Evolutionary analysis of base-pairing interactions in DNA and RNA secondary structures

Michael Golden\textsuperscript{1}, Ben Murrell\textsuperscript{2}, Darren Martin\textsuperscript{3}, and Jotun Hein\textsuperscript{1}

\textsuperscript{1}Department of Statistics, University of Oxford, UK
\textsuperscript{2}Department of Medicine, University of California San Diego, California, USA
\textsuperscript{3}Department of Integrative Biomedical Sciences, Computational Biology Group, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa

September 16, 2018

Abstract

Pairs of nucleotides within biologically functional nucleic acid secondary structures often exhibit evidence of coevolution that is consistent with the maintenance of canonical base-pairing. MESSI is a sequence evolution model that infers substitution rates associated with base-paired sites in alignments of DNA or RNA sequences. MESSI can estimate these whilst simultaneously accounting for the uncertainty associated with an unknown RNA or DNA secondary structure shared across an alignment of sequences. Moreover, the unknown structure can be predicted, or a base-pairing probability matrix calculated. MESSI optionally leverages CUDA GPU parallelism to accelerate inference. MESSI was used to infer coevolution rates associated with GC, AU (AT in DNA), GU (GT in DNA) pairs in non-coding RNA alignments, and single-stranded RNA and DNA virus alignments. Inferred rates of GU pair coevolution were found to be higher at base-paired sites in single-stranded RNA viruses and non-coding RNAs than those of GT pairs in single-stranded DNA viruses, suggesting that GT pairs do not stabilise DNA secondary structures to the same extent as GU pairs in RNA. The relative coevolution rates associated with GC, AU, and GU pairs were largely consistent with their relative chemical base-pairing stabilities (GC base-pairs being more stable than AU base-pairs, and AU base-pairs being more stable than GU base-pairs). Additionally, MESSI estimates the degrees of coevolution at individual base-paired sites in an alignment. These estimates were computed for a SHAPE-MaP-determined HIV-1 NL4-3 RNA secondary structure and two corresponding alignments. MESSI’s estimates of coevolution were significantly more strongly correlated with experimentally-determined SHAPE-MaP pairing scores as compared to three non-evolutionary measures of base-pairing covariation. Finally, to assist researchers in prioritising substructures with potential biological functionality, MESSI automatically identifies substructures and ranks them by degrees of coevolution at base-paired sites within them. Such a ranking was created for an HIV-1 subtype B alignment, revealing an excess of top-ranking substructures that have been previously identified in the literature as having structure-related functional importance, and a number of top-ranking structures that have not yet been characterised.
1 Introduction

The primary role of nucleic acid molecules, such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), is encoding genetic information for storage and transfer. However, both types of molecules can form structures with additional functions (Mattick, 2003). DNA is ordinarily thought of as a double-stranded molecule forming the now iconic double helical configuration (Watson and Crick 1953), although many viral genomes consist entirely of single-stranded DNA or RNA molecules. Such single-stranded nucleic acid molecules are far less constrained than double-stranded nucleic acid molecules in the variety of structures that they can form. For example, the Rev response element (RRE) within the single-stranded HIV RNA genome plays a crucial role in the regulation of HIV virion expression by binding the HIV Rev protein to facilitate the transfer of HIV genomes from the nucleus to the cytoplasm where translation and virion packaging occur (Heaphy et al., 1990; Daugherty et al., 2010).

The structures that nucleic acid molecules form are commonly referred to as their secondary or tertiary structures. Secondary structure is defined as the set of hydrogen bonding interactions between the constituent bases of a nucleic acid molecule; tertiary structure is defined as the arrangement of the constituent atoms of a nucleic acid molecule in three-dimensional space. This study focuses exclusively on RNA and DNA secondary structures.

Both computational (Markham and Zuker, 2008; Sükösd et al., 2012; Bernhart et al., 2006) and hybrid experimental-computational techniques (Wilkinson et al., 2006) for secondary structure prediction exist. However, even if the secondary structure of an RNA sequence can be accurately determined, this does not immediately say anything about the potential functional or biological importance of the identified structure. Many RNA secondary structures are known to have specific biological functions, and it is expected that these structures might detectably impact patterns of evolution in the sequences in which they occur.

One evolutionary signal that can be leveraged to identify selectively maintained secondary structures is nucleotide coevolution. Nucleotide coevolution is expected at base-paired nucleotide positions within these structures (Eddy and Durbin, 1994; Tuplin et al., 2002; Cheng et al., 2012). Many pairs of nucleotides within RNA molecules exhibit evidence of coevolution, such that whenever a substitution occurs in one partner of the pair, complementary substitutions are selected for in the other partner in a manner that is consistent with the selective maintenance of canonical base-pairing (Cheng et al., 2012). In this study we consider the canonical RNA base-pairs to be the two Watson-Crick base-pairs, GC and AU, and the weaker GU wobble base-pair (GC, AT, and GT base-pairs in DNA, respectively).

Methods for detecting coevolution, such as mutual information (Eddy and Durbin, 1994; Lindgreen et al., 2006), can be used to aid the computational inference of secondary structures. Accordingly, some RNA comparative secondary structure prediction approaches, such as PPFold (Sükösd et al., 2012), use information about coevolving nucleotides inferred from sequence alignments to more accurately predict secondary structures. Conversely, within a given secondary structural element, evidence that paired bases are coevolving is evidence of the functional importance of that element (Tuplin et al., 2002; Cheng et al., 2012; Muhire et al., 2014).

Standard approaches for measuring coevolution (or more accurately: covariation), such as mutual information, are non-evolutionary in that they do not take into account the phylogenetic relationships of the sequences being analysed. Founder substitutions can, by chance, induce correlations between bases in a large number of observed species (Bhattacharya et al., 2007), which may be mistaken for strong evidence of coevolution if the phylogeny is not accounted for. Substitution models provide a statistical framework for modelling of both phylogenetic relationships and underlying substitution processes. However, the computational cost of applying such models can severely limit their utility.
In this study, we extend the M95 (Muse 1995) paired site model of RNA base-pairing evolution and implement a new software tool called MESSI (Modelling Evolution of Secondary Structure Interactions). In what follows, the underlying models and methodologies are detailed. Relative rates of coevolution amongst different base-pairs are measured, degrees of coevolution at base-paired sites are measured, secondary structure uncertainty is accounted for and predicted, and substructures are identified and ranked by their expected structure-related biological functionality.

2 Methods

2.1 The Muse 1995 model

Muse (1995) developed a paired site model, henceforth referred to as the M95 model. M95 accounts for RNA base-pairing constraints by modeling pairs of nucleotide positions using a 16 × 16 matrix. The model generalises upon standard 4 × 4 nucleotide substitution models, such as the GTR model, by introducing a coevolution parameter, λ, that is intended to capture substitutions at paired positions that are consistent with the maintenance of canonical RNA base-pairing. We define the set of canonical base-pairs as follows:

\[ \mathcal{C} = \{ \text{GC, CG, AU, UA, GU, UG} \} \]  \hspace{1cm} (1)

Here we present a version of the original M95 paired model based on a GTR model \( Q \) and a set of canonical base-pairs \( \mathcal{C} \):

\[
M_{ab} = \begin{cases} 
q_{ab} / \lambda & \text{pairing lost} \\
q_{ab} & \text{pairing unchanged} \\
q_{ab} \lambda & \text{pairing gained}
\end{cases}
\]

\( a \notin \mathcal{C} \) and \( b \notin \mathcal{C} \), e.g. \( a=AC \rightarrow b=AU \) \( a, b \notin \mathcal{C} \) or \( a, b \in \mathcal{C} \), e.g. \( a=AU \rightarrow b=GU \) \( a \in \mathcal{C} \) and \( b \notin \mathcal{C} \), e.g. \( a=AU \rightarrow b=AC \) \( q_{ab} \), e.g. \( a=AU \rightarrow b=GC \) \[ 2 \text{ differences} \]

Where \( a \) and \( b \) are nucleotide pairs, \( q_{ab} \) is the entry of the GTR matrix \( Q \) corresponding to the nucleotide position within the nucleotide pair that underwent a substitution, and \( \lambda \) is a parameter capturing the degree of RNA coevolution; i.e. the degree to which canonical RNA base-pairing is evolutionary maintained (\( \lambda > 1 \)) or disrupted (\( \lambda < 1 \)). Note that \( \lambda = 1 \) represents the neutral case where each of the two nucleotide positions in a pair are treated as evolving independently under the GTR model specified by \( Q \).

