SpliceGrapher User Guide

Release 0.2.4

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December 14, 2013

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CHAPTER

ONE

INTRODUCTION

1.1 Overview

SpliceGrapher predicts alternative splicing patterns and produces splice graphs that capture in a single structure the ways a gene's exons may be assembled. It enhances gene model annotations using evidence from next-generation sequencing and EST alignments. With *SpliceGrapherXT* (version 0.2.3), we introduced the ability to convert splice graph predictions into transcript predictions using IsoLasso or PSGInfer.

1.2 Downloading and Installation

- You may download *SpliceGrapher* from http://sourceforge.net/projects/splicegrapher
- Requires PyML version 0.7.9 or higher for users who wish to classify splice sites.
- Requires matplotlib version 1.1.0 or higher for users who wish to use the extensive graphics tools.
- Requires pysam version 0.5 or higher for users who wish to work with both BAM and SAM formats.
- The optional IsoLasso pipeline requires IsoLasso version 2.6.1 or higher, along with the gtfToGenePred and genePredToBed programs from UCSC
- The optional PSGInfer pipeline requires PSGInfer version 1.1.3

Currently Unix/Linux and Mac OS-X are supported. A `setup.py` script is provided so installation is standard for python:

```
python setup.py build
python setup.py install
```

To check that SpliceGrapher is installed correctly, run the python interpreter and type

```
>>> import SpliceGrapher
>>> SpliceGrapher.__version__
'0.2.4'
```

To test your installation more thoroughly, use the test script in the *SpliceGrapher* `examples` sub-directory:

```
cd examples
run_tests.sh
```

CHAPTER

TWO

TUTORIAL

Before getting into SpliceGrapher's nuts and bolts, we begin with a few examples to demonstrate its key features. In the directory where you installed SpliceGrapher you will find a sub-directory called `tutorial` that contains all the data you will need for the following examples. Be sure that SpliceGrapher's scripts are in your path.

Note that all of the examples use the `SpliceGrapher.cfg` file in the `tutorial` directory. This contains the paths to the default reference genome (`a_thaliana.fa.gz`) and the default gene models (`a_thaliana.gff3.gz`). See *The SpliceGrapher Environment* for details.

Example 1: Create Classifiers

In this example you will create accurate models for canonical (GT-AG) and semi-canonical (GC-AG) splice sites for the plant *Arabidopsis thaliana*. To reduce classifier training time you may also want to set the number of examples to something relatively small such as `-n 400` (the default is 2000). Create the classifiers by running the `build_classifiers.py` script as follows:

```
build_classifiers.py -d gt,gc -n 400
```

The program shows you the SpliceGrapher scripts it uses for each step in the process. Below is output from a typical run:

```
Creating SVM models for splice sites
  09:45:10 generate_splice_site_data.py -d gt -n 200 -o gt_don_training.fa -r splice_site_report.txt
  09:45:20 generate_splice_site_data.py -d gt -n 200 -o gt_don_neg.fa -N
  09:45:29 cat gt_don_neg.fa >> gt_don_training.fa
  09:45:29 rm gt_don_neg.fa
  09:45:51 generate_splice_site_data.py -d gc -n 200 -o gc_don_training.fa
  09:46:01 generate_splice_site_data.py -d gc -n 200 -o gc_don_neg.fa -N
  09:46:10 cat gc_don_neg.fa >> gc_don_training.fa
  09:46:10 rm gc_don_neg.fa
  09:46:31 generate_splice_site_data.py -a -d ag -n 200 -o ag_acc_training.fa
  09:46:40 generate_splice_site_data.py -a -d ag -n 200 -o ag_acc_neg.fa -N
  09:46:49 cat ag_acc_neg.fa >> ag_acc_training.fa
  09:46:49 rm ag_acc_neg.fa
  09:47:12 zip classifiers.zip ??_???.cfg ??_???.svm ??_???.fa
  09:47:12 rm ??_tmp_e*_i*.fa
Finished.
```

When the script finishes, you may check classifier performance by looking for the ROC scores in their corresponding `.cfg` files:

```
grep roc *.cfg
```

Scores typically will be above 0.90:

```
ag_acc.cfg:roc_score = 0.935625
gc_don.cfg:roc_score = 0.94925
gt_don.cfg:roc_score = 0.969875
```

The script packages these classifiers into the `classifiers.zip` file in the local directory.

Example 2: Filter Alignments

For this example you may use the classifiers you created in Example 1, or you may simply copy the `Arabidopsis_thaliana.zip` file from `classifiers` sub-directory of your SpliceGrapher distribution. This example uses the following files:

- Alignment output (SAM format): `alignments.sam.gz`
- *A. thaliana* classifiers (ZIP format): `classifiers.zip` (from Example 1 above) or `Arabidopsis_thaliana.zip`

To filter the alignment output, run the `sam_filter.py` script:

```
sam_filter.py alignments.sam.gz classifiers.zip -o filtered.sam
```

For this example we included several false-positive alignments to illustrate the filtering process. If you run `sam_filter.py` with *verbose* turned on (`-v` option) you will see that these have been filtered out of the output file.

Example 3: Predict a Splice Graph

This example demonstrates SpliceGrapher's prediction modules. In this case we will use it to predict a graph for the gene **AT2G04700** that we selected as an illustrative example. This example uses the following files:

- Alignment output (SAM format): `filtered.sam`
- Plotter configuration file: `AT2G04700_plot.cfg`

To predict a graph for this gene, run the `predict_splicegraph.py` script:

```
predict_splicegraph.py AT2G04700 -d filtered.sam -o AT2G04700.gff
```

Predicting a graph for a single gene is useful, but in most cases you will want to make predictions for a collection of genes (or genome-wide). In that case you will want to use the *predict_graphs.py* script that is part of the extended example in section *RNA-Seq Analysis Example*.

There are two ways to view the resulting graph: `view_splicegraphs.py` and `plotter.py`. The first script allows you to view a splice graph on your screen or, if you prefer, save it to a file. `plotter.py` is designed for file output and provides a more extensive set of options. We have provided an example plotter configuration file to get you started:

```
view_splicegraphs.py AT2G04700.gff -L
plotter.py AT2G04700_plot.cfg
```

2.1 RNA-Seq Analysis Example

On the SpliceGrapher SourceForge website we have included *RNA-Seq-tutorial.tgz* that contains files so that you can perform a complete RNA-Seq analysis to get better acquainted with the software. In this tutorial you will filter alignments, generate predictions from the alignments, generate plots for a couple of genes and compare statistics for the original gene models with the predicted graphs. We recommend that you create a separate directory to run this example to make it easier to keep track of what you're doing.

2.1.1 Download and unpack the tar file

First download the tutorial file from SourceForge into the directory you are using to run the example and unpack the tarball as follows:

```
tar xfz RNA-Seq-tutorial.tgz
```

You should find the following files for the model plant *A. thaliana*:

```
accepted_hits.sam
Arabidopsis_thaliana.zip
a_thaliana.fa
a_thaliana_reduced.gtf
a_thaliana_reference.gtf
AT5G18280.cfg
```

The *accepted_hits.sam* file contains alignments we produced by running *Tophat* on a set of simulated reads. *Arabidopsis_thaliana.zip* contains the files for splice-site classifiers used in the filtering step. The file *a_thaliana.fa* containes the reference genome in FASTA format. There are two files containing gene models: *a_thaliana_reduced.gtf* contains a single representative transcript for 1000 randomly-sampled genes from *A. thaliana*, while *a_thaliana_reference.gtf* contains the complete TAIR10 gene models for these same genes. Finally, *AT5G18280.cfg* is a configuration file to use with the *plotter.py* script once splice graphs have been created.

2.1.2 Filter Tophat alignments

Given a set of alignments in *accepted_hits.sam*, the first step is to filter the alignments to eliminate possibly spurious spliced alignments. The *sam_filter.py* script is used to perform this task:

```
sam_filter.py accepted_hits.sam Arabidopsis_thaliana.zip -f a_thaliana.fa
              -m a_thaliana_reference.gtf -v -o filtered.sam
```

The output should look similar to the following:

```
Loading FASTA records from a_thaliana.fa
Found 7 FASTA records in a_thaliana.fa
loaded classifier for 'ag' acceptor sites
loaded classifier for 'gc' donor sites
loaded classifier for 'gt' donor sites
Loading and validating gene models from a_thaliana_reference.gtf
..
Validating junctions in accepted_hits.sam
....|....|....|....|....|....|....|....
Found 554,708 ungapped and 271,298 spliced alignments in accepted_hits.sam
7,654 (99.4%) TP and 45 (0.6%) FP junction alignments out of 7,699 total
40 (47.1%) TP and 45 (52.9%) FP novel junction alignments out of 85 total

Also found 78 intergenic and 40 transgenic junctions
```

2.1.3 Predict splice graphs

Next predict splice graphs using the reduced gene models and the filtered alignments created in the previous step. When predicting graphs for multiple genes, use the *predict_graphs.py* script as follows:

```
predict_graphs.py filtered.sam -m a_thaliana_reduced.gtf -v -d predicted
```

The reduced gene models in *a_thaliana_reduced.gtf* consist of a single representative transcript per gene, while the SAM alignments represent a much broader set of transcripts from the complete gene models. Later we will compare the predicted graphs with graphs generated from the complete gene models in *a_thaliana_reference.gtf*. Output from the *predict_graphs.py* script should appear as follows:

```
Loading and validating gene models from a_thaliana_reduced.gtf
Initializing SAM input from filtered.sam
Found 5 chromosomes in common between a_thaliana_reduced.gtf and filtered.sam:
1, 2, 3, 4, 5
Generating predictions:
  starting chromosome 1
    loading alignment records from filtered.sam
    predicting splice graphs .
  starting chromosome 2
    loading alignment records from filtered.sam
    predicting splice graphs
  starting chromosome 3
    loading alignment records from filtered.sam
    predicting splice graphs
  starting chromosome 4
    loading alignment records from filtered.sam
    predicting splice graphs
  starting chromosome 5
    loading alignment records from filtered.sam
    predicting splice graphs .
Finished: splice graphs were modified for 911 / 1,000 genes.
```

2.1.4 Generate plots

Now you can experiment with the plotting utility *plotter.py* using the plotter configuration file *AT5G18280.cfg* provided in the tar file. Use the *-v* option to monitor its progress:

```
plotter.py AT5G18280.cfg -v
```

Notice that this command takes some time to run. A significant part of the time is taken reading the SAM input file. To speed this process, you can split the SAM file into smaller pieces, one file per chromosome:

```
sam_split.py filtered.sam
```

This should create the files *1.sam*, *2.sam*, *3.sam*, *4.sam* and *5.sam*. To improve plotting speed, modify the *AT5G18280.cfg* file and change the name of the SAM file it uses. There are two locations in the file where you will want to change *filtered.sam* to *5.sam*. Both lines appear as follows:

```
source_file   = 5.sam
```

The plotter script produces an output file *AT5G18280.pdf*. If you look at the PDF file you will notice that the figure includes the splice graph for the reduced gene model, the spliced alignments and the read coverage. At this point it is instructive to add the predicted graph to the plot, which you can do by adding the following section to the configuration file (assuming you placed your predicted graphs in the *predicted* directory):

```
[MyPrediction]
plot_type     = splice_graph
source_file   = predicted/5/AT5G18280.gff
title_string  = AT5G18280 Prediction
```

To make the splice graphs adjacent in the final figure, insert this section right before the *[SpliceJunctions]* section in the configuration file. Now the plot will show the splice graph for the reduced gene model as well as the predicted splice graph, allowing you to see the exons added by the prediction software.

2.1.5 Depths files

We can now repeat the exercises in the last two sections using SpliceGrapher depths files in place of SAM files. First convert the filtered SAM alignments into a depths file:

```
sam_to_depths.py filtered.sam -o filtered.depths
```

If you do a directory listing you will see that the depths file is much smaller than the SAM file, meaning that predictions and plotting should take less time. Now you can use the depths file in place of the SAM file to predict splice graphs:

```
predict_graphs.py filtered.depths -m a_thaliana_reduced.gtf -v -d depths_predicted
```

You may also wish to rerun `plotter.py` by first modifying the configuration file and replacing all references to SAM files with `filtered.depths`.

2.1.6 Compare predictions with complete gene models

We can also compare the predicted splice graph with the complete gene models. The SAM alignments were simulated from the complete gene models, so we would like to know how closely the predicted graphs match the complete gene models. The full gene models for these genes are in the fila *a_thaliana_reference.gtf*. To add these to our plot, we first need to generate splice graphs from the gene models using the *gene_model_to_splicegraph.py* script:

```
gene_model_to_splicegraph.py -m a_thaliana_reference.gtf -a -S -d reference
```

This will create splice graphs in the same kind of directory structure as for a set of predictions. We can now add the splice graph for the complete gene model to the plot configuration file by adding a new section:

```
[CorrectGraph]
plot_type     = splice_graph
source_file   = reference/5/AT5G18280.gff
title_string  = AT5G18280 Gene Model
```

As before, to keep the splice graphs adjacent in the final figure, insert this section right before the *[SpliceJunctions]* section in the configuration file. Now when you generate a plot, you can see the representative transcript, the predicted graph and the complete gene model all on the same figure.

2.1.7 Generate statistics

In addition to viewing individual genes, it is also worthwhile to generate statistics from a set of splice graphs using the *splicegraph_statistics.py* script. This script accepts a list of splice graphs as input, that we can create easily using the Linux *find* command:

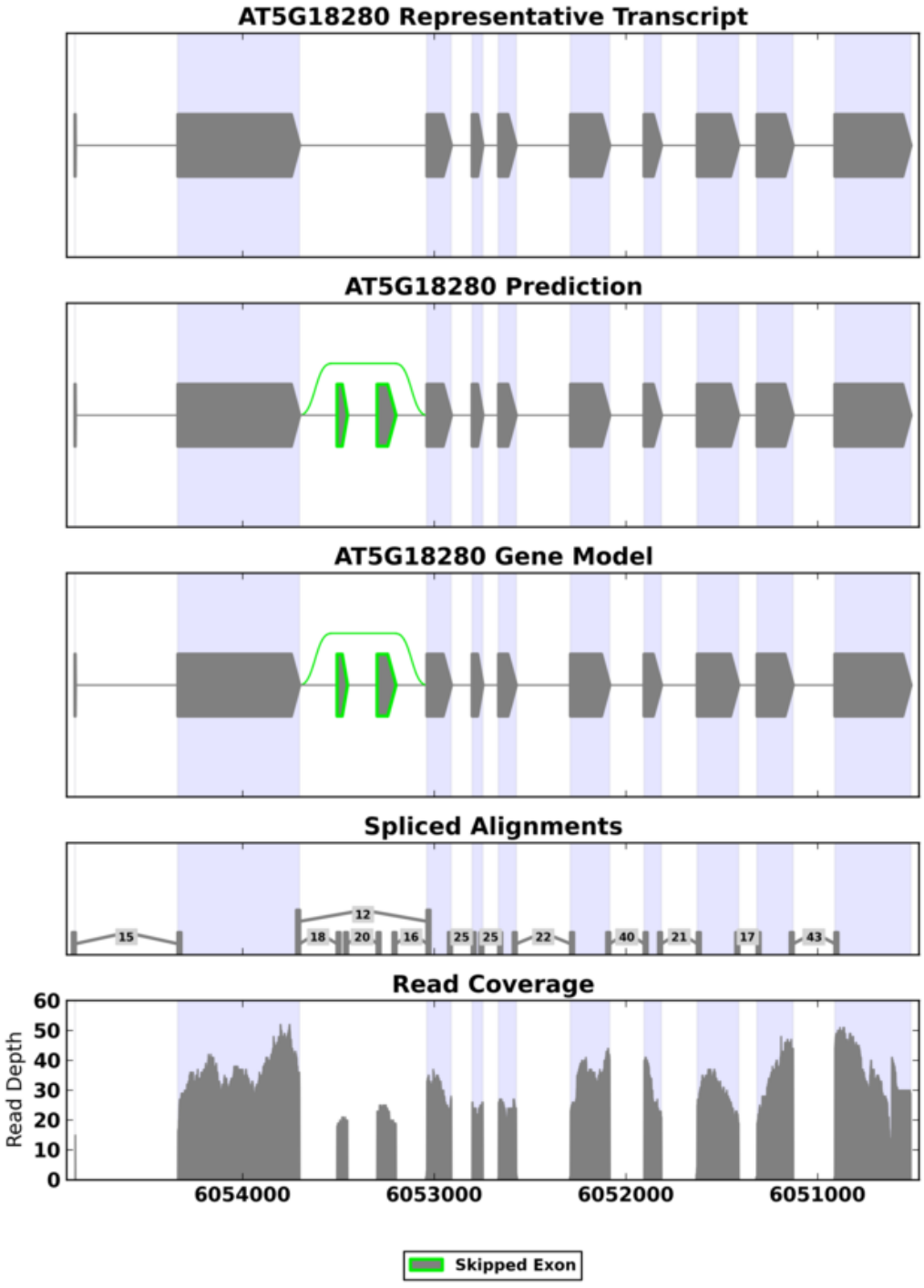
```
find predicted -name "*.gff" > predicted.lis
find reference -name "*.gff" > reference.lis
```

Once we have created these lists, we can generate statistics for the two sets of graphs. The output from these commands should be similar to the following for the predicted graphs:

```
splicegraph_statistics.py predicted.lis

Alternative Splicing Statistics for 1,000 Graphs (780 with AS)
Intron Retention  Skipped Exon  Alt. 5'      Alt. 3'      Total
345 (28.7%)       180 (15.0%)   257 (21.4%)  420 (34.9%)  1,202
```

For the reference gene models, the statistics are different:


AT5G18280 Representative Transcript
AT5G18280 Prediction
AT5G18280 Gene Model
Spliced Alignments
15
12
18
20
16
25
25
22
40
21
17
43
Read Coverage
Read Depth
60
50
40
30
20
10
0
6054000
6053000
6052000
6051000
Skipped Exon

```
splicegraph_statistics.py reference.lis

Alternative Splicing Statistics for 1,000 Graphs (863 with AS)
Intron Retention  Skipped Exon  Alt. 5′      Alt. 3′      Total
443 (29.7%)       184 (12.3%)   327 (21.9%)  536 (36.0%)  1,490
```

Note: the statistics for SpliceGrapherXT's predicted graphs are slightly lower than for the complete gene models. This is because predictions were based on a single representative transcript for each gene (in other words, no AS events to start with), and SAM alignments that were simulated from the complete gene models. Thus the predictions reflect only those known AS events that were present in the simulated alignments.

2.1.8 Generate plots for other genes

Earlier we developed a configuration file for the gene *AT5G18280*. Assuming this configuration has the characteristics we want, it is relatively simple to use the same configuration for another gene. First, copy the configuration file to create a new one:

```
cp AT5G18280.cfg AT2G45960.cfg
```

Next, replace every instance of *AT5G18280* with *AT2G45960* in the new file. You will also need to replace every reference to the chromosome subdirectory */5/* with */2/*:

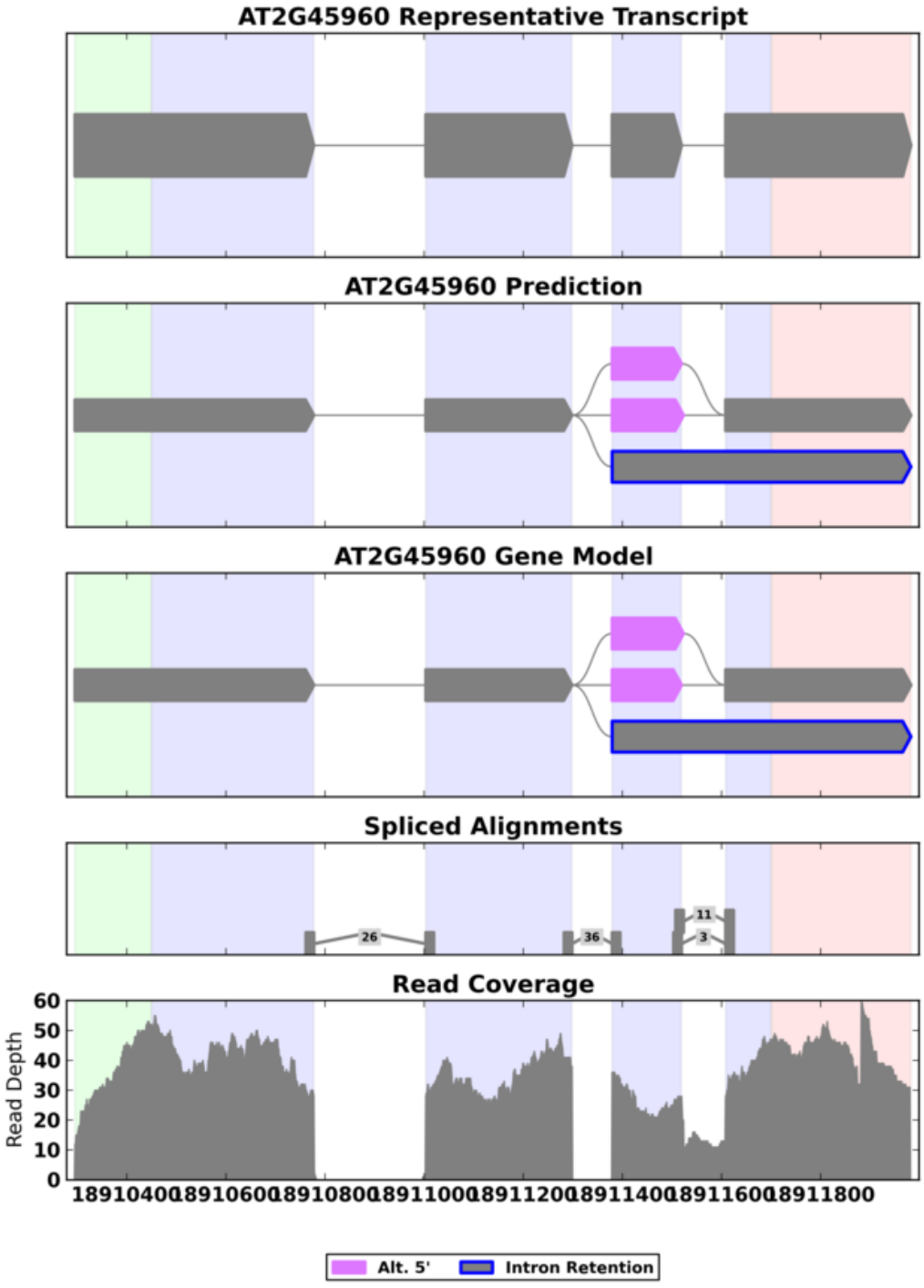
```
predicted/2/AT2G45960.gff
reference/2/AT2G45960.gff
```

Finally, replace every instance of *5.sam* with *2.sam*:

```
source_file   = 2.sam
```

Now you can generate a new figure for the gene:

```
plotter.py AT2G45960.cfg
```


AT2G45960 Representative Transcript
AT2G45960 Prediction
AT2G45960 Gene Model
Spliced Alignments
26
36
11
3
Read Coverage
Read Depth
60
50
40
30
20
10
0
18910400
18910600
18910800
18911000
18911200
18911400
18911600
18911800
Alt. 5'
Intron Retention

CHAPTER

THREE

THE *SPLICEGRAPHER* ENVIRONMENT

Throughout the this guide we describe a variety of tasks that require different input files. By far the most common files are a reference sequence (a FASTA file) and a gene model (either GFF3 or GTF format).

3.1 Environment Variables

You may specify default paths for these files by setting the environment variables `SG_FASTA_REF` and `SG_GENE_MODEL`. For example, in C shell this might look like:

```
setenv SG_FASTA_REF "/home/o_sativa/sequences/o_sativa.fasta"
setenv SG_GENE_MODEL "/home/o_sativa/annotations/o_sativa.gff3"
```

SpliceGrapher will use these default paths when they are not provided to a script on the command line or in a configuration file.

3.2 SpliceGrapher Configuration File

SpliceGrapher programs and modules also use the configuration file `SpliceGrapher.cfg` to locate the genomic reference and gene model files. Below is an example of a configuration file.

```
[SpliceGrapher]
GENE_MODEL = /home/o_sativa/annotations/o_sativa.gff3
FASTA_REFERENCE = /home/o_sativa/sequences/o_sativa.fasta
```

SpliceGrapher scripts look for `SpliceGrapher.cfg` in your `PATH`. When working with data for different organisms, it is often convenient to place a `SpliceGrapher.cfg` file for each organism in its own directory. Then one can change *SpliceGrapher*'s defaults simply by changing directories. *SpliceGrapher* scripts will first use any paths specified on the command line, followed by those found in a configuration file and then those given as environment variables.

CHAPTER

FOUR

SPLICE SITE CLASSIFIERS

For RNA-Seq data we are concerned with two kinds of alignments: *ungapped* alignments and *splice junction* alignments. An ungapped alignment is one in which a read aligns completely within an exonic region in the reference sequence. Most short-read alignment algorithms can perform exonic alignments. A splice junction alignment is one in which part of a read aligns to the 3' end of one exon and the remainder aligns to the 5' end of another exon, usually within the same gene. However, many spliced alignment programs use only rudimentary heuristics to identify correct splice sites. *SpliceGrapher's* approach is to filter these junctions using highly accurate splice-site classifiers.

SpliceGrapher comes with over 100 pre-built classifiers for a variety of different species. These are provided as `.zip` files located in the `classifiers` directory. You may also build your own classifiers using the `build_classifiers.py` script.

4.1 Building Classifiers

To simplify the process of constructing classifiers and to provide an example script you may use to create your own pipeline, *SpliceGrapher* includes the script `build_classifiers.py`. Assuming you have established paths for a FASTA reference sequence a gene model file `SpliceGrapher.cfg` file, you can generate a complete set of classifiers for filtering spliced alignments with one command:

```
build_classifiers.py
```

The script performs the following steps:

1. Generates training data for each splice site dimer.
2. Selects optimal parameters for each classifier.
3. Stores the data for each classifier in a `.zip` file

The script accepts the following options:

Option	Value	Description
–commands		Show but don't run commands
-a	dimer list	Acceptor site dimers to predict
-d	dimer list	Donor site dimers to predict
-f	file path	Fasta reference file
-m	file path	GFF3 gene model annotation file
-n	examples	Total number of examples to use for training
-l	file path	Optional output log file

By default the script builds classifiers only for canonical GT donor sites and AG acceptor sites. To build a classifier for semi-canonical GC donor sites as well, simply provide a list to the script:

```
build_classifiers.py -d gt,gc
```

In each step of this pipeline, the main script calls other *SpliceGrapher* scripts to generate data and train classifiers. By using the `--commands` option, you can see the commands that would be generated without actually running them. This is instructive when learning how to use each of the scripts individually. Each of the scripts used in `build_classifiers.py` is described in the following sections.

(For more information on how these classifiers are constructed, please see [BENHUR2008] and http://pyml.sourceforge.net/).

For the splice site prediction modules *SpliceGrapher* uses support vector machines (SVM) implemented in the PyML package. To create a set of splice-site classifiers, we suggest taking the following steps:

1. Identify most common splice-site dimers for the species
2. Generate positive, negative training data sets
3. Find optimal classifier parameters for each splice site

Each of these procedures is described in the following sections.

4.1.1 Identifying Splice-Site Dimers

The workhorse script `generate_splice_site_data.py` can identify frequently-occurring splice-site dimers and generate positive and negative example sequences for any given dimer. The script can produce a report of all splice site dimers and splice junctions found in a set of gene models. The most frequently occurring splice site dimers are then candidates for constructing classifiers for predicting novel splice sites in a genome. The command is simply:

```
generate_splice_site_data.py -r
```

Below is an example of a report, truncated for readability:

```
Breakdown of donor sites for 122535 introns:
 GT:  121165 (98.88%)
 GC:    1248 ( 1.02%)
 AT:      84 ( 0.07%)
 GA:      13 ( 0.01%)
 TT:       8 ( 0.01%)
Breakdown of acceptor sites for 122535 introns:
 AG:  122419 (99.91%)
 AC:      76 ( 0.06%)
 AT:       9 ( 0.01%)
Breakdown of splice junctions:
 GT-AG:  121132 (98.8550%)
 GC-AG:    1248 ( 1.0185%)
 AT-AC:      73 ( 0.0596%)
 GA-AG:      13 ( 0.0106%)
 TT-AG:       8 ( 0.0065%)
```

Given a list of splice-site dimers and their frequencies, one can then choose an optimal set that balances the potential number of successful predictions with the overhead of training a classifier for each dimer.

4.1.2 Generating Training Data

The `generate_splice_site_data.py` script is also used to generate positive and negative examples for training SVMs. Positive examples are simply examples of the given dimer found at splice sites in the gene model. To generate negative examples *SpliceGrapher* uses the procedure outlined in [RATSCH2005]: it looks for all examples of the given dimer within each gene and uses as negative examples those that don't appear in splice sites.

To generate positive examples for a given donor-site dimer such as `GT`, use the following command:

```
generate_splice_site_data.py -o gt_positive.fa -d GT
```

To generate examples for acceptor sites, use the `-a` option:

```
generate_splice_site_data.py -o ag_positive.fa -d AG -a
```

As with other scripts, the default gene model and FASTA reference are provided in `SpliceGrapher.cfg` variables, and output is written to `stdout` by default.

To generate negative examples, simply add the `-N` option to the command:

```
generate_splice_site_data.py -o gt_negative.fa -d GT -N
```

By default, output splice site sequences will include 100nt on either side of a splice-site dimer, but will not include the dimer itself. To include splice-site dimers in output sequences, use the `-D` option. To change the window size (for example, 30nt on either side), use the `-W` option:

```
generate_splice_site_data.py -o gt_positive.fa -d GT -W 30
```

Note that the window size will reduce the number of possible SVM parameter combinations (described in *Optimizing Classifiers*) but may also reduce SVM performance if the windows are too small. Positive examples for any dimer tend to be relatively rare, while negative examples are plentiful. In fact, there may orders of magnitude more negative examples than positive, which would be overkill for training a good classifier. To set a limit on the number of negative examples generated, use the `-n` option. When a limit is set, the script will perform the following random selection procedure a fixed number of times.

First it selects a gene at random from the genome. Once a gene has been selected, it samples positions at random from within the gene until it finds the appropriate dimer at a position other than a known splice site. Once the appropriate number of negative examples have been randomly selected, the script halts. Thus to generate a sample of 10,000 negative `GT` examples, use the command:

```
generate_splice_site_data.py -o gt_negative.fa -d GT -N -n 10000
```

4.1.3 Optimizing Classifiers

SpliceGrapher uses support-vector machines (SVMs) to classify splice sites. More specifically it uses SVMs with a weighted-degree kernel that has proved especially successful at discriminating real splice sites from spurious ones. While these models are well-suited for predicting novel splice sites, there are a number of parameters that that can influence SVM performance for a specific kind of splice site. Briefly, these are:

- intronic and exonic sequence length on either side of a site
- range of k-mer sizes to use
- size of shifts to allow when assessing k-mers
- number of mismatches to allow
- SVM regularization parameter, **C**

For an overview and tutorial on SVMs with weighted-degree kernels, see [BENHUR2008].

Option	Value	Description
-a		Interpret splice sites as acceptors
-e	EXONSIZE	List of exon sizes to try (e.g., '20,25,30')
-C	CLIST	List of regularization constants for SVM (e.g., '0.1,1.0,10.0')
-i	INTRONSIZE	List of intron sizes to try (e.g., '20,25,30')
-l	LOGFILE	Optional log file for tracking performance
-k	KFOLDS	Number of folds to run cross-validation
-m	MINK	Set of minimum k values for kernel (e.g, '1,2,3')
-M	MAXK	Set of maximum k values for kernel (e.g., '3,4,5')
-N		Turn normalization OFF
-p		Apply a mismatch profile
-P	PREFIX	Optional prefix for output files
-S	SHIFT	List of maximum shift values

To simplify this process, *SpliceGrapher* includes the script `select_model_parameters.py`. This script trains a weighted-degree kernel SVM on labeled FASTA sequences generated by `generate_splice_site_data.py` and runs cross-validation for a variety of parameter settings. As the program proceeds, it saves the best model and parameter configuration. The basic format of this command is

```
select_model_parameters.py FASTA-training-data dimer
```

For example:

```
select_model_parameters.py gt_training.fa gt
```

This runs cross-validation on a model with just the default parameters (exon lengths of 8nt, 12nt and 16nt; intron lengths of 15nt, 20nt and 25nt; minimum k-mer size 1; maximum k-mer 1, 2 or 3; no mismatches; shift sizes 0 and 1, and C = {0.01,0.1,1,10,100}). By default, cosine normalization is applied to kernel vectors, but this may be turned off using the `-N` flag.

The script accepts a list of values to try for every SVM parameter. For example, to try several shift values, one might use `'-S 0,1,2,3'`. Note, however, that the number of parameter combinations grows geometrically with each set of values. Since it can take a long time to run cross-validation on an elaborate model, the program accepts a maximum of 100 parameter combinations per run.

4.1.4 Intron and Exon Sequence Lengths

When `generate_splice_site_data.py` generates training data, it uses the same window size on either side of a splice site. This permits the model selection program to try different combinations of intron and exon sequence lengths up to this window size.

Note: in theory, one could use windows that are hundreds or thousands of nucleotides long. But as window sizes increase, the time required for cross-validation and for classification can increase dramatically. Experience has shown that classifier performance often does not improve significantly beyond a range of 30-40nt on either side of a splice site. The parameter selection script imposes a hard limit of 50nt on either side of a junction. If you wish to experiment with longer sequences you will need to change the hard-coded limit.

To identify the best intron and exon sequence lengths, run the model selection algorithm with a series of possible lengths for each. For example, if one wishes to find the best model performance for a donor `GT` splice site with exon sequence sizes from 10 to 30 and intron sequence sizes from 10 to 40, the command would then be:

```
select_model_parameters.py gt_training.fa gt -e 10,20,30 -i 10,20,30,40
```

To experiment with parameters, simply include in the same command values for those parameters you wish to try:

```
select_model_parameters.py gt_training.fa gt -e 10,20,30 -i 10,20,30,40 -m 1,2 -M 2,3,4
```

Note: despite one's best efforts, some parameter settings may cause an SVM to fail to converge. Often this may be corrected by adding training data or by using different parameter values. For this reason we recommend working with only a few parameters at a time, taking care to save the resulting output files.

4.2 Classifier Data

SpliceGrapher creates three files for each classifier: a `.svm` file that contains the trained classifier parameters; a `.fa` file that contains the sequences used for training, and a `.cfg` file that contains meta-data about the classifier including the parameters optimized by `select_model_parameters.py` and its ROC score. To avoid having to track three files for every classifier, *SpliceGrapher* usually stores these files in a single convenient `.zip` archive.

4.3 Generating ROC Curves

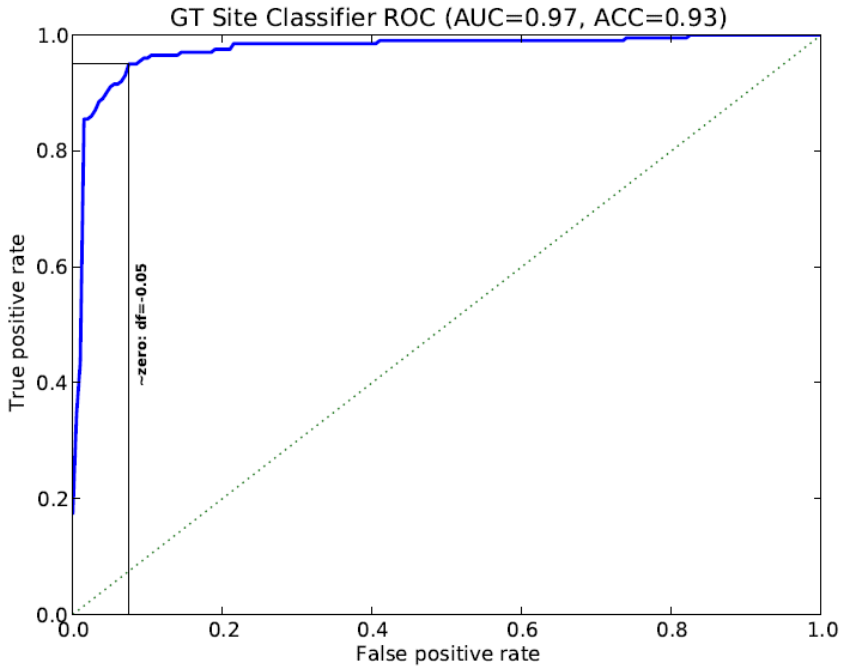


Figure 4.1: Example of an ROC curve generated by `generate_roc.py` for a GT classifier.

SpliceGrapher includes a script for generating ROC curves for classifiers. The basic command syntax is `generate_roc.py config-file`. This script reads the files generated by the parameter selection program, performs 5-fold cross- validation, and generates an ROC curve. For example:

```
generate_roc.py gt_don.cfg -o gt_roc.pdf
```

Example output is shown in the figure. It may not always be possible to train a classifier with strong ROC scores. In these cases it may be useful to set a decision function threshold for classifying splice sites. The `generate_roc.py` script can aid in this process. Using the `-D` option, one can see the True Positive rate associated with different decision function values. One can then set an appropriate threshold value in the classifier configuration (`.cfg`) file for classifying splice sites.

CHAPTER

FIVE

PREDICTING SPLICE GRAPHS

Splice graph prediction is *SpliceGrapher*'s main purpose. This process involves two key aspects: validating the available evidence and making confident predictions from all available evidence.

5.1 Filtering Spliced Alignments

A key aspect of splice graph prediction is ensuring the quality of the evidence being used. To this end, *SpliceGrapher* includes scripts for filtering spliced alignments in the SAM format. The first is `sam_filter.py` (which replaces `filter_sam_jct.py` in older versions). It takes as input a SAM file and a set of classifiers and produces a copy of the SAM file with false-positive sites removed:

```
sam_filter.py alignments.sam classifiers.zip -o filtered.sam
```

The script accepts the following options:

Option	Value	Description
-F	SAM file	File for storing false-positive records
-o	OUTPUT	Output file
-r	REPORT	Write classifier scores to file
-z		Apply gzip compression to output

By default, SAM records that include false-positive sites will be discarded. You may save these records to a separate file using the `-F` option. The `-r` option allows you to see the classifier scores for each splice site found in the input file: negative values show which sites were predicted as false-positives.

5.1.1 Collating SAM Files

For large volumes of RNA-Seq data, a single SAM file may be unwieldy. For this reason *SpliceGrapher* includes the script `sam_collate.py` that reads a SAM alignment file and writes the output to separate files, one for each chromosome.

```
sam_collate.py *.sam
```

Because SAM files can be large, the script can read gzipped files and with the `-z` option, it can write them as well.

Note: Most SpliceGrapher scripts expect SAM files to be sorted by chromosome and position within the chromosome. A command for sorting a SAM file is:

```
sort -k 3,3 -k 4,4n unsorted.sam > sorted.sam
```

Alternately you may use samtools for manipulating files prior to collating.

5.1.2 SpliceGrapher Depths Files

With version 0.2.4 we introduced SpliceGrapher *depths* files. These files encapsulate the coverage information from a SAM or BAM file in a compact format that SpliceGrapher can then use to make predictions or to generate plots. These depths files may be up to two orders of magnitude smaller than the original SAM or BAM file (e.g., from 5GB to 50MB) and thus can dramatically reduce running times for other SpliceGrapher scripts. To create a depths file from a sorted SAM or BAM file, use the `sam_to_depths.py` script:

```
sam_to_depths.py accepted_hits.bam -o accepted_hits.depths
```

Once a depths file has been created, you may use it in place of SAM files in other SpliceGrapher scripts, for example:

```
predict_graphs.py accepted_hits.depths -m h_sapiens.gtf -d predicted
```

Similarly, you may use a depths file in a `plotter.py` configuration file:

```
[Reads]
plot_type     = read_depth
source_file   = accepted_hits.depths
title_string  = %gene Read Coverage
```

Thus, `sam_to_depths.py` creates compact files that allow other scripts to run faster, and permits you to use SpliceGrapher equally well with either BAM or SAM files.

Note: to use `sam_to_depths.py` you must install the pysam package, available from code.google.com/p/pysam.

5.2 Creating Splice Graphs from Gene Models

Splice graphs are a key data structure for the *SpliceGrapher* package. They can be used to depict a gene model, a collection of EST alignments and of course, they provide the foundation for splice graph predictions. SpliceGrapher files are stored in a GFF format that is specific to these graphs.

The script `gene_model_to_splicegraph.py` accepts a GFF3 gene model annotation file as input and generates splice graphs as output. The output file may contain all the genes in the gene model file, or you may specify an output directory and write a separate file for each gene. For example, to generate splice graphs for all gene models in the `GENE_MODEL` file (from `SpliceGrapher.cfg`), the command would be:

```
gene_model_to_splicegraph.py -o gene_models.gff
```

To generate the same graphs into separate files in a directory `/home/mygraphs`, the command would be:

```
gene_model_to_splicegraph.py -d /home/mygraphs
```

It is often useful to organize splice graphs by chromosome, since reference sequences are separated that way. For example, instead of having a single top- level directory, you may have a directory for each chromosome, such as `/home/mygraphs/chr1, /home/mygraphs/chr2, ...`. In this case, simply use the `-c` option to specify the chromosome for which you want to generate graphs:

```
gene_model_to_splicegraph.py -d /home/mygraphs/chr3 -c chr3
```

Finally, you may use the script to generate splice graphs for a specific set of genes using the `-g` option. For example, if you were interested in generating graphs only for the Arabidopsis genes AT3G01100, AT3G01120, AT3G01460 and AT3G01490 into the local directory you would enter:

```
gene_model_to_splicegraph.py -d . -g AT3G01100,AT3G01120,AT3G01460,AT3G01490
```

Note: although GFF3 gene model annotations are becoming more standardized, not all organisms adhere to the same naming conventions. For example, UTR records may be given as 'three_prime_UTR' and 'five_prime_UTR', or

simply 'UTR' in which case the relative gene position must be inferred. Thus you may need to modify the GFF3 annotations prior to converting them to splice graphs if you want access to all gene features. (See the GeneModel module for more information.)

5.2.1 Converting GTF Annotations

GTF file functionality allows users to work easily with gene models in either GTF or GFF3 formats. We have replaced the `gtf2gff.py` script with `convert_models.py` that can convert gene models in GTF format to GFF3 format and vice versa. To convert from GTF to GFF3, the command is:

```
convert_models.py Homo_sapiens.gtf --gff3=Homo_sapiens.gff3
```

To convert from GFF3 to GTF, the command is:

```
convert_models.py Homo_sapiens.gff3 --gtf=Homo_sapiens.gtf
```

Note: for both GTF and GFF3 files, SpliceGrapher expects curated gene models from sites such as UCSC, ENSEMBL or the TAIR website. In many cases it can accept GTF files output from tools like Cufflinks, but this behavior is not guaranteed, as not all GTF or GFF3 files represent gene models. Also, some tools may not adhere strictly to the GTF or GFF3 standards. For more on these file formats, see GTF.

5.3 Creating Splice Graphs from ESTs

Popular EST alignment algorithms such as BLAST, BLAT and GMAP can produce output in PSL format. Consequently *SpliceGrapher* includes a script, `ests_to_splicegraph.py` that converts EST alignments in PSL format to a splice graph. The script operates very much like the `gene_model_to_splicegraph.py` script outlined above except that you must specify a PSL file:

```
ests_to_splicegraph.py gmap_alignments.psl -d est_splicegraphs
```

As before, you may specify a single output file or an output directory, and you may request specific gene names.

Alignment programs sometimes yield introns that are too short (for example exons may abut one another) and that may lead to spurious alternative splicing predictions. To address this issue, the script allows you to specify a minimum allowed intron length (default is 4nt). For example, if the smallest known intron for an organism is 10nt long, you might use:

```
ests_to_splicegraph.py gmap_alignments.psl -d est_splicegraphs -i 10
```

Alignments in the PSL file that infer introns shorter than this will not be included in the splice graphs.

5.4 `predict_graphs.py`

SpliceGrapher allows you to predict the splice graphs for every gene in a file of gene models at once. The `predict_graphs.py` script provides a simple interface for loading RNA-Seq alignments along with gene models to generate predictions for all genes that have read coverage. The predicted graphs will be created in files stored in subdirectories named for each chromosome in the gene models.

The basic format of the command is:

```
predict_graphs.py alignments-file [options]
```

Here, `alignments-file` may either be a SAM file, or starting with version 0.2.4 you may use a SpliceGrapher *depths* file (see *SpliceGrapher Depths Files*). This program accepts the following parameters:

Option	Value	Description
-d	Output dir	Top-level directory to hold output graphs
-m	Gene models	short-read alignments
-J	JMINDEPTH	Minimum coverage required across splice junctions
-T	THRESHOLD	Minimum depth threshold for accepting short-read clusters

Note: when used with SAM files, `predict_graphs.py` loads the complete file along with a complete set of gene models, so it can require a lot of memory (40GB or more for predictions across the human genome). To avoid this issue, first convert the SAM file into a SpliceGrapher depths file (*SpliceGrapher Depths Files*). Alternately you may split the SAM file (see *Collating SAM Files*) and gene models by chromosome and run `predict_graphs.py` on each chromosome separately.

5.5 predict_splicegraph.py

To predict splice graphs from diverse evidence sources, *SpliceGrapher* uses the script `predict_splicegraph.py`. Given annotations for a particular gene along with additional evidence such as EST alignments, ungapped short read alignments and splice junction alignments, it combines the information and uses inference rules to predict an output graph.

The program starts with a splice graph created from a gene model. To this you may add additional splice graphs (such as those generated from EST alignments) or short read alignment data. The output is a single splice graph that incorporates all of the data sources into a single predicted graph.

The basic format of the command is:

```
predict_splicegraph.py gene-model [options]
```

This program accepts the following parameters:

Option	Value	Description
-d	SAM file	short-read alignments
-J	JMINDEPTH	Minimum coverage required across splice junctions
-M	MINANCHOR	Minimum anchor size required for junction evidence
-s	GRAPHS	Comma-separated list of EST splice graph files
-T	THRESHOLD	Minimum depth threshold for accepting short-read clusters

Use the `-s` option to include additional splice graphs from EST alignments. For example, for the Human gene HES4, you might use:

```
predict_splicegraph.py HES4_model.gff -s HES4_ests.gff
```

To include RNA-Seq reads, specify a SAM alignment file using the `-d` option:

```
predict_splicegraph.py HES4_graph.gff -d chr1.sam.gz
```

The `-M` option provides flexibility beyond the anchor constraints placed on spliced alignments. Even if you specify a minimum anchor size for spliced alignment, the distribution of reads across a junction may not be uniform. This option tells the prediction program to use only junctions supported by at least one read with this many bases on each side:

```
predict_splicegraph.py HES4_graph.gff -d chr1.sam.gz -M 12
```

Finally, the `-T` option provides the prediction program with a threshold for treating short-read clusters as background noise. By default, *SpliceGrapher* accepts all short-read clusters as possible evidence.

5.5.1 Finding Splice Forms

SpliceGrapher also has the ability to identify annotated splice forms that are represented in a set of RNA-Seq data. It uses existing gene models to establish a set of known transcripts that are comprised of lists of nodes from a splice graph. By identifying splice junctions and nodes unique to each transcript, it can infer which transcripts are present in a set of aligned reads. The `find_splice_forms.py` script accepts as input a SAM file of RNA-Seq alignments and reports splice forms from annotated gene models where the RNA-Seq data provide sufficient evidence for them.:

```
find_splice_forms.py SAM-file [options]
```

The script uses the following parameters to assess all genes in a gene model file:

Option	Value	Description
-d	OUTDIR	Output directory (overrides -o)
-j	MINJCT	Minimum junction threshold
-o	OUTPUT	Output file
-O	OVERLAP	Minimum number of bases that a read cluster must overlap a feature
-t	THRESHOLD	Minimum average read coverage for clusters

Note that a gene model is absolutely required for making these predictions. The input gene model file may either be given as a command-line option (`-m`) or specified in your `SpliceGrapher.cfg` file.

Each splice form is represented by splice junctions or exons unique to that form. `find_splice_forms.py` loads RNA-Seq alignments and converts them into clusters of overlapping reads. When a cluster overlaps sequence that is unique to a single exon and that exon is unique to a single splice form, the splice form will be included in the final graph. Similarly, when spliced reads fall across a splice junction that is unique to a particular splice form, the splice form will be included in the final graph.

Three parameters control the sensitivity/specificity of this program: minimum average cluster read coverage (`-t`), a minimum number of splice junction reads (`-j`), and the minimum required cluster overlap (`-O`). Read clusters must have the desired minimum coverage before they are used to identify splice forms. Likewise, splice junctions must have the given minimum number of reads before they are used to identify forms. In cases where the RNA-Seq coverage does not uniquely cover any particular splice forms, no output graph will be produced.

You may specify an output file (`-o`), in which case all splice graphs will be written to the same file. The recommended approach is instead to specify an output directory (`-d`) in which case a separate file will be written for each gene for which a graph is produced.

Features That Uniquely Identify Splice Forms

In this context, a *feature* is that part of an exon that is completely unique to a particular splice form. Clusters must overlap unique exon *features* by a minimum amount (the default is 1, but a higher number is recommended for better specificity). For example, an exon that represents a retained intron will typically have as its unique feature that region from the start to the end of the associated intron. Some unique features may be smaller than the minimum overlap, for example, when the minimum is set to 10 and an alternative 3' site is a NAGNAG site whose unique feature is just 3nt long. For this reason, SpliceGrapher uses the smaller of the overlap size or the feature length.

CHAPTER

SIX

VIEWING SPLICE GRAPHS

6.1 Simple Viewing Tool

SpliceGrapher includes two scripts for viewing splice graphs. These scripts also serve as examples of how to use the SpliceGrapher viewing modules, many of which have been encapsulated in the `ViewerUtils` module. The simplest way to view one or more splice graphs is to use the `view_splicegraphs.py` script. For example, to view the graph stored in `gene_graph.gff`, simply enter:

```
view_splicegraphs.py gene_graph.gff
```

This produces a splice graph with minimal annotations. The script will accept more than one file on the command line. For example, to plot the files `AT1G01020.gff`, `AT1G01030.gff`, `AT1G01040.gff` and `AT1G01060.gff`, one might enter either of the following commands:

```
view_splicegraphs.py AT1G010[2346]0.gff
view_splicegraphs.py AT1G010*.gff
```

This can be powerful if you want to compare graph predictions for the same gene across different data sets (such as for different tissue samples or mutants):

```
view_splicegraphs.py data/var[1-3]/AT1G01020.gff
```

The script provides a selection of options that allow you to make the output graph as elaborate as you like:

Option	Value	Description
-a		(Re)annotate graphs
-m	MODEL	GFF gene model reference
-o	OUTPUT	Output file
-x		Show genomic positions of graph elements
-E	EDGE	Intron edge weight
-H	HEIGHT	Display area height, in inches
-W	WIDTH	Display area width, in inches
-L		Add legend to splice graph plot
-F	FONTSIZE	Font size for plot titles
-X		Use the same genomic position range for each plot
-S		Shrink introns

For example, to generate a more elaborate plot for the comparison above and write the output to a PDF file, one could enter:

```
view_splicegraphs.py data/var[1-3]/AT1G01020.gff -xL -t var1,var2,var3 -o comparison.pdf
```

6.1.1 Shrinking Introns

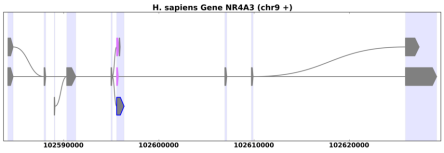


Figure 6.1: Example of a splice graph for a human gene with large introns.

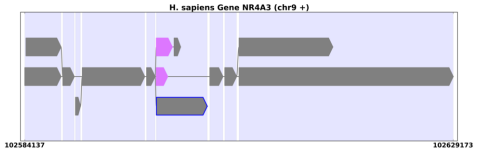


Figure 6.2: The same human gene with introns reduced to emphasize exons.

Many organisms, especially mammals, have introns that are much longer than exons. This can make it difficult to see alternative splicing behavior on these plots. Figure 8.1 shows an example of a human gene that has large introns relative to its exons as generated from the command:

```
view_splicegraphs.py -m chr9.gff.gz NR4A3
```

You can change the way these appear in `view_splicegraphs.py` using the `-S` option (Figure 8.2):

```
view_splicegraphs.py -m chr9.gff.gz NR4A3 -S
```

6.1.2 Plot Types

SpliceGrapher can produce different kinds of plots for different kinds of data. These are: splice graphs, isoform plots, read coverage plots, splice junction plots and X-Y plots. These are described in more detail below:

6.1.3 Splice Graphs

Splice graphs are *SpliceGrapher's* principal plot type. These depict splice graphs that represent all the different ways in which a gene's exons may be combined.

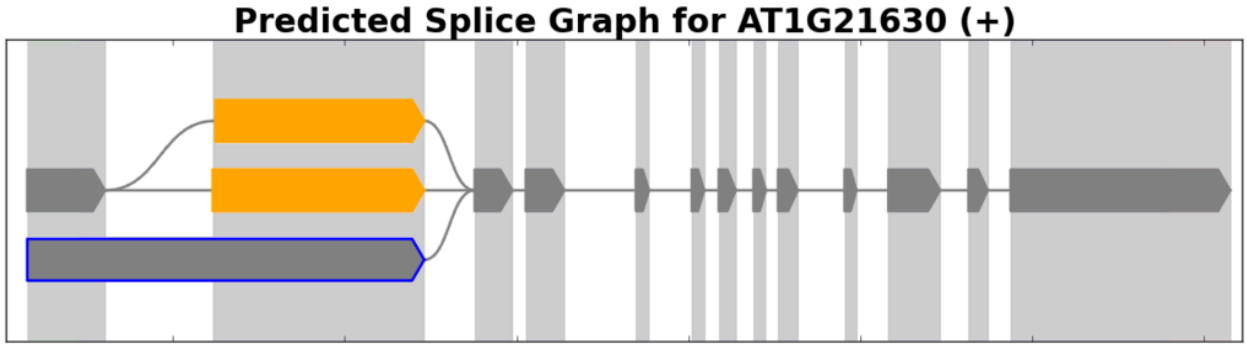


Figure 6.3: Example of a splice graph for a gene from the plant *A. thaliana*.

6.1.4 Isoforms

Isoform graphs are similar to splice graphs in the way that introns and exons are depicted. However, instead of plotting the graph in its compact representation, isoform graphs show each possible splice form from a graph. These are particularly useful for showing splice forms represented in RNA-Seq data (see *Finding Splice Forms*).

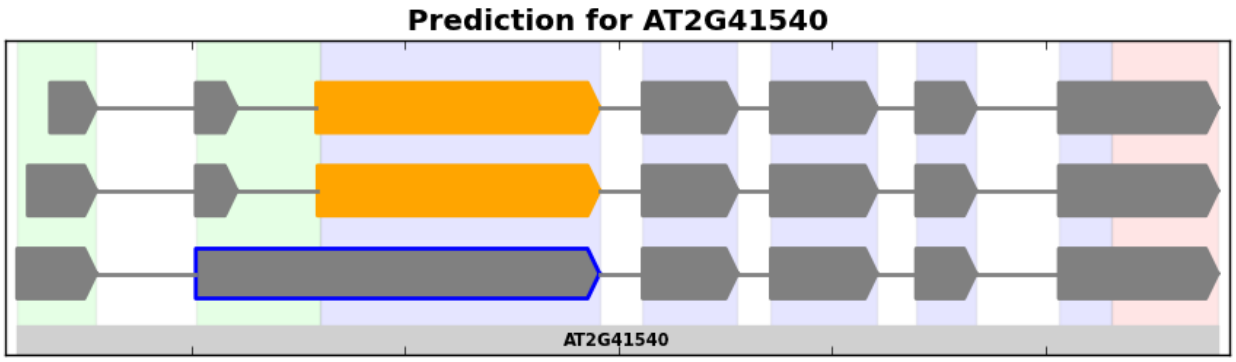


Figure 6.4: Example of an isoform graph for a gene from the plant *A. thaliana*.

6.1.5 Read Coverage

Read coverage graphs depict the way RNA-Seq reads aligned to a gene region. The height of the graph at any given position represents the number of reads whose alignments overlap at that point.

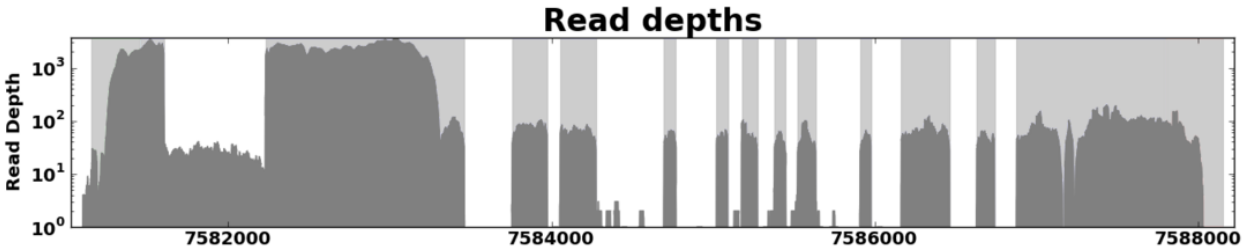


Figure 6.5: Example of a read coverage graph for a gene from *A. thaliana*.

6.1.6 Splice Junction Coverage

Splice junction coverage graphs depict the way RNA-Seq reads aligned across splice junctions within a gene region. Splice junctions are depicted as '^'-shaped widgets flanked by bars that depict the anchor regions on either side of a junction. Labels for each splice junction show the number of reads that aligned across each junction.

6.1.7 X-Y Plots

SpliceGrapher also provides a generic X-Y plot that can depict arbitrary values across a gene region. These could be used, for example, to plot values from other forms of genomic activity (such as methylation) to see if there is any correlation between alternative splicing and these other phenomena.

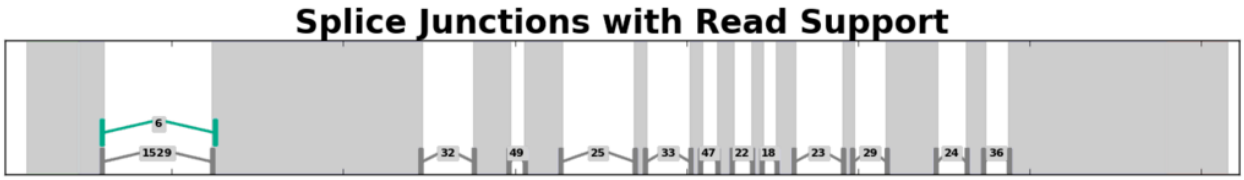


Figure 6.6: Example of a splice junction coverage graph for a gene from *A. thaliana*.

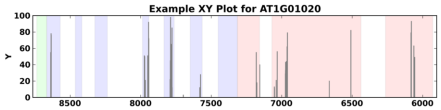


Figure 6.7: Example of an X-Y graph for a gene from *A. thaliana* using randomly-generated values between 1 and 100 to simulate activity within the gene.

6.2 Multiple Plots

For more elaborate plots, *SpliceGrapher* includes a second script designed to show a predicted splice graph along with its accompanying evidence. It accepts a variety of data formats as input and can produce pages with one to four plots. Each of these plots requires a separate input file.

- Predicted splice graph (*SpliceGrapher* GFF format)
- Original gene model splice graph (*SpliceGrapher* GFF format)
- Short read depths (SAM format)
- (Optional) splice junctions with short read support (SAM format)

6.2.1 File Options

The options for this script are organized into file options and display options. File options tell the script what the kinds of input files to expect and how to render the output graphs. You may include any combination of input files and display them in any order (see Display Options below).

File Options	Value	Description
-m	MODEL	Gene models (GTF or GFF3 format)
-o	OUTPUT	Output plot file (infers format from file extension)
-d	DEPTH_FILE	SAM alignments (ungapped and spliced reads)
-s	SPLICE_GRAPH	GFF splice graph file
-G	ORIG_GRAPH	Optional original graph file
-X	XYDATA	Data file of X,Y value pairs for a simple graph

Note: SAM alignments may be specified in two ways. Some pipelines may produce separate files for ungapped alignments and spliced alignments, in which case both the -d and -j options should be used. Other methods (notably *TopHat*) produce a single file that contains both ungapped and spliced alignments, in which case just the -d option should be used.

6.2.2 Display Options

The display options are nearly the same as those for `view_splicegraphs.py`:

Display Options	Value	Description
-c		Add read coverage labels to junctions
-x		Add genomic position labels to plots
-E	EDGE	Minimum edge thickness
-J	MINJCT	Minimum junction depth
-L		Add a legend to graph
-H	HEIGHT	Display window height (inches)
-W	WIDTH	Display window width (inches)
-F	FONTSIZE	Font size for plot titles
-S		Shrink introns relative to exons
-D	DISPLAY	Display order string for plots

As an example, suppose we have used *SpliceGrapher* to predict a splice graph for the gene G123. We used `gene_model_to_splicegraph.py` to generate a splice graph for the gene model and stored it in `G123m.gff`, generated splice junction and read depth files in SAM format and used `predict_splicegraph.py` to generate a splice graph prediction and stored it in `G123p.gff`. To produce a four-panel plot of the predicted graph along with its evidence, we could enter:

```
view_splicegraph_multiplot.py G123 -d chr1.sam -G G123m.gff -s G123p.gff
```

Note that the background for each of the panels includes a color-coded synopsis of the gene model. Thus we may choose to omit the gene model graph from the output and focus instead on the prediction and its evidence. In addition, suppose we wish to see the evidence at the top of the page and the predicted splice graph at the bottom. We may change all of these characteristics in a single step using the `-D` option. It accepts a string that determines the display order and whether or not to display each plot. Each character represents a specific plot: 'O' for the original gene model, 'P' for the predicted splice graph, 'J' for the splice junction plot, and 'R' for the read depth plot. The default display order is 'OPJR', so to modify a graph to omit the original graph and change the order, simply enter the string 'RJP':

```
view_splicegraph_multiplot.py G123 -d chr1.sam -G G123m.gff -s G123p.gff -D RJP
```

6.3 High-Quality Plotting

In addition to viewing utilities, *SpliceGrapher* provides plotting utilities that allow you to tune your plotting parameters for a specific purpose (such as figures suitable for a talk or paper) and save your configuration for later use. The program is called simply `plotter.py` and has the following format:

```
plotter.py gene.cfg
```

The plotting utility has a few options that allow you to try different output parameters such as page dimensions, but because the program provides a rich set of options, it is more convenient to store these in a configuration file. Here is an example:

```
[SinglePlotConfig]
legend         = True
shrink_introns = True
height         = 5.0
width          = 18.0
fontsize       = 16
output_file    = NR4A3.pdf

[GeneModelGraph]
plot_type     = gene
gene_name     = NR4A3
relative_size = 10.0
```

```
source_file   = ${GENE_MODELS}/chr9.gff3.gz
file_format   = gene_model
title_string  = Gene Model for %gene

[SpliceGrapher]
plot_type     = splice_graph
relative_size = 10.0
source_file   = ${GRAPHS}/NR4A3_pred.gff
title_string  = SpliceGrapher Prediction for %gene

[Reads]
plot_type     = read_depth
relative_size = 8.0
source_file   = ${SAM}/chr9.sam.gz
title_string  = %gene Read Coverage
```

6.3.1 Main Section

The only required section is the main `[SinglePlotConfig]` section that provides general parameters for producing a single plot of one or more panels. The remaining sections each identify a distinct panel that will appear on the same plot in the output file. You may specify as many of these panels as you wish, provided each one has a unique name. The main difference between the graph sections and the main section is the parameters that are allowed for each section. *SpliceGrapher* enforces strict typing on these parameters and values.

Parameters for the `[SinglePlotConfig]` section are:

Name	Type	Description
fontsize	int	Font size for title strings
legend	boolean	Include legend on all plots
log_file	string	Write error messages to the given log file instead of stderr
height	float	Page height (in inches)
output_file	string	Path to output file
shrink_introns	boolean	Shrink introns relative to exons
width	float	Page width (in inches)

6.3.2 Plot Sections

Parameters for individual graphs are:

Name	Type	Description
acceptors	string	Comma-delimited list of acceptor sites to highlight (junctions only)
background	boolean	Show the gene model in the plot background
clusters	boolean	Show read depths as whole clusters (read_depth only)
donors	string	Comma-delimited list of donor sites to highlight (junctions only)
edge_weight	int	Edge weight to use when drawing intron edges
file_format	string	Type of data file to use (gene_plot only)
gene_name	string	Gene name to use (gene_plot only)
hide	boolean	Use the gene information but do not display the plot (gene_plot only)
highlight	string	Comma-delimited list of regions to highlight (read_depth only)
labels	boolean	Plot-dependent labeling: exon ids for splice graphs, read coverage for junctions
min_coverage	float	Minimum read coverage required for showing junctions
relative_size	float	Relative plot size
plot_type	string	One of: gene/isoforms/junctions/read_depth/splice_graph/xy_plot
source_file	string	Path to file containing data for this plot
title_string	string	Text to use for plot title
x_labels	boolean	Show positions for all features in plot

Note: A gene model section is required for all plots that use shaded bars in the background. To prevent the gene model itself from plotting, use the `hide` option.

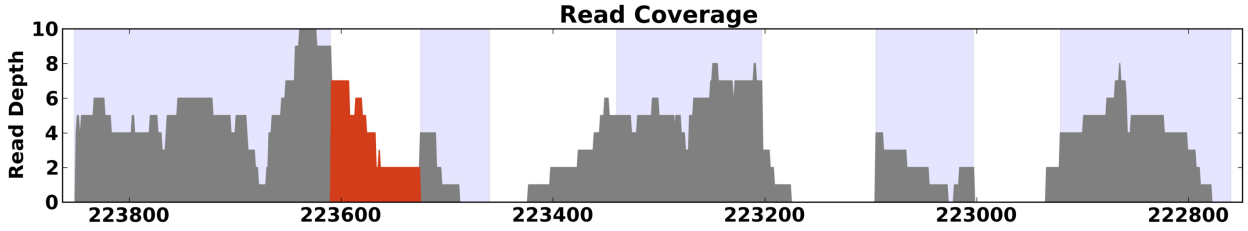


Figure 6.8: Example of read depth plot with highlighting turned on to highlight possible intron retention activity.

6.3.3 Source Files for Plots

Each plot type is associated with specific file formats:

Plot Type	File Format	Description
gene	gene model GFF	Plots a gene model as a splice graph
isoforms	splice graph GFF	Plots elements in a splice graph as distinct splice forms
junctions	SAM/depths	Depicts all splice junctions from alignments
read_depth	SAM/depths	Depicts read coverage based on alignments
splice_graph	splice graph GFF	Plots a splice graph
xy_plot	CSV	Plots X-Y values

Note: if you use SAM files, the plotting tools expect them to be sorted. If you provide an unsorted SAM file to `plotter.py`, you may see nothing in the splice junctions or read depth sections of your plot. See *Collating SAM Files* for information on how to collate and sort SAM files.

6.3.4 Output Options

Although the configuration file allows you to specify output settings such as page width and height and font size, you may wish to try different settings to get a figure to look the way you want. Thus `plotter.py` provides command-line options to change these settings:

Option	Value	Description
-F	FONTSIZE	Font size (12-point)
-H	HEIGHT	Plotting area width (11.0 inches)
-W	WIDTH	Plotting area width (8.5 inches)
-o	OUTPUT	Output file

By default, a plot will appear on the screen, but if you specify an output file, `plotter.py` will output to the file, using the file extension to determine the file format. Valid file extensions are: emf, eps, pdf, png, ps, raw, rgba, svg and svgz. These command-line settings will override settings in the configuration file. If no settings are specified in either place, the defaults are used.

CHAPTER

SEVEN

REALIGNMENT PIPELINE

Initially, SpliceGrapherXT predicts novel exons conservatively and records information about those it could not resolve due to ambiguous combinations of acceptor and donor sites. We wish to resolve those ambiguous loci by realigning RNA-Seq reads to a database of putative transcripts in order to find compelling evidence for exons that SpliceGrapherXT could not resolved in its inference step. We thus created a pipeline that constructs putative transcripts from unresolved exons, aligns reads to these transcripts, and resolves exons that have sufficient coverage from the alignments.

The pipeline proceeds in three stages: first it generates putative transcripts using unresolved exons; next it realigns reads to the putative transcripts using BWA, and finally it resolves novel exons when realigned reads completely span the exon and its boundaries.

The script for running the pipeline is `realignment_pipeline.py` and has the following format:

```
realignment_pipeline.py original-dir
```

Here the original directory is the directory containing original predictions created with `predict_graphs.py`. The realignment pipeline may be used with either single-end or paired-end reads, as follows. For single-end read, merely specify a single FASTQ file:

```
realignment_pipeline.py original_predictions -1 myreads.fq
```

For paired-end reads, specify two FASTQ files, where the second file contains the mate pairs for the reads in the first file:

```
realignment_pipeline.py original_predictions -1 myreads_1.fq -2 myreads_2.fq
```

The pipeline script has just a few options:

Option	Value	Description
-1	FASTQ	Reads file or first set of mate pairs
-2	FASTQ	Second set of mate pairs (paired-end only)
-d	OUTDIR	Output directory for updated predictions
-f	FASTA	Genome reference file
-m	MODEL	Gene model annotations

Below is output from an example run on 100-nt paired-end reads (some paths have been shortened for readability)

```
realignment_pipeline.py sgxt_pred -1 reads_1.fq -2 reads_2.fq -v -d realigned

03:46:33 realignment_pipeline.py Started

  Generating putative transcript database
    03:46:33 generate_putative_sequences.py sgxt_pred.lis -Uo putative.fa
                    -m putative_transcripts.map
```

```
Running BWA alignments on FASTQ files using 4/8 CPUs
  03:46:35 bwa aln -f reads_1.aln -t 4 -M 8 -R 2 -o 0 -e 0 -d 0
                   -i 100 putative.fa reads_1.fq
  03:46:38 bwa aln -f reads_2.aln -t 4 -M 8 -R 2 -o 0 -e 0 -d 0
                   -i 100 putative.fa reads_2.fq
  03:46:42 bwa sampe -n 2 -N 2 -P -f pair_1.raw putative.fa
              reads_1.aln reads_2.aln reads_1.fq reads_2.fq
  03:46:49 get_good_pairs.py pair_1.raw pair_1.pairs
Updating original predictions.
  03:47:10 fix_unresolved.py sgxt_pred.lis putative_transcripts.map
              filtered_bwa.sam -f putative.fa -c -d realigned -v
Finished.
```

The pipeline script shows each of the commands it uses as it runs. Note that it expects the BWA alignment software to be in the user's path. The first step generates a putative transcript database that consists of two files: `putative.fa` is a FASTA file that contains the putative sequences along with metadata in the sequence headers such as unique identifiers that aid in post-processing. `putative_transcripts.map` provides a mapping between the transcript identifiers and the splice graph nodes from which they were generated.

The second step is the BWA alignment phase, in which FASTQ reads are aligned to the putative transcriptome. In the case of paired-end reads, there is an additional step to ensure that read alignments adhere to strict constraints. The final step updates the graphs by searching for unresolved exons that may be resolved when the aligned reads completely cover an exon and its boundaries. The updated graphs are then placed in the output directory. See [ROGERS2013] for more details on the realignment procedure.

CHAPTER

EIGHT

TRANSCRIPT PREDICTION PIPELINES

SpliceGrapherXT also provides two methods for predicting transcripts that are novel relative to the input gene models. The basis of these methods is to use predicted splice graphs to generate a set of putative transcripts that we can provide to one of two other methods—PSGInfer or IsoLasso—to predict transcript frequencies or expression levels. SpliceGrapherXT then predicts novel transcripts whenever a putative transcript has a substantial frequency or estimated expression level. The approach is similar to the realignment procedure, but instead of generating putative transcripts only for graphs with unresolved nodes, it generates transcripts by traversing all the paths in every graph that has multiple paths. In this case, these putative transcripts are stored as a set of gene models that are then provided to other tools to predict either probabilistic splice graphs or transcript expression levels.

In principle this approach could used with any tool that predicts transcript expression levels. SpliceGrapherXT provides pipelines for two such approaches: PSGInfer (see [LEGAULT2013]) and IsoLasso (see [LI2011]).

8.1 PSGInfer Pipeline

The PSGInfer pipeline uses a list of splice graphs and two FASTQ files as input, and produces splice graphs that include novel predicted transcript annotations:

```
psginfer_pipeline.py graph-list fastq1 fastq2
```

Example:

```
psginfer_pipeline.py initial.lis reads_1.fq reads_2.fq
```

The script accepts the following options:

Option	Value	Description
-d	OUTDIR	Output directory for updated predictions
-f	FASTA	Genome reference file
-l	LOGFILE	Optional log file [default: none]
-t	THRESH	Probability threshold [default: 0.01]

Below is output from an example run on 100-nt paired-end reads (some paths have been shortened for readability):

```
psginfer_pipeline.py sgxt_pred.lis reads_1.fq reads_2.fq -d psg_updated
              -t 0.15 -v -l psginfer_update.log
  05:45:41 psginfer_pipeline.py Started
  05:45:51 generate_putative_sequences.py sgxt_pred.lis -M putative_forms.gtf
              -m putative_forms.map -UA
Changing directories to /tmp/test/FASTA_REF
  05:45:54 psg_prepare_reference.py putative_forms.gtf putative_forms_psg_reference
  05:46:43 psg_infer_frequencies.py reads_1.fq reads_2.fq
              putative_forms_psg_reference putative_forms_psg_results --num-CPUs=2
```

```
Changing directories back to /tmp/test
  05:52:01 psginfer_update_graphs.py sgxt_pred.lis putative_forms.map
              FASTA_REF/putative_forms_psg_results/isoform.results.txt
              -vd psg_updated -t 0.15
Finished.
```

The first task in this pipeline is to generate a set of putative transcripts, including those that involve unresolved exons. As in the realignment pipeline, this creates a FASTA file of transcript sequences, along with a file that maps transcript identifiers to their corresponding splice graph nodes. Next it runs the PSGInfer scripts that prepare reference graphs and infer frequencies over those graphs from the RNA-Seq data (for details, see [LEGAULT2013]). In the last step, SpliceGrapherXT infers legitimate transcripts using the frequencies assigned by PSGInfer.

Note: the SpliceGrapherXT pipeline requires PSGInfer version 1.1.3.

8.2 IsoLasso Pipeline

The IsoLasso pipeline is similar to the PSGInfer pipeline, but uses alignments in SAM format instead of short reads:

```
isolasso_pipeline.py graph-files SAM-file
```

The pipeline provides access to two IsoLasso methods: the original method and a newer CEM algorithm. Both methods are highly dependent on their input parameters. The pipeline uses default parameters that have been tuned extensively on our own data; however, the pipeline includes a pass-through mechanism that allows users to access IsoLasso parameters if desired.

Option	Value	Description
-C		Use CEM instead of LASSO
-d	OUTDIR	Output directory
-p	PARAMS	Additional parameters for IsoLasso
-t	THRESH	Minimum FPKM threshold
-U		Include unresolved transcripts

Example using the original IsoLasso method:

```
isolasso_pipeline.py sgxt_pred.lis filtered.sam -d psg_updated -t 100
```

Example using the IsoLasso/CEM method:

```
isolasso_pipeline.py sgxt_pred.lis filtered.dam -Cd cem_updated -t 100
```

Example output:

```
  11:15:00 isolasso_pipeline.py Started
  11:15:00 generate_putative_sequences.py sgxt_pred.lis -AU
            -M initial_predictions_forms.gtf -m initial_predictions_forms.map
  11:15:02 gtfToGenePred initial_predictions_forms.gtf stdout | genePredToBed |
            sort -k1,1 -k2,2n > initial_predictions_forms.bed
  11:15:03 runlasso.py -x initial_predictions_forms.bed --forceref --useem
            -o cem_update filtered.sam
  11:15:14 isolasso_update_graphs.py initial_predictions.lis initial_predictions_forms.map
            cem_update.pred.gtf -t 1.00 -d cem_update
Finished.
```

Both the PSGInfer and IsoLasso pipelines will add new transcript identifiers to the splice graphs whenever the associated method predicts a sufficient expression level for a novel transcript (based on the threshold). These updated graphs may then be converted back into gene models and used, for example, to assess differential expression levels.

CHAPTER

NINE

FILE FORMATS

Given the variety of data used to generate predictions, *SpliceGrapher* uses several different file formats to process data.

9.1 Gene Model Files

9.1.1 GFF3 Files

Gene models are often stored using the GFF3 format and may contain a wide variety of different record types, though *SpliceGrapher* uses only a subset of these types:

- *chromosome* records are used to establish boundaries for all genes within a chromosome, but may also be inferred from the genes found within a chromosome.
- *gene* records are required to establish parent/child relationships within a gene using the "ID" attribute in the last column. A gene record is assumed to have one or more children given as exon records.
- *mRNA* records are used to infer exon and intron boundaries as well as parent/child relationships. Each record must have an "ID" attribute and a "Parent" attribute that refers to its gene. An mRNA record is assumed to have one or more children given as UTR or CDS records.
- *exon* records are used to infer exon and intron boundaries as well as parent/child relationships. Each record must have an "ID" attribute and a "Parent" attribute that refers to its gene.
- *CDS* records (CDS, UTR, FIVE_PRIME_UTR, or THREE_PRIME_UTR) are used to infer exon and intron boundaries as well as parent/child relationships. Each record must have an "ID" attribute and a "Parent" attribute that refers to its mRNA parent.

9.1.2 GTF Files

The annotations for many organisms (notably those on the ENSEMBL website) use GTF format instead. *SpliceGrapher* can load either GFF3 or GTF files directly.

9.2 Splice Graph Files

SpliceGrapher also uses the GFF format to store splice graphs. The GFF format is a convenient way to express all the parent/child relationships in a graph, along with annotations for alternative splicing analysis and visualization purposes. A *SpliceGrapher* GFF file uses the following record types:

- *graph* records define a splice graph. These records include attributes that include the gene name and a brief description of how the splice graph differs from the gene models. Note that this replaces the old *cluster* name, which has been deprecated.
- *parent* records define exons in a splice graph that act as root nodes in the graph.
- *child* records define all other exons in a graph, including leaf nodes.

Both *parent* and *child* records include attributes that describe unique identifiers for each exon, the splicing forms that include them, and the alternative splicing forms associated with them. Child records also include their parents' unique identifiers. Many of these attributes are used by *SpliceGrapher's* visualization modules.

9.3 SAM and BAM Files

SAM files are similar to GFF files but are used to store short-read depths after reads have been aligned to a genome (for a complete description, see http://samtools.sourceforge.net/SAM1.pdf). BAM files are simply compressed, binary versions of SAM files. With version 0.2.4, SpliceGrapher provides the `sam_to_depths.py` script for converting SAM or BAM files into an even more compact *depths* format (see *SpliceGrapher Depths Files*). *SpliceGrapher* uses short read alignments stored in the SAM format or depths format to predict splice graphs and to display read depths and splice junctions on plots.

9.4 BED and WIG Files

Some spliced alignment tools use BED and WIG files to store information about short-read alignments and splice junctions that have short-read support. *SpliceGrapher* can read these files to predict splice graphs and to display read depths and splice junctions on plots. For more information on these file formats, see http://genome.ucsc.edu/FAQ/FAQformat.html.

9.5 PSL Files

Programs that perform EST or cDNA alignments, such as BLAST and GMAP, can produce alignment information in PSL format. *SpliceGrapher* uses PSL files to construct splice graphs from these alignments that may then be merged with other graphs to make predictions. For details on the PSL file format, see http://fungi.ensembl.org/info/website/upload/psl.html.

CHAPTER

TEN

PRE-BUILT CLASSIFIERS

SpliceGrapher comes with a set of pre-built classifiers for over 100 different species. Only classifiers with adequate performance are included, so some species will have classifiers for canonical (GT donors/AG acceptors) and semi-canonical (GC donors) sites while others will have classifiers only for canonical sites. This table below shows the ROC scores corresponding to the each of the pre-built classifiers:

Species	GT	GC	AG
Acyrthosiphon pisum	0.96	0.94	
Aedes aegypti	0.96	0.95	
Amphimedon queenslandica	0.99	0.99	
Anolis carolinensis	0.98	0.96	
Anopheles gambiae	0.96	0.95	
Apis mellifera	0.98	0.96	
Arabidopsis thaliana	0.97	0.95	
Ashbya gossypii	0.99	0.96	
Aspergillus clavatus	0.98	0.93	
Aspergillus flavus	0.96	0.92	
Aspergillus fumigatus	0.97	0.90	
Aspergillus fumigatusa1163	0.95	0.92	
Aspergillus nidulans	0.96	0.91	
Aspergillus terreus	0.97	0.93	
Bombyx mori	0.98	0.98	
Bos taurus	0.98	0.96	
Botryotinia fuckeliana	0.98	0.95	
Brachypodium distachyon	0.98	0.96	
Caenorhabditis brenneri	0.97	0.98	
Caenorhabditis briggsae	0.95	0.97	
Caenorhabditis elegans	0.97	0.98	
Caenorhabditis remanei	0.97	0.98	
Canis familiaris	0.98	0.95	
Cavia porcellus	0.97	0.96	
Choloepus hoffmanni	0.98	0.96	
Ciona intestinalis	0.98	0.96	
Culex quinquefasciatus	0.96	0.97	
Danaus plexippus	0.97	0.96	
Danio rerio	0.98	0.96	
Daphnia pulex	0.98	0.95	
Dasypus novemcinctus	0.97	0.96	
Dictyostelium discoideum	0.99	0.99	

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Table 10.1 – continued from previous page

Species	GT	GC	AG
Dipodomys ordii	0.98	0.97	
Drosophila ananassae	0.98	0.96	
Drosophila erecta	0.98	0.97	
Drosophila grimshawi	0.98	0.96	
Drosophila melanogaster	0.98	0.96	
Drosophila mojavensis	0.97	0.96	
Drosophila persimilis	0.96	0.96	
Drosophila pseudoobscura	0.98	0.96	
Drosophila sechellia	0.97	0.96	
Drosophila simulans	0.97	0.96	
Drosophila virilis	0.98	0.97	
Drosophila willistoni	0.97	0.96	
Drosophila yakuba	0.98	0.97	
Echinops telfairi	0.98	0.96	
Entamoeba histolytica	0.99	0.96	
Equus caballus	0.95	0.95	
Erinaceus europaeus	0.98	0.96	
Felis catus	0.98	0.96	
Fusarium oxysporum	0.96	0.92	
Gadus morhua	0.98	0.96	
Gallus gallus	0.97	0.95	
Gasterosteus aculeatus	0.96	0.95	
Gibberella moniliformis	0.97	0.93	
Gibberella zeae	0.98	0.94	
Gorilla gorilla	0.97	0.95	
Heliconius melpomene	0.96	0.96	
Homo sapiens	0.98	0.96	
Ixodes scapularis	0.94	0.93	
Latimeria chalumnae	0.97	0.94	
Loxodonta africana	0.96	0.95	
Macaca mulatta	0.95	0.93	
Macropus eugenii	0.97	0.96	
Magnaporthe oryzae	0.97	0.93	
Meleagris gallopavo	0.97	0.94	
Microcebus murinus	0.97	0.96	
Mus musculus	0.98	0.96	
Myotis lucifugus	0.97	0.97	
Nematostella vectensis	0.97	0.96	
Neosartorya fischeri	0.98	0.93	
Neurospora crassa	0.96	0.94	
Nomascus leucogenys	0.96	0.94	
Ochotona princeps	0.98	0.96	
Ornithorhynchus anatinus	0.96	0.94	
Oryctolagus cuniculus	0.97	0.96	
Oryza sativa	0.95	0.94	
Oryzias latipes	0.96	0.95	
Otolemur garnettii	0.97	0.96	
Pan troglodytes	0.98	0.96	
Petromyzon marinus	0.95	0.93	

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Table 10.1 – continued from previous page

Species	GT	GC	AG
Physcomitrella patens	0.98	0.95	
Plasmodium berghei	0.94	0.99	
Plasmodium chabaudi	0.96	0.98	
Plasmodium falciparum	0.96	0.98	
Pristionchus pacificus	0.94	0.98	
Procavia capensis	0.98	0.96	
Pteropus vampyrus	0.98	0.97	
Pythium ultimum	0.95	0.95	
Rattus norvegicus	0.95	0.93	
Saccharomyces cerevisiae	0.99	0.92	
Sarcophilus harrisii	0.97	0.95	
Schistosoma mansoni	0.96	0.94	
Schizosaccharomyces pombe	0.99	0.95	
Sclerotinia sclerotiorum	0.95	0.90	
Sorex araneus	0.98	0.96	
Sorghum bicolor	0.97	0.96	
Sus scrofa	0.97	0.96	
Takifugu rubripes	0.96	0.92	
Tarsius syrichta	0.99	0.97	
Tetraodon nigroviridis	0.96	0.95	
Toxoplasma gondii	0.98	0.98	
Tribolium castaneum	0.96	0.96	
Trichoplax adhaerens	0.96	0.93	
Tupaia belangeri	0.97	0.96	
Tursiops truncatus	0.98	0.97	
Vicugna pacos	0.98	0.96	
Xenopus tropicalis	0.96	0.94	
Zea mays	0.96	0.94	

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