PETcofold: predicting conserved interactions and structures of two multiple alignments of RNA sequences
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ABSTRACT
Motivation: Predicting RNA–RNA interactions is essential for determining the function of putative non-coding RNAs. Existing methods for the prediction of interactions are all based on single sequences. Since comparative methods have already been useful in RNA structure determination, we assume that conserved RNA–RNA interactions also imply conserved function. Of these, we further assume that a non-negligible amount of the existing RNA–RNA interactions have also acquired compensating base changes throughout evolution. We implement a method, PETcofold, that can take covariance information in intra-molecular and inter-molecular base pairs into account to predict interactions and secondary structures of two multiple alignments of RNA sequences.
Results: PETcofold's ability to predict RNA–RNA interactions was evaluated on a carefully curated dataset of 32 bacterial small RNAs and their targets, which was manually extracted from the literature. For evaluation of both RNA–RNA interaction and structure prediction, we were able to extract only a few high-quality examples: one vertebrate small nucleolar RNA and four bacterial small RNAs. For these we show that the prediction can be improved by our comparative approach. Furthermore, PETcofold was evaluated on controlled data with phylogenetically simulated sequences enriched for covariance patterns at the interaction sites. We observed increased performance with increased amounts of covariance. Availability: The program PETcofold is available as source code and can be downloaded from http://rth.dk/resources/petcofold.
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1 INTRODUCTION
Non-coding RNAs (ncRNAs) are receiving rapidly growing attention; they are present in large numbers as observed by The ENCODE Project Consortium (2007) and the FANTOM2 project (Ravasi et al., 2006), and they appear in many unexpected cases (e.g. Mercer et al., 2008). The potential of functional ncRNAs is further supported by the observation that the amount of non-protein-coding DNA increases with organismal complexity, whereas the amount of protein-coding DNA is relatively constant (Tait et al., 2007). A substantial number of putative ncRNAs has emerged from several genomic in silico screens for RNA structure taking compensatory base pair changes into account (Torarinsson et al., 2006, 2008; Washietl et al., 2007; Will et al., 2007). Deep sequencing approaches are another growing source of ncRNAs (e.g. Sharma et al., 2010).

One step towards assigning functions to these putative ncRNAs is to consider RNA interactions. An important class of these are RNA–RNA interactions; many ncRNAs base pair with other RNAs, such as the majority of characterized bacterial small regulatory RNAs (sRNAs) (Waters and Storz, 2009). Besides the well-known examples of microRNAs (miRNAs) and small interfering RNAs (siRNAs), there are also many longer eukaryotic ncRNAs that act via RNA–RNA interaction. Examples are small nucleolar RNAs ( snoRNAs), of which the main function is to guide the editing of ribosomal RNA (Bachellerie et al., 2002). However, there is growing evidence that other long ncRNAs might act via base pair interactions as well. For example, it is likely that certain long ncRNAs modulate the activity of miRNAs by forming RNA–RNA interactions (Wilusz et al., 2009).

Even though most screens for ncRNA candidates involve mammalian genomes (Gorodkin et al., 2010) we predominantly found with the exception of a few snoRNA examples, bacterial examples of verified RNA–RNA interactions involving the structure of both RNAs. This pattern might be the reason that many of the existing methods for the prediction of RNA–RNA interactions have been designed to work on bacterial sRNAs [see Backofen and Hess (2010) for a review]. Furthermore, extensive curated data for RNA–RNA interactions are limited because this effort is, just as
for RNA structural alignments, rarely acknowledged (Menzel et al., 2009). Recent interest in prediction of RNA–RNA interactions has led to four main classes of computational methods to solve this task. Initial approaches evaluated only base pairs involved in duplex formation (e.g. Rehmsmeier et al., 2004; Tifer and Hofacker, 2008).

The second class consists of methods that predict a joint secondary structure of two interacting RNAs by folding their concatenated RNA sequences (e.g. Andronescu et al., 2005; Bernhart et al., 2006; Dirks et al., 2007). This concatenation approach considers only a restricted set of interaction types, and therefore, the third class evolved. Here, all interactions with one site are allowed and interaction site accessibility is calculated by the partition function of the structure ensemble (Bomphalinererer et al., 2008; Busch et al., 2008; Mückstein et al., 2006, 2008; Richter et al., 2010). The final class of methods handles more complex joint structures with multiple interaction sites (Alkan et al., 2006; Chitsaz et al., 2009a, b; Huang et al., 2009, 2010; Pervouchine, 2004; Saliari et al., 2010a, b). However, these methods are very resource-demanding, which makes them not applicable for genome-wide scans. Furthermore, all aforementioned methods evaluate only interactions between a pair of single sequences.

