BIOL*3300 Lab8 F21

Variant Calling Practice on Compute Canada

Background review

In this lab, we will continue the process of characterizing genotypes. This series of labs is based on the published protocol:

Rochette, N.C. and Catchen, J.M., 2017. Deriving genotypes from RAD-seq short-read data using Stacks. Nature Protocols, 12(12), pp.2640-2659.

In the last lab, you mapped reads to a fish reference genome. To do this, we have downloaded the reference genome and created the index. The software bwa was used to align reads. The output .bam/.sam file told you where each read aligned and the details of that alignment.

The purpose of this lab is to identify a sample's genotype at different positions across the reference genome. We will use the pstacks program from the stacks software suite to do the analysis. The pstacks software collects all the reads that overlap a nucleotide in the reference genome. From these reads, the genotype is determined at a nucleotide site.

Step7 Pstacks analysis of SNPs and alleles

Obtaining reads and alignments

Move into the stacks_ref directory and load a version of the stacks software that supports pstacks.

```
cd ~/scratch/Biol3300/SNP_lab/stacks_ref
module load nixpkgs/16.09 gcc/5.4.0 stacks/1.46
```

Previously, we only mapped some of the samples reads. If you recall, in the raw folder, we had three lanes of reads. Lane 1 contains 10 files of sequences that are all from the same fish population, South Jetty, but we only used one file. To get all the reads that map to a sample, we would need to sorting the barcodes from all the 10 files and align them to the genome.

In order to analyze a sample’s genotype at different positions across the reference genome, we demultiplexed and mapped reads from all 10 fastq files (001-010.fq.gz) of lane 3 in the raw directory. One of fish samples, **cs_1335.01** from the lane 3 folder, will be used in this lab. The process of mapping all reads to this sample and aligning reads to the genome were exactly the same as we previously did on lab 6 and lab 7. To save time, you can copy sample **cs_1335.01.fq.gz** and its **bam file** to your directories:

```
cp /scratch/hchang02/Biol3300/SNP_lab/cleaned/cs_1335.01.fq.gz ~/scratch/Biol3300/SNP_lab/cleaned
cp /scratch/hchang02/Biol3300/SNP_lab/alignments/cs_1335.01.bam ~/scratch/Biol3300/SNP_lab/alignments
```

Running pstacks

We will first define some arguments for pstacks.

```
sample=cs_1335.01
sample_index=1
bam_file=../alignments/$sample.bam
pstacks -f $bam_file -i $sample_index -o ./
```

sample is our sample name
sample_index is the unique identifier for the sample. We only have one sample, but we would set the sample index to '1' for the first sample file, '2' for the second, and so on if had more.

This will give you the following output:

```
pstacks parameters selected:
  Alignments file: ./cs_1335.01.aligned.sorted.bam
  Output directory: ./
  Sample ID: 1
  Min locus depth: 3
  Max clipped proportion: 0.15
  Min mapping quality: 10
  Model: snp
    Model alpha: 0.05
Reading alignments...
Done reading alignment records:
  Kept 1088586 primary alignments
  Skipped 153728 (12.2%) primary alignments with insufficient mapping qualities
  Skipped 21608 (1.7%) excessively soft-clipped primary alignments
  Skipped 7995 secondary alignments
  Skipped 0 supplementary alignments
  Skipped 38245 unmapped reads
Collapsed reads into 132496 stacks.
Created 42145 loci; mean coverage is 25.83 (stdev: 15.87, max: 1038).
Discarded 2671 low coverage loci comprising 3451 (0.3%) reads.
Kept 39474 loci; mean coverage is 27.49 (stdev: 15.01, max: 1038).
Identifying polymorphic sites and calling consensus sequences...done. (0 loci were blacklisted.)
Writing tags, models, snps, and alleles files...
pstacks is done.
```

From the log files of pstacks, the number of primary alignments (i.e., 1088586) were kept for the analysis. Dividing this number by the initial number of reads (i.e, 1302521) of the sample gives the percentage of reads that remain after alignment and filtering. This percentage is one of the main statistics to consider in assessing the effectiveness of an alignment protocol. In our case, the percentage of used reads is 83.57%.

Count the number of reads that are in the file 'cs_1335.01.fq.gz'. There are different ways of doing this. I'd recommend matching the number of lines in the file that start with @ and counting them. The initial number of reads (1302521).

```
    zless ../cleaned/cs_1335.01.fq.gz | grep '^@' | wc -l
    zgrep -c '^@' ../cleaned/cs_1335.01.fq.gz
```

Note: Percentages of aligned reads under 50% should be a trigger to reconsider the alignment protocol. Such low numbers may be caused, notably, by a misuse of the alignment program, a poor choice of parameters, or a very incomplete and/or distant reference genome.

Still in the log files of pstacks, check the average per-locus coverage. These numbers should provide an estimate of the actual sequencing coverage that was achieved. Per-locus coverage for sample cs_1335.01 was 27.49.

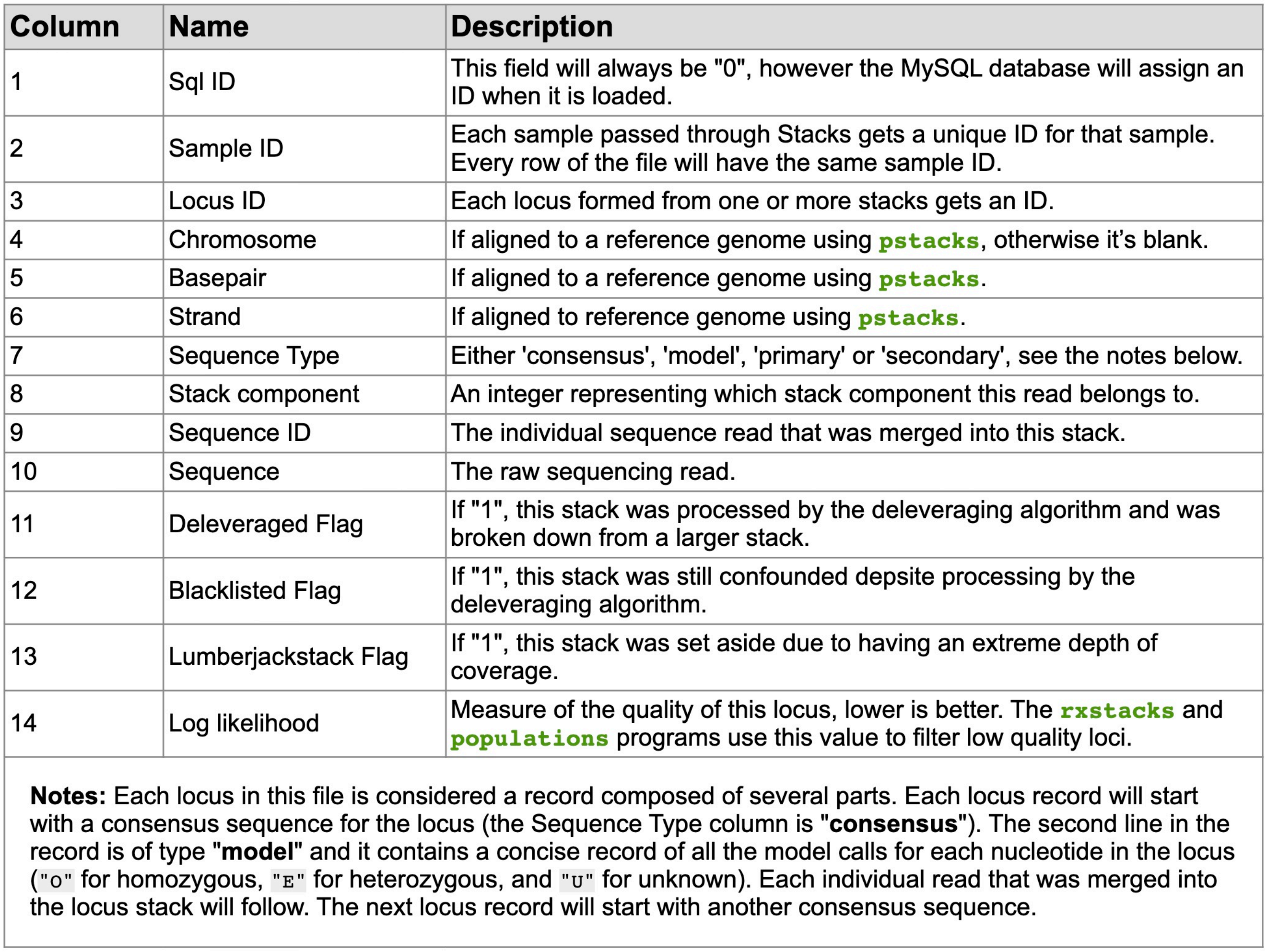
Note: Coverage, or the number of reads that map to a reference genome position, strongly affects the quality of genotype calls. If samples have low-coverage, genotypes may be called as "unknown" or be miscalled.

Step8 Reading the fields mean in Stacks output files

After its successful completion, pstacks should have created four files for each sample in addition to the screen output ($sample.tags.tsv.gz, $sample.models.tsv.gz, $sample.snps.tsv.gz, and $sample.alleles.tsv.gz).

1. sample.tags.tsv: Assembled loci

A 'tag' is another name for a 'read'. This file shows where every tag aligns, e.g. at which locus, and the genotype call.



Column	Name	Description
1	Sql ID	This field will always be "0", however the MySQL database will assign an ID when it is loaded.
2	Sample ID	Each sample passed through Stacks gets a unique ID for that sample. Every row of the file will have the same sample ID.
3	Locus ID	Each locus formed from one or more stacks gets an ID.
4	Chromosome	If aligned to a reference genome using `pstacks`, otherwise it's blank.
5	Basepair	If aligned to a reference genome using `pstacks`.
6	Strand	If aligned to reference genome using `pstacks`.
7	Sequence Type	Either 'consensus', 'model', 'primary' or 'secondary', see the notes below.
8	Stack component	An integer representing which stack component this read belongs to.
9	Sequence ID	The individual sequence read that was merged into this stack.
10	Sequence	The raw sequencing read.
11	Deleveraged Flag	If "1", this stack was processed by the deleveraging algorithm and was broken down from a larger stack.
12	Blacklisted Flag	If "1", this stack was still confounded depsite processing by the deleveraging algorithm.
13	Lumberjackstack Flag	If "1", this stack was set aside due to having an extreme depth of coverage.
14	Log likelihood	Measure of the quality of this locus, lower is better. The `rxstacks` and `populations` programs use this value to filter low quality loci.

Notes: Each locus in this file is considered a record composed of several parts. Each locus record will start with a consensus sequence for the locus (the Sequence Type column is "**consensus**"). The second line in the record is of type "**model**" and it contains a concise record of all the model calls for each nucleotide in the locus (`"O"` for homozygous, `"E"` for heterozygous, and `"U"` for unknown). Each individual read that was merged into the locus stack will follow. The next locus record will start with another consensus sequence.

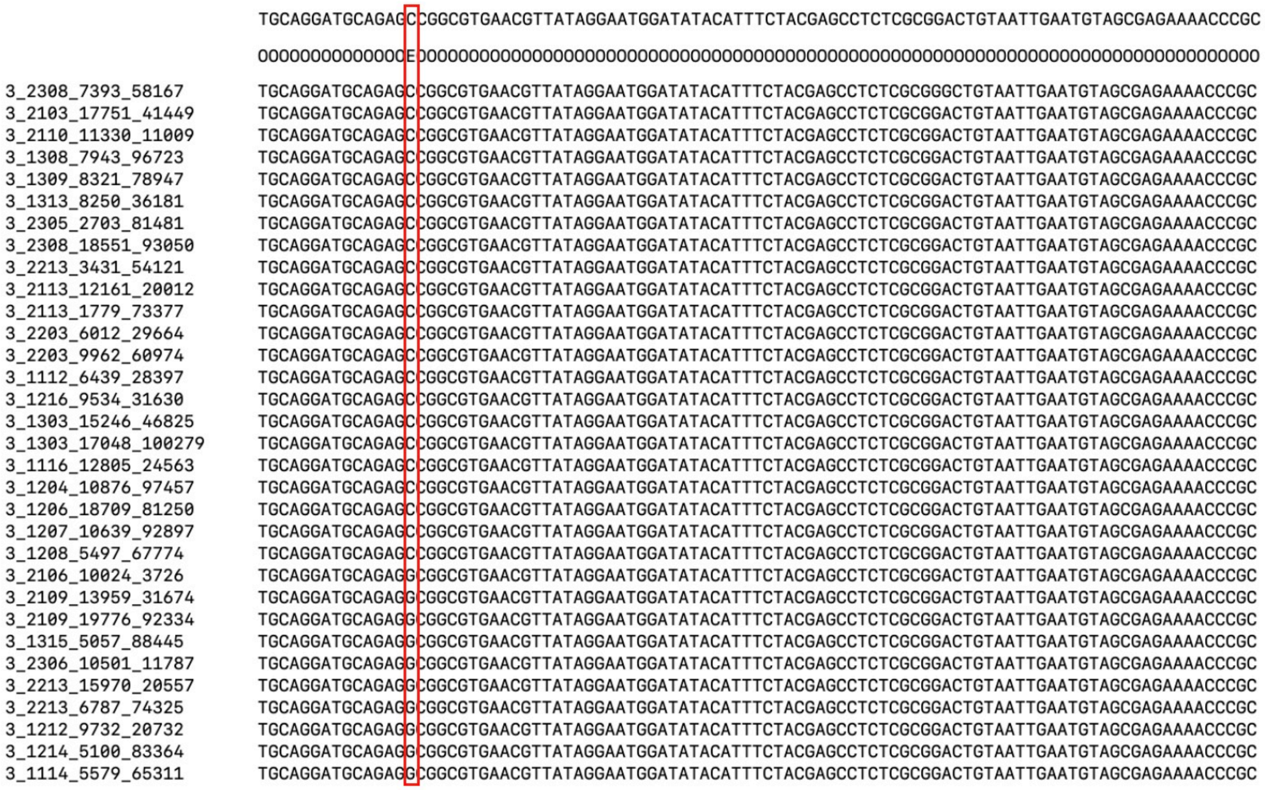
We can check the file with:

```
zless cs_1335.01.tags.tsv.gz
```

Several reads/tags such as primary 0, primary 1 etc. aligned to the same genomic location on chromosome/contig "groupIII" from the reference genome at position 10,005,320. The consensus sequence for this sample, based on all reads, at this site is shown.

The predicted genotype of this individual is shown on the line 'model'. The model is "O" for homozygous, "E" for heterozygous, and "U" for unknown. It is hard to see, but position 15 is deemed heterozygous with a 'E' labeled. Investigate this record on your machine by looking at more of the aligned reads.

A figure of the first locus can help you understand:



```
                           TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC

                           00000000000000E00000000000000000000000000000000000000000000000000000000000000000000000000000000

3_2308_7393_58167          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGGCTGTAATTGAATGTAGCGAGAAAACCCGC
3_2103_17751_41449         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2110_11330_11009         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1308_7943_96723          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1309_8321_78947          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1313_8250_36181          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2305_2703_81481          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2308_18551_93050         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2213_3431_54121          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2113_12161_20012         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2113_1779_73377          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2203_6012_29664          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2203_9962_60974          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1112_6439_28397          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1216_9534_31630          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1303_15246_46825         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1303_17048_100279        TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1116_12805_24563         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1204_10876_97457         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1206_18709_81250         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1207_10639_92897         TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1208_5497_67774          TGCAGGATGCAGAGCCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2106_10024_3726          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2109_13959_31674         TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2109_19776_92334         TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1315_5057_88445          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2306_10501_11787         TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2213_15970_20557         TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_2213_6787_74325          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1212_9732_20732          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1214_5100_83364          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
3_1114_5579_65311          TGCAGGATGCAGAGGCGGCGTGAACGTTATAGGAATGGATATACATTTCTACGAGCCTCTCGCGGACTGTAATTGAATGTAGCGAGAAAACCCGC
```

2. sample.snps.tsv: Model calls from each locus

This file does not include the reads. It just has the summary of model calls as seen in the tags file.

Column	Name	Description
1	Sql ID	This field will always be "0", however the MySQL database will assign an ID when it is loaded.
2	Sample ID	The numerical ID for this sample, matches the ID in the `tags` file.
3	Locus ID	The numerical ID for this locus, in this sample. Matches the ID in the `tags` file.
4	Column	Position of the nucleotide within the locus, reported using a zero-based offset (first nucleotide is enumerated as 0)
5	Type	Type of nucleotide called: either heterozygous (`"E"`), homozygous (`"O"`), or unknown (`"U"`).
6	Likelihood ratio	From the SNP-calling model.
7	Rank_1	Majority nucleotide.
8	Rank_2	Alternative nucleotide.
9	Rank_3	Third alternative nucleotide (only possible in the `batch_X.catalog.snps.tsv` file).
10	Rank_4	Fourth alternative nucleotide (only possible in the `batch_X.catalog.snps.tsv` file).
Notes: There will be one line for each nucleotide in each locus/stack.		

We see the nucleotide calls for cs_1335.01 in this case at locus 1. We see the heterozygote again.

```
    zless cs_1335.01.snps.tsv.gz | head -n 20
```

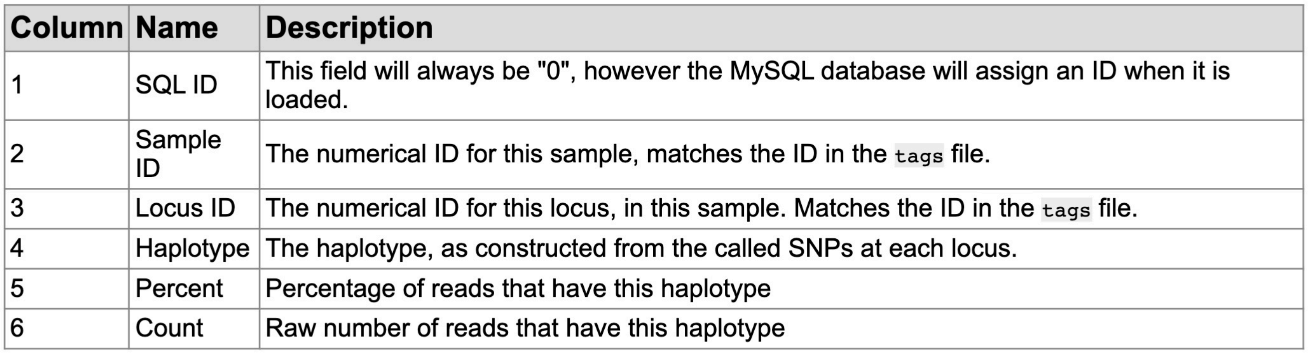
The output is:

```
0        1        1        0        O        44.36        T        -
0        1        1        1        O        44.36        G        -
0        1        1        2        O        44.36        C        -
0        1        1        3        O        44.36        A        -
0        1        1        4        O        44.36        G        -
0        1        1        5        O        44.36        G        -
0        1        1        6        O        44.36        A        -
0        1        1        7        O        44.36        T        -
0        1        1        8        O        44.36        G        -
0        1        1        9        O        44.36        C        -
0        1        1        10        O        44.36        A        -
0        1        1        11        O        44.36        G        -
0        1        1        12        O        44.36        A        -
0        1        1        13        O        44.36        G        -
0        1        1        14        E        -17.36        C        G
0        1        1        15        O        44.36        C        -
0        1        1        16        O        44.36        G        -
0        1        1        17        O        44.36        G        -
0        1        1        18        O        44.36        C        -
```

As we found, the SNP site is on the 15th nucleotide (Note here starts with 0).

3. sample.alleles.tsv: Haplotypes/alleles recorded from each locus

This file lists the different haplotypes/alleles recorded from each locus.



Column	Name	Description
1	SQL ID	This field will always be "0", however the MySQL database will assign an ID when it is loaded.
2	Sample ID	The numerical ID for this sample, matches the ID in the `tags` file.
3	Locus ID	The numerical ID for this locus, in this sample. Matches the ID in the `tags` file.
4	Haplotype	The haplotype, as constructed from the called SNPs at each locus.
5	Percent	Percentage of reads that have this haplotype
6	Count	Raw number of reads that have this haplotype

```
    zless cs_1335.01.alleles.tsv.gz | head
```

The output is:

```
0        1        1        C        68.75        22
0        1        1        G        31.25        10
0        1        4        CC        42.86        15
0        1        4        GT        57.14        20
0        1        13        C        39.47        15
0        1        13        T        60.53        23
0        1        15        A        72.73        8
0        1        15        G        27.27        3
0        1        20        A        41.18        14
```

At locus 1, there were two alleles at position 15. These are represented here. In the tags file, the first locus has an allele G found on 10 reads compared to C on 22 reads.

Assignment for Lab 8

Please perform the pstacks unit of Stacks on sample cs_1335.02.fq.gz with its bam file. You need to copy cs_1335.02.fq.gz and cs_1335.02.bam from my directories as the example cs_1335.01 in this tutorial.

Give the aligned reads percentage and per-locus coverage on sample cs_1335.02.fq.gz. How does this sample compare to sample cs_1335.01?
Using the output files, find a heterozygous site within the sample and give its genomic position.

Reference

These lab materials are from the following papers and website:

1. Rochette, N.C. and Catchen, J.M., 2017. Deriving genotypes from RAD-seq short-read data using Stacks. Nature Protocols, 12(12), pp.2640-2659.
2. The population structure and recent colonization history of Oregon threespine stickleback determined using restriction-site associated DNA-sequencing. Mol. Ecol. 22, 2864–2883 (2013).
3. https://catchenlab.life.illinois.edu/stacks/manual-v1/