DNA metabarcoding diet analysis in reindeer is quantitative and integrates feeding over several weeks

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Abstract

Filtering of the SPER01 DNA metabarcoding raw data.

1. Setting up the R environment

1.1. Install missing packages

```
packages <- c("igraph", "tidyverse", "devtools",
              "R.utils","vegan","vctrs","magrittr")
install.packages(setdiff(packages,
                         rownames(installed.packages())),
                 dependencies = TRUE
                )
```

1.2. Loading of the R libraries

- `ROBITools` package is used to read result files produced by OBITools.
- `ROBITaxonomy` package provides function allowing to query OBITools formated taxonomy.

```
if (!"ROBITools" %in% rownames(installed.packages())) {
  # ROBITools are not available on CRAN and have to be installed
  # from http://git.metabarcoding.org using devtools

  metabarcoding_git <- "https://git.metabarcoding.org/obitools"
```

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```
  devtools::install_git(paste(metabarcoding_git,
                              "ROBIUtils.git",
                              sep="/"))

  devtools::install_git(paste(metabarcoding_git,
                              "ROBITaxonomy.git",
                              sep="/"))
  devtools::install_git(paste(metabarcoding_git,
                              "ROBITools.git",
                              sep="/"))
}

library(ROBITools)
library(ROBITaxonomy)
```

- `tidyverse`[1] provides various method for efficient data manipulation and plotting via `ggplot2`[2]

```
library(tidyverse)
```

```
library(R.utils)
```

```
library(vegan)
```

```
library(magrittr)
```

```
source("methods.R")
```

```
Attaching package: 'matrixStats'
The following object is masked from 'package:dplyr':

    count

Attaching package: 'vctrs'
The following object is masked from 'package:dplyr':

    data_frame
The following object is masked from 'package:tibble':

    data_frame
```

2. Loading the data

2.1. Load the NCBI taxonomy

```
if (! file.exists("Data/ncbi20210212.adx")) {
  gunzip("Data/ncbi20210212.adx.gz",remove=FALSE)
  gunzip("Data/ncbi20210212.ndx.gz",remove=FALSE)
  gunzip("Data/ncbi20210212.rdx.gz",remove=FALSE)
  gunzip("Data/ncbi20210212.tdx.gz",remove=FALSE)
}
```

```
taxo <- read.taxonomy("Data/ncbi20210212")
```

2.2. Loading the metabarcoding data

```
if (! file.exists("Data/Rawdata/SPER01_all_paired.ali.assigned.ann.diag.uniq.ann.c1.l10.clean.EMBL.
    gunzip("Data/Rawdata/SPER01_all_paired.ali.assigned.ann.diag.uniq.ann.c1.l10.clean.EMBL.tag.ann


SPER01.raw = import.metabarcoding.data("Data/Rawdata/SPER01_all_paired.ali.assigned.ann.diag.uniq.a
```

2.3. Loading the metadata

```
samples.metadata = read_csv("Data/Faeces/sampling_dates.csv",
                            show_col_types = FALSE)
```

3. Sample description

Normalization of samples names

Extract information relative to PCR replicates and sample names.

```
sample_names_split = strsplit(as.character(sample_names), "_R")

replicate = sapply(sample_names_split, function(x) x[length(x)])
sample_id = sapply(sample_names_split, function(x) x[1])

samples_desc = data.frame(name = samples(SPER01.raw)$sample, replicate = replicate, sample_id = sam

SPER01.raw@samples = samples_desc
```

3.1. Categorize MOTUs

DNA Sequence of the 6 synthetic sequences used as SPER01 positive controls.

```
Standard1 = "taagtctcgcactagttgtgacctaacgaatagagaattctataagacgtgttgtcccat"
Standard2   = "gtgtatggtatatttgaataatattaaatagaatttaatcaatctttacatcgcttaata"
Standard3   = "cacaatgctcggtaactagaagcatttgta"
Standard4   = "attgaatgaaaagattattcgatatagaat"
Standard5   = "agaacgctagaatctaagatgggggggggatgagtaagatatttatcagtaacatatga"
```

```
Standard6    = "atttttgtaactcattaacaatttttttttgatgtatcataagtactaaactagttact"
```

- Identify which MOTUs are corresponding to these positive control sequences and associated them to their corresponding category.
- All the MOTUs exhibiting a similarity with one of the reference SPER01 database greater than 95% is tagged as `SPER01`
- The remaining sequences are tagged as `Unknown`

```
sequence_type = rep("Unknown", nrow(motus(SPER01.raw)))
sequence_type[which(motus(SPER01.raw)$`best_identity:db_GH`> 0.95)] = "SPER01"
sequence_type[which(motus(SPER01.raw)$sequence == Standard1)] = "standard1"
sequence_type[which(motus(SPER01.raw)$sequence == Standard2)] = "standard2"
sequence_type[which(motus(SPER01.raw)$sequence == Standard3)] = "standard3"
sequence_type[which(motus(SPER01.raw)$sequence == Standard4)] = "standard4"
sequence_type[which(motus(SPER01.raw)$sequence == Standard5)] = "standard5"
sequence_type[which(motus(SPER01.raw)$sequence == Standard6)] = "standard6"

SPER01.raw@motus$sequence_type = as.factor(sequence_type)
table(SPER01.raw@motus$sequence_type)
```

```
  SPER01 standard1 standard2 standard3 standard4 standard5 standard6   Unknown
   44125         1         1         1         1         1         1     36419
```

4. Curation procedure

4.1. Select motus occuring at least at 1% in at least one PCR

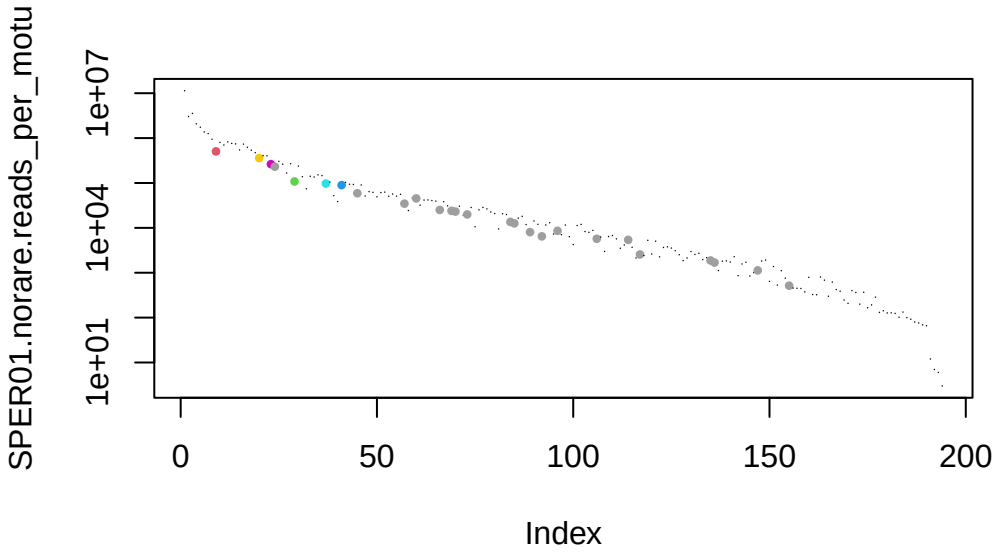
Only MOTUs occurring at least at one percent in at least one PCR are conserved. The others are discarded and correspond to few rare taxa, and many spurious MOTUs generated by PCR artefacts.

