Stacks Manual

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1. Introduction [↑top]

Several molecular approaches have been developed to focus short reads to specific, restriction-enzyme anchored positions in the genome. Reduced representation techniques such as CRoPS, RAD-seq, GBS, double-digest RAD-seq, and 2bRAD effectively subsample the genome of multiple individuals at homologous locations, allowing for single nucleotide polymorphisms (SNPs) to be identified and typed for tens or hundreds of thousands of markers spread evenly throughout the genome in large numbers of individuals. This family of reduced representation genotyping approaches has generically been called genotype-by-sequencing (GBS) or Restriction-site Associated DNA sequencing (RAD-seq). For a review of these technologies, see Davey et al. 2011 or Andrews, et al., 2016.

Stacks is designed to work with any restriction-enzyme based data, such as GBS, CRoPS, and both single and double digest RAD. Stacks is designed as a modular pipeline to efficiently curate and assemble large numbers of short-read sequences from multiple samples. Stacks identifies loci in a set of individuals, either de novo or aligned to a reference genome (including gapped alignments), and then genotypes each locus. Stacks incorporates a maximum likelihood statistical model to identify sequence polymorphisms and distinguish them from sequencing errors. Stacks employs a Catalog to record all loci identified in a population and matches individuals to that Catalog to determine which haplotype alleles are present at every locus in each individual.

Stacks is implemented in C++ with wrapper programs written in Perl. The core algorithms are multithreaded via OpenMP libraries and the software can handle data from hundreds of individuals, comprising millions of genotypes.

Stacks proceeds in six major stages. First, reads are demultiplexed and cleaned by the process_radtags program. The next three stages comprise the main Stacks pipeline: building loci (**`ustacks`**), creating the catalog of loci (**`cstacks`**), and matching against the catalog (**`sstacks`**). In the fifth stage, the **`gstacks`** program is executed to assemble and merge paired-end contigs, call variant sites in the population and genotypes in each sample. In the final stage, the **`populations`** program is executed, depending on the type of input data. This flow is diagrammed in the following figure.

2. Installation [↑top]

2.1. Prerequisites

Stacks should build on any standard UNIX-like environment (Apple OS X, Linux, etc.) Stacks is an independent pipeline and can be run without any additional external software.

2.2. Build the software

Stacks uses the standard autotools install:

```
% tar xfvz stacks-2.xx.tar.gz
% cd stacks-2.xx
% ./configure
% make

(become root)
# make install

(or, use sudo)
% sudo make install
```

You can change the root of the install location (`/usr/local/` on most operating systems) by specifying the `--prefix` command line option to the configure script.

```
% ./configure --prefix=/home/smith/local
```

You can speed up the build if you have more than one processor:

```
% make -j 8
```

A default install will install files in the following way:

`/usr/local/bin` Stacks executables and Perl scripts.

The pipeline is now ready to run.

3. What types of data does *Stacks 2.0* support? [↑top]

Stacks is designed to process data that stacks together. Primarily this consists of restriction enzyme-digested DNA. There are a few similar types of data that will stack-up and could be

processed by Stacks, such as DNA flanked by primers as is produced in metagenomic 16S rRNA studies.

The goal in Stacks is to assemble loci in large numbers of individuals in a population or genetic cross, call SNPs within those loci, and then read haplotypes from them. Therefore Stacks wants data that is a uniform length, with coverage high enough to confidently call SNPs. Although it is very useful in other bioinformatic analyses to variably trim raw reads, this creates loci that have variable coverage, particularly at the 3' end of the locus. In a population analysis, this results in SNPs that are called in some individuals but not in others, depending on the amount of trimming that went into the reads assembled into each locus, and this interferes with SNP and haplotype calling in large populations.

3.1. Protocol Type

Stacks supports all the major restriction-enzyme digest protocols such as RAD-seq, double-digest RAD-seq, and a subset of GBS protocols, among others.

3.2. Sequencer Type

Stacks is optimized for short-read, Illumina-style sequencing. There is no limit to the length the sequences can be, although there is a hard-coded limit of 1024bp in the source code now for efficency reasons, but this limit could be raised if the technology warranted it.

Stacks can also be used with data produced by the Ion Torrent platform, but that platform produces reads of multiple lengths so to use this data with Stacks the reads have to be truncated to a particular length, discarding those reads below the chosen length. The **process_radtags** program can truncate the reads from an Ion Torrent run.

Other sequencing technologies could be used in theory, but often the cost versus the number of reads obtained is prohibitive for building stacks and calling SNPs.

3.3. Paired-end Reads

Stacks directly supports paired-end reads, for both single and double digest protocols. In the case of a single-digest protocol, Stacks will use the staggered paired-end reads to assemble a contig across all of the individuals in the population. For double-digest RAD, both the single-end and paired-end reads are anchored by a contig and Stacks will assemble them into two loci. In both cases, the paired-end contig/locus will be merged with the single-end locus. If the loci do not overlap, they will be merged with a small buffer of Ns in between them.

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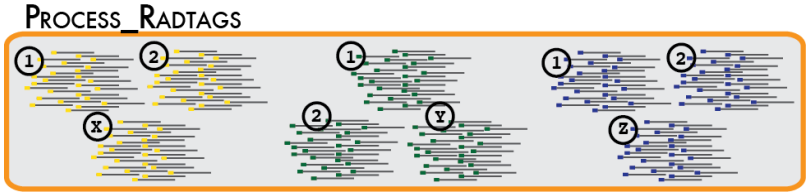
4. Running the pipeline [↑top]

4.1. Clean the data

In a typical analysis, data will be received from an Illumina sequencer, or some other type of sequencer as FASTQ files. The first requirement is to demultiplex, or sort, the raw data to recover the individual samples in the Illumina library. While doing this, we will use the Phred scores provided in the FASTQ files to discard sequencing reads of low quality. These tasks are accomplished using the **process_radtags** program.



Some things to consider when running this program:

- **process_radtags** can handle both single-end or paired-end Illumina sequencing.
- The raw data can be compressed, or gzipped (files end with a "`.gz`" suffix).
- You can supply a list of barcodes, or indexes, to **process_radtags** in order for it to demultiplex your samples. These barcodes can be single-end barcodes or combinatorial barcodes (pairs of barcodes, one on each of the paired reads). Barcodes are specified, one per line (or in tab separated pairs per line), in a text file.
    - If, in addition to your barcodes, you also supply a sample name in an extra column within the barcodes file, **process_radtags** will name your output files according to sample name instead of barcode.
- If you believe your reads may contain adapter contamination, **process_radtags** can filter it out.
- You can supply the restriction enzyme used to construct the library. In the case of double-digest RAD, you can supply both restriction enzymes.
- If instructed, (`-r` command line option), **process_radtags** will correct barcodes and restriction enzyme sites that are within a certain distance from the true barcode or restriction enzyme cutsite.

4.1.1 Understanding barcodes/indexes and specifying the barcode type

Genotype by sequencing libraries sample the genome by selecting DNA adjacent to one or more restriction enzyme cutsites. By reducing the amount of total DNA sampled, most researchers will multiplex many samples into one molecular library. Individual samples are demarcated in the library by ligating an oligo barcode onto the restriction enzyme-associated DNA for each sample. Alternatively, an index barcode is used, where the barcode is located upstream of the sample DNA within the sequencing adaptor. Regardless of the type of barcode used, after sequencing, the data must be demultiplexed so the samples are again separated. The `process_radtags` program will perform this task, but we much specify the type of barcodes used, and where to find them in the sequencing data.

There are a number of different configurations possible, each of them is detailed below.

1. If your data are **single-end** or **paired-end**, with an inline barcode present only on the single-end (marked in red):

```
@HWI-ST0747:188:C09HWACXX:1:1101:2968:2083 1:N:0:
TTATGATGCAGGACCAGGATGACGTCAGCACAGTGCGGGTCCTCCATGGATGCTCCTCGGTCGTGGTTGGGGGAGGAGGCA
+
@@@DDDDDBHHFBF@CCAGEHHHBFGIIFGIIGIEDBBGFHCGIIGAEEEDCC;A?;;5,:@A?=B5559999B@BBBBBA
@HWI-ST0747:188:C09HWACXX:1:1101:2863:2096 1:N:0:
TTATGATGCAGGCAAATAGAGTTGGATTTTGTGTCAGTAGGCGGTTAATCCCATACAATTTTACACTTTATTCAAGGTGGA
+
CCCFFFFFHHHHHJJGHIGGAHHIIGGIIJDHIGCEGHIFIJIH7DGIIIAHIJGEDHIDEHJJHFEEECEFEFFDECDDD
@HWI-ST0747:188:C09HWACXX:1:1101:2837:2098 1:N:0:
GTGCCTTGCAGGCAATTAAGTTAGCCGAGATTAAGCGAAGGTTGAAAATGTCGGATGGAGTCCGGCAGCAGCGAATGTAAA
```

Then you can specify the `--inline_null` flag to **process_radtags**. This is also the default behavior and the flag can be ommitted in this case.

