Genome Annotation

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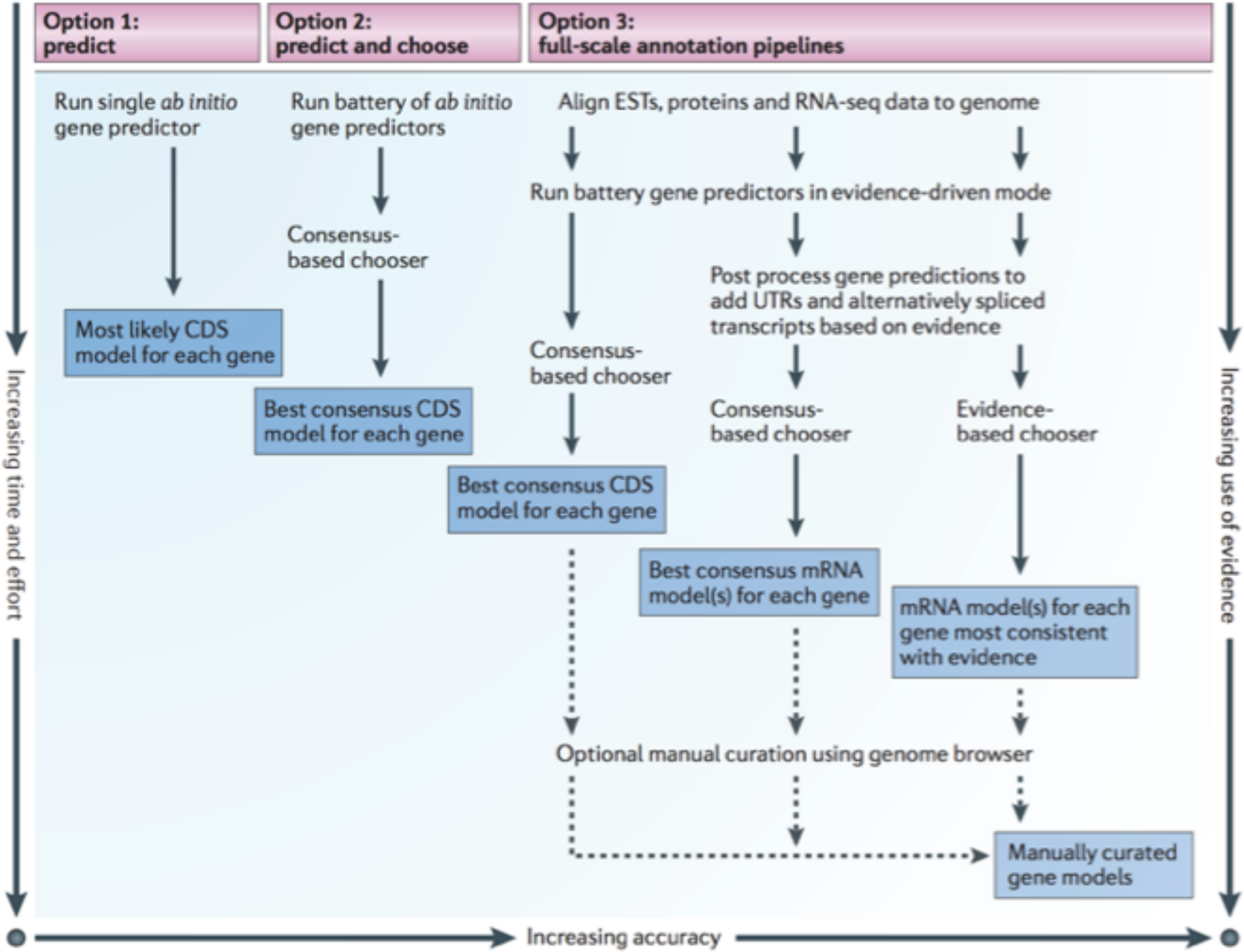
What is genome annotation?

- Genome annotation is the process of identifying different elements in a genome assembly:
  - Structural
    - Repetitive elements
    - Genes (with introns and exons)
  - Functional
    - What does each gene do?

Gene prediction: ab initio vs evidence driven

ab initio	**evidence-driven**
• use only the query sequence	• use external evidence

Combining both approaches is the best option


Option 1: predict
Option 2: predict and choose
Option 3: full-scale annotation pipelines
Run single ab initio gene predictor
Most likely CDS model for each gene
Run battery of ab initio gene predictors
Consensus-based chooser
Best consensus CDS model for each gene
Align ESTs, proteins and RNA-seq data to genome
Run battery gene predictors in evidence-driven mode
Consensus-based chooser
Best consensus CDS model for each gene
Post process gene predictions to add UTRs and alternatively spliced transcripts based on evidence
Consensus-based chooser
Best consensus mRNA model(s) for each gene
Evidence-based chooser
mRNA model(s) for each gene most consistent with evidence
Optional manual curation using genome browser
Manually curated gene models
Increasing time and effort
Increasing use of evidence
Increasing accuracy

Chromosome 3R: 23,049,569-23,054,170

ATGTTTGCAAAATATGACAACTCTTTAATAAATTCACTTTTGGCTTTTTTTGTACAGAGTATGTTTGTTGTGGTAGACAAAAGTTACGTAGTCACATAGAACTGCAGTT
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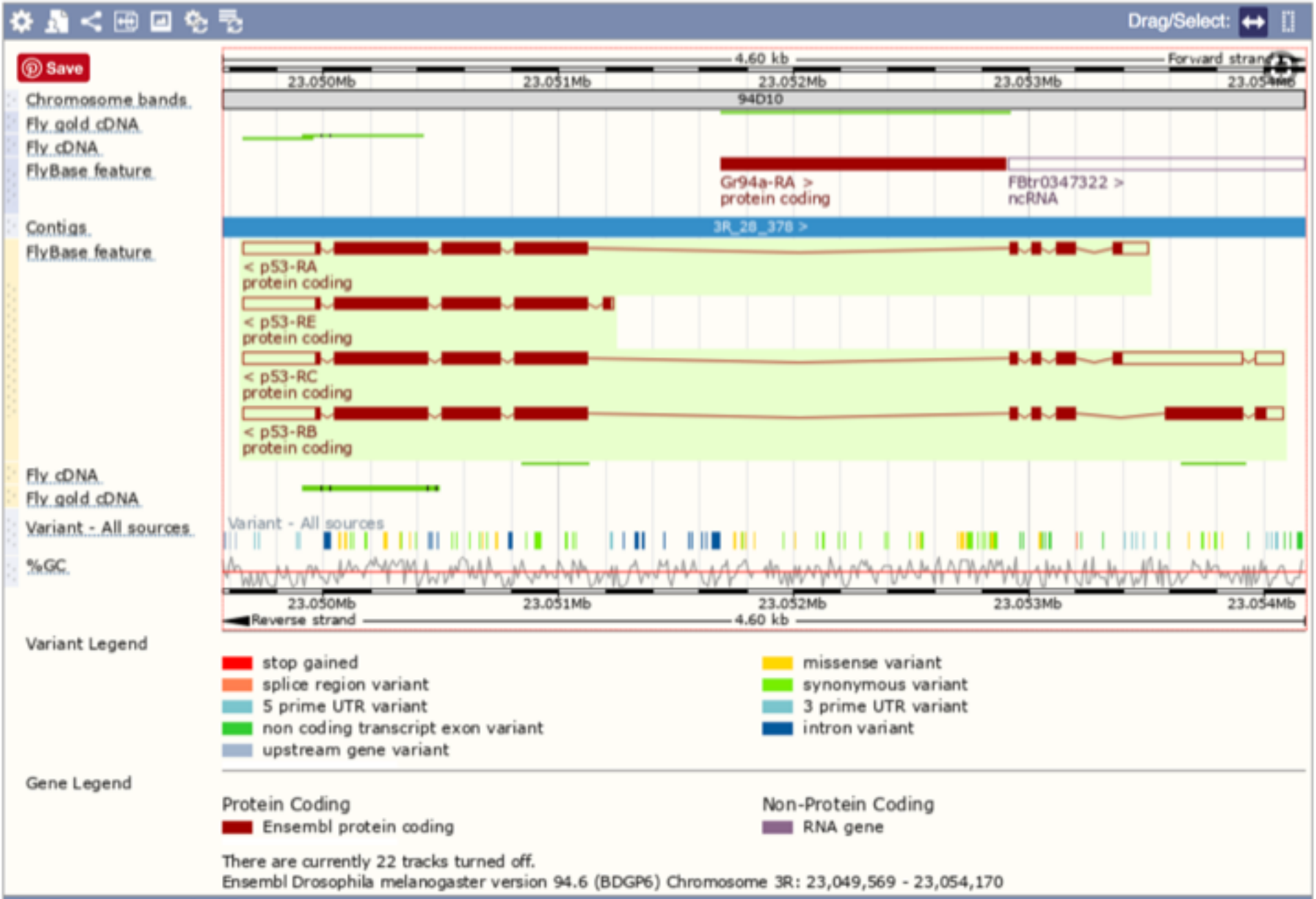
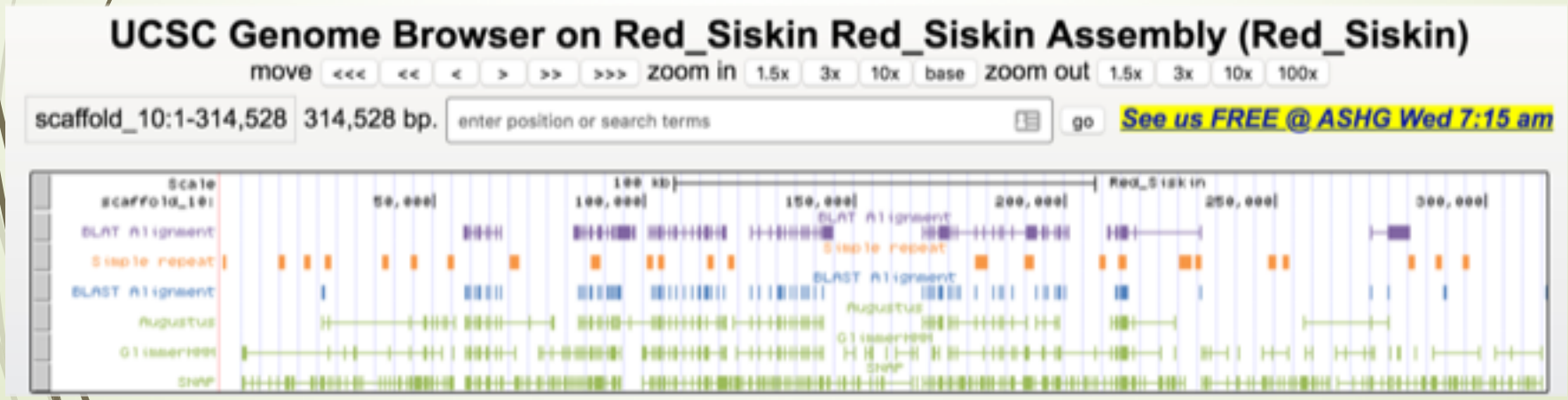


