# CALLING VARIANTS TUTORIAL: GATK

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Mechanisms of AdaptatioN and GenOmics

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# INTRODUCTION

Genome-wide variant detection involves the identification of single nucleotide polymorphisms (SNPs) which are changes of one nucleotide in a region of the genome and small insertions and deletions called indels from next generation sequencing data.

This tutorial will focus on the different parts of SNP detection from the mapping step to variant calling and the statistical analyses needed for false positive filtering.

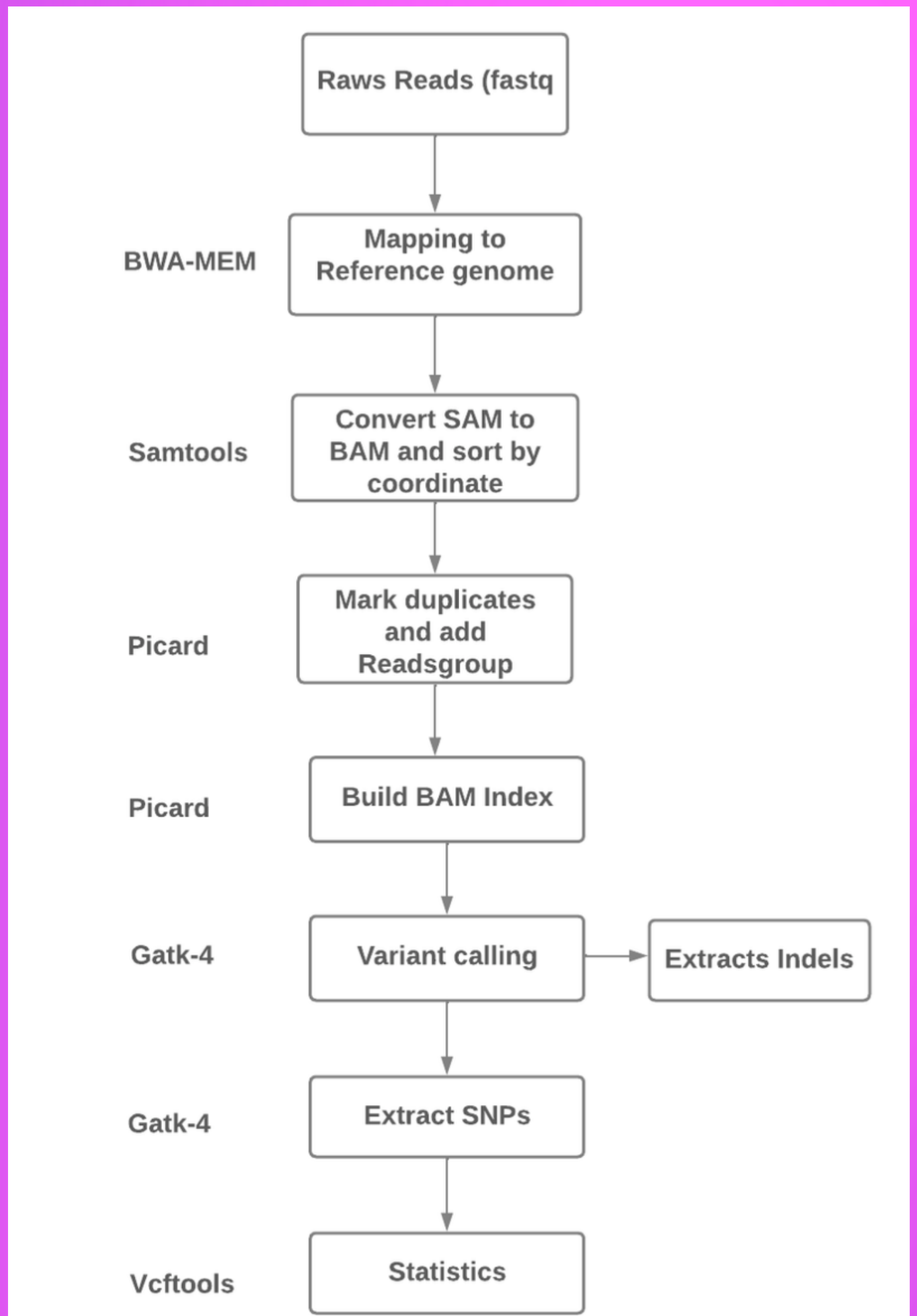
# Principle of this tutorial

The goal of this work is to allow any user to perform variant detection analysis (SNP or INDELS) using GATK and picard tools without having any knowledge in bioinformatics.

# TOOLS REQUIRED

- Bwa 0.7.8
- Picard tools
- Gatk 4
- Samtools
- vcftools
- IGV
- Bcftools

# Example of Workflow



# DATA SETS

We use here the dataset of the At66 collection of Valérie HINOUX. This collection is composed of 173 individuals of ***Arabidopsis thaliana*** sequenced in short illumina reads. We have chosen only one individual which is PO10C to realize this tutorial.

The ***Arabidopsis thaliana*** reference genome was used to map the paired short reads.

# Pre-treatement

First you need to create all the folders to store the output data and declare all the file paths that you will need in the different stages of the analysis.

## Creation of index file with reference genome and reference dictionary

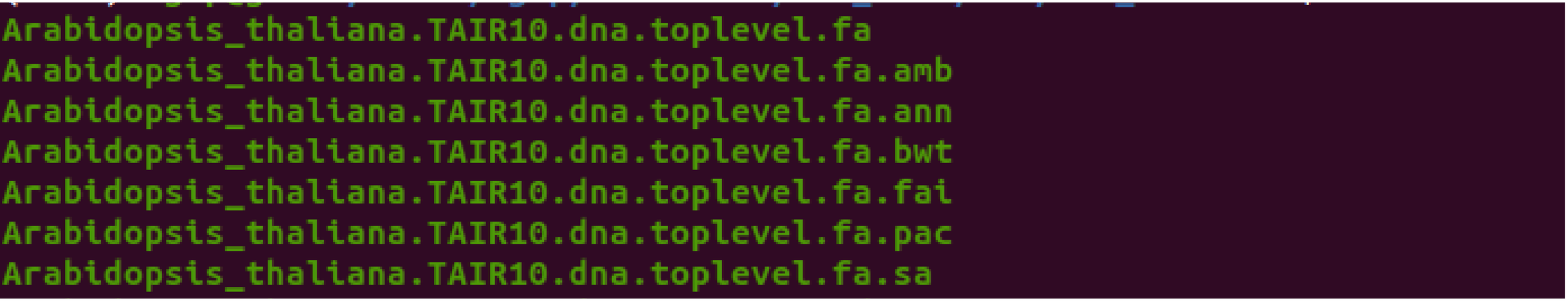
With the help of the reference sequence, we can generate the index files and a dictionary sequence that are essential for the execution of GATK. They can be created with the following two command lines:

```
samtools faidx reference.fasta

bwa index reference.fasta
```

# Output of the commands

To know if the commands worked, we should expect the following output obtained with the **ls** command.



