 Hierarchical methods

The hierarchical clustering methods used were:

1. *Single linkage clustering*, where the minimum distance between elements of each cluster is taken as clustering criteria.

$$D(X, Y) = \min\{d(x_i, y_j) : x_i \in X, y_j \in Y\} \quad (\text{A.1})$$

where X and Y are vectors, and $d(x_i, y_j)$ is the distance between cluster elements.

2. *Complete linkage clustering*, where the maximum distance between cluster elements is the clustering criteria.

$$D(X, Y) = \max\{d(x_i, y_j) : x_i \in X, y_j \in Y\} \quad (\text{A.2})$$

3. *Average linkage clustering*, the mean distance between elements of each cluster is taken as clustering criteria.

$$D(X, Y) = \frac{1}{N_x * N_y} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} d(x_i, y_j) \quad (\text{A.3})$$

where N_x and N_y are the number of elements in respective clusters.

4. *Centroid linkage clustering*, uses the distance between cluster centroids, as clustering criteria.

$$D(X, Y) = d(\bar{x}, \bar{y}) \quad (\text{A.4})$$

$$\bar{x} = \frac{1}{N_x} \sum_{i=1}^{N_x} x_i \quad (\text{A.5})$$

$$\bar{y} = \frac{1}{N_y} \sum_{i=1}^{N_y} y_i \quad (\text{A.6})$$

$$(A.7)$$

Structure	Property I	Property II
1	1.00	5.00
2	-2.00	6.00
3	2.00	-2.00
4	-2.00	-3.00
5	3.00	-4.00

Table A.1: Example of structures, considered as bidimensional vectors, to be clustered using the average linkage method and the Manhattan distance.

5. *Ward's Method*, uses the error sum of squares (ESS).

$$D(X, Y) = ESS(XY) - [ESS(X) + ESS(Y)] \quad (\text{A.8})$$

$$ESS(X) = \sum_{i=1}^{N_x} \left| x_i - \frac{1}{N_x} \sum_{j=1}^{N_x} x_j \right|^2 \quad (\text{A.9})$$

As an example lets think of a case where we have five structures. Each one of them is described by a bidimensional vector as illustrated in Table A.1.

The first step is to chose a distance definition. We chose Manhattan and the distance values between structures can be displayed in a lower triangular matrix as seen in equation A.10

$$d(X, Y) = \begin{vmatrix} & 1 & 2 & 3 & 4 \\ 1 & & & & \\ 2 & 4 & & & \\ 3 & 8 & 12 & & \\ 4 & 11 & 9 & 5 & \\ 5 & 11 & 15 & 3 & 6 \end{vmatrix} \quad (\text{A.10})$$

Let's calculate explicitly the Manhattan distance between structures 2 and 3,

$$d(2, 3) = | -2.00 - 6.00 | + | 2.00 - -2.00 | = 12 \quad (\text{A.11})$$

Now that we have calculated the distances we need a clustering method, in this case, we will use the average linkage clustering method. There are two hierarchical techniques called agglomerative, or bottom-up, and divisive, or top-down. We will use the agglomerative technique, that is, going from the bottom where no objects are grouped, to the top, where all objects constitute one final group. The first step is then to group whatever structures are closer, that is, structures 3 and 5 ($d(3, 5) = 3$). Now we find the mean distance between the elements of this cluster and the remaining unclustered structures, that is, structures 1, 2 and 4, we obtain the following mean distances

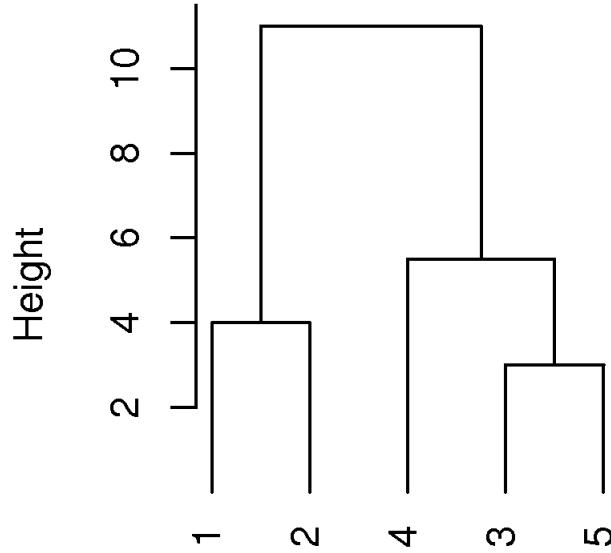
$$D(\{3, 5\}, 1) = \frac{1}{2 * 1} * (8 + 11) = 4.5 \quad (\text{A.12})$$

$$D(\{3, 5\}, 2) = \frac{1}{2 * 1} * (12 + 15) = 13.5 \quad (\text{A.13})$$

$$D(\{3, 5\}, 4) = \frac{1}{2 * 1} * (5 + 6) = 5.5 \quad (\text{A.14})$$

Since the distances between $\{3, 5\}$ and all remaining unclustered vectors is higher than the distance between vectors 1 and 2 ($d(1, 2) = 4$) then $\{1, 2\}$ are grouped. The following value, in hierarchical

