AftrRAD Version 5.0 User Manual

AftrRAD is a bioinformatic pipeline consisting of perl and R scripts that is designed to aid in analyzing genotyping-by-sequencing (GBS) data such as that produced by restriction site associated DNA sequence (RADseq) methods. If you have questions about AftrRAD that are not addressed in this manual, two additional resources include the FAQ file and the AftrRAD google group (https://groups.google.com/forum/#!forum/aftrrad).

Notable updates associated with AftrRAD Version 5.0…

- Reduced run times, including a new option for parallel analyses.
- Reduced RAM requirements (especially applicable to datasets that are demultiplexed prior to running AftrRAD).
- New output options including Fasta, TreeMix, Migrate, and folded and multidimensional site frequency spectra for FastSimCoal.

**See the version history information for a complete list of updates*.

Contents

Before you start using AftrRAD, make sure…

1.) You're using a Mac or Linux machine. Both work, but the program usually runs faster on Macs. Windows version may eventually be available, but not currently.
2.) You're analyzing single-end reads. There is currently no option for paired end data.
3.) Your reads are all the same length. If you have multiple sequencing runs of varying lengths, I'd suggest trimming them all down to the shortest for AftrRAD analyses.
4.) You have plenty of free hard disk space available on your computer to store large temporary files created during the run (on the order of 10's to 100's of GB to be safe). See FAQ document for more information about memory requirements.
5.) You have the following dependencies correctly installed and working…
    a. ACANA -> Download this program from: https://www.niehs.nih.gov/research/resources/software/biostatistics/acana/index.cfm and copy the two executable files 'ACANA' and 'dnaMatrix' into your working AftrRAD directory. In a Terminal window, move to this directory and type ./ACANA to make sure it's working.
    b. Mafft -> Get it at: http://mafft.cbrc.jp/alignment/software/. It should be in your path after you download it. To make sure it's working, simply type mafft in the terminal window from any directory and the program should run (it should ask for an input file).
    c. R -> Available from www.rproject.org

6.) If running version 5.0 or greater, you have installed the Perl module Parallel:ForkManager (not necessary for versions prior to 5.0). This can often be installed with the command 'sudo cpan Parallel:ForkManager' and following the prompts.

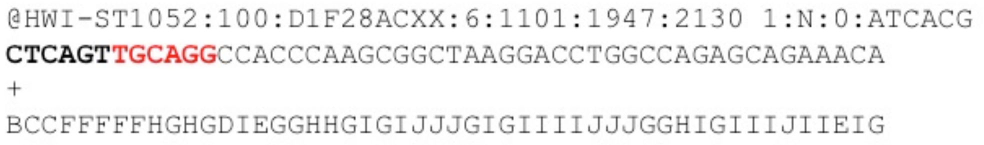
*A small example data file is available, along with its associated barcode file, if you want to try out AftrRAD and make sure everything is installed correctly before running your (presumably larger) dataset. See README file included with the example data for more information.

After everything is downloaded and installed, do one of the following:

A.) If your data are not already demultiplexed, and have inline barcodes as in Fig 1:

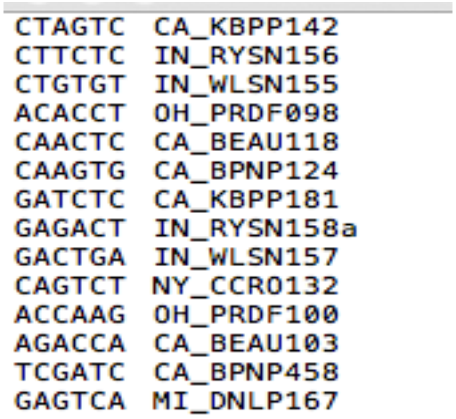
1.) Add all of your data files (in fastq format) to the “Data” subdirectory (you may have to create this Data directory in your working AftrRAD directory).

2.) Add all of your barcode files to the “Barcodes” subdirectory (again, create it in the working AftrRAD directory if necessary). These barcode files are text files that contain barcodes and their associated sample names, separated by a tab (**Figure 2**). **The barcode files must have the exact same name as the data file they are associated with (including the extension)**, and there should be nothing else in the barcode directory (extra files in the Data directory should be OK). Note that hidden characters can cause problems with how AftrRAD reads the barcode files, so we suggest a good text editor such as JEdit, TextWrangler, or nano in Terminal to create these files.



```
@HWI-ST1052:100:D1F28ACXX:6:1101:1947:2130 1:N:0:ATCACG
CTCAGTTGCAGGCCACCCAAGCGGCTAAGGACCTGGCCAGAGCAGAAACA
+
BCCFFFFFHGHGDIEGGHHGIGIJJJGIGIIIIJJJGGHIGIIIJIIEIG
```

Figure 1. Example read in fastq format. In default mode, AftrRAD expects each read to begin with an in-line barcode (bold, black), followed by a restriction enzyme recognition site (red, bold). However, data that are already demultiplexed can be analyzed by setting the command line argument *dplexedData* to ‘1’.



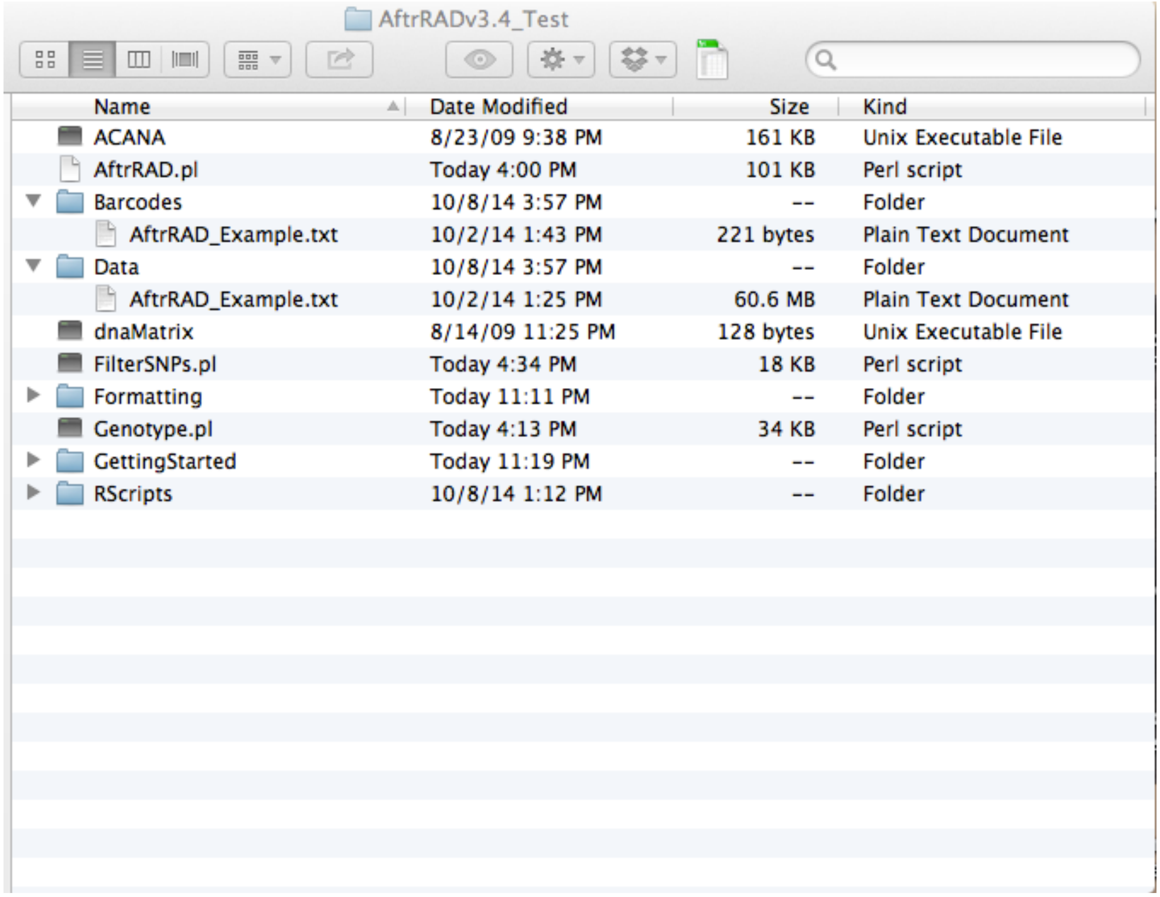
```
CTAGTC  CA_KBPP142
CTTCTC  IN_RYSN156
CTGTGT  IN_WLSN155
ACACCT  OH_PRDF098
CAACTC  CA_BEAU118
CAAGTG  CA_BPNP124
GATCTC  CA_KBPP181
GAGACT  IN_RYSN158a
GACTGA  IN_WLSN157
CAGTCT  NY_CCR0132
ACCAAG  OH_PRDF100
AGACCA  CA_BEAU103
TCGATC  CA_BPNP458
GAGTCA  MI_DNLP167
```

Figure 2. Example format of a barcode file - plain text file with barcode <tab> sample name <return>.

B.) If your data are already demultiplexed, and do not contain inline barcodes such as those shown in Fig 1:

1.) Create a folder in your working directory named ‘DemultiplexedFiles’ and add all of your demultiplexed data files (in fastq format) to this folder. I suggest naming these files with their respective sample name, as these will be used to create the barcode file.
2.) When you run AftrRAD.pl (see below), include the command line argument ‘dplexedData-1’.

At this point, your working directory (with undemultiplexed data) should look something like this…



Name	Date Modified	Size	Kind
ACANA	8/23/09 9:38 PM	161 KB	Unix Executable File
AftrRAD.pl	Today 4:00 PM	101 KB	Perl script
▼ Barcodes	10/8/14 3:57 PM	--	Folder
AftrRAD_Example.txt	10/2/14 1:43 PM	221 bytes	Plain Text Document
▼ Data	10/8/14 3:57 PM	--	Folder
AftrRAD_Example.txt	10/2/14 1:25 PM	60.6 MB	Plain Text Document
dnaMatrix	8/14/09 11:25 PM	128 bytes	Unix Executable File
FilterSNPs.pl	Today 4:34 PM	18 KB	Perl script
▶ Formatting	Today 11:11 PM	--	Folder
Genotype.pl	Today 4:13 PM	34 KB	Perl script
▶ GettingStarted	Today 11:19 PM	--	Folder
▶ RScripts	10/8/14 1:12 PM	--	Folder

Figure 3. Example of working directory before running AftrRAD with undemultiplexed data. If your data are already demultiplexed, the files should be in a folder named DemultiplexedFiles, and the Barcodes and Data folders in the figure are not needed.

*****If you have questions beyond what is covered in this manual, please check the FAQ document and/or the AftrRAD google group.**

Running AftrRAD

Part I: AftrRAD.pl

In the terminal window, cd to your working directory. AftrRAD can be run by typing “**perl**<*space*>**AftrRAD.pl**”. There are several parameters that need to be set for each run. All have default values, which can be edited by including arguments on the command line when starting an AftrRAD run. The parameters are…

re The restriction enzyme recognition sequence that occurs in your reads. In Figure 1 above, this is TGCAGG, which corresponds to a digestion with the enzyme SbfI. This is the default value. If there is no restriction enzyme recognition site in your reads, enter 0.

minQual The minimum quality (Phred) score for retaining reads. Reads that contain bases with scores below this value are removed from the analysis. The default value is 20.

minDepth The minimum mean nonzero read depth to retain a read. For each unique read in the dataset, the counts of that read will be obtained for each sample. The average of the nonzero counts will be obtained, and checked against the value entered here. Any reads with values less than this threshold are assumed to be error and are eliminated from the dataset. Default value is 5. See Figs S2 and 5 in MER paper for more details.

minIden The minimum percent identity (after alignment) to consider two reads to be alternative alleles from the same locus. The default is 90%.

numIndels The maximum number of indels allowed between any two reads to consider them alternative alleles from the same locus. The default is 3.

P2 The beginning of the P2 adaptor sequence. Reads containing this string are removed from the analysis. If you don’t want to search for the P2 adaptor, you can enter “noP2” here, or any other string of characters that will not be found in any of your reads. The default is ATTAGATC.

minParalog The minimum number of reads that must occur at a third allele in an individual to flag a locus as paralogous. Default is 5.

Phred The quality score methodology used in the sequencing. Most often Phred33, which is the default. An alternative is Phred64.

dplexedData If data are demultiplexed prior to the AftrRAD run, set this to ‘1’. In this case, the data files must be stored in a folder named ‘DemultiplexedFiles’. If running demultiplexed data, use the default for “DataPath”.

stringLength Reads containing strings of homopolymers of this length will be removed. Default is 15.

DataPath Path to directory containing fastq data files for the run. Default is the Data directory in the AftrRAD working directory.

BarcodePath Path to directory containing barcode files. Default is the Barcodes directory in the AftrRAD working directory.

MaxH Maximum proportion of samples allowed to be heterozygous at a locus. Loci for which heterozygosity exceeds this value will be flagged as paralogous. Default is 90%.

maxProcesses The maximum number of processors to use in a parallel run.

Arguments to change any of the default values are entered after the 'perl AftrRAD.pl' command, with the argument and it's value separated by a dash. For example, the following command would start a parallelized AftrRAD run using 6 cores, and would change the defaults for the restriction enzyme recognition sequence and the maximum number of indels allowed between two alleles at a locus to ATCACG and 4, respectively…

```
perl AftrRAD.pl  re-ATCACG  numIndels-4 maxProcesses-6
```

The first step in AftrRAD.pl is quality filtering of the reads (among a few other things). An update ("Filtering sequence X") will print to the screen after completion of every 1000000 reads. The first update will usually appear within the first minute. After the initial quality filtering, the main function of this script is locus identification. Runtimes can range from minutes to days, depending on a number of factors including the size of the dataset, the number of loci sampled, the platform (i.e. Mac/Linux), and whether parallel analysis is used.

Part II: Genotype.pl

After running the AftrRAD.pl script, run Genotype.pl by typing “**perl**<*space*>**Genotype.pl**”. The following parameters can be set for this script in the same way as above.

MinReads The minimum coverage required at a locus in an individual to apply a binomial test and call a genotype. Loci with fewer reads than this value will be scored as missing data for the sample. Default is 10.

pvalLow For each locus in each individual, a binomial test is applied to score the sample as heterozygous or homozygous. The assumption is that the two alleles in a heterozygote will be sequenced in equal frequencies. So, for a locus at which an individual has read counts of 35 and 50 for two alleles, respectively, a binomial test with a probability of success of 0.5 gives a p-value of 0.064. If this value is greater than pvalLow, the locus is scored as heterozygous. Alternatively for two read counts of 25 and 1, the p-value is 7.75e-7, and this will be scored as homozygous for the allele with 25 reads, as long as the pvalLow is >7.75e-7. Default is 0.0001.

pvalHigh Same as pvalLow, but allows for a different p-value threshold for loci that have relatively high total counts. For example, a locus with read counts of 350 and 500 gives a p-value of 1.50e-7, which is close to the 1 vs 25 test above. pvalHigh is applied when the total number of reads at the locus is greater than or equal to pvalThresh. Default is 0.00001.

pvalThresh The threshold number of reads at a locus in each binomial test that determines whether pvalLow or pvalHigh is used as the critical p-value. Default is 100. With this default, tests based on <100 reads will use the p-value set as ‘pvalLow’, while those with >100 read will use ‘pvalHigh’.

subset Option to genotype only a subset of the individuals in the dataset. Default is ‘0’, and includes all samples. If set to '1', a text file must be provided with the names of the samples to include. The default name for this file is 'SamplesForSubset.txt', and it should be located in the main AftrRAD directory (the same place as Genotypes.pl).

subsetfile File name/path containing file with sample names to include in genotyping. Only applies if subset argument is set to ‘1’. Default is file 'SamplesForSubset.txt', in the main AftrRAD directory.

maxProcesses The maximum number of processors to use in a parallel run.

The first major function of this script is to score genotypes for all loci in each individual. These genotyped loci are printed to a file named Genotypes.txt in the TempFiles directory.

Next, the script will quantify the amount of missing data at polymorphic loci in each of the samples, and flag any samples that have >2 standard deviations more than the average amount of missing data. You’ll be asked whether to include these samples in the remainder of the analyses. **Use the flagged samples as a guide only.** The proportions of missing data for each individual are recorded in “MissingDataProportions.txt”, and counts of loci with missing data for each individual are plotted in a PDF file named "MissingDataCounts". Both of these are in the "Output/RunInfo" directory. It's a good idea to check these files, and base your decisions off of them. There may be additional samples you want to remove (i.e. – just under 2 stdev greater than the mean), or you may decide to keep some of the flagged samples. You'll have the option to do this. If you choose to remove additional individuals, simply enter the names of the samples you want to remove, with each separated by a tab. Note that 'Individual' is added to the beginning of each name in some of the output files – don't include this when entering samples to remove.

Third, this script produces a file named 'SNPLocations.pdf' in the Output/RunInfo folder that plots the locations of each SNP along the read. This provides some insight into whether artifactual SNPs have built up toward the end of the reads, and if so, at what read position this begins to be a problem (see MER paper Fig 2 for more information). This information is used when running the next script, FilterSNPs.pl

Total runtime for Genotype.pl is usually <30 min, but depends on the number of individuals/loci in the dataset.

Part III: FilterSNPs.pl

After running the Genotypes.pl script, run “FilterSNPs.pl” by typing “**perl**<*space*>**FilterSNPs.pl**” at the command prompt. The following parameters can be set for this script.

pctScored Percent of individuals that must be genotyped in order to retain the locus (1-100). The default is 100, which means all samples must be genotyped in order to retain the locus.

maxSNP The maximum location along the reads to score SNPs. This value should be chosen based on the plot in the file Output/RunInfo/SNPLocations.pdf. The default is 0, which prints all SNPs.

MinReads As in Genotypes.pl, the minimum coverage required at a locus in an individual to score a genotype. In FilterSNPs.pl, this value is needed when deciding which monomorphic loci to output. Default is to use the value used in the most recent run of Genotypes.pl.

FilterSNPs.pl filters the data based on two criteria that are set by the pctScored and maxSNP arguments, respectively. The script outputs the major results from the AftrRAD run. Each time FilterSNPs.pl is run, it will output three files in the Output/Genotypes directory. The first is called SNPMatrix_X.Y.txt, with X being the percent entered, and Y being the max read location for SNPs selected. This file contains inferred genotypes for all of the SNPs identified (note that if a given locus has multiple SNPs, each will appear in a separate column in the matrix, but they will share the same locus number). The second file is called Haplotypes_X.Y.txt, which is similar to SNPMatrix, but combines the SNPs at each locus into a haplotype. Both of these files are tab delimited, and can be viewed easily in a program such as Excel. The third file is named Monomorphics_X.txt, and it contains all of the monomorphic loci that were scored in at least X% of the samples, along with information on the read counts for each sample for these loci.

Suggestions for evaluating your dataset

AftrRAD outputs a variety of files that can be helpful in evaluating the quality of your RAD run(s). Some of these are described below…

1.) Output/RunInfo-> “BarcodeInfoX”: Provides information on the number of reads assigned to each individual (barcode) in the run (demultiplexing). There will be one of these files for each fastq file you started with. The main thing to look for here is evenness in counts across individuals. Samples with extremely low counts will probably need sequenced deeper, or possibly re-prepped, depending on the reason for the low counts. Any samples with extremely low read counts are likely to have been flagged as potentially bad samples during the Genotype.pl run, and may be best omitted from the analyses.

2.) Output/RunInfo-> “ReportX”: Provides information relating to the initial quality filtering of the data (i.e. numbers of retained reads used for analyses). Again, there will be one for each fastq file you started with.

3.) Output/RunInfo-> “MasterReport.txt”: This file reports the total number of monomorphic, polymorphic, and paralogous loci identified, and also the average and median read depth across genotyped loci.

4.) TempFiles/RawReadCountFiles-> “RawReadCount_NonParalogous_X”: The program creates one of these files for each sample analyzed. The counts for each allele are the only thing that will vary for the different samples. These are the counts used in the binomial tests for genotyping (the two highest if there are more than two). Scrolling through these files can give you a good sense of what kind of read depths you have. Note the current default in Genotype.pl is to score genotypes only if there is a minimum of 10 reads at the locus. So, in this case, an individual with two alleles with counts 6 and 2, respectively, would not be genotyped (scored as missing data). If you have a lot of missing data in your dataset, patterns observed in these files can be extremely valuable in pinpointing the reason for the missing data. As you scroll through a sample, if most of the loci have data, but the read depths are relatively low (i.e less then 10 reads at the locus, as described above), then simply resequencing the library to add more depth will likely address the issue. However, another pattern that sometimes occurs is that many loci will have total depths of zero, while the loci that are scored have relatively high read counts. This suggests the missing data is due to problems with the library prep, and resequencing the library is probably not going to benefit you very much.

5.) Output/Results-> “Haplotypes_X.Y.txt”: You can have multiple Haplotypes files, with different values of “X” and “Y” - you will get one of these files each time you run FilterSNPs.pl. The “X” and “Y” in the file names refer to the criteria you set for the minimum proportion of individuals genotyped (X), and the maximium SNP position along the read (Y) in each run of FilterSNPs.pl. This file can be opened in Excel, or any text editor. Comparing the number of loci (columns in the Excel file), to the total number of polymorphic loci from “MasterReport.txt” above will give you some indication of the level of missing data in your dataset (a more direct place to look for this information is the "MissingDataProportions.txt" file, and it's associated plot "MissingDataCounts.pdf", which are both in the RunInfo folder).

6.) Output/RunInfo->"MissingDataProportions.txt": This file reports the proportion of missing data in each sample based on polymorphic loci. Interpreting the absolute values of these data can sometimes be a little tricky. If the numbers are all relatively low (i.e. <5-10%), this is good (there will always be some level of missing data due to null alleles caused by polymorphism in restriction sites). If most samples have relatively low amounts of missing data, but a subset of samples have higher levels, this likely suggests there's a problem with those specific samples, and you might consider leaving them out of the analyses. However, sometimes the missing data values can be relatively high across all samples. There are a number of reasons for this. First, it could be insufficient sequencing coverage (see item 4 above). Second, it could result from bad library preps (again, see 4 above). A final possibility is that one, or a few samples in the dataset have a large number of unique loci, elevating the missing data values for the other samples. As a hypothetical example, imagine a scenario where a dataset contains 20 samples, 19 of which were size selected for fragment sizes of 300-450 bp. Size selection for the 20th sample inadvertently targeted 300-550. In this case, all of the loci between 450 and 550 have been introduced to the dataset, but will not be scored in the majority of the samples, raising their level of missing data.

If you are comfortable with your dataset based on the above checks, you can use the scripts in the Formatting directory to prepare input files for a variety of downstream analyses.

Formatting Data Files

A number of scripts are provided in the Formatting directory that prepare input files for a variety of downstream analyses. Move to the Formatting directory and simply call the script you want to run with "perl<*space*>ScriptName.pl". Most of these scripts use files in the Output/Genotypes folder as starting points. If you have run FilterSNPs.pl multiple times with different values for missing data and SNP locations, the script will likely prompt you to specify which file you want to format. This will be one of the Haplotypes files (i.e. Haplotypes_100.35.txt) or one of the SNPMatrix files (i.e. SNPMatrix_100.35.txt). Some of these will currently only work with data that that are scored in all individuals. Some brief descriptions of the files currently available…

OutputStructure.pl
Uses a Haplotypes_X.Y.txt file as input, and allows for missing data, which is coded in the Structure input files as "-9". Output is file called 'StructureInput.txt', which should be ready to load into Structure (each sample is represented with two lines). Order of the samples in the StructureInput.txt file will be the same as that in the Haplotypes file used. Samples can be sorted alphabetically by sample name with the Sort_Haplotypes.pl script prior to running this script.

OutputGenepop.pl
Uses a Haplotypes_X.Y.txt file as input, and allows for missing data. Output is file called 'GenepopInput.txt'. As with the Structure script, samples can be sorted alphabetically with the Sort_Haplotypes.pl script prior to running this script. This can be especially helpful if the samples are named with this in mind, as samples must be ordered by population in the Genepop input file. At least a bit of manual editing is required before loading the GenepopInput.txt file into Genepop. First, if your samples are not arranged by population, you'll have to do this rearrangement manually. Then, separate each population with the POP flag. See Genepop documentation for more information.

OutputSNAPP.pl
Uses a SNPMatrix_X.Y.txt file as input, and allows for missing data. Produces a nexus file named "Beauti_Infile.nex" that can be loaded into Beauti to create an xml file to run in SNAPP. Arguments include…

subset — If set to '1', limits the SNAPP file to a subset of your samples. Requires a text file named "IncludedSamples.txt" in the Formatting directory that specifies sample names to include.

SNPsOnly — Set to '1' to print only variable sites to the SNAPP input file. Default is '0', which prints all sites.

unlinked — Flag indicating whether to print all or unlinked SNPs. Default '1' prints only unlinked SNPs.

OutputFastSimCoal_SinglePop.pl

Uses a SNPMatrix_X.Y.txt file as input to produce a single population site frequency spectrum for FastSimCoal analysis. Does not allow any missing data. You will likely have to do some manual editing to the SNPMatrix file prior to running this script. If creating an unfolded SFS, the outgroup sample must be the first sample in the SNPMatrix file (directly below the line with the locus names). If you have more than one population, use OutputFastSimCoal.pl (details below). Name of the output file will depend on the specific arguments used when running the script, and will be printed to the screen when the script completes. You'll need to add a prefix to this name that corresponds to the name of the model you are running – see FSC documentation for more info. Command line arguments include…

resamp Flag indicating whether to create resampled datasets. If set to '1', loci will be resampled according to arguments passed with the "num", "rep", and "pct" flags.

num Number of resampled datasets to create (Default is 1).

pct Percent of loci sampled to create each resampled dataset (1-100, default is 50).

rep Flag indicating whether to sample with replacement when generating resampled datasets. Default is '0' (sample loci without replacement).

unlinked Flag indicating whether to include only unlinked SNPs in the site frequency spectrum. Default is '0', which prints all SNPs.

folded Flag indicating the site frequency spectrum will be folded (the identity of the "derived" allele at each locus is inferred as the minor allele in the dataset). Default is '0', which requires that an outgroup sample is included as the first sample in the SNPMatrix file.

MonoScaled Flag indicating whether to scale the number of monomorphic sites based on the proportion of SNPs retained after removing linked SNPs. Default is '0', meaning all monomorphic sites are counted. If set to '1', the proportion of total SNPs that are unlinked (occurring in different reads) is calculated, and the total number of monomorphic sites is scaled by this proportion.

OutputFastSimCoal.pl.

Uses a SNPMatrix_X.Y.txt file as input to produce either joint or multidimensional site frequency spectra for FastSimCoal analyses from data with 2 or more populations. Does not allow any missing data. Samples must be arranged by population in the SNPMatrix file. Keep track of the number of samples (diploid individuals – not alleles) in each population, as you'll be prompted to enter these numbers during the run. Name of the output file(s) will depend on the specific arguments used when running the script, and will be printed to the screen when the script completes. If creating joint SFS, one file is created for each pairwise comparison of populations. Command line arguments include…

resamp Flag indicating whether to create resampled datasets. If set to '1', loci will be resampled according to arguments passed with the "num", "rep", and "pct" flags.

num Number of resampled datasets to create (Default is 1).

pct Percent of loci sampled to create each resampled dataset (1-100, default is 50).

rep Flag indicating whether to sample with replacement when generating resampled datasets. Default is '0' (sample loci without replacement).

unlinked Flag indicating whether to include only unlinked SNPs in the site frequency spectrum. Default is '0', which prints all SNPs.

folded Flag indicating the site frequency spectrum will be folded (the identity of the "derived" allele at each locus is inferred as the minor allele in the dataset). Default is '0', which requires that an outgroup sample is included as the first sample in the SNPMatrix file.

MonoScaled Flag indicating whether to scale the number of monomorphic sites based on the proportion of SNPs retained after removing linked SNPs. Default is '0', meaning all monomorphic sites are counted. If set to '1', the proportion of total SNPs that are unlinked (occurring in different reads) is calculated, and the total number of monomorphic sites is scaled by this proportion.

multi Flag indicating that a single multidimensional SFS will be produced. Default is '0' (pairwise joint spectra will be output). Note that multidimensional SFS are necessary if performing model choice with FastSimCoal data (i.e. AIC) – see FSC google group discussions for more info.

OutputFasta.pl
Outputs data in fasta format. Can handle missing data. Command line arguments include…

SNPsOnly Flag to print only SNPs (variable sites) to the fasta file. Default is '0' (all sites are included). Set to '1' to print just SNPs.

unlinked Flag to print only unlinked SNPs. Only applies if SNPsOnly flag is set to '1'. Default is '0' (all variable sites are included).

ambig Flag to print only line per sample, with ambiguity codes used for heterozygous sites. In the case of sites heterozygous for an indel, the base is printed, and the indel is ignored. Default is 0 (two lines are printed for each sample in the fasta file).

OutputTreeMix.pl
Creates input file for TreeMix. Similar to OutputFastSimCoal.pl, requires that samples be ordered by population in the SNPMatrix file. Can handle missing data. Command line arguments include…

linked Flag indicating whether to include all SNPs, or only unlinked SNPs in the TreeMix file. Default is '0' (includes only unlinked SNPs).

OutputMigrate.pl
Creates input file for Migrate. Requires a text file named 'PopulationsForMigrate.txt' in the Formatting directory that has 'Population' on the first line, and a population designation for each sample on individual lines below that. These population designations need to be in the same order as samples appear in the file 'TempFiles/GenotypesUpdate.txt'. You need a population designation for each line in the GenotypesUpdate.txt file, so if you have diploid samples, this 'PopulationsForMigrate.txt' file will have a total of 2N+1 lines, where N is the number of individuals in the dataset.

Sort_SNPMatrix.pl
Script to sort samples in the SNPMatrix_X_Y.txt file alphabetically by sample name. If sample locations are reflected in the sample names, this may be useful prior to running scripts such as OutputFastSimCoal.pl or OutputTreeMix.pl, which require that samples are grouped by population in this SNPMatrix file.

Sort_Haplotypes.pl
Similar to the Sort_SNPMatrix.pl script, but works on the Haplotypes_X_Y.txt file.

Calculate_Heterozygosity.pl

Script calculates and outputs the proportion of loci that are heterozygous. Output is a text file named "Heterozygosity_Results.txt" in the Formatting folder, which includes heterozygosity values for each sample, and optional output of population-level heterozygosity. Heterozygosity values are calculated across all loci sampled, including monomorphic loci. Command line arguments include…

MinReads The minimum number of reads necessary to score a monomorphic locus in an individual. Default is to use the same value used in the most recent Genotypes.pl run for genotyping polymorphic loci.

PopSizes An ordered vector of population sizes for calculation of population heterozygosities. Populations must be ordered in the Haplotypes_X_Y.txt file prior to running this script if using this option. Separate population sizes with a comma. If this argument is given, the script will print heterozygosities for each population in addition to the defaults of individual heterozygosities and overall heterozygosity.

Calculate_DStat_Pop.pl

Uses population allele frequency data to calculate a D statistic according to Equation 2 of Durand et al (2011) *Mol. Biol. Evol.* 28(8):2239–2252. Also includes an option for generating a series of D statistics based on allele frequencies that are resampled from a binomial distribution parameterized by the number of samples and the observed allele frequencies at each locus. Results are printed to file "DStat_pop_out.txt" in the Formatting directory. Command line arguments include…

bootstrap The number of datasets to generate for calculating D statistics based on resampled data.

ScoreDuplicates.pl

If a subset of the samples in your dataset have been run in duplicate, this script can help to assess levels of repeatability in your genotyping. To run it, the duplicate samples need to have different names in the barcodes file of the AftrRAD run. For example, say you run one library that contains 'Sample1' with the barcode ATACAT. Then you run a second library that has the same individual, but this time, it has the barcode CCAGAG. If you want to treat these as separate samples for the purpose of evaluating repeatability, then the barcode files should have them labeled with different names, such as 'Sample1' and 'Sample1b'. After the AftrRAD run, create a file called "Replicates.txt" that identifies pairs of replicate samples. Each pair of names is on a separate line, and the two names are separated with a tab.

```
Sample1     Sample1b
Sample2     Sample2b
```

Put this file in the Formatting directory with the script “ScoreDuplicates.pl” and then run this script. The output file will be called “Duplicate_Report.txt”. It will have the following columns…

Comparison: The two sample names being compared.

Sites Compared: The number of sites compared – sites with missing data in one or both of the samples are not included so this value will usually vary slightly from one comparison to another.

Number Matches: The number of matching genotype calls.

Proportion Matches: The proportion of scored sites called consistently in the two samples.

Mismatches_Homozygous_In_Sample1: In most cases, mismatches will be homozygous in one of the two samples (they can be genotyped as heterozygous for different alleles, but this is rare). The expectation is that the mismatches observed will be roughly evenly distributed between the two replicates in terms of which sample is homozygous (based on our experience, the homozygote is generally the incorrect call). Regardless, comparing this value to the comparable one for Sample 2 can be helpful, as biases toward one or the other may indicate problems with a sample, and possibly a library.

Mismatches_Homozygous_In_Sample2: Similar to above.

Loci_With_Both_Reps_Homozygous: Loci that were compared in which both replicate samples were homozygous.

Loci_With_At_Least_One_Het: Loci that were compared in which at least one of the two replicates was heterozygous. This value could be used (conservatively) as the denominator when calculating the proportion of mismatches.