Evaluation of coding gene annotations: tools and measurements

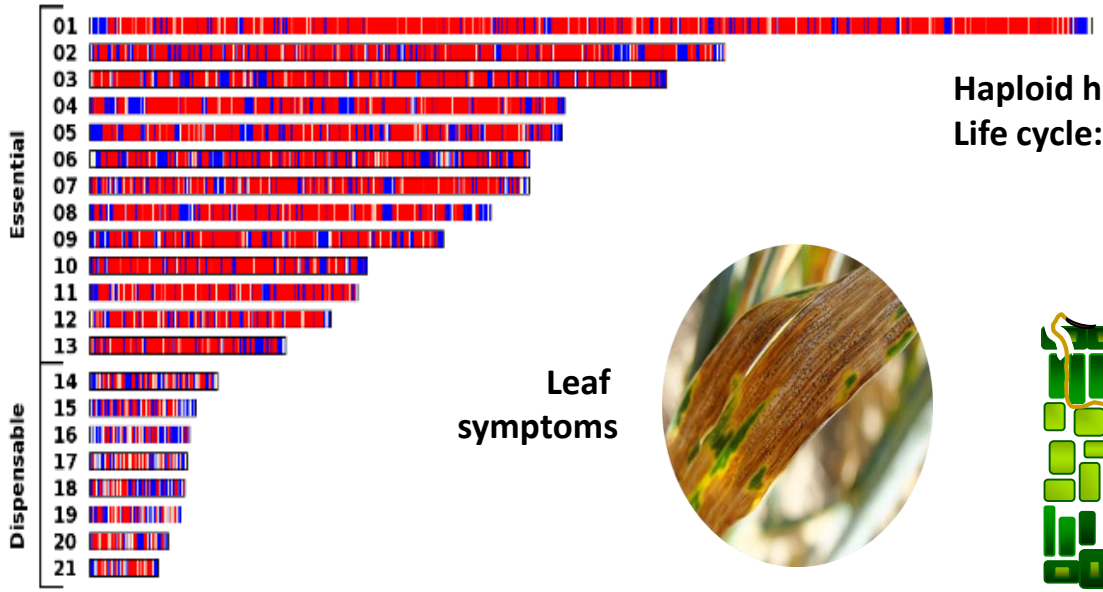
Nicolas Lapalu –PEPI IBIS

14-15th September 2023

Biological context: *Zymoseptoria tritici,* a wheat pathogen

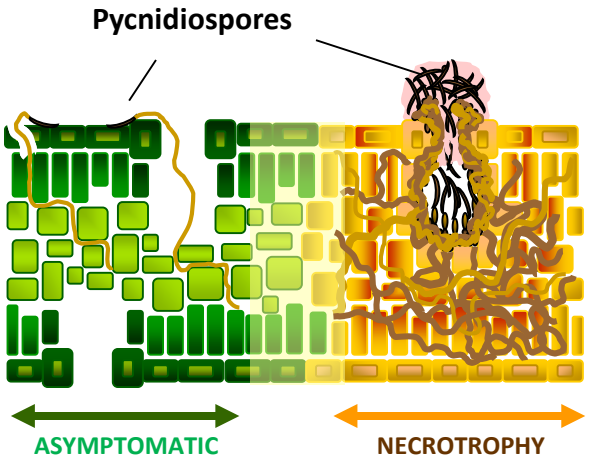
Fully sequenced genome* 21 chromosomes,

39.7 Mb, 18 % transposons



Haploid hemi-biotrophic ascomycete
Life cycle: sexual and asexual reproduction

Leaf symptoms



* Goodwin, et al 2011. Finished genome of the fungal wheat pathogen Mycosphaerella graminicola reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. PLoS Genet.

Genome annotation releases of *Z.tritici* IPO323

Several annotations were obtained, each with a specific method (ab initio prediction software, evidence used, pipeline)

1) JGI: Release 1, GeneWise and FGenesh ab initio software, EST and protein evidence

Published in 2011*, 10849 genes

2) MPI: Release 2, EVM, PASA and GeneWise ab initio software, RNA-Seq and protein evidence, JGI predictions kept

Published in 2015*, 11712 genes

3) - CURTIN, 13922 genes, CodingQuarry ab initio software using RNA-Seq data, RNA-Seq and protein evidence

- RRES, 13583 genes , Maker2 ab initio software, RNA-Seq and protein evidence

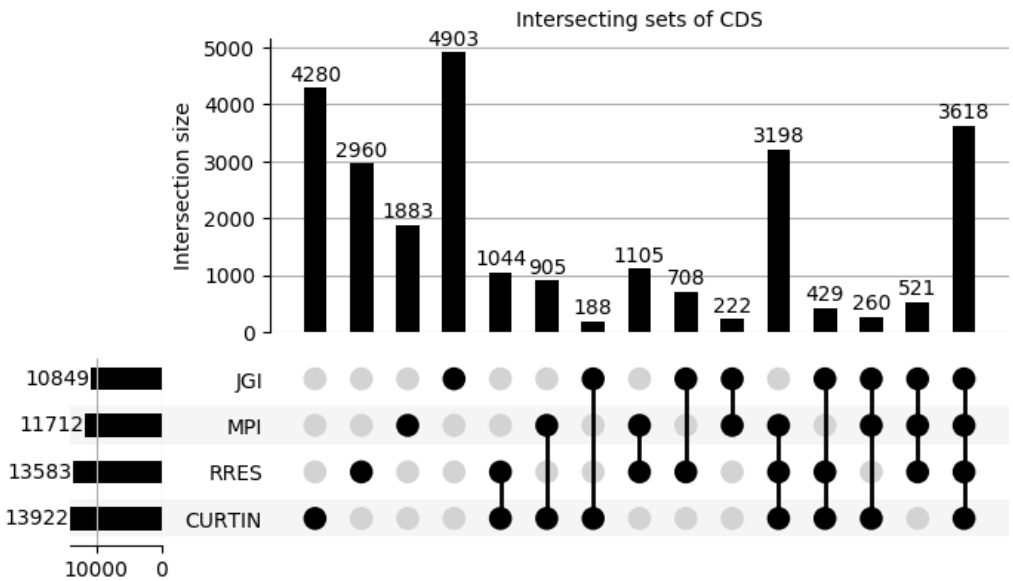
Why another annotation ?

* Goodwin, et al 2011. Finished genome of the fungal wheat pathogen Mycosphaerella graminicola reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. PLoS Genet. 7

**Grandaubert et al2015. RNA-seq-Based gene annotation and comparative genomics of four fungal grass pathogens in the genus Zymoseptoria identify novel orphan genes and species-specific invasions of transposable elements. G3

Genome annotation releases of *Z.tritici* IPO323

CoDing Sequences (CDS) congruence



		JGI	MPI	RRES	CURTIN
pairwise identical CDS	JGI	*10849*			
	MPI	4621	*11712*		
	RRES	5276	8442	*13583*	
	CURTIN	4495	7981	8289	*13922*
dissimilar CDS (same locus)		4752	1871	2367	3844
specific CDS (specific locus)		151	12	593	436

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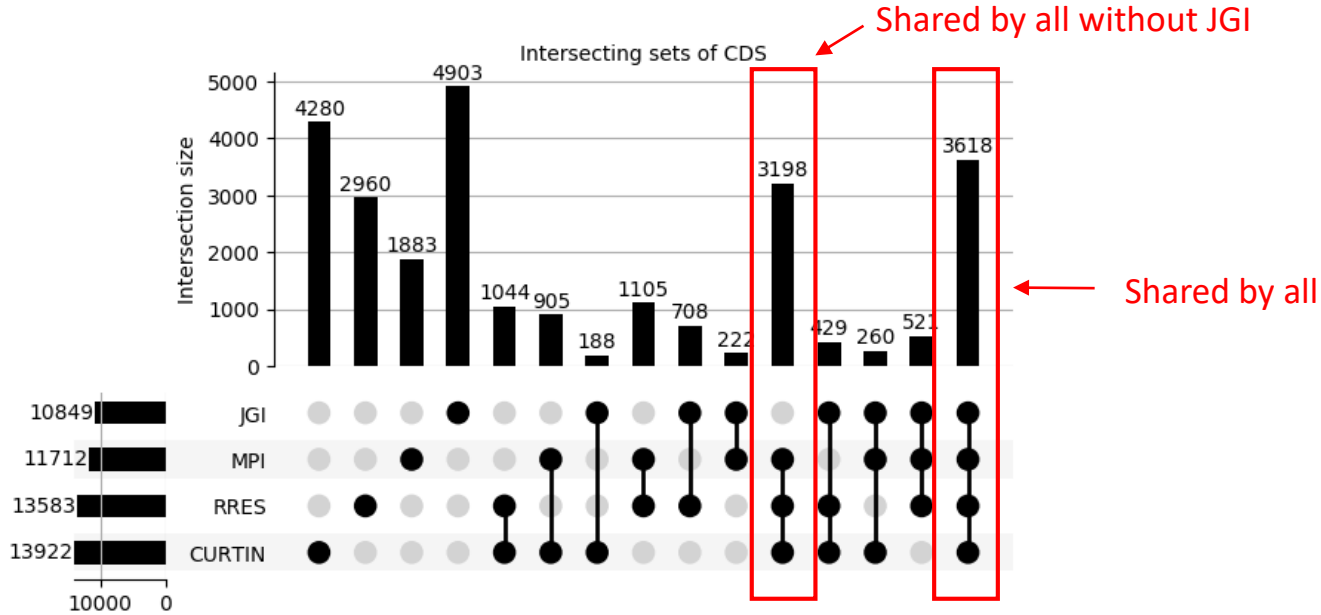

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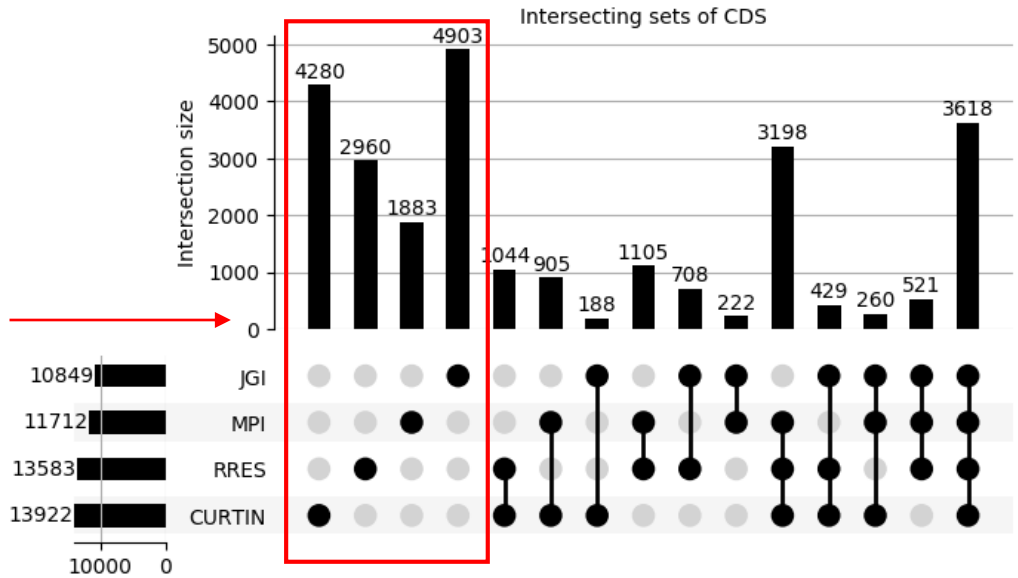
Annotation specific



