



B- Training on Galaxy: Metabarcoding

December 2021 - Webinar

FROGS Practice on 16S data

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Objectives



Objectives: a count table

	Affiliation	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
OTU1	Species A	0	100	0	45	75	18645
OTU2	Species B	741	0	456	4421	1255	23
OTU3	Species C	12786	45	3	0	0	0
OTU4	Species D	127	4534	80	456	756	108
OTU5	Species E	8766	7578	56	0	0	200

Why FROGS was developed ?

Most solutions are often **designed for specialists** making access difficult for the whole community (command lines).

We developed the pipeline **FROGS: « Find Rapidly OTU with Galaxy Solution »** usable with command lines or within interface.

Who is in the current FROGS group?



Maria BERNARD



Olivier RUÉ

Developers



Lucas AUER



**Laurent
CAUQUIL**

Biology experts



Patrice Déhais

Galaxy
support



**Mahendra
MARIADASSOU**

Statistical expert

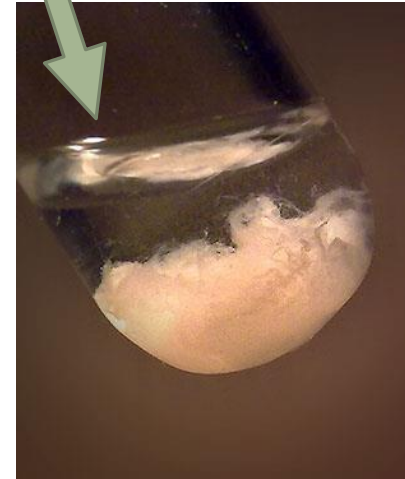
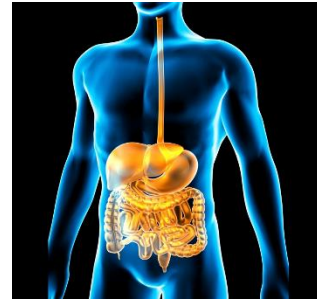


**Géraldine
PASCAL**

Coordinator

Material

Sample collection and DNA extraction



« Meta-omics » using next-generation sequencing (NGS)



DNA

RNA

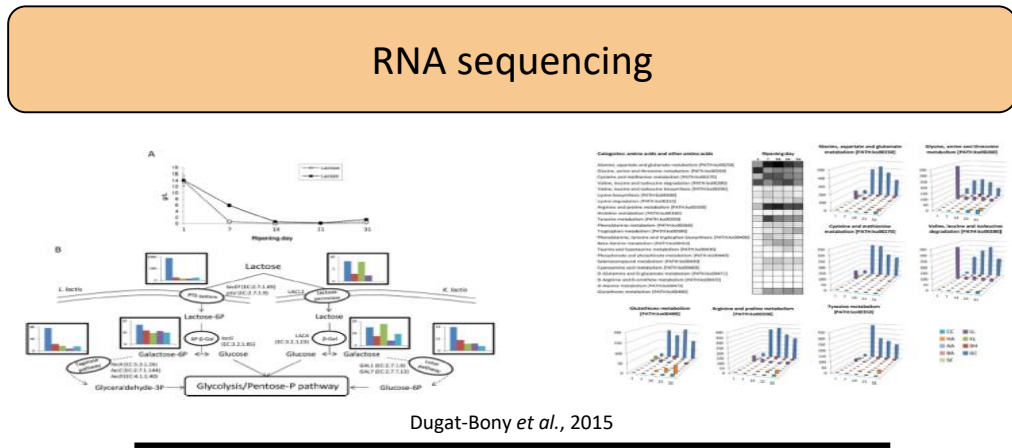
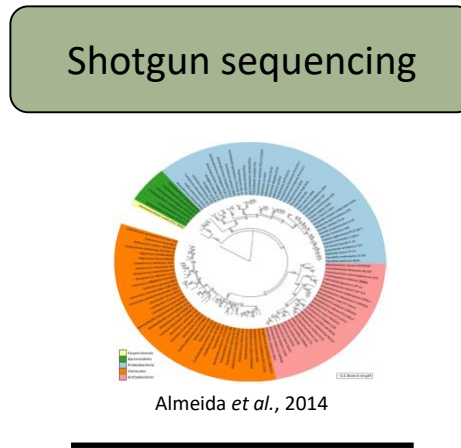
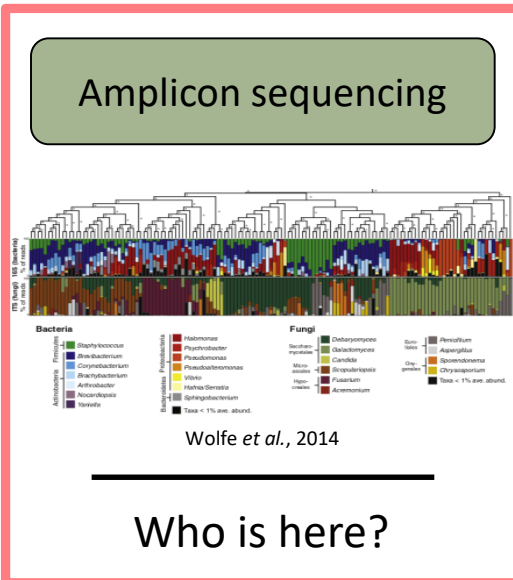
Metagenomics

Metatranscriptomics

Amplicon sequencing

Shotgun sequencing

RNA sequencing



The gene encoding the small subunit of the ribosomal RNA

The most widely used gene in **molecular phylogenetic** studies

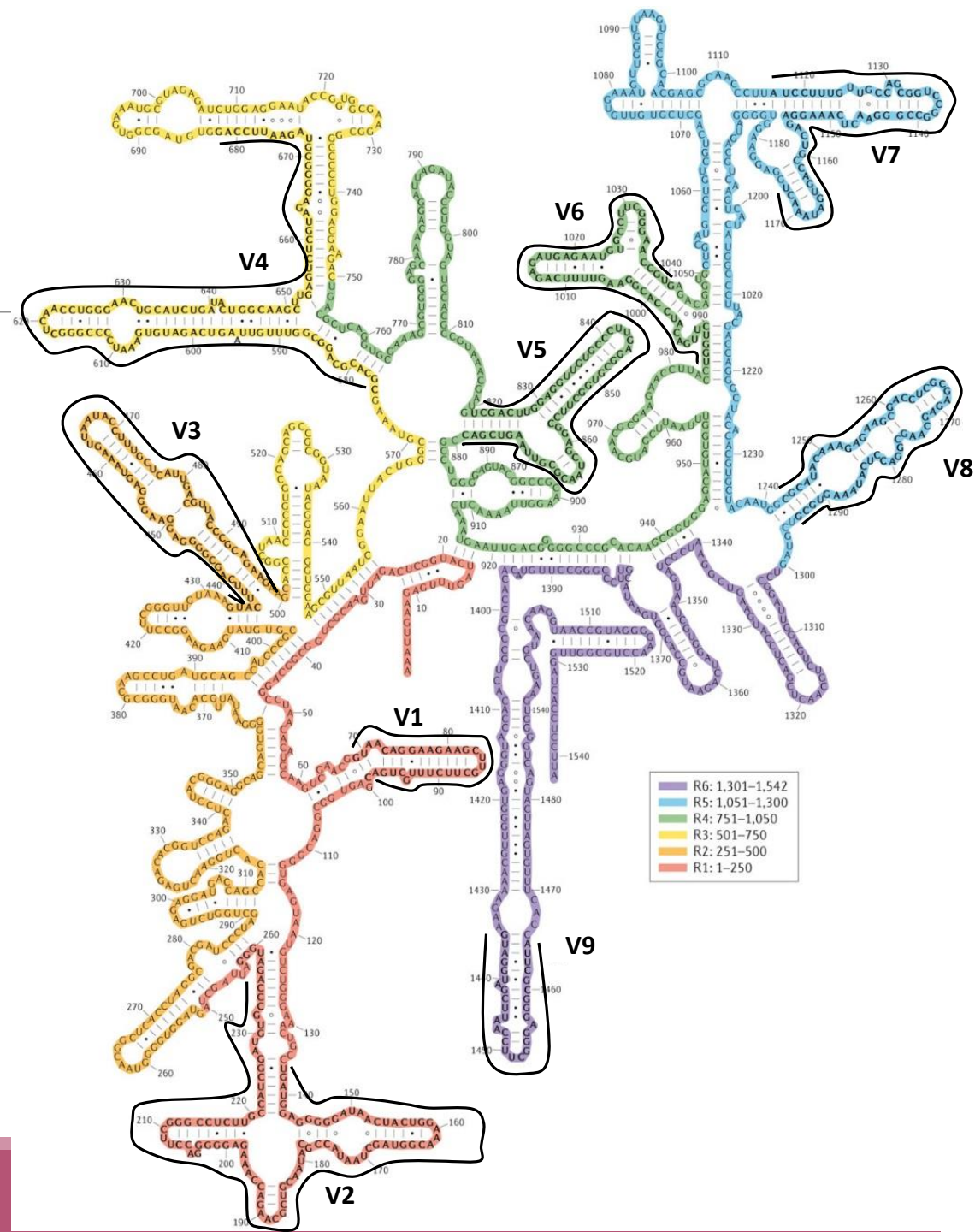
Ubiquist gene : **16S rDNA** in prokaryotes ; **18S rDNA** in eukaryotes

Gene encoding a ribosomal RNA : non-coding RNA (not translated), part of the small subunit of the ribosome which is responsible for the translation of mRNA in proteins

Not submitted to lateral gene transfer

Availability of databases facilitating comparison

(Silva v138.1 - 2021: available SSU/LSU sequences to over **10,700,000**)



Secondary structure of the 16S rRNA of *Escherichia coli*

In red, fragment R1 including regions V1 and V2;
 in orange, fragment R2 including region V3;
 in yellow, fragment R3 including region V4;
 in green, fragment R4 including regions V5 and V6;
 in blue, fragment R5 including regions V7 and V8;
 and in purple, fragment R6 including region V9.

Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences
 Pablo Yarza, et al.
 Nature Reviews Microbiology 12, 635–645
 (2014) doi:10.1038/nrmicro3330

The gene encoding the small subunit of the ribosomal RNA



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

Other targets

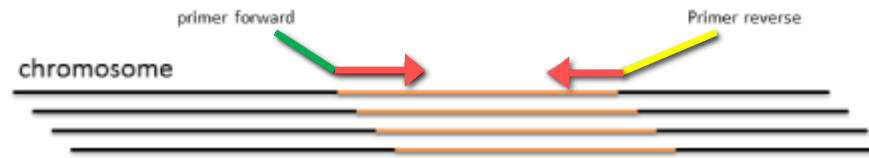
Bacterial lineages vary in their genomic contents, which suggests that different genes might be needed to resolve the diversity within certain taxonomic groups.

The genes that have been proposed for this task include those encoding :

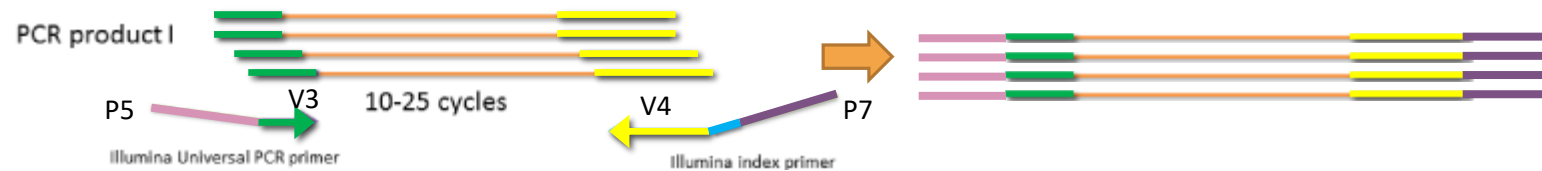
- 23S rRNA,
- DNA gyrase subunit B (*gyrB*),
- RNA polymerase subunit B (*rpoB*),
- TU elongation factor (*tuf*),
- DNA recombinase protein (*recA*),
- protein synthesis elongation factor-G (*fusA*),
- dinitrogenase protein subunit D (*nifD*),
- Internal Transcribed Spacer (ITS) for Fungi.

Steps for Illumina sequencing

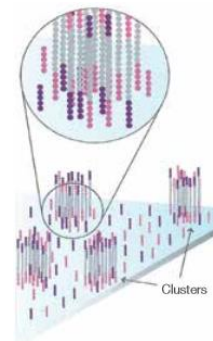
- 1st step : one PCR



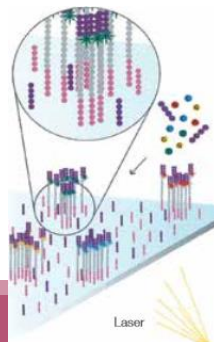
- 2nd step: one PCR



- 3rd step: on flow cell, the cluster generations



- 4th step: sequencing



Amplification and sequencing

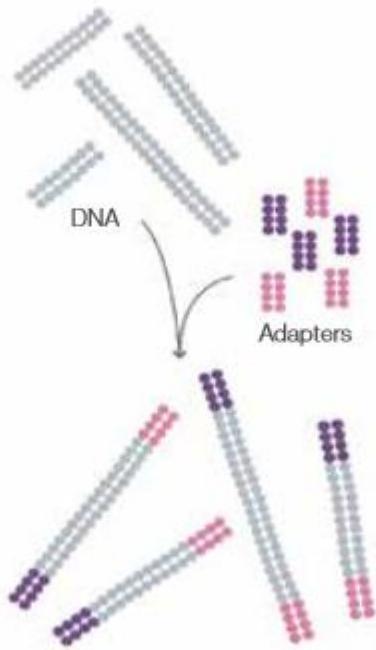
« **Universal** » primer sets are used for **PCR amplification** of the phylogenetic biomarker

The primers contain **adapters** used for the sequencing step and **barcodes** (= tags = MIDs) to distinguish the samples (multiplexing = sequencing several samples on the same run)



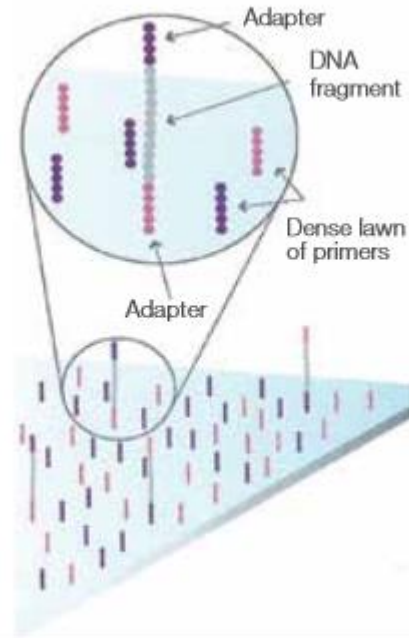
Cluster generation

Prepare Genomic DNA Sample



Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

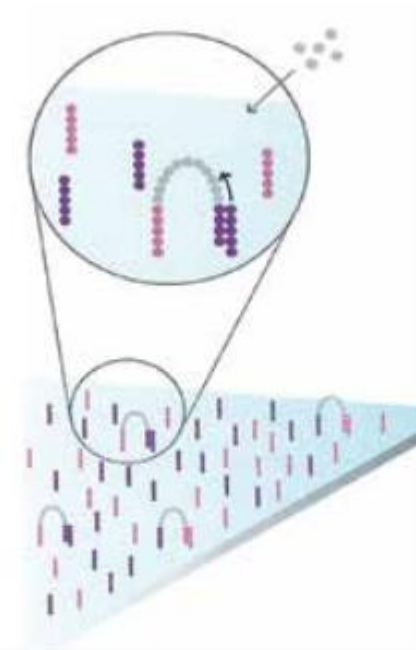
Attach DNA to Surface



Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

Attach DNA to surface

Bridge Amplification

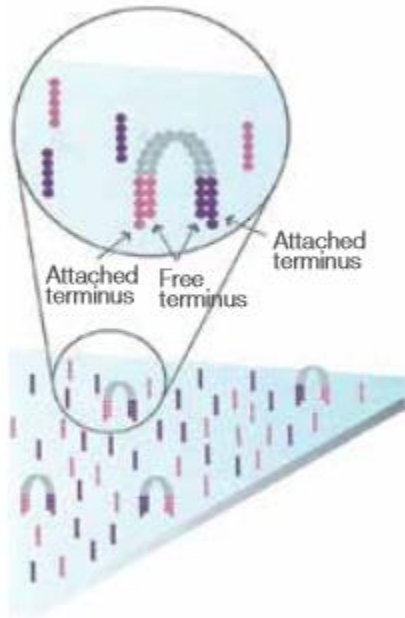


Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

Bridge amplification

Cluster generation

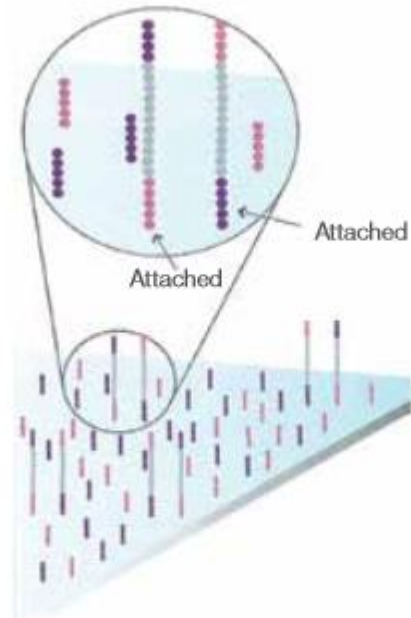
Fragments Become Double Stranded



The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.

Fragments become double stranded

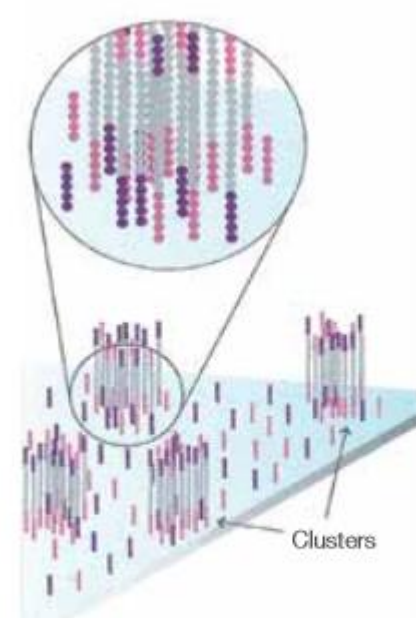
Denature the Double-Stranded Molecules



Denaturation leaves single-stranded templates anchored to the substrate.

Denature the double-stranded molecule

Complete Amplification

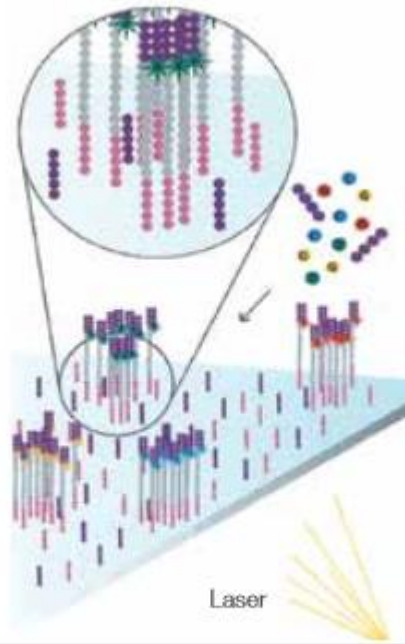


Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

Cycle of new strand synthesis and denaturation to make multiple copies of the same sequence (amplification)
Reverse strands are washed

Sequencing by synthesis

Determine First Base



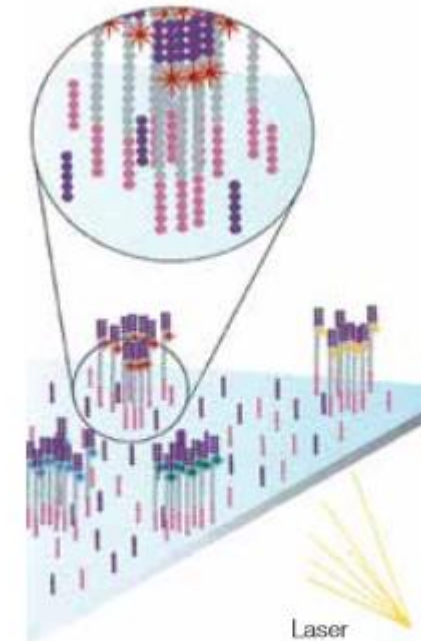
The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.
Light signal is more strong in cluster

Image First Base



After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

Determine Second Base



The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

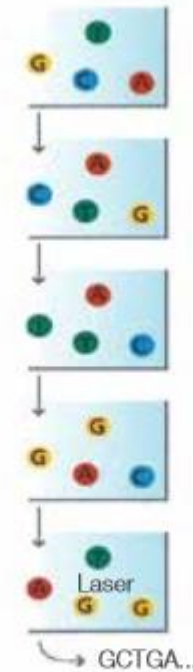
Sequencing by synthesis

Image Second Chemistry Cycle



After laser excitation, the image is captured as before, and the identity of the second base is recorded.

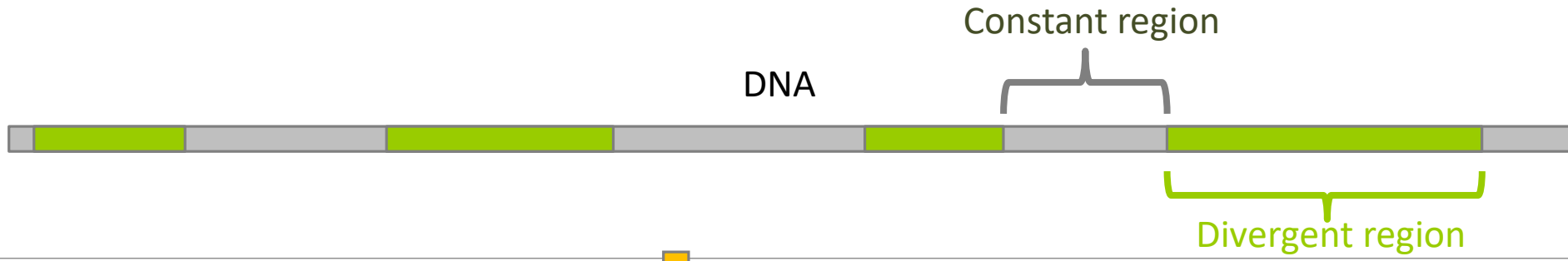
Sequencing Over Multiple Chemistry Cycles



The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

Barcode is read, so cluster is identified.

After first sequencing (250 or 300 nt of Reverse strand), fragment form bridges again and Forward strand can be sequenced also.



↓ PCRs

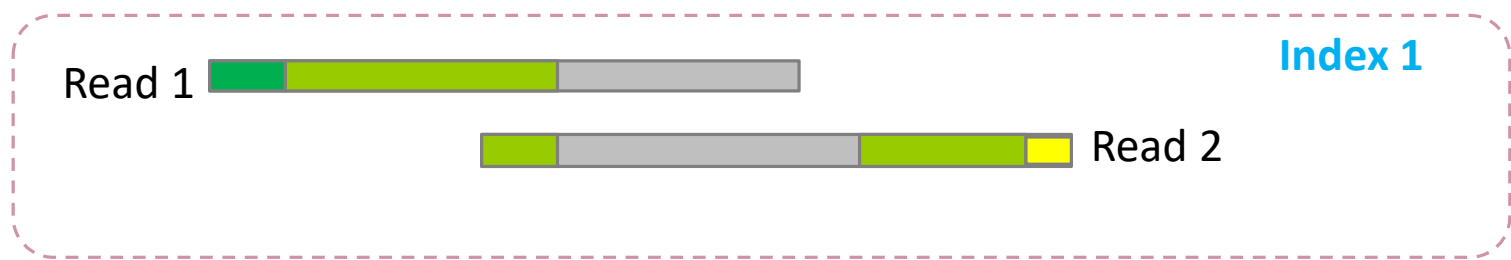
Illumina index



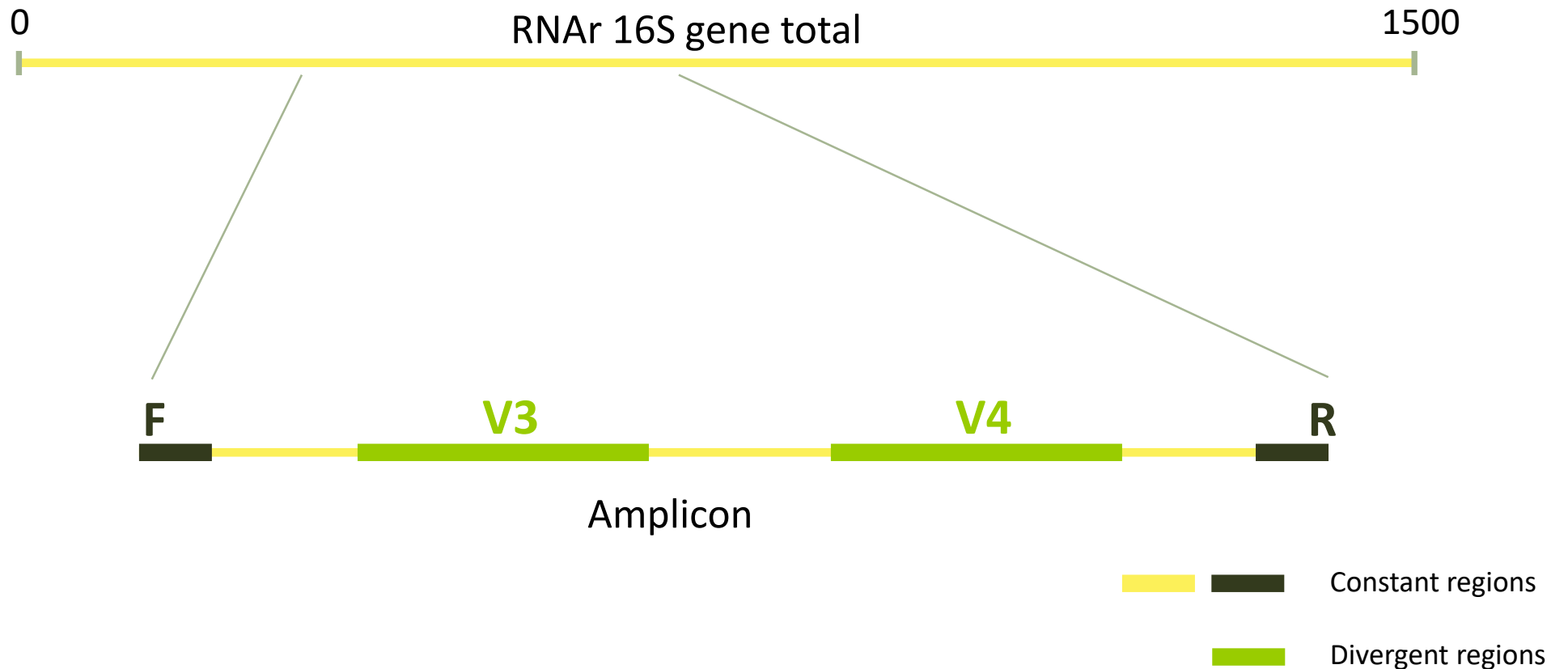
Illumina adapter

Illumina adapter

↓ Sequencing



Identification of bacterial populations may be not discriminating



Amplification and sequencing

Sequencing is generally performed on Roche-454 (obsolete now) or Illumina MiSeq platforms or Oxford Nanopore Technology platform.

Read quantity: ~10 000 reads per sample (454), ~30 000 reads per sample (MiSeq), up to several Tera of data (ONT).

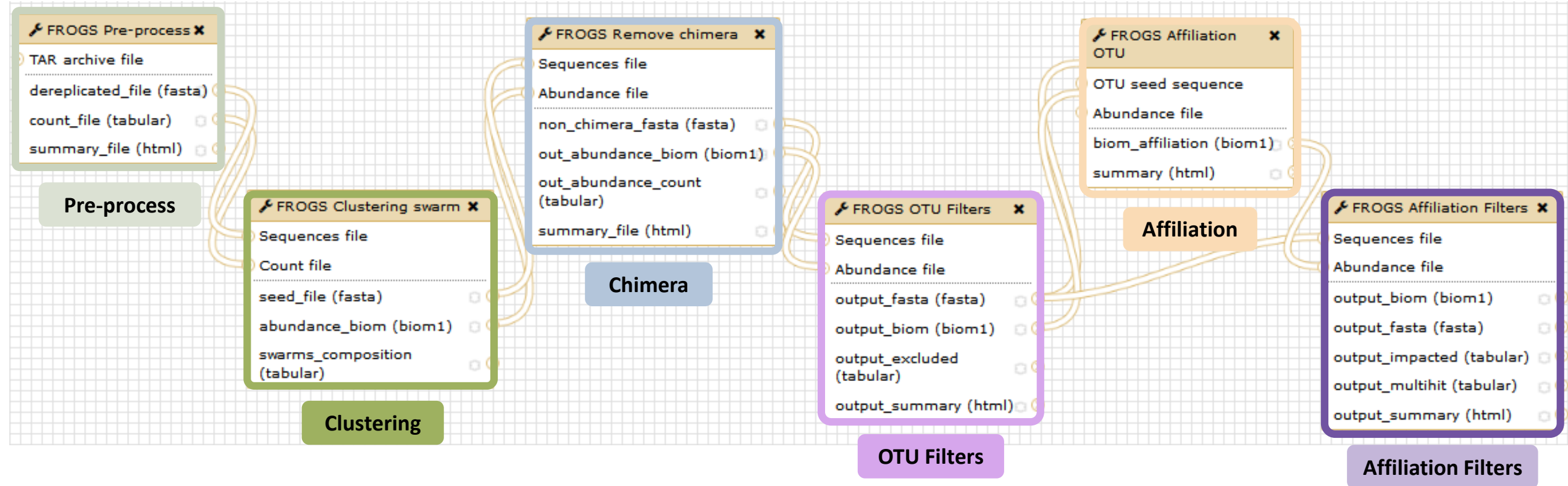
Sequence lengths: >650 bp (Roche-454), 2 x 250 bp or 2 x 300 bp (MiSeq), Longest read > 2Mb (ONT)