# Reads Alignment

We use in this step **BWA-MEM** to align the paired reads to a reference genome and **Samtools** to convert the **SAM file** to **BAM** format and index it.

NB: Other alternatives can be used such as Bowtie2.

Once we have the reference index, we can proceed to the alignment step. We run BWA as follows:

```
bwa mem -t 8 reference.fasta reads_1.fastq reads_2.fastq | samtools sort -@ 8
-o output.sorted.bam

samtools index output.sorted.bam
```

# Alignment statistics

This part will allow us to see the percentage of reads that have been mapped to the reference genome. We use Samtools flagstats for this.

```
samtools flagstat output.sorted.sam > alignment_metrics.txt
```

## Output exemple :

```
3769644 + 0 in total (QC-passed reads + QC-failed reads)
3618048 + 0 primary
 + 0 secondary
51596 + 0 supplementary
 + 0 duplicates
 + 0 primary duplicates
2643954 + 0 mapped (95.26% : N/A)
2492358 + 0 primary mapped (95.23% : N/A)
3618048 + 0 paired in sequencing
1809024 + 0 read1
1809024 + 0 read2
1665798 + 0 properly paired (91.73% : N/A)
2408684 + 0 with itself and mate mapped
3674 + 0 singletons (0.35% : N/A)
46680 + 0 with mate mapped to a different chr
50634 + 0 with mate mapped to a different chr (mapQ>=5)
```

# Marking and removing duplicates with Picard tools

This step consists in removing duplicates often produced during the construction of the libraries using PCR. We use Picard tools markduplicate to mark these duplicates and remove them in the BAM files.

```
java -jar picard.jar MarkDuplicates --INPUT output.sorted.bam --OUTPUT output.markdup.bam
--METRICS_FILE output.metrics.txt --VALIDATION_STRINGENCY SILENT --CREATE_INDEX true --
REMOVE_DUPLICATES true
```

# Output with picard MarkDuplicates

If the command is executed correctly, we should see an output like this:

```
INFO    2022-08-22 11:00:01     MarkDuplicates  After generateDuplicateIndexes f
reeMemory: 6134522248; totalMemory: 8048345088; maxMemory: 8048345088
INFO    2022-08-22 11:00:01     MarkDuplicates  Marking 4101430 records as dupli
cates.
INFO    2022-08-22 11:00:01     MarkDuplicates  Found 121385 optical duplicate c
lusters.
INFO    2022-08-22 11:00:01     MarkDuplicates  Reads are assumed to be ordered
by: coordinate
INFO    2022-08-22 11:01:49     MarkDuplicates  Written    10 000 000 records.
Elapsed time: 00:01:47s.  Time for last 10 000 000:  107s.  Last read position:
Chr4:2 626 752
INFO    2022-08-22 11:03:28     MarkDuplicates  Writing complete. Closing input
iterator.
INFO    2022-08-22 11:03:28     MarkDuplicates  Duplicate Index cleanup.
INFO    2022-08-22 11:03:28     MarkDuplicates  Getting Memory Stats.
INFO    2022-08-22 11:03:28     MarkDuplicates  Before output close freeMemory:
8055760232; totalMemory: 8110735360; maxMemory: 8110735360
INFO    2022-08-22 11:03:28     MarkDuplicates  Closed outputs. Getting more Mem
ory Stats.
INFO    2022-08-22 11:03:28     MarkDuplicates  After output close freeMemory: 8
126539112; totalMemory: 8181514240; maxMemory: 8181514240
[Mon Aug 22 11:03:28 CEST 2022] picard.sam.markduplicates.MarkDuplicates done. E
lapsed time: 4,87 minutes.
Runtime.totalMemory()=8181514240
```

# Add of read groups

The read groups are provided as a string. This information is essential for using GATK, as it does not work without a read group tag.

```
java -jar picard.jar AddOrReplaceReadGroups --INPUT output.sorted.markdup.bam
--OUTPUT output.sorted.markdup.RG.bam --RGSM {NAME} --RGID {NAME} --RGLB
lib1 --RGPL illumina --RGPU 2
```

# Output with picad reads groups

We will have to have an exit like this if everything works out well :

```
INFO    2022-08-22 11:16:21     AddOrReplaceReadGroups  Processed    10 000 000 records.
Elapsed time: 00:01:47s.  Time for last 1 000 000:   10s.  Last read position: Chr4:2 626
752
INFO    2022-08-22 11:16:32     AddOrReplaceReadGroups  Processed    11 000 000 records.
Elapsed time: 00:01:59s.  Time for last 1 000 000:   11s.  Last read position: Chr4:8 328
169
INFO    2022-08-22 11:16:42     AddOrReplaceReadGroups  Processed    12 000 000 records.
Elapsed time: 00:02:09s.  Time for last 1 000 000:   10s.  Last read position: Chr4:17 489
 661
INFO    2022-08-22 11:16:52     AddOrReplaceReadGroups  Processed    13 000 000 records.
Elapsed time: 00:02:19s.  Time for last 1 000 000:    9s.  Last read position: Chr5:7 946
489
INFO    2022-08-22 11:17:04     AddOrReplaceReadGroups  Processed    14 000 000 records.
Elapsed time: 00:02:30s.  Time for last 1 000 000:   11s.  Last read position: Chr5:15 323
 829
INFO    2022-08-22 11:17:14     AddOrReplaceReadGroups  Processed    15 000 000 records.
Elapsed time: 00:02:41s.  Time for last 1 000 000:   10s.  Last read position: Chr5:24 585
 944
INFO    2022-08-22 11:17:23     AddOrReplaceReadGroups  Processed    16 000 000 records.
Elapsed time: 00:02:50s.  Time for last 1 000 000:    9s.  Last read position: ChrPt:20 03
1
INFO    2022-08-22 11:17:32     AddOrReplaceReadGroups  Processed    17 000 000 records.
Elapsed time: 00:02:59s.  Time for last 1 000 000:    8s.  Last read position: ChrPt:73 31
4
INFO    2022-08-22 11:17:40     AddOrReplaceReadGroups  Processed    18 000 000 records.
Elapsed time: 00:03:07s.  Time for last 1 000 000:    8s.  Last read position: ChrPt:123 8
96
INFO    2022-08-22 11:17:50     AddOrReplaceReadGroups  Processed    19 000 000 records.
Elapsed time: 00:03:17s.  Time for last 1 000 000:    9s.  Last read position: */*
[Mon Aug 22 11:17:58 CEST 2022] picard.sam.AddOrReplaceReadGroups done. Elapsed time: 3,42
 minutes.
Runtime.totalMemory()=397934592
```

