Empirical Models for Substitution in Ribosomal RNA

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Running head: Empirical rRNA substitution model

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Abstract

Empirical models of substitution are often used in protein sequence analysis because the large alphabet of amino acids requires that many parameters be estimated in all but the simplest parametric models. When information about structure is used in the analysis of substitutions in structured RNA, a similar situation occurs. The number of parameters necessary to adequately describe the substitution process increases in order to model the substitution of paired bases.

We have developed a method to obtain substitution rate matrices empirically from RNA alignments that include structural information in the form of base pairs. Our data consisted of alignments from the European ribosomal RNA database of Bacterial and Eukaryotic Small Subunit and Large Subunit ribosomal RNA (Wuyts et al., 2001a; Wuyts et al., 2002). Using secondary structural information, we converted each sequence in the alignments into a sequence over a 20-symbol code: one symbol for each of the 4 individual bases, and one symbol for each of the 16 ordered pairs. Substitutions in the coded sequences are defined in the natural way, as observed changes between two sequences at any particular site. For given ranges (windows) of sequence divergence, we obtained substitution frequency matrices for the coded sequences. Using a technique originally developed for modelling amino acid substitutions (Veerassamy et al., 2003), we were able to estimate the actual evolutionary distance for each window. The actual evolutionary distances were used to derive instantaneous rate matrices, and from these we selected a universal rate matrix.

The universal rate matrices were incorporated into the Phylip package (Felsenstein, 2002), and we analyzed the ribosomal RNA alignments using both distance and maximum likelihood methods. The empirical substitution models performed well on simulated data,
and produced reasonable evolutionary trees for 16S ribosomal RNA sequences from sequenced Bacterial genomes.

Empirical models have the advantage of being easily implemented, and the fact that the code consists of 20 symbols makes the models easily incorporated into existing programs for protein sequence analysis. In addition, the models are useful for simulating the evolution of RNA sequence and structure simultaneously.
Introduction

The conserved nature of ribosomal RNA structure, facilitated by compensating substitutions of paired bases, means that the evolution of bases in structurally related positions may be highly dependent. Ribosomal RNA (rRNA) sequences thus violate a key assumption of many phylogeny methods: that different sites in a sequence have independent evolutionary rates (Tillier & Collins, 1995). Because many rRNA structures are well known, we are now in a position to incorporate structural information into the analysis of rRNA. Indeed, several methods and models have already done so (reviewed in Savill et al., 2001, recognizing the likely evolutionary dependence between sites.

Probabilistic methods of phylogenetic analysis, such as Bayesian phylogeny (Yang & Rannala, 1997), maximum likelihood (Felsenstein, 1981) and modified distance measures (Kimura, 1981), make use of substitution models that define probabilities for the transition from one base to another. When base substitution is adequately modelled using a low number of states (or under restricting assumptions), parametric models are feasible. Simultaneously modelling the evolution of sequence and structure has been done with several parametric models, but this approach has been shown to be accurate only when a large number of parameters are used (Savill et al., 2001). In the common case where a reasonable description of the substitution process requires a large number of parameters, e.g. for models of amino acid substitution, empirical models are often used. For empirical models, the relative substitution probabilities are derived from databases of alignments; the PAM model of amino acid substitution is a well known example (Dayhoff et al., 1978). The estimation of empirical substitution rates in rRNA has been
previously been attempted (Tillier & Collins, 1998; Higgs, 2000).

Databases of rRNA sequences are now sufficiently large (with over 50,000 sequences represented) to form the basis of an empirical model of substitution for rRNA. Here we present an empirical model of substitution for both individual and paired bases in structurally annotated rRNA. Our model is based on sequence alignments from RNA databases and conserved reference secondary structures. The method we used to obtain substitution matrices from rRNA data is an extension of a procedure we recently applied to derive models of amino acid substitution (Veerassamy et al., 2003). We accomplish this by converting the rRNA sequences into sequences over a 20-symbol code. While the 20-symbol code reflects the number of individual bases and ordered pairs of bases, the size of the alphabet is fortuitous: the resulting rRNA models have $20 \times 20$ substitution rate matrices, and can be used in many applications designed to analyze the evolution of proteins using $20 \times 20$ amino acid substitution rate matrices. Such programs were modified to allow the analysis of rRNA sequences with the same methods.