For an approach that makes use of multiple alignments, our assumption is that a non-negligible amount of the existing RNA–RNA interaction contains compensatory changes across the binding sites. Whereas this is a motivating aspect, the general variation in the sequences with even a completely conserved interaction site might also yield prediction improvements. Such an improvement over single sequence-based methods can be obtained by taking the overall sequence variation into account for the joint framework of energy folding and covariation.

The literature contains only limited examples of conserved RNA–RNA interactions with likely compensating base pair changes, such as the MicA–ompA interaction, where base pairing is preserved by compensatory changes in several enterobacterial species (Udekwu et al., 2005). These authors also developed a method for predicting interactions that takes phylogenetic conservation into account, but to our knowledge, they have not yet published it. One explanation for the limited amount of data containing covariance information might well be that most existing data have been found using sequence similarity-based methods such as BLAST (Altschul et al., 1997) to find homologs of the interacting RNAs. Such an approach is expected to lead to a collection of RNAs that are highly conserved in the primary sequence rather than structure. Although in these cases it is likely that the interaction pattern is conserved as well, only a small number of compensatory base pair changes is expected. Therefore, we also include simulated data based on substitution statistics of interacting base pairs. Applying simulated data to make controlled tests or to supplement existing data has been done several times before (e.g. Kolbe and Eddy, 2009).

To summarize, there are three main problems for the prediction of RNA–RNA interactions: (i) the small number of examples with mapped interactions, (ii) most existing computational methods work on single sequences and therefore suffer from a low specificity and (iii) the search for complex types of interactions is expensive if a guaranteed maximum score is to be obtained. The last is discussed in more details in Seemann et al. (2010), where we propose an algorithm for searching for RNA–RNA interactions between two multiple RNA sequence (or sequence-structure) alignments. Our algorithm can compute a semi-optimal combination of intra- and inter-molecular base pairs to predict a joint secondary structure of two multiple alignments of RNA sequences, each representing its own evolutionary and structurally conserved RNA. The method makes use of the idea of a linker from RNAcofold (Bernhart et al., 2006) by concatenating both RNA sequences, but employs this idea in the context of PETfold (Seemann et al., 2008) along with a strategy for hierarchical folding (e.g. Gaspin and Westhof, 1995). PETfold is based on Pfold (Knudsen and Hein, 2003), which provides reliabilities for evolutionarily conserved base pairs, and unifies them with folding energies in one model. Hierarchical folding allows for the prediction of pseudoknots between intra- and inter-molecular base pairs but is still fast enough for genome-scale applications. Here we present PETcofold, and an in-depth performance evaluation. A key motivation for implementing a method that can predict RNA–RNA interactions between two multiple alignments of RNA sequences is the existence of many ncRNA candidates identified by genome-wide in silico screens as mentioned before. These de novo predicted RNA structures exist already as multiple alignments and the PETcofold implementation presented here can readily be employed to further analyse these candidates for potential interaction partners.

2 METHODS

2.1 Algorithm and implementation

The algorithm presented in Seemann et al. (2010) is implemented here in the PETcofold program. The input consists of two RNA alignments A1 and A2, and the program performs hierarchical folding in two steps. This multi-step approach is motivated by the observation that interaction formation is often initiated at well-accessible intra-molecular structures such as hairpin loops (Brunel et al., 2002). Furthermore, several existing methods are based on this observation and assume that the interaction sites are made accessible to allow for hybridization of the two RNAs (e.g. Busch et al., 2008; Mückstein et al., 2006). The workflow of the PETcofold pipeline is shown in Figure 1. Supplementary Figure S1 shows a flow chart of the different programs integrated in PETcofold.