Furthermore, let \( \pi^{\text{dinuc}} \) denote a length 16 vector of paired frequencies. \( \pi^{\text{dinuc}} \) is the union of two mutually exclusive cases: \( \pi^{\text{dinuc}} = \pi^{\text{unpaired}} \cup \pi^{\text{paired}} \), \( \pi^{\text{unpaired}} \) represents the cases where the target pair \( d_{ij} \) is not in the set of canonical base-pairs (\( d_{ij} \notin \mathcal{C} \)), and \( \pi^{\text{paired}} \) represents the cases where the target pair \( d_{ij} \) is in the set of canonical base-pairs (\( d_{ij} \in \mathcal{C} \)), respectively:

\[
\pi^{\text{unpaired}}_{d_{ij}} = \kappa_1^{-1} \pi_i \pi_j \quad \text{and} \quad \pi^{\text{paired}}_{d_{ij}} = \kappa_1^{-1} \pi_i \pi_j \lambda^2,
\]

Note that \( i \) and \( j \) correspond to the first and second positions of the target pair, respectively. Where \( \pi_i \) is the equilibrium frequency under the GTR model, \( Q \), of the nucleotide in the first position of the target pair \( d_{ij} \), and similarly \( \pi_j \) is the equilibrium frequency of the nucleotide in the second position. \( \kappa_1 = 1 + 2(\pi_G \pi_C + \pi_A \pi_U + \pi_G \pi_U)(\lambda^2 - 1) \) is a normalising constant that ensures the entries of \( \pi^d \) sum to one.

Note that within the set of canonical base-pairs, \( \mathcal{C} \) (defined in Equation 1), there are three pairs of symmetrical base-pairs: \{GC, CG\}, \{AU, UA\}, and \{GU, UG\}. It is assumed that each base-pair within a symmetrical pair has the same fitness. This is a reasonable assumption as it treats the evolution of nucleotides towards the 5’ end of the sequence the same as nucleotides towards the 3’ end. From this point forward we assume this symmetry and refer to the three pairs of symmetrical base-pairs as the three canonical base-pairs.

In the formulation of the original M95 model in Equation 2, all three canonical base-pairs in the set \( \mathcal{C} \) are treated as having equal fitness. However, there
is good evidence that GU base-pairings in RNA, for example, are deleterious evolutionary intermediates relative to GC and AU (Rousset et al., 1991). In light of this, in the next section we extend the M95 model such that substitutions affecting the three canonical base-pairs are not constrained to have the same co-evolution rate.

2.2 Differentiating between types of base-pairing substitutions

We extend the M95 model to differentiate between the three different canonical base-pairs, by introducing potentially distinct coevolution rates ($\lambda_{GC}$, $\lambda_{AU}$, and $\lambda_{GU}$) for each of three different base-pairs (GC, AU, and GU, respectively). Using similar notation as before the extended rate matrix is given as follows:

\[
M_{ab} = \begin{cases} 
q_{ab}\lambda_{GC} & \text{GC pairing gained} \\
q_{ab}\lambda_{AU} & \text{AU pairing gained} \\
q_{ab}\lambda_{GU} & \text{GU pairing gained} \\
q_{ab}/\lambda_{GC} & \text{GC pairing lost} \\
q_{ab}/\lambda_{AU} & \text{AU pairing lost} \\
q_{ab}/\lambda_{GU} & \text{GU pairing lost} \\
q_{ab}\lambda_{GU}/\lambda_{GC} & \text{GC to GU} \\
q_{ab}\lambda_{GC}/\lambda_{GU} & \text{GU to GC} \\
q_{ab}\lambda_{AU}/\lambda_{GU} & \text{AU to GU} \\
q_{ab}\lambda_{AU}/\lambda_{GU} & \text{GU to AU} \\
q_{ab} & \text{pairing unchanged} \\
0 & 2 \text{ differences}
\end{cases}
\]

where $a$ and $b$ represent nucleotide pairs. The conditions in (5) and (6) were verified using the symbolic math package, SymPy (Joyner et al., 2012), as implemented in the musesymbolic.py script in the Supplementary Material.

2.3 Stationarity and time-reversibility

We show that the paired frequencies, $\pi$, given in (4) correspond to the stationary distribution of $M$ by verifying that:

\[\pi M = 0,\]

and that time-reversibility of $M$ holds:

\[\pi_a M_{ab} = \pi_b M_{ba} \quad \forall_{ab}\]

where $a$ and $b$ represent nucleotide pairs. The conditions in (5) and (6) were verified using the symbolic math package, SymPy (Joyner et al., 2012), as implemented in the musesymbolic.py script in the Supplementary Material.

2.4 Modelling variable degrees of co-evolution

In the M95 model (2) the rate of coevolution, as specified by $\lambda_{GC}$, $\lambda_{AU}$, and $\lambda_{GU}$, was assumed to be the same at each base-paired site within a secondary structure $S$. However, it is expected that the strength of the selective forces maintaining canonical base-pairing will vary amongst base-paired sites in $S$. In this section, we extend the M95 model such that the degree of coevolution, denoted by $\eta_{q,r}$, is able to vary from base-paired site to base-paired site. $\eta_{q,r}$ is drawn independently for each base-paired site (described in the next section), and acts to scale the

and the corresponding paired frequencies are:

\[
\begin{align*}
\pi_{\text{unpaired}} &= \kappa_2^{-1} \pi_i \pi_j \\
\pi_{GC} &= \kappa_2^{-1} \pi_G \pi_C \lambda_{GC}^2 \\
\pi_{AU} &= \kappa_2^{-1} \pi_A \pi_U \lambda_{AU}^2 \\
\pi_{GU} &= \kappa_2^{-1} \pi_G \pi_U \lambda_{GU}^2
\end{align*}
\]

Where $\kappa_2 = 1 + 2[\pi_{GC} \pi_C (\lambda_{GC}^2 - 1) + \pi_{AU} \pi_U (\lambda_{AU}^2 - 1) + \pi_{GU} \pi_U (\lambda_{GU}^2 - 1)]$
three coevolution rates as follows:

\[
\begin{align*}
\lambda_{q,r}^{GC} &= (\lambda_{GC} - 1)\eta_{q,r} + 1 \\
\lambda_{q,r}^{AU} &= (\lambda_{AU} - 1)\eta_{q,r} + 1 \\
\lambda_{q,r}^{GU} &= (\lambda_{GU} - 1)\eta_{q,r} + 1
\end{align*}
\]

(7)

where \(\lambda_{GC} \geq 1\), \(\lambda_{AU} \geq 1\), and \(\lambda_{GU} \geq 1\) are the base-pairing substitution rates shared across all paired sites. This parametrisation was chosen so that \(\lambda_{q,r}^{GC} = \lambda_{q,r}^{AU} = \lambda_{q,r}^{GU} = 1\) when \(\eta_{q,r} = 0\).

In addition to allowing the rate of coevolution, \(\eta\), to vary across base-paired sites, we also allow substitution rates to vary from site to site following the gamma distributed sites rate approach of (Yang, 1993, 1994). For unpaired sites this is modelled using a standard GTR+Γ model. For base-paired sites slightly more care needs to be taken (see Supplementary Section 2.5 Testing neutrality of coevolution)

To test the hypothesis that two nucleotide positions within a particular base-paired site are evolving neutrally, i.e. the substitutions at each of the two sites are occurring independently rather than actively favouring the maintenance canonical base-pairing, we assume that the degree of coevolution, \(\eta_{q,r}\), at each base-paired site is distributed as follows: \(\eta_{q,r} = 0\) with probability \(w_{\eta}\) (the neutral, independent case), or \(\eta_{q,r}\) is drawn from a M-way discretised gamma distribution with probability \(1 - w_{\eta}\) (the dependent case). Note that \(\eta_{q,r} \geq 0\) and therefore the case where substitutions are acting to disrupt canonical RNA base-pairing is not considered, i.e. the case where the coevolution parameters are between 0 and 1. For all analyses a discretisation of \(M = 4\) was used, resulting in five rate categories: one neutral category with probability \(w_{\eta}\), and four positive categories each with probability \(\frac{1-w_{\eta}}{4}\).

### 2.6 Priors

This section lists the priors (and hyperpriors) over parameters used in the most general version of the implemented model (the unconstrained model). Note that for some analyses we perform Bayesian inference, whereas for others we perform maximum likelihood (ML) inference. The priors over the parameters specified here are those used for Bayesian inference, however, we also indicate how the parameters are treated during ML inference (either estimated whilst ignoring the prior, or using the prior and fully marginalised).

