# Amplicomsat: Scoring microsatellite genotypes from amplicon sequencing and eDNA samples

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## Background

Amplicon sequencing represents an efficient way to genotype microsatellite markers, allowing for increased multiplexing of samples and markers. A particular case where amplicon sequencing is favorable is when more than two microsatellite alleles occur in each sample locus combination. Examples of such samples are forensic analysis with possibly more than one offender DNA mixed or eDNA analysis where several individual microscopic life stages might be present in each sample (e.g., kelp gametophytes). Amplicomsat can output the observed (sequenced) counts of alleles (OAC) produced from such forensic or eDNA samples. Alleles can be scored based on their fragment size or sequence variation. Genotype tables for classical microsatellite analysis can also be produced. A variety of diagnostic plots are created throughout the pipeline.

## Setup

We start by loading the package

The Amplicomsat package can be installed from GitHub

```r
# We need package devtools to install from GitHub
library(devtools)
install_github("UWMAlberto-Lab/Amplicomsat")
```

We can now load the package

```r
# Loading the package
library(Amplicomsat)
```

Two set-up files and one directory, where sample fastq files are saved, are required.

### Required files:

1) A tab-delimited file with primer information saved in the working directory is required. The format is shown in Table 1 for an example with 11 markers. The *nmotif* (last column) indicates how many microsatellite sequence motif repeats a read should have to be retained. Consider changing this for different types of microsatellites (di-nucleotides, three-nucleotides, might require larger numbers).

Table 1: Argument LocusInfo table format, containing loci name, FWD and RVS primer sequence, and microsatellite repeat motif

| Locus | Fprimer | Rprimer | Motif | nmotif |
|---|---|---|---|---:|
| Nl21 | AACGCGTCATAAATACATACCAGGT | AACCTGCGAATCCAAATTGCACGCT | ACAT | 2 |
| Nl24 | TGGGAGCAAGGAATTAGGACGACCC | AGGTACATTCTTGTTCGTCTTGGTT | AGAT | 2 |
| Nl25 | CGGTGACCGTAAGCGAAGAGCCTTC | TAGCGCCATCGTGAGGTTAACCTGC | CCTG | 2 |
| Nl26 | CGTCAACAATTTGAAAGTGACACCC | AGCAACAGGATCTAGGCTGTAACCT | ATCC | 2 |
| Nl27 | ACCTCTGCTGTGAACCGGAGAAACGA | AACAGAGGCGGTGAGAGCGAGCTAA | ACAT | 2 |

| | | | | |
|---|---|---|---|---|
| Nl30 | GAAGCATCTCCACTGTGCGACCGAG | TCGTGCTCATTTCGTCTTCTTCCTT | GTTT | 2 |
| Nl40 | CTTCTTGCCGCCTTTGCCCTAGCTC | TTGTAGGGAGATAAGGCGTGCGGGA | GCCT | 2 |
| Nl47 | ACTGTAGCGCCTGAAGCTTCTGAGGT | ACGTAACCCGCCGTCAATAGG | AAAC | 2 |
| Nl50 | CGAGATGAACAACTCCGCGGCGAAA | GGGTTACCAGGTACCGGGCAGTCAA | GCCT | 2 |
| Nl59 | TGCTGATAATGACTGCTGTTTGCTCC | GAGAGCGGTAGTGGCAAACGAACGG | GCTT | 2 |
| Ner2 | GAAGAGGTGCGGTGGCTT | GGAATCGGAACCCAAAATTAGT | ATTCGG | 2 |

2) A calibration file for the allele-scoring algorithm, saved as a tab-delimited file in the R working directory. The example in Table 2 shows the first two columns (loci) of a calibration file needed for running the *GenotypeModel* function. The default name for the file is “CalibrationSamples.txt”.

Table 2: Example of the first two columns (loci) in the calibration file

| Nl21 | Nl24 |
|---|---|
| 100-240 | 100-210 |
| KELP0004_S4.extendedFrags.F.fastq | KELP0005_S5.extendedFrags.F.fastq |
| KELP0013_S12.extendedFrags.F.fastq | KELP0010_S9.extendedFrags.F.fastq |
| KELP0014_S13.extendedFrags.F.fastq | KELP0019_S18.extendedFrags.F.fastq |
| KELP0015_S14.extendedFrags.F.fastq | KELP0026_S25.extendedFrags.F.fastq |
| KELP0017_S16.extendedFrags.F.fastq | KELP0066_S66.extendedFrags.F.fastq |
| KELP0018_S17.extendedFrags.F.fastq | KELP0067_S67.extendedFrags.F.fastq |
| 110 | 100 |

The first row should have the loci names. The loci names should be written exactly as in the lociInfo object in Table 1. The second row has the range in bp to look for alleles. Here, the advice is to balance a too-narrow range that might miss alleles and a too-broad range that will slow down the code. The following rows contain file names for samples that represent a homozygous pattern. Utilizing a few (4-6) to calibrate the algorithm is better to average out any minor differences between the relative frequency of sequences with different sizes that constitute the “phenotype” of a single microsatellite allele. The last row should contain a coverage threshold for each loci. Sequences with coverage below this value will not be counted as possible alleles. However, to identify homozygous samples for each locus, the appropriate coverage thresholds, and the allele ranges, we still need to visualize the amplification patterns for each locus before completing this calibration file.