# BAM file index with Picard tools

This indexer file is important to do before using GATK, because it does not work without the indexed BAM file.

```
java -jar picard.jar BuildBamIndex --INPUT output.sort.bowtie.markdup.RG.bam
```

**Here is the resulting output:**

```
11:30:56.669 INFO  NativeLibraryLoader - Loading libgkl_compression.so from jar:file:/home/lgdp/pi
card.jar!/com/intel/gkl/native/libgkl_compression.so
[Mon Aug 22 11:30:56 CEST 2022] BuildBamIndex --INPUT PO10C-bwa.RG.bam --VERBOSITY INFO --QUIET fa
lse --VALIDATION_STRINGENCY STRICT --COMPRESSION_LEVEL 5 --MAX_RECORDS_IN_RAM 500000 --CREATE_INDE
X false --CREATE_MD5_FILE false --GA4GH_CLIENT_SECRETS client_secrets.json --help false --version
false --showHidden false --USE_JDK_DEFLATER false --USE_JDK_INFLATER false
[Mon Aug 22 11:30:56 CEST 2022] Executing as lgdp@gwas on Linux 5.4.0-124-generic amd64; OpenJDK 6
4-Bit Server VM 1.8.0_152-release-1056-b12; Deflater: Intel; Inflater: Intel; Provider GCS is not
available; Picard version: Version:2.27.1
INFO    2022-08-22 11:31:36     BuildBamIndex   Successfully wrote bam index file PO10C-bwa.RG.bai
[Mon Aug 22 11:31:36 CEST 2022] picard.sam.BuildBamIndex done. Elapsed time: 0,66 minutes.
Runtime.totalMemory()=756023296
```

# Variants Calling : SNP et Indels

We use GATK HaplotypeCaller to perform variant calling.
GATK HaplotypeCaller is able to call SNPs and Indels simultaneously using the reference sequence and the BAM file.

```
java -jar gatk-package-4.2.6.1-local.jar HaplotypeCaller -R reference.fasta -I output.sorted.markdup.RG.bam -O output_variants.vcf
```

# Filtering SNPs AND Indels

This step will allow us to filter the SNPs from the Indels in order to process them independently using GATK SelectVariants. Here, we are only interested in SNPs.

## SNP Filtering :

```
java -jar gatk-package-4.2.6.1-local.jar -T SelectVariants -R reference.fasta -V
output_variants.vcf -selectType SNP -o output_snps.vcf
```

## SNP Filtering :

```
java -jar gatk-package-4.2.6.1-local.jar -T SelectVariants -R reference.fasta -V
output_variants.vcf -selectType indels -o output_snps.vcf
```

# GATK script on Population

In the case of a population, the script remains the same for each individual. The difference is made after obtaining the vcf files of each individual which will be merged thereafter with the function CombineGVCFs of gatk.

```
#!/usr/bash

Ref=/path/reference.fasta
Bam_file=/path/Bam_file
Output=/path/outDir

#Index Reference Sequence
samtools faidx reference.fasta
bwa index reference.fasta
java -jar picard.jar CreateSequenceDictionary R=reference.fasta O=reference.fasta.dict

for fq1 in /path/file_R1.fq
do
fq2=$(echo $fq1 | sed -e "s/_R1.fq.*/_R2*/g")
OUT=$(basename $fq1 | awk -F "_" '{print $1}')

bwa mem -t 8 $REF $fq1 $fq2 |samtools sort -@ 8 -o $Bam_file/$OUT.sorted.bam -

samtools flagstats $Bam_file/$OUT.sorted.bam > $output/$OUT.stats.txt

#Mark the duplicate with picard tools
java -jar picard.jar MarkDuplicates --INPUT $Bam_file/$OUT.sorted.bam --OUTPUT $Bam_file/$OUT.markdup.bam
--VALIDATION_STRINGENCY SILENT --CREATE_INDEX true --REMOVE_DUPLICATES true

#Add the read groups on the bam file
java -jar picard.jar AddOrReplaceReadGroups --INPUT $Bam_file/$OUT.sorted.markdup.bam
--OUTPUT $Bam_file/$OUT.markdup.RG.bam --RGSM $OUT --RGID $OUT --RGLB lib1 --RGPL illumina --RGPU 2

#Sort bam markdup with picard
java -jar picard.jar SortSam --INPUT $Bam_file/$OUT.markdup.RG.bam --OUTPUT $Bam_file/$OUT.sorted.markdup.RG.bam
--VALIDATION_STRINGENCY SILENT --SORT_ORDER coordinate

#index bam
java -jar picard.jar BuildBamIndex --INPUT $Bam_file/$OUT.sorted.markdup.RG.bam

#Called the Variants (indels and SNP)
java -jar gatk-package-4.2.6.1-local.jar HaplotypeCaller -R $REF -I $Bam_file/$OUT.sorted.markdup.RG.bam
-O $Output/$OUT.POP.g.vcf.gz -ERC GVCF

rm -r $Bam_file/$OUT.markdup.bam*
rm -r $Bam_file/$OUT.markdup.RG.bam*

done

#Create vcf file list
ls $vcf_file/*.g.vcf.gz > $vcf_file/vcfs.list

#Merged per samples gVCF files produced by HaplotypeCaller into a multi-sample gVCF file
java -jar gatk-package-4.2.6.1-local.jar CombineGVCFs -R $REF --variant $vcf_file/vcfs.list
-O $Output/$OUT.POP.merged.g.vcf.gz

#GenotypeGVCFs the multi-sample gVCF file produced by CombineGVCFs
java -jar gatk-package-4.2.6.1-local.jar GenotypeGVCFs -R $Ref -V $Output/$OUT.POP.merged.g.vcf.gz
-O $Output/$OUT.POP.genotype.vcf

#Filter only SNP with gatk on multi-sample file
java -jar gatk-package-4.2.6.1-local.jar SelectVariants -R $Ref -V $Output/$OUT.POP.genotype.vcf
--select-type-to-include SNP -O $Output/$OUT.POP.SNP.vcf

#Filter only INDEL with gatk on multi-sample file
java -jar gatk-package-4.2.6.1-local.jar SelectVariants -R $Ref -V $Output/$OUT.POP.genotype.vcf
--select-type-to-include INDEL -O $Output/$OUT.POP.indels.vcf
```

# Use GATK on cluster

The execution of a script on a cluster is not always the same when you are on a personal machine. The difference can be noticed in the writing of the script or in the execution. For the case of variant calls like gatk, it is necessary to create two scripts, a run which contains the code to be executed and a launch which contains the path of the target files and the script to be executed. In our case, we write a script for an OpenPBS cluster with the qsub function which allows to execute the scripts.

# run_gatk.sh Script on cluster

```
#PBS -N name_your_run
#PBS -l nodes=1:ppn=6
#PBS -q lmem
#PBS -M email.fr
#PBS -m e

#samtools v1.13
module load samtools/1.15
module load bowtie2/2.4.4
module load bwa/0.7.17
module load gatk/4.2.5.0
module load picard/

Ref=/path/reference.fasta
Bam_file=/path/Bam_file
Output=/path/outDir

FQ1=$file
FQ2=$(echo $file | sed -e "s/_R1.fq.*/_R2*/g")
OUT=$(basename $file | awk -F "_" '{print $1}')


#file=$file
#OUT=$(basename $file | awk -F "." '{print $1}')

#bowtie2 --very-sensitive-local -p 4 -x $REF -1 $fq1 -2 $fq2 -S $Bam_file/$OUT-bowtie.sam
#samtools reheader -P $Bam_file/$OUT-bowtie.sam $Bam_file/$OUT-bowtie.bam


bwa mem -t 8 $REF $fq1 $fq2 |samtools sort -@ 8 -o $Bam_file/$OUT.sorted.bam -

samtools flagstats $Bam_file/$OUT.sorted.bam > $output/$OUT.stats.txt

#Mark the duplicate with picard tools
java -jar picard.jar MarkDuplicates --INPUT $Bam_file/$OUT.sorted.bam --OUTPUT $Bam_file/$OUT.markdup.bam
--VALIDATION_STRINGENCY SILENT --CREATE_INDEX true --REMOVE_DUPLICATES true

#Add the read groups on the bam file
java -jar picard.jar AddOrReplaceReadGroups --INPUT $Bam_file/$OUT.sorted.markdup.bam
--OUTPUT $Bam_file/$OUT.markdup.RG.bam --RGSM $OUT --RGID $OUT --RGLB lib1 --RGPL illumina --RGPU 2

#Sort bam markdup with picard
java -jar picard.jar SortSam --INPUT $Bam_file/$OUT.markdup.RG.bam --OUTPUT $Bam_file/$OUT.sorted.markdup.RG.bam
--VALIDATION_STRINGENCY SILENT --SORT_ORDER coordinate

#index bam
java -jar picard.jar BuildBamIndex --INPUT $Bam_file/$OUT.sorted.markdup.RG.bam

#Called the Variants (indels and SNP)
java -jar gatk-package-4.2.6.1-local.jar HaplotypeCaller -R $REF -I $Bam_file/$OUT.sorted.markdup.RG.bam
-O $Output/$OUT.POP.g.vcf.gz -ERC GVCF


rm -r $Bam_file/$OUT.markdup.bam*
rm -r $Bam_file/$OUT.markdup.RG.bam*
```

# launch_gatk.sh Script on a cluster

```
#!/bin/bash

#path your fastq file
list_file=$(ls /path/fastq_file)

#Loupe on fastq file
for file in $list_file
do

#qsub allows to run the script
qsub -v file=$file /path/run_gatk.sh
done
```

# Script gatk_merge.sh on cluster

This script merges all the vcf files obtained with gatk HaplotypeCaller into one file, then genotypes and filters them to separate SNPs from Indels.

```
#PBS -N gatk_merge
#PBS -l nodes=1:ppn=6
#PBS -q lmem
#PBS -M email@.fr
#PBS -m e

module load gatk/4.2.5.0

Ref=/path/reference.fasta
vcf_file=/path/vcf_file
Output=/path/outDir

#Create list vcf_file
ls $vcf_file/*.g.vcf.gz > $vcf_file/vcfs.list


#Merged per samples gVCF files produced by HaplotypeCaller into a multi-sample gVCF file
java -jar gatk-package-4.2.6.1-local.jar CombineGVCFs -R $REF --variant $vcf_file/vcfs.list
-O $Output/$OUT.POP.merged.g.vcf.gz

#GenotypeGVCFs the multi-sample gVCF file produced by CombineGVCFs
java -jar gatk-package-4.2.6.1-local.jar GenotypeGVCFs -R $Ref -V $Output/$OUT.POP.merged.g.vcf.gz
-O $Output/$OUT.POP.genotype.vcf

#Filter only SNP with gatk on multi-sample file
java -jar gatk-package-4.2.6.1-local.jar SelectVariants -R $Ref -V $Output/$OUT.POP.genotype.vcf
--select-type-to-include SNP -O $Output/$OUT.POP.SNP.vcf

#Filter only INDEL with gatk on multi-sample file
java -jar gatk-package-4.2.6.1-local.jar SelectVariants -R $Ref -V $Output/$OUT.POP.genotype.vcf
--select-type-to-include INDEL -O $Output/$OUT.POP.indels.vcf
```

The command to run for this script is as follows:

**qsub gatk_merge.sh**

# Variant Statistics

We use here tools able to filter variants from a vcf file to make distributions in the form of plot with R namely vcftools and RStudio.
The objective of these statistics is to bring a decision support on the choice of the filters to be applied on the vcf brût.

We can make analyses on several parameters such as the average depth of coverage of the variants, on the qualities of variants, on the frequency of minor alleles (MAF) or on the missing data. And in order to do this, we use R scripts to make plots.

These statistics can be applied on a population, here we use the whole At66 collection.

# Average depth of coverage per individual

This part will allow us to calculate the average depth of coverage of each individual in the collection.

This calculation is done with vcftools thanks to its --depth function.

The command used is the following:

```
vcftools --vcf output_snps.vcf --depth --out output_snp
```

Here we obtain a file with an extension .idepht.mean containing the name of the individual, the number of variants and the average depth of coverage.

# example of average coverage output per individual

| INDV | N_SITES | MEAN_DEPTH | |
|---|---|---|---|
| PO01A-vs-TAIR10 | 4455580 | 15.4357 | |
| PO01B-vs-TAIR10 | 4455531 | 16.3596 | |
| PO01C-vs-TAIR10 | 4455667 | 13.3344 | |
| PO02A-vs-TAIR10 | 4455334 | 15.1015 | |
| PO02B-vs-TAIR10 | 4455660 | 14.8374 | |
| PO02C-vs-TAIR10 | 4455594 | 16.8962 | |
| PO02D-vs-TAIR10 | 4455727 | 15.0376 | |
| PO02E-vs-TAIR10 | 4455458 | 19.4515 | |
| PO02F-vs-TAIR10 | 4455534 | 18.8342 | |
| PO02G-vs-TAIR10 | 4455162 | 15.4907 | |
| PO02H-vs-TAIR10 | 4455254 | 13.8889 | |
| PO02I-vs-TAIR10 | 4455497 | 16.0763 | |
| PO02J-vs-TAIR10 | 4455689 | 15.7405 | |
| PO03A-vs-TAIR10 | 4455692 | 16.537 | |
| PO03B-vs-TAIR10 | 4455737 | 14.4205 | |
| PO03C-vs-TAIR10 | 4456088 | 19.1175 | |
| PO03D-vs-TAIR10 | 4455609 | 16.3083 | |
| PO03E-vs-TAIR10 | 4455757 | 16.7391 | |
| PO03F-vs-TAIR10 | 4456027 | 17.673 | |
| PO03G-vs-TAIR10 | 4455884 | 16.918 | |
| PO03H-vs-TAIR10 | 4455203 | 14.4419 | |
| PO03I-vs-TAIR10 | 4455729 | 15.3194 | |
| PO04A-vs-TAIR10 | 4455818 | 17.6617 | |

# Average depth of coverage per variants

The filter on the average depth of the variants will allow us to eliminate the false positive calls, i.e. the variants with a low or very high coverage.

This calculation is done with **vcftools** thanks to its **--site-mean-depth** function.
The command used is the following:

```
vcftools --vcf output_snps.vcf --site-mean-depth --out output_snps
```

Here we obtain a file with an extension .ldepht.mean containing the position of each variant, its average coverage depth and the variation of the coverage depth within the individual.

# example of average coverage output by variant

| CHROM | POS | MEAN_DEPTH | VAR_DEPTH |
|---|---|---|---|
| Chr1 | 55 | 2.24277 | 14.4989 |
| Chr1 | 56 | 2.16763 | 12.7915 |
| Chr1 | 60 | 2.46821 | 12.4597 |
| Chr1 | 73 | 2.23121 | 11.5509 |
| Chr1 | 115 | 7.78035 | 31.0678 |
| Chr1 | 138 | 12.0173 | 43.1102 |
| Chr1 | 194 | 3.75145 | 32.4553 |
| Chr1 | 201 | 4.12139 | 40.0259 |
| Chr1 | 214 | 4.55491 | 43.6438 |
| Chr1 | 216 | 4.61272 | 42.9829 |
| Chr1 | 237 | 6.74566 | 68.8768 |
| Chr1 | 253 | 8.31214 | 86.588 |
| Chr1 | 346 | 18.237 | 29.3679 |
| Chr1 | 353 | 25.4798 | 188.053 |
| Chr1 | 425 | 24.5145 | 190.391 |
| Chr1 | 429 | 24.3988 | 193.125 |
| Chr1 | 437 | 24.1387 | 190.597 |
| Chr1 | 471 | 23.3988 | 175.055 |
| Chr1 | 502 | 16.3699 | 19.1996 |
| Chr1 | 508 | 16.3179 | 19.439 |
| Chr1 | 524 | 16.3526 | 29.6249 |
| Chr1 | 551 | 16.0751 | 30.1629 |
| Chr1 | 553 | 16.0809 | 30.5632 |
| Chr1 | 560 | 16.0058 | 31.3081 |
| Chr1 | 564 | 16.0636 | 31.8157 |

**What do you think is due to the huge difference in coverage in some sites?**

# Variants quality

The calculation of the quality of the variants gives us information on the quality score of each variant. This will then allow us to apply filters to eliminate poor quality variants.

The calculation is done with **vcftools** with its **--site-quality** function using the following command:

```
vcftools --vcf output_snps.vcf --site-quality --out output_snps
```

Here we get a file with a .lqual extension containing the position of each variant and the quality value.

# example of output on the quality variants

| CHROM | POS | QUAL |
|---|---|---|
| Chr1 | 55 | 134.57 |
| Chr1 | 56 | 164.29 |
| Chr1 | 60 | 133.01 |
| Chr1 | 73 | 111.84 |
| Chr1 | 115 | 390.63 |
| Chr1 | 138 | 5123.24 |
| Chr1 | 194 | 919.43 |
| Chr1 | 201 | 2333.5 |
| Chr1 | 214 | 2184.99 |
| Chr1 | 216 | 1339.75 |
| Chr1 | 237 | 2277.6 |
| Chr1 | 253 | 2076.1 |
| Chr1 | 346 | 109125 |
| Chr1 | 353 | 4916.78 |
| Chr1 | 425 | 4957.5 |
| Chr1 | 429 | 1142.1 |
| Chr1 | 437 | 2543.19 |
| Chr1 | 471 | 4722.46 |
| Chr1 | 502 | 111344 |

# Minors allele frequencies

We calculate here the frequency of minor alleles of each variant with **vcftools** with the function **--freq2**.

The command used is the following:

```
vcftools --vcf output_snps.vcf --freq2 --out output_snps
```

We obtain here a file with a .freq extension containing the position of each variant and the frequency of minor alleles.

# Example of output on the Minors frequencies

| CHROM | POS | N_ALLELES | N_CHR | {FREQ} | |
|---|---|---|---|---|---|
| Chr1 | 55 | 2 | 70 | 0.971429 | 0.0285714 |
| Chr1 | 56 | 2 | 38 | 0.947368 | 0.0526316 |
| Chr1 | 60 | 2 | 88 | 0.977273 | 0.0227273 |
| Chr1 | 73 | 2 | 18 | 0.888889 | 0.111111 |
| Chr1 | 115 | 2 | 40 | 0.85 | 0.15 |
| Chr1 | 138 | 2 | 78 | 0.435897 | 0.564103 |
| Chr1 | 194 | 2 | 34 | 0.941176 | 0.0588235 |
| Chr1 | 201 | 2 | 34 | 0.882353 | 0.117647 |
| Chr1 | 214 | 2 | 70 | 0.942857 | 0.0571429 |
| Chr1 | 216 | 2 | 40 | 0.95 | 0.05 |
| Chr1 | 237 | 2 | 64 | 0.9375 | 0.0625 |
| Chr1 | 253 | 2 | 222 | 0.981982 | 0.018018 |
| Chr1 | 346 | 2 | 346 | 0.147399 | 0.852601 |
| Chr1 | 353 | 2 | 346 | 0.959538 | 0.0404624 |
| Chr1 | 425 | 2 | 346 | 0.959538 | 0.0404624 |
| Chr1 | 429 | 2 | 346 | 0.988439 | 0.0115607 |
| Chr1 | 437 | 2 | 346 | 0.976879 | 0.0231214 |
| Chr1 | 471 | 2 | 342 | 0.959064 | 0.0409357 |
| Chr1 | 502 | 2 | 330 | 0.1 | 0.9 |
| Chr1 | 524 | 2 | 344 | 0.994186 | 0.00581395 |
| Chr1 | 560 | 2 | 344 | 0.994186 | 0.00581395 |
| Chr1 | 564 | 2 | 342 | 0.994152 | 0.00584795 |
| Chr1 | 572 | 2 | 324 | 0.987654 | 0.0123457 |
| Chr1 | 573 | 2 | 344 | 0.997093 | 0.00290698 |
| Chr1 | 581 | 2 | 344 | 0.994186 | 0.00581395 |
| Chr1 | 583 | 2 | 328 | 0.981707 | 0.0182927 |
| Chr1 | 584 | 2 | 344 | 0.997093 | 0.00290698 |

# Missing data

There are two ways to calculate missing data. One can calculate the proportion of missing data per sample with the **--missing-ind** function of **vcftools** or per variant with **--missing-site.**

The commands used are the following:

```
vcftools --vcf output_snps.vcf --missing-ind --out output_snps
vcftools --vcf output_snps.vcf --missing-site --out output_snps
```

We obtain here two files, the first with an .imiss extension (for the individuals) and another with an lmiss extension (for the variants) containing the position of each variant or each individual as well as the percentage of missing data.

# Example of output on missing data

| CHR | POS | N_DATA | N_GENOTYPE_FILTERED | N_MISS | F_MISS |
|---|---|---|---|---|---|
| Chr1 | 55 | 346 | 0 | 276 | 0.797688 |
| Chr1 | 56 | 346 | 0 | 308 | 0.890173 |
| Chr1 | 60 | 346 | 0 | 258 | 0.745665 |
| Chr1 | 73 | 346 | 0 | 328 | 0.947977 |
| Chr1 | 115 | 346 | 0 | 306 | 0.884393 |
| Chr1 | 138 | 346 | 0 | 268 | 0.774566 |
| Chr1 | 194 | 346 | 0 | 312 | 0.901734 |
| Chr1 | 201 | 346 | 0 | 312 | 0.901734 |
| Chr1 | 214 | 346 | 0 | 276 | 0.797688 |
| Chr1 | 216 | 346 | 0 | 306 | 0.884393 |
| Chr1 | 237 | 346 | 0 | 282 | 0.815029 |
| Chr1 | 253 | 346 | 0 | 124 | 0.358382 |
| Chr1 | 346 | 346 | 0 | 0 | 0 |
| Chr1 | 353 | 346 | 0 | 0 | 0 |
| Chr1 | 425 | 346 | 0 | 0 | 0 |
| Chr1 | 429 | 346 | 0 | 0 | 0 |
| Chr1 | 437 | 346 | 0 | 0 | 0 |
| Chr1 | 471 | 346 | 0 | 4 | 0.0115607 |
| Chr1 | 502 | 346 | 0 | 16 | 0.0462428 |
| Chr1 | 508 | 346 | 0 | 16 | 0.0462428 |
| Chr1 | 524 | 346 | 0 | 2 | 0.00578035 |
| Chr1 | 551 | 346 | 0 | 22 | 0.0635838 |
| Chr1 | 553 | 346 | 0 | 22 | 0.0635838 |
| Chr1 | 560 | 346 | 0 | 2 | 0.00578035 |
| Chr1 | 564 | 346 | 0 | 4 | 0.0115607 |
| Chr1 | 572 | 346 | 0 | 22 | 0.0635838 |
| Chr1 | 573 | 346 | 0 | 2 | 0.00578035 |
| Chr1 | 581 | 346 | 0 | 2 | 0.00578035 |
| Chr1 | 583 | 346 | 0 | 18 | 0.0520231 |
| Chr1 | 584 | 346 | 0 | 2 | 0.00578035 |
| Chr1 | 586 | 346 | 0 | 0 | 0 |
| Chr1 | 611 | 346 | 0 | 18 | 0.0520231 |
| Chr1 | 614 | 346 | 0 | 20 | 0.0578035 |
| Chr1 | 615 | 346 | 0 | 20 | 0.0578035 |
| Chr1 | 631 | 346 | 0 | 18 | 0.0520231 |

# Plot on statistical data

To create diagrams, we use the **RStudio** programming language with the **tidyverse library**. To do this, we will load the package on **RStudio** with the following command:

```
library(tidyverse)
```

# Statistics on the average depth of individuals

Here we examine the average depth of each variant. This is the number of reads mapped to that position.
To do this, we must first read the file on R by positioning on the data directory.

```
stat_depth <- read_delim("./output_snps.idepht", delim = "\t",
                         col_names = c("chr", "pos", "mean_depth", "var_depth"), skip = 1)
```

We then look at the median, mean and standard deviation with the summary command

```
summary(stat_depth$mean_depth)
```

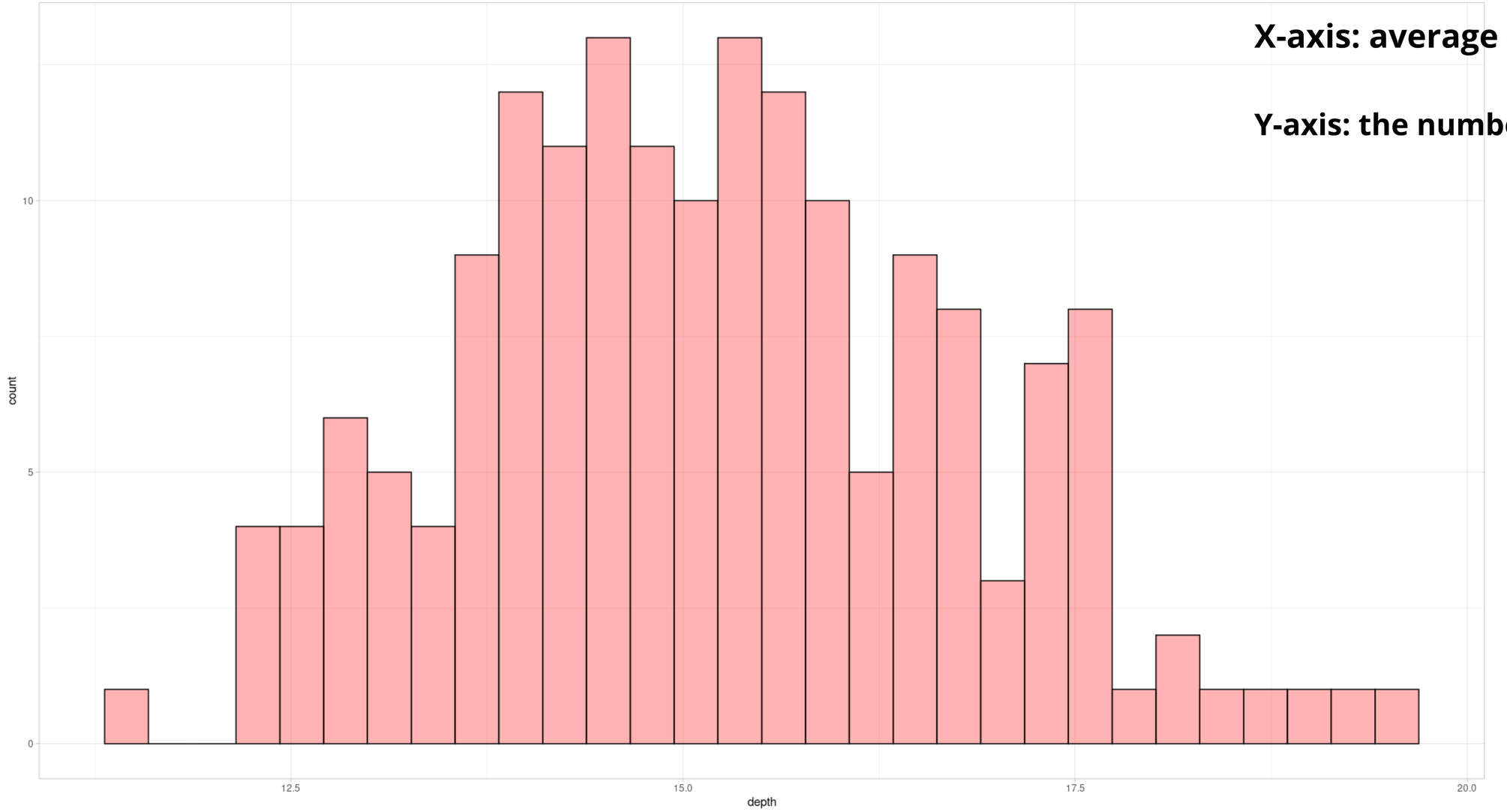
# Statistics on the average depth of individuals

We use the **ggplot** package to build graphs on R.

```
plot_depth<- ggplot(stat_depth, aes(mean_depth)) +
geom_density(fill = "dodgerblue1", colour = "black", alpha = 0.3)

plot_depth + theme_light() + xlim(0, 100)
```

# Histogramm on depht coverage per individual




**X-axis: average coverage**

**Y-axis: the number of individuals**

# Statistics on variant quality

Variant quality refers to the measure of confidence we have in variant calls.
First, we read from the site quality report that we generated using the following command:

```
stats_qual <- read_delim("./output_snps.lqual", delim = "\t",
                         col_names = c("chr", "pos", "qual"), skip = 1)
```

We then look at the median, mean and standard deviation with the summary command :

```
summary(stat_qual$qual)
```

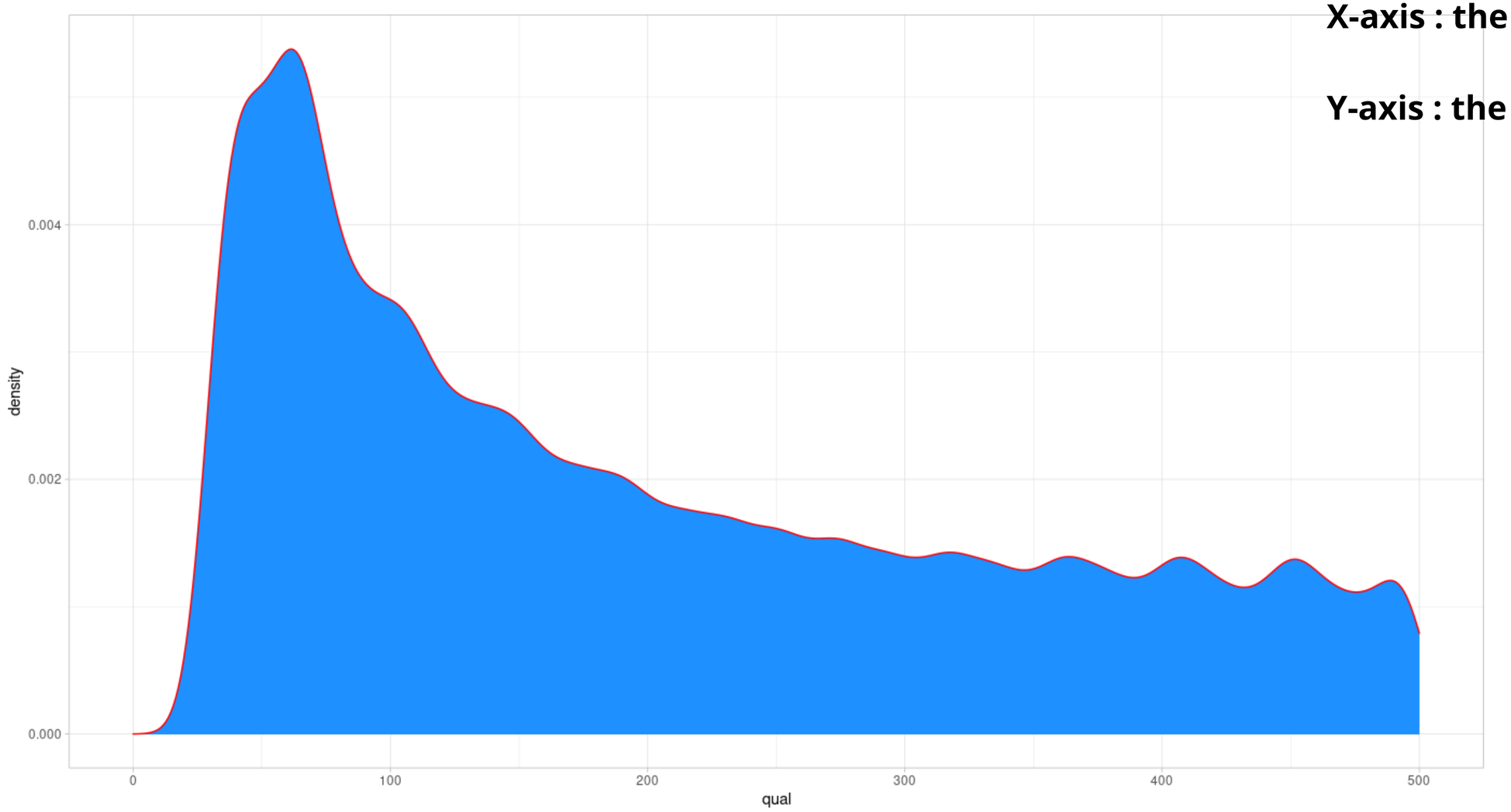
# Statistics on variant quality

We use the ggplot package to build graphs on R.

```
plot_qual <- ggplot(var_qual, aes(qual)) + geom_density(fill = "dodgerblue1", colour = "black", alpha = 0.3)

plot_qual + theme_light() + xlim(0, 500) #xlim à définir selon la taille des echantillons
```

# Graph on variants quality




**X-axis : the variant quality**

**Y-axis : the density**

# Statistics on Minors allele frequencies

We will examine the distribution of allele frequencies to set the minimum MAF threshold.

```
stat_freq <- read_delim("./output_snps.frq", delim = "\t",
                        col_names = c("chr", "pos", "nalleles", "nchr", "a1", "a2"), skip = 1)

# find minor allele frequency
stat_freq$maf <- var_freq %>% select(a1, a2) %>% apply(1, function(z) min(z))
```

We then look at the median, mean and standard deviation with the summary command.

```
summary(stat_freq$freq)
```

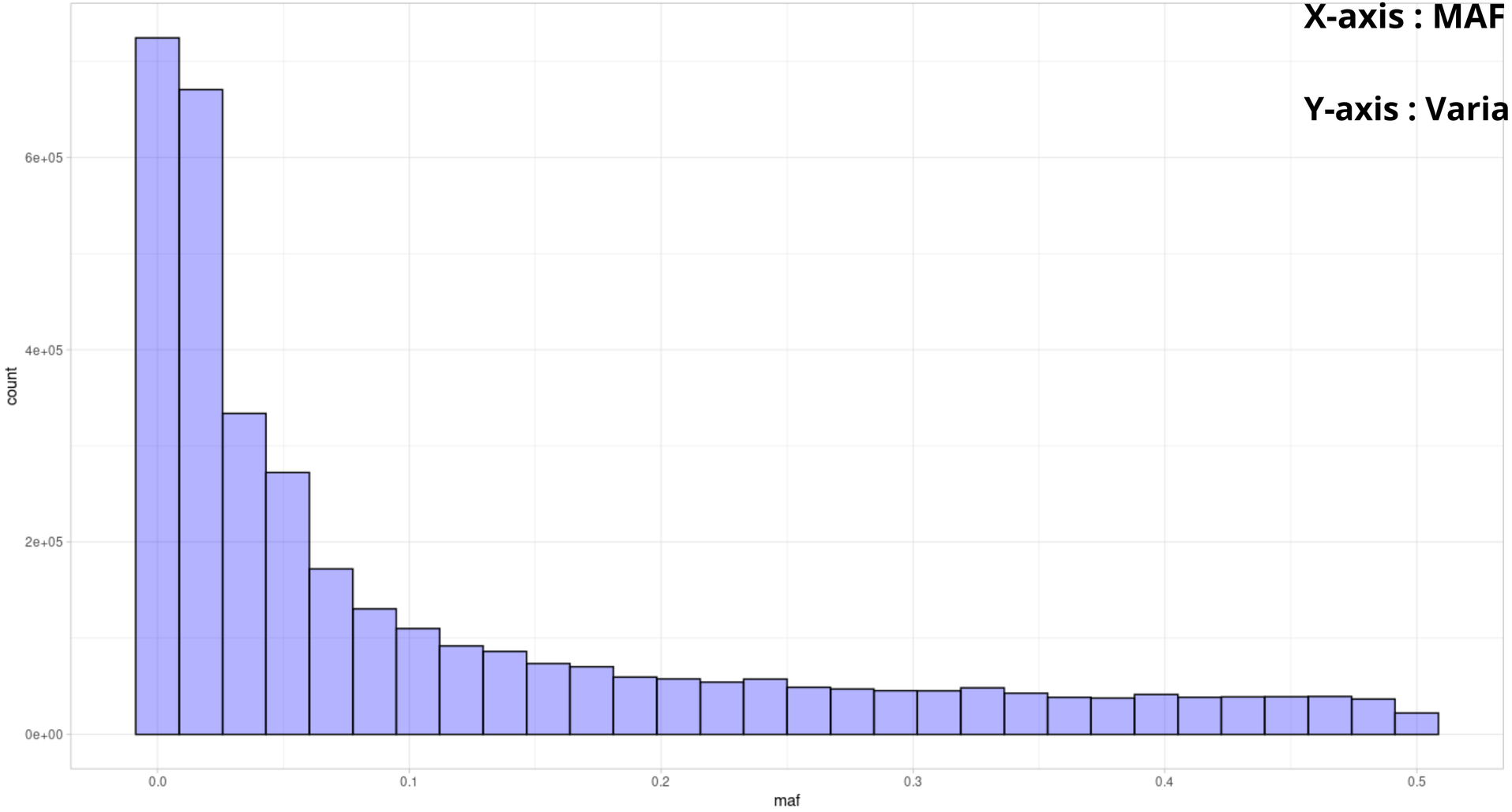
# Statistics on Minors allele frequencies

Here we plot the distribution of minor allelic frequencies using ggplot.

```
plot_maf <- ggplot(stat_freq, aes(maf)) + geom_density(fill = "dodgerblue1", colour = "black", alpha = 0.3)

plot_qual + theme_light()
```

# Graph on Minors allele frequencies MAF




**X-axis : MAF value la valeur de MAF**

**Y-axis : Variant number**

# Filtering of the vcf with the parameters defined with the stats

After the obtained statistics, we will be able to define the values of the minimum and maximum average depth of each variant, the quality of the variants and the **MAF** defined by the respective terms **--min-meanDP/--max-meanDP, --minQ, --maf**. Don't forget also to define the minimum value of missing data which is interesting for the **GWAS step**.

The command used is the following:

```
vcftools --vcf output_snps.vcf --max-missing INT --min-meanDP/--max-meanDP INT --minQ INT --maf INT  --recode --stdout
```


Thank
you