In step 1, we search for highly reliable base pairs in both RNA alignments, these are interpreted as being not accessible for the RNA–RNA interaction. We apply the maximum expected accuracy approach of PETfold separately on the alignments A1 and A2 to calculate reliabilities unifying the thermodynamic probabilities from RNAfold (Hofacker et al., 1994) and the evolutionary reliabilities from Pfold. The set of all base pairs with high base pair reliability RBP in the folding of the individual alignments, i.e. RBP > δ, forms a partial structure σψ and will be denoted as σ1 and σ2 for A1 and A2, respectively. To ensure that the highly reliable base pairs of the partial structure σψ are also part of the final (consensus) structure, they should make up a significant portion of the probability Pr[E(σψ)] in either the thermodynamic or the evolutionary model, where E(σψ) is the ensemble of structures that are compatible with the partial structure σψ. A high value of Pr[E(σψ)] is guaranteed by the introduction of a second threshold γ. The threshold δ for highly reliable base pairs is now increased until the probability of E(σψ) exceeds γ in the evolutionary or the thermodynamic model or both. In addition, constrained stems in the partial structure σψ are extended by inner and outer base pairs if the average reliability of the extended stem is larger than δ and if the partial structure probability Pr[E(σψ)] exceeds γ. This feature is enabled by the option -exist. In step 2, we concatenate the input alignments A1 and A2 with a linker symbol ‘&’ to search for conserved interactions and structures of
the sequences of the two alignments. On the concatenated alignment, we apply an adapted PETfold model that can handle fixed partial structures \( \sigma^1 \) and \( \sigma^2 \) from the first step by constrained expected accuracy scoring, which is an extension of PETfold’s maximum expected accuracy scoring for constrained folding. We search for a joint structure \( \sigma \) of the combined alignment that extends both \( \sigma^1 \) and \( \sigma^2 \). Although PETfold cannot handle pseudoknots and the linker in step 2 forbids pseudoknots in the concatenated sequences, i.e. the resulting structure has to be nested, the hierarchical folding strategy of PETfold allows pseudoknots between intra-molecular base pairs from step 1 and inter-molecular (as well as intra-molecular) base pairs from step 2 by restricting the positions of the concatenated alignments that are covered by base pairs from \( \sigma^1 \) and \( \sigma^2 \) to be single stranded. Under these constraints, the thermodynamic probabilities are calculated with RNAfold, and the evolutionary reliabilities are calculated with an adapted Folded using constrained folding, which yields the structure \( \sigma_{\text{int}} \). This constrained folding results in raw probabilities, which are then weighted by the product of partial structure probabilities \( \Pi(\sigma(i)) \) from step 1. However, to avoid underestimating the probabilities of step 2, we here replace the product with the geometric mean of partial structure probabilities. The constrained expected accuracy structure \( \sigma_{\text{int}} \) is calculated by a Nussinov-style algorithm and might contain both intra-molecular as well as inter-molecular base pairs. The final consensus structure including the interaction is then

\[
\sigma = \sigma^1 \cup \sigma^2 \cup \sigma_{\text{int}}
\]

In the final scoring, the reliabilities of all base pairs from \( \sigma^1 \) and \( \sigma^2 \) are equal to the partial structure probabilities \( \Pi(\sigma(i)) \) and the \( \Pi(\sigma(i)) \), respectively.

The algorithm, which is described in more detail in Seemann et al. (2010), has a time complexity of \( O(N \times L^3) \), where \( N \) is the number of sequences, \( L \) is the number of interactions in the adaptation of \( \delta \) to find probable partial structures and \( L \) is the sum of the sequence lengths of both alignments.

2.2 Datasets and processing

The evaluation of PETcofold was performed using datasets extracted from the literature combined with simulated data, in which the degree of compensating base changes is controlled.

First, a dataset of interactions between bacterial sRNAs and their target mRNAs was extracted. Starting from a set of experimentally verified interactions used by Busch et al. (2008), we included further sRNA–mRNA interactions with experimental support from the literature. The final dataset contained 13 different sRNAs and 32 interactions from Escherichia coli K12 (E.coli), Salmonella typhimurium LT2 and Staphylococcus aureus N315 (see Supplementary Table S1).

For each sRNA, the RNA family sequence alignment was downloaded from the Rfam database 9.1 (Gardner et al., 2009). Orthologs of target genes were predicted in species with available complete genomes according to the Rfam annotation. The prediction of orthologous genes was carried out with OrthoMCL (Li et al., 2003) using the default parameters. For each target gene, a 250 nt subsequence was extracted (150 nt upstream and 100 nt downstream of the annotated translational start site) because all interactions occurred from \(-132\) nt to \(+56\) relative to the start codon. The sets of putative orthologous target sequences were locally aligned with MAFFT (using option L-INS-i for generalized affine gap costs) (Katoh and Toh, 2008).