Average linkage example tree



Manhattan distance

Figure A.1: Clustering tree for 5 bidimensional vectors using the Manhattan distance definition and the average linkage clustering method.

increasing order is 4.5 between $\{3, 5\}$ and 1 (see equation A.12), but since 1 and 2 are already grouped we can't group $\{3, 5\}$ with 1. The next value, following the lower to higher hierarchy, is 5 ($d(3, 4) = 5$), but we have already grouped 3 with 5, so we have to keep advancing in the hierarchy. The next value is 5.5, which corresponds to grouping $\{3, 5\}$ with 4, so we cluster them. The only remaining possibility for grouping is, group $\{1, 2\}$ and $\{4, 3, 5\}$, so we do it as illustrated in Figure A.1.

Supplement A

Figure Supplements

S1 Supplement Figures for Chapter 2

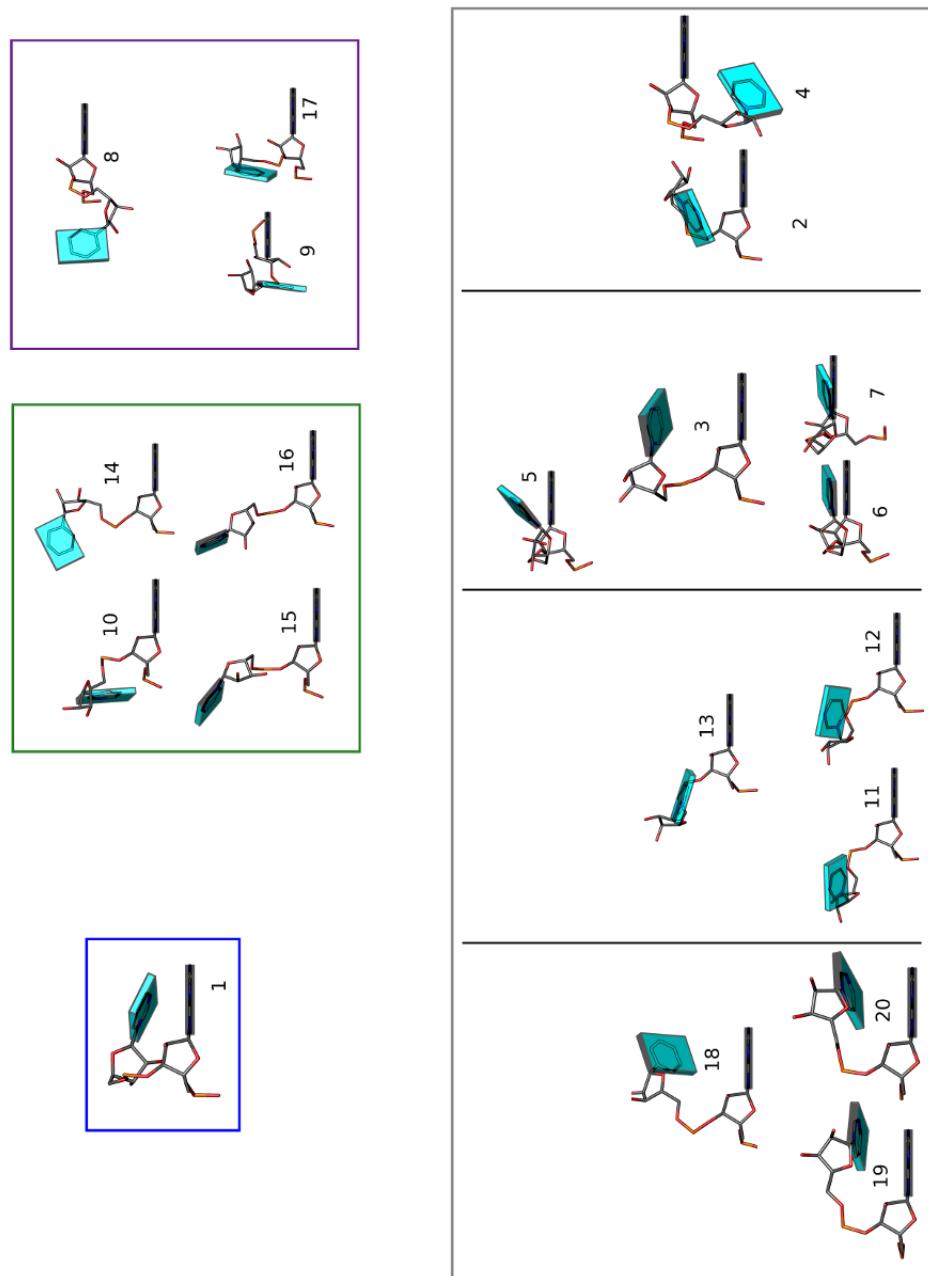


Figure S1: Non A-RNA Type base steps centered on the standard reference frame of Adenine. Top view with the Minor Groove side of Adenine pointing down the page and the Major Groove pointing up.

