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Rtools: a web server for various secondary structural analyses on single RNA sequences

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INTRODUCTION

The primary sequences of RNAs are important for their functions because the base-parings of the reverse complementary sub-sequences identify the target RNAs. At the same time, those RNAs frequently conserve their secondary structures. The secondary structures reflect the tertiary structures and also related to the free sub-sequences which can interact with the reverse complementary sub-sequences of the other RNAs. While high-throughput experimental methods have still limitations, it is desirable to characterize the secondary structures of RNAs in silico.

In silico prediction of RNA secondary structures is often referred as ‘unreliable,’ often failing to predict well-known secondary structures like the clover-leaf of tRNA e.g. (1). Although the prediction gives approximately ‘correct’ secondary structures in considerable frequency, the predicted structures almost always include many errors.

The probability distribution of the secondary structure θ of an RNA sequence x is denoted as Boltzmann distribution (2):

\[ p(\theta|x) = \frac{1}{Z(x)} \exp \left( -\frac{E(\theta|x)}{RT} \right), \]  

(1)

where \( E(\theta|x) \) is the free energy of the secondary structure \( \theta \), \( R \) is the ideal gas constant, \( T \) is a temperature and \( Z(x) \) is the partition function,

\[ Z(x) = \sum_{\theta} \exp \left( -\frac{E(\theta|x)}{RT} \right). \]  

(2)

The nearest neighbor energy model defines \( E(\theta|x) \) as the summation of the free energy of all the loops, which is calculated according to the combinations of the nearest nucleotides of the loops. The experimentally identified parameters are used for this calculation.

The number of possible secondary structures of an RNA is huge, and the probability of the predicted structure (with respect to the Boltzmann distribution) is very small, around...
1% even in the fortunate cases. For example, it is known that the structure of an rRNA with the highest probability is about $10^{-22}$ (3). Therefore, it is dangerous to proceed to further biological inferences based on the predicted structures.

The in silico secondary structural analyses are, however, not useless but productive if the combination of the tools are appropriately utilized. Although the total probability of a precise secondary structure of an RNA sequence is very small, various marginal probabilities with respect to the distribution $p(\theta|x)$, which are not necessarily very small, can be introduced. For example, the ‘base-pairing probability matrix’ (BPPM), where each component $p_{ij}$ is the marginal probability that $x_i$ and $x_j$ form a base-pair (called ‘base-pairing probability’) reflects the landscape of the probability distribution of the secondary structures. BPPM can be computed efficiently in $O(L^2)$ time ($L$ is the length of RNA sequence) by using a dynamic programming (DP) based on the McCaskill model (2) (cf. Figure 2). Similar algorithms give us the marginal probabilities of a variety of local structural features, such as loop probabilities and accessibility (4,5).

The extensions of DP algorithms also enable us to extract different types of secondary structural information. For example, the energy change by all the possible base mutations (including the combination of base mutations) can be calculated (6). The validation of the predicted structure is also important. Because the probability of the structure itself is very small, more reasonable measures are required. The credibility limit (7) is the measure of the concentration of the probabilities around the predicted structures, which reflects the stability and the reliability of the predicted structure.

**DESIGN OF Rtools WEB SERVER**

In Rtools web server, we implemented seven tools described in the next section, all of which focus on analyzing secondary structures for single RNA sequences, based on probability distribution of RNA secondary structures. Due to the reason, an interface of Rtools is quite simple, where users paste an RNA sequence in the text-form and press ‘Submit’ button; then Rtools quickly returns various analyses for the sequence. The results (e.g. base-pairing probability matrix and graphs) are offered as the following formats: EPS, PNG and JPEG, which can be easily utilized in the research paper. Additionally, users can easily adjust internal parameters in each tool through web interface (Figure 1). In Rtools, we implemented a routine for visualizing base-pairing probability matrix in colors. Additionally, users obtain ‘Request ID’ in the results page and can always access the results by using the ID.

**ALGORITHMS AND SOFTWARE**

Given an RNA sequence $x$, a set of possible secondary structures of $x$ is denoted by $S(x)$, where a secondary structure $\theta \in S(x)$ is represented as a (upper-)triangular binary-valued matrix $\{\theta_{ij}\}_{i<j}$: $\theta_{ij} = 1$ if the $i$-the nucleotide in $x$ (denoted by $x_i$) forms a base-pair with $x_j$. In addition, a probability distribution of RNA secondary structure of $x$ is denoted by $p(\theta|x)$ ($\theta \in S(x)$), where $\sum_{\theta \in S(x)} p(\theta|x)$ holds.

Several models for $p(\theta|x)$ have been proposed (as described in Introduction section). By using this notation, a marginal probability that $x_i$ and $x_j$ form a base-pair (a base-pairing probability described in Introduction) is written as follows:

$$p_{ij} = \sum_{\theta \in S(x)} I(\theta_{ij} = 1) p(\theta|x) \quad (3)$$

where $I(\cdot)$ is the indicator function and $\theta_{ij}$ is $(i,j)$-element of $\theta \in S(x)$ i.e., $I(\theta_{ij} = 1)$ is equal to 1 only when $x_i$ and $x_j$ form a base-pair ($I(\theta_{ij} = 1) = 0$ otherwise).

Each tool implemented in Rtools web server is closely related to marginal probabilities (including base-pairing probability) with respect to a distribution of RNA secondary structures, as described in the following.

**CentroidFold**

CentroidFold (8) predicts a pseudo-knot-free RNA secondary structure from an individual RNA sequence. The algorithm of CentroidFold is based on $\gamma$-centroid estimators (9), a type of maximizing expected accuracy (MEA) estimators. The $\gamma$-centroid estimators are known to have better expected accuracy than the minimum free energy (MFE) predictions, in terms of the number of true positives and true negatives of base pairs (8). On average, it is known that CentroidFold achieves better performance with respect to accurate predictions of base-pairs than other tools (10).

The time complexity of CentroidFold using base-pairing probability matrix $\{p_{ij}\}_{i<j}$ (Equation 3) is $O(L^2)$ for an RNA sequence of length $L$. As probabilistic models for RNA secondary structures, CentroidFold can utilize one of the following models: McCaskill model (2) with Turner 2004 energy parameters (11) that were determined experimentally; McCaskill model with Boltzmann Likelihood (BL) parameters (12) obtained by retraining energy parameters using machine learning procedures; CONTRAfold model (13) based on a machine learning based model called conditional random fields (CRFs). The interface allows us to select the energy model between McCaskill and CONTRAfold. In CentroidFold, ViennaRNA package (14) is utilized to compute base-pairing probability matrix for McCaskill model.

**CentroidHomfold**

To improve the accuracy of secondary structure predictions, comparative approaches with homologous sequences are often utilized because important structures are conserved evolutionarily. CentroidHomfold (15) aims to predict secondary structures more accurately (than CentroidFold) by using the information of the homologous sequences. The initial version of CentroidHomfold requires not only target RNA sequence whose secondary structure is to be predicted but also its homologous sequences. However, CentroidHomfold in Rtools does not need homologous sequences because Rtools automatically collects homologous sequences of the input from RNA sequence database (constructed based on Rfam database) (16).
Figure 1. A snapshot of the Rtools web server (http://rtools.cbrc.jp/). Users can paste RNA sequence in the text form (located in the top part) and press the submit button, then the server quickly returns various analyses based on secondary structures for the sequence (not shown); the results are downloadable with a few formats: TEXT, PNG, PDF and EPS. Additionally, users can easily adjust several internal parameters in each tool through web interface (shown in the bottom part).

**IPKnot**

IPKnot (17) predicts RNA secondary structures including pseudo-knot, while CentroidFold predicts only pseudo-knot-free RNA secondary structures. As with CentroidFold, IPKnot employs the maximum expected accuracy estimators in combination with integer programming for optimizing the score function with base-pairing probabilities derived from the principle of MEA. In general, computational costs should be much higher than secondary structure prediction without pseudo-knot. Although the worst case time complexity of IPKnot is not polynomial order, it has been empirically proven that IPKnot is fast enough for long RNA sequences.

A predicted secondary structure by IPKnot is visualized using VARNA (18). ViennaRNA package (14) are utilized to compute base-pairing probability matrix for McCaskill model. Additionally, NUPACK (Dirks-Pierce model) (19) is utilized for computing base-pairing probability matrix with considering pseudo-knot structures.

**CapR**

CapR (4) efficiently computes marginal probabilities that each RNA base position is located within each secondary structural context, where six categories of RNA structures were taken into account: (i) stem, (ii) hairpin, (iii) bulge, (iv) internal loop, (v) multi-branch loop and (vi) exterior loop (cf. Figure 3). In CapR, those marginal probabilities are computed according to a probability distribution of secondary structures of a given RNA sequence, provided by McCaskill (energy) model.

In order to reduce computational time, CapR introduces a maximal span of base-pairs, leading to the computational time of $O(Lw^2)$, where $w$ is a maximal span of base-pair and $L$ is the length of input sequence.

**Raccess**

Raccess (5) computes accessibility (the marginal probability of forming a single strand with respect to a probability distribution of secondary structures) of every region of RNA sequences. In Raccess, McCaskill model is uti-
Figure 2. The upper triangular matrix shows a base-pairing probability matrix (BPPM) of a tRNA sequence (having a ‘clover-leaf’ secondary structure), produced by Rtools web server (with CentroidFold). A base-pairing probability is a marginal probability (with respect to a probability distribution of secondary structures) that a pair of nucleotides form a base-pair. The colors of plots show the values of probabilities. On the other hand, the lower triangle shows the optimal RNA secondary structure predicted by CentroidFold, using the base-pairing probability matrix shown in the upper triangle.

Figure 3. Results of CapR for a tRNA sequence. The horizontal axis shows nucleotide position of the input sequence and the vertical axis shows marginal probabilities for structural contexts (bulge, exterior, hairpin, internal, multi-branch and stem) with respect to a probability distribution of secondary structures (cf. Figure 5). Here, the distribution is provided by McCaskill model (Equation 1), which is based on experimentally determined energy parameters.

Given an input sequence \( x \), the accessibility of the segment defined by \( [a, b] \) is

\[
\text{access}_\text{energy}(a, b) = -RT \log(p([a, b]))
\]

where

\[
p([a, b]) = \frac{\sum_{\theta \in S[a, b]} \exp(-E(\theta)/RT)}{\sum_{\theta \in S(x)} \exp(-E(\theta)/RT)}
\]

In the above, \( S[a, b] \) is all the secondary structures having range \( [a, b] \) as loop region. Clearly, \( S[a, b] \subseteq S(x) \) holds. Accessible energy is considered to be energy that is required to keep range \( [a, b] \) being accessible. Note that accessibility is closely related to marginal probabilities with respect to a probability distribution of secondary structures (cf. Equation 5).

**Rchange**

\( R\text{change} \) (6) computes entropy and internal energy changes of secondary structures for mutated sequences. Given an input RNA sequence \( x \) and its mutated sequence \( x' \), the relative change of ensemble free energy is defined by

\[
\frac{dF}{|F|} = \frac{F(x) - F(x')}{|F(x)|}
\]

where

\[
F(x) = -RT \log(Z(x)).
\]

In the above, \( Z(x) \) is the partition function (defined in Equation 2). In Rtools, the maximum and minimum values of Equation 6 with respect to SNPs for each position in the sequence are provided (cf. Figure 5).

Recently, ‘riboSNitch’ has been receiving great attentions (20) and \( R\text{change} \) will be useful for computational analysis for riboSNitch.

**RintD**

\( R\text{intD} \) (1) provides an efficient method to summarize the probability distribution \( p(\theta | x) \), which cannot be summarized (visualized) easily due to its high dimensionality of each solution.
where \( \mathbb{N} \) is the set of all natural numbers. In other words, \( \alpha \% \) credibility limit is the minimum Hamming distance radius that contain \( \alpha \% \) of the possible RNA secondary structures (7). Clearly, the credibility limit for the target sequence can be computed by utilizing \( \{ p_d \}_{d=0,1,...} \). Note that the smaller credibility limit indicates that the reference structure is more stable (reliable) in the ensemble of secondary structures.

\[ R_{\text{intD}} \equiv \{ p_d \}_{d=0,1,...} \text{ and credibility limits of the reference structure. Moreover, } R_{\text{intD}} \text{ can efficiently compute the distribution from two reference RNA secondary structures (cf. (1)), which is also implemented in } R_{\text{tools}} \text{ (cf. Figure 6).} \]

**AN EXAMPLE OF PERFORMING Rtools WEB SERVER**

As a case study of our web server (Rtools), we took a transfer RNA (tRNA) sequence (AL138651.1/64525-64597): GGGGAUGUAUCUAAUGGUAGCCGUCGCUUUG CAUGCAGAGGCACAGGGUUUGG AUUCCCCGUACUC CA, and analyzed it by using Rtools. As a result, the execution time of Rtools was within 1 min.

A base-pairing probability matrix and the optimal secondary structure predicted by CentroidFold of the tRNA sequence are shown in upper and lower matrices in Figure 2, respectively. The figure shows that CentroidFold successfully predicted ‘clover-leaf secondary structure’ of tRNAs for this sequence. Although the BPPM does not predict RNA secondary structures, it includes richer information of secondary structures than the optimal secondary structure. Moreover, Figure 3 indicates the results for CapR, where the marginal probabilities of six structural contexts (Bulge, Exterior, Hairpin, Internal, Multiblanch and stem) in each position are shown. We emphasize that those structural information includes richer information than conventional base-pairing probability matrix.

Figure 4 shows the accessibility (Equation 4) of the sequence, where three accessible lengths are shown. Users can find accessible region from this figure; in this case the figure suggests that the loop region of ‘clover-leaf secondary structure’ is accessible.

Figure 5 shows the results of Rchange for the same tRNA sequence, where the upper bound and lower bound of \( \Delta F/|l| \) in Equation 6 are plotted in each position. By utilizing this figure, users can easily find nucleotides whose SNPs greatly affect the RNA secondary structures. Additionally, it would be useful to see predicted secondary structures (by CentroidFold and context profiles (by CapR)) in combination with the results of Rchange.

Figure 6 (the results of RintD) shows the distribution of secondary structures from the reference structure (the optimal structure predicted by CentroidFold). Specifically, it plots \( d, p_d \) for \( d = 0, 1, 2, ... \) where \( p_d \) is defined in Equation 7. The figure shows that there exists a peak of probability mass around \( d = 25 \). Moreover, the credibility limits of the optimal secondary structure (\( CL(50), CL(90) \) and \( CL(95) \) in Equation 8) are relatively large, indicating that the sum of the probabilities of secondary structures near the optimal secondary structure is not dominant in the ensem-

![Figure 5](image1.png)

**Figure 5.** Results of Rchange for a tRNA sequence. The upper and lower bounds of the relative changes of the ensemble free energy (\( \Delta F/|l| \)) in Equation 6. Because there are three mutated nucleotides for each position, the upper bound values (shown in black line) indicate the largest energy increase caused by a single mutation, and the lower bound values (shown in red line) indicate the largest energy decrease caused by a single mutation.

![Figure 6](image2.png)

**Figure 6.** The reference secondary structure was obtained by CentroidFold. The horizontal axis indicates the (hamming) distance of the reference secondary structure \( d \) in Equation 7, and the vertical axis shows marginal probabilities \( p_d \) in Equation 7. The dashed lines indicate \( \alpha \% - \) credibility limits (7). Clearly, the credibility limit for the target sequence can be computed by utilizing \( \{ p_d \}_{d=0,1,...} \). Note that the smaller credibility limit indicates that the reference structure is more stable (reliable) in the ensemble of secondary structures.

Given a reference (or target) secondary structure \( y \in S(x) \) of interest, \( S_d \) denotes a set of RNA secondary structures whose hamming distance from \( y \) is equal to \( d \). More formally, we define

\[ S_d = \{ \theta \in S(x) \mid d = \sum_{i<j} I(\theta_{ij} \neq y_{ij}) \} \]

where \( I(\cdot) \) is the indicator function. Then, \( R_{\text{intD}} \) can efficiently compute \( \{ p_d \}_{d=0,1,...} \) where \( p_d \) is a marginal probability as follows:

\[ p_d = \sum_{\theta \in S_d} p(\theta | x) \tag{7} \]

In \( R_{\text{intD}} \), McCaskill model is utilized as \( p(\theta | x) \).

For a given RNA secondary structure \( y \), \( \alpha \% - \) credibility limit (7) (denoted by \( CL(\alpha) \)) is defined as follows:

\[ CL(\alpha) = \min \left\{ d \in \mathbb{N} \cup \{0\} \mid \sum_{d' < d} p_{d'} \geq \frac{\alpha}{100} \right\} \tag{8} \]
ble distribution of secondary structures. (Note that all the figures above were generated by Rtools web server.)

In summary, by utilizing Rtools, users can obtain not only secondary structures but also various information including marginal probabilities of structural context (computed by CapR), accessibility (computed by Raccess), the influence of single nucleotide mutation (computed by Rchange) and the summary of the distribution with respect to the predicted secondary structure (computed by RintD).

DISCUSSION

There exists several related web servers such as RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (14), RNAstructure web server (21) and Sfold web server (22). As described in previous sections, Rtools include several features that are different from those existing web servers.

One direction to improve the web server is to incorporate experimental probing information (23) into our structural analyses. Because there exist several methods to incorporate the probing information into RNA secondary structure prediction e.g. (24), we plan to implement one (or a few) of those methods in the future.

CONCLUSION

In this study, we have developed a novel web server, Rtools (http://rtools.cbrc.jp/), to perform various secondary structural analyses on single RNA sequences by Centroid-Fold, CentroidHomfold, IPKnot, CapR, Rchange, Raccess and RintD (all of which were published in reliable journals). The all tools in Rtools are closely related to marginal probabilities with respect to probability distribution of RNA secondary structures of given RNA sequence. By ‘one-click’, users can conduct various analyses of single RNA sequences that they are interested in, and obtain many results which will be utilized for their research paper and/or their further study.

ENDNOTES

In general, marginal probabilities are computed by the following form: \( p = \sum_{\theta \in S} p(\theta|x) \) where \( S \) is a subset of \( S(x) \) (\( S \subset S(x) \)). Various marginal probabilities are introduced according to \( S \).

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REFERENCES

1. Mori,R., Hamada,M. and Asai,K. (2014) Efficient calculation of exact probability distributions of integer features on RNA secondary structures. BMC Genomics, 15(Suppl. 10), S6.

2. McCaskill,J.S. (1990) The equilibrium partition function and base pair binding probabilities for RNA secondary structure. Biopolymers, 29, 1105–1119.

3. Hamada,M. (2014) Fighting against uncertainty: an essential issue in bioinformatics. Brief. Bioinformatics, 15, 748–767.

4. Fukunaga,T., Ozaki,H., Terai,G., Asai,K., Iwashaki,W. and Kiyryu,H. (2014) CapR: revealing structural specificities of RNA-binding protein target recognition using CLIP-seq data. Genome Biol., 15, R16.

5. Kiyryu,H., Terai,G., Imamura,O., Yoneyama,H., Suzuki,K. and Asai,K. (2011) A detailed investigation of accessibilities around target sites of siRNAs and miRNAs. Bioinformatics, 27, 1788–1797.

6. Kiyryu,H. and Asai,K. (2012) Rchange: algorithms for computing energy changes of RNA secondary structures in response to base mutations. Bioinformatics, 28, 1093–1101.

7. Webb-Robertson,B.J., McCue,L.A. and Lawrence,C.E. (2008) Measuring global credibility with application to local sequence alignment. PLoS Comput. Biol., 4, e1000077.

8. Hamada,M., Kiyryu,H., Sato,K., Mituyama,T. and Asai,K. (2009) Prediction of RNA secondary structure using generalized centroid estimators. Bioinformatics, 25, 465–473.

9. Hamada,M., Kiyryu,H., Iwashaki,W. and Asai,K. (2011) Generalized centroid estimators in bioinformatics. PLoS ONE, 6, e16450.

10. Puton,T., Kozlowski,L.P., Rother,K.M. and Bujnicki,J.M. (2013) ComparaRNA: a server for continuous benchmarking of automated methods for RNA secondary structure prediction. Nucleic Acids Res., 41, 4307–4322.

11. Turner,D.H. and Mathews,D.H. (2010) NNDB: the nearest neighbor database for predicting stability of nucleic acid secondary structure. Nucleic Acids Res., 38, D280–D282.

12. Andronescu,M.S., Pop,C. and Condon,A.E. (2010) Improved free energy parameters for RNA pseudoknotted secondary structure prediction. RNA, 16, 26–42.

13. Do,C.B., Woods,D.A. and Batzoglou,S. (2006) CONTRAfold: RNA secondary structure prediction without physics-based models. Bioinformatics, 22, c90–e98.

14. Lorenzo,R., Bernhart,S.H., Honer Zu Siederdissen,C., Tafer,H., Flamm,C., Studler,P.F. and Hofacker,I.L. (2011) ViennaRNA Package 2.0. Algorithms Mol. Biol., 6, 26.

15. Hamada,M., Sato,K., Kiyryu,H., Mituyama,T. and Asai,K. (2009) Predictions of RNA secondary structure by combining homologous sequence information. Bioinformatics, 25, i330–i338.

16. Hamada,M., Yamada,K., Sato,K., Frith,M.C. and Asai,K. (2011) CentroidHomfold-LAST: accurate prediction of RNA secondary structure using automatically collected homologous sequences. Nucleic Acids Res., 39, W100–W106.

17. Sato,K., Kato,Y., Hamada,M., Akutsu,T. and Asai,K. (2011) IPKnot: fast and accurate prediction of RNA secondary structures with pseudoknots using integer programming. Bioinformatics, 27, 85–93.

18. Darty,K., Denise,A. and Ponty,Y. (2009) VARNA: Interactive drawing and editing of the RNA secondary structure. Bioinformatics, 25, 1974–1975.

19. Zadeh,J.N., Steenberg,C.D., Bois,J.S., Wolfe,B.R., Pierce,N.A., Khan,A.R., Dirks,R.M. and Pierce,N.A. (2011) NUPACK: analysis and design of nucleic acid systems. J. Comput. Chem., 32, 170–173.

20. Solem,A.C., Halvorsen,M., Ramos,S.B. and Laederach,A. (2015) The potential of the riboSNitch in personalized medicine. Wiley Interdiscip. Rev. RNA, 6, 517–532.

21. Bellaoussof,S., Reuter,J.S., Seetin,M.G. and Mathews,D.H. (2013) RNAstructure: web servers for RNA secondary structure prediction and analysis. Nucleic Acids Res., 41, W471–W474.

22. Ding,Y., Chan,C.Y. and Lawrence,C.E. (2004) Sfold web server for statistical folding and rational design of nucleic acids. Nucleic Acids Res., 32, W135–W141.

23. Mortimer,S.A., Kidwell,M.A. and Doudna,J.A. (2014) Insights into RNA structure and function from genome-wide studies. Nat. Rev. Genet., 15, 469–479.

24. Quarrier,S., Martin,J.S., Davis-Neulander,L., Beauregard,A. and Laederach,A. (2010) Evaluation of the information content of RNA structure mapping data for secondary structure prediction. RNA, 16, 1108–1117.