SPLASH-structure: a statistical approach to identify RNA secondary structures from raw sequencing data, bypassing multiple sequence alignment

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Abstract

RNA secondary and tertiary structure is critically involved in ribozyme and ribosomal rRNA function, as well as viral and cellular regulation. Traditional experimental methods for RNA structure determination such as X-ray crystallography or chemical mapping are incisive; however, these approaches suffer from low-throughput and low-dimensionality, respectively. Computational approaches, leveraging evolutionary signals from correlated positions' mutations, provide an alternative means to infer RNA structures. However, these methods require assembly, and face challenges due to statistical biases inherent in multiple sequence alignment (MSA). Furthermore, these methods cannot make use of the full spectrum of natural variations seen for a given RNA element. Here, we introduce SPLASH-structure, a direct assembly-free, MSA-free, and metadata-free statistical method for identifying conserved RNA structures by analyzing raw sequencing data, quantifying compensatory mutations or stem variation exclusion in the putative RNA structures. We show SPLASH-structure rediscovers known HIV structural elements and identifies conserved rRNA structures in metatranscriptomics samples. Moreover, SPLASH-structure finds Culex narnavirus 1, Gordis virus, and Culex mosquito virus 4, as well as previously unannotated viral genomes in mosquito metatranscriptomics samples *de novo*, highlighting the method's potential for viral discovery. SPLASH-structure is an ultra-fast, easy to use, and robust tool that excels in high-throughput RNA structure prediction and hypothesis generation, presenting a novel approach for discovering structural RNA elements.

Introduction

Highly conserved RNA secondary and tertiary structures are a hallmark of functional RNAs across life (Rivas 2021). Examples include rRNA, tRNA, and the 7SL RNA and its derived SINE elements (Gundelfinger et al. 1984). Many other examples, from ribozymes (e.g., the group I intron catalytic RNA), to riboswitches (Breaker 2012), to crRNA that direct Cas proteins to their targets (Jackson et al. 2014) exhibit structural conservation. Experimentally, RNA structures can be determined directly (e.g. small angle X-ray scattering, X-ray crystallography, or cryogenic electron microscopy), or indirectly via biochemical probing (e.g. RNase footprinting (Peng, Soper, and Woodson 2012), chemical mapping via SHAPE (Deigan et al. 2009), and NGS methods (Spitale and Incarnato 2023)). These methods have proven to be very productive (Zhang et al. 2022) but cannot scale with the present pace of biological sequence discovery (Charon et al. 2024). While seminal developments have been made to identify and infer RNA secondary structures from sequence data (Rivas, Clements, and Eddy 2017; Rivas 2021; 2020), these methods still fail to keep pace with the sequence discovery rate.

Evolutionary signatures of biomolecular structures have proven to be potent signals for protein structure inference (Jumper et al. 2021). Analogously, comparative genomic analysis of orthologous RNA sequences can be used to infer RNA secondary structures. The first evidence for such an RNA structure signal came from the analysis of compensatory mutations in multiple sequence alignment (Noller et al. 1981). The logic for this idea is as follows: assuming an RNA's secondary structure is necessary for cellular fitness, a single base-pair-disrupting base change in such an RNA's sequence will be deleterious to fitness, however, a compensating mutation will "rescue" the structure and have no or little net fitness cost. Thus, observing compensatory mutations (in the form of co-varying

positions in a multiple sequence alignment of orthologous RNAs) in an area of inferred local structure can be both a signature of secondary structure and of selection for said structure. This idea has been explored extensively and adapted to experimental techniques for RNA secondary structure inference (Cheng et al. 2017; Rivas 2021; Deigan et al. 2009).

Rigorous analytic methods to infer RNA structure through multiple sequence alignment (MSA) have been developed and used to discover novel structural elements, most notably through tools such as R-scape (Rivas, Clements, and Eddy 2017). These authors have also provided insights into the inferential pitfalls of some current methods based on MSA, including false positive calls of structural covariation (Gao, Yang, and Rivas 2023). First, MSA reduce the rich variation in microbial or even eukaryotic genome diversity, within and between hosts, to representative sequences. Second, these alignments are themselves computational procedures; thus, inference on them is conditional on the algorithmic output, that is, potentially statistically biased. This bias has been articulated in previous work (Gao, Yang, and Rivas 2023) and includes (i) some extensive compensatory mutations that may be censored out of MSA, (ii) MSA biases towards including sequences with covariation in their alignment, (iii) MSA inference may suffer from evolutionary confounding as described in a rich body of work: the existence of covariation as typically quantified must be taken to be conditional on evolutionary history (Rivas, Clements, and Eddy 2017). Proposed methods seek to remove confounding by descent/evolutionary history. However, in the microbial world, phylogenetic trees cannot fully capture genetic diversity partly due to the prevalence of horizontal gene transfer (Garcia-Vallvé, Romeu, and Palau 2000), and to rapid mutation rates within hosts (Sanjuán et al. 2010), in the case of RNA viruses. In summary, classical analysis of covariation are conditional procedures, biased by the process of genome assembly and MSA.

Critical obstacles inherent to existing multi-step bioinformatic processes preclude the high throughput and precise *de novo* computational prediction of RNA structures. Namely: 1) genomes or genome fragments must be assembled; 2) a multiple sequence alignment on them must be performed; 3) and a statistical decision and scoring of putative compensatory mutations must be performed without a closed form *p*-value and one that is confounded by phylogeny (Rivas, Clements, and Eddy 2017)). With rare exceptions (e.g. a genomic approach relying on a reference genome that yielded new riboswitches (Stav et al. 2019)), all of these steps have created a bottleneck for discovery.

In addition, RNA structures within RNA viruses are especially important with known functions (e.g. in poliovirus (Burrill et al. 2013), SARS-CoV-2 (Andrews et al. 2021), flavivirus (Lee et al. 2021)) that are relatively conserved in a background of rapid genomic variation. At the same time, RNA viruses are characterized by high mutation rates, exist as quasispecies (Lauring and Andino 2010) and can rapidly evolve within and between hosts, making assembly difficult. Raw sequences, not assemblies, are the evolutionary “tinder” of viruses, and provide an insight into the "fitness landscape" they explore and which are selected.

Here, we introduce a direct statistical approach, SPLASH-structure (https://github.com/juliefrwang/splash-structure), to detect conserved secondary structures from sequencing data, focusing on conserved elements despite nucleotide variations across samples (**Figure 1A**). The method both quantifies compensatory mutations in stem-loops, and additionally detects cases of apparent negative selection *against* putatively deleterious structure-disrupting

variants. SPLASH-structure is based on the SPLASH paradigm (Chaung et al. 2023), a method that directly analyzes raw sequencing data using a statistical test to detect sample-specific sequence variation. SPLASH-structure builds on SPLASH using it as an analytical pipeline for the inference of potential compensatory mutations under selection and provides a direct statistical test for compensating mutations or absent mutations in stems to detect RNA secondary structure, bypassing MSA and its accompanying statistical and bioinformatic pitfalls (Gao, Yang, and Rivas 2023). SPLASH-structure does not require a reference genome and enables statistical prioritization of sequences which exhibit higher levels of compensatory variation compared to a null distribution, providing efficiency and convenience over other RNA structure detection methods (**Figure 1B**). We show SPLASH-structure provides precise *p*-values by theory and by simulation as well as demonstrate its performance on real world data.

We first show SPLASH-structure can rediscover known structural elements on a purely statistical basis, without relying on sequence alignment. We ran SPLASH-structure on longitudinal HIV sampling from an HIV-infected cohort (Antar et al. 2020) on a host-by-host basis to control for confounding covariation caused by phylogenetic relations (Rivas, Clements, and Eddy 2017). Without a reference sequence, SPLASH-structure recovers well-known structures including the ribosomal frameshift site, psi packaging signal, and trans-activating response element (Watts et al. 2009). The power of SPLASH-structure should be realized in its ability to predict conserved RNA structures missing from reference genomes. To test this, we ran SPLASH-structure on the complex, multi-organism sequencing mixture from a mosquito metagenomics sample. Here, SPLASH-structure prioritizes known structures in rRNA as a positive control, while predicting structures in viruses where structural covariation has not been described, as well as novel predictions in sequences missing from NCBI nucleotide databases. We thus present SPLASH-structure as one of a growing number of tools (Lin et al. 2023) that predict structure from sequence.

Results

SPLASH-structure is an alignment-free statistical approach to identify RNA secondary structures, bypassing MSA

SPLASH-structure is a statistical approach designed to identify RNA secondary structures by focusing on conserved elements despite nucleotide variations across samples. We reasoned that if an RNA structure is functional, its overall structure would be conserved despite base changes in its nucleotide sequences, and sequences near this structure should be called by SPLASH.

SPLASH processes raw sequencing data and generates analyzable units known as anchors and targets across the samples. An anchor is any specific *k*-mer sequence in a read, with each *k*-mer at a fixed offset downstream from said anchor the target. A given anchor may have multiple targets. SPLASH-structure then detects patterns in target sequence variations across samples that could be explained by pressure to conserve secondary structures. The algorithm begins by selecting the most abundant target of an anchor as the base target, T_0, and aims to identify a “stem-loop” configuration within T_0 of each anchor (**Figure 2A**). Specifically, SPLASH-structure looks for a consecutive *k*-mer (a minimum of 5 bases in practice) from the start of T_0 and a reverse complement of this *k*-mer further downstream that does not overlap with the original *k*-mer. The reverse complement is defined

according to Watson-Crick-Franklin base-pairing rules. The identified *k*-mer and its reverse complement are defined as the stems, and the intervening sequences are the loop. While RNA can fold following non-Watson-Crick-Franklin base-pairing rules (Vicens and Kieft 2022), we only consider Watson-Crick-Franklin base-pairing to simplify the statistical formulation. As a result, our statistical tests tend to be conservative at identifying structures. Only anchors with a base target featuring such a stem-loop structure are kept for further analysis. One limitation is that this approach primarily focuses on the simplest RNA secondary structure, stem-loops, and thus neglects more nuanced secondary structures such as bulges and pseudoknots.

SPLASH-structure proceeds by assessing variation in the rest of the targets against the base target (**Figure 2B)**. The algorithm tallies mismatches located within or outside the stems, and determines the compensatory mutations that occur in stems. For instance, if a nucleotide A within stems of a base target changes to G in another target, and the corresponding T changes to C, this is considered a compensatory mutation pair that is consistent with a conserved structure, which we refer as compensatory mutations or base-pair covariation (BPC). Another mechanism that is consistent with conserved structures involves the absence of mismatches in the stems between targets, despite their presence elsewhere. We call this mechanism stem-variation exclusion (SVE).

For each varied target, SPLASH-structure combines the BPC and SVE tests. Each is tested against the null hypothesis that mismatches are uniformly distributed across any positions within the target and that these mismatches occur independently, given a fixed sequence length (k), stem length (L), and number of mismatches (v, Hamming distance from the base target). The target p-value, p_{tar} is defined for the two alternatives separately: 1) For BPC: p_{tar} is defined as the likelihood of having at least as many compensatory mutations as observed, given the predetermined parameters k, L, and v. 2) For SVE: p_{tar} is the likelihood of all mismatches occurring outside the stem regions. Both p_{tar} can be computed in closed form (**Methods**). The two target p-values are combined using an indicator function that checks for mismatches in the stem regions. If there are no mismatches, p_{tar} for SVE is used. Otherwise, p_{tar} for BPC is used.

Then for each anchor, we construct an aggregate test statistic, anchor score, defined as the weighted mean of p_{tar} of all targets of an anchor. We can derive the distribution of the anchor score, compute a p-value for each anchor (**Methods**), and adjust p-values to control false discovery rate (FDR) through Benjamin-Hochberg (BH) procedure. With this hypothesis test, a low BH-adjusted p-value associated with an anchor suggests the presence of a stem-loop structure that is consistent with BPCs or SVE among varied targets in samples.

We specified the method described so far as SPLASH-structure (target mode), as by far, we have been detecting structures in target sequences. We note that a stem-loop can be located distant from an anchor. Furthermore, stem-loop structures in nature can vary substantially in length, and many structural elements possess greater complexities than simple stem-loops, such as internal loops (**Figure 3C**). To comprise longer sequences and complexities, we adopted a similar analytical framework on compactors, i.e. SPLASH-structure (compactor mode) (**Methods**; **Suppl. Figure 1A)**. Compactors are longer local anchor-seeded assemblies sequences generated using probabilistic methods, and each seed-anchor can have multiple compactors (**Suppl. Figure 1B**) (Henderson et al. 2024). In this study, we generated compactors that are 81nt long. Anchors are included at the beginning as the first 27-mer of these compactors, and we call the 54-mer following the anchor the

“trimmed compactor” (**Figure 3A**). The support of a compactor measures the count of occurrences in samples of all sequences that it represents. The trimmed compactor of the compactor with the highest support is defined as the base trimmed compactor.

SPLASH-structure (compactor mode) adopts a split-and-concatenate method for analyzing longer sequences, allowing for the identification of two pairs of complementary sequences in a compactor. The principle idea is to split a trimmed compactor into four nearly equal-length sub-sequences, and then concatenate any two of these segments in an order-preserving manner (**Figure 3A**). This means that if one segment precedes another within the original compactor, it will also precede it in the new composite sequences. As a result, there are three ways to recombine four sub-sequences. In the study, we split a 54nt long trimmed compactor into 14, 14, 13 and 13 nt long sub-sequences and the resulting recombined sequences have 28, 27 or 26 nucleotides (**Methods**). To detect structure, we treat each resulting recombined sequence just like a target, allowing previously described processes in “target mode" to be directly applied to compactors. But now because sequences are permuted, nested stems can be detected. This includes two adjacent stem-loops, a stem-loop with a longer loop, and a longer stem-loop with an internal loop and, in principle, pseudoknots (**Figure 3B**, **Suppl. Figure 2**). Next, for each split-and-concatenate permutation, the same statistical measure is applied to evaluate an anchor, with parameters being the aggregate of the two recombined sequences. This split-and-concatenate method can be expanded by dividing a long sequence into more than four sub-sequences. This increases the number of possible ways to reassemble the sequences, allowing for the detection of structures of more possibilities in future work (**Methods**). As we show in later sections, SPLASH-structure (compactor mode) is more flexible and powerful than the target mode for detecting stem-loop structures. Therefore, we will primarily focus on reporting results from the compactor mode.

We designate anchors with a BH-adjusted p-value below 0.05 in target mode or compactor mode of SPLASH-structure as structure-significant anchors. This distinguishes them from SPLASH-significant anchors, which are identified by the SPLASH algorithm as having significant variations across different samples. In the remainder of this paper, “significant anchors” refers to the structure-significant anchors, unless explicitly specified as SPLASH-significant anchors.

