

# Analyzing Variant Call results using EuPathDB Galaxy, Part II

In this exercise, we will work in groups to examine the results from the SNP analysis workflow that we started yesterday. *The first step is to share your SNP workflow histories with the rest of the workshop participants:*

1. Give your workflow a meaningful name, eg. The sample or group name.
2. Click on the on the 'History options' link and select the 'share or Publish option'.
3. On the next page click on the 'Make History Accessible and Publish' link.

**1**

History

search datasets

ENU-mutant RH clone resistant to IBET-151 1C6

7 shown, 12 hidden

52.95 GB

17: SnpEff on data 15

16: SnpEff on data 15

13: BAM to BigWig on data 12

9: FastQC on data 2: Webpage

3: FastQC on data 1: Webpage

2: SRR5123637\_2.fastq.gz

1: SRR5123637\_1.fastq.gz

**2**

History

HISTORY LISTS

Saved Histories

Histories Shared with Me

CURRENT HISTORY

Create New

Copy History

Copy Datasets

Share or Publish

Extract Workflow

Dataset Security

Resume Paused Jobs

Collapse Expanded Datasets

Unhide Hidden Datasets

Delete Hidden Datasets

Purge Deleted Datasets

Show Structure

Export Citations

Export to File

Delete

Delete Permanently

OTHER ACTIONS

**3**

Share or Publish History 'ENU-mutant RH clone resistant to IBET-151 1C6'

Make History Accessible via Link and Publish It

This history is currently restricted so that only you and the users listed below can access it. You can:

Make History Accessible via Link

Generates a web link that you can share with other people so that they can view and import the history.

Make History Accessible and Publish

Makes the history accessible via link (see above) and publishes the history to Galaxy's Published Histories section, where it is publicly listed and searchable.

Share History with Individual Users

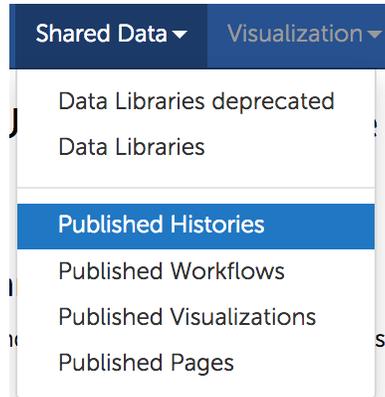
You have not shared this history with any users.

Share with a user

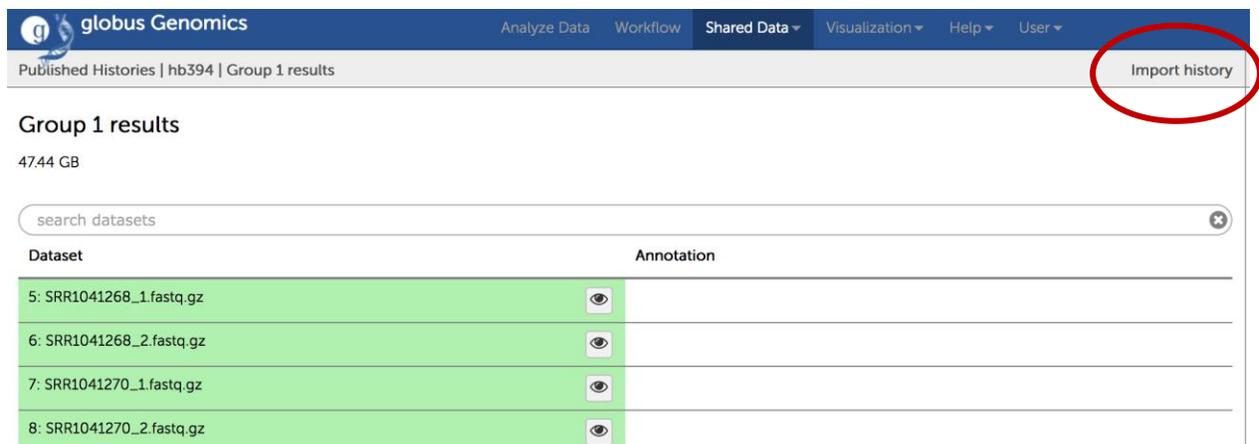
Back to Histories List

To import a shared history into your workspace follow these steps:

1. Select 'Published Histories' from the Shared data menu.



2. From the list of shared histories click on the one you want to import and on the next page select the 'Import' link in the upper right hand side.