The resulting dataset was processed by homology reduction and removal of sequences that were very distant to the reference, i.e. the organism in which the interaction was detected. This step aims to remove false positive predicted orthologs and was achieved by excluding all mRNA sequences with a low pairwise sequence identity (\(\Pi_{\text{seq}}\)) at the interaction site and within 10 nt of the flanking sequences compared with the reference sequence. \(\Pi_{\text{seq}}\) thresholds of 40, 50 and 60% were applied. To avoid a bias caused by overestimating redundant sequence information, mRNA sequences were clustered with the BLASTClust tool (Altschul et al., 1997) using a word size of 8 and a percent identity threshold of 100% over an area covering 90% of each sequence. The sequence with the lowest \(\Pi_{\text{seq}}\) was taken from each cluster. Further details on the preparation of the dataset are given in the Supplemental Material.

To evaluate the covariance of a dataset, the arithmetic mean of compensatory base pair (CBP) exchanges of all interactions was computed. For a specific interaction, the CBP is computed as the average number of compensatory base pairs in all interacting alignment columns, where a compensatory base pair is distinct from the bases involved in the pairing of the reference sequence. Thus, the maximal value of the CBP is 5. Another covariance measure is the probability \(\Pi(\sigma_{\text{int}}(i),T,M)\) of a base pair \((i,j)\) calculated by Folded. It is more accurate because it takes into account, by the tree \(T\), the evolutionary distance of the sequences in the alignment \(A\). The model \(M\) describes the substitution rates of base pairs and unpaired bases and the probabilities of secondary structure production rules (Knudsen and Hein, 1999); however, the bias introduced by \(M\) can be ignored. To measure the covariance of a full interaction, we use the normalized Folded reliability \(\overline{\Pi}_{\text{int}}(i,\text{ant})\), which is the mean of all base pair probabilities in the interaction.

2.3 Simulated data

The dataset described above is based on sRNA–mRNA interactions that were experimentally validated in one reference organism. The incorporation of homologous sequences from Rfam families and computational prediction of orthologous genes is often used. However, this approach could be limited in two respects. From a biological point of view, our method assumes that the homologous sRNAs regulate the same targets by the same mechanism in all organisms that are contained in the sequence sets. Although this assumption probably holds true for many interactions, it does not have to apply to all of them. Furthermore, the target orthologs might contain false positive predictions with different physiological functions and regulatory mechanisms. From a technical point of view, weak covariance at the interaction sites limits the full potential of PETcofold. For example, the most conserved region of the sRNA SgrS is involved in base pairing at its target (Hotter and Vanderpool, 2009).

Thus, we created a simulated dataset with increased covariance. We used SiSST (Gesell and von Haeseler, 2006) to simulate sequence data with site-specific interactions annotated by the sRNA–mRNA interactions of our dataset along phylogenetic trees. To be biologically relevant, we estimated each phylogenetic tree from the corresponding alignment using a maximum likelihood method with an independent model. For tree
reconstructions, iQPNNI 3.3.1 (Vinoh and von Heuser, 2004) was used with a F81 model (Felsenstein, 1981), the default settings were used. To specify the rate matrices, we simply counted the frequencies of the nucleotides [A, C, G, U] for sites evolving independently and doublet frequencies for the distant RNA interaction pairs. For each of the 32 alignments, we performed 20 simulations with the same length and in the context of the annotations of the sRNA–mRNA interactions using a Markov model of nucleotide sequence evolution (Gnesl and von Heuser, 2006) with rate matrix types of the F81 model. We initially started simulations along the estimated phylogenetic trees. Then, to increase the covariance, we multiplied each branch length by a scaling factor. The simulation runs were repeated under the same parameters with eight different scaling values as shown in Table 1.