The prior probability of neutral coevolution, \(w_{\eta}\) (estimated during ML inference whilst ignoring the prior), is drawn from a beta distribution:

\[w_{\eta} \sim \text{Beta}(2,2)\]  

(8)

For each paired position \(q, r\) a Bernoulli random variable, \(X_{q,r}\) (marginalised during ML inference), indicating neutral coevolution is drawn from a Bernoulli distribution with probability \(w_{\eta}\):

\[X_{q,r} \sim \text{Bernoulli}(w_{\eta})\]  

(9)

A tied shape and rate parameter, \(c\) (estimated during ML inference whilst ignoring the prior), specifying a prior over the variation in coevolution rates is drawn from an exponential distribution:

\[c \sim \text{Exponential}(\frac{1}{10})\]  

(10)

For each paired position \(q, r\) the coevolution rate, \(\eta_{q,r}\), is set equal to zero if \(X_{q,r} = 1\), otherwise if \(X_{q,r} = 0\) the coevolution rate, \(\eta_{q,r}\) (marginalised during ML inference), is drawn from a discretised gamma distribution with shape \(c\) and rate \(c\):

\[\eta_{q,r} = \begin{cases} 
0 & \text{if } X_{q,r} = 1 \\
\eta_{q,r} \sim \text{DiscretisedGamma}_M(c, c) & \text{otherwise}
\end{cases}\]  

(11)

A tied shape and rate parameter, \(d\) (estimated during ML inference whilst ignoring the prior), specifying a prior over the variation in site-to-site substitution rates is drawn from an exponential distribution:

\[d \sim \text{Exponential}(\frac{1}{10})\]  

(12)
For each nucleotide position \( q \) a substitution rate, \( \rho_q \) (marginalised during ML inference), is drawn from a discretised gamma distribution with shape \( d \) and rate \( d \):

\[
\rho_q \sim \text{DiscretisedGamma}_K(d,d) \quad (13)
\]

The prior probabilities of the four nucleotides, \( \pi \) (estimated during ML inference), is given by a flat Dirichlet distribution:

\[
(\pi_A, \pi_C, \pi_G, \pi_T) \sim \text{Dirichlet}(1,1,1,1) \quad (14)
\]

The six symmetric nucleotide substitution rates (estimated during ML inference whilst ignoring the prior) are each drawn from exponential distributions:

\[
\begin{align*}
q_{AC} & \sim \text{Exponential} \left( \frac{1}{10} \right) \\
q_{AG} & \sim \text{Exponential} \left( \frac{1}{10} \right) \\
q_{AT} & \sim \text{Exponential} \left( \frac{1}{10} \right) \\
q_{CG} & \sim \text{Exponential} \left( \frac{1}{10} \right) \\
q_{CT} & \sim \text{Exponential} \left( \frac{1}{10} \right) \\
q_{GT} & \sim \text{Exponential} \left( \frac{1}{10} \right)
\end{align*}
\]

\quad (15)

The three coevolution rates (estimated during ML inference whilst ignoring the prior) corresponding to the three canonical base-pairs are each drawn from exponential distributions right shifted by 1:

\[
\begin{align*}
\lambda_{GC} & \sim \text{Exponential} \left( \frac{1}{10} \right) + 1 \\
\lambda_{AT} & \sim \text{Exponential} \left( \frac{1}{10} \right) + 1 \\
\lambda_{GT} & \sim \text{Exponential} \left( \frac{1}{10} \right) + 1
\end{align*}
\]

\quad (16)

The secondary structure, \( S \) (marginalised during ML inference), is drawn from the KH99 SCFG (discussed in Section 2.9):

\[
S \sim \text{KH99} \quad (17)
\]

Finally, the phylogenetic tree, \( \hat{T} \), relating the alignment of sequences, \( D \), for both Bayesian and ML inference is estimated in advance and fixed apriori using FastTree [Price et al., 2010] under a GTR+CAT model.

\[\text{Secondary structure without a pseudoknot}\]

A. Dot bracket notation

\[
\text{CUAACACUAGCAGCAGCGUAGC}
\]

\[
(((...)))..(((...).))
\]

B. VARNA visualisation

C. Circular visualisation

\[\text{Pseudoknotted secondary structure}\]

D. Extended dot bracket notation

\[
\text{CUAACACUAGCAGCAGCGUAGC}
\]

\[
(((...<)))..(((>..).))
\]

E. VARNA visualisation

F. Circular visualisation

Figure 1: Examples of secondary structure representations. Above (A) is a dot bracket representation of a secondary structure, and the corresponding VARNA and circular visualisations (B and C, respectively) produced by VARNA [Darty et al., 2009]. Below (D) is an extended dot bracket notation format with an additional bracket type, \(<\), that allows a pseudoknotted structure to be represented unambiguously. E and F are the corresponding VARNA visualisations for D. Note how the overlapping bonds in the circular visualisation (F) demonstrate that the secondary structure is pseudoknotted.

2.7 Computer representations of secondary structure

To model nucleic acid secondary structure a suitable definition of secondary structure is required. We use the definition outlined in Moulton et al. (2000): a secondary structure, \( S \), for a nucleic acid molecule consisting of \( N \) nucleotides is a simple graph specified by the vertex set \( [N] := \{1, \ldots, N\} \) and an edge set \( B_S \). Where each vertex in \( [N] \) corresponds to a nucleotide
and each edge in the edge set $B_S$ corresponds to a base-pair. $S$ is such that if $\{i,j\}, \{k,l\} \in B_S$ with $i < j$ and $k < l$ then:

i. $i = k$ if and only if $j = l$, and

ii. $k \leq j$ implies that $i < k < l < j$

Vertices that are not contained within the edge set $B_S$ are termed unpaired. Condition (i) implies that each vertex (nucleotide) belongs to at most one base-pair. Condition (ii) prevents pseudoknotting, i.e. non-nested base-pairs.

Note that pseudoknotting is physically possible in both real RNA and DNA structures, but is excluded in many definitions of secondary structures as efficient algorithms exist for marginalising or maximising over secondary structures when assuming (ii). MESSI permits a canonical secondary structure with pseudoknots to be specified a priori, however, if the user instead treats the structure as unknown, MESSI will strictly sum over non-pseudoknotted structures only.

Figure 1 gives a computational format for representing secondary structures. The dot-bracket format (Figure 1A) is a natural and compact way of representing non-pseudoknotted secondary structures. Matching brackets represent base-paired nucleotide positions and dots represent unpaired (singled-stranded) nucleotide positions. To represent pseudoknotted structures (structures that violate condition (ii)) additional bracket types are required (Figure 1D).

### 2.8 Likelihood

Conditioned on a secondary structure, $S$, unpaired nucleotide positions within $S$, denoted by $q$, and base-paired nucleotide positions within $S$, denoted by $\hat{q}, \hat{r}$, are assumed to be independent. The likelihood of an alignment, $D$, is given by a simple product of unpaired and paired site likelihoods:

$$ p(D|S, \hat{T}, \theta) = \prod_{q \in S} p(D_q|q, \hat{T}, \theta) \prod_{\hat{q}, \hat{r} \in S} p(D_{\hat{q}, \hat{r}}|\hat{q}, \hat{r}, \hat{T}, \theta) $$

(18)

Where $\hat{T}$ is a phylogenetic tree and Felsenstein’s pruning algorithm \cite{Felsenstein1981} was used to calculate both the unpaired site likelihoods, $p(D_q|q, \hat{T}, \theta)$, and the paired site likelihoods, $p(D_{\hat{q}, \hat{r}}|\hat{q}, \hat{r}, \hat{T}, \theta)$. Paired sites were modelled using the unconstrained M95 model, whereas unpaired sites were modelled using the GTR+Γ model that is nested within the unconstrained M95 model.

### 2.9 Prior over RNA secondary structures

Equation 18 assumes that the secondary structure $S$ is known a priori either through experimental or computational methods of structure prediction. However, it also possible to treat the secondary structure as unknown, by placing a prior probability distribution, $p(S)$, over secondary structures and marginalising $S$.

One way of introducing a prior over secondary structures is by using a Stochastic Context Free Grammar (SCFG). A SCFG is probabilistic extension of a context-free grammar (CFG). A CFG is a type of grammar that defines a set of rules for generating all possible strings in a given formal language. A SCFG extends this notion by assigning probabilities to each possible string in the given language. RNA SCFGs are SCFGs that give probability distributions over strings of base-paired and unpaired nucleotides representing RNA secondary structures \cite{Anderson2014}.

#### 2.9.1 The KH99 grammar

The KH99 SCFG \cite{KnudsenHein1999} was chosen as a prior over secondary structures. The rules
and associated probabilities are given as follows:

\[
S \rightarrow \bullet \quad \text{or} \quad LS \quad \text{or} \quad (F) \\
\begin{array}{ccc}
0.118 & 0.869 & 0.014
\end{array}
\]

\[
G_{\text{KH99}} = L \rightarrow \bullet \quad \text{or} \quad (F) \\
\begin{array}{ccc}
0.895 & 0.105
\end{array}
\]

\[
F \rightarrow (F) \quad \text{or} \quad LS \\
\begin{array}{ccc}
0.788 & 0.212
\end{array}
\]

Note that \( S \) is the start symbol.

The KH99 assigns probabilities to all strings of a specified length that can be written in dot-bracket notation, with at least two unpaired nucleotides separating every base-pair.

