RNA secondary structures having a compatible sequence of certain nucleotide ratios

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Abstract

Given a random RNA secondary structure, $S$, we study RNA sequences having fixed ratios of nucleotides that are compatible with $S$. We perform this analysis for RNA secondary structures subject to various base pairing rules and minimum arc- and stack-length restrictions. Our main result reads as follows: in the simplex of the nucleotide ratios there exists a convex region in which, in the limit of long sequences, a random structure a.a.s. has compatible sequence with these ratios and outside of which a.a.s. a random structure has no such compatible sequence. We localize this region for RNA secondary structures subject to various base pairing rules and minimum arc- and stack-length restrictions. In particular, for GC-sequences having a ratio of $G$ nucleotides smaller than $1/3$, a random RNA secondary structure without any minimum arc- and stack-length restrictions has a.a.s. no such compatible sequence. For sequences having a ratio of $G$ nucleotides larger than $1/3$, a random RNA secondary structure has a.a.s. such compatible sequences. We discuss our results in the context of various families of RNA structures.

Keywords: RNA secondary structure, compatible sequence, nucleotide ratio, generating function, singularity analysis.

1. Introduction

This paper is motivated by the question whether there is “more” to DNA sequence data than the mere sequence of nucleotides, identified by alignment methods. As DNA is transcribed into RNA and subsequently processed in various ways, we ask here the question what effect the frequencies of the nucleotides have on the variety of RNA structures that are compatible with the given sequence. Sequences are called compatible if they satisfy the base pairing rules for all bonds of the structure. In particular, for RNA secondary structures the work of McCaskill [McCaskill, 1990], allows to efficiently Boltzmann-sample such configurations. Simple quantities such as the nucleotide ratios could be used to identify the functionality of embedded DNA sequences.

This paper provides the mathematical analysis of the fact that in the simplex of the nucleotide ratios there exists a convex region in which, in the limit of long sequences, a
random structure a.a.s. has compatible sequences with these ratios and outside of which a.a.s. a random structure has no such compatible sequence. Here a.a.s. stands for asymptotically almost surely, i.e. in the limit of long sequences, the probability tends to 1. The nucleotide ratios are a discriminant of whether or not a random secondary structure has such a compatible sequence and is thus accessible by means of folding. By random we mean a secondary structure that, in the limit of long sequences, has the average number of arcs, see Theorem 8 for details. On the flip side, given a biologically relevant sequence, whose ratios are not within this window, we can test for whether or not functionalities are achieved by other means, i.e. for instance by interactions with either Proteins or other RNA structures.

Before we proceed by providing some background on RNA secondary structures as contact structures and diagrams, we validate our modeling Ansatz. As we shall show in Section 4 the key lies in a central limit theorem for the number of arcs in biologically “realistic” RNA secondary structures. Here realistic is tantamount to RNA secondary structures having minimal free energy. These structures do not contain bonds that force an extreme bending of the backbone and they contain base pairs organized in stacks of length at least three. The latter reflects the fact that the main contribution of lowering free energy stems from electron delocalization between stacked bonds (Hunter and Sanders, 1990; ˇSponer et al., 2001, 2013).

The space of minimum free energy (mfe) RNA secondary structures can only be understood by means of extensive computation. The space of all RNA secondary structures that satisfy the above mentioned constraints, however, can be mathematically analyzed. It comes down to the question of whether or not the number of arcs of mfe-structures can be obtained by uniformly sampling RNA secondary structures satisfying minimum stack-length three and arc-length four constraints. In Figure 1 we contrast these two distributions. The similarity of the two distributions allows us to pass to combinatorics and thus to prove statements in the larger space.

An RNA sequence is described by its primary structure, a linear oriented sequence of the nucleotides and can be viewed as a string over the alphabet \{A, U, G, C\}. An RNA strand folds by forming hydrogen bonds between pairs of nucleotides according to Watson-Crick A-U, C-G and wobble U-G base-pairing rules. The secondary structure encodes this bonding information of the nucleotides irrespective of the actual spacial embedding. More than three decades ago, Waterman and colleagues pioneered the combinatorics and prediction of RNA secondary structures (Waterman, 1978, 1979; Smith and Waterman, 1978; Howell et al, 1980; Schmitt and Waterman, 1994; Penner and Waterman, 1993).
RNA secondary structures can be represented as diagrams, see Fig. 2. A diagram is a labeled graph over the vertex set \( \{1, \ldots, n\} \) whose vertices are arranged in a horizontal line and arcs are drawn in the upper half-plane. Clearly, vertices and arcs correspond to nucleotides and base pairs, respectively. The number of nucleotides is called the length of the structure. The length of an arc \((i, j)\) is defined as \( j - i \) and an arc of length \( k \) is called a \( k \)-arc. The backbone of a diagram is the sequence of consecutive integers \( \{1, \ldots, n\} \) together with the edges \( \{(i, i+1) \mid 1 \leq i \leq n-1\} \). We shall distinguish the backbone edge \( \{i, i+1\} \) from the arc \((i, i+1)\), which we refer to as a 1-arc. Two arcs \((i_1, j_1)\) and \((i_2, j_2)\) are crossing if \( i_1 < i_2 < j_1 < j_2 \). An RNA secondary structure is defined as a diagram satisfying the following three conditions (Waterman, 1978):

1. **non-existence of 1-arcs**: if \((i, j)\) is an arc, then \( j - i \geq 2 \),
2. **non-existence of base triples**: any two arcs do not have a common vertex,
3. **non-existence of pseudoknots**: any two arcs are non-crossing, i.e., for two arcs \((i_1, j_1)\) and \((i_2, j_2)\) where \( i_1 < i_2, i_1 < j_1, \) and \( i_2 < j_2 \), we have either \( i_1 < j_1 < i_2 < j_2 \) or \( i_1 < i_2 < j_2 < j_1 \).
Fig. 2. An RNA secondary structure represented as a contact graph (a) with hydrogen bonds as red edges and as a diagram (b) having minimum stack-length 4 and arc-length 5. In (b), we display three compatible sequences having the AUGC-ratios (0.088, 0.099, 0.417, 0.396), (0.340, 0.000, 0.330, 0.330) and (0.296, 0.242, 0.242, 0.220), respectively.

More succinctly, an RNA secondary structure expressed as a diagram has exclusively non-crossing arcs in the upper half-plane. Pairs of nucleotides may form Watson-Crick A-U, C-G and wobble U-G bonds labeling the above mentioned arcs.

This paper is organized as follows: In Section 2, we provide some basic facts of singularity analysis of power series. In Section 3, we compute the bivariate generating function of RNA secondary structures of length \( n \) having \( l \) arcs and derive its asymptotic expansion as well as the central limit theorem for the distribution of arcs. In Section 4, we analyze the fraction of RNA secondary structures having compatible sequences and prove our main results. We conclude with Section 5, where we integrate and discuss our findings.

### 2. Singularity analysis

RNA secondary structures as combinatorial objects give rise to generating functions, i.e. formal power series whose coefficients count the number of structures for given sequence length. The estimation of these coefficients will be at the heart of this analysis and we next discuss some basic facts of how to compute them. The key tool here is singularity analysis: facilitated by Cauchy-integration along certain contours passing close by the dominant critical point(s). Singularity analysis is described in great detail in the book
by Flajolet and Sedgewick (Flajolet and Sedgewick, 2009). In the following, we showcase all facts needed to exact the asymptotics of coefficients from our generating functions.

Let \( f(x) = \sum_{n \geq 0} a_n x^n \) be a combinatorial generating function. We are interested in the calculation of \( a_n \) in terms of their exponential growth rate, \( \gamma \) and their subexponential factor \( P(n) \), that is, \( a_n \sim P(n) \gamma^n \). The key for obtaining the asymptotic information about the coefficients of a generating function is to locate its dominant singularities.

