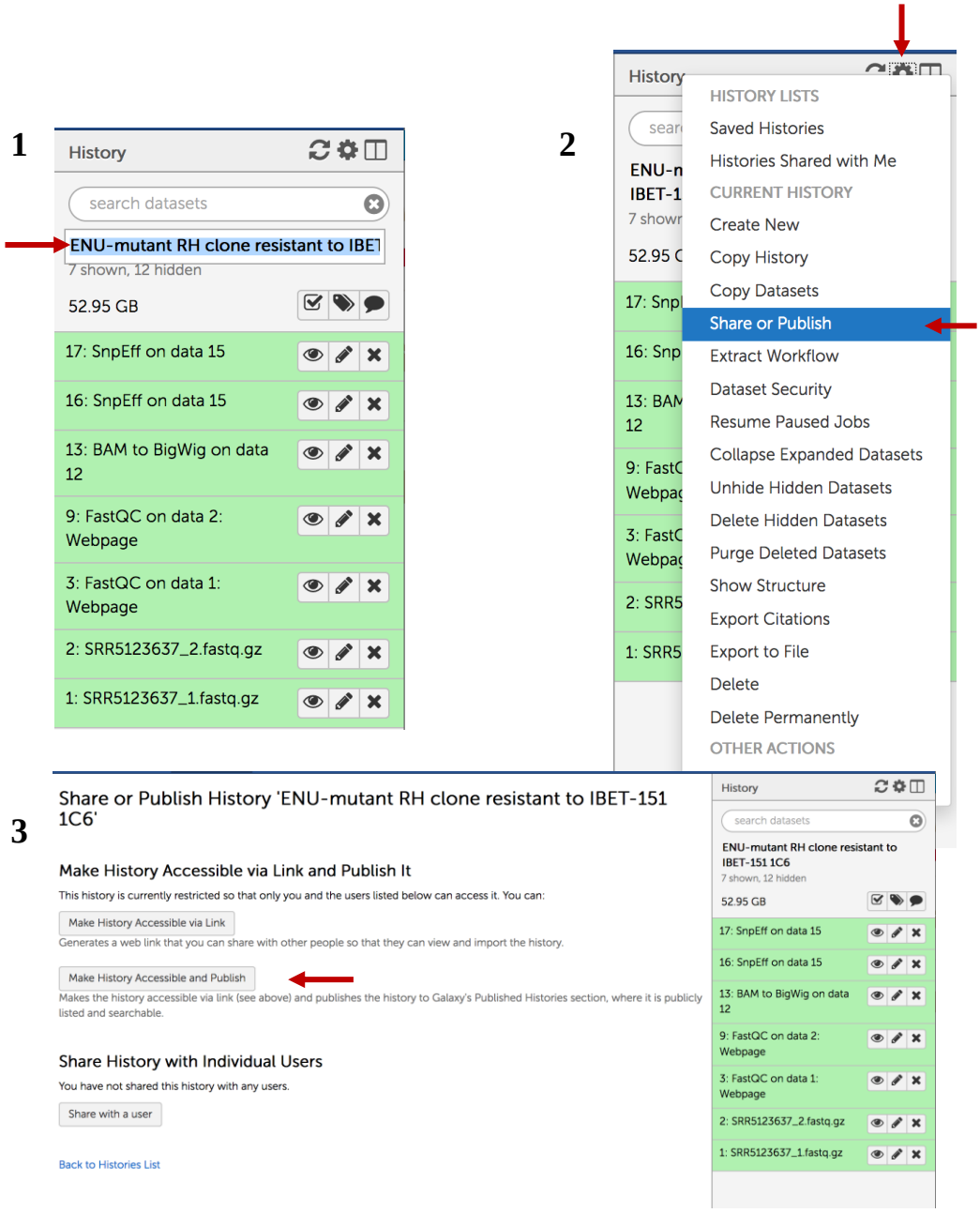
# Analyzing Variant Call results using EuPathDB Galaxy, Part II

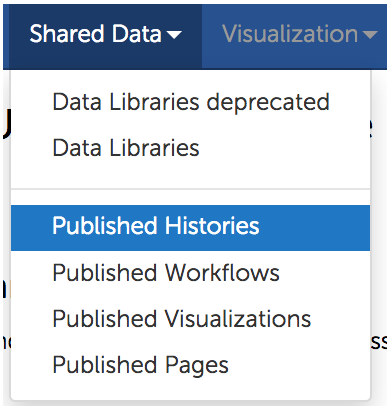
In this exercise, we will work in groups to examine the results from the SNP analysis workflow that we started yesterday. *The first step is to share your SNP workflow histories with the rest of the workshop participants:*

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.