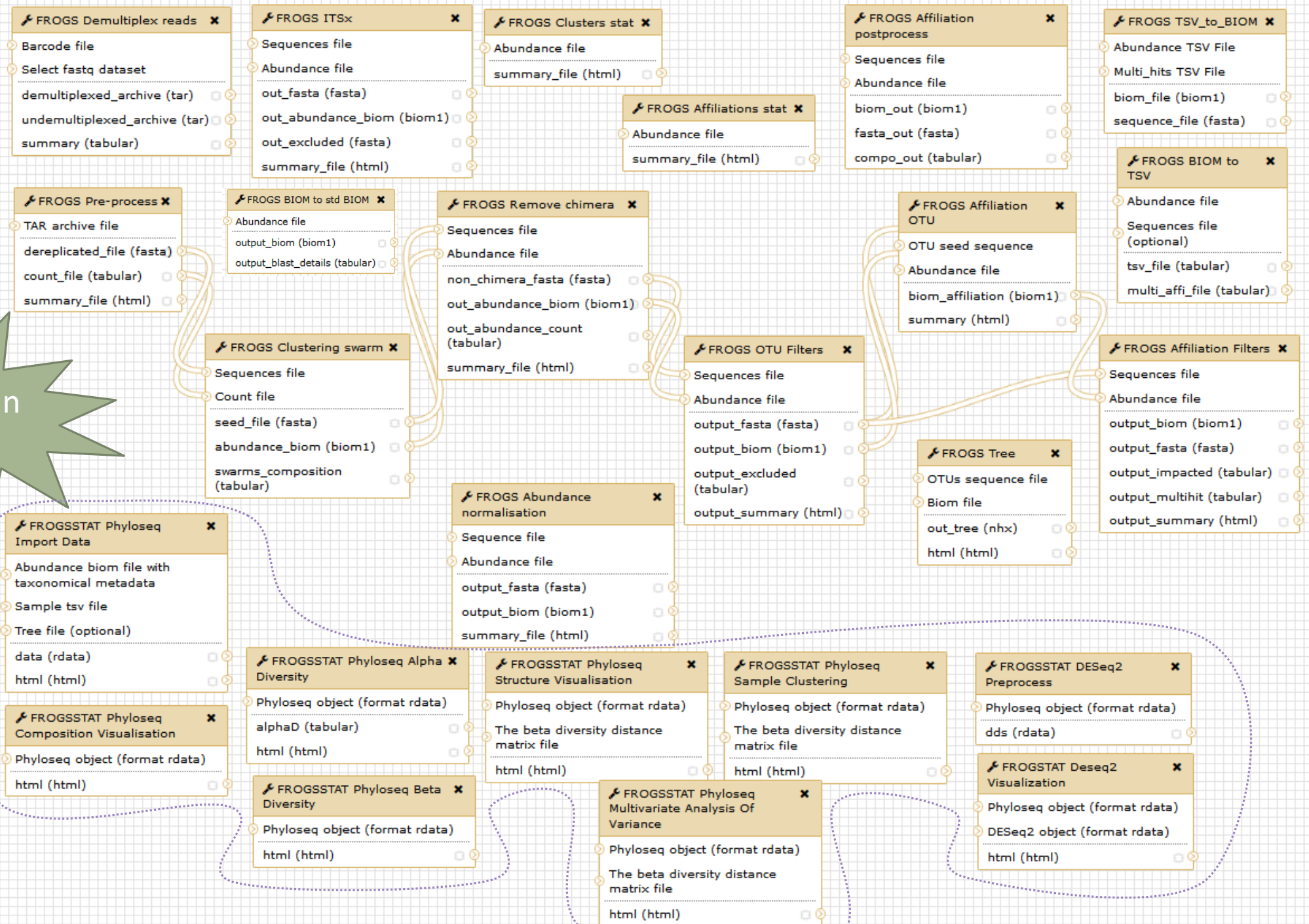


Methods



FROGS Pipeline

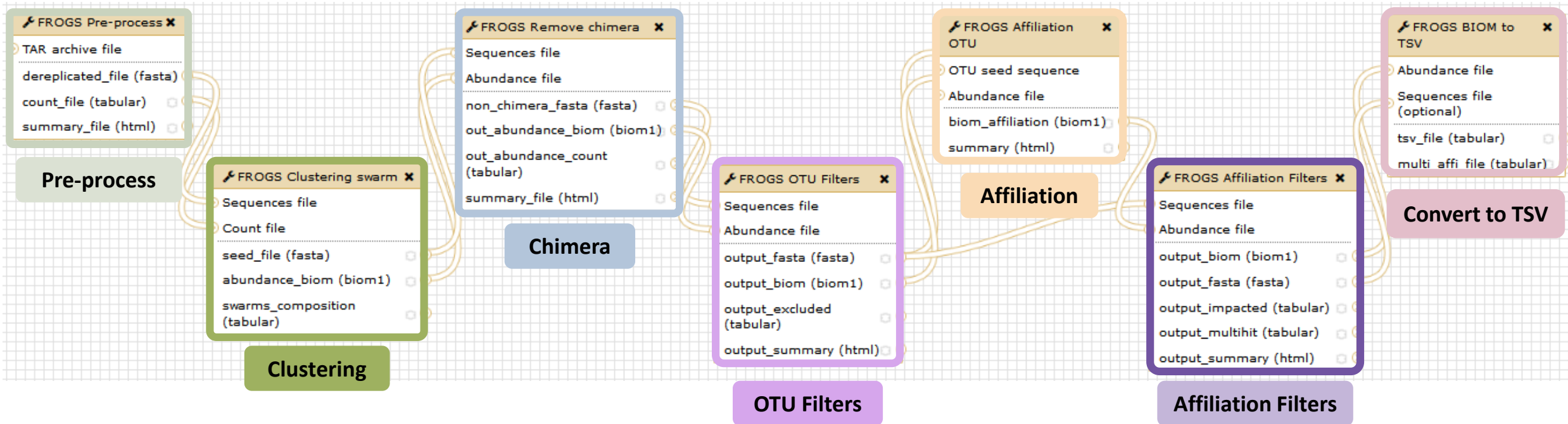


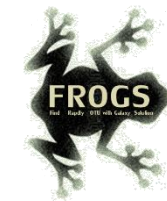




FROGS Pipeline

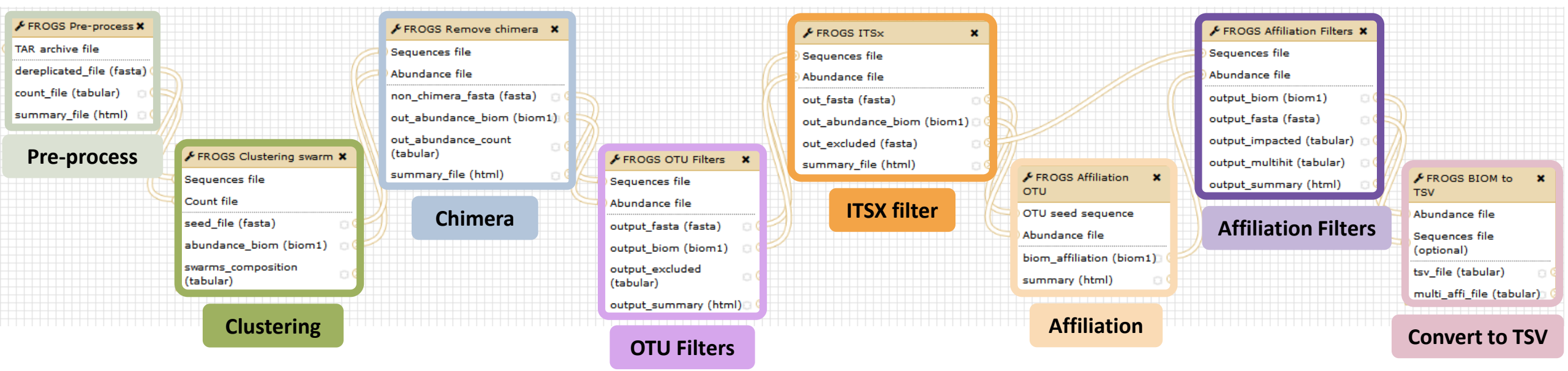
Minimal pipeline for bacterial amplicon analyses





FROGS Pipeline

Minimal pipeline for ITS amplicon analyses



FROGS Tools for Bioinformatics analyses

The screenshot displays the Galaxy web interface. The main panel shows the configuration for the 'FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication' tool. The configuration includes a dropdown for 'Sequencer' (Illumina), 'Input type' (Files by samples), and 'Reads already contiged?' (No). There are input fields for 'Samples' (Name) and 'Reads 1'/'Reads 2' (FASTQ files). Below these are fields for 'Reads 1 size', 'Reads 2 size', and 'Expected amplicon size'. A '+ Insert Samples' button is also present.

On the left, a 'Tools' sidebar lists various FROGS tools under the 'METAGENOMICS' category. On the right, a 'History' panel shows a sequence of 25 analysis steps, each with a status icon (eye, lock, or X) and a file name.

Overlaid on the interface are several colored callout boxes:

- Demultiplexing** (yellow)
- Pre-process** (grey)
- Clustering** (green)
- Chimera** (blue)
- OTU Filters** (purple)
- ITSX** (orange)
- Affiliation** (yellow)
- Affiliation filter** (purple)
- Affiliation postprocess** (yellow)
- Normalization** (green)
- Phylogenetics Tree** (red)
- Cluster Stat** (blue)
- Affiliation Stat** (blue)
- Waiting to run** (grey)
- Currently running** (yellow)
- Result files** (green)
- BIOM to std BIOM** (blue)
- BIOM to TSV** (blue)
- TSV to BIOM** (blue)

Tools List (Left Sidebar):

- FROGS Demultiplex reads**: Attribute reads to samples in function of inner barcode.
- FROGS Pre-process**: merging, denoising and dereplication.
- FROGS Clustering swarm**: Single-linkage clustering on sequences.
- FROGS Remove chimera**: Remove PCR chimera in each sample.
- FROGS OTU Filters**: Filters OTUs on several criteria.
- FROGS ITSx**: Extract the highly variable ITS1 and ITS2 subregions from ITS sequences.
- FROGS Affiliation OTU**: Taxonomic affiliation of each OTU's seed by RDPtools and BLAST.
- FROGS Affiliation Filters**: Filters OTUs on several affiliation criteria.
- FROGS Affiliation postprocess**: Optional step to resolve inclusive amplicon ambiguities and to aggregate OTUs based on alignment metrics.
- FROGS Abundance normalisation**: Normalize OTUs abundance.
- FROGS Tree**: Reconstruction of phylogenetic tree.
- FROGS Clusters stat**: Process some metrics on clusters.
- FROGS Affiliations stat**: Process some metrics on taxonomies.

History Panel (Right):

- FROGS analysis** (444.7 MB)
- 25: FROGS** (Affiliations_stat: summary.html)
- 24: FROGS BIOM to std BIOM: blast_metadata.tsv**
- 23: FROGS BIOM to std BIOM: abundance.biom**
- 22: FROGS BIOM to TSV: multi_hits.tsv**
- 21: FROGS BIOM to TSV: abundance.tsv**
- 20: FROGS** (Affiliations_stat: summary.html)
- 19: FROGS Clusters** (stat: summary.html)
- 18: FROGS Affiliation OTU: report.html**
- 17: FROGS Affiliation OTU: affiliation.biom**
- 16: FROGS Clusters** (stat: summary.html)
- 15: FROGS Filters: report.html**
- 14: FROGS Filters: excluded.tsv**
- 13: FROGS Filters: abundance.biom**
- 12: FROGS Filters: sequences.fasta**

Configuration Fields (Main Panel):

- Sequencer**: Illumina
- Input type**: Files by samples
- Reads already contiged ?**: No
- Samples**: 1: Samples
- Name**: [Empty field]
- Reads 1**: [No fastq dataset available.]
- reads 2**: [No fastq dataset available.]
- Reads 1 size**: [Empty field]
- Reads 2 size**: [Empty field]
- Expected amplicon size**: [Empty field]

Callout Boxes:

- Waiting to run**: Grey box
- Currently running**: Yellow box
- Result files**: Green box
- BIOM to std BIOM**: Blue box
- BIOM to TSV**: Blue box
- TSV to BIOM**: Blue box

FROGS Tools for Statistic analyses

The screenshot displays the Galaxy web interface for a FROGS Pre-process tool. The tool is titled "FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0)".

Tool Configuration:

- Sequencer:** Illumina
- Input type:** Files by samples
- Reads already contiged ?** No
- Samples:** 1: Samples
 - Name:** (empty field)
 - Reads 1:** No fastq dataset available.
 - Reads 2:** No fastq dataset available.
- Reads 1 size:** (empty field)
- Reads 2 size:** (empty field)
- Expected amplicon size:** (empty field)

History Panel:

- FROGS analysis** (444.7 MB)
 - 25: FROGS Affiliations stat: summary.html
 - 24: FROGS BIOM to std BIOM: blast_metadata.tsv
 - 23: FROGS BIOM to std BIOM: abundance.biom
 - 22: FROGS BIOM to TSV: multi_hits.tsv
 - 21: FROGS BIOM to TSV: abundance.tsv
 - 20: FROGS Affiliations stat: summary.html
 - 19: FROGS Clusters stat: summary.html
 - 18: FROGS Affiliation OTU: report.html
 - 17: FROGS Affiliation OTU: affiliation.biom
 - 16: FROGS Clusters stat: summary.html
 - 15: FROGS Filters: report.html
 - 14: FROGS Filters: excluded.tsv
 - 13: FROGS Filters: abundance.biom
 - 12: FROGS Filters: sequences.fasta

Annotations:

- Waiting to run:** A grey box highlighting steps 20-25 in the history panel.
- Currently running:** A yellow box highlighting steps 17 and 18 in the history panel.
- Result files:** A green box highlighting steps 12-16 in the history panel.

Tool Categories (Left Panel):

- OTUS STRUCTURE AND COMPOSITION ANALYSIS:**
 - FROGSSTAT Phyloseq Import Data from 3 files: biomfile, samplefile, treefile
 - FROGSSTAT Phyloseq Composition Visualisation with bar plot and composition plot
 - FROGSSTAT Phyloseq Alpha Diversity with richness plot
 - FROGSSTAT Phyloseq Beta Diversity distance matrix
 - FROGSSTAT Phyloseq Structure Visualisation with heatmap plot and ordination plot
 - FROGSSTAT Phyloseq Sample Clustering of samples using different linkage methods
 - FROGSSTAT Phyloseq Multivariate Analysis Of Variance
- DIFFERENTIAL ABUNDANCE ANALYSIS:**
 - FROGSSTAT DESeq2 Preprocess import a Phyloseq object and prepare it for DESeq2 differential abundance analysis
 - FROGSSTAT Deseq2 Visualization to extract and visualize differentially abundant OTUs

Navigation Buttons (Left Side):

- Import R data
- Composition visualisation
- Alpha diversity
- Beta diversity
- Structure visualisation
- Sample clustering
- Multivariate analysis of variance
- DESeq2 preprocess
- DESeq2 Visualisation

What kind of data ?

2 Histories

16S fastq sequences in an archive
tar.gz

Food environment

chailou_withprimers_64renamedsa
mples_V1V3_10000seq_R1R2.tar.gz

ITS data

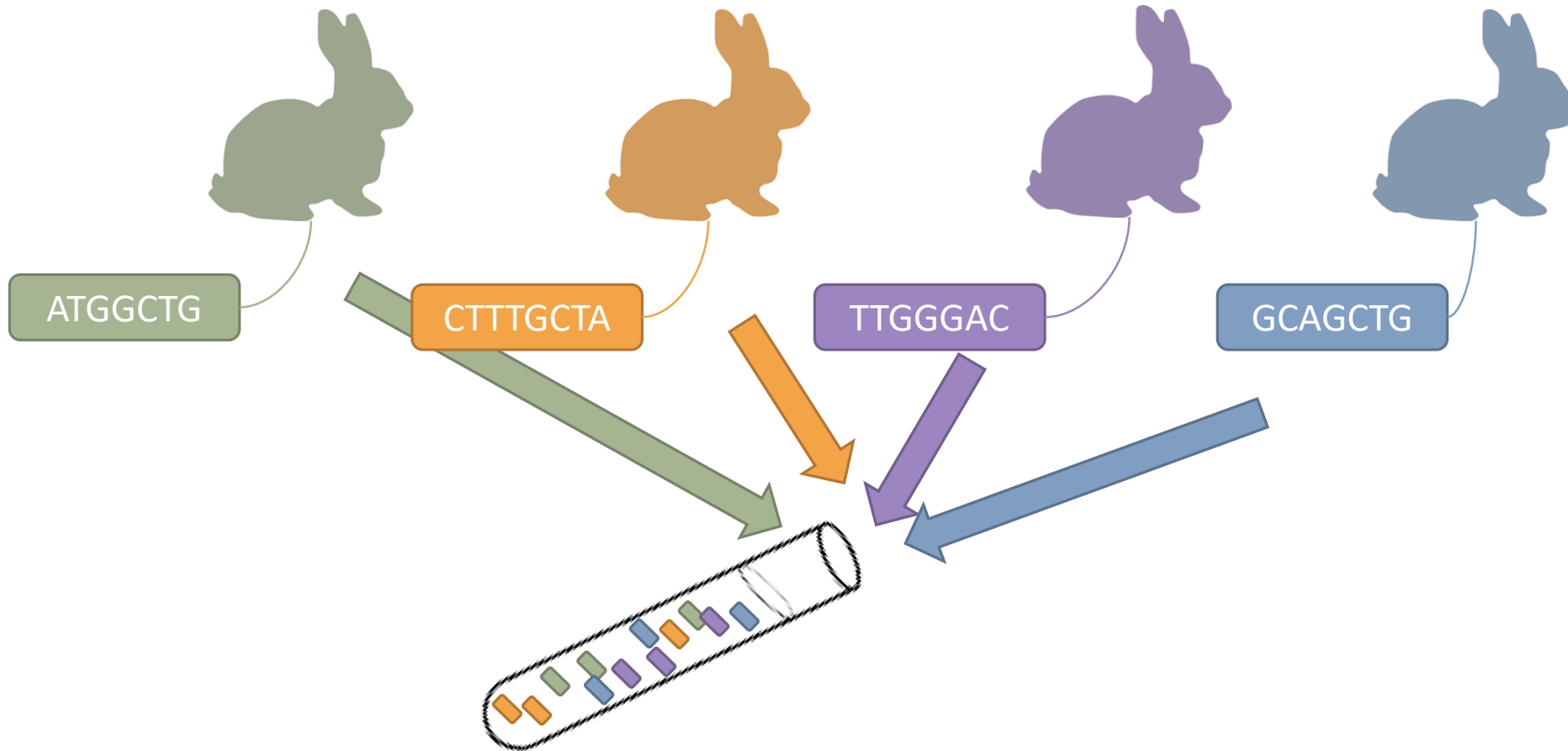
METABARFOOD
project

ITS.tar.gz



Demultiplexing tool

Barcoding ?



Demultiplexing

Sequence demultiplexing in function of barcode sequences :

- In forward
- In reverse
- In forward and reverse

Remove unbarcoded or ambiguous sequences

Demultiplexing forward



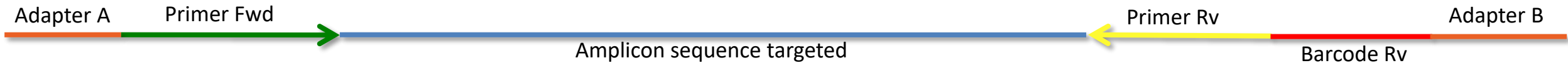
Single-end sequencing



Paired-end sequencing



Demultiplexing reverse



Single end sequencing



Paire end sequencing



Demultiplexing forward and reverse



Single end sequencing



Paire end sequencing



The tool parameters depend on the input data type

FROGS Demultiplex reads (version 1.1.0)

Barcode file:
1: barcode.tabular
This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads:
Single
Select between paired and single end data

Select fastq dataset:
Specify dataset of your single end reads

barcode mismatches:
0
Number of mismatches allowed in barcode

barcode on which end ?:
Forward
Forward at the beginning of the forward end or of the reverse end or both?
Reverse
Both ends
Execute

Where is the barcode seq on the reads?

You have only R1 seq.

FROGS Demultiplex reads (version 1.1.0)

Barcode file:
1: barcode.tabular
This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads:
Paired
Select between paired and single end data

Select first set of reads:
Specify dataset of your forward reads

Select second set of reads:
Specify dataset of your reverse reads

barcode mismatches:
0
Number of mismatches allowed in barcode

barcode on which end ?:
Forward
Forward at the beginning of the forward end or of the reverse end or both?
Reverse
Both ends
Execute

You have R1 and R2 seq.

FROGS Demultiplex reads

- Barcode file
- Select fastq dataset
- demultiplexed_archive (data)
- undemultiplexed_archive (data)
- summary (tabular)

Demultiplexing

FROGS Demultiplex reads Attribute reads to samples in function of inner barcode. (Galaxy Version 2.0.0) Options

Barcode file

This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads

Single

Select between paired and single-end data

Select fastq dataset

Specify dataset of your single end reads

Barcode mismatches

Number of mismatches allowed in barcode

Barcode on which end ?

Forward

The barcode is placed either at the beginning of the forward end or of the reverse end or both?

Input example

MgArd0001	ACAGCGT
MgArd0009	ACAGTAG
MgArd0017	ACGTCAG
MgArd0029	ACTCAGT
MgArd0038	ACTCGTC
MgArd0046	AGCAGTC
MgArd0054	AGCTATG
MgArd0062	AGCTCGC
MgArd0073	AGTATCT
MgArd0081	AGTCTGC




if index is in only at forward:
tabular file with 2 columns
sample names + barcodes




Advices




For your own data

- Do not forget to indicate barcode sequence as they are in the fastq sequence file, especially if you have data multiplexed via the reverse strand.
- For the mismatch threshold, we advised you to let the threshold to 0, and if you are not satisfied by the result, try with 1. The number of mismatch depends on the length of the barcode, but often those sequences are very short so 1 mismatch is already more than the sequencing error rate.
- If you have different barcode lengths, you must demultiplex your data in different times beginning by the longest barcode set and used the "unmatched" or "ambiguous" sequence with smaller barcode and so on.
- If you have Roche 454 sequences in sff format, you must convert them with some program like [sff2fastq](#)

Outputs

9: FROGS Demultiplex   
reads: report

8: FROGS Demultiplex   
reads: undemultiplexed.tar.gz

7: FROGS Demultiplex   
reads: demultiplexed.tar.gz

A tar archive is created by grouping one (or a pair of) fastq file per sample with the names indicated in the first column of the barcode tabular file.

1	2
#sample	count
ambiguous	0
MgArd0009	91
MgArd0017	166
MgArd0038	1208
MgArd0029	193
unmatched	245
MgArd0001	119
MgArd0081	246
MgArd0046	401
MgArd0054	243
MgArd0073	474
MgArd0062	1127

With barcode mismatches >1 sequence can correspond to several samples. Sequences that match at only one sample are affected to this sample but the others (ambiguous) are not re-affected to a sample.

Sequences without known barcode. So these sequences are non-affected to a sample.

Format: Barcode

BARCODE FILE is expected to be **tabulated**:

- first column corresponds to the sample name (unique, without space)
- second to the forward sequence barcode used (None if only reverse barcode)
- optional third is the reverse sequence barcode (optional)

Take care to indicate sequence barcode in the strand of the read, so you may **need to reverse complement** the reverse barcode sequence. Barcode sequence must have the same length.

Example of barcode file.

The last column is optional, like this, it describes sample multiplexed by both fragment ends.

```
MgArd00001      ACAGCGT      ACGTACA
```

Format : FastQ

FASTQ : Text file describing biological sequence in 4 lines format:

- first line start by "@" correspond to the sequence identifier and optionally the sequence description. "@Sequence_1 description1"
- second line is the sequence itself. "ACAGC"
- third line is a "+" following by the sequence identifier or not depending on the version
- fourth line is the quality sequence, one code per base. The code depends on the version and the sequencer

```
@HNSHOSKD01ALD0H  
ACAGCGTCAGAGGGGTACCAGTCAGCCATGACGTAGCACGTACA  
+  
CCCFHHHHHHJJJJHHFF@DEDDDDDDDD@CDDDDACDD
```

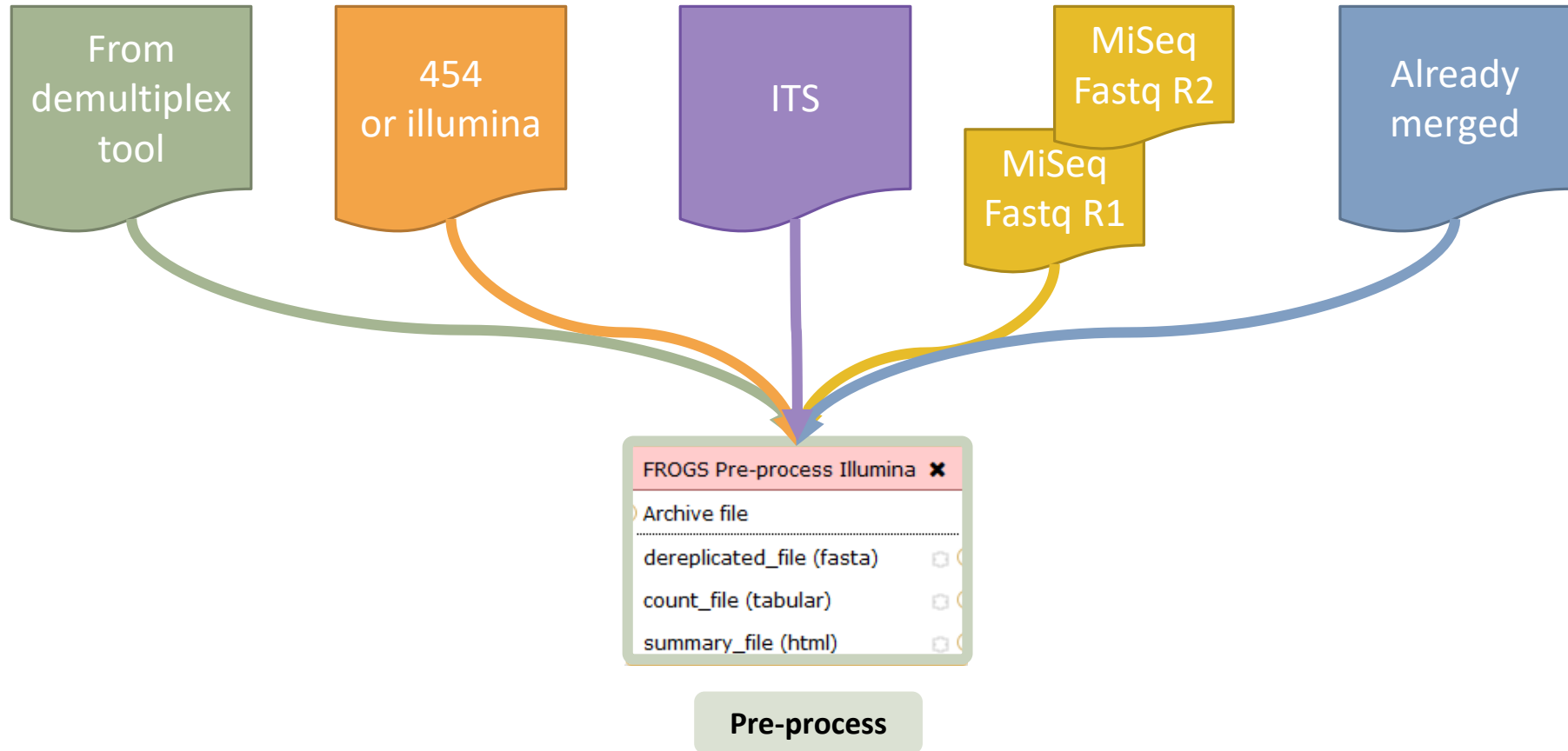
How it works ?

For each sequence or sequence pair the sequence fragment at the beginning (forward multiplexing) of the (first) read or at the end (reverse multiplexing) of the (second) read will be compared to all barcode sequences.

If this fragment is equal (with less or equal mismatch than the threshold) to one (and only one) barcode, the fragment is trimmed and the sequence will be attributed to the corresponding sample.

Finally fastq files (or pair of fastq files) for each sample are included in an archive, and a report describes how many sequences are attributed for each sample.

Pre-process tool



Pre-process

- Merging of R1 and R2 reads
- Delete sequences without good primers
- Finds and removes adapter sequences
- Delete sequence with not expected lengths
- Delete sequences with ambiguous bases (N)
- Dereplication

- + removing homopolymers (size = 8) for 454 data
- + quality filter for 454 data

Example for:

- Illumina MiSeq data
- 1 sample
- Non joined

Pre-process example 1

FROGS Pre-process merging, denoising and dereplication. (Galaxy Version r3.0-3.0) Options

Sequencer
Illumina
Select the sequencing technology used to produce the sequences.

Input type
Files by samples
Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Reads already contiged ?
No
The inputs contain 1 file by sample : R1 and R2 are already merged by pair.

Samples

1: Samples

Name
sampleA
The sample name.

Reads 1
1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/FROGS_ini/DATA/sampleA_R1.fastq
R1 FASTQ file of paired-end reads.

reads 2
2: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/FROGS_ini/DATA/sampleA_R2.fastq
R2 FASTQ file of paired-end reads.

+ Insert Samples

Reads 1 size
250
The maximum read1 size.

Reads 2 size
250
The maximum read2 size.

mismatch rate.
0.1
The maximum rate of mismatches in the overlap

Merge software
Vsearch
Select the software to merge paired-end reads.

Would you like to keep unmerged reads?
Yes No
No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

Parameters for the merging

Minimum amplicon size
340
The minimum size for the amplicons.

Maximum amplicon size
450
The maximum size for the amplicons.

Sequencing protocol
Illumina standard
The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer
GTGCCAGCMGCCGCGTAA
The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer
ATTAGAWACCCBDGTAGTCC
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Execute

V4-16S variability
Mean size = 390 ncl.

Primer sequences

degenerate primer
are accepted
(IUPAC code)

Pre-process example 1

Example for:

- Roche 454 data
- 1 sample
- Only one read (454 process)

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0) Options

Sequencer
454
Select the sequencer family used to produce the sequences.

Input type
One file by sample
Samples files can be provided in single archive or with one file by sample.

Samples
1: Samples

Name
my_sample
The sample name.

Sequence file
1: /work/formation/FROGS/454.fastq.gz
FASTQ file of sample.

Minimum amplicon size
380
The minimum size for the amplicons (with primers).

Maximum amplicon size
500
The maximum size for the amplicons (with primers).

5' primer
ACGGGAGGCAGCAG
The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer
AGGATTAGATACCCTGGTA
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Execute

[V3 – V4] 16S variability

Primer sequences

Pre-process example 2

Example for:

- Illumina MiSeq data
- 9 samples in 1 archive
- Joined
- Without sequenced PCR primers (Kozich protocol)

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0) Options

Sequencer
Illumina **Sequencing technology**
Select the sequencer family used to produce the sequences.

Input type
Archive **One file per sample and all files are contained in a archive**
Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Archive file
1: /work/project/frogs/Formation/100spec_90000seq_9samples_Hantagulomic.tar.gz
The tar file containing the sequences file(s) for each sample.

Reads already contiged ?
Yes **Paire-end sequencing all ready joined**
The archive contains 1 file by sample : Reads 1 and Reads 2 are already contiged by pair.

Minimum amplicon size
380 **[V3 – V4] 16S variability**
The minimum size for the amplicons.

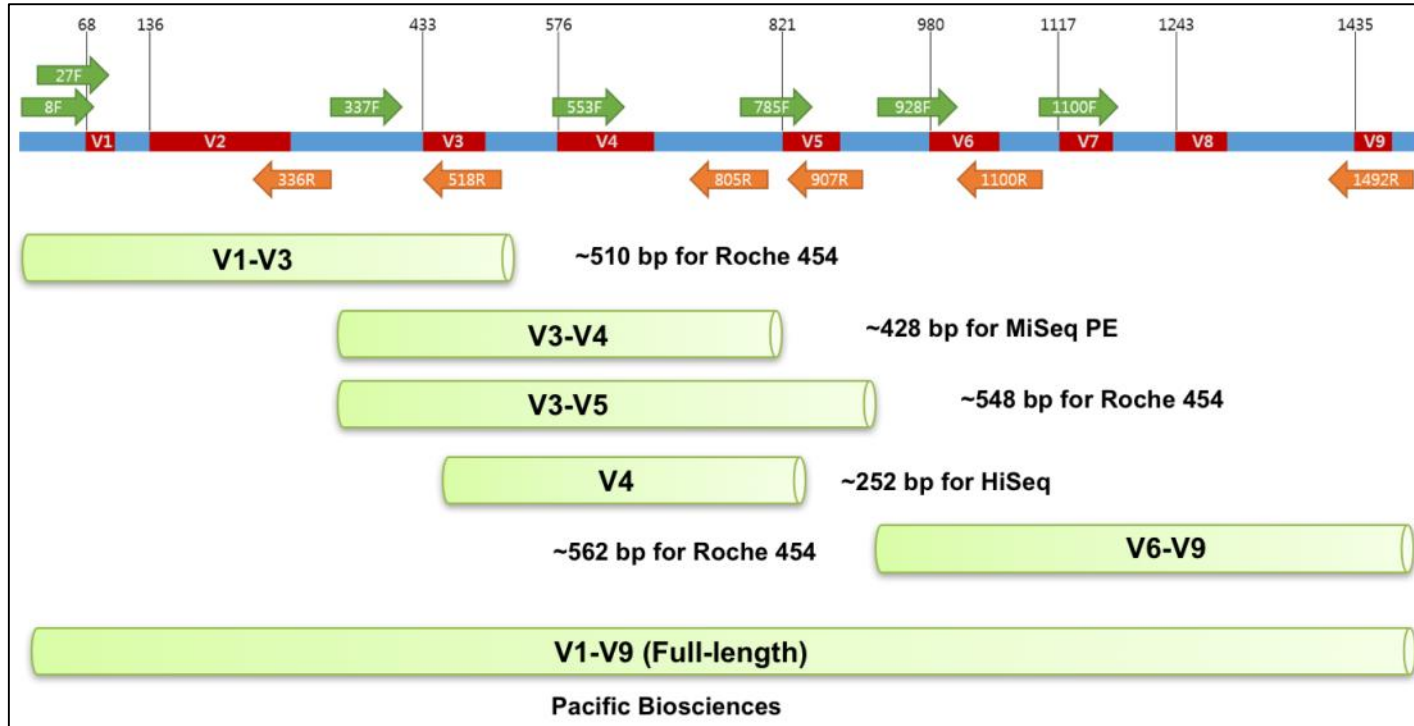
Maximum amplicon size
500
The maximum size for the amplicons.

Sequencing protocol
Custom protocol (Kozich et al. 2013) **No more primers**
The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

Execute

Pre-process example 3

Which primers for 16S ?



Name of primer F=forward, R=reverse	Sequence
8F	AGAGTTTGATCCTGGCTCAG
27F	AGAGTTTGATCMTGGCTCAG
336R	ACTGCTGCSYCCCGTAGGAGTCT
337F	GACTCCTACGGGAGGCWGCAG
337F	GACTCCTACGGGAGGCWGCAG
341F	CCTACGGGNGGCWGCAG
515FB	GTGYCAGCMGCCGCGGTAA
518R	GTATTACCGGGCTGCTGG
533F	GTGCCAGCMGCCGCGGTAA
785F	GGATTAGATACCCTGGTA
805R	GACTACHVGGGTATCTAATCC
806RB	GGACTACNVGGGTWTCTAAT
907R	CCGCAATTCCTTTRAGTTT
928F	TAAACTYAAAKGAATTGACGGG
1100F	YAACGAGCGCAACCC
1100R	GGGTTGCGCTCGTTG
1492R	CGGTTACCTTGTACGACTT

NGS platforms	16S region	PCR primers	Estimated insert size to read (E. coli)	Sequencing
Illumina MiSeq PE (Pair End)	V3V4	341F & 805R	427 bp	250 bp x 2 or 300 bp x 2
Illumina HiSeq/iSeq100 (Earth Microbiome Project)	V4	515FB & 806RB	250 bp	150 x 2

What does the Pre-process tool do?