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median_CDS_length, bp	1071	1203	1044	1041
min_CDS_length, bp	150	150	96	93
max_CDS_length, bp	13842	18297	18423	14523
nb_exons	28313	29728	30772	30564
average_exons_per_CDS	2.6	2.5	2.2	2.2
average_exon_length, bp	531	577	570	586
min_exon_length	2	1	1	1
max_exon_length	12888	12975	18423	9987
nb_transcript_mono_exon	3153	3746	**5233**	**5594**
nb_introns	17464	18016	17189	16642
average_introns_per_transcript	1.6	1.5	1.2	1.2
average_intron_length	**133**	93	109	92
min_intron_length	11	23	4	10
max_intron_length	**42135**	7292	**59574**	5000

Longer CDS

More mono-exons

Longer intron

Very long introns

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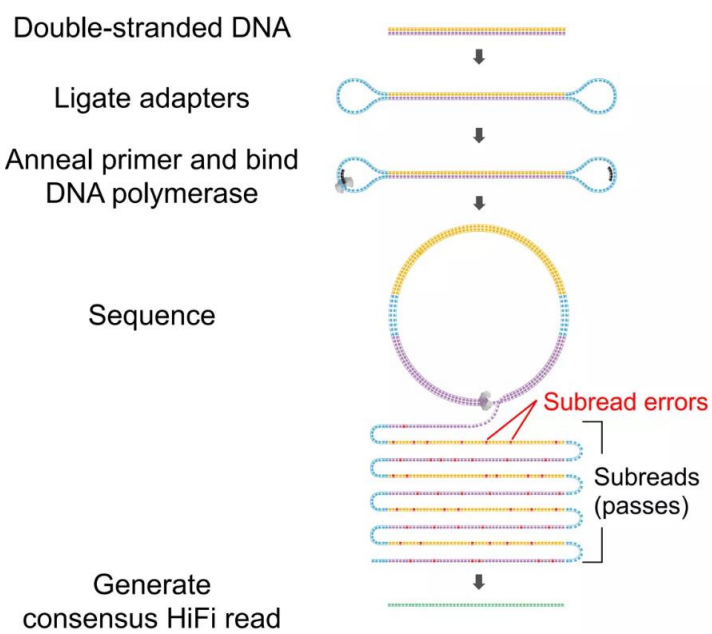
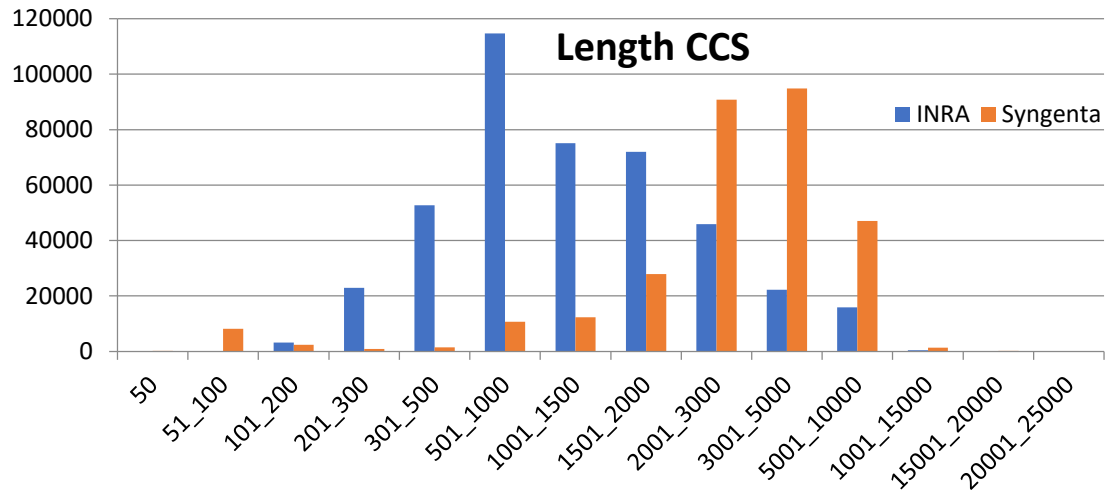
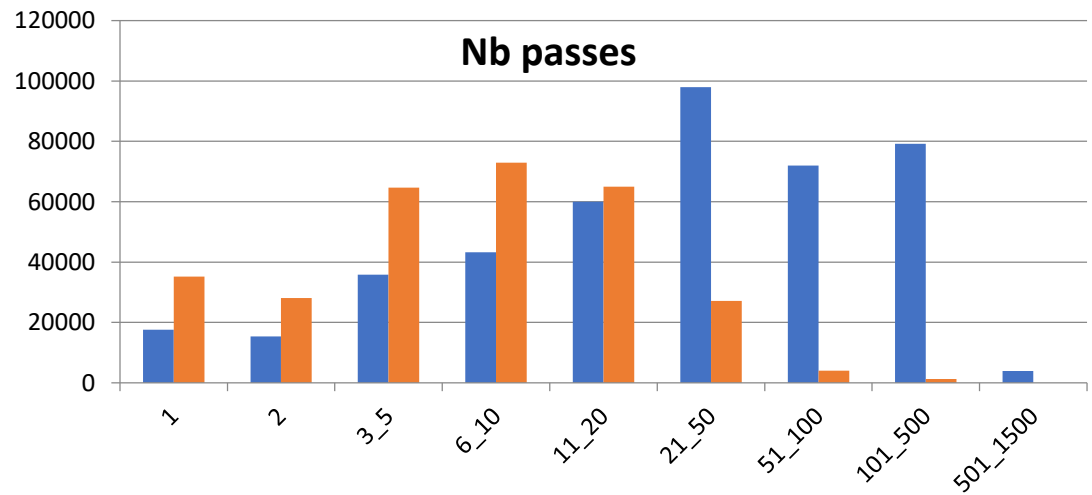
Goal: providing a new release with the best from each annotation !

Iso-Seq long reads: new source of evidence

Add new source of evidence to fix common errors and facilitate the selection of the best model

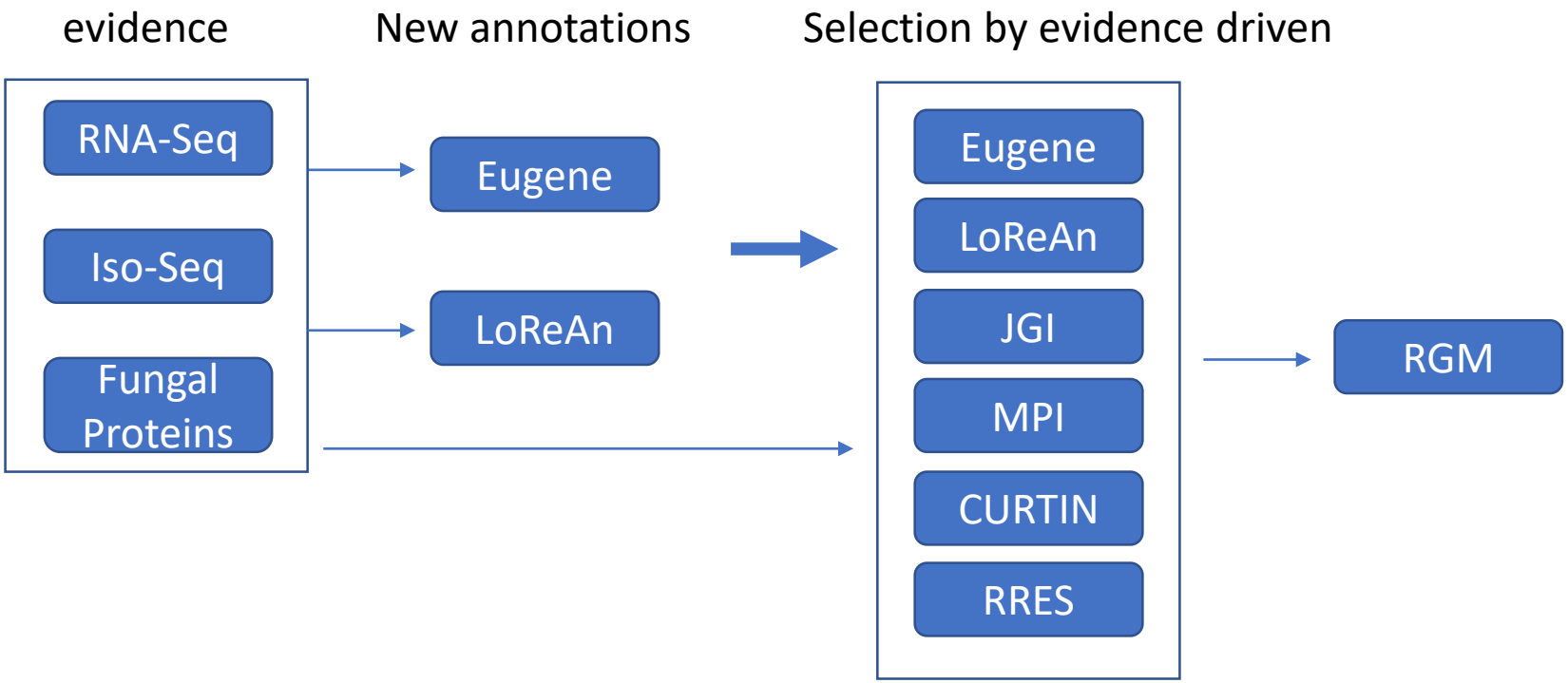






Reannotated Gene Models (RGM)

Generate new gene predictions using Iso-Seq and RNA-Seq data (Eugene, LoReAn) and select the best gene models using all annotations and Iso-Seq, RNA-Seq, protein evidence



*Sallet, E. et al 2019. EuGene: An automated integrative gene finder for eukaryotes and prokaryotes. Methods in Molecular Biology
** Cook et al. 2019. Long-Read Annotation: Automated Eukaryotic Genome Annotation Based on Long-Read cDNA Sequencing. Plant Physiol

Reannotated Gene Models (RGM)

Integration of different sources / usefull tools:

- EVidenceModeler (EVM) –> done with MPI annotation, need to specify a weight for each source.