SPLASH-structure *de novo* rediscovers conserved HIV-1 structural RNA elements

We tested SPLASH-structure on human immunodeficiency virus (HIV). HIV is a single-stranded, positive-sense RNA virus characterized by a small genome, high mutation rates, and many secondary structures crucial to viral replication and host defense mechanisms (Watts et al. 2009). These RNA structural motifs play critical regulatory roles, including promoting transcription or translation, and direct virion assembly (dimerization and packaging) (Watts et al. 2009; Lu, Heng, and Summers 2011; Ye et al. 2022). The extensive variety of known structural motifs and the presence of short stem-loops with considerable nucleotide variation in HIV make it an ideal case for SPLASH-structure to detect RNA structures independently of traditional assembly and MSA techniques.

We analyzed publicly available Illumina short-read sequencing datasets of provirus sequences from a longitudinal study involving individuals infected with HIV-1 subtype B+ (Antar et al. 2020).We applied SPLASH-structure separately on seven hosts (Hosts 1124, 1194, 1211, 1532, 548, 583 and 746)t to detect evolutionary conserved RNA structures, testing for significance within a host and

controlling for confounding covariation caused by phylogenetic relations (Rivas, Clements, and Eddy 2017). SPLASH-structure (target mode) identified 13, 5, 10, 7, and 3 significant anchors (BH-adjusted *p*-values < 0.05) in Host 1124, Host 1194, Host 1211, Host 583 and Host 746 respectively, with no significant anchors detected in the other hosts. In Host 1124, all significant anchors match the HIV ribosomal frameshift signal element (RF00480) of the Rfam database, and the structural element is detected to be conserved through SVE. We show a detected example of a frameshift element in **Figure 4A**. Base-pairing within predicted stem regions shows consistency across various targets. Significant anchors detected in other hosts align to HIV genomes by BLASTn, but do not match any structure in the Rfam database.

SPLASH-structure (target mode) is expected to overlook elements due to the algorithm's design, which disregards sequences with minimal variations across samples, consequently missing structures in highly conserved regions. Additionally, the target mode may miss structural elements for following reasons: 1) Targets (length 27 bps, maximum 31 bps in SPLASH) are too short to accommodate structural elements with longer stems or larger loops. 2) False negative calls of anchors in SPLASH (**Methods**). We chose not to investigate the latter in depth because it's tangential to this study. We therefore expect using SPLASH-structure (compactor mode) to find more structural elements of HIV.

SPLASH-structure (compactor mode) identifies a median of 2.2% structure-significant anchors relative to the total number of SPLASH-significant anchors equipped with compactors across all hosts. We found hits to the retroviral psi packaging element stem-loop 3 (SL3; RF01381) in 4 out of 7 hosts (except for Host 1194, 548 and 1532), one the most frequently detected. SL3 is a 14 nt highly conserved stem-loop, and SPLASH-structure (compactor mode) precisely identifies the stem sequences conserved through SVE in compactors (**Figure 4B**). SPLASH-structure detected sequence variations outside the stem-loops, particularly on both sides of the stem-loop, making it less possible to be identified near an anchor in the target mode.

SPLASH-structure (compactor mode) also detects the trans-activation response element (TAR; RF00250) in 4 out of 7 hosts. The TAR has a hairpin structure essential for viral replication that contains 24 base pairs, a loop of 6 bases, and three bulges in the stems. The nearest bulge to the loop is positioned 4 nucleotides away (**Figure 4C**). In target mode, the SPLASH-structure is unable to detect the stem-loop due to its requirement for a minimum stem length of 5 nucleotides. Consequently, the bulge must be at least 5 nucleotides away from the loop for target mode to successfully identify the stem-loop. Compactor mode has the advantage of detecting compensatory stems that are further apart and away from the loop, making detection of TAR possible. Overall, we observe that SPLASH-structure (compactor mode) exhibits greater ability to identify conserved structures; therefore, we will focus on the results from the compactor mode.

SPLASH-structure identifies conserved rRNA structures in mosquito metatranscriptomics

Mosquitoes serve as vectors and hosts for a variety of microorganisms, including eukaryotic, prokaryotic, and viral agents. Metatranscriptomic sequencing of individual mosquitoes offers a single assay capable of sampling from very diverse organisms, many of which likely lack references. We sought to identify functionally important elements within complex metatranscriptomics data by prioritizing regions under selection for conserved structural elements in order.

We analyzed a publically available mosquito metatranscriptomics dataset which profiled 148 diverse wild-caught mosquitoes (Adult *Aedes*, *Culex*, and *Culiseta* mosquito species) collected in California (Batson et al. 2021). SPLASH-structure (target mode) identified 687 significant anchors in all reads and SPLASH-structure (compactor mode) identified 4718 significant anchors. We searched for matches of the combined anchor and base target sequences, as well as base compactors, in the Rfam database. Our findings revealed that under compactor mode, base compactors of 190 significant anchors match ribosomal RNA (rRNA) in Rfam, a positive control for SPLASH-structure, because rRNA has highly conserved structures across diverse organisms, despite variations in primary sequences. Among the top 10 most significant anchors identified by the lowest *p*-values that have their base compactors matched to rRNA, two match with eukaryotic large subunit rRNA (28S rRNA), four match the eukaryotic small subunit rRNA (18S rRNA), two match bacterial large subunit rRNA (23S rRNA), and two match bacterial small subunit rRNA (16S rRNA).

Figure 5A shows a match to 16S rRNA. Within the base compactor, two stem-loop structures of stem length of 5 and 6, respectively, were detected. BPCs occur at different locations in the stems, and these changes can vary across compactors. For example, the base pair G-C (nucleotides at index 52 and 64) in the base compactor has a compensatory change to A-T in the first compactor followed, but remains unchanged in the next compactor and only has one base change in the last one. The base pair A-T (nucleotides at index 48 and 68) in the base compactor changes to T-A changes to T-A in all of the three compactors. Similarly, SPLASH-structure observed covariations of base pairs in the compactors that match 28S rRNA (**Figure 5B**).

SPLASH-structure identifies RNA viral structures in mosquito metatranscriptomics

4113/4718 (87.18%) base compactors aligned (BLASTn) to *Arthropda* phylum genomes, 55/4718 (1.17%) to bacteria, 51/4718 (1.08%) to *chordata* phylum, 46/4718 (0.96%) to viruses, 28/4718 (0.59%) to fungi, and other organisms (**Figure 6A**), with 391/4718 (8.28%) having unaligned base compactors. Given the myriad functions of RNA structures in regulating virus replication, we first focused on sequences that align with viruses in the BLAST results.

SPLASH-structure detects five viruses without relying on assembly of reads and provides statistical evidence of potentially functional elements within them: Culex narnavirus 1, Gordis virus, Culex mosquito virus 4, Aedes vigilax sobemo-like virus 1 and Wuhan mosquito virus 4. Notably, Aedes vigilax sobemo-like virus 1 and Wuhan mosquito virus 4 were not reported in previous analyses, highlighting SPLASH-structure's ability to detect RNA viruses without references. Culex narnavirus 1 had the highest number of aligned base compactors (40 out of 46). For all viruses except Wuhan mosquito virus 4, the base compactors aligned with over 80% coverage and high sequence similarity. The base compactors aligned to Wuhan mosquito virus 4 showed only 29 out of 81 bases with 100% sequence similarity to the subject sequence (**Figure 6B**). We queried other compactors of the same anchor,

We show evidence of BPCs at various positions in predicted stem-loops of Culex narnavirus 1. In **Figure 7A**, the G-C base pair (nucleotides at indices 46 and 51) in the base compactor changes to A-T in 3 out of 4 subsequent compactors. The only compactor without BPCs at these positions exhibits another pair of compensatory changes – A-T to G-C at indices 44 and 53. The extensive BPCs also make it possible to detect structures detectable in the target mode (**Figured 7B**). We also show examples of the statistical detection of structures of Gordis virus and Culex mosquito virus 4 by SVE (**Figures 7C** and **7D**). Although the detected structures are located near the anchor (within 27 bases

downstream), the lack of sequence variations causes them to be ignored in target mode. Variations occurring at the ends of the compactor sequences make these sequences be analyzed under compactor mode.

SPLASH-structure detects eukaryotic mRNA structures with statistical evidence of BPC or SVE across species in mosquito metatranscriptomics

Eukaryotic mRNAs have structure that is important for their function (Passmore and Coller, 2022, Biziaev et al., 2022). Because SPLASH-structure weights stem-loop covariation, and the genes encoding eukaryotic mRNAs are not expected to rapidly accumulate mutations (structurally compensatory or otherwise), we did not generally expect it to detect mRNA structures, unless multiple related species separated by large evolutionary distances were present. Interestingly, the Batson et al. dataset provided sequences from 10 *Aedes*, *Culex*, and *Culiseta* related mosquito species (Batson et al. 2021)**.** After running SPLASH-structure, 1132 out of 4718 (23.99%) base compactors of the significant anchors had BLASTn aligned to mRNA (**Figure 6C**; **Methods**). This finding supports the hypothesis that eukaryotic mRNAs with statistical evidence of BPC or SVE can be identified using SPLASH-structure when applied to datasets that contain mRNA sequences from multiple related but sufficiently divergent species.

SPLASH-structure detects novel sequences by statistically evident structures with BPC or SVE in mosquito metatranscriptomics

Unaligned (by BLASTn) base compactors suggest the presence of novel sequences that are not currently documented in the existing nucleotide database. SPLASH-structure identified them with statistically evident RNA structures. To investigate and interpret these unexplored sequences, we generated longer compactors (over 81 nucleotides) for the significant unaligned anchors (see **Methods**). In total, 669 compactors were created, with lengths ranging from 135 to 459 nucleotides (**Suppl. Figure 3**). Re-running BLASTn with these extended compactors led to the identification of sequences that aligned with Culex Bunyavirus 2 and Culex narnavirus 1 (**Figure 8A** and **8B**). This suggests that the previously unannotated anchors of these compactors may correspond to viral genomes that have not yet been documented, highlighting SPLASH-structure's ability to identify novel viral sequences with statistical evident structures.

For compactors that remained unaligned after the second round of BLASTn, we performed *in silico* translation and queried protein databases using BLASTx. We found sequences whose translated proteins matched uncharacterized proteins at various *loci* in mosquitos. This suggests that SPLASH-structure can potentially uncover novel sequences linked to proteins.

Discussion

Amid an explosion in available RNA-sequencing data, and an ongoing need to understand the RNA structurome across the tree of life, scalable sequence-based RNA structure prediction algorithms are needed. We developed a RNA structure prediction method, SPLASH-structure, that takes raw sequencing data, infers sequences in a reference-free manner using SPLASH, and then interrogates these sequences for predicted stem-loop RNA structures. In leveraging the ability of SPLASH to identify sequence variations, SPLASH-structure values base-pair covariations (BPC) and stem

variation exclusion (SVE) that appear to preserve predicted structures. As a result, biologically meaningful RNA structures are more likely to be identified. Unlike existing sequence-based RNA structure prediction approaches, SPLASH-structure does not rely on multiple sequence alignment (MSA), neither alignment nor assembly, and can be used to identify RNA structures from a single unknown sequencing sample. Furthermore, though structure prediction might be more accurately performed with long-read sequencing data, since most publicly available data consists of short reads, SPLASH-structure is advantageous as it effectively predicts structure from short-read datasets and provides critical statistics to address whether predicted structures are conserved.

We tested SPLASH-structure in isolated HIV-1 provirus sequencing and mosquito metatranscriptome datasetsWithout providing any information other than short-read sequencing data from HIV amplicons generated from patients' blood, SPLASH-structure identified several known HIV genome RNA secondary structures, including the frameshift signal, the psi packaging signal, and the TAR. SPLASH-structure did not pick up all of the predicted or known structures of HIV, but the top hits of SPLASH-structure were all RNA structures important for the HIV life cycle.

In RNA-sequenced whole mosquitoes we found that SPLASH-structure identified ribosomal subunit RNAs. SPLASH-structure also identified structured RNA sequences that, when queried against known sequences, were found to belong to RNA viruses. This finding highlights the unique power of SPLASH-structure in identifying RNA viruses in mixed or unknown sequencing samples. Furthermore, our algorithm identified several sequences that align to viruses not identified in the Batson et al. analysis. These new hits could be due to the longer query sequences generated by the compactor assembly function of SPLASH, as several were incompletely aligned. Intriguingly, these could actually represent related viruses not yet added to the NCBI reference sequence database and remain to be worked up. Indeed, reference genomes for Aedes vigilax sobemo-like virus 1 and Wuhan mosquito virus 4 became available after the Batson et al. analysis was published which may have prevented their identification.

Despite SPLASH-structure's effectiveness in identifying known structured RNAs, and generating hypotheses about new regions of RNA structure, we recognize the following limitations in its current implementation:

1. Bulges and conserved bulges: SPLASH-structure, adopting a SPLASH viewpoint, views sequencing data as anchor-target pairs to explore various target sequences linked to the same anchor. The method identifies the perfect reverse complement sequence within the most abundant target (base target) and counts several types of mismatches observed in different targets associated with the same anchor. This approach, primarily focused on the simplest RNA secondary structures such as stem-loops, may neglect the potential presence of bulges in double-helical structures.

2. Watson-Crick-Franklin base-pairing: In the probability formulations, SPLASH-structure adopts canonical Watson-Crick-Franklin base-pairing to identify potential stem-loops. While essential, Watson-Crick-Franklin base-pairing often receives excessive focus, overshadowing other crucial interactions in RNA structure, such as non-Watson-Crick-Franklin pairings that are vital for RNA three-dimensional structure and function. The overemphasis on Watson-Crick-Franklin base-pairing and the underemphasis on non-base-pairing interactions

in predicting RNA structures can lead to an incomplete understanding of RNA's structural and functional capabilities (Vicens and Kieft 2022).

3. Choice of sample: SPLASH-structure is best suited to sequencing data derived from organisms with structured RNA and rapidly-changing RNA, which emphasizes its use in detecting RNA viruses and suggesting structured elements in an unknown mixture. For example, though human polyadenylated mRNAs have secondary structure, mutations in the genomic DNA template occur very slowly, making compensatory mutations in stem-loop structures unlikely. Although SPLASH-structure identified some mRNA stem-loops when applied to metatranscriptomics dataset that contains mRNA sequences from related but sufficiently divergent mosquito species, we therefore recommend SPLASH-structure is best utilized in settings where the source RNA sequences are hypothesized to contain some variation, ideally 1) the presence of known or unknown RNA viruses or viroids, or 2) DNA-templated RNA from related organisms across evolutionary time (e.g., RNA-seq from related primates (or mosquitoes), or within a species separated by many generations).