### 2.9.2 Structure-integrated likelihood

Using Bayes’ rule, the probability of a secondary structure, \( S \), conditional on the data, \( D \), and phylogenetic parameters, \( \theta \), is given by:

\[
p(S|D, \theta) = \frac{p(D|S, \theta)p_G(S)}{\sum_S p(D|S, \theta)p_G(S)} \tag{20}
\]

The **structure-integrated likelihood** term in the denominator, \( p_S(D|\theta) = \sum_S p(D|S, \theta)p_G(S) \), requires summing over all possible secondary structures, which grows exponentially with the length of the alignment \( L \). Fortunately, there exists an \( O(L^3) \) polynomial-time algorithm, the inside algorithm, for summing the probabilities of all derivations of an SCFG (all valid secondary structures in the case of RNA SCFG). By analogy to the forward algorithm for HMMs, the inside algorithm allows the structure-integrated likelihood, \( p_S(D|\theta) \) (the analogue of the forward likelihood for HMMs), to be efficiently computed. The structure-integrated likelihood is given by element \( I(S, 1, L) \) of the inside probability matrix, where \( S \) is the start symbol of the KH99 grammar.

Likewise, by analogy to the backward algorithm for HMMs, there exists an ‘outside algorithm’, which together with the inside probabilities allows the posterior marginals of the hidden variables to be computed (in the case of an RNA SCFG: positional emission probabilities of base-pair and unpaired terminal symbols – see Section).

Note that structure-integrated likelihood in the denominator of (20) is given by:

\[
p(D|\theta) = \sum_S p(D|S)p_G(S) = I(S, 1, L) \tag{21}
\]

A recursive formula for calculating the outside probabilities is as follows:

\[
O(U, q, r) = \begin{cases}
    1 & \\
    \sum_{V \rightarrow W} p[V \rightarrow UW] \sum_{k>q} O(V, q, k) I(W, r+1, k) + \\
    \sum_{V \rightarrow WU} p[V \rightarrow WU] \sum_{k<q} O(V, k, r) I(W, q-1, k-1) + \sum_{V \rightarrow (U)} p[V \rightarrow (U)] O(V, q-1, r+1, q-1, r + 1) & \text{if } \frac{r}{q} \neq 1 \end{cases}
\]

where \( G \) is a RNA secondary structure grammar.

### 2.9.3 Parallelisation of the inside and outside algorithms

Figure [illustrates the calculation of the inside probability matrix, showing the order in which elements are computed and the data dependencies required to compute a particular element. Using these patterns, Sükösrd et al. (2011) developed a strategy for CPU parallelism, whereby blocks of elements running diagonally along the inside matrix can be computed in parallel, as they do not have data dependencies. We implemented a similar scheme for the CUDA GPU architecture, whereby instead of blocks, each element along a diagonal is computed in parallel. This can be done because each element along a diagonal is independent of all other elements on the same diagonal. For large alignments \( (L > 1000) \) this implies thousands of computational threads executing the same set of instructions in parallel, but on differ-
ent data (different elements of a particular diagonal), this is known as SIMD (Single Instruction Multiple Data) parallelism and is the regime of parallelism for which GPU architectures are tailored. As far as we are aware this is the first GPU implementation of the inside and outside algorithms.

2.10 Paired site likelihoods

Because the inside and outside algorithms consider every possible base-pairing they require a matrix $B$ of paired site likelihoods. Each element $B_{qr}$ of $B$ corresponds to a paired site likelihood $p(D_{q,r} | \hat{q}, \hat{r}, \hat{T}, \theta)$ for a pair of sites, $q$ and $r$, in the alignment $D$, which can be calculated using Felsenstein’s algorithm. Since the diagonal of $B$ is ignored and $B_{qr} = B_{rq}$ (i.e. $B$ is symmetric), $\binom{L}{2}$ paired site likelihoods need to be calculated. Whilst, the number of computational steps is only $O(L^2)$ in the alignment length $L$, compared to $O(L^3)$ for the inside and outside algorithms, the amount of time per computational step for computing the paired site likelihoods is substantially higher due the use of Felsenstein’s algorithm. To ameliorate this bottleneck, we use the partial site caching strategy of Pond and Muse (2004) to reduce the number of likelihood calculations required and developed a CUDA GPU implementation.

Note that the inside and outside algorithms also require a vector, $S$, of length of $L$ single site likelihoods, where each element corresponds to $p(D_q | \hat{q}, \hat{T}, \theta)$. However, this is fast to compute compared to $B$.

2.11 Sampling secondary structure configurations

The inside probability matrix can be used to sample secondary structure configurations:

$$\hat{S} \sim p(S | D, \theta)$$

Sampling terminal strings (secondary structures in our case) using an SCFG is analogous to sampling hidden state sequences using the forward-filtering backward-sampling algorithm for HMMs (Frühwirth-...
An algorithmic description for sampling secondary structures from an RNA SCFG is given in the Supplementary Methods Section.

**2.12 Bayesian posterior inference**

The posterior distribution of the continuous-parameters, \( \theta \), conditional on the data \( D \) and a secondary structure \( S \) can be sampled using the Metropolis-Hastings algorithm and the relationship given by Bayes' formula:

\[
p(\theta|D,S) \propto p(D|S,\theta)p(\theta)
\]

(24)

Where the likelihood term, \( p(D|S,\theta) \), is given by (18) and \( p(\theta) \) is the prior.

We can also treat the secondary structure as unknown and assume a RNA SCFG prior, \( p_G(S) \), over secondary structures. Using the structure-integrated likelihood \( p_S(D|\theta) \):

\[
p(\theta|D) \propto p_S(D|\theta)p(\theta)
\]

(25)

However, note that the structure-integrated likelihood term is computed every time a new set of parameters is proposed. As mentioned previously, this requires computing a matrix \( B \) of paired site likelihoods and running the inside algorithm, the Metropolis-Hastings step (27) only requires \( O(L) \) operations and can be repeated for multiple iterations following the Gibbs sampling step. In our implementation we repeat the Metropolis-Hastings step 50 times following the Gibbs sampling step. Together these give a Markov Chain Monte Carlo algorithm whose stationary distribution, \( p(S,\theta|D) \), and associated marginals, \( p(S|D) \) and \( p(\theta|D) \), are the distributions of interest.

**2.13 Maximum likelihood inference**

The COBYLA optimization algorithm (Powell, 1994) in the NLOpt library (Johnson, 2014) was used to find the maximum likelihood (ML) parameters via the structure-integrated likelihood (20). Note that when doing so the priors over the continuous parameters were either ignored and estimated using ML, or the priors were used and the parameters were fully marginalised (as specified in the Priors section).

**2.14 Site permutations**

To test whether secondary structure dependencies present in real datasets influence model fit, each alignment was taken and its sites randomly permuted. Two such permuted datasets \( (p_1 \text{ and } p_2) \) were generated for each real dataset. ML estimation using the structure-integrated likelihood was used to fit the parameters of each permuted dataset under the unconstrained model and the secondary structure information entropy was calculated.

**3 Results and Discussion**

**3.1 Site permutation benchmarks**

To assess the degree to which secondary structure dependencies present in real datasets influence model fit, ML inference was performed on real and permuted datasets, and their structure-integrated likelihoods using the Metropolis-Hastings algorithm. Whilst the Gibbs sampling step (26) still requires computing a matrix \( B \) of paired site likelihoods and running the inside algorithm, the Metropolis-Hastings step (27) only requires \( O(L) \) operations and can be repeated for multiple iterations following the Gibbs sampling step.
and structure information entropies were compared (Section 2.14 in Methods). The structure-integrated likelihoods for the permuted datasets were expected to be lower than those of the real datasets. Conversely, the structure information entropies were expected to be higher for the permuted datasets than for the real datasets. Unlike the real datasets, the patterns of coevolution in the permuted datasets were not expected to coincide with stable secondary structure configurations, thereby spreading the probability mass over a larger number of secondary structure configurations.

The maximum likelihood estimates of the structure-integrated likelihoods were indeed lower for the permuted datasets in every instance (Supplementary Table 3.2).

### 3.2 Benchmarks of RNA structure prediction

Whilst, our model was not designed to predict RNA secondary structure, the expected base-pairing and unpairing probabilities can be calculated (see Supplementary Section).

MESSI has lower precision but higher recall than the other two methods, implying that it predicts more base-pairs (higher recall), but with a higher number of false-positives (lower precision; Figure 3). For the F1-score and MCC measures, both of which combine precision and recall, MESSI performs slightly better than RNAalifold and PPfold. MESSI also performs marginally better with respect to the mountain similarity measure – a measure that takes into account the overall 'shape' of the secondary structures being compared, rather than the exact matching of base-pairs.