Red siskin genome browser

- Multiple sources of evidence:
  - BLAT alignment
  - Repetitive regions
  - BLAST alignment
  - Gene annotator and predictors

Galaxy PROJECT



Genome Annotation

- Augustus
- SNAP
- GlimmerHMM
- Genemark-ES
- FGenesh
- Gnomon

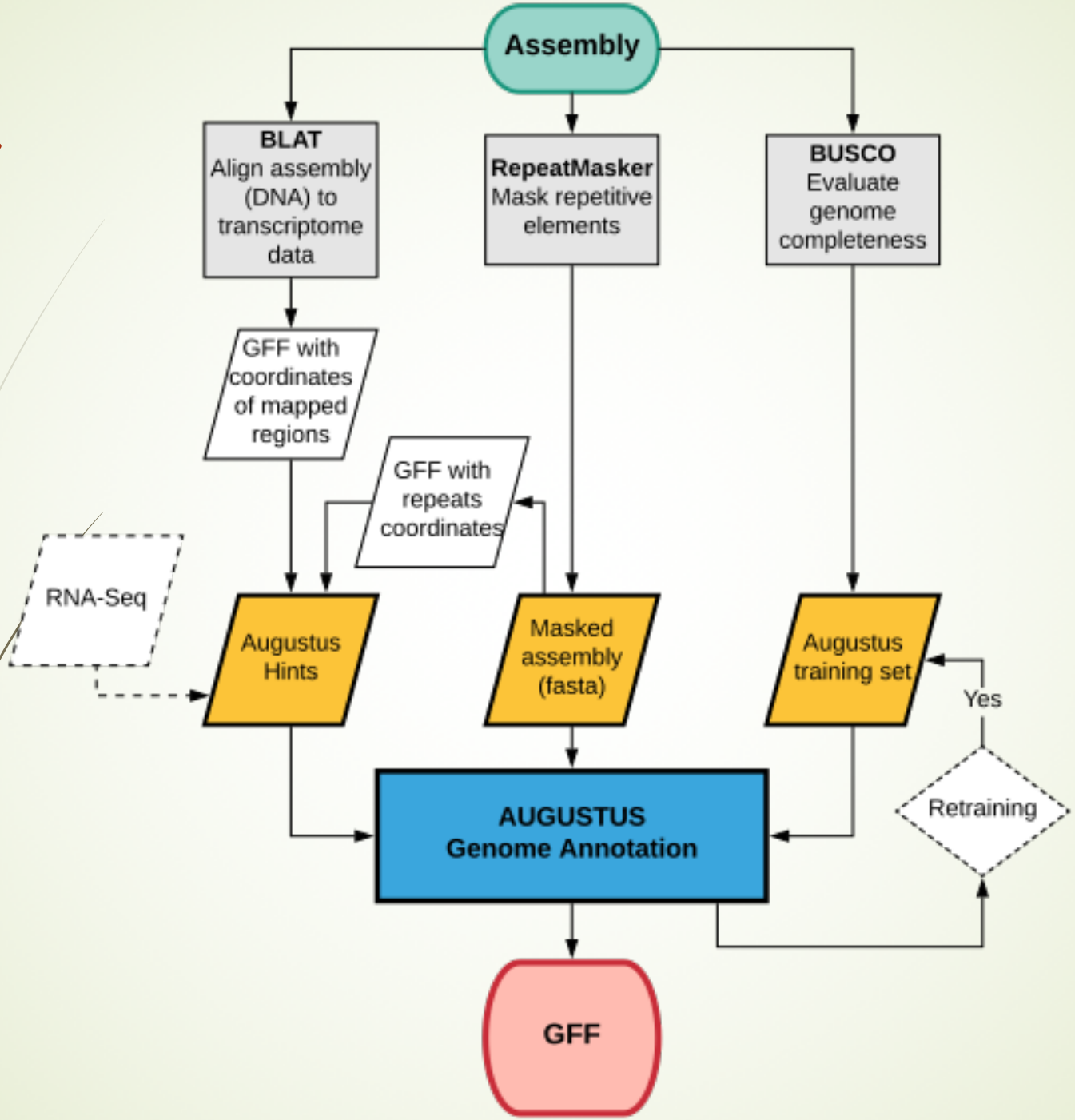
Other alternatives

Pros

- Standardized
- Free

Limitations

- Quality requirements
- Taxonomic priorities


Assembly
BLAT
Align assembly (DNA) to transcriptome data
RepeatMasker
Mask repetitive elements
BUSCO
Evaluate genome completeness
GFF with coordinates of mapped regions
GFF with repeats coordinates
RNA-Seq
Augustus Hints
Masked assembly (fasta)
Augustus training set
Yes
Retraining
AUGUSTUS
Genome Annotation
GFF

Folder structure

- Create the following folders:
  - assembly
  - busco ***
  - repeatmasker
  - augustus ***
  - blast

***

You should have your augustus and busco folders

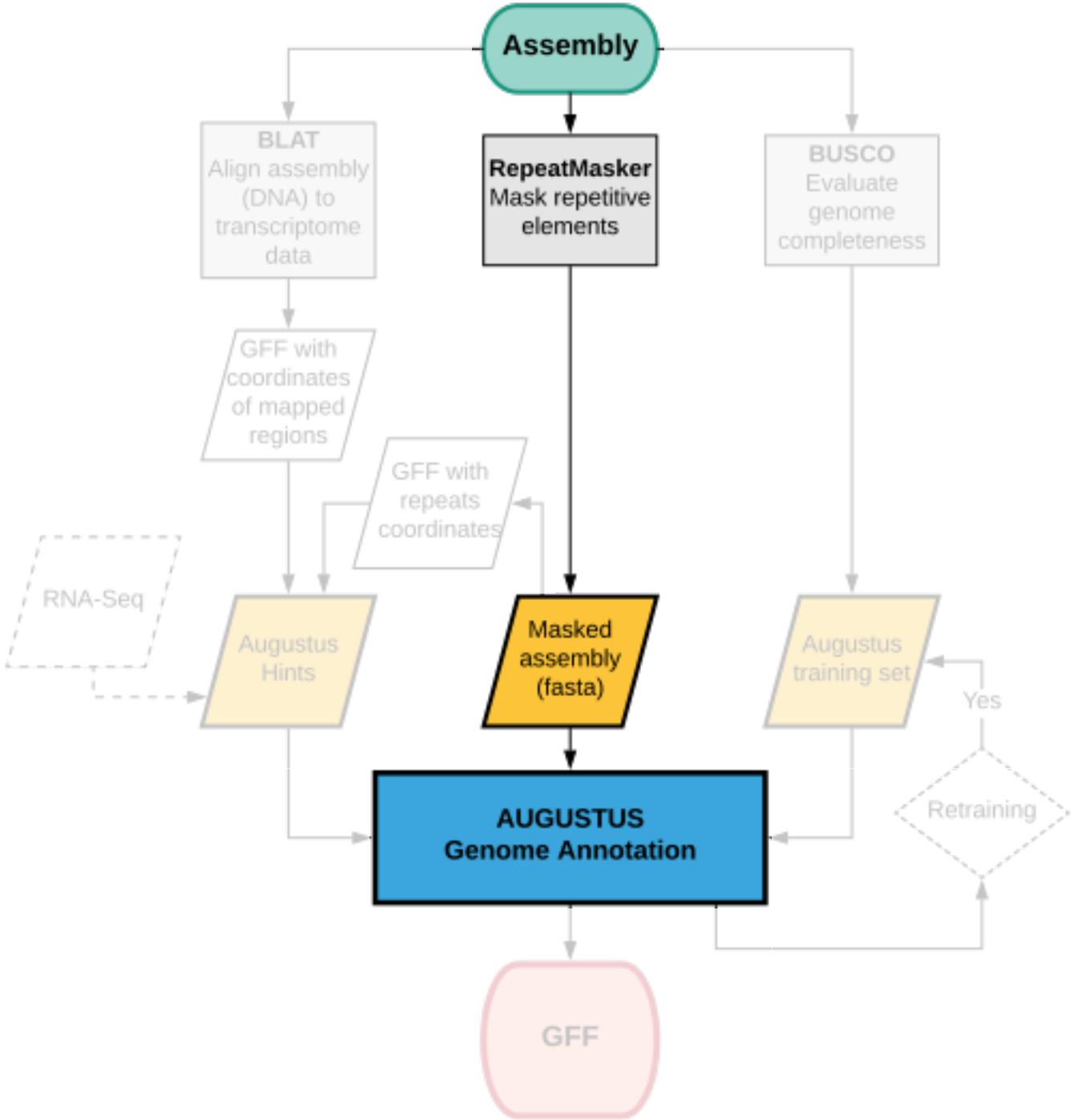
***

- Each job will be executed from its specific folder.

Why?
It is easier to find everything later.