We plan to address these limitations in future work. Ultimately, SPLASH-structure builds on the SPLASH statistical framework to 1) add RNA secondary structure detection, 2) add within-structure sequence covariation analysis, and 3) provide statistical parameters and sequence outputs for exploration of results, all in a reference sequence-agnostic manner. In doing so, it may help discover new biology hiding in sequencing datasets, especially the presence of RNA viruses in which secondary structures play an outsize role in the life cycle. Downstream, after identification and validation, targeting of RNA structures (including viral structures) with small molecules is an emerging human disease therapeutic area (Childs-Disney et al. 2022). We encourage the use of SPLASH-structure on RNA sequencing datasets, whether derived from known viral RNA inputs or from complex mixtures of organisms.

Methods

Generating SPLASH-significant anchors and their targets

The SPLASH algorithm was applied on the raw FASTQ sequences of each dataset to generate `anchors` and `targets` (both 27 nt long) (Chaung et al. 2023; Kokot et al. 2024). SPLASH version 2.1.15 was run with the following command, taking `samplesheet.csv` as an argument, which stores paths to FASTQ sequences:

```
$ splash --outname_prefix result --anchor_len 27 --gap_len 0
--target_len 27 --poly_ACGT_len 8 --max_pval_opt_for_Cjs 0.1
--n_threads_stage_1 4 --n_threads_stage_2 32 --n_bins 128
--n_most_freq_targets 10 --kmc_max_mem_GB 12 samplesheet.csv
```

The output file `results.after_correction.scores.tsv` contains SPLASH-significant anchors – anchors with the corrected *p*-values `pval_opt_corrected` less than 0.05 – and their top 10 targets, specified by `--n_most_freq_targets 10`. A lower `pval_opt_corrected` indicates

higher significance for an anchor. Note that derivation of the SPLASH's test statistic involves splitting data into train and test set, which introduces inherent randomness and can lead to false negatives.

Generating compactors from SPLASH-significant anchors

Compactors were generated using software “compactors”, a local assembly method based on a probabilistic model included in SPLASH version 2.1.15 (Henderson et al. 2024). The following command extends seed anchors from `anchor_list.txt`, queries the raw FASTQ files specified in `samplesheet.csv`, and outputs the compactors for seed anchors and their support (number of sequences that a compactor represents) to `compactors.tsv`. Compactors of each anchor are ordered by descending support descending. Compactors of 81 nucleotides in length are specified by `--num_kmers 2` and `--max_length 81`. For each anchor, up to 10 compactors were generated, as set by `--max_anchor_compactors 10`.

```
$ compactors --kmer_len 27 --num_kmers 2 --epsilon 0.05 --beta 0.4
--lower_bound 1 --max_mismatch 4 --poly_threshold 6 --max_length 81
--max_anchor_compactors 10 --num_threads 16 --reads_buffer_gb 48
samplesheet.csv anchor_list.txt compactors.tsv
```

In cases where we needed to extend the compactors to interpret the sequences, we adjusted the `--max_length` flag to 1000 to generate longer compactors, capping their length at 1000 nucleotides.

Removing contaminant sequences

We implemented filtering procedures on the anchors to eliminate genomic artifacts and PCR primers, ensuring that SPLASH-structure results were free from common sequencing contaminants. An internal pipeline was applied to efficiently index reference sequences and query every k-mer present in combined anchor and target sequences and compactors against these references. For each queried sequence, the output includes a comprehensive list of matching references of each k-mer. We set a threshold for the maximum allowable counts of a contaminant reference and filtered out sequences containing more hits than this threshold. Additionally, for the HIV datasets, sequences containing PCR primers used in a specialized PCR protocol were filtered out (Bruner et al. 2016). This is accomplished by using the command-line `grep -v` to exclude sequences that match the primers.

Filtering out low-abundance and low-variability sequences

As specified, up to the top 10 most abundant targets or compactors are generated for each anchor. Targets or compactors with less than 5% abundance relative to the generated sequences were discarded for better computation efficiency. Next, anchors with fewer than three varied targets or compactors, excluding the base target or base compactor, were also excluded from the analysis. Under compactor mode, we also filtered out anchors whose second most abundant compactor has only support 1, i.e. the sequence that it represents only shows up once in the dataset. This step ensured that only anchors with sufficient variability in their targets or compactors were considered. As a result, short stem-loops in highly conserved sequences may be intentionally overlooked under these settings.

SPLASH-structure (target mode)

1. Likelihood of observed sequence variations in a target: target *p*-value, p_{tar}

Consider the most abundant target of an anchor base target, T_0. We search for a stem-loop structure with a minimum stem length of 5 in T_0. The algorithm achieves this by selecting a 5-mer from the beginning of T_0 and searches for its reverse complement from the base immediately following the selected 5-mer, adhering to the canonical Watson-Crick-Franklin base-pairing rules of A to T and C to G. If no reverse complement is found downstream, the algorithm steps one base forward and repeats the search for the next 5-mer. If a reverse complement is located, the algorithm extends the current k-mer and its reverse complement by one base and verifies if the extended sequences remain complementary. This recurring process halts either when the extended sequences no longer match or they begin to overlap, whichever occurs first.

The length of the stem is L ($L \geq 5$). We then iterate all targets T_i ($i = 1, \cdots, K$, K is the number of targets of an anchor besides T_0), and calculate the number of single-nucleotide mismatches between T_0 and T_i, denoted as Hamming distance v. We then compute how the mismatches are distributed in the stem-loop.

Under the null hypothesis, with fixed target length k, stem length, L and number of mismatches v, we assume that mismatches occur uniformly are uniformly distributed across any v positions out of k in the target T_i. Furthermore, each mismatched base is independent of others, and transitions to any of the other three bases with equal probability (for example, A changes to C, G, or T with equal likelihood).

The alternative hypothesis proposes that a stem-loop structure is preserved in two mechanisms. The first mechanism preserves the stem-loop structure through mismatches that form compensatory mutations within the stems, termed BPC. The second mechanism considers that mismatches are selectively excluded from the stems, called stem conservation.

In the context of BPC, we define the conditional likelihood of having at least as many compensatory mutation pairs C as the observed value c, given the predetermined parameters target length k, stem length L, and number of mismatches v, as the first target *p*-value, denoted as $p_{tar}^{(1)}$,

$$\begin{aligned} p_{\text{tar}}^{(1)} &= \mathbb{P}(C \geq c \mid L, v, k) \\ &= \sum_{h=2c}^{\min(v,2L)} \mathbb{P}(C \geq c \mid L, h)\, \mathbb{P}(H = h \mid L, v, k). \end{aligned}$$

where the last line is allowed by the law of total probability. H denotes the variable representing the number of mismatches observed in stems, and h denotes the possible values that H can take on.

In the preceding equation, the second term can be modeled with a hypergeometric distribution,

$$\mathbb{P}(H = h \mid L, v, k) = \frac{\binom{2L}{h}\binom{k-2L}{v-h}}{\binom{k}{v}}.$$

The first term can be decomposed into the summation of discrete cases of g pairs of compensatory mutations existent in a target, and it can be further decomposed and computed in a closed form (see **Supplementary Methods**).

$$\mathbb{P}(C \geq c \mid L, h) = \sum_{g=c}^{\lfloor h/2 \rfloor} \mathbb{P}(g \mid L, h).$$

To have statistical power against another alternative, where mutations are absent in stem regions (SVE), we define another statistic, $p_{tar}^{(2)}$, representing the probability of v mismatches all occurring outside stems,

$$p_{\text{tar}}^{(2)} = \frac{\binom{k-2L}{v}}{\binom{k}{v} 3^v}.$$

The final likelihood, denoted p_{tar}, combines the two alternative hypotheses of the preservation mechanisms of stem-loops via an indicator function. The observed number of mismatches in stems, s, serves to distinguish between the two mutually exclusive cases: BPC ($s > 0$) and stem conservation ($s = 0$). Accordingly, we employ $p_{tar}^{(1)}$ in cases where mismatches are present in stems, $p_{tar}^{(2)}$ when mismatches are absent in stems. Formally, p_{tar} is given by

$$p_{\text{tar}} = \mathbf{1}_{(s>0)} p_{\text{tar}}^{(1)} + \mathbf{1}_{(s=0)} p_{\text{tar}}^{(2)}$$

which follows a uniform distribution, in a discrete sense. The exact distribution of p_{tar} can be computed as there is a limited number of possible combinations for the locations and types of mutations in a target.

2. Test statistics, anchor score R

To capture the likelihood of preserving a stem-loop structure among varied targets, we assign a single statistic to an anchor, termed anchor score, R. We define R as a linear combination of each target p_{tar} weighted by their abundance. Formally, consider an anchor with K targets $T_1, \ldots, T_k$. Define the weight of the i-th target, w_i (where $i = 1, \cdots, K$), as the proportion of counts of that target among all K targets. Represent the weights of the K targets with the vector $w = (w_1, \cdots, w_K)^{\top}$ with $\sum_{i=1}^{K} w_i = 1$, and the corresponding p_{tar} as $p = (p_{tar_1}, \cdots, p_{tar_K})^{\top}$. Then the anchor score R is

$$R = p \cdot w$$

3. *p*-value

To obtain a *p*-value for the test that measures the global conservation of stem-loops, we need the null distribution of the test statistic, anchor score, R. Since R is the weighted mean of K discrete uniformly distributed random variables, p_{tar}, the distribution of random variable R can be derived using convolution of discrete variables. For example, $K = 2$, we expand R,

$$R = \underbrace{p_{\mathrm{tar}_1} w_1}_{X} + \underbrace{p_{\mathrm{tar}_2} w_2}_{Y},\ X \perp\!\!\!\perp Y$$

where w_1 and w_2 are constants and p_{tar_1} and p_{tar_2} are PMF of known distributions. Define two independent random variables, $X = p_{tar_1} w_1$ and $Y = p_{tar_2} w_2$, supported on R_X and R_Y respectively, with PMF $p_X(x)$ and $p_Y(y)$. The distribution of anchor score S can then be derived via convolutions of the two independent random variables, X and Y, i.e.,

$$p_R(r) = \sum_{x \in R_X} p_X(x) p_Y(r - x),$$

where r is the observed value of the anchor score variable R. Now a *p*-value is computed as the lower-tail probability,

$$p\text{-value} = \mathbb{P}(R \leq r).$$

Since we conduct a hypothesis test and compute a *p*-value for each anchor, to control false discovery rate (FDR), we apply Benjamini-Hochberg procedure over the list of *p*-values from the multiple hypothesis tests. We report the list of corrected *p*-value through Python functionality `statsmodels.stats.multitest.multipletests` with FDR controlled at $\alpha = .05$.

Simulation of anchor score, R

To study the distribution of test statistics (anchor score, R) across multiple samples under the null hypothesis, and to validate the BH-adjusted *p*-value computation, we performed simulations of anchor score under null and compared the empirical cumulative density function (ECDF) of *p*-values with the empirical data.

The simulation procedure is as follows. Given a pre-processed dataset containing anchors, each anchor has a base target T_0 (a stem-loop was detected in T_0), and targets T_i ($i = 1, \cdots, K$). For each anchor, we have also computed targets weights $w = (w_1, \cdots, w_K)^\top$ and tallied the number of mismatches in each target $v = (v_1, \cdots, v_K)^\top$. Subsequently, for each anchor, we simulated K targets. The i-th simulated target was generated by randomly selecting v_i positions in the base target, T_0, and replacing the nucleotide at those positions with one of the other three nucleotide types, each with equal probability. We then computed a conditional likelihood for each simulated target given fixed stem length L, number of mismatches v and target length k, same as the computation of p_{tar}. Next, we performed the same process to compute the simulated test statistics, anchor score, R and *p*-value for each anchor in the given dataset (**Suppl. Figure 4A**).

We conducted this simulation on the pre-processed mosquito metatranscriptomics dataset, repeating the process 1000 times. The empirical cumulative distribution function (ECDF) of the simulated *p*-values was then plotted and compared with the empirical data (**Suppl. Figure 4B**).

SPLASH-structure (compactor mode)

The compactor mode of SPLASH-structure operates on compactors generated from SPLASH-significant anchors. Anchors are included as the first 27-mer of these compactors, and the 54-mers that follow are referred to as trimmed compactors. Define the trimmed compactor of the most supported compactor as the base trimmed compactor.

1. Split-and-concatenate method to process compactors

SPLASH-structure adopts a split-and-concatenate method to process trimmed compactors, in order to incorporate longer and more complex structures in our predictions. We split a 54nt long trimmed compactor into four almost equal-length sub-sequences of 14, 14, 13 and 13 nt long, following index slicing: [0:14), [14:28), [28:41), [41:54). These sub-sequences are designated as $D^{(1)}$, $D^{(2)}$, $D^{(3)}$ and $D^{(4)}$. The sub-sequences are then concatenated into two recombined sequences in an order-preserving manner, ensuring that sub-sequences with smaller indices precede those with larger indices. As a result, we have following three ways to obtain two recombined sequences:

- $D^{(1)}$ – $D^{(2)}$: 28nt, $D^{(3)}$ – $D^{(4)}$: 26nt
- $D^{(1)}$ – $D^{(3)}$: 27nt, $D^{(2)}$ – $D^{(4)}$: 27nt
- $D^{(1)}$ – $D^{(4)}$: 28nt, $D^{(2)}$ – $D^{(3)}$: 27nt

Such split-and-concatenate methods can be expanded by dividing a long sequence into more than four sub-sequences. This increases the number of possible ways to reassemble the sequences, allowing for the detection of structures of more possibilities. For example, split a trimmed compactor into 8 nearly equal-length sub-sequences. Concatenating any two sub-sequences with their orders preserved will result in $\binom{7}{1}\binom{5}{1}\binom{3}{1}$= 105 combinations.

2. Computing anchor score under compactor mode

Under each way of split-and-concatenate, each trimmed compactor turns into two recombined sequences as described in the previous section, and we try to find a stem-loop within each of them. If no stem-loop is found in any of the two, this split-and-concatenate way is discarded for the currently evaluated anchor.

We treat compactors under each split-and-concatenate way as targets, allowing all established algorithms for the target mode to be directly applied to compactors. To map the treated compactors to our target framework, we separately compare the two recombined sequences of a trimmed compactor to their corresponding indices in the base trimmed compactor and compute the parameters necessary computing p_{tar}, including stem length L_j, number of mismatches v_j, number of compensatory mutation pairs c_j, and number of mutations in the stem regions s_j, where j denote the specific recombined sequence ($j = 1\ or\ 2$).