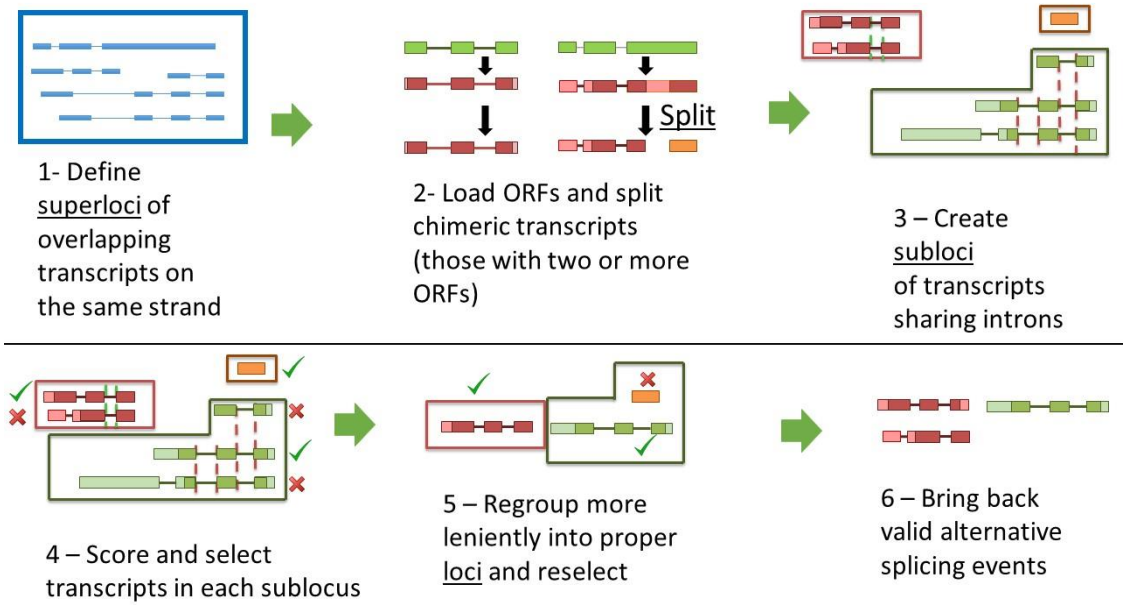
```
ABINITIO_PREDICTION      augustus                      1
ABINITIO_PREDICTION      twinscan                      1
ABINITIO_PREDICTION      glimmerHMM                    1
PROTEIN                  spliced_protein_alignments    1
PROTEIN                  genewise_protein_alignments   2
TRANSCRIPT               spliced_transcript_alignments 1
TRANSCRIPT               PASA_transcript_assemblies    10
OTHER_PREDICTION         PASA_transdecoder             5
```

$$Score(a,b) = \sum_{a<=i<=b} ScoringVector[i] + \sum_{\substack{evidence\_end5'=a \\ evidence\_end3'=b}} featureLength * weight(evidence)$$

Reannotated Gene Models (RGM)

Integration of different sources / usefull tools:

- Mikado 2 (2019) -> integration multiple sources, compare structure. Main goal, improve RNA-Seq transcripts with fusion detection. Complete and complexe configuration (external score available)

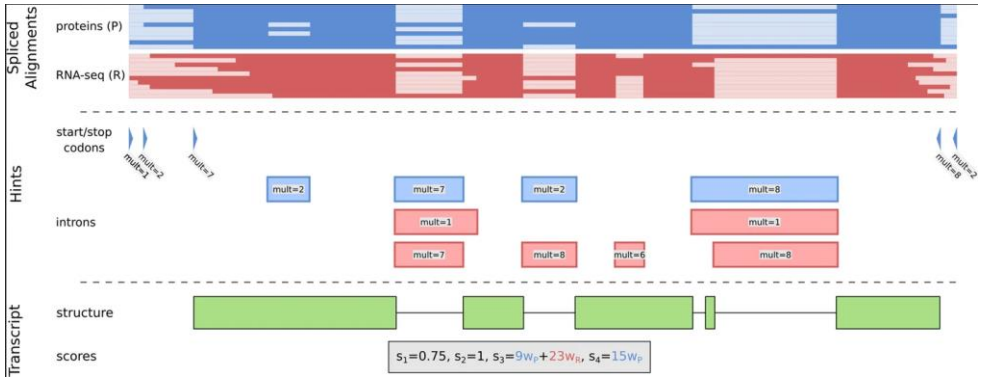


https://mikado.readthedocs.io/en/stable/Scoring_files/#scoring-files

Reannotated Gene Models (RGM)

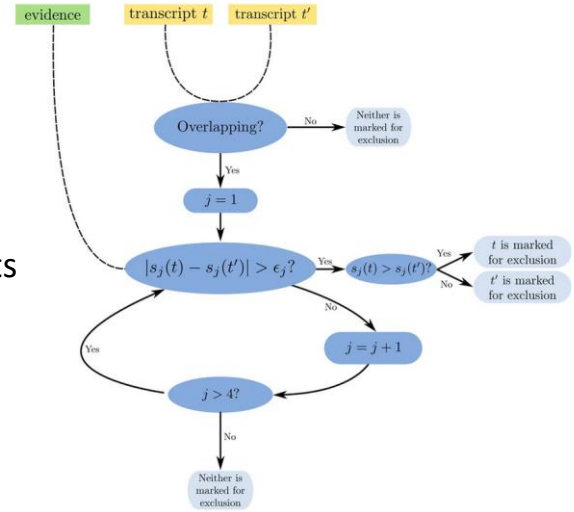
Integration of different sources / usefull tools:

- TSEBRA (2021) -> based on BRAKER outputs, improve selection of genes comparing BRAKER1/BRAKER2 outputs. => new preprint BRAKER3 (2023), fully automated pipeline ; BRAKER (AUGUSTUS, GeneMark.hmm)



transcript scores quantify the support of the transcript structure

Comparison rule for two transcripts



How to evaluate annotation improvement ?

With reference !

TSEBRA, MIKADO:

```
Command line:
/usr/local/bin/mikado compare -r reference.gff3 -p mikado.loci.gff3 -o compare -l compare.log
7 reference RNAs in 5 genes
15 predicted RNAs in  8 genes
--------------------------------- |   Sn |   Pr |   F1 |
                       Base level: 85.74  64.73  73.77
            Exon level (stringent): 63.83  42.86  51.28
              Exon level (lenient): 80.00  52.94  63.72
                      Intron level: 89.47  59.65  71.58
                Intron chain level: 33.33  14.29  20.00
      Transcript level (stringent): 0.00  0.00  0.00
  Transcript level (>=95% base F1): 28.57  13.33  18.18
  Transcript level (>=80% base F1): 42.86  20.00  27.27
         Gene level (100% base F1): 0.00  0.00  0.00
        Gene level (>=95% base F1): 40.00  25.00  30.77
        Gene level (>=80% base F1): 60.00  37.50  46.15

#   Matching: in prediction; matched: in reference.

            Matching intron chains: 2
             Matched intron chains: 2
   Matching monoexonic transcripts: 1
    Matched monoexonic transcripts: 1
        Total matching transcripts: 3
         Total matched transcripts: 3

          Missed exons (stringent): 17/47  (36.17%)
           Novel exons (stringent): 40/70  (57.14%)
            Missed exons (lenient): 9/45  (20.00%)
             Novel exons (lenient): 32/68  (47.06%)
                    Missed introns: 4/38  (10.53%)
                     Novel introns: 23/57  (40.35%)

                Missed transcripts: 0/7  (0.00%)
                 Novel transcripts: 6/15  (40.00%)
                      Missed genes: 0/5  (0.00%)
                       Novel genes: 2/8  (25.00%)
```

- Exons, ok
- Transcripts, UTRs, Isoforms ?
- UTRs, CDS ?

How to evaluate annotation improvement ?

Without reference ? Find metrics/measurement usefull to compare annotations

- Eugene: gff tags (Field 9) est_cons=74.8;est_incons=0.0, CDS (est_cons = adequation with splicing site, est_incons = presence of different splicing site/non-splicing site)
- Augustus: gff score, CDS , 0->1, posterior probability, ex 0.8 = sampling 80/100
- TSEBRA: transcript score
- Maker: AED = Annotation Edit Distance

AED:0.41 eAED:0.41 QI:0|0.5|0.4|1|1|1|5|0|278

- Length of the 5 UTR
- Fraction of splice sites confirmed by an EST alignment
- Fraction of exons that overlap an EST alignment
- Fraction of exons that overlap EST or Protein alignments
- Fraction of splice sites confirmed by a SNAP prediction
- Fraction of exons that overlap a SNAP prediction
- Number of exons in the mRNA
- Length of the 3 UTR
- Length of the protein sequence produced by the mRNA

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D. S. Standage and V. P. Brendel, “ParsEval: Parallel comparison and analysis of gene structure annotations,” *BMC Bioinformatics*, vol. 13, no. 1, p. 187, Aug. 2012, doi: 10.1186/1471-2105-13-187.

Comparison annotations vs ref, or annotation versions. Comparison of structures, no measurement of biological relevance

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D. S. Standage, “AEGeAn: an integrated toolkit for analysis and evaluation of annotated genomes,” 2015. http://standage.github.io/AEGeAn. => GAEVAL

Compute coverage score: percentage of nucleotides in exons that have coverage from one or more transcript alignments.

Compute integrity score:

- A: the percentage of introns confirmed by an alignment gap; for single-exon gene predictions lacking introns, A represents the ratio of the observed CDS length to the expected CDS length (with a maximum of 1.0)
- B: the exon coverage
- Γ: the ratio of the observed 5’ UTR length to the expected 5’ UTR length (with a maximum of 1.0)
- E: the ratio of the observed 3’ UTR length to the expected 3’ UTR length (with a maximum of 1.0)

A weight is applied to each of these 4 values, and the final integrity score Φ is computed as follows.

$$\Phi = \alpha A + \beta B + \gamma \Gamma + \epsilon E$$

The sum of the weights must be 1.0, and their default values are as follows.

- $\alpha = 0.6$
- $\beta = 0.3$
- $\gamma = 0.05$
- $\epsilon = 0.05$

Expected lengths for UTRs and CDSs should be determined empirically. The original GAEVAL tool calculated these values as the length achieved by 95% of the evaluated features.

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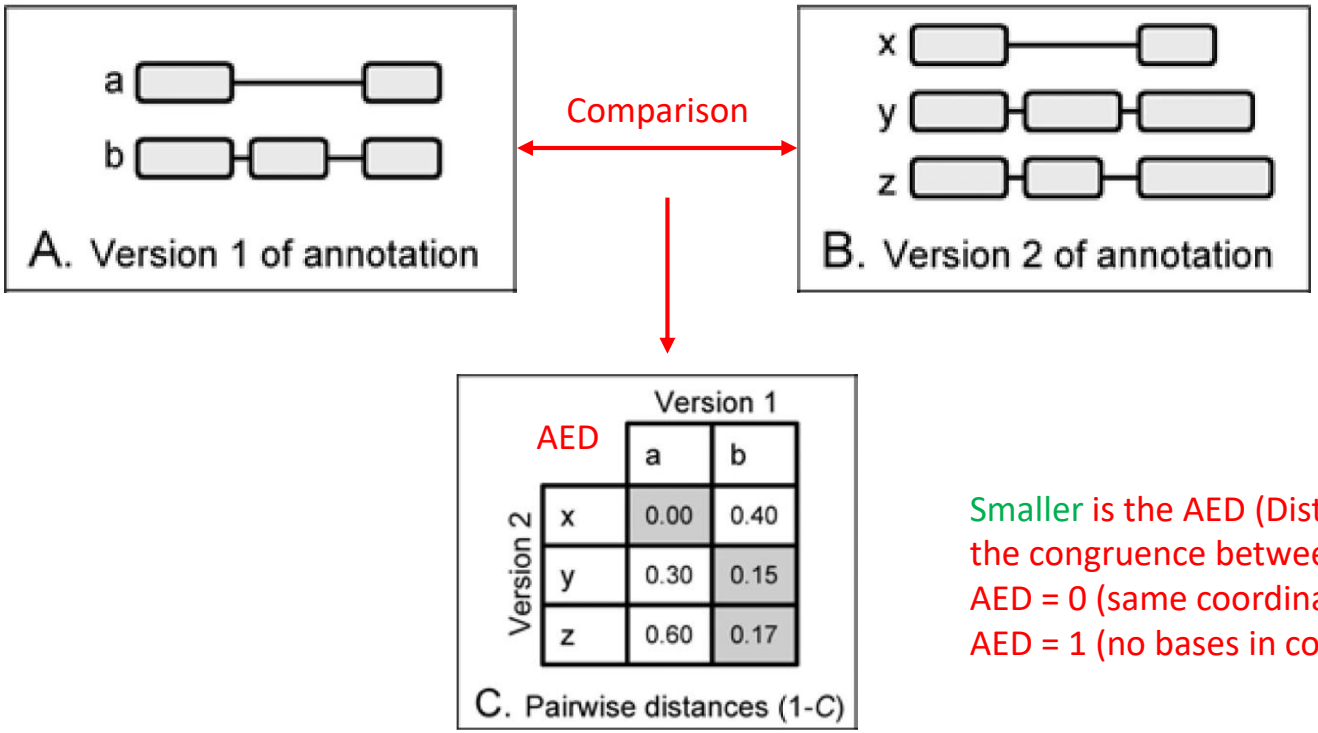
Annotation Edit Distance (AED)