2.4 Performance evaluation

All predictions were evaluated by calculating their correlations to the structures from literature ignoring non-canonical base pairs. We used the Matthews correlation coefficient (Matthews, 1975) defined as

\[
\text{MCC} = \frac{TP \times TN - FP \times FN}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}}
\]

where TP is the number of mutual base pairs in the two assignments (true positives), TN are the true negatives, FP is the number of predicted base pairs not in the annotated assignment (false positives) and FN are the false negatives. For RNA secondary structures, the geometric mean of sensitivity (SE = TP/(TP+FN)) and positive predictive value (PPV = TP/(TP+FP)), \(\sqrt{\text{SE} \times \text{PPV}}\), is a good approximation of the MCC (Gorodkin et al., 2001) and, here, is used to compare predicted joint secondary structures with the annotations. The MCC should be maximized to achieve the best possible trade-off between sensitivity and PPV.

The MCC of the prediction to the annotation is computed for each interaction site accessibility. In real applications, sequence-based alignments are often used with PIint of 60%, we achieve the best performance for 0 ≤ γ ≤ 0.9 (see Supplementary Table S2). For multiple alignments with PIint of 60%, we achieve the best performance for 0 ≤ γ ≤ 0.5. In real applications, sequence-based alignments are often used as input, which perform satisfactorily for pairwise sequence

Fig. 2. The performance of PETcofold while varying the parameters \(\delta\) (maximum intra-molecular base pair reliability) and \(\gamma\) (minimal partial structure probability). The 3D plot shows the mean MCC of 32 interactions using input sequences with a minimal pairwise mRNA interaction site sequence identity to the reference of 60%. Predictions were carried out without the option -noLP. The maximal MCC is marked with ‘*’.
identities above 60%. For the prediction of joint secondary structures, we use a rather restrictive $\gamma$ of 0.1 to allow many constraints for intra-molecular base pairs. Furthermore, on the limited set of joint structures presented in Section 3.3, we achieved no performance increase by a higher value for $\gamma$ (see Supplementary Table S3).

3.2 Simulated interactions with increased covariance

The average number of compensatory interaction base pair exchanges (CBP) in the 32 interactions from Section 3.1 was rather low with values of 0.1–0.2. Therefore, we created a simulated sequence dataset based on these interactions as described in Section 2.3. Here, covariance at the interaction sites was increased by scaling the branch lengths of the phylogenetic trees that guided the simulation. As shown in Table 1, the minimal CBP computed from the simulated data was 0.8, which was higher than the CBP of the real data. This was due to the fact that the simulations only covered a subset of the evolutionary constraints on the sequences. For example, only the interactions without thermodynamics were used as structural constraints. Other evolutionary pressures on the sequences were neglected. However, taking into account all aspects in the simulations with a corresponding maximum likelihood framework is beyond the scope of this article. At this point, we focused on evaluating PETcofold’s performance on datasets with increased covariance.

We applied PETcofold to these datasets and computed the mean interaction MCC of all 20 simulation runs for 9 different phylogenetic scaling factors. Figure 3 shows the mean interaction MCC plotted over the phylogenetic scaling factor for PETcofold predictions with $\delta = 0.9$, $\gamma = 0.1$ and the option noLP. The mean MCC of the predicted interactions was 0.505 for scaling factor 1. The prediction accuracy of PETcofold in terms of MCC increased with increasing scaling factors. A mean MCC of 0.821 was achieved for scaling factor 200. Table 1 shows for all scaling factors the mean interaction MCC of all 20 simulation runs for 9 different phylogenetic scaling factors. Figure 3 shows the mean interaction MCC of the predicted interactions was 0.505 for scaling factor 1. PETcofold was called with parameters $\delta = 0.9$, $\gamma = 0.1$ and option noLP. MCC evaluates only the interaction performance in the simulated sequence data. PETcofold was called with parameters $\delta = 0.9$, $\gamma = 0.1$ and option noLP.

Section 2.2. Regarding the sRNAs, the Rfam entry of RyhB (RF00057) missed the first 29 nt of the E.coli RyhB sequence. However, this subsequence is involved in forming the secondary structures. The mRNA and sRNA alignments of the four examples were hand curated by removal of redundant sequences and of sequences that were very distinct from the reference organism E.coli, in which the interaction models were experimentally determined.