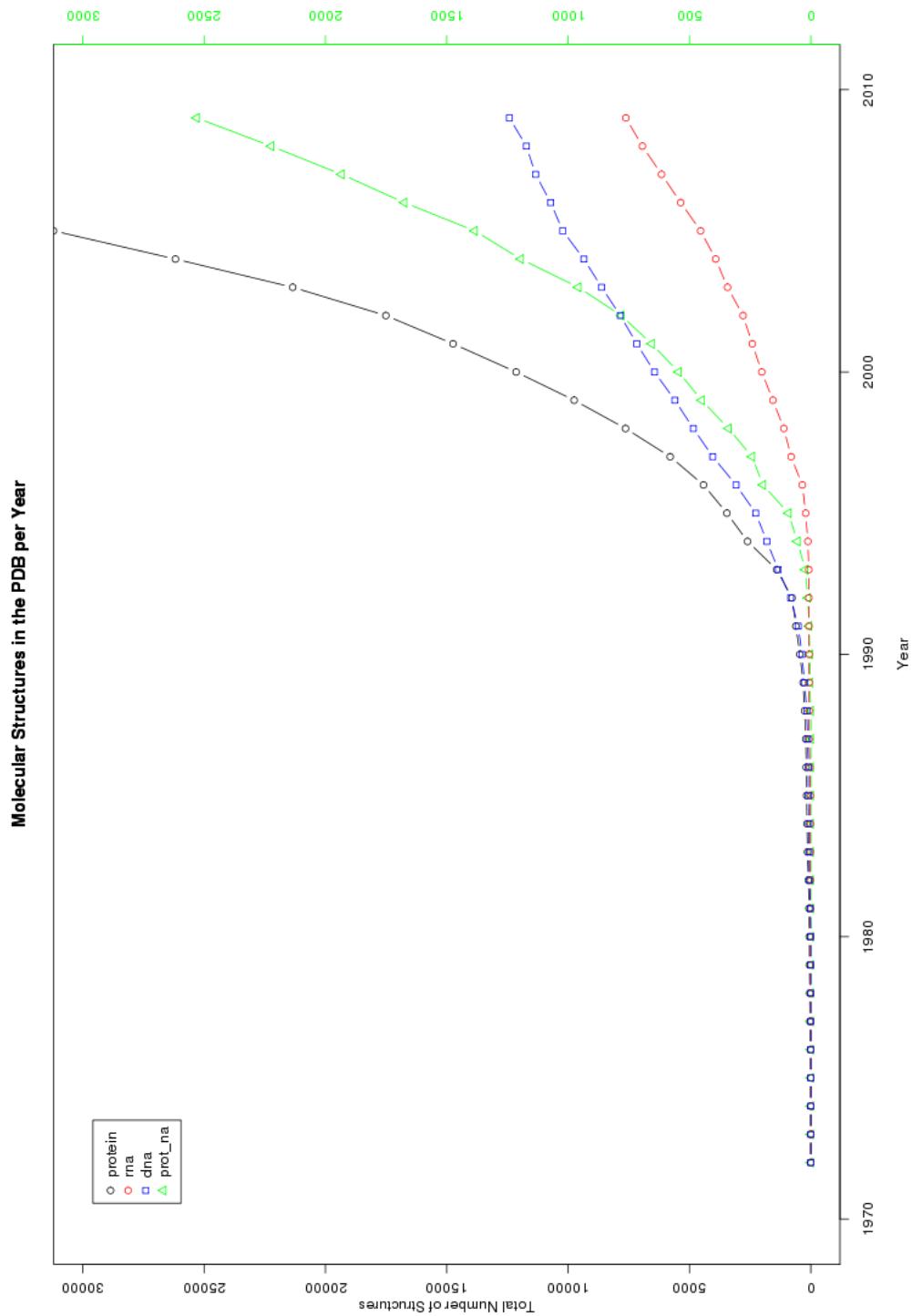


Figure S2: The total number of structures available in the pdb up to the end of year 2009. The scale of the axis in the left (in black), is ten times that in the right (in green). The black y-axis sets the scale for the number of protein structures available in the PDB up to the end of the year 2009. The green y-axis sets the scale for the number of molecular structures containing, rna only (in red), dna only (in blue), and protein plus nucleic acid (in green). One can clearly see that the total number of protein, rna, and protein plus nucleic acid structures is growing exponentially. It is also clear that the number of DNA structures is perhaps tending toward a constant number, that is, it might not be growing. It is also interesting to see how the number of RNA structures really lifts off in the middle of the nineties, whereas for DNA the growth started earlier and is settling down.