AED proposed by MAKER* developers to evaluate the match between different gene predictions



Smaller is the AED (Distance), stronger is the congruence between annotations.
AED = 0 (same coordinates) = identical
AED = 1 (no bases in common)

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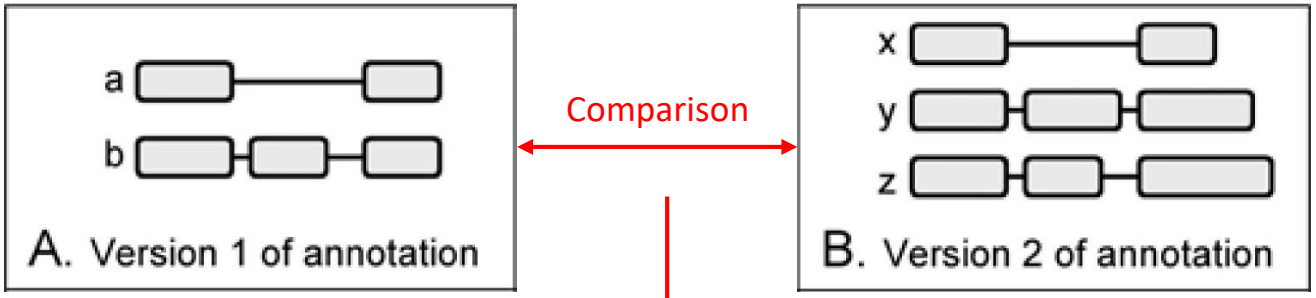
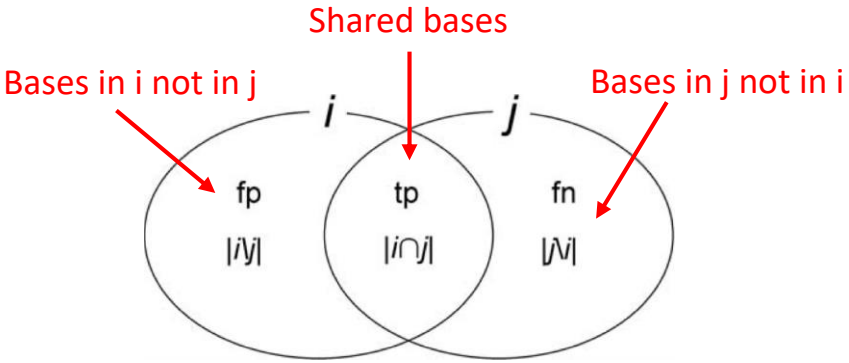


AED

Version 2 \ Version 1	a	b
x	0.00	0.40
y	0.30	0.15
z	0.60	0.17

C. Pairwise distances (1-*C*)



Sensibility, Specificity, Accuracy

*Eilbeck, K., Moore, B., Holt, C., and Yandell, M. 2009. Quantitative measures for the management and comparison of annotated genomes. BMC Bioinformatics. 10:67

AED in InGenAnnot (Inspection of Gene Annotation)

Adapted AED to evaluate distance between gene models and evidence

Selection of best gene models according to AED scores

Selected annot

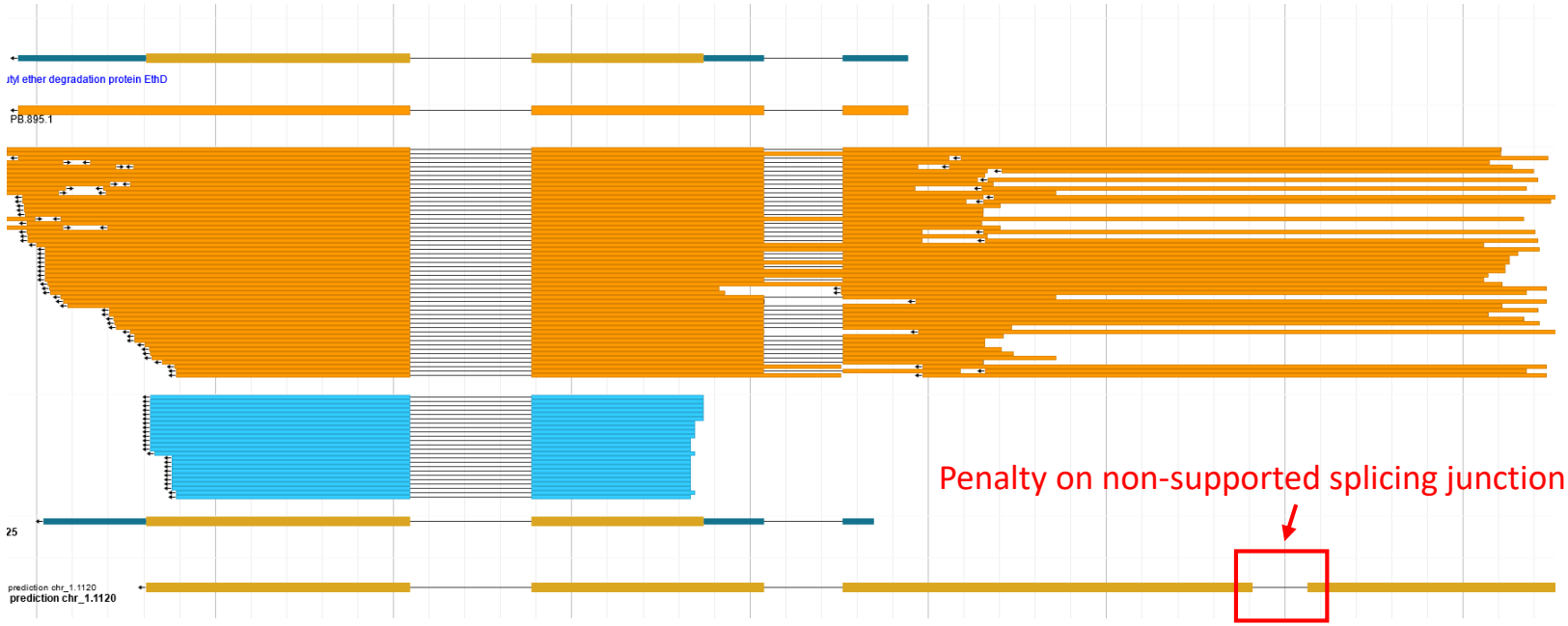
Iso-Seq

RNA-Seq transcripts

Protein (DB) matches

Annot 1

Annot 2



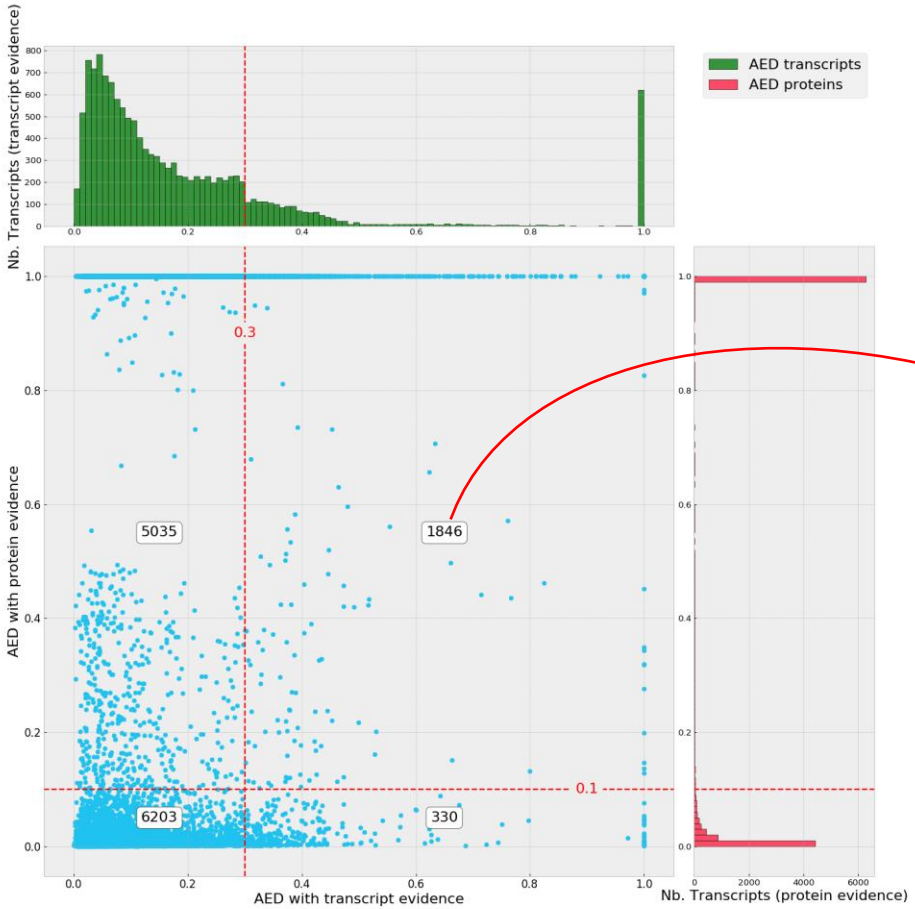
Penalty on non-supported splicing junction

	Annot 1	Annot 2
AED_RNA-Seq	0.00738 (penalty=NO)	0.3007 (penalty=YES)
AED_protein	0.0049	0.3164
AED_long-read	0.1839 (penalty=NO)	0.6042 (penalty=YES)

Ranking → Annot1, Annot2

New annotation release driven by evidence

RGM release (Reannotation Gene Models) => 13414 gene models



Selection thresholds:
AED transcripts <= 0.3
AED proteins <= 0.1
11568 genes

1846 rescued genes (locus supported by 4 annotations)

⇒ 574 genes no AED (fully ab-initio) , 224 co-localized in a specific region of chromosome 7 (repressive histone marks* as mini-chromosomes)

*Schotanus et al. 2015. Histone modifications rather than the novel regional centromeres of Zymoseptoria tritici distinguish core and accessory chromosomes. Epigenetics and Chromatin

Genome annotation release comparison

RGM displays coherent statistics

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max_intron_length	**42135**	7292	**59574**	5000	3166

