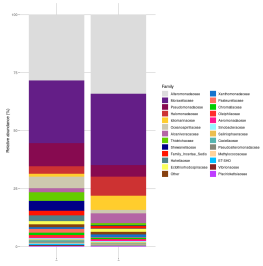
Aug 27, 2025

Bioinformatic workflow for Nanopore metabarcoding analysis



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Protocol status: Working
We use this protocol and it's working

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Disclaimer

None

Abstract

This protocol is aimed to help scientists process an Oxford Nanopore sequencing run from a 16S meta-barcoding experiment to produce a rarefaction curve, an alpha diversity analysis between samples and a stacked bar plot of the most represented taxons.

Guidelines

If its your first time using a Linux environment, you should check a basic Linux tutorial first or ask for help to a colleague.

Materials

Software used in this specific workflow:

- **A Linux/Mac terminal with Ubuntu 22.04.5**

(In case you are using Windows: a Linux virtual machine will work (But you may need to manually install some dependencies that are installed by default in Linux/Mac)).

- ***R v. 4.4.2***
- ***R Studio v. 2024.04.2***
- ***Perl v 5.32.1***
- ***Porechop v. 0.2.4***
- ***FastQC v. 0.11.9***
- ***Chopper v. 0.8.0***
- ***Parallel-meta 2 v. 2.4.1***
- ***Metaxa2 Database v. 2.2.3***

The R libraries used in this analysis are:

- RColorBrewer
- vegan
- tidyverse
- tibble
- cowplot
- ggh4x
- factoextra
- reshape2
- dplyr
- microbiome
- ggplot2
- ranacapa
- microViz
- microbiomeutilities

Safety warnings

Running Parallel-meta in a low-specs machine may cause trouble to the hardware.

Considerations

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- ***Metaxa2 Database v. 2.2.3***
- ***Apptainer v.*** **1.4.0**
- ***Taxonkit*** **v.0.18.0**

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- microViz
- microbiomeutilities

Software

NAME	
R programming language	
DEVELOPER	
The R Foundation	
REPOSITORY	
Comprehensive R Archive Network	
SOURCE LINK	
https://cran.r-project.org/	

To install any library you need to use the following command in the R terminal:

Command

new command name

```
install.packages("NAME")
```

3 go to step #26 is very computational demanding.
If you dont't run Parallel-meta2 in a high-end computer or in a cluster, consider that the processing may take a long time.

4 When the commands in this tutorial have a name (The ones that end with ".sh" or ".pl"), you need to download them and run the command in your terminal.
If the command doesn't have a name, then you can simply copy and paste it directly at the terminal.

5 A sample data set to follow along this protocol can be obtained in the following repository:
https://github.com/Zurita-Artaloitia/taxonomic-annotation-protocol/tree/main/sample_dataset

Pre-processing data

6 IMPORTANT: If your data isn't coming from a Nanopore sequencer, and you already have the fastqs, skip to "Primer Cleaning".

7 After you extract the fastQ files from the MinION device, locate the files in the drive in your machine.

Note

The MinION device gives you the option to automatically "base call" the reads, meaning it does the signals interpretation as nucleotides. In case you didn't select that option or want to manually do the base calling, then you can do it using a software like Dorado for the task.
Available online at: https://github.com/nanoporetech/dorado

Copy only the desired barcodes files into your local PC. For example: the barcodes 12, 24, 36 and 48.

Command

new command name

```
cp -r barcode12 barcode24 barcode36 barcode48 ~/PATH-to-store-barcodes
```

8 Verify that the directories aren't empty and have the right amount of files (Relative to the the sequencing run time).

Note

The Nanopore sequencing will usually generate a lot of barcodes because of sequencing errors. But this can be easily identified by entering the non-desired directories and checking the number of files and the number of lines in each file.
For example:
The line count for the files in the barcode02 goes from 1 to 9.
The line count for the barcode12 goes from 2,835 to 2,659,653
Inside each file you can check this lengths using the command:
wc -l *

Optional: Verify that the files are actual Nanopore reads.

Command

new command name

```
zcat FAW16131_pass_barcode12_1dba8d5b_de32243e_0.fastq.gz | head -2
```

The result should look like this:

@90c28ef6-45c8-4631-8851-2a67f74df137
runid=de32243e9f191a1a220f17ceaf869f23af588111 read=30 ch=324 start_time=2024-06-14T08:27:37.190699-06:00 flow_cell_id=FAW16131 protocol_group_id=Sigsbee24 sample_id=1 barcode=barcode12 barcode_alias=barcode12 parent_read_id=90c28ef6-45c8-4631-8851-2a67f74df137 basecall_model_version_id=dna_r9.4.1_e8_fast@v3.4
GCCGTCCATGGCCGCCGTTCCAGTTACGTATTGCCAATTGCTGCAGGTAGAAAACAGAATC
GGATTAACCTACTTGCCTGTCGCTCTATCTTCCGGTTACCTTGTTACGACTTCCACCCCAGT
CATGAACCACACCGTGAAATCGTCCCTCCCGAAGGTTAGACTAACTACTTCTGGTGCAATC
CCGCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGAC
ATTCTGATTCGCGATTTTAAGCGATTCCGACTTCGGAGTCGAGTTGCAGACTCCGATCCGG
ACTACGACGCGTTTTAAAGGGATTGGCTCACTCTCGCGAGTTGGCAGCCCCTCTGTACGCG
CCATTGTAGCACGTGTGTAGCCCCTGGCCGTAGGGTGACCTGACGTCATCCCCACCTTCCC
CTCGGTTTGTCACCGGCAGTCTCCCTGGAGTTCTCAGCATTACCTGCTAGCAACCAGGGAT
AGGGGTTATACGACACGAGCTGACGACGGCCATGCAGCACCTATCCCTGGTTGCTGGCAGG
CTCGGCGTTGCACCTAAATGACCTGCTCTTGGCTCCGCCTCTTCCGTGGCTGGCTGTCGC
GAATCATCACCCATTCCTTCAGATGGTACTGAAATGACCAGTCGGAACATTTCCCGGTGTGG
TGTCATGTTCATGACCGAATTCTGCCTAACTACTACAGCTTTCAACTTC

9 Concatenate all the files.

Note

Make sure you are inside the correct directory and name the concatenated output accordingly.

Command

new command name

```
zcat * > barcode12.fastq.gz
```

Once you have them all concatenated with each file being named after the barcode, store them into the same directory.

Primer cleaning

10 Proceed to removing the Nanopore adapter sequences from our files.

11 Install the following software.

Software

Porechop	NAME
CentOS 7.4.1708	OS
Ryan Wick	DEVELOPER
https://github.com/rrwick/Porechop	REPOSITORY
https://github.com/rrwick/Porechop	SOURCE LINK

12 Run this loop. Which iterates over all the fastq files and removes the adapters, generating for each an output with the "pc" name, meaning that they have been through Porechop.

Command

Run Porechop

```
for name in ./*.fastq.gz; do

porechop-runner.py -i "$name" -o "${name}.pc.fastq.gz"

done
```

Quality check

13 To assure the quality of the reads, a QC step is necessary.
Install the following software:

Software

FastQC — NAME

Simon Andrews — DEVELOPER

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ — SOURCE LINK

14 Run the following command.

Command

new command name

```
fastqc -o ./FastQC_results *.pc.fastq.gz
```

Now check your quality results and consider if the length of your results is adequate, the quality and length of the reads.

Note

If your reads are not bigger than 1,000 bp those may not be complete 16S reads.
If your reads are bigger than 2,000 bp, those may be 16S chimeras.

Sequence filtering

15 Filter your sequence length with Chopper.

16

Software

Chopper	NAME
Ubuntu 22.04	OS
Wouter De Coster	DEVELOPER
https://github.com/wdecoster/chopper	REPOSITORY
https://github.com/wdecoster/chopper	SOURCE LINK

17 Here, we are selecting the sequences that have a have a minimum length of 1000 and a maximum length of 2000. These lengths were selected because they are within the common lengths of 16S genes.

Command

new command name

```
chopper --minlength 1000 --maxlength 2000 -i $FILE > $output
```

Note

The filters can be variable. Depending on the quality and lengths you are looking for. This is just an example of how to filter with these parameters.
If desired, a quality filter can be done with the option:
--quality 6
(In this example, we use reads with a Phred score above 6)
We did not consider quality in this protocol because Parallel-Meta 2 has an internal filter that removes sequences that do not resemble 16S genes.

18 Optional: After this step run FASTQC again, go to step #14 to ensure that chopper worked.

Running parallel-meta

19 Now run the taxonomical analysis with Parallel-Meta 2, and the database Metaxa2.

20 Install the following software:

Software

NAME	OS	DEVELOPER
Parallel-Meta	CentOS 7.4.1708	Xiaoquan Su

21 As this software is no longer available, an image was uploaded here: https://services.ibt.unam.mx/uusmb/r/zU8_totDs9#eE9davcUQGqihcKtuqLWWeWRag7HINWUeoF3z0VcrIY=

22 The image can be run with Apptainer, which can be downloaded here: **https://apptainer.org/**

23 Then, this is how it can run:

Command

new command name

```
$ apptainer exec ./pm2.sif parallel-meta -h
```

24 Download the DB Metaxa2. Assing as "X" in the database index.

Dataset

NAME	LINK
Metaxa2	https://microbiology.se/software/metaxa2/

25 Unzip your data before using parallel-meta.

Command

new command name

```
gunzip *
```

26 Run the following command.

Note

This process is very computational demanding. Make sure to run it in a powerful enough computer or ask assistance from the computer expert in your research center.
A cluster with a scheduling system is highly recommended.

Command

new command name

```
for name in ./*.fastq; do

parallel-meta -r ${name}.fastq -d X -e 1e-30 -o "${name}_parallel"

done
```

27 The previous step generates a directory for each barcode/sample.

Data parsing

28 For the next step, we need to make a loop that produces a count matrix for each classification file.
Now we edit the name of each file with the sample name, making sure each has a the termination "classification.txt"

29 First, make sure to be in the directory that contains all the directories created in the go to step #27 .
After, create a file named classification_list in which all the paths to the classification files are saved.

Command

new command name

```
find . -name '*classification.txt' | sed 's/.txt//' >
classification_list
```

30 Now, we use this command to make the loop and prepare the count matrix for each classification file.

Command

count.matrix.sh

```
#!/bin/bash

while read name; do

cut -f5 ${name}.txt | grep -v 'Classification' |sort | uniq -c | sort
-nr | sed 's/; /;/g; s/;$//' | sed 's/^ \+//' | sed 's/ /\t/' | awk -
F '\t' '{print $2 "\t" $1}' > ${name}_count_matrix.txt


echo "Finished for ${name}"

done < classification_list
```

31 Now we do the same that in step 23, making now a path list file but for each matrix.

Command

new command name

```
find . -name '*classification_count_matrix.txt' > matrix_list.txt
```

32

Command

matrix.integrator.perl

```
#!/usr/bin/perl
use strict;
use warnings;

scalar@ARGV == 1 || die "usage: $0 <file_list.txt>
      file_list.txt      List of input files, each one is a matrix
with the same format:
                         - No header
                         - Lineage is in the first column separated
by ';'
                         - Abundance in integer numbers in the second
column
";

my $files = $ARGV[0];
my @names;
my %each_matrix;
open (VALUES, $files) or die ("I can not open the file $files\n") ;
while (<VALUES>) {
        chomp;
        my $name = $_;
        PARSE($name);
        push(@names, $name);
}
close(VALUES);

## Print the matrix
open MATCOUNT, ">tmp" or die ("I can not create the file
integrated_matrix.txt, check write permissions on current
directory\n");
my $header = join("\t", @names);
print MATCOUNT "SampleID\t$header\n"; #<STDIN>;

my $lastname=scalar@names;
my $cont_names=1;
foreach my $tax (sort keys %each_matrix) {
        print MATCOUNT "$tax\t";
        foreach my $file (@names) {
                if ($each_matrix{$tax}{$file}) {
                        print MATCOUNT "$each_matrix{$tax}{$file}\t";
```

```
                } else {
                        print MATCOUNT "0\t";
                }
        }
        print MATCOUNT "\n";
}
close(MATCOUNT);

system("sed 's/\t\$//' tmp > integrated_matrix.txt && rm tmp");

### Subroutine to parse the file
sub PARSE {
        my $file=shift;
#       my @new_array=();
        open (FILE, $file) or die ("I can not open the file $file\n")
;
        while (<FILE>) {
                chomp;
                my($taxo, $abundance)=split('\t', $_);
#               my@lineage=split(/;/, $taxo);
#               foreach my$each_name (@lineage){
#                       if ($each_name !~ /epsilon_subdivisions/ and
$each_name !~ /[Gg]roup/  and $each_name !~ /unclassified/ and
$each_name !~ /[Ii]ncertae_[Ss]edis/ ) {
#                               push(@new_array, $each_name);
#                       }
#               }
#               if (scalar@new_array == 6) {
#                       my$sp=join("_", $new_array[-1], "sp\.");
#                       push(@new_array, $sp);
#                       $taxo=join(";", @new_array);
#               }else{
#                       $taxo=join(";", @new_array);
#               }
                $each_matrix{$taxo}{$file}=$abundance;
#               @new_array=();
        }
        close(FILE);
}
```

33 Run with the following

Command

new command name

```
perl matrix.pl matrix_list.txt
```

34 Next, we filter the whole table to delete the hits for eukarya, mitochondria, chloroplast and other lines that we dont need.

Note

As we are only focused in 16S diversity, we are removing the chloroplast, mitochondria and eukarya sequences.

Command

new command name

```
sed 's/classification_count_matrix.txt//g ; s/[./]//g ;'s/\'//g' ;
s/_S[0-9][0-9][0-9]//g ; s/_S[0-9][0-9]//g ; s/_S[0-9]//g ;
/Eukaryota/d;/Mitochondria/d;/Chloroplast/d' integrated_matrix.txt >
integrated_matrix_good.txt
```

35

Command

taxa_levels.pl

```
#!/usr/bin/perl
use strict;
use warnings;

scalar@ARGV == 2 || die "usage: $0 <matrix.txt> <counts|abun>

      Programa que separa una matrix de abundancias o conteos en
niveles taxonomicos

      matrix.txt      Matriz de abundancias donde la primer columna
es la asignacion
                      taxonomica y el resto son las abundancias para
cada muestra

      counts|abun     Especificar si la matriz es de conteos crudos o
abundancias, en
                      el segundo caso, reporta la proporcion de
abundancia asignada a
                      niveles taxonomicos mas altos del corte dado

";

my $file=$ARGV[0];
my $tipo_mat=$ARGV[1];

unless ($tipo_mat =~ "counts"  or $tipo_mat =~ "abun") {
        die "El tipo de matriz solo puede ser counts o abun en
minusculas\n";
}

## Armando el hash de la matriz de abundancia original
my %matrix=();
open (FILE, $file) or die ("No puedo abrir el archivo $file\n");
my $header=<FILE>;
while (<FILE>) {
        chomp;
        my@line=split('\t', $_);
        my$tax_name=shift@line;
        if (exists $matrix{$tax_name}){
                my@sums=();
```

```
                for (my $i=0; $i<scalar@line; $i++){
                        my$add=$matrix{$tax_name}[$i]+$line[$i];
                        push(@sums, $add);
                }
                $matrix{$tax_name}=[@sums];
        }else{
                $matrix{$tax_name}=[@line];
        }
}
close(FILE);

my @samples=split("\t", $header);
my $samples_number=scalar@samples -1 ;

## Armando las matrices para cada nivel taxonomico
my %domain=SEPARA(\%matrix, 1, $samples_number, $tipo_mat);
open DOM, ">dom_tmp.txt";
print DOM "$header";
foreach my $dom (keys %domain){
        print DOM "$dom\t" ;
        foreach my $count (@{ $domain{$dom} }) {
                print DOM "$count\t";
        }
                print DOM "\n";
}
close(DOM);
system("sed 's/\t\$//' dom_tmp.txt > domain_matrix.txt");
system("rm dom_tmp.txt");

my %phylum=SEPARA(\%matrix, 2, $samples_number, $tipo_mat);
open PHY, ">phy_tmp.txt";
print PHY "$header";
foreach my $phy (keys %phylum){
        print PHY "$phy\t" ;
        foreach my $count (@{ $phylum{$phy} }) {
                print PHY "$count\t";
        }
                print PHY "\n";
}
close(PHY);
system("sed 's/\t\$//' phy_tmp.txt > phylum_matrix.txt");
system("rm phy_tmp.txt");

my %class=SEPARA(\%matrix, 3, $samples_number, $tipo_mat);
open CLASS, ">class_tmp.txt";
print CLASS "$header";
```

```
foreach my $cla (sort keys %class){
        print CLASS "$cla\t" ;
        foreach my $count (@{ $class{$cla} }) {
                print CLASS "$count\t";
        }
                print CLASS "\n";
}
close(CLASS);
system("sed 's/\t\$//' class_tmp.txt > class_matrix.txt");
system("rm class_tmp.txt");

my %order=SEPARA(\%matrix, 4, $samples_number, $tipo_mat);
open ORD, ">order_tmp.txt";
print ORD "$header";
foreach my $ord (sort keys %order){
        print ORD "$ord\t" ;
        foreach my $count (@{ $order{$ord} }) {
                print ORD "$count\t";
        }
                print ORD "\n";
}
close(ORD);
system("sed 's/\t\$//' order_tmp.txt > order_matrix.txt");
system("rm order_tmp.txt");

my %family=SEPARA(\%matrix, 5, $samples_number, $tipo_mat);
open FAM, ">family_tmp.txt";
print FAM "$header";
foreach my $fam (sort keys %family){
        print FAM "$fam\t" ;
        foreach my $count (@{ $family{$fam} }) {
                print FAM "$count\t";
        }
                print FAM "\n";
}
close(FAM);
system("sed 's/\t\$//' family_tmp.txt > family_matrix.txt");
system("rm family_tmp.txt");

my %genus=SEPARA(\%matrix, 6, $samples_number, $tipo_mat);
open GEN, ">genus_tmp.txt";
print GEN "$header";
foreach my $gen (sort keys %genus){
        print GEN "$gen\t" ;
        foreach my $count (@{ $genus{$gen} }) {
                print GEN "$count\t";
        }
```

```
        }
                print GEN "\n";
}
close(GEN);
system("sed 's/\t\$//' genus_tmp.txt > genus_matrix.txt");
system("rm genus_tmp.txt");

my %species=SEPARA(\%matrix, 7, $samples_number, $tipo_mat);
open SPC, ">species_tmp.txt";
print SPC "$header";
foreach my $spc (sort keys %species){
        print SPC "$spc\t" ;
        foreach my $count (@{ $species{$spc} }) {
                print SPC "$count\t";
        }
                print SPC "\n";
}
close(SPC);
system("sed 's/\t\$//' species_tmp.txt > species_matrix.txt");
system("rm species_tmp.txt");

my %subspecies=SEPARA(\%matrix, 8, $samples_number, $tipo_mat);
open SSPC, ">subspecies_tmp.txt";
print SSPC "$header";
foreach my $subspc (sort keys %subspecies){
        print SSPC "$subspc\t" ;
        foreach my $count (@{ $subspecies{$subspc} }) {
                print SSPC "$count\t";
        }
                print SSPC "\n";
}
close(SPC);
system("sed 's/\t\$//' subspecies_tmp.txt > subspecies_matrix.txt");
system("rm subspecies_tmp.txt");

sub SEPARA {
        my %matriz_sub=%{$_[0]};
        my $level=$_[1];
        my $muestras=$_[2];
        my $type_matrix=$_[3];
        my %regresa=();
        my(@sumas, @taxa_levels, @diferencia);
        my($level_key, $pos, $diff,$taxa2print);
        my$suma_val=0;

        foreach my $llaves (sort keys %matriz_sub) {
                @taxa levels=split(';', $llaves);
```

```
                my $tamano=scalar@taxa_levels;
                if ($tamano >= $level) {
                        $pos=$level-1;

                        if ($taxa_levels[$pos] =~ /no_rank/){
                                $pos=$level;
                        }

                        $taxa2print=join(';',
@taxa_levels[0..$pos]); ### variable nueva para imprimir

                        if (!exists($regresa{$taxa2print})) {

$regresa{$taxa2print}=$matriz_sub{$llaves};
                        }else{

                                for (my $i=0; $i<$muestras; $i++) {

$suma_val=$regresa{$taxa2print}[$i]+$matriz_sub{$llaves}[$i];
                                        push(@sumas, $suma_val);
                                }
                        $regresa{$taxa2print}=[@sumas];
                        @sumas=();
                        }
                }
        }

        if ($type_matrix =~ 'abun'){
                my @suma_per_sample=();
                my $suma_val=0;

                for (my $i=0; $i<$muestras; $i++) {
                        foreach my $key (sort keys %regresa){
                                $suma_val=$regresa{$key}
[$i]+$suma_val;
                        }
                        push(@suma_per_sample, $suma_val);
                        $suma_val=0;
                }

                foreach my $cada_suma (@suma_per_sample){
                        my $resta=100-$cada_suma;
                        if ($resta > 0) {
                                my $higher_level= sprintf "%.6f",
$resta;
```

```
                                push(@diferencia, $higher_level);
                        }else{
                                push(@diferencia, "0");
                        }
                }
                $regresa{"Classified_at_a_higher_level"}=
[@diferencia];
                return %regresa;
        }else{
                return %regresa;
        }
}
```

And its run adding "counts" at the end.

Command

new command name

```
perl taxa_levels.pl integrated_matrix_good.txt counts
```

36 We format the data for phyloseq.

Command

new command name

```
#!/bin/bash

awk '{print $1}' species_matrix.txt > 7taxonomy_table.txt

cut -f1 species_matrix.txt > 7taxonomy_table.txt
sed 's/;/\t/g' 7taxonomy_table.txt | sed
's/SampleID/Domain\tPhylum\tClass\tOrder\tFamily\tGenus\tSpecie/g' >
7_taxonomy_table1.txt
awk 'BEGIN{ FS = OFS = "\t" } { print (NR==1? "TAXA" : "TAXA"NR-1),
$0 }' 7_taxonomy_table1.txt > taxonomy_table.txt
cut -f2- species_matrix.txt > 7_otu_table.txt
awk 'BEGIN{ FS = OFS = "\t" } { print (NR==1? "TAXA" : "TAXA"NR-1),
$0 }' 7_otu_table.txt > otu_table.txt
```

37 As metaxa2 database has some outdated taxas, we will use the software Taxonkit in order to update them.

Software

Taxonkit NAME

https://bioinf.shenwei.me/taxonkit/ SOURCE LINK

Command

new command name

```
taxonkit name2taxid --data-dir $PATH/taxonkit/database/ -i 8 --show-rank taxonomy_table.txt > out_species.txt
```

38 After this step, its necessary to check for the taxons that weren't detected by taxonkit.

Command

new command name

```
awk -F'\t' '$9 == ""' out_species.txt
```

39 Manually look up for the species name in the Taxonomy browser from NCBI (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) and change the species name in the 8th column to the current one in the taxonomy_table.txt.

In the example above, the species *Pseudomonas alcaligenes* needs to be changed to: *Aquipseudomonas alcaligenes*

40 After all the species names are corrected, run again go to step #37 and go to step #38 to make sure that all the taxons were successfully corrected by Taxonkit.
Then, run again this command:

Command

new command name

```
taxonkit reformat --data-dir /home/pardolab/Apps/taxonkit/database/ -
I 9 -f "{K}|{p}|{c}|{o}|{f}|{g}|{s}" -F out_species.txt >
species_corrected.txt
```

41 Then, transform the previous output into the taxonomy table file.

Command

new command name

```
cut -f1,11 species_corrected.txt  | sed 's/|/\t/g' | sed "s/'//g" |
sed 's/TAXA\t/TAXA    Domain  Phylum  Class   Order   Family  Genus
Specie/' > taxonomytable.txt
```

42 Now we create a file named metadata.txt with the following structure example (each column must be separated by a tab):
SampleID Origin
ID1 Water
ID2 Sediment

ID3 Soil
ID4 Saliva

A metadata file can be created with the following command (note: you can add as many meta information as necessary, as shown in the example above):

Command

new command name

```
head -1 integrated_matrix_good.txt | tr "\t" "\n" > metadata.txt
```

...

Diversity analysis

43 As a result, we should have 3 files:
otu_table.txt
taxonomytable.txt
metadata.txt

44

Software

Phyloseq NAME

https://joey711.github.io/phyloseq/ SOURCE LINK

45 Now paste this command in the R Studio file.
Be sure to add the working directory you are in before running it in this part:
setwd("/PATH").

The code should be copied into an Rstudio session, so each line is ran individually. This is because some lines need to be modified.

For each abundance, search for the following 4 lines:

Command

new command name

```
Class_1up = filter_taxa(Class_perc, function(x) sum(x) > 0.1, TRUE)
Class_1up
#40
Class_top = aggregate_top_taxa2(Class_perc,top = 40, level = "Class")
```

For each, run the first line to define the cutoff of your relative abundance.
The second line will tell how many taxas are considered after your cut off. Try to use a number between 40-60 to avoid having too many or to little taxas.
You can register this number in the third line.
And in the fourth line register this number in a variable to be used in the plot.

Also, in the line:

Command

new command name

```
  facet_nested(. ~ Origin + Depth + Platform , scales =
"free_x",space="free_x") +
```

Replace "Origin", "Depth" and "Platform" with your metadata.

46

Command

new command name

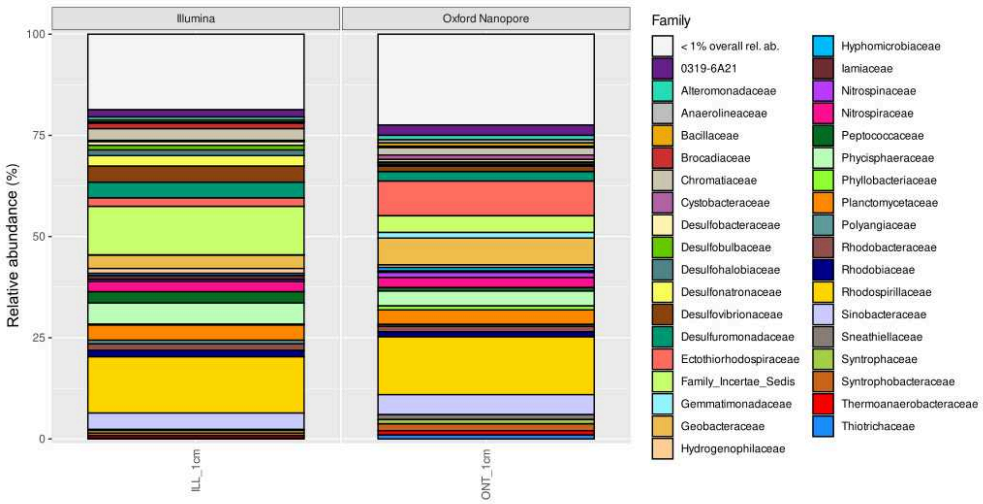
```
#libraries
library(phyloseq)
library(dplyr)
library(vegan)
library(tidyverse)
library(tibble)
library(cowplot)
library(ggh4x)
library(factoextra)
library(reshape2)
library(dplyr)
library(microbiome  )
library(ggplot2)
library(ranacapa)
library(microViz)
library(microbiomeutilities)

#2 change working directory
setwd("$PATH_to_files")

#########################################################################
##########
#3 importar data

#16S
otu16s_df<-read.table("otu_table.txt",
                      header=TRUE, row.names=1, check.names=FALSE)
otu16s_mat<-as.matrix(otu16s_df)
tax16s_df<-read.table("taxonomytable.txt",
                      header=TRUE, row.names=1, check.names=FALSE,
sep = '\t')
tax16s_mat<-as.matrix(tax16s_df)
metadata16s_df<-read.table("metadata.txt",
                           sep="\t", header=TRUE,  row.names=1)
OTU16s = otu_table(otu16s_mat, taxa_are_rows = TRUE)
TAX16s = tax_table(tax16s_mat)
ps_16s = phyloseq(OTU16s,TAX16s, sample_data(metadata16s_df))
ps_16s

ps_16s_genus = ps_16s %>% aggregate_taxa(level = "Genus")
```

```
##### Curves ###############
fuente_rare = theme(text = element_text(size= 12, face="bold"),
                    axis.text.x = element_text(size = 12,face="bold"),
                    axis.text.y = element_text(size = 12,face="bold"),
                    panel.background = element_rect(fill =
"white",colour = "grey50"),
                    panel.grid.major =
element_blank(),panel.grid.minor = element_blank())

#genus
ggrare(ps_16s_genus, step = 209, plot = T,
       parallel = T, se = F, color = "Sample" ) +
  fuente_rare + labs(y="Géneros observados", x="Tamaño de la
muestra")


##################alpha#######################3
alphadiv <- plot_richness(ps_16s, x = "Sample",
                          measures=c("Observed","Chao1", "Shannon",
"Simpson"), color = "Sample") +
  labs(x="Origin", y="Alpha diversity")
alphadiv

##############
#16s
genus_comp = ps_16s_genus %>% transform(transform = "compositional")
genus_perc = transform_sample_counts(genus_comp, function(x) x*100)
genus_perc
genus_1up = filter_taxa(genus_perc, function(x) sum(x) > 1, TRUE)
genus_1up
#70
genus_5up = filter_taxa(genus_perc, function(x) sum(x) > 2, TRUE)
genus_5up
#30

genus_top = aggregate_top_taxa2(genus_perc,top = 32, level = "Genus")
genus_top
data = psmelt(genus_top) # create dataframe from phyloseq object
data$Genus <- as.character(data$Genus) #convert to character
data$Genus[data$Genus == "Other"] <- "< 2% overall rel. ab."

mycolors= c(
"#F6F6F6","#68228B","#2BDEBB","#C0C0C0","#EEAD0E","#CD3333",
            "#CDC8B1","#B565A7","#fff5b5","#66CD00","#53868B",
```

```
"#fcff5d","#8B4513","#009B77","#FF6F61","#CAFF70",
```

47 This is the results for the family levels taxa, but stacked bar plots can be made in different taxa levels.

Expected result




```
ps_16s_Phylum = ps_16s %>% aggregate_taxa(level = "Phylum")

Phylum_comp = ps_16s_Phylum %>% transform(transform = "compositional")
Phylum_perc = transform_sample_counts(Phylum_comp, function(x) x*100)
Phylum_perc
Phylum_1up = filter_taxa(Phylum_perc, function(x) sum(x) > 1, TRUE)
Phylum_1up
#30 y 10
Phylum_top = aggregate_top_taxa2(Phylum_perc,top = 30, level =
"Phylum")
Phylum_top
data = psmelt(Phylum_top) # create dataframe from phyloseq object
data$Phylum <- as.character(data$Phylum) #convert to character
data$Phylum[data$Phylum == "Other"] <- "< 2% overall rel. ab."
bars_Phylum_top <- ggplot(data, aes(x=Sample, y=Abundance,
fill=Phylum)) +
  geom_bar(aes(), stat="identity", position="stack", color="black") +
  facet_nested(. ~ Origin + Platform, scales =
"free_x", space="free_x") +
```

```
 free_x",space="free_x") +
  labs(x = "", y = "Abundancia relativa (%)") +
  scale_fill_manual(values=mycolors) + guides(x = guide_axis(angle =
90)) +
  theme(panel.spacing=unit(0.1,"lines"),
        strip.background=element_rect(color="grey30", fill="grey90"),
        panel.border=element_rect(color="grey90", fill = NA),
        axis.ticks.x=element_blank()) +
  scale_y_continuous(expand = c(0.01, 0.01))
bars_Phylum_top

######################################################################
######

###############Class####################
ps_16s_Class = ps_16s %>% aggregate_taxa(level = "Class")

Class_comp = ps_16s_Class %>% transform(transform = "compositional")
Class_perc = transform_sample_counts(Class_comp, function(x) x*100)
Class_perc
Class_1up = filter_taxa(Class_perc, function(x) sum(x) > 0.1, TRUE)
Class_1up
#64 y 40
Class_top = aggregate_top_taxa2(Class_perc,top = 40, level = "Class")
Class_top
data = psmelt(Class_top) # create dataframe from phyloseq object
data$Class <- as.character(data$Class) #convert to character
data$Class[data$Class == "Other"] <- "< 0.5% overall rel. ab."
bars_Class_top <- ggplot(data, aes(x=Sample, y=Abundance,
fill=Class)) +
  geom_bar(aes(), stat="identity", position="stack", color="black") +
  facet_nested(. ~ Origin + Platform, scales =
"free_x",space="free_x") +
  labs(x = "", y = "Abundancia relativa (%)") +
  scale_fill_manual(values=mycolors) + guides(x = guide_axis(angle =
90)) +
  theme(panel.spacing=unit(0.1,"lines"),
        strip.background=element_rect(color="grey30", fill="grey90"),
        panel.border=element_rect(color="grey90", fill = NA),
        axis.ticks.x=element_blank()) +
  scale_y_continuous(expand = c(0.01, 0.01))
bars_Class_top

######################################################################
######

###############Order####################
```

```
ps_16s_Order = ps_16s %>% aggregate_taxa(level = "Order")

Order_comp = ps_16s_Order %>% transform(transform = "compositional")
Order_perc = transform_sample_counts(Order_comp, function(x) x*100)
Order_perc
Order_1up = filter_taxa(Order_perc, function(x) sum(x) > 1, TRUE)
Order_1up
#42
Order_top = aggregate_top_taxa2(Order_perc,top = 42, level = "Order")
Order_top
data = psmelt(Order_top) # create dataframe from phyloseq object
data$Order <- as.character(data$Order) #convert to character
data$Order[data$Order == "Other"] <- "< 1% overall rel. ab."
bars_Order_top <- ggplot(data, aes(x=Sample, y=Abundance,
fill=Order)) +
  geom_bar(aes(), stat="identity", position="stack", color="black") +
  facet_nested(. ~ Origin + Platform, scales =
"free_x",space="free_x") +
  labs(x = "", y = "Abundancia relativa (%)") +
  scale_fill_manual(values=mycolors) + guides(x = guide_axis(angle =
90)) +
  theme(panel.spacing=unit(0.1,"lines"),
        strip.background=element_rect(color="grey30", fill="grey90"),
        panel.border=element_rect(color="grey90", fill = NA),
        axis.ticks.x=element_blank()) +
  scale_y_continuous(expand = c(0.01, 0.01))
bars_Order_top

#########################################################################
######

###############Family###################
ps_16s_Family = ps_16s %>% aggregate_taxa(level = "Family")

Family_comp = ps_16s_Family %>% transform(transform = "compositional")
Family_perc = transform_sample_counts(Family_comp, function(x) x*100)
Family_perc
Family_1up = filter_taxa(Family_perc, function(x) sum(x) > 2, TRUE)
Family_1up
#30 y 10
Family_top = aggregate_top_taxa2(Family_perc,top = 35, level =
"Family")
Family_top
data = psmelt(Family_top) # create dataframe from phyloseq object
data$Family <- as.character(data$Family) #convert to character
data$Family[data$Family == "Other"] <- "< 2% overall rel. ab."
```

```
bars_Family_top <- ggplot(data, aes(x=Sample, y=Abundance,
fill=Family)) +
  geom_bar(aes(), stat="identity", position="stack", color="black") +
  facet_nested(. ~ Origin + Platform, scales =
"free_x",space="free_x") +
  labs(x = "", y = "Abundancia relativa (%)") +
  scale_fill_manual(values=mycolors) + guides(x = guide_axis(angle =
90)) +
  theme(panel.spacing=unit(0.1,"lines"),
        strip.background=element_rect(color="grey30", fill="grey90"),
        panel.border=element_rect(color="grey90", fill = NA),
        axis.ticks.x=element_blank()) +
  scale_y_continuous(expand = c(0.01, 0.01))
bars_Family_top

######################################################################
######

###############Specie###################
ps_16s_Specie = ps_16s %>% aggregate_taxa(level = "Specie")

Specie_comp = ps_16s_Specie %>% transform(transform = "compositional")
Specie_perc = transform_sample_counts(Specie_comp, function(x) x*100)
Specie_perc
Specie_1up = filter_taxa(Specie_perc, function(x) sum(x) > 1, TRUE)
Specie_1up
#30 y 10
Specie_top = aggregate_top_taxa2(Specie_perc,top = 54, level =
"Specie")
Specie_top
data = psmelt(Specie_top) # create dataframe from phyloseq object
data$Specie <- as.character(data$Specie) #convert to character
data$Specie[data$Specie == "Other"] <- "< 1% overall rel. ab."
bars_Specie_top <- ggplot(data, aes(x=Sample, y=Abundance,
fill=Specie)) +
  geom_bar(aes(), stat="identity", position="stack", color="black") +
  facet_nested(. ~ Origin + Platform, scales =
"free_x",space="free_x") +
  labs(x = "", y = "Abundancia relativa (%)") +
  scale_fill_manual(values=mycolors) + guides(x = guide_axis(angle =
90)) +
  theme(panel.spacing=unit(0.1,"lines"),
        strip.background=element_rect(color="grey30", fill="grey90"),
        panel.border=element_rect(color="grey90", fill = NA),
        axis.ticks.x=element_blank()) +
  scale_y_continuous(expand = c(0.01, 0.01))
bars_Specie_top
```

```
bars_specie_top

#genus###################
ps_16s_genus
genus_df <- data.frame(phyloseq::otu_table(ps_16s_genus))
#write.table(genus_transpose,file="genus_nmds.txt", sep = "\t",
row.names = TRUE, col.names = NA)
genus_transpose_m <- as.data.frame(t(as.matrix(genus_df)))
#write.table(genus_transpose,file="genus_nmds_transpose.txt", sep =
"\t", row.names = TRUE, col.names = NA)

genus_nmds <- metaMDS(genus_transpose_m, k=2, trymax=100, sfgrmin =
1e-9)
genus_nmds

stressplot(genus_nmds) # Produces a Shepards diagram
plot(genus_nmds)
genus_nmds
ordiplot(genus_nmds,type="n")
orditorp(genus_nmds,display="sites",cex=1.25,air=0.01)
```