**Methods**

**Data**

Sequence alignments were obtained from the European databases on Small and Large Subunit ribosomal RNAs (Wuyts et al., 2001a; Wuyts et al., 2002). For each alignment, a reliable reference sequence, with a known secondary structure, was selected from the comparative RNA website (CRW) database (Cannone et al., 2002). The reference structures we used were *Escherichia coli* (J01695) for both the Large Subunit (LSU) and Small Subunit (SSU) Bacterial
alignments, *Mit. Zea mays* (X00794) for the SSU Mitochondrial alignment, *Mit. Xenopus laevis* (M10217) for the LSU Mitochondrial alignment, *Saccharomyces cerevisiae* (U53879) for both SSU and LSU Eukaryotic alignments, and *Methanococcus jannaschii* (U67517) for the SSU Archaeal alignment (due to insufficient data, we did not apply our method to the LSU Archaeal alignment). We used the secondary structure as a reference to identify paired bases in all sequences in the alignments. This assumes that the ribosomal RNA structures are similar for species within an alignment.

**Sequence Coding**

We converted each rRNA sequence into a sequence over a 20-symbol alphabet in order to treat both single stranded and double-stranded bases uniformly. The code is given in Figure 1(a), and an example conversion is shown in Figure 1(b). The code uses 4 symbols to represent the single stranded bases: the unpaired bases or bases paired with a gap, (which are also single stranded), and 16 symbols to represent ordered pairs of bases, as given by the reference secondary structure. Bases that were not A, C, G, U or – were coded as unknowns (X), as were pairs having the form (–,–), and unknowns were ignored in subsequent analyses. The symbol for each pair appears exactly once in the converted sequence, and the symbols are ordered according to the position of the 3’ base in a pair.
Deriving the Empirical Model

Our model was constructed using an approach similar to that of Veerassamy et al. (2003), designed to obtain an amino acid substitution model from the BLOCKS database (Henikoff & Henikoff, 1992). The model uses empirical data in the form of matrices that count substitutions between pairs of coded sequences (from the alignment) having a distance falling within a particular range (window). We were able to obtain count matrices for the sliding windows, each containing data corresponding to a different degree of sequence divergence. As described in more detail below, the count matrices are converted into mutability matrices. Each mutability matrix is a function of two unknowns: a universal rate matrix, and an actual evolutionary distance. The approach first estimates the actual evolutionary distance using a differential equation. The estimated actual evolutionary distance is then used to derive an instantaneous rate matrix for each window. From the set of instantaneous rate matrices, we select one as our estimate of the universal rate matrix.

Substitution Frequency Matrices and Observed Distances

From the alignments, each pair of converted sequences was compared and their observed sequence divergence (frequency of substitution) was recorded. Those sites containing X were disregarded. Sequence pairs were disregarded altogether when there were fewer than 200 comparable sites between them. This was done to prevent the artificial situation of observing very high divergence between two sequences purely because very few sites were comparable.

A series of overlapping windows representing the full range of sequence divergence were
defined. The first window included all sequence pairs with divergence less than or equal to the fixed window size $w$. The $i^{th}$ window included all pairwise sequence comparisons with a relative divergence between $i$ and $w + i$. For each alignment, each type of substitution was counted for all of the sequence pairs in each window. In this manner, a count matrix was obtained for each window, and these were converted into frequency matrices. Because direction of substitution is not known, each substitution was counted for both directions and the diagonal entries in the matrix were doubled. Using this method, consecutive windows are expected to contain a nearly identical set of pairs, and windows corresponding to sufficiently high divergence are expected to be empty. The sliding window strategy allowed us to ensure that we had observations from many different levels of sequence divergence. By manipulating the window size we were able to control the amount of data in each window. The sizes for the sliding windows were selected according to the quality of matrices produced for the sizes (see below).

**Estimating Actual Evolutionary Distances**

Each count matrix was converted to a frequency matrix, denoted by $F$. From the frequency matrices, we calculated the vector $\pi$ of observed frequencies for each code symbol

$$
\pi_i = \frac{\sum_{j=1}^{20} F_{ij}}{\sum_{i' = 1}^{20} \sum_{j=1}^{20} F_{i'j}},
$$