- Merging of R1 and R2 reads with **vsearch**, **flash** or **pear** (only in command line)
- Delete sequences without good primers
- Finds and removes adapter sequences with **cutadapt**
- Delete sequence with not expected lengths
- Delete sequences with ambiguous bases (N)
- Dereplication
- + removing homopolymers (size = 8) for 454 data
- + quality filter for 454 data

VSEARCH: a versatile open source tool for metagenomics.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F.
PeerJ. 2016 Oct 18;4:e2584. eCollection 2016.

Bioinformatics (2011) 27 (21):2957-2963. doi:10.1093/bioinformatics/btr507

FLASH: fast length adjustment of short reads to improve genome assemblies

TanjaMagoc, Steven L. Salzberg

Bioinformatics (2014) 30 (5):614–620 doi.org/10.1093/bioinformatics/btt593

PEAR: a fast and accurate Illumina Paired-End reAd merger

J. Zhang, K. Kobert, T. Flouri, A. Stamatakis,

EMBnet Journal, Vol17 no1. doi : 10.14806/ej.17.1.200

Cutadapt removes adapter sequences from high-throughput sequencing reads

Marcel Martin

How work reads merging ?

WITH VSEARCH

The aim of Vsearch is to merge R1 with R2

Case of a sequencing of overlapping sequences: case of 16S V3-V4 amplicon MiSeq sequencing:

Imagine a real amplicon sequence of 400bp

400bp



Imagine a Miseq paired sequencing of 2x250bp

R1 : 250bp



R2 : 250bp



Reconstructing amplicon sequence is possible thanks to the overlap region



Merged sequence length : 400bp, with 100bp overlap

The aim of Vsearch is to merge R1 with R2

Case of a sequencing of over-overlapping sequences:

Imagine a real amplicon sequence of 200bp

200bp



Imagine a Miseq paired sequencing of 2x250bp

R1 : 250bp

R2 : 250bp



FROGS takes in charge this case in trimming over bases

200bp



Merged sequence length : 200bp, with 100% overlap

Practice:

Exercise

Go to « [16S](#) » history

Launch the pre-process tool on that data set

→ objective: understand Vsearch software

16S dataset presentation:

A real analysis provided by Stéphane Chaillou *et al.*

Comparison of meat and seafood bacterial communities.

8 environment types (EnvType) :

- Meat → Ground Beef, Ground veal, Poultry sausage, Diced bacon
- Seafood → Cooked schrimps, Smoked salmon, Salmon filet, Cod filet



16S dataset presentation:



From Chaillou paper, we produced simulated data:

- 64 samples of 16S amplicons
- R1 and R2 overlapping reads of 300 bases.
- 8 replicates per condition
- with errors among the linear curve $2.54e-1$ $2.79e-1$

- with 10% chimeras
- Primers for V1-V3:
 - 5' AGAGTTTGATCCTGGCTCAG 3'
 - 5' CCAGCAGCCGCGGTAAT 3'

Chaillou, S. et al (2015). Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. ISME J, 9(5):1105-1118.

Sequencer

Illumina

Select the sequencing technology used to produce the sequences.

Input type

TAR Archive

Samples files can be provided in a single TAR archive or sample by sample (with one or two files each).

TAR archive file 1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/Webinar_data/chaillou_withprimers_64renamedsam...

The TAR file containing the sequences file(s) for each sample.

Are reads already merged ?

No

The archive contains 1 file by sample : R1 and R2 pair are already merged in one sequence.

Reads 1 size

300

The maximum read1 size.

Reads 2 size

300

The maximum read2 size.

Mismatch rate.

0.1

The maximum rate of mismatch in the overlap region

Merge software

Vsearch

Select the software to merge paired-end reads.

Would you like to keep unmerged reads? Yes No

No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

Vsearch is recommended (in command line, prefer pear)

Minimum amplicon size

The minimum size for the amplicons (with primers).

Maximum amplicon size

The maximum size for the amplicons (with primers).

Sequencing protocol

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

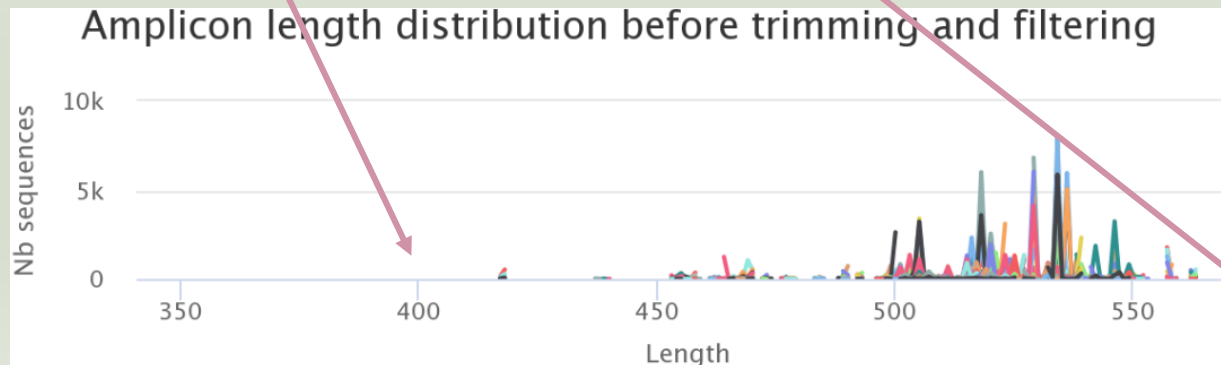
5' primer

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

✓ Execute



Minimum amplicon size

The minimum size for the amplicons (with primers).

Maximum amplicon size

The maximum size for the amplicons (with primers).

Sequencing protocol

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

N.B.
Primers in 5' → 3' sens

Ex: read R1

@63_0 reference=otu_00517 position=1..300

AGAGTTTGATCCTGGCTCAGgatgaacgctagcgggaggcttaacacatgcaagccgagggg
tagaattagcttgctaattgagaccggcgacgggtgcgtaacgcgtatgcaacttgcctactgaaa
ggatagcccagagaaatttgattaatactttataatagactgaatggcatcatttagttttgaaagattt
atcgcagtaggataggcatgcgtaagattagatagttggtagagtaacggctcaccaagtcgacgatct
ttagggggcctgagagggtgaaccccca

Ex: read R2




@63_0 reference=otu_00517 position=1..300 errors=5%G

ATTAGCGCGGCTGCTGGcacggagttagccggtgcttattcttctggtaccttcagctacttacac
gtaagtaggtttatccccagataaaaagtagtttacaaccataaggccgctacctacacgcgggatggc
tggatcaggctccaccattgtccaatattcctcactgctgctcccgtaggagtctggtccgtgtctcag
taccagtgtgggggttcaccctctcaggccccctaaagatcgtcgacttggtgagccgttacctcacca
ctatctaattcttacgcatgcct



R2 primer must be reverse transcribed

Exercise

1. Do you understand how enter your primers ?
2. What is the « FROGS Pre-process: dereplicated.fasta » file ? 
3. What is the « FROGS Pre-process: count.tsv » file ? 
4. Explore the file « FROGS Pre-process: report.html » 
5. *Who loose a lot of sequences ?*

Exercise

6. How many sequences are there in the input file ?
7. How many sequences did not have the 5' primer?
8. How many sequences still are after pre-processing the data?
9. How much time did it take to pre-process the data ?
10. What is the length of your merged reads before preprocessing ?
11. What can you tell about the samples, based on amplicon size distributions ?

Q1: Do you understand how enter your primers ?

Minimum amplicon size

The minimum size for the amplicons (with primers).

Maximum amplicon size

The maximum size for the amplicons (with primers).

Sequencing protocol

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters' help section.

✓ Execute

N.B.
Primers in 5' → 3' sens



R2 primer must be reverse transcribed
Use <https://www.bioinformatics.nl/cgi-bin/emboss/revseq>

Q2: What is the « FROGS Pre-process: dereplicated.fasta » file ?

Q3: What is the « FROGS Pre-process: count.tsv » file ?

```
>06_5949;size=4 reference=otu_00680 position=1..300 errors=20%T
AGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>56_3551;size=1 reference=otu_00680 position=1..300 errors=21%A
AAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>53_322;size=1 reference=otu_01408,otu_00680 amplicon=1..300,1..300 position=1..300
ATTGAACGGTGGCGGCATGCCTACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>56_2589;size=1 reference=otu_00680 position=1..300 errors=21%C
CAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>56_7560;size=1 reference=otu_00680 position=1..300 errors=21%C
CAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>36_626;size=1 reference=otu_00680 position=1..300 errors=21%C
CAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>53_6128;size=1 reference=otu_00231,otu_00941,otu_00680 amplicon=1..300,1..300,1..30
CTGGCTCAGGATGAACGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
>51_6860;size=1 reference=otu_00799,otu_00680 amplicon=1..300,1..300 position=1..300
GACGAAAGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTTCACAGAGGGATAGCCCAGAGAAAATTTGGATTAATACCTCATA
```

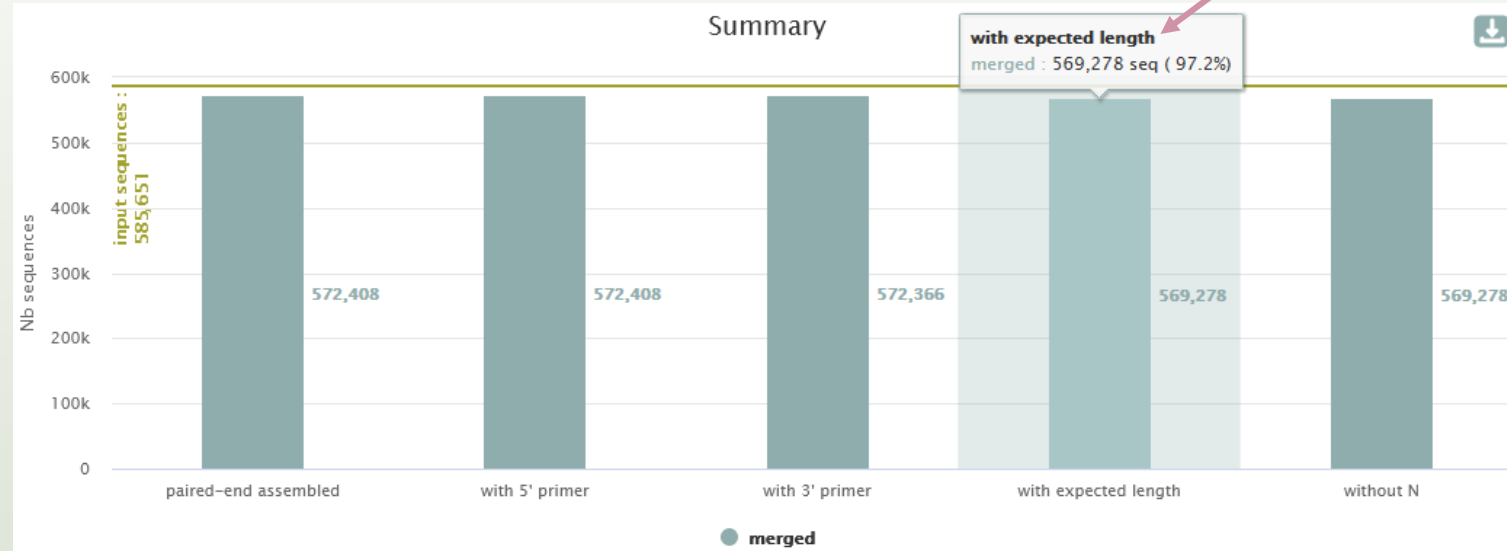
#id	BHT0.LOT01	BHT0.LOT03	BHT0.LOT04	BHT0.LOT05	BHT0.LOT06	BHT0.LOT07
06_5949	0	0	0	0	0	0
56_3551	0	0	0	0	0	0
53_322	0	0	0	0	0	0
56_2589	0	0	0	0	0	0
56_7560	0	0	0	0	0	0
36_626	0	0	0	0	0	0
53_6128	0	0	0	0	0	0
51_6860	0	0	0	0	0	0
56_6896	0	0	0	0	0	0
56_3997	0	0	0	0	0	0
59_6	0	0	0	0	0	191
59_5144	0	0	0	0	0	1
59_5852	0	0	0	0	0	1
60_1696	0	0	0	0	0	0
59_6656	0	0	0	0	0	1
59_1182	0	0	0	0	0	1

Fasta sequence of all clean and dereplicated sequence *i.e.* only one copy of each sequence is kept

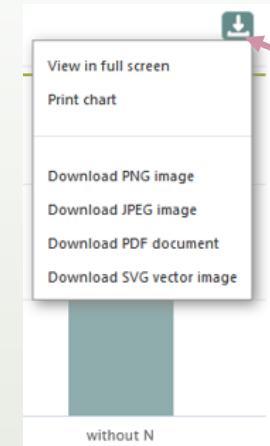
count table for each sequence in each sample

Answer 4

Q4: Explore the file « FROGS Pre-process: report.html »



By moving the mouse over the graphic, new information appears



You can download graphics or table in different formats

Details on merged sequences

Show 10 entries

Search:

CSV

Samples	before process	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
BHT0.LOT01	9,282	97.90	9,087	9,087	9,087	9,087	9,087
BHT0.LOT03	9,173	97.83	8,984	8,984	8,984	8,974	8,974
BHT0.LOT04	9,171	97.79	8,969	8,969	8,968	8,968	8,968

You can sort data in the table by clicking on the column headers

Answer 5

Q5: Who loose a lot of sequences ?

53: FROGS Pre-process: report.html

error
An error occurred with this dataset:

```
## Application  
Software: preprocess.py (version: 3.2.2)  
Command: /galaxydata/galaxy-preprod/my_tools/FROGS
```

52: FROGS Pre-process: count.tsv

51: FROGS Pre-process: dereplicated.fasta

If your outputs are red, click on the bug to read the error message

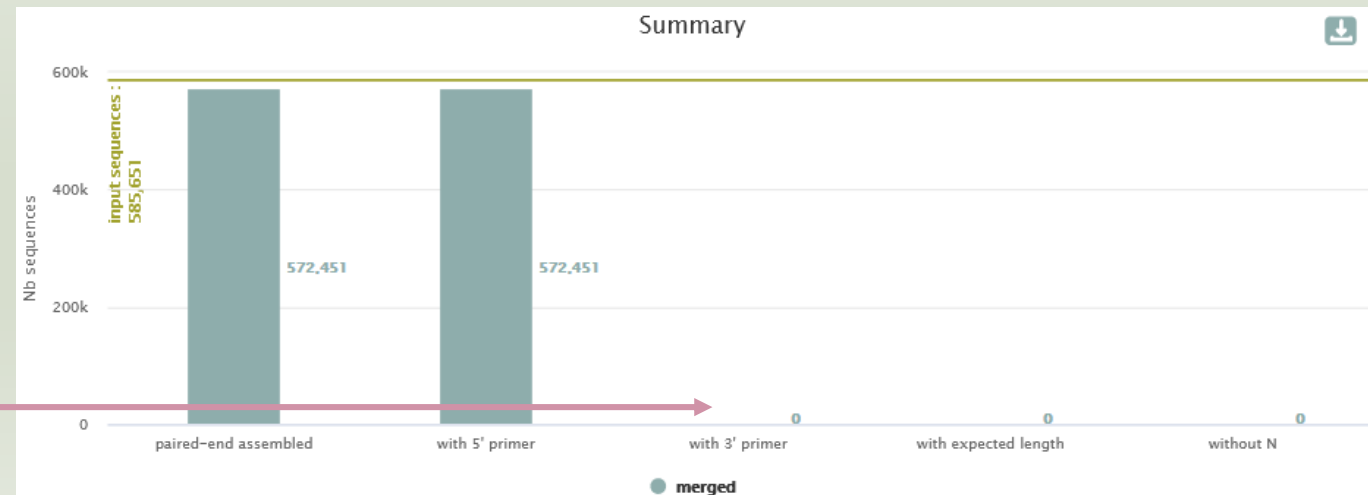
Dataset generation errors

Dataset 53: FROGS Pre-process: report.html

Tool execution generated the following error message:

```
Fatal error: Exit code 1 ()  
Traceback (most recent call last):  
  File "/galaxydata/galaxy-preprod/my_tools/FROGS_dev/app/preprocess.py", line 1290, in <module>  
    process( args )  
  File "/galaxydata/galaxy-preprod/my_tools/FROGS_dev/app/preprocess.py", line 1141, in process  
    raise_exception( Exception( "\n\n#ERROR : The filters have eliminated all sequences (see summary for more details).\n\n" ))  
  File "/galaxydata/galaxy-preprod/my_tools/FROGS_dev/lib/frogsUtils.py", line 45, in raise_exception  
    raise exception  
Exception:  
#ERROR : The filters have eliminated all sequences (see summary for more details).
```

it is likely that you did not enter the 3' primer in the right direction



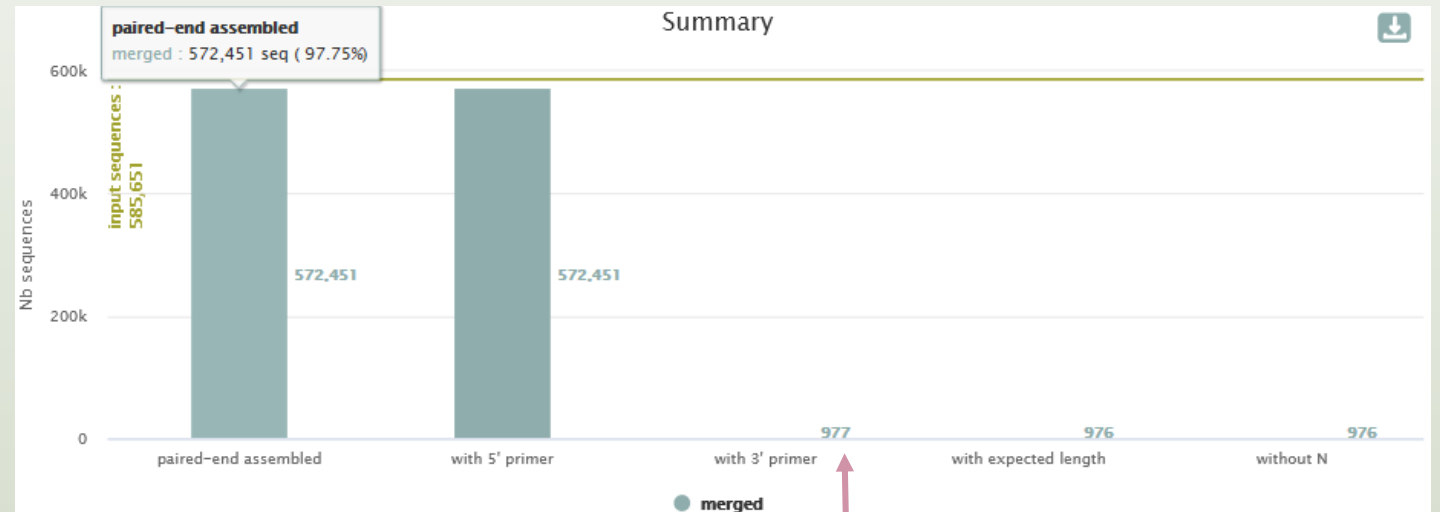
Answer 5

All outputs are green
but check the
report.html

[65: FROGS Pre-process: report.html](#)

[64: FROGS Pre-process: count.tsv](#)

[63: FROGS Pre-process: dereplicated.fasta](#)



Error in 3' primer sequence.
Primers must be similar with 10% of
errors (~1 or 2 bases per primer)

Answer 5

FROGS Pre-process merging, denoising and dereplication. (Galaxy Version 3.2.1)

Options

Sequencer

ILLUMINA

Select the sequencing technology used to produce the sequences.

Input type

TAR Archive

Samples files can be provided in a single TAR archive or sample by sample (with one or two files each).

TAR archive file

1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/Webinar_data/chaillou_withprimers_64renamedsam...

The TAR file containing the sequences file(s) for each sample.

Are reads already merged ?

No

The archive contains 1 file by sample : R1 and R2 pair are already merged in one sequence.

Reads 1 size

300

The maximum read1 size.

Reads 2 size

300

The maximum read2 size.

Mismatch rate.

0.1

The maximum rate of mismatch in the overlap region

Merge software

Vsearch

Select the software to merge paired-end reads.

Would you like to keep unmerged reads?

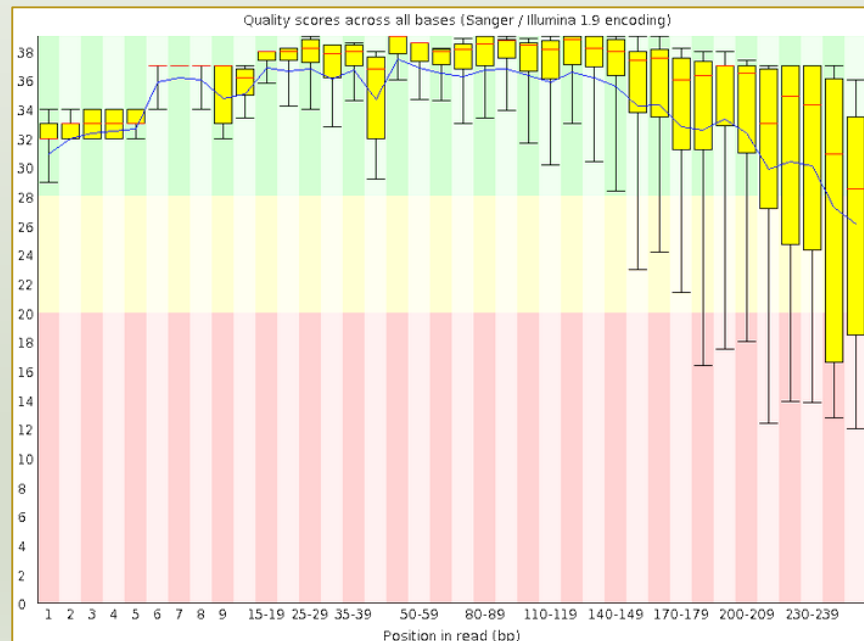
Yes No

No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

To check the sequence quality use
FASTQC (present in galaxy tools)

FastQC: fastq/sam/bam

FastQC:Read QC reports using
FastQC

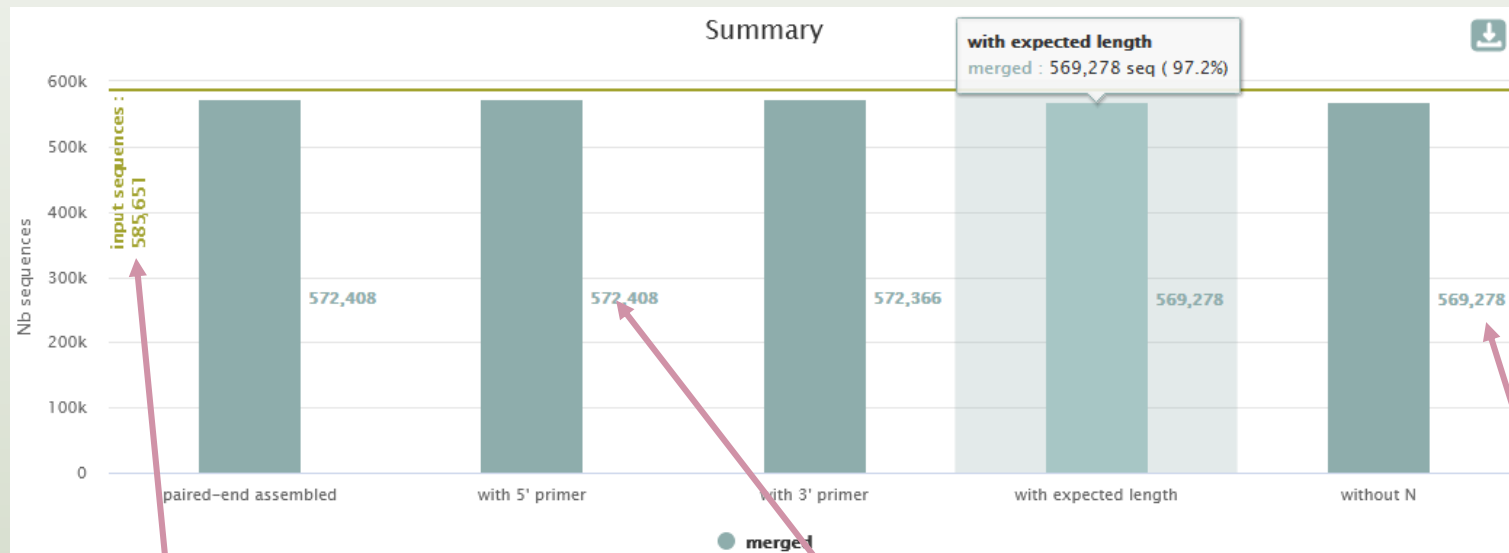


Answer 6, 7 & 8

Q6: How many sequences are there in the input file ?

Q7: How many sequences did not have the 5' primer?

Q8: How many sequences still are after pre-processing the data?



Total number of sequences before preprocessing: 585 651

All sequences have the 5' primer

569 321 sequences are still after preprocessing

Answer 9

Q9: How much time did it take to pre-process the data ?

3: FROGS Pre-process: dereplicated.fasta

287,252 sequences
format: **fasta**, database: ?

```
## Application
Software: preprocess.py (version: 3.2.2)
Command: /galaxydata/galaxy-preprod
/my_tools/FROGS_dev/app/preprocess.py
illumina --output-dereplicated /galaxydata
/galaxy-prod/my_job_working_directory
/000/380/380454
/galaxy_dataset_731997.dat --ou
```

Click on « i »

Tool: FROGS Pre-process	
Name:	FROGS Pre-process: dereplicated.fasta
Created:	Tue 09 Mar 2021 05:00:30 PM (UTC)
Filesize:	152.4 MB
Dbkey:	?
Format:	fasta
Galaxy Tool ID:	FROGS_preprocess_3_2_2
Galaxy Tool Version:	3.2.2
Tool Version:	
Tool Standard Output:	stdout
Tool Standard Error:	stderr
Tool Exit Code:	0
History Content API ID:	d7ff127129900fa8
Job API ID:	45c5decf7bd90ae1
History API ID:	96f266d5ffa0ae3
UUID:	58d5bf75-595e-412b-8c08-a16dbbe9110a

Input Parameter	Value
Sequencer	illumina
Input type	archive
TAR archive file	1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/Webinar_data/chaillou_withprimers_64renamedsamples_V1V3_10000seq_R1R2.tar.gz
Are reads already merged ?	paired
Reads 1 size	300
Reads 2 size	300
Mismatch rate.	0.1
Merge software	vsearch
Would you like to keep unmerged reads?	False
Minimum amplicon size	400
Maximum amplicon size	580
Sequencing protocol	standard
5' primer	AGAGTTTGATCCTGGCTCAG
3' primer	CCAGCAGCCGCGGTAAT

Retrieve the tool parameters

Stdout contains FROGS command lines and time execution

Answer 10

Q10: What is the length of your merged reads before preprocessing ?

Show entries Search:

<input checked="" type="checkbox"/>	Samples	process	kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
<input checked="" type="checkbox"/>	BHT0.LOT01	9,282	97.90	9,087	9,087	9,087	9,087	9,087
<input checked="" type="checkbox"/>	BHT0.LOT03	9,173	97.83	8,984	8,984	8,984	8,974	8,974
<input checked="" type="checkbox"/>	BHT0.LOT04	9,171	97.79	8,969	8,969	8,968	8,968	8,968
<input checked="" type="checkbox"/>	BHT0.LOT05	9,109	97.56	8,890	8,890	8,888	8,887	8,887
<input checked="" type="checkbox"/>	BHT0.LOT06	9,193	97.86	8,996	8,996	8,996	8,996	8,996

Select all samples

Answer 10

Q10: What is the length of your merged reads before preprocessing ?

<input type="checkbox"/>	VHT0.LOT07	9,337	97.03	9,064	9,064	9,064	9,060	9,060
<input checked="" type="checkbox"/>	VHT0.LOT08	9,436	97.33	9,192	9,192	9,192	9,184	9,184
<input type="checkbox"/>	VHT0.LOT10	9,165	97.64	8,983	8,983	8,982	8,949	8,949

With selection:

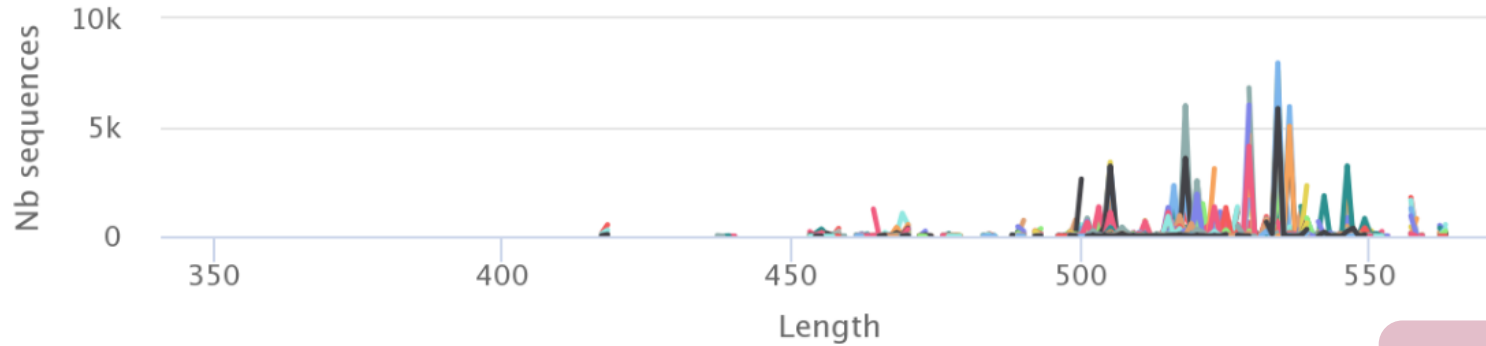
Display amplicon lengths

Display preprocessed amplicon lengths

at the bottom of the table

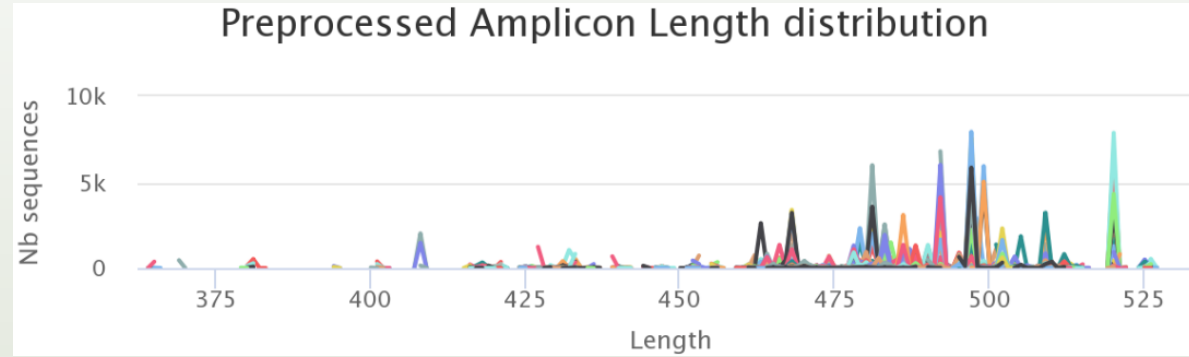
Q10: What is the length of your merged reads before preprocessing ?

Amplicon length distribution before trimming and filtering

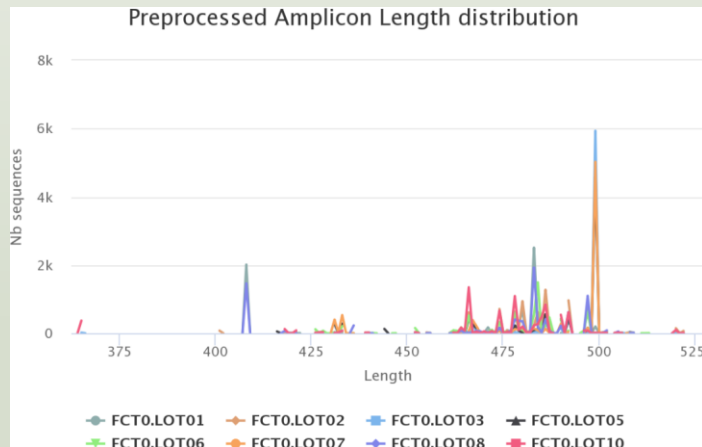


Before preprocessing:
343 < sequence length < 570

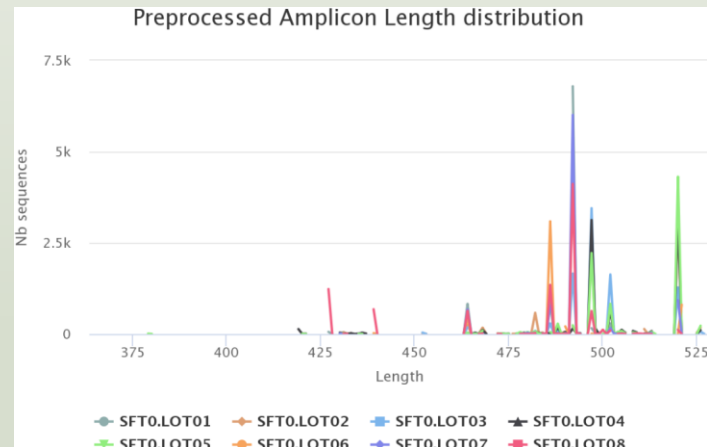
Q11: What can you tell about the samples, based on amplicon size distributions ?



