CiLiQuant: quantification of RNA junction reads based on their circular or linear transcript origin

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Abstract

Distinguishing circular RNA (circRNA) reads from reads derived from the linear host transcript is a challenging task because of sequence overlap. We developed a computational approach, CiLiQuant, that determines the relative circular and linear abundance of transcripts and gene loci using backsplice and forward splice junction reads generated by existing mapping and circRNA discovery tools.

Availability & Implementation: CiLiQuant is implemented in Python, source code and documentation are freely available via GitHub (https://github.com/OncoRNALab/CiLiQuant).

Keywords: bioinformatics, pipeline, circRNA

Introduction

Circular RNAs (circRNAs) are a novel class of non-coding RNAs found in eukaryotic transcriptomes that result from a process called backsplicing during RNA maturation. In recent years, circRNAs are attracting considerable research attention and evidence of their involvement in normal development and disease has been reported [1–3]. Due to their stable, circular conformation, tissue-specific expression patterns and abundance in biofluids, circRNAs are emerging as potential biomarker candidates in minimally-invasive liquid biopsies [4–6]. As circRNAs share most of their sequence with their linear counterparts, it is impossible to distinguish linear from circular RNA reads that do not include the backsplice sequence itself. This hampers calculations of the relative contribution of circRNAs to aggregated gene counts and can obscure differential expression analyses. To this end, we developed a computational pipeline that determines the linear or ambiguous character of forward junctions and propose two strategies to determine the circular and linear contribution.

Statement of need

Several RNA sequencing library preparation methods can pick up both linear and circular RNA transcripts but contrary to the clear circular RNA origin of backsplice reads, the linear or circular origin of forward splice junctions is not always obvious. CiLiQuant classifies forward splice junction reads as linear only or ambiguous depending on the overlap with detected circRNA transcripts. By correcting the sum of junction reads for the number of unique junctions, the linear only and backsplice junction reads can be directly compared. The entire pipeline can be initiated with a single command and only requires Python and the Pandas package [7]. Unlike other strategies that determine circular-to-linear RNA ratios [8,9], the flexible input format of CiLiQuant allows the usage of junction count files from various combinations of mappers and circRNA quantification tools. This enables users to look at circRNA transcript fractions using their preferred mapping and circRNA detection strategy. Moreover, the dual approach allows users to look at the circular versus linear RNA abundance from different perspectives. The confidence interval provides the user with a level of uncertainty on the calculated fraction; e.g. a ratio of 2/2 has higher uncertainty than a ratio of 20/20 reads. As the output table also includes the original counts, the user can still impose a minimum count threshold and look at absolute count differences. Possible applications include, but are not limited to, comparing linear-to-circular RNA enrichment between tissues, biofluids, healthy and disease state; identifying genes or regions with abundant circRNA expression; discovering interesting circRNAs for biomarker purposes. Finally, the ambiguous

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category can help to determine the relative level of mixed (linear and circular RNA) signal in aggregated gene counts. In case of sufficient sequencing depth, the linear only classification can be used as a starting point for differential expression analysis of linear RNA read counts only. This approach would be very similar to the current backsplice junction count differential expression for circRNAs. In conclusion, the CiLiQuant pipeline distinguishes linear only from potentially mixed junction reads and determines the circular and linear contribution in RNA sequencing data in a systematic and uniform way.

Implementation

The pipeline requires three input files in tab-separated format. Any combination of existing mapping and circRNA discovery tools can be used to generate the first two files: one file for forward splice junctions, e.g. from STAR [10], and one for backsplice junctions e.g. from CIRCexplorer2 [11]. The only requirements are that these junction files were generated from the same sequencing data and contain information about the coordinates of the junctions and their respective read counts in separate columns. The third input file should contain start and stop coordinates of the genes (or exons) of interest. More details and example input files can be found on GitHub.

For each gene, the pipeline classifies forward splice junctions as having a linear or ambiguous origin based on their overlap with detected backsplice junctions (figure 1A). A forward splice junction that starts and stops in between any detected backsplice junction is considered ambiguous because both linear and circular RNA can contribute to the read counts of this junction. In case the forward splice junctions do not (completely) fall within the start and stop of any detected backsplice junction, they are classified as linear. Using this information, our method determines the relative circular to linear RNA abundance at two levels, backsplice and gene level (figure 1B). At the individual backsplice level, backsplice reads are compared to the linear forward splice junction reads that are directly flanking the backsplice. Of note, sometimes this information is not available – either because there are no flanking reads or because those reads are classified as ambiguous as they could be derived from another circRNA. Therefore, an alternative calculation using the average of all linear only junction reads in the gene is provided as well. At the gene level, the average number of backsplice junction reads is compared to the average number of linear only junction reads per junction. For each circRNA fraction, an Agresti-Coull 95% confidence interval is calculated [12].

Availability of source code and requirements

Project name: CiLiQuant

Project home page: https://github.com/almorlio/CiLiQuant

Operating system: Platform independent

Programming language: Python

Other requirements: Python 3.6 or higher, Pandas 1.0.5 or higher

RRID: SCR_019319

https://bio.tools/ciliquant

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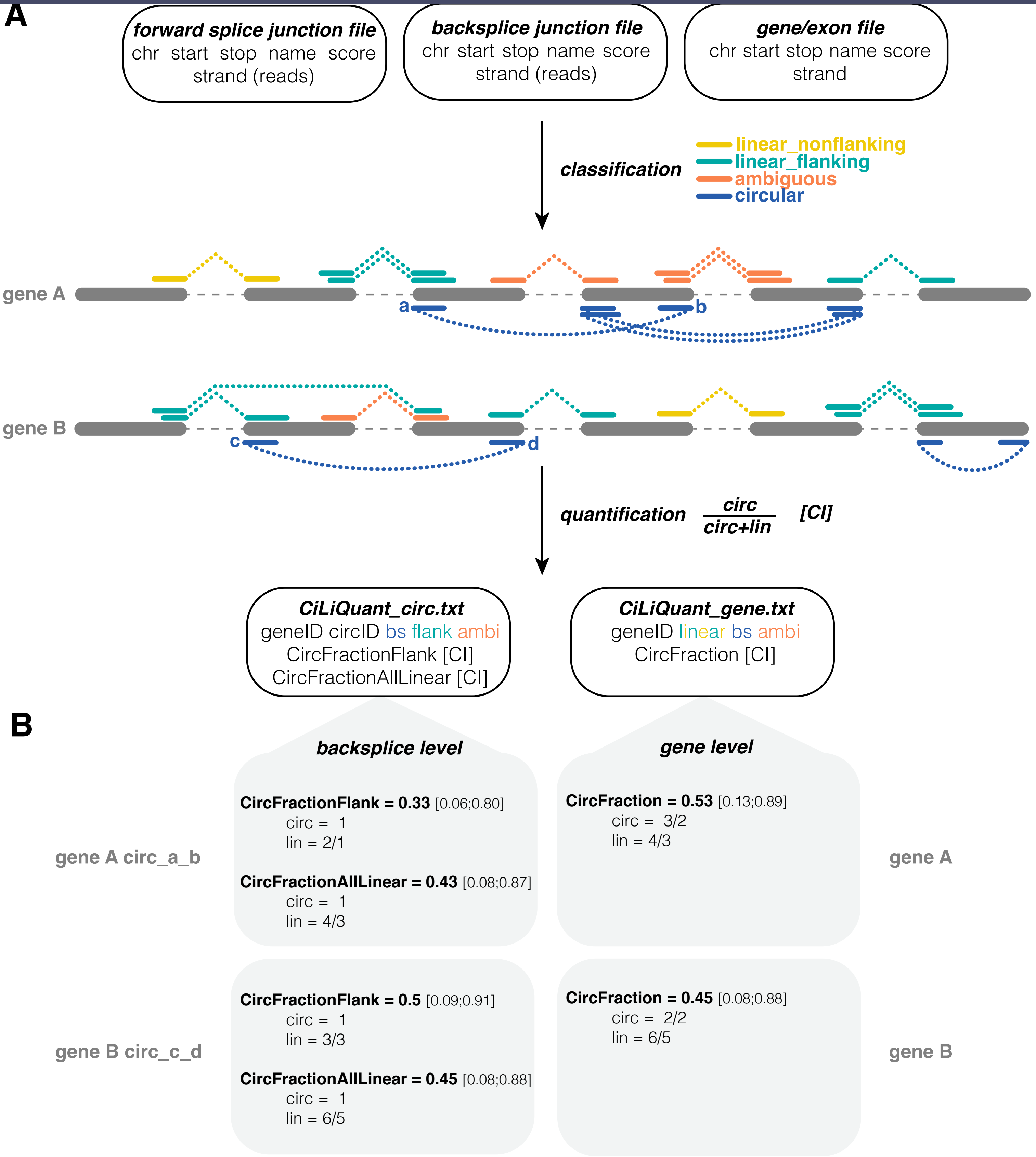
Figures

Figure 1. CiLiQuant classifies splice junctions based on their linear or circular origin and determines circRNA fractions. A) Input, processing and output of the pipeline. Three input files required (number of junction reads may be in separate column or in score column), junctions are divided into four types based on overlap with detected circRNAs, quantification both at backsplice and gene level; B) Examples of circRNA fraction calculations. CircFractionFlank only considers linear junctions directly next to the backsplice of interest while CircFractionAllLinear and CircFraction consider all linear (flanking and non-flanking) junctions in the gene. In each calculation the sum of junction reads is corrected for the number of distinct junctions. Note that the counts in this example are rather low resulting in large confidence intervals. Test case with real sequencing data on GitHub. bs: backsplice; ambi: ambiguous (circular or linear origin); CI: Agresti-Coull 95% confidence interval.


A
forward splice junction file
chr start stop name score strand (reads)
backsplice junction file
chr start stop name score strand (reads)
gene/exon file
chr start stop name score strand
classification
linear_nonflanking
linear_flanking
ambiguous
circular
gene A
a
b
gene B
c
d
quantification
circ
circ+lin
[CI]
CiLiQuant_circ.txt
geneID circID bs flank ambi
CircFractionFlank [CI]
CircFractionAllLinear [CI]
CiLiQuant_gene.txt
geneID linear bs ambi
CircFraction [CI]
B
backsplice level
gene level
gene A circ_a_b
CircFractionFlank = 0.33 [0.06;0.80]
circ = 1
lin = 2/1
CircFractionAllLinear = 0.43 [0.08;0.87]
circ = 1
lin = 4/3
CircFraction = 0.53 [0.13;0.89]
circ = 3/2
lin = 4/3
gene A
gene B circ_c_d
CircFractionFlank = 0.5 [0.09;0.91]
circ = 1
lin = 3/3
CircFractionAllLinear = 0.45 [0.08;0.88]
circ = 1
lin = 6/5
CircFraction = 0.45 [0.08;0.88]
circ = 2/2
lin = 6/5
gene B

Declarations

List of abbreviations

ambi: ambiguous (circular or linear origin)

bs: backsplice

CI: Agresti-Coull 95% confidence interval

circRNA: circular RNA

Competing interests

The author(s) declare that they have no competing interests.

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