Overall, our method performs on a par with the other two well-established methods of comparative RNA structure prediction. This was surprising given that the model was not tuned for secondary structure prediction. Maximum likelihood inference was used to determine the parameters, where the coevolution parameters ($\lambda_{GC}$, $\lambda_{AU}$, and $\lambda_{GU}$) were free to vary with the only restriction being: $\lambda_{GC} \geq 1$, $\lambda_{AU} \geq 1$, and $\lambda_{GU} \geq 1$. Although not tested here, it might be possible to improve the predictive accuracy of MESSI’s structure predictions by performing Bayesian or MAP inference of the parameters using a prior learnt from a training dataset of alignments and corresponding structures.

### 3.3 CPU and GPU timing benchmarks

The two computational bottlenecks in performing both maximum likelihood and Bayesian inference are computing paired site likelihood matrices (computed using an iterative version of Felsenstein’s algorithm) and computing inside probability matrices (using an iterative version of the inside algorithm); both of which are required repeatedly. Although optimised CPU implementations written in Julia were created for both of these steps, these were still relatively slow. Therefore GPU implementations written in CUDA were implemented for both.

The number of computational steps is expected to grow linearly with the number of unique paired site patterns and hence this was chosen as a predictor of the computational time required (Figure 4). Compared to the single-threaded CPU implementation a
\[ \sim 50 \times \text{speed-up} \] was achieved with the GPU implementation across most datasets.

The number of computational steps for the inside algorithm is expected to grow \( O(L^3) \) where \( L \) is the number of alignment sites in a particular dataset (Figure 5). A 50 to 200 fold speed-up for the paired site likelihood calculations was achieved for moderate dataset sizes, with the fold speed-ups for larger datasets being bigger, due to larger datasets better saturating the GPU.

The speed-ups seen here are significant, enabling us to analyse datasets which would typically be considered intractable. Note that CPU and GPU implementations were also developed for the outside algorithm with similar speed-ups obtained (Figure 3).

### 3.4 The role of GU and GT base-pairs in single-stranded RNA and DNA

It is well-established that GU pairs can hydrogen bond in RNA to form base-pairs, although they are chemically weaker than GC and AU base-pairings \cite{Rousset1991}. The relative chemical strengths of GC, AU, and GU base-pairs are partially due to the number of hydrogen bonds that form between their constituent bases: three for GC base-pairs, two for AU base-pairs, and two for GU base-pairs. Although GU pairs form the same number of hydrogen bonds as in AU pairs, the geometry of the bases leads to the GU pairing being substantially weaker than the AU pairing \cite{Varani2000}.

Despite the weaker chemical interaction, GU base-pairings are known to be involved in functional RNA structures \cite{Gautheret1995}. Less well understood is the role of GT base-pairings in DNA. There are some reports of GT base-pairings in double-stranded DNA helices \cite{Early1978, Ho1985}, but none that were found in a literature search of GT base-pairings in single-stranded DNA. Whilst, we were unable to measure the chemical strength of these base-pairing interactions in the present study, we used MESSI to analyse alignments for evidence of evolutionary forces favouring the maintenance of
Table 1: Tests of GU/GT neutral hypothesis across 15 datasets

| Dataset    | Type     | Number of sites | GU (GT) neutral log-likelihood (M0) | Unconstrained log-likelihood (M1) | Delta M1-M0 log-likelihood | LRT p-value (λ) | λGU (λGT) |
|------------|----------|-----------------|-------------------------------------|-----------------------------------|--------------------------|-----------------|-----------|
| RF01846    | ncRNA    | 624             | -11101.49                           | -11043.98                         | 57.51                    | ***            | 2.64      |
| RF00379    | ncRNA    | 335             | -10523.44                           | -10470.32                         | 53.12                    | ***            | 3.57      |
| RF00010    | ncRNA    | 996             | -85067.98                           | -84007.62                         | 1060.36                  | ***            | 3.01      |
| RF00003    | ncRNA    | 203             | -4711.20                            | -4653.04                          | 58.16                    | ***            | 2.97      |
| RF00001    | ncRNA    | 230             | -28077.49                           | -27897.66                         | 179.83                   | ***            | 2.25      |
| FMDV       | ssRNA    | 8349            | -114986.98                          | -114610.85                        | 376.12                   | ***            | 5.21      |
| H. poliovirus 1 | ssRNA | 7668          | -65013.50                           | -64966.32                         | 47.19                    | ***            | 5.60      |
| Tobamovirus | ssRNA   | 6849            | -88906.32                           | -88767.09                         | 139.23                   | ***            | 7.53      |
| Rhinovirus A | ssRNA  | 7308            | -219614.83                          | -217136.20                        | 2478.62                  | ***            | 21.92     |
| Hepatitis A | ssRNA   | 7572            | -63755.35                           | -63755.31                         | 0.04                     | n.s.           | 1.00      |
| MSV        | ssDNA    | 2755            | -15345.85                           | -15341.48                         | 4.37                     | **             | 1.57      |
| TYLCV      | ssDNA    | 2925            | -40743.07                           | -40743.03                         | 0.04                     | n.s.           | 1.00      |
| BCTV       | ssDNA    | 3215            | -32094.09                           | -32093.56                         | 0.53                     | n.s.           | 1.18      |
| Bocavirus  | ssDNA    | 5577            | -31987.81                           | -31985.98                         | 1.84                     | n.s.           | 1.25      |
| WDV        | ssDNA    | 2755            | -10733.63                           | -10731.59                         | 2.04                     | *              | 1.57      |

* p < 0.05; ** p < 0.005; *** p < 0.0005; n.s. = not significant

formation of GT pairs at base-paired positions.

For all five non-coding RNA datasets (RF00001, RF00003, RF00010, RF00379, and RF01846) likelihood ratio tests (LRTs) rejected the GU neutral model in favour of the unconstrained model (p < 0.0005. See Table 1, with ML estimates for λGU in the range 2.25 – 3.57. This is evidence of many GU pairs being under selective maintenance in the five non-coding RNA datasets tested.

For four of the five RNA virus datasets tested (Rhinovirus A, Tobamovirus, human poliovirus 1, and foot-and-mouth disease virus. See Table 1) LRTs rejected the GU neutral model in favour of the unconstrained model (p < 0.0005 in all four cases), with ML estimates for λGU in the range 5.21 – 21.92. Curiously, the GU neutral model could not be rejected in favour of the unconstrained model for the Hepatitis A virus dataset (Table 1), with the ML estimate for λGU = 1.

Three of the five DNA virus genome datasets tested (Human bocavirus, beet curly top virus, and tomato yellow leaf curl virus in Table 1) showed no significant difference between the unconstrained model and a GU (GT) neutral model (λGU := 1). With the Wheat Dwarf Virus dataset meeting the p < 0.05 significance threshold for rejecting the GT neutral model, and the Maize Streak Virus dataset meeting the p < 0.005 significance threshold for rejecting the GT neutral model. ML estimates for λGT were in the range 1.0 - 1.57, which is low compared to those determined for the non-coding RNA and RNA virus datasets.

The LRT results and the ML estimates for λGU (λGT) suggest that GT pairs are under weak selective maintenance in DNA virus genomes, and strong selective maintenance in RNA virus genomes and non-coding RNAs. This may indicate that GT base-pairings in DNA are chemically weaker relative to GU base-pairings in RNA and hence do not stabilise DNA secondary structures to the same extent as GU base-pairings in RNA.

3.5 Relative coevolution rates

The relative selective strengths of the coevolution rates associated with GC, AU and GU pairs were compared across both DNA and RNA virus genomes. The original M95 model assumed that λGC := λAU and λGC := 1, however, experimental evidence shows that GC base-pairings are chemically stronger than AU base-pairings in RNA [Mathews et al., 1999].
To assess whether $\lambda_{GC} := \lambda_{AU}$ is a reasonable assumption, we performed LRTs comparing the unconstrained model to a $\lambda_{GC} := \lambda_{AU}$ constrained model. For 14 of the 15 datasets, LRTs rejected the GC-AU constrained model in favour of the unconstrained model (results not shown). The only exception was the human poliovirus 1 dataset, where the GC-AU constrained model could not be rejected.

This was explored further by comparing the inferred relative magnitudes of the rates associated with GC, AU (AT) and GU (GT) dinucleotides. If the fitness value of a RNA secondary structure element is positively correlated with its chemical stability, it is expected that the relative chemical stabilities associated with the three canonical base-pairs would be reflected in the relative magnitudes of the coevolution rates inferred by MESSI.

MESSI’s Bayesian posterior inference mode was applied to 15 datasets, five from each of three dataset types. Posterior probabilities associated with all six possible orderings of the three base coevolution rates were estimated for each dataset (Figure 6). Given the relative chemical base-pairing stabilities, the dominant ordering for the base coevolution rates was expected to be $\lambda_{GC} > \lambda_{AU} > \lambda_{GU}$. For all five ncRNA datasets, all five ssDNA virus datasets, and two of the five ssRNA virus datasets the posterior probability associated with the $\lambda_{GC} > \lambda_{AU} > \lambda_{GU}$ ordering was indeed 1.0.

