

Supplementary Information

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1 Introduction

In this experiment, we used quaddRAD library prep to prepare the sample DNA. This means that there were both two unique outer barcodes (typical Illumina barcodes) *AND* two unique inner barcodes (random barcode bases inside the adapters) for each sample - over 1700 to be exact!

The sequencing facility demultiplexes samples based on the outer barcodes (typically called 5nn and i7nn). Once this is done, each file still contains a mix of the inner barcodes. We will refer to these as “sublibraries”

because they are sort of halfway demultiplexed. We separate them out bioinformatically later.

1.1 Raw Data File Naming - Sublibraries

Here's a bit of information on the file name convention. The typical raw file looks like this:

`AMH_macro_1_1_12px_S1_L001_R1_001.fastq.gz`

- These are author initials and “macro” stands for “Macrosystems”. These are on every file.

`AMH_macro`

- The first number is the *i5nn* barcode for the given sublibrary. We know all these samples have a *i5nn* barcode “1”, so that narrows down what they can be. The second number is the *i7nn* barcode for the given sublibrary. We know all these samples have a *i7nn* barcode “1”, so that further narrows down what they can be.

`1_1`

- This refers to how many samples are in the sublibrary. “12px” means 12-plexed, or 12 samples. In other words, we will use the inner barcodes to further distinguish 12 unique samples in this sublibrary.

`12px`

- This is a unique sublibrary name. `S1` = 1 *i5nn* and 1 *i7nn*.

`S1`

- This means this particular file came from lane 1 of the NovaSeq. There are four lanes. All samples should appear across all four lanes.

`L001`

- This is the first (R1) of two paired-end reads (R1 and R2).

`R1`

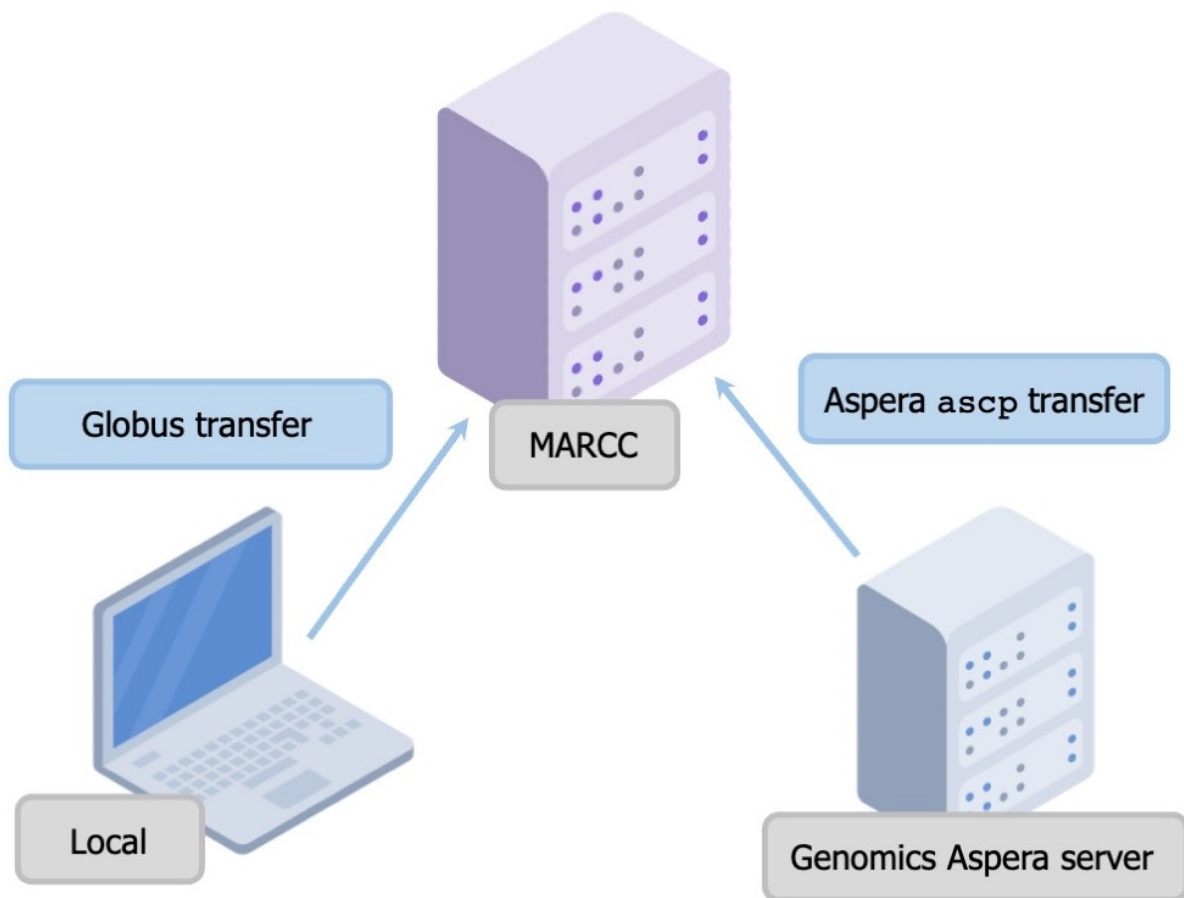
- The last part doesn't mean anything - it was just added automatically before the file suffix (`fastq.gz`)

`001.fastq.gz`

1.2 A Note on File Transfers

There are three main systems at play for file transfer: the local machine, the sequencing facility's (GRCF) Aspera server, and [MARCC](#). The Aspera server is where the data were/are stored immediately after sequencing. MARCC is where we plan to do preprocessing and analysis. Scripts and text files are easy for me to edit on my local machine. We used [Globus](#) to transfer these small files from my local machine to MARCC.

Midway through this analyses, we transitioned to another cluster, JHU's Rockfish. Scripts below, with the exception of file transfer from the Aspera server, should reflect the new filesystem, though you will have to adjust the file paths accordingly.



2 Preprocessing

2.1 Step 1 - Transfer Files

Files can be found in the `01_transfer_files/` directory.

This directory contains files named in this convention: `01-aspera_transfer_n.txt`. These are text files containing the *names* of `fastq.gz` files that we wanted to transfer from the sequencing facility's Aspera server to the computing cluster (MARCC). This was to maximize ease of transferring only certain files over at once, since transferring could take a long time. We definitely did this piecemeal. Possible file names shown in [Aspera Transfer File Names](#). There are multiple of these files so that we could parallelize (replace `n` with the correct number in the command used below). This text file will need to be uploaded to your scratch directory in MARCC.

Files were then transferred using the following commands. Before starting, make sure you are in a data transfer node. Then, load the aspera module. Alternatively, you can install the Aspera transfer software and use that.

```
module load aspera
```

Initiate the transfer from within your scratch directory:

```
ascp -T -l8G -i /software/apps/aspera/3.9.1/etc/asperaweb_id_dsa.openssh  
--file-list=01-aspera_transfer_n.txt  
--mode=recv --user=<aspera-user> --host=<aspera-IP> /scratch/users/<me>@jhu.edu
```

2.2 Step 2 - Concatenate Files and Install Stacks

Files can be found in the 02_concatenate_and_check/ directory.

2.2.1 Step 2a - Concatenate Files for each Sublibrary

We ran my samples across the whole flow cell of the NovaSeq, so results came in 8 files for each demultiplexed sublibrary (4 lanes * paired reads). For example, for sublibrary 1_1, we'd see the following 8 files:

```
AMH_macro_1_1_12px_S1_L001_R1_001.fastq.gz
AMH_macro_1_1_12px_S1_L001_R2_001.fastq.gz
AMH_macro_1_1_12px_S1_L002_R1_001.fastq.gz
AMH_macro_1_1_12px_S1_L002_R2_001.fastq.gz
AMH_macro_1_1_12px_S1_L003_R1_001.fastq.gz
AMH_macro_1_1_12px_S1_L003_R2_001.fastq.gz
AMH_macro_1_1_12px_S1_L004_R1_001.fastq.gz
AMH_macro_1_1_12px_S1_L004_R2_001.fastq.gz
```

The 02_concatenate_and_check/02-concat_files_across4lanes.sh script finds all files in the working directory with the name pattern *_L001_*.fastq.gz and then concatenates across lanes 001, 002, 003, and 004 so they can be managed further. The "L001" part of the filename is then eliminated. For example the 8 files above would become:

```
AMH_macro_1_1_12px_S1_R1.fastq.gz
AMH_macro_1_1_12px_S1_R2.fastq.gz
```

Rockfish uses [slurm](#) to manage jobs. To run the script, use the `sbatch` command. For example:

```
sbatch ~/code/02-concat_files_across4lanes.sh
```

This command will run the script from within the current directory, but will look for and pull the script from the code directory. This will concatenate all files within the current directory that match the loop pattern.

2.2.2 Step 2b - Download and Install Stacks

On Rockfish, [Stacks](#) will need to be downloaded to each user's code directory. Stacks, and software in general, should be compiled in an interactive mode or loaded via module. For more information on interactive mode, see `interact --usage`.

```
interact -p debug -g 1 -n 1 -c 1
module load gcc
```

Now download Stacks. We used version 2.60.

```
wget http://catchenlab.life.illinois.edu/stacks/source/stacks-2.60.tar.gz
tar xfvz stacks-2.60.tar.gz
```

Next, go into the stacks-2.60 directory and run the following commands:

```
./configure --prefix=/home/<your_username>/code4-<PI_username>
make
make install
export PATH=$PATH:/home/<your_username>/code4-<PI_username>/stacks-2.60
```

The filesystem patterns on your cluster might be different, and you should change these file paths accordingly.