Next, we compute the likelihood of the observed variations in compactors as p_{tar}, using the aggregated parameters: total stem length ($L = L_1 + L_2$), total number of mismatches ($v = v_1 + v_2$), number of compensatory mutation pairs ($c = c_1 + c_2$), number of mutations in the stem regions (

$s = s_1 + s_2$), and the combined lengths of k-mers ($k = k_1 + k_2 = 54$). Finally, anchor score R and *p*-value for compactor mode are computed analogously as they are for targets.

p-value distribution from datasets

We ran SPLASH-structure on a mosquito metatranscriptomics dataset and seven HIV Hosts' provirus sequencing data (Table 1). The empirical distributions of SPLASH-structure BH-adjusted *p*-values of anchors validates that SPLASH-structure assigns significance to sequences containing conserved stem-loops, either through BPC or SVE (**Suppl. Figure 5**). It also validates that SPLASH-structure effectively identifies significant sequences of structural RNA, including rRNA or RNA viruses within real sequencing data.

Datasets and software included in the study

This study analyzes existing publicly available data, listed in Table 1 with accession numbers. Software and algorithms used in this study are also provided in Table 1.

Query of structure match in the Rfam database

We conducted a query of structure matches to the Rfam database for compactors (from compactor mode), and combined anchor and target sequences (from target mode) from the HIV datasets and the mosquito metatranscriptomics dataset. The sequences were saved to FASTA files. The search utilized software Infernal (version 1.1.4), which implements covariance modes (CMs) to search for similar RNA structure in the Rfam database.

```
$ cmscan -Z $TOTAL_RESIDUAL --notextw --rfam --cut_ga --nohmmonly
--cpu 32 --tblout <OUTPUT_PATH> --fmt 2 --clanin Rfam.clanin Rfam.cm
<FASTA_PATH>
```

Exploratory sequence querying strategy for mosquito metatranscriptomics dataset

We ran remote BLASTn to query base compactors of structure-significant anchors against the nucleotide database `nt`. The queried sequences were saved to FASTA files. BLASTn (remote) was run using the following command (E-value threshold: 1E-3, word size: 11):

```
$ blastn -remote -db nt -query <FASTA_PATH> -evalue 1e-3 -out
<OUTPUT_PATH> -task blastn -outfmt 6 -max_target_seqs 4 -dust no -word_size
11
```

The sequence hit is chosen based on the lowest E-value. Species are determined using the hit's accession number and categorized based on their superkingdom (for bacteria and viruses) or phylum (for eukaryote). The categories include arthropoda, bacteria, chordata, viruses, fungi, plants, other invertebrates, euglenozoa, and others. BLASTn hits were also classified by RNA – rRNA, mRNA, RNA virus and others – according to the subject sequence title to which a query sequence aligns.

For base compactors that did not return any BLASTn hit from the previous query, we generated longer compactors (length longer than 81 nts) for these anchors. In the result outputs, compactors of length 81 are excluded and the rest are named long compactors.

We then did a second round of sequence querying against the `nt` database for long compactors using BLASTn. For sequences that still don't have any hits, we ran BLASTx that translates the nucleotide query sequence in six reading frames, resulting in six protein sequences. These translated sequences are searched against the nr database, with combined statistics across the six frames to assess statistical significance. The BLASTx command:

```
$ blastx -remote -db nr -query <FASTA_PATH> -evalue 1e-3 -out
<OUTPUT_PATH> -task blastx -outfmt 6 -max_target_seqs 6 -word_size 6
```

Acknowledgements

We thank members of Salzman lab for feedback and discussions throughout the project. We thank Ivan Nikolay Zheludev for helpful discussions, valuable comments on methods and results, and for reviewing the manuscript. We thank George Henderson for code prototyping and discussion of derivation of statistics. We thank Erin Modecai for the feedback on the manuscript. This work was supported by K08 AI163369 (A.R.) and R35GM139517 (J.S.).

Figures

Figure 1 SPLASH-structure vs other RNA detection methods. (A) Compared with existing methods, SPLASH-structure detects structures based on the sequence variation from SPLASH (a reference-free, statistical genomic algorithm) outputs with rigorous statistics, and post-hoc interpretations include mapping to Rfam database and BLAST to nucleotide database. **(B)** SPLASH-structure is independent of assembly and MSA, which is computationally intensive and is not statistically tractable.

Figure 2 SPLASH-structure Procedures. (A) The SPLASH-structure process consists of three key steps. In the first step, the SPLASH algorithm identifies fixed sequences, or anchors, that are followed by statistically significant sequence variations (targets). In the toy example, an anchor of length 16 (highlighted in blue) is followed by multiple targets (highlighted in yellow, pink, and green) of the same length, offset by 1 base. The second step involves identifying a stem-loop structure (illustrated with a *forna* RNA structure drawing) in the base target T_0 and computing parameters (*c*, *v*, *s*) for the targets T_1 and T_2. These parameters are then used in the third step - statistical tests for detecting conserved structures (see Methods). SPLASH-structure prioritizes conserved structures that exhibit sequence variation across different samples or over time. **(B)** Toy example of computation of SPLASH-structure statistics. Stems in T_0 are depicted as light gray boxes, with arrows indicating reverse complementarity. In the rest targets, SPLASH-structure finds conserved base-pairs (cartoon boxes in red) and tallies parameters *v*, *s*, and *c* (described in Figure 2A). For each target, SPLASH-structure tests the sequence for BPC or SVE, based on the number of variations in stems (*s*). The test statistic, anchor score is computed as the weighted average of these values and SPLASH-structure reports a closed-formed *p*-value.

Figure 3 SPLASH-structure applied to compactors. (A) SPLASH-structure adopts a split-and-concatenate method for analyzing compactors. The process breaks a compactor into four

distinct subsequences (colored by blue, yellow, green, and red), and pairwise concatenates any two of these sub-sequences together. Three ways of concatenation are depicted in green, orange and blue curves. **(B)** Toy example of 3 ways of split-and-concatenation of compactors. Each way results in two composite sequences. SPLASH-structure aims to identify a stem-loop in each composite sequence. Thus, with three options of concatenations, compactors can be used to model more complex RNA structures: double stem-loops, stem-loops with large loop regions or stem-loops with a bulge. *forna* RNA drawing of such structures are given for each case.

Figure 4: Detected HIV structural elements. **(A) - (C)** show *forna* drawings of the detected structures that match HIV ribosomal frameshift element, HIV Psi packaging signal SL3 and HIV TAR, respectively, accompanied with the secondary structure in Rfam, *p*-value and counts of targets or compactors in samples. In *forna* drawings, nucleotides within the stems are colored in green and those outside stems are in blue. **(A)** An example of the HIV frameshift element detected by SPLASH-structure (target mode), conserved through SVE. The leftmost structure drawing is the base target. The rest to the right of the base target are varied targets. **(B)** An example of the HIV Psi packaging signal SL3 detected by SPLASH-structure (compactor mode), conserved through SVE. The *forna* drawing is the base compactor. The aligned nucleotide sequences of varied compactor sequences are displayed. Capital letters and green highlights denote unvaried nucleotides in stems. Contrived bases are in red text color with orange highlight, and all other variations are shaded in gray. **(C)** Similar to (B) an example of the HIV TAR detected by SPLASH-structure (compactor mode), conserved through SVE in T_1 and T_2, and BPC in T_3 and T_4.

Figure 5: Detected rRNA structures in Mosquito Metatranscriptomics dataset. (A) An example of the detected structures that match 23S rRNA by SPLASH-structure (compactor mode), conserved through both SVE and BPCs across targets. **(B)** An example of the detected structures that match 28S rRNA by SPLASH-structure (compactor mode), conserved through BPCs. The zoom-ins show the contrived base pairs in the varied sequence of the detected stem-loop.

Figure 6: BLASTN hits of base compactors of the structure-significant anchors of analyzed mosquito metatranscriptomics. (A) Each point represents a unique structure-significant base compactor detected by SPLASH-structure (compactor mode), with colors indicating taxonomy obtained from the BLASTn hit with the lowest E-value. The plot does not show the relative abundance of compactors across samples. Query coverage refers to the percentage of the query sequence aligned with the subject sequence. Percentage identity indicates the proportion of identical nucleotides in the aligned region. **(B)** Each point represents a distinct structure-significant base compactor aligned to viral genomes using BLASTn. These points correspond to the viral taxonomy group “Viruses” shown in Figure A. Sequence that aligned with Wuhan Mosquito Virus 4 has low coverage but 100% similarity. **(C)** Each point represents a unique structure-significant base compactor under SPLASH-structure (compactor mode), as in the previous plots, but points are categorized by RNA type. BLASTn hits with the smallest E-value are used for labeling. Query coverage and percentage identity are defined as in the previous plot.

Figure 7: Detected RNA virus structures in Mosquito Metatranscriptomics dataset. (A) - (B) show *forna* drawings of detected structures that are mapped to the Culex narnavirus 1, separately identified under compactor mode and target mode of SPLASH-structure, accompanied with *p*-value and counts of targets or compactors in samples. The structure detected in compactor mode is

conserved through BPC. The structure detected in target mode is conserved through SVE in T_1, and BPC in T_2 and T_3. **(C)** SPLASH-structure (compactor mode) detected Gordis virus via structure conserved through SVE. The *forna* drawing is the base compactor. The aligned nucleotide sequences of varied compactor sequences are displayed. Capital letters and green highlights denote unvaried nucleotides in stems. Conserved bases are in red text color with orange highlight, and all other variations are shaded in gray. We chose to show a stem with a length of 4 nucleotides and a tetraloop, rather than the 5-nucleotide stem with a 2-nucleotide loop detected by the algorithm, because tetraloop sequences are more stable and avoid the increased strain from with the sharp bend in a 2-nucleotide loop. **(D)** SPLASH-structure (compactor mode) detected Culex mosquito virus 4 via structure conserved through SVE. The color scheme convention is the same as in (C).

Figure 8: Alignment between extended compactors and RNA virus. (A) An extended compactor of 135 nt of an unaligned 81nt compactor by BLASTn aligns with a subject sequence of Culex Bunyanirus 2. The alignment between the subject sequence (highlighted in blue) and the extended long compactor (highlighted in light purple) is shown, along with BLASTn E-value, identities and coverage. The anchor sequence, marked with orange whiskers, and the compactor where SPLASH-structure searches for stem-loops, marked in blue, are indicated. Nucleotides in the detected stems are colored green and shown in uppercase. **(B)** Similar to (A), using the same color scheme and marker notation, an extended compactor of 135 nt from an unaligned 81nt compactor, aligns with a subject sequence from Culex narnavirus 1.

Suppl. Figure 1: SPLASH-structure (compactor mode) workflow and compactor generations. (A) The raw sequencing data in FASTQ format is initially analyzed by SPLASH, generating an output file significant_anchors.csv that contains the SPLASH-significant anchors and their targets. Compactors are then constructed by using the SPLASH-significant anchors as seeds to query the original FASTQ file. The targets and compactors derived from the SPLASH-significant anchors are subsequently used as inputs for the two modes of SPLASH-Structure: Target Mode and Compactor Mode, respectively. Dotted lines represent file dependencies between the processes. **(B)** Compactors are local anchor-seeded assemblies of regions that vary across samples. They are generated iteratively based on a probabilistic model.

Suppl. Figure 2: Toy example of SPLASH-structure (compactor mode) detecting a pseudoknot. In principle, SPLASH-structure can detect pseudoknots in sequences. Toy example demonstrates that a trimmed compactor adopts the second way of split-and-concatenation as illustrated in Figure 3. In the resulting two composite sequences, compensatory stems are identified in both. A *forna* drawing of the detected pseudoknot is provided, with nucleotides in stems highlighted in green circles and pseudoknot links represented by gray straight lines.

Suppl. Figure 3: Length distribution of extended compactors over 81 nts. To interpret unannotated base compactors (81nt) using BLASTn, longer compactors with lengths capped at 1000 nucleotides are generated for the anchors of these base compactors. The length distribution of these compactors is visualized in the bar plot.

Suppl. Figure 4: Comparison of *p*-value distribution of SPLASH-structure ran on empirical and simulated data. **(A)** A toy example illustrating the simulation procedure of test statistic, anchor score, in SPLASH-structure (target mode). The process begins with an anchor and its associated targets,

treating each as an anchor-targets simulation unit. The real dataset consists of anchors for which a stem-loop structure is detected in their base targets. For each anchor-targets simulation unit, nucleotides are randomly mutated at a number of positions in each target. In the example, the anchor has three targets, shown in the orange-shaded box. The green squares represent the stem locations, and the number of mismatches (*v*) in each target comes from the real data. For simulation, the corresponding *v* nucleotide positions in each target are randomly mutated (denoted as gray squares in the blue-shaded box), and a simulated *p*-value is computed. This process is repeated 1000 times for all anchor-targets units in the dataset. **(B)** ECDFs of *p*-values (unadjusted) obtained from real data (SPLASH-structure output of mosquito metatranscriptomics dataset, Batson, et al.) and 1000 simulated dataset are presented. The empirical cumulative distribution functions (ECDFs) show unadjusted *p*-values obtained from the SPLASH-structure output of mosquito metatranscriptomics dataset (Batson et al.) alongside 1000 simulated datasets. Each data point corresponds to an anchor where a stem-loop is detected in the base compactor. The zoomed-in view highlights the enrichment of small *p*-values in the empirical dataset, indicating that SPLASH-structure effectively identifies conserved stem-loops in RNA sequences.

Suppl. Figure 5: ECDF of BH-adjusted *p*-values in Mosquito Metatranscriptomics and HIV. **(A) and (B)** show ECDF of adjusted *p*-values stratified across various RNA sequence categories obtained from different datasets using SPLASH-structure on targets and compactors. For targets, each data point in the ECDF represents an anchor's adjusted *p*-value. For compactors, each data point in the ECDF represents an anchor's *p*-value for a way of split-and-concatenation of compactors. **(A)** For mosquito metatranscriptomics, all extendors (combined anchor and target sequences) and compactors undergo alignment with the Rfam database and a variety of viral genomes. Sequences that do not correspond with known rRNA sequences or match RNA virus genomes are categorized as "Unannotated RNA." Sometimes different extendors (or compactors) linked to a single anchor get different annotations; for instance, if four out of five extendors associated with one anchor are identified as rRNA while one remains unannotated, we apply a majority rule approach for labeling the anchor, in this case as rRNA. The sequences are also classified based on the type of structural variation they exhibit, which is detailed in Figure 1C. The categories include BPC, SVE, and sequences with "Neither" (noting no conservation nor covariation in their stem regions). Similarly, the label for each anchor is determined by the majority annotation of its data points. **(B)** In the dataset of HIV, all extendors and compactors are aligned to the Rfam database. Sequences are classified by whether it aligns to a HIV structural element and by mechanisms of conserved structures. We employed the same majority rule to label anchors.