2. If your data are **single-end** or **paired-end**, with a single index barcode (in blue):

```
@9432NS1:54:C1K8JACXX:8:1101:6912:1869 1:N:0:ATGACT
TCAGGCATGCTTTCGACTATTATTGCATCAATGTTCTTTGCGTAATCAGCTACAATATCAGGTAATATCAGGCGCA
+
CCCFFFFFHHHHHJJJJJJJJIJJJJJJJJJJJHIIJJJJJJIJJJJJJJJJJJJJJJJJJJGIJJJJJJHHHFF
@9432NS1:54:C1K8JACXX:8:1101:6822:1873 1:N:0:ATGACT
CAGCGCATGAGCTAATGTATGTTTTACATTCCAGAAAGAGAGCTACTGCTGCAGGTTGTGATAAAATAAAGTAAGA
+
B@@FFFFFHFFHHJJJJFHIJHGGGHIJIIJIJCHJIIGGIIIGGIJEHIJJHII?FFHICHFFGGHIIGG@DEHH
@9432NS1:54:C1K8JACXX:8:1101:6793:1916 1:N:0:ATGACT
TTTCGCATGCCCTATCCTTTTATCACTCTGTCATTCAGTGTGGCAGCGGCCATAGTGTATGGCGTACTAAGCGAAA
+
@C@DFFFFHGHHHGIGHHJJJJJJJGIJIJJIGIJJJJHIGGGHGII@GEHIGGHDHEHIHD6?493;AAA?;=;=
```

Then you can specify the `--index_null` flag to **process_radtags**.

3. If your data are **single-end** with both an inline barcode (in red) and an index barcode (in blue):

```
@9432NS1:54:C1K8JACXX:8:1101:6912:1869 1:N:0:ATCACG
TCACGCATGCTTTCGACTATTATTGCATCAATGTTCTTTGCGTAATCAGCTACAATATCAGGTAATATCAGGCGCA
+
CCCFFFFFHHHHHJJJJJJJJIJJJJJJJJJJJHIIJJJJJJIJJJJJJJJJJJJJJJJJJJGIJJJJJJHHHFF
@9432NS1:54:C1K8JACXX:8:1101:6822:1873 1:N:0:ATCACG
GTCCGCATGAGCTAATGTATGTTTTACATTCCAGAAAGAGAGCTACTGCTGCAGGTTGTGATAAAATAAAGTAAGA
+
B@@FFFFFHFFHHJJJJFHIJHGGGHIJIIJIJCHJIIGGIIIGGIJEHIJJHII?FFHICHFFGGHIIGG@DEHH
@9432NS1:54:C1K8JACXX:8:1101:6793:1916 1:N:0:ATCACG
GTCCGCATGCCCTATCCTTTTATCACTCTGTCATTCAGTGTGGCAGCGGCCATAGTGTATGGCGTACTAAGCGAAA
+
@C@DFFFFHGHHHGIGHHJJJJJJJGIJIJJIGIJJJJHIGGGHGII@GEHIGGHDHEHIHD6?493;AAA?;=;=
```

Then you can specify the `--inline_index` flag to **process_radtags**.

4. If your data are **paired-end** with an inline barcode on the single-end (in red) and an index barcode (in blue):

```
@9432NS1:54:C1K8JACXX:7:1101:5584:1725 1:N:0:CGATGT
ACTGGCATGATGATCATAGTATAACGTGGGATACATATGCCTAAGGCTAAAGATGCCTTGAAGCTTGGCTTATGTT
+
#1=DDDFFHFHFHIFGIEHIEHGIIHFFHICGGGIIIIIIIIAEIGIGHAHIEGHHIHIIGFFFGGIIIGIIIEE7
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 1:N:0:CGATGT
TTCGACATGTGTTTACAACGCGAACGGACAAAGCATTGAAAATCCTTGTTTTGGTTTCGTTACTCTCTCCTAGCAT
+
#1=DFFFFHHHHHJJJJJJJJJJJJJJJJJIIJIJJJJJJJJJJIIJJHHHHHFEFEEDDDDDDDDDDDDDDDD@


@9432NS1:54:C1K8JACXX:7:1101:5584:1725 2:N:0:CGATGT
AATTTACTTTGATAGAAGAACAACATAAGCCAAGCTTCAAGGCATCTTTAGCCTTAGGCATATGTATCCCACGTTA
+
@@@DFFFFHGHDHIIJJJGGIIIEJJJCHIIIGIJGGEGGIIGGGIJIJIHIIJJJJIJJJIIIGGIIJJJIICEH
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 2:N:0:CGATGT
AGTCTTGTGAAAAACGAAATCTTCCAAAATGCTAGGAGAGAGTAACGAAACCAAAACAAGGATTTTCAATGCTTTG
+
C@CFFFFFHHHHHJJJJJJIJJJJJJJJJJJJJJIJJJHIJJFHIIJJJJIIJJJJJJJJJHGHHHHFFFFFFFED
```

Then you can specify the `--inline_index` flag to **process_radtags**.

5. If your data are **paired-end** with indexed barcodes on the single and paired-ends (in blue):

```
@9432NS1:54:C1K8JACXX:7:1101:5584:1725 1:N:0:ATCACG+CGATGT
ACTGGCATGATGATCATAGTATAACGTGGGATACATATGCCTAAGGCTAAAGATGCCTTGAAGCTTGGCTTATGTT
+
#1=DDDFFHFHFHIFGIEHIEHGIIHFFHICGGGIIIIIIIIAEIGIGHAHIEGHHIHIIGFFFGGIIIGIIIEE7
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 1:N:0:ATCACG+CGATGT
TTCGACATGTGTTTACAACGCGAACGGACAAAGCATTGAAAATCCTTGTTTTGGTTTCGTTACTCTCTCCTAGCAT
+
#1=DFFFFHHHHHJJJJJJJJJJJJJJJJJIIJIJJJJJJJJJJIIJJHHHHHFEFEEDDDDDDDDDDDDDDDD@


@9432NS1:54:C1K8JACXX:7:1101:5584:1725 2:N:0:ATCACG+CGATGT
AATTTACTTTGATAGAAGAACAACATAAGCCAAGCTTCAAGGCATCTTTAGCCTTAGGCATATGTATCCCACGTTA
```

```
+
@@@DFFFFHGHDHIIJJJGGIIIEJJJCHIIIGIJGGEGGIIGGGIJIJIHIIJJJJIJJJIIIGGIIJJJIICEH
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 2:N:0:ATCACG+CGATGT
AGTCTTGTGAAAAACGAAATCTTCCAAAATGCTAGGAGAGAGTAACGAAACCAAAACAAGGATTTTCAATGCTTTG
+
C@CFFFFFHHHHHJJJJJJIJJJJJJJJJJJJJIJJJHIJJFHIIJJJJIIJJJJJJJJJHGHHHHFFFFFFFED
```

Then you can specify the `--index_index` flag to **process_radtags**.

6. If your data are **paired-end** with inline barcodes on the single and paired-ends (in red):

```
@9432NS1:54:C1K8JACXX:7:1101:5584:1725 1:N:0:
ACTGGCATGATGATCATAGTATAACGTGGGATACATATGCCTAAGGCTAAAGATGCCTTGAAGCTTGGCTTATGTT
+
#1=DDDFFHFHFHIFGIEHIEHGIIHFFHICGGGIIIIIIIIAEIGIGHAHIEGHHIHIIGFFFGGIIIGIIIEE7
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 1:N:0:
TTCGACATGTGTTTACAACGCGAACGGACAAAGCATTGAAAATCCTTGTTTTGGTTTCGTTACTCTCTCCTAGCAT
+
#1=DFFFFHHHHHJJJJJJJJJJJJJJJJIIJIJJJJJJJJJJIIJJHHHHHFEFEEDDDDDDDDDDDDDDDD@


@9432NS1:54:C1K8JACXX:7:1101:5584:1725 2:N:0:
AATTTACTTTGATAGAAGAACAACATAAGCCAAGCTTCAAGGCATCTTTAGCCTTAGGCATATGTATCCCACGTTA
+
@@@DFFFFHGHDHIIJJJGGIIIEJJJCHIIIGIJGGEGGIIGGGIJIJIHIIJJJJIJJJIIIGGIIJJJIICEH
@9432NS1:54:C1K8JACXX:7:1101:5708:1737 2:N:0:
AGTCTTGTGAAAAACGAAATCTTCCAAAATGCTAGGAGAGAGTAACGAAACCAAAACAAGGATTTTCAATGCTTTG
+
C@CFFFFFHHHHHJJJJJJIJJJJJJJJJJJJJIJJJHIJJFHIIJJJJIIJJJJJJJJJHGHHHHFFFFFFFED
```

Then you can specify the `--inline_inline` flag to **process_radtags**.

4.1.2 Specifying the barcodes

The **process_radtags** program will demultiplex data if it is told which barcodes/indexes to expect in the data. It will also properly name the output files if the user specifies how to translate a particular barcode to a specific output file neam. This is done with the barcodes file, which we provide to **process_radtags** program. The barcode file is a very simple format — one barcode per line; if you want to rename the output files, the sample name prefix is provided in the second column.

```
% cat barcodes_lane3
CGATA<tab>sample_01
CGGCG     sample_02
GAAGC     sample_03
GAGAT     sample_04
TAATG     sample_05
TAGCA     sample_06
AAGGG     sample_07
ACACG     sample_08
ACGTA     sample_09
```

The sample names can be whatever is meaningful for your project:

```
% more barcodes_run01_lane01
CGATA<tab>spruce_site_12-01
CGGCG     spruce_site_12-02
GAAGC     spruce_site_12-03
GAGAT     spruce_site_12-04
TAATG     spruce_site_06-01
TAGCA     spruce_site_06-02
AAGGG     spruce_site_06-03
ACACG     spruce_site_06-04
```

Combinatorial barcodes are specified, one per column, separated by a tab:

```
% cat barcodes_lane07
CGATA<tab>ACGTA<tab>sample_01
CGGCG     ACGTA     sample_02
GAAGC     ACGTA     sample_03
GAGAT     ACGTA     sample_04
CGATA     TAGCA     sample_05
CGGCG     TAGCA     sample_06
GAAGC     TAGCA     sample_07
GAGAT     TAGCA     sample_08
```

1. If you don't want **process_radtags** to rename your samples, simply do not specify the last column in the barcodes file, and the output files will instead be named after the barcode.

2. Often, sequencing centers will return data from indexed libraries already demultiplexed. In this case, omit the barcodes file and `process_radtags` will not attempt to demulitplex the data, but can still be used to clean the data.

4.1.3 Running process_radtags

Here is how single-end data received from an Illumina sequencer might look:

```
% ls ./raw
lane3_NoIndex_L003_R1_001.fastq.gz  lane3_NoIndex_L003_R1_006.fastq.gz  lane3_NoIndex_L003_R1_011.fastq.gz
lane3_NoIndex_L003_R1_002.fastq.gz  lane3_NoIndex_L003_R1_007.fastq.gz  lane3_NoIndex_L003_R1_012.fastq.gz
lane3_NoIndex_L003_R1_003.fastq.gz  lane3_NoIndex_L003_R1_008.fastq.gz  lane3_NoIndex_L003_R1_013.fastq.gz
lane3_NoIndex_L003_R1_004.fastq.gz  lane3_NoIndex_L003_R1_009.fastq.gz
lane3_NoIndex_L003_R1_005.fastq.gz  lane3_NoIndex_L003_R1_010.fastq.gz
```

Then you can run **process_radtags** in the following way:

```
% process_radtags -p ./raw/ -o ./samples/ -b ./barcodes/barcodes_lane3 \
                  -e sbfI -r -c -q
```

I specify the directory containing the input files, `./raw`, the directory I want **process_radtags** to enter the output files, `./samples`, and a file containing my barcodes, `./barcodes/barcodes_lane3`, along with the restrction enzyme I used and instructions to clean the data and correct barcodes and restriction enzyme cutsites (`-r`, `-c`, `-q`).

Here is a more complex example, using paired-end double-digested data (two restriction enzymes) with combinatorial barcodes, and gzipped input files. Here is what the raw Illumina files may look like:

```
% ls ./raw
GfddRAD1_005_ATCACG_L007_R1_001.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_001.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_002.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_002.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_003.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_003.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_004.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_004.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_005.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_005.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_006.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_006.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_007.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_007.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_008.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_008.fastq.gz
GfddRAD1_005_ATCACG_L007_R1_009.fastq.gz  GfddRAD1_005_ATCACG_L007_R2_009.fastq.gz
```

Now we specify both restriction enzymes using the `--renz_1` and `--renz_2` flags along with the type combinatorial barcoding used. Here is the command:

```
% process_radtags -P -p ./raw  -b ./barcodes/barcodes -o ./samples/ \
                  -c -q -r --inline_index --renz_1 nlaIII --renz_2 mluCI
```

The output of process_radtags

The output of the **process_radtags** differs depending if you are processing single-end or paired-end data. In the case of single-end reads, the program will output one file per barcode into the output directory you specify. If the data do not have barcodes, then the file will retain its original name.

If you are processing paired-end reads, then you will get four files per barcode, two for the single-end read and two for the paired-end read. For example, given barcode ACTCG, you would see the following four files:

```
sample_ACTCG.1.fq
sample_ACTCG.rem.1.fq
```

```
sample_ACTCG.2.fq
sample_ACTCG.rem.2.fq
```

The **process_radtags** program wants to keep the reads in phase, so that the first read in the `sample_XXX.1.fq` file is the mate of the first read in the `sample_XXX.2.fq` file. Likewise for the second pair of reads being the second record in each of the two files and so on. When one read in a pair is discarded due to low quality or a missing restriction enzyme cut site, the remaining read can't simply be output to the `sample_XXX.1.fq` or `sample_XXX.2.fq` files as it would cause the remaining reads to fall out of phase. Instead, this read is considered a remainder read and is output into the `sample_XXX.rem.1.fq` file if the paired-end was discarded, or the `sample_XXX.rem.2.fq` file if the single-end was discarded.

Modifying how process_radtags executes

The **process_radtags** program can be modified in several ways. If your data do not have barcodes, omit the barcodes file and the program will not try to demultiplex the data. You can also disable the checking of the restriction enzyme cut site, or modify what types of quality are checked for. So, the program can be modified to only demultiplex and not clean, clean but not demultiplex, or some combination.

There is additional information available in **process_radtags** manual page.

4.2. Align data against a reference genome

If a reference genome is available for your organism, once you have demultiplexed and cleaned your samples, you will align these samples using a standard alignment program, such as GSnap, BWA, or Bowtie2.

Performing this alignment is outside the scope of this document, however there are several things you should keep in mind:

- Stacks can read BAM and SAM files, so you can use any aligner that can generate these standard format output files.
- Stacks can understand gapped alignments. It will interpret the alignment according to the CIGAR string in the SAM/BAM file.
- Make sure you produce unique alignments, or randomly place repetitive alignments.
- Be careful of **terminal alignments**. Several alignment programs will create terminal alignments: if an aligner cannot find a full-length alignment it will soft-mask the portion of the read it cannot align and report the fragment that does align. Stacks will convert these soft-masked regions to Ns, since they are not part of the alignment and if the soft-masked region is too long, the read will be discarded by **gstacks**.
- Use a program like Samtools or Picard to measure how many of your raw reads aligned to the reference genome. If too few align, you should consider the alignment parameters, or, you should also do a de novo analysis with which to comapre your results. If too few reads align, it could indicate the quality of your reference is low, or a lot of the true genome is missing from the reference, or it may indicate you are using a reference that is too evolutionarily distant from your focal organism.

4.3. Run the pipeline

The simplest way to run the pipeline is to use one of the two wrapper programs provided: if you do not have a reference genome you will use **denovo_map.pl**, and if you do have a reference genome use **ref_map.pl**. In each case you will specify a list of samples that you demultiplexed in the first step to the program, along with several command line options that control the internal algorithms.

4.3.1 **denovo_map.pl** versus **ref_map.pl**

Here are some key differences in running **denovo_map.pl** versus **ref_map.pl**:

- Both pipelines can be run for either a genetic map or population analysis.
- **denovo_map.pl** takes data in FASTQ, or FASTA format, compressed or uncompressed.
- **denovo_map.pl** will execute the pipeline, running **ustacks** to assemble loci in each individual de novo, calling SNPs in each assembled locus. It will then executing **cstacks** to build the catalog followed by **sstacks** to match either the parents and progeny, or all the generic samples against the catalog. Next, it will run **tsv2bam** to transpose data from being store per-sample to be stored per-locus, then it will run **gstacks** to assemble paired-end contigs (if paired-end data is provided) and re-call SNPs using the population-wide data.
- **ref_map.pl** expects data that has been aligned to a reference genome, and accepts either SAM or BAM files.
- **ref_map.pl** will execute the pipeline, running **gstacks** to build and genotype single- or paired-end data and call SNPs using the population-wide data per locus.

4.3.2 The Population Map

A population map is a text file containing two columns: the prefix of each sample in the analysis in the first column, followed by an integer or string in the second column indicating the population.

Most of the Stacks component programs make use of the population map as a mechanism to list all of the files in an analysis. All of the programs (with the exception of **ustacks**) make use of the population map to specify all of the samples in an analysis. It is often convenient to generate several initial population maps just to control execution of the pipeline. For example, **cstacks** will take a population map as a means to specify which samples to build the catalog from. If you want to include only a subset of samples in your catalog, you can specify a particular population map for that purpose. Or, if you want to perform a search of parameters to set the de novo assembly parameters, you likely only want to conduct the parameter search on a small subset of your individuals, which can be specified in its own population map. In these cases, the second column, containing the population designation is not used. But, as you will see, this column is very important for the **populations** program.

The **populations** program uses the population map to determine which groupings to use for calculating summary statistics, such as heterozygosity, π, and F_{IS}. If no population map is supplied the **populations** program computes these statistics with all samples as a single group. With a population map, we can tell **populations** to instead calculate them over subsets of individuals. In addition, if enabled, F_{ST} will be computed for each pair of populations supplied in the population map.

Here is a very simple population map, containing six individuals and two populations (the integer indicating the population can be any unique number, it doesn't have to be sequential):

```
% cat popmap
indv_01<tab>6
indv_02     6
indv_03     6
indv_04     2
indv_05     2
indv_06     2
```

Alternatively, we can use strings instead of integers to enumerate our populations:

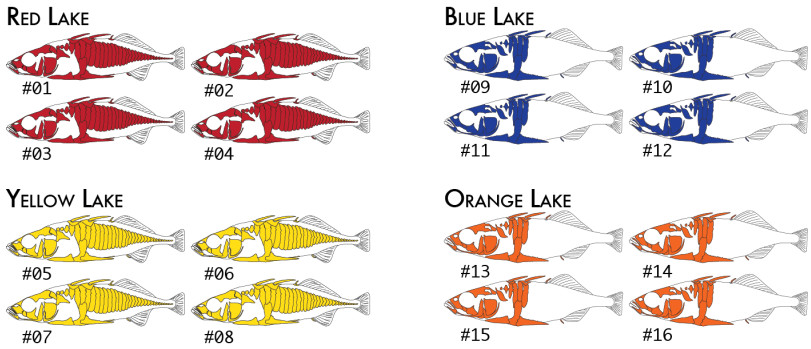
```
% cat popmap
indv_01<tab>fw
indv_02     fw
```

```
indv_03     fw
indv_04     oc
indv_05     oc
indv_06     oc
```

These strings are nicer because they will be embedded within various output files from the pipeline as well as in names of some generated files.

Analyzing the same data set with multiple population maps

Here is a more realistic example of how you might analyze the same data set using more than one poulation map. In our initial analysis, we have four populations of stickleback each collected from a different lake and each population consisting of four individuals.



We would code these 16 individuals into a population map like this:

```
% cat popmap_geographical
sample_01<tab>red
sample_02     red
sample_03     red
sample_04     red
sample_05     yellow
sample_06     yellow
sample_07     yellow
sample_08     yellow
sample_09     blue
sample_10     blue
sample_11     blue
sample_12     blue
sample_13     orange
sample_14     orange
sample_15     orange
sample_16     orange
```

This would cause summary statistics to be computed four times, once for each of the red, yellow, blue, and orange groups. F_{ST} would also be computed for each pair of populations: red-yellow, red-blue, red-orange, yellow-blue, yellow-orange, and blue-orange.

And, we would expect the prefix of our Stacks input files to match what we have written in our population map. So, a listing of our paired-end samples directory would look something like this:

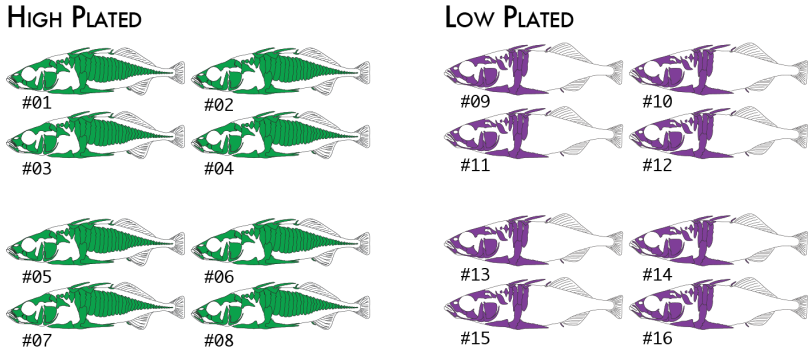
```
% ls -1 ./samples/
sample_01.1.fq.gz
sample_01.2.fq.gz
sample_02.1.fq.gz
sample_02.2.fq.gz
sample_03.1.fq.gz
sample_03.2.fq.gz
...
sample_16.1.fq.gz
sample_16.2.fq.gz
```

Or, in the case of referenced aligned data (single or paired-end) it would look something like this:

```
% ls -1 ./aligned/
sample_01.bam
sample_02.bam
```

```
sample_03.bam
...
sample_16.bam
```

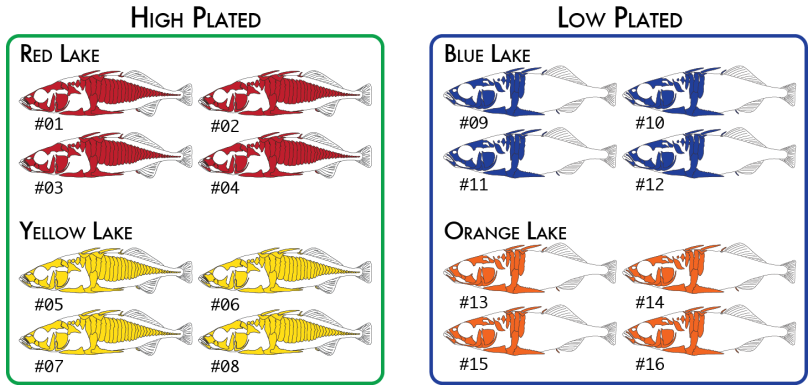
We might decide to regroup our samples by phenotype (we will use high and low plated stickleback fish as our two phenotypes), and re-run just the **populations** program, which is the only component of the pipeline that actually uses the population map. This will cause pairwise F_{ST} comparisons to be done once between the two groups of eight individuals (high and low plated) instead of previously where we had six pairwise comparisons between the four geographical groupings.



Now we would code the same 16 individuals into a population map like this:

```
% cat popmap_phenotype
sample_01<tab>high
sample_02     high
sample_03     high
sample_04     high
sample_05     high
sample_06     high
sample_07     high
sample_08     high
sample_09     low
sample_10     low
sample_11     low
sample_12     low
sample_13     low
sample_14     low
sample_15     low
sample_16     low
```

Finally, for one type of analysis we can include two levels of groupings. The **populations** program will calculate F_{ST} based on RAD haplotypes in addition to the standard SNP-based F_{ST} calculation. An analysis of molecular variance (AMOVA) is used to calculate F_{ST} in this case on all populations together (it is not a pairwise calculation), and it is capable of up to two levels of grouping, so we could specify both the geographical groupings and phenotypical groupings like this:



Once again we would code the same 16 individuals into a population map like this:

```
% cat popmap_both
sample_01<tab>red<tab>high
sample_02     red      high
```

```
sample_03     red      high
sample_04     red      high
sample_05     yellow   high
sample_06     yellow   high
sample_07     yellow   high
sample_08     yellow   high
sample_09     blue     low
sample_10     blue     low
sample_11     blue     low
sample_12     blue     low
sample_13     orange   low
sample_14     orange   low
sample_15     orange   low
sample_16     orange   low
```

And then we could run the **populations** a third time. Only the AMOVA haplotype-F_{ST} calculation will use both levels. Other parts of the program will continue to only use the second column, so summary statistics and pairwise F_{ST} calculations would use the geographical groupings.

4.3.3 Examples

In each of these examples, we assume that a population map has been created that lists each sample file in the analysis and assigns it to a population. Here is an example running **denovo_map.pl** for a set of single-end data:

```
% denovo_map.pl -T 8 -m 3 -M 4 -n 4 -o ./stacks/ --samples ./samples --popmap ./popmaps/popmap
```

Here is an example running **denovo_map.pl** for a set of paired-end data:

```
% denovo_map.pl -T 8 -m 3 -M 4 -n 4 -o ./stacks/ --samples ./samples --popmap ./popmaps/popmap --paired
```

It is assumed that your files are named properly in the population map and on the file system. So, for a paired-end analysis, given `sample_2351` listed in the population map, **denovo_map.pl** expects to find files `sample_2351.1.fq.gz` and `sample_2351.2.fq.gz` in the directory specified with`--samples`.

Here is an example running **ref_map.pl** for a paired-end population analysis:

```
% ref_map.pl -T 8 --popmap ./popmaps/popmap -o ./stacks/ --samples ./aligned
```

ref_map.pl will read the file names out of the population map and look for them in the directory specified with `--samples`. The **ref_map.pl** program expects, given `sample_2351` listed in the population map, to find a `sample_2351.bam` file containing both single and paired-reads aligned to the reference genome and sorted.

4.3.4 Choosing parameters for building stacks de novo

If you do not have a reference genome and are processing your data de novo, the following tutorial describes how to set the major parameters that control stack formation in **ustacks**:

- How do the major Stacks parameters control the de novo formation of stacks and loci?

To optimize those parameters we recommend using the r80 method. In this method you vary several de novo parameters and select those values which maximize the number of polymorphic loci found in 80% of the individuals in your study. This process is outlined in the paper:

- Lost in parameter space: a road map for Stacks. **Methods in Ecology and Evolution** 2017.

Another method for parameter optimization has been published by Mastretta-Yanes, et al. They provide a very nice strategy of using a small set of replicate samples to determine the best de novo parameters for assembling loci. Have a look at their paper:

- RAD sequencing, genotyping error estimation and de novo assembly optimization for population genetic inference. **Molecular Ecology Resources.**

4.4. Run the pipeline by hand

In certain situations it doesn't make sense to use the wrappers and you will want to run the individual components by hand. Here are a few cases to consider running the pipeline manually:

1. You have an extremely large number of samples and access to a large computer cluster. In this case, you can split up the running of **ustacks** and **sstacks** (but not **cstacks**) among your different nodes. Most queuing systems support job arrays, which work very well for processing large numbers of samples.

2. You have a more complex experimental design. Let's say you have generated three maps, each with two parents, from the same organism. Generating genotypes for any single map requires no more than two parents and the corresponding progeny, however, you want to have the same catalog loci in all three maps, so you can cross-reference common loci across the maps. In this case, you feed all six parents to **cstacks** to build your catalog, but then match your data with **sstacks** in three, separate batches: once for each set of parents and progeny for each map.

3. You have a very large number of samples from a set of populations, but no reference genome. In this case placing all of your samples into the catalog may create too many loci to process given the size of your computer, or, it may create too many loci that are found in just one or two individuals in the analysis. To speed things up, given that you are only concerned (in this case) with loci that are found widely in the population, you could simply supply the first ten individuals from each population to **cstacks**, knowing that the vast majority of widely available loci will be found in the first ten individuals of each population and will appear in the catalog.

4.4.1 De novo **Data**

Here is an example shell script for de novo aligned data that uses shell loops to easily execute the pipeline by hand:

```
#!/bin/bash

src=$HOME/research/project

files="sample_01
sample_02
sample_03"

#
# Build loci de novo in each sample for the single-end reads only. If paired-end reads are available,
# they will be integrated in a later stage (tsv2bam stage).
# This loop will run ustacks on each sample, e.g.
#   ustacks -f ./samples/sample_01.1.fq.gz -o ./stacks -i 1 --name sample_01 -M 4 --gapped -p 8
#
id=1
for sample in $files
do
    ustacks -f $src/samples/${sample}.1.fq.gz -o $src/stacks -i $id --name $sample -M 4 --gapped -p 8
    let "id+=1"
done

#
# Build the catalog of loci available in the metapopulation from the samples contained
# in the population map. To build the catalog from a subset of individuals, supply
# a separate population map only containing those samples.
#
cstacks --gapped -n 6 -P $src/stacks/ -M $src/popmaps/popmap -p 8

#
# Run sstacks. Match all samples supplied in the population map against the catalog.
#
```

```
sstacks --gapped -P $src/stacks/ -M $src/popmaps/popmap -p 8

#
# Run tsv2bam to transpose the data so it is stored by locus, instead of by sample. We will include
# paired-end reads using tsv2bam. tsv2bam expects the paired read files to be in the samples
# directory and they should be named consistently with the single-end reads,
# e.g. sample_01.1.fq.gz and sample_01.2.fq.gz, which is how process_radtags will output them.
#
tsv2bam -P $src/stacks/ -M $src/popmaps/popmap --pe-reads-dir $src/samples -t 8

#
# Run gstacks: build a paired-end contig from the metapopulation data (if paired-reads provided),
# align reads per sample, call variant sites in the population, genotypes in each individual.
#
gstacks -P $src/stacks/ -M $src/popmaps/popmap -t 8

#
# Run populations. Calculate Hardy-Weinberg deviation, population statistics, f-statistics
# export several output files.
#
populations -P $src/stacks/ -M $src/popmaps/popmap -r 0.65 --vcf --genepop --structure --fstats --hwe -t 8
```

4.4.2 Reference-aligned Data

Here is an example shell script for reference-aligned data that uses shell loops to easily execute the pipeline by hand:

```
#!/bin/bash

src=$HOME/research/project
bwa_db=$src/bwa_db/my_bwa_db_prefix

files="sample_01
sample_02
sample_03"

#
# Align paired-end data with BWA, convert to BAM and SORT.
#
for sample in $files
do
    bwa mem -t 8 $bwa_db $src/samples/${sample}.1.fq.gz $src/samples/${sample}.2.fq.gz |
      samtools view -b |
      samtools sort --threads 8 > $src/aligned/${sample}.bam
done

#
# Run gstacks to build loci from the aligned paired-end data. We have instructed
# gstacks to remove any PCR duplicates that it finds.
#
gstacks -I $src/aligned/ -M $src/popmaps/popmap --rm-pcr-duplicates -O $src/stacks/ -t 8

#
# Run populations. Calculate Hardy-Weinberg deviation, population statistics, f-statistics and
# smooth the statistics across the genome. Export several output files.
#
populations -P $src/stacks/ -M $src/popmaps/popmap -r 0.65 --vcf --genepop --fstats --smooth --hwe -t 8
```

4.4.3 Whitelists and Blacklists

The **populations** and **genotypes** programs allow the user to specify a list of catalog locus IDs (also referred to as markers) to the two programs. In the case of a whitelist, the program will only process the loci provided in the list, ignoring all other loci. In the case of a blacklist, the listed loci will be excluded, while all other loci will be processed. These lists apply to the entire locus, including all SNPs within a locus if they exist.

A whitelist or blacklist are simple files containing one catalog locus per line, like this:

```
% cat whitelist
3
7
521
11
46
103
972
2653
22
```

Whitelists and blacklists are processed by the **populations** program immediately. All loci destined not to be processed are never read into memory, before any calculations are made on the remaining data. So, once the whitelist or blacklist is implemented, the other data is not present and will not be seen or interfere with any downstream calculations, nor will they appear in any output files.

SNP-specific Whitelists

In the **populations** program it is possible to specify a whitelist that contains catalog loci and specific SNPs within those loci. This is useful if you have a specific set of SNPs for a particular dataset that are known to be informative; perhaps you want to see how these SNPs behave in different population subsets, or perhaps you are developing a SNP array that will contain a specific set of data.

To create a SNP-specific whitelist, simply add a second column (separated by tabs) to the standard whitelist where the second column represents the column within the locus where the SNP can be found. Here is an example:

```
% cat whitelist
1916<tab>12
517	14
517	76
1318
1921	13
195	28
260	5
28	44
28	90
5933
1369	18
```

You can include all the SNPs at a locus by omitting the extra column, and you can include more than one SNP per locus by listing a locus more than once in the list (with a different column). The column is a zero-based coordinate of the SNP location, so the first nucleotide at a locus is labeled as column zero, the second position as column one. These coordinates correspond with the column reported in the `populations.sumstats.tsv` file as well as in several other output files from **populations**.

Why do loci drop out of the analysis despite being on the whitelist?

Loci, or SNPs within a locus can still drop out from an analysis despite being on the whitelist. This can happen for several reasons, including:

1. The filters in the **populations** and **genotypes programs** are still applied after the whitelist is applied. A locus must still pass the filters to be retained. Once you have created a whitelist, it is normal to turn off most or all other filters.
2. If you change your population map after creating the whitelist, you may see SNPs drop out of the analysis because introducing a population map may change if a locus is fixed. In a large, single population a locus may be polymorphic, but once you subset your data into multiple populations that locus may become fixed in one or more subpopulations and will not be output in those populations.
3. If you add populations to your population map, you may find a small number of loci where additional populations bring a third or fourth allele into a particular SNP position, causing that position to fail the infinite alleles assumption of the software and be dropped.

4.5. Exporting data from Stacks

Stacks, through the **populations** program, is able to export data for a wide variety of downstream analysis programs. Here we provide some advice for programs that can be tricky to get running with Stacks data.

4.5.1 Exporting data for STRUCTURE

While STURCTURE is a very useful analysis program, it is very unforgiving during its import process and does not provide easily decipherable error messages. The **populations** program can export data directly for use in STRUCTURE, although there are some common errors that occur.

1. Data for STRUCTURE is exported by supplying the `--structure` command line option to the **populations** program. Since STRUCTURE does not want linked loci, you will typically also supply the `--write_single_snp` flag so that you do not get more than one SNP per RAD locus (SNPs at the same RAD locus are almost certainly linked). If you aligned your data to a reference genome you may also want to use the `--ordered_export` option, because if two RAD loci overlap in the genome, this option will ensure that only one of the overlapping SNPs is output, again ensuring SNPs are not linked. A file will be output with a name similar to `populations.structure.tsv`.

2. Stacks places a comment line at the top of the file to date-stamp the file and supply the program version that generated it. This is done so that if there are problems we can know exactly which set of code exported the file and when the export was done. STRUCTURE is unable to ignore comment lines so you will need to delete this line before using the file with STRUCTURE.

3. STRUCTURE requires you to provide a configuration file, e.g. `mainparams`, that contains, among other things, the number of indiviudals ("`#define NUMINDS XX`") and the number of loci ("`#define NUMLOCI YY`") in the analysis (or enter them into the graphical user interface). One of the most common problems is that these numbers do not match those supplied in the file exported by Stacks. When this problem occurs, STRUCTURE will output many lines of errors, but at the end of the errors, you should see a message telling you that the file does not have the expected number of columns in it:

```
-----------------------------------
There were errors in the input file (listed above). According to
"mainparams" the input file should contain one row of markernames with 1100 entries,
32 rows with 1102 entries .

There are 33 rows of data in the input file, with an average of 1001.94
entries per line.  The following shows the number of entries in each
line of the input file:

# Entries:   Line numbers
1000:   1
1002:   2--33
-----------------------------------
```

   In this particular example, I have 16 individuals in the analysis and should have 1100 loci; STRUCTURE expects a header line containing the 1100 loci (in 1100 columns), and then two lines per individual (with 1102 columns -- the 1100 loci, the sample name, and the population ID) for a total of 33 lines (header + (16 individuals x 2)). The error says that instead the header line has 1000 entries and the 32 additional rows have 1002 entries, so there are 100 missing loci. This usually happens because the Stacks filters have removed more loci than you thought they would.

   The simplest way to fix this is to just change the STRUCTURE configuration file to expect 1000 entries instead of 1100. I can also use some UNIX to check exactly how many loci are in the STRUCTURE file that Stacks exported:

```
% head -n 1 populations.structure.tsv | tr "\t" "\n" | grep -v "^$" | wc -l
```

4. Another common problem is that while Stacks allows you to label populations in the population map using any string of characters, STRUCTURE requires populations to be labeled with integer numbers only. Stacks will pass the population names into the STRUCTURE output file (column 2). This can be fixed by creating a second population map where you use numbers instead of strings to label the populations. Or, you can use some quick UNIX to fix the problem after export. In this example, I have two populations, `fw` and `oc`, and I will just replace these names with `1` and `2` in the STRUCTURE input file:

```
% cat populations.structure.tsv |
  sed -E -e 's/	fw	/	1	/' -e 's/	oc	/	2	/' > populations.s
```

Be careful, as those are tab characters surrounding the 'fw' and 'oc' strings.

4.5.2 Exporting data for Adegenet

The simplest method to get genotypes exported from Stacks into the Adegenet program is to use the GenePop export option (`--genepop`) for the **`populations`** program. Adegenet requires a GenePop file to have an `.gen` suffix, so you can rename the Stacks file before trying to use it. You can then import the data into Adegenet in R like this:

```
> library("adegenet")
> x <- read.genepop("populations.gen")
```

4.6. Evaluating sequencing covergae

In our experience, the most important factor that correlates with the success of a RAD project is sequencing coverage. While the Stacks variant and genotype calling model can handle low coverage, the quality of individual SNP genotypes is closely related to coverage level, as is the availability of SNPs and haplotypes across most of your samples. Therefore, this is the most imporant aspect of your data to track when doing a RAD analysis, especially for the first time in a new organism.

There are several stages of the pipeline when you can track the coverage of your data.

1. The first is monitoring the raw read counts of your demultiplexed samples, as produced by **`process_radtags`**. While these raw counts aren't coverage per se, you want to be aware of what percentage of your raw data was successfully cleaned and demultiplexed. Were there any particular samples that had extremely low coverage relative to the others? One could consider excluding samples that weren't properly multiplexed during the construction of the RAD library.

2. The **`ustacks`** program will print the final coverage for the single sample it processed at the end of execution. It looks like this:

   ```
   Final coverage: mean=45.02; stdev=30.06; max=928; n_reads=2419464(74.8%)
   ```

   If you are running the **`denovo_map.pl`** wrapper, the program will pull the coverages for all samples out and print them in a table on the screen and it will be stored in the `denovo_map.log` file. I looks like this:

   ```
   Depths of Coverage for Processed Samples:
   sample_11072: 47.16x
   sample_11073: 45.02x
   sample_11074: 50.83x
   sample_11075: 49.67x
   sample_11076: 51.04x
   sample_11077: 48.99x
   sample_11078: 54.59x
   sample_11079: 40.95x
   sample_11080: 47.58x
   ...
   ```

3. The gstacks module will also calculate depth of coverage along with locus size and the amount of each locus that can be genotyped. It is stored in the `gstacks.log` file and looks like this:

   ```
   Genotyped 427033 loci:
     effective per-sample coverage: mean=55.9x, stdev=28.8x, min=17.6x, max=177.2x (per locus where sample
     mean number of sites per locus: 304.8
   ```

4. Finally, the populations program does not calculate depth of coverage but will calculate the mean locus size and the amount of the locus that can be genotyped, after applying

the specified filters. It is stored in the `populations.log` file and looks like this:

```
Number of loci with PE contig: 45771.00 (100.0%);
  Mean length of loci: 520.45bp (stderr 0.29);
Number of loci with SE/PE overlap: 45764.00 (100.0%);
  Mean length of overlapping loci: 530.43bp (stderr 0.29); mean overlap: 152.39bp (stderr 0.03);
Mean genotyped sites per locus: 530.15bp (stderr 0.29).
```

4.7. A full protocol for running a RAD analysis

We have published a full protocol for conducting a RAD analysis including cleaning the data, optimizing parameters, a de novo assembly, as well as reference alignment and a reference-guided assembly. The protocol includes sample data that you can download for practice purposes.

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

5. Pipeline Components [↑top]

The Stacks pipeline is designed modularly to perform several different types of analyses. Programs listed under Raw Reads are used to clean and filter raw sequence data. Programs under Core represent the main Stacks pipeline — building single-end loci (**`ustacks`**), creating a catalog of loci (**`cstacks`**), and matching samples back against the catalog (**`sstacks`**), transposing the data to be organized from sample to instead being organized by locus (**`tsv2bam`**), assembling the paired-end contig, calling variable sites in the population and genotyping each sample at those sites (**`gstacks`**). Finally. **`populations`** performs a population genomics analysis. Programs under Execution Control will run the whole pipeline.

Raw Reads

- `process_radtags`
- `process_shortreads`
- `clone_filter`
- `kmer_filter`

Core

- `ustacks`
- `cstacks`
- `sstacks`
- `tsv2bam`
- `gstacks`
- `populations`

Execution control

- `denovo_map.pl`
- `ref_map.pl`

6. What do the fields mean in *Stacks* output files? [↑top]

These are the current Stacks file formats as of version 2.0.

6.1. ustacks

6.1.1 XXX.tags.tsv: Assembled loci

Column	Name	Description
1	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.
2	Locus ID	Each locus formed from one or more stacks gets an ID.
3	Sequence Type	Either 'consensus', 'model', 'primary' or 'secondary', see the notes below.
4	Stack component	An integer representing which stack component this read belongs to.
5	Sequence ID	The individual sequence read that was merged into this stack.
6	Sequence	The raw sequencing read.
7	Deleveraged Flag	If "1", this stack was processed by the deleveraging algorithm and was broken down from a larger stack.
8	Blacklisted Flag	If "1", this stack was still confounded depsite processing by the deleveraging algorithm.
9	Lumberjackstack Flag	If "1", this stack was set aside due to having an extreme depth of coverage.

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.

6.1.2 XXX.snps.tsv: Model calls from each locus

Column	Name	Description
1	Sample ID	The numerical ID for this sample, matches the ID in the `tags` file.
2	Locus ID	The numerical ID for this locus, in this sample. Matches the ID in the `tags` file.
3	Column	Position of the nucleotide within the locus, reported using a zero-based offset (first nucleotide is enumerated as 0)
4	Type	Type of nucleotide called: either heterozygous (`"E"`), homozygous (`"O"`), or unknown (`"U"`).
5	Likelihood ratio	From the SNP-calling model.
6	Rank_1	Majority nucleotide.
7	Rank_2	Alternative nucleotide.
8	Rank_3	Third alternative nucleotide (only possible in the `batch_X.catalog.snps.tsv` file).
9	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.

6.1.3 XXX.alleles.tsv: Haplotypes/alleles recorded from each locus

Column	Name	Description
1	Sample ID	The numerical ID for this sample, matches the ID in the `tags` file.
2	Locus ID	The numerical ID for this locus, in this sample. Matches the ID in the `tags` file.
3	Haplotype	The haplotype, as constructed from the called SNPs at each locus.
4	Percent	Percentage of reads that have this haplotype
5	Count	Raw number of reads that have this haplotype

6.2. `cstacks`

The **`cstacks`** files are the same as those produced by the **`ustacks`** program although they are named as `batch_X.catalog.tags.tsv`, and similarly for the SNPs and alleles files.

6.3. `sstacks`

6.3.1 XXX.matches.tsv: Matches to the catalog

Column	Name	Description
1	Catalog ID	ID of the catalog locus matched against.
2	Sample ID	Sample ID matched to the catalog.
3	Locus ID	ID of the locus within this sample matched to the catalog.
4	Haplotype	Matching haplotype.
5	Stack Depth	number or reads contained in the locus that matched to the catalog.
6	CIGAR string	Alignment of matching locus to the catalog locus.

Notes: Each line in this file records a match between a catalog locus and a locus in an individual, for a particular haplotype. The Catalog ID represents a unique locus in the entire population, while the Sample ID and the Locus ID together represent a unique locus in an individual sample.

6.4. `populations`

6.4.1 populations.sumstats.tsv: Summary statistics for each population

The **`populations`** program will calculate a standard set of population genetic statistics for population in the set of data it processes. These values are calculated at every variant site in the metapopulation (that means a site may be fixed in one or more populations, but is variant in at least one population, or across populations). Each variant site will be listed in the file on one line, for each population. If there are three populations in the analysis, each variant site will be listed on three lines, once per population.

- If the analysis is de novo, then the chromosome will be listed as "un" which is short for "unknown" and the basepair will arbitrarily ordered.
- If smoothing is enabled, with the `--smooth`, or more specific, `--smooth_popstats` option, then the smoothed columns will have values, otherwise they will be blank. The

chromosome and basepair fields will also be populated.

- If bootstrapping is enabled, smoothed windows will be resampled to generate a p-value indicating significance for each of the smoothed statistics, otherwise these columns will be blank.

Column	Name	Description
1	Locus ID	Catalog locus identifier.
2	Chromosome	If aligned to a reference genome.
3	Basepair	If aligned to a reference genome. This is the basepair for this particular SNP.
4	Column	The nucleotide site within the catalog locus, reported using a zero-based offset (first nucleotide is enumerated as 0).
5	Population ID	The ID supplied to the populations program, as written in the population map file.
6	P Nucleotide	The most frequent allele at this position in this population.
7	Q Nucleotide	The alternative allele.
8	Number of Individuals	Number of individuals sampled in this population at this site.
9	P	Frequency of most frequent allele.
10	Observed Heterozygosity	The proportion of individuals that are heterozygotes in this population.
11	Observed Homozygosity	The proportion of individuals that are homozygotes in this population.
12	Expected Heterozygosity	Heterozygosity expected under Hardy-Weinberg equilibrium.
13	Expected Homozygosity	Homozygosity expected under Hardy-Weinberg equilibrium.
14	π	An estimate of nucleotide diversity.
15	Smoothed π	A weighted average of π depending on the surrounding 3σ of sequence in both directions.
16	Smoothed π P-value	If bootstrap resampling is enabled, a p-value ranking the significance of π within this population.
17	F_{IS}	The inbreeding coefficient of an individual (I) relative to the subpopulation (S). Derived from Hartl & Clark, Principles of Population Genetics, fourth edition, equation 6.4, page 264.
18	Smoothed F_{IS}	A weighted average of F_{IS} depending on the surrounding 3σ of sequence in both directions.
19	Smoothed F_{IS} P-value	If bootstrap resampling is enabled, a p-value ranking the significance of F_{IS} within this population.
20	HWE P-value	The probability that this variant site deviates from Hardy-Weinberg equilibrium. Calculated via an exact test.
21	Private allele	True (1) or false (0), depending on if this allele is only occurs in this population.

6.4.2 populations.sumstats_summary.tsv: Summary of summary statistics for each population

The **`populations`** program will summarize the standard set of population genetic statistics across the dataset. These values can be replicated by summing the columns in the `populations.sumstats.tsv` file. For example, the mean value of Π is obtained by summing column 14 in the `populations.sumstats.tsv` file for one of the populations and dividing by the number of rows.

Column	Name	Description

1	Pop ID	Population ID as defined in the Population Map file.
2	Private	Number of private alleles in this population.
3	Number of Individuals	Mean number of individuals per locus in this population.
4	Variance	
5	Standard Error	
6	P	Mean frequency of the most frequent allele at each locus in this population.
7	Variance	
8	Standard Error	
9	Observed Heterozygosity	Mean obsered heterozygosity in this population.
10	Variance	
11	Standard Error	
12	Observed Homozygosity	Mean observed homozygosity in this population.
13	Variance	
14	Standard Error	
15	Expected Heterozygosity	Mean expected heterozygosity in this population.
16	Variance	
17	Standard Error	
18	Expected Homozygosity	Mean expected homozygosity in this population.
19	Variance	
20	Standard Error	
21	Π	Mean value of π in this population.
22	Π Variance	
23	Π Standard Error	
24	F_{IS}	Mean measure of F_{IS} in this population.
25	F_{IS} Variance	
26	F_{IS} Standard Error	

Notes: There are two tables in this file containing the same headings. The first table, labeled "Variant" calculated these values at only the variable sites in each population. The second table, labeled "All positions" calculted these values at all positions, both variable and fixed, in each population.

6.4.3 populations.fst_Y-Z.tsv: F_{ST} calculations for each pair of populations

If `--fstats` is specified to the **`populations`** program, then F_{ST} statistics will be calculated for each pair of populations, as defined in the population map.

- F_{ST} will be calculated for each variable site between the pair of populations (different pairs of populations may have different numbers of variable sites).
- A p-value indicating a statistically significant difference in allele frequencies (that is a p-value for the F_{ST} measure), is provided by Fisher's Exact Test in the "FET p-value" column.
- If a reference genome is available and `--smooth` is specified, these values will be smoothed across chromosomes and those smoothed values will be stored in the "Smoothed AMOVA $_{ST}$" column below.

- If bootstrapping is enabled, then it will be used to generate p-values for each smoothing window and stored in the "Smoothed AMOVA F_{ST} P-value" column.

Column	Name	Description
1	Locus ID	Catalog locus identifier.
2	Population ID 1	The ID supplied to the populations program, as written in the population map file.
3	Population ID 2	The ID supplied to the populations program, as written in the population map file.
4	Chromosome	If aligned to a reference genome.
5	Basepair	If aligned to a reference genome.
6	Column	The nucleotide site within the catalog locus, reported using a zero-based offset (first nucleotide is enumerated as 0).
7	Overall π	An estimate of nucleotide diversity across the two populations.
8	AMOVA F_{ST}	Analysis of Molecular Variance F_{ST} calculation. Derived from Weir, Genetic Data Analysis II, chapter 5, "F Statistics," pp166-167.
9	FET p-value	P-value describing if the F_{ST} measure is statistically significant according to Fisher's Exact Test.
10	Odds Ratio	Fisher's Exact Test odds ratio.
11	CI High	Fisher's Exact Test confidence interval.
12	CI Low	Fisher's Exact Test confidence interval.
13	LOD Score	Logarithm of odds score.
14	Corrected AMOVA F_{ST}	AMOVA F_{ST} with either the FET p-value, or a window-size or genome size Bonferroni correction.
15	Smoothed AMOVA F_{ST}	A weighted average of AMOVA F_{ST} depending on the surrounding 3σ of sequence in both directions.
16	Smoothed AMOVA F_{ST} P-value	If bootstrap resampling is enabled, a p-value ranking the significance of F_{ST} within this pair of populations.
17	Window SNP Count	Number of SNPs found in the sliding window centered on this nucleotide position.

6.4.4 populations.hapstats.tsv: Haplotype-based summary statistics for each locus in each population

The **`populations`** program will calculate a standard set of population genetic statistics for population in the set of data it processes. These values are calculated at every variant locus in the metapopulation (that means a RAD locus may be fixed in one or more populations, but is variant in at least one population, or across populations). In these statistics, the phased SNPs are taken as a set of haplotypes. Each locus will be listed in the file on one line, for each population. If there are three populations in the analysis, each locus will be listed on three lines, once per population.

- If the analysis is de novo, then the chromosome will be listed as "un" which is short for "unknown" and the basepair will arbitrarily ordered.
- If smoothing is enabled, with the `--smooth`, or more specific, `--smooth_popstats` option, then the smoothed columns will have values, otherwise they will be blank. The chromosome and basepair fields will also be populated.
- If bootstrapping is enabled, smoothed windows will be resampled to generate a p-value indicating significance for each of the smoothed statistics, otherwise these columns will be blank.

Column	Name	Description
1	Locus ID	Catalog locus identifier.
2	Chromosome	If aligned to a reference genome.
3	Basepair	If aligned to a reference genome.
4	Population ID	The ID supplied to the populations program, as written in the population map file.
5	N	Number of alleles/haplotypes present at this locus.
6	Haplotype count	
7	Gene Diversity	A measure of locus haplotype richness, similar to nucleotide-level π. In this measure, loci are considered as either the same or different, regardles of how different they are.
8	Smoothed Gene Diversity	
9	Smoothed Gene Diversity P-value	
10	Haplotype Diversity	A measure of locus haplotype richness that takes into account how different haplotypes are from one another in terms of nucleotide distance.
11	Smoothed Haplotype Diversity	
12	Smoothed Haplotype Diversity P-value	
13	HWE P-value	The probability that this locus deviates from Hardy-Weinberg equilibrium. Calculated using Guo and Thompson's MCMC walk.
14	HWE P-value SE	The standard error for the HWE p-value.
15	Haplotypes	A semicolon-separated list of haplotypes/haplotype counts in the population.

6.4.5 populations.phistats_Y-Z.tsv: Haplotype-based F_{ST} calculations for each pair of populations

If `--fstats` is specified to the **`populations`** program, then F_{ST} statistics will be calculated for each pair of populations, as defined in the population map.

- F_{ST} will be calculated for each variable locus between the pair of populations (different pairs of populations may have different numbers of variable loci).
- If a reference genome is available and `--smooth` is specified, these values will be smoothed across chromosomes and those smoothed values will be stored in the smoothed columns below.
- If bootstrapping is enabled, then it will be used to generate p-values for each smoothing window and stored in the smoothed p-value column.

Column	Name	Description
1	Locus ID	Catalog locus identifier.
2	Population ID 1	The ID supplied to the populations program, as written in the population map file.

3	Population ID 2	The ID supplied to the populations program, as written in the population map file.
4	Chromosome	If aligned to a reference genome.
5	Basepair	If aligned to a reference genome.
6	Φ_{ST}	Φ_{ST} is an AMOVA-based measure of F_{ST} intended for haplotpye analysis. Stacks' implementation is based on Excoffier, et al. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. **Genetics**.
7	Smoothed Φ_{ST}	
8	Smoothed Φ_{ST} P-value	
9	F_{ST}'	F_{ST}' is a haplotype measure of F_{ST} that is scaled to the theoretical maximum F_{ST} value at this locus. Depending on how many haplotypes there are, it may not be possible to reach an F_{ST} of 1, so this method will scale the value to 1. Stacks' implementation is taken from Meirmans. (2006) Using the AMOVA framwwork to estimate a standardized genetic differentiation measure. **Evolution**.
10	Smoothed F_{ST}'	
11	Smoothed F_{ST}' P-value	
12	D_{EST}	D_{EST} is an orthogonal measure of locus differentiation, using an entirely different menthod than F_{ST} calculations. Stacks implementation is found in Jost. (2008) GST and its relatives do not measure differentiation, **Molecular Ecology**.
13	Smoothed D_{EST}	
14	Smoothed D_{EST} P-value	

6.4.6 populations.phistats.tsv: Haplotype-based F_{ST} calculations for all populations

Haplotype-based AMOVA statistics can be calculated at several different hierarchical levels. While Stacks calculates haplotype-based F-statistics per pair of populations, it also calculates the share of variance attributable to all populations considered together.

Column	Name	Description
1	Locus ID	Catalog locus identifier.
2	Chromosome	If aligned to a reference genome.
3	Basepair	If aligned to a reference genome.
4	PopCnt	The number of populations included in this calculation. Some loci will be absent in some populations so the calculation is adjusted when populations drop out at a locus.
5	Φ_{ST}	Φ_{ST} is an AMOVA-based measure of F_{ST} intended for haplotpye analysis. Stacks' implementation is based on Excoffier, et al. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. **Genetics**.
6	Smoothed Φ_{ST}	
7	Smoothed	

	Φ_{ST} P-value	
8	Φ_{CT}	
9	Smoothed Φ_{CT}	
10	Smoothed Φ_{CT} P-value	
11	Φ_{SC}	
12	Smoothed Φ_{SC}	
13	Smoothed Φ_{SC} P-value	
14	D_{EST}	D_{EST} is an orthogonal measure of locus differentiation, using an entirely different menthod than F_{ST} calculations. Stacks implementation is found in Jost. (2008) GST and its relatives do not measure differentiation, **Molecular Ecology**.
15	Smoothed D_{EST}	
16	Smoothed D_{EST} P-value	

6.4.7 Per-locus consensus sequence: populations.loci.fa

The per-locus consensus FASTA output, generated by the **`populations`** program by specifying the `--fasta_loci` option, will write one consensus sequence (majority rules allele for each variant position) per RAD locus. The "CLocus_X" refers to catalog locus X, also known as the population-wide locus ID, in the data set.

```
>CLocus_1
TGCAGGGTACACAGTCTGCAGAGGACCACCACTGTGTCCCTAAAGGACCGTGGTTTGAAAACGTTCTGGCTAAATGTGGAGAGATTGTTGGCACT
>CLocus_2
TGCAGGTGTTCTCATAATCAGAGTTAGATTTCAGCTTAAAGTGTTACTTAAACACTATTTATCAGAACTTTACTGTAACTAAAAGCTGCAGGTGA
>CLocus_3
TGCAGGTACACCCATCAGACACGTGGAGCACATCACATCCATTTATATTAGTATAAAAAAAGCTTTAAAGAAATCTTTGTGGTTTTAATTTTGTTT
>CLocus_4
TGCAGGACTTGTTCGCCTCCAGGACTCTGAGGAGGGAAGGAGAGATCGTCGCCGACCCCTCTCACCATCAGGACTAAGACCTCCCGCCACACGAA
>CLocus_5
TGCAGGAAAACAAACAAACAAACAACAAAAACGGGAGTTGAAAAACAGAAAACAAACAGCAGCTGAACTTGATCATTTTATGATGCAAGATGATCAAAT
```

6.4.8 Per-locus, per-haplotype sequences: populations.samples.fa

The per-locus, per-haplotpye FASTA output from the **`populations`** program, generated by specifying the `--fasta_samples` option, provides the full sequence from the RAD locus, for each haplotype, from every sample in the population, for each catalog locus. This output is identical to the `populations.haplotypes.tsv` output, except it provides the variable sites embedded within the full consensus seqeunce for the locus. The output looks like this:

```
>CLocus_10056_Sample_934_Locus_12529_Allele_0 [sample_934; groupI, 49712]
TGCAGGCCCCAGGCCACGCCGTCTGCGGCAGCGCTGGAAGGAGGCGGTGGAGGAGGCGGCCAACGGCTCCCTGCCCCAGAAGGCCGAGTTCACCG
>CLocus_10056_Sample_934_Locus_12529_Allele_1 [sample_934; groupI, 49712]
TGCAGGCCCCAGGCCACGCCGTCTGCGGCAGCGTTGGAAGGAGGCGGTGGAGGAGGCGGCCAACGGCTCCCTGCCCCAGAAGGCCGAGTTCACCG
>CLocus_10056_Sample_935_Locus_13271_Allele_0 [sample_935; groupI, 49712]
TGCAGGCCCCAGGCCACGCCGTCTGCGGCAGCGCTGGAAGGAGGCGGTGGAGGAGGCGGCCAACGGCTCCCTGCCCCAGAAGGCCGAGTTCACCG
>CLocus_10056_Sample_935_Locus_13271_Allele_1 [sample_935; groupI, 49712]
TGCAGGCCCCAGGCCACGCCGTCTGCGGCAGCGTTGGAAGGAGGCGGTGGAGGAGGCGGCCAACGGCTCCCTGCCCCAGAAGGCCGAGTTCACCG
>CLocus_10056_Sample_936_Locus_12636_Allele_0 [sample_936; groupI, 49712]
TGCAGGCCCCAGGCCACGCCGTCTGCGGCAGCGCTGGAAGGAGGCGGTGGAGGAGGCGGCCAACGGCTCCCTGCCCCAGAAGGCCGAGTTCACCG
```

The first number in red is the catalog locus, or the population-wide ID for this locus. The second number in green is the sample ID, of the individual sample that this locus originated from. Each sample you input into the pipeline is assigned a numeric ID by either the **`ustacks`** or **`gstacks`** programs. This ID is embedded in all the internal Stacks files and is

used internally by the pipeline to track the sample. The third number, in blue, is the locus ID for this locus within the individual. The fourth number is the allele or haplotype number, in purple, the sample name is shown in brackets, and if this data was aligned to a reference genome, the alignment position is provdied as a FASTA comment.

6.4.9 Per-locus, raw sequences: populations.samples-raw.fa

The full sequence from each RAD locus, regardless of whether the number of alleles is biologically plausible, can be generated by the **populations** program by specifying the `--fasta_samples_raw` option. This raw output can contain multiple haplotypes per individual, some of which may be weakly supported and will have been filtered from the other outputs from the **populations** program.

```
>CLocus_5_Sample_28_Locus_5_Allele_0 [rb_2217.014]
TGCAGGAAAACAAACAAACAAACAACAAAAACGGGAGTTGAAAAACAGAAAACAAACAGCAGCTGAACTTGATCATTTTATGATGCAAGATGATCAAAT
>CLocus_5_Sample_28_Locus_5_Allele_1 [rb_2217.014]
TGCAGGAAAACAAACAAACAAACAACAAGAACGGGAGTTGAAAAACAGAAAACAAACAGCAGCTGAACTTGATCATTTTATGATGCAAGATGATCAAAT
>CLocus_5_Sample_28_Locus_5_Allele_3 [rb_2217.014]
TGCAGGAAAACAAACAAACAAACAACAAGAACGGGAGTTGAAAAACAGAAAACAAACAGCAGCTGAACTTGATCATTTTATGATGCAAGATTATCAAAT
```