(1)
for \(1 \leq i \leq 20\). For each row of the frequency matrix, we divide the entries in that row by the row sum, resulting in mutability matrix \(M\):

\[
M_{ij} = \frac{F_{ij}}{\sum_{j'=1}^{20} F_{ij'}},
\]

where \(1 \leq i, j \leq 20\). Mutability matrices were only computed for windows where the frequency matrices had nonzero row sums in order to prevent division by zero in Equation 2. Mutability matrices describe the frequency of any symbol being substituted by any other (including itself). Because each element of a mutability matrix falls between 0 and 1, and the sum of the elements in each row is equal to 1, mutability matrices are stochastic matrices. The mutability matrices derived from the frequency matrices are reversible and thus fulfill the detailed balance equation:

\[
\pi_i M_{ij} = \pi_j M_{ji}.
\]

The average substitution frequency can be calculated from the mutability matrices and the frequency of the code symbols using the following formula:

\[
D(M) = 1 - \sum_{i=1}^{20} \pi_i M_{ii}.
\]
from which $M$ was derived, we let $M(P)$ denote the mutability matrix $M$ as a function of evolutionary distance $P$. The value of $P$ is unknown since sequences ancestral to those in the alignment are unknown and because multiple substitutions may have actually occurred at any site. Since actual evolutionary distance is additive, taking the square (square root) of a mutability matrix will double (halve) the actual evolutionary distance. In general, for any mutability matrix $M$, and any number $n$,

$$M(P)^n = M(nP), \quad (5)$$

which expresses a special form of the Chapman-Kolmogorov equation for Markov chains. We used this property to derive the instantaneous rate matrix from the mutability matrices. Because this required taking fractional powers and logarithms of the mutability matrices, the mutability matrices were used only if their eigenvalues were all non-negative.

We estimated the derivatives of the observed distances with respect to actual distance numerically using the 5-point formula (Burden & Faires, 1985),

$$\frac{dD(M(P))}{dP} = \frac{1}{12(\lambda)P} \left(D(M((1 - 2\lambda)P)) - 8D(M((1 - \lambda)P)) + 8D(M((1 + \lambda)P)) - D(M((1 + 2\lambda)P))\right) \quad (6)$$
with $\lambda = 0.01$:

$$\frac{dD(M(P))}{dP} = \frac{1}{12(0.01)} P \left( D(M(0.98P)) - 8D(M(0.99P)) + 8D(M(1.01P)) - D(M(1.02P)) \right).$$ \hspace{1cm} (7)

We used the additivity of mutability matrices and the memoryless property of Markov chains (Equation 5) to obtain the following equivalent formula:

$$P \frac{dD(M(P))}{dP} = \frac{1}{0.12} \left( D(M(P)^{0.98}) - 8D(M(P)^{0.99}) + 8D(M(P)^{1.01}) - D(M(P)^{1.02}) \right).$$ \hspace{1cm} (8)

The expression on the right can be estimated by a polynomial in $D(M(P))$. For notational convenience, let $D = D(M(P))$. We found that a cubic polynomial with four unknown coefficients was sufficient to characterize the observed data (Figure 2), yielding the differential equation:

$$P \frac{dD}{dP} \approx a_3D^3 + a_2D^2 + a_1D + a_0,$$ \hspace{1cm} (9)

We also know the initial conditions since, at sufficiently low distance there are no overlapping substitutions and the number of actual substitutions is equal to the number of observed substitutions. Therefore,

$$\lim_{P \to 0} D(M(P)) = 0 \hspace{1cm} \text{and} \hspace{1cm} \lim_{P \to 0} \frac{dD(M(P))}{dP} = 1.$$ \hspace{1cm} (10)
allowing us to conclude that $a_0 = 0$ and $a_1 = 1$. The differential simplifies to the quadratic

$$\frac{P \, dD}{D \, dP} \approx c_2 D^2 + c_1 D + 1,$$

(11)

with only two unknown coefficients, $c_2$ and $c_1$. The differential is separable and easily solved. The solution defines a multiple-hit correction formula from which the estimated actual $P$ distances can be estimated from observed $D$ distances

$$P = \text{corrected}(D)$$

(12)

The correction formula depends on the dataset and several examples are shown in Figure 3.

**Instantaneous and Universal Rate Matrices**

Once the actual evolutionary distances have been estimated, we had sufficient information to derive instantaneous rate matrices for each window. The following formula was used to obtain the instantaneous rate matrix:

$$A = \ln(M)/P.$$  

(13)

Equation 13 can only be used when the logarithm is well defined, which requires that all eigenvalues of $M$ be non-negative. A valid instantaneous rate matrix is required to have both a corresponding valid mutability matrix, and also not have any negative values off the main diagonal.