UTRs

Genome annotation release comparison

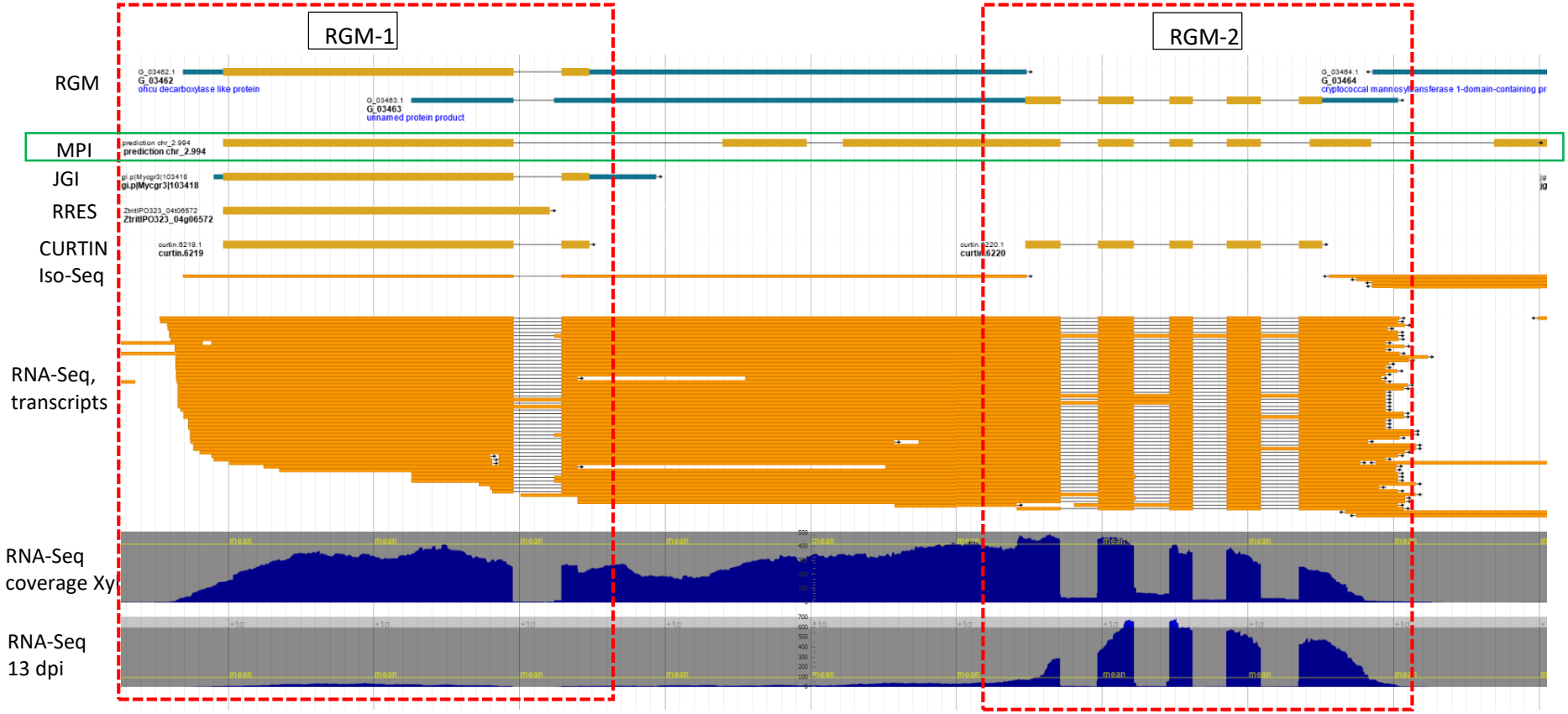
BUSCO

BUSCO category	JGI	MPI	CURTIN	RRES	RGM
Complete BUSCOs (C)	1633	1679	1681	1693	1696
Complete BUSCOs (C) %	95.7%	98.4%	98.5%	99.2%	99.4%
Complete and single-copy BUSCOs (S)	1632	1678	1615	1692	1695
Complete and duplicated BUSCOs (D)	1	1	66	1	1
Fragmented BUSCOs (F)	25	3	8	5	2
Missing BUSCOs (M)	48	24	17	8	8
Total BUSCO groups			1706		

BUSCO: not enough to evaluate improvement !

Gene model Improvements

Fixing fused genes specific of MPI annotation



Iso-Seq (RGM-1) and RNA-Seq (infection 13 dpi, RGM-2) identified two distinct genes

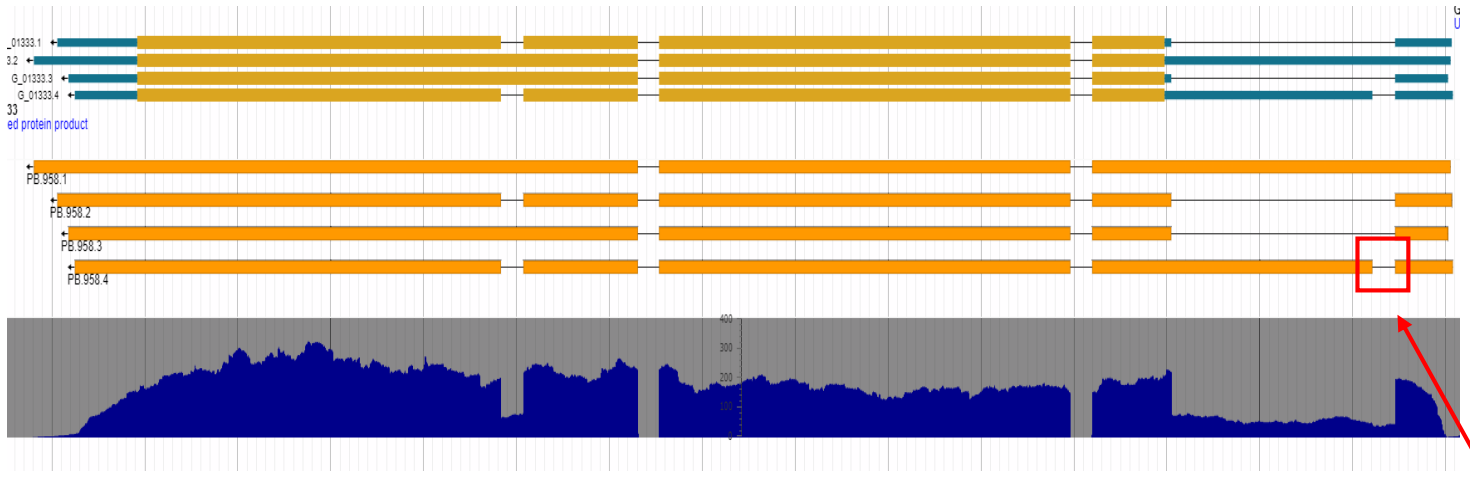
Alternative splicing: Iso-Seq + RNA-Seq

Iso-Seq detection of transcript isoforms validated by RNA-Seq (expression level)

RGM with isoforms

Iso-Seq

RNA-Seq coverage
Xylose medium



Splicing site not supported by RNA-Seq data

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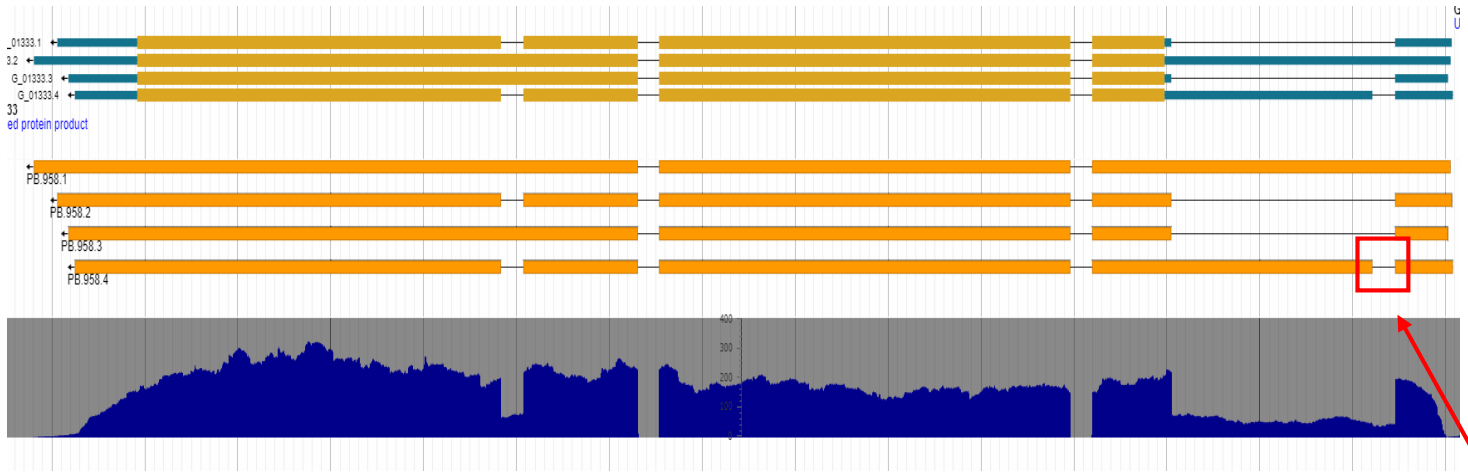
Iso-Seq detection of transcript isoforms validated by RNA-Seq (expression level)

RGM with isoforms

Iso-Seq

RNA-Seq coverage Xylose medium



categories	counts
genic[1]	664
Intron retention (IR)	1571
novel_in_catalog (NIC)[2]	7
novel_not_in_catalog (NNC)[3]	474

1342 genes with at least one isoform supported by Iso-Seq

Splicing site not supported by RNA-Seq data

[1]: partial overlaps of intron and exons not compliant with intron/exons coordinates
[2]: use combination_of_known_splice sites
[3]: at_least_one_novel_splice site detected

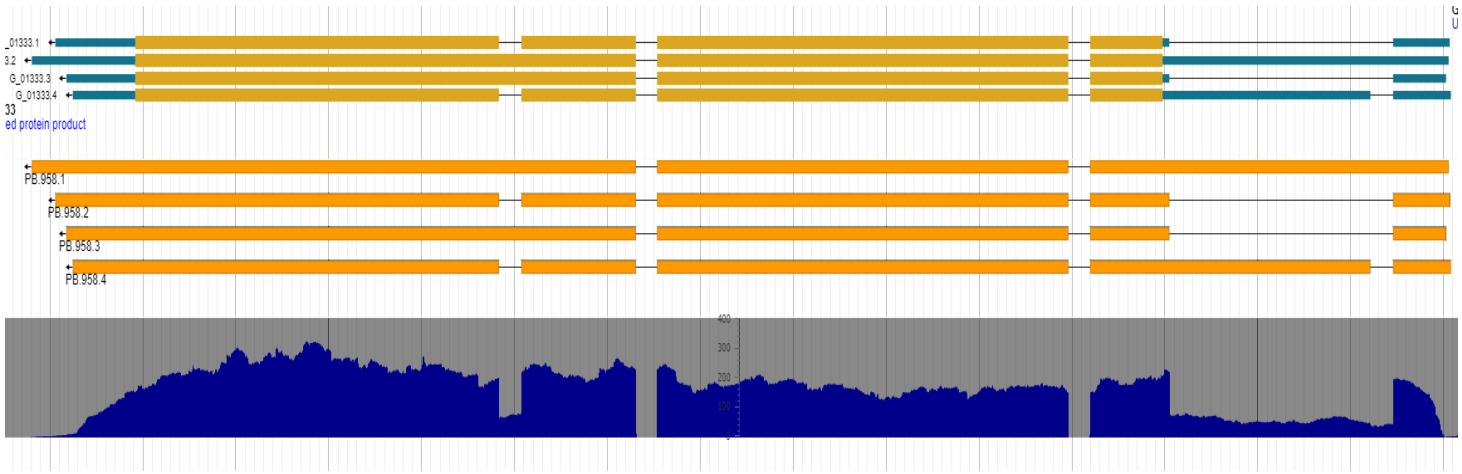
Alternative splicing: Iso-Seq + RNA-Seq

Iso-Seq detection of transcript isoforms validated by RNA-Seq (expression level)