Tables

Table 1 Dataset and software

RESOURCE	SOURCE	IDENTIFIER
Public data		
HIV-1	(Antar et al. 2020)	BioProject accession: PRJNA613107

PCR primers for HIV-1 provirus extraction	(Bruner et al. 2016)	
Mosquito metatranscriptomics	(Batson et al. 2021)	BioProject accession: PRJNA605178
Rfam database	(Kalvari et al. 2021)	https://rfam.org/
Software and algorithms		
SPLASH-structure	This study	
SPLASH (v2.1.15)	(Chaung et al. 2023; Kokot et al. 2024)	https://github.com/refresh-bio/SPLASH
SPLASH: compactors (v2.1.15)	(Henderson et al. 2024)	https://github.com/refresh-bio/SPLASH/blob/master/COMPACTORS.md
BLAST	(Camacho et al. 2009)	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Infernal (v1.1.4)	(Nawrocki and Eddy 2013)	http://eddylab.org/infernal/
forna	(Kerpedjiev, Hammer, and Hofacker 2015)	http://rna.tbi.univie.ac.at/forna/

Suppl. Files

Suppl. File 1 Supplementary Methods

Suppl. File 2 Supplementary Tables

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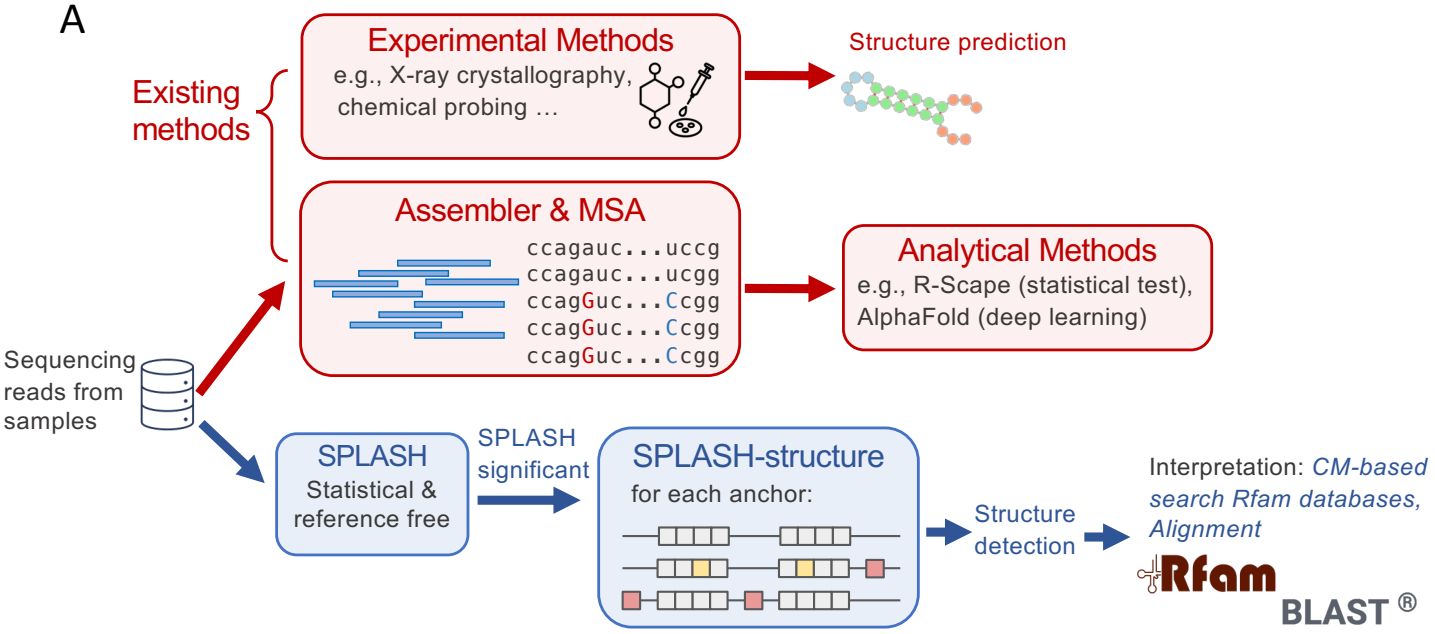
Figure 1

A

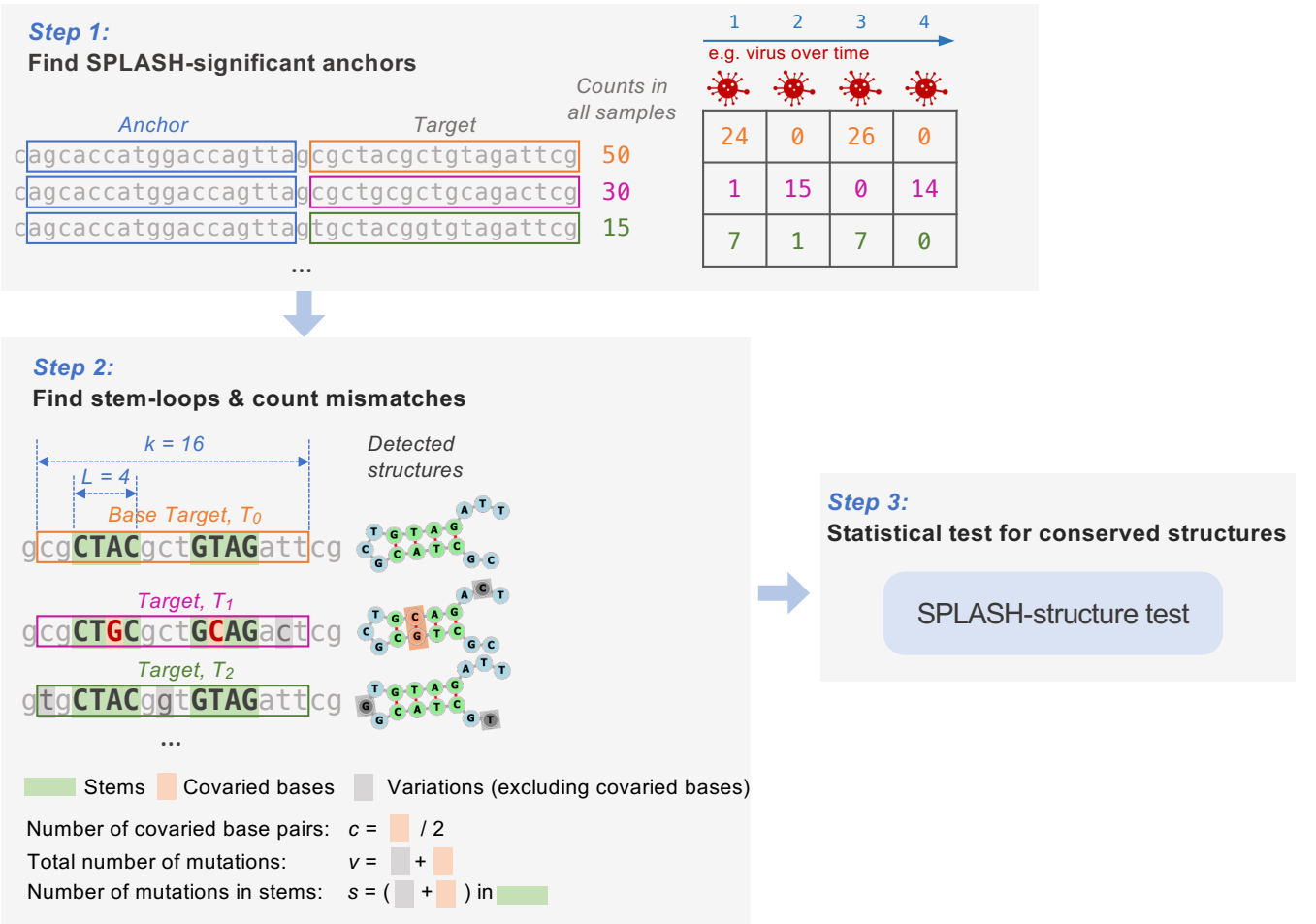


B

	Scale up to large sequencing data	Statistical significance testing	Independent of multiple sequence alignment (MSA) ccagauc...uccg ccagauc...ucgg ccagGuc...Ccgg ccagGuc...Ccgg	Time saving / computation-efficient
Experimental methods (e.g., x-ray crystallography, chemical probing)	✗	✗	✓	✗
Analytical methods (e.g. R-scape)	✓	✓	✗	✗
SPLASH-structure	✓	✓	✓	✓

Figure 2

A SPLASH-structure Concept



B SPLASH-structure test statistic and p-value

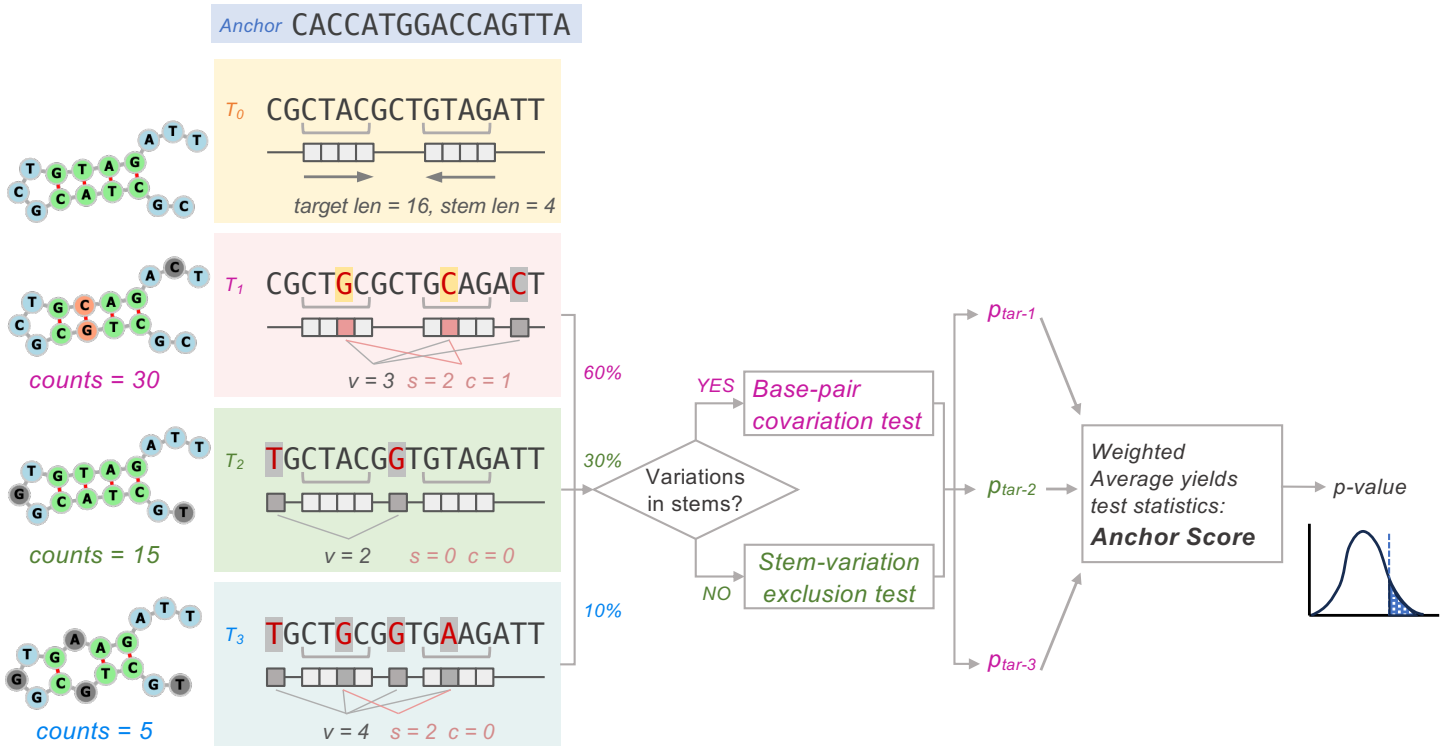
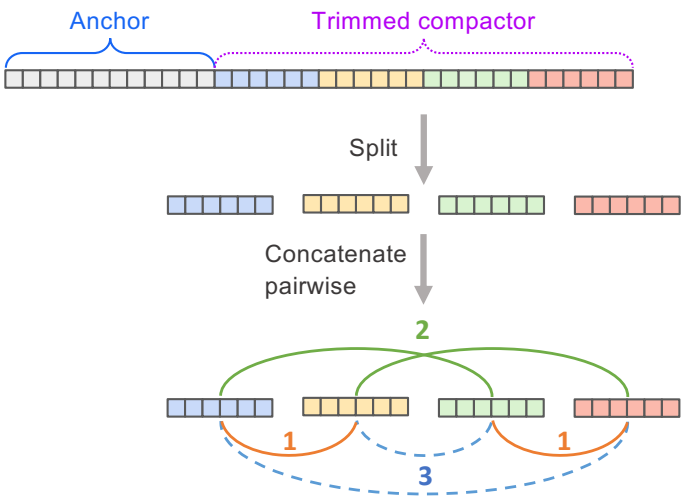