2.3 Step 3 - Remove PCR Clones

Files can be found in the 03_clone_filter/ directory.

2.3.1 Step 3a - Run PCR Clone Removal Script

The `03-clone_filter.sh` script runs `clone_filter` from [Stacks](#). The program was run with options `--inline_inline --oligo_len_1 4 --oligo_len_2 4`. The `--oligo_len_x 4` options indicate the 4-base pair degenerate sequence was included on the outside of the barcodes for detecting PCR duplicates. The script uses the file name prefixes listed for each single sub-pooled library in `03-clone_filter_file_names.txt` and loops to run `clone_filter` on all of them. Possible file names shown in [clone_filter File Names](#).

2.3.2 Step 3b - Parse PCR Clone Removal Results

If you want to extract descriptive statistics from the `clone_filter` output, you can use the `03.5-parse_clone_filter.py` script to do so. It can be run on your local terminal after transferring the `clone_filter.out` logs to your local computer.

```
source("03_clone_filter/examine_clones.R")
make_cloneplot()
```

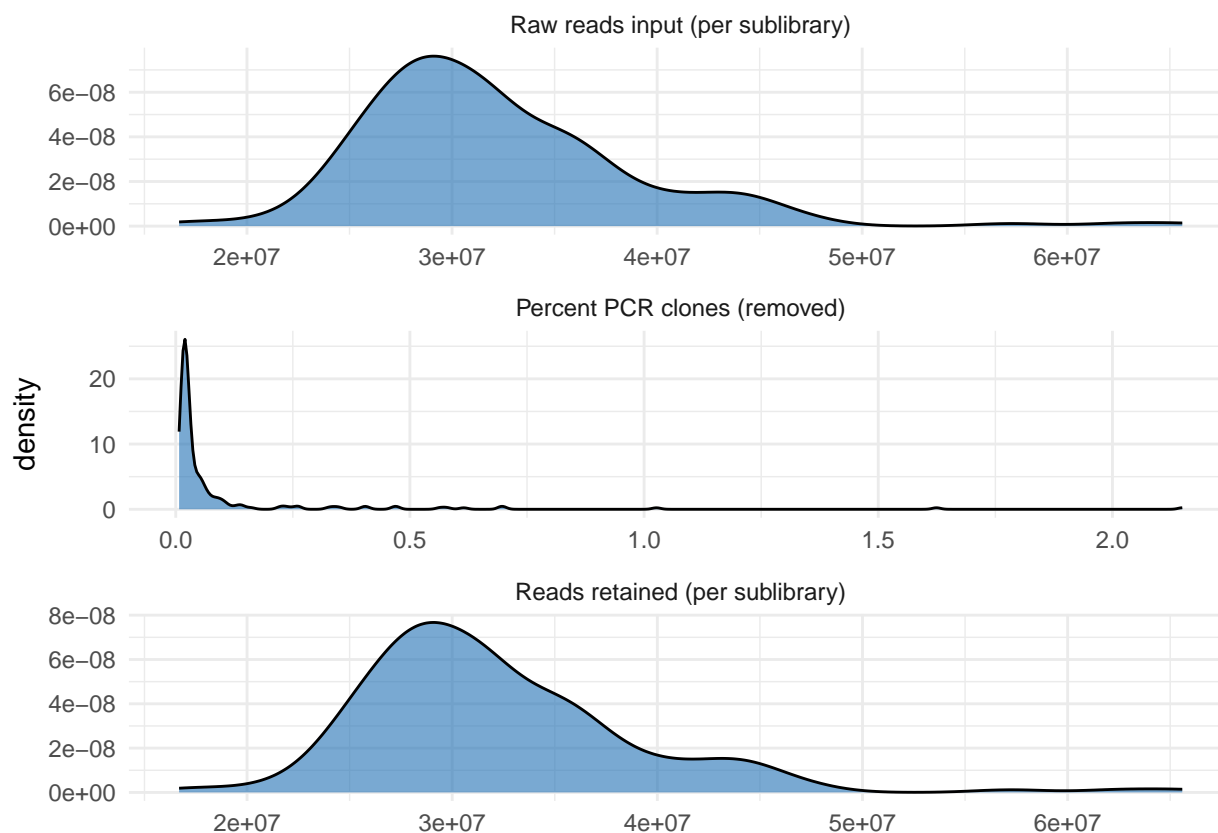


Figure S1: PCR clone removal statistics

2.4 Step 4 - Demultiplexing and Sample Filtering

Files can be found in the `04_demux_filter/` directory.

2.4.1 Step 4a - Demultiplex and Filter

The `04-process_radtags.sh` script runs `process_radtags` from [Stacks](#). The program was run with options `-c -q --inline_inline --renz_1 pstI --renz_2 mspI --rescue --disable_rad_check`. The script uses the same file prefixes as [Step 3 - 03-clone_filter.sh](#). Each sub-pooled library has a forward

and reverse read file that was filtered in the previous step. Like the [above section](#), the script uses the file name prefixes listed for each single sub-pooled library in `04-process_radtags_file_names.txt` and loops to run `process_radtags` on all of them. Possible file names shown in [clone_filter File Names](#).

Each sub-pooled library also has a demultiplexing file (`04-demux/` directory) that contains the sample names and inner(i5 and i7) barcodes. For example, the sublibrary 1_1, we'd see the following barcode file:

```
ATCACG AGTCAA DS.BA.PIK.U.1
CGATGT AGTTCC DS.BA.PIK.U.2
TTAGGC ATGTCA DS.BA.PIK.U.3
TGACCA CCGTCC DS.BA.PIK.U.4
ACAGTG GTCCGC DS.BA.PIK.U.5
GCCAAT GTGAAA DS.BA.DHI.U.1
CAGATC GTGGCC DS.BA.DHI.U.2
ACTTGA GTTTCG DS.BA.DHI.U.3
GATCAG CGTACG DS.BA.DHI.U.4
TAGCTT GAGTGG DS.BA.DHI.U.5
GGCTAC ACTGAT DS.BA.GA.U.1
CTTGTA ATTCCT DS.BA.GA.U.2
```

The 'process_radtags' command will demultiplex the data by separating out each sublibrary into the individual samples. It will then clean the data, and will remove low quality reads and discard reads where a barcode was not found.

2.4.2 Step 4b - Organize files

In a new directory, make sure the files are organized by species. In the `process_radtags` script, we specified that files be sent to `~/scratch/demux/*sublibrary_name*` (reasoning for this is in [Step 4c](#)), but files should manually be organized into species folders (i.e., `~/scratch/demux/*SPP*`) after `process_radtags` is performed. For example, the file "DS.MN.L01-DS.M.1.1.fq.gz" should be sent to the `~/scratch/demux/DS` directory.

Note: this is not automated at this point but it would be nice to automate the file moving process so it's not forgotten at this point.

2.4.3 Step 4c - Assess the raw, processed, and cleaned data

In the script for [Step 4](#), we have specified that a new output folder be created for each sublibrary. The output folder is where all sample files and the log file will be dumped for each sublibrary. It is important to specify a different output folder if you have multiple sublibraries because we will be assessing the output log for each sublibrary individually (and otherwise, the log is overwritten when the script loops to a new sublibrary).

The utility `stacks-dist-extract` can be used to extract data from the log file. First, we examined the library-wide statistics to identify sublibraries where barcodes may have been misentered or where sequencing error may have occurred. We used:

```
stacks-dist-extract process_radtags.log total_raw_read_counts
```

to pull out data on the total number of sequences, the number of low-quality reads, whether barcodes were found or not, and the total number of retained reads per sublibrary. Look over these to make sure there are no outliers or sublibraries that need to be checked and rerun.

Next, we used:

```
stacks-dist-extract process_radtags.log per_barcode_raw_read_counts
```

to analyze how well each sample performed. There are three important statistics to consider for each sample.

1. *The proportion of reads per sample for each sublibrary* indicates the proportion that each individual was processed and sequenced within the overall library. This is important to consider as cases where a single sample dominates the sublibrary may indicate contamination.
2. *The number of reads retained for each sample* can be an indicator of coverage. It is most likely a good idea to remove samples with a very low number of reads. Where you decide to place the cutoff for low coverage samples is dependent on your dataset. For example, a threshold of 1 million reads is often used but this is not universal.
3. *The proportion of reads retained for each sample* can also indicate low-quality samples and will give an idea of the variation in coverage across samples.

Output for sublibraries for this step are summarized in [process_radtags-library_output.csv](#).

Output for individual samples for this step are summarized in [process_radtags-sample_output.csv](#).