This scheme can provide up to $n(1 - w) + 1$ instantaneous rate matrices, but much fewer
were found to be valid. Both the situations of negative eigenvalues in mutability matrices, and negative off-diagonal rates in instantaneous rate matrices, have been found to be associated with insufficient observations in the count matrix and/or high divergence.

From the set of valid instantaneous rate matrices, one was selected as the universal rate matrix. The selection criteria was based on how well an instantaneous matrix could predict the observed mutability matrices, given the estimated actual evolutionary distance. For each valid instantaneous rate matrix $A$, we evaluated the sum of relative residuals. This is calculated as the difference in the norm (largest singular value) between the exponential of the estimated log of the matrix and the original matrix:

$$
\sum_i \frac{\| \exp(AP_i) - M_i \|}{\| M_i \|},
$$

where the sum is over all valid mutability matrices. The instantaneous matrix minimizing this quantity was selected as the universal rate matrix. Examples of rate matrices are shown in Figure 4.

**Simulations Experiments**

Experiments were conducted to assess how well the matrices could recover phylogenetic trees obtained from simulated data. Our Bacterial 16S rRNA model was incorporated into the PSeq-Gen program (Grassly *et al.*, 1997). The PSeq-Gen program was designed to generate protein sequences from a given tree and an amino-acid substitution model such as PAM (Dayhoff *et al.*, 1978). Since the matrices that describe our model have the same dimensions as amino acid substitution matrices, programs using amino acid substitution matrices are easily modified to
implement our empirical model instead. We modified the PSeq-Gen program to incorporate the SSU bacterial empirical rRNA substitution model.

We simulated sequences using a 20-species tree (see Figure 5) and the resulting sequences were analyzed with our own programs, and programs from the Phylip package (Felsenstein, 2002). We used both the dnaml program and the combination of the dnadist and neighbor programs from Phylip, on sequences obtained by converting the simulated sequences (over the 20-symbol code) back into the 4 bases of RNA (using our program called 20to4). We also used the programs rrnadist and rrnaml, which are modified versions of the Phylip protdist and protml that incorporate our 16S rRNA matrices. Finally, our own distance program rnadist, which implements the parametric model OTRNA from Tillier and Collins (1998), was also used.

**Example Data Set: Bacterial Phylogeny**

We selected 16S rRNA sequences from the set of completely sequenced genomes for which there was a representative alignment in the European ribosomal RNA database (a total of 70 full length sequences). We used *E.coli* as a reference sequence and the secondary structure obtained from the RNA database (Cannone et al., 2002), and converted the sequences to the 20 letter code with our program 4to20. We analyzed this data set using the same programs listed above for the simulated data. We allowed for gamma- distributed rates, with a gamma parameter of 1, for all methods; for the maximum likelihood methods, we used 2 categories in the HMM. All trees were bootstrapped 100 times. The trees obtained using the maximum likelihood methods are shown in Figure 6.
Results

The rRNA Matrices

We applied our method to several alignments from the European RNA databases (Wuyts et al., 2001a; Wuyts et al., 2002) attempting to obtain matrices specific to Bacterial, Eukaryotic, Archeal and Mitochondrial ribosomal RNAs, for both the Small Subunit and the Large Subunit. Some properties of these alignments are given in Table 1.

The alignments were of varying sizes. The number of observations that can be extracted from an alignment is proportional to the number of pairs of sequences and the number of comparable sites in each pair. The number of sequences in the alignments ranged from 156 for Eukaryote LSU, to 12107 for Bacterial SSU. The alignment lengths ranged from 1000 to 2500 bases, but the number of comparable sites varied considerably, even within an alignment. The most important factor affecting the number of observations for any alignment is the number of sequences in that alignment. Only four alignments (Bacterial, Mitochondrial, Eukaryote SSU and Eukaryote LSU) produced a universal rate matrix. For the remainder, the alignments were too small (too few observations). The Eukaryote LSU alignment is an exception to this trend. Having only 156 sequences, this alignment still produced many valid instantaneous rate matrices from which to select a universal rate matrix.

Window sizes were selected to produce the greatest number of valid matrices. For the Bacterial, Mitochondrial and Archaeal alignments, a window size of 10% of the alignment length was found to produce very good results, and deviation from this size did not produce significantly more valid matrices. For Eukaryote (SSU and LSU) alignments, increasing the
window size resulted in improvements up to 20%. Since we wish to cover the largest range of actual divergences, the window size was selected to balance the sometimes conflicting requirements of producing instantaneous rate matrices corresponding to large divergence, and also to keep the residuals low.