**Samples directories required**

By default, Amplicomsat expects a directory written in the working directory, named “samples,” containing the combined fastq files. A single merged file per sample is expected. Paired-end reads can be merged using Flash (https://ccb.jhu.edu/software/FLASH/) or similar software.

eDNA samples, where multiple alleles (possibly more than two) are expected per sample loci combination, should be saved in a directory written in the R working directory called, by default, “samplesF.MA” (more details below).

When using Amplicomsat, almost no objects are written to the R session. The code mostly writes and reads files from the working directory and creates a few new directories in there, too:

**Directories created:**

“samplesF” (filtered fastq reads) created by function *read.quality.filter* containing fastq files filtered for sequence quality. A file named “filterLog” is also written by function *read.quality.filter* with the proportions of reads filtered out for each sample.

The directory “ReadSizesRepeatNumbers” contains files with information on the size of each read, filtered by sample and loci combination, and the number of msat motif repeats for all reads where the two primers and a user-selected number of msat motif repeats are found.

The directory “Plots” will contain plots with the distribution of reads along the range, as created by function *reads.plots*. A single plot per locus with all samples is produced. Two plots per sample; in addition to the above plot, a second plot with read size in bp vs number of msat motif repeat number can be produced (optionally).

The directory “Model Tables” will contain tables with expected relative frequencies of reads for different combinations of possible diploid genotypes. The files in this folder are for internal use of the functions scoring alleles.

The directory “GenotypeOut” will contain the genotype tables in two formats (one or two columns per locus).

The directory “AllelesBySeq” will contain a) a genotype file for sequence-variation-based allele scoring (Genotypes.by.Sequence.txt); b) a database of allele sizes, sequences, and numerical code translation (Allele DataBase.txt); and a file with a genotype table coded using numerical codes, as most pop gen software requires this (Genotype Codes.txt). The correspondence between sequence-based, size-only, and numerical codes is available in the Allele DataBase.txt file.

Most of these folders contain information of little interest to users. The “Plots,” “GenotypeOut,” and “AllelesBySeq” directories contain information for users.

## Running the Amplicomsat pipeline

### flash example for one sample

#run this in the shell after instaling flash

flash –max-overlap 300 KELP0001_S1_L001_R1_001.fastq KELP0001_S1_L001_R2_001.fastq -o KELP0001

#The parallel way to run all samples at once (requires sample.names file, here file.names.txt)

cat file.names.txt | parallel -j 4 flash –max-overlap 300 {}_L001_R1_001.fastq.gz {}_L001_R2_001.fastq.gz -o {}

### Filtering reads for phred 30 or higher

```
read.quality.filter(sampleDir = "./samples", filterDir = "samplesF")
```

The fastq merged files will be read; only reads with all bases with phred 30 or higher are retained. (The quality level of reads should be user-defined at some point, as this might be too restrictive.) The filtered reads are written in a ” sampleF ” directory created by the function in the R working directory. A log file, “filterLog” is also written to the working directory with information on the number and proportion of reads removed.

### Finding the reads with primers and microsatellite repeat motif

The next step is to find reads for each primer that contain the microsatellite motif (repeated *nmotif* times) while saving some summary stats (read size and repeat number).

```
read.size.freqs(locusInfo = "primers", sampleDir = "./samplesF")
```

The *locusInfo* is the path to a file containing loci names: fwd primers, rvs primers, microsatellite motif sequences, and *nmotif* values. See the file format above in the required files section. The *sampleDir* argument indicates the folder where the files are stored.

## Plotting the distribution of the read sequence sizes

Now, we can use the *reads.plot* function to plot the distribution of read sequence size (x-axis bp range) by filtered read coverage (y-axis).

```
reads.plots(sampleDir = "./samplesF", locusInfo = "primers", x.range = c(90, 350))
```

The most important arguments are shown; check the function manual for other options. The plots are created inside the directory *Plots* written by the function in the working directory. Examples of these plots for a heterozygous and homozygous sample are shown in Fig. 1 and 2, respectively. The function will produce a single PDF file per locus containing all samples scored.

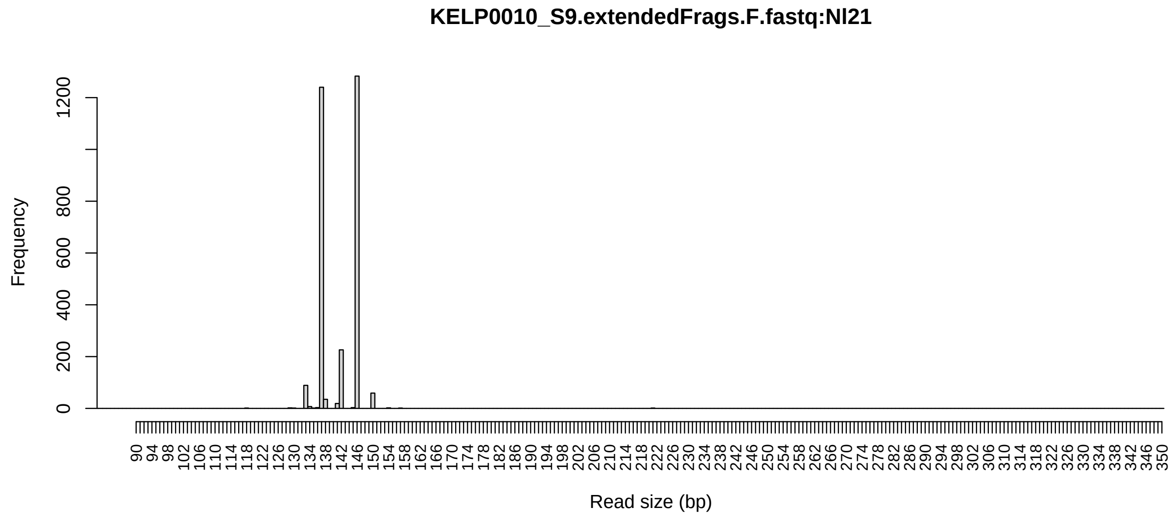


Figure 1: Example of the read distribution plot for one heterozygous sample in locus Nl21.