A screenshot of the 'Group 1 results' page in the globus Genomics interface. The page header includes the globus Genomics logo and navigation links for 'Analyze Data', 'Workflow', 'Shared Data', 'Visualization', 'Help', and 'User'. Below the header, the page title is 'Published Histories | hb394 | Group 1 results'. A red circle highlights the 'Import history' button in the top right corner. The main content area shows 'Group 1 results' with a size of '47.44 GB'. There is a search bar for datasets. Below the search bar is a table with two columns: 'Dataset' and 'Annotation'. The table contains four rows of dataset information, each with a green background and an eye icon in the 'Annotation' column.

Dataset	Annotation
5: SRR1041268_1.fastq.gz	
6: SRR1041268_2.fastq.gz	
7: SRR1041270_1.fastq.gz	
8: SRR1041270_2.fastq.gz	

## Examining your results:

1. Click on the hidden files link in the history panel to reveal all workflow output files.
2. Examine the output files. What does the tool FASTQC do? What about Sickle?

The image displays two side-by-side screenshots of a workflow history panel. The left screenshot shows a dataset titled "B. micro Wisconsin single" with 4 shown files and 7 hidden files. A red circle highlights the "7 hidden" link, with a red arrow pointing to the right screenshot. The right screenshot shows the same dataset with all 11 files revealed. The files are listed in descending order of step number: 11: SnpEff on data 9, 10: SnpEff on data 9, 9: Filter variants by quality on data 8: filtered by quality, 8: FreeBayes on data 7 (variants), 7: Sort on data 6: sorted BAM, 6: Bowtie2 on data 4: aligned reads, and 1: ERR1349056.fastq.gz. Each hidden file is preceded by an orange warning box that says "This dataset has been hidden" and "Unhide it".

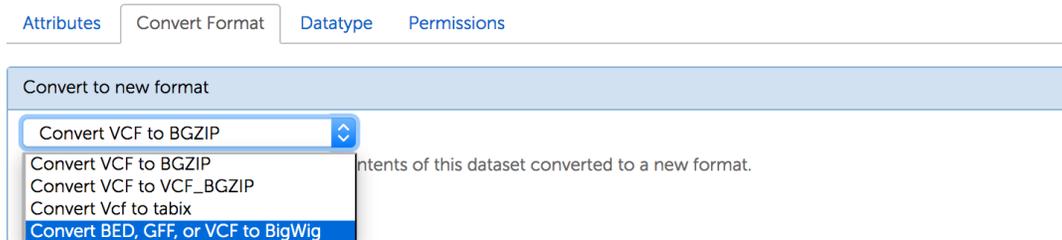
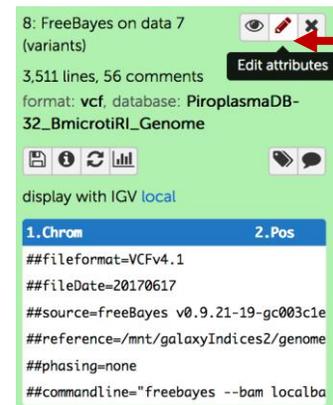
3. The output of Sickle is used by a program called Bowtie2. What does this tool do? Bowtie generates a file called a BAM file. Whenever dealing with sequence alignment files you will likely hear of file formats called SAM or BAM. SAM stands for Sequence Alignment/Map format, and BAM is the binary version of a SAM file.

- Many of the downstream analysis programs that use BAM files require a sorted BAM file. This allows access to reads to be done more efficiently.
- The sorted BAM file is the input for a program called FreeBayes. This program is a Bayesian genetic variant detector designed to find small polymorphisms, specifically SNPs (single-nucleotide polymorphisms), indels (insertions and deletions), MNPs (multi-nucleotide polymorphisms), and complex events (composite insertion and substitution events) smaller than the length of a short-read sequencing alignment. The output for many variant callers is a file called a VCF file. VCF stands for variant interchange format.
- Examine the VCF file in your results (click on the eye icon to view its contents). Detailed information about VCF file content is available here: <https://samtools.github.io/hts-specs/VCFv4.2.pdf>
- What does tool SnpEFF do? SnpEff is a variant annotation and effect prediction tool. It annotates and predicts the effects of variants on genes (such as amino acid changes).

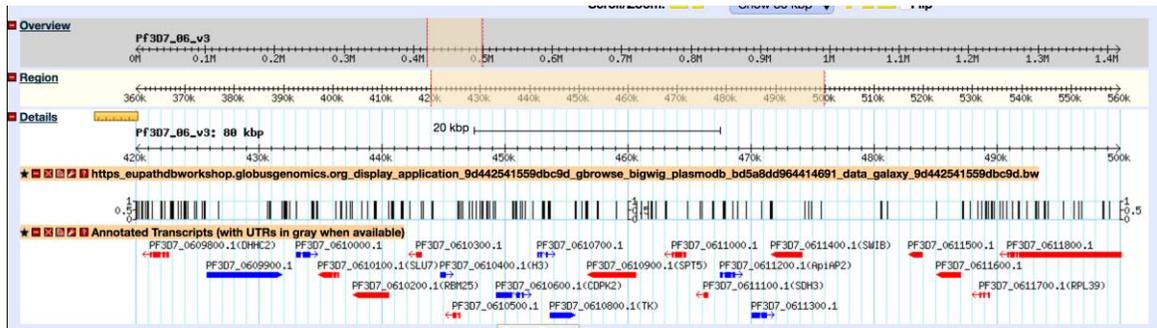
### Viewing VCF file results in a genome browser:

*In order to view a VCF file in GBrowse, it first has to be converted to a format that GBrowse can understand like Big Wig. To do this follow these steps:*

- Click on the edit attributes icon on the FreeBayes VCF output file.
- In the central window click on the 'Convert Format' tab.
- Next select the 'Convert BED, GFF or VCF to BigWig' option and click on the 'Convert' link.
- Notice a new step will appear in you history for the conversion step.



- Once the conversion is done, you can export the BigWig file(s) to you EuPathDB dataset page



## Filtering data in VCF files:

VCF files contain a lot of data about variants and their positions. SnpEff generates various analyses/summaries of VCF files (including GeneIDs that overlap variant positions). However, it is often necessary to filter VCF files further to obtain useful information for your specific question. For example, you may want to filter out SNP positions that have an impact on the coding sequence. One tool that can be used is called SnpSift Filter. This tool allows you to write complex expressions to filter a VCF file.

CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO
PF3D7_0L_v3	30	-	A	G	106.836	.	AB=0;ABP=0;AC=0;AF=0;AN=2;AO=0
PF3D7_0L_v3	415	-	G	C	100.39	.	AB=0;ABP=0;AC=0;AF=0;AN=2;AO=0
PF3D7_0L_v3	421	-	CCTA	ATTC	93.0313	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=1
PF3D7_0L_v3	466	-	T	A	211.522	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=7
PF3D7_0L_v3	704	-	T	C	73.1939	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=7
PF3D7_0L_v3	709	-	G	C	179.817	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=1
PF3D7_0L_v3	737	-	C	G	52.3887	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=1
PF3D7_0L_v3	781	-	GTTA	CTTA	69.6111	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=2
PF3D7_0L_v3	977	-	G	C	169.554	.	AB=0;ABP=0;AC=2;AF=1;AN=2;AO=5

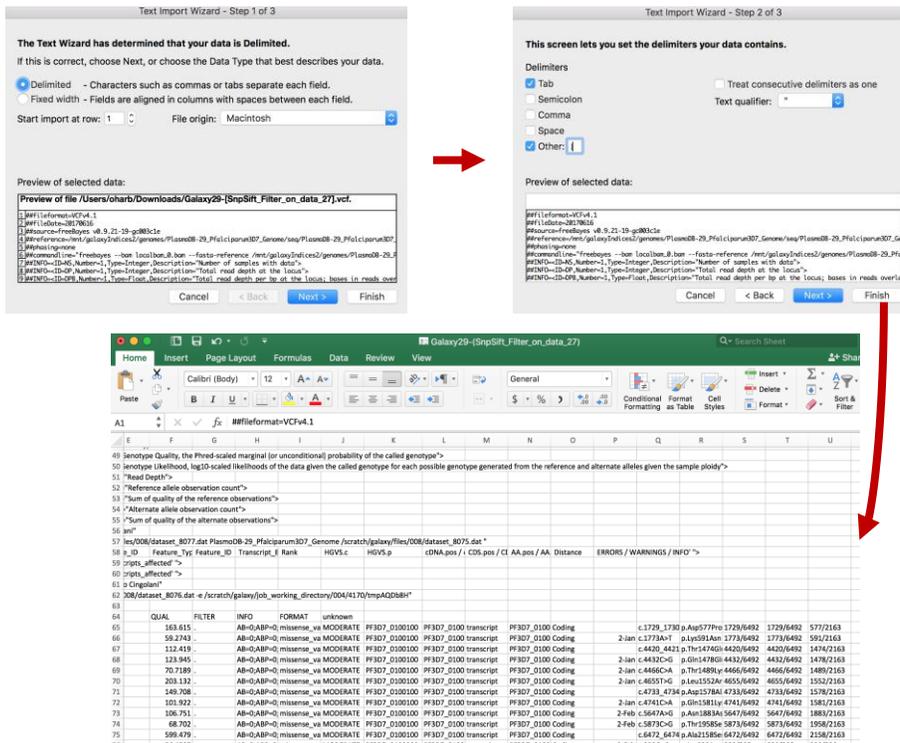
The galaxy workflow we used already includes SnpSift Filter as the final step using the following expression:  
 (((ANN[\*].IMPACT has 'HIGH') | (ANN[\*].IMPACT has 'MODERATE')) & ((na FILTER) | (FILTER = 'PASS')))

- Examine the filtered VCF file. Notice that the GeneIDs are buried in the file but the file has some structure which means you can extract them either programmatically or using a program like Excel.

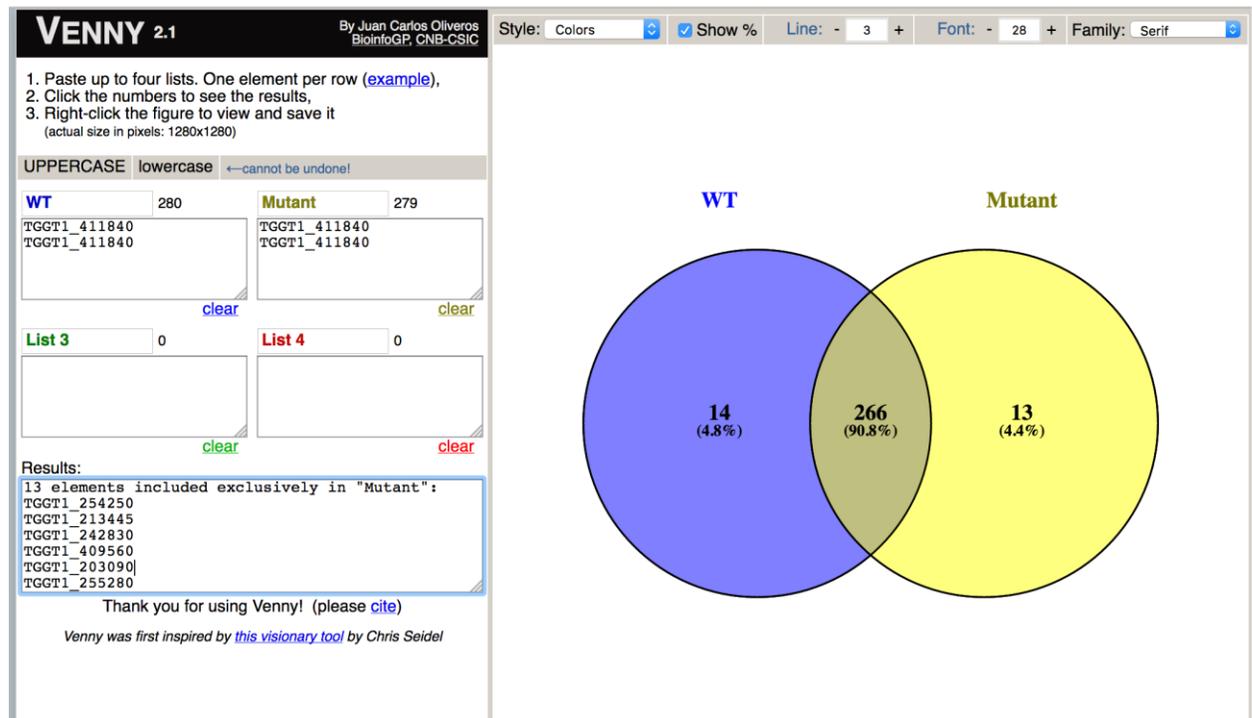


Here are some steps you can take to extract Gene IDs from two VCF files then compare them to identify genes that are in common or that distinguish the two files.

1. Download the SnpSift Filter output by clicking on the save icon
2. Open this file using excel and make sure you select tabs and | as column delimiters



- Now you can look for Gene IDs of interest in the excel file. For example, if this is a known drug resistant line you can find the gene(s) that might be responsible for the resistance and see what kinds of SNPs are present.
- If you are comparing a mutant and a wild type or two different strains you can extract gene IDs from both VCF files and use a website like <http://bioinfoqp.cnb.csic.es/tools/venny/>



\*Note that in the above steps you are ultimately comparing gene IDs – do you think you might be missing some important polymorphisms using this method? Of course, the answer is yes 😊

It is quite possible that a gene with a SNP in the WT and a SNP in the mutant that will be in the intersection of the two gene lists, contains different SNPs – you will miss this by doing the above steps. Below is a description of steps you can take to create a list of unique IDs for SNPs. This list of unique IDs can then be used in Venny.

- Start with the same excel files that you opened in the above section.
- To create a unique ID for SNPs we will combine information from multiple columns to create something that looks like this: chromosome:position:geneID
- To do this you will use the concatenate function in Excel:  
`=concatenate(cell#1,":",cell#2,":",cell#3)`  
 Cell#1 = cell with chromosome number

Cell#2 = cell with position

Cell#3 = cell with GeneID

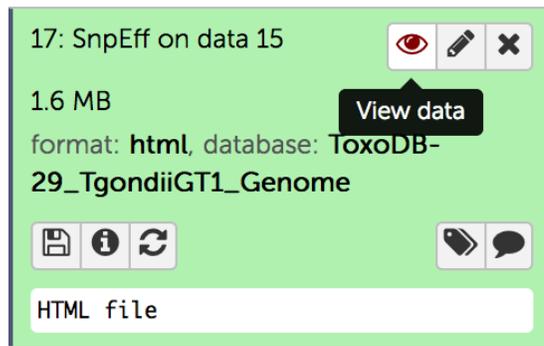
#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	unknown
TGGT1_chrla	227230		A	C	1156.55		AB=0;ABP=0;missense_va	MODERATE	TGGT1_293300
TGGT1_chrla	1340271		G	C	2387.77		AB=0;ABP=0;missense_va	MODERATE	TGGT1_295040
TGGT1_chrla	1396177		A	C	387.162		AB=0;ABP=0;missense_va	MODERATE	TGGT1_295125
TGGT1_chrlb	78769		A	G	1780.8		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207440
TGGT1_chrlb	153771		T	G	1414.57		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207480
TGGT1_chrlb	276348		T	G	2066.14		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207750
TGGT1_chrlb	622140		G	C	2335.06		AB=0;ABP=0;missense_va	MODERATE	TGGT1_208310
TGGT1_chrlb	1446003		C	T	60.6579		AB=0;ABP=0;missense_va	MODERATE	TGGT1_209755B
TGGT1_chrlb	1446022		G	T	82.4046		AB=0;ABP=0;missense_va	MODERATE	TGGT1_209755B

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	unknown
TGGT1_chrla	227230		A	C	1156.55		AB=0;ABP=0;missense_va	MODERATE	TGGT1_293300
TGGT1_chrla	1340271		G	C	2387.77		AB=0;ABP=0;missense_va	MODERATE	TGGT1_295040
TGGT1_chrla	1396177		A	C	387.162		AB=0;ABP=0;missense_va	MODERATE	TGGT1_295125
TGGT1_chrlb	78769		A	G	1780.8		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207440
TGGT1_chrlb	153771		T	G	1414.57		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207480
TGGT1_chrlb	276348		T	G	2066.14		AB=0;ABP=0;missense_va	MODERATE	TGGT1_207750
TGGT1_chrlb	622140		G	C	2335.06		AB=0;ABP=0;missense_va	MODERATE	TGGT1_208310
TGGT1_chrlb	1446003		C	T	60.6579		AB=0;ABP=0;missense_va	MODERATE	TGGT1_209755B
TGGT1_chrlb	1446022		G	T	82.4046		AB=0;ABP=0;missense_va	MODERATE	TGGT1_209755B

- You should get unique SNP IDs that look like this (for example):  
TGGT1\_chrlb:1446003:TGGT1\_209755B
- Copy this function to the rest of the column to replicate the concatenate function.
- Copy these newly generated unique IDs into Venny and compare the mutant and wild type.

### Examining SnpEff summary:

- Click on the view icon (eye) in the SnpEff output file that has the html format.



- This will open the html file right in galaxy where you can view it.
- The header contains a short summary and information about the run and it has several major components:

1. Summary table that warns about possible genomic annotation errors or inconsistencies identified in the reference genome. If there are many, use caution interpreting results and examine associated gff files for any issues (ex. missing feature values in gff files, incomplete gene sequences, more than one stop codon per gene, etc.).
2. Summary statistics for variant types

Number variants by type

Type	Total
SNP	114,034
MNP	12,864
INS	6,907
DEL	7,304
MIXED	2,180
INTERVAL	0
<b>Total</b>	<b>143,289</b>

Here is an example of variant calls and what they mean in terms of nucleotide changes:

Type	What is means	Example
SNP	Single-Nucleotide Polymorphism	Reference = 'A', Sample = 'C'
Ins	Insertion	Reference = 'A', Sample = 'AGT'
Del	Deletion	Reference = 'AC', Sample = 'C'
MNP	Multiple-nucleotide polymorphism	Reference = 'ATA', Sample = 'GTC'
MIXED	Multiple-nucleotide and an InDel	Reference = 'ATA', Sample = 'GTCAGT'

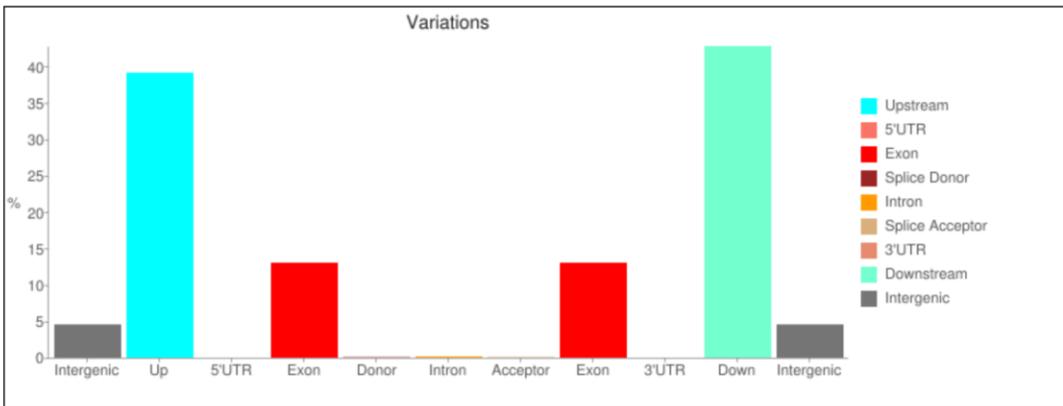
### 3. Statistics for the variant effects and impacts:

Number of effects by functional class

Type (alphabetical order)	Count	Percent
MISSENSE	21,588	35.949%
NONSENSE	131	0.218%
SILENT	38,332	63.832%

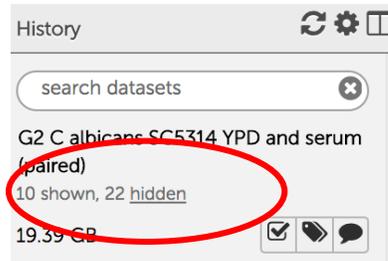
Type (alphabetical order)	Count	Percent
DOWNSTREAM	321,858	40.292%
EXON	67,505	8.451%
INTERGENIC	74,749	9.358%
INTRON	1,064	0.133%
NONE	1	0%
SPLICE_SITE_ACCEPTOR	5	0.001%
SPLICE_SITE_DONOR	4	0.001%
SPLICE_SITE_REGION	176	0.022%
TRANSCRIPT	12	0.002%
UPSTREAM	333,432	41.741%

Base changes summary. SnpEff.html files provides a break down of SNPs across gene features:



The SNP workflow you are using is set up to generate certain files that will provide you with the information you can export and use further in your analysis (yellow stars).

If you select certain options they will be shown in your history. If you do not select to display these files, you can view the output by clicking on displaying the hidden files from the history menu:



Now, let's take a look at the files generated by the workflow and steps that you can take to further evaluate them.

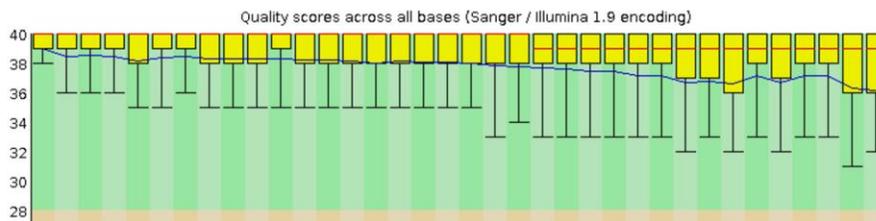
1. Examine sequence quality based on FastQC quality scores.

FastQC provides an easy-to-navigate visual representation sequencing data quality and distribution of nucleotides per read position.

**Basic Statistics**

Measure	Value
Filename	SRR298691.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	4887868
Sequences flagged as poor quality	0
Sequence length	36
%GC	58

**Per base sequence quality**



2. Download vcf files and evaluate workflow results.

The vcf file generated by SnpEff contains information about SNPs and the genomic location.

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	unknown
CM001231	189057	.	AG	CT	787.449	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:143:0:0:143:5341:-207.887,-43.0473,0		
CM001231	483825	.	G	A	64.8756	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:4:0:0:4:146:-10.0999,-1.20412,0		
CM001231	518226	.	G	C	51.7908	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:0:0:7:276:-11.5007,-2.10721,0		
CM001231	574021	.	C	G	237.265	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:17:0:0:17:583:-39.079,-5.11751,0		
CM001231	609879	.	GAA	CAG	55.2785	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:32:8:277:22:861:-18.1711,-0.694735,0		
CM001231	1090073	.	G	T	79.4156	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:2:75:6:238:-11.5539,-1.36362,0		
CM001231	1090104	.	A	T	70.961	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:6:0:0:6:220:-12.5146,-1.80618,0		
CM001231	1153611	.	CCTC	GCTG	111.123	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:5:188:3:97:-9.30616,-6.1461,0		
CM001231	1159150	.	CT	GC	126.126	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:31:0:0:19:741:-29.7713,-5.71957,0		
CM001231	1159438	.	C	G	82.3312	.	AB=0;ABP=0;GT:DP:RO:Qf 0/0:47:30:1092:17:640:0,-9.53002,-3.50705		
CM001231	1159465	.	G	C	249.656	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:126:47:1770:79:3013:-53.8644,-25.2134,0		
CM001231	1159499	.	T	C	124.95	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:143:32:1167:111:4248:-76.1575,-33.4865,0		
CM001231	1181576	.	CC	TG	191.675	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:27:0:0:25:924:-41.7448,-7.52575,0		
CM001231	1293309	.	C	G	51.22	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:2:0:0:2:78:-6.92763,-0.60206,0		
CM001231	1323058	.	TT	GC	71.3001	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:6:0:0:6:223:-12.5485,-1.80618,0		
CM001231	1485397	.	A	G	3558.42	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:499:0:0:497:18671:-804.678,-149.612,0		
CM001231	1485429	.	G	A	3783.33	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:517:1:38:516:20010:-843.425,-151.978,0		

Post-processing of SNP data is normally required to make sense of thousands of SNPs and to decide which ones have biological and functional importance. Data processing can help you to extract SNP distribution and parse associated data including GeneIDs, protein-coding annotations, and effects in sequence ontology terms such as missense or synonymous variants, stop codon gain, etc. and also link changes to the genome model.

#### Summary

<b>Genome</b>	ToxoDB-29_TgondiiGT1_Genome
<b>Date</b>	2017-06-17 05:56
<b>SnEff version</b>	SnEff 4.11 (build 2015-10-03), by Pablo Cingolani
<b>Command line arguments</b>	SnEff -i vcf -o vcf -stats /scratch/galaxy/files/008/dataset_8107.dat ToxoDB-29_TgondiiGT1_Genome /scratch/galaxy/files/008/dataset_8105.dat
<b>Warnings</b>	3,941
<b>Errors</b>	0
<b>Number of lines (input file)</b>	8,411
<b>Number of variants (before filter)</b>	8,483
<b>Number of not variants (i.e. reference equals alternative)</b>	0
<b>Number of variants processed (i.e. after filter and non-variants)</b>	8,483
<b>Number of known variants (i.e. non-empty ID)</b>	0 (0%)
<b>Number of multi-allelic VCF entries (i.e. more than two alleles)</b>	72
<b>Number of effects</b>	14,149
<b>Genome total length</b>	63,945,332
<b>Genome effective</b>	