Figure 3

A Split-and-concatenate concept



B Toy example of SPLASH-structure compactor mode

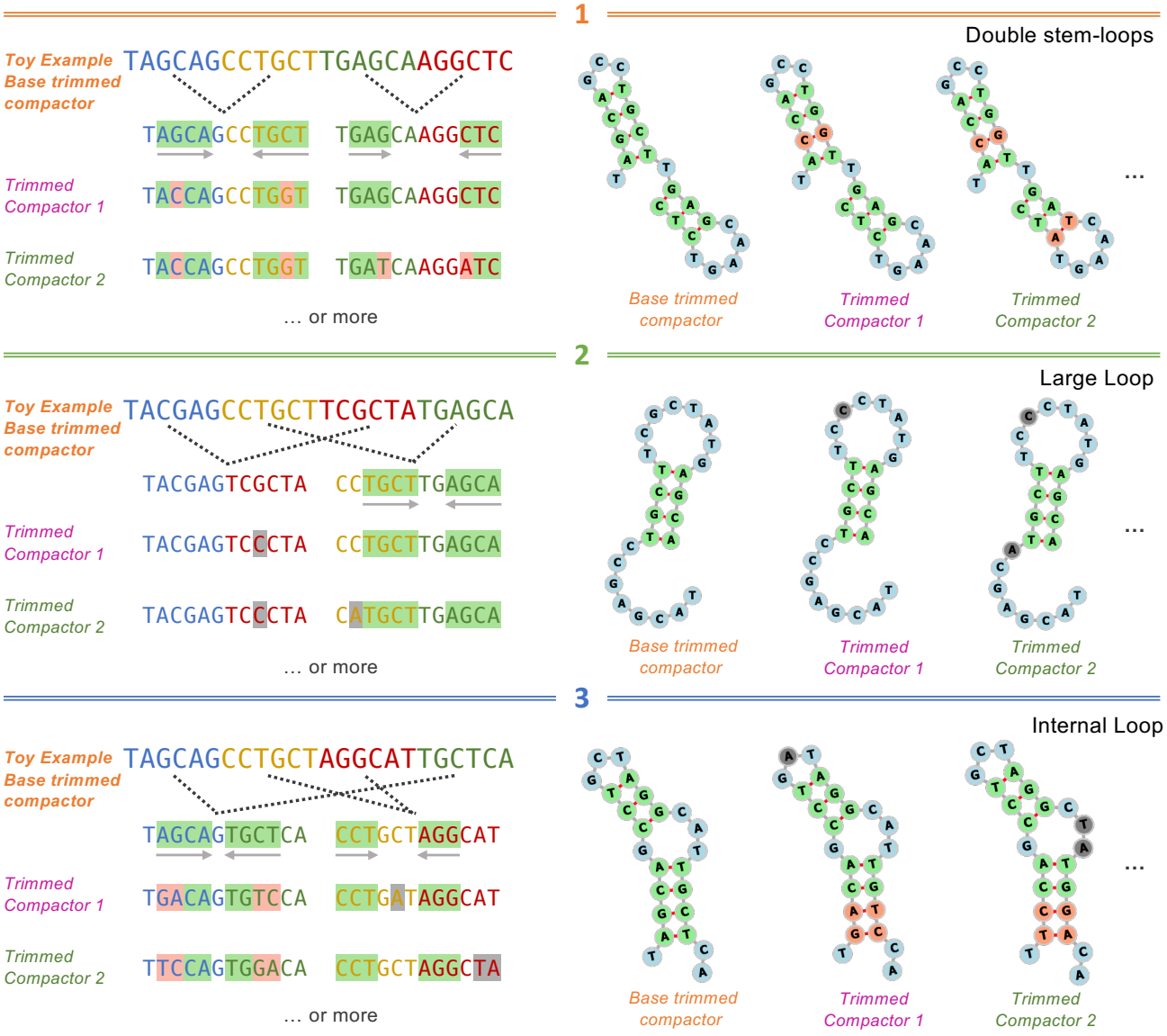
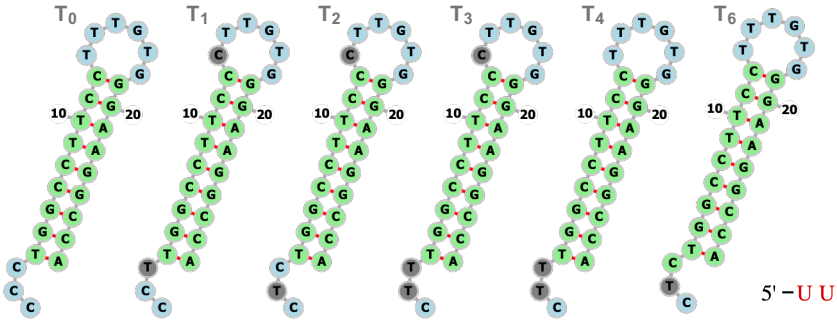


Figure 4

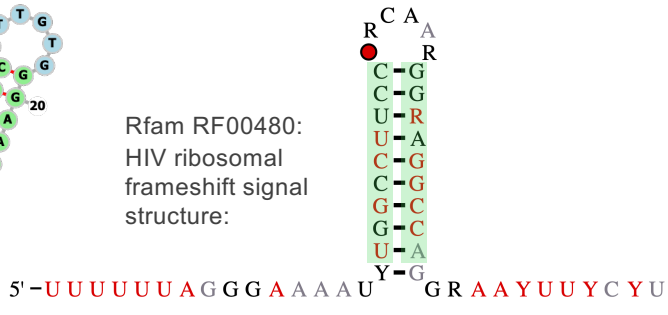
A Statistical detection of HIV ribosomal frameshift element within Host 1124 (Target mode)



SVE

BH-adjusted p-value = 0.018 < 0.05

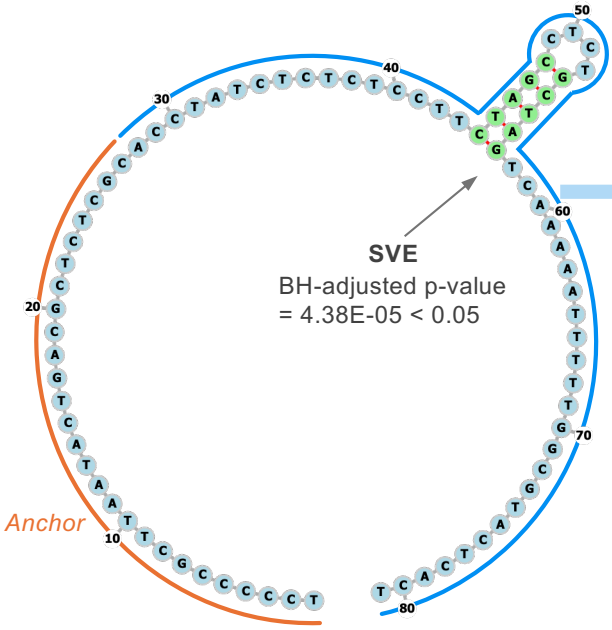
Rfam RF00480: HIV ribosomal frameshift signal structure:



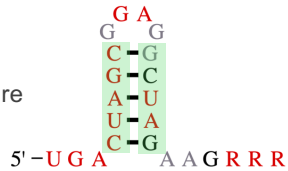
Counts within Host 1124

T_0	T_1	T_2	T_3	T_4	T_5
77	74	58	54	26	20

B Statistical detection of HIV Psi packaging signal SL3 within Host 746 (Compactor mode)



Rfam RF01381: SL3 structure

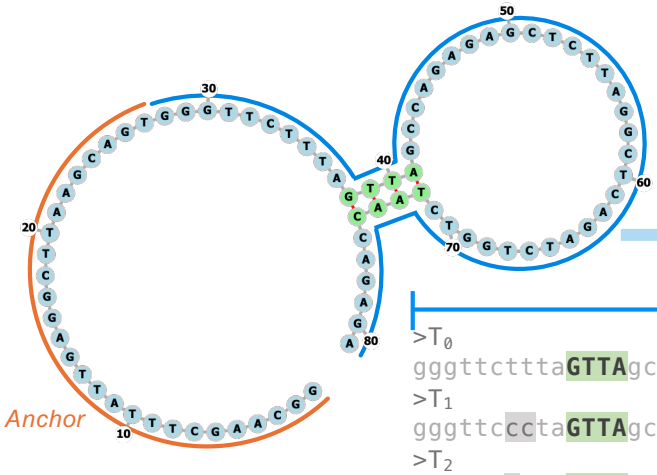


Counts within Host 746

T_0	T_1	T_2	T_3	T_4
7	4	4	2	1

```
>T0
acctatctctctccttCTAGCctctGCTAGtcaaaaattttggcgtactcact
>T1
acctatctctctcctgCTAGCttctGCTAGtcaaaaatttcagcgtactcactt
>T2
acctatctctctccttCTAGCctctGCTAGtcaaaatttcagcgtactcacct
>T3
acccatctctctccttCTAGCctccGCTAGtcaaaaatttcagcgtactcactt
>T4
acccatctctctccttCTAGCctccGCTAGtcaaaaagttcggcgtctgtctct
```

C Statistical detection of HIV TAR within Host 1124 (Compactor mode)

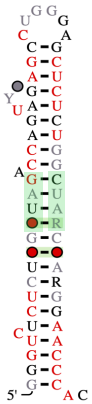


Counts within Host 1124

T_0	T_1	T_2	T_3	T_4
62	55	31	28	22

BH-adjusted p-value = 0.037 < 0.05

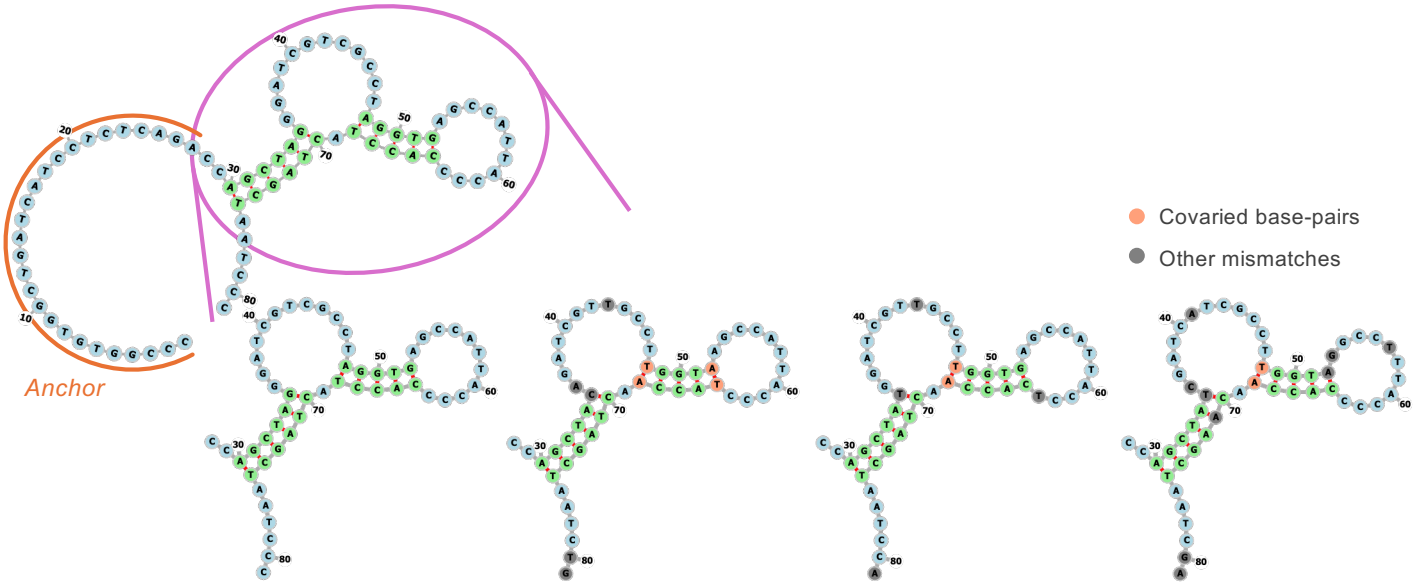
Rfam RF00250: TAR structure



```
>T0
gggttctttaGTTAgccagagagctcttaggctcagatctggtcTAACcagaga
>T1
gggttcccctaGTTAgccagagagctcccaggctcagatctggtcTAACcagaga (SVE)
>T2
gggttccttaGTTAgccagagagctccctaggctcagatctggtcTAACtagaga (SVE)
>T3
gggttccttgATcctcgcctactaacaggcggccttaaccgtagcAcTggtgaa (BPC)
>T4
ggactaatggGAAAatttaaagtgcaaccaatctgagtcaacagaTTtcttcca (BPC)
```

Figure 5

A Statistical detection of 16S rRNA (Compactor mode)



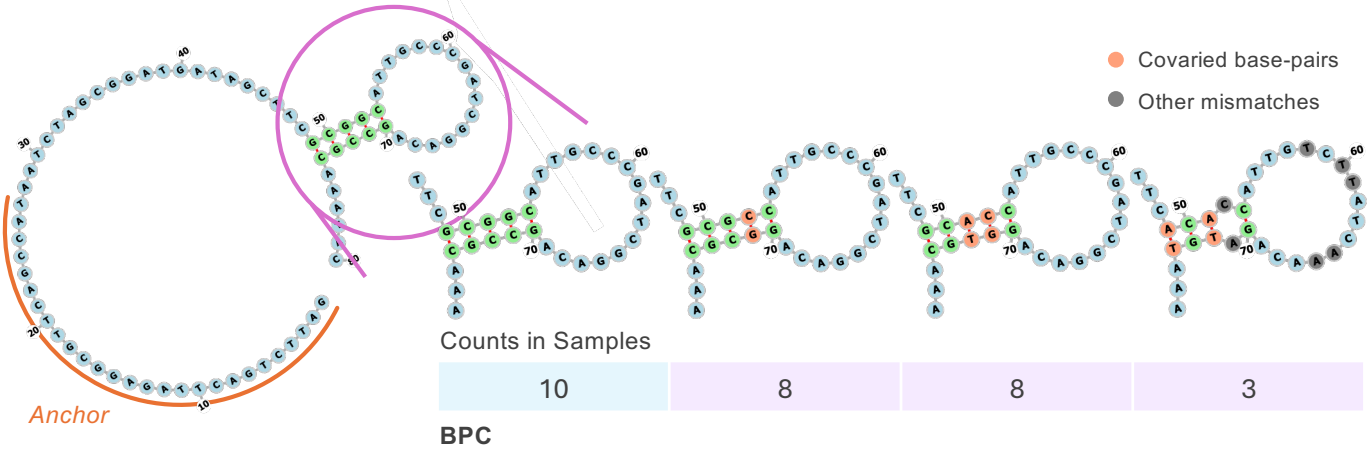
Counts in Samples

45	10	10	4

BPC

BH-adjusted p-value = 0.0028 < 0.05

B Statistical detection of 28S rRNA (Compactor mode)



Counts in Samples

10	8	8	3

BPC

BH-adjusted p-value = 3.38E-06 < 0.05

Figure 6

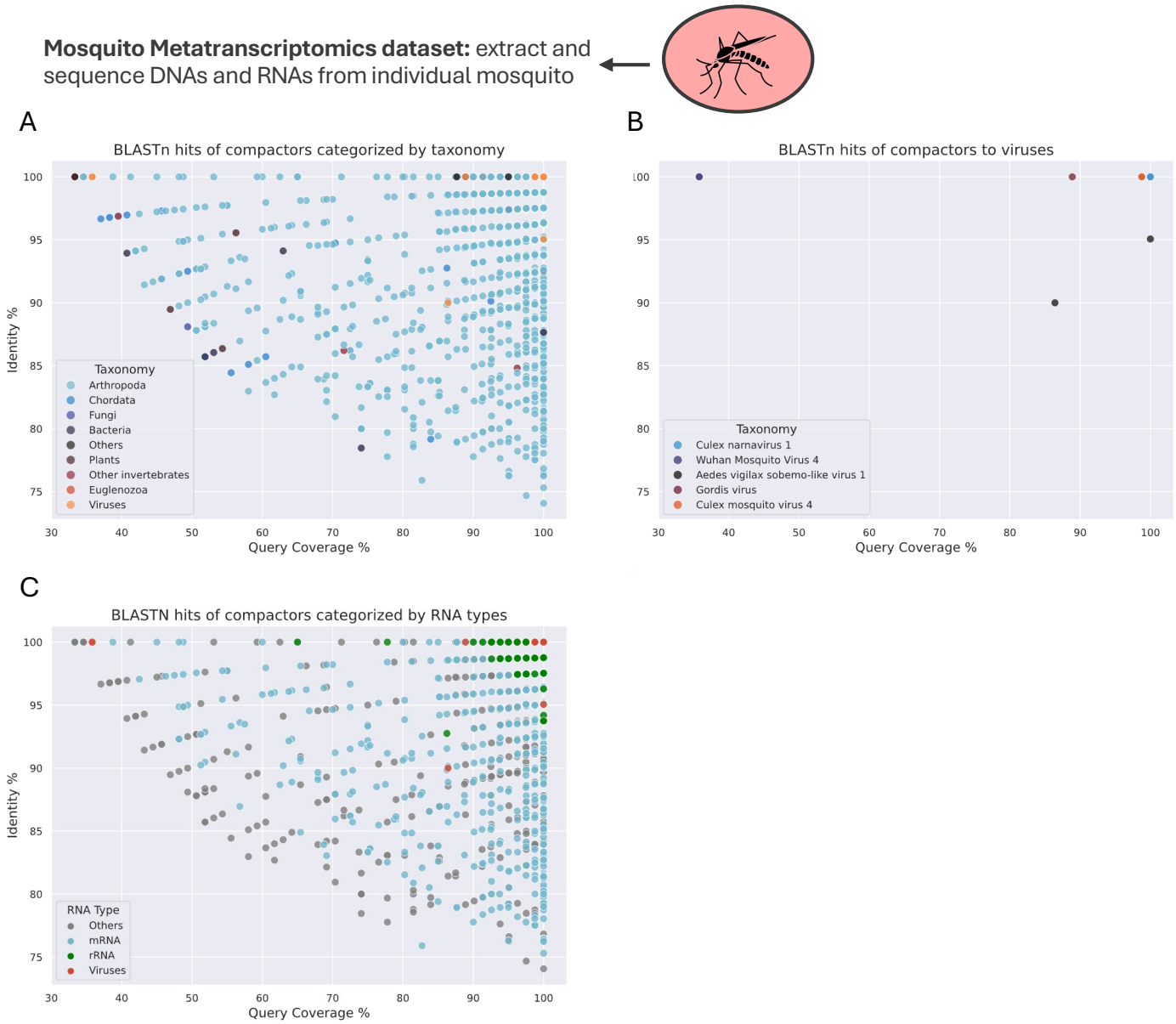
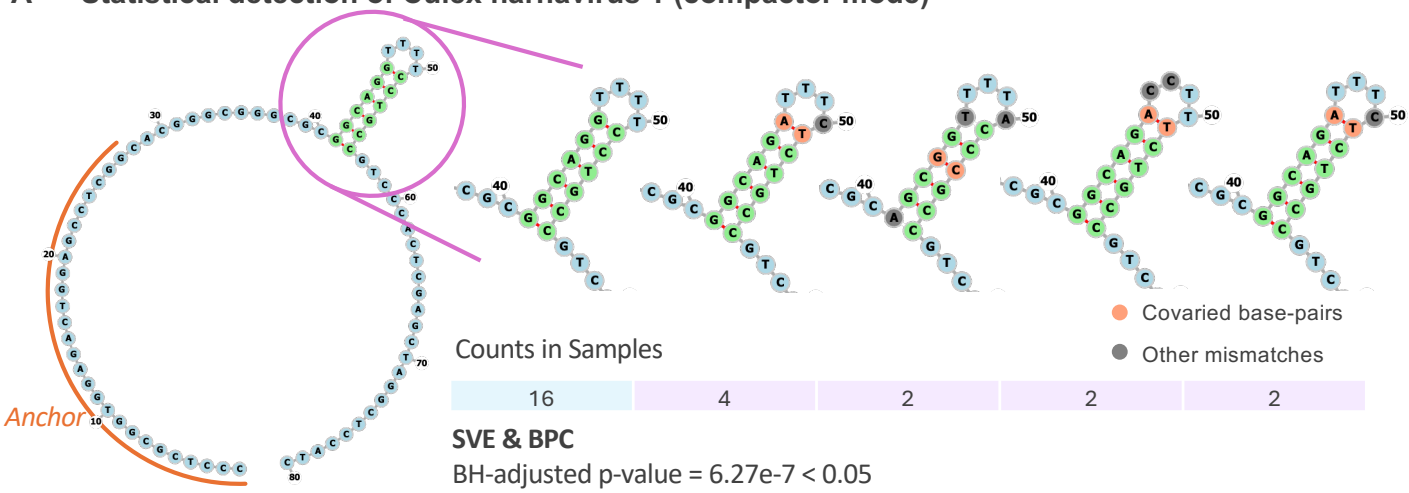
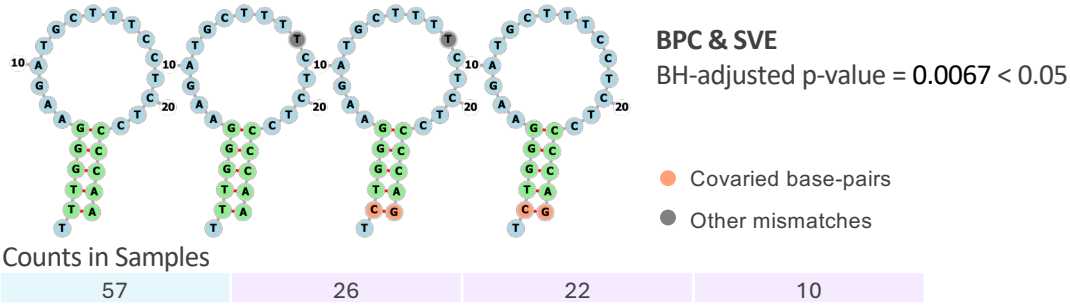

Mosquito Metatranscriptomics dataset: extract and sequence DNAs and RNAs from individual mosquito
A
BLASTn hits of compactors categorized by taxonomy
Identity %
Query Coverage %
Taxonomy
Arthropoda
Chordata
Fungi
Bacteria
Others
Plants
Other invertebrates
Euglenozoa
Viruses
B
BLASTn hits of compactors to viruses
Identity %
Query Coverage %
Taxonomy
Culex narnavirus 1
Wuhan Mosquito Virus 4
Aedes vigilax sobemo-like virus 1
Gordis virus
Culex mosquito virus 4
C
BLASTN hits of compactors categorized by RNA types
Identity %
Query Coverage %
RNA Type
Others
mRNA
rRNA
Viruses

Figure 7

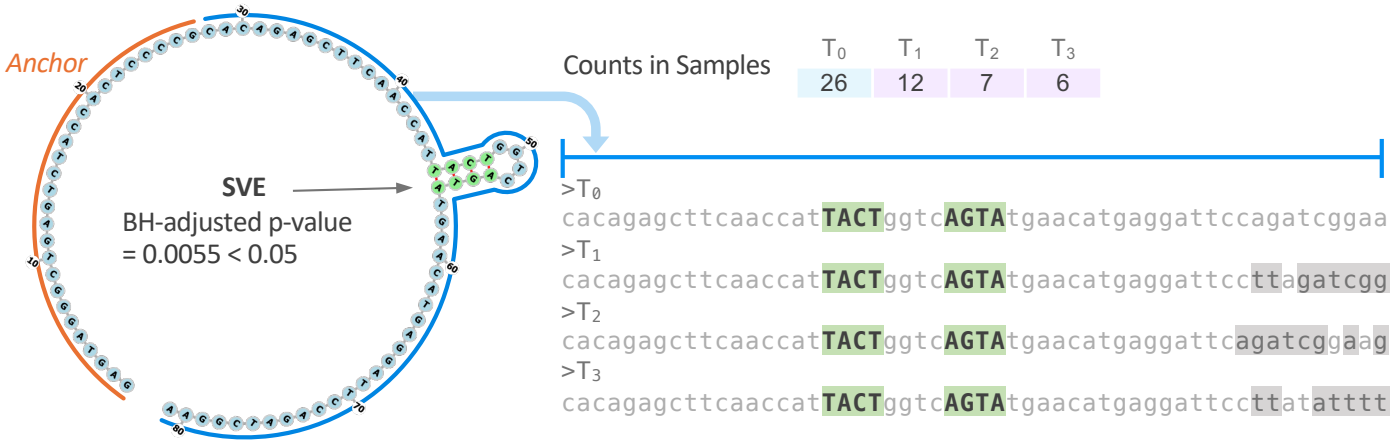
A Statistical detection of Culex narnavirus 1 (compactor mode)



B Statistical detection of Culex narnavirus 1 (target mode)



C Statistical detection of Gordis virus (compactor mode)



D Statistical detection of Culex mosquito virus 4 (compactor mode)

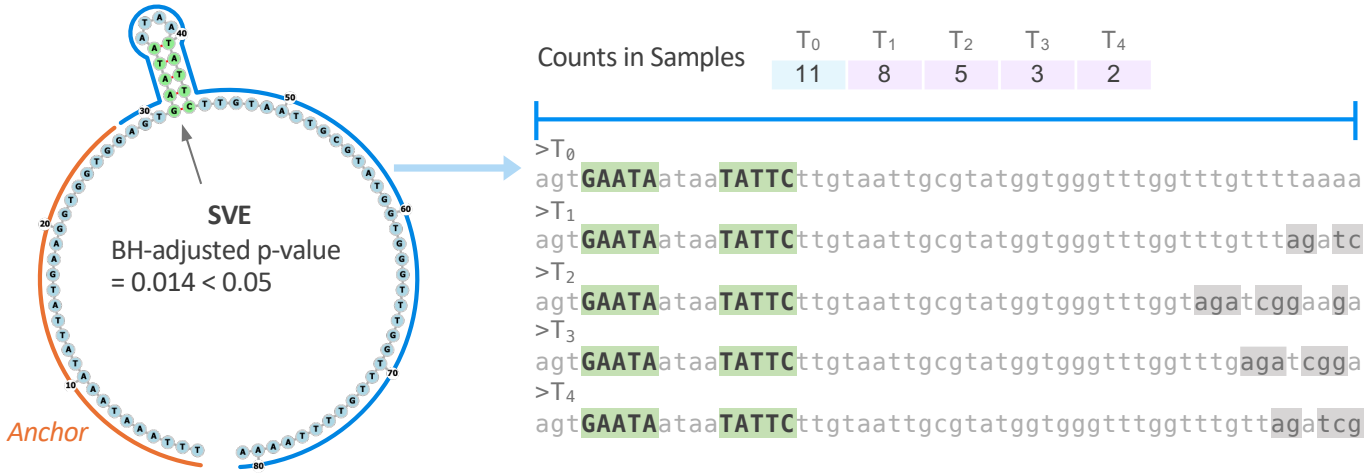


Figure 8

A Extended compactor aligned with Culex Bunyanirus 2

SPLASH-structure:

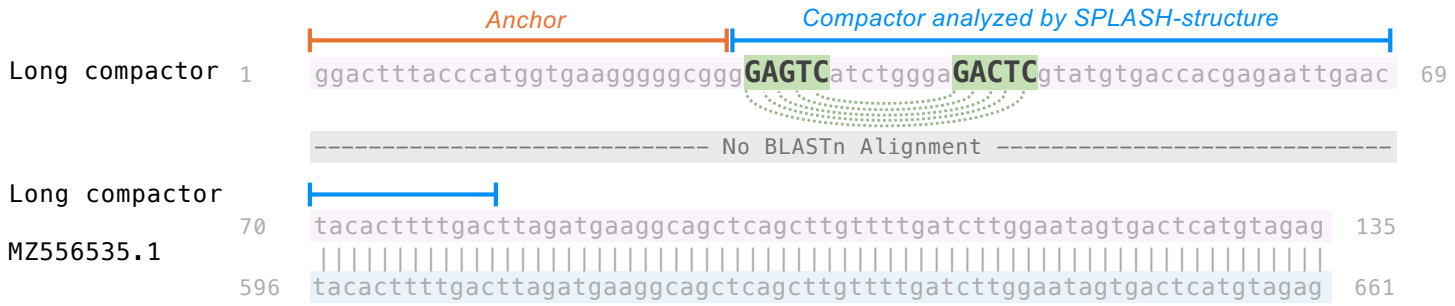
Structure conserved by SVE

BH-adjusted p-value: 0.018

BLASTn:

Subject MZ556535.1: MAG: Culex Bunyavirus 2 isolate CMS002_029d_WVAL glycoprotein gene, complete cds

E-value: 5e-23, Identities: 66/66(100%), Coverage: 66/135(48%)



B Extended compactor aligned with Culex narnavirus 1

SPLASH-structure:

Structure conserved by SVE

BH-adjusted p-value: 0.033

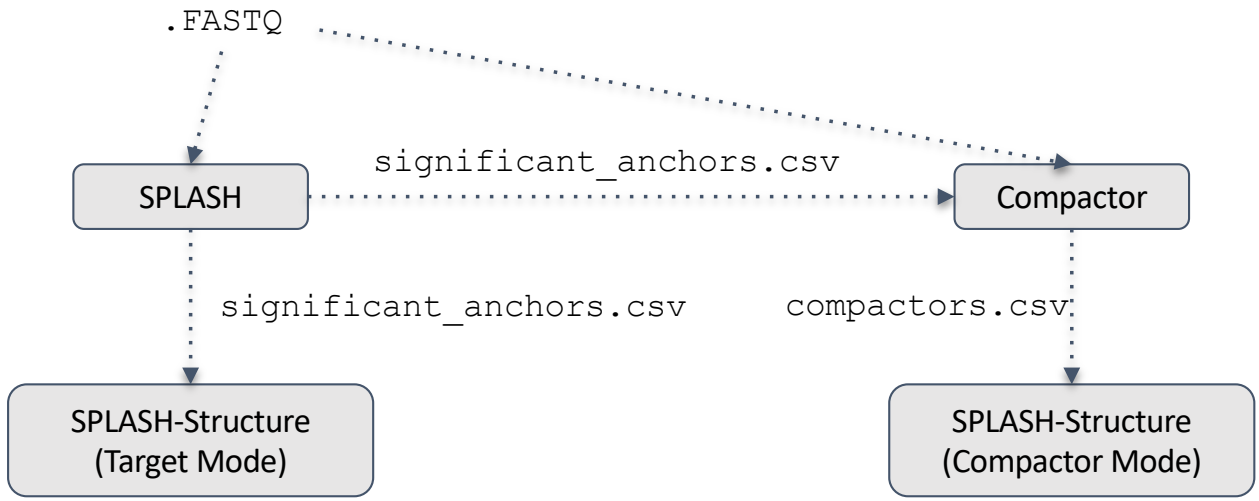
BLASTn:

Subject MW434196.1: MAG: Culex narnavirus 1 isolate CMS001_044_ALCO genomic sequence, sequence

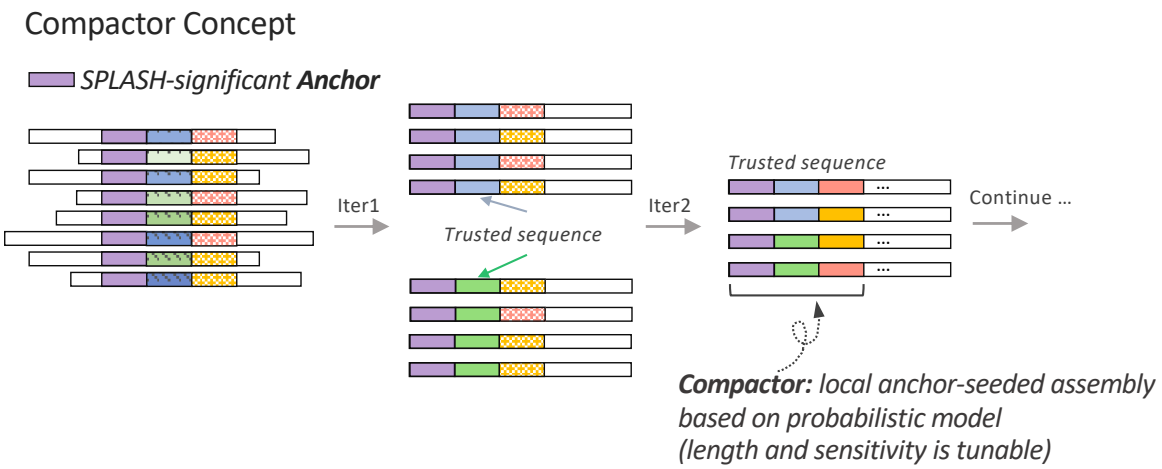
E-value: 5e-16, Identities: 53/53(100%), Coverage: 53/135(39%)

Supplementary Figure 1

A

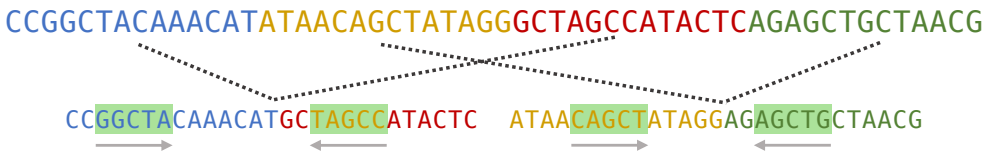


B

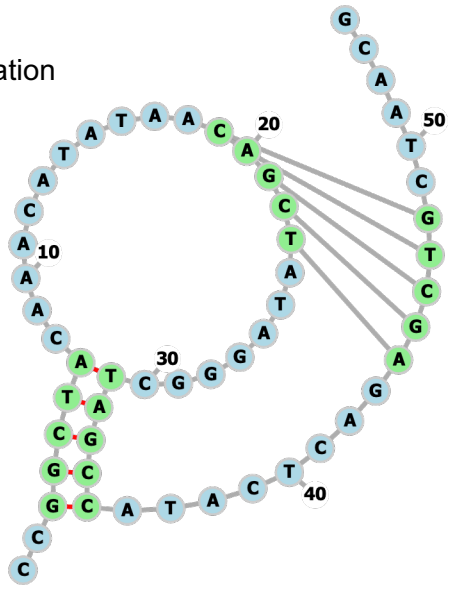


Supplementary Figure 2

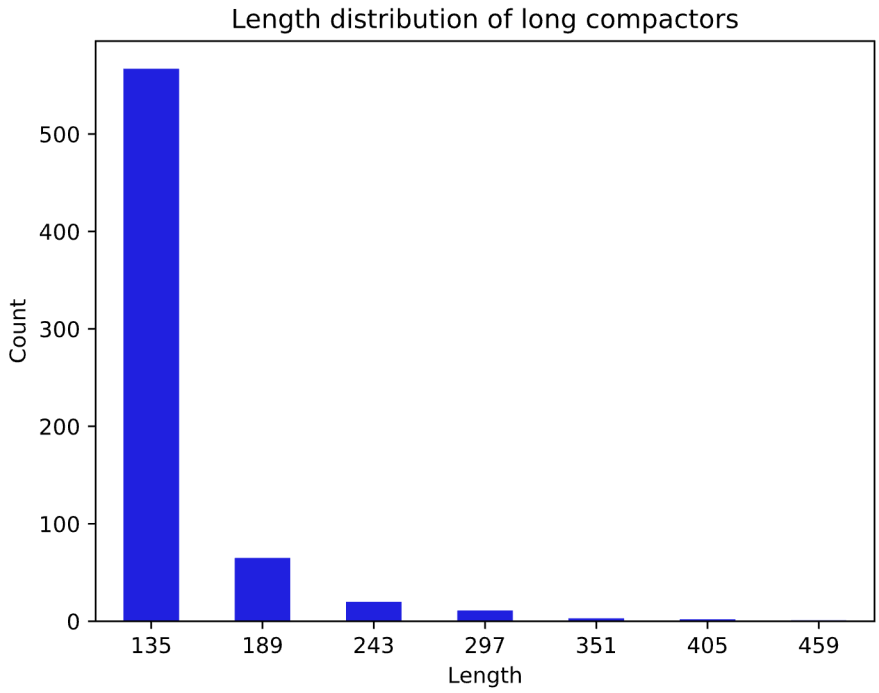
Toy example of SPLASH-structure (compactor mode) detecting a pseudoknot



Structure illustration

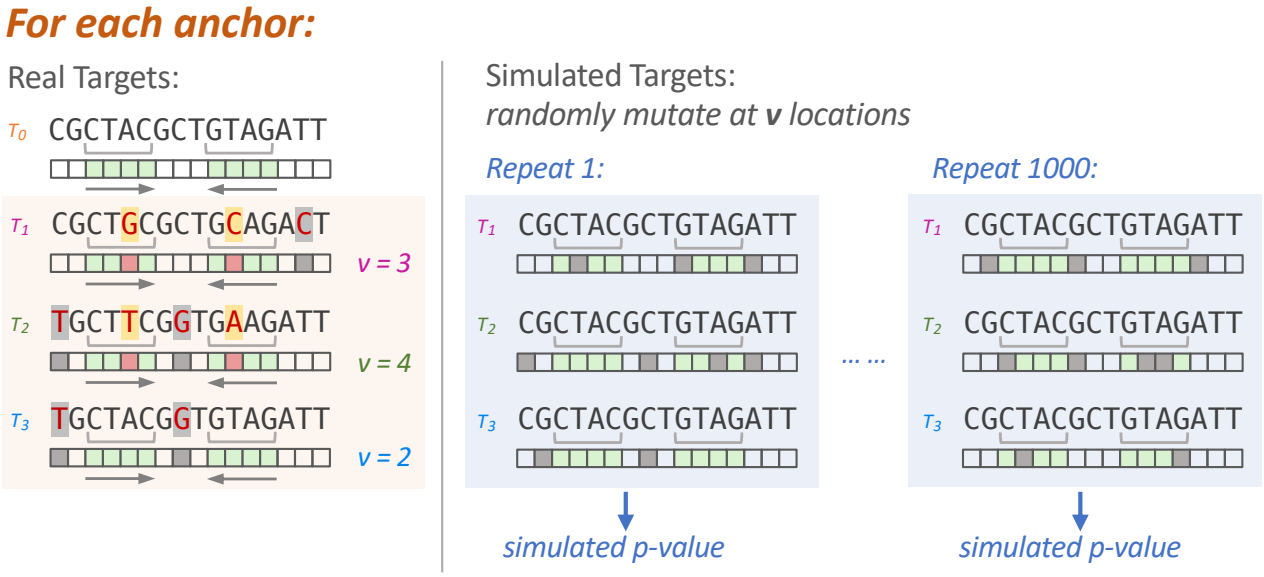


Supplementary Figure 3

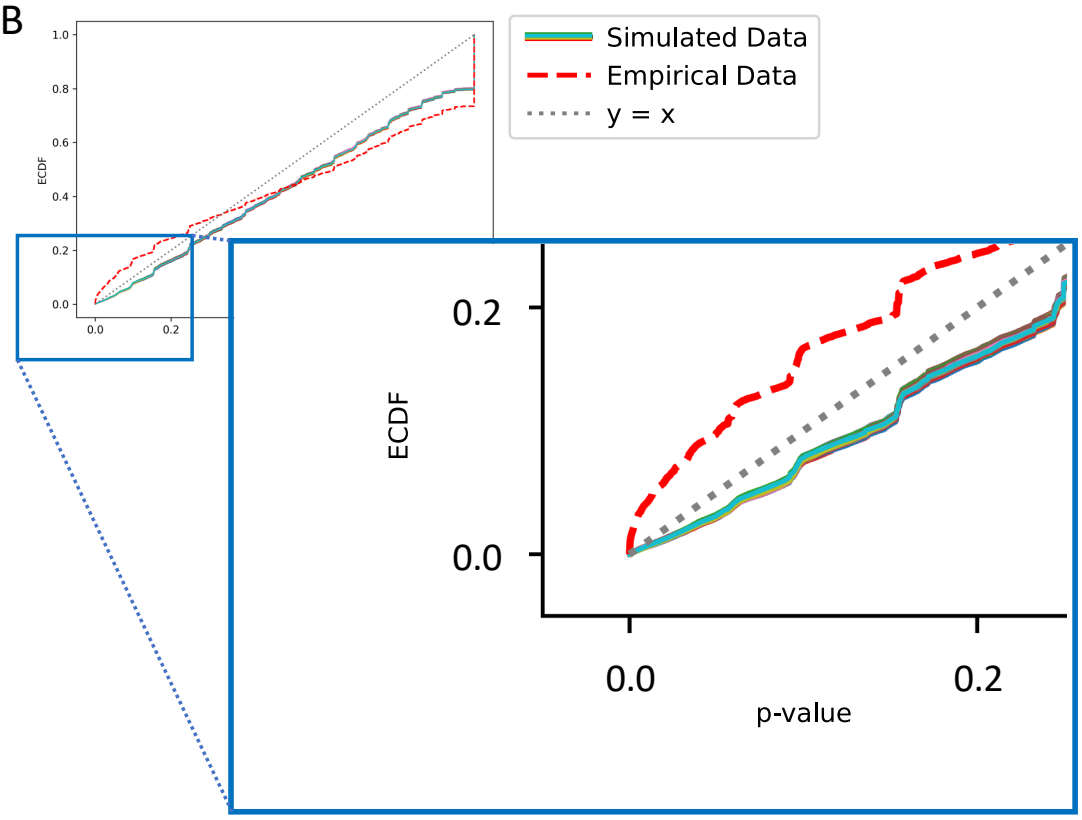


Supplementary Figure 4

A **Simulation Toy Example**

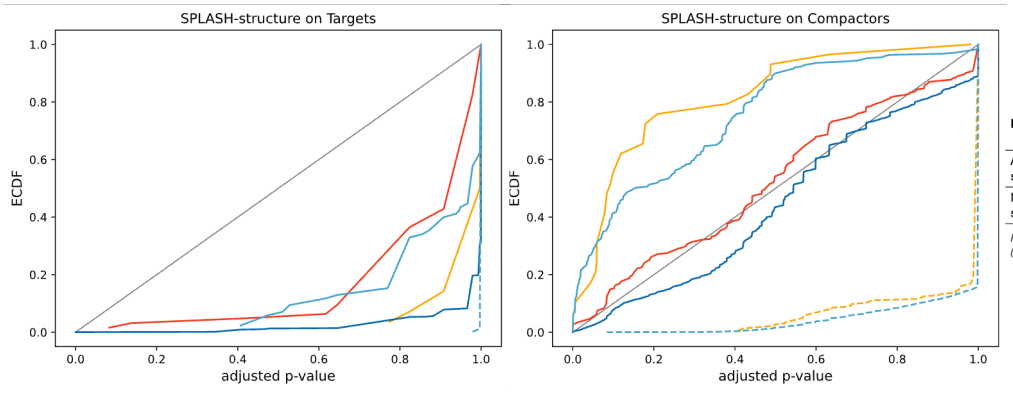


B



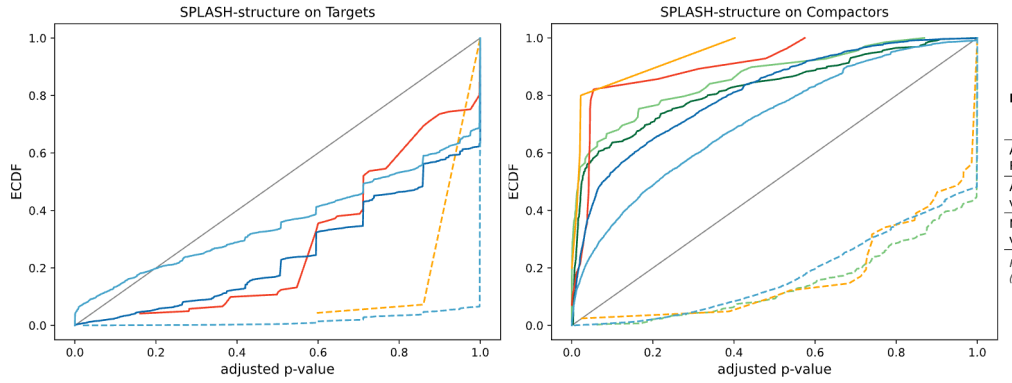
Supplementary Figure 5

A Example HIV dataset (Host 1211)



Legend		Stem Conservation	Base-pair Covariation	Neither	Fraction (%) targets	Fraction (%) compactors
Aligned to HIV structure by Rfam					2.82	5.45
Not aligned to HIV structure by Rfam					97.18	94.55
Fraction (%)	targets	41.56	3.55	54.89		
	compactors	32.47	5.57	61.96		

B Mosquito Metatranscriptomics



Legend		Stem mutation exclusion	Base-pair Covariation	Neither	Fraction (%) targets	Fraction (%) compactors
Aligned to rRNA by Rfam					0	1.81
Aligned to RNA virus by BLAST					0.36	0.15
Not aligned to RNA virus / rRNA					99.64	98.04
Fraction (%)	targets	35.55	5.63	58.82		
	compactors	18.50	20.86	60.64		