RGM with isoforms

Iso-Seq

RNA-Seq coverage Xylose medium



categories	counts
genic[1]	664
Intron retention (IR)	1571
novel_in_catalog (NIC)[2]	7
novel_not_in_catalog (NNC)[3]	474

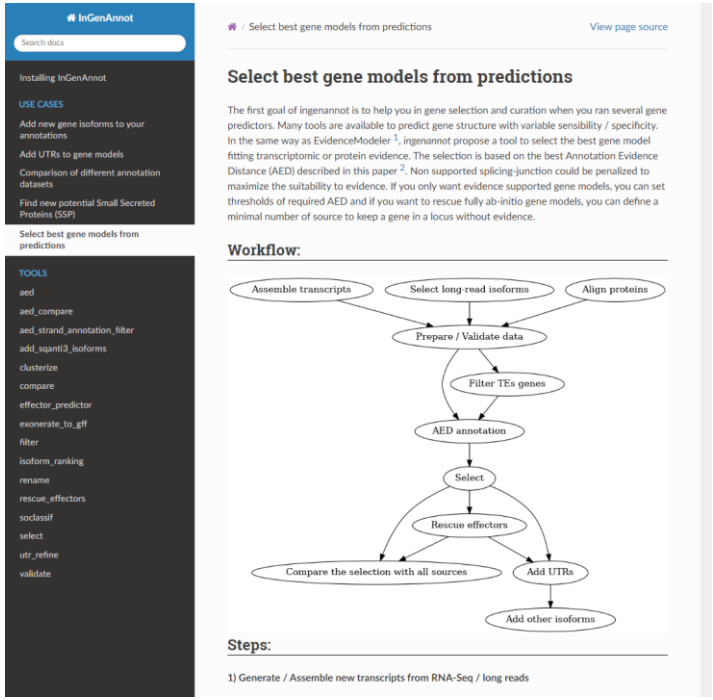
→ Differential Isoform Usage (DIU) → Few DIU statistically validated between in vitro and infection. Only few reads during infection: bias No clear signals

[1]: partial overlaps of intron and exons not compliant with intron/exons coordinates
[2]: use combination_of_known_splice sites
[3]: at_least_one_novel_splice site detected

InGenAnnot

Tool suite to select, compare and filter gene annotation

https://bioger.pages.mia.inra.fr/ingenannot



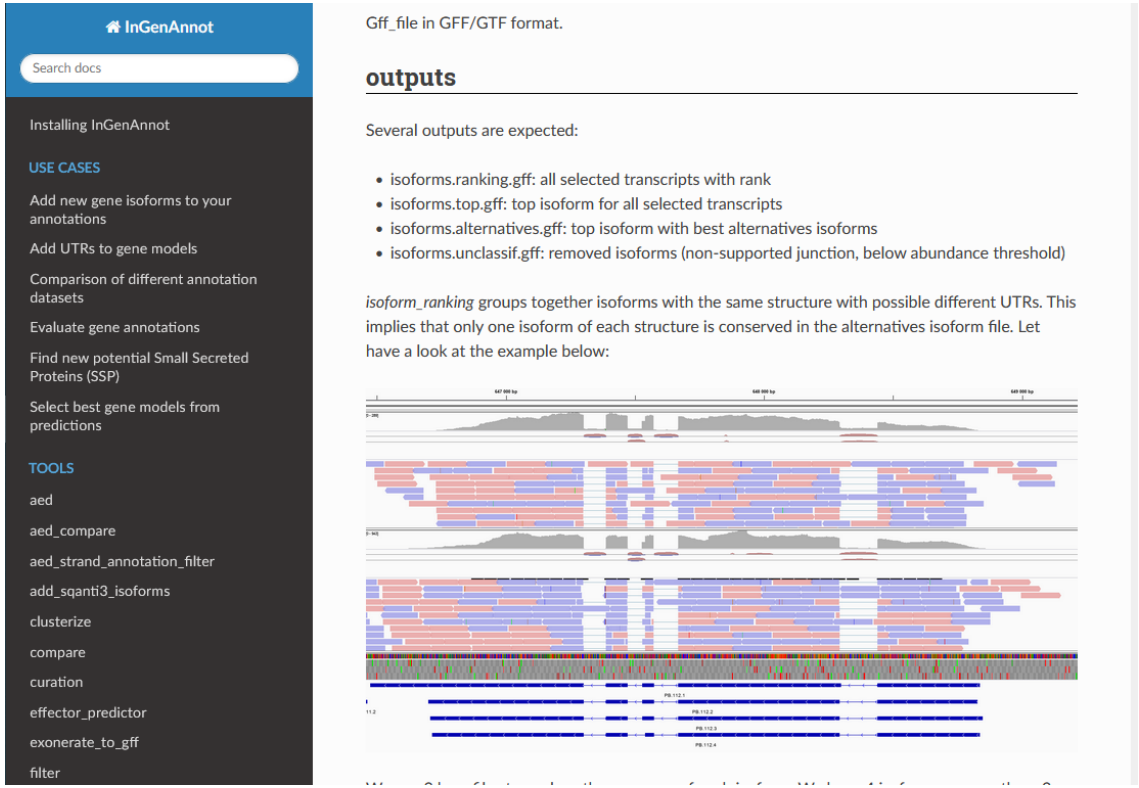
https://pypi.org/project/ingenannot/

SO classification, Isoform selection, UTR, rescue effectors …

InGenAnnot

Tool suite to select, compare and filter gene annotation

https://bioger.pages.mia.inra.fr/ingenannot https://pypi.org/project/ingenannot/

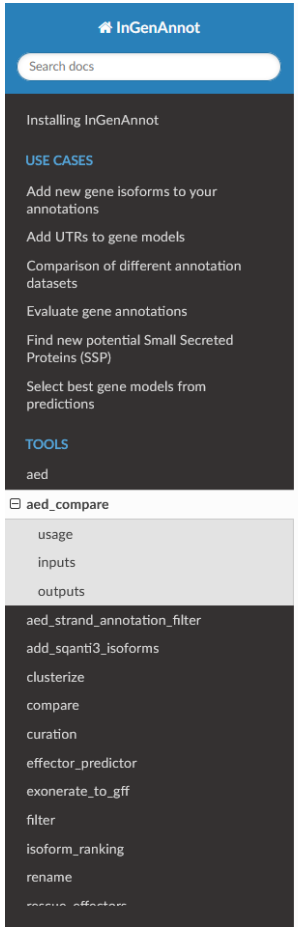


Isoform ranking: select best isoform to predict gene models

InGenAnnot

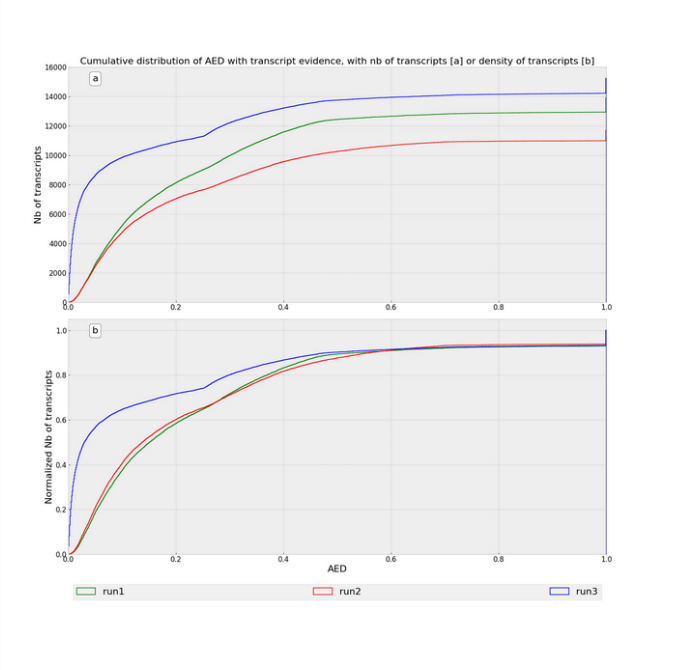
Tool suite to select, compare and filter gene annotation

https://bioger.pages.mia.inra.fr/ingenannot https://pypi.org/project/ingenannot/



comparison of aed score between datasets. You can compute some metrics as ... with *–statistics*

Output example of plot showing cumulative transcript aed scores:



Output of statistics:

#	mean (tr)	median (tr)	stdev (tr)	mean (pr)	median (pr)	stdev (pr)	median
run1	0.244	0.152	0.258	0.478	0.176	0.480	0.176
run2	0.239	0.138	0.255	0.444	0.089	0.476	0.154

AED comparison between several annotation sets. Cumulative AED like in MAKER2 publication: 10.1186/1471-2105-12-491

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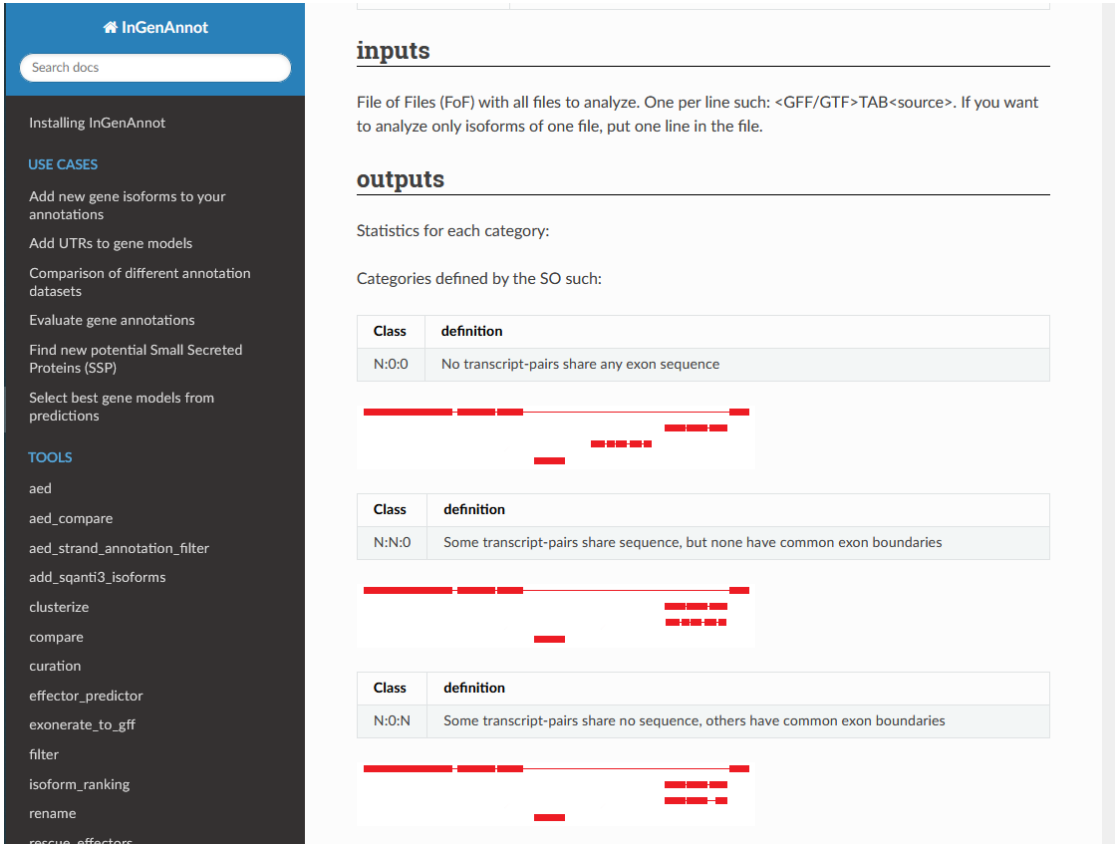