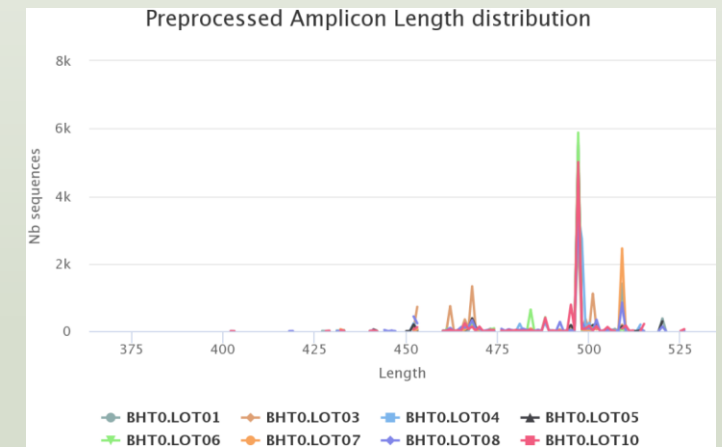
« Filet Cabillaud » samples



« Saumon Fumé » samples



« Bœuf Haché » samples



For each EnvType, we can observe different amplicon sizes. They correspond to different species.

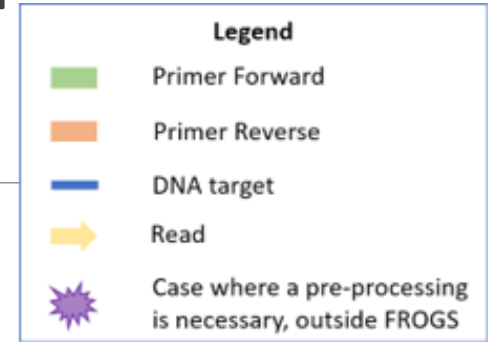
N.B. amplicons with same size can represent different species.

Preprocess tool in brief

	Take in charge
Illumina	✓
454	✓
Merged data	✓
Not merged data	✓
Without primers	✓
Only R1 or only R2	⊘
Too distant R1 and R2 to be merged	✓
Over-overlapping R1 R2	✓

	Take in charge
Archive .tar.gz	✓
Fastq	✓
Fasta	⊘
With only 1 primer	⊘
Multiplexed data	⊘
Demultiplexed data	✓

Processed data by FROGS in brief



454



illumina

Standard sequencing protocol

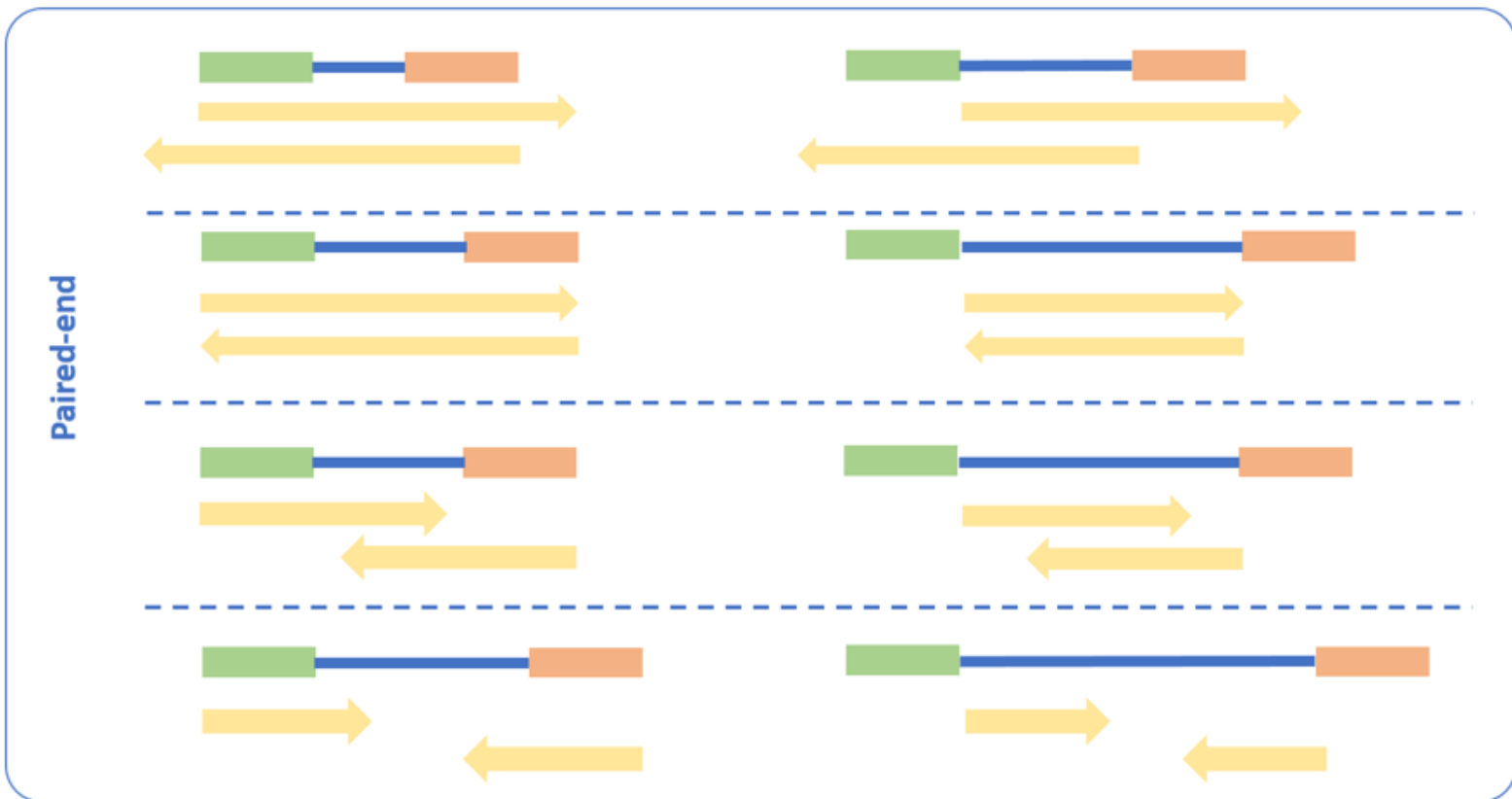
Kozich protocol : primers are not included in reads



→ Remove reverse primer before FROGS processing

Legend

- Primer Forward
- Primer Reverse
- DNA target
- Read
- Case where a pre-processing is necessary, outside FROGS



Length of the sequenced target < length of one read

Supported since version 3.0

Length of the sequenced target < the sum of the lengths of the two reads

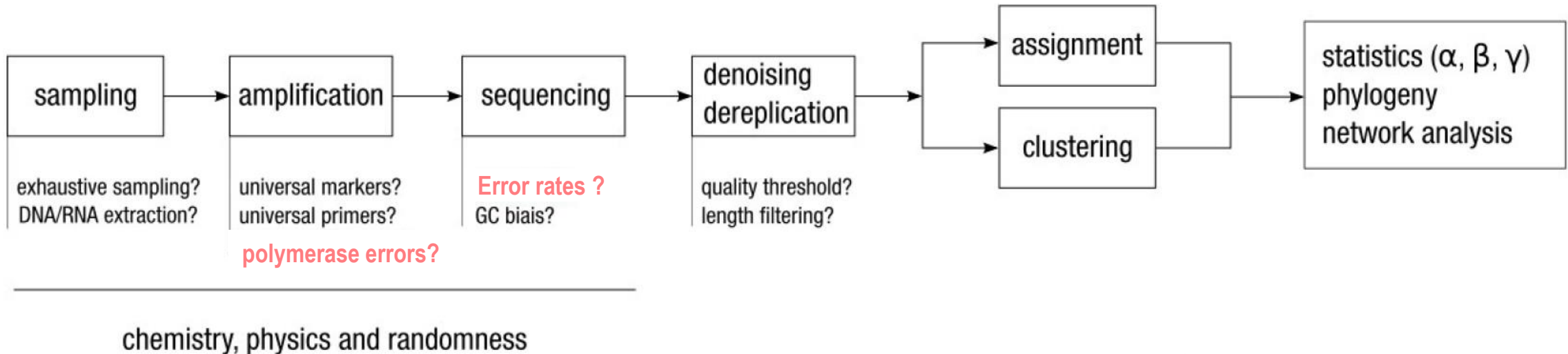
Length of the sequenced target >= the sum of the lengths of the two reads

Supported since version 3.0 with option "keep unmerged reads" in preprocess Tool

Clustering tool

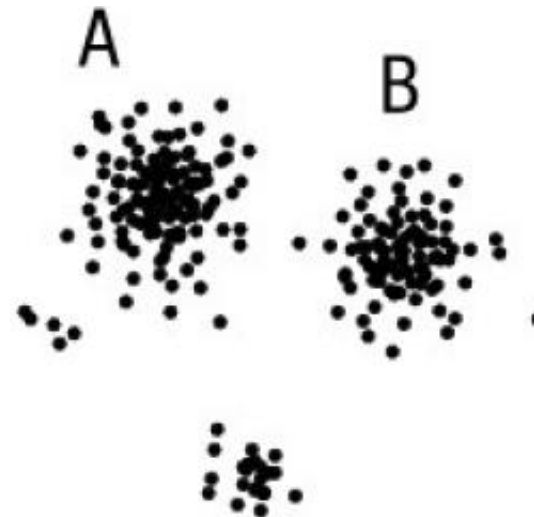
Why do we need clustering ?

Amplification and sequencing and are not perfect processes



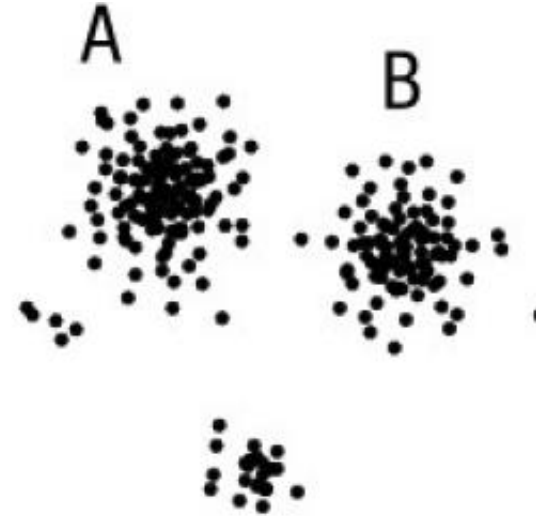


Expected



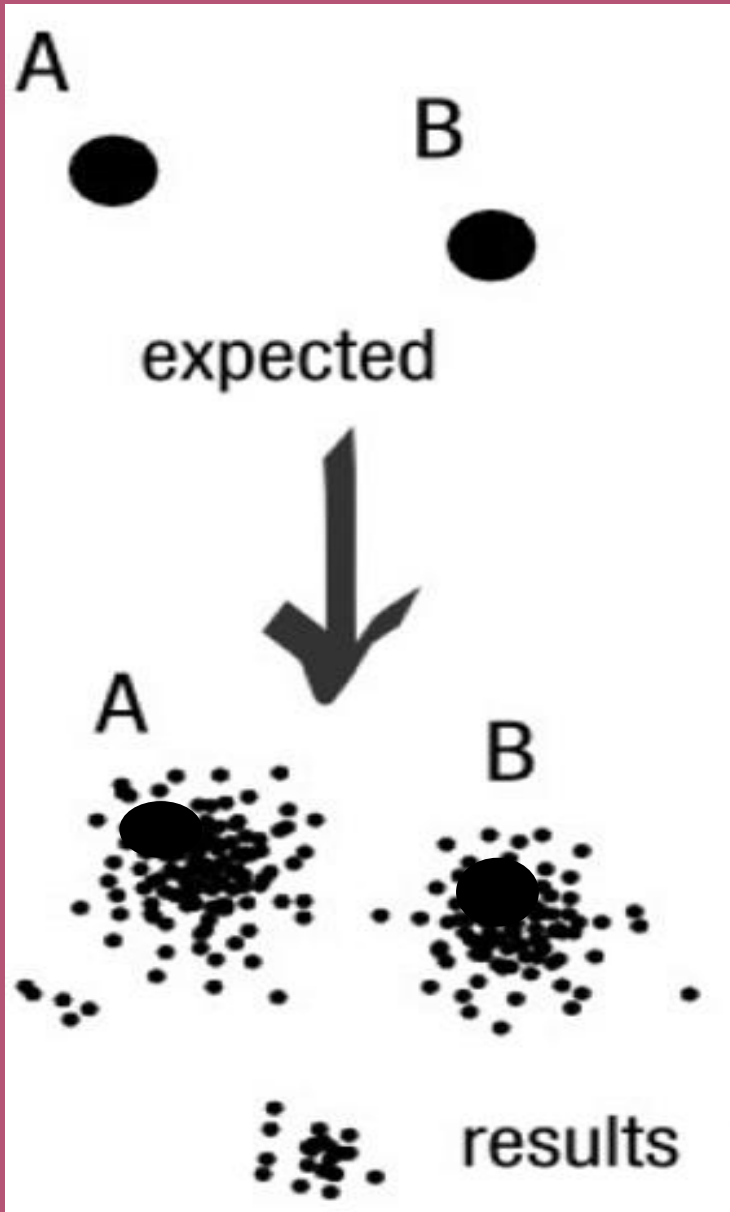
Results

Natural variability?
Technical noise?
Contaminant?
Chimeras?



Natural variability ?
Technical noise?
Contaminant?
Chimeras?

16S variability
Cf. RRNDB (ribosomal RNA operons database)
max. 21 copies of 16S in bacteria (*Photobacterium damsela*)
ex. *E. coli* 7 copies



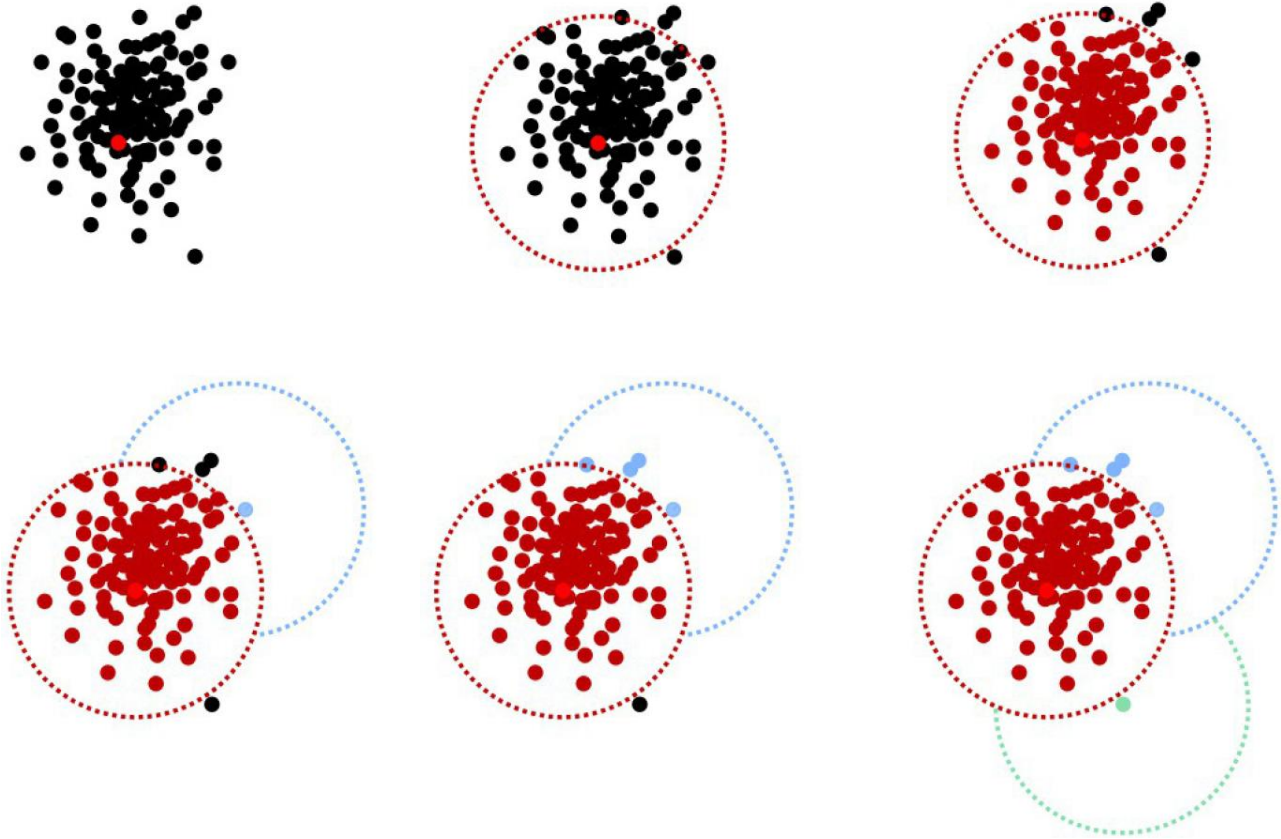
To have the best accuracy:

Method: All against all

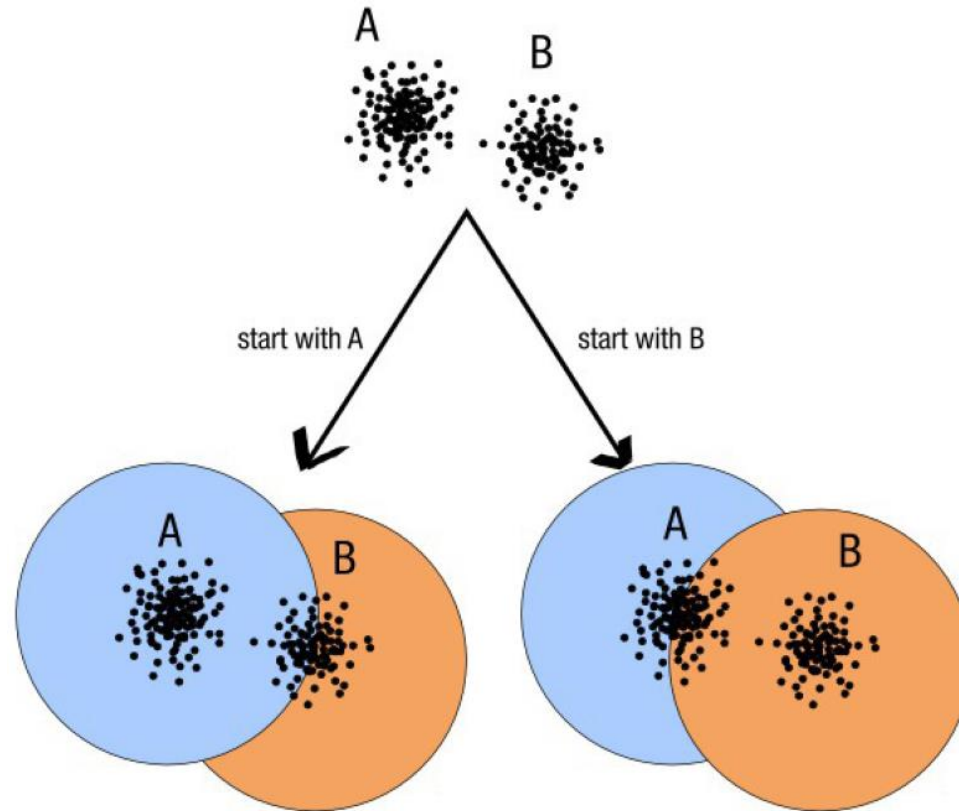
- Very accurate
- Requires a lot of memory and/or time

=> Impossible on very large datasets without strong filtering or sampling

How traditional clustering works ?

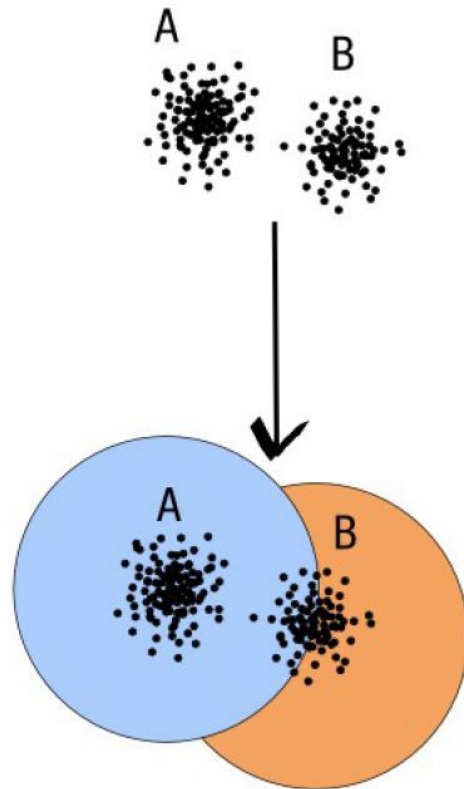


Input order dependent results

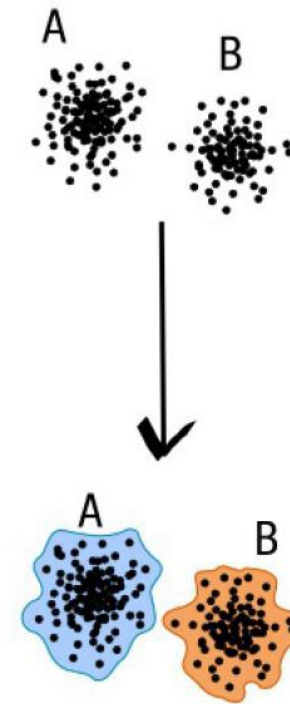


decreasing length,
decreasing abundance,
external references

Single a priori clustering threshold

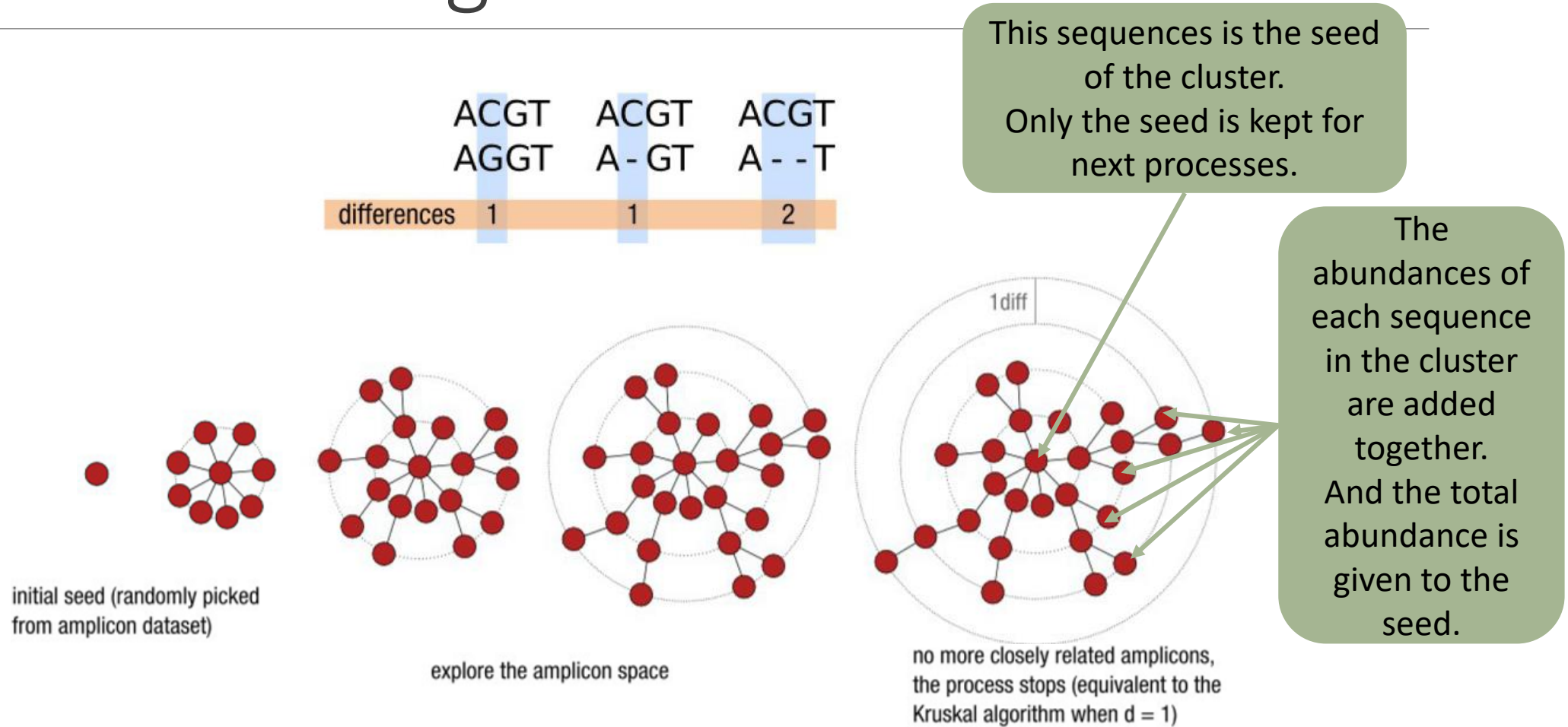


compromise threshold
unadapted threshold

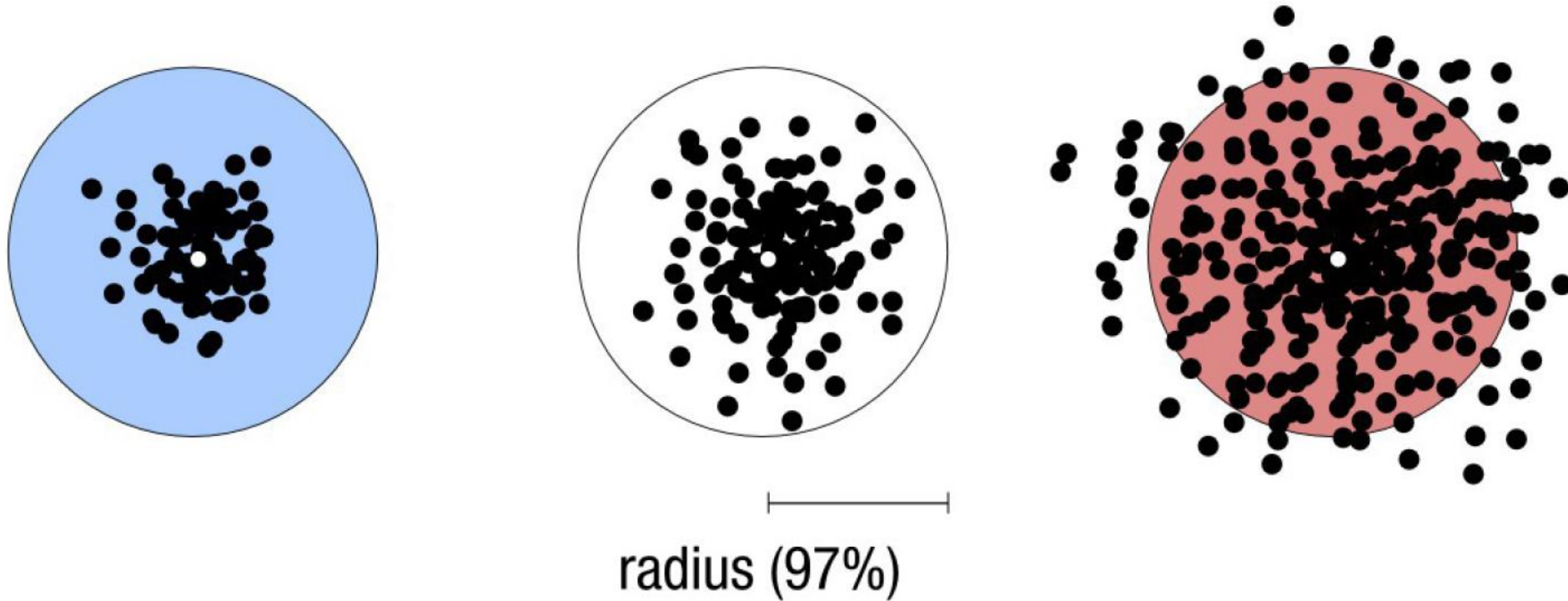


natural limits of clusters

Swarm clustering method

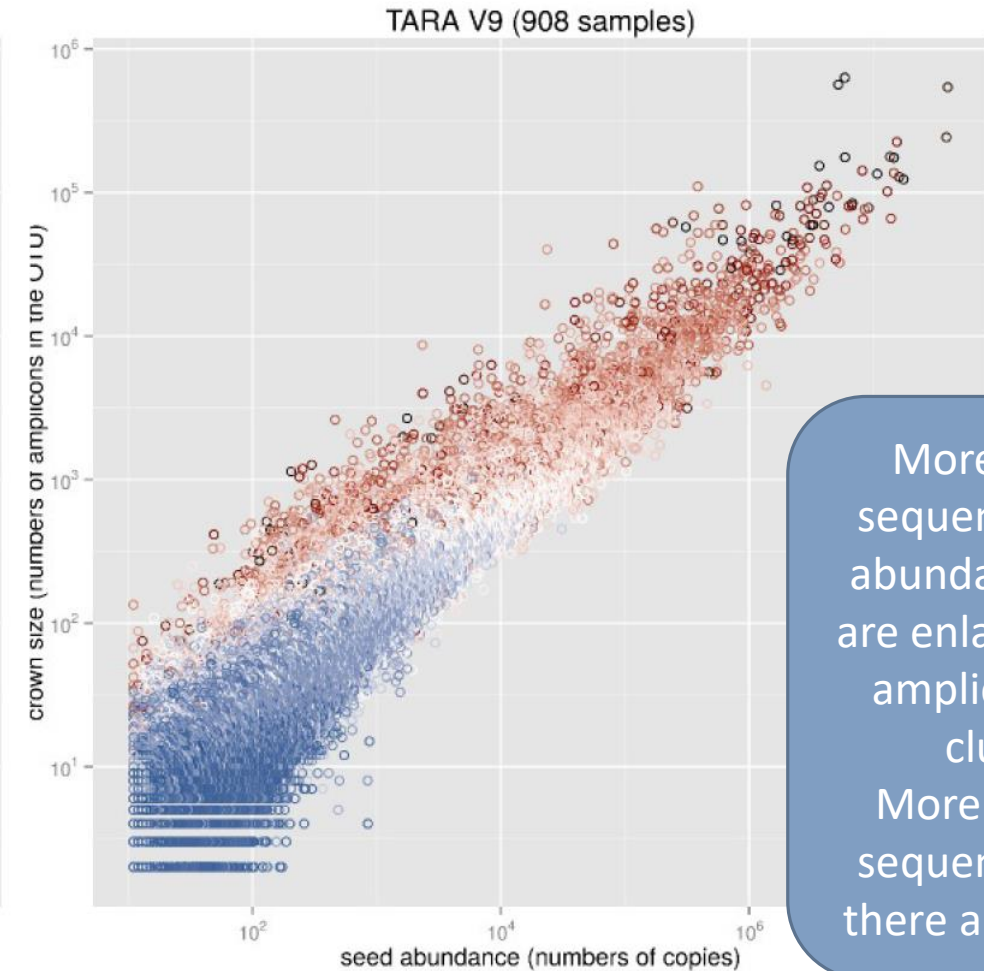
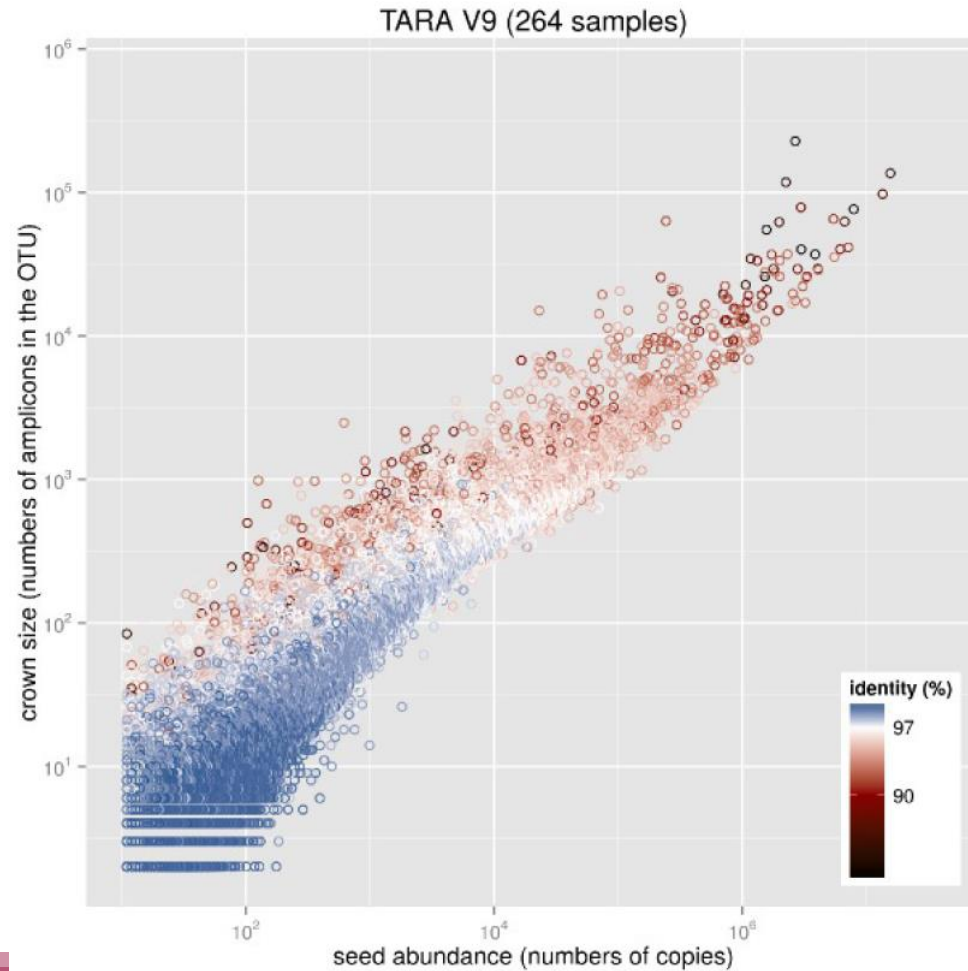


Comparison Swarm and 3% clusterings



Radius expressed as a percentage of identity with the central amplicon (97% is by far the most widely used clustering threshold)

Comparison Swarm and 3% clusterings



More there is sequences, more abundant clusters are enlarged (more amplicon in the cluster).
More there are sequences, more there are artefacts

SWARM

A **robust** and **fast** clustering method for amplicon-based studies.