Interestingly, an unexpected ordering, $\lambda_{GC} > \lambda_{GU} > \lambda_{AU}$, emerged for three of the ssRNA with a posterior probability of 1.0 for the Rhinovirus A dataset and posterior probabilities of 0.81 and 0.55 for the Human poliovirus 1 and Tobamovirus datasets, respectively. Possible explanations for this result include: (i) more stable structures are not necessarily selectively favoured over less stable structures (ii) datasets with coding regions have additional constraints on synonymous and non-synonymous substitutions, and these constraints might mislead MESSI, (iii) for some datasets it is not valid to assume a canonical secondary structure that is shared across the entire phylogeny. Two or more parts of the phylogeny may have different mutually exclusive secondary structures, giving rise to misleading patterns of pair evolution.

Figure 6: Estimated posterior probabilities for all six orderings of the three base coevolution rates across 15 datasets.
3.6 Degrees of coevolution are correlated with experimental SHAPE-MaP quantities

A notable example of a large RNA structure that has been partially experimentally-determined is that of the HIV-1M subtype B NL4-3 isolate (Watts et al., 2009; Siegfried et al., 2014). Rather than relying solely on computational techniques for the determination of RNA secondary structure of the 9173 nucleotide NL4-3 genome, the hybrid experimental-computational SHAPE-MaP (Selective 2'-hydroxyl acylation analysed by primer extension and mutational profiling; Siegfried et al. (2014)) approach was used to model the structure. The SHAPE-MaP approach preferentially mutates unpaired nucleotides, allowing the mutated nucleotides to be identified using DNA sequencing following reverse transcription. The SHAPE-MaP reactivity information is then used to constrain a thermodynamic RNA folding algorithm, enabling the construction of a secondary structure model which is reflective of the experimental data.

Three non-evolutionary computational measures of covariation (A. Mutual information, B. RNAAlifold mutual information, and C. Mutual information with stacking) as described in Lindgreen et al. (2006) and two evolutionary measures of coevolution inferred by MESSI (D. Posterior probability $\eta \neq 1$, and E. Posterior mean $\eta$), were compared to experimental SHAPE-MaP reactivities and partially experimental SHAPE-MaP pairing probabilities at base-paired sites corresponding to three different datasets: an HIV 1b dataset, an HIV group 1M dataset, and a Simian Immunodeficiency Virus (SIV) dataset. The SHAPE-MaP reactivities, SHAPE-MaP pairing probabilities and base-pairings were derived from a SHAPE-MaP analysis of the HIV NL4-3 sequence (Watts et al., 2009) when analysing the two HIV datasets and a SHAPE-MaP analysis of the SIVmac239 sequence (Pollom et al., 2013) when analysing the SIV dataset. Given that high SHAPE-MaP reactivities indicate unpairing, it was expected

Figure 7: Spearman’s correlations between five different measures of coevolution and base-pair averaged $\eta_{5}$ SHAPE-MaP reactivities (a, c, and e), and the same five measures and base-pair averaged SHAPE-MaP pairing probabilities (b, d, and e).
that degrees of coevolution (or covariation) would be negatively correlated with SHAPE-MaP reactivities. Conversely, given that some paired nucleotides are expected to be selectively maintained due to structure-related functional importance, a positive correlation between degrees of coevolution (or covariation) and SHAPE-MaP pairing probabilities was expected.

For all three datasets the two measures of coevolution (D and E) were significantly correlated with both the SHAPE-MaP reactivities and SHAPE-MaP pairing probabilities using Spearman’s rank correlation test, and the correlations were in the expected direction (negatively correlated for SHAPE-MaP reactivities and positively correlated for SHAPE-MaP pairing probabilities; Figure 7). For all three datasets the correlation coefficients were significantly stronger in the expected direction for the two coevolution measures (D and E) compared to the three covariation measures (A, B, and C; see the 95% confidences intervals for Spearman’s rho). It should be noted that whilst many of the correlations were statistically significant, the magnitudes of the correlations were weak, such that the correlations were not visually discernible in scatterplots comparing coevolution or covariation and the SHAPE-MaP quantities.

Curiously, for the SIV dataset, SHAPE-MaP reactivities were significantly positively correlated with the three measures of covariation (A, B, and C in Figure 7e), rather than negatively correlated as expected. There is broad evidence to suggest that base-paired sites in a functionally important RNA structure tend to be more conserved (less variable) due to being under selective constraint (Muhire et al. 2014, Tuplin et al. 2004) and that double-stranded RNA (i.e. base-paired positions) is less susceptible to basal mutational processes (Lindahl and Nyberg 1974). Conversely, unpaired sites are expected to undergo relatively higher rates of mutation. These higher rates of mutation may cause the three non-evolutionary measures of covariation to be erroneously inflated, given that they do not fully account for site-to-site rate variation (see Supplementary Section).

Overall, these results provide some reassurance that our method is performing as expected and that the evolutionary measures of coevolution are more reliable than the three measures of covariation that do not take into account evolutionary dependencies amongst the sequences being analysed. The detected degrees of coevolution suggest that a large proportion of the predicted base-pairings in the SHAPE-MaP structures have been selectively maintained since the common ancestors of the sequences being analysed in each of the three datasets.

### 3.7 Ranking and visualisation of sub-structures

Rather than considering the entire secondary structure of a large sequence, it is often useful to consider individual substructures. There are two primary reasons for considering substructures: (i) smaller regions are more easily conceptualised, and (ii) the functional components of secondary structures tend to correspond to sub-regions of a secondary structure.

MESSI automatically ranks substructures by degrees of coevolution between their constituent nucleotides (see Supplementary Methods Section).

The highest ranked substructure in both the SHAPE and consensus rankings was the RRE (SHAPE RRE visualised in Figure 8). The RRE occurs in the genomes of all known HIVs and plays a crucial role in the regulation of HIV virion expression (Heaphy et al. 1990, Daugherty et al. 2010).

The longest continuous helix identified in both the SHAPE-MaP and MESSI structures, was ranked 2nd in the SHAPE ranking and 8th in the consensus ranking, respectively. The SHAPE-MaP analysis revealed that this helix is highly stable, although its function is unknown. The significant degrees of coevolution detected at base-paired sites within this substructure and the fact that MESSI detects it as conserved across all HIV-1 subtype sequences provides further evidence of its likely functional importance.

Portions of the 3’ and 5’ untranslated regions (UTRs) were ranked 3rd and 4th in the SHAPE ranking, respectively. This was not surprising given that...
Table 2: SHAPE structure ranking. Top 10 of 86 non-overlapping HIV NL4-3 substructures ranked from highest to lowest z-score based on the estimated degrees of coevolution within an alignment of HIV-1 subtype B sequences. Where the HIV NL4-3 SHAPE-MaP secondary structure was used as the canonical structure.

| Rank | Alignment position | NL4-3 position | Length | Name | Median degree of coevolution | z-score |
|------|--------------------|----------------|--------|------|-----------------------------|---------|
| 1    | 8233 - 8582        | 7249 - 7595    | 350    | Rev Response element (RRE)  | 5.38    | 5.02 |
| 2    | 2608 - 2943        | 1991 - 2326    | 336    | Longest continuous helix    | 5.17    | 2.92 |
| 3    | 10155 - 10383      | 8982 - 9170    | 229    | 3' Untranslated region (3’UTR) | 5.27    | 2.69 |
| 4    | 588 - 838          | 105 - 344      | 251    | 5' Untranslated region (5’UTR) | 5.65    | 2.61 |
| 5    | 9570 - 9584        | 8440 - 8454    | 15     | 5.91    | 2.29 |
| 6    | 860 - 979          | 366 - 485      | 120    | 5.65    | 2.28 |
| 7    | 1710 - 1845        | 1177 - 1312    | 136    | 5.17    | 2.28 |
| 8    | 2115 - 2301        | 1561 - 1711    | 187    | Gag-pol frameshift           | 5.31    | 2.21 |
| 9    | 1479 - 1490        | 946 - 957      | 12     | 5.85    | 2.04 |
| 10   | 3886 - 3907        | 3269 - 3290    | 22     | 5.80    | 2.01 |

Table 3: Consensus structure ranking. Top 10 of 118 non-overlapping HIV consensus substructures ranked from highest to lowest z-score based on their degrees of coevolution within an alignment of HIV-1 subtype B sequences. Where the canonical structure was treated as unknown and a consensus structure predicted by MESSI.