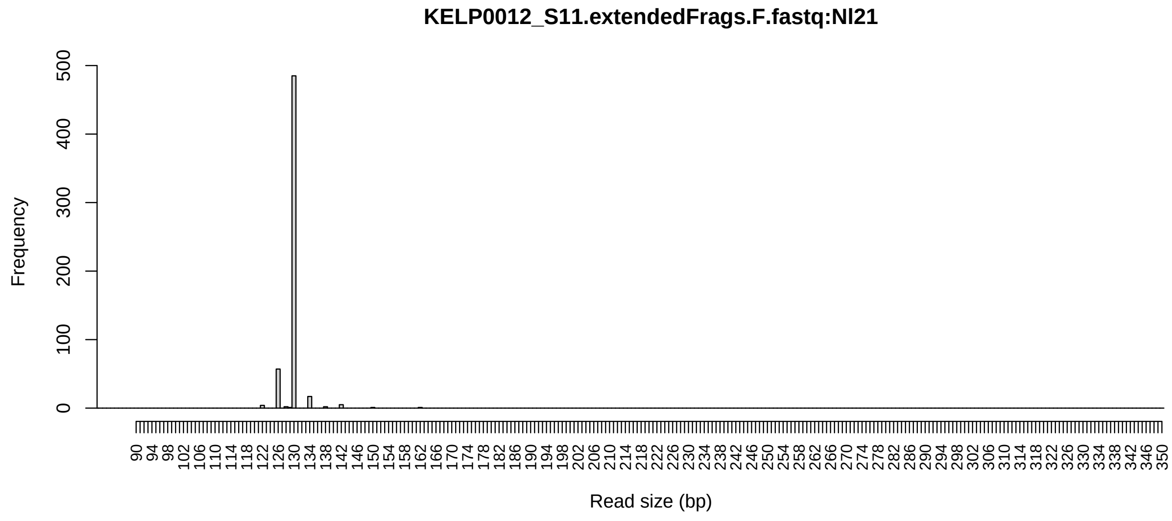


Figure 2: Example of the read distribution plot for one homozygous sample in locus Nl21.

## Creating tables with expected relative frequency of reads for all combinations of possible genotypes

These expected frequency tables will be matched to the observed relative frequencies (the sequencing data for each sample) to score alleles when using function *alleleFinder*. A few calibration homozygous samples are necessary to generate these tables. Select one to six homozygous samples by analyzing the plots created in the previous step. Figure 2 shows an example of a homozygous pattern for locus Nl21. Write the names of these samples in the different rows of the calibration file (see Required Files above). (tip: copy the name from the plot directly). The function will use this information of intervals between amplified reads for a single allele to generate the expectation for all possible combinations of alleles in a diploid genotype within the allele range supplied in the calibration file. Upon observing the plots created in the previous step, 1) select an allele range to bind the left and right limit of alleles that the function will consider. Scrutinize all samples so that the selected range does not exclude possible alleles. However, use a range that is not much wider than necessary because this will slow down the code. Write the range for each locus in the second row of the calibration file (see required files above, e.g., 100-250); and 2) inspect the patterns of possible alleles and determine a level of coverage (y-axis) that captures reads you believe are alleles. Write down this coverage in each locus's last row of the calibration file. Reads scored as alleles need coverage larger than this value; otherwise, they are written as missing data NA.

We can now run the *GenotypeModel* function.

```r
GenotypeModel(sampleDir = "./samplesF", locusInfo = "primers",
    calibrationFiles = "CalibrationSamples.txt")
```

## Scoring alleles

With the model tables produced, we can now score fragment size-based alleles. The function will also export a genotype table to the local environment and save two files inside the "GenotypesOut" directory created in the working directory. Both files have the diploid genotypes but one or two columns per locus (one: locus table or two: alleles table).

```r
genos.DB <- alleleFinder(sampleDir = "./samplesF", locusInfo = "primers",
    calibrationFiles = "CalibrationSamples.txt")
```

At this stage, recheck the genotype tables and plots to ensure that alleles were not missed. If adjustments are needed, edit the respective loci's coverage value in the calibration file and rerun alleleFinder.