To predict the joint structures, PETcofold was called with parameters $\delta = 0.9$, $\gamma = 0.1$, option noLP and optionally with option -extrin. Our evaluation included the following single sequence-based methods: the sparsified version of interRNA (Salari et al., 2010b), Pairfold (Andronescu et al., 2005), RactIP (Kato et al., 2010), all with default parameters, and RNAcofold (Bernhart et al., 2006) using parameters -d2 = noLP. All methods apart from RactIP predict minimum free energy joint secondary structures. interRNA is based on the model by Chitsaz et al. (2009b), whereas Pairfold and RNAcofold are

### Table 1. Prediction performance of PETcofold on simulated sequence data

| Scaling factor | Mean branch length (nt) | R (inter) | CBP | Mean MCC |
|----------------|-------------------------|----------|-----|----------|
| 1              | 0.03                    | 0.486    | 0.832 | 0.505    |
| 5              | 0.15                    | 0.665    | 1.818 | 0.677    |
| 10             | 0.3                     | 0.699    | 2.182 | 0.717    |
| 25             | 0.75                    | 0.732    | 2.550 | 0.743    |
| 50             | 1.5                     | 0.775    | 2.755 | 0.773    |
| 75             | 0.75                    | 0.791    | 2.858 | 0.783    |
| 100            | 3.0                     | 0.804    | 2.908 | 0.790    |
| 150            | 4.5                     | 0.822    | 2.988 | 0.802    |
| 200            | 6.0                     | 0.831    | 3.040 | 0.821    |

Scale factor multiplies each branch length of the phylogenetic tree. Mean branch length denotes the mean of the branch lengths for all 32 phylogenetic trees. $R$(inter) denotes the mean base pair probability at the interaction sites (calculated by PETcofold). CBP denotes the average number of compensatory interaction base pair exchanges in the simulated sequence data. MCC evaluates only the interaction performance in the simulated sequence data. PETcofold was called with parameters $\delta = 0.9$, $\gamma = 0.1$ and option noLP.
Table 2. Performance of PETcofold and other joint structure prediction methods on four sRNA–mRNA examples

| sRNA-target pair | MCC PETcofold | MCC inteRNA | MCC Pairfold | MCC RactIP | MCC RNAcofold | Run time (s) PETcofold | Run time (s) inteRNA | Run time (s) Pairfold | Run time (s) RactIP | Run time (s) RNAcofold |
|------------------|---------------|-------------|--------------|------------|--------------|------------------------|---------------------|---------------------|---------------------|----------------------|
| -extstem         | -             | -           | -            | -          | -            |                        |                     |                     |                     |                      |
| 80%              | 100%          | 80%         | 100%         | 80%        | 100%         |                        |                     |                     |                     |                      |
| MicA-ompA        | 0.87          | 0.83        | 0.69         | 0.57       | 0.80         | 0.64                   | 28.7                | 28.4                | 69493.1             | 3.2                  | 3.0                  | 0.2                  |
| OxyS-fhlA        | 0.80          | 0.82        | 0.64         | 0.61       | 0.48         | 0.61                   | 20.6                | 19.3                | 129636.7            | 1.9                  | 2.0                  | 0.2                  |
| RyhB-uof-fur     | 0.13          | 0.13        | 0.12         | 0.21       | 0.19         | 0.21                   | 26.4                | 25.3                | 65599.2             | 2.6                  | 2.7                  | 0.2                  |
| RyhB-sodB        | 0.67          | 0.71        | 0.70         | 0.65       | 0.65         | 0.65                   | 15.4                | 15.2                | 25759.3             | 1.7                  | 2.0                  | 0.1                  |
| Average          | 0.62          | 0.62        | 0.49         | 0.58       | 0.52         | 0.47                   | 22.8                | 22.1                | 72077.1             | 2.4                  | 2.5                  | 0.2                  |

The MCC evaluates the joint structure, i.e. both the interaction between the two RNAs and the secondary structure of each single RNA. PETcofold was called with parameters \( \alpha = 0.9, \beta = 0.1, \gamma = 0.1 \) and optionally with option -extstem. inteRNA, Pairfold, and RactIP were called with default parameters. RNAcofold was called with options -g2 -m0 -n0LP. The columns 80% and 100% give the result for the consensus structure with base pairs that occur in 80 and 100%, respectively, of the single structures. The run time of all single sequence-based approaches is the sum for all input sequences.

Fig. 4. Joint secondary structure of the sRNA MicA and the mRNA ompA. The alignment shows the two input alignments concatenated by the linker symbol ‘\&’. The joint structure predicted by PETcofold (with parameters \( \beta = 0.9, \gamma = 0.1 \) and option -extstem) is visualized with Jalview (Waterhouse et al., 2009).

