Tutorial - R Dada2 metabarcode analyis

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Contents

1 Aim

This tutorial explain how to process Illumina data with the Dada2 suite as implemented in R (dada2 is also implemented in Qiime). It is adapted from : https://benjjneb.github.io/dada2/tutorial.html

2 Directory structure

Relative to the main directory from GitHub

- **../fastq** : fastq files
- **../fastq_filtered** : fastq files after filtration
- **../qual_pdf** : qual pdf files
- **../dada2** : dada2 processed files
- **../databases** : PR2 database files (contains PR2 database formatted for dada2 - https://github.com/pr2database/pr2database/releases/)
- **../blast** : BLAST files output
- **../img** : Images
- **../R_dada2** : This tutorial for Illumina files

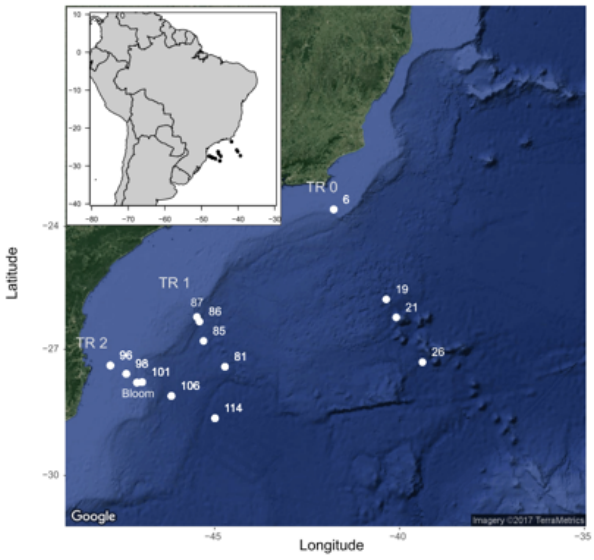
3 Downloads

Install the following software :

- R : https://pbil.univ-lyon1.fr/CRAN/
- R studio : https://www.rstudio.com/products/rstudio/download/#download
- Download and install the following libraries by running under R studio the following lines

```
install.packages("readr")      # To read and write files
install.packages("readxl")     # To read excel files

install.packages("dplyr")      # To manipulate dataframes
install.packages("tibble")     # To work with data frames
install.packages("tidyr")      # To work with data frames

install.packages("stringr")    # To manipulate strings

install.packages("ggplot2")    # To do plots


source("https://bioconductor.org/biocLite.R")
biocLite('dada2')              # metabarcode data analysis
biocLite('phyloseq')           # metabarcode data analysis
biocLite('Biostrings')         # needed for fastq.geometry
```

4 Data used



The samples originate from the CARBOM cruise (2013) off Brazil.

Samples have been sorted by flow cytometry and 3 genes have been PCR amplified :

- 18S rRNA - V4 region
- 16S rNA with plastid
- nifH

The PCR products have been sequenced by 1 run of Illumina 2*250 bp. The data consist of the picoplankton samples from one transect and fastq files have been subsampled with 1000 sequences per sample.

4.1 References

- Gerikas Ribeiro C, Marie D, Lopes dos Santos A, Pereira Brandini F, Vaulot D. (2016). Estimating microbial populations by flow cytometry: Comparison between instruments. Limnol Oceanogr Methods 14:750–758.
- Gerikas Ribeiro C, Lopes dos Santos A, Marie D, Brandini P, Vaulot D. (2018). Relationships between photosynthetic eukaryotes and nitrogen-fixing cyanobacteria off Brazil. ISME J in press.
- Gerikas Ribeiro C, Lopes dos Santos A, Marie D, Helena Pellizari V, Pereira Brandini F, Vaulot D. (2016). Pico and nanoplankton abundance and carbon stocks along the Brazilian Bight. PeerJ 4:e2587.

5 Tutorial description

5.1 Load the necessary libraries**

```
library("dada2")
library("phyloseq")
library("Biostrings")

library("ggplot2")

library("dplyr")
library("tidyr")
library("tibble")

library("readxl")
library("readr")

library("stringr")

library("kableExtra")  # necessary for nice table formatting with knitr
```

5.2 Set up directories

Create directories that will be used to stored the files at the different stage of the processing

```
# change the following line to the path where you unzipped the tutorials


fastq_dir <- "../fastq/"  # fastq directory
filtered_dir <- "../fastq_filtered/"  # fastq filtered
qual_dir <- "../qual_pdf/"  # qual pdf
dada2_dir <- "../dada2/"  # dada2 results
blast_dir <- "../blast/"  # blast2 results
database_dir <- "../databases/"  # databases

dir.create(filtered_dir)
dir.create(qual_dir)
dir.create(dada2_dir)
dir.create(blast_dir)
```

5.3 Primers

Note that the primers are degenerated. Dada2 has an option to remove primers (FilterandTrim) but this function will not accept degeneracy.

```
primer_set_fwd = c("CCAGCAGCCGCGGTAATTCC", "CCAGCACCCGCGGTAATTCC", "CCAGCAGCTGCGGTAATTCC",
    "CCAGCACCTGCGGTAATTCC")
primer_set_rev = c("ACTTTCGTTCTTGATYRATGA")
primer_length_fwd <- str_length(primer_set_fwd[1])
primer_length_rev <- str_length(primer_set_rev[1])
```

5.4 PR2 tax levels

```
PR2_tax_levels <- c("Kingdom", "Supergroup", "Division", "Class", "Order", "Family",
    "Genus", "Species")
```

5.5 Examine the fastQ files

5.5.1 Construct a list of the fastq files

It is assumed that the sample names are at the start of file name and separated by _.

```
# get a list of all fastq files in the ngs directory and separate R1 and R2
fns <- sort(list.files(fastq_dir, full.names = TRUE))
fns <- fns[str_detect(basename(fns), ".fastq")]
fns_R1 <- fns[str_detect(basename(fns), "R1")]
fns_R2 <- fns[str_detect(basename(fns), "R2")]

# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
sample.names <- str_split(basename(fns_R1), pattern = "_", simplify = TRUE)
sample.names <- sample.names[, 1]
```

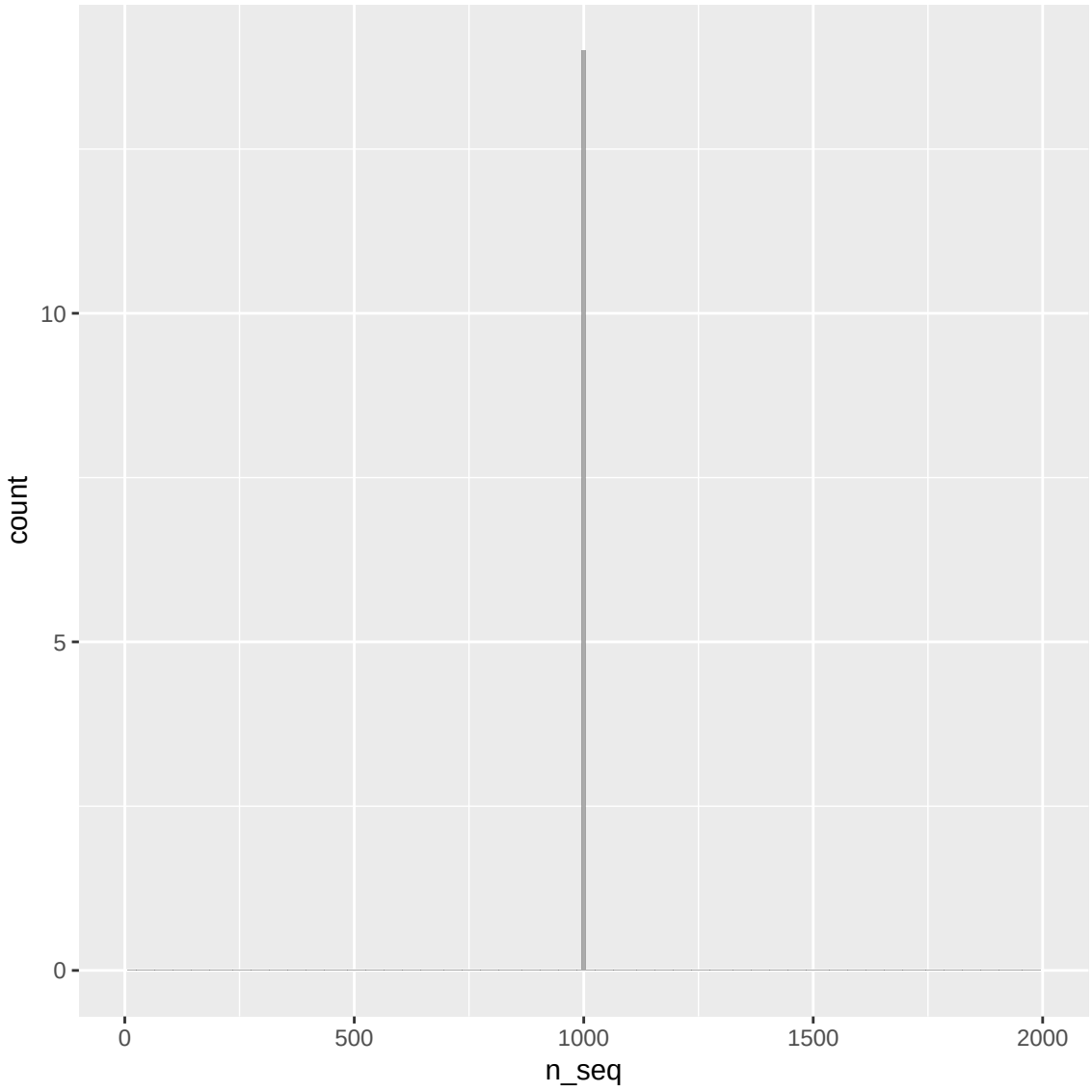
5.5.2 Compute number of paired reads

```
# create an empty data frame
df <- data.frame()

# loop through all the R1 files (no need to go through R2 which should be
# the same)

for (i in 1:length(fns_R1)) {

    # use the dada2 function fastq.geometry
    geom <- fastq.geometry(fns_R1[i])

    # extract the information on number of sequences and file name
    df_one_row <- data.frame(n_seq = geom[1], file_name = basename(fns[i]))

    # add one line to data frame
    df <- bind_rows(df, df_one_row)
}
```

```r
# display number of sequences and write data to small file
knitr::kable(df)
```

n_seq	file_name
1000	120p_S39_R1.subsample.fastq
1000	120p_S39_R2.subsample.fastq
1000	121p_S57_R1.subsample.fastq
1000	121p_S57_R2.subsample.fastq
1000	122p_S4_R1.subsample.fastq
1000	122p_S4_R2.subsample.fastq
1000	125p_S22_R1.subsample.fastq
1000	125p_S22_R2.subsample.fastq
1000	126p_S40_R1.subsample.fastq
1000	126p_S40_R2.subsample.fastq
1000	140p_S5_R1.subsample.fastq
1000	140p_S5_R2.subsample.fastq
1000	141p_S23_R1.subsample.fastq
1000	141p_S23_R2.subsample.fastq

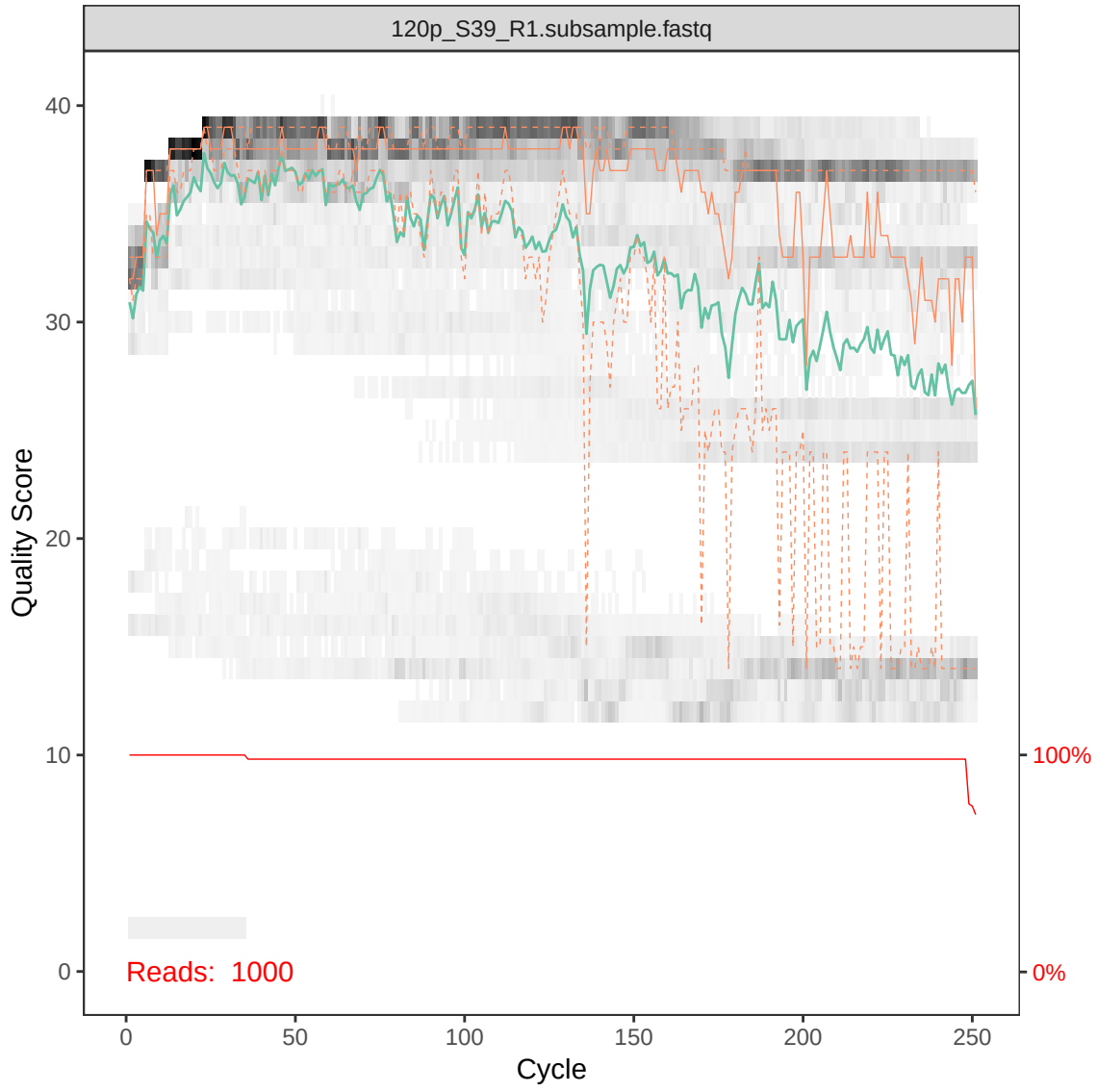
```r
# write.table(df, file = 'n_seq.txt', sep='\t', row.names = FALSE, na='',
# quote=FALSE)

# plot the histogram with number of sequences
ggplot(df, aes(x = n_seq)) + geom_histogram(alpha = 0.5, position = "identity",
    binwidth = 10) + xlim(0, 2000)
```

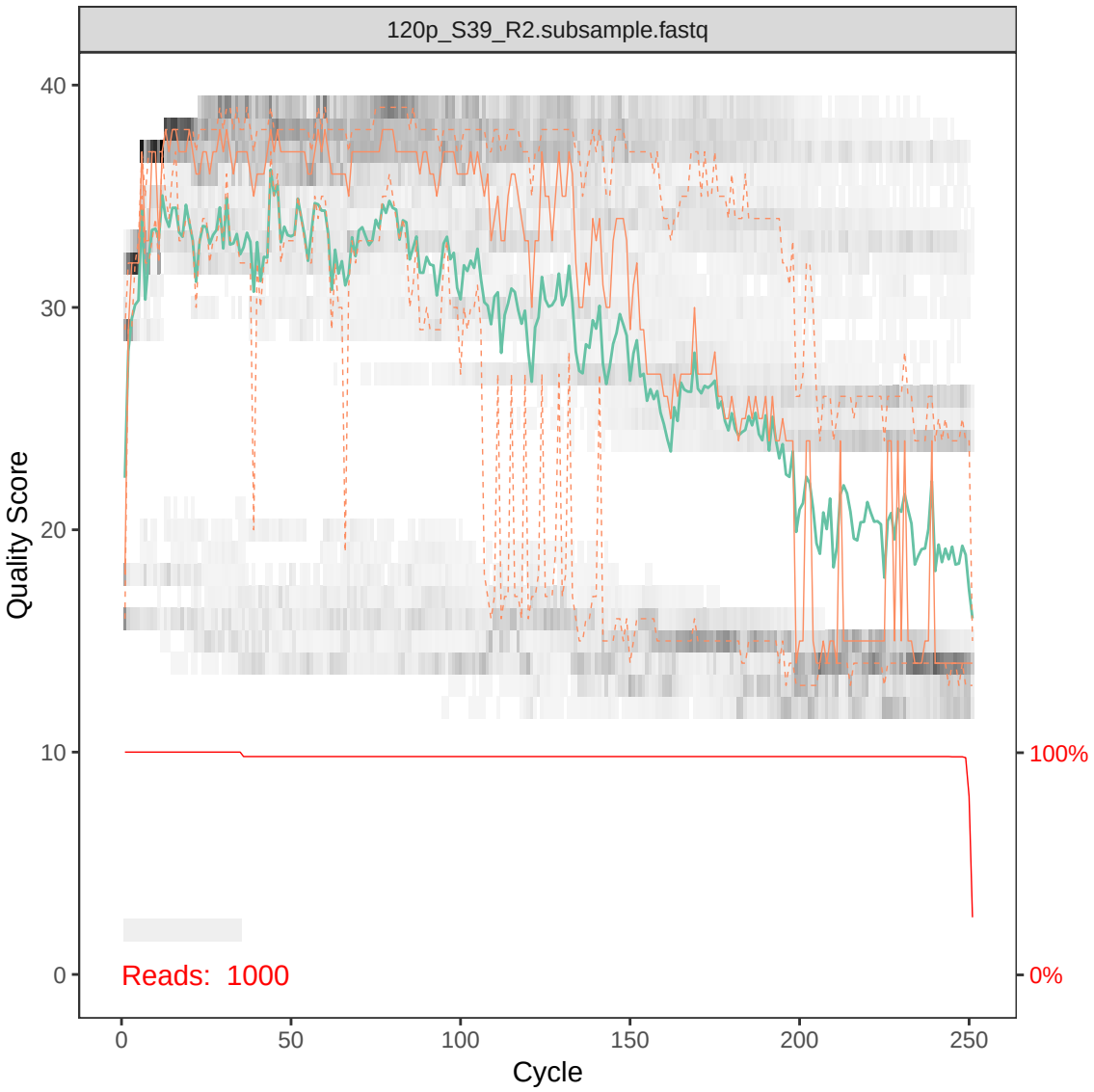


5.5.3 Plot quality for reads

```
for (i in 1:length(fns)) {

    # Use dada2 function to plot quality
    p1 <- plotQualityProfile(fns[i])

    # Only plot on screen for first 2 files
    if (i <= 2) {
        print(p1)
    }

    # save the file as a pdf file (uncomment to execute)
    p1_file <- paste0(qual_dir, basename(fns[i]), ".qual.pdf")
    ggsave(plot = p1, filename = p1_file, device = "pdf", width = 15, height = 15,
```

```
        scale = 1, units = "cm")
}
```





5.6 Filter and Trim the reads

The dada2 algorithm requires primers to be removed prior to processing.

- Using dada2 there are 2 possibilities
  - Remove by sequence, but dada2 does not allow for ambiguities
  - Remove by position, which is not a problem for Illumina sequences but is a problem for 454
- For complex situation we recommend to use **cutadapt** to remove the primers : http://cutadapt.readthedocs.io/en/stable/guide.html#.
  The program is really very powerful.

5.6.1 Create names for the filtered files

We create the name of the files that will be generated by the `filterAndTrim` function in the step below. These names are composed by the path name ("../fastq_filtered/"), the sample names, the read number (R1 or R2) and a "_filt" suffix.

```
filt_R1 <- str_c(filtered_dir, sample.names, "_R1_filt.fastq")
filt_R2 <- str_c(filtered_dir, sample.names, "_R2_filt.fastq")
```

5.6.2 Removing the primers by sequence (DO NOT EXECUTE THIS STEP)

- Go to next step

The next piece of code could be used to remove the primers by **sequence**. The dada2 package does not allow for primer degeneracy. Since our forward primer is degenerated at two positions, all four combinations need to be tested. However it will be necessary to re-assemble after that the 4 fastQ files created (which has not to done). So the better strategy is to remove primer by trunction (see next step).

```
# On Windows set multithread=FALSE

out_all <- data.frame(id = length(fns_R1))
for (i in 1:4) {
    out <- filterAndTrim(fns_R1, filt_R1, fns_R2, filt_R2, truncLen = c(250,
        240), trimLeft = c(0, 0), maxN = 0, maxEE = c(Inf, Inf), truncQ = 10,
        rm.phix = TRUE, primer.fwd = primer_set_fwd[i], compress = FALSE, multithread = FALSE)
    out_all <- cbind(out_all, out)

}
```

```
knitr::kable(out_all, "latex") %>% kable_styling(bootstrap_options = "striped",
    font_size = 7)
```

5.6.3 Remove primers by truncation and filter

Filter all sequences with N, truncate R2 to 240 bp

```
out <- filterAndTrim(fns_R1, filt_R1, fns_R2, filt_R2, truncLen = c(250, 240),
    trimLeft = c(primer_length_fwd, primer_length_rev), maxN = 0, maxEE = c(2,
        2), truncQ = 10, rm.phix = TRUE, compress = FALSE, multithread = FALSE)
```

5.7 Dada2 processing

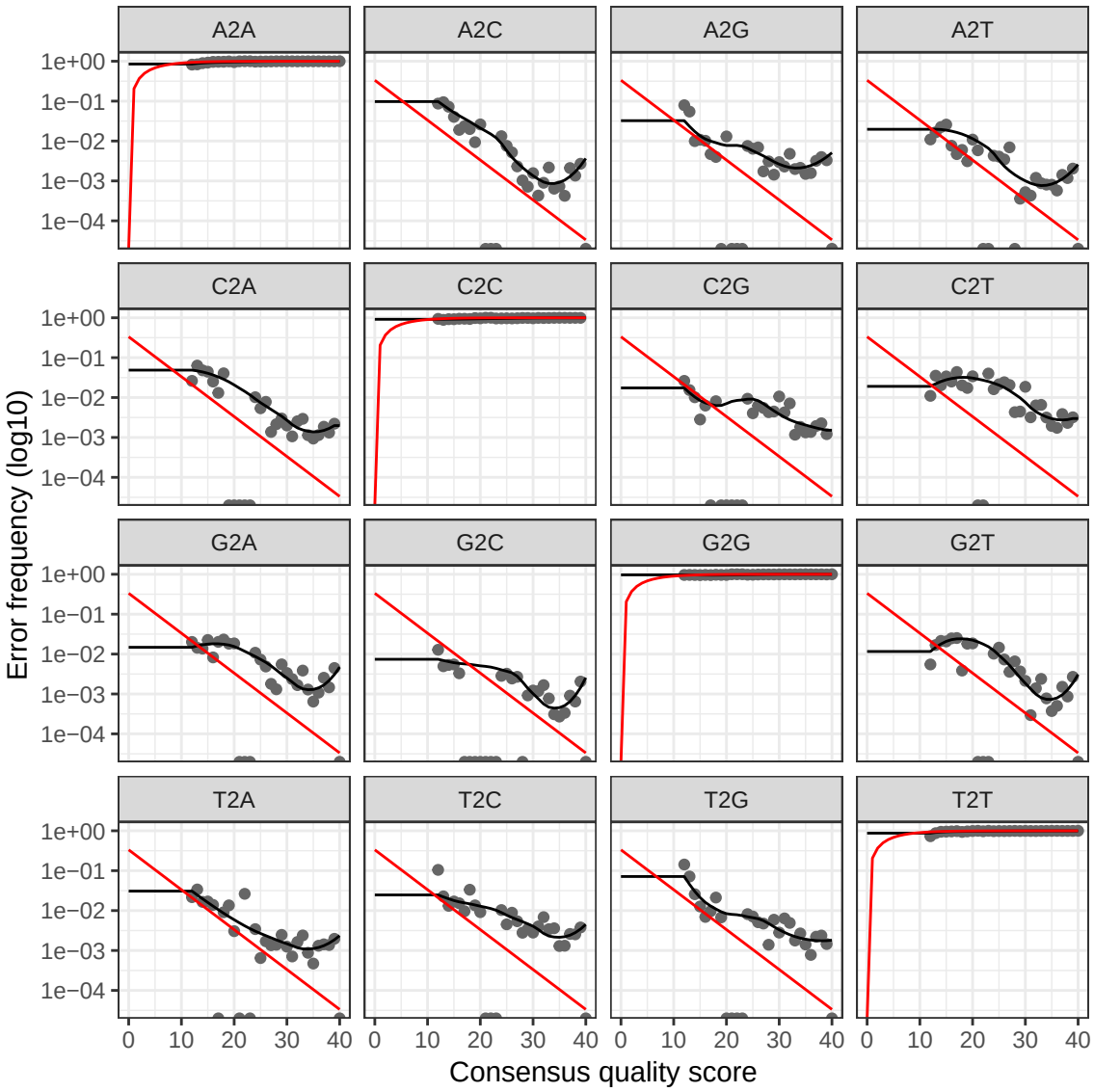
5.7.1 Learn error rates

The error rates are plotted.

```
err_R1 <- learnErrors(filt_R1, multithread = FALSE)
```

```
1581480 total bases in 6876 reads from 14 samples will be used for learning the error rates.
Initializing error rates to maximum possible estimate.
selfConsist step 1 ..............
   selfConsist step 2
   selfConsist step 3
Convergence after  3  rounds.
```

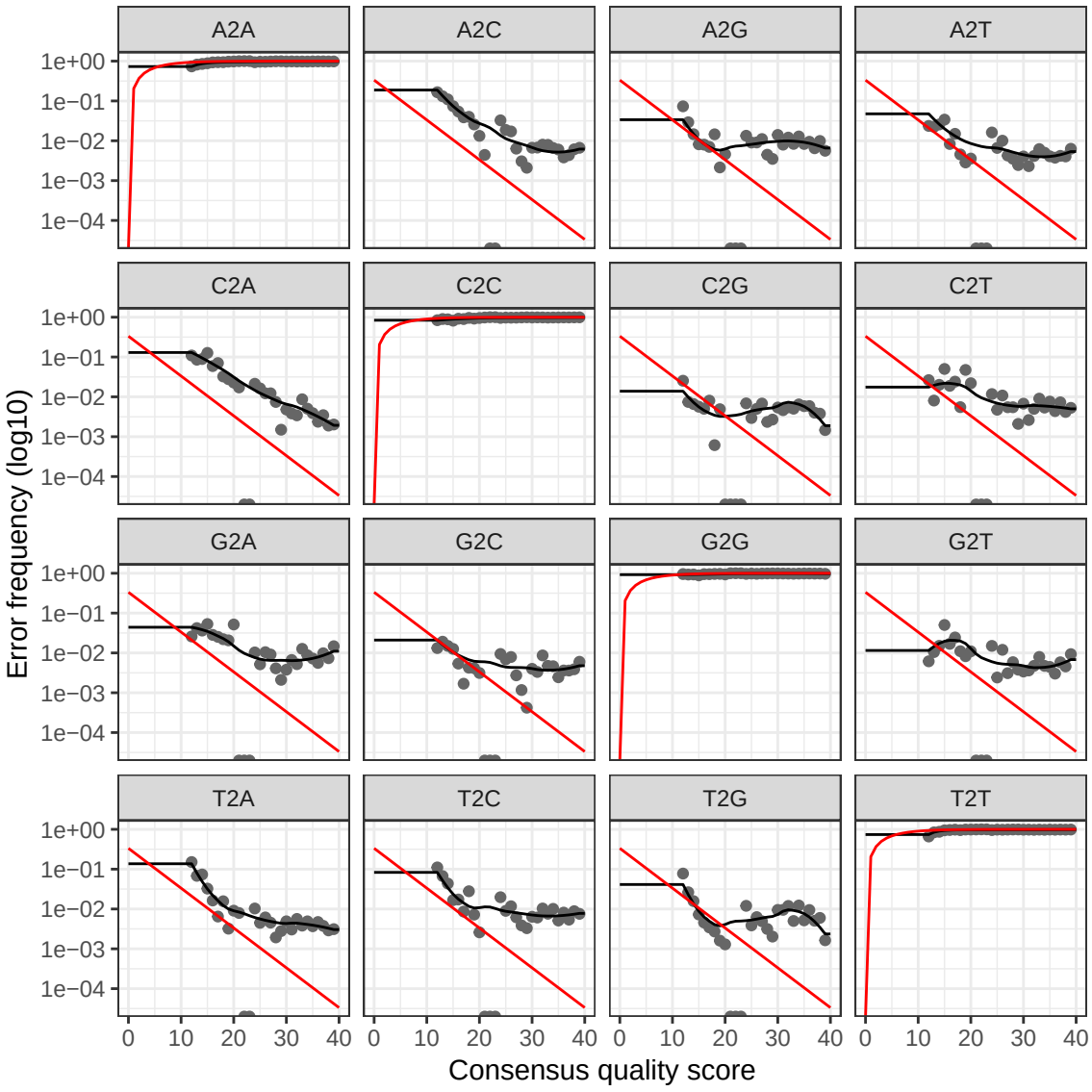
```
plotErrors(err_R1, nominalQ = TRUE)
```




```
err_R2 <- learnErrors(filt_R2, multithread = FALSE)
```

```
1505844 total bases in 6876 reads from 14 samples will be used for learning the error rates.
Initializing error rates to maximum possible estimate.
selfConsist step 1 ..............
   selfConsist step 2
   selfConsist step 3
Convergence after  3  rounds.
```

```
plotErrors(err_R2, nominalQ = TRUE)
```



5.7.2 Dereplicate the reads

```
derep_R1 <- derepFastq(filt_R1, verbose = FALSE)
derep_R2 <- derepFastq(filt_R2, verbose = FALSE)

# Name the derep-class objects by the sample names
names(derep_R1) <- sample.names
names(derep_R2) <- sample.names
```

5.7.3 Sequence-variant inference algorithm to the dereplicated data

```
dada_R1 <- dada(derep_R1, err = err_R1, multithread = FALSE, pool = FALSE)
```

```
Sample 1 - 256 reads in 93 unique sequences.
```

```
Sample 2 - 457 reads in 166 unique sequences.
Sample 3 - 407 reads in 128 unique sequences.
Sample 4 - 553 reads in 220 unique sequences.
Sample 5 - 508 reads in 219 unique sequences.
Sample 6 - 456 reads in 147 unique sequences.
Sample 7 - 473 reads in 180 unique sequences.
Sample 8 - 583 reads in 211 unique sequences.
Sample 9 - 528 reads in 172 unique sequences.
Sample 10 - 530 reads in 211 unique sequences.
Sample 11 - 513 reads in 177 unique sequences.
Sample 12 - 521 reads in 199 unique sequences.
Sample 13 - 519 reads in 172 unique sequences.
Sample 14 - 572 reads in 170 unique sequences.
```

```
dada_R2 <- dada(derep_R2, err = err_R2, multithread = FALSE, pool = FALSE)
```

```
Sample 1 - 256 reads in 236 unique sequences.
Sample 2 - 457 reads in 391 unique sequences.
Sample 3 - 407 reads in 299 unique sequences.
Sample 4 - 553 reads in 409 unique sequences.
Sample 5 - 508 reads in 451 unique sequences.
Sample 6 - 456 reads in 335 unique sequences.
Sample 7 - 473 reads in 347 unique sequences.
Sample 8 - 583 reads in 458 unique sequences.
Sample 9 - 528 reads in 418 unique sequences.
Sample 10 - 530 reads in 404 unique sequences.
Sample 11 - 513 reads in 384 unique sequences.
Sample 12 - 521 reads in 395 unique sequences.
Sample 13 - 519 reads in 396 unique sequences.
Sample 14 - 572 reads in 400 unique sequences.
```

```
dada_R1[[1]]
```

```
dada-class: object describing DADA2 denoising results
5 sequence variants were inferred from 93 input unique sequences.
Key parameters: OMEGA_A = 1e-40, OMEGA_C = 1e-40, BAND_SIZE = 16
```

```
dada_R2[[1]]
```

```
dada-class: object describing DADA2 denoising results
3 sequence variants were inferred from 236 input unique sequences.
Key parameters: OMEGA_A = 1e-40, OMEGA_C = 1e-40, BAND_SIZE = 16
```

5.7.4 Merge sequences

```
mergers <- mergePairs(dada_R1, derep_R1, dada_R2, derep_R2, verbose = TRUE)

# Inspect the merger data.frame from the first sample
knitr::kable(head(mergers[[1]]))
```

sequence
AGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAACGCTCGTAGTCGGATTTCGGGGCAGGTTTGCCGGTC
CACACGTCTAATGTTGCATTGTAAAGCACAAACCACTGTTTTACATTTAGCTGCAGAAAGAGGAACTGTAGAAC
AGCTCCAATAGCGTATACTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGGTCGAAGCAGCCGCTC

5.7.5 Make sequence table

```
seqtab <- makeSequenceTable(mergers)

dim(seqtab)
```

```
[1] 14 58
```

```
# Make a transposed of the seqtab to make it be similar to mothur database
t_seqtab <- t(seqtab)

# Inspect distribution of sequence lengths
table(nchar(getSequences(seqtab)))
```

```
318 351 360 363 367 369 371 372 373 375 376 377 378 379 380 382 383 384
  3   1   1   3   1   1   2   1   2   1   3   6  12   4   4   2   5   2
387 388
  1   3
```

5.7.6 Remove chimeras

Note that remove chimeras will produce spurious results if primers have not be removed. The parameter `methods` can be `pooled` or `consensus`

```
seqtab.nochim <- removeBimeraDenovo(seqtab, method = "consensus", multithread = FALSE,
    verbose = TRUE)

# Compute % of non chimeras
paste0("% of non chimeras : ", sum(seqtab.nochim)/sum(seqtab) * 100)
```

```
[1] "% of non chimeras : 100"
```

```
paste0("total number of sequences : ", sum(seqtab.nochim))
```

```
[1] "total number of sequences : 5869"
```

In our case there were no chimeras found. It is noteworthy that the total number of sequences is almost twice that what is recovered with **mothur** which is **2573**

5.7.7 Track number of reads at each step

```
# define a function
getN <- function(x) sum(getUniques(x))

track <- cbind(out, sapply(dada_R1, getN), sapply(mergers, getN), rowSums(seqtab),
    rowSums(seqtab.nochim))

colnames(track) <- c("input", "filtered", "denoised", "merged", "tabled", "nonchim")
rownames(track) <- sample.names

knitr::kable(track)
```

	input	filtered	denoised	merged	tabled	nonchim
120p	1000	256	241	223	223	223
121p	1000	457	446	397	397	397
122p	1000	407	396	357	357	357
125p	1000	553	551	464	464	464
126p	1000	508	493	340	340	340
140p	1000	456	441	427	427	427
141p	1000	473	460	381	381	381
142p	1000	583	567	495	495	495
155p	1000	528	524	445	445	445
156p	1000	530	525	426	426	426
157p	1000	513	507	438	438	438
165p	1000	521	509	442	442	442
166p	1000	519	510	478	478	478
167p	1000	572	563	556	556	556

```
write_tsv(data.frame(track), str_c(dada2_dir, "read_numbers_dada2.tsv"))
```

5.7.8 Transforming and saving the ASVs sequences

In the OTU put of dada2, otu names are the sequences. We change to give a Otuxxx name and the sequences are stored in the taxonomy table.

```
seqtab.nochim_trans <- as.data.frame(t(seqtab.nochim)) %>% rownames_to_column(var = "sequence") %>%
    rowid_to_column(var = "OTUNumber") %>% mutate(OTUNumber = sprintf("otu%04d",
    OTUNumber)) %>% mutate(sequence = str_replace_all(sequence, "(-|\\.)", ""))

df <- seqtab.nochim_trans
seq_out <- Biostrings::DNAStringSet(df$sequence)

names(seq_out) <- df$OTUNumber

Biostrings::writeXStringSet(seq_out, str_c(dada2_dir, "CARBOM_ASV_no_taxo.fasta"),
    compress = FALSE, width = 20000)
```

5.7.9 Assigning taxonomy

This step is quite long... If you want to skip please go to next step.

```
pr2_file <- paste0(database_dir, "pr2_version_4.72_dada2.fasta.gz")
taxa <- assignTaxonomy(seqtab.nochim, refFasta = pr2_file, taxLevels = PR2_tax_levels,
    minBoot = 0, outputBootstraps = TRUE, verbose = TRUE)
saveRDS(taxa, str_c(dada2_dir, "CARBOM.taxa.rds"))
```

5.7.10 Export data as produced by Dada2

```
taxa <- readRDS(str_c(dada2_dir, "CARBOM.taxa.rds"))
write_tsv(as.tibble(taxa$tax), path = str_c(dada2_dir, "taxa.txt"))
write_tsv(as.tibble(taxa$boot), path = str_c(dada2_dir, "taxa_boot.txt"))
write_tsv(as.tibble(seqtab.nochim), path = str_c(dada2_dir, "seqtab.txt"))
```

5.7.11 Appending taxonomy and boot to the sequence table

```
taxa_tax <- as.data.frame(taxa$tax)
taxa_boot <- as.data.frame(taxa$boot) %>% rename_all(funs(str_c(., "_boot")))
seqtab.nochim_trans <- taxa_tax %>% bind_cols(taxa_boot) %>% bind_cols(seqtab.nochim_trans)
```

5.7.12 Filter for 18S

Remember that we sequenced 3 genes (18S, 16S plastid and nifH. We remove the sequences are not 18S by selecting only bootstrap value for Supergroup in excess of 80.

```
bootstrap_min <- 80

# Filter based on the bootstrap
seqtab.nochim_18S <- seqtab.nochim_trans %>% dplyr::filter(Supergroup_boot >=
    bootstrap_min)

# Create a database like file for dada2
write_tsv(seqtab.nochim_18S, str_c(dada2_dir, "CARBOM_dada2.database.tsv"))
```

5.7.13 Write FASTA file for BLAST analysis with taxonomy

Use the Biostrings library

```
df <- seqtab.nochim_18S
seq_out <- Biostrings::DNAStringSet(df$sequence)

names(seq_out) <- str_c(df$OTUNumber, df$Supergroup, df$Division, df$Class,
    df$Order, df$Family, df$Genus, df$Species, sep = "|")

Biostrings::writeXStringSet(seq_out, str_c(blast_dir, "CARBOM_ASV.fasta"), compress = FALSE,
    width = 20000)
```

This file can be sent to a server and a BLAST analysis can be done using the following qsub file

```
#!/bin/bash
# Commands starting with  '#$' are interpreted by SGE
# Shell to be used for the job
#$ -S /bin/bash
# User to be informed
#$ -M vaulot@sb-roscoff.fr
# Export all environment variable
#$ -V
# Send a message by email  at beginning (b), end (e) and abort (a) of job
#$ -m bea
# Standard output.  Can use '-j y' to add stderr with stdout
#$ -o repl
# Send the commande from the curent directory where the script reside
#$ -cwd
# Define environmental variables

# submitted with
```

```
# qsub -q short.q qsub_blast_antar.sh

# Replace the next line by the location of the directory where you have your data
DIR_PROJECT="/projet/sbr/ccebarcodep1408/workshop_nz_2018/blast/"

cd $DIR_PROJECT

FILE="CARBOM_ASV"

FASTA=$DIR_PROJECT$FILE".fasta"
BLAST_TSV=$DIR_PROJECT$FILE".blast.tsv"

OUT_FMT="6 qseqid sseqid sacc stitle sscinames staxids sskingdoms sblastnames pident slen length mismat

blastn -max_target_seqs 100 -evalue 1.00e-10 -query $FASTA -out $BLAST_TSV -db /db/blast/all/nt -outfmt
```

5.8 Phyloseq

- Create and save a phyloseq object from dada2 results

```
samdf <- data.frame(sample_name = sample.names)
rownames(samdf) <- sample.names

OTU <- seqtab.nochim_18S %>% column_to_rownames("OTUNumber") %>% select_if(is.numeric) %>%
    select(-contains("_boot")) %>% as.matrix() %>% otu_table(taxa_are_rows = TRUE)

TAX <- seqtab.nochim_18S %>% column_to_rownames("OTUNumber") %>% select(Kingdom:Species) %>%
    as.matrix() %>% tax_table()

ps_dada2 <- phyloseq(OTU, sample_data(samdf), TAX)
saveRDS(ps_dada2, str_c(dada2_dir, "CARBOM_phyloseq.rds"))
```