The purpose of **swarm** is to provide a novel clustering algorithm to handle **large sets of amplicons**.

swarm results are **resilient to input-order changes** and rely on a **small local linking threshold d** , the maximum number of differences between two amplicons.

swarm forms stable high-resolution clusters, with a high yield of biological information.

Swarm: robust and fast clustering method for amplicon-based studies.
Mahé F, Rognes T, Quince C, de Vargas C, Dunthorn M.
PeerJ. 2014 Sep 25;2:e593. doi: 10.7717/peerj.593. eCollection 2014.
PMID:25276506

FROGS Clustering swarm Single-linkage clustering on sequences (Galaxy Version 3.2.1) Options

Sequences file

 The dereplicated sequences file (format: fasta).

Count file

 It contains the count by sample for each sequence (format: TSV).

FROGS guidelines version

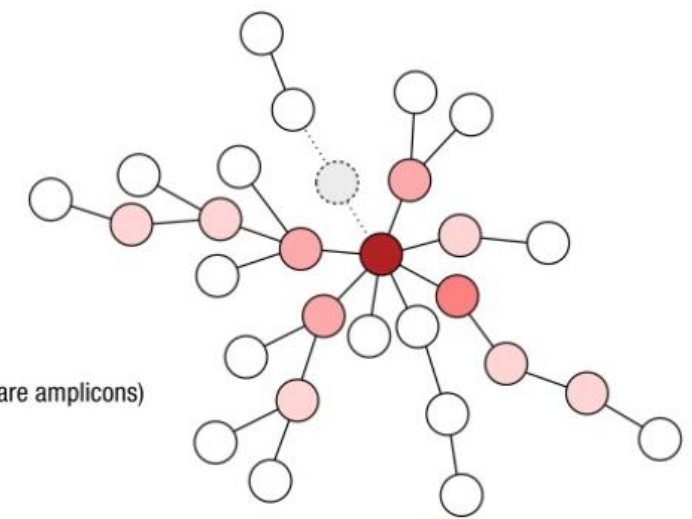
 Denoising step prior to a d3 clustering is no more recommended since FROGS 3.2, but you can still choose it.

Aggregation distance clustering

 Maximum number of differences between sequences in each aggregation swarm step. (recommended d=1)

Refine OTU clustering
 Yes No
 Clustering will be performed with the swarm , which is recommended and only usable in association with a distance of 1 (default and recommended: Yes)

longer but more accurate




Cluster stat tool

FROGS Clusters stat Process some metrics on clusters. (Galaxy Version 3.2.1)

Options

Abundance file

   6: FROGS Clustering swarm: abundance.biom

Clusters abundance (format: BIOM).

Execute

Practice:

LAUNCH CLUSTERING AND CLUSTERSTAT TOOLS

Exercise

Go to « 16S » history

Launch the Clustering SWARM tool on that data set with guideline 3.2 *i.e. aggregation distance =1*

→ objectives :

- understand the outputs from clustering
- understand the ClusterStat utility

Exercise

1. How many clusters do you get ?

Launch FROGS **Cluster Stat tools** on the previous abundance biom file

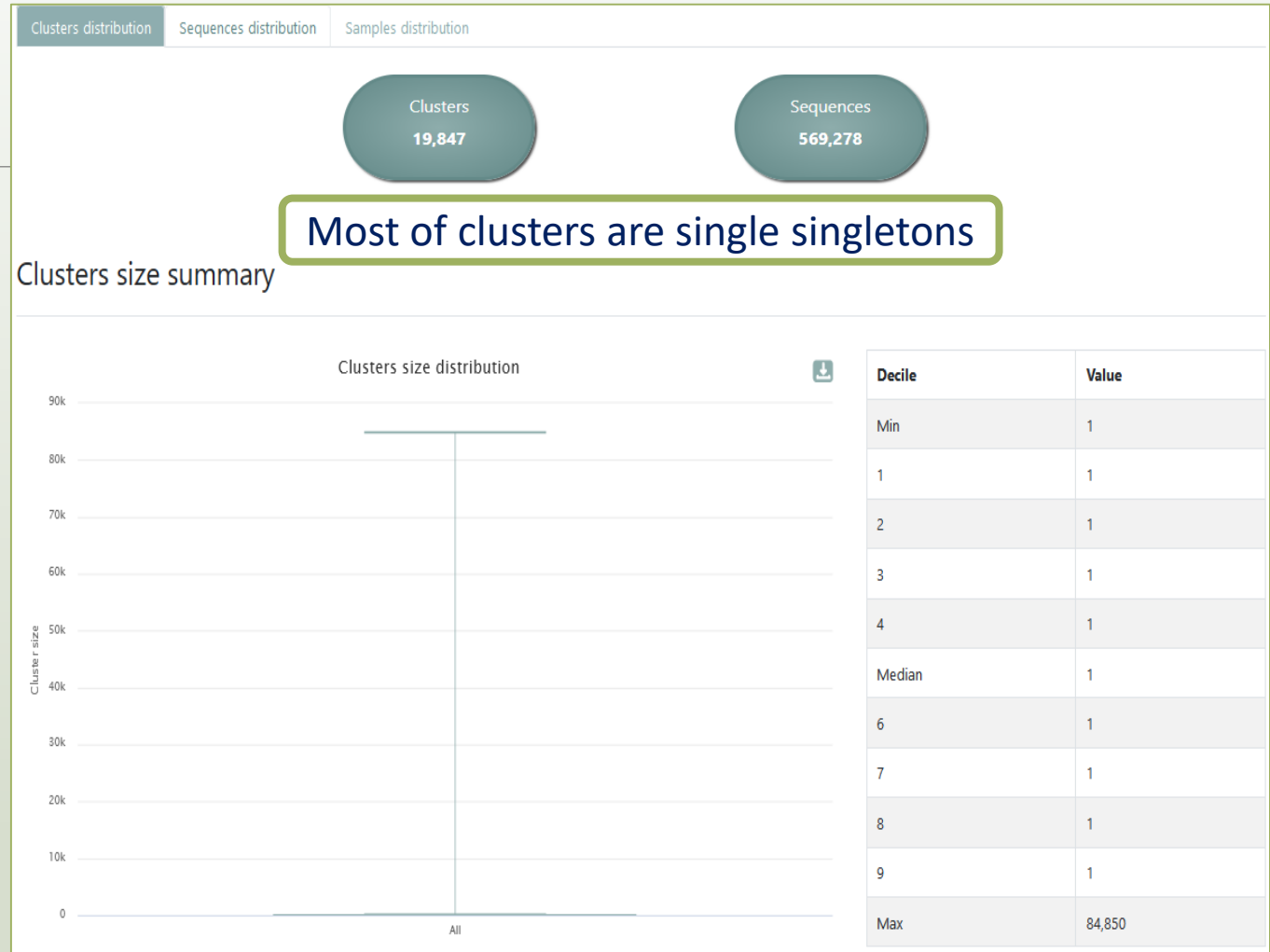
FROGS Clusters stat Process
some metrics on clusters.

Exercise

2. Interpret the boxplot: **Clusters size summary**
3. Interpret the table: **Clusters size details - How many single singletons do you find?**
4. What can we say by observing the **sequence distribution**?
5. How many clusters share “BHT0.LOT08” with at least one other sample?
6. How many clusters could we expect to be shared ?
7. How many sequences represent the 106 specific clusters of “CDT0.LOT06”?
8. This represents what proportion of “CDT0.LOT06”?
9. What do you think about it?
10. How do you interpret the « Hierarchical clustering » ?

Answer 1, 2
& 3

- Q1: How many clusters do you get ?
- Q2: Interpret the boxplot: **Clusters size summary**
- Q3: Interpret the table: **Clusters size details** -
How many single singletons do you find?



Answer 5 to 9

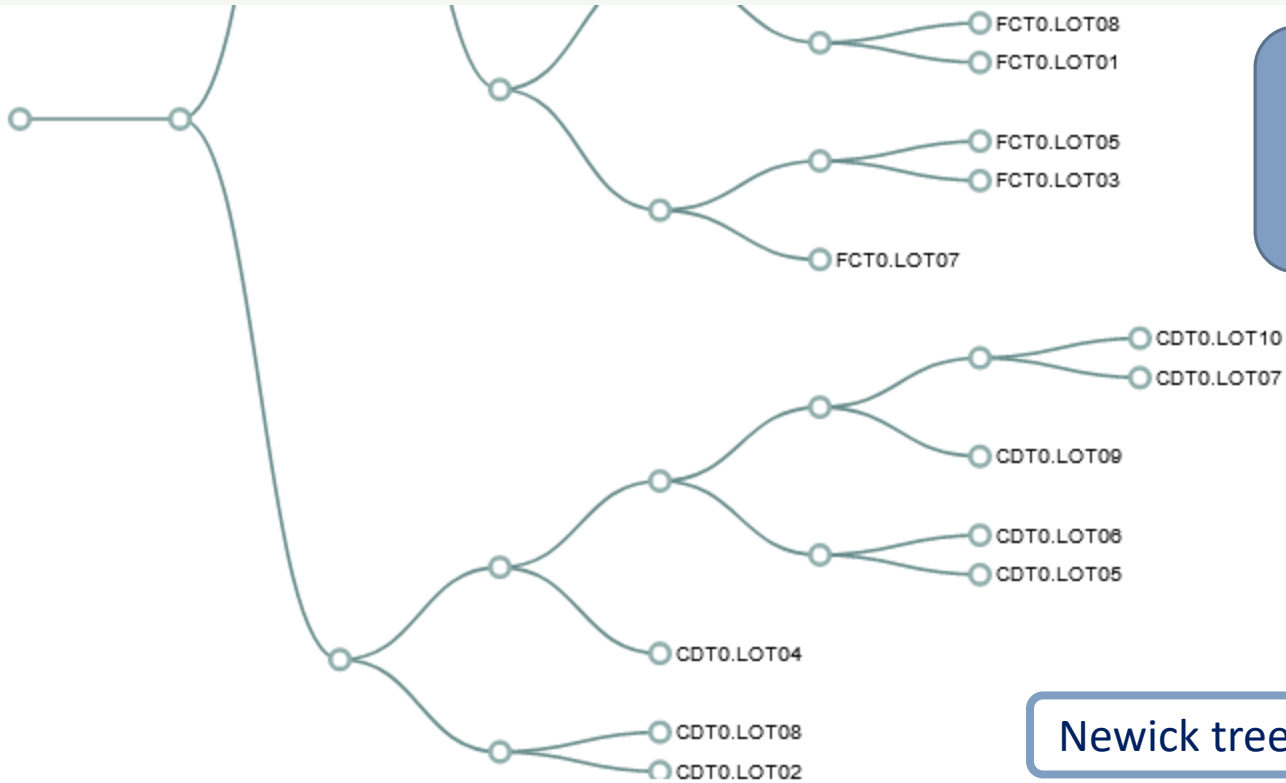
	Total clusters	Shared clusters	Own clusters	Total sequences	Shared sequences	Own sequences
BHT0.LOT01	491	114	377	9,087	8,709	378
BHT0.LOT03	433	140	293			
BHT0.LOT04	474	152	322			
BHT0.LOT05	475	153	322			
BHT0.LOT06	490	156	334	8,996	8,662	334
BHT0.LOT07	531	165	366	9,059	8,690	369
BHT0.LOT08	430	201	229	8,715	8,486	229
BHT0.LOT10	401	100	308	8,938	8,630	308
CDT0.LOT02			490	9,259	8,767	492
CDT0.LOT04			302	8,917	8,609	308
CDT0.LOT05	380	241	139	8,516	8,377	139
CDT0.LOT06	362	256	106	8,370	8,264	106
CDT0.LOT07	489	100	389			389
CDT0.LOT08	556	162	394			398
CDT0.LOT09	456	150	306			308
CDT0.LOT10	465	157	308			308

Q5: How many clusters share "BHT0.LOT08" with at least one other sample?
 Q6: How many clusters could we expect to be shared ?
 Q7: How many sequences represent the 106 specific clusters of "CDT0.LOT06"?
 Q8: This represents what proportion of "CDT0.LOT06"?
 Q9: What do you think about it?

201 clusters of BHT0.LOT08 are common at least once with another sample

~30 % of the specific clusters of CDT0.LOT06 represent around ~1% of sequences
 Could be interesting to remove if individual variability is not the concern of user

Q10: How do you interpret the « Hierarchical clustering » ?



The « Hierarchical clustering » is established with a Bray Curtis distance particularly well adapted to abundance table of very heterogenous values (very big and very small figures).

Newick tree available too, can be copied and pasted an tree viewer

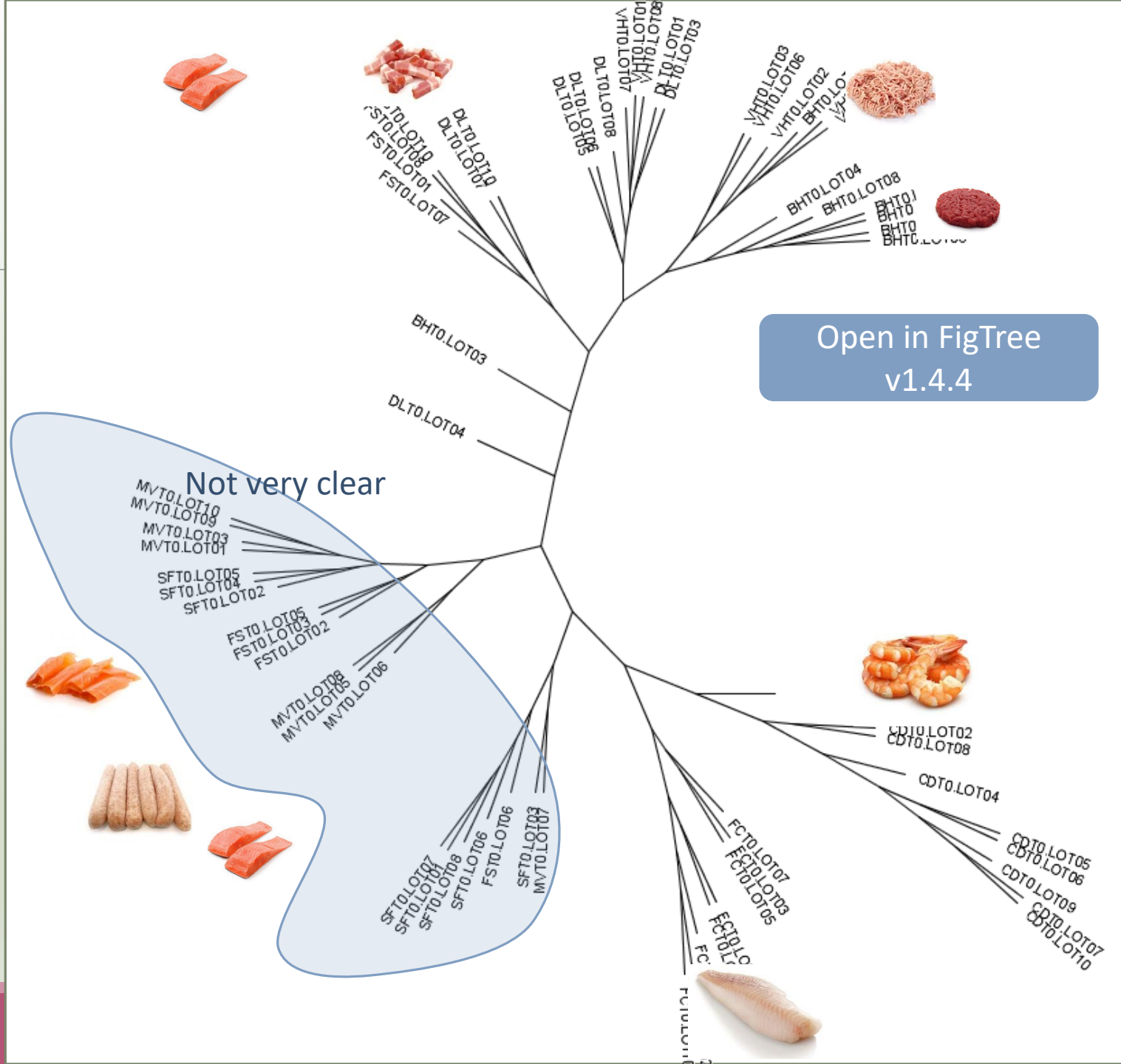
Newick

```
(((CDT0.LOT02,CDT0.LOT08):0.312,(CDT0.LOT04,((CDT0.LOT05,CDT0.LOT06):0.518,(CDT0.LOT09,(CDT0.LOT07,CDT0.LOT10):0.533):0.582):0.757):0.816):0.840,(((FCT0.LOT07,(FCT0.LOT03,FCT0.LOT05):0.257):0.262,((FCT0.LOT01,FCT0.LOT08):0.352,(FCT0.LOT06,(FCT0.LOT02,FCT0.LOT10):0.427):0.631):0.805):0.832,(((MVT0.LOT07,SFT0.LOT03):0.493,(FST0.LOT06,(SFT0.LOT06,(SFT0.LOT08,(SFT0.LOT01,SFT0.LOT07):0.132):0.345):0.354):0.570):0.655,(((MVT0.LOT06,(MVT0.LOT05,MVT0.LOT08):0.439):0.511,((FST0.LOT02,(FST0.LOT03,FST0.LOT05):0.147):0.179,((SFT0.LOT02,(SFT0.LOT04,SFT0.LOT05):0.211):0.227,((MVT0.LOT01,MVT0.LOT03):0.161,(MVT0.LOT09,MVT0.LOT10):0.341):0.466):0.526):0.661):0.681,(DLT0.LOT04,(((DLT0.LOT05,DLT0.LOT06):0.173,(DLT0.LOT08,((VHT0.LOT07,(VHT0.LOT01,VHT0.LOT08):0.095):0.184,(DLT0.LOT01,DLT0.LOT03):0.231):0.267):0.325):0.411,((BHT0.LOT04,(BHT0.LOT08,((BHT0.LOT01,BHT0.LOT07):0.224,(BHT0.LOT05,BHT0.LOT06):0.231):0.309):0.352):0.462,((VHT0.LOT03,VHT0.LOT06):0.387,(VHT0.LOT02,(BHT0.LOT10,(VHT0.LOT04,VHT0.LOT10):0.272):0.336):0.401):0.463):0.590):0.711,(BHT0.LOT03,((FST0.LOT07,(FST0.LOT01,(FST0.LOT08,FST0.LOT10):0.254):0.388):0.408,(DLT0.LOT07,DLT0.LOT10):0.440):0.666):0.734):0.745):0.827):0.856):0.875):0.911):0.938);
```

Answer 10

Q10: How do you interpret the « Hierarchical clustering » ?

N.B.: Hierarchical clustering is not all a phylogenetic tree ! Please consult with caution.



Chimera removal tool

What is chimera ?

PCR-generated chimeras are typically created when an aborted amplicon acts as a primer for a heterologous template. Subsequent chimeras are about the same length as the non-chimeric amplicon and contain the forward (for.) and reverse (rev.) primer sequence at each end of the amplicon.

Chimera: from 5 to 45% of reads (Schloss 2011)

Fichot and Norman *Microbiome* 2013 **1**:10
doi:10.1186/2049-2618-1-10

aborted amplification



next cycle's "primer"



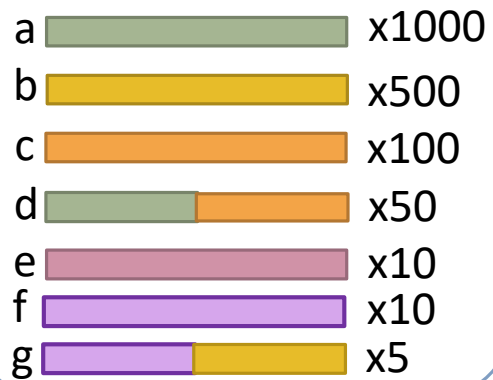
chimeric sequence



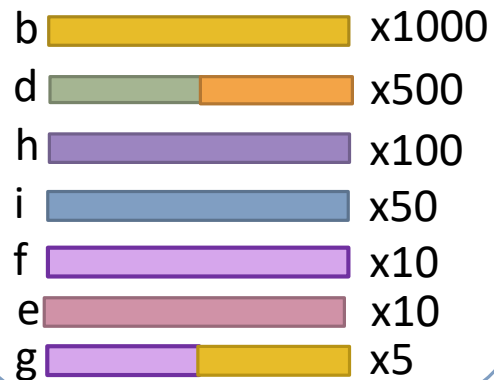
A smart removal chimera to be accurate

We use a sample cross-validation

Sample A



Sample B



“d” is view as chimera by Vsearch
Its “parents” are presents

“d” is view as normal sequence by Vsearch
Its “parents” are absents

- ⇒ For FROGS “d” is not a chimera
- ⇒ For FROGS “g” is a chimera, “g” is removed
- ⇒ FROGS increases the detection specificity

Practice:

LAUNCH THE REMOVE CHIMERA TOOL

Exercise

Go to « 16S » history

Launch the « FROGS Remove Chimera » tool

Follow by the « FROGS ClusterStat » tool

→ objectives :

- understand the efficiency of the chimera removal
- make links between small abundant OTUs and chimeras

FROGS Remove chimera Remove PCR chimera in each sample. (Galaxy Version 3.2.1)

Options

Sequences file

5: FROGS Clustering swarm: seed_sequences.fasta

The sequences file (format: fasta).

Abundance type

BIOM file

Select the type of file where the abundance of each sequence by sample is stored.

Abundance file

6: FROGS Clustering swarm: abundance.biom

It contains the count by sample for each sequence.

Execute

Exercise

1. Understand the « FROGS remove chimera : report.html»
 - a. How many clusters are kept after chimera removal?
 - b. How many sequences that represent ? So what abundance?
 - c. What do you conclude ?
2. What is the size of the largest removed cluster of chimeras?

Exercise

3. Rename html output in Chimera_report.html

Launch « FROGS ClusterStat » tool on non_chimera_abundance.biom

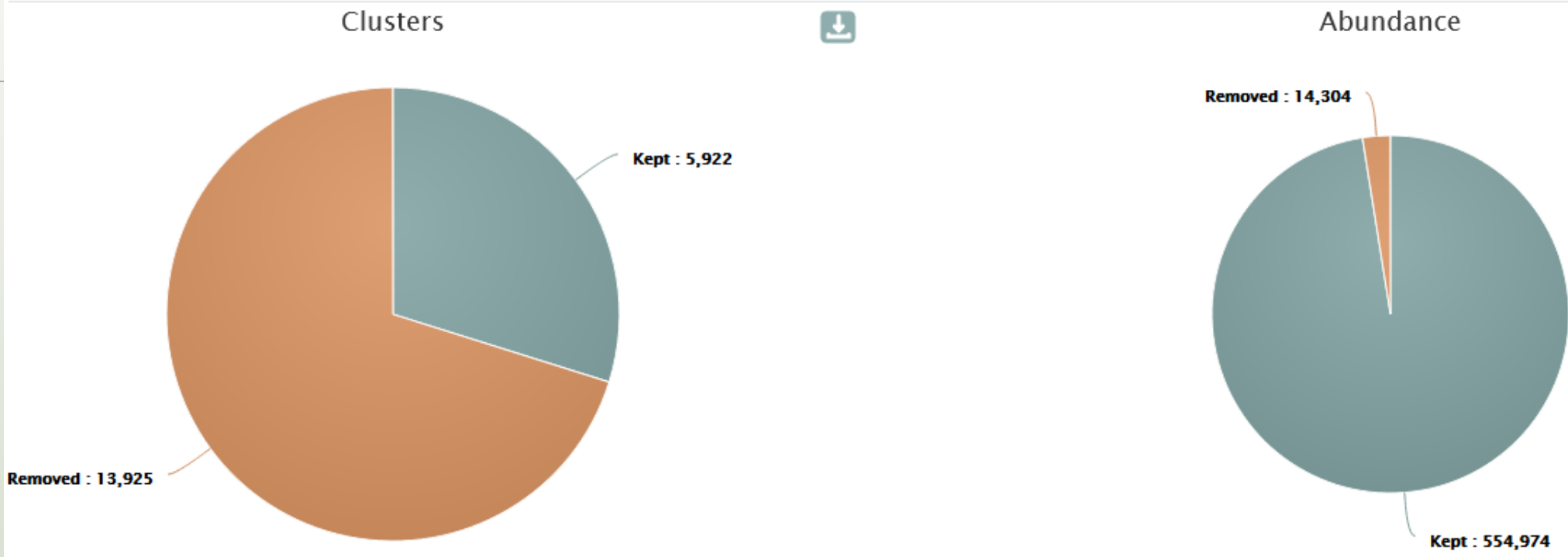
4. Compare the HTML files

- a. Of what are mainly composed singleton ? (compare with previous report.html)
- b. What are their abundance?
- c. What do you conclude ?

Answer 1

Q1a: How many clusters are kept after chimera removal?
Q1b: How many sequences that represent ? So what abundance?
Q1c: What do you conclude ?

Remove summary



5922 clusters are kept.
The 13925 removed clusters represent ~2.5 % of sequences

Here, chimera clusters represent many clusters ~70% but very few sequences.

Removed clusters are low abundance clusters.

Answer 2

Q2: What is the size of the largest removed cluster of chimeras?

Sample	Clusters kept	Cluster abundance kept	Chimeric clusters removed	Chimeric abundance removed	Abundance of the most abundant chimera removed	Individual chimera detected	Individual chimera abundance detected	Abundance of the most abundant individual chimera detected
VHT0.LOT02	205	8,862	366	410	19	372	446	19
MVT0.LOT10	253	9,312	16	16	10	169	304	92
VHT0.LOT08	261	8,852	10	10	10	310	344	1
VHT0.LOT01	197	8,831	8	8	8	365	382	8

The largest cluster of chimeras contained 19 sequences.

92 chimeras are detected but only 10 are removed because 82 have been invalidated by the cross validation

Q3: Rename html output in Chimera_report.html

Answer 3

11: FROGS Remove chimera: report.html



Attributes

Convert Format

Data

Edit Attributes

Name:

Chimera_report.html

Info:

Application

Software :/gal

/galaxy-preprod/my_tools

11: Chimera_report.html



Answer 4

Q4a: Of what are mainly composed singleton ? (compare with previous report.html)

Q4b: What are their abundance?

Q4c: What do you conclude ?

Cluster size	↑↓	Number of cluster	↑↓	% of all clusters	↑↓
1		19,078		96.13	
2		148		0.75	
3		22		0.11	
4		10		0.05	

Cluster_Stat report after clustering

Most small clusters are composed of chimeras

Cluster size	↑↓	Number of cluster	↑↓	% of all clusters	↑↓
1		5,287		89.28	
2		48		0.81	
3		15		0.25	
4		7		0.12	

Cluster_Stat report after chimera removing

OTU Filter tool

OTU Filter

Goal: This tool deletes OTU among conditions enter by user. If an OTU reply to at least 1 criteria, the OTU is deleted.

Criteria:

The OTU prevalence: The number of times the OTU is present in the environment, *i.e.* the number of samples where the OTU must be present.

OTU size: An OTU that is not large enough for a given proportion or count will be removed.

Biggest OTU: Only the X biggest are conserved.

Contaminant: If OTU sequence matches with phiX, chloroplastic/mitochondrial 16S of A. Thaliana or your own contaminant sequence.

One tool, 4 criteria

1

2

3

4

FROGS OTU Filters Filters OTUs on several criteria. (Galaxy Version 3.2.2)

Options

Sequences file

9: FROGS Remove chimera: non_chimera.fasta

The sequence file to filter (format: fasta).

Abundance file

10: FROGS Remove chimera: non_chimera_abundance.biom

The abundance file to filter (format: BIOM).

Minimum prevalence

4

Fill the field only if you want this treatment. Keep OTU if it is present in at least this number of samples.

Minimum OTU abundance as proportion or count. We recommend to use a proportion of 0.00005.

as proportion

Minimum proportion of sequences abundance to keep OTU

0.00005

Fill the field only if you want this treatment. Example: 0.00005, recommended by Bokulich et al 2013, to keep OTU with at least 0,005% of all sequences).

N biggest OTUs

Fill the fields only if you want this treatment. Keep the N biggest OTU.

Search for contaminant OTU.

Use contaminant fasta file from the server

Either you use your own contaminant fasta file or you select one among available ones.

Contaminant databank

phiX

For example the phiX databank (the phiX is a control added in Illumina sequencing technologies).

Execute

1

FROGS OTU Filters Filters OTUs on several criteria. (Galaxy Version 3.2.2) Options

Sequences file

9: FROGS Remove chimera: non_chimera.fasta

The sequence file to filter (format: fasta).

Abundance file

10: FROGS Remove chimera: non_chimera_abundance.biom

The abundance file to filter (format: BIOM).

Minimum prevalence

Prevalence

Fill the field only if you want this treatment. Keep OTU if it is present in at least this number of samples.

Here, user wants that each OTU are present in at least 4 samples.

Minimum OTU abundance as proportion or count. We recommend to use a proportion of 0.00005.

as proportion

Minimum proportion of sequences abundance to keep OTU

0.00005

Fill the field only if you want this treatment. Example: 0.00005, recommended by Bokulich et al 2013, to keep OTU with at least 0,005% of all sequences).

OR

Minimum OTU abundance as proportion or count. We recommend to use a proportion of 0.00005.

as count

Minimum number of sequences to keep OTU

2

Fill the field only if you want this treatment. Ex: 2 to keep OTU with at least 2 sequences, so remove single singleton.

2

Here, user wants that each OTU has an abundance representing at least 0.005% of total number of sequences.

Here, user wants that each OTU has an abundance at least equals to 2 sequences -> single singleton will be removed.

3

N biggest OTUs

50

Fill the fields only if you want this treatment. Keep the N biggest OTU.

Here, user wants to keep the 50 biggest OTU.

Search for contaminant OTU.

Use contaminant fasta file from the server

Either you use your own contaminant fasta file or you select one among available ones.

Contaminant databank

phiX

For example the phiX databank (the phiX is a control added in Illumina sequencing technologies).

Remove phiX sequence (use as buffer while sequencing)

OR

Search for contaminant OTU.

Use contaminant fasta file from the server

Either you use your own contaminant fasta file or you select one among available ones.

Contaminant databank

Arabidopsis TAIR10 Chloroplast and mitochondrie

For example the phiX databank (the phiX is a control added in Illumina sequencing technologies).

Remove chloroplastic and mitochondrial 16S sequences of *A. Thaliana*

OR

Search for contaminant OTU.

Use contaminant fasta file from the history

Either you use your own contaminant fasta file or you select one among available ones.

Select a contaminante reference from history

31: contaminant.fasta

Place in your history (with getadata tool) your own file of contaminant sequences in fasta format.

Practice:

LAUNCH THE OTU FILTER TOOL

Exercise:

Go to history « 16S » history

Launch « OTU Filter » tool with non_chimera_abundance.biom, non_chimera.fasta

Use 3 criteria to filter OTUs:

- OTU must be present at least in 4 samples
- Each OTU must represented a minimum of 0.005 % ⁽¹⁾ of the totality of the sequences
- OTU of phiX ⁽²⁾ must be removed

→ objective : play with filters, understand their impacts on false-positives OTUs

⁽¹⁾ *Nat Methods*. 2013 Jan;10(1):57-9. doi: 10.1038/nmeth.2276. Epub 2012 Dec 2.
Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing.
Bokulich NA1, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG.