Data prep

- **ASSEMBLY**:
  - cd to your **assembly folder** and copy the same contig you used as reference for for the variant calling pipeline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Augustus training set
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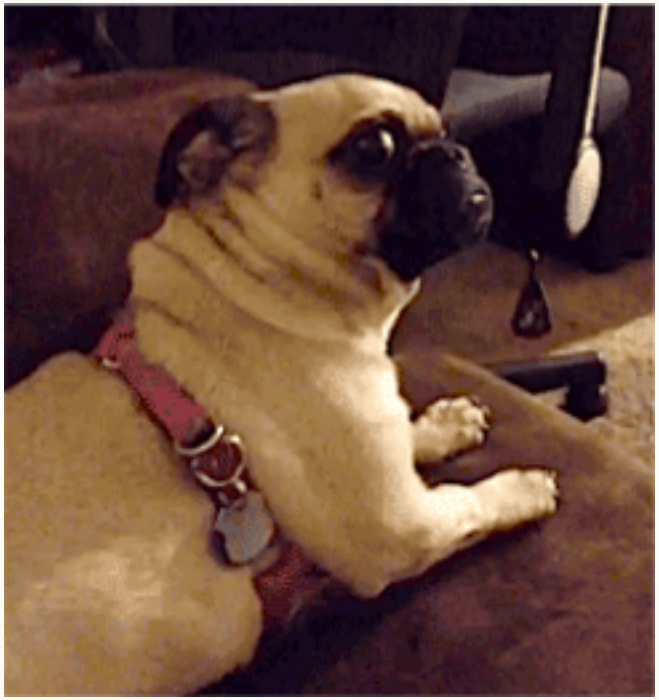
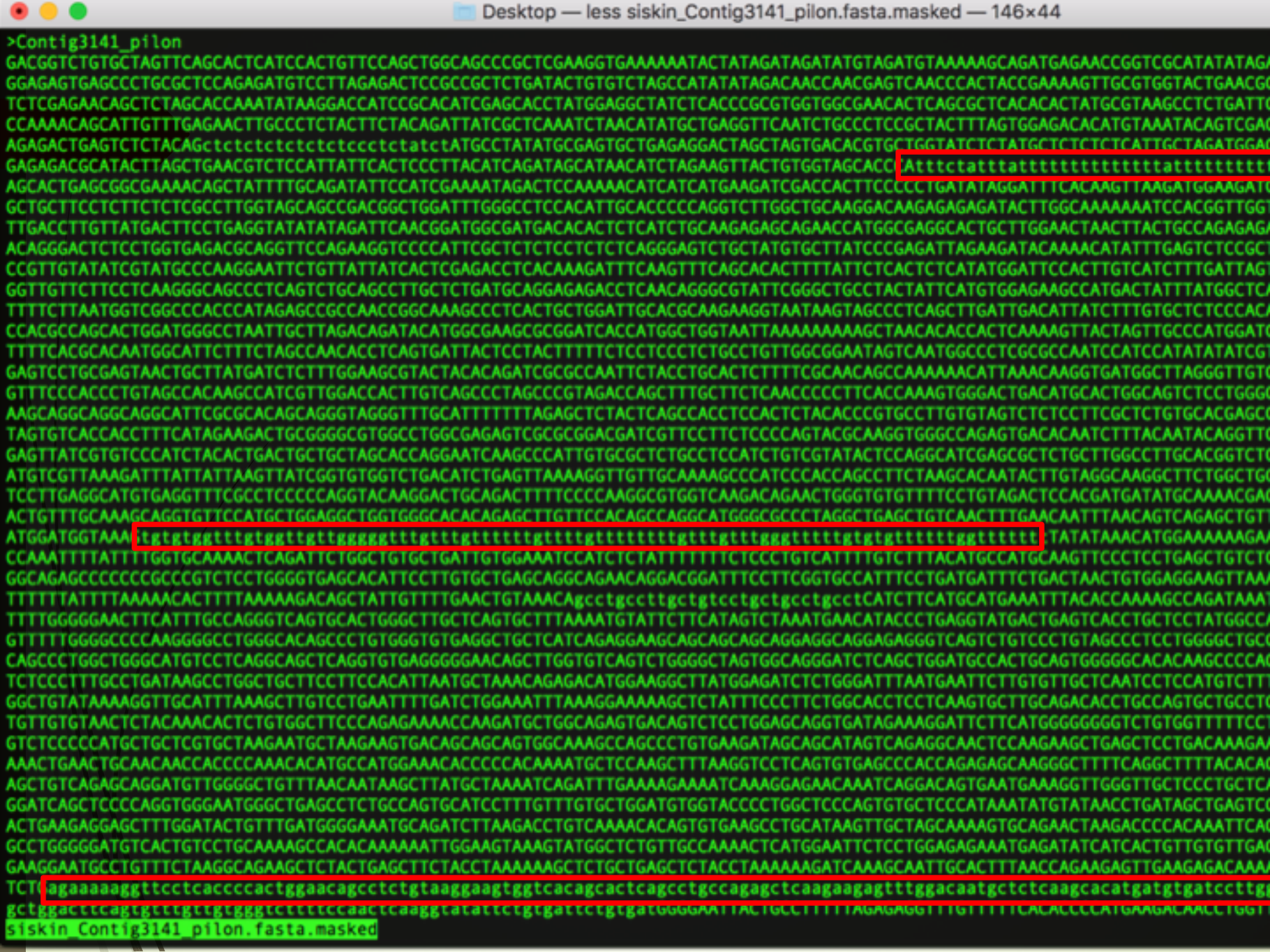
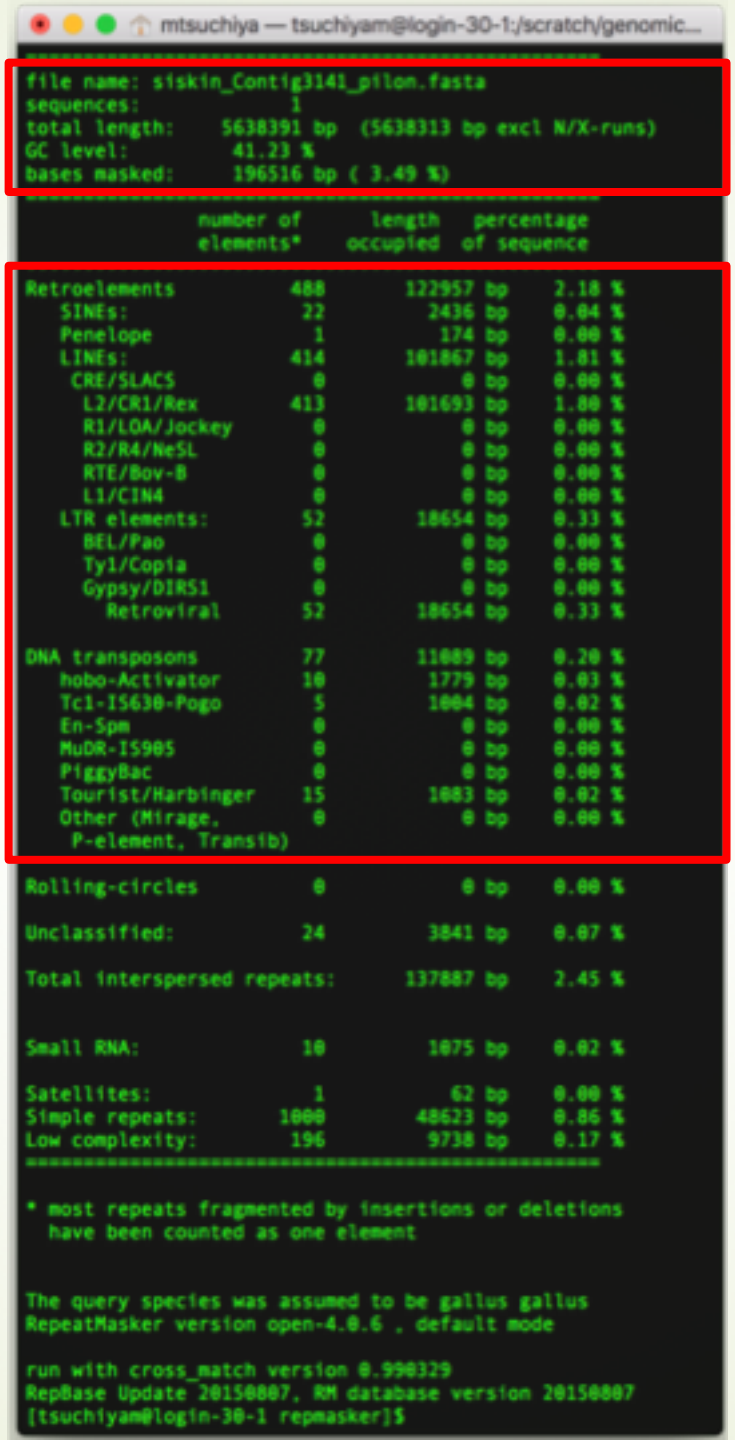
RepeatMasker

RepeatMasker is a program that screens DNA sequences for interspersed repeats and low complexity DNA sequences.

(from the RepeatMasker website)




Desktop — less siskin_Contig3141_pilon.fasta.masked — 146×44
>Contig3141_pilon
siskin_Contig3141_pilon.fasta.masked



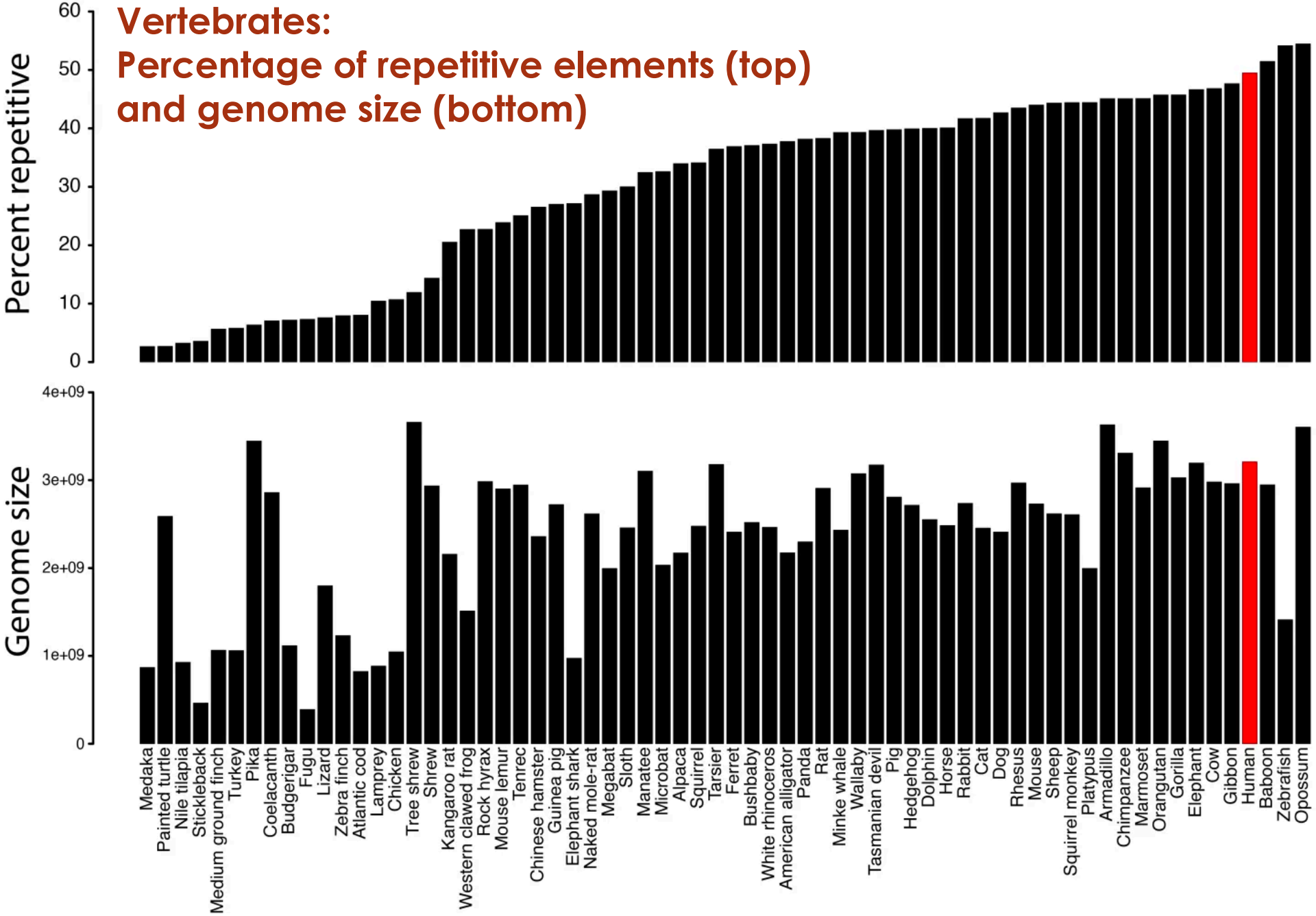
Run information: input file, total length, % bases masked