The script `04c-process_radtags_stats.R` was used to create many plots for easily assessing each statistic. Output from this step can be found in `figures/process_radtags/` where figures are organized by species.

```
source("04_demux_filter/04c-radtags_filter_summary.R")
make_filterplot()
```

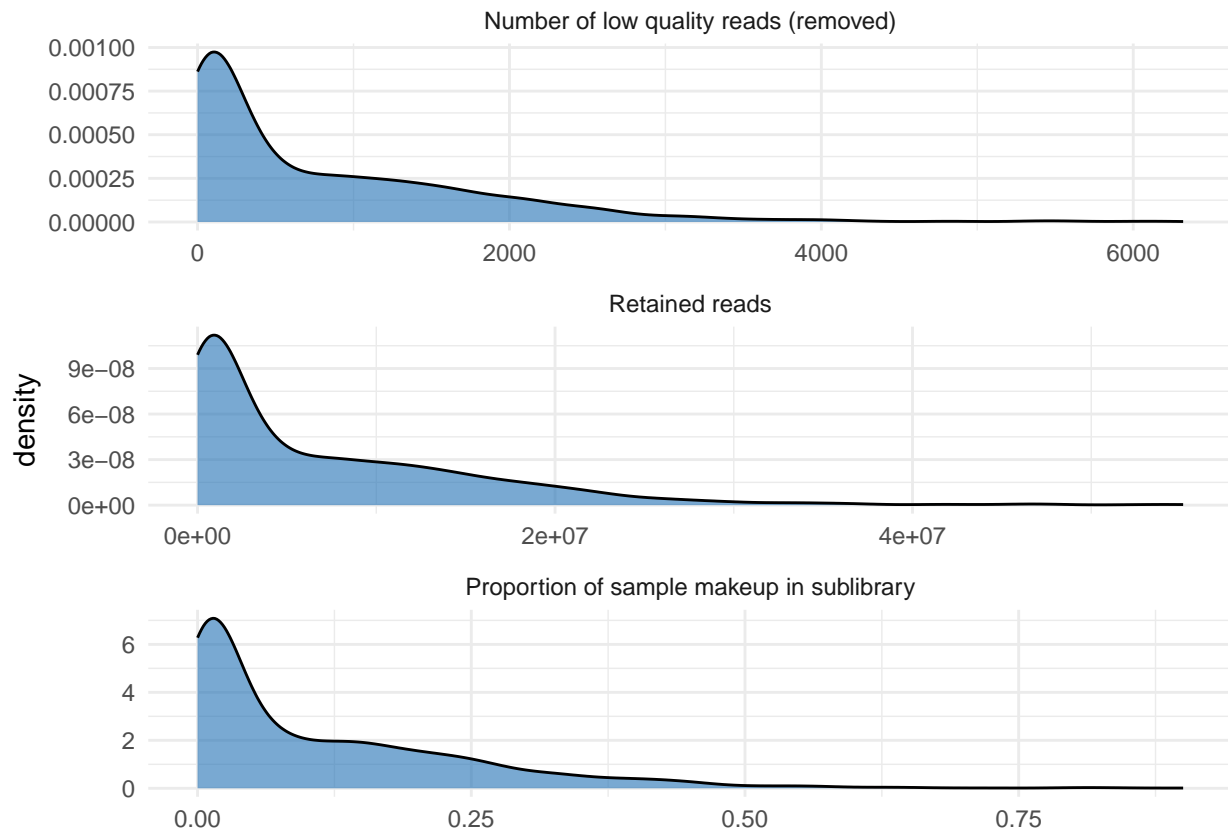


Figure S2: RAD tag processing statistics

2.4.4 Step 4d - Identify low-coverage and low-quality samples from downstream analysis

Using the same output log and the above statistics, we removed low-coverage and low-quality samples that may skew downstream analyses.

Samples were identified and removed via the following procedure:

1. First, samples that represented less than **1% of the sequenced sublibrary** were identified and removed. These samples correlate to low-read and low-coverage samples.
2. Next, a threshold of **1 million retained reads per sample** was used to remove any remaining low-read samples. Low-read samples correlate to low coverage and will lack enough raw reads to contribute to downstream analyses.

Good/kept samples are summarized in [process_radtags-kept_samples.csv](#).

Discarded samples are summarized in [process_radtags-discarded_samples.csv](#).

```
source("04_demux_filter/04c-radtags_filter_summary.R")
make_manual_discard_plot()
```

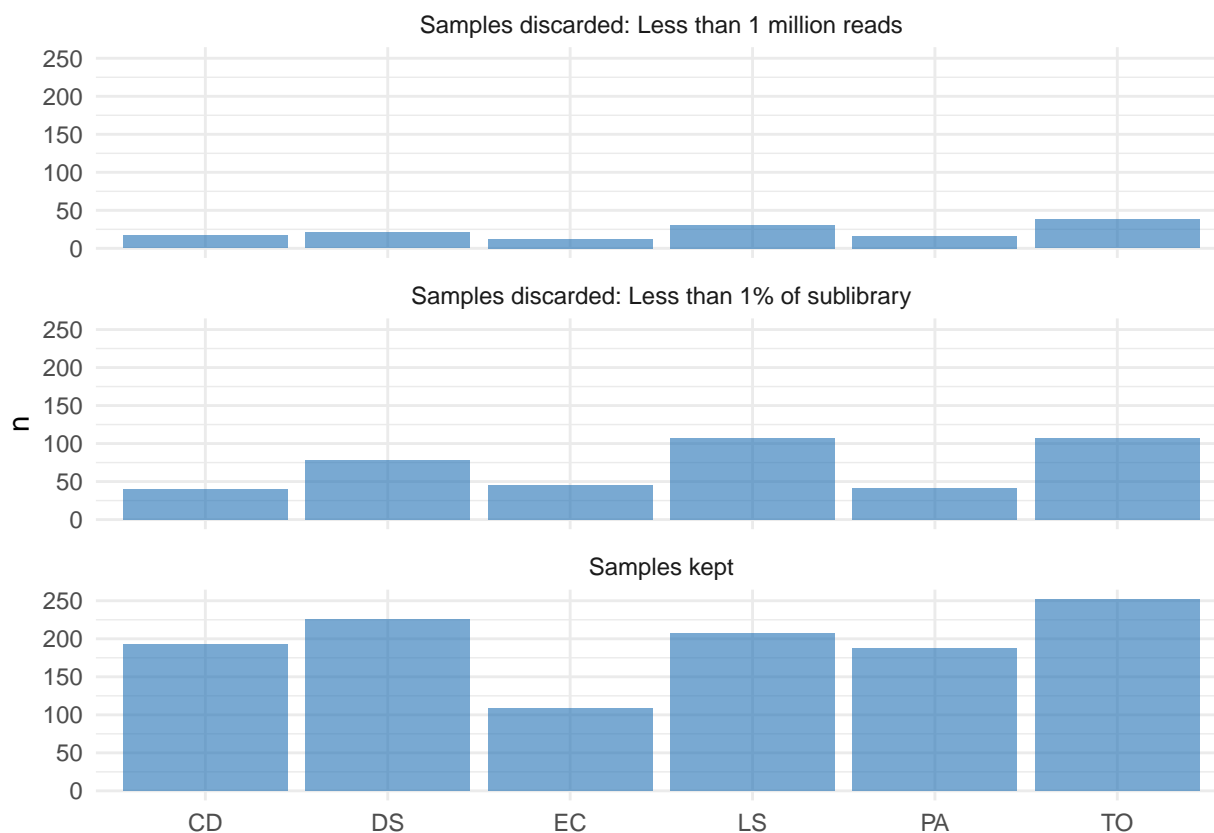


Figure S3: RAD tag manual filtering summary

Note: At this point, we started using Stacks 2.62 for its multi-threading capabilities. Functionality of the previous steps should be the same, however.

3 Generating Stacks Catalogs and Calling SNPs

3.1 Step 5 - Metapopulation Catalog Building and Parameter Search

Files can be found in the `05_ustacks_and_params/` directory.

Going forward, when we use the term **metapopulation**, we are referring to the collection of all samples within species among all cities where the species was present.

It is important to conduct preliminary analyses that will identify an optimal set of parameters for the dataset (see [Step 5a](#)). Following the parameter optimization, the program `ustacks` can be run to generate a catalog of loci.

3.1.1 Step 5a - Run `denovo_map.sh`

Stack assembly will differ based on several different aspects of the dataset (such as the study species, the RAD-seq method used, and/or the quality and quantity of DNA used). So it is important to use parameters that will maximize the amount of biological data obtained from stacks.

There are three main parameters to consider when doing this:

1. m = controls the minimum number of raw reads required to form a stack (implemented in `ustacks`)
2. M = controls the number of mismatches between stacks to merge them into a putative locus (implemented in `ustacks`)
3. n = controls the number of mismatches allowed between stacks to merge into the catalog (implemented in `cstacks`)

There are two main ways to optimize parameterization:

1. an iterative method where you sequentially change each parameter while keeping the other parameters fixed (described in *Paris et al. 2017*), or
2. an iterative method where you sequentially change the values of M and n (keeping $M = n$) while fixing $m = 3$, and then test $m = 2, 4$ once the optimal $M = n$ is determined (described in *Rochette and Catchen 2017, Catchen 2020*).

We performed the second method and used the `denovo_map.sh` script to run the `denovo_map.pl` command to perform iterations. This script requires that we first choose a subset of samples to run the iterations on. The samples should be representative of the overall dataset; meaning they should include all populations and have similar read coverage numbers. Read coverage numbers can be assessed by looking at the descriptive statistics produced from [Step 4c](#).

Place these samples in a text file (`popmap_test_samples.txt`) with the name of the sample and specify that all samples belong to the same population. For example, `popmap_test_samples.txt` should look like...

```
DS.BA.GA.U.1    A
DS.PX.BUF.M.5    A
DS.BO.HC4.M.1    A
...
```

It is important to have all representative samples treated as one population because you will assess outputs found across 80% of the individuals. The script will read this text file from the `--popmap` argument.

The script also requires that you specify an output directory after `-o`. This should be unique to the parameter you are testing... for example, if you are testing $M = 3$, then you could make a subdirectory labeled `stacks.M3` where all outputs from `denovo_map.sh` will be placed. Otherwise, for each iteration, the outputs will be overwritten and you will lose the log from the previous iteration. The `denovo_map.sh` script also requires that you direct it toward where your samples are stored, which is your directory built in [Step 4b](#). Make sure to run the `--min-samples-per-pop 0.80` argument.

To decide which parameters to use, examine the following from each iteration:

1. the average sample coverage: This is obtained from the summary log in the `ustacks` section of `denovo_map.log`. If samples have a coverage $< 10\times$, you will have to rethink the parameters you use here.
2. the number of assembled loci shared by 80% of samples: This can be found in the `haplotypes.tsv` by counting the number of loci: `cat populations.haplotypes.tsv | grep -v ^"# " | wc -l`

3. the number of polymorphic loci shared by 80% of samples: This can be found in `populations.sumstats.tsv` or by counting `populations.hapstats.tsv`: `cat populations.hapstats.tsv | grep -v "^#" | wc -l`
4. the number of SNPs per locus shared by 80% of samples: found in `denovo_map.log` or by counting the number of SNPs in `populations.sumstats.tsv`: `populations.sumstats.tsv | grep -v "^#" | wc -l`

The script `05a-param_opt-figures_script.R` was used to create plots for assessing the change in shared loci across parameter iterations.

Based on this optimization step, we used the following parameters:

Table S1: Final parameter optimization values for the Stacks pipeline.

Species	M (locus mismatches)	n (catalog mismatches)	m (minimum reads)
CD	8	8	3
DS	10	10	3
EC	8	8	3
LS	7	7	3
PA	5	5	3
TO	6	6	3

3.1.2 Step 5b - Run ustacks

`ustacks` builds *de novo* loci in each individual sample. We have designed the `ustacks` script so that the process requires three files:

- `05-ustacks_n.sh` : the shell script that executes `ustacks`
- `05-ustacks_id_n.txt` : the sample ID number
- `05-ustacks_samples_n.txt` : the sample names that correspond to the sample IDs

The sample ID should be derived from the `order_id` column (first column) on the master spreadsheet. It is unique (1-1736) across all of the samples.

The sample name is the corresponding name for each sample ID in the spreadsheet. E.g., sample ID “9” corresponds to sample name “DS.BA.DHI.U.4”. Sample naming convention is species.city.site.management_type.replicate_plant.

`05-ustacks_n.sh` should have an `out_directory` (`-o` option) that will be used for all samples (e.g., `stacks/ustacks`). Files can be processed piecemeal into this directory. There should be three files for every sample in the output directory:

- `<samplename>.alleles.tsv.gz`
- `<samplename>.snps.tsv.gz`
- `<samplename>.tags.tsv.gz`

Multiple versions of the `05-ustacks_n.sh` script can be run in parallel (simply replace `n` in the three files above with the correct number).

A small number of samples (13) were discarded at this stage as the `ustacks` tool was unable to form any primary stacks corresponding to loci. See [output/ustacks-discarded_samples.csv](#).

3.1.3 Step 5c - Correct File Names

This step contains a script `05b-fix_filenames.sh` which uses some simple regex to fix filenames that are output in previous steps. Stacks adds an extra “1” at some point at the end of the sample name which is not meaningful. The following files:

- DS.MN.L02-DS.M.3.1.alleles.tsv.gz
- DS.MN.L03-DS.U.2.1.tags.tsv.gz
- DS.MN.L09-DS.U.1.1.snps.tsv.gz

become:

- DS.MN.L02-DS.M.3.alleles.tsv.gz
- DS.MN.L03-DS.U.2.tags.tsv.gz
- DS.MN.L09-DS.U.1.snps.tsv.gz

The script currently gives some strange log output, so it can probably be optimized/improved. The script should be run from the directory where the changes need to be made. Files that have already been fixed will not be changed.

3.1.4 Step 5d - Choose catalog samples/files

In the next step, we will choose the files we want to go into the catalog. This involves a few steps:

1. Create a meaningful directory name. This could be the date (e.g., `stacks_22_01_25`).
2. Copy the `ustacks` output for all of the files you want to use in the reference from Step 5b. Remember this includes three files per sample. So if you have 20 samples you want to include in the reference catalog, you will transfer $3 \times 20 = 60$ files into the meaningful directory name. The three files per sample should follow this convention:
 - `<samplename>.alleles.tsv.gz`
 - `<samplename>.snps.tsv.gz`
 - `<samplename>.tags.tsv.gz`
3. Remember the meaningful directory name. You will need it in Step 6.

3.2 Step 6 - Metapopulation catalog with `cstacks`

Files can be found in the `06_cstacks/` directory.

`cstacks` builds the locus catalog from all the samples specified. The accompanying script, `cstacks_SPECIES.sh` is relatively simple since it points to the directory containing all the sample files. It follows this format to point to that directory:

```
cstacks -P ~/directory ...
```

Make sure that you use the meaningful directory from Step 5c and that you have copied all the relevant files over. Otherwise this causes [problems downstream](#). For example, you might edit the code to point to `~/scratch/stacks/stacks_22_01_25`.

```
cstacks -P ~/scratch/stacks/stacks_22_01_25 ...
```

The tricky thing is ensuring enough compute memory to run the entire process successfully. There is probably space to optimize this process.

The `cstacks` method uses a “population map” file, which in this project is `cstacks_popmap_SPECIES.txt`. This file specifies which samples to build the catalog from and categorizes them into your ‘populations’, or in this case, cities using two tab-delimited columns, e.g.:

```
DS.BA.GA.U.1    Baltimore
DS.BA.GA.U.2    Baltimore
DS.BA.GA.U.3    Baltimore
DS.BA.GA.U.4    Baltimore
DS.BA.GA.U.5    Baltimore
...
```

Make sure the samples in this file correspond to the input files located in e.g., `~/scratch/stacks/stacks_22_01_25`.

`cstacks` builds three files for use in all your samples (in this pipeline run), mirroring the sample files output by `ustacks`:

- `catalog.alleles.tsv.gz`
- `catalog.snps.tsv.gz`
- `catalog.tags.tsv.gz`

Table S2: Subset of samples used in SNP catalog creation.

Sample	Species	City
DS.BA.PIK.U.1	DS	BA
DS.BA.GA.U.4	DS	BA
DS.BA.LH-1.M.4	DS	BA
DS.BA.LH-3.M.1	DS	BA
DS.BA.WB.U.2	DS	BA
DS.BA.LL-4.M.5	DS	BA
DS.BA.LH-2.M.5	DS	BA
DS.BA.TRC.U.3	DS	BA
DS.BA.W3.M.2	DS	BA
DS.BA.RG-1.M.1	DS	BA
DS.BA.LL-3.M.3	DS	BA
DS.BA.RG-2.M.4	DS	BA
DS.BO.HC1.M.3	DS	BO
DS.BO.HC4.M.5	DS	BO
DS.BO.LC1.M.3	DS	BO
DS.BO.LC2.M.2	DS	BO
DS.BO.LC3.M.5	DS	BO
DS.BO.WL1.M.2	DS	BO
DS.BO.WL2.M.1	DS	BO
DS.BO.WL3.M.5	DS	BO
DS.BO.I4.U.1	DS	BO
DS.BO.R1.U.4	DS	BO
DS.BO.R2.U.2	DS	BO
DS.BO.R4.U.4	DS	BO
DS.MN.L05-DS.M.3	DS	MN
DS.MN.L09-DS.M.3	DS	MN
DS.MN.L11-DS.M.1	DS	MN
DS.MN.L02-DS.U.1	DS	MN
DS.MN.L02-DS.M.4	DS	MN
DS.MN.L03-DS.U.3	DS	MN
DS.MN.L04-DS.U.5	DS	MN
DS.MN.L06-DS.U.3	DS	MN
DS.MN.L07-DS.U.3	DS	MN
DS.MN.L09-DS.U.3	DS	MN
DS.MN.L11-DS.U.1	DS	MN
DS.MN.L11-DS.U.5	DS	MN
DS.PX.BUF.M.1	DS	PX
DS.PX.PIE.M.2	DS	PX
DS.PX.ALA.M.1	DS	PX
DS.PX.MTN.M.6	DS	PX
DS.PX.LAP.M.3	DS	PX
DS.PX.NUE.M.4	DS	PX
DS.PX.WES.M.2	DS	PX

Sample	Species	City
DS.PX.DF1.M.1	DS	PX
DS.PX.ENC.M.1	DS	PX
DS.PX.DOW.M.1	DS	PX
DS.PX.DOW.M.4	DS	PX
DS.PX.DF2.M.3	DS	PX
CD.BA.LA.U.2	CD	BA
CD.BA.TRC.U.3	CD	BA
CD.BA.WGP.M.2	CD	BA
CD.BA.LH-2.M.2	CD	BA
CD.BA.LL-4.M.1	CD	BA
CD.BA.PIK.U.2	CD	BA
CD.BA.WB.U.2	CD	BA
CD.BA.CP.U.4	CD	BA
CD.BA.FH.U.1	CD	BA
CD.BA.PSP.M.4	CD	BA
CD.BA.AA.U.4	CD	BA
CD.BA.RG-1.M.2	CD	BA
CD.BA.W3.M.3	CD	BA
CD.BA.GA.U.3	CD	BA
CD.BA.WBO.U.5	CD	BA
CD.LA.WHI.M.3	CD	LA
CD.LA.SEP.M.3	CD	LA
CD.LA.SEP.M.4	CD	LA
CD.LA.ROS.M.5	CD	LA
CD.LA.MR2.M.2	CD	LA
CD.LA.ALL.M.2	CD	LA
CD.LA.ALL.M.5	CD	LA
CD.LA.VAL.M.5	CD	LA
CD.LA.HAR.M.4	CD	LA
CD.LA.LUB.M.3	CD	LA
CD.LA.GLO.M.4	CD	LA
CD.LA.ZOO.M.3	CD	LA
CD.LA.NWH.M.5	CD	LA
CD.LA.KIN.M.3	CD	LA
CD.LA.KIN.M.5	CD	LA
CD.PX.CAM.U.5	CD	PX
CD.PX.MON.U.5	CD	PX
CD.PX.PKW.U.5	CD	PX
CD.PX.LAP.M.4	CD	PX
CD.PX.NES.U.4	CD	PX
CD.PX.PAL.M.3	CD	PX
CD.PX.ASU.M.1	CD	PX
CD.PX.NUE.M.5	CD	PX
CD.PX.WES.M.3	CD	PX
CD.PX.MAN.M.4	CD	PX
CD.PX.CLA.M.3	CD	PX
CD.PX.DF1.M.5	CD	PX
CD.PX.COY.M.5	CD	PX
CD.PX.RPC.M.3	CD	PX
CD.PX.ENC.M.2	CD	PX
EC.BA.LH-2.M.2	EC	BA
EC.BA.WBO.U.4	EC	BA

Sample	Species	City
EC.BA.WB.U.5	EC	BA
EC.BA.FH.U.3	EC	BA
EC.BA.CP.U.2	EC	BA
EC.BA.TRC.U.3	EC	BA
EC.BA.LL-4.M.4	EC	BA
EC.BA.WB.U.1	EC	BA
EC.BA.PIK.U.5	EC	BA
EC.BA.PSP.M.4	EC	BA
EC.BA.GA.U.2	EC	BA
EC.BA.LL-3.M.3	EC	BA
EC.BA.ML.U.1	EC	BA
EC.BA.TRC.U.5	EC	BA
EC.BA.ML.U.3	EC	BA
EC.LA.SGB.U.2	EC	LA
EC.LA.SGB.U.5	EC	LA
EC.LA.DUR.U.2	EC	LA
EC.LA.HOW.U.2	EC	LA
EC.LA.SAN.U.2	EC	LA
EC.LA.VER.U.1	EC	LA
EC.LA.VER.U.4	EC	LA
EC.LA.VB2.U.4	EC	LA
EC.LA.AC2.U.2	EC	LA
EC.LA.AC1.U.1	EC	LA
EC.LA.VB1.U.1	EC	LA
EC.LA.VB1.U.3	EC	LA
EC.LA.SGR.U.4	EC	LA
EC.LA.SGR.U.5	EC	LA
EC.LA.HOW.U.3	EC	LA
EC.PX.BUF.M.1	EC	PX
EC.PX.BUF.M.3	EC	PX
EC.PX.ALA.M.3	EC	PX
EC.PX.MTN.M.2	EC	PX
EC.PX.WES.M.1	EC	PX
EC.PX.WES.M.2	EC	PX
EC.PX.MAN.M.1	EC	PX
EC.PX.CLA.M.1	EC	PX
EC.PX.PSC.M.1	EC	PX
EC.PX.DF1.M.1	EC	PX
EC.PX.DOW.M.1	EC	PX
EC.PX.DOW.M.2	EC	PX
EC.PX.COY.M.2	EC	PX
EC.PX.COY.M.3	EC	PX
EC.PX.ALA.M.5	EC	PX
LS.BA.WB.U.1	LS	BA
LS.BA.WB.U.2	LS	BA
LS.BA.DHI.U.2	LS	BA
LS.BA.GA.U.1	LS	BA
LS.BA.PIK.U.3	LS	BA
LS.BA.PIK.U.5	LS	BA
LS.BA.CP.U.2	LS	BA
LS.BA.ML.U.2	LS	BA
LS.BA.WBO.U.3	LS	BA

Sample	Species	City
LS.BO.WL3.M.4	LS	BO
LS.BO.I1.U.1	LS	BO
LS.BO.I2.U.1	LS	BO
LS.BO.WL2.M.2	LS	BO
LS.BO.R1.U.2	LS	BO
LS.BO.R2.U.4	LS	BO
LS.BO.R3.U.3	LS	BO
LS.BO.HC4.M.3	LS	BO
LS.BO.LC4.M.2	LS	BO
LS.LA.VET.M.4	LS	LA
LS.LA.SSV.M.1	LS	LA
LS.LA.NAV.M.4	LS	LA
LS.LA.SHO.M.2	LS	LA
LS.LA.WES.M.3	LS	LA
LS.LA.GLO.M.3	LS	LA
LS.LA.HOW.U.5	LS	LA
LS.LA.SAN.U.2	LS	LA
LS.LA.ARR.U.2	LS	LA
LS.MN.L06-LS.U.2	LS	MN
LS.MN.L06-LS.U.5	LS	MN
LS.MN.L07-LS.U.4	LS	MN
LS.MN.L08-LS.U.5	LS	MN
LS.MN.L09-LS.U.3	LS	MN
LS.MN.L01-LS.M.4	LS	MN
LS.MN.L01-LS.U.3	LS	MN
LS.MN.L02-LS.U.1	LS	MN
LS.MN.L05-LS.U.2	LS	MN
LS.PX.MON.U.2	LS	PX
LS.PX.PKW.U.5	LS	PX
LS.PX.PIE.M.4	LS	PX
LS.PX.ALA.M.3	LS	PX
LS.PX.PAL.M.3	LS	PX
LS.PX.MAN.M.2	LS	PX
LS.PX.NUE.M.1	LS	PX
LS.PX.ENC.M.4	LS	PX
LS.PX.COY.M.3	LS	PX
PA.BA.PIK.U.1	PA	BA
PA.BA.LH-3.M.2	PA	BA
PA.BA.LH-3.M.3	PA	BA
PA.BA.WB.U.1	PA	BA
PA.BA.AA.U.1	PA	BA
PA.BA.WGP.M.3	PA	BA
PA.BA.LL-4.M.3	PA	BA
PA.BA.LA.U.2	PA	BA
PA.BA.LH-2.M.2	PA	BA
PA.BA.W3.M.3	PA	BA
PA.BA.RG-1.M.2	PA	BA
PA.BA.LL-3.M.5	PA	BA
PA.BO.I2.U.3	PA	BO
PA.BO.HC1.M.4	PA	BO
PA.BO.R3.U.2	PA	BO
PA.BO.HC4.M.5	PA	BO

Sample	Species	City
PA.BO.R4.U.2	PA	BO
PA.BO.WL2.M.5	PA	BO
PA.BO.WL4.M.4	PA	BO
PA.BO.LC4.M.4	PA	BO
PA.BO.HC2.M.1	PA	BO
PA.BO.R1.U.2	PA	BO
PA.BO.WL1.M.1	PA	BO
PA.BO.I1.U.5	PA	BO
PA.LA.ALL.M.5	PA	LA
PA.LA.SEP.M.1	PA	LA
PA.LA.SEP.M.5	PA	LA
PA.LA.WHI.M.2	PA	LA
PA.LA.ROS.M.5	PA	LA
PA.LA.LUB.M.2	PA	LA
PA.LA.GLO.M.2	PA	LA
PA.LA.ZOO.M.4	PA	LA
PA.LA.ZOO.M.5	PA	LA
PA.LA.NWH.M.2	PA	LA
PA.LA.KIN.M.4	PA	LA
PA.LA.POP.M.4	PA	LA
PA.PX.BUF.M.3	PA	PX
PA.PX.PIE.M.4	PA	PX
PA.PX.LAP.M.5	PA	PX
PA.PX.ALA.M.1	PA	PX
PA.PX.PAP.M.2	PA	PX
PA.PX.PAP.M.5	PA	PX
PA.PX.DF1.M.2	PA	PX
PA.PX.RPP.U.3	PA	PX
PA.PX.ENC.M.4	PA	PX
PA.PX.ENC.M.5	PA	PX
PA.PX.COY.M.1	PA	PX
PA.PX.BUF.M.2	PA	PX
TO.BA.WBO.U.4	TO	BA
TO.BA.CP.U.1	TO	BA
TO.BA.FH.U.1	TO	BA
TO.BA.LH-3.M.4	TO	BA
TO.BA.WGP.M.3	TO	BA
TO.BA.GA.U.4	TO	BA
TO.BA.PIK.U.4	TO	BA
TO.BA.PSP.M.1	TO	BA
TO.BA.RG-2.M.2	TO	BA
TO.BO.HC1.M.4	TO	BO
TO.BO.HC2.M.5	TO	BO
TO.BO.HC3.M.1	TO	BO
TO.BO.HC4.M.5	TO	BO
TO.BO.LC1.M.1	TO	BO
TO.BO.LC2.M.5	TO	BO
TO.BO.LC3.M.1	TO	BO
TO.BO.WL2.M.1	TO	BO
TO.BO.I2.U.3	TO	BO
TO.LA.WHI.M.5	TO	LA
TO.LA.HAR.M.4	TO	LA

Sample	Species	City
TO.LA.MR1.M.1	TO	LA
TO.LA.GLO.M.5	TO	LA
TO.LA.ZOO.M.1	TO	LA
TO.LA.NWH.M.4	TO	LA
TO.LA.VNS.M.2	TO	LA
TO.LA.PEP.M.5	TO	LA
TO.LA.COM.M.4	TO	LA
TO.MN.L11-TO.M.3	TO	MN
TO.MN.L02-TO.U.1	TO	MN
TO.MN.L04-TO.U.1	TO	MN
TO.MN.L06-TO.U.2	TO	MN
TO.MN.L08-TO.U.5	TO	MN
TO.MN.L09-TO.U.2	TO	MN
TO.MN.L11-TO.U.3	TO	MN
TO.MN.L05-TO.M.5	TO	MN
TO.MN.L08-TO.M.5	TO	MN
TO.PX.BUF.M.1	TO	PX
TO.PX.ALA.M.2	TO	PX
TO.PX.LAP.M.4	TO	PX
TO.PX.WES.M.1	TO	PX
TO.PX.CLA.M.1	TO	PX
TO.PX.DF1.M.1	TO	PX
TO.PX.DF2.M.1	TO	PX
TO.PX.COY.M.1	TO	PX
TO.PX.COY.M.6	TO	PX

3.3 Step 7 - Metapopulation locus matching with **sstacks**

Files can be found in the 07_**sstacks**/ directory.

All samples in the population (or all samples you want to include in the analysis) are matched against the catalog produced in **cstacks** with **sstacks**, run in script **stacks_SPECIES.sh** and **stacks_SPECIES_additional.sh**. It runs off of the samples based in the output directory *and* the listed samples in **sstacks_samples_SPECIES.txt** and **sstacks_samples_SPECIES_additional.txt** (respectively), so make sure all your files (sample and catalog, etc.) are there and match. **sstacks_samples_SPECIES.txt** takes the form:

```
DS.BA.GA.U.1
DS.BA.GA.U.2
DS.BA.GA.U.3
DS.BA.GA.U.4
DS.BA.GA.U.5
...
```

There should be a new file produced at this step for every sample in the output directory:

- `<samplename>.matches.tsv.gz`

A small number of samples generated very few matches to the catalog (such as only 4 loci matching, obviously not enough to draw any conclusions) and therefore aren't used in the next step. See [output/sstacks-discarded_samples.csv](#).

3.4 Step 8 - Genotype probabilities with **polyRAD**

Files can be found in the 08_**polyRAD**/ directory.

3.4.1 Make RADdata object

We used the [polyRAD package](#) to call genotypes because many of our species are polyploid or have historical genome duplication. PolyRAD takes the catalog output (`catalog.alleles.tsv.gz`) and accompanying matches to the catalog (e.g., `CD.BA.AA.U.1.matches.tsv.gz`) to create genotype likelihoods for species with diploidy and/or polyploidy. We used the catalog and match files to create a RADdata object class in R for each species. We ran this on the Rockfish compute cluster, with the `make_polyRAD_<spp>.R` script doing the brunt of the work. The R script was wrapped by `polyrad_make_<spp>.sh` to submit the script to the SLURM scheduler.

Relevant Parameters:

- `min.ind.with.reads` was set to 20% of samples. This means we discarded any loci not found in at least 20% of samples for each species.
- `min.ind.with.minor.allele` was set to 2. This means a locus must have at least this many samples with reads for the minor allele in order to be retained.

Requires:

- `popmap_<spp>_polyrad.txt`, a list of samples and population
- output from `sstacks`

Outputs:

- `<spp>_polyRADdata.rds`, RDS object (the RADdata object)

3.4.2 Calculate overdispersion

Next, we calculated overdispersion using the `polyRAD_overdispersion_<spp>.R` script, wrapped by `polyrad_overd_<spp>.sh` to submit the script to the SLURM scheduler.

Requires:

- `popmap_<spp>_polyrad.txt`, a list of samples and population
- `<spp>_polyRADdata.rds`, RDS object (the RADdata object) output from the previous step

Outputs:

- `<spp>_overdispersion.rds`, RDS object (the overdispersion test output)

3.4.3 Estimate genotypes

Next, we calculated filtered loci based on the expected Hind/He statistic and estimated population structure/genotypes using the `polyRAD_filter_<spp>.R` script, wrapped by `polyrad_filt_<spp>.sh` to submit the script to the SLURM scheduler.

We used the table in [this tutorial](#), which estimated an inbreeding based on the ploidy, optimal overdispersion value, and mean Hind/He. These values are hardcoded in `polyRAD_filter_<spp>.R`.

Requires:

- `popmap_<spp>_polyrad.txt`, a list of samples and population
- `<spp>_polyRADdata.rds`, RDS object (the RADdata object) output from the previous step
- `<spp>_overdispersion.rds`, RDS object (the overdispersion test output) output from the previous step

Outputs:

- `<spp>_filtered_RADdata.rds`, RDS object (RADdata object filtered for appropriate Hind/He)
- `<spp>_IteratePopStructPCA.csv`, data output from the genotype estimate PCA, suitable for plotting
- `<spp>_estimatedgeno_RADdata.rds`, RDS object (RADdata object with genotype estimates)

3.4.4 Final filter and file cleanup

The output `<spp>_estimatedgeno_RADdata.rds` needs to be converted to `genind` and `structure` format for further analysis and steps. There is a little cleanup involved so the population information is retained. For example, `Structure` needs the population identity to be an integer, not a string. This set of functions can be run on a laptop.

At this stage, we also visually assessed the H_{ind}/H_e statistic versus the locus depth (see `check_coverage` inside the `convert_genomics.R` script). We removed the following samples from further analysis:

Table S3: Subset of samples discarded after genotype estimation using polyRAD.

Sample
CD.BA.PSP.M.1
CD.BA.DHI.U.2
CD.BA.DHI.U.3
CD.BA.RG-1.M.5
CD.BA.RG-1.M.4
DS.BO.WL1.M.4
DS.BO.I1.U.3
EC.BO.R4.U.1
LS.BO.HC2.M.5
LS.BO.LC4.M.3
LS.BO.R2.U.4
LS.BO.R2.U.1
PA.BA.LH-3.M.4
PA.BA.AA.U.3
PA.BA.AA.U.4
PA.PX.RPP.U.2
PA.BO.HC2.M.4
PA.PX.RPP.U.1
TO.BA.TRC.U.1
TO.BA.TRC.U.3
TO.BO.R4.U.1
TO.BA.TRC.U.2
TO.BO.R4.U.2
TO.BO.R2.U.2

```
source("08_polyRAD/convert_genomics.R")
```

```
convert_all()
```

3.5 Step 9 - Populations with Structure

Files, including model parameters, can be found in the `09_structure/` directory.

`Structure` documentation can be found [here](#).

3.5.1 Running Structure

polyRAD outputs genotype probabilities in a format suitable for `Structure`. These files were named as:

```
CD_estimatedgeno.structure  
DS_estimatedgeno.structure
```

```
EC_estimatedgeno.structure
LS_estimatedgeno.structure
PA_estimatedgeno.structure
TO_estimatedgeno.structure
```

We ran all species using a naive approach (not using prior information) with $K = 1, 2, 3, 4, 5$ (MAXPOPS argument). To search for the most appropriate K , We ran Structure through 5 replicate runs for each combination of species and K , with 10000 iterations discarded as burn-in and retained 20000 iterations. These runs created files that look like:

```
structure_out_CD1_naive_f      // K = 1, rep 1
structure_out_CD1_naive_rep2_f // K = 1, rep 2
structure_out_CD1_naive_rep3_f // K = 1, rep 3
structure_out_CD1_naive_rep4_f // K = 1, rep 4
structure_out_CD1_naive_rep5_f // K = 1, rep 5
structure_out_CD2_naive_f      // K = 2, rep 1
structure_out_CD2_naive_rep2_f // K = 2, rep 2
structure_out_CD2_naive_rep3_f // K = 2, rep 3
...
```

Within each species, we compressed the result files for all K and reps and submitted to [Structure Harvester](#) to choose the optimal K using the Delta- K method (see <https://link.springer.com/article/10.1007/s12686-011-9548-7>). Once the optimal K was selected per species, we re-ran Structure using a greater number of iterations (100000) for final output and plotting.

4 Analysis

4.1 NLCD Data

From the USGS:

The U.S. Geological Survey (USGS), in partnership with several federal agencies, has developed and released four National Land Cover Database (NLCD) products over the past two decades: NLCD 1992, 2001, 2006, and 2011. This one is for data from 2016 and describes urban imperviousness.

<https://www.mrlc.gov/data/type/urban-imperviousness>

NLCD imperviousness products represent urban impervious surfaces as a percentage of developed surface over every 30-meter pixel in the United States. NLCD 2016 updates all previously released versions of impervious products for CONUS (NLCD 2001, NLCD 2006, NLCD 2011) along with a new date of impervious surface for 2016. New for NLCD 2016 is an impervious surface descriptor layer. This descriptor layer identifies types of roads, core urban areas, and energy production sites for each impervious pixel to allow deeper analysis of developed features.

First, we trimmed the large data. This makes a smaller `.rds` file for each city.

```
source("R/trim_NLCD_spatial_data.R")
```

```
create_spatial_rds_files()
```

4.2 Make maps of sampling locations

Next, we made plots for each city's sampling locations. Note that these only include sites that had viable SNPs.

```
source("R/plot_map_of_samples.R")
```

```
make_all_urban_site_plots()
```

4.3 Continental population structure: population statistics by species

We used `polyrad::calcPopDiff()` to calculate continental population statistics for each species.

```
source("R/calc_continental_stats.R")
```

```
do_all_continental_stats()
```

```
# CD as an example
```

```
read.csv("output/population_stats/CD_continental_stats.csv")
```

```
##   X statistic      value
## 1 1      JostD 0.30579677
## 2 2      Gst  0.02735719
## 3 3      Fst  0.02812163
```

4.4 Continental population structure: Structure software results

Within each species, we compressed the result files for all K and reps and submitted to [Structure Harvester](https://link.springer.com/article/10.1007/s12686-011-9548-7) to choose the optimal K using the Delta-K method (see <https://link.springer.com/article/10.1007/s12686-011-9548-7>).

The results were:

CD: K=3 DS: K=3 EC: K=2 LS: K=3 PA: K=4 TO: K=3

4.5 Continental population structure: Structure plots

```
source("R/plot_structure.R")
```

```
make_structure_multi_plot()
```

4.6 Local: F_{IS} - Homozygosity within population

We used [GenoDive v. 3.0.6](#) to calculate F_{IS} . This gives a good estimate of whether there are more homozygotes than expected (positive number) or more heterozygotes than expected (negative number). Notably, GenoDive accommodates polyploids and reduces the bias on F_{IS} by performing a permutation test. By default, there are 999 permutations.

```
head(read.csv("output/population_stats/genodive_output_Fis.csv"))
```

```
##   Species Population   Fis
## 1      CD          BA 0.166
## 2      CD          LA 0.186
## 3      CD          PX 0.200
## 4      CD    Overall 0.187
## 5      DS          BA 0.208
## 6      DS          B0 0.252
```

4.7 Local: ρ - Pairwise comparison

We used [GenoDive v. 3.0.6](#) to calculate pairwise ρ (rho) among cities within species. We used the following script to clean up the results.

```
source("R/rho.R")
compile_rho_table()

# LS as an example
read.csv("output/population_stats/rho_all.csv")
```

##	rowname	spp	variable	value
## 1	LA	CD	BA	0.046
## 2	PX	CD	BA	0.050
## 3	PX	CD	LA	0.015
## 4	BO	DS	BA	0.018
## 5	MN	DS	BA	0.031
## 6	PX	DS	BA	0.012
## 7	MN	DS	BO	0.007
## 8	PX	DS	BO	-0.002
## 9	PX	DS	MN	-0.002
## 10	LA	EC	BA	0.038
## 11	PX	EC	BA	0.098
## 12	PX	EC	LA	0.087
## 13	BO	LS	BA	0.032
## 14	LA	LS	BA	0.038
## 15	MN	LS	BA	0.009
## 16	PX	LS	BA	0.077
## 17	LA	LS	BO	0.010
## 18	MN	LS	BO	0.021
## 19	PX	LS	BO	0.056
## 20	MN	LS	LA	0.039
## 21	PX	LS	LA	0.061
## 22	PX	LS	MN	0.069
## 23	BO	PA	BA	0.008
## 24	LA	PA	BA	0.011
## 25	PX	PA	BA	0.015
## 26	LA	PA	BO	0.024
## 27	PX	PA	BO	0.028
## 28	PX	PA	LA	-0.002
## 29	BO	TO	BA	0.008
## 30	LA	TO	BA	0.011
## 31	MN	TO	BA	0.001
## 32	PX	TO	BA	0.023
## 33	LA	TO	BO	0.009
## 34	MN	TO	BO	0.008
## 35	PX	TO	BO	0.013
## 36	MN	TO	LA	0.009
## 37	PX	TO	LA	0.009
## 38	PX	TO	MN	0.015

4.8 Local: \bar{r}_d - Linkage disequilibrium

We used `poppr::ia()` to calculate the standardized index of association of loci in the dataset (\bar{r}_d or `rbarD`). We use the standardized index of association to avoid the influence of different sample sizes, as described by [Agapow and Burt 2001](#).

When `p.rD` is small (<0.05) and `rbarD` is (relatively) higher, that is a sign that the population could be in linkage disequilibrium.

An interesting note from the documentation:

It has been widely used as a tool to detect clonal reproduction within populations. Populations whose members are undergoing sexual reproduction, whether it be selfing or out-crossing, will produce gametes via meiosis, and thus have a chance to shuffle alleles in the next generation. Populations whose members are undergoing clonal reproduction, however, generally do so via mitosis. This means that the most likely mechanism for a change in genotype is via mutation. The rate of mutation varies from species to species, but it is rarely sufficiently high to approximate a random shuffling of alleles. The index of association is a calculation based on the ratio of the variance of the raw number of differences between individuals and the sum of those variances over each locus. You can also think of it as the observed variance over the expected variance.

There is a nice description [here](#).

```
source("R/rbarD.R")
calc_rbarD()
```

Table S4: rbarD statistics for species and cities in this study.

spp	city	n	Ia	p.Ia	rbarD	p.rD
CD	BA	55	664.76547	0.001	0.2950534	0.001
CD	LA	48	470.59126	0.001	0.2070064	0.001
CD	PX	82	634.57866	0.001	0.2792566	0.001
DS	BA	55	557.73341	0.001	0.2123881	0.001
DS	BO	52	896.09061	0.001	0.3398873	0.001
DS	MN	81	578.24942	0.001	0.2192197	0.001
DS	PX	36	965.88065	0.001	0.3995368	0.001
EC	BA	41	130.00266	0.001	0.1016315	0.001
EC	LA	37	138.01718	0.001	0.1075911	0.001
EC	PX	29	283.04879	0.001	0.2296380	0.001
LS	BA	20	44.25346	0.001	0.0805235	0.001
LS	LA	60	59.62442	0.001	0.1078184	0.001
LS	BO	34	129.90835	0.001	0.2386926	0.001
LS	MN	30	61.84565	0.001	0.1122845	0.001
LS	PX	40	127.15860	0.001	0.2329002	0.001
PA	BA	47	230.66552	0.001	0.3142042	0.001
PA	LA	38	95.28187	0.001	0.1292179	0.001
PA	BO	61	362.49100	0.001	0.4991782	0.001
PA	PX	30	160.33901	0.001	0.2174130	0.001
PA	MN	2	NA	NA	NA	NA
TO	BA	60	84.26479	0.001	0.1260217	0.001
TO	LA	29	125.25324	0.001	0.1874654	0.001
TO	BO	58	113.07839	0.001	0.1689793	0.001
TO	MN	75	70.93300	0.001	0.1059746	0.001
TO	PX	16	261.79575	0.001	0.3991308	0.001

```
head(read.csv("output/population_stats/rbarD.csv"))
```

```
##   spp city  n      Ia  p.Ia    rbarD  p.rD
## 1  CD   BA 55 664.7655 0.001 0.2950534 0.001
## 2  CD   LA 48 470.5913 0.001 0.2070064 0.001
## 3  CD   PX 82 634.5787 0.001 0.2792566 0.001
## 4  DS   BA 55 557.7334 0.001 0.2123881 0.001
## 5  DS   BO 52 896.0906 0.001 0.3398873 0.001
```

```
## 6 DS MN 81 578.2494 0.001 0.2192197 0.001
```

4.9 Isolation by distance

We assessed isolation by distance by comparing genetic distance to geographic distance. Specifically, we took the traditional approach of comparing a geographic dissimilarity matrix (based on latitude and longitude) to a genetic dissimilarity matrix. We calculated the genetic dissimilarity matrix with the `dist.genpop` function in the `ade4` package. We use the [Cavalli-Sforza](#) distance metric, or `method = 2` argument for the `dist.genpop` function.

Note that for this analysis, we treated each *sampling site* as a distinct location. There would not be enough power to do a distance matrix among 3-5 cities.

```
source("R/isolation_by_distance.R")
```

```
extract_ibd_stats_and_plots()
```

```
# Across all cities
```

```
extract_ibd_stats_and_plots(TRUE, "BA")
```

```
extract_ibd_stats_and_plots(TRUE, "PX")
```

```
extract_ibd_stats_and_plots(TRUE, "LA")
```

```
extract_ibd_stats_and_plots(TRUE, "BO")
```

```
extract_ibd_stats_and_plots(TRUE, "MN")
```

Here are the results of the mantel test:

Table S5: Statistics from running 9999 permutations via mantel test.

Species	Observation	Hypothesis	Reps	Std.Obs	Expectation	Variance	p-value
Bermuda grass (CD)	0.4476430	greater	9999	12.305181	-0.0005079	0.0013264	1e-04
crabgrass (DS)	0.3299992	greater	9999	8.314767	-0.0001454	0.0015766	1e-04
horseweed (EC)	0.4028339	greater	9999	9.109720	0.0000800	0.0019547	1e-04
prickly lettuce (LS)	0.1939607	greater	9999	7.705054	0.0000806	0.0006332	1e-04
bluegrass (PA)	0.2821562	greater	9999	8.227910	-0.0001475	0.0011772	2e-04
dandelion (TO)	0.3101457	greater	9999	7.283013	0.0000965	0.0018123	1e-04

We also repeated this within city. Code for generating stats from the Mantel test and creating network plots can be found in the source code below.

```
source("R/isolation_by_distance_by_city.R")
```

Table S6: Statistics from running 9999 permutations via mantel test, limited to within city.

Observation	Hypothesis	Reps	Std.Obs	Expectation	Variance	p.value	spp	city
0.0772829	greater	9999	0.4913316	0.0017290	0.0236464	0.2617	CD	BA
0.0233043	greater	9999	0.1719811	-0.0022465	0.0220723	0.2689	CD	LA
0.0959610	greater	9999	0.7582701	0.0005926	0.0158183	0.2162	CD	PX
-0.0399862	greater	9999	-0.3088894	-0.0002184	0.0165752	0.5243	DS	BA
-0.0958390	greater	9999	-0.5390954	0.0005071	0.0319402	0.6478	DS	BO
-0.0920704	greater	9999	-0.5427566	-0.0007803	0.0282903	0.6489	DS	MN
-0.1214396	greater	9999	-0.5656858	-0.0008503	0.0454430	0.6200	DS	PX
-0.1086321	greater	9999	-0.5396215	-0.0012475	0.0396009	0.6214	EC	BA
0.0192066	greater	9999	0.1302304	-0.0012068	0.0245701	0.4168	EC	LA

Observation	Hypothesis	Reps	Std.Obs	Expectation	Variance	p.value	spp	city
-0.2059353	greater	9999	-1.1259800	0.0004167	0.0335858	0.9493	EC	PX
0.1417600	greater	9999	0.5926773	-0.0045816	0.0609676	0.3399	LS	BA
-0.1144810	greater	9999	-0.7579351	0.0000753	0.0228441	0.7559	LS	BO
0.1899000	greater	9999	1.2753662	-0.0001752	0.0222117	0.0747	LS	LA
0.0093208	greater	9999	0.0431229	0.0015094	0.0328129	0.3584	LS	MN
-0.1887409	greater	9999	-0.8855957	0.0003189	0.0455751	0.8656	LS	PX
0.0177684	greater	9999	0.1524460	-0.0006379	0.0145782	0.3930	PA	BA
-0.0932886	greater	9999	-0.8697657	-0.0004552	0.0113921	0.8896	PA	BO
-0.0178809	greater	9999	-0.0896877	-0.0014221	0.0336767	0.3976	PA	LA
0.3215745	greater	9999	1.6008704	0.0010999	0.0400751	0.0705	PA	PX
-0.1141184	greater	9999	-0.6790755	-0.0003391	0.0280731	0.8038	TO	BA
-0.1540646	greater	9999	-0.8420713	-0.0013214	0.0329023	0.8708	TO	BO
0.1380763	greater	9999	0.7578447	0.0005027	0.0329541	0.2443	TO	LA
0.0443237	greater	9999	0.7413905	-0.0002560	0.0036156	0.2118	TO	MN
-0.1136297	greater	9999	-0.5078763	-0.0002439	0.0498427	0.6079	TO	PX

5 Appendix

5.1 SessionInfo()

```
sessionInfo()
```

```
## R version 4.3.1 (2023-06-16)
## Platform: aarch64-apple-darwin20 (64-bit)
## Running under: macOS Ventura 13.4.1
##
## Matrix products: default
## BLAS: /Library/Frameworks/R.framework/Versions/4.3-arm64/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.3-arm64/Resources/lib/libRlapack.dylib; LAPACK v
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## time zone: America/New_York
## tzcode source: internal
##
## attached base packages:
## [1] stats      graphics  grDevices  utils      datasets  methods   base
##
## other attached packages:
## [1] adegenet_2.1.10 ade4_1.7-22 ggh4x_0.2.5 here_1.0.1
## [5] lubridate_1.9.2 forcats_1.0.0 purrr_1.0.1 tibble_3.2.1
## [9] tidyverse_2.0.0 polysat_1.7-7 cowplot_1.1.1 viridis_0.6.3
## [13] viridisLite_0.4.2 raster_3.6-20 sp_1.6-1 stringr_1.5.0
## [17] readr_2.1.4 polyRAD_2.0.0 dplyr_1.1.2 magrittr_2.0.3
## [21] tidyr_1.3.0 ggplot2_3.4.2
##
## loaded via a namespace (and not attached):
## [1] tidyselect_1.2.0 farver_2.1.1 fastmap_1.1.1 promises_1.2.0.1
## [5] digest_0.6.31 timechange_0.2.0 mime_0.12 lifecycle_1.0.3
## [9] cluster_2.1.4 ellipsis_0.3.2 terra_1.7-29 compiler_4.3.1
```

```
## [13] rlang_1.1.1      tools_4.3.1      igraph_1.5.0     utf8_1.2.3
## [17] yaml_2.3.7       knitr_1.43       labeling_0.4.2   bit_4.0.5
## [21] plyr_1.8.8       withr_2.5.0      grid_4.3.1      fansi_1.0.4
## [25] xtable_1.8-4     colorspace_2.1-0 scales_1.2.1     MASS_7.3-60
## [29] cli_3.6.1        rmarkdown_2.22   vegan_2.6-4     crayon_1.5.2
## [33] ragg_1.2.5       generics_0.1.3   rstudioapi_0.14 reshape2_1.4.4
## [37] tzdb_0.4.0       ape_5.7-1        splines_4.3.1   parallel_4.3.1
## [41] vctrs_0.6.2      Matrix_1.5-4.1   hms_1.1.3       bit64_4.0.5
## [45] seqinr_4.2-30    systemfonts_1.0.4 glue_1.6.2       codetools_0.2-19
## [49] stringi_1.7.12   gtable_0.3.3     later_1.3.1     munsell_0.5.0
## [53] pillar_1.9.0     htmltools_0.5.5  R6_2.5.1        textshaping_0.3.6
## [57] rprojroot_2.0.3  vroom_1.6.3      evaluate_0.21    shiny_1.7.4
## [61] lattice_0.21-8   highr_0.10        httpuv_1.6.11   Rcpp_1.0.10
## [65] fastmatch_1.1-3  permute_0.9-7     gridExtra_2.3    nlme_3.1-162
## [69] mgcv_1.8-42      xfun_0.39         pkgconfig_2.0.3
```

5.2 File Organization

All data files for the Macrosystems project are permanently stored under Meghan Avolio's group resources in the Johns Hopkins University Rockfish computing cluster. Files are stored under the 'data' directory under the following subdirectories:

- **01-raw_data:** This folder contains the raw, unprocessed data files that were obtained directly from the sequencing server. There are eight **fastq.gz** files per sublibrary that correspond to the four sequencing lanes for each read direction.
- **02-concatenated_data:** This folder contains the concatenated, unprocessed files for each sublibrary (i.e., the files containing the sequences for each lane were combined to create one file per read direction).
- **03-pcr_filtered_data:** Here, you will find the resulting data files from the **clone_filter** program, where pcr replicates/clones have been removed from the raw sequences. There are two **fq.gz** files per sublibrary.
- **04-process_radtags:** This folder contains various subdirectories that correspond to the **process_radtags** program that demultiplexes and cleans the data. The **demux_txt_files** folder contains the .txt files used to identify barcodes and separate out the individual samples from each sublibrary. The resulting data files from the **process-radtags** program are separated by individual and can be found in the relevant species folder (i.e., CD, DS, EC, LS, PA, TE, TO). Each individual sample has four data files; **sampleID.1.fq.gz** and **sampleID.2.fq.gz** correspond to the forward and reverse reads for each sample and **sampleID.rem,1/2.fq.gz** contain the remainder reads that were cleaned and removed from the data sequence.
- **05-ustacks-denovo_data:** This folder contains species subdirectories that store the resulting data files from the **ustacks** program for each individual. There are three files per individual; **sampleID.alleles.tsv.gz**, **sampleID.snps.tsv.gz**, and, **sampleID.tags.tsv.gz**. These files should be permanently stored here and copied to a new directory for any new catalogs and/or when a group of samples are being aligned to a new catalog.
- **catalogs_by_city:** For any given species within city, there is likely to be a slightly different set of SNPs compared to the whole metapopulation of five cities. We examined 24 sets of species-city combinations. These catalogs are permanently stored here.
- **catalogs_by_species:** Metapopulation catalogs are stored within this folder for each species. The metapopulation catalog was created using samples from all populations to create a national catalog.

Some notes about catalog directories:

- Catalogs contain three files; **catalog.alleles.tsv.gz**, **catalog.snps.tsv.gz**, and **catalog.tafs.tsv.gz**. If you would like to use the catalog on a new project, you will

- need to copy all three files to a new project folder.
- You can determine which individuals were used to create the catalog by looking at the `cstacks_popmap.txt` found within each folder. Specifically for the metapopulation catalogs, this information is also found in the [cstacks-metapop-catalog_samples-included.csv](#)
- You can determine which individuals were subsequently aligned to the catalog and used in the subsequent stacks analysis by looking at the `popmap*.txt` found within each folder.
- Each folder also contains the relevant ustacks and stacks pipeline scripts and output files (i.e., from `cstacks`, `gstacks`, `stacks`, `tsv2bam`, and `populations`),

5.3 Aspera Transfer File Names

See [data/aspera_transfer_file_names.csv](#). Preview:

```
readLines("data/aspera_transfer_file_names.csv", 10)
```

```
## [1] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L001_R1_001.fastq.gz"
## [2] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L001_R2_001.fastq.gz"
## [3] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L002_R1_001.fastq.gz"
## [4] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L002_R2_001.fastq.gz"
## [5] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L003_R1_001.fastq.gz"
## [6] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L003_R2_001.fastq.gz"
## [7] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L004_R1_001.fastq.gz"
## [8] "/Hoffman_macrosystems/AMH_macro_1_1_12px_S1_L004_R2_001.fastq.gz"
## [9] "/Hoffman_macrosystems/AMH_macro_1_10_8px_S10_L001_R1_001.fastq.gz"
## [10] "/Hoffman_macrosystems/AMH_macro_1_10_8px_S10_L001_R2_001.fastq.gz"
```

5.4 clone_filter File Names

See [data/clone_filter_file_names.csv](#). Preview:

```
readLines("data/clone_filter_file_names.csv", 10)
```

```
## [1] "AMH_macro_1_1_12px_S1" "AMH_macro_1_10_8px_S10" "AMH_macro_1_11_8px_S11"
## [4] "AMH_macro_1_12_8px_S12" "AMH_macro_1_13_8px_S13" "AMH_macro_1_14_8px_S14"
## [7] "AMH_macro_1_2_12px_S2" "AMH_macro_1_3_12px_S3" "AMH_macro_1_4_12px_S4"
## [10] "AMH_macro_1_5_8px_S5"
```