⁽²⁾ <https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html>

Exercise:

1. What are the output files of “OTU Filter” ?
2. Explore “FROGS Filter : report.html” file. How many OTUs have you removed ? How many OTUs do they remain ? Which sample loses the most OTUs and for what reason?
3. Build the Venn diagram on the two filters. How many OTUs have you removed with each filter ?
4. How many own OTU remains in BHT0.LOT08 ? To retrieve this information, which tool do you need to launch previously ?

Answer 1

FROGS OTU Filters Filters OTUs on several criteria. (Galaxy Version 3.2.2)

Options

Sequences file

9: FROGS Remove chimera: non_chimera.fasta

The sequence file to filter (format: fasta).

Abundance file

10: FROGS Remove chimera: non_chimera_abundance.biom

The abundance file to filter (format: BIOM).

Minimum prevalence

4

Fill the field only if you want this treatment. Keep OTU if it is present in at least this number of samples.

Minimum OTU abundance as proportion or count. We recommend to use a proportion of 0.00005.

as proportion

Minimum proportion of sequences abundance to keep OTU

0.00005

Fill the field only if you want this treatment. Example: 0.00005, recommended by Bokulich et al 2013, to keep OTU with at least 0,005% (all sequences).

0.005% = 0.00005

Keep N biggest OTUs

Fill the fields only if you want this treatment. Keep the N biggest OTU.

Search for contaminant OTU.

Use contaminant fasta file from the server

Either you use your own contaminant fasta file or you select one among available ones.

Contaminant databank

phiX

For example the phiX databank (the phiX is a control added in Illumina sequencing technologies).

Execute

Outputs

16: FROGS OTU Filters: report.html



15: FROGS OTU Filters: excluded.tsv



14: FROGS OTU Filters: abundance.biom

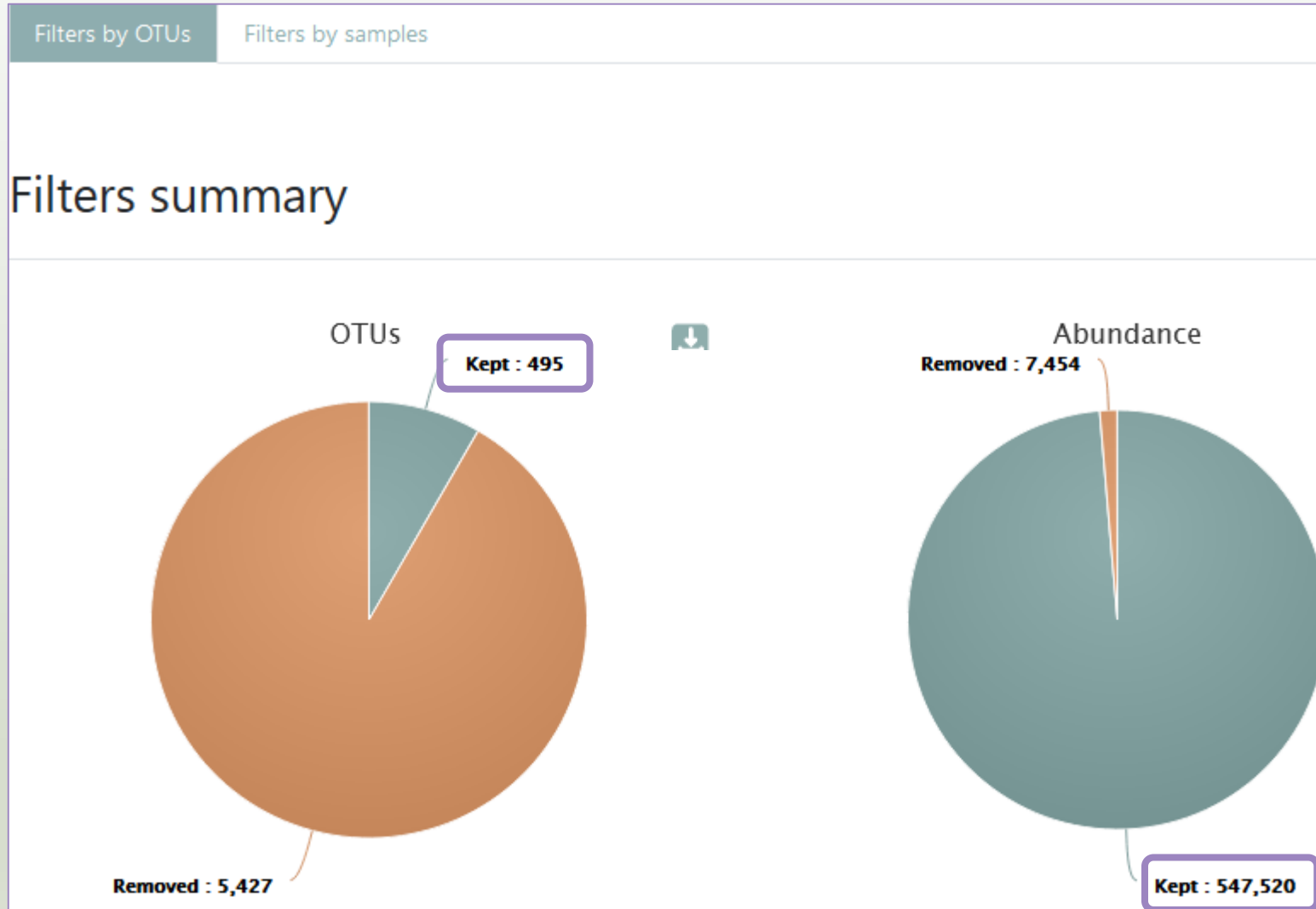


13: FROGS OTU Filters: sequences.fasta



Answer 2

To tabs to explore



Answer 2

Filters by OTUs **Filters by samples**

Details by samples

Show 10 entries **Sort by Kept to find the answer** [Download CSV](#) Search:

Sample name	Initial	Kept	Present in less than 4 samples	Abundance < 0.005% (i.e 28 sequences)	Present in databank of contaminants
SFT0.LOT06	433	34	376	398	0
SFT0.LOT07	275	66	188	209	0
SFT0.LOT01	308	70	216	238	0
SFT0.LOT08	324	88	215	236	0
CDT0.LOT02	234	92	141	142	0
MVT0.LOT10	253	96	155	157	0
SFT0.LOT03	196	97	92	98	0
BHT0.LOT01	172	98	72	74	0
CDT0.LOT07	187	99	87	88	0
SFT0.LOT05	214	105	107	108	0

Showing 1 to 10 of 64 entries

Previous 1 2 3 4 5 6 7 Next

This sample have only very small clusters that are shared by very few other samples.

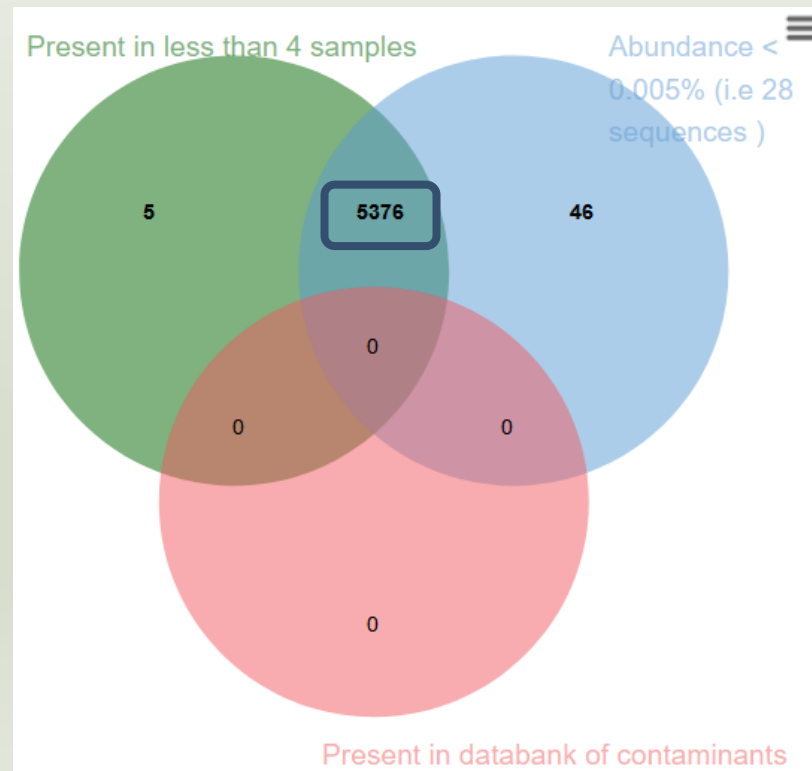
Answer 3

Filters intersections

Draw a Venn to see which OTUs had been deleted by the filters chosen (Maximum 6 options):

- Present in less than 4 samples
- Abundance < 0.005% (i.e 28 sequences)
- Present in databank of contaminants

Venn



- No phiX sequence.
- Most clusters are both small and not shared by 4 samples.

Answer 4

report.html of ClusterStat tool

Because of the "prevalence = 4" criterion, there is no longer an "own cluster" for any sample.

Clusters distribution Sequences distribution **Samples distribution**

Sequences count

Show entries Search: [↓ CSV](#)

Sample	Total clusters	Shared clusters	Own clusters	Total sequences	Shared sequences	Own sequences
BHT0.LOT01	98	98	0	8,690	8,690	0
BHT0.LOT03	135	135	0	8,377	8,377	0
BHT0.LOT04	150	150	0	8,643	8,643	0
BHT0.LOT05	140	140	0	8,544	8,544	0
BHT0.LOT06	145	145	0	8,646	8,646	0
BHT0.LOT07	150	150	0	8,671	8,671	0
BHT0.LOT08	195	195	0	8,479	8,479	0
BHT0.LOT10	165	165	0	8,606	8,606	0
CDT0.LOT02	92	92	0	8,750	8,750	0
CDT0.LOT04	161	161	0	8,605	8,605	0

Showing 1 to 10 of 64 entries Previous **1** 2 3 4 5 6 7 Next

Affiliation tool

FROGS Affiliation OTU Taxonomic affiliation of each OTU's seed

Using reference database

Select reference from the list

Also perform RDP assignment?

 Yes No

Optional

Taxonomy affiliation will be performed thanks to Blast. This option is optional.

Taxonomic ranks

The ordered taxonomic ranks levels stored in the taxonomical reference database.

OTU seed sequence

OTU sequences (format: fasta).

Abundance file

OTU abundances (format: BIOM).



silva138.1 16S
silva138.1 pintail100 16S
silva138.1 pintail80 16S
silva138.1 pintail50 16S
silva138.1 18S
silva138.1 23S
silva138.1 28S
silva138 16S
silva138 pintail100 16S
silva138 pintail80 16S
silva138 pintail50 16S
silva138 18S
silva138 SSU
silva132 LSU
silva132 28S
silva132 16S
silva132_pintail100 16S
silva132_pintail80 16S
silva132_pintail50 16S
silva132 18S
silva132 23S
greengenes13_5
midas_S132_3.6
midas_S123_2.1.3
Pyringae CTS 20200131
pr2_4.12.0
rpoB_122017
Unite_Fungi_8.2_20200204
Unite_Euka_8.2_20200204
Unite_Fungi_8.0_18112018
Unite_Euka_8.0_18112018
RSyst_Diatom_7

on 3.2.3)

Options

DAIRYdb_v1.1.2
EZBioCloud_052018
PHYMYCO-DB_2013
BOLD_COI-5P_022019
BOLD_COI-5P_1percentN_022019
MIDORI_UNIQUE_COI_20180221
MIDORI_UNIQUE_COI_MARINE_20180221
silva128 16S
silva128_pintail100 16S
silva128_pintail80 16S
silva128_pintail50 16S
silva128 18S
silva128 23S
silva123 16S
silva123 23S
silva123 18S
midas_S119_1.20
pr2_4.11.0
pr2_gb203_4.5
Unite_s_7.1_20112016



For more details on FROGS databanks:

http://genoweb.toulouse.inra.fr/frogs_databanks/assignation/readme.txt

1 Cluster = 2 affiliations

RDPClassifier*: one affiliation with bootstrap, on each taxonomic subdivision.

Bacteria;(1.0);Actinobacteriota;(1.0);Actinobacteria;(1.0);Propionibacteriales;(1.0);Propionibacteriaceae;(1.0);Cutibacterium;(1.0);Cutibacterium acnes;(0.57);

NCBI Blastn+** : one affiliation with identity %, coverage %, e-value, alignment length and a special tag “**Multi-affiliation**”.

Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Multi-affiliation

Identity: 100% and Coverage: 100%

* Appl. Environ. Microbiol. August 2007 vol. 73 no. 16 5261-5267. doi : 10.1128/AEM.00062-07
Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy.
Qiong Wang, George M.Garrity, James M. Tiedje and James R. Cole

** BMC Bioinformatics 2009, 10:421. doi:10.1186/1471-2105-10-421
BLAST+: architecture and applications
Christiam Camacho, George Coulouris, Vahram Avagyan, Ning Ma, Jason Papadopoulos, Kevin Bealer and Thomas L Madden

Affiliation Strategy of FROGS

Blastn+ with “**Multi-affiliation**” management

Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus

Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus saprophyticus

Strictly identical (V1-V3 amplification) on 499 nucleotides

Which one to choose?

Affiliation Strategy of FROGS

Blastn+ with “**Multi-affiliation**” management

Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus

Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus saprophyticus

Strictly identical (V1-V3 amplification) on 499 nucleotides



Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;**Multi-affiliation**

We cannot choose without preconceived ideas.

Practice:

LAUNCH THE FROGS AFFILIATION TOOL

Exercice:

Go to history « 16S » history

Launch the « FROGS Affiliation » tool with

- SILVA 138.1 16S database pintail 100

→ objectives :

- understand abundance tables columns
- understand the BLAST affiliation

FROGS Affiliation OTU Taxonomic affiliation of each OTU's seed by RDPtools and BLAST (Galaxy Version 3.2.2)

Options

Using reference database

silva138.1 pintail100 16S

Select reference from the list

Also perform RDP assignment?

Yes

No

Taxonomy affiliation will be perform thanks to Blast. This option allow you to perform it also with RDP classifier (default No)

OTU seed sequence

32: FROGS OTU Filters: sequences.fasta

OTU sequences (format: fasta).

Abundance file

33: FROGS OTU Filters: abundance.biom

OTU abundances (format: BIOM).

Execute

Exercise

1. What are the « **FROGS Affiliation tool** » output files ?
2. How many sequences are affiliated by BLAST ?
3. How many OTU have a “multiaffiliation” at Order ranks ?
4. Click on the « eye » button on the BIOM output file, what do you understand ?



Exercise

Use the **Biom_to_TSV** tool on this last file and click again on the "eye" on the new output generated.



FROGS BIOM to TSV Converts a BIOM file in TSV file. (Galaxy Version 3.2.2) Options

Abundance file
37: FROGS Affiliation OTU: affiliation.biom
The BIOM file to convert (format: BIOM).

Sequences file (optional)
32: FROGS OTU Filters: sequences.fasta
The sequences file (format: fasta). If you use this option the sequences will be add in TSV.

Extract multi-alignments
 Yes No
If you have used FROGS ... out multiple alignments in a second TSV.

Execute

Transform the biom file in tsv file (easy to manipulate on excel or R)

Optional but very useful, insert sequence of OTU in the abundance table

Build the multi_affiliations.tsv: the list of possible affiliations for each ambiguous OTU with multi-affiliation

[FROGS Affiliation OTU](#)
Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

[FROGS Affiliation Filters](#)
Filters OTUs on several affiliation criteria.

[FROGS Affiliation postprocess](#)
Optionnal step to resolve inclusive amplicon ambiguities and to aggregate OTUs based on alignment metrics

[FROGS Abundance normalisation](#)
Normalise OTUs abundance.

[FROGS Tree](#)
Reconstruction of phylogenetic tree

[FROGS Clusters stat](#)
Process some metrics on clusters.

[FROGS Affiliations stat](#)
Process some metrics on taxonomies.

[FROGS BIOM to std BIOM](#)
Converts a FROGS BIOM in fully compatible BIOM.

[FROGS BIOM to TSV](#)
Converts a BIOM file in TSV file.

[FROGS TSV to BIOM](#)
Converts a TSV file in a BIOM file.

Exercise



5. Click again on the "eye" on the new output generated.



Or open it in your favorite spreadsheet (Excel, google sheet, Calc...) !

Now, what do you think about the file format? What does it contain?

Exercise

6. Observe and describe

- In FROGS BIOM to TSV: abundance_silva.tsv, the different columns of cluster 3
 - a. how would you qualify the alignment between the OTU3 seed and the sequences of the silva database?
 - b. What does it mean e-value = 0 ?
 - c. What is the header of column that shows the sequence of OTU seed ?
 - d. How many sequences have OTU3 in total ?
 - e. How many sequences have OTU3 in MVT0.LOT10 ? What is the sample where OTU3 is absent ?

Exercise

7. Observe and describe

- In FROGS BIOM to TSV: multi_affiliations.tsv, identifies the lines corresponding to cluster3
 - a. Why cluster3 has a multi-affiliation for species ?
 - b. Why “Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus;Lactobacillus sakei” is present 74 times ?

Q1: What are the « **FROGS Affiliation tool** » output files ?

Q2: How many sequences are affiliated by BLAST ?

Exercise

Answer 1

19: FROGS Affiliation OTU: report.html

18: FROGS Affiliation OTU: affiliation.biom

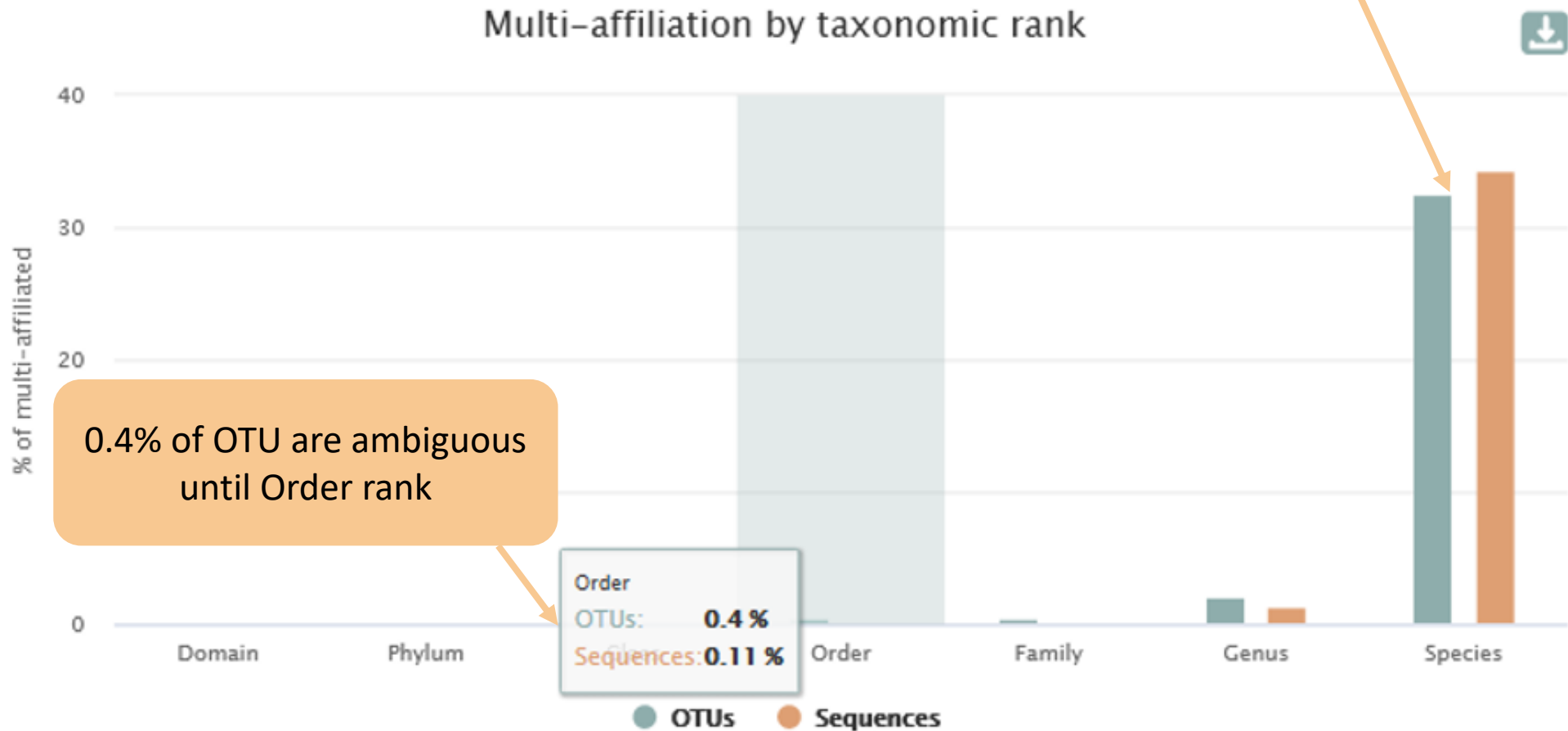
Answer 2



All sequences have a blast affiliation

Blast multi-affiliation summary

Most of OTUs are ambiguous at species rank.
For this study, V1V3 amplicon is not resolvable enough to identify the species.



Q4: Click on the « eye » button on the BIOM output file, what do you understand ?

```
{
  "matrix_type": "sparse",
  "shape": [495, 64],
  "date": "2021-03-03T11:57:55",
  "matrix": [
    [2, 23], [1, 3, 18], [1, 4, 19], [1, 5, 20], [1, 6, 29], [1, 7, 3], [1, 8, 1], [1, 9, 69], [2, 30, 98], [2, 31, 93], [2, 32, 38], [2, 33, 1682], [2, 34, 1598], [2, 35, 846], [3, 44, 210], [3, 45, 190], [3, 46, 122], [3, 47, 13], [3, 48, 3], [3, 49, 4, 61, 335], [4, 62, 540], [4, 63, 1943], [5, 0, 2408], [5, 1, 603], [5, 2, 1372], [5, 3, 7, 7, 24], [7, 9, 139], [7, 11, 7], [7, 12, 1], [7, 13, 37], [7, 14, 4], [7, 15, 46, 1], [9, 47, 4], [9, 51, 7], [9, 52, 4], [9, 56, 4], [9, 59, 4], [9, 60, 3], [9, 61, 1], [11, 47, 236], [11, 49, 24], [11, 50, 26], [11, 51, 44], [11, 52, 30], [11, 54, 1], [11, 55, 59, 71], [12, 60, 119], [12, 61, 16], [12, 62, 92], [12, 63, 272], [13, 0, 19], [13, 1, 27, 2], [14, 28, 3], [14, 29, 6], [14, 30, 8], [14, 31, 3], [14, 32, 10], [14, 33, 9], [17, 4, 17], [17, 5, 17], [17, 6, 20], [17, 7, 14], [17, 8, 3], [17, 9, 9], [17, 10, 18, 21, 34], [18, 22, 40], [18, 23, 105], [18, 25, 152], [18, 26, 2], [18, 27, 25], [18, 28, 20, 16, 16], [20, 17, 5], [20, 18, 1064], [20, 19, 12], [20, 20, 30], [20, 21, 33], [20, 22, 33, 43], [21, 34, 52], [21, 35, 59], [21, 36, 48], [21, 37, 44], [21, 38, 45], [21, 39, 21, 40], [23, 6, 16], [23, 7, 2], [23, 9, 2], [23, 10, 12], [23, 11, 27], [23, 12, 1], [23, 13, 2], [25, 30, 5], [25, 31, 23], [25, 36, 2], [25, 37, 16], [25, 38, 39], [25, 39, 4], [25, 40, 7, 16, 25], [27, 17, 7], [27, 18, 60], [27, 19, 40], [27, 20, 74], [27, 21, 41], [27, 22, 29, 23, 15], [29, 24, 4], [29, 25, 519], [29, 26, 1], [29, 27, 79], [29, 28, 1318], [29, 29, 31, 43, 16], [31, 44, 36], [31, 45, 91], [31, 46, 11], [31, 47, 2], [31, 56, 5], [31, 57, 76], [35, 12, 42], [35, 13, 2], [35, 14, 33], [35, 15, 78], [36, 0, 7], [36, 3, 1], [36, 4, 38, 28, 295], [38, 29, 45], [38, 30, 135], [38, 31, 566], [38, 32, 3], [38, 36, 3], [38, 37, 41], [41, 17, 2], [41, 20, 5], [41, 21, 4], [41, 22, 1], [41, 23, 9], [41, 28, 1], [41, 29, 1], [43, 38, 8], [43, 40, 2], [43, 42, 7], [43, 44, 3], [43, 46, 3], [43, 56, 2], [43, 57, 7, 11, 14], [47, 12, 1], [47, 13, 2], [47, 14, 1], [47, 15, 1], [47, 20, 2], [47, 21, 500], [50, 25, 21], [50, 26, 1], [50, 27, 1], [50, 28, 7], [50, 30, 6], [50, 31, 2], [50, 32, 84], [52, 29, 3], [52, 30, 2], [52, 31, 21], [52, 32, 1], [52, 33, 6], [52, 34, 3], [52, 35, 1], [54, 52, 1], [54, 55, 1], [54, 58, 3], [54, 60, 2], [55, 3, 8], [55, 4, 7], [55, 5, 2, 2], [57, 6, 2], [57, 7, 2], [57, 8, 1], [57, 10, 16], [57, 11, 22], [57, 12, 10]
```

The biom file is not a human readable format. It is only very useful for bioinformaticians. To read the abundance table you have to transform the BIOM file in TSV file thanks to **BIOM_to_TSV tool**.

Answer 5

Q5: what do you think about the TSV file format? What does it contain?

The TSV format: tabular separated Value.
Universal format, ideal for different spreadsheets.

This file contain the abundance table and information about affiliation of OTUs.

#comment	blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Listeriaceae;Brochothrix;Brochothrix thermosphacta	multi-subject	100	100
no data	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Vibrionaceae;Photobacterium;unknown species	FJ456662.1.1555	100	100
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Latilactobacillus;Multi-affiliation	multi-subject	100	100
no data	Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Multi-affiliation	multi-subject	100	100
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Leuconostoc;Multi-affiliation	multi-subject	100	100
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus;Lactococcus piscium	AM943029.1.1242	99.799	100
no data	Bacteria;Firmicutes;Bacilli;Erysipelotrichales;Erysipelotrichaceae;ZOR0006;unknown species	HG792212.1.1536	94.203	100
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus;Multi-affiliation	multi-subject	100	100
no data	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Weissella;Weissella ceti	FN813251.1.1761	99.799	100

blast_evalue	blast_aln_length	seed_id	seed_sequence	observation_name	observation_sum	BHT0.LOT01	BHT0.LOT03	BHT0.LOT04	BHT0.LOT05	BHT0.LOT06	BHT0.LOT07	BHT0.LOT08
0	497	17_41	GACGAACGCTGGCGGC...	Cluster_1	84849	791	402	433	911	1232	653	441
0	492	17_611	ATTGAACGCTGGCGGC...	Cluster_2	31333	22	4	23	18	19	20	29
0	520	17_595	GACGAACGCTGGCGGC...	Cluster_3	40711	342	70	71	218	81	199	114
0	468	17_257	GACGAACGCTGGCGGC...	Cluster_4	22275	146	1251	263	327	180	118	293
0	497	17_4	GATGAACGCTGGCGGC...	Cluster_5	29355	1842	217	1243	1799	1623	1374	954
0	497	17_23	GACGAACGCTGGCGGC...	Cluster_6	21301	2408	603	1372	2231	2597	2218	1981
0	483	57_5	GATGAACGCTGGCGGC...	Cluster_7	15272	0	0	0	0	0	0	0
0	499	17_420	GACGAACGCTGGCGGC...	Cluster_8	16252	54	33	51	10	72	1	50
0	497	57_3	TGCAAGTCGAACGCAC...	Cluster_9	11525	0	0	0	0	0	0	0

Answer 6

- a. how would you qualify the alignment between the OTU3 seed and the sequences of the silva database?

Alignment is perfect ! 100% identity and 100% coverage between OTU3 seed and the 520 nucleotides of sequence from silva database

- b. What does it mean e-value = 0 ?

The expect value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower the e-value, or the closer it is to zero, the more "significant" the match is.

- c. What is the header of column that shows the sequence of OTU seed ?

Seed_sequence

- d. How many sequences have OTU3 in total ?

40711 found in column " observation_sum"

- e. How many sequences have OTU3 in MVT0.LOT10 ? What is the sample where OTU3 is absent ?

MVT0.LOT10	CDT0.LOT02
4	64
0	1
6722	0
13	0
20	3

We can remark that OTU3 is particularly present in MV samples and rare in CD samples

Answer 7

- a. Why cluster3 has a multi-affiliation for species ?

In multi-affiliations.tsv file, for cluster_3, we observe that 75 affiliations are possible for this OTU at species rank.

All strictly equivalent 100% identity and 100% coverage with 75 different sequences of silva database.

ctobacillus;Lactobacillus sakei	CP025206.1448122.1449699	100	100	0	520
ctobacillus;Lactobacillus sakei	CP020806.1000690.1002267	100	100	0	520
ctobacillus;Lactobacillus sakei	CP025839.1959094.1960671	100	100	0	520
ctobacillus;unknown species	KF601977.1.1550	100	100	0	520
ctobacillus;Lactobacillus sakei	CP020806.811637.813214	100	100	0	520
ctobacillus;Lactobacillus sakei	CP020806.1103805.1105382	100	100	0	520
ctobacillus;Lactobacillus sakei	CP020806.1109220.1110797	100	100	0	520

- b. Why “Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus;Lactobacillus sakei” is present 74 times ?

Because these are 74 different strains of *L. sakei*. They have blast ID different.

Silva pintail or not pintail ?

Pintail* represents the probability that the rRNA sequence contains anomalies or is a chimera, where 100 means that the probability for being anomalous or chimeric is low.

4 ranks of available databases in FROGS: 50 pintail, 80 pintail or 100 pintail or no pintail filter.

silva138.1 16S
silva138.1 pintail100 16S
silva138.1 pintail80 16S
silva138.1 pintail50 16S
silva138.1 18S
silva138.1 23S
silva138.1 28S



Only for 16S !

* <http://aem.asm.org/content/71/12/7724.abstract>

Exemple between silva 138.1 and silva 138.1 pintail 100

130 identical blast best hits on SILVA 138.1 pintail 100 databank

- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes 6609
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes C1
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes KPA171202
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes SK137
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeIA2 P.acn17
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeIA2 P.acn31
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeIA2 P.acn33

Exemple between silva 138.1 and silva 138.1 pintail 100

267 identical blast best hits on SILVA 138.1 full databank

- ? Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Corynebacteriales;Corynebacteriaceae;Corynebacterium;unknown species
- ? Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Aureobasidium melanogenum
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes 266
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes 6609
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes C1
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes hdn-1
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes HL096PA1
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes KPA171202
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes SK137
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;unknown species
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeA2 P.acn17
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeA2 P.acn31
- Cluster_4 Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Cutibacterium acnes TypeA2 P.acn33
- ? Cluster_4 Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Dolosigranulum;unknown species

Induces a multi-affiliation up to phylum rank



accession number	organism name	sequence length	sequence quality	alignment quality	pintail quality	SILVA taxonomy
<input type="checkbox"/> KF100699	<i>uncultured bacterium</i>	1341	<div style="width: 100%; height: 10px; background-color: green;"></div>	<div style="width: 100%; height: 10px; background-color: green;"></div>	<div style="width: 0%; height: 10px; background-color: gray;"></div>	Bacteria > Firmicutes > Bacilli...

How choose the good affiliation ?

Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	D83374.1.1477	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.2831760.2833315	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1649831.1651386	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1426849.1428404	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1544187.1545742	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	LT963439.723352				
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.158796				
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2356345.2857902	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.22851139.2852696	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2904966.2906523	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2899760.2901317	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.1470936.1472493	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.1685669.1687226	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus saprophyticus	EU855225.1.1531	100	100	0	499

2 choices for cluster 64

How choose the good affiliation ?

Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	D83374.1.1477	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.2831760.2833315	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1649831.1651386	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1426849.1428404	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP007208.1544187.1545742	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	LT963439.723352.724884	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.1587968.1589525	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2856345.2857902	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2851139.2852696	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2904966.2906523	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.2899760.2901317	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.1470936.1472493	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus xylosus	CP013922.1685669.1687226	100	100	0	499
Cluster_64	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus saprophyticus	EU855225.1.1531	100	100	0	499

- you have a preconceived notion
- you are familiar with the environment being studied
- you are looking for specific organisms as pathogens
- you collect bibliographical information

Ex:

Staphylococcus saprophyticus is a bacterium that can cause urinary tract infections in young women

and

Staphylococcus xylosus exists as a commensal on the skin of humans and animals and in the environment. It appears to be much more common in animals than in humans. *S. xylosus* has very occasionally been identified as a cause of human infection.