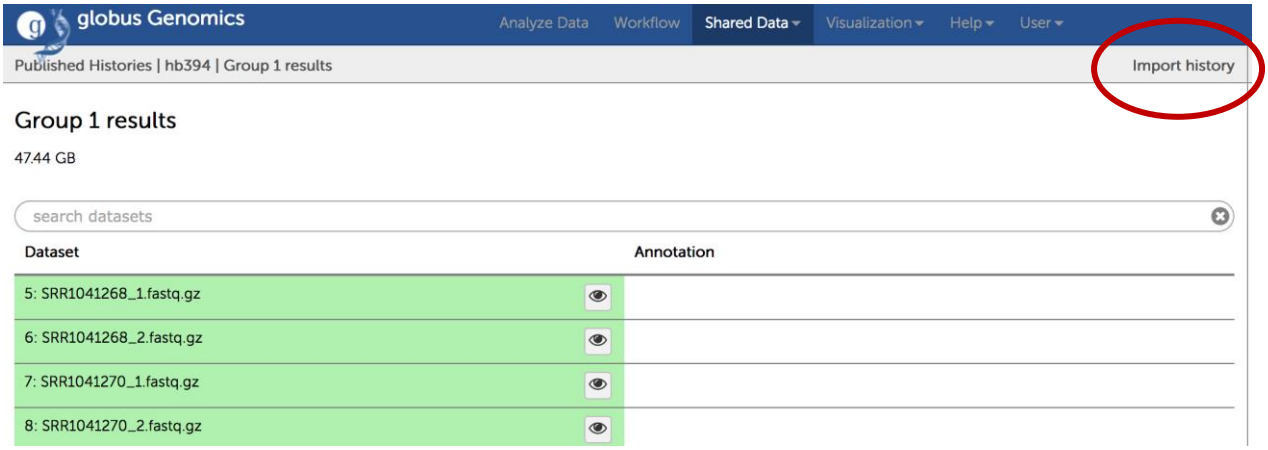


To import a shared history into your workspace follow these steps:

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

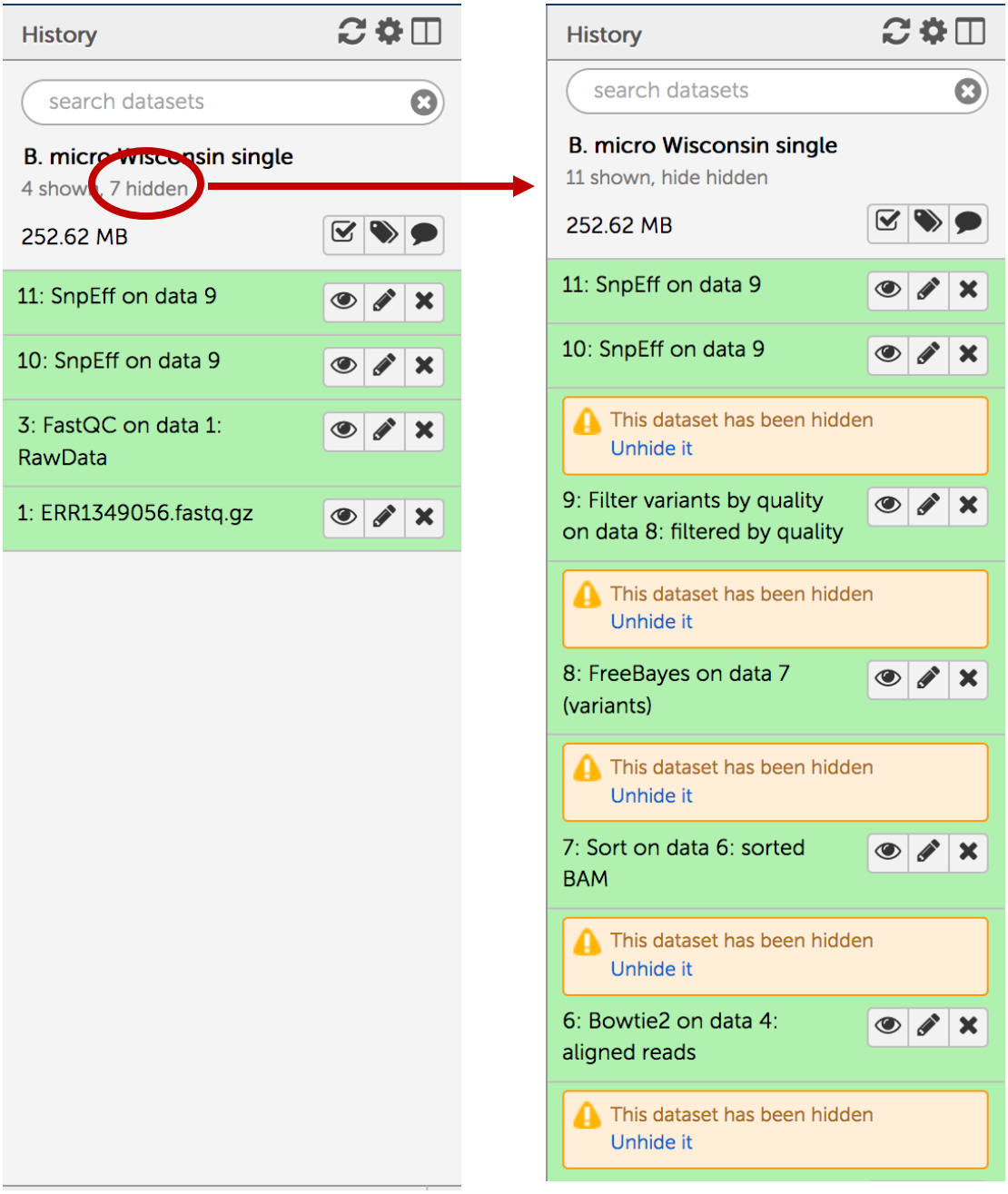



2. From the list of shared histories click on the one you want to import and on the next page select the 'Import' link in the upper right hand side.



## 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?

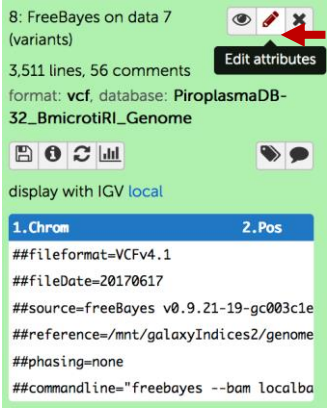



3. The output of Sickle is used by a program called Bowtie2. What does this tool do? Bowtie generates a file called a BAM file. Whenever dealing with sequence alignment files you will likely hear of file formats called SAM or BAM. SAM stands for Sequence Alignment/Map format, and BAM is the binary version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

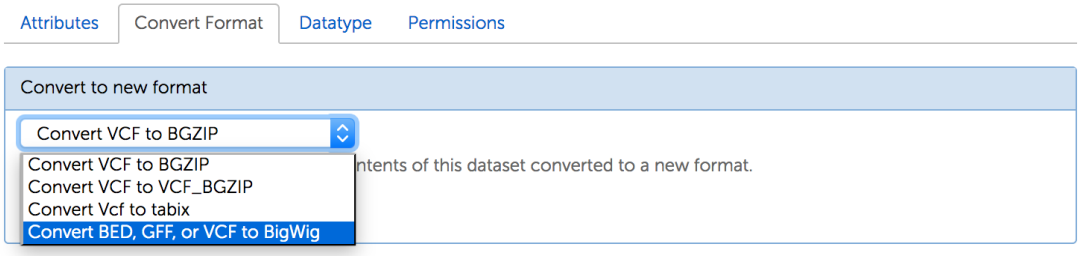
4. Many of the downstream analysis programs that use BAM files require a sorted BAM file. This allows access to reads to be done more efficiently.
5. The sorted BAM file is the input for a program called FreeBayes. This program is a Bayesian genetic variant detector designed to find small polymorphisms, 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.
6. 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
7. 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:**

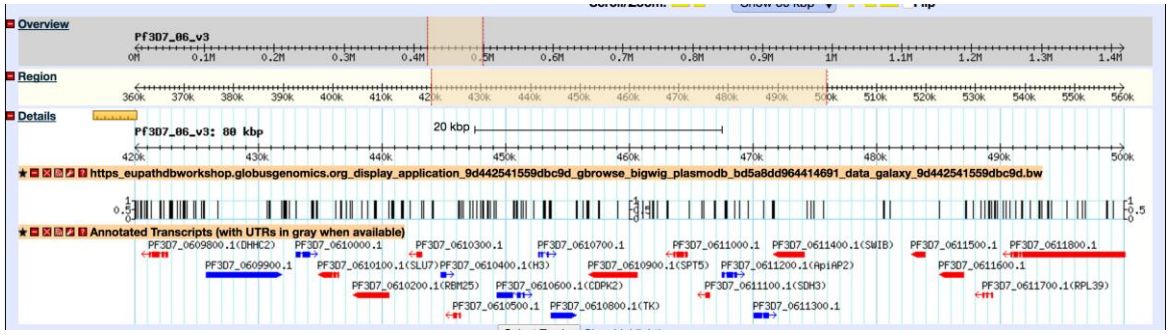
*In order to view a VCF file in GBrowse, it first has to be converted to a format that GBrowse can understand like BigWig. To do this follow these steps:*




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

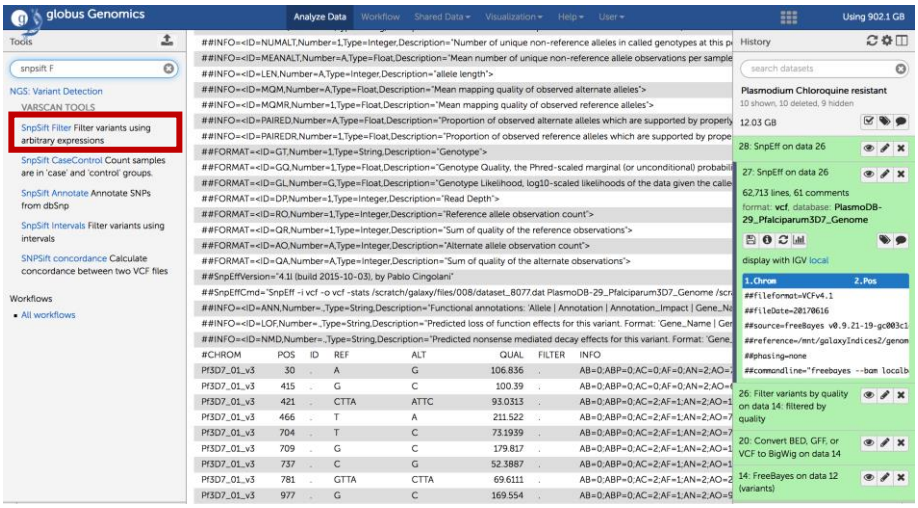



5. Once the conversion is done, you can export the BigWig file(s) to you EuPathDB dataset page




## 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.

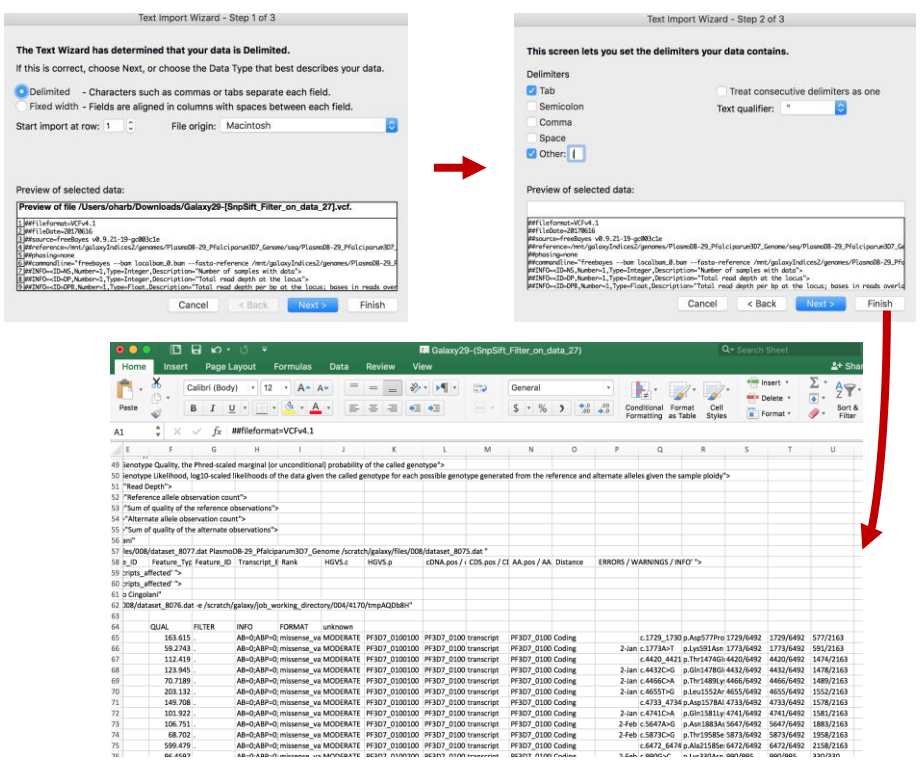



The galaxy workflow we used already includes SnpSift Filter as the final step using the following expression:
(((ANN[*].IMPACT has 'HIGH') | (ANN[*].IMPACT has 'MODERATE')) & ((na FILTER) | (FILTER = 'PASS')))

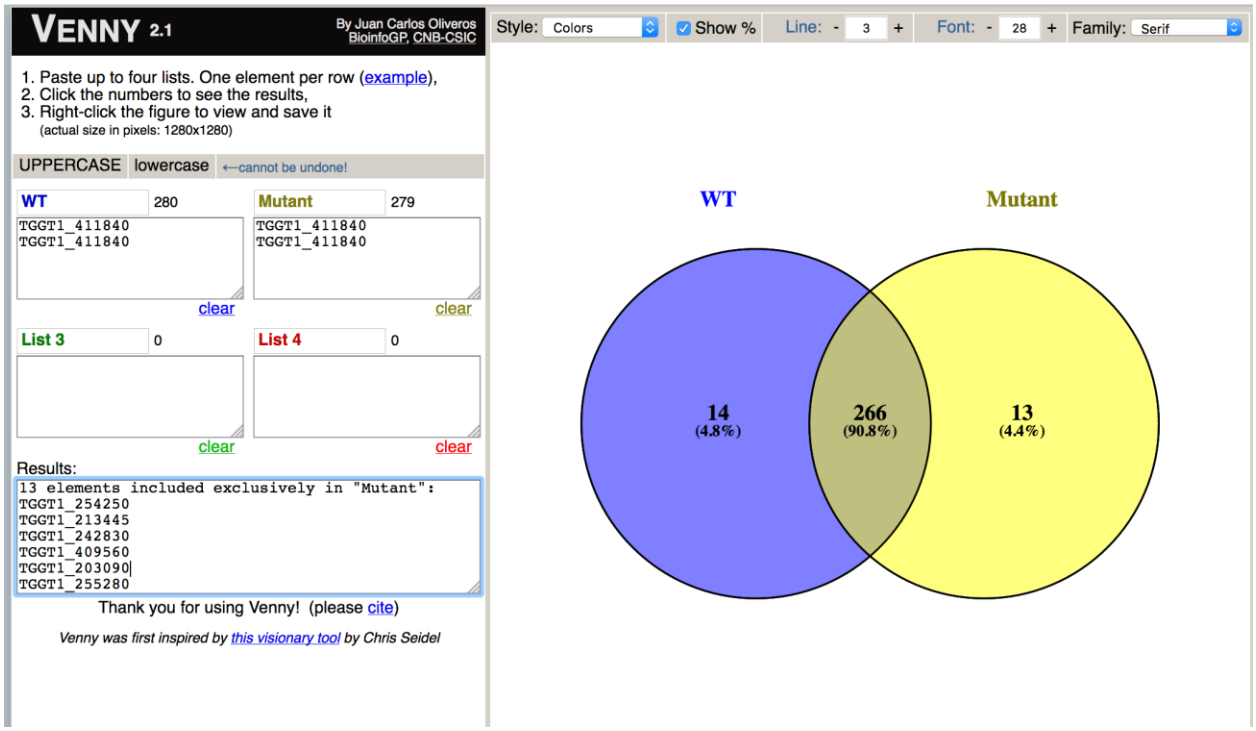
- Examine the filtered VCF file. Notice that the GeneIDs are buried in the file but the file has some structure which means you can extract them either programmatically or using a program like Excel.


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



3. 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.
4. 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://bioinfogp.cnb.csic.es/tools/venny/](http://bioinfogp.cnb.csic.es/tools/venny/)

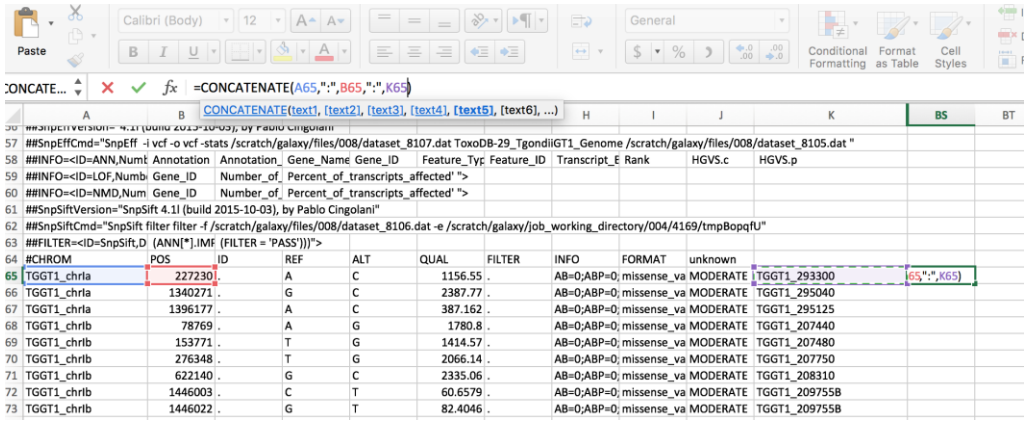


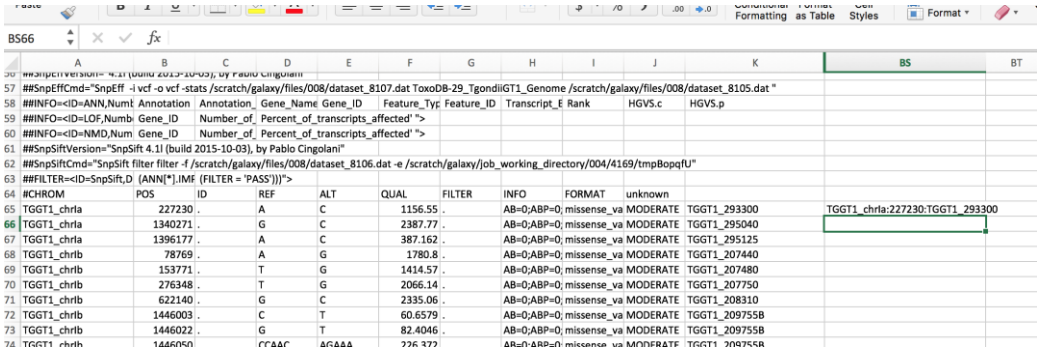

*Note that in the above steps you are ultimately comparing gene IDs – do you think you might be missing some important polymorphisms using this method? Of course, the answer is yes☺

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.

1. Start with the same excel files that you opened in the above section.
2. To create a unique ID for SNPs we will combine information from multiple columns to create something that looks like this: chromosome:position:geneID
3. 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

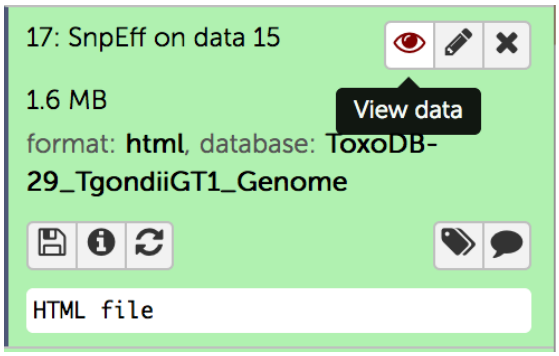



4. You should get unique SNP IDs that look like this (for example): TGGT1_chrIb:1446003:TGGT1_209755B
5. Copy this function to the rest of the column to replicate the concatenate function.
6. Copy the these newly generated unique IDs into Venny and compare the mutant and wild type.

**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 variantss by type**

| Type | Total |
|---|---|
| SNP | 114,034 |
| MNP | 12,864 |
| INS | 6,907 |
| DEL | 7,304 |
| MIXED | 2,180 |
| INTERVAL | 0 |
| Total | 143,289 |

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' |

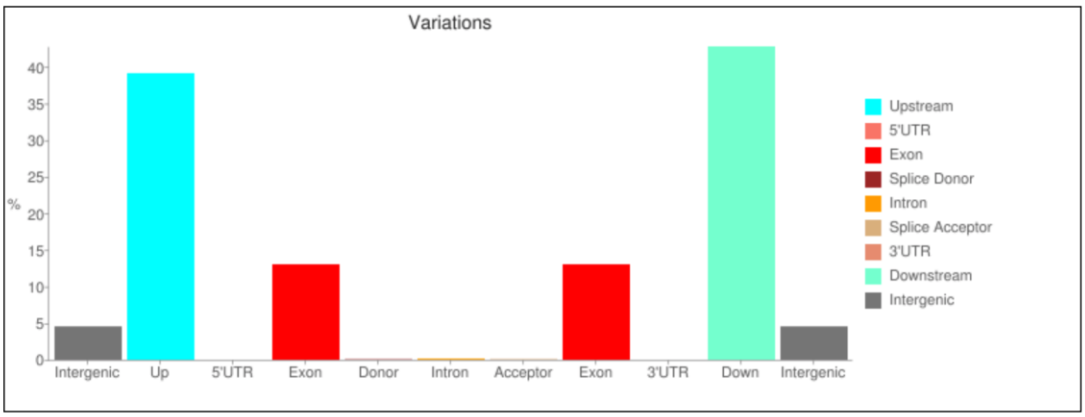
3. Statistics for the variant effects and impacts:

**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% |

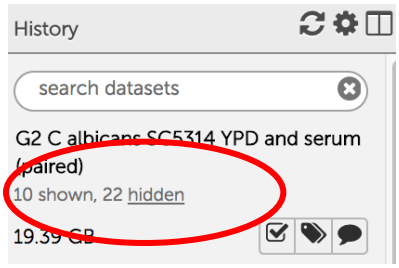
| Type (alphabetical order) | Count | Percent |
|---|---|---|
| DOWNSTREAM | 321,858 | 40.292% |
| EXON | 67,505 | 8.451% |
| INTERGENIC | 74,749 | 9.358% |
| INTRON | 1,064 | 0.133% |
| NONE | 1 | 0% |
| SPLICE_SITE_ACCEPTOR | 5 | 0.001% |
| SPLICE_SITE_DONOR | 4 | 0.001% |
| SPLICE_SITE_REGION | 176 | 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, lets take a look at the files generated by the workflow and steps that you can take to further evaluate them.

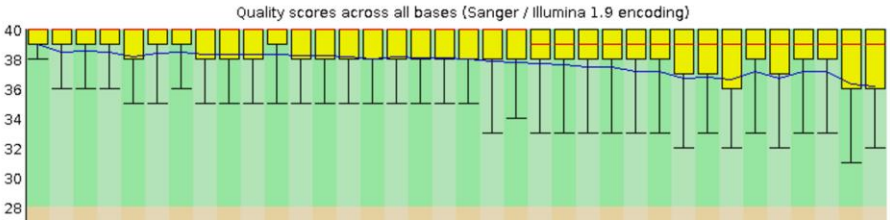
1. Examine sequence quality based on FastQC quality scores.

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**

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