```
norare = apply(decostand(reads(SPER01.raw),method = "total"),
               MARGIN = 2,
               FUN = max) >= 0.01
table(norare)
```

```
norare
FALSE  TRUE
80356   194
```

```
SPER01.norare <- SPER01.raw[,which(norare)]
```

```
SPER01.norare.reads_per_motu = colSums(reads(SPER01.norare))
plot(SPER01.norare.reads_per_motu,log="y",
     cex=0.1 + 0.5 * (SPER01.norare@motus$sequence_type!="SPER01"),
     col = as.integer(SPER01.norare@motus$sequence_type), pch=16)
```



4.2. Analysis of the PCR Positive controls

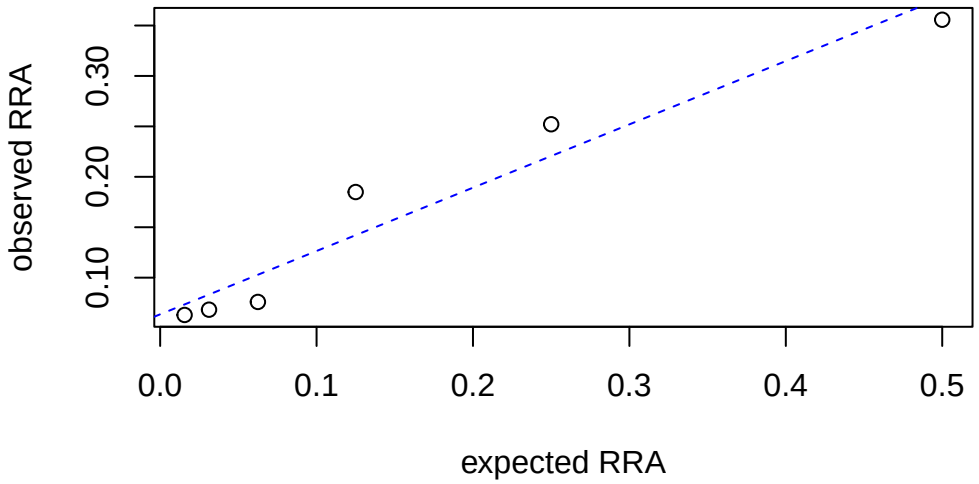
Synthetic MOTUs are extracted from the data set and their relative read abundances (RRA) is plotted as a function of their theoretical abundances to check the quality of the PCR.

```
rp <- SPER01.norare.reads_per_motu[! SPER01.norare@motus$sequence_type  %in% c("SPER01","Unknown")]
rp/sum(rp)
```

```
GHP2_00000016 GHP3_00000014 GHP3_00000156 GHP2_00000010 GHP3_00000144
   0.35574603    0.25211192    0.18491040    0.07594321    0.06826363
GHP2_00000144
   0.06302481
```

```
expectedRRA <- 1/2^(1:6)
observedRRA <- rp/sum(rp)

plot(expectedRRA,observedRRA,
     xlab="expected RRA",
     ylab="observed RRA")
abline(lm(observedRRA ~ expectedRRA),
       col = "blue", lty = 2)
```



4.3. Filtering for PCR outliers

Only library 1 and 2 have individually tagged PCR replicates.

```
library_3.ids = read.csv("Data/samples_library_3.txt",
                         stringsAsFactors = FALSE,
                         header = FALSE)[,1]
```

```
library3.keep = gsub("_R.?$","_R",rownames(SPER01.norare)) %in% library_3.ids
SPER01.lib3 = SPER01.norare[library3.keep,]
SPER01.lib12= SPER01.norare[!library3.keep,]

dim(SPER01.lib3)
```

```
[1]  65 194
```

```
dim(SPER01.lib12)
```

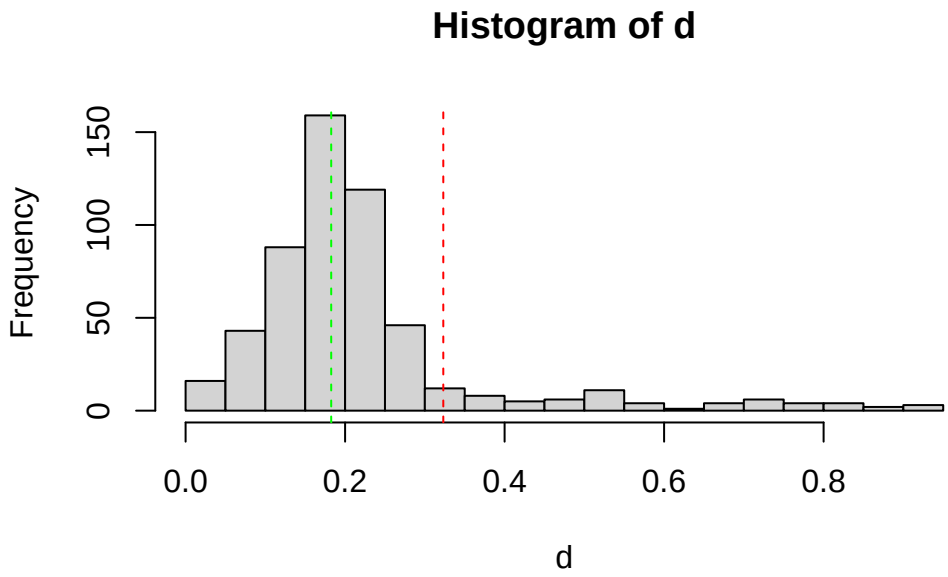
```
[1] 541 194
```

Load the script containing the selection procedure implemented in function `tag_bad_pcr`.

```
source("select_pcr.R")
```

4.3.1. First selection round

```
keep1 = tag_bad_pcr(samples = samples(SPER01.lib12)$sample_id,
                    counts = reads(SPER01.lib12),
                    plot = TRUE
                   )
```



Histogram shows the empirical distribution of the PCR replicate distances. The red vertical dashed line indicates the threshold used to discard outlier PCRs. The green vertical dashed line indicates the mode of the observed distribution.

```
table(keep1$keep)
```

```
FALSE  TRUE
   44   497
```

`FALSE`is the count of PCR to discard, `TRUE` the count of PCR conserved at the end of this selection round.

```
samples(SPER01.lib12)$name[!keep1$keep]
```

```
 [1] "DNANC12_R3"  "DNANC14_R3"  "DNANC15_R3"  "DNANC_10_R2" "DNANC_11_R3"
 [6] "DNANC_13_R3" "DNANC_14_R3" "DNANC_15_R3" "DNANC_7_R2"  "DNANC_8_R2"
[11] "DNANC_9_R2"  "PCRNC_3_R3"  "PCRNC_6_R3"  "PCRPOS_3_R3" "PCRPOS_4_R2"
[16] "PCRPOS_5_R3" "PCRPOS_6_R3" "X_29_R1"     "X_38_R3"     "X_50_R2"
[21] "X_64_R1"     "X_66_R1"     "Y_24_R2"     "Y_28_R2"     "Y_2_R1"
[26] "Y_33_R2"     "Y_36_R2"     "Y_44_R1"     "Y_45_R1"     "Y_46_R3"
[31] "Y_47_R1"     "Y_48_R1"     "Y_48_R2"     "Y_49_R2"     "Y_51_R2"
[36] "Y_52_R1"     "Y_56_R3"     "Z_20_R1"     "Z_30_R1"     "Z_36_R1"
```

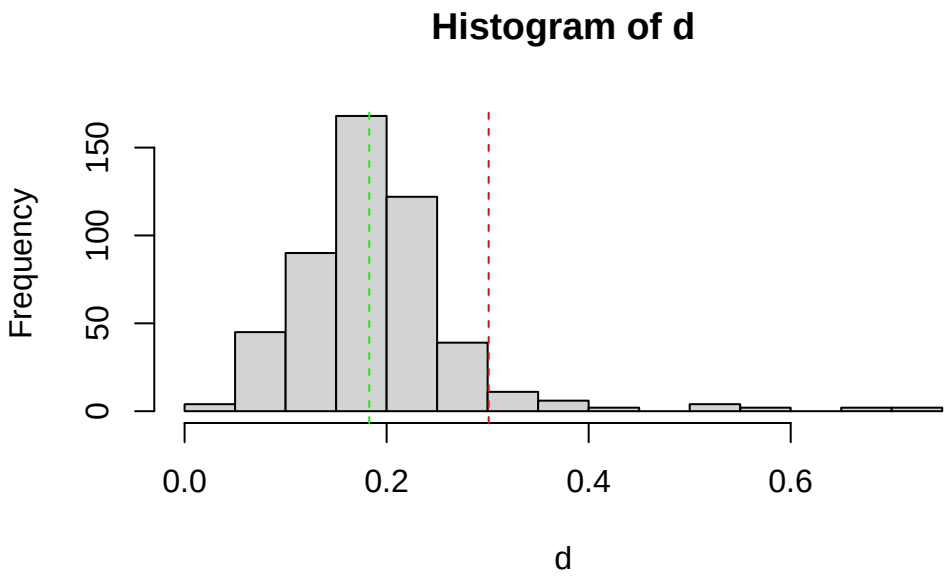
```
[41] "Z_45_R2"      "Z_53_R1"      "Z_5_R1"       "Z_61_R3"
```

Above is the list of the ids of the discarded PCRs.

```
SPER01.lib12.k1 = SPER01.lib12[keep1$keep,]
```

4.3.2. Second selection round

```
keep2 = tag_bad_pcr(samples = samples(SPER01.lib12.k1)$sample_id,
                    counts = reads(SPER01.lib12.k1),
                    plot = TRUE
                   )
```




```
table(keep2$keep)
```

```
FALSE  TRUE
   24   473
```

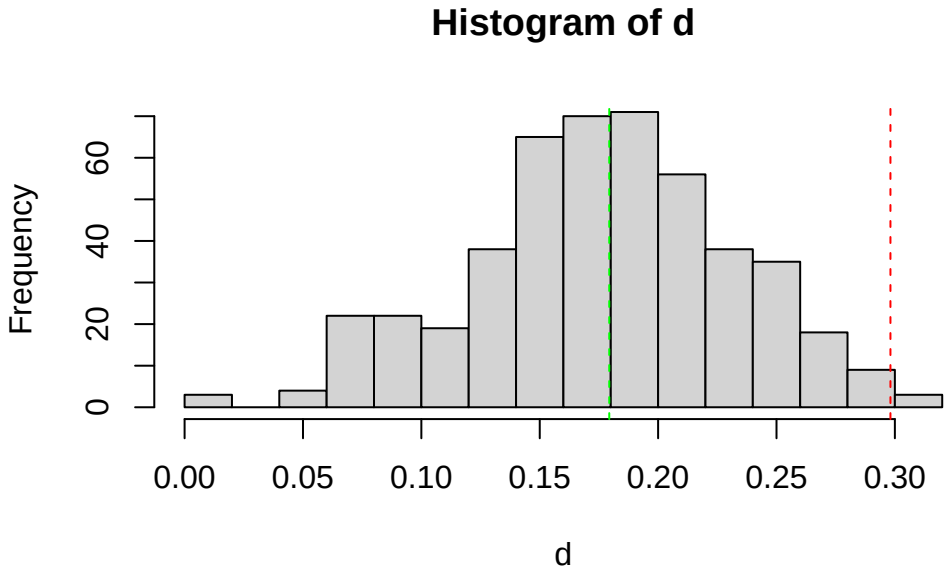
```
samples(SPER01.lib12.k1)$name[!keep2$keep]
```

```
 [1] "PCRPOS_3_R2" "X_50_R1"     "X_50_R3"     "Y_18_R1"     "Y_2_R2"
 [6] "Y_2_R3"      "Y_31_R1"     "Y_44_R2"     "Y_46_R1"     "Y_46_R2"
[11] "Y_47_R2"     "Y_49_R1"     "Y_49_R3"     "Y_56_R1"     "Y_56_R2"
[16] "Y_71_R1"     "Z_30_R2"     "Z_30_R3"     "Z_45_R1"     "Z_49_R1"
[21] "Z_53_R2"     "Z_53_R3"     "Z_61_R1"     "Z_61_R2"
```

```
SPER01.lib12.k2 = SPER01.lib12.k1[keep2$keep,]
```

4.3.3. Third selection round

```
keep3 = tag_bad_pcr(samples = samples(SPER01.lib12.k2)$sample_id,
                    counts = reads(SPER01.lib12.k2),
                    plot = TRUE
                   )
```




```
table(keep3$keep)
```

```
FALSE  TRUE
    7   466
```

```
keep3[!keep3$keep,]
```

```
        samples  distance   maximum repeats  keep
X_14_R3    X_14 0.2982691 0.2982691       3 FALSE
Y_44_R3    Y_44 0.0000000 0.0000000       1 FALSE
Y_47_R3    Y_47 0.0000000 0.0000000       1 FALSE
Y_50_R3    Y_50 0.2986271 0.2986271       3 FALSE
Y_52_R3    Y_52 0.3004914 0.3004914       2 FALSE
Z_45_R3    Z_45 0.0000000 0.0000000       1 FALSE
Z_55_R3    Z_55 0.3004595 0.3004595       3 FALSE
```

```
SPER01.lib12.k3 = SPER01.lib12.k2[keep3$keep,]
```

4.3.4. Merge remaining PCR replicates

```
freq = decostand(reads(SPER01.lib12.k3),
                 method = "total")
SPER01.lib12.k3$count = reads(SPER01.lib12.k3)
SPER01.lib12.k3@reads = freq

SPER01.merged = aggregate(SPER01.lib12.k3, MARGIN = 1, by = list(sample_id=samples(SPER01.lib12.k3)
```

Look for controls left in library 1 and 2

```
rownames(SPER01.merged)
```

```
  [1] "X_10" "X_11" "X_12" "X_14" "X_15" "X_16" "X_17" "X_18" "X_19" "X_2"
 [11] "X_20" "X_21" "X_22" "X_23" "X_24" "X_25" "X_26" "X_27" "X_28" "X_29"
 [21] "X_3"  "X_30" "X_31" "X_33" "X_34" "X_35" "X_36" "X_37" "X_38" "X_39"
 [31] "X_4"  "X_41" "X_42" "X_44" "X_51" "X_53" "X_54" "X_56" "X_57" "X_59"
 [41] "X_60" "X_63" "X_64" "X_65" "X_66" "X_68" "X_70" "X_74" "X_75" "X_76"
 [51] "X_77" "X_78" "X_79" "X_80" "X_9"  "Y_1"  "Y_11" "Y_13" "Y_14" "Y_18"
 [61] "Y_21" "Y_23" "Y_24" "Y_25" "Y_26" "Y_28" "Y_29" "Y_3"  "Y_31" "Y_32"
 [71] "Y_33" "Y_34" "Y_36" "Y_38" "Y_39" "Y_4"  "Y_40" "Y_41" "Y_42" "Y_43"
 [81] "Y_45" "Y_5"  "Y_50" "Y_51" "Y_52" "Y_53" "Y_57" "Y_58" "Y_59" "Y_6"
 [91] "Y_61" "Y_69" "Y_7"  "Y_70" "Y_71" "Y_72" "Y_74" "Y_8"  "Y_9"  "Z_1"
[101] "Z_10" "Z_11" "Z_12" "Z_13" "Z_14" "Z_15" "Z_16" "Z_17" "Z_18" "Z_19"
[111] "Z_21" "Z_22" "Z_23" "Z_24" "Z_25" "Z_27" "Z_28" "Z_3"  "Z_31" "Z_32"
[121] "Z_33" "Z_34" "Z_35" "Z_36" "Z_37" "Z_38" "Z_4"  "Z_40" "Z_42" "Z_43"
[131] "Z_44" "Z_46" "Z_48" "Z_49" "Z_5"  "Z_51" "Z_52" "Z_54" "Z_55" "Z_56"
[141] "Z_59" "Z_6"  "Z_60" "Z_62" "Z_63" "Z_65" "Z_66" "Z_67" "Z_68" "Z_69"
[151] "Z_7"  "Z_70" "Z_71" "Z_72" "Z_73" "Z_74" "Z_75" "Z_76" "Z_77" "Z_78"
[161] "Z_79" "Z_8"  "Z_80"
```

4.4. Merge lib 1,2 and 3

4.4.1. Remove controls in library 3

```
rownames(SPER01.lib3)
```

```
  [1] "DNANC_3_R1"  "DNANC_4_R1"  "DNANC_5_R1"  "DNANC_6_R1"  "DNANC_6_R2"
  [6] "PCRNC_1_R1"  "PCRNC_2_R1"  "PCRNC_2_R2"  "PCRPOS_1_R1" "PCRPOS_2_R1"
 [11] "PCRPOS_2_R2" "X_1_R1"      "X_32_R1"     "X_40_R1"     "X_43_R1"
 [16] "X_45_R1"     "X_46_R1"     "X_47_R1"     "X_48_R1"     "X_49_R1"
 [21] "X_52_R1"     "X_55_R1"     "X_58_R1"     "X_5_R1"      "X_62_R1"
 [26] "X_67_R1"     "X_69_R1"     "X_6_R1"      "X_71_R1"     "X_72_R1"
 [31] "X_73_R1"     "X_7_R1"      "X_8_R1"      "Y_10_R1"     "Y_12_R1"
 [36] "Y_15_R1"     "Y_16_R1"     "Y_17_R1"     "Y_19_R1"     "Y_20_R1"
 [41] "Y_22_R1"     "Y_27_R1"     "Y_30_R1"     "Y_35_R1"     "Y_37_R1"
 [46] "Y_54_R1"     "Y_55_R1"     "Y_60_R1"     "Y_62_R1"     "Y_63_R1"
 [51] "Y_64_R1"     "Y_65_R1"     "Y_66_R1"     "Y_67_R1"     "Y_68_R1"
 [56] "Y_73_R1"     "Z_26_R1"     "Z_29_R1"     "Z_2_R1"      "Z_41_R1"
 [61] "Z_47_R1"     "Z_50_R1"     "Z_57_R1"     "Z_58_R1"     "Z_9_R1"
```

```
  SPER01.lib3.samples = SPER01.lib3[-(1:11),]
  rownames(SPER01.lib3.samples@reads) = sub("_R.?$","",rownames(SPER01.lib3.samples))
  rownames(SPER01.lib3.samples)
```

```
 [1] "X_1"  "X_32" "X_40" "X_43" "X_45" "X_46" "X_47" "X_48" "X_49" "X_52"
[11] "X_55" "X_58" "X_5"  "X_62" "X_67" "X_69" "X_6"  "X_71" "X_72" "X_73"
[21] "X_7"  "X_8"  "Y_10" "Y_12" "Y_15" "Y_16" "Y_17" "Y_19" "Y_20" "Y_22"
[31] "Y_27" "Y_30" "Y_35" "Y_37" "Y_54" "Y_55" "Y_60" "Y_62" "Y_63" "Y_64"
[41] "Y_65" "Y_66" "Y_67" "Y_68" "Y_73" "Z_26" "Z_29" "Z_2"  "Z_41" "Z_47"
[51] "Z_50" "Z_57" "Z_58" "Z_9"
```

4.4.2. Merge library 1, 2 and 3

```
  SPER01.lib123.reads = rbind(SPER01.merged@reads,
                              decostand(SPER01.lib3.samples@reads,method = "total"))

  common = intersect(names(SPER01.merged@samples),
                     names(SPER01.lib3.samples@samples))

  SPER01.lib123.samples = rbind(SPER01.merged@samples[,common],
                            SPER01.lib3.samples@samples[,common])

  SPER01.lib123 = metabarcoding.data(reads = decostand(SPER01.lib123.reads,method = "total"),
                                  samples = SPER01.lib123.samples,
                                  motus = SPER01.merged@motus)

  dim(SPER01.lib123)
```

```
[1] 217 194
```

```
  SPER01.lib123@samples$animal_id = sapply(SPER01.lib123@samples$sample_id,
                                           function(x) strsplit(as.character(x),"_")[[1]][1])
```

4.4.3. Check for empty MOTUs

Look at MOTUs still present in the data matrix, but represented by no more reads because of the filtering procedure.

```
  zero = colSums(reads(SPER01.lib123)) == 0
  table(zero)
```

```
zero
FALSE  TRUE
  184    10
```

```
  SPER01.nozero = SPER01.lib123[,!zero]
```

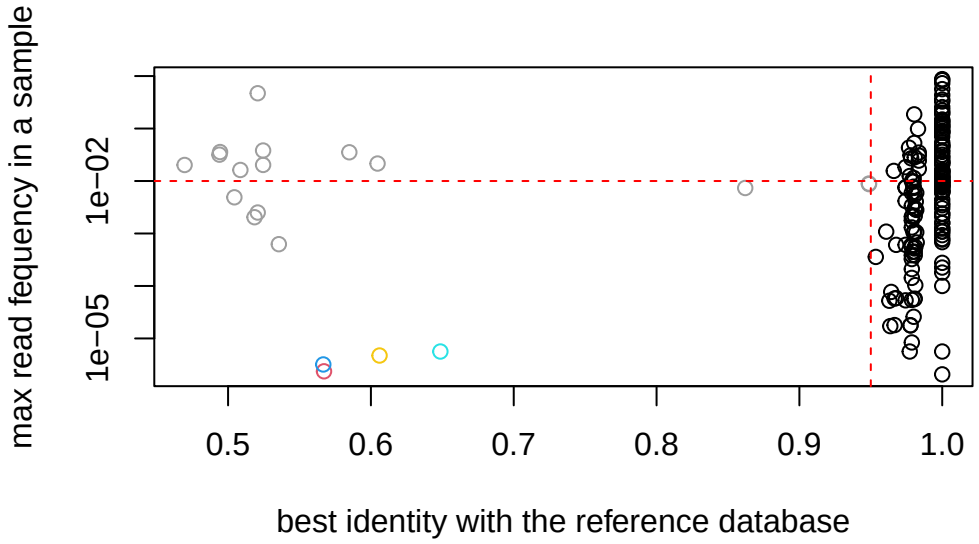
```
  table(SPER01.nozero@motus$sequence_type)
```

```
   SPER01 standard1 standard2 standard3 standard4 standard5 standard6   Unknown
```

```
164          1          0          1          1          0          1         16
```

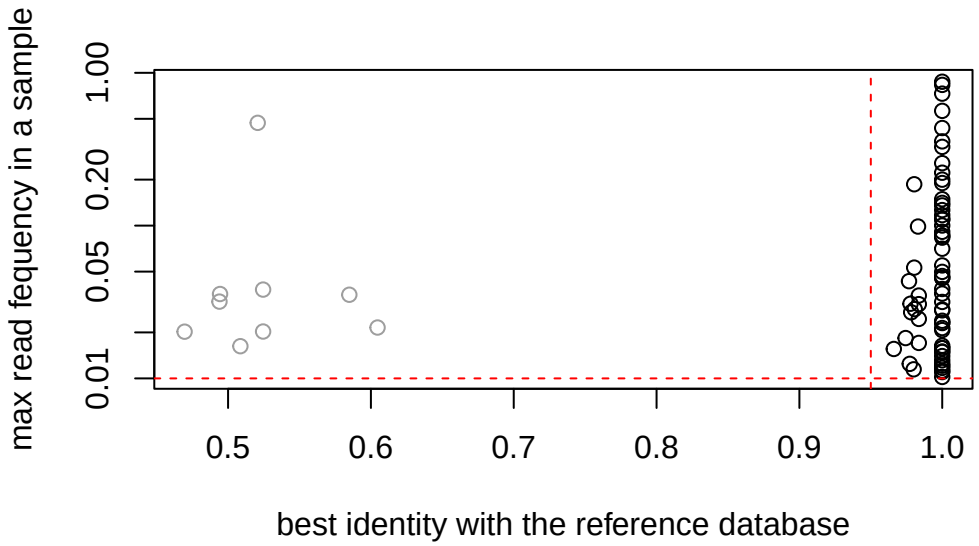
5. Filter out rare species

```
plot(SPER01.nozero@motus$`best_identity:db_GH`,
     apply(reads(SPER01.nozero),2,max),
     col=as.factor(SPER01.nozero@motus$sequence_type),
     log="y",
     ylab="max read fequency in a sample",
     xlab="best identity with the reference database")
abline(h=0.01,col="red",lty=2)
abline(v=0.95,col="red",lty=2)
```




```
SPER01.merged3 = SPER01.lib123[, apply(reads(SPER01.lib123),2,max) > 0.01]
```

```
plot(SPER01.merged3$motus$`best_identity:db_GH`,
     apply(reads(SPER01.merged3),2,max),
     col=as.factor(SPER01.merged3$motus$sequence_type),
     log="y",
     ylab="max read fequency in a sample",
     xlab="best identity with the reference database")
abline(h=0.01,col="red",lty=2)
abline(v=0.95,col="red",lty=2)
```




```
which(SPER01.merged3@motus$sequence_type == "Unknown" & apply(reads(SPER01.merged3),2,max) > 0.4)
```

```
GHP3_00000038
           21
```

```
SPER01.merged3@motus[21,"sequence"]
```

```
[1] "tatagggttttcttggtgtatttcacaccgaaccaggatgggcatgcaaaacaggttggtctcggtagttcagccctcgccatcggcaggggatttt
```

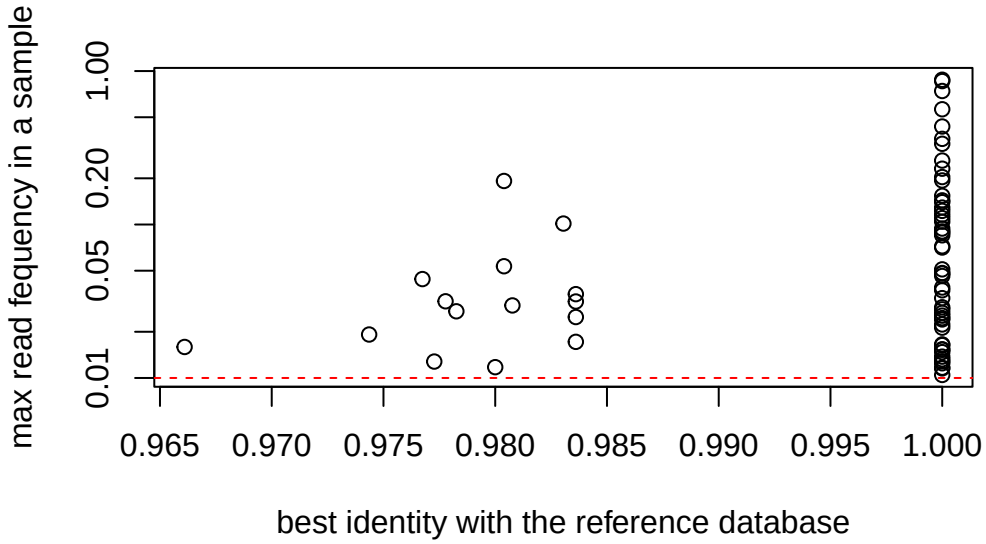
Looks like nothing at embl by blast

5.1. Keep only MOTUs Strictly identical to one of the reference sequence

5.1.1. First level stringency filter (95% identity)

```
SPER01.merged4 = SPER01.merged3[,SPER01.merged3@motus$`best_identity:db_GH` > 0.95]
SPER01.merged4@reads = decostand(SPER01.merged4@reads,method = "total")
SPER01.merged4@motus <- SPER01.merged4@motus %>% select(-starts_with("obiclean_status:"))
```

```
plot(SPER01.merged4$motus$`best_identity:db_GH`,
     apply(reads(SPER01.merged4),2,max),
     col=as.factor(SPER01.merged4$motus$sequence_type),
     log="y",
     ylab="max read fequency in a sample",
     xlab="best identity with the reference database")
abline(h=0.01,col="red",lty=2)
```



5.1.2. High stringency filtering (100% identity)

```
spermatophyta.taxid <- ecofind(taxo,patterns = "^Spermatophyta$")
SPER01.merged4@motus$is_spermatophyta <- is.subcladeof(taxo,SPER01.merged4@motus$taxid,spermatophyt
table(SPER01.merged4@motus$is_spermatophyta)
```

```
FALSE  TRUE
    6    60
```

```
SPER01.merged4@motus %>% filter(!is_spermatophyta)
```

```
                       id best_identity:db_GH best_match:db_GH  count  family
GHP1_00000082 GHP1_00000082                  1         KY427333 137903 1203520
GHP1_00000361 GHP1_00000361                  1         AF515231  49169 1203520
GHP3_00000569 GHP3_00000569                  1         AF192562  56685   13803
GHP3_00000419 GHP3_00000419                  1         HE993635  22646 1203500
GHP1_00000874 GHP1_00000874                  1         AJ133265  13008    3250
GHP1_00008815 GHP1_00008815                  1         AF023727   2987   52989
                  family_name genus   genus_name match_count:db_GH      rank
GHP1_00000082      Athyriaceae 32109     Athyrium                1   species
GHP1_00000361      Athyriaceae 32109     Athyrium                1     genus
GHP3_00000569      Sphagnaceae 13804     Sphagnum                1     genus
GHP3_00000419 Cystopteridaceae 32115 Gymnocarpium                1   species
GHP1_00000874    Lycopodiaceae    NA         <NA>                1 subfamily
GHP1_00008815  Orthotrichaceae    NA         <NA>                1    family
                     scientific_name species          species_list:db_GH
GHP1_00000082        Athyrium sinense  672195        ['Athyrium sinense']
GHP1_00000361                Athyrium      NA                          []
```

```
GHP3_00000569                Sphagnum      NA                           []
GHP3_00000419 Gymnocarpium dryopteris   32116 ['Gymnocarpium dryopteris']
GHP1_00000874          Lycopodioideae      NA                           []
GHP1_00008815         Orthotrichaceae      NA                           []
                         species_name   taxid
GHP1_00000082        Athyrium sinense  672195
GHP1_00000361                    <NA>   32109
GHP3_00000569                    <NA>   13804
GHP3_00000419 Gymnocarpium dryopteris   32116
GHP1_00000874                    <NA> 1965347
GHP1_00008815                    <NA>   52989
                                            sequence sequence_type
GHP1_00000082 atcttgtattattcggatgaatttcgggcgatgaggcga        SPER01
GHP1_00000361 atcttgtattattcagatgaatttcgggcgatgaggcga        SPER01
GHP3_00000569                atcttgttttcataacataaatgg        SPER01
GHP3_00000419 atcttgtattactcaaatgaatttcgggcaatgaggcaa        SPER01
GHP1_00000874                   atcctgtttagcaaatggcgg        SPER01
GHP1_00008815                  atattattttatttaaaaataa        SPER01
              is_spermatophyta
GHP1_00000082            FALSE
GHP1_00000361            FALSE
GHP3_00000569            FALSE
GHP3_00000419            FALSE
GHP1_00000874            FALSE
GHP1_00008815            FALSE
```

```
musaceae.taxid <- ecofind(taxo,patterns = "^Musaceae$")
to_keep <- ! (is.subcladeof(taxo,SPER01.merged4@motus$taxid,musaceae.taxid) | SPER01.merged4@motus$
              is.subcladeof(taxo,SPER01.merged4@motus$taxid,spermatophyta.taxid) &
              SPER01.merged4@motus$`best_identity:db_GH` == 1
table(to_keep)
```

```
to_keep
FALSE  TRUE
   22    45
```

```
SPER01.merged4@motus %>% filter(!to_keep)
```

```
                       id best_identity:db_GH best_match:db_GH   count
GHP2_00000044 GHP2_00000044          0.9803922         AB817362 2080755
GHP2_00000355 GHP2_00000355          0.9807692         AF098856  303606
GHP2_00000136 GHP2_00000136          0.9767442         DQ359689  261620
GHP1_00000082 GHP1_00000082          1.0000000         KY427333  137903
GHP2_00000549 GHP2_00000549          0.9830508         KX872610  135866
GHP1_00000014 GHP1_00000014          0.9803922         AB817372   52144
GHP2_00000171 GHP2_00000171          0.9743590         AC183493   90296
GHP2_00000260 GHP2_00000260          0.9777778         EF440558   51279
GHP2_00000820 GHP2_00000820          0.9772727         AJ505541   61937
GHP1_00000361 GHP1_00000361          1.0000000         AF515231   49169
GHP2_00000554 GHP2_00000554          0.9782609         AJ430966   62557
GHP3_00000569 GHP3_00000569          1.0000000         AF192562   56685
```

```
GHP1_00000681 GHP1_00000681           0.9836066         AB979732   24268
GHP3_00000153 GHP3_00000153           0.9836066         AB979732   19101
GHP1_00003018 GHP1_00003018           0.9836066         AB979732   10572
GHP1_00000030 GHP1_00000030           0.9836066         AB979732   25179
GHP3_00000419 GHP3_00000419           1.0000000         HE993635   22646
GHP1_00000874 GHP1_00000874           1.0000000         AJ133265   13008
GHP2_00008966 GHP2_00008966           0.9661017         KX872610    7043
GHP2_00009529 GHP2_00009529           0.9800000         AY344156    6375
GHP1_00008815 GHP1_00008815           1.0000000         AF023727    2987
GHP3_00027523 GHP3_00027523           1.0000000         AB817687    2256
               family      family_name genus   genus_name match_count:db_GH
GHP2_00000044      NA             <NA>    NA         <NA>                33
GHP2_00000355    4210       Asteraceae    NA         <NA>                 6
GHP2_00000136    3440    Ranunculaceae  3445   Ranunculus                 1
GHP1_00000082 1203520      Athyriaceae 32109     Athyrium                 1
GHP2_00000549    4479          Poaceae    NA         <NA>                NA
GHP1_00000014    3745         Rosaceae    NA         <NA>                14
GHP2_00000171    3700     Brassicaceae    NA         <NA>                 1
GHP2_00000260    3318         Pinaceae  3337        Pinus                 2
GHP2_00000820    4136        Lamiaceae    NA         <NA>                 2
GHP1_00000361 1203520      Athyriaceae 32109     Athyrium                 1
GHP2_00000554      NA             <NA>    NA         <NA>                15
GHP3_00000569   13803      Sphagnaceae 13804     Sphagnum                 1
GHP1_00000681    3514       Betulaceae    NA         <NA>                 1
GHP3_00000153    3514       Betulaceae    NA         <NA>                 1
GHP1_00003018    3514       Betulaceae    NA         <NA>                 1
GHP1_00000030    3514       Betulaceae    NA         <NA>                 1
GHP3_00000419 1203500 Cystopteridaceae 32115 Gymnocarpium                 1
GHP1_00000874    3250    Lycopodiaceae    NA         <NA>                 1
GHP2_00008966    4479          Poaceae    NA         <NA>                11
GHP2_00009529   14101        Juncaceae 13578       Juncus                 2
GHP1_00008815   52989  Orthotrichaceae    NA         <NA>                 1
GHP3_00027523    4637         Musaceae    NA         <NA>                 1
                  rank                          scientific_name species
GHP2_00000044    order                                Asterales      NA
GHP2_00000355 subfamily                            Asteroideae      NA
GHP2_00000136    genus                              Ranunculus      NA
GHP1_00000082  species                     Athyrium sinense  672195
GHP2_00000549     <NA>                                  <NA>      NA
GHP1_00000014    tribe                                 Maleae      NA
GHP2_00000171    tribe                              Brassiceae      NA
GHP2_00000260 subgenus                                   Pinus      NA
GHP2_00000820    tribe                               Mentheae      NA
GHP1_00000361    genus                               Athyrium      NA
GHP2_00000554    order                               Asterales      NA
GHP3_00000569    genus                                Sphagnum      NA
GHP1_00000681   family                               Betulaceae      NA
GHP3_00000153   family                               Betulaceae      NA
GHP1_00003018   family                               Betulaceae      NA
GHP1_00000030   family                               Betulaceae      NA
GHP3_00000419  species                Gymnocarpium dryopteris   32116
GHP1_00000874 subfamily                        Lycopodioideae      NA
```

```
GHP2_00008966   no rank Poeae Chloroplast Group 1 (Aveneae type)      NA
GHP2_00009529     genus                                   Juncus      NA
GHP1_00008815    family                          Orthotrichaceae      NA
GHP3_00027523    family                                  Musaceae      NA

GHP2_00000044
GHP2_00000355
GHP2_00000136
GHP1_00000082
GHP2_00000549
GHP1_00000014                                                    ['Photinia loriformis', 'Docynia
GHP2_00000171
GHP2_00000260
GHP2_00000820
GHP1_00000361
GHP2_00000554 ['Cymbonotus lawsonianus', 'Saussurea nematolepis', 'Helichrysum glumaceum', 'Villarsia
GHP3_00000569
GHP1_00000681
GHP3_00000153
GHP1_00003018
GHP1_00000030
GHP3_00000419
GHP1_00000874
GHP2_00008966                                                                ['Chascolytrum itatiaiae', '
GHP2_00009529
GHP1_00008815
GHP3_00027523
                      species_name   taxid
GHP2_00000044                 <NA>    4209
GHP2_00000355                 <NA>  102804
GHP2_00000136                 <NA>    3445
GHP1_00000082     Athyrium sinense  672195
GHP2_00000549                 <NA>      NA
GHP1_00000014                 <NA>  721813
GHP2_00000171                 <NA>  981071
GHP2_00000260                 <NA>  139271
GHP2_00000820                 <NA>  216718
GHP1_00000361                 <NA>   32109
GHP2_00000554                 <NA>    4209
GHP3_00000569                 <NA>   13804
GHP1_00000681                 <NA>    3514
GHP3_00000153                 <NA>    3514
GHP1_00003018                 <NA>    3514
GHP1_00000030                 <NA>    3514
GHP3_00000419 Gymnocarpium dryopteris   32116
GHP1_00000874                 <NA> 1965347
GHP2_00008966                 <NA> 1652080
GHP2_00009529                 <NA>   13578
GHP1_00008815                 <NA>   52989
GHP3_00027523                 <NA>    4637
                                                                sequence
GHP2_00000044            atcacgtttccgaaaacaaacaaaggttcagaaagcgaaaataaaaaag
```

```
GHP2_00000355           atcacgttttccgaaaacaaacaaaggttcagaaagcgaaaagaaaaaaaa
GHP2_00000136                   atcctgctttcagaaaacaaaaagagggttcagaaagcaaagg
GHP1_00000082                       atcttgtattattcggatgaatttcgggcgatgaggcga
GHP2_00000549    atccgtgttttgagaaaacaaaggggttctcgaatcgaactataatacaaaggaaaag
GHP1_00000014           atcctgttttatgaaaataaacaagggtttcataaaccgaaaataaaaaag
GHP2_00000171                       atcatgggttacgcgaacaaaccaaagtttagaaagcgg
GHP2_00000260                atccggttcatgaagacaatgtttcttctcctaagataggaaggg
GHP2_00000820                  atcctgttttcccaaaacaaaggtttcaaaaaacgaaaaaaag
GHP1_00000361                       atcttgtattattcagatgaatttcgggcgatgaggcga
GHP2_00000554               atcacgttttccgaaaacaaaggttcagaaagcgaaaatcaaaaag
GHP3_00000569                                       atcttgttttcataacataaatgg
GHP1_00000681 gtcctgttttccgaaaacaaataaaacaaatttaagggttcataaagtgagaataaaaaag
GHP3_00000153 ctcctgttttccgaaaacaaataaaacaaatttaagggttcataaagtgagaataaaaaag
GHP1_00003018 ttcctgttttccgaaaacaaataaaacaaatttaagggttcataaagtgagaataaaaaag
GHP1_00000030  tcctgttttccgaaaacaaataaaacaaatttaagggttcataaagtgagaataaaaaag
GHP3_00000419                       atcttgtattactcaaatgaatttcgggcaatgaggcaa
GHP1_00000874                                         atcctgtttagcaaatggcgg
GHP2_00008966    atccgtgttttgagaaaacaaaggggttctcaaatcgaactataatacaaaggaaaag
GHP2_00009529            gtctttattttgataaaatttgtttttatagaaaaattcaaatcaaaaaa
GHP1_00008815                                       atattattttatttaaaaataa
GHP3_00027523              atccttattttgagaaaacaaaggtttataaaactagaatttaaaag
              sequence_type is_spermatophyta
GHP2_00000044        SPER01             TRUE
GHP2_00000355        SPER01             TRUE
GHP2_00000136        SPER01             TRUE
GHP1_00000082        SPER01            FALSE
GHP2_00000549        SPER01               NA
GHP1_00000014        SPER01             TRUE
GHP2_00000171        SPER01             TRUE
GHP2_00000260        SPER01             TRUE
GHP2_00000820        SPER01             TRUE
GHP1_00000361        SPER01            FALSE
GHP2_00000554        SPER01             TRUE
GHP3_00000569        SPER01            FALSE
GHP1_00000681        SPER01             TRUE
GHP3_00000153        SPER01             TRUE
GHP1_00003018        SPER01             TRUE
GHP1_00000030        SPER01             TRUE
GHP3_00000419        SPER01            FALSE
GHP1_00000874        SPER01            FALSE
GHP2_00008966        SPER01             TRUE
GHP2_00009529        SPER01             TRUE
GHP1_00008815        SPER01            FALSE
GHP3_00027523        SPER01             TRUE
```

```
SPER01.final <- SPER01.merged4[,which(to_keep)]
SPER01.final@reads <- decostand(SPER01.final@reads,method = "total")
```

6. Saving the filtered dataset

6.1. Updating the sample metadata

6.1.1. Adding samples metadata

```
metadata <- read_csv("Data/Faeces/metadata.csv",
                     show_col_types = FALSE)
```

```
SPER01.final@samples %<>%
  select(sample_id,animal_id) %>%
  left_join(metadata,by = "sample_id") %>%
  mutate(id = sample_id) %>%
  column_to_rownames("id") %>%
  select(sample_id,animal_id,Sample_number,Date,Sample_time,times_from_birch, Fed_biomass)
```

6.1.2. Homogenize time from burch

Adds : - 6 hours to animal X, - 3 hours to animal Y, - 4 hours to animal 2

```
SPER01.final@samples %<>%
  mutate(times_from_birch = times_from_birch +
           ifelse(animal_id == "X",6,
         ifelse(animal_id == "Y",3,4)))
```

```
SPER01.final@samples %<>%
  mutate(Animal_id = ifelse(animal_id == "X","9/10",
         ifelse(animal_id == "Y","10/10","12/10")))
```

6.1.3. Adds pellets consumption data

```
pellets <- read_tsv("Data/pellet_weigth.txt", show_col_types = FALSE) %>%
           mutate(Date = str_replace(Date,"2018","18")) %>%
           separate(Date, c("d","m","y"),sep = "/") %>%
           mutate(d = as.integer(d)+1,
                  m = as.integer(m),
                  m = ifelse(d==32,m+1,m),
                  d = ifelse(d==32,1,d),
                  d = sprintf("%02d",d),
                  m = sprintf("%02d",m)) %>%
           unite(col="Date",d,m,y,sep="/") %>%
           pivot_longer(-Date,names_to = "Animal_id",values_to = "pellets")

SPER01.final@samples %<>%
   left_join(pellets)
```

```
Joining with `by = join_by(Date, Animal_id)`
```

6.2. Only keep samples

```
SPER01.final <- SPER01.final[which(str_detect(SPER01.final@samples$sample_id,"^[XYZ]")),]
```

6.3. Updating count statistics

```
SPER01.final %<>%
  update_motus_count() %>%
  update_samples_count() %>%
  clean_empty()
```

6.4. Add MOTUs Metadata

```
SPER01.final@motus %<>%
  mutate(category = ifelse(is.subcladeof(taxo,taxid,spermatophyta.taxid),
                           "Plant",
                           "Lichen"))
```

6.5. Write CSV files

```
write_csv(SPER01.final@samples,
          file = "Data/Faeces/FE.Spermatophyta.samples.samples.csv")
write_csv(SPER01.final@motus,
          file = "Data/Faeces/FE.Spermatophyta.samples.motus.csv")
write_csv(SPER01.final@reads %>%
            decostand(method = "total") %>%
            as.data.frame()%>%
            rownames_to_column("id"),
          file = "Data/Faeces/FE.Spermatophyta.samples.reads.csv")
```

References

[1] H. Wickham, M. Averick, J. Bryan, W. Chang, L. McGowan, R. François, G. Grolemund, A. Hayes, L. Henry, J. Hester, M. Kuhn, T. Pedersen, E. Miller, S. Bache, K. Müller, J. Ooms, D. Robinson, D. Seidel, V. Spinu, K. Takahashi, D. Vaughan, C. Wilke, K. Woo, H. Yutani, Welcome to the tidyverse, Journal of open source software 4 (43) (2019) 1686. doi:10.21105/joss.01686.
URL https://joss.theoj.org/papers/10.21105/joss.01686

[2] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York, 2016.
URL https://ggplot2.tidyverse.org