Types of repetitive elements, quantity, length and percentage of sequence

```
[tsuchiyam@login-30-1 repmasker]$ head -n 200 *.out
   SW   perc perc perc  query             position in query           matching       repeat              position in repeat
score   div. del. ins.  sequence          begin    end       (left)   repeat         class/family      begin  end    (left)   ID

   17    8.8  0.0  0.0  Contig3141_pilon      604     627 (5637764) + (CT)n          Simple_repeat         1     24     (0)    1
   26   11.3  0.0  0.0  Contig3141_pilon      819     856 (5637535) + (T)n           Simple_repeat         1     38     (0)    2
   30   20.8  3.4  2.2  Contig3141_pilon     3372    3459 (5634932) + (TGTT)n        Simple_repeat         1     89     (0)    3
   12    8.2  3.6  3.6  Contig3141_pilon     3852    3879 (5634512) + (GCCT)n        Simple_repeat         1     28     (0)    4
  780   23.1  5.7  2.9  Contig3141_pilon     5845    6049 (5632342) + CR1-X2         LINE/CR1           3923   4133     (6)    5
   18   18.1  0.0  0.0  Contig3141_pilon     6253    6290 (5632101) + (ATT)n         Simple_repeat         1     38     (0)    6
   14   21.8  0.0  4.5  Contig3141_pilon    17123   17168 (5621223) + G-rich         Low_complexity        1     44     (0)    7
  459   24.3  0.0  1.8  Contig3141_pilon    23515   23627 (5614764) + CR1-X1         LINE/CR1           4023   4133     (4)    8
 1488   27.5  1.5  1.2  Contig3141_pilon    28192   28600 (5609791) C CR1-F2         LINE/CR1            (16)   4497   4088    9
  254   24.1  0.0  0.0  Contig3141_pilon    28926   28983 (5609408) C UCON24         Unknown             (111)    263    206   10
   20   22.2  0.0  3.4  Contig3141_pilon    30324   30384 (5608007) + A-rich         Low_complexity        1     59     (0)   11
   21    0.0  0.0  0.0  Contig3141_pilon    40281   40303 (5598088) + (T)n           Simple_repeat         1     23     (0)   12
  969   24.0  2.5  5.5  Contig3141_pilon    42725   43050 (5595341) C CR1-X2         LINE/CR1            (41)   4098   3783   13
   15    8.0  0.0  3.6  Contig3141_pilon    44280   44308 (5594083) + (TCTTC)n       Simple_repeat         1     28     (0)   14
   32    0.0  0.0  0.0  Contig3141_pilon    44314   44343 (5594048) + (T)n           Simple_repeat         1     30     (0)   15
   13   18.4  7.0  0.0  Contig3141_pilon    44531   44573 (5593818) + (CTGCTG)n      Simple_repeat         1     46     (0)   16
   13   18.0  0.0  3.0  Contig3141_pilon    47527   47560 (5590831) + (CCTCCC)n      Simple_repeat         1     33     (0)   17
  631   24.5  6.6  4.2  Contig3141_pilon    51189   51380 (5587011) + CR1-H          LINE/CR1           4602   4798   (14)   18
   19    0.0  0.0  3.7  Contig3141_pilon    52831   52858 (5585533) + (AACA)n        Simple_repeat         1     27     (0)   19
   15    5.6  0.0  0.0  Contig3141_pilon    57134   57152 (5581239) + (T)n           Simple_repeat         1     19     (0)   20
 1008   13.1  9.6  0.5  Contig3141_pilon    60288   60487 (5577904) + CR1-C4         LINE/CR1           4289   4508    (3)   21
   16    0.0  0.0  0.0  Contig3141_pilon    64723   64739 (5573652) + (T)n           Simple_repeat         1     17     (0)   22
   15   19.9  0.0  0.0  Contig3141_pilon    64868   64896 (5573495) + A-rich         Low_complexity        1     29     (0)   23
   12    3.5  5.7  8.8  Contig3141_pilon    65872   65906 (5572485) + (CTTTA)n       Simple_repeat         1     34     (0)   24
   47    0.0  0.0  0.0  Contig3141_pilon    72051   72093 (5566298) + (T)n           Simple_repeat         1     43     (0)   25
   47   39.1  0.7  2.2  Contig3141_pilon    72913   73192 (5565199) + (GT)n          Simple_repeat         1    276     (0)   26
  429   25.7  6.4  4.8  Contig3141_pilon    76071   76299 (5562092) C GGLTR8B        LTR/ERVL            (837)    234      2   27
   38    2.3  0.0  0.0  Contig3141_pilon    78999   79042 (5559349) + (A)n           Simple_repeat         1     44     (0)   28
   28   14.7  0.0  0.0  Contig3141_pilon    80244   80288 (5558103) + (A)n           Simple_repeat         1     45     (0)   29
   35   10.5  0.0  0.0  Contig3141_pilon    80590   80640 (5557751) + (A)n           Simple_repeat         1     51     (0)   30
 1021   23.5  6.7  1.7  Contig3141_pilon    84211   84687 (5553704) + CR1-C4         LINE/CR1           3956   4516   (27)   31
  235   29.2  3.0  0.0  Contig3141_pilon    90620   90684 (5547707) + Chompy-2_Croc  DNA/PIF-Harbinger     6     72     (0)   32
   13    9.8  0.0  0.0  Contig3141_pilon    92076   92097 (5546294) + (GGA)n         Simple_repeat         1     22     (0)   33
  513   15.5  1.1  0.0  Contig3141_pilon    93425   93538 (5544853) + CR1-F2         LINE/CR1           3888   3982   (531)   34
   12    8.1  3.6  3.6  Contig3141_pilon    97420   97447 (5540944) + GA-rich        Low_complexity        1     28     (0)   35
   12   12.7  2.5  7.9  Contig3141_pilon   105002  105041 (5533350) + (TGTATA)n      Simple_repeat         1     38     (0)   36
   17   12.5  0.0  0.0  Contig3141_pilon   109381  109406 (5528985) + (T)n           Simple_repeat         1     26     (0)   37
   13   11.1  0.0  6.5  Contig3141_pilon   110496  110528 (5527863) + (TATGCA)n      Simple_repeat         1     31     (0)   38
  263   28.0  9.1  1.0  Contig3141_pilon   123790  123890 (5514501) C LFSINE_Vert    SINE/tRNA           (307)    152     43   39
   20   10.0  0.0  0.0  Contig3141_pilon   124173  124204 (5514187) + (ATA)n         Simple_repeat         1     32     (0)   40
  916   19.7 11.7  0.5  Contig3141_pilon   132695  132913 (5505478) C CR1-C4         LINE/CR1             (7)   4504   4258   41
   13   11.7  3.6  0.0  Contig3141_pilon   143379  143406 (5494985) + (CTGTG)n       Simple_repeat         1     29     (0)   42
   13    0.0  0.0  0.0  Contig3141_pilon   146450  146466 (5491925) + (GCA)n         Simple_repeat         1     17     (0)   43
  871   26.1  0.0  4.6  Contig3141_pilon   154617  154877 (5483514) C MER126         DNA                 (199)    250      2   44
   15   19.9  0.0  0.0  Contig3141_pilon   155007  155035 (5483356) + A-rich         Low_complexity        1     29     (0)   45
   20   16.4  0.0  0.0  Contig3141_pilon   158673  158706 (5479685) + (T)n           Simple_repeat         1     34     (0)   46
   19    4.5  0.0  0.0  Contig3141_pilon   162947  162969 (5475422) + (T)n           Simple_repeat         1     23     (0)   47
   19    0.0  0.0  0.0  Contig3141_pilon   163664  163684 (5474707) + (TA)n          Simple_repeat         1     21     (0)   48
  311   21.1  3.1  6.9  Contig3141_pilon   170755  170856 (5467535) C CR1-8_Crp      LINE/CR1            (813)   2045   1948   49
   16   18.9  0.0  0.0  Contig3141_pilon   173044  173073 (5465318) + (AC)n          Simple_repeat         1     30     (0)   50
   12   14.4  3.0  3.0  Contig3141_pilon   173908  173940 (5464451) + (AATGAA)n      Simple_repeat         1     33     (0)   51
  400   18.4  0.9  0.2  Contig3141_pilon   175213  175287 (5463104) C CR1-X1         LINE/CR1             (1)   4136   4071   52
   14   27.1  0.0  0.0  Contig3141_pilon   176602  176645 (5461746) + A-rich         Low_complexity        1     44     (0)   53
   12    7.8  3.2  6.7  Contig3141_pilon   187232  187262 (5451129) + (ATAA)n        Simple_repeat         1     30     (0)   54
   12   26.5  0.0  5.8  Contig3141_pilon   191939  191993 (5446398) + (TTTC)n        Simple_repeat         1     52     (0)   55
```

Vertebrates: Percentage of repetitive elements (top) and genome size (bottom)



https://davetang.org/muse/2014/05/22/repetitive-elements-in-vertebrate-genomes/

RepeatMasker

- RepeatMasker has several repetitive elements databases (Eukaryotic species only)

- Commonly used species include:

mammal, carnivore, rodentia, rat, cow, pig, cat, dog, chicken, fugu, danio, "ciona intestinalis" drosophila, anopheles, elegans, diatoaea, artiodactyl, arabidopsis, rice, wheat, and maize

RepeatMasker – job

- Create a symlink of the assembly in the repeatmasker folder:
  ln -s ../assembly/myassembly.fasta .