For the particular case of power series with nonnegative coefficients with a radius of convergence \( R > 0 \), Pringsheim’s theorem (Flajolet and Sedgewick, 2009; Titchmarsh, 1939) guarantees a (unique) positive, real, dominant singularity at \( z = R \). In our case all generating functions are by construction combinatorial, whence we always have such a positive real, dominant, singularity.

A function \( f(x) \) is \( \Delta_\rho \) analytic at its dominant singularity \( x = \rho \), if it is analytic in some domain \( \Delta_\rho(\phi, d) = \{ x \mid |x| < d, x \neq \rho, |\text{Arg}(x - \rho)| > \phi \} \), for some \( \phi, d \), where \( d > |\rho| \) and \( 0 < \phi < \frac{\pi}{2} \). We set

\[
(f(x) = O(g(x)) \text{ as } x \to \rho) \iff (f(x)/g(x) \text{ is bounded as } x \to \rho),
\]

Noting that for any \( \gamma \in \mathbb{C} \setminus 0 \),

\[
[x^n]f(x) = \gamma^n [x^n] f\left(\frac{x}{\gamma}\right),
\]

we can, without loss of generality, reduce our analysis to the case where \( x = 1 \) is the unique dominant singularity. The following transfer-theorem allows us to derive the asymptotics of coefficients from the asymptotic expansion of its generating function around its dominant singularity.

**Theorem 1** (Flajolet and Sedgewick (2009), Theorem VI.3, pp. 390). Let \( f(x) \) be a \( \Delta_1 \) analytic function at its unique dominant singularity \( x = 1 \). Let

\[
g(x) = (1 - x)^\alpha \log^\beta \left(\frac{1}{1-x}\right), \quad \alpha, \beta \in \mathbb{R}.
\]

That is we have in the intersection of a neighborhood of 1

\[
f(x) = O(g(x)) \text{ for } x \to 1.
\]

Then we have

\[
[x^n]f(x) = O([x^n]g(x)).
\]
Theorem 2 (Flajolet and Sedgewick (2009)). Suppose \( f(x) = (1 - x)^{-\alpha}, \alpha \in \mathbb{C} \setminus \mathbb{Z}_{\leq 0}, \) then

\[
[x^n]f(x) \sim \frac{n^{\alpha-1}}{\Gamma(\alpha)} \left[ 1 + \frac{\alpha(\alpha - 1)}{2n} + \frac{\alpha(\alpha - 1)(\alpha - 2)(3\alpha - 1)}{24n^2} + \frac{\alpha^2(\alpha - 1)^2(\alpha - 2)(\alpha - 3)}{48n^3} + O\left(\frac{1}{n^4}\right) \right].
\]

The next result extracts asymptotics of generating functions, satisfying polynomial equations. It is based on the method of Newton polygons and derives the Newton-Puiseux expansion and the resultant of two polynomials to locate the dominant singularity. The resultant of two polynomials \( f(z) = a_n \prod_{i=1}^{n} (z - \alpha_i) \) and \( g(z) = b_m \prod_{j=1}^{m} (z - \beta_j) \) is given by

\[
R(f, g, z) = a_n^m b_m^n \prod_{i=1}^{n} \prod_{j=1}^{m} (\alpha_i - \beta_j).
\]

Theorem 3. Let \( z(x) = \sum_{n \geq 0} z_n x^n \) be a generating function, analytic at 0, that satisfies the polynomial equation \( \Phi(x, z) = 0 \). Let \( \Delta(x) = R\left(\Phi(x, z), \frac{\partial}{\partial z} \Phi(x, z), z\right) \). Suppose

(1) there exist positive numbers \( \rho \) and \( \pi = z(\rho) \), such that \( \rho \) is a root of the resultant \( \Delta(x) \) and \( (\rho, \pi) \) satisfies the system of equations,

(2.1) \( \Phi(\rho, \pi) = 0, \Phi_z(\rho, \pi) = 0, \)

(2) \( \Phi(x, z) \) satisfies the conditions:

(2.2) \( \Phi_x(\rho, \pi) \neq 0, \Phi_{zz}(\rho, \pi) \neq 0, \)

(3) \( z(x) \) is aperiodic, i.e., there exist three indices \( i < j < k \) such that \( z_iz_jz_k \neq 0 \) and \( \gcd(j - i, k - i) = 1. \)

Then \( \rho \) is the dominant singularity of \( z(x) \) and \( z(x) \) has the following expansion at \( \rho \)

(2.3) \( z(x) = \pi + \delta(\rho - x)^{-\frac{3}{2}} + O(\rho - x), \) for some nonzero constant \( \delta. \)

Furthermore the coefficients of \( z(x) \) satisfy

\[
[x^n]z(x) \sim c n^{-\frac{3}{2}} \rho^{-n}, \quad n \to \infty,
\]

for some constant \( c > 0. \)

The proof can be found in Flajolet and Sedgewick (2009) or alternatively in Hille (1962, pp. 103). The key point here is, that when applying Newton’s polygon method to determine the type of expansion of \( z(x) \): conditions (1) and (2) guarantee the first exponent of \( x \) to be \( \frac{1}{2} \). The asymptotics of the coefficients follows then from eq. (2.3) as an application of the transfer theorem \(1\) and theorem \(2\).
For a bivariate generating function $F(x, y)$, satisfying a functional equation, the next theorem allows us to obtain key information about the singular expansion of $F(x, y)$, considering the latter as a univariate generating function parameterized by $y$.

**Theorem 4** (Flajolet and Sedgewick [2009]). Let $F(x, y)$ be a bivariate function that is analytic at $(0, 0)$ and has non-negative coefficients. Suppose that $F(x, y)$ is one of the solutions $z$ of a polynomial equation

$$\Phi(x, y, z) = 0,$$

where $\Phi$ is a polynomial of degree $\geq 2$ in $z$, such that $\Phi(x, 1, z)$ satisfies the conditions of Theorem [A]. Let

$$\Delta(x, y) = R\left(\Phi(x, y, z), \frac{\partial}{\partial z}\Phi(x, y, z), z\right)$$

and let $\rho$ be the root of $\Delta(x, 1)$, such that $z(x) := F(x, 1)$ is singular at $x = \rho$ and $z(\rho) = \pi$. Suppose there exists some $\rho(y)$ being the unique root of the equation

$$\Delta(\rho(y), y) = 0,$$

analytic at 1 and such that $\rho(1) = \rho$.

Then $F(x, y)$ has the singular expansion

$$F(x, y) = \pi(y) + \delta(y) (\rho(y) - x)^{1/2} (1 + o(1)),$$

where $\pi(y)$ and $\delta(y)$ are analytic at 1 such that $\pi(1) = \pi$ and $\delta(1) \neq 0$. In addition we have

$$[x^n]F(x, y) = c(y) n^{-\frac{1}{2}} \rho(y)^{-n} \left(1 + O\left(\frac{1}{n}\right)\right),$$

uniformly for $y$ restricted to a small neighborhood of 1, where $c(y)$ is continuous and nonzero near 1.

Theorem 4 is implied by Proposition IX. 17 and Theorem IX. 12 of Flajolet and Sedgewick [2009].

We shall end this section by stating the following central limit theorem due to Bender [Bender, 1973]:

**Theorem 5** (Bender [1973]). Suppose we are given the bivariate generating function

$$f(x, u) = \sum_{n,t \geq 0} f(n, t) x^n u^t,$$

where $f(n, t) \geq 0$ and $f(n) = \sum_t f(n, t)$. Let $X_n$ be a r.v. such that $\mathbb{P}(X_n = t) = f(n, t)/f(n)$. Suppose

$$[x^n]f(x, e^s) = c(s) n^\alpha \gamma(s)^{-n} \left(1 + O\left(\frac{1}{n}\right)\right),$$
uniformly in $s$ in a neighborhood of 0, where $c(s)$ is continuous and nonzero near 0, $\alpha$ is a constant, and $\gamma(s)$ is analytic near 0.

Then there exists a pair $(\mu, \sigma)$ such that the normalized random variable

$$\bar{X}_n^* = \frac{X_n - \mu n}{\sqrt{n} \sigma},$$

converges in distribution to a Gaussian variable with a speed of convergence $O(n^{-\frac{1}{2}})$. That is we have

$$\lim_{n \to \infty} \mathbb{P}(\bar{X}_n^* < x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{x} e^{-\frac{1}{2}t^2} dt,$$

where $\mu$ and $\sigma^2$ are given by

$$\mu = -\frac{\gamma'(0)}{\gamma(0)} \quad \text{and} \quad \sigma^2 = \left(\frac{\gamma'(0)}{\gamma(0)}\right)^2 - \frac{\gamma''(0)}{\gamma(0)}.$$

### 3. RNA secondary structures

We consider secondary structures subject to minimum arc-length restrictions, arising from the rigidity of the backbone. RNA secondary structures having minimum arc-length two were studied by Waterman (Waterman, 1978). Arguably, the most realistic cases is $\lambda = 4$ (Stein and Waterman, 1979), and RNA folding algorithms, generating minimum free energy structures, implicitly satisfy this constraint for energetic reasons.

A stack of length $r$ is a maximal sequence of ”parallel” arcs, $((i, j), (i+1, j-1), \ldots, (i+(r-1), j-(r-1)))$. Stacks of length 1 are energetically unstable and we find typically stacks of length at least two or three in biological structures (Waterman, 1978). A secondary structure, $S$, is $r$-canonical if its stack-length satisfies $\geq r$. An RNA sequence is compatible with a structure $S$, if and only if for any $S$-arc $(i, j)$, the pair $(x_i, x_j)$ forms a base pair (Reidys et al., 1997).

A rainbow is an arc connecting the first and last vertices in a structure. A secondary structure is called reducible if it does not contain a rainbow. Let $s^{[r]}(n)$ and $t^{[r]}(n)$ denote the numbers of $r$-canonical secondary structures and reducible secondary structures of $n$ nucleotides with minimum arc-length $\lambda$, respectively. Let furthermore $s^{[r]}(n, l)$ and $t^{[r]}(n, l)$ denote the numbers of $r$-canonical secondary structures and reducible secondary structures, filtered by the number of arcs. Let $S^{[r]}_\lambda(x, y) = \sum_{n,l} s^{[r]}(n, l)x^ny^l$ and $T^{[r]}_\lambda(x, y) = \sum_{n,l} t^{[r]}(n, l)x^ny^l$ denote the corresponding bivariate generating functions.

First we compute the generating function $S^{[r]}_\lambda(x, y)$.

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1each hairpin loop contains at least three unpaired bases
Theorem 6. For any $\lambda, r \in \mathbb{N}$, the generating function $S_\lambda^r(x,y)$ satisfies the functional equation

\[(3.1) \quad (x^2y)^r S_\lambda^r(x,y)^2 - B_\lambda^r(x,y) S_\lambda^r(x,y) + A_\lambda^r(x,y) = 0,\]

where

\[
A_\lambda^r(x,y) = 1 - x^2y + (x^2y)^r,
\]

\[
B_\lambda^r(x,y) = (1 - x) A_\lambda^r(x,y) + (x^2y)^r \sum_{i=0}^{\lambda-2} x^i.
\]

Explicitly, we have

\[
S_\lambda^r(x,y) = \frac{B_\lambda^r(x,y) - \sqrt{B_\lambda^r(x,y)^2 - 4(x^2y)^r A_\lambda^r(x,y)}}{2(x^2y)^r},
\]

\[
S_\lambda^r(x,y) = \frac{A_\lambda^r(x,y)}{B_\lambda^r(x,y)} C \left( \frac{(x^2y)^r A_\lambda^r(x,y)}{B_\lambda^r(x,y)^2} \right),
\]

where $C(x)$ denotes the generating function for the Catalan numbers and is given by $\frac{1 - \sqrt{1 - 4x}}{2x}$. In particular, for $r = 1$,

\[(3.3) \quad s_\lambda^1(n, l) = \sum_{k=1}^{l} N(l, k) \binom{n - (\lambda - 1)k}{2l},\]

where $N(l, k) = \frac{1}{l} \binom{l}{k} \binom{l-1}{k-1}$ is the Narayana number, counting the number of plane trees of $l$ edges with $k$ leaves.

Proof. First we derive a functional equation satisfied by $S_\lambda^r(x,y)$ and $T_\lambda^r(x,y)$ via a decomposition for an $r$-canonical secondary structure with minimum arc-length $\lambda$. Any given structure must belong to one of the following three classes:

1. the empty structure it corresponds to the coefficient 1;
2. the collection of structures, starting with an unpaired vertex, are counted by $x S_\lambda^r(x,y)$;
3. structures starting with a paired vertex induce a maximum stack containing this base pair, and two segments, one being the nested substructure and the other the concatenated substructure. An arc corresponds to $x^2y$ and a stack of size at least $r$ corresponds to $\frac{(x^2y)^r}{1-x^2y}$. The nested segment must be reducible and contains at least $\lambda - 1$ vertices, as a result of the arc-length restriction. Since any segment with at most $\lambda - 2$ vertices corresponds to the term $\sum_{i=0}^{\lambda-2} x^i$, the
The decomposition of secondary structure and irreducible structure (reducible structures are colored in blue).

Fig. 3. The decomposition of secondary structure and irreducible structure (reducible structures are colored in blue).

The nested segment gives rise to $T^{[r]}_\lambda(x, y) - \sum_{i=0}^{\lambda-2} x^i$. The concatenated segment gives rise to the term $S^{[r]}_\lambda(x, y)$. Thus we arrive at

$$\frac{(x^2 y)^r}{1 - x^2 y} S^{[r]}_\lambda(x, y) \left( T^{[r]}_\lambda(x, y) - \sum_{i=0}^{\lambda-2} x^i \right).$$

The decomposition is illustrated in Fig. 3. Accordingly we obtain the functional equation:

\begin{equation}
S^{[r]}_\lambda(x, y) = 1 + x S^{[r]}_\lambda(x, y) + \frac{(x^2 y)^r}{1 - x^2 y} S^{[r]}_\lambda(x, y) \left( T^{[r]}_\lambda(x, y) - \sum_{i=0}^{\lambda-2} x^i \right).
\end{equation}

Now we decompose an irreducible secondary structure deriving a second functional equation, relating $S^{[r]}_\lambda(x, y)$ and $T^{[r]}_\lambda(x, y)$. Given an irreducible structure, it can decomposed as a maximum stack containing its rainbow and the nested substructure, see Fig. 3. The nested segment has to be reducible and has length at least $\lambda - 1$, i.e. $T^{[r]}_\lambda(x, y) - \sum_{i=0}^{\lambda-2} x^i$. The irreducible structures have the generating function $S^{[r]}_\lambda(x, y) - T^{[r]}_\lambda(x, y)$, whence

\begin{equation}
S^{[r]}_\lambda(x, y) - T^{[r]}_\lambda(x, y) = \frac{(x^2 y)^r}{1 - x^2 y} \left( T^{[r]}_\lambda(x, y) - \sum_{i=0}^{\lambda-2} x^i \right).
\end{equation}

Solving eq. (3.5) for $T^{[r]}_\lambda(x, y)$ and substituting the solution into eq. (3.4), we obtain eq. (3.1) which immediately implies eqs. (3.2). It remains to prove eq. (3.3). Given a secondary structure $S$ having $n$ vertices and $l$ arcs, removing any unpaired vertices induces the diagram $S'$, i.e. a matching. Suppose $S'$ has $k$ 1-arcs. It is well-known that the set of matchings with $l$ arcs and $k$ 1-arcs corresponds to the set of plane trees of $l$ edges with $k$ leaves, which is counted by the Narayana number $N(l, k) = \frac{1}{l+1} \binom{l+1}{k}$ (see e.g. Stanley (2001)). In view of minimum arc-length constraints, in order to recover $S$ from $S'$, we need to guarantee that each 1-arc contains at least $\lambda - 1$ unpaired vertices. Accordingly, the number of all possible insertions is given by $\binom{n-(\lambda-1)k}{2l}$, i.e eq. (3.3).

Remark: Eq. (3.3) generalizes the closed formula $s_2^{[1]}(n, l) = \frac{1}{l+1} \binom{n-l}{l-1} \binom{n-l-1}{l-1}$ proved in Schmitt and Waterman (1994) by establishing a bijection between secondary structures and linear trees.
Interpreting the indeterminant $y$ as a parameter, we consider $S^{[r]}_{\lambda}(x, y)$ as a univariate powerseries. One key question then is to compute the asymptotic behaviour of the coefficients.

**Theorem 7.** For $1 \leq \lambda \leq 4$ and $1 \leq r \leq 3$, the coefficients of $S^{[r]}_{\lambda}(x, y)$ are asymptotically given by

$$[x^n]S^{[r]}_{\lambda}(x, y) = c^{[r]}(y)n^{-\frac{3}{2}}(\rho^{[r]}(y))^{-n} (1 + O(n^{-1})).$$

As $n \to \infty$, uniformly, for $y$ restricted to a small neighborhood of 1, where $c^{[r]}(y)$ is continuous and nonzero near 1, and $\rho^{[r]}(y)$ is the minimal positive, real solution of $B^{[r]}_{\lambda}(x, y)^2 - 4(x^2y)^2A^{[r]}(x, y) = 0$, for $y$ in a neighborhood of 1.

In particular, we have

$$\rho^{[r]}_1(y) = \frac{1 - 2\sqrt{y}}{1 - 4y}, \quad \rho^{[r]}_2(y) = \frac{1 + 2\sqrt{y} - \sqrt{1 + 4\sqrt{y}}}{2y}.$$

**Proof.** We shall employ Theorem 4.

**Step 1:** we consider first the monovariate power series $S^{[r]}_{\lambda}(x, 1)$:

In view of eq. (3.11) of Theorem 3 and setting $y = 1$, we see that $S^{[r]}_{\lambda}(x, 1)$ satisfies the equation

$$\Phi(x, S^{[r]}_{\lambda}(x, 1)) = x^{2r}S^{[r]}_{\lambda}(x, y)^2 - B^{[r]}_{\lambda}(x, 1)S^{[r]}_{\lambda}(x, 1) + A^{[r]}(x, 1) = 0,$$

where $\Phi(x, z) = x^{2r}z^2 - B^{[r]}_{\lambda}(x, 1)z + A^{[r]}(x, 1)$. Next we observe that

$$\Delta(x) = R \left( \Phi(x, z), \frac{\partial}{\partial z} \Phi(x, z) \right) = -x^{2r}(B^{[r]}_{\lambda}(x, 1)^2 - 4x^{2r}A^{[r]}(x, 1)),$$

where $R(f, g, z)$ is the resultant of two polynomials $f(z) = a_n \prod_{i=1}^n (z - \alpha_i)$ and $g(z) = b_m \prod_{j=1}^m (z - \beta_j)$, $R(f, g, z) = a_n b_m \prod_{i=1}^n \prod_{j=1}^m (\alpha_i - \beta_j)$.

**Ad (1),** we inspect for $1 \leq \lambda \leq 4$ and $1 \leq r \leq 3$, that there exist positive numbers $\rho^{[r]}_{\lambda}$ and $\pi^{[r]}_{\lambda} = S^{[r]}_{\lambda}(\rho^{[r]}_{\lambda}, 1)$, such that $\rho^{[r]}_{\lambda}$ is a root of the resultant $\Delta(x)$ and $(\rho^{[r]}_{\lambda}, \pi^{[r]}_{\lambda})$ and furthermore $\Phi(x, \rho^{[r]}_{\lambda}, \pi^{[r]}_{\lambda}) = 0$ as well as $\Phi_z(\rho^{[r]}_{\lambda}, \pi^{[r]}_{\lambda}) = 0$. In fact, $\rho^{[r]}_{\lambda}$ is the minimal positive real solution of the $\Delta(x)$-divisor $B^{[r]}_{\lambda}(x, 1)^2 - 4x^{2r}A^{[r]}(x, 1)$.

As for (2), we verify $\Phi_x(\rho^{[r]}_{\lambda}, \pi^{[r]}_{\lambda}) \neq 0$ and $\Phi_{zz}(\rho^{[r]}_{\lambda}, \pi^{[r]}_{\lambda}) \neq 0$.

**Ad (3),** for any $\lambda$ there exists some $i$, such that $s^{[r]}_{\lambda}(i), s^{[r]}_{\lambda}(i + 1), s^{[r]}_{\lambda}(i + 2) \neq 0$, i.e. $S^{[r]}_{\lambda}(x, 1)$ is aperiodic.

Theorem 3 implies that $\rho^{[r]}_{\lambda}$ is the dominant singularity and that the singular expansion of $S^{[r]}_{\lambda}(x, 1)$ is given by

$$S^{[r]}_{\lambda}(x, 1) = \pi^{[r]}_{\lambda} + \delta^{[r]}_{\lambda} (\rho^{[r]}_{\lambda} - x)^{\frac{1}{2}}(1 + o(1)),$$
where $\delta_{\lambda}^{[r]}$ is a positive real number.

**Step 2:** we are now in position to employ Theorem 4. By eq. (3.1), $S_{\lambda}^{[r]}(x, y)$ satisfies

$$(x^2 y)S_{\lambda}^{[r]}(x, y)^2 - B_{\lambda}^{[r]}(x, y) S_{\lambda}^{[r]}(x, y) + A^{[r]}(x, y) = 0$$

and $\Phi(x, y, z) = (x^2 y)z^2 - B_{\lambda}^{[r]}(x, y)z + A^{[r]}(x, y)$ is a polynomial in $z$ of degree two. $\Delta(x, y) = R(\Phi(x, y, z), \frac{\partial}{\partial z}\Phi(x, y, z), z)$ satisfies $\Delta(x, 1) = \Delta(x)$ and $\rho_{\lambda}^{[r]}$ is a root of $\Delta(x, 1)$. Step 1 implies that $z(x) = S(x, 1)$ has the unique dominant singularity $\rho_{\lambda}$ and $z(\rho) = \pi_{\lambda}$. We compute

$$\Delta(x, y) = R\left(\Phi(x, y, z), \frac{\partial}{\partial z}\Phi(x, y, z), z\right) = -(x^2 y)^r(B_{\lambda}^{[r]}(x, y)^2 - 4(x^2 y)^r A^{[r]}(x, y))$$

and check that there exists for $1 \leq \lambda \leq 4$ and $1 \leq r \leq 3$ a unique $\rho_{\lambda}^{[r]}(y)$, such that $\Delta(\rho_{\lambda}^{[r]}(y), y) = 0$ and $\rho_{\lambda}^{[r]}(1) = \rho_{\lambda}^{[r]}$. $\rho_{\lambda}^{[r]}(y)$ is the minimal, positive, real solution of $B_{\lambda}^{[r]}(x, y)^2 - 4(x^2 y)^r A^{[r]}(x, y) = 0$ for $y$ in a neighborhood of 1.

By Theorem 4 the singular expansion of $S_{\lambda}^{[r]}(x, y)$ is given by

$$S_{\lambda}^{[r]}(x, y) = \pi_{\lambda}^{[r]}(y) + \delta_{\lambda}^{[r]}(y)(\rho_{\lambda}^{[r]}(y) - x)^{\frac{1}{2}}(1 + o(1)),$$

where $\pi_{\lambda}^{[r]}(y)$ and $\delta_{\lambda}^{[r]}(y)$ are analytic at 1 and $\delta_{\lambda}^{[r]}(1)$ is a positive, real number and furthermore

$$[x^n]S_{\lambda}^{[r]}(x, y) = c_{\lambda}^{[r]}(y)n^{-\frac{1}{2}}\left(\rho_{\lambda}^{[r]}(y)\right)^{-n}(1 + O(n^{-1})).$$

We next analyze the random variable $Y_{\lambda,n}^{[r]}$, counting the numbers of arcs in RNA secondary structures. By construction we have

$$P(Y_{\lambda,n}^{[r]} = l) = \frac{s_{\lambda}^{[r]}(n, l)}{s_{\lambda}^{[r]}(n)},$$

where $l = 0, 1, \ldots, \lfloor \frac{n+1-\lambda}{2} \rfloor$.

Theorem 7 and Theorem 4 immediately imply

**Theorem 8.** There exists a pair $(\mu_{\lambda}^{[r]}, \sigma_{\lambda}^{[r]})$ such that the normalized random variable

$$y_{n,\lambda}^{[r],*} = \frac{Y_{\lambda,n}^{[r]} - \mu_{\lambda}^{[r]} n}{\sqrt{n(\sigma_{\lambda}^{[r]})^2}}$$
converges in distribution to a Gaussian variable with a speed of convergence $O(n^{-\frac{1}{2}})$. That is, we have

$$\lim_{n \to \infty} P\left( \frac{Y_{\lambda,n}^r - \mu_{\lambda}^r n}{\sqrt{n (\sigma_{\lambda}^r)^2}} < x \right) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{x} e^{-\frac{1}{2}t^2} dt,$$

where $\mu_{\lambda}^r$ and $\sigma_{\lambda}^r$ are given by

$$\mu_{\lambda}^r = -\frac{\theta'(0)}{\theta(0)}, \quad (\sigma_{\lambda}^r)^2 = \left( \frac{\theta'(0)}{\theta(0)} \right)^2 - \frac{\theta''(0)}{\theta(0)},$$

where $\theta(s) = \rho_{\lambda}^r(e^s)$.

In Table 1 we list the values of $\mu_{\lambda}^r$ and $(\sigma_{\lambda}^r)^2$ for $1 \leq \lambda \leq 4$ and $1 \leq r \leq 3$.

**Remark.** The expectation and variance of the number of arcs in secondary structures has been studied computationally in Fontana et al. (1993) for mfe structures and combinatorially in Hofacker et al. (1998) for 1-canonical structures with minimum arc-length $\lambda$. In Jin and Reidys (2008) more general results on local and global limit theorems for $k$-noncrossing structures are proved. These imply, setting $k = 2$, Theorem 8. The above approach, in light of the combinatorial proof of the recursion in Theorem 6, provides an elementary derivation.

**Table 1.** The central limit theorem for the number of arcs. We list the values of $\mu_{\lambda}^r$ and $(\sigma_{\lambda}^r)^2$ derived from eq. (3.8).

| $r$ = 1 | $r$ = 2 | $r$ = 3 |
|---------|---------|---------|
| $\mu_{\lambda}^r$ | $(\sigma_{\lambda}^r)^2$ | $\mu_{\lambda}^r$ | $(\sigma_{\lambda}^r)^2$ | $\mu_{\lambda}^r$ | $(\sigma_{\lambda}^r)^2$ |
| $\lambda$ = 1 | 0.3333 | 0.0556 | 0.3484 | 0.0719 | 0.3582 | 0.0852 |
| $\lambda$ = 2 | 0.2764 | 0.0447 | 0.3172 | 0.0643 | 0.3364 | 0.0791 |
| $\lambda$ = 3 | 0.2500 | 0.0442 | 0.2983 | 0.0631 | 0.3215 | 0.0778 |
| $\lambda$ = 4 | 0.2367 | 0.0469 | 0.2865 | 0.0651 | 0.3113 | 0.0793 |

Theorem 8 follows from Theorem 7 and Theorem 4, setting $f(x, e^s) = S_{\lambda}^r(x, e^s)$.

We display the distribution of the numbers of arcs for sequence length $n = 400$ in Fig. 4, observing that the expected number of arcs drops from 111 to 95, when increasing the minimum arc-length from 2 to 4.
4. Main results

4.1. Structures having Purine-Pyrimidine base pairs. We consider RNA structures, $S$, over sequences having only two types of nucleobases: purines ($R$) and pyrimidines ($Y$), having the base pairs $(R, Y)$, having the base pairs $(R, Y)$ and $(Y, R)$.

For any $p \in [0, 1]$ and $n \in \mathbb{N}$, let $s^{[r]}_{p,\lambda}(n)$ denote the number of $r$-canonical RNA secondary structures for which there exists at least one compatible sequence, containing $\lfloor pn \rfloor$ purine bases and $\lceil (1 - p)n \rceil$ pyrimidine bases, respectively. Let furthermore $s^{[r]}_{p,\lambda}(n, l)$ denote the corresponding number of structures, filtered by the number of arcs.

Remark 1. In view of $\lfloor pn \rfloor / n = p + O(n^{-1})$, $s^{[r]}_{p,\lambda}(n)$ or $s^{[r]}_{p,\lambda}(n, l)$, count in the limit of large sequence lengths, secondary structures of sequences whose percentage of purine bases equals $p$.

Proposition 1. For any $n, r, l, \lambda \in \mathbb{N}$ and $p \in [0, 1[$, we have

\[
\begin{align*}
  s^{[r]}_{p,\lambda}(n, l) &= s^{[r]}_{\lambda}(n, l) & \text{if } l \leq \min(\lfloor pn \rfloor, \lceil (1 - p)n \rceil), \\
  s^{[r]}_{p,\lambda}(n, l) &= 0 & \text{if } l > \min(\lfloor pn \rfloor, \lceil (1 - p)n \rceil),
\end{align*}
\]
and

\( s_{p,\lambda}^{[r]}(n) = \sum_{l=0}^{\min([pn],[1-(1-p)n])} s_{\lambda}^{[r]}(n, l). \) \tag{4.2} 

**Proof.** We shall show that any such structure having \( l \) arcs is counted by \( s_{p,\lambda}^{[r]}(n, l) \) iff \( l \leq [pn] \) and \( l \leq [(1-p)n] \). Given a structure \( S \) having \( l \) arcs, each arc corresponds to a pair \((R, Y)\) or \((Y, R)\) for any compatible sequence, while each unpaired vertex could be either \( R \) or \( Y \). Accordingly, in any compatible sequence, the number of purine bases is at least \( l \). For \( S \) to be counted by \( s_{p,\lambda}^{[r]}(n, l) \) the existence of \( l \) arcs thus implies \( l \leq [pn] \).

Since we have \([ (1-p)n ] \) pyrimidine bases in such a sequence, we observe for the same reason \( l \leq [(1-p)n] \). In case of \( l > \min([pn], [(1-p)n]) \) we either have not enough purine or pyrimidine bases, whence \( s_{p,\lambda}^{[r]}(n, l) = 0 \) and the proposition follows. \( \square \)

**Theorem 9.** For \( 1 \leq \lambda \leq 4, 1 \leq r \leq 3, p \in [0, \frac{1}{2}] \) and \( n \in \mathbb{N} \), we have

\( s_{p,\lambda}^{[r]}(n) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{(p-\mu^{[r]}_{\lambda})\sigma^{[r]}_{\lambda}/\sqrt{\pi}} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}), \) \tag{4.3} 

where \( \mu^{[r]}_{\lambda}, \sigma^{[r]}_{\lambda} \) are the mean and standard deviation of \( \mathbb{Y}_{\lambda,n}^{[r]} \), respectively.

Equivalently, for \( p \in [\mu^{[r]}_{\lambda}, \frac{1}{2}] \), a random structure a.a.s. has a compatible sequence with nucleotide ratio \( p \). Conversely, in case of \( p \in [0, \mu^{[r]}_{\lambda}] \), a.a.s. no random structure has a compatible sequence.

**Proof.** For \( p \in [0, \frac{1}{2}] \), we have

\[ \frac{s_{p,\lambda}^{[r]}(n)}{s_{\lambda}^{[r]}(n)} = \sum_{l=0}^{\min([pn],[1-(1-p)n])} \frac{s_{\lambda}^{[r]}(n, l)}{s_{\lambda}^{[r]}(n)} = \sum_{l=0}^{[pn]} \frac{s_{\lambda}^{[r]}(n, l)}{s_{\lambda}^{[r]}(n)} = \mathbb{P}(\mathbb{Y}_{\lambda,n}^{[r]} \leq [pn]), \]

where the first equation employs eq. \((4.1)\). Theorem \( 8 \) allows us to estimate \( \mathbb{P}(\mathbb{Y}_{\lambda,n}^{[r]} \leq [pn]) \) by the integral of the Gaussian density function with an \( O(n^{-\frac{1}{2}}) \) error term. Specifically, setting \( x = \frac{[pn]-\mu^{[r]}_{\lambda}}{\sigma^{[r]}_{\lambda}/\sqrt{\pi}} \) in eq. \((3.7)\), we obtain

\[ \mathbb{P}(\mathbb{Y}_{\lambda,n}^{[r]} \leq [pn]) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\frac{[pn]-\mu^{[r]}_{\lambda}}{\sigma^{[r]}_{\lambda}/\sqrt{\pi}}} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\frac{(p-\mu^{[r]}_{\lambda})\sigma^{[r]}_{\lambda}/\sqrt{\pi}}{\sqrt{\pi}}} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}), \]
where the last equality is implied by

\[
\left| \int_{-\infty}^{\infty} e^{-\frac{t^2}{2\sigma^2}} dt - \int_{-\infty}^{\frac{\mu_n - \mu}{\sigma_n \sqrt{n}}} e^{-\frac{t^2}{2\sigma^2}} dt \right| \leq \frac{1}{\sigma^2} \cdot e^{-\frac{t^2}{2}} \bigg|_{t=0} = O(n^{-\frac{1}{2}}),
\]

whence eq. (4.3). For \( p \in \mu_{\lambda}, \frac{1}{2} \)

\[\frac{s_{p,\lambda}^{[r]}(n)}{s_{\lambda}^{[r]}(n)} \geq \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} e^{-\frac{t^2}{2\sigma^2}} dt + O(n^{-\frac{1}{2}}) \to 1, \quad \text{as } n \to \infty,\]

implying that asymptotically, almost surely any structure has a compatible sequence with nucleotide ratio \( p \) and the theorem follows. \( \square \)

For any Gaussian distributed random variable, \( X \), we have

\[
\mathbb{P}(\mu - 2\sigma \leq X \leq \mu + 2\sigma) = \frac{1}{\sqrt{2\pi}} \int_{\mu-2\sigma}^{\mu+2\sigma} e^{-\frac{(t-\mu)^2}{2\sigma^2}} \approx 0.9545,
\]

\[
\mathbb{P}(\mu - 3\sigma \leq X \leq \mu + 3\sigma) = \frac{1}{\sqrt{2\pi}} \int_{\mu-3\sigma}^{\mu+3\sigma} e^{-\frac{(t-\mu)^2}{2\sigma^2}} \approx 0.9973,
\]

i.e. the fraction of its values within two and three standard deviations away from its mean is approximately 0.9545 and 0.9973, respectively. In Fig. 5 we display the limit distribution.

### 4.2. Watson-Crick base pairs.

We next consider RNA secondary structures realized by sequences composed by the four nucleotides \( \text{A, U, C, G} \), assuming Watson-Crick (\( \text{A-U, C-G} \)) base pairs.

For any \( n \in \mathbb{N} \) and \( p = (p_1, p_2, p_3, p_4) \), where \( p_i \in [0, 1] \) and \( \sum_{i=1}^{4} p_i = 1 \), let \( s_{p,\lambda}^{[r]}(n) \) denote the number of \( r \)-canonical RNA secondary structures for which there exists at least one compatible \( \text{AUCCG} \)-sequence containing \( [p_1 n], [p_2 n], [p_3 n] \), and \( n - [p_1 n] - [p_2 n] - [p_3 n] \) nucleotides, respectively. Let \( s_{p,\lambda}^{[r]}(n, l) \) be defined analogously. Obviously, as observed for two letter sequences, as the sequence length approaches infinity,

\[
\left( \frac{[p_1 n]}{n}, \frac{[p_2 n]}{n}, \frac{[p_3 n]}{n}, \frac{n - ([p_1 n] + [p_2 n] + [p_3 n])}{n} \right) = (p_1, p_2, p_3, p_4) + O(n^{-1}).
\]

**Proposition 2.** For any \( n, r, l, \lambda \in \mathbb{N} \) and \( p \), let

\[l_0 = \min([p_1 n], [p_2 n]) + \min([p_3 n], n - ([p_1 n] + [p_2 n] + [p_3 n])).\]
Fig. 5. LHS: $\frac{s^{[r]}_p(n)}{s^{[r]}_\lambda(n)}$ as a function of $p$ for GC-sequences of length $n = 400$ and 3-canonical structures subject to minimum arc-length restrictions $\lambda = 2$ (green), 3 (blue) and 4 (red), respectively. RHS: $\frac{s^{[r]}_{p,\lambda}(n)}{s^{[r]}_\lambda(n)}$ as a function of $p$ for GC-sequences of length $n = 400$ and $r$-canonical structures with minimum arc-length 4 subject to minimum stack-length restrictions $r = 1$ (green), 2 (blue) and 3 (red), respectively.

Then we have

\begin{equation}
\begin{align*}
  s^{[r]}_{p,\lambda}(n, l) &= s^{[r]}_\lambda(n, l) & \text{if } l \leq l_0, \\
  s^{[r]}_{p,\lambda}(n, l) &= 0 & \text{otherwise},
\end{align*}
\end{equation}

and

\begin{equation}
  s^{[r]}_{p,\lambda}(n) = \sum_{l=0}^{l_0} s^{[r]}_{p,\lambda}(n, l).
\end{equation}

Proof. We prove that any structure having $l$ arcs is counted by $s^{[r]}_{p,\lambda}(n, l)$ if $l \leq l_0$. Given a structure $S$ having $l$ arcs, each arc corresponds to an A-U pair or a C-G pair for any compatible sequence, while each unpaired vertex could be either one of the four bases. Suppose we have $l_1$ A-U and $l_2$ C-G base pairs. Then $l_1 \leq \lfloor p_1 n \rfloor$ and since $\lfloor p_2 n \rfloor$ is the number of U nucleotides, we have $l_1 \leq \lfloor p_2 n \rfloor$. The case of C-G pairs is treated analogously. Thus if $l \leq l_0$ $S$ is counted by $s^{[r]}_{p,\lambda}(n, l)$. We inspect that then for any $l' \leq l$ any structure containing $l'$ arcs is counted by $s^{[r]}_{p,\lambda}(n, l)$.
We thus arrive at the following integer linear programming problem:

maximize: \[ l = l_1 + l_2 \]
subject to \[ l \leq |p_1 n|, l_1 \leq |p_2 n|, l_2 \leq |p_3 n|, l_2 \leq n - (|p_1 n| + |p_2 n| + |p_3 n|) \]
and \[ l_1 \geq 0, l_2 \geq 0, l_1, l_2 \in \mathbb{Z}. \]

Here \( l_0 = \min(|p_1 n|, |p_2 n|) + \min(|p_3 n|, n - (|p_1 n| + |p_2 n| + |p_3 n|)) \) is a solution that is by construction unique. In case of \( l > l_0, l \) is of the form \( l = l_1 + l_2 \) such that \( l_1 \geq |p_1 n| \) or \( l_1 \geq |p_2 n| \) or \( l_2 \geq |p_3 n| \), or \( l_2 \geq n - (|p_1 n| + |p_2 n| + |p_3 n|) \). This means however, that we have not enough of one type of the four bases to satisfy all \( l \) base pairings of the structure, whence \( s_{p_\lambda}^r(n, l) = 0 \).

\[ \square \]

**Theorem 10.** Given any \( 1 \leq \lambda \leq 4 \), \( 1 \leq r \leq 3 \), any \( n \in \mathbb{N} \) and \( p \), let \( p_0 = \min(p_1, p_2) + \min(p_3, p_4) \). Then we have

\[
(4.6) \quad \frac{s_{p_\lambda}^r(n)}{s_{\lambda}^r(n)} = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{(p_0 - \mu_{\lambda}^r)n/\sigma_{\lambda}^r} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}),
\]

where \( \mu_{\lambda}^r, \sigma_{\lambda}^r \) are the mean and standard deviation of \( \mathbb{Y}_{\lambda,n}^r \).

Equivalently, for \( p_0 \in (\mu_{\lambda}^r, \frac{1}{2}] \), a random structure a.a.s. has a compatible sequence with nucleotide ratio \( p \). Conversely, in case of \( p_0 \in [0, \mu_{\lambda}^r] \), a.a.s. no random structure has a compatible sequence.

**Proof.** Given \( p = (p_1, p_2, p_3, p_4) \), analogous to the proof of Theorem 9, we derive by eq. (4.4)

\[
\frac{s_{p_\lambda}^r(n)}{s_{\lambda}^r(n)} = \sum_{l=0}^{l_0} \frac{s_{\lambda}^r(n, l)}{s_{\lambda}^r(n)} = \mathbb{P}(\mathbb{Y}_{\lambda,n}^r \leq l_0) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{(l_0 - \mu_{\lambda}^r)n/\sigma_{\lambda}^r} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\frac{(l_0 - \mu_{\lambda}^r)n}{\sigma_{\lambda}^r\sqrt{n}}} e^{-\frac{t^2}{2}} dt + O(n^{-\frac{1}{2}}),
\]

where the last equality is implied by the inspecting \( |l_0 - p_0 n| \leq 2 \) and

\[
\left| \int_{-\infty}^{(l_0 - \mu_{\lambda}^r)n/\sigma_{\lambda}^r\sqrt{n}} e^{-\frac{t^2}{2}} dt - \int_{-\infty}^{\frac{(l_0 - \mu_{\lambda}^r)n}{\sigma_{\lambda}^r\sqrt{n}}} e^{-\frac{t^2}{2}} dt \right| = \left| \int_{\frac{(l_0 - \mu_{\lambda}^r)n}{\sigma_{\lambda}^r\sqrt{n}}}^{\frac{(l_0 - \mu_{\lambda}^r)n}{\sigma_{\lambda}^r\sqrt{n}}} e^{-\frac{t^2}{2}} dt \right| \leq \frac{2}{\sigma_{\lambda}^r\sqrt{n}} \cdot e^{-\frac{1}{2}} \left| t=0 \right| = O(n^{-\frac{1}{2}}),
\]

whence eq. (4.6) and the theorem is proved. \( \square \)
4.3. **Watson-Crick and wobble base pairs.** We consider RNA secondary structures over four letter sequences, having Watson-Crick (A-U, C-G) and wobble (U-G) base pairs.

For any $n \in \mathbb{N}$ and $p = (p_1, p_2, p_3, p_4)$, where $p_i \in [0,1]$ and $\sum_{i=1}^{4} p_i = 1$, let $s^{[r]}_{p,\lambda}(n)$ denote the number of RNA secondary structures for which there exists at least one compatible AUCG-sequence containing $[p_1n], [p_2n], [p_3n]$, and $n - [p_1n] - [p_2n] - [p_3n]$ nucleotides, respectively. Let $\bar{s}^{[r]}_{p,\lambda}(n, l)$ be this quantity, filtered by the number of arcs.

**Proposition 3.** For any $n, r, l, \lambda \in \mathbb{N}$ and $p$, let

$$\bar{l}_0 = \min\{\min\{[p_1n], [p_2n]\} + n - ([p_1n] + [p_2n] + [p_3n]), \min\{[p_3n], n - ([p_1n] + [p_2n] + [p_3n])) + [p_2n]\}.$$

Then we have

$$\bar{s}^{[r]}_{p,\lambda}(n, l) = s^{[r]}_{\lambda}(n, l) \quad \text{if } l \leq \bar{l}_0,$$

and

$$\bar{s}^{[r]}_{p,\lambda}(n, l) = 0 \quad \text{otherwise,}$$

and

$$\bar{s}^{[r]}_{p,\lambda}(n) = \sum_{l=0}^{\bar{l}_0} \bar{s}^{[r]}_{p,\lambda}(n, l).$$

**Proof.** We first prove that any structures having $l$ arcs are counted by $\bar{s}^{[r]}_{p,\lambda}(n, l)$ if $l \leq \bar{l}_0$.

Suppose we have $l_1$ A-U pairs, $l_2$ C-G pairs and $l_3$ U-G pairs. In order to be counted by $\bar{s}^{[r]}_{p,\lambda}(n, l)$, the number of U is at least $l_1 + l_3$, i.e. we have, $l_1 + l_3 \geq [p_2n]$ and since we have $[p_1n]$ A nucleotides $l_1 \leq [p_1n]$. The situation for C and G is analogous. As long as $l_1, l_2$ and $l_3$ satisfy these conditions, we can always construct a compatible p-sequence for a structure $S$ having $l = l_1 + l_2 + l_3$ arcs. Clearly, this holds for any $l' < l$, whence a structure having $l$ arcs is counted by $\bar{s}^{[r]}_{p,\lambda}(n, l)$ if the number of arcs is not greater than the maximum of $l_1 + l_2 + l_3$ subject to the constraints of the following integer linear programming problem:

**maximize:** $\quad l = l_1 + l_2 + l_3$

**subject to** $\quad l_1 \leq [p_1n], l_1 + l_3 \leq [p_2n], l_2 \leq [p_3n], l_2 + l_3 \leq n - ([p_1n] + [p_2n] + [p_3n])$

and $\quad l_1, l_2, l_3 \geq 0, l_1, l_2, l_3 \in \mathbb{Z}$.

Its unique solution is

$$\bar{l}_0 = \min\{\min\{[p_1n], [p_2n]\} + n - ([p_1n] + [p_2n] + [p_3n]), \min\{[p_3n], n - ([p_1n] + [p_2n] + [p_3n])) + [p_2n]\}.$$
and in case of \( l > \bar{l}_0 \), we observe as in Proposition 2 that we have not enough of at least one of the four nucleotide types, whence \( \bar{s}_p \bar{\lambda}(n, l) = 0 \) and the proposition follows. \( \square \)

As a result we derive

**Theorem 11.** For \( 1 \leq \lambda \leq 4, 1 \leq r \leq 3, n \in \mathbb{N} \) and \( p \), let \( \bar{p}_0 = \min(\min(p_1, p_2) + p_4, \min(p_3, p_4) + p_2) \). Then we have

\[
\frac{\bar{s}_p \bar{\lambda}(n)}{s_\lambda^{(r)}(n)} = \frac{1}{2\pi} \int_{-\infty}^{(\bar{p}_0 - \mu_\lambda^{(r)}) / \sigma_\lambda^{(r)}} e^{-\frac{t^2}{2}} \, dt + O(n^{-\frac{1}{2}}),
\]

where \( \mu_\lambda^{(r)}, \sigma_\lambda^{(r)} \) are the mean and standard deviation of \( \bar{Y}_{\lambda, n} \).

Equivalently, for \( \bar{p}_0 \in [\mu_\lambda^{(r)}, \frac{1}{2}] \), a random structure a.a.s. has a compatible sequence with nucleotide ratio \( p \). Conversely, in case of \( \bar{p}_0 \in ]0, \mu_\lambda^{(r)}[ \), a.a.s. no random structure has a compatible sequence.

**Proof.** The proof is analogous to that of Theorem 10, the only difference being that \(|\bar{l}_0 - \bar{p}_0 n| \leq 2\). \( \square \)

In Fig. 1 from Supplementary Material, we display \( \bar{s}_p \bar{\lambda}(n) \) as a function of \( p_1 \) and \( p_3 \), employing the average AUCG-ratios found in RNA databases. We contrast the cases of Watson-Crick and Watson-Crick together with wobble base pairs.

5. Discussion

In this section we discuss our findings in the context of nucleotide percentages observed in RNA databases (Berman et al., 2000) and provide some implications of our results. Using the data from the RCSB PDB database (Berman et al., 2000), we list in Table 2 the average nucleotide ratios for several classes of RNA and the corresponding values of \( p_0 \) and \( \bar{p}_0 \) (see Theorem 10 and Theorem 11).

The observed average AUCG-ratio of all RNA families is \((0.208, 0.200, 0.271, 0.321)\) facilitating according to Theorem 10 and Theorem 11 the formation of asymptotically almost all structures, see Fig. 1 from Supplementary Material.

Table 2 shows, that the average ratio for mRNAs is significantly different from that of other families and can thus be used as a discriminat for the mRNAs that can easily be obtained at the time of sequencing. With a ratio of \( A \) being 0.412 and a 2 : 1 ratio of \( G \) to \( C \) the mRNA family exhibits particular nucleotide ratios making it more difficult to form configurations having low free energies. For mRNA, \( p_0 \) and \( \bar{p}_0 \) are relatively
close to the critical value $\mu_4^{[3]} = 0.3113$ which motivates to look in more detail at mRNA structures, in particular in the context of mRNA redesign with the objective to achieve a mfe-structure without affecting the induced amino acid sequence (Gaspar et al., 2013). Buratti and Baralle (2004) formulates the hypothesis that pre-mRNA molecules behaved essentially as a linear structure, or “tape” so to speak. In particular, our results show that pre-mRNAs with $p_0 < \mu_4^{[3]}$ almost surely have a linear structure.

We observe that for mRNAs whose critical values $p_0$ are greater than $\mu_4^{[3]}$, fold. For instance, mRNA HP210 (Mahen et al., 2010) with $p_0 = \bar{p}_0 = 0.45$ folds into several distinct confirmations and interchanges them quickly (in vivo).

mRNA sequences with $p_0 < \mu_4^{[3]}$, have a diminished capability of folding and typically interact with other biopolymers: for instance, the two synthetic messenger RNAs

$5'$-UUUUUUUUUUUUUUUGGCAAGGAGGUUUUUUUUUUUUUUUUU-3',

having $p_0 = 0.08$, $\bar{p}_0 = 0.18$ and

$5'$-GGCAAGGAGGUAUUUGAAAAUGAAAAAA-3',

having $p_0 = \bar{p}_0 = 0.11$ are used to study the interaction of mRNA with the ribosome at different states of translation (Yusupova et al., 2006). During the translation initiation these mRNAs combine with 16s ribosomal RNA, forming ribosome complexes, see Fig. 6.

A second instance of such interactions is reported in Rozov et al. (2015). The messenger RNA

$5'$-GGCAAGGAGGUAUUUGAAAAUGAAAAAA-3',

($p_0 = \bar{p}_0 = 0.133$), pairs, as a result of a G-U mismatch, with an incorrect tRNA and as a result produces an incorrect amino acid.

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**Table 2.** The average nucleotide ratios and the values of $p_0$, $\bar{p}_0$ for different families of RNA structures. The theoretical value with whom $p_0$ and $\bar{p}_0$ have to be compared is $\mu_4^{[3]} = 0.3113$.

|        | A | U | C | G | $\sigma^2$ | $p_0$  | $\bar{p}_0$ |
|--------|---|---|---|---|-----------|------|------|
| mRNA   | 0.412 | 0.265 | 0.110 | 0.213 | 0.092 | 0.375 | 0.375 |
| tRNA   | 0.189 | 0.201 | 0.292 | 0.318 | 0.004 | 0.481 | 0.493 |
| 5S ribosomal RNA | 0.208 | 0.165 | 0.304 | 0.323 | 0.006 | 0.470 | 0.470 |
| 16S ribosomal RNA | 0.214 | 0.165 | 0.268 | 0.353 | 0.004 | 0.433 | 0.433 |
| 23S ribosomal RNA | 0.246 | 0.180 | 0.244 | 0.330 | 0.006 | 0.424 | 0.424 |
| Bacteria | 0.238 | 0.197 | 0.247 | 0.317 | 0.010 | 0.444 | 0.444 |
| Eukaryota | 0.232 | 0.237 | 0.238 | 0.293 | 0.021 | 0.470 | 0.475 |
| Viruses | 0.182 | 0.205 | 0.298 | 0.315 | 0.015 | 0.480 | 0.497 |
| RNA    | 0.208 | 0.200 | 0.271 | 0.321 | 0.052 | 0.471 | 0.471 |
Fig. 6. Interactions between mRNAs and 16s ribosomal RNA.

Of course nucleotide ratios are a rather coarse criterion: within sequences of a fixed nucleotide ratio structural conformations depend on additional factors: Seffens and Digby (1999) showed that mRNAs from databases have lower mfe than random RNA sequences of the same nucleotide ratio. Since mfe critically depends on the stacking of base pairs and loops, Workman and Krogh (1999) was able to show that random RNA sequences, generated with the same dinucleotide ratio, have not significantly higher mfe values. Accordingly, the dinucleotide ratio is the adequate equivalence concept when studying mfe configurations.

Irrespective of RNA family, if for an RNA sequence $p_0$ and $\bar{p}_0$ are smaller than $\mu^{[3]}_4 = 0.3113$, it is likely that it interacts with other biopolymers. In the following we list several instances of this phenomenon, see Fig. 7.

1. RNAs found in the bluetongue virus (Diprose et al., 2002), having three distinct segments of 412, 276, and 265 bp, each of which comprised of two chains of A-sequences and U-sequences ($p_0 = \bar{p}_0 = 0$) binding to each other by A-U base pairs;
2. the consensus sequence 5′-UACUAACACC-3′ ($p_0 = \bar{p}_0 = 0.2$) of the precursor (pre)mRNA intron, interacting with the short region 5′-GGUGUAGUA-3′ ($p_0 = 0.222, \bar{p}_0 = 0.333$) of the U2 small nuclear (sn)RNA and forming a complementary helix of seven base pairs with a single, unpaired A-residue (Newby and Greenbaum, 2002);
3. the secondary structure of the minimal, hinged hairpin ribozyme, formed by the interaction between the interdomain linker strand 5′-CGGUGAGAAGGGXGGCAGAGAAACACGA-3′, ($p_0 = \bar{p}_0 = 0.2$) and two other strands (MacElrevey et al., 2008).

We display the above three examples in Fig. 7.

The mathematical analysis presented here is currently extended to topological RNA structures, i.e. RNA structures having pseudoknots. These RNA structures are filtered by the topological genus of their corresponding fatgraphs. In this framework the concept of diagrams is enriched by passing from graphs the fatgraphs. This is achieved by ordering
Fig. 7. LHS: the double-stranded structure \cite{diprose2002} comprised of an A-sequence and an U-sequence. M: the complementary helix \cite{newby2002} formed by the (pre)mRNA intron and U2 small nuclear (sn)RNA. RHS: the minimal, hinged hairpin ribozyme \cite{macelrevey2008} consisting of three interacting strands.

the edges around a vertex and the secondary structures discussed here are just topological RNA structures of genus zero.

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The authors declare that no competing financial interests exist.

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