| Rank | Alignment position | NL4-3 position | Length | Name | Median degree of coevolution | z-score |
|------|--------------------|----------------|--------|------|-----------------------------|---------|
| 1    | 8240 - 8577        | 7256 - 7590    | 338    | Rev Response element (RRE)  | 5.64    | 6.53 |
| 2    | 2202 - 2229        | 1645 - 1672    | 28     | Gag-pol frameshift           | 8.17    | 4.56 |
| 3    | 1710 - 1845        | 1177 - 1312    | 136    | 6.44    | 4.50 |
| 4    | 4751 - 4833        | 4134 - 4216    | 83     | 6.47    | 3.97 |
| 5    | 4505 - 4709        | 3888 - 4092    | 205    | 5.22    | 3.21 |
| 6    | 591 - 939          | 108 - 445      | 349    | 5' Untranslated region (5’UTR) | 5.38    | 3.16 |
| 7    | 133 - 151          | NA             | 19     | 6.85    | 2.94 |
| 8    | 2564 - 2890        | 1947 - 2273    | 327    | Longest continuous helix     | 4.44    | 2.62 |
| 9    | 9782 - 9800        | 8645 - 8663    | 19     | 6.92    | 2.55 |
| 10   | 3612 - 3623        | 2995 - 3006    | 12     | 6.74    | 2.50 |
these are both non-coding regions. The 5’ UTR is involved in regulation of translation (Damgaard et al. 2004), whereas the 3’ UTR is believed to be involved in regulation of transcription (Watts et al. 2009). A 5’ UTR substructure at a similar position is ranked 6th in the consensus ranking, whereas a 3’ UTR substructure at a similar position was not detected in the consensus structure. This may be explained by the large number of UTR missing sequences and high degrees of alignment uncertainty in the HIV-1 subtype B alignment in the UTR regions; factors which would both decay support for the predicted base-pairings in the consensus structure.

An uncharacterised substructure (Alignment position: 1710-1845) ranked 7th in the SHAPE structure ranking and 3rd in the consensus structure ranking (Figure 8). This substructure warrants further study, given the supporting evidence from experimental SHAPE-MaP reactivities, MESSI’s coevolution estimates, and MESSI’s evidence of conservation across HIV-1 subtype B sequences. Despite MESSI predicting the same helix as in the SHAPE-MaP model at the base of this substructure, the remainder of the substructure is different in the SHAPE-MaP model. It is likely that the SHAPE-MaP model of this substructure is more accurate in this instance.

Interestingly, an additional uncharacterised substructure (Alignment position: 4751-4833) ranked 4th in consensus ranking, but was not present in the HIV-1 NL4-3 SHAPE structure and hence was not present in the SHAPE structure ranking (Consensus rank 4 in Figure 8). Overlaid SHAPE-MaP reactivities from the HIV N4L-3 SHAPE model provide some support for MESSI’s prediction; particularly at unpaired positions which are supported by high SHAPE-MaP reactivities (indicating single-strandedness). It is possible that either MESSI’s or SHAPE-MaP’s prediction is wrong, or that the particular conformation predicted by MESSI is conserved amongst a subset of HIV-1 subtype B sequences that excludes NL4-3. It is also possible that this substructure exists in alternative conformations depending on in vivo or in vitro conditions.

Finally, the gag-pol frameshift-associated substructure was ranked 8th in the SHAPE ranking and 2nd in the consensus rankings. This substructure regulates the ratio of HIV gag/gag-pol that is expressed. Ribosomal synthesis of the gag-pol polyprotein requires a -1 ribosomal frameshift, without which translation ends in synthesis of the gag protein alone.

Overall, there is an excess of substructures amongst the top ranking that have been identified in the literature as having structure-related importance. This is particularly evident in the SHAPE-MaP structure ranking. The use of the experimentally-determined SHAPE-MaP structure as the canonical structure strongly informs the SHAPE structure ranking, but has the disadvantage that it is based on the HIV NL4-3 sequence rather than being representative of base-pairings conserved across all sequences within the HIV-1 subtype B alignment. On the other hand, the consensus ranking canonical structure is predicted by MESSI and is based solely on evolutionary information, rather than experimental or thermodynamic information. In the future, we hope to extend MESSI by adding both experimental constraints from experiments such as SHAPE-MaP and thermodynamic constraints from folding software such as Vienna RNAfold. This is expected to improve estimates of coevolution and the overall ranking provided by MESSI.

4 Concluding remarks

MESSI was developed for modelling substitutions that are consistent with the maintenance of canonical base-pairing at paired sites within alignments of DNA and RNA sequences. To achieve this, an existing model, M95 (Muse 1995), was extended in four ways. The first was to allow the three different canonical base-pairs to have different rates of coevolution instead of a single shared rate. The second was to allow substitution rates to vary across sites, including allowing the two positions in a nucleotide pair each to have a potentially different substitution rate. This was done to account for variable substitution rates,
Figure 8: Visualisation of several top ranking substructures in the SHAPE-MaP structure and consensus structure rankings. NL4-3 SHAPE-MaP experimental reactivities are mapped and visually overlaid using the same colour scheme as in [Watts et al., 2009]. Depicted within each nucleotide is a sequence logo summarising the nucleotide composition at the corresponding alignment position. Mean degrees of coevolution inferred using MESSI are depicted for each base-pair using coloured links (blue-green-yellow gradient).
such as those expected within coding sequences. The third was to permit the strength of coevolution to vary across base-paired sites, enabling the identification of sites that are evolving independently versus those that are coevolving in a manner that favours canonical base-pairing. The fourth and final extension was to allow for an unknown secondary structure by using an RNA SCFG prior over secondary structures, and marginalising over structures using the inside algorithm during ML inference or a Metropolis-within-Gibbs procedure during Bayesian inference.

Extending the model to permit an unknown secondary structure posed significant computational challenges. The first challenge was the need to compute likelihoods using Felsentein’s algorithm for all $\binom{n}{2}$ paired sites. Fortunately, a large number of redundant calculations could be avoided due to a large proportion of paired sites sharing the same partial sites patterns [Pond and Muse (2004)], resulting in at least a $5 \times$ speed-up. Additionally, a further $50 \times$ speed-up was achieved using a GPU implementation of Felsenstein’s algorithm. The second bottleneck was the need to marginalise an unknown secondary structure using the inside algorithm. Computational speed-ups by factors of $50 \times - 200 \times$ were achieved using a GPU implementation of the inside algorithm. For Bayesian inference a Metropolis-within-Gibbs procedure was implemented to further avoid calculating the paired matrix likelihoods and inside probabilities at every iteration.

Both ML and Bayesian inference were used for different analyses. ML inference allowed us to perform likelihood ratio tests of various hypotheses, where Bayesian model comparison was computationally intractable. Bayesian inference was used to obtain posterior distributions over various parameters, including the rates of coevolution associated with the canonical base-pairs, and posterior probabilities and degrees of coevolution at base-pair sites.

To perform an initial validation of our model, site permutations of alignment datasets were performed to disrupt the secondary structure dependencies expected in real datasets. Consistent with the model behaving desirably, the structure-integrated maximum likelihood values were lower, and the structure information entropy values higher for the permuted datasets overall.

The ability to marginalise an unknown secondary structure shared amongst an alignment of sequences, implies that MESSI is also capable of secondary structure prediction. Although MESSI was not designed with structure prediction in mind, we found that it performed similarly to two popular comparative secondary structure prediction methods: RNAalifold (Hofacker 2009) and PPfold (Sikósi et al. 2012). This result served to further validate our model.

Strong evidence was found for GU pairs being selectively favoured at base-paired sites in five non-coding RNA datasets and four of five RNA virus genome datasets. Whereas strong evidence for GT pairs being selectively favoured at base-paired sites only found for one out of five of the DNA virus dataset tested. The notion that GU pairs play a role in stabilizing RNA secondary structures is consistent with numerous phylogenetic, and experimental analyses of RNA molecules (Woese et al. 1980; Eddy and Durbin 1994; Deigan et al. 2009). The role of GT base-pairings in stabilizing DNA genomic secondary structures remains unclear.

The model was applied to the HIV-1 NL4-3 secondary structure and two corresponding alignment datasets containing large numbers of HIV-1 sequences, and an SIVmac239 secondary structure and a corresponding alignment of SIV sequences. It was found that correlations between the SHAPE-MaP-determined quantities and degrees of coevolution as detected using MESSI were stronger than correlations detected when comparing the determined quantities and degrees of covariation, as measured using three non-evolutionary methods.

Interactive visualisations of the HIV-1 NL4-3 SHAPE-MaP and consensus secondary structures with the inferred degrees of coevolution overlaid were automatically generated by MESSI. Two rankings of substructures based on inferred degrees of coevolu-
tion within an alignment of HIV-1 subtype B sequences demonstrated an excess of high ranking substructures that have been commonly cited in the literature as having structure-related importance.

The ability to infer degrees of coevolution in a statistically rigorous manner, together with MESSI's visualisation and ranking features is expected to assist researchers in focusing their experimental analyses on those portions of large RNA structures that are most likely to be biologically relevant.

5 Software availability

Julia code (compatible with Windows and Linux) is available at: [https://github.com/michaelgoldendev/MESSI](https://github.com/michaelgoldendev/MESSI)

6 Acknowledgements

MG is supported by the ERC under the European Unions Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement no. 614725-PATHPHYLODYN.

References

Anderson, J. 2014. Stochastic Context-Free Grammars and RNA Secondary Structure Prediction. In M. S. Poptsova, editor, Genome Analysis: Current Procedures and Applications, pages 339–66. Caister Academic Press: England.

Bernhart, S. H., Tafer, H., Mückstein, U., Flamm, C., Stadler, P. F., and Hofacker, I. L. 2006. Partition function and base pairing probabilities of RNA heterodimers. Algorithms for Molecular Biology, 1(1): 3.

Bhattacharya, T., Daniels, M., Heckerman, D., Foley, B., Frahm, N., Kadie, C., Carlson, J., Yusim, K., McMahon, B., Gaschen, B., et al. 2007. Founder effects in the assessment of hiv polymorphisms and hla allele associations. Science, 315(5818): 1583–1586.

Burge, S. W., Daub, J., Eberhardt, R., Tate, J., Barquist, L., Nawrocki, E. P., Eddy, S. R., Gardner, P. P., and Bateman, A. 2012. Rfam 11.0: 10 years of RNA families. Nucleic acids research, 41(D1): D226–D232.

Cheng, N., Mao, Y., Shi, Y., and Tao, S. 2012. Coevolution in RNA molecules driven by selective constraints: evidence from 5S rRNA. PloS one, 7(9): e44376.

Damgaard, C. K., Andersen, E. S., Knudsen, B., Gorodkin, J., and Kjems, J. 2004. RNA interactions in the 5 region of the HIV-1 genome. Journal of molecular biology, 336(2): 369–379.

Darty, K., Denise, A., and Ponty, Y. 2009. VARNA: Interactive drawing and editing of the RNA secondary structure. Bioinformatics, 25(15): 1974.

Daugherty, M. D., Liu, B., and Frankel, A. D. 2010. Structural basis for cooperative RNA binding and export complex assembly by HIV Rev. Nature structural & molecular biology, 17(11): 1337–1342.

Deigan, K. E., Li, T. W., Mathews, D. H., and Weeks, K. M. 2009. Accurate SHAPE-directed RNA structure determination. Proceedings of the National Academy of Sciences, 106(1): 97–102.

Early, T. A., Olmsted III, J., Kearns, D. R., and Lezious, A. G. 1978. Base pairing structure in the poly d (GT) double helix: wobble base pairs. Nucleic acids research, 5(6): 1955–1970.

Eddy, S. R. and Durbin, R. 1994. RNA sequence analysis using covariance models. Nucleic acids research, 22(11): 2079–2088.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of molecular evolution, 17(6): 368–376.

Fruhwaert-Schnatter, S. 1994. Data augmentation and dynamic linear models. Journal of time series analysis, 15(2): 183–202.
Gautheret, D., Konings, D., and Gutell, R. R. 1995. GU base pairing motifs in ribosomal RNA. *Rna*, 1(8): 807–814.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Kern, J., Lowe, A. D., Singh, M., and Skinner, M. A. 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. *Cell*, 60(4): 685–693.

Ho, P., Frederick, C., Quigley, G., Van der Marel, G., Van Boom, J., Wang, A., and Rich, A. 1985. GT wobble base-pairing in Z-DNA at 1.0 Å atomic resolution: the crystal structure of d(CGCGTG). *The EMBO journal*, 4(13A): 3617.

Hofacker, I. L. 2009. RNA secondary structure analysis using the Vienna RNA package. *Current Protocols in Bioinformatics*, pages 12–2.

Johnson, S. G. 2014. The NLopt nonlinear-optimization package.

Joyner, D., Čertík, O., Meurer, A., and Granger, B. E. 2012. Open source computer algebra systems: SymPy. *ACM Communications in Computer Algebra*, 45(3/4): 225–234.

Knudsen, B. and Hein, J. 1999. RNA secondary structure prediction using stochastic context-free grammars and evolutionary history. *Bioinformatics*, 15(6): 446–454.

Lindahl, T. and Nyberg, B. 1974. Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochemistry*, 13(16): 3405–3410.

Lindgreen, S., Gardner, P. P., and Krogh, A. 2006. Measuring covariation in RNA alignments: physical realism improves information measures. *Bioinformatics*, 22(24): 2988–2995.

Markham, N. R. and Zuker, M. 2008. UNAFold. In *Bioinformatics*, pages 3–31. Springer.

Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of molecular biology*, 288(5): 911–940.

Mattick, J. S. 2003. Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. *Bioessays*, 25(10): 930–939.

Moulton, V., Zuker, M., Steel, M., Pointon, R., and Penny, D. 2000. Metrics on RNA secondary structures. *Journal of Computational Biology*, 7(1-2): 277–292.

Muhire, B. M., Golden, M., Murrell, B., Lefeuvre, P., Lett, J.-M., Gray, A., Poon, A. Y., Ngandu, N. K., Semegni, Y., Tanov, E. P., et al. 2014. Evidence of pervasive biologically functional secondary structures within the genomes of eukaryotic single-stranded DNA viruses. *Journal of virology*, 88(4): 1972–1989.

Muse, S. V. 1995. Evolutionary analyses of DNA sequences subject to constraints of secondary structure. *Genetics*, 139(3): 1429–1439.

Pollom, E., Dang, K. K., Potter, E. L., Gorelick, R. J., Burch, C. L., Weeks, K. M., and Swanstrom, R. 2013. Comparison of SIV and HIV-1 genomic RNA structures reveals impact of sequence evolution on conserved and non-conserved structural motifs. *PLoS pathogens*, 9(4): e1003294.

Pond, S. L. K. and Muse, S. V. 2004. Column sorting: Rapid calculation of the phylogenetic likelihood function. *Systematic biology*, 53(5): 685–692.

Powell, M. J. D. 1994. *A Direct Search Optimization Method That Models the Objective and Constraint Functions by Linear Interpolation*, pages 51–67. Springer Netherlands.

Price, M. N., Dehal, P. S., and Arkin, A. P. 2010. FastTree 2—approximately maximum-likelihood trees for large alignments. *PloS one*, 5(3): e9490.

Rousset, F., Pelandakis, M., and Solignac, M. 1991. Evolution of compensatory substitutions through...
GU intermediate state in Drosophila rRNA. *Proceedings of the National Academy of Sciences*, 88(22): 10032–10036.

Siegfried, N. A., Busan, S., Rice, G. M., Nelson, J. A., and Weeks, K. M. 2014. RNA motif discovery by SHAPE and mutational profiling (SHAPE-MaP). *Nature methods*, 11(9): 959–965.

Sükösd, Z., Knudsen, B., Værum, M., Kjems, J., and Andersen, E. S. 2011. Multithreaded comparative RNA secondary structure prediction using stochastic context-free grammars. *BMC bioinformatics*, 12(1): 103.

Sükösd, Z., Knudsen, B., Kjems, J., and Pedersen, C. N. 2012. PPfold 3.0: fast RNA secondary structure prediction using phylogeny and auxiliary data. *Bioinformatics*, 28(20): 2691–2692.

Tuplin, A., Wood, J., Evans, D. J., Patel, A. H., and Simmonds, P. 2002. Thermodynamic and phylogenetic prediction of RNA secondary structures in the coding region of hepatitis C virus. *RNA*, 8(6): 824–841.

Tuplin, A., Evans, D., and Simmonds, P. 2004. Detailed mapping of RNA secondary structures in core and NS5B-encoding region sequences of hepatitis C virus by RNase cleavage and novel bioinformatic prediction methods. *Journal of General Virology*, 85(10): 3037–3047.

Varani, G. and McClain, W. H. 2000. The G-U wobble base pair. *EMBO reports*, 1(1): 18–23.

Watson, J. D. and Crick, F. H. 1953. Molecular structure of nucleic acids. *Nature*, 171(4356): 737–738.

Watts, J. M., Dang, K. K., Gorelick, R. J., Leonard, C. W., Bess Jr, J. W., Swanstrom, R., Burch, C. L., and Weeks, K. M. 2009. Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature*, 460(7256): 711–716.

Wilkinson, K. A., Merino, E. J., and Weeks, K. M. 2006. Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. *Nature protocols*, 1(3): 1610–1616.

Woese, C., Magrum, L., Gupta, R., Siegel, R., Stahl, D., Kop, J., Crawford, N., Brosius, R., Gutell, R., Hogan, J., *et al*. 1980. Secondary structure model for bacterial 16S ribosomal RNA: phylogenetic, enzymatic and chemical evidence. *Nucleic acids research*, 8(10): 2275–2294.

Yang, Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution*, 10(6): 1396–1401.

Yang, Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *Journal of Molecular evolution*, 39(3): 306–314.