Maybe, for this cluster, *S. xylosus* is better

Affiliation explorer

<https://shiny.migale.inrae.fr/app/affiliationexplorer>

Affiliation explorer

Upload Biom File
Browse... Galaxy37-[f]
Upload complete

Optional: upload Fasta File
Browse... Galaxy32-[f]
Upload complete

Upload MultiHits TSV File
Browse... Galaxy42-[f]
Upload complete

Download

Affiliation selection Affiliation edition

Select OTU
Cluster_3
Update OTU Skip OTU

Cluster_3 - 2 conflicting affiliations, ambiguity at rank Species

Select new affiliation by clicking on a row (double click on a cell to edit its content).
Click "Update OTU" to update affiliation (with selected row) or "Skip OTU" to move to the next one.

Show 10 entries

	Kingdom	Phylum	Class	Order	Family	Genus	Species	Blast ID	%id	%cov
1	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Latilactobacillus	Lactobacillus sakei	CP032640.225274.226851	100	100
2	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Latilactobacillus	unknown species	KF601977.1.1550	100	100

Showing 1 to 2 of 2 entries

Previous 1 Next

Show sequence

A very user-friendly tool, developed by Mahendra Mariadassou and his collaborators (Maiage unit - INRAE Jouy-en-Josas). It allows to modify very simply the affiliations of an abundance table from FROGS.

Affiliation explorer

<https://shiny.migale.inrae.fr/app/affiliationexplorer>

Demo
video

The screenshot shows a web browser window displaying the 'Affiliation explorer' Shiny application. The browser's address bar shows the URL: <https://hub.gke2.mybinder.org/user/mahendra-mariad-liationexplorer-4jqjb7jw/rstudio/?token=r0mZweROqcCzicA5hQm8IA&view=shiny>. The application interface has a dark blue header with the title 'Affiliation explorer' and a hamburger menu icon. On the left side, there is a dark sidebar with three sections: 'Upload Biom File', 'Optional: upload Fasta File', and 'Upload MultiHits TSV File'. Each section contains a 'Browse...' button and a 'No file sele...' button. The main content area has a light blue background and features two tabs: 'Affiliation selection' (active) and 'Affiliation edition'. Below the tabs, a text box contains the instruction: 'Please upload your data (Biom file and MultiHits TSV file)'. The browser window also shows standard navigation and window control buttons.

Divergence on the composition of microbial communities at the different taxonomic ranks

With the first versions of FROGS where multi-affiliation did not yet exist.

Affiliations and abundances of FROGS OTUs are they reliable ?

Taxonomic ranks	Average divergence of the affiliations of the 10 samples (%) 500setA	Average divergence of the affiliations of the 10 samples (%) 100setA
Kingdom	0.00	0.00
Phylum	0.46	0.41
Class	0.64	0.50
Order	0.94	0.68
Familly	1.18	0.78
Genus	1.76	1.30
Species	23.87	34.80

Affiliation was chosen with arbitrary criterion among all strictly equivalent affiliation

solution

Report on abundance table, the multiple identical affiliations

Only one best hit

Taxonomic ranks	Average divergence of the affiliations of the 10 samples (%) 500setA	Average divergence of the affiliations of the 10 samples (%) 100setA
Kingdom	0.00	0.00
Phylum	0.46	0.41
Class	0.64	0.50
Order	0.94	0.68
Familly	1.18	0.78
Genus	1.76	1.30
Species	23.87	34.80

Multiple best hit

Taxonomic ranks	Median divergence of the affiliations of the 10 samples (%) 500setA	Median divergence of the affiliations of the 10 samples (%) 100setA
Kingdom	0.00	0.00
Phylum	0.46	0.41
Class	0.64	0.50
Order	0.93	0.68
Familly	1.17	0.78
Genus	1.60	1.00
Species	6.63	5.75



With the FROGS guideline
OTU filter on abundance < 0.005%

Taxonomic ranks	Median divergence of the affiliations of the 10 samples (%) 500setA filter: 0.005% - 505 OTUs	Median divergence of the affiliations of the 10 samples (%) 100setA filter: 0.005% - 100 OTUs
Kingdom	0.00	0.00
Phylum	0.38	0.38
Class	0.57	0.48
Order	0.81	0.64
Familly	1.08	0.74
Genus	1.43	0.76
Species	1.53	0.78



Affiliation Stat

FROGS Affiliations stat Process some metrics on taxonomies. (Galaxy Version 3.2.2)

Options

Abundance file

18: FROGS Affiliation OTU: affiliation.biom

OTUs abundances and affiliations (format: BIOM).

Taxonomic ranks

Domain Phylum Class Order Family Genus Species

The ordered taxonomic ranks levels stored in BIOM. Each rank is separated

Rarefaction ranks

Class Order Family Genus Species

The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed

FROGS blast

Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute

If your OTU are affiliated with less taxonomic ranks (species is missing for example), change it.

Practice:

LAUNCH THE FROGS AFFILIATION STAT TOOL

Exercice:

Go to history « 16S » history

Launch the « FROGS Affiliation Stat » tool on last affiliation_abundance.biom

→ objectives :

understand rarefaction curves and the diversity diagram

Exercise:

1. Build the **rarefaction** curve on genus rank with the 10 samples that contain the least number of different genus.
2. SFT0.LOT06 and MVT0.LOT10 have they been sequenced deeply enough?
3. Build the **distribution** on FC samples *i.e.* “Filet de Cabillaud”
4. How many sequences are some *Brochothrix thermosphacta* ?
5. On the total of sequences, what is the proportion affiliated to the Firmicutes?
6. Among Firmicutes, how many are Bacilli ?
7. But what is the proportion of Firmicutes in the total of sequence of all sample ?
8. How many OTUs are align perfectly with a database sequence ?

Answer 1

Q1: Build the **rarefaction** curve on genus rank with the 10 samples that contain the least number of different genus.

<input type="checkbox"/>	Samples	Nb domain	Nb phylum	Nb class	Nb order	Nb family	Nb genus	Nb species	Nb sequences
<input checked="" type="checkbox"/>	SFT0.LOT06	1	4	5	9	14	35	57	8,821
<input checked="" type="checkbox"/>	SFT0.LOT01	1	4	6	13	27	39	63	8,859
<input checked="" type="checkbox"/>	FCT0.LOT01	1	5	6	13	24	41	96	8,504
<input checked="" type="checkbox"/>	SFT0.LOT05	1	5	7	18	32	50	95	8,728
<input checked="" type="checkbox"/>	SFT0.LOT08	1	4	6	13	33	53	77	8,788
<input checked="" type="checkbox"/>	BHT0.LOT01	1	7	9	20	35	55	83	8,750
<input checked="" type="checkbox"/>	SFT0.LOT04	1	6	8	17	34	55	83	8,750
<input checked="" type="checkbox"/>	SFT0.LOT03	1	5	8	17	34	55	83	8,750
<input checked="" type="checkbox"/>	SFT0.LOT02	1	6	7	17	34	55	83	8,750
<input type="checkbox"/>	MVT0.LOT10	1	4	5	17	31	57	83	9,143
<input type="checkbox"/>	CDT0.LOT02	1	6	8	22	36	58	85	8,750

1. Sort the table by genus number

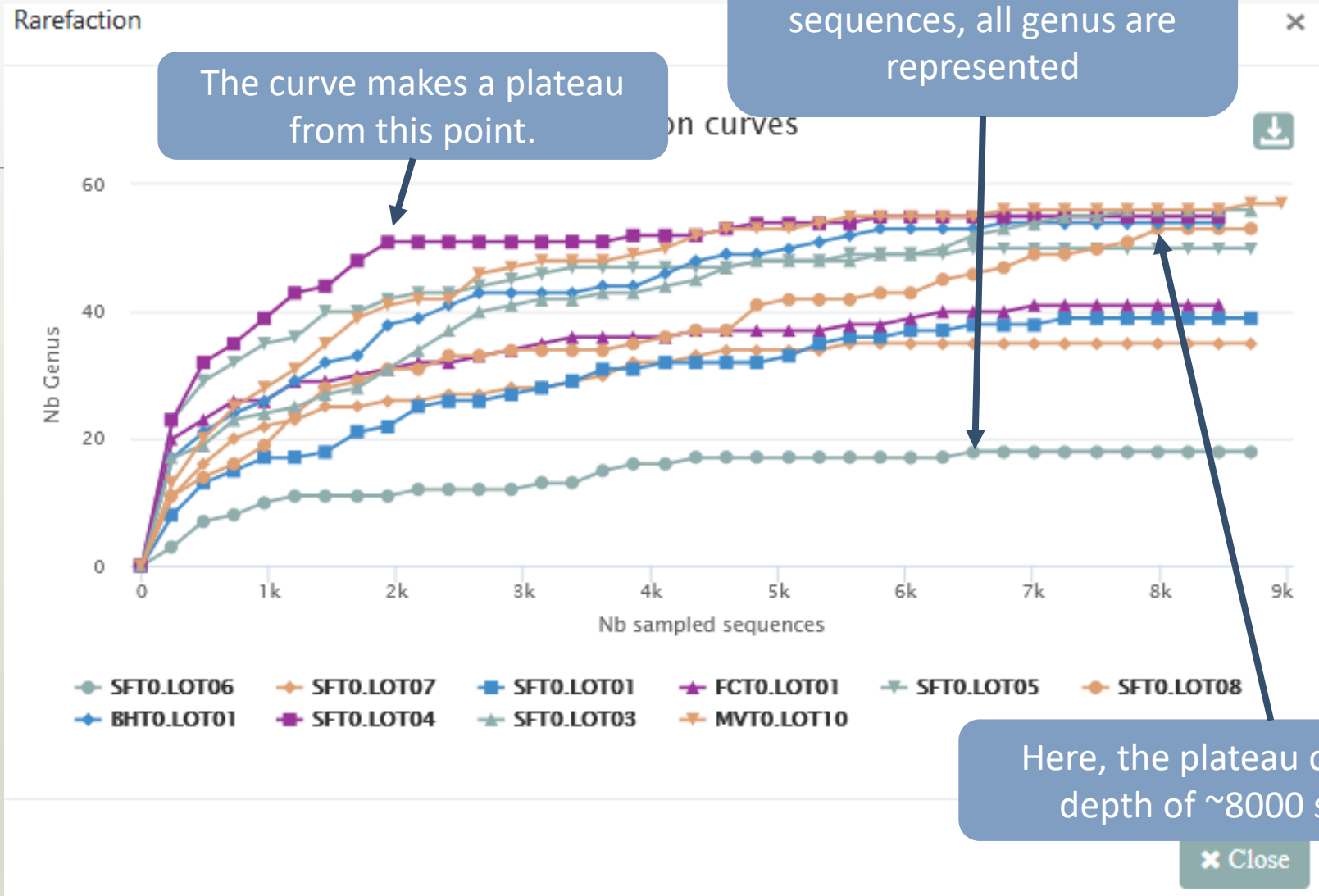
2. Select the 10 first samples

3. At the bottom of the table click on

With selection: Genus

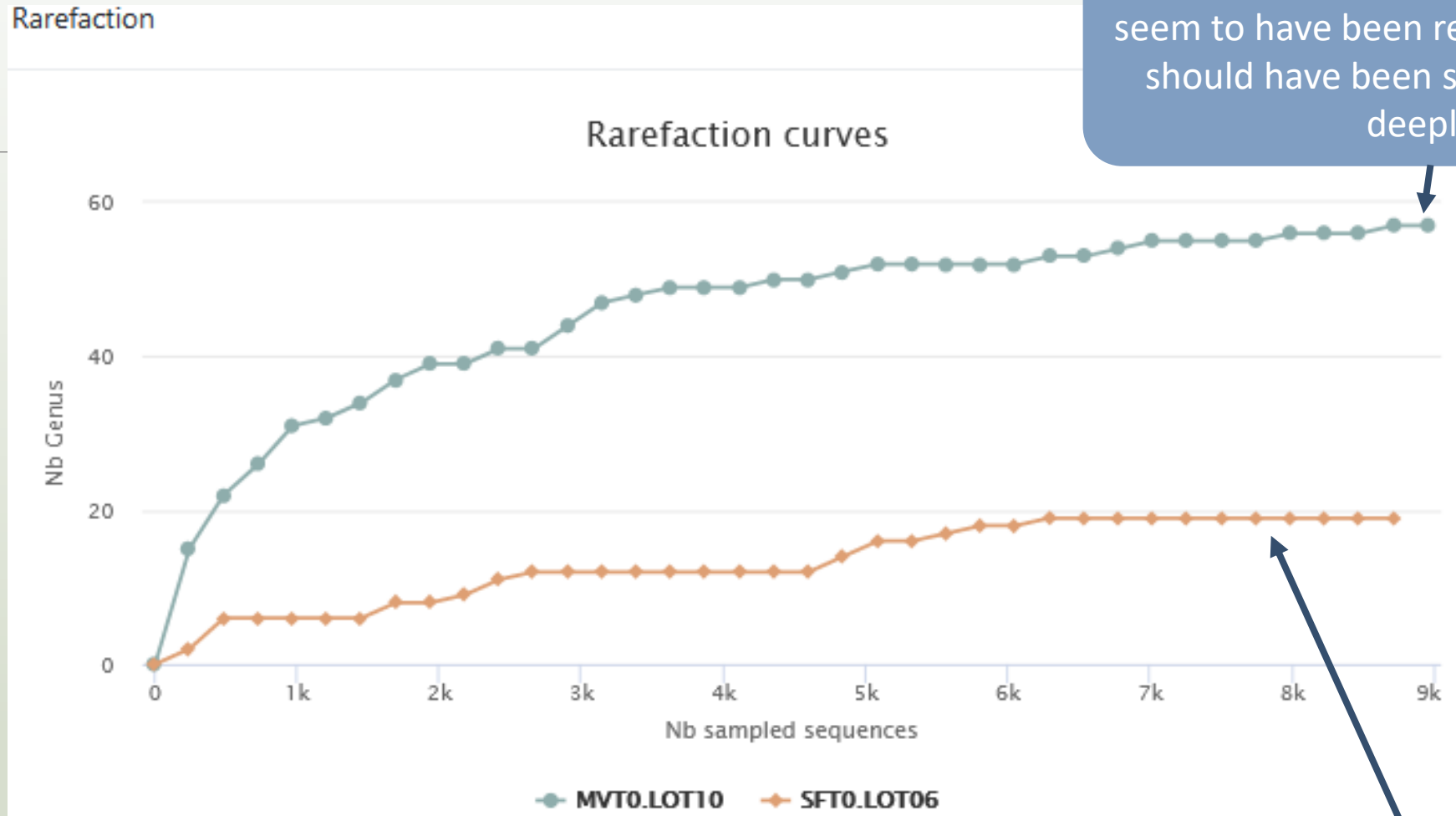
Answer 2

Q2: SFT0.LOT06 and MVT0.LOT10 have they been sequenced deeply enough?



Answer 2

Q2: SFT0.LOT06 and MVT0.LOT10 have they been sequenced deeply enough?



For MVT0.LOT10, the plateau does not seem to have been reached. Perhaps they should have been sequenced more deeply?

With ~8000 sequences, all genus for this species are represented

Q3: Build the **distribution** on FC samples *i.e.* "Filet de Cabillaud"

Use search to find only FC samples

Show Select the 8 samples of FC Search [CSV](#)

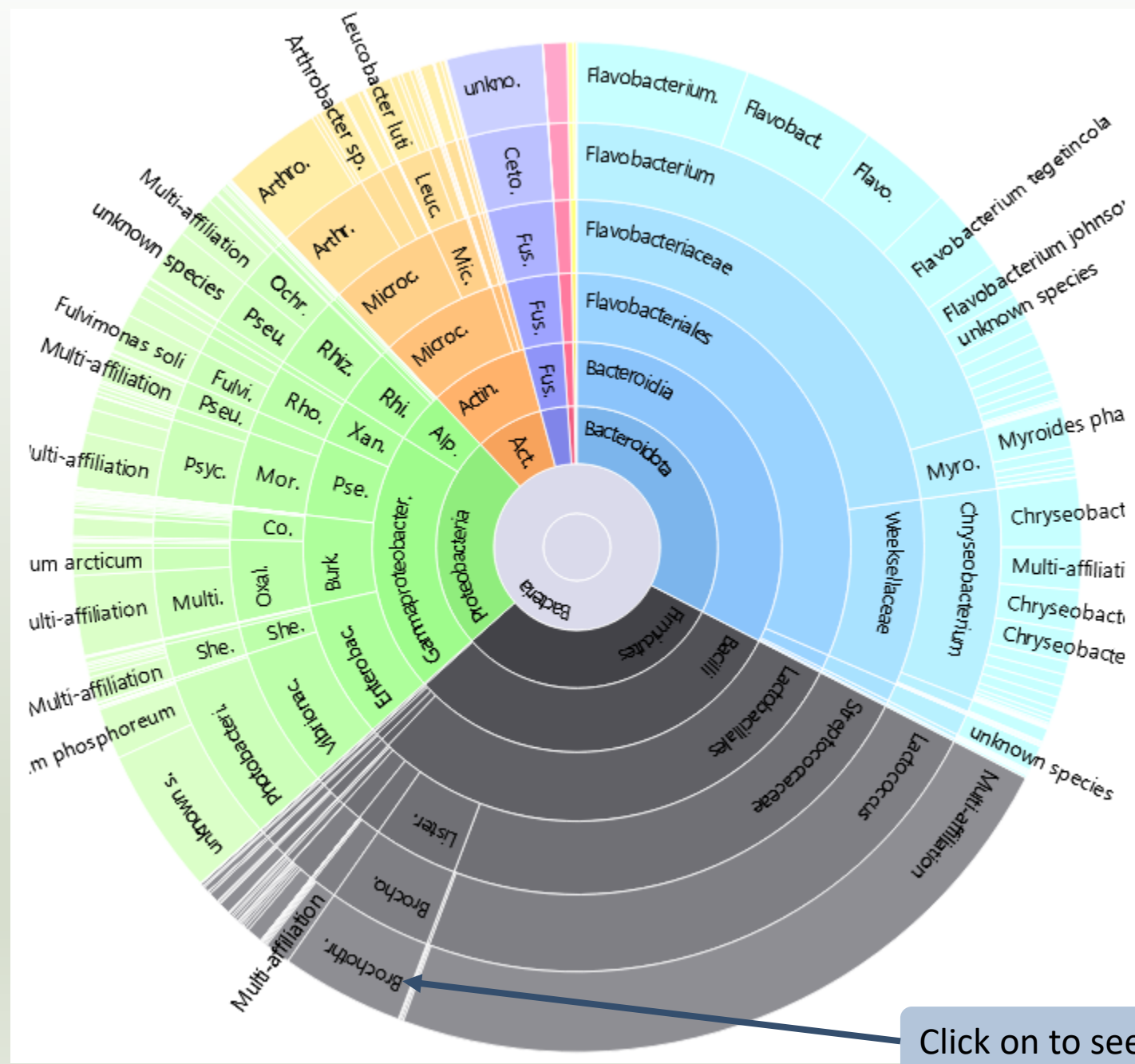
<input checked="" type="checkbox"/>	Samples	Nb domain	Nb phylum	Nb class	Nb order	Nb family	Nb genus	Nb species	Nb sequences
<input checked="" type="checkbox"/>	FCT0.LOT01	1	5	6	13	24	41	96	8,504
<input checked="" type="checkbox"/>	FCT0.LOT02	1	6	8	23	40	67	126	7,638
<input checked="" type="checkbox"/>	FCT0.LOT03	1	8	10	26	45	71	122	8,608
<input checked="" type="checkbox"/>	FCT0.LOT05	1	8	10	25	44	78	139	8,577
<input checked="" type="checkbox"/>	FCT0.LOT06	1	8	10	29	53	97	151	8,577
<input checked="" type="checkbox"/>	FCT0.LOT07	1	5	7	24	46	80	126	8,577
<input checked="" type="checkbox"/>	FCT0.LOT08	1	7	9	27	53	97	151	8,577
<input checked="" type="checkbox"/>	FCT0.LOT10	1	7	9	27	53	97	151	8,577

At the bottom of the table click on

With selection: Genus [Display rarefaction](#) [Display distribution](#)

Answer 3 4 & 5

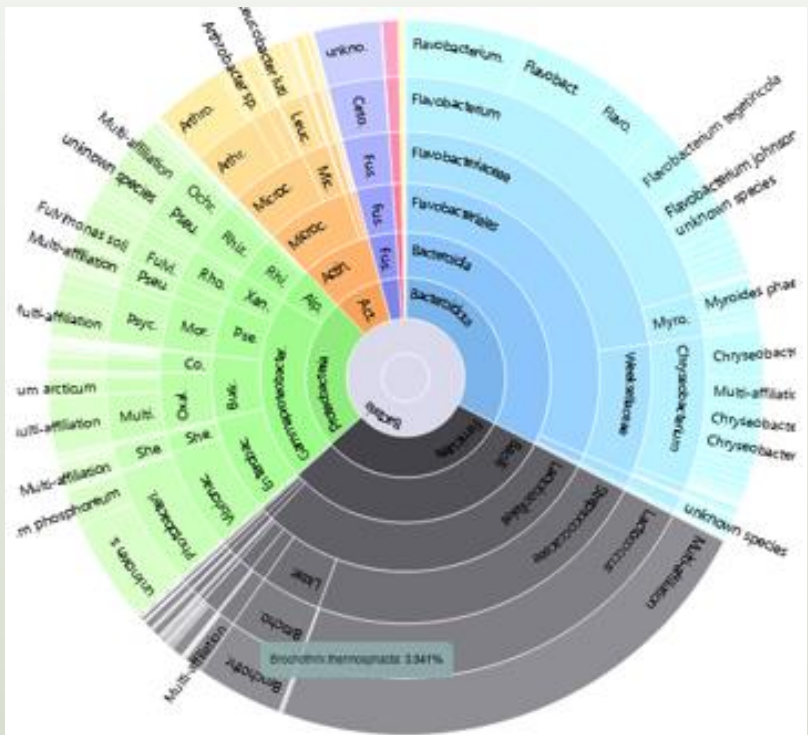
Q3: Build the distribution on FC samples *i.e.* "Filet de Cabillaud"



Click on to see *Brochothrix thermosphacta*

Answer 3,
4, 5 & 6

Q4: How many sequences are some *Brochothrix thermosphacta* ?
 Q5: On the total of sequences, what is the proportion affiliated to the Firmicutes?
 Q6: Among Firmicutes, how many are Bacilli ?



Detail on selected:

Name	Size	Global %	Parent %
root	67211		
Bacteria	67211	100.000	100.000
Firmicutes	20741	30.860	30.860
Bacilli	20658	30.736	99.600
Lactobacillales	19871	29.565	96.190
Listeriaceae	2649	3.941	13.331
Brochothrix	2649	3.941	100.000
Brochothrix thermosphacta	2649	3.941	100.000

Brochothrix thermosphacta nb children: 0

A table appears

Name	Size	Global %	Parent %
root	67211		
Bacteria	67211	100.000	100.000
Firmicutes	20741	30.860	30.860
Bacilli	20658	30.736	99.600
Lactobacillales	19871	29.565	96.190
Listeriaceae	2649	3.941	13.331
Brochothrix	2649	3.941	100.000
Brochothrix thermosphacta	2649	3.941	100.000

Brochothrix thermosphacta nb children: 0

- 2649 sequences are some *Brochothrix thermosphacta*
- Firmicutes represent ~30% of total of sequences of these samples
- 99.6% of Firmicutes are Bacilli

Answer 7

Q7: But what is the proportion of Firmicutes in the total of sequence of all sample ?

Taxonomy distribution Alignment distribution

At the top of the page, click on [Display global distribution](#)

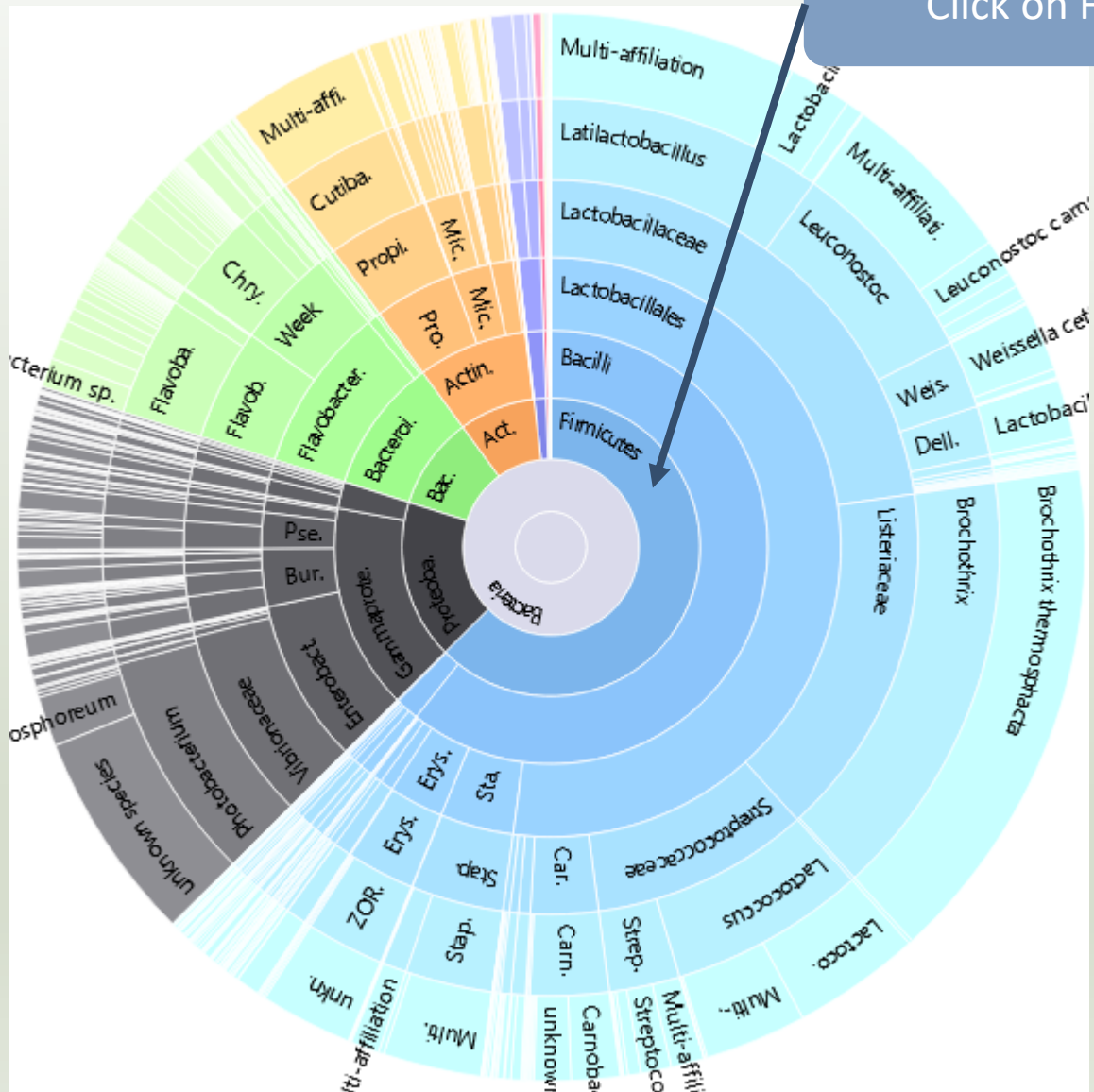
Show entries Search:

<input type="checkbox"/>	Samples ^{↑↓}	Nb domain ^{↑↓}	Nb phylum ^{↑↓}	Nb class ^{↑↓}	Nb order ^{↑↓}	Nb family ^{↑↓}	Nb genus ^{↑↓}	Nb species ^{↑↓}	Nb sequences ^{↑↓}
<input type="checkbox"/>	BHT0.LOT01	1	7	9	20	35	54	77	8,690
<input type="checkbox"/>	BHT0.LOT03	1	5	8	25	46	88	120	8,377
<input type="checkbox"/>	BHT0.LOT04	1	7	10	27	51	89	126	8,643
<input type="checkbox"/>	BHT0.LOT05	1	5	7	22	40	69	116	8,544
<input type="checkbox"/>	BHT0.LOT06	1	6	10	28	47	91	125	8,646
<input type="checkbox"/>	BHT0.LOT07	1	6	9	28	51	90	124	8,671
<input type="checkbox"/>	BHT0.LOT08	1	6	9	27	53	109	166	8,479
<input type="checkbox"/>	BHT0.LOT10	1	4	7	26	50	106	144	8,606
<input type="checkbox"/>	CDT0.LOT02	1	6	8	22	36	58	85	8,750
<input type="checkbox"/>	CDT0.LOT04	1	5	7	22	41	74	138	8,605

With selection:

Q7: But what is the proportion of Firmicutes in the total of sequence of all sample ?

Click on Firmicutes



Name	Size	Global %	Parent %
root	547518		
Bacteria	547518	100.000	100.000
Firmicutes	342409	62.538	62.538

Firmicutes represent 62% of Bacteria

Q8: How many OTUs are align perfectly with a database sequence ?

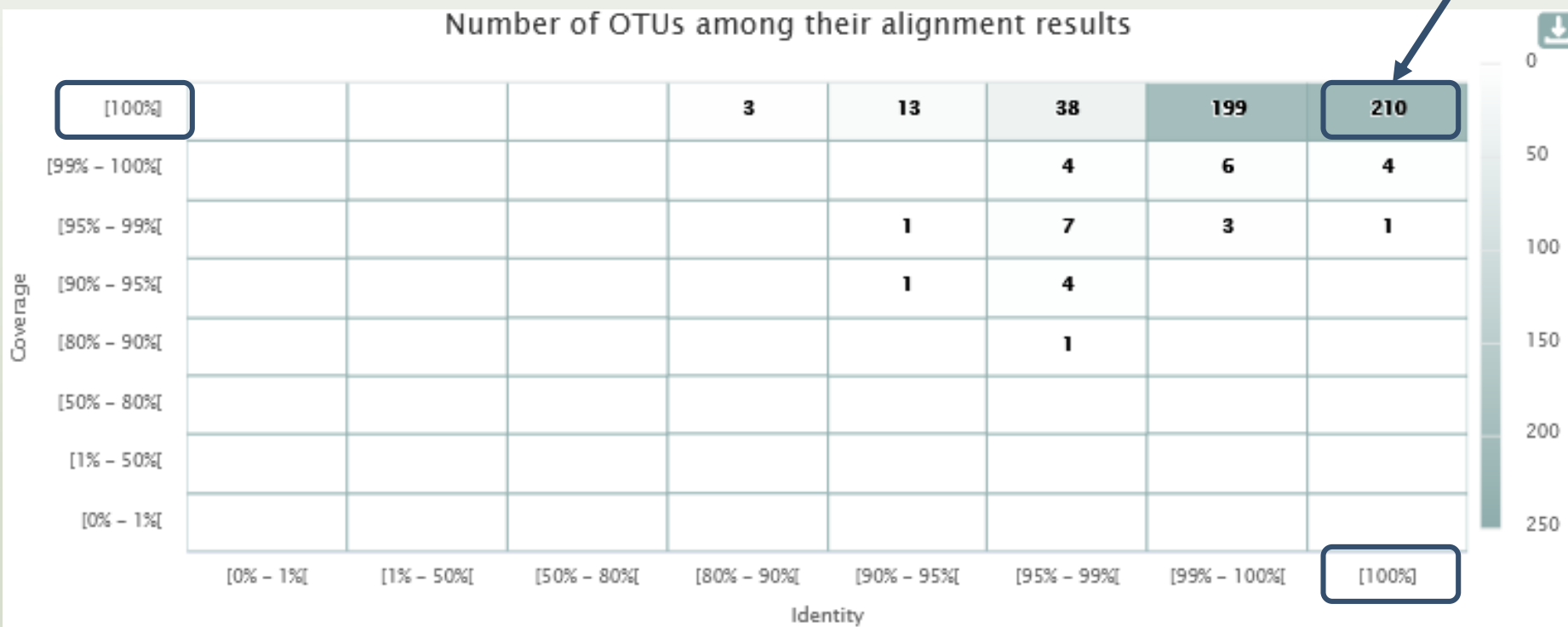
At the top of the page, click on this tab

Taxonomy distribution

Alignment distribution

210 sequences are aligned with 100% identity and 100% coverage with a sequence of silva.

Number of OTUs among their alignment results



by OTUs

by sequences

Filters on affiliations

FROGS Affiliation Filters Filters OTUs on several affiliation criteria. (Galaxy Version 3.2.2) Options

Sequences file
 13: FROGS OTU Filters: sequences.fasta
 The sequence file to filter (format: fasta).

Abundance file
 18: FROGS Affiliation OTU: affiliation.biom
 The abundance file to filter (format: BIOM).

Taxonomic ranks

 The ordered taxonomic ranks levels stored in BIOM. Each rank is separated by one space.

Filtering mode
 Hidding mode
 Deleting mode
 Do you want to delete OTUs or hide affiliations

Filter on Blast affiliations

Maximum e-value (between 0 and 1)

 Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)

 Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)

 Fill the field only if you want this treatment

Minimum alignment length

 Fill the field only if you want this treatment

Filter blast affiliations including these taxon / word

1: Filter blast affiliations including these taxon / word trash

Full or partial taxon name

 ex: "unknown species" or "subsp."

2: Filter blast affiliations including these taxon / word

Full or partial taxon name

 ex: "unknown species" or "subsp."

3: Filter blast affiliations including these taxon / word

Full or partial taxon name

 ex: "unknown species" or "subsp."

Filter on RDP affiliations

Taxonomical rank on which to apply bootstrap filter

 One of the available taxonomical rank name. Ex: Species

Minimum bootstrap % (between 0 and 1)

 Fill these two fields if you want this treatment.

Careful, it is case sensitive.
 Firmicutes it's different of firmicutes !

Not open by default

2 modes: hidding or deleting mode.
 All affiliations that enter in criteria of filter will be either hidden or deleted

- hidding: affiliation counting are not affected, affiliation are simply hidden
- deleting: all abundancies are computed again, affiliation have disappeared

Practice:

LAUNCH THE FROGS AFFILIATION FILTER TOOL

Exercise:

1. Apply filters to keep only sequences with perfect alignment with Silva sequences and affiliations without « unknown species » and « Firmicutes » terms. (deleting mode)
2. Apply filters to hide OTU affiliations that have not a perfect alignment with Silva sequences and the affiliations without « unknown species » and « Firmicutes » terms.
3. In deleting mode:
 - How many OTUs remain?
 - Among OTUs with multiaffiliation, How many were impacted/modified ?
4. In hiding mode:
 - What outputs change between deleted mode and hiding mode ?

FROGS Affiliation Filters Filters OTUs on several affiliation criteria. (Galaxy Version 3.2.2) Options

Sequences file
13: FROGS OTU Filters: sequences.fasta
The sequence file to filter (format: fasta).

Abundance file
18: FROGS Affiliation OTU: affiliation.biom
The abundance file to filter (format: BIOM).

Taxonomic ranks
Domain Phylum Class Order Family Genus Species
The ordered taxonomic ranks levels stored in BIOM. Each rank is separated by one space.

Filtering mode
 Hidding mode
 Deleting mode
Do you want to delete OTU or hide affiliations

Filter on Blast affiliations

Maximum e-value (between 0 and 1)
Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)
1
Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)
1
Fill the field only if you want this treatment

Minimum alignment length
Fill the field only if you want this treatment

Filter blast affiliations including these taxon / word

1: Filter blast affiliations including these taxon / word
Full or partial taxon name
unknown species
ex: "unknown species" or "subsp."

2: Filter blast affiliations including these taxon / word
Full or partial taxon name
Firmicutes
ex: "unknown species" or "subsp."

+ Insert Filter blast affiliations including these taxon / word

Filter on RDP affiliations

Execute

Answer 1

FROGS Affiliation Filters Filters OTUs on several affiliation criteria. (Galaxy Version 3.2.2) Options

Sequences file
13: FROGS OTU Filters: sequences.fasta
The sequence file to filter (format: fasta).

Abundance file
18: FROGS Affiliation OTU: affiliation.biom
The abundance file to filter (format: BIOM).

Taxonomic ranks
Domain Phylum Class Order Family Genus Species
The ordered taxonomic ranks levels stored in BIOM. Each rank is separated by one space.

Filtering mode
 Hidding mode
 Deleting mode
Do you want to delete OTU or hide affiliations

Filter on Blast affiliations

Maximum e-value (between 0 and 1)
Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)
Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)
Fill the field only if you want this treatment

Minimum alignment length
Fill the field only if you want this treatment

Filter blast affiliations including these taxon / word

2: Filter blast affiliations including these taxon / word
Full or partial taxon name
Firmicutes
ex: "unknown species" or "subsp."

+ Insert Filter blast affiliations including these taxon / word

Filter on RDP affiliations

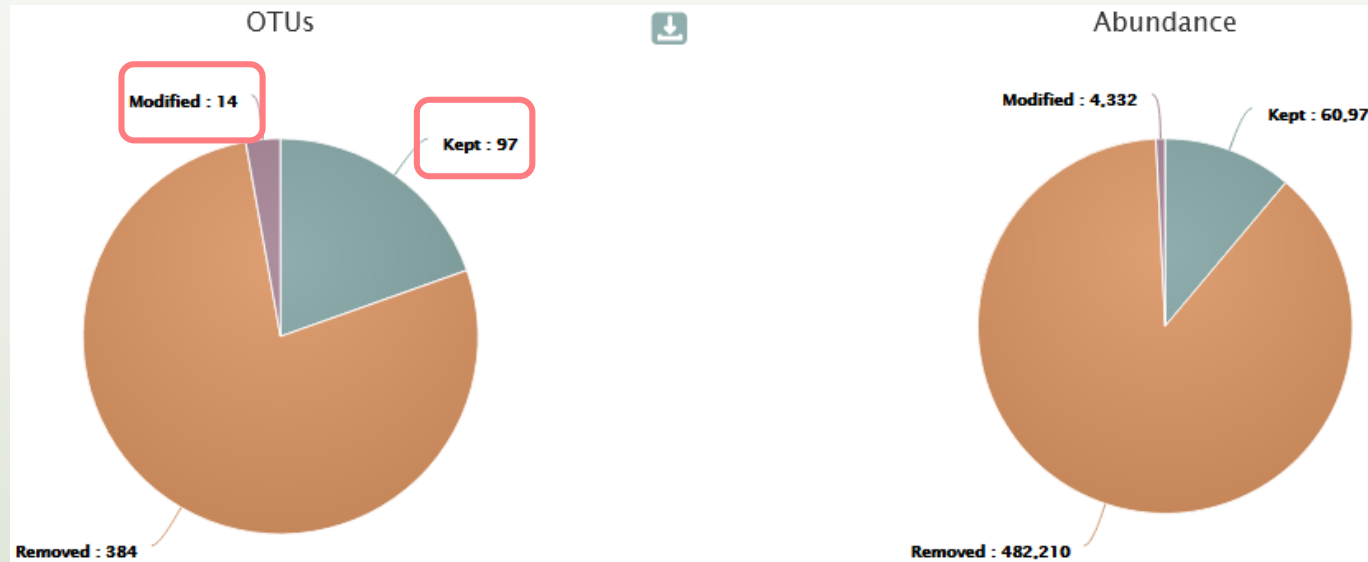
Execute

Answer 2

we want to keep the OTUs that have aligned perfectly with a sequence of the silva bank i.e. 100% identity and 100% coverage

Enter key word

Q3: In deleting mode:
- How many OTUs remain?



- Only 97 OTUs are kept without modification.
- 14 OTUs with multi-affiliation were impacted/modified (all affiliations in the multi_affiliations with key words “unknown species” or “Firmicutes” were deleted).
The consequences are either OTU have less multi-affiliations, or all multi-affiliations are impacted and OTU is deleted.
The list of blast affiliations for multi-affiliated impacted OTUs are in **impacted_OTU.multi-affiliation.tsv**
- So, **111 OTUs** remains after filtering

[: FROGS Affiliation Filters: report.html](#)

[FROGS Affiliation Filters: impacted_OTU.multi-affiliations.tsv](#)

[FROGS Affiliation Filters: impacted_OTU.tsv](#)

[FROGS Affiliation Filters: sequences.fasta](#)

[FROGS Affiliation Filters: abundance.biom](#)

Q3: In deleting mode:

Among OTUs with multi-affiliation, How many multi-affiliation were impacted/modified ?

570 affiliations at species rank disappeared after filtering, including multi-affiliations of 54 OTUs.

Taxon lost summary

Filtering criteria are applied by affiliation. So for blast, filters are not only applied on the blast consensus taxonomy but on each blast hit (cf multihit.tsv file). For each OTU, none, part or all blast affiliations may be removed, resulting in unchanged / updated or deleted blast consensus taxonomy. The detailed number of lost affiliations (not only the consensus) by rank are summarised. It may also precise if blast consensus multi-affiliation are lost.

Affiliation method	Domain	Phylum	Class	Order	Family	Genus	Species
Blast	0	7	12	31 (including 1 multi-affiliation(s))	56 (including 1 multi-affiliation(s))	164 (including 4 multi-affiliation(s))	570 (including 54 multi-affiliation(s))

In addition to the Firmicutes phylum that was deleted, there are 6 others that are deleted (unknow species or %id %cov)

31 Orders were deleted and 1 was a OTU with a multi-affiliation (-> Cluster_451)

Cluster_451 Bacteria;Firmicutes;Bacilli;Multi-affiliation;Multi-affiliation;Multi-affiliation;Multi-affiliation

Cluster_451	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus aureus	CP026068.13	100	100	0	497
Cluster_451	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus aureus	CP029082.38	100	100	0	497
Cluster_451	Bacteria;Firmicutes;Bacilli;Paenibacillales;Paenibacillaceae;Paenibacillus;Staphylococcus aureus	MIZO010000	100	100	0	497
Cluster_451	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus aureus	CP029030.22	100	100	0	497
Cluster_451	Bacteria;Firmicutes;Bacilli;Staphylococcales;Staphylococcaceae;Staphylococcus;Staphylococcus aureus	CP029671.97	100	100	0	497

Answer 3

- [: FROGS Affiliation Filters: report.html](#)
- [FROGS Affiliation Filters: impacted_OTU.multi-affiliations.tsv](#)
- [FROGS Affiliation Filters: impacted_OTU.tsv](#)
- [FROGS Affiliation Filters: sequences.fasta](#)
- [FROGS Affiliation Filters: abundance.biom](#)

N.B. The abundancy table (TSV format) of all deleted (or hidden according to the tool parameters) or modified OTUs are kept in **impacted_OTU.tsv**



#comment	status	blast_taxonomy
undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Listeriaceae;Brochothrix;Brochothrix thermosphacta
undesired_tax_in_blast	OTU_deleted	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Vibrionaceae;Photobacterium;unknown species
undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Latilactobacillus;Multi-affiliation
undesired_tax_in_blast	Blast_taxonomy_changed	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Multi-affiliation
blast_identity_lt_1.0;undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus;Lactococcus piscium
blast_identity_lt_1.0;undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Erysipelotrichales;Erysipelotrichaceae;ZOR0006;unknown species
undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus;Multi-affiliation
blast_identity_lt_1.0;undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Weissella;Weissella ceti
blast_identity_lt_1.0	OTU_deleted	Bacteria;Bacteroidota;Bacteroidia;Flavobacteriales;Flavobacteriaceae;Flavobacterium;Flavobacterium sp.
blast_identity_lt_1.0	OTU_deleted	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Vibrionaceae;Photobacterium;Photobacterium phosphoreum
blast_identity_lt_1.0;blast_coverage_lt_1.0;undesired_tax_in_blast	OTU_deleted	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Dellaglioia;Lactobacillus algidus

In impacted_OTU.tsv

- #comment: the reason(s) why OTU was deleted (or hidden)
- #status: for deleted OTU (or hidden OTU), or for OTU with modified consensus taxonomy with affiliation (or multi-affiliation) was modified

Q4: In hiding mode: What outputs change between deleted mode and hiding mode ?

- [FROGS Affiliation Filters: report.html](#)
- [FROGS Affiliation Filters: impacted_OTU.multi-affiliations.tsv](#)
- [FROGS Affiliation Filters: impacted_OTU.tsv](#)
- [FROGS Affiliation Filters: abundance.biom](#)

In hidden mode: no **sequence.fasta** as output because none OTU was deleted

In hidden mode: **abundance.biom** contains all OTU but 111 have their affiliation that is hidden

#comment	blast_taxonomy	blast_subject	blast_percent_identity	blast_percent_identity	blast_evalue	blast_align	seed_id	observation
undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_41	Cluster_1
undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_611	Cluster_2
undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_595	Cluster_3
undesired_tax_in_blast	Bacteria;Actinobacteriota;Actinobacteria;Propionibacteriales;Propionibacteriaceae;Cutibacterium;Multi-affiliation	multi-subjec	100	100	0	468	17_257	Cluster_4
undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_4	Cluster_5
blast_identity_lt_1.0;undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_23	Cluster_6
blast_identity_lt_1.0;undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	57_5	Cluster_7
undesired_tax_in_blast	no data	no data	no data	no data	no data	no data	17_420	Cluster_8

« no data » appears in hiding mode

To see the content, think to transform the BIOM to TSV file with **BIOM_to_TSV tool**



Normalization



Normalization

Conserve a predefined number of sequence per sample:

- update Biom abundance file
- update seed fasta file

May be used when :

- Low sequencing sample
- Required for some statistical methods to compare the samples in pairs

Exercise 8

1. What is the smallest sequenced samples ?
2. Normalize your data from Affiliation based on this number of sequence
3. Explore the report HTML result.

Answer 1

Q1: What is the smallest sequenced samples ?

Clusters distribution Sequences distribution **Samples distribution**

Sequences count

Show entries [Download CSV](#)

Sample	Total clusters	Shared clusters	Own clusters	Total sequences	Shared sequences	Own sequences
FCT0.LOT02	162	162	0	7,638	7,638	0
FST0.LOT03	152	152	0	7,778	7,778	0
FST0.LOT05	158	158	0	7,908	7,908	0
FST0.LOT02	149	149	0	7,956	7,956	0
CDT0.LOT06	253	253	0	8,257	8,257	0
DLT0.LOT10	222	222	0	8,331	8,331	0
DLT0.LOT07	263	263	0	8,338	8,338	0
CDT0.LOT05	240	240	0	8,376	8,376	0
BHT0.LOT03	135	135	0	8,377	8,377	0
MVT0.LOT05	158	158	0	8,378	8,378	0




Showing 1 to 10 of 64 entries [Previous](#) [1](#) [2](#) [3](#) [4](#) [5](#) [6](#) [7](#) [Next](#)

Thanks to Clusterstat output, you can know what is the size of the smallest sample.
Sort by **Total sequences**
i.e. 7638 sequences

7638 is the maximal size that you can ask for normalizing the sample sizes.




FROGS Abundance normalisation Normalise OTUs abundance. (Galaxy Version 3.2.2) Options

Sequence file

   32: FROGS OTU Filters: sequences.fasta ▼

Sequence file to normalise (format: fasta).

Abundance file

   37: FROGS Affiliation OTU: affiliation.biom ▼

Abundance file to normalise (format: BIOM).

Number of reads

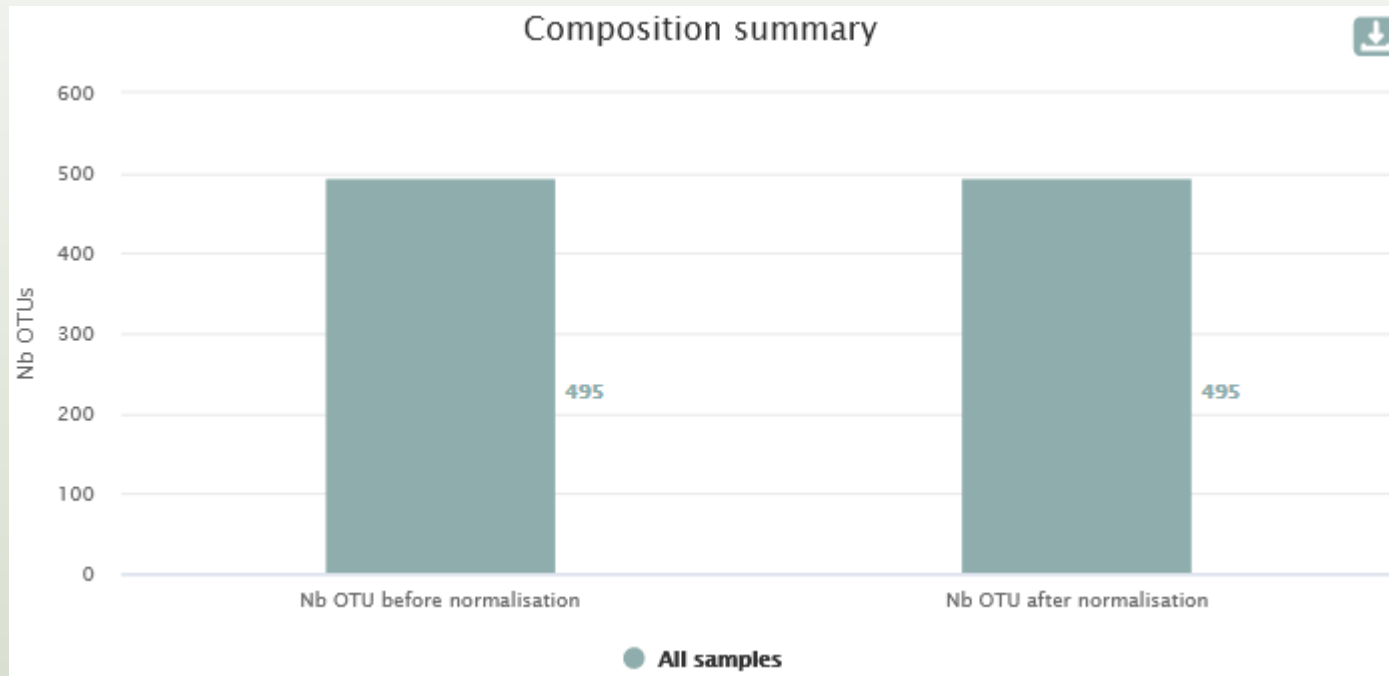
7638

The final number of reads per sample.

Execute

Answer 3

Q3: Explore the report HTML result.



N.B. if you normalize this datasets at 5000 or even 2000 sequences threshold, curiously you will not loose OTUs
But, **careful!** Generally, **more you normalize at low threshold, more you loose OTUs**

This reduction of data has not as consequence to loose OTUs

FROGS Tree

CREATE A PHYLOGENETICS TREE OF OTUS

FROGS Tree

This tool builds a phylogenetic tree thanks to affiliations of OTUs contained in the BIOM file
It uses MAFFT for the multiple alignment and FastTree for the phylogenetic tree.

FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 3.2.2) Options

OTUs sequence file

14: FROGS OTU Filters: sequences.fasta

OTUs sequence file (format: fasta). Warning: FROGS Tree does not work on more than 10000 sequences!

Biom file

19: FROGS Affiliation OTU: affiliation.biom

The abundance table of OTUs (format: biom).

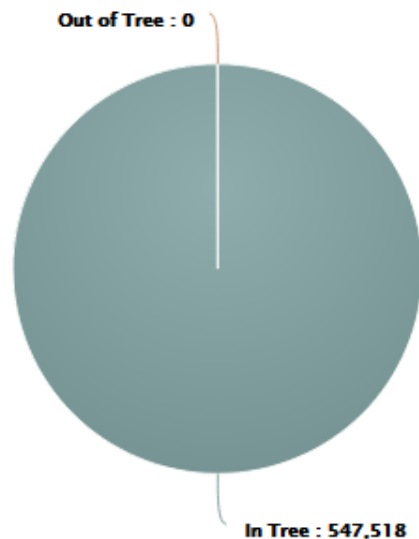
2 outputs:

FROGS Tree: report.html

FROGS Tree: tree.nwk

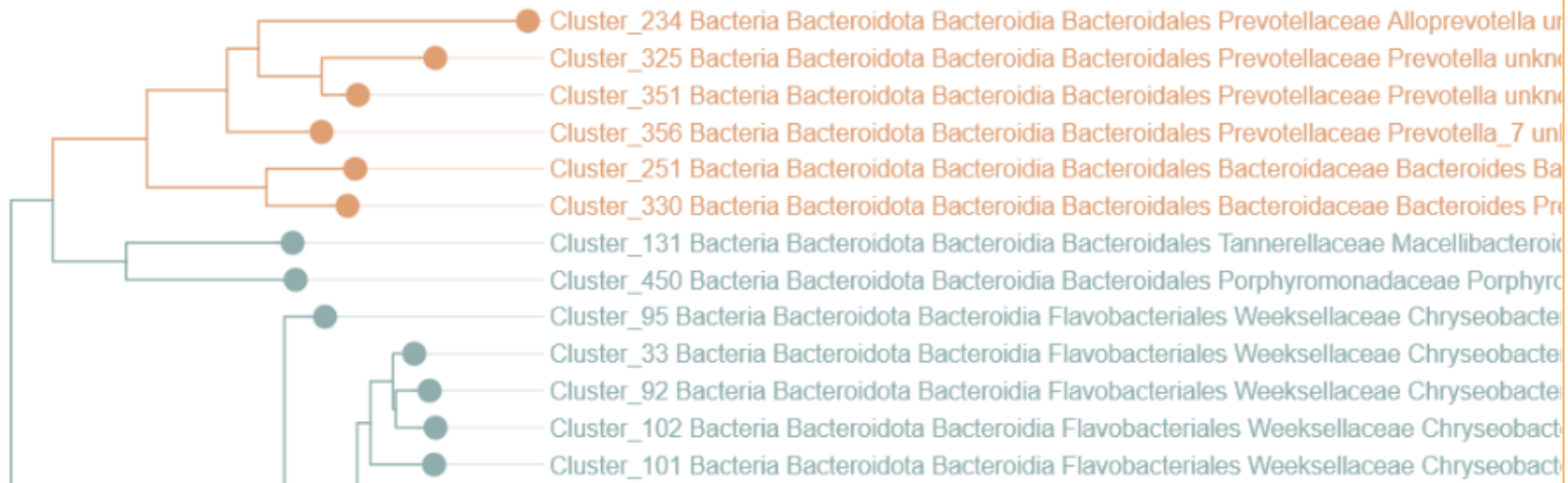
OTUs

Abundance

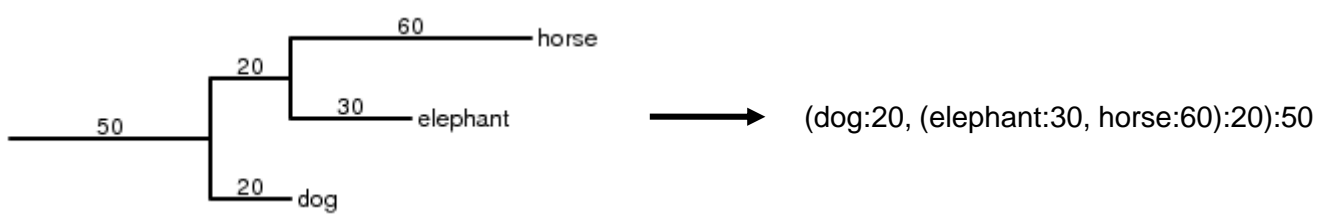


Tree View

Enabling zoom:



The phylogenetic tree in Newick format *i.e.* each node is represented between brackets. This format is universal and can be used with all tree viewer



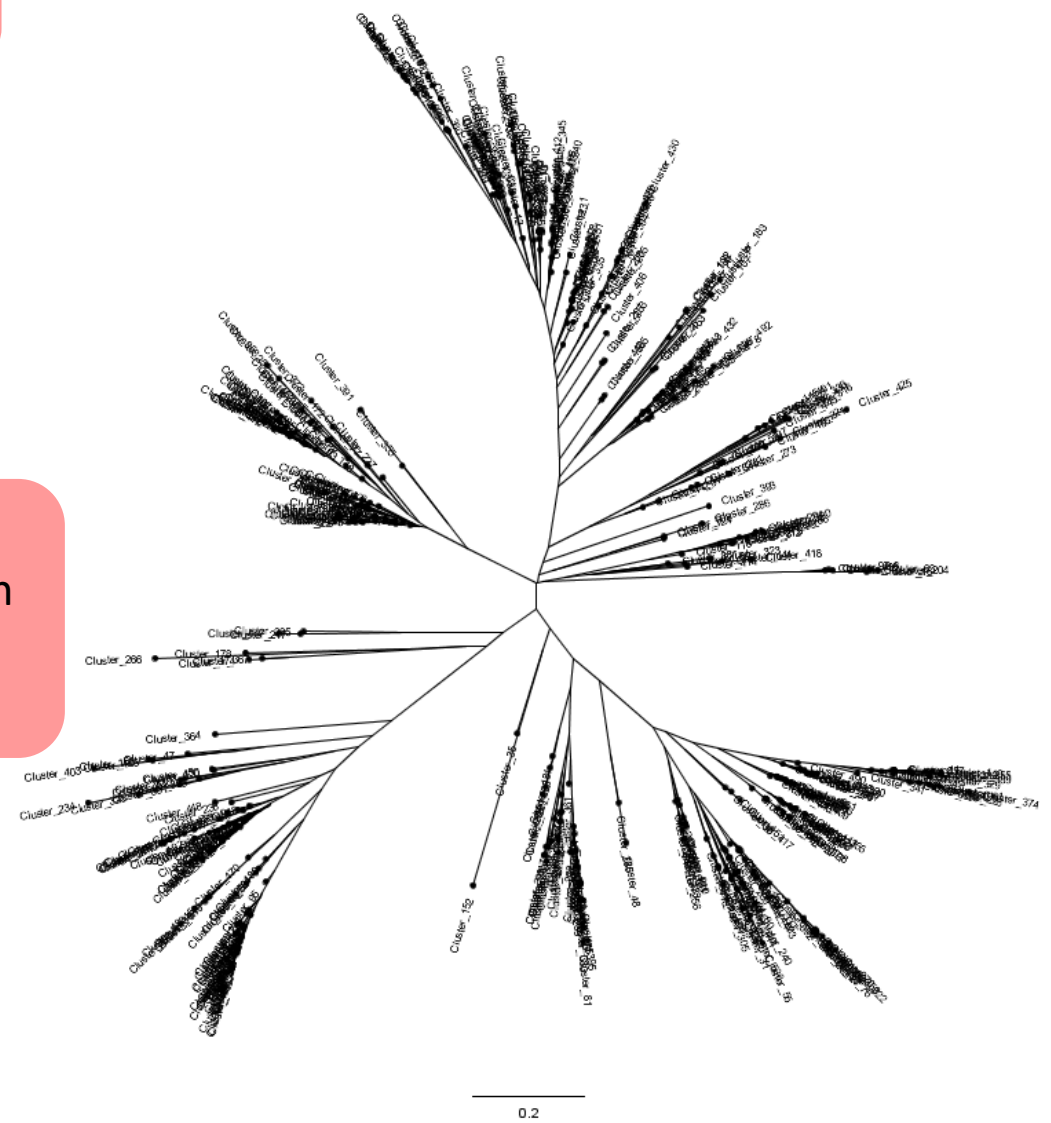
Our tree in nhx (= nwk) format

Exemple of visualization in FigTree from nhx file

```

((((((((((((Cluster_234:0.25278,(Cluster_325:0.09784,Cluster_67):0.972:0.02504,(Cluster_468:0.0269,(Cluster_138:0.0016782:0.00832,Cluster_277:0.01601)1.000:0.06764,Cluster_47:0.13954,(Cluster_166:0.16129,(Cluster_403:0.2293472:0.01332,(Cluster_400:0.00545,Cluster_473:0.01483)1.000)0.829:0.01282,Cluster_240:0.12227)0.717:0.02027)0.981:0.00055,(Cluster_193:0.00055,Cluster_359,Cluster_484:0.01913)0.880:0.03155)0.993:0.08088)0.450989)0.827:0.01144)0.870:0.01235,((Cluster_81:0.08926,Cluster_105)0.862:0.00658,(Cluster_303:0.04337,Cluster_398:0.0311237)0.953:0.01895,(Cluster_346:0.0235,((Cluster_369:0.0100054,(Cluster_402:0.12402,(Cluster_309:0.02202,(Cluster_284:0.00054,(Cluster_427:0.00054,(Cluster_14:0.00402,Cluster_10:0.791:0.02141,(Cluster_93:0.00054,Cluster_340:0.01463)0.03373)0.847:0.03692,Cluster_406:0.16125)0.831:0.03655)0.04264)0.321:0.00907)0.487:0.01277,Cluster_129:0.06386)0.2802)0.763:0.02715,(Cluster_16:0.1183,(Cluster_63:0.062

```






Practice:

Exercise:

1. Create the phylogenetic tree that will be used for statistical analyses.




FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 3.2.2)

OTUs sequence file

   14: FROGS OTU Filters sequences.fasta

OTUs sequence file (format: fasta). Warning: FROGS Tree does not work on more than 10000 sequences!

Biom file

   19: FROGS Affiliation OTU affiliation.biom

The abundance table of OTUs (format: biom).

Execute

*For tutorial, we ask you to create a phylogenetic tree on affiliation.biom **before** “affiliation filter” process. Otherwise on your own data, create the phylogenetic tree on cleaned affiliation.biom*

Download your data

In order to share resources as well as possible, files that have not been accessed for more than 120 days are regularly purged. The backup of data generated using of Galaxy is your responsibility.

You have 2 backup possibilities:

1. Save your datasets one by one using the "floppy disk" icon.



```
55: FROGS Affiliation [eye] [edit] [close]
OTU:
excluded_data_report.html
11.4 KB
format: html, database: ?
## Application Software:
affiliation_OTU.py (version: 0.4.0)
Command: /usr/local/bioinfo
/src/galaxy-test/galaxy-dist/tools
/FROGS/affiliation_OTU.py
--reference /save/galaxy-
test/bank/FROGS/silva_119-1
/prokaryotes
/silva_119-1_prokaryotes.fasta
--abundance
[save] [info] [refresh] [share] [print]
HTML file
```

2. Or export each history.

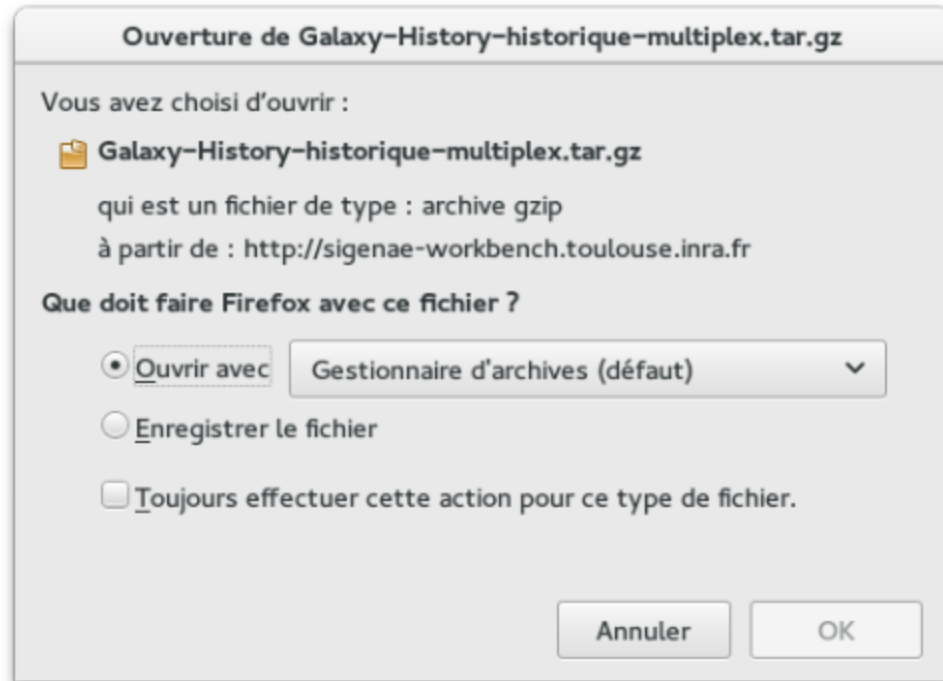
To export a history, from the "History" menu, click on the wheel, then "Export History to File":



- History
- HISTORY LISTS
 - Saved Histories
 - Histories Shared with Me
- HISTORY ACTIONS
 - Create New
 - Copy History
 - Share or Publish
 - Show Structure
 - Extract Workflow
 - Delete
 - Delete Permanently
- DATASET ACTIONS
 - Copy Datasets
 - Dataset Security
 - Resume Paused Jobs
 - Collapse Expanded Datasets
 - Unhide Hidden Datasets
 - Delete Hidden Datasets
 - Purge Deleted Datasets
- DOWNLOADS
 - Export Tool Citations
 - Export History to File**
- OTHER ACTIONS
 - Import from File

To retrieve your history, click on the http link that appears automatically:

It is then possible to record the data :



This directory contains :



1. in the "datasets" directory: Your Galaxy files.
2. in the files "-attrs.txt" : Metadata about your datasets, your jobs and your history.

How to cite FROGS

Frédéric Escudié, Lucas Auer, Maria Bernard, Mahendra Mariadassou, Laurent Cauquil, Katia Vidal, Sarah Maman, Guillermina Hernandez-Raquet, Sylvie Combes, Géraldine Pascal.

"FROGS: Find, Rapidly, OTUs with Galaxy Solution." *Bioinformatics*, Volume 34, Issue 8, 15 April 2018, Pages 1287–1294

Maria Bernard, Olivier Rué, Mahendra Mariadassou and Géraldine Pascal; **FROGS**: a powerful tool to analyse the diversity of fungi with special management of internal transcribed spacers, *Briefings in Bioinformatics* 2021, 10.1093/bib/bbab318

Sequence analysis

FROGS: Find, Rapidly, OTUs with Galaxy Solution

Frédéric Escudié^{1,†}, Lucas Auer^{2,†}, Maria Bernard³, Mahendra Mariadassou⁴, Laurent Cauquil⁵, Katia Vidal⁶, Sarah Maman⁶, Guillermina Hernandez-Raquet⁶, Sylvie Combes⁶ and Géraldine Pascal^{2,*}

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[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

Associate Editor: Bonnie Berger

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Abstract

Motivation: Metagenomics leads to major advances in microbial ecology and biