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Graph-Theoretic Partitioning of RNAs and Classification of Pseudoknots-II

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Abstract—**Dual graphs have been applied to model RNA secondary structures with pseudoknots, or intertwined base pairs. In previous works, a linear-time algorithm was introduced to partition dual graphs into maximally connected components called blocks and determine whether each block contains a pseudoknot or not. In addition, we have extended the partitioning algorithm by classifying pseudoknots as either recursive or non-recursive. In this paper we present a methodology that uses our previous results and classify pseudoknots into the classical H,K,L, and M types, based upon a novel representation of RNA secondary structures as dual directed graphs (i.e., digraphs). This classification would help with the systematic analysis of RNA structure and prediction as for example the implementation of more accurate folding algorithms.**

Keywords: Graph Theory, RNA Secondary Structures, Partitioning, Bi-connectivity, Pseudoknots.

1 Introduction

Let $G = (V, E)$ be undirected graph composed of by a finite set of *vertices* V and a set E of unordered pairs $e = (v_1, v_2)$ of vertices. called *edges*, where each edge represents a relation between two vertices.

In 2003, Gan et al. [5] introduced dual graphs to model RNA secondary structures (2D). The 2D elements of RNA molecules consist of double-stranded (stem) regions defined by base pairing such as Adenine-Uracil, Guanine-Cytosine, Guanine-Uracil, and single stranded loops; stems and loops are mapped to the vertices and edges of the corresponding dual graph, respectively (later we present an alternative definition of dual graphs).

Dual graphs can represent complex RNA structures called pseudoknots (*PKs*), which results when two base-paired regions intertwine. Pseudoknots have been associated with a diverse range of important RNA activities as for example in viral gene expression and genome replication (e.g., hepatitis C, and SARS-CoV viruses). Even though emphasis has been recently placed on viral translational initiation and elongation, the broader roles of pseudoknots are well-documented [4, 13].

In [17, 18] a linear-time partitioning algorithm was introduced based on the dual graph representation of RNA 2Ds. This algorithm partitions a dual graph into connected components called *blocks* and then determines whether each block contains a pseudoknot or is a regular region. Thus our procedure provides a systematic approach to partition an RNA 2D, into smaller classified regions, while providing a topological perspective for the analysis of RNAs.

In [19] pseudoknots were classified into two main groups: *recursive* and *non-recursive* pseudoknot. The former is distinguished from the latter because it contains an internal pseudoknotted or regular region that does not intertwine with external stems within the PK. In addition, if the PK is recursive, the partitioning algorithm uniquely identifies each recursive region.

In the classical literature, pseudoknots have been classified and predicted by folding algorithms into four types: H,K,L, and M [12]. Even though each type is defined in terms of how few stems intertwine, pseudoknots can be complex structures, recursive, and be comprised of several stems. In this paper we show that based on the directed graphs modeling of RNA 2Ds, each PK type can be identified through a series of reductions to a unique representative. This methodology will allow us to systematically analyze thousands of motifs and develop more precise RNA folding algorithms. Moreover, representation of RNA secondary structures based on dual directed graphs, is more precised than its dual undirected counterpart, as it is possible, as will be discussed in Section 3, that a dual undirected graph can model two or more distinct RNA 2Ds; this conflict is avoided by the novel representation.

The stimulatory nature of PKs in viral replication, and specifically in frameshifting, has been widely studied (see for example [2] and [4]). In a recent publication (2021), Bhatt et al. [3] presented a detailed study of programmed ribosomal frameshifting in translation of the SARS-CoV-2 virus. Interesting enough evidences show that the simplest H-type pseudoknot (or related structures, see Section 3) are predominantly present in eukaryotic families

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of viral mRNAs. Related questions follow from this observation;

1. Are there families of viruses or retroviruses in which other types of PKs (i.e., K, L, M) stimulate frameshifting.
2. Are there structural differences in pseudoknots comprising the FSEs (i.e., frameshifting element) in eukaryotic cells versus prokaryotic cells.
3. Are there viral mRNAs in which PKs are not present.

Our methodology will be able to shed some light on these outstanding questions.

In the next section, we present background material and definitions relevant to this paper, and we review the partitioning algorithm introduced in [17, 18], as well as its applications, as for example the development of a library of building blocks for RNA design by fragment assembly [9, 10]; we also discuss how the partitioning algorithm can detect recursive PKs and its recursive regions. In Section 3, we show how the aforementioned PK types are identified from dual digraphs. In Section 4 we summarize the findings and describe ongoing and future work.

2 Background

2.1 Biological and Topological Definitions

In 2003, Gan et al. [5] introduced *dual* graph-theoretic representations of RNA 2D motifs in a framework called RAG (RNA-As-Graphs) [14].

We define our biological variables as follows.

Definition 1. *General terms:*

a. RNA primary sequence: a sequence of linearly ordered bases $x_1, x_2, \ldots, x_r$, where $x_i \in \{A, U, C, G\}$.

b. canonical base pair: a base pair $(x_i, x_j) \in \{(A,U), (U,A), (C,G), (G,C), (G,U), (U,G)\}$.

c. RNA secondary structure without pseudoknot - or regular structure, encapsulated in the region $(i_0, \ldots, k_0)$: an RNA 2D structure in which no two base pairs $(x_i, x_j), (x_l, x_m)$, satisfy $i_0 \leq i < l < j < m \leq m_0$ (i.e., no two base pairs intertwined).

d. a base pair stem: a tuple $(x_i, x_{i+1}, \ldots, x_{i+r}, x_{j-r}, \ldots, x_{j-1}, x_j)$ in which (x_i, x_j), $(x_{i+1}, x_{j-1}), \ldots, (x_{i+r}, x_{j-r})$ form base pairs.

e. segment region: is a tuple $(x_i, x_{i+1}, \ldots, x_{i+r})$ in which (x_i, x_j) is not a base pair whenever $j - i \geq 1$.

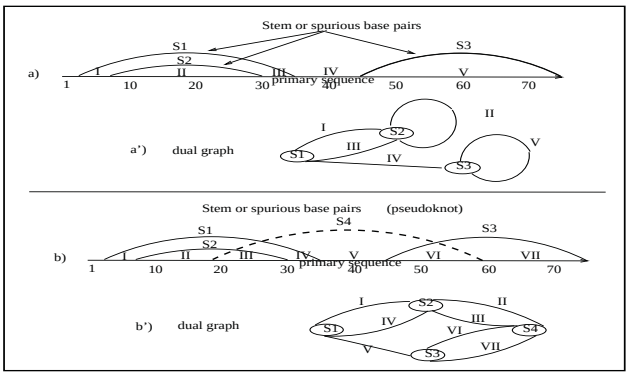

Figure 1: *Graphical and dual graph representations of an RNA 2D structure. (a) graphical representation of a pseudoknot-free RNA primary sequence and embedded stems or base pairs; (a′) corresponding dual graph representation. (b) graphical representation of a pseudoknotted RNA 2D structure; (b′) corresponding dual graph. This figure was originally depicted in [17].*

f. a pseudoknot encapsulated in the region $(i_0, \ldots, k_0)$: if $\exists l, m, (i_0 < l < m < k_0)$ such that (x_{i_0}, x_m) and (x_l, x_{k_0}) are base pairs (i.e., at least two base pairs intertwined).

A dual graph can be easily derived from the graphical representation of an RNA 2D structure: each stem is modeled by a vertex of the dual graph, and following the primary sequence in linear order (i.e., from the 5' end to the 3' end), a segment between stems S_i and S_j is represented by an edge (S_i, S_j) in the dual graph (see Fig. 1).

In the next section we present our partitioning approach of a dual graph G, into subgraphs $G' \subseteq G$, called blocks.

2.2 Graph Partitioning Algorithm

The graph-theoretic partitioning algorithm is based on identifying *articulation points* of the dual graph representation of an RNA 2D. An articulation point is a vertex of a graph whose deletion disconnects a graph or an isolated vertex remains. Articulation points allow us to identify blocks (see Fig. 2); since a block is a maximally non-separable component, a pseudoknot cannot be then contained in two different blocks. Thus identification of these block components allows us to isolate pseudoknots (as well as pseudoknot-free blocks), without breaking their structural properties.

An algorithm for identifying (bi-connected) block components in a graph was introduced by John Hopcroft and Robert Tarjan (1973, [7]), and runs in linear computational time.

A *hairpin* loop occurs when two regions of the same

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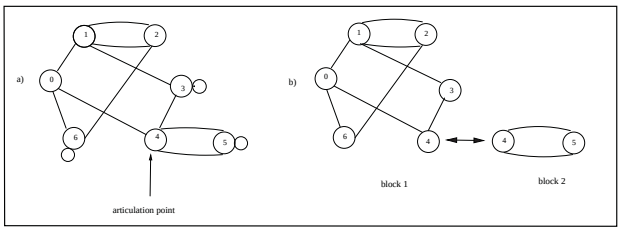

Figure 2: *Identification of a) articulation points and b) partitioning of a dual graph.*

strand, usually complementary in nucleotide sequence when read in opposite directions, base-pair to form a double helix that ends in an unpaired loop. A self-loop in the dual graph, i.e., an edge having the same vertex as the end-points, represents a hairpin, and as it does not connect two different vertices (i.e., stems), it is formally deleted from the dual graph.

From Definition 1-c, an RNA 2D structure is a regular-region (pseudoknot-free) and encapsulated in a region $(i_0, \ldots, k_0)$, if no two base pairs $(x_i, x_j), (x_l, x_m)$, satisfy $i < l < j < m$, $i_0 \leq i, j, l, m \leq m_0$, otherwise the region is a pseudoknot; this definition yields the following main result.

Corollary 1. *[17, 18] Given a dual graph representation of RNA 2D structure, a block represents a pseudoknot if and only if the block has a vertex of degree (Definition 1-f) at least* 3 *where the degree of a vertex u is the number of edges incident at u.*

Corollary 1 yields the following algorithm,

Algorithm 1. Partitioning

1. Partition the dual graph into blocks by application of Hopcroft and Tarjan's algorithm.

2. Analyze each block to determine whether contains a vertex of degree at least 3. If that is the case then the block contains a pseudoknot, according to Corollary 1. If not then the block represents a pseudoknot-free structure.

Consider as an example the dual graph shown in Figure 2. This graph is decomposed into 2 blocks. According to Corollary 1, block 1 is a pseudoknot as it has a vertex of degree at least 3, while block 2, a cycle, corresponds to a regular region.

In the next section we extend our algorithm to classify PKs as either recursive or non-recursive; the algorithm can also identify each recursive region.

2.3 Classification of pseudoknots as either recursive or non-recursive and identification of each recursive region

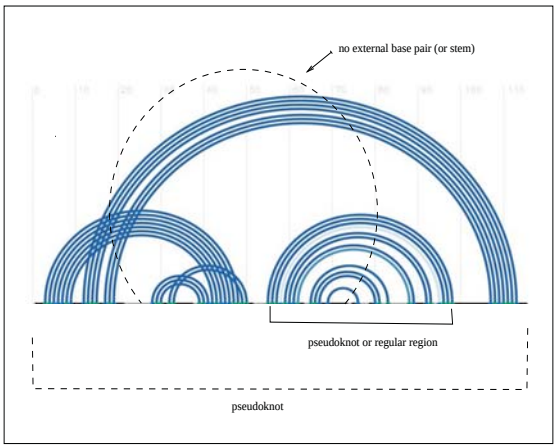

Figure 3: *Recursive pseudoknot.*

The RNA 2D dual graph and graphical representations depicted in this section are based upon New York University's *RAG*-database [8], and *R-Chie* visualization software [15], respectively.

A recursive pseudoknot is a pseudoknot $M_{i,j}$ in a region $[i, j]$ that contains a pseudoknotted or regular region $M_{k,l}$, $i < k < l < j$, and there does not exist a base pair (x_c, x_d), such that x_d is a base of $M_{k,l}$, and x_c is a base of $M_{i,j}$ external to $M_{k,l}$ (see Fig. 3).

A pseudoknotted block can be classified as recursive by determining the edge-connectivity of the block. The *edge-connectivity* is defined as the minimum number of edges that if they are deleted then the resulting graph is disconnnected. As an example consider the *Hepatitis Delta Virus Ribozyme* (see Fig. 4), necessary for viral replication. The stem labeled 4 in the graphical representation (or vertex labeled 4 in the dual graph) is attached to the pseudoknot by the segments a and b in its graphical representation, or edges labeled a and b in the dual graph representation. As by deleting two edges in the dual graph, vertex labeled 4 becomes disconnected, then stem 4 is a recursive region of the PK, thus the dual graph edge-connectivity is 2. The proof of the following lemma was shown in [19].

Lemma 1. *The dual graph representation of a pseudoknotted block is recursive if and only if the block has edge-connectivity 2.*

It can be determined that a pair if edges is a disconnecting set by application of Depth-First-Search [6] in time $(|E|^3)$, allowing us to find every internal recursive region of a recursive pseudoknot, if such pair of edges exist.

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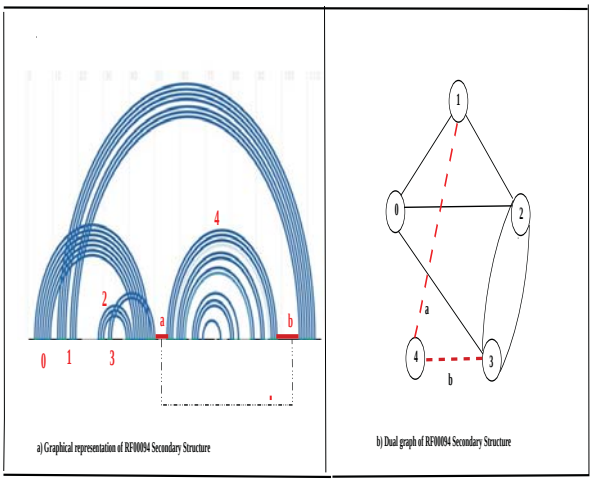

Figure 4: *Hepatitis Delta Virus Ribozyme secondary structure. a) Graphical representation. b) Dual graph representation.*

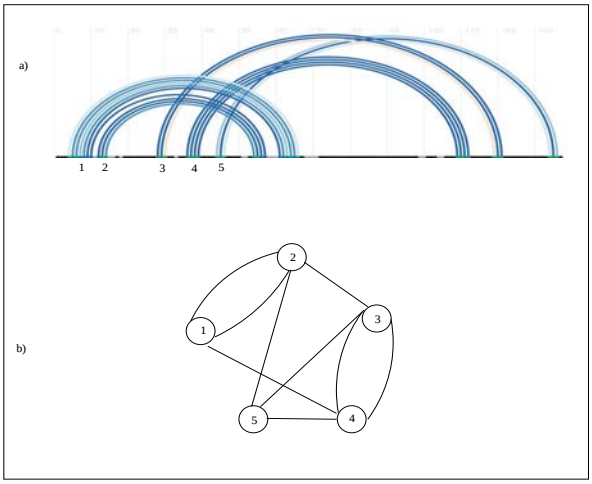

Figure 5: *Translational repression of the Escherichia coli alpha operon mRNA*. a) Graphical representation; b) Dual graph representation.

The following is the partitioning and classification of pseudoknots algorithm.

Algorithm 2. Partitioning and Classification of PKs

i. Input dual graph $G = (V, E)$ as the Adjacency Matrix, of a RNA 2D.

ii. Output partitioning of the RNA 2D into recursive PK, non-recursive PK, and regular regions.

1. Partition the dual graph into blocks by application of Hopcroft and Tarjan's algorithm;
2. Analyze each block to determine whether each contains a vertex of degree at least 3*;*
3. **IF** *the block has a vertex of degree* ≥ 3 *then the block is a* **pseudoknot;**
   - *Apply Depth-First-Search to find possible pairs of edges that disconnect the graph (i.e., edge-connectivity equals 2);*
   - *if edge-connectivity = 2 then the block is a* **recursive pseudoknot;**
     else the **pseudoknot is not recursive;**
4. **ELSE** *the* **block is a regular region;**.

As an example of a non-recursive pseudoknot consider the *Translational repression of the Escherichia coli alpha operon mRNA* ([22]), illustrated in Fig. 5. The dual graph representation of this motif 2D has edge-connectivity 3, thus it is not a recursive PK.

The algorithm is written in C++ and is archived for public use [20].

3 Classification of H, K, L, M pseudoknots types

In the classical literature, pseudoknots have been classified and predicted by folding algorithms into four types: H,K,L, and M [12]. Even though each type is defined in terms of how few stems intertwined, pseudoknots can be complex structures, recursive, and be comprised of several stems. In this paper we show based on dual directed graphs, each PK type can be identified through a series of reductions to a unique representative. This methodology will allow us to systematically analyze thousands of motifs and develop more precise RNA folding algorithms. The results and algorithms discussed in Section 2.2 and Section 2.3, based upon undirected dual graphs, can be extended to dual directed graphs, as the direction of a directed edge can be ignored. Moreover, representation of RNA secondary structures based on dual directed graphs, is more precised than its dual undirected counterpart, as a dual undirected graph can model two or more distinct RNA 2Ds; this conflict is avoided by the novel representation. For example, the distinct directed graphs representing K and L types (see Fig. 8), have the same corresponding dual undirected graph, i.e., by replacing directed edges by undirected edges.

In this section we follow the definitions of pseudoknots as stated by Antczak et al. [1], and Kucharik et al. [12].

A H-type pseudoknot occurs when a nucleotide of a loop or bulge pairs with a nucleotide of a single-stranded region outside the loop; this type can be alternatively illustrated from the graphical representation (see Fig. 6-a) by the intertwining of two stems.

A K-type PK results when two nucleotides from different

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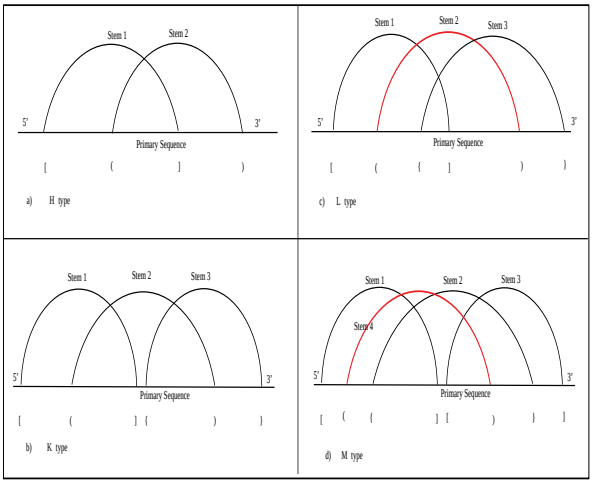

Figure 6: *Graphical representation of the H,K,L, and M types. a) H-type. b) K-type, c) L-type, and d) M-type.*

loops (or bulges) pair to form a double helical segment (see Fig. 6-b). Similarly we can also describe L and M types pseudoknots, derived from the H and K types, respectively, by addition of a stem (colored red) as shown in Figure 6-c and Figure 6-d.

Even though the different PK types are defined in terms of few stems, each type may be composed of several stems and also they could be recursive (see section 2.3). For example Figure 7 illustrates two H-type PKs, PKB70 (i.e., the Legionella pneumophilia tmRNA), causative agent of the Legionannary's disease [11], composed of 5 stems, and recursive PKB259 (i.e., potato yellow vein virus [16]) comprising a recursive region and 3 stems. Even though two PKs of the same type of maybe structurally different, in the following section we show how using dual digraphs each motif type can be reduced to a unique digraph type-representative.

3.1 Dual digraphs and reductions to unique PK type identifiers

In this section we used dual digraphs and based upon two graph-theoretical transformations, these digraphs can be reduced to a unique representative identifying H,K,L, and M pseudoknot types.

A dual digraph can be easily derived from the graphical representation of a RNA 2D structure: each stem is modeled by a vertex of the dual digraph, and following the primary sequence in linear order (i.e., from the 5' end to the 3' end), a segment between stems S_i and S_j is represented by an directed edge (S_i, S_j) in the dual digraph. Moreover each directed edge is assigned a weight corresponding to the order in which the segment is reached following the primary sequence (see Fig. 7-b); this weight is represented as $w(S_i, S_j)$.

From a RNA 2D perspective, we informally define a *super-stem* as a stem that completely contains another stem (*sub-stem*). Formally a super-stem is a n-tuple $(x_i, x_{i+1}, \ldots, x_{i+r}, x_{j-r} \ldots, x_{j-1}, x_j)$, $n = j - i + 1$; in which $(x_{i+k}, x_{j-k}), k \leq r$ forms a base-pair (see definition 1-d), if there exist another stem (i.e., sub-stem) in the region $(x_l, x_{l+1}, \ldots, x_{l+s}, x_{m-s}, \ldots, x_{m-1}, x_m)$, $l > i + r$, and $m < j - r$. For example in Stem S_0 shown is Figure 7-a (i.e., PKB70) is a super-stem of S_1 and S_2, and stem S_1 is a super-stem of S_2; similarly stem S_3 is a super-stem with respect to S_4. For ease of notation let $S_i > S_2$ represent the case when S_i is a super-stem of S_j. The following proposition describes how a super-stem S_i and its corresponding sub-stem S_j can be detected in a dual digraph,

Proposition 1. *If there are exist exactly two anti-parallel directed edges (S_i, S_j) and (S_j, S_i) between vertices S_i and S_j of the dual digraph, with $w(S_j, S_i) - w(S_i, S_j) \geq 2$, then S_i is a super-stem of S_j.*

Proof: Without loss of generality, let $w(S_j, S_i) > w(S_i, S_j)$. Since $w(S_j, S_i) - w(S_i, S_j) \geq 2$, implies that if there is either an eternal stem S_k, intertwining S_i **and** S_j, or, S_j forms a self-loop; if there exist an external stem S_k intertwining S_i and S_j, this stem does not intercept the primary sequence between S_i and S_j. If $w(S_j, S_i) - w(S_i, S_j) = 1$, then S_j is not fully contained in S_i (i.e., intertwines S_j) $\square$

As an example consider stems labeled S_0 and S_1 for PKB270 shown in Figure 7-a. In the corresponding dual digraph (Fig. 7-b) the vertices labeled S_0 and S_1 are connected by exactly two anti-parallel edges with $w(S_1, S_0) - w(S_0, S_1) = 6 \geq 2$. Please note that the converse it is not necessarily true, that is, if S_i is a super-stem of S_j, then there exist exactly two anti-parallel edges between vertices S_i and S_j in the dual digraph with $w(S_1, S_0) - w(S_0, S_1) \geq 2$; for example in PKB259 (Fig. 7-e), stem S_0 is a super-stem of S_1, however there are no anti-parallel edges between the corresponding vertices in the dual digraph (Fig. 7-f). This has to do with the fact that another stem (i.e., S_3) intercepts the primary sequence between S_0 and S_1. From Proposition 1 we introduce the following graph reduction,

Reduction 1. *Let $G = (V, E)$ be a dual digraph.If there are exist exactly two anti-parallel directed edges (S_i, S_j) and (S_j, S_i) between vertices S_i and S_j of G, with $w(S_j, S_i) - w(S_i, S_j) \geq 2$, then transform G into G', where S_1 and S_j are identified into a single vertex $[S_i, S_j]$ and any directed edge in G, (S_k, S_i), (S_k, S_j), $k \neq i$ or $k \neq j$, will result in an directed edge $(S_k, [S1, S2])$ in G', while keeping the same weights from the edges of G. Similarly an edge (S_i, S_k), or (S_j, S_k) in G will have the corresponding edge $([S1, S2], S_k)$ in G', while maintaining the same weights on the original edges (see Fig. 7-b-c).*

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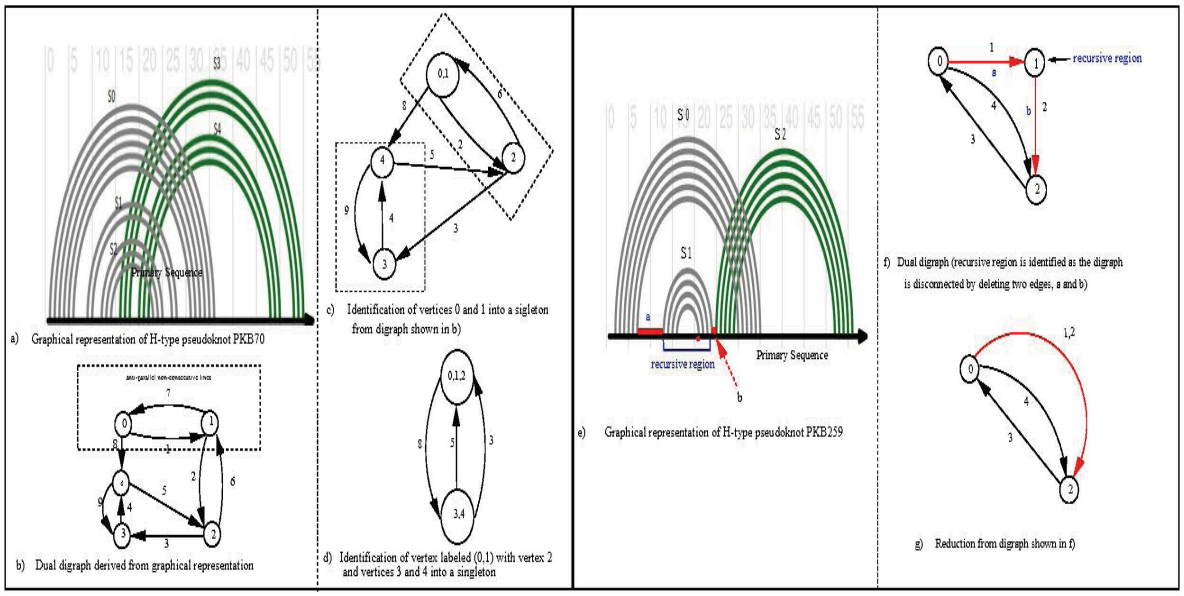

Figure 7: *Reduction of H-type pseudoknots for RNAs PKB70 and PKB259 to the dual digraph representative of a H-type PK.*

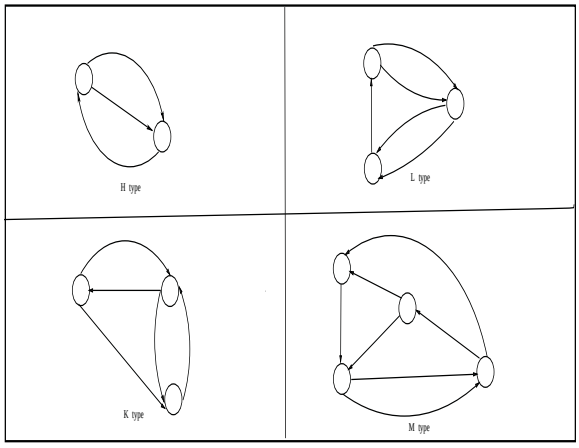

Figure 8: *By application of Reductions 1 and 2, a directed graph representing a RNA motif, will be reduced to a unique representative of the 4 different types (H,K,L,M).*

A recursive PK can be classified by our partitioning algorithm and a recursive fragment can be identified (see Section 2.2). For example in the graphical representation of PKB259 (Fig. 7-e), segments a and b isolate the recursive fragment Stem S_1, or equivalently, deleting exactly two edges of the dual digraph (connectivity 2), a and b, identified by Algorithm 2, disconnects the dual digraph. The following graph reduction follows from the algorithm,

Reduction 2. *Let $G = (V, E)$ be a dual digraph, and G_0 be a recursive fragment adjacent to Stem S_0 by direct segment a from Stem S_0 to G_0, and by segment b from G_0 to Stem S_1 (i.e., deleting segments a and b isolates G_0), then transform G into G', by deleting segments a and b, recursive fragment G_0, and by inserting a direct segment from S_0 to S_1 (see Fig. 7-e-f).*

By application of Reductions 1 and 2, a directed weighted dual graph modeling a pseudoknotted region of a RNA 2D (recognized by partitioning Algorithm 2, Section 2.3) will be reduced to one types depicted in Fig 8, otherwise the PK is none of these types.

4 Conclusions and Ongoing Work

The Covid-19 pandemic accelerated the study of viral replication and the need to develop therapeutics to control infectivity. The importance of the three-stemmed pseudoknot-dependent ribosomal frameshifting for the propagation of SARS-related coronaviruses is well-established. We suggest that pseudoknots not only play a significant role, but a predominant one in viral transmissibility for most viruses, and our proposed techniques aim to shed some light in this area, as well to better understand the roles of PKs in general RNA functionality. Evidences show that the simplest H-type pseudoknot (or related structures) are predominantly present in eukaryotic families of viral mRNAs, and our proposed techniques could make substantial progress on this area of research.

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Acknowledgments

An earlier version of this work was archived in [21].

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