Based on folding of the concatenated input sequences using the model of Zuker and Stiegler (1981), RactIP uses integer linear programming to maximize an objective function that is based on internal and external base pair probabilities. All computations were performed on a machine with AMD Opteron 2356 processor (2.3 GHz) and 16 GB RAM.

All resulting consensus structures were compared with the joint structure from the literature using the approximated MCC. Table 2 lists the MCCs of all four examples for PETcofold and the compared methods.

For the interactions of OxyS-fhlA and RyhB-sodB, the MCC of the PETcofold predictions was slightly higher when using the option -extstem. The opposite applies to MicA-ompA. On the non-curated alignments, the MCC was up to 0.2 lower (data not shown), which emphasized the importance of high-quality input alignments for our method. However, the option -extstem seemed to improve the prediction when using low-quality input alignments by extension of imperfectly (structurally) conserved stems.

When comparing PETcofold with the other methods, our method overall showed a better performance in predicting the joint structures. The prediction quality of the RyhB-uof-fur joint structure is very low for all compared approaches (maximal MCC of 0.21), which implies that the published interaction model is not predictable with the evaluated computational approaches. Thus, the prediction for this example is not reliable. When excluding RyhB-uof-fur, our approach gives consistently more reliable predictions than the single sequence-based methods (see Table 2.)

For comparison to the complex joint secondary structure prediction methods with high resource consumption (both high time and memory complexity), we were only able to compare to inteRNA, as this is currently the only method with a sufficiently low resource consumption. However, the evaluation shows that this (minimum free energy structure) prediction approach is not very reliable without homology information. Thus, one has to resort to the more complex partition function approaches, which, however, have drastically larger time and memory requirements. For example, it was not possible to obtain predictions from RNAripl (Huang et al., 2010) with reasonable resources. In comparison, PETcofold gave reliable predictions within seconds, which makes it also fast enough for genome-scale applications.

Figure 4 shows the annotation and PETcofold prediction for MicA-ompA together with the sequence alignments used as input. PETcofold correctly predicted all interaction base pairs from the annotation. The interaction site is highly conserved in both RNAs and contains only one compensatory mutation for the pairing between alignment positions 16 and 126. The intra-molecular structures contain compensatory mutations, for instance, between alignment positions 60 and 65.

Alignments and joint structures of OxyS-fhlA, RyhB-sodB and RyhB-uof-fur are given in Supplementary Figure S3. Two
The interactions are located in highly conserved regions for methods predicted further binding sites in addition to the reported structure conserved in 80% as well as 100% of sequences. Both performed with an approximated MCC of 0.51 for the consensus predicted by Figure 5 shows the joint structure including a pseudoknot as Xenopus showed that depletion of the C/D box snoRNA mgU6-77 from O

secondary structures and the predicted interaction. methylated and marked by 'm'. We used in green. The cytosine at alignment position 78 (pos. 77 in mouse) is 2´-et al. snoRNA structure, the interaction in mouse (Tycowski U6 RNAcofold and the PET cofold seed alignment from Rfam. The joint structure consisting of the known snoRNA as

inter-molecular structures of the two RNAs and the interactions between them. Furthermore, PETcofold allows for the prediction of pseudoknots between intra- and inter-molecular base pairs.

We have shown in controlled runs on simulated data that the covariance information improves the prediction ability for RNA–RNA interactions. We have also shown both for a vertebrate snoRNA and for four bacterial sRNAs that the addition of evolutionary information from multiple sequence alignments improves the performance in comparison to methods based on single sequences. This implies that single sequence-based methods could perform better if the comparative information is also taken into account. As for other RNA structure prediction methods making use of sequence-based alignments, it is well-documented that these work best with an average pairwise sequence identity above 60% (Gardner et al., 2005; Washietl and Hofacker, 2004). In the process of cleaning up the (already sparse) bacterial data, we took this into account by removing more distant sequences to keep the balance of accurate assignment and covariance patterns of base pairs.

Many genomic screens for ncRNAs predict secondary structures with compensatory base pair changes on RNA alignments. These intra-molecular structures can be used as input of step 1 of the PETcofold pipeline to identify highly reliable substructures, which are then constrained for step 2. For example, PETcofold could be applied to predict RNA–RNA interactions on the CMfinder-generated structure-based alignments of de novo predicted RNA motifs in the ENCODE regions (Torarinsson et al., 2008).

The sparse amount of known examples of sequences with RNA–RNA interactions and the paucity of covariations were the reasons why we introduced simulated data. Some of the known examples of RNA–RNA interactions, e.g. from bacterial sRNA–mRNA and eukaryotic miRNA–mRNA interactions, tend to be rather conserved. Even in cases with little or no compensating base pairs (in interaction sites or in the intra-molecular structure), any given variation will collectively contribute to the calculation of the reliabilities and thereby to the overall structure of the interaction complex. Hence, in the complete lack of covariation, PETcofold reduces to an improved energy folding approach, which also has impact on matching up the interacting base pairs. The sRNA–mRNA interactions studied here exhibit only a small number of compensatory base pair changes, which might be because sRNA sequences often show poor conservation across distant bacterial species. Thus, the regulators might be recently acquired and rapidly evolving (Waters and Storz, 2009). Nevertheless, many of the homologous ncRNAs and mRNAs have been found based on sequence similarity, which leads to highly identical sequences and thereby also to highly conserved interactions. PETcofold considers sequence conservation, but its full power is only revealed when the input data contains structural covariance. In the future, we expect that deep sequencing approaches will give rise to many more

4 DISCUSSION

Here, we presented PETcofold, the first comparative method for the prediction of a joint secondary structure of two interacting RNAs. The method identifies evolutionary conserved structures and can exploit the information from compensating base changes in the intra-molecular structures of the two RNAs and the interactions between them. Furthermore, PETcofold allows for the prediction of pseudoknots between intra- and inter-molecular base pairs.

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characterized transcriptomes, which will increase the amount of data available for analysis including RNAs containing compensating base pair changes.

Certain RNA-RNA interactions, e.g. the functionally important interactions between the 16S and 23S rRNAs in the ribosome, involve non-canonical base pairs. Our method looks primarily for canonical, e.g. Watson-Crick, base pairs and G-U wobble base pairs. However, the Pfold model includes equilibrium distributions for the frequencies of all possible 16 bp and substitution rates for all possible base pair substitutions (16 x 16 matrix), which have been estimated from given trusted alignments of RNA and rRNAs including non-canonical base pairs (Knuusen and Hein, 1999). The probabilities of non-canonical base pairs are low compared with Watson-Crick and Wobble base pairs and, thus, in practice, non-canonical base pairs are only found together with canonical base pairs. Nevertheless, the equilibrium distributions in the evolutionary model of PECofold could be adapted to increase the impact of non-canonical base pairs by using a different training set.

Future improvements can include explicit handling of redundant sequence information. Currently, redundant sequences contribute equally to the scoring to directly reflect the input data instead of overweighting outliers in a dense evolutionary tree. Thus, datasets with highly redundant sequences should be cleaned prior to usage of the program.

Long RNA-RNA interactions form helices similar to the DNA double strand (Watson and Crick, 1953). These helices pose inter- and intra-molecular topological challenges. Due to the turn of the helix (i) the length of the interaction between the two RNAs is constrained and (ii) a certain number of unpaired bases on either side of the interaction are necessary to enable the first enclosing intra-molecular base pair (Pervouchine, 2004). In practice, this means that the computational methods can predict longer helices, because the outer bases are complementary, but in the three-dimensional topology these base pairings would be spatially unfeasible. However, taking topological constraints into account would be excessively time costly. Our method, and all other methods capable of genome-scale analysis, work only at the level of secondary structures. However, in the future it would be useful to accommodate this.

In some cases, the hierarchical folding approach predicts RNA-RNA interactions with reduced accuracy because the intra-molecular constraints overlap with the interaction sites. In these cases, the interaction site accessibility model of PECofold is too strict and overestimates the stability of intra-molecular structures. As an example, one of the two OxyS-phi sites is not predicted because the stem enclosing the second interaction site has a high reliability and, thus, gets constrained. In Seemann et al. (2010), the input alignment consisted of more distant sequences, which reduced the reliability of this stem and made the interaction site accessible. For avoiding too restrictive constraints, we introduced a parameter γ when reliable alternative structures exist. It is planned that a future version of PECofold will take the cost of opening stems into account as e.g. in RNAup and IntaRNA.

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