Table 1 also shows the number of windows in each alignment from which observations were made. The total number of windows is the number of count matrices with non-zero row sums. All of these were used in our analysis when testing the universal rate matrices according to predictive ability. Not all of these could be used when fitting the curves needed to estimate the actual distances. Those windows for which the mutability matrix had negative eigenvalues could not be used in Equation 8. We also required that the numerically estimated derivatives from Equation 8 be non-negative. The mutability matrices corresponding to all windows fitting this criterion were used to fit the curve given by Equation 11. Figure 2 gives the curves for Bacterial SSU, Mitochondrial SSU, and Eukaryote SSU and LSU, where the data points used to estimate the curve are plotted, along with the resulting curve. The fitted curve is shown over the range of divergence for non-empty windows. Figure 3 shows the resulting distance correction for the different datasets.

The valid windows are those that meet the requirements for fitting the curve, and also produced a valid instantaneous rate matrix with no negative values off the diagonal. Table 1 shows that the Eukaryote alignments produced many valid windows, while the mitochondrial alignment produced very few.

The most important statistic in Table 1 is the average relative residual of the observed mutability matrices with the mutability matrices predicted using the universal rate matrix. The
relative residual measures how well the selected universal rate matrix can predict the observed data (the mutability matrices). We calculated residuals predicting mutability matrices from all non-empty windows, also for predicting mutability matrices from those windows with a valid instantaneous rate matrix. When predicting mutability matrices for those windows producing valid instantaneous matrices, the universal rate matrix had residuals less than 0.1. The residual for predicting Bacterial matrices was based on 144 comparisons. For the Mitochondrial SSU matrix, the residual with respect to valid matrices was 0.048, but averaged over only 19 matrices, each corresponding to a window close to the one used to obtain the universal rate matrix. The Eukaryotic alignments produced a large number of valid matrices.

**Simulations**

Because of the unusual nature of our parametric substitution model for recoded RNA sequences, and because we would be using protein analysis programs for the analysis of rRNA data, we first established the feasibility of our approach using simulated sequences. The Bacterial SSU rRNA matrix was incorporated into PSeq-Gen (Grassly et al., 1997) and used to generate 100 simulated data sets which we then analyzed with several methods and the results are given in Figure 5. The values on the branches are the number of simulations in which the correct tree (given in Figure 5(f)) was found. The topology and branch lengths of the tree in Figure 5(f) were chosen to reflect a range for the degree of difficulty in correctly identifying the branching by standard methods. The rRNA methods (*rrnaml* (Figure 5(b)) and *rrnadist* (Figure 5(a)) using our Bacterial SSU rRNA matrix with default parameters) did well in recovering the trees. This is not surprising since these methods applied the same model as was employed to generate the
sequences. Nevertheless, this result means that such models are useful for the simulation of rRNA evolution and for phylogenetic analysis of such sequences. The simulated sequences were also analyzed with the distances calculated according to the OTRNA model (Figure 5(c)), which is a parametric model for RNA evolution (Tillier & Collins, 1998). Although rnadist does not do as well as the rrnadist (Figure 5(a)), it does perform better than the standard DNA methods dnadist (Figure 5(e)) and dnaml (Figure 5(d)) on the same data. The DNA methods were applied after reverse coding the sequences back to the 4-base RNA code, so that the secondary structure information was lost. The observed reduction in the methods’ ability to obtain the correct tree shows that the consideration of structural information is important for obtaining the correct phylogeny.

**Bacterial phylogeny**

Since the rrnadist and rrnaml programs were successful in simulations, we were confident in applying the empirical models to actual rRNA data. We chose the sequenced Bacterial genomes since we have interests in these genomes and their true phylogeny is unknown. The resulting rrnaml tree is compared to the dnaml tree in Figure 6. The trees are remarkably similar, but with generally lower bootstrap values for the rrnaml tree. The distance trees obtained with dnadist, rnadist, and rrnadist are also not significantly different from one another (data not shown).
Discussion

Obtaining the matrices that describe our model required making several methodological assumptions and also making assumptions and approximations on the datasets we used.