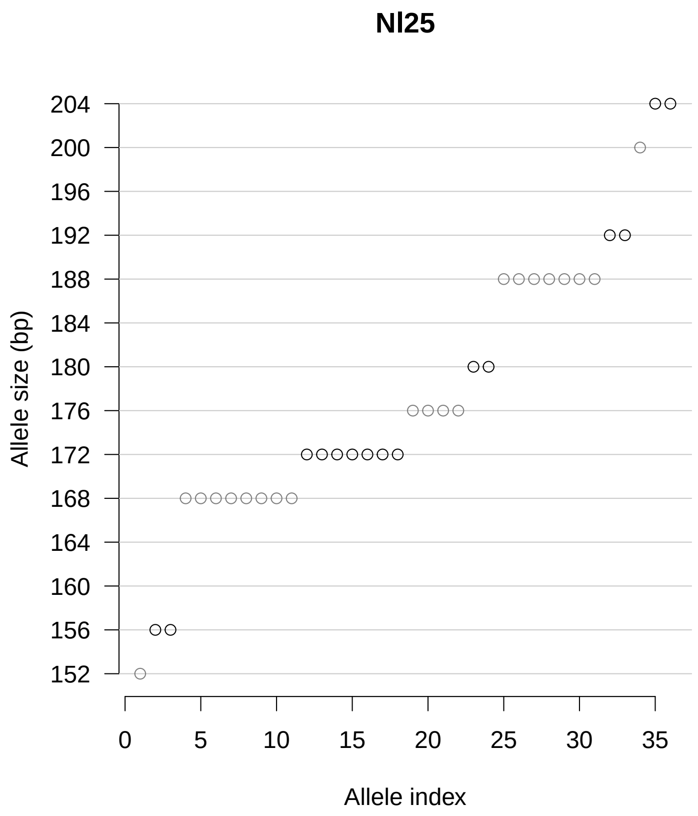
## Plotting read distributions for all loci and samples

We can now visualize the alleles scored for each loci using cumulative distribution plots (similar to those produced by MsatAllele [Alberto 2009]).

```r
# reading the table with the scored alleles that was produced by alleleFinder
genos.DB <- read.delim("./GenotypesOut/Genotypes allele table.txt")

AlleleCum(GenotypeA.DF = genos.DB, locus = "Nl25", locusInfo = "primers", ymin = NULL,
    ymax = NULL)
```

Figure 3 shows the plot produced by the code above. The example is for locus Nl25 for all alleles contained in 10 samples. When plotting much larger data sets, the *ymin* and *ymax* arguments can be used to zoom in.



Further interaction with the plot is possible using the function *getpoints*, which will require the user to click the mouse pointer twice on the plot to retrieve the names of samples carrying a particular range of alleles. The first click is below the desired range of alleles, and the second is above it. Below, we show the output of running *getpoints* and clicking below and above the 168 bp allele (Table 3).

```
getpoints(GenotypeA.DF = genos.DB, locus = "Nl25")
```

Table 3: Example of samples carrying allele 168 for locus Nl25, as retrieved by interacting with the cumulative allele distribution plot for this locus (Fig.3) using function getpoints

| Sample | Nl25.a1 | Nl25.a2 |
|---|---|---|
| KELP0017_S16.extendedFrags.F.fastq | 168 | 168 |
| KELP0022_S21.extendedFrags.F.fastq | 168 | 188 |
| KELP0023_S22.extendedFrags.F.fastq | 168 | 176 |
| KELP0027_S26.extendedFrags.F.fastq | 168 | 168 |
| KELP0029_S28.extendedFrags.F.fastq | 168 | 168 |

## Visualizing a single sample.

After visualizing the list of samples carrying a specific allele, the user may want to inspect the distribution of reads for a specific sample. One option is to look up the sample in the Plots directory. Alternatively, the function *sampleDist* can plot the distribution of reads for a single sample and locus. Notice that the argument *sample* takes a string with the sample name and locus name separated by a colon. The *xlim* argument can be used to zoom in on the reads (alleles) for that locus sample combination.

```r
sampleDist(sample = "KELP0011_S10.extendedFrags.F.fastq_Nl25", xlim = c(134, 210))
```

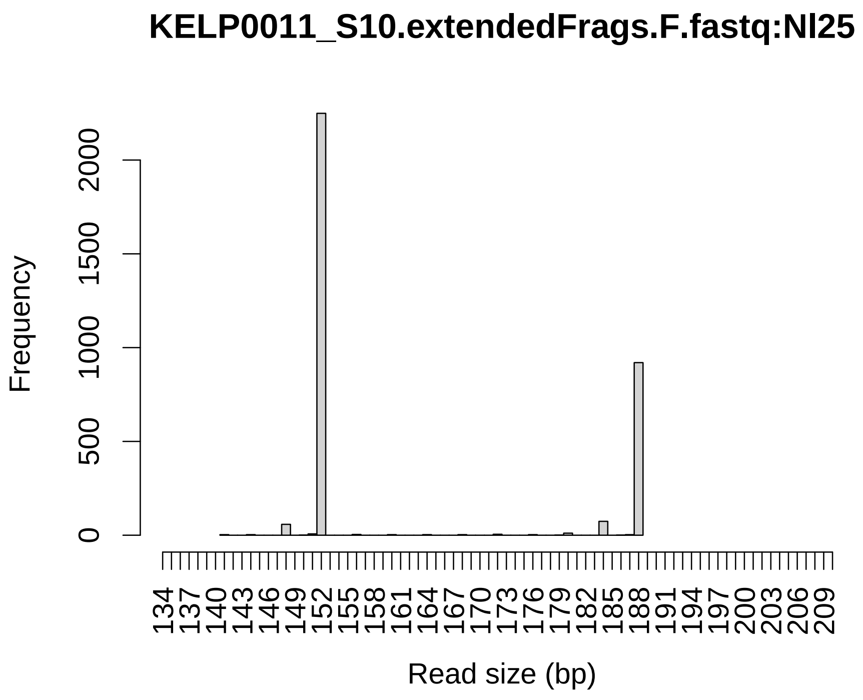


Figure 3: A plot of the cumulative distribution of reads (alleles) for a single sample using function sampleDist

## Scoring sequence based alleles

So far, the pipeline has identified only alleles based on read size. We will now identify alleles based on sequence composition. When using amplicon sequencing, these are alleles of the same size that differ in their sequence and are hereafter called sequence variants.

```r
score.seq.variants(locusInfo = "primers", RefGenotypes = "./GenotypesOut/Genotypes allele table.txt",
    sampleDir = "./samplesF", HeteroThreshold = 0.4, alleleDB = NULL,
    calibrationFiles = "CalibrationSamples.txt")
```

There are three new function arguments to mention here. *RefGenotypes* takes the "Genotype allele table.txt" written into the "GenotypeOut" directory by function *alleleFinder*. Note that the function will only look for sequence-variant alleles "within" the size-based alleles written to "Genotype allele table.txt".

When sequence variation exists within reads of the same size for the same loci, two variants will dominate the read frequencies, with many more in low frequency due to sequencing errors. The *HeteroThreshold* argument refers to the proportion of reads in the second most frequent variant compared to the first. The value sets the minimum allowed proportion required to score the second allele as a sequence-based variant allele, i.e., not a sequencing error. It defaults to 0.4.

*alleleDB* is the file name for a tab-delimited file containing the allele database with information on allele size, sequence variation, sequence-variant allele code, and the nucleotide sequence (Table 4). The default is NULL, in which case the function will produce the database from the data supplied. When a database is supplied, the function will update it with any new sequence variants that might be identified. The database is organized with different sequence variants by row and the following required columns:

Locus: The locus name for the sequence-based allele. Needs to match the loci names supplied in argument locusInfo;

SIZE: The size in bp for the sequence-based allele;

VARIANT: A low case letter code to distinguish the different sequence-based alleles that share the same sequence size;

SEQ: The nucleotide sequence for each sequence-based allele;

AlleleCode: A numeric code used to write alleles in a format most population genetics software requires.

The function writes three output files in a folder created in the R working directory named *AlleleBySeq*.

The files are “Allele DataBase.txt” (table 4), containing the reference database of allele sequence variants found;

“Genotypes.by.Sequence.txt” (Table 5) contains a population genetics file with two columns per locus and alleles represented by the concatenation of allele size and variant letter code (e.g., 150a, 150b);

“Genotype Codes.txt” contains a population genetics file with new numeric allele codes, one column per locus, required for most population genetics software.

The allele database file contains the correspondence between the different allele code formats. A “warningLog” file is also written directly in the working directory, reporting cases where more than two alleles were found. When this happens, the diploid genotype is ambiguous; thus, NAs are written to the output files.

Table 4: The database required by argument alleleDB in function score.seq.variants should have the format in this table. Note that sequence information in SEQ column is trimmed to fit the table. When a database is not supplied to the function, one is created as an output.

| Locus | SIZE | VARIANT | AlleleCode | SEQ |
|---|---|---|---|---|
| Nl21 | 114 | a | 100 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 122 | a | 101 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 122 | b | 102 | ...CGTACATATATACATACATACATACA... |
| Nl21 | 126 | a | 103 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 130 | a | 104 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 134 | a | 105 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 134 | b | 106 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 137 | a | 107 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 137 | b | 108 | ...ATACGTACGTACATACATACATACAT... |
| Nl21 | 138 | a | 109 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 138 | b | 110 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 141 | a | 111 | ...ATACGTACGTACATACATACATACAT... |
| Nl21 | 142 | a | 112 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 142 | b | 113 | ...CGTACATACATACATACATACATACA... |
| Nl21 | 142 | c | 114 | ...CGTACATACATACATACATACATACA... |

Table 5: View of the first three loci in the Genotypes.by.sequence.txt output file. Sequence-variant alleles are coded by concatenating allele size with a letter code. For example, for the first locus Nl21 (columns 2 and 3), we can see three sequence-variant alleles for size 150 (a, b, and c) in locus Nl21.

| Sample | Nl21.a1 | Nl21.a2 | Nl24.a1 | Nl24.a2 | Nl25.a1 | Nl25.a2 |
|---|---|---|---|---|---|---|
| KELP0010_S9.extendedFrags.F.fastq | 137a | 137a | 166a | 166a | NA | NA |
| KELP0011_S10.extendedFrags.F.fastq | 130a | 194a | 149a | 166a | 152a | 152a |
| KELP0012_S11.extendedFrags.F.fastq | 130a | 130a | 158a | 166a | 188a | 188a |
| KELP0013_S12.extendedFrags.F.fastq | 154a | 154a | 150a | 170a | 188a | 188a |
| KELP0014_S13.extendedFrags.F.fastq | 182a | 182a | 162a | 173a | NA | NA |
| KELP0015_S14.extendedFrags.F.fastq | 154a | 154a | 149a | 166a | NA | NA |
| KELP0016_S15.extendedFrags.F.fastq | 142a | 150a | 157a | 169a | 156a | 156a |
| KELP0017_S16.extendedFrags.F.fastq | 150b | 150b | 157a | 169a | 168a | 168a |
| KELP0018_S17.extendedFrags.F.fastq | 138a | 138a | 150a | 177a | 176a | 180a |
| KELP0019_S18.extendedFrags.F.fastq | 150b | 158a | 161a | 161a | 172a | 172a |
| KELP0020_S19.extendedFrags.F.fastq | 130a | 130a | 149b | 169a | 176b | 188a |
| KELP0021_S20.extendedFrags.F.fastq | 138a | 138a | 150a | 177a | 176a | 180a |
| KELP0022_S21.extendedFrags.F.fastq | 142a | 142a | 170a | 173a | 168a | 188a |
| KELP0023_S22.extendedFrags.F.fastq | 146a | 146a | 153a | 170a | 168a | 176a |
| KELP0024_S23.extendedFrags.F.fastq | 142b | 142b | 153a | 169a | 172a | 192a |
| KELP0025_S24.extendedFrags.F.fastq | 142a | 182a | 157a | 169a | 192b | 200a |
| KELP0026_S25.extendedFrags.F.fastq | 146a | 158a | 166a | 166a | 172a | 172a |
| KELP0027_S26.extendedFrags.F.fastq | 142a | 150c | 170a | 174a | 168a | 168a |
| KELP0028_S27.extendedFrags.F.fastq | NA | NA | NA | NA | NA | NA |
| KELP0029_S28.extendedFrags.F.fastq | 134a | 182a | 165a | 169a | 168a | 168a |
| KELP0030_S29.extendedFrags.F.fastq | 130a | 150c | 169a | 170a | 172a | 172a |

## Working with eDNA or forensic samples with more than two alleles per sample

The function searches samples for multiple alleles of the same loci. Examples of such samples come from forensic analysis, where DNA from multiple individuals might be present, or eDNA samples containing alleles from several individuals. Only alleles present in the reference population can be detected.

### Scoring multiple size-based alleles per sample locus combination

Using a similar approach as above, we first score size-based alleles, allowing for multiple alleles to be scored for the same locus in one sample, and then use the resulting data to search for additional sequence-based variants.

Again, we start by filtering the reads for sequencing quality using the function *read.quality.filter* (see code above). In this case, the filtered reads were then copied to a directory we created in the working directory of the R session named "samplesF.MA."

Next, we use again *read.size.freqs* to find and filter reads in these samples. The code for these two steps is not shown below, but it is similar to the examples above while changing the names parsed to arguments if necessary.

we can use the function *M.allele at this stage.Finder* to score all alleles from each locus in our samples.

The argument *RefGenotypes* takes the two-column per locus genotype table of the reference population, as produced by the *alleleFinder* function (see above). Only alleles present in a reference population can be scored as multiple alleles in any sample; thus, the genotype file for such a population should be used here.

The argument *ReadSizesRepeatNumbers* takes the path to the folder created by function *read.size.freqs* above.

The function returns a list with the alleles found for each locus and individual. The function also saves to the R session working directory a pdf file for each sample containing plots of read (allele) distribution, with separate plots per page showing the different loci and red vertical lines indicating the alleles scored (Figure 4).

```
AllelesSample <- M.allele.Finder(sampleDir = "./samplesF.MA",
    locusInfo = "primers", calibrationFiles = "CalibrationSamples.txt",
    ReadSizesRepeatNumbers = "./ReadSizesRepeatNumbers/", RefGenotypes = "./GenotypesOut/Genotypes alle
```

```
## Inspecting the list with the multiple alleles scored for
## each loci in the first sample
AllelesSample["S.1.simulated.Forensics.fastq"]
```

```
## $S.1.simulated.Forensics.fastq
## $S.1.simulated.Forensics.fastq$Nl21
## [1] 126 130 134 137 138 142 146
##
## $S.1.simulated.Forensics.fastq$Nl24
## [1] 158 165 166 169 177
##
## $S.1.simulated.Forensics.fastq$Nl25
## [1] 180 184 188
##
## $S.1.simulated.Forensics.fastq$Nl26
## [1] 175 187 191 195 199 203
##
## $S.1.simulated.Forensics.fastq$Nl27
## [1] 150 154 158 162 166
##
## $S.1.simulated.Forensics.fastq$Nl30
## [1] 142 148 162 164 166 198
##
## $S.1.simulated.Forensics.fastq$Nl40
## [1] 172
##
## $S.1.simulated.Forensics.fastq$Nl47
## [1] 155 163 167
##
## $S.1.simulated.Forensics.fastq$Nl50
## [1] 170 178 182 186
##
## $S.1.simulated.Forensics.fastq$Nl59
## [1] 120 128 136
##
## $S.1.simulated.Forensics.fastq$Ner2
## [1] 260
```

The code below can be used to count the number of alleles per locus found for a given sample. This is the observed allele count (OAC) required as an imput to package GenotypeQuant. The OAC can be used to estimate the number of genotypes in the sample trough Maximum likelihood estimation (Liggan et al. submitted, see https://github.com/UWMAlberto-Lab/GenotypeQuant)

```
## Inspecting the list with the multiple alleles scored for
## each loci in the first sample
```

```
sapply(AllelesSample["S.1.simulated.Forensics.fastq"][[1]], length)
```

```
## N121 N124 N125 N126 N127 N130 N140 N147 N150 N159 Ner2
##    7    5    3    6    5    6    1    3    4    3    1
```

Or for all samples at once.

```
FileNames <- list.files("./samplesF.MA")
locusNames <- read.delim("primers")[, 1]
n.locus <- length(locusNames)
n.files <- length(FileNames)
AlleleCounts <- data.frame(matrix(nrow = n.files, ncol = n.locus +
    1))
AlleleCounts[, 1] <- FileNames
names(AlleleCounts) <- c("Samples", locusNames)

for (f in 1:n.files) {
    AlleleCounts[f, 2:ncol(AlleleCounts)] <- as.numeric(sapply(AllelesSample[FileNames[f]][[1]],
        length))
}

AlleleCounts
```

```
##                           Samples N121 N124 N125 N126 N127 N130 N140 N147 N150
## 1   S.1.simulated.Forensics.fastq    7    5    3    6    5    6    1    3    4
## 2  S.10.simulated.Forensics.fastq    7    9    3    5    4    3    0    3    6
## 3   S.2.simulated.Forensics.fastq    5    7    3    5    7    5    2    3    5
## 4   S.3.simulated.Forensics.fastq    8    9    3    3    6    4    2    5    6
## 5   S.4.simulated.Forensics.fastq    3    8    5    3    5    5    2    3    5
## 6   S.5.simulated.Forensics.fastq    7    8    3    3    3    3    1    5    4
## 7   S.6.simulated.Forensics.fastq    6   10    3    4    4    3    3    5    6
## 8   S.7.simulated.Forensics.fastq    4    8    4    3    4    4    2    5    2
## 9   S.8.simulated.Forensics.fastq    6    7    3    2    5    2    0    4    4
## 10  S.9.simulated.Forensics.fastq    7    8    1    2    4    3    1    3    5
##    N159 Ner2
## 1     3    1
## 2     3    1
## 3     2    1
## 4     6    2
## 5     3    1
## 6     5    1
## 7     7    0
## 8     5    1
## 9     3    1
## 10    4    1
```

## Scoring multiple sequence-based alleles per sample locus combination

Finally, we use function *M.allele.seq* to score sequence variant alleles that may occur within the multiple size-based alleles identified in the previous step. The user must supply the list of multiple alleles created using *M.allele.Finder* (previous step) to argument *AlleleList* below. Also required is the database of sequence variant alleles, like the one created by function *score.seq.variants* above.

The function does not create a database by itself because this type of forensics or DNA analysis often requires allele frequencies from a reference population that would be first analyzed with *score.seq.variants* creating a database to use here. However, the database might be updated while running *M.allele.seq*. This will happen

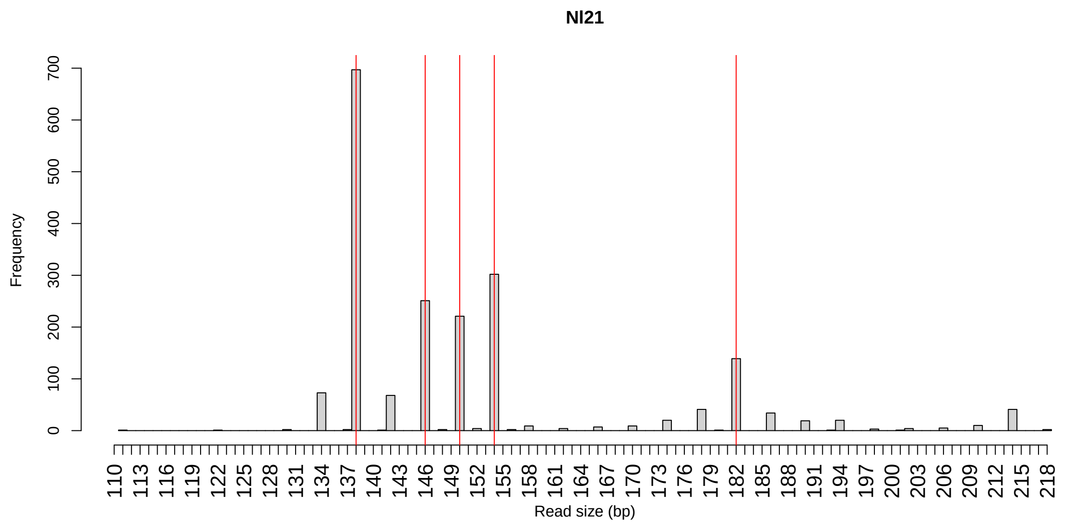


Figure 4: Read distribution with multiple alleles called (red vertical lines). This is the example of the plot for a single locus in one sample.

if the differences in the read coverage filter are different between function uses (inspect values and plots), resulting in new sequence variants that pass the coverage filter. If the database of sequence variant alleles is updated, the new database is written to the working directory and named "Allele DataBase UPDATED.txt." The new entries to the database are also written in a file named "DataBase new entries.txt." This file might be useful in investigating the origin of the new alleles.

```
List.seq.basedAlleles <- M.allele.seq.variant(sampleDir = "./samplesF.MA",
    AlleleList = AllelesSample, locusInfo = "primers",
    alleleDB = "./AlleleBySeq/Allele DataBase.txt",
    HeteroThreshold = 0.4, calibrationFiles = "CalibrationSamples.txt")
```

The function returns a list with the multiple sequence variant alleles found for each sample and locus. The list has a similar format to the one produced by *M.allele.Finder*; we adapt the previous code below to summarize the results.

```
## Inspecting the list with the multiple alleles scored for
## each loci in the first sample
List.seq.basedAlleles["S.1.simulated.Forensics.fastq"]
```

```
## $S.1.simulated.Forensics.fastq
## $S.1.simulated.Forensics.fastq$Nl21
## [1] "126a" "130a" "134a" "137b" "138a" "142b" "146a"
##
## $S.1.simulated.Forensics.fastq$Nl24
## [1] "158b" "165a" "166a" "169a" "177a"
##
## $S.1.simulated.Forensics.fastq$Nl25
## [1] "180e" "184a" "188e"
##
## $S.1.simulated.Forensics.fastq$Nl26
## [1] "175a" "187a" "191a" "195c" "199a" "203b"
##
## $S.1.simulated.Forensics.fastq$Nl27
## [1] "150a" "154b" "154a" "158a" "162b" "166b"
##
## $S.1.simulated.Forensics.fastq$N130
## [1] "142a" "148a" "162a" "164a" "166a" "198a"
##
## $S.1.simulated.Forensics.fastq$N140
## [1] "172a"
##
## $S.1.simulated.Forensics.fastq$N147
## [1] "155e" "163a" "167a"
##
## $S.1.simulated.Forensics.fastq$N150
## [1] "170a" "178a" "182a" "186a"
##
## $S.1.simulated.Forensics.fastq$N159
## [1] "120a" "128a" "136a"
##
## $S.1.simulated.Forensics.fastq$Ner2
## [1] "260a"
```

The code below can be used to count the number of sequence variant alleles found for a given sample (OAC).

```
## Inspecting the list with the multiple alleles scored for
## each loci in the first sample
sapply(List.seq.basedAlleles["S.1.simulated.Forensics.fastq"][[1]],
    length)
```

```
## N121 N124 N125 N126 N127 N130 N140 N147 N150 N159 Ner2
##    7    5    3    6    6    6    1    3    4    3    1
```

Or for all samples at once.

```
Seq.Based.AlleleCounts <- data.frame(matrix(nrow = n.files, ncol = n.locus +
    1))
Seq.Based.AlleleCounts[, 1] <- FileNames
names(Seq.Based.AlleleCounts) <- c("Samples", locusNames)

for (f in 1:n.files) {
    AlleleCounts.f <- as.numeric(sapply(List.seq.basedAlleles[FileNames[f]][[1]],
        length))
    Seq.Based.AlleleCounts[f, 2:ncol(Seq.Based.AlleleCounts)] <- AlleleCounts.f
}

Seq.Based.AlleleCounts
```

```
##                           Samples N121 N124 N125 N126 N127 N130 N140 N147 N150
## 1   S.1.simulated.Forensics.fastq    7    5    3    6    6    6    1    3    4
## 2  S.10.simulated.Forensics.fastq    8    9    3    5    4    3    0    3    7
## 3   S.2.simulated.Forensics.fastq    7    7    3    5    8    5    2    3    6
## 4   S.3.simulated.Forensics.fastq    9    9    4    3    7    4    2    5    6
## 5   S.4.simulated.Forensics.fastq    3    8    5    3    5    5    2    3    5
## 6   S.5.simulated.Forensics.fastq    7    8    3    3    3    3    1    5    4
## 7   S.6.simulated.Forensics.fastq    6   10    3    4    7    3    3    5    8
## 8   S.7.simulated.Forensics.fastq    4    8    4    3    6    4    2    5    2
## 9   S.8.simulated.Forensics.fastq    6    7    3    2    7    2    0    4    4
## 10  S.9.simulated.Forensics.fastq    7    8    1    2    6    3    1    4    5
##    N159 Ner2
## 1     3    1
## 2     3    1
## 3     2    1
## 4     6    3
## 5     3    1
## 6     5    1
## 7     7    0
## 8     5    1
## 9     3    1
## 10    4    1
```

A quick subtraction of sequence-based allele counts by size-based alleles gives the difference in number of alleles revealed by each method per sample and loci combination.

```
Seq.Based.AlleleCounts[, 2:12] - AlleleCounts[, 2:12]
```

```
##    N121 N124 N125 N126 N127 N130 N140 N147 N150 N159 Ner2
## 1     0    0    0    0    1    0    0    0    0    0    0
## 2     1    0    0    0    0    0    0    0    1    0    0
## 3     2    0    0    0    1    0    0    0    1    0    0
## 4     1    0    1    0    1    0    0    0    0    0    1
## 5     0    0    0    0    0    0    0    0    0    0    0
## 6     0    0    0    0    0    0    0    0    0    0    0
## 7     0    0    0    0    3    0    0    0    2    0    0
## 8     0    0    0    0    2    0    0    0    0    0    0
## 9     0    0    0    0    2    0    0    0    0    0    0
## 10    0    0    0    0    2    0    0    1    0    0    0
```

## Additional functions

The pipeline relies on filtering reads by primer and microsatellite sequencing, and most functions look for exact matches in the primer sequences. Fastq files might show primer sequences that differ from those in the user-supplied loci information file for various reasons, leading to a failure to extract read information for affected loci. If there is doubt that the loci information file might not precisely match the sequences in the fastq files, the function *Edit.Primer.File* can search primer sequences, allowing for mismatches of up to a user-determined number of base pairs. If any mismatches are found, the function writes an edited primer info file for use in the pipeline above. Because this function searches all reads, allowing for mismatches, it is the slowest function in the package.