- Parameters:
  - Short
  - 6 GB memory
  - Serial
  - Module: repeatmasker
  - Command:

WHY?
RepeatMasker saves all output files in the folder where the assembly is.

RepeatMasker -species chicken -xsmall -gff -pa $NSLOTS assembly.fasta

RepeatMasker command

RepeatMasker

-species chicken: RepBase species

-xsmall : soft-masking (repetitive elements are masked in low caps instead of replaced by N)

-gff: additional output in gff2 format

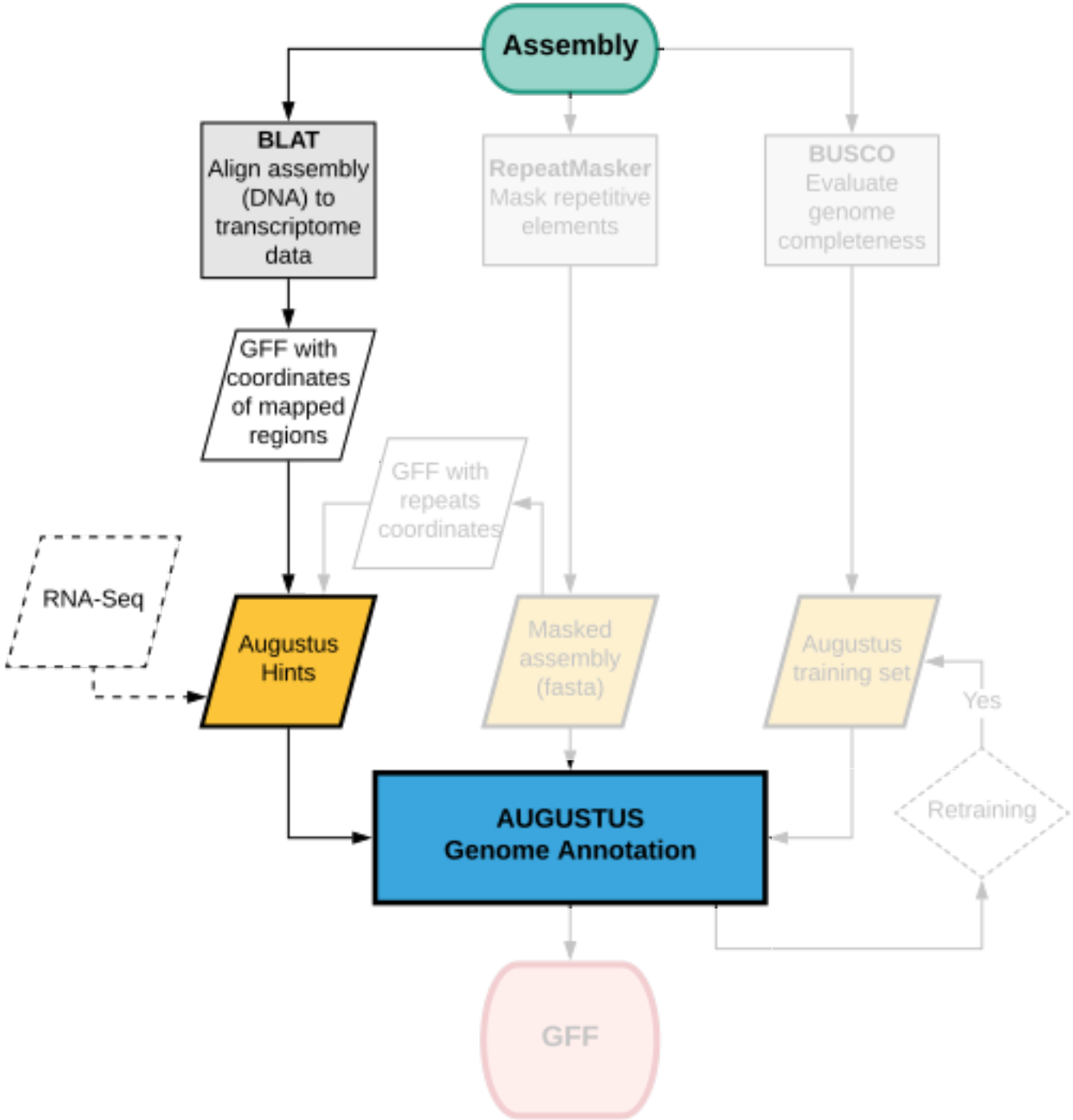
-pa $NSLOTS: number of cpus

assembly.fasta: input file

Other sources of evidence

- RNA-Seq
- Transcriptomes

Today we will use the transcriptome of a different species to generate another source of information for the annotation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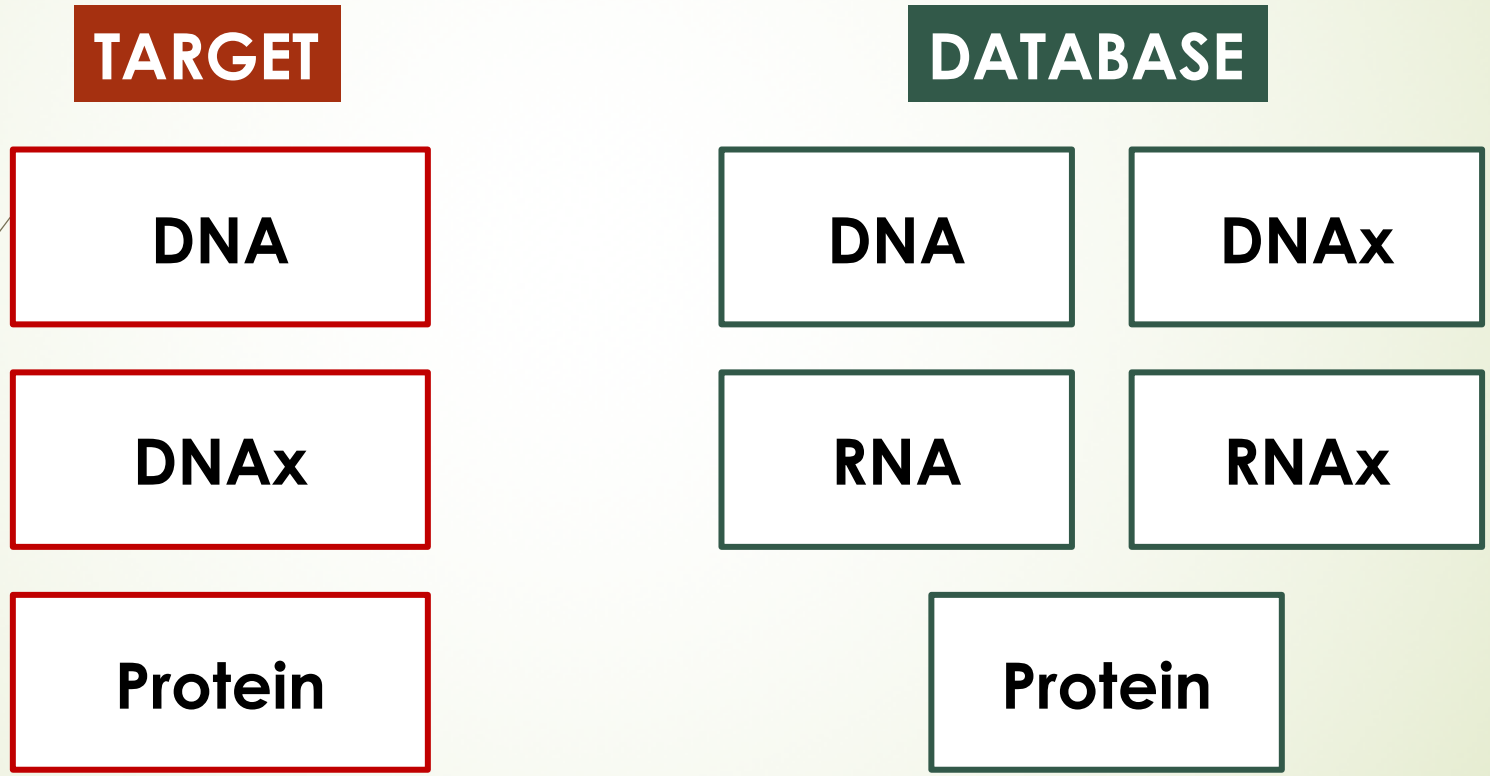
AUGUSTUS
Genome Annotation
GFF

BLAT

- BLAT-like Alignment Tool



DNAx and RNAx correspond to 6-frame translated sequences

BLAT - what does it tell us?

- It will provide information regarding exons and introns, based on the alignment of the transcriptome sequence to our assembly.

Augustus – hints - Blat

- From the /pool/genomics/user/augustus folder:
  - Create blat folder and cd to it:
    mkdir blat && cd blat
  - Download the chicken transcript file (reference):

    wget
    ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/315/GCF_000002315.5_GRCg6a/GCF_000002315.5_GRCg6a_rna.fna.gz

  - Unzip: gunzip
    GCF_000002315.5_GRCg6a_rna.fna.gz

Augustus hints: Blat

- Create the job file using the Qsub generator

- Command:
  blat -t=dna -q=rna \
  ../../repmasker/your_assembly.masked \
  GCF_000002315.5_GRCg6a_rna.fna \
  siskin_blat.psl

BUSCO

- **B**enchmarking **U**niversal **S**ingle-**C**opy **O**rthologs

What is a ortholog?