The assumptions of our method for deriving the empirical matrix have largely been addressed in Veerassamy et al. (2003). Some minor methodological changes were made to apply the method to the coded rRNA sequences. Unlike the amino acid substitution frequency matrices from the BLOCKS database (Henikoff & Henikoff, 1992), the rRNA substitution frequency matrices often had negative eigenvalues for higher sequence divergence levels and thus could not be used for estimating a rate matrix. Negative eigenvalues in mutability matrices are often associated with an insufficient number of observations in the corresponding count matrix. Instead of using the clustering approach of Blosum (Henikoff & Henikoff, 1992), we used a sliding window strategy. The purpose of the sliding window was to obtain a large number of count matrices, corresponding to many different levels of sequence divergence, and to ensure that each count matrix contained a high number of counts.

Negative eigenvalues are also observed when the frequency matrix is too far from identity (Devauchelle et al., 2002). Biologically speaking, this is the situation of too many overlapping substitutions to determine a set of positive substitution rates that produced the observed data. This phenomena has been previously observed and accounts for the difficulty in obtaining distance estimates from rRNA (Hoyle & Higgs, 2003). This happens earlier (i.e. at lower levels of observed divergence) in the Bacterial data set than in the Eukaryotic data set. A likely cause is higher rates of simultaneous compensatory base substitutions in Bacteria, and stronger
conservation of secondary structure in Bacteria.

Certain assumptions underlying our model are independent of our methods. The most evident assumption is that the sequence data and the alignments used are correct. The huge alignments from the rRNA databases represent solutions to an extremely difficult global multiple sequence alignment problem, and cannot realistically be expected to be optimal with respect to any measure of alignment quality. The nature of our empirical model requires that very large multiple alignments be used, so we must assume that the alignment given is sufficiently accurate to form a basis of inference.

We also analyzed alignments obtained from the RDPII database (Maidak & others (13 coauthors), 2001), and obtained similar results (data not shown). We chose to focus on the European rRNA database, so that we could refine our methods for a specific database. The European rRNA database was more complete in terms of 23S sequences. Also, in the RDPII database, the crucial distinction between deleted nucleotides and unknown (un-sequenced) nucleotides was not always clear.

Other assumptions had to do with the correctness of the reference structures, and how well conserved is the secondary structure in any of the alignments used. The structures we used were obtained from CRW database (Cannone et al., 2002). The structures were largely obtained by comparative sequence analysis (Gutell et al., 2002) which also assumes conserved structures throughout sequences. Although the structure of rRNA is quite conserved, it is not immutable. Particularly the Eukaryote sequences have shown changes in structure (Wuyts et al., 2001b). This is reflected in the universal rate matrix we derived for them, which showed some significant substitution rates between paired and unpaired bases (see Figure 4). These rates reflect changes
in structure or problems with the alignments. The matrices will be refined by considering more reference secondary structures in an alignment. The same methods can easily be applied in the extreme case where each sequence has a known secondary structure.

The idea that the base pairs rather than the bases in the sequences are the independently evolving units in an structured RNA sequence has been used before (Tillier & Collins, 1995) and sophisticated parametric models considering up to the 16 base combinations possible in a pairing have been developed. The disadvantages of these models are that they can be slow and difficult to implement, although some such models have been implemented in a Bayesian phylogenetic framework (PHASE) (Jow et al., 2002; Hudelot et al., 2003), and for distance analyses (rrnadist). It has also been shown that RNA models models require many free parameters to be accurate (Savill et al., 2001) because of differences in the rates of substitution between the base pairs. However, the models are usually applied only to rRNA molecules for phylogenetic analysis. The large size of the rRNA database makes it possible to empirically derive the substitution rates between the base-pairs, and including the single-stranded bases in the model allowed us to easily implement the combined analysis of single and double stranded regions. This task was made even easier by the fact that $20 \times 20$ empirical matrices have commonly been implemented for the analysis of protein sequences.

A recoding approach could be applied to other RNA molecules (tRNAs for example), but different recoding alphabets might also be used on other types of sequences. For example it is conceivable to derive an empirical model for codon evolution (a $64 \times 64$ matrix). The method used to then derive a matrix is independent of the recoding. Our method for deriving the empirical matrices (from Veerassamy et al., 2003), requires large amounts of data (as does the
approach of Muller & Vingron (2000)). Other approaches for deriving matrices could be used in more specific cases with smaller datasets (Arvestad & Bruno, 1997; Whelan & Goldman, 2001; Devauchelle et al., 2002).

The phylogenetic trees obtained with the new method show reduced levels of statistical support, as expected due to the reduced number of independently evolving characters considered in the recoded sequences (Tillier & Collins, 1995). The new method therefore does not allow better resolution of the tree, rather it gives a more accurate estimate of the (generally low) confidence in the branch estimates. The branching order is not significantly different from the one obtained with the standard DNA approaches however. This is reassuring in two ways, the first being that the standard DNA methods on this dataset do show some robustness with respect to the violation of the assumption of independence of sites, and secondly because it shows that the methods using our empirical models do at least as well as the parametric approaches.

We have shown that empirical matrices can be used for the study of rRNA evolution with both simulations and with an example phylogenetic analysis of Bacterial 16S rRNA sequences. The ability to analyze and simulate rRNA sequence evolution including secondary structure constraints should be very useful in other studies.

**Supplementary Material**

All programs and matrices developed here are available from URL:

http://www.uhnres.utoronto.ca/tillier/rRNA/rna.html which is password protected until publication (username: ribosome, password: matrix).
References


Figures Legends

**Figure 1. rRNA sequence conversion.** In panel (a) is given the conversion code for the rRNA single-stranded bases and double-stranded base-pairs to the 20-symbol code. In panel (b) is given an example of how a sequence and its structure are denoted with the new code.

**Figure 2. Graph of $PdD/dP$ vs $D$.** These graphs indicate the quality of fit, and the range of valid data compared with the range of observable data.

**Figure 3. Corrected distances.** The corrected evolutionary distance is shown as a function of the observed evolutionary distance for 3 SSU rRNA datasets and 1 LSU rRNA dataset.

**Figure 4. Rate matrices for SSU rRNA.** The entries of instantaneous rate matrices derived for the bacteria and eukaryote data sets are divided by the frequency of the base/ base pair for that column. The resulting matrices are symmetric and the Bacterial matrix is shown on the upper right and the Eukaryotic matrix in the lower left. The order of the rows/columns have been changed to show them in order of single-stranded bases, Watson-Crick and GU base pairs and non- Watson-Crick pairings.

**Figure 5. Simulation results.** The numbers at the nodes indicate how often the correct branching was found out of 100 simulations using 5 different analysis methods. The simulated tree topology is shown in panel 5(f).
**Figure 6. Bacterial SSU rRNA trees.** Results of 100 bootstrap samples for the bacterial 16S rRNA sequences using maximum likelihood methods. \texttt{dnaml} was run with a discrete approximation of gamma-distribution with gamma parameter of 1 and 4 categories of sites. \texttt{rrnaml} was run with the same gamma parameter and only 2 categories. The values at the nodes indicate the number of bootstrap samples with the corresponding branching. Although the trees are almost identical, the bootstrap values for the \texttt{dnaml} tree are generally higher.
Table 1: Properties of alignments used to derive rRNA matrices. The reference structures were: *E. coli* (J01695) for Bacteria, *Mit. Z. mays* for Mitochondria (SSU) and *Mit. X. laevis* for Mitochondria (LSU), *S. cerevisiae* (U53879) for Eukaryote, and *M. jannaschii* (U67517) for Archaea.

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Figure 1:

(a) RNA Code:

\[
\begin{align*}
A & \mapsto A & AA & \mapsto C & CA & \mapsto H & GA & \mapsto M & UA & \mapsto T \\
C & \mapsto R & AC & \mapsto Q & CC & \mapsto I & GC & \mapsto F & UC & \mapsto W \\
G & \mapsto N & AG & \mapsto E & CG & \mapsto L & GG & \mapsto P & UG & \mapsto Y \\
U & \mapsto D & AU & \mapsto G & CU & \mapsto K & GU & \mapsto S & UU & \mapsto V
\end{align*}
\]

(b) Example Recoding:

Original: \text{GCGU–XGCAUXC–ACUAAGA}

\[\downarrow\]

Converted: \text{NLMTDXMRADXRA}
Figure 2:

- **Bacteria (SSU)**
- **Mitochondria (SSU)**
- **Eukaryote (SSU)**
- **Eukaryote (LSU)**
Figure 3:

- Bacteria (SSU)
- Mitochondria (SSU)
- Eukaryote (SSU)
- Eukaryote (LSU)
## Figure 4:

### Bacteria SSU rRNA

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### Eukaryote SSU rRNA
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Figure 6: