# GBS SNP Calling Reference Optional Pipeline (GBS-SNP-CROP)

## User Manual v1.0

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## INTRODUCTION

The GBS SNP Calling Reference Optional Pipeline (GBS-SNP-CROP) is executed via a sequence of seven PERL scripts which integrate custom parsing and filtering procedures with well-known, vetted bioinformatic tools, giving the user full access to all intermediate files. By employing a novel strategy of SNP calling based on the correspondence of within-individual to across-population patterns of polymorphism, the pipeline is able to identify and distinguish high-confidence SNPs from both sequencing and PCR errors. The pipeline adopts a clustering strategy to build a population-tailored "Mock Reference" using the same GBS data for downstream SNP calling and genotyping. Designed for libraries of paired-end (PE) read of arbitrary lengths, GBS-SNP-CROP maximizes data usage by eliminating unnecessary data culling due to imposed length uniformity requirements. GBS-SNP-CROP is a complete bioinformatics pipeline developed primarily to support curation, research, and breeding programs wishing to utilize GBS for the cost-effective genome-wide characterization of plant genetic resources, mainly in the absence of a reference genome. The pipeline, however, can also be used when a reference genome is available, either as a standalone analysis or as a complement to reference-based analyses via alternative pipelines (e.g. TASSEL-GBS) or indeed its own reference-independent analysis.

## HOW TO CITE

Melo et al. (2015) GBS-SNP-CROP: A reference-optional pipeline for SNP discovery and plant germplasm characterization using genotyping-by-sequencing data. BMC Bioinformatics. DOI XX.

## PIPELINE WORKFLOW

SNP-GBS-CROP is accomplished via the following series of seven steps (i.e. PERL scripts), functionally grouped into four main stages of the analysis:

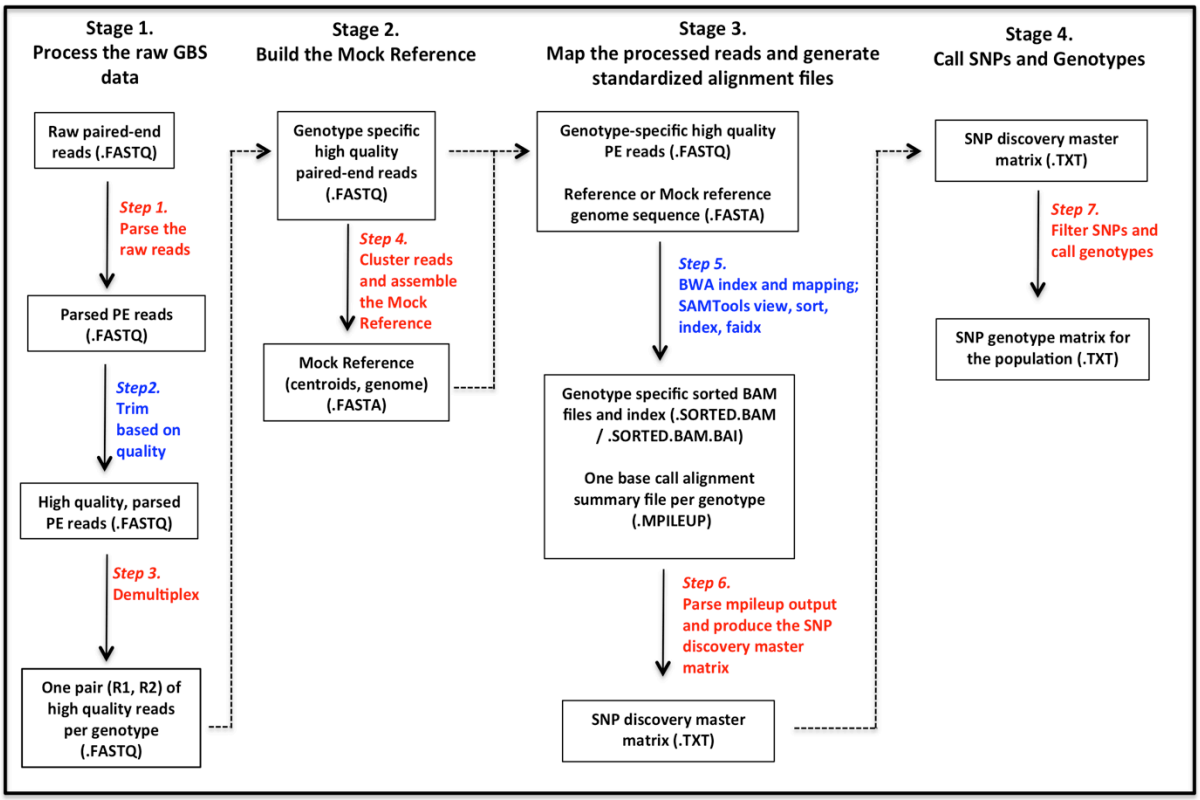
**Stage 1. Process the raw GBS data**
- Step 1: Parse the raw reads
- Step 2: Trim based on quality
- Step 3: Demultiplex

**Stage 2. Build the Mock Reference**
- Step 4: Cluster reads and assemble the Mock Reference

**Stage 3. Map the processed reads and generate standardized alignment files**
- Step 5: Align with BWA-mem and process with SAMTools
- Step 6: Parse mpileup output and produce the SNP discovery master matrix

**Stage 4. Calling SNPs and Genotypes**
- Step 7: Filter SNPs and call genotypes

Below is a schematic of the workflow, with inputs and outputs indicated for each step (arrow), Red steps (1, 3, 4, and 7) are executed via custom PERL scripts, while blue steps (2 and 5) call upon familiar bioinformatic tools.




**REPOSITORY**

https://github.com/iagohale/GBS-SNP-CROP.git

**STAGE 1. PROCESS THE RAW GBS DATA**

**Step 1: Parsing the raw reads**

**PERL script:**
**GBS-SNP-CROP-1.pl**

**Arguments (all required):**

| Flag | Explanation |
|---|---|
| -b | Barcode-ID file name (.txt file). See Appendix 1 |
| -fq | FASTQ file name seed (string). Ex: *FileNameSeed*_R1_001.fastq.gz |
| -s | Start number of FASTQ files (numeric). Ex: FileNameSeed_R1_00*1*.fastq.gz |

| | |
|---|---|
| -e | End number of FASTQ files (numeric). Ex: FileNameSeed_R1_0*34*.fastq.gz |
| -enz1 | Enzyme 1 restriction site residue sequence (string). Ex: *TGCA* (PstI) |
| -enz2 | Enzyme 2 restriction site residue sequence (string). Ex: *CGG* (MspI) |

**UNIX command example:**
perl /*path to GBS_SNP_CROP*/GBS-SNP-CROP-1.pl –b barcodesID.txt –fq *FileNameSeed* –s 1 –e 34 –enz1 TGCA –enz2 CGG

**FASTQ file naming convention:**

| | |
|---|---|
| *FileNameSeed*_R1_001.fastq.gz | *FileNameSeed*_R2_001.fastq.gz |
| *FileNameSeed*_R1_002.fastq.gz | *FileNameSeed*_R2_002.fastq.gz |
| *FileNameSeed*_R1_003.fastq.gz | *FileNameSeed*_R2_003.fastq.gz |

etc.

**Inputs:**
- CASAVA-generated paired-end (R1, R2) files (.fastq.tar.gz)
- Barcode-ID file (.txt)

**Outputs:**
Output files are placed in the following four directories:

| | |
|---|---|
| ./parsed: | Parsed paired-end fastq files, with barcode-annotated headers |
| ./singles: | Parsed, unpaired R1 reads, with barcode-annotated headers |
| ./summaries: | Text files containing parsing summary information |
| ./distribs: | Text files containing read length distribution summaries |

**Details:**

The code associated with Step 1 is compatible with Illumina1.8+ sequencing data, where the input files are assumed to be CASAVA-processed, paired-end (i.e. R1 and R2), compressed (*.tar.gz) FASTQ files. As per the protocol developed by Poland et al. (2012), these FASTQ files are assumed to contain multiplexed reads from a barcoded library of genotypes. To execute this stage of the pipeline, an auxiliary text file is required that associates each barcode with its corresponding genotype ID (see example "Barcode-ID" file in Appendix 1). The script for Step 1 processes the raw reads in a relatively standard manner, beginning by searching the R1 read for a high-confidence barcode sequence (i.e. no more than one mismatch, relative to the provided list of barcodes) immediately preceding the expected cut site remnant of the more frequent cutter. If both barcode and cut site are found, they are trimmed from the read, the barcode is appended to the header of both the R1 and R2 reads, and the pair is retained for further processing. This first parsing script then searches for the 3'-ends of the GBS fragment, indicated by the in-line presence of the Illumina common adapter, coupled with the appropriate cut site residue. If found, the reads are truncated appropriately. Finally, all reads consisting of a majority of uncalled bases (i.e. N's) are discarded.

## Step 2: Trim based on quality

**PERL script:**
**GBS-SNP-CROP-2.pl**

**Arguments (all required):**

| Flag | Explanation |
|---|---|
| -fq | FASTQ file name seed (string). Ex: *FileNameSeed*_R1_001.fastq.gz |

| | |
|---|---|
| -t | Number of threads used by Trimmomatic (numeric) |
| -ph | Trimmomatic phred scale: 33 or 64 (numeric) |
| -l | Trimmomatic LEADING parameter value (numeric) |
| -sl | Trimmomatic SLIDINGWINDOW parameter values (colon-separated numeric) |
| -tr | Trimmomatic TRAILING parameter value (numeric) |
| -m | Trimmomatic MINLEN parameter value (numeric) |

**UNIX command example:**
perl */path to GBS_SNP_CROP*/GBS-SNP-CROP-2.pl –fq L001 –t 10 –l 30 –sl 4:30 –tr 30 –m 32

**Inputs:**
- Parsed PE read file pair (.fastq), created in Step 1.

**Outputs:**
- High quality, parsed files of PE reads (.fastq)
- High quality, parsed files of orphan reads/singletons (.fastq)

Examples:

*FileNameSeed*_PE_R1parsed.fastq *FileNameSeed*_PE_R2parsed.fastq
*FileNameSeed*_SR_R1parsed.fastq *FileNameSeed*_SR_R2parsed.fastq

**Requirements:**
Java 7 or higher
Trimmomatic (Bolger et al., 2014)

**Details:**
Trimmomatic performs a variety of useful trimming tasks for Illumina paired-end data, of which only a subset are called upon by GBS-SNP-CROP. What follows is a description of the parameters provided to Trimmomatic via the command line:

LEADING: Trim bases from the beginning of a read, if below the specified quality threshold
SLIDINGWINDOW: Scan the read with a sliding window and truncate once the average quality within the window falls below the specified threshold.
TRAILING: Trim bases from the end of a read, if below the specified quality threshold
MINLEN: Cull a trimmed read entirely if its length is below a specified minimum

More information about Trimmomatic, including detailed descriptions of parameters, can be found at http://www.usadellab.org/cms/?page=trimmomatic

Please note that users may use read-cleaning bioinformatics tools other than those specified in the Steps 1 and 2 above. In this case, we encourage users to concatenate all surviving R1 reads into a single FASTQ file per library/lane, and the same for the corresponding R2 reads, because Step 3 (Demultiplex) requires this arrangement.

## Step 3: Demultiplex

**PERL script**
**GBS-SNP-CROP-3.pl**

**Arguments (all required):**

| Flag | Explanation |
|---|---|
| -b | Barcode-ID file name (.txt file). See Appendix 1 |
| -fqN | FASTQ file name (file). Ex: *FileNameSeed*_PE_R1parsed.fastq |
| -r | The type of reads being demultiplexed, R1 or R2 (string) |

**UNIX command example:**
perl /*path to GBS_SNP_CROP*/GBS-SNP-CROP-3.pl –b barcodesID.txt –fqN *FileNameSeed*_PE_R1parsed.fastq –r R1

**Inputs:**
- Barcode-ID file (.txt)
- One pair (R1, R2) of high-quality files (.fastq) per library, created in Step 2.

**Outputs:**
- One pair (R1, R2) of high-quality files (.fastq) per genotype, with all reads associated with that genotype placed inside a daughter directory named ./demultiplexed

Example:

| | |
|---|---|
| *TaxaName1*.R1.fastq | *TaxaName1*.R2.fastq |
| *TaxaName2*.R1.fastq | *TaxaName2.*R2.fastq |
| *TaxaName3*.R1.fastq | *TaxaName*3.R2.fastq |

**Details:**

This script creates a separate pair of FASTQ files (R1 and R2) for each genotype. Specifically, the script processes each entry of the high quality PE files, searches for the barcode appended to each header, and sends the reads for each index to its own genotype-specific FASTQ file. The final output from this step is a separate pair of FASTQ files (R1 and R2) for each genotype, containing all reads associated with that genotype, and featuring the ID name in the filename. Please note that it is necessary to run this script twice for each pair of PE files (PE_R1 and PE_R2).

# STAGE 2. BUILD THE MOCK REFERENCE

## Step 4: Cluster reads and assemble the Mock Reference

**PERL script:**
**GBS-SNP-CROP-4.pl**

**Arguments (all required):**

| Flag | Explanation |
|---|---|
| -b | Barcode-ID file name (.txt file). See Appendix 1 |
| -rl | Raw GBS read lengths (numeric). Ex: *100* bp, *150* bp |

| | |
|---|---|
| -pl | Minimum length required after merging to retain read (numeric) |
| -p | p-value for PEAR (Zhang et al. 2014) (numeric) |
| -id | Nucleotide identity value required for USEARCH read clustering (numeric) |
| -t | Number of threads dedicated to USEARCH clustering (Edgar, 2010) (numeric) |
| -MR | Mock Reference name (string) |

**UNIX command example:**
perl */path to GBS_SNP_CROP*/GBS-SNP-CROP-4.pl –b barcodeID.txt –rl 150 –pl 32 –p 0.01 –id 0.93 –t 10 –MR *MockRefName*

**Inputs:**
- Barcode-ID file (.txt)
- Genotype-specific high quality PE files (.fastq), created in Step 3.

**Outputs:**
- A daughter directory named ./fastqForRef which contains the PEAR-assembled and manually-stitched FASTQ files used for building the Mock Reference
- Two different Mock Reference FASTA files:
  *MockRefName*.MockRef_Genome.fasta (used for alignment)
  *MockRefName*.MockRef_Clusters.fasta (used for optional downstream analysis)

**Requirements:**
PEAR (Zhang et al., 2014)
USEARCH (Edgar, 2010)

**Details:**

If a suitable reference genome is available for the target population, one may move directly to Stage 3 of the pipeline, skipping Stage 2 entirely; although it may be worth conducting a Mock Reference analysis in any case, to complement the results of reference-based approaches. If a reference is unavailable, however, Stage 2 is necessary. In it, the parsed and quality-filtered reads from Stage 1 are used to build a GBS-specific, reduced-representation reference (Mock Reference) to enable GBS read mapping and facilitate SNP discovery.

First, the pipeline calls upon the PEAR software package to merge the processed paired-end reads into single reads spanning the full GBS fragment lengths, wherever sequence overlap is sufficient (≥ 10 bp) between the paired reads to justify merging. For each genotype designated in the Barcode-ID file to contribute to the Mock Reference assembly (see Appendix 1), this step generates four different FASTQ files: An “assembled” file, containing successfully merged reads; two “unassembled” files (R1 and R2), comprised of sequentially paired R1 and R2 reads which could not be merged; and a “discarded” file, containing assembled reads that failed to pass PEAR's user-specified minimum sequence length requirement.

Second, the pipeline stitches together all unmerged reads by joining pairs of sufficiently long "unassembled" R1 and R2 sequences together with an intermediate run of 20 high-quality A's, thus producing a FASTQ file of "stitched" R1+R2 reads:

```
@header with barcode
R1+AAAAAAAAAAAAAAAAAAAA+R2
+
R1quality+IIIIIIIIIIIIIIIIII+R2 quality
```

Representing the reduced genomic space targeted by the GBS restriction protocol, these PEAR-assembled and manually-stitched reads are then concatenated into a single FASTQ

file per genotype.

Third, GBS-SNP-CROP calls upon the USEARCH software package to cluster these "assembled" and "stitched" reads based on a user-specified similarity threshold, thereby producing a reduced list of non-redundant consensus sequences (centroids) that span the GBS fragment space. To accomplish this, the USEARCH clustering procedure is executed first within each selected genotype and subsequently, if more than one genotype is selected to build the Mock Reference, across all selected genotypes. It is this final list of non-redundant clusters that are linked together, end-to-end, to constitute the Mock Reference.

## STAGE 3. MAP THE PROCESSED READS AND GENERATE STANDARDIZED ALIGNMENT FILES

### Step 5: Align with BWA-mem and process with SAMTools

**PERL script**
**GBS-SNP-CROP-5.pl**

**Arguments:**

| Flags | Explanation |
|---|---|
| -b | Barcode-ID file name (.txt file). See Appendix 1 |
| -ref | Reference FASTA file, either Mock Reference or true reference (file) |
| -samf | SAMTools flags controlled by small f (numeric) |
| -samF | SAMTools flags controlled by CAPS F (numeric) |
| -t | Number of threads dedicated to BWA-mem mapping (numeric) |

**UNIX command example:**
Using both "f" and "F" flags from SAMTools:
perl */path to GBS_SNP_CROP*/GBS-SNP-CROP-5.pl –b barcodeID.txt –ref *MockRefName*.MockRef_Genome.fasta –samf 2 –samF 2308 –t 10

Using only the "f" flag from SAMTools:
perl */path to GBS_SNP_CROP*/GBS-SNP-CROP-5.pl –b barcodeID.txt –ref *MockRefName*.MockRef_Genome.fasta –samF 0 –samf 2 –t 10

**Inputs:**
- Barcode-ID file (.txt)
- Genotype-specific high quality PE files (.fastq), created in Stage 1.
- Reference genome or Mock Reference [genome], created in Stage 2 (.fasta).

**Outputs:**
- Aligned and filtered reads (.bam)
- Sorted BAM files (.sorted.bam)
- Indexed BAM files (.sorted.bam.bai)
- Indexed reference or Mock Reference (.fasta.idx)
- Base call alignment summary file (.mpileup)

Note: One complete set of the above output files is created for each genotype in the population.

**Requirements:**

BWA-mem algorithm (Li & Durbin 2009)
SAMtools (Li et al., 2009)

**Details:**

In this step, the BWA-mem algorithm (Li & Durbin 2009) is called upon to align the Stage 1 processed reads, genotype-by-genotype, to the reference/Mock Reference. SAMtools (Li et al., 2009) is then used to accomplish the following steps: 1) Filter the mapped reads via SAMtools flags, retaining only those which map appropriately as pairs without potentially confounding secondary or supplementary alignments to the reference; 2) Convert the filtered SAM files to BAM files; 3) Index and sort the BAM files; and 4) Index the FASTA reference sequence. These five steps (BWA-mem alignment and the four SAMtools procedures) are carried out individually for each genotype.

After alignment, we encourage users to advance only those reads for further analysis that map in proper pairs (-f2) with no supplementary alignments (-F2308), criteria applied via samtools view flags. For more detailed explanation about SAMTools flags, please refer to https://broadinstitute.github.io/picard/explain-flags.html.

Finally, the SAMtools mpileup algorithm is used to make a liberal identification of all potential SNPs based on the individual alignments of each genotype considered separately, producing a base call alignment summary (mpileup file) for each genotype. In this step, we encourage the use of SAMTools flags –B (to reduce false SNP calling caused by misalignment) and –C50 (to delete reads with excessive mismatches).

## Step 6: Parse mpileup output and produce the SNP discovery master matrix

**PERL script:**
**GBS-SNP-CROP-6.pl**

**Arguments:**

| Flag | Explanation |
|---|---|
| -b | Barcode-ID (file). See Appendix 1 |
| -out | SNP discovery master matrix (.txt file). Should be text tab delimited file |

**UNIX command example:**
perl /*path to GBS_SNP_CROP*/GBS-SNP-CROP-6.pl –b barcodeID.txt –out SNPs_master_matrix.txt

**Inputs:**
- Barcode_ID file (.txt)
- One base call alignment summary file (.mpileup) per genotype, created in Step 5

**Outputs:**
- One reduced base call alignment summary count file (.txt) per genotype
- The SNP discovery master matrix (.txt)

**Details:**

The Step 6 script scans the genotype-specific mpileup files from Step 5 and extracts the necessary information to create distilled "count" files, text files containing four essential tab-delimited columns: (1) Reference genome/chromosome identifier; (2) SNP position; (3) Reference base at that position; and (4) A comma-delimited string containing

alignment information at that position (i.e. depths of A, C, G, and T reads). Example:

| | | | |
|---|---|---|---|
| chr01 | 71019 | A | 0,15,0,0 |
| chr01 | 135677 | C | 0,89,0,0 |
| chr01 | 135714 | T | 0,0,115,0 |

Each genotype-specific count file is then parsed, such that only those rows containing reads polymorphic to the reference sequence are retained. Once this is completed for each genotype separately, Step 6 proceeds by mining the full set of reduced genotype-specific count files to generate a single, non-redundant master list of all potential SNP positions throughout the target population. Comprehensive alignment information is then extracted from the original count files for each genotype for all potential SNP positions in the master list and the data organized into a "master matrix" for the population.

## STAGE 4. CALLING SNPS AND GENOTYPES

### Step 7: Filter SNPs and call genotypes

**Perl script:**
**GBS-SNP-CROP-7.pl**

**Arguments:**

| Flag | Explanation |
|---|---|
| -in | SNP discovery master matrix input file (file). The output from last step |
| -out | SNP genotyping matrix for the population output file (file) |
| -mnHoDepth | Minimum depth required for homozygote call (numeric) |
| -mnHeDepth | Minimum depth required for heterozygote call (numeric) |
| -mnAlleleProp | Minimum threshold of ratio of lower-depth to higher-depth allele (numeric) |
| -call | Minimum acceptable percentage of genotyped individuals to retain a SNP (numeric) |
| -mnAvgDepth | Minimum acceptable average SNP depth (numeric) |
| -mxAvgDepth | Maximum acceptable average SNP depth (numeric) |

**UNIX command example:**
perl */path to GBS_SNP_CROP*/GBS-SNP-CROP-7.pl –in SNPs_master_matrix.txt –out SNPs_genotyping_matrix.txt –mnHoDepth 7 –mnHeDepth 2 –mnAlleleProp 0.1 –call 0.25 –mnAvgDepth 4 –mxAvgDepth 200

**Inputs:**
- SNP discovery master matrix (.txt), created in Step 6

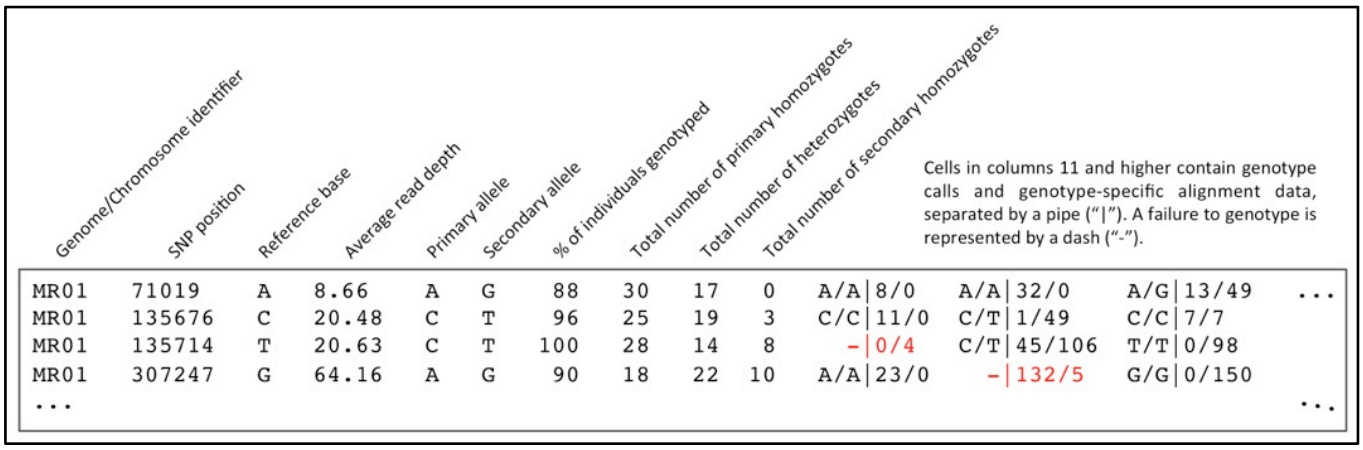
**Outputs:**
- Final SNP genotyping matrix (.txt)

**Details:**
In this step, the master matrix created in Step 6 is systematically pared down based on a series of SNP-culling filters to arrive at a final "SNP genotyping matrix" containing only high-confidence SNPs and genotypes. First, the master list of potential SNPs is parsed

based upon a flat criteria of independence, namely that a SNP is retained for further consideration if and only if there exist independent reads of the putative secondary allele, at a specified minimum depth (-mnHeDepth), across at least three genotypes. This simple requirement for independent instances of the secondary allele is an essential strategy for minimizing false SNP declarations due to sequencing and PCR errors.

For calling heterozygotes, a given genotype must have a user-specified minimum read depth for each allele (-mnHeDepth); and the depth ratio of the lower-depth to the higher-depth allele must exceed a ploidy-appropriate threshold (-mnAlleleProp). The GBS-SNP-CROP genotyping criterion for homozygotes is more stringent, recommending a relatively high minimum depth (e.g. ≥ 7 for -mnHoDepth) in an effort to reduce the rate of erroneous calls. Next, in an effort to retain only broadly informative SNPs, the matrix is further reduced such that all SNPs (i.e. rows) are discarded for which less than some minimum proportion of population (-call) have genotype calls. Finally, users are able to retain only those SNPs with an average depth (D) within a specified range, as a means of deleting all markers with low coverage as well as those that are over-represented. In our analysis, we accept only those SNPs such that $3 < D < 200$.

To facilitate the characterization of those high-confidence SNPs that satisfy the above filters and criteria, the final SNP genotyping matrix contains both summary statistics as well as complete genotype-specific alignment data for each retained SNP. The first ten columns of the matrix feature the following information: 1) Genome/chromosome identifier; 2) SNP position; 3) Reference base; 4) Average read depth at that SNP position across the population; 5) Primary allele (i.e. the most frequent allele at that position, based on read depth across the population); 6) Secondary allele (i.e. the less frequent allele at that position); 7) Percentage of individuals genotyped at that position; 8) Total number of potential homozygotes for the primary allele; 9) Total number of potential heterozygotes; and 10) Total number of potential homozygotes for the secondary allele. Columns 11 and higher contain the complete alignment data for each individual genotype for all possible SNP positions. The structure of the final SNP genotyping matrix is shown in the figure below:




**DOWNSTREAM TOOL**

**PERL script:**
**GBS-SNP-CROP-8.pl**

**Function:**

To transform the final SNP genotyping matrix into forms appropriate for downstream analysis via familiar software packages, specifically R (input matrix), TASSEL GUI (HapMap file), and/or PLINK (.tped file).

**Arguments:**

| Flag | Explanation |
|---|---|
| -in | Final SNP genotyping matrix input file (file) |
| -out | Output label name without extension (string) |
| -b | Barcode-ID file name (.txt file). See Appendix 1 |
| -formats | The name(s) of the software packages (R, Tassel, Plink) for which an input-compatible file format should be created. If more than one format is desired, the names should be separated by commas, as in the examples shown below. (string) |

**UNIX command example:**
**Creating input files for R, TASSEL GUI, and PLINK**
perl /*path to GBS_SNP_CROP*/GBS-SNP-CROP-8.pl –in SNPs_genotyping_matrix.txt –out SNP_genotyping –b barcodeID.txt –formats R,Tassel,Plink

**Creating input file only for Tassel**
perl /*path to GBS_SNP_CROP*/GBS-SNP-CROP-8.pl –in SNPs_genotyping_matrix.txt –out SNP_genotyping –b barcodeID.txt –formats Tassel

**Inputs:**
- Final SNP genotyping matrix (.txt), created in Step 7

**Outputs:**
- R-compatible final SNP genotyping matrix ("*GBS-SNP-CROP*_R_in.txt")
- TASSEL GUI-compatible final SNP genotyping matrix ("*GBS-SNP-CROP*_Tassel_in.hmp.txt")
- PLINK-compatible final SNP genotyping matrix ("*GBS-SNP-CROP*_Plink_in.tped")

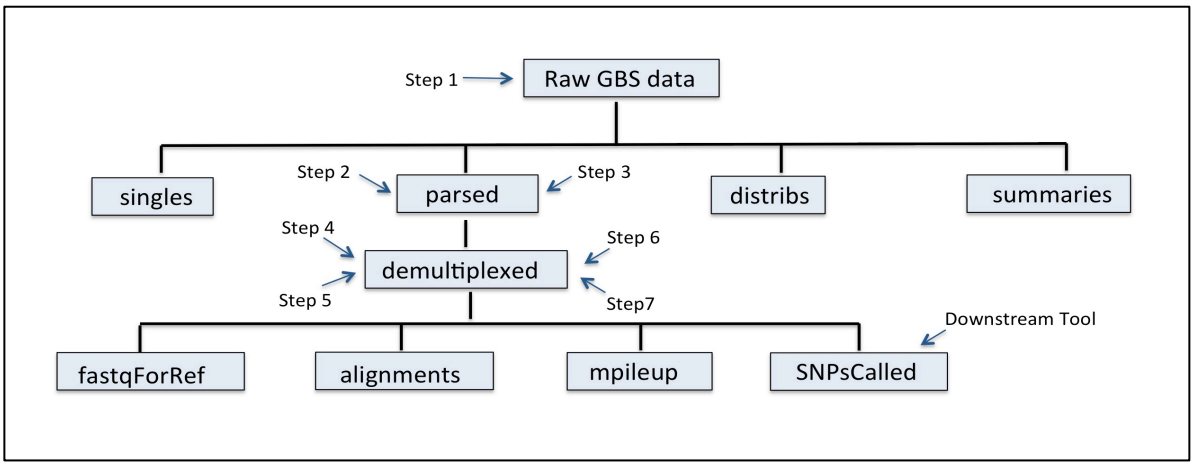
**Details:**

Based on the output formats specified by the user, this script converts the final SNP genotyping matrix from Step 7 into:
- A version appropriate for diversity analyses within R (R Development Core Team, 2013) (e.g. calculating distance metrics, generating cladograms, etc.), achieved by replacing primary homozygotes with 0, heterozygotes with 0.5, secondary homozygotes with 1, and unassignable genotypes with "NA";
- A HapMap file for use as input into Tassel GUI (Glaubitz et al., 2014), allowing users to easily access the functionality of that software package for forward analysis; and/or
- The transposed .tped file required by the whole genome association analysis toolset PLINK (Purcell et al., 2007).

## DIRECTORY STRUCTURE CREATED BY GBS-SNP-CROP

The figure below shows the structure of all directories created by GBS-SNP-CROP over the course of the analysis and is intended to help users navigate efficiently during pipeline execution. Starting in a directory with the "Raw GBS data" (FASTQ), users should move to the "parsed" directory to execute steps 2 and 3. Afterwards, inside the "demultiplexed" directory, users will run all further steps (4, 5, 6 and 7). In end, both the

SNP master matrix and the SNP genotyping matrix can be found inside the "SNPsCalled" directory, within which the three optional downstream scripts can be executed.

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**APPENDIX 1: BARCODE-ID file example**

The Barcode-ID file is a simple tab-delimited text file required by GBS-SNP-CROP for the purpose of associating barcodes with genotype names. This file also allows users to specify which individuals from the target population are to be used for building the Mock Reference.

The file itself contains only three tab-delimited fields per line (i.e. three columns), with one line needed for each unique genotype*barcode combination in the population. The first field (column) contains the 6-10 bp barcode sequences, or index, the second contains the genotype name associated with that barcode, and the third contains a simple flag ( “YES” or “NO”) indicating if the genotype is to be used to build the Mock Reference ("YES") or not ("NO"). The file requires no header. Example of the appearance of the file in Excel:

| Barcode | Genotype name | Used for building the Mock Reference? |
|---|---|---|
| TGACGCCA | Lib1_01 | YES |
| CAGATA | Lib1_02 | YES |
| GAAGTG | Lib2_05 | YES |
| TAGCGGAT | Lib3_10 | NO |
| TATTCGCAT | Lib3_11 | YES |

The final file, saved without a header as a tab-delimited text file:

```
TGACGCCA    Lib1_01    YES
CAGATA      Lib1_02    YES
GAAGTG      Lib2_05    YES
TAGCGGAT    Lib3_10    NO
TATTCGCAT   Lib3_11    YES
```

As discussed in the accompanying manuscript (Melo et al., 2015), users will need to complete Stage 1 of the pipeline to decide, based on number of recovered reads per genotype, which individual(s) should be used to build the Mock Reference. In this situation, users should modify column 3 of the Barcode-ID file before running Step 4 of GBS-SNP-CROP. In other words, users can initialize column 3 with all "YES" flags in Stage 1 of the pipeline and then simply update the “YES/NO” column before Stage 2, using the result of Stage 1 to decide how many and which genotypes to use for building the Mock Reference.