SO classification : gene overlaps, aberrant mRNA isoforms

InGenAnnot

Tool suite to select, compare and filter gene annotation

https://bioger.pages.mia.inra.fr/ingenannot https://pypi.org/project/ingenannot/

Manual curation: prioritization

Conclusions & Perspectives

Iso-Seq sequencing method is a complementary method to RNA-Seq:

- Robust isoform detection
- UTR inference
- Resolution of overlapping genes coordinates (dense genome)
- Full lncRNA sequencing

Drawbacks:

- highlights rare transcripts / transcription machinery errors -> need quantitative control (RNA-Seq)

Zymoseptoria tritici IPO323 new release: RGM (preprint + InGenAnnot)
https://doi.org/10.1101/2023.04.26.537486

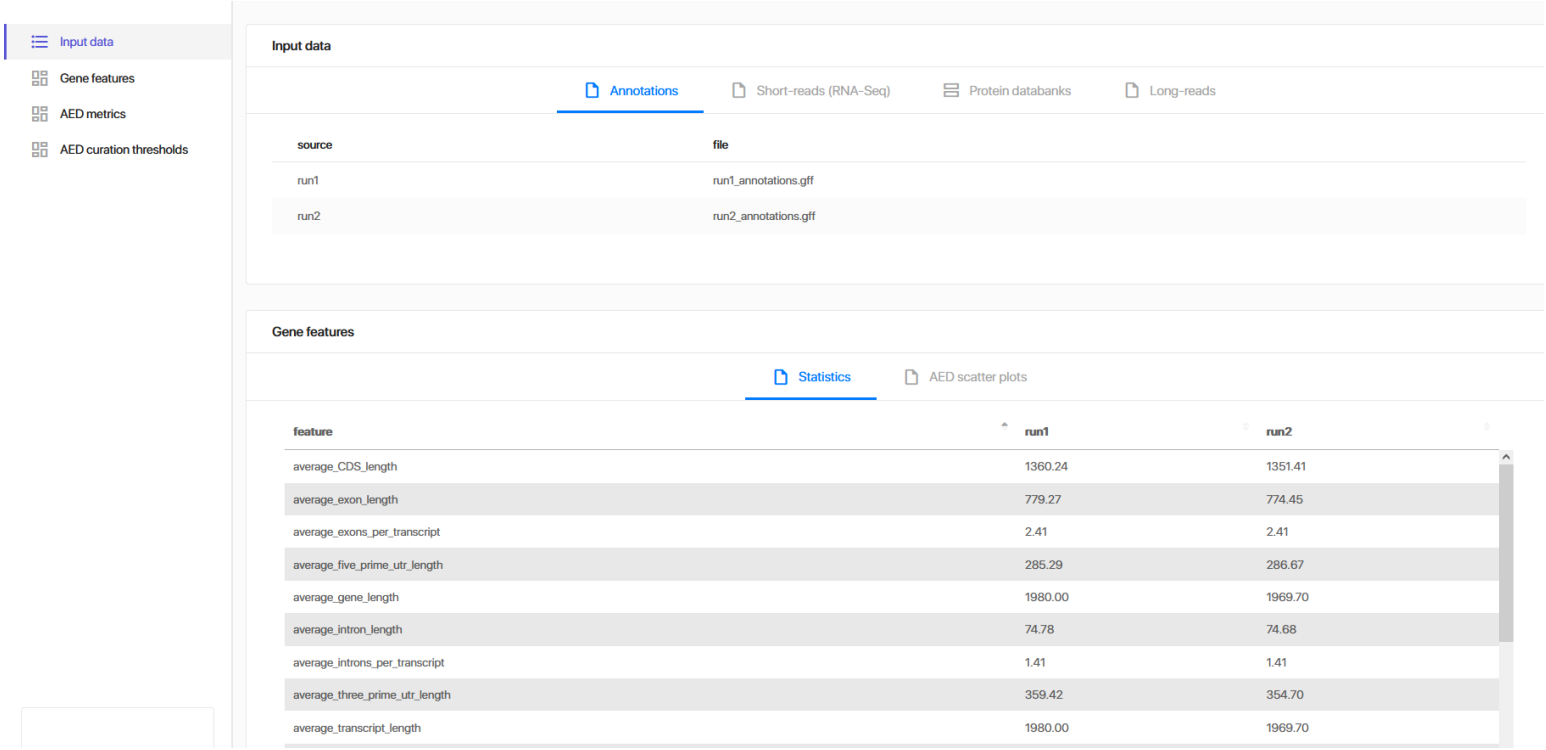
InGenAnnot: new suite of tools to deal with gene annotations.

Benchmark analysis in progress versus EvidenceModeler, TSEBRA, gaeval (AEGeAn) , FINDER.

Snakemake workflow in progress: comparison of annotations or evaluation of an annotation release.

Conclusions & Perspectives

Snakemake workflow in progress: comparison of annotations or evaluation of an annotation release.



VARUS: Stanke, M., Bruhn, W., Becker, F. *et al.* VARUS: sampling complementary RNA reads from the sequence read archive. *BMC Bioinformatics* **20**, 558 (2019). https://doi.org/10.1186/s12859-019-3182-x

Conclusions & Perspectives

https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/aps3.11533

APPLICATION ARTICLE | Open Access

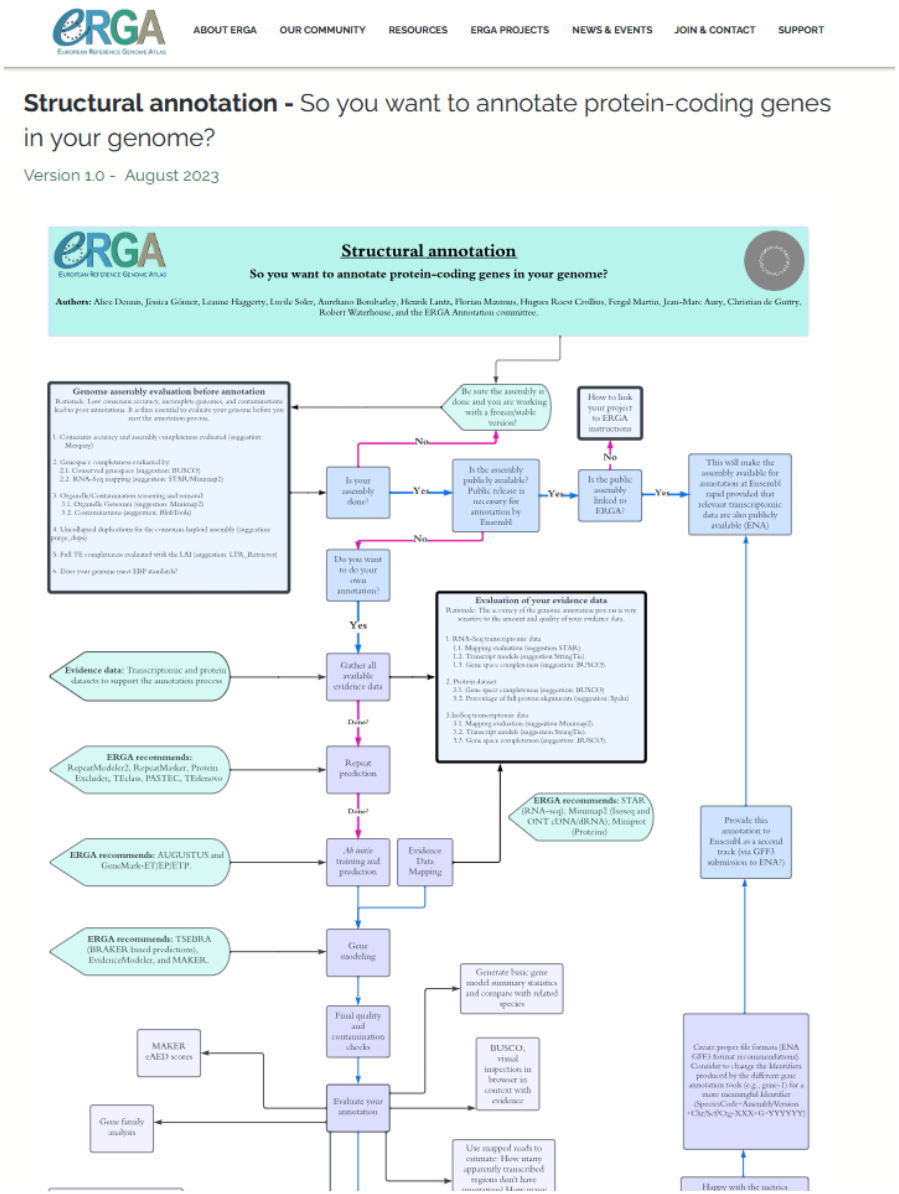
Welcome to the big leaves: Best practices for improving genome annotation in non-model plant genomes

Vidya S. Vuruputoor, Daniel Monyak, Karl C. Fetter, Cynthia Webster, Akriti Bhattarai, Bikash Shrestha, Sumaira Zaman, Jeremy Bennett, Susan L. McEvoy, Madison Caballero, Jill L. Wegrzyn

First published: 08 August 2023 | https://doi.org/10.1002/aps3.11533 | Citations: 2

Conclusions & Perspectives

https://www.erga-biodiversity.eu/structural-annotation



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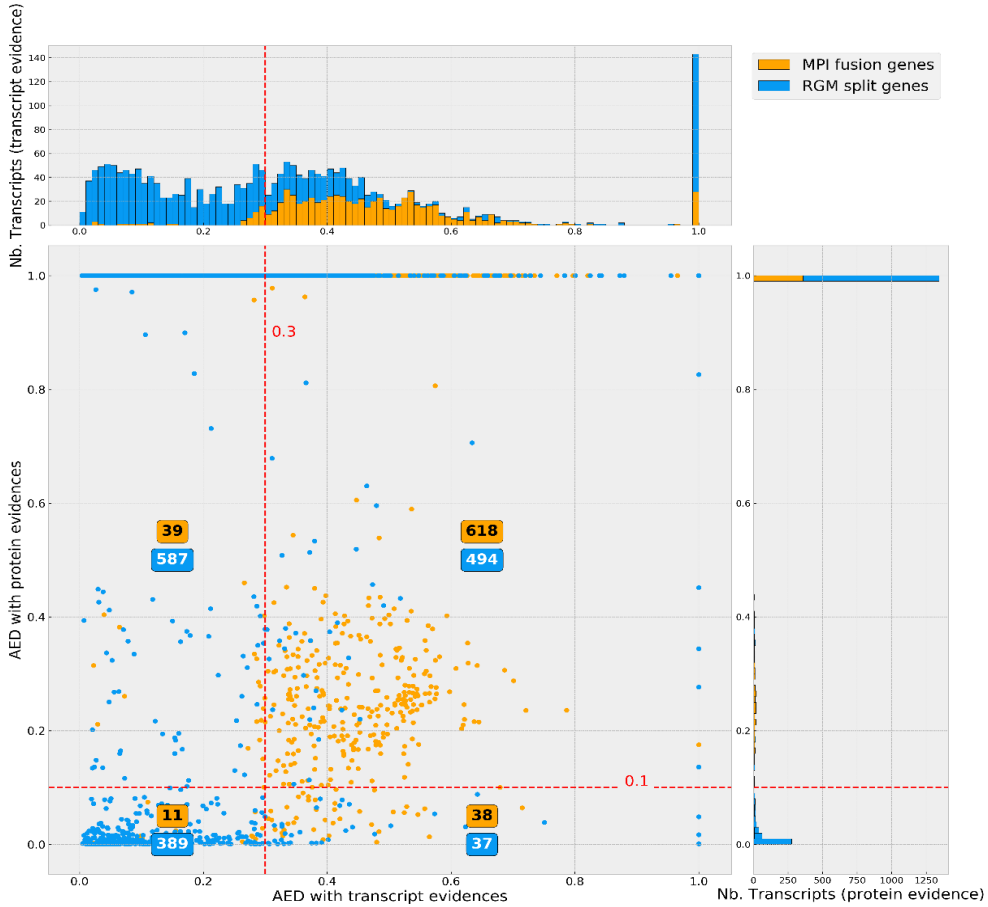
BIOGER

Gene model Improvements

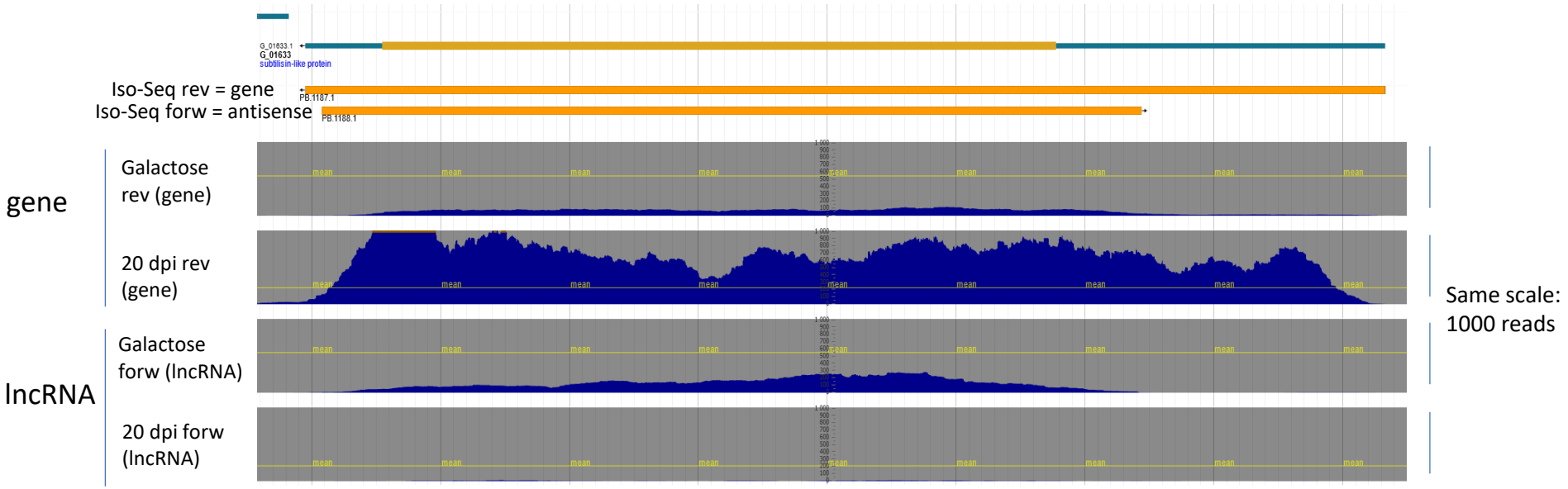
Fixed many fused genes in MPI annotation



MPI 706 genes
vs
RGM 1507 genes

Identification of Long Non-Coding RNAs using Iso-Seq

Identification of 51 reliable Long Non-Coding RNA (lncRNA) validated by RNA-Seq (mainly antisense transcripts)



Negative correlation between the expression of the subtilisin-like coding gene and its antisense:

Infection: subtilisin (up) antisense (down)

In vitro: subtilisin (down) antisense (up)

Hypothesis: Negative control of subtilisin-like coding gene expression by the antisense lncRNA.

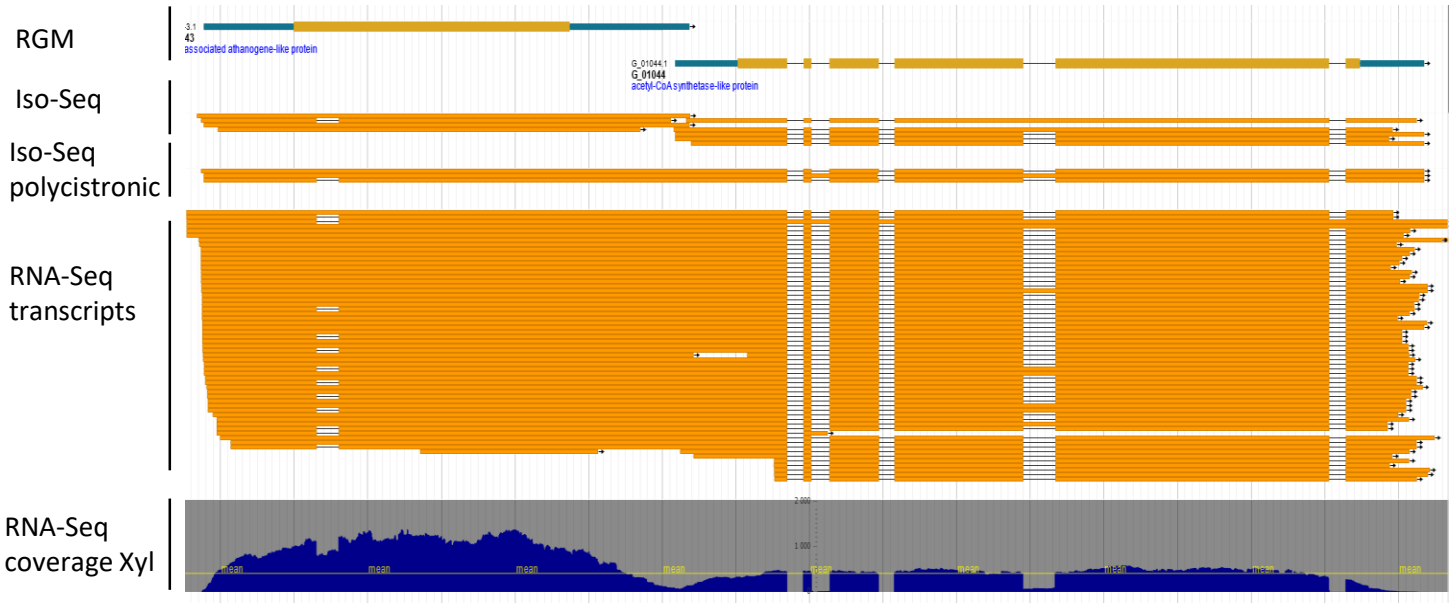
Subtilisins are secreted proteases playing an important role in plant infection*, **.

* Li, J. et al. 2010. New insights into the evolution of subtilisin-like serine protease genes in Pezizomycotina. BMC Evol. Biol. 10

** Figueiredo,et al, 2014. Subtilisin-like proteases in plant–pathogen recognition and immune priming: A perspective. Front. Plant Sci. 5

Identification of Polycistronic mRNAs using Iso-Seq

Identification of polycistronic mRNAs validated by the occurrence of independent long Iso-Seq molecules and RNA-Seq



2.625 potential polycistronic mRNA (224 validated by Independent Iso-Seq molecules)

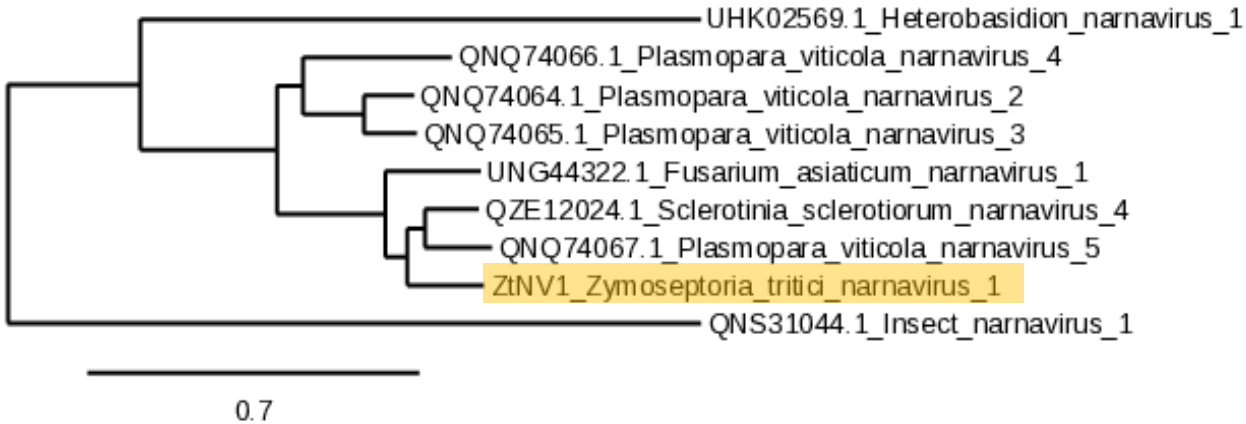
Polycistronic mRNAs observed in *Agaromycetes** and *F.graminearum***. *Agaromycetes* polycitronic mRNAs found for secondary metabolite gene clusters => stop codon, prevent for intermediate metabolite accumulation ?

* P. Lu *et al.*, “Landscape, complexity and regulation of a filamentous fungal” *bioRxiv*, Nov. 2021

** S. P. Gordon *et al.*, “Widespread Polycistronic Transcripts in Fungi Revealed by Single-Molecule mRNA Sequencing,” *PLoS One*, 2015

Identification of RNA Mycovirus using Iso-Seq

Detection of a new RNA mycovirus from narnavirus : ZtNV1



ZtNV1: 3091 nt (986 aa)
Iso-Seq detects rare Long RNAs -> Internal priming.

Not detected by RNA-Seq transcript assembly*, ratio 1/70000 compared to ZtFV1 virus

* Gilbert, K. B., Holcomb, E. E., Allscheid, R. L., and Carrington, J. C. 2019. Hiding in plain sight: New virus genomes discovered via a systematic analysis of fungal public transcriptomes. PLoS One. 14