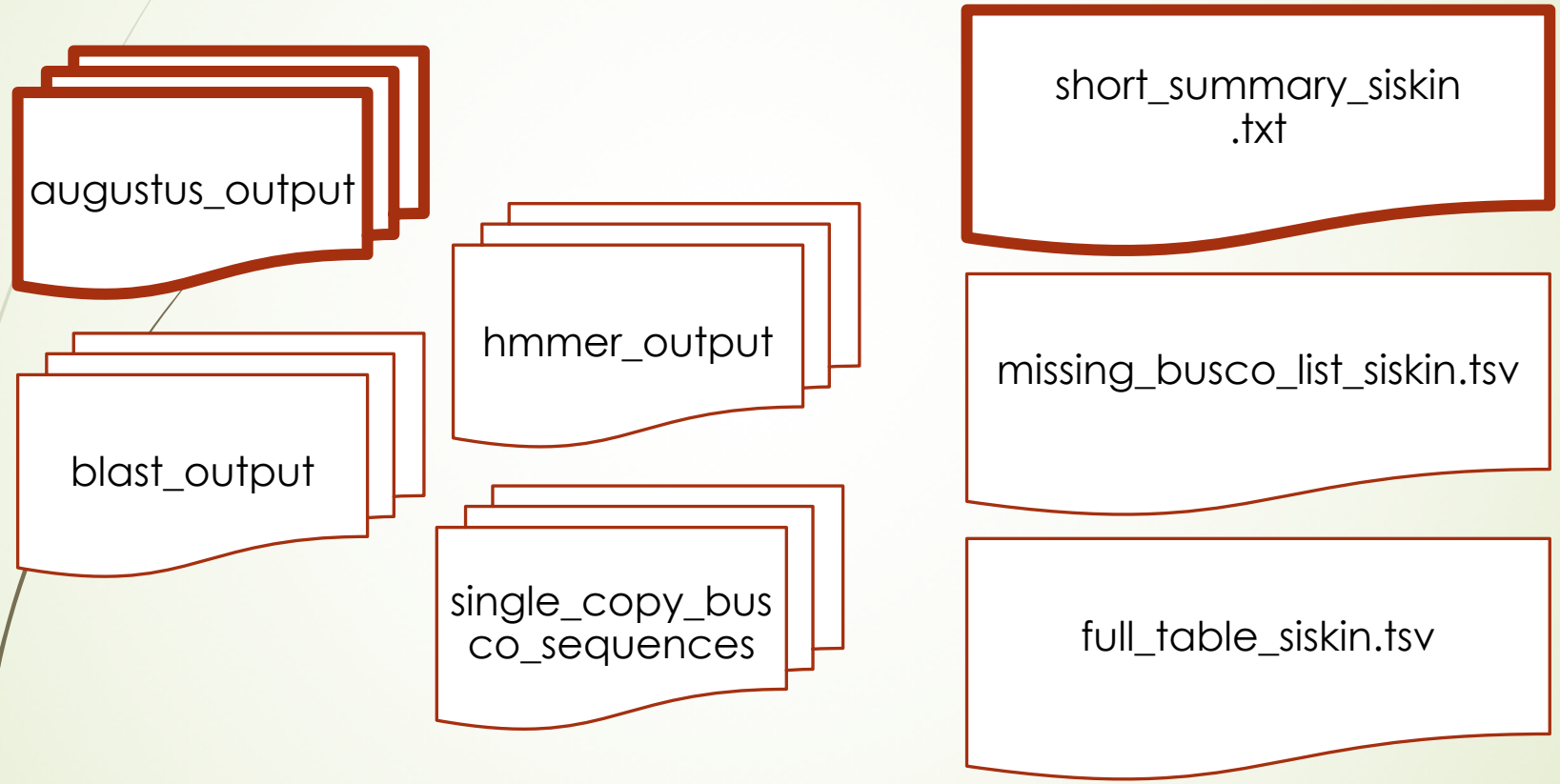
Orthologs are genes in different species that evolved from a common ancestral gene by speciation.

BUSCO: What did we do?

- Input: red siskin assembly (full)
- Database: aves_odb9
- Mode: genome

```
export AUGUSTUS_CONFIG_PATH="/pool/genomics/user_id/augustus/config"
#
run_BUSCO.py --long \
-o siskin \
-i /data/genomics/dikowr/SMSC/finished_assembly/masurca_siskin.fasta \
-l ./aves_odb9 \
-c 1 \
-m genome
```

BUSCO Output - run_siskin_busco



BUSCO - results:

1. Check the results of your BUSCO run in the short_summary_siskin.txt

    a. How many Complete (C), Duplicated (D), Fragmented (F) and Missing (M)?

    C: 4610

    D: 63

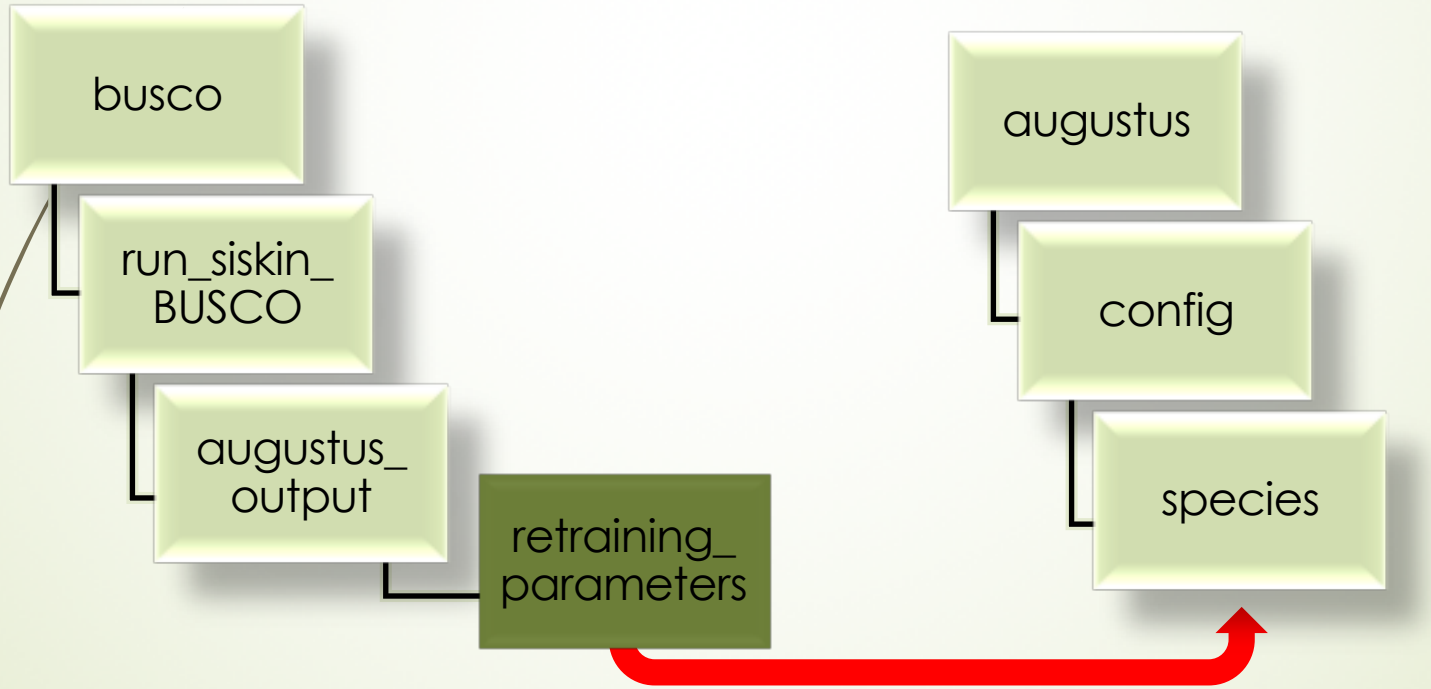
    F: 162

    M: 143

    a. Do you think this is a good or a bad assembly?

From BUSCO to Augustus

- Copy the folder retraining_parameters to augustus/config/species



From BUSCO to Augustus

- cd to augustus/config/species/retraining_parameters

- ls to check the names of all files:

```
BUSCO_siskin_busco_2684346740_metapars.cgp.cfg
BUSCO_siskin_busco_2684346740_metapars.utr.cfg
BUSCO_siskin_busco_2684346740_parameters.cfg
BUSCO_siskin_busco_2684346740_weightmatrix.txt
```

Rename your retraining parameters folder

- Copy the prefix that appears in all files
- Go one level above (cd ..) and change the name of the retraining_parameters folder to the prefix:

  mv retraining_parameters BUSCO_siskin_busco_2684346740

Creating hints for Augustus

Augustus hints: Blat

- Convert blat results to hints **(from the interactive node: qrsh)**
  - Sort the .psl file
    cat siskin_blat.psl | sort -n -k 16,16 | sort -n -k 14,14 > siskin_blat_srt.psl
  - Load the augustus/3.3 module
    module load bioinformatics/augustus/3.3
  - Run the script blat2hints.pl
    blat2hints.pl --in=siskin_blat_srt.psl --out=siskin_blat_hints.out

Augustus hints: RepeatMasker

- cd to your repeatmasker folder
- load the module repeatmasker
  module load bioinformatics/repeatmasker

- Use the script rmOutToGFF3.pl to convert your .out file into GFF3:

  rmOutToGFF3.pl <input.out> > <output>.gff3

Augustus hints: RepeatMasker

- Copy the gff2hints.pl script
  cp /pool/genomics/tsuchiyam/gff2hints.pl .

- Now let's convert the gff3 into a hints file:

  perl gff2hints.pl --in=input.gff3 --source=RM --out=hints_RM.out

Augustus: Combining hints

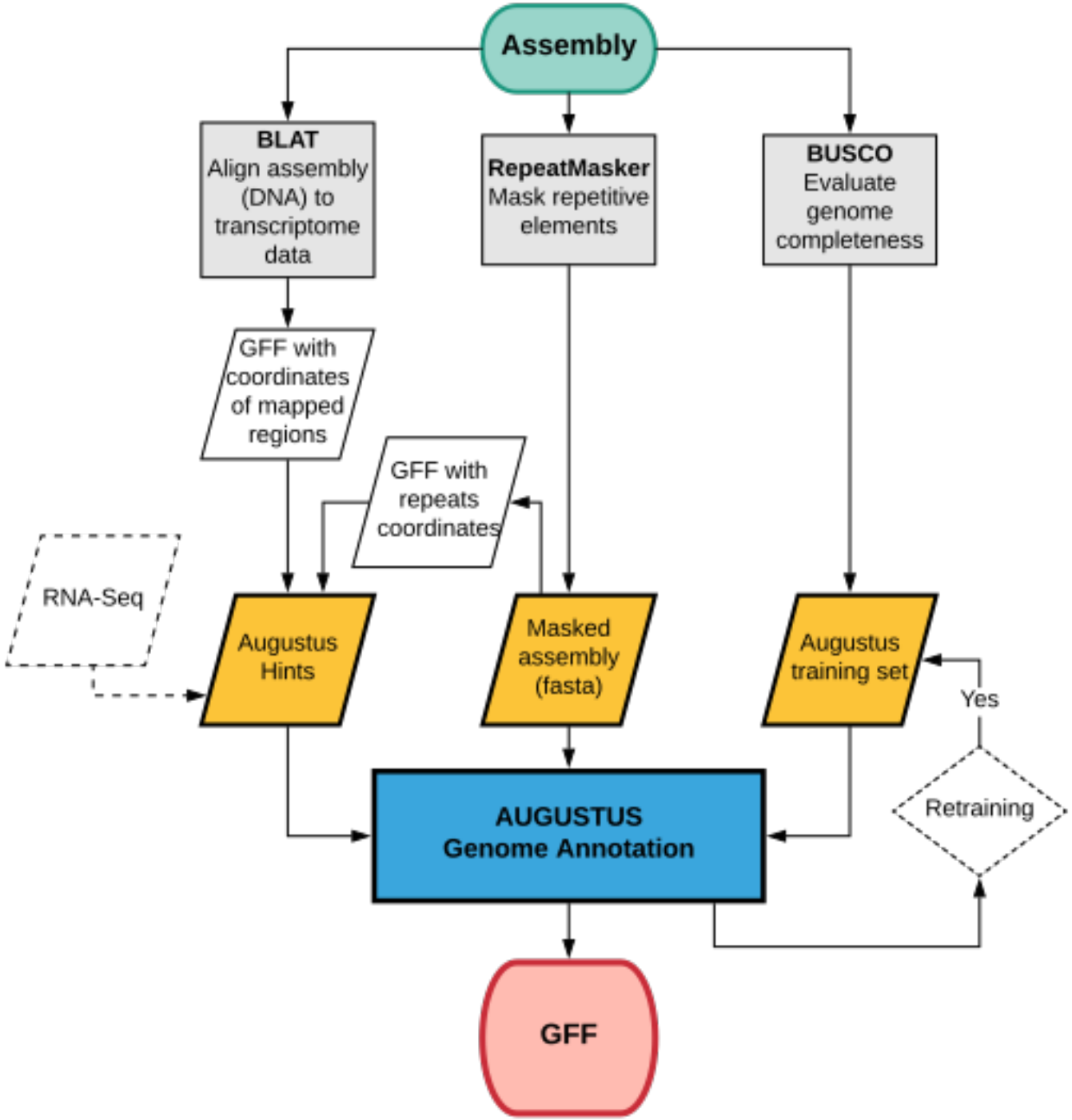
- Merge both files:

1. from your augustus folder
2. merge both hints files

```
cat ../repeatmasker/hints_RM.out
blat/siskin_blat_hints.out | sort -n -k 4,4 | sort -n
-k 5,5 > siskin_RM_E.hints
```

Phew…

- What do we have now?
  - Masked fasta from RepeatMasker
  - Hints file
  - Training set from BUSCO
- What else do we need?


Assembly
BLAT
Align assembly (DNA) to transcriptome data
RepeatMasker
Mask repetitive elements
BUSCO
Evaluate genome completeness
GFF with coordinates of mapped regions
GFF with repeats coordinates
RNA-Seq
Augustus Hints
Masked assembly (fasta)
Augustus training set
Yes
Retraining
AUGUSTUS
Genome Annotation
GFF

Augustus

- *ab initio (*internal) + evidence-driven(external)

AUGUSTUS is based on a generalized hidden Markov model (GHMM), which defines probability distributions for the various sections of genomic sequences. Introns, exons, intergenic regions, etc. correspond to states in the model and each state is thought to create DNA sequences with certain pre-defined emission probabilities.

(Mario Stanke, Burkhard Morgenstern; AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints, *Nucleic Acids Research*, Volume 33, Issue suppl_2, 1 July 2005, Pages W465–W467, https://doi.org/10.1093/nar/gki458)

Augustus

- Augustus needs to be trained:
  - Training consists on the generation of a training set that will more accurately predict genes.

(BUSCO solved the training issue for us)

Augustus

- Inputs:
  - Masked fasta (repeatmasker output)
  - Hints file
  - Training set

Augustus

- Masked fasta:
  - Copy the file from the repeatmasker

cp ../repeatmasker/assembly.fasta.masked .

Alternatively, we cam create a symlink instead.

ln -s ../repeatmasker/assembly.masked .

Augustus extrinsic file

- Defines how the information from the hints will be weighted:
- It assigns "bonus" and "malus" (penalty) values to each hint used
  - M: manual annotation
  - W: RNA-Seq coverage information
  - E: EST/cDNA database hit
  - R: retroposed genes
  - RM: repeat masking

```
[SOURCES]
M RM E

#
# individual_liability: Only unsatisfiable hints are disregarded. By default this flag is not set
# and the whole hint group is disregarded when one hint in it is unsatisfiable.
# 1group1gene: Try to predict a single gene that covers all hints of a given group. This is relevant for
# hint groups with gaps, e.g. when two ESTs, say 5' and 3', from the same clone align nearby.
#
[SOURCE-PARAMETERS]


#   feature        bonus         malus   gradelevelcolumns
#               r+/r-
#
# the gradelevel colums have the following format for each source
# sourcecharacter numscoreclasses boundary    ...  boundary    gradequot  ...  gradequot
#

[GENERAL]
      start      1          1  M    1  1e+100  RM  1     1    E 1    1
       stop      1          1  M    1  1e+100  RM  1     1    E 1    1
        tss      1          1  M    1  1e+100  RM  1     1    E 1    1
        tts      1          1  M    1  1e+100  RM  1     1    E 1    1
        ass      1      1 0.1  M    1  1e+100  RM  1     1    E 1    1
        dss      1      1 0.1  M    1  1e+100  RM  1     1    E 1    1
   exonpart      1  .992 .985  M    1  1e+100  RM  1     1    E 1  1e2
       exon      1          1  M    1  1e+100  RM  1     1    E 1  1e4
 intronpart      1          1  M    1  1e+100  RM  1     1    E 1    1
     intron      1        .34  M    1  1e+100  RM  1     1    E 1  1e6
    CDSpart      1     1 .985  M    1  1e+100  RM  1     1    E 1    1
        CDS      1          1  M    1  1e+100  RM  1     1    E 1    1
    UTRpart      1     1 .985  M    1  1e+100  RM  1     1    E 1    1
        UTR      1          1  M    1  1e+100  RM  1     1    E 1    1
     irpart      1          1  M    1  1e+100  RM  1     1    E 1    1
nonexonpart      1          1  M    1  1e+100  RM  1     1.15 E 1    1
  genicpart      1          1  M    1  1e+100  RM  1     1    E 1    1

#
# Explanation: see original extrinsic.cfg file
#
```

Extrinsic file

- For practical purposes, copy the extrinsic file below to your augustus/config/extrinsic folder:

/scratch/genomics/tsuchiyam/augustus/config/extrinsic/extrinsic.M.RM.E.cfg

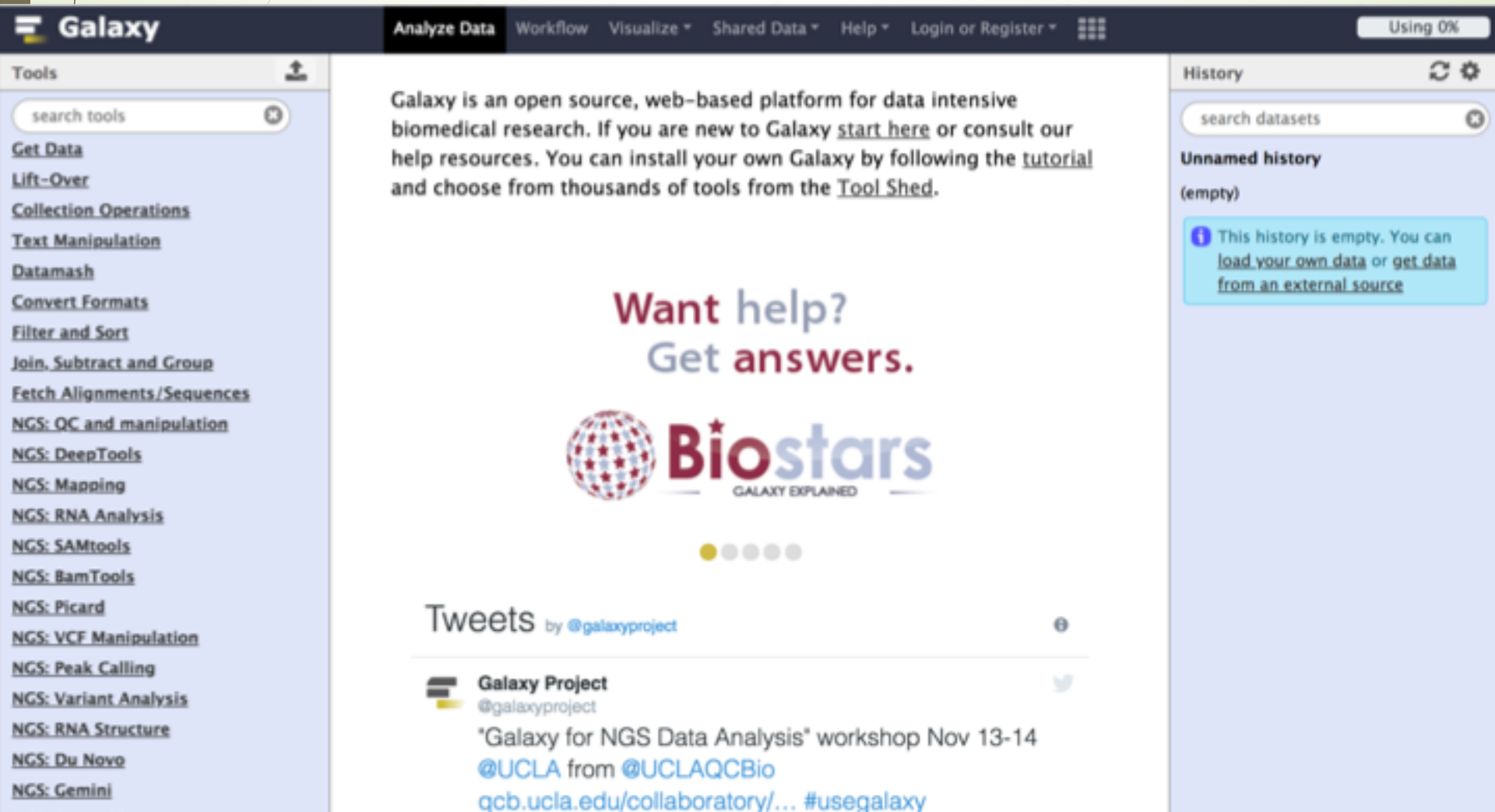
Let's run Augustus

```
augustus --strand=both --singlestrand=true \
   --hintsfile= siskin_RM_E.hints \
   --extrinsicCfgFile=extrinsic.M.RM.E.cfg \
   --alternatives-from-evidence=true \
   --gff3=on \
   --uniqueGeneId=true \
   --softmasking=1 \
   --species= BUSCO_siskin_busco_2684346740 \
   assembly.fasta.masked > siskin_Contig3141.gff
```

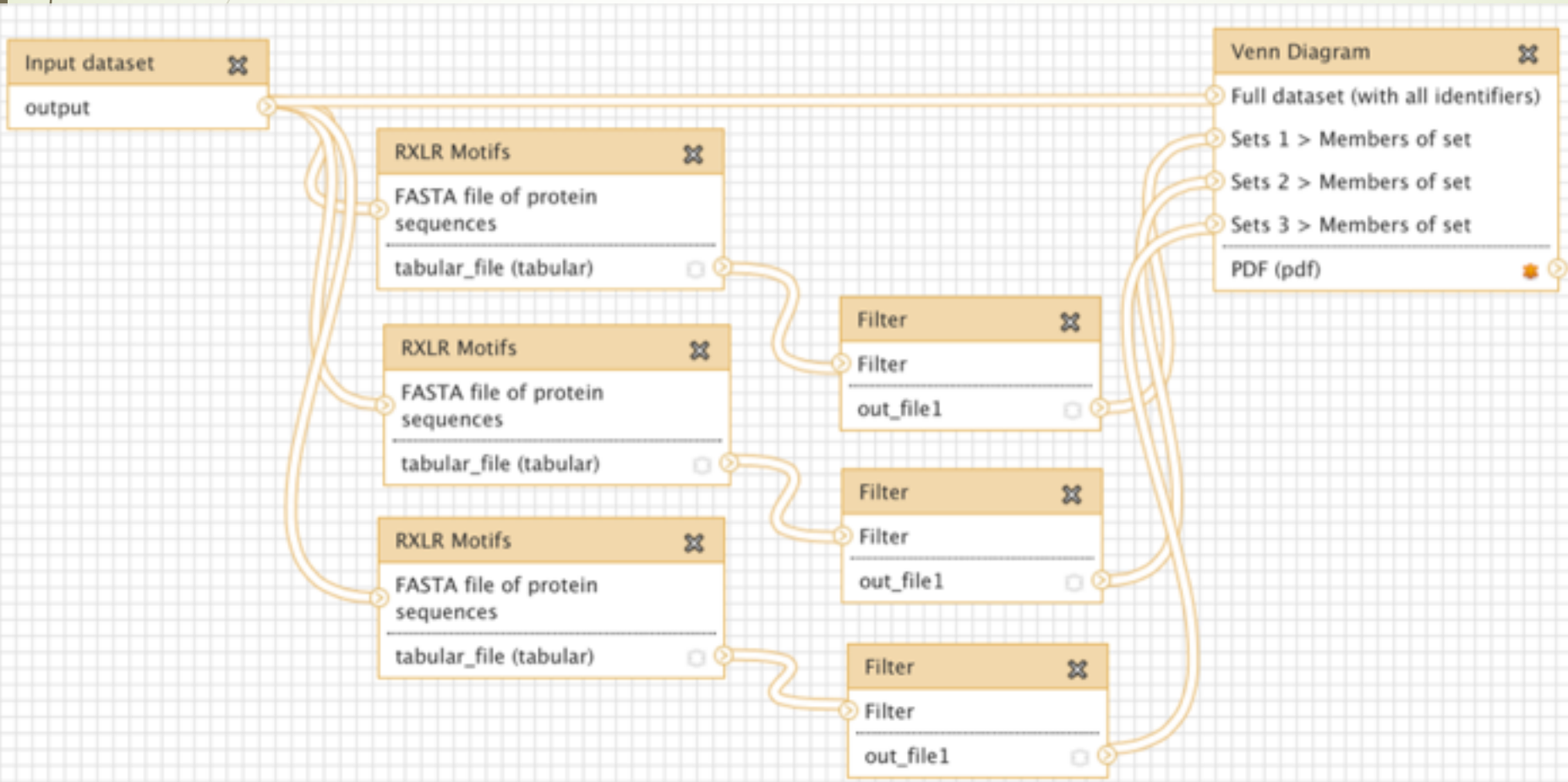
What do we do with the annotation?

- The next step is to understand the functions of the genes we identified
- For functional annotation, we used both BLAST and BLAST2GO

Galaxy PROJECT



Galaxy PROJECT

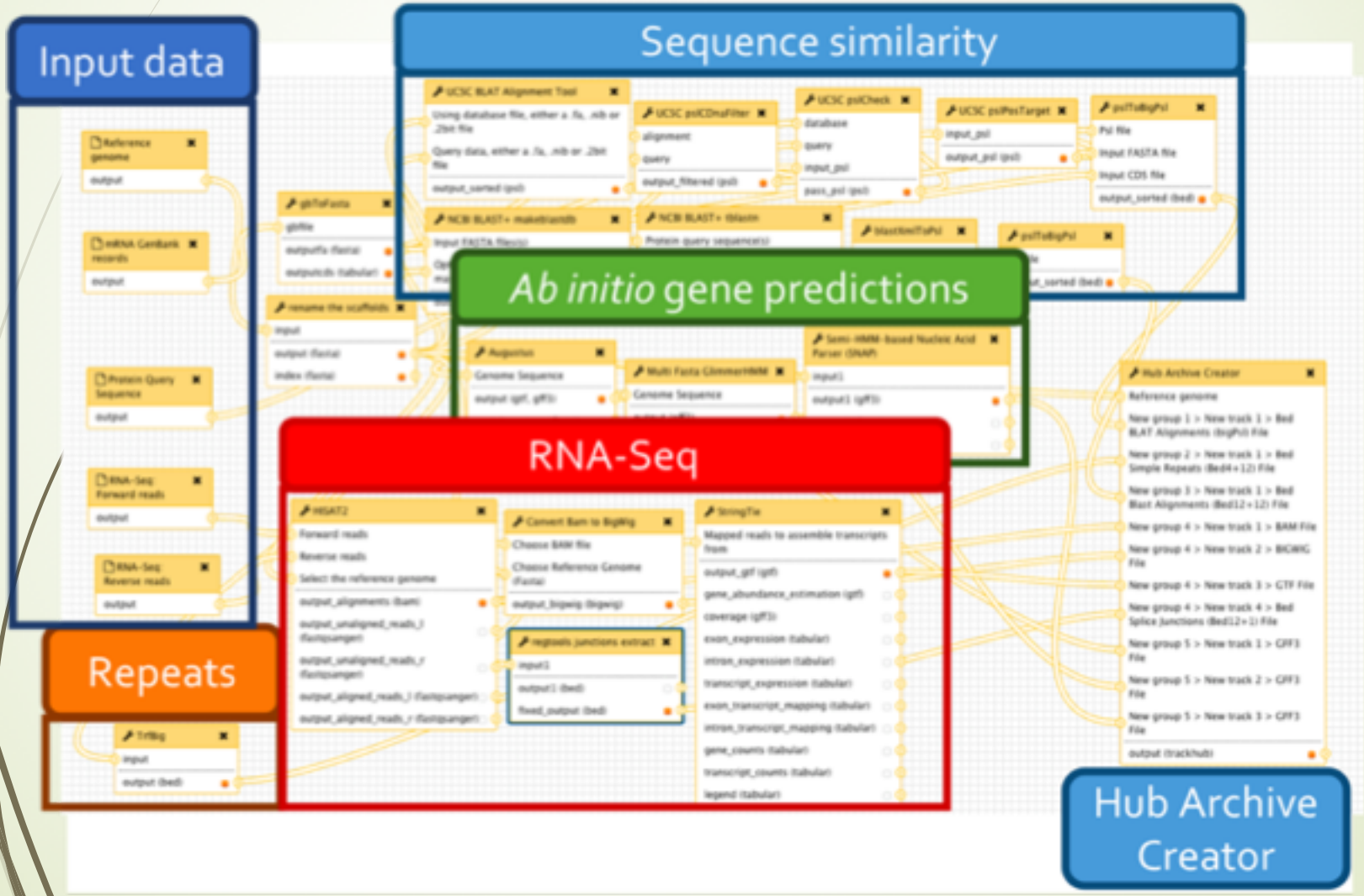


Galaxy PROJECT

- Usegalaxy.org
- Core group + Galaxy community (very active)
- At Smithsonian:
  - Galaxy Server (connected to Hydra)
    - Powerful
    - Behind firewall, intranet only
  - Galaxy on the cloud
    - Accessible from anywhere

Galaxy PROJECT

G-OnRamp

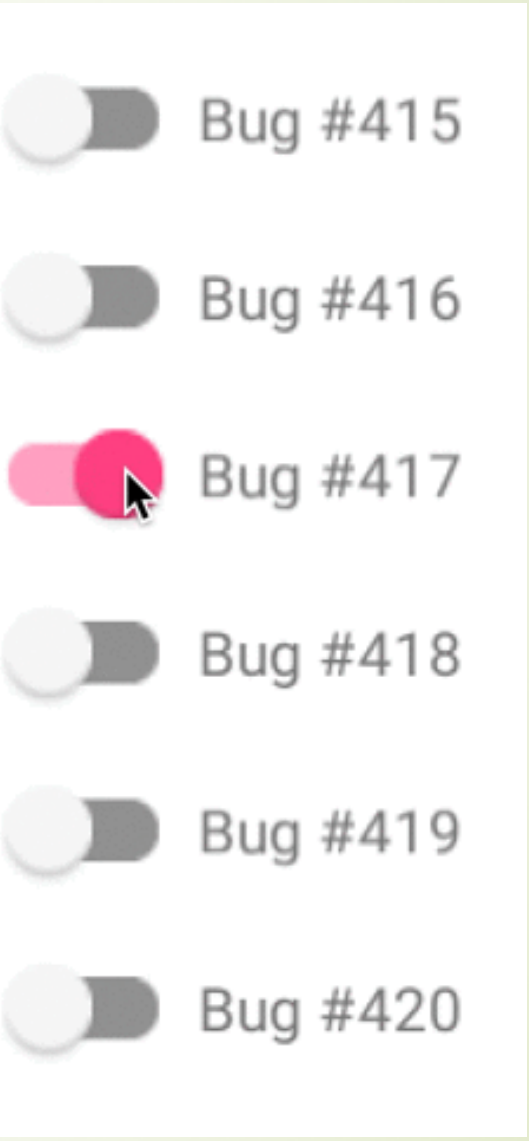


Galaxy PROJECT

Galaxy Annotation: https://usegalaxy.eu/

Thank you


99 little bugs in the code.
99 little bugs in the code.
Take one down, patch it around.
127 little bugs in the code...


Bug #415
Bug #416
Bug #417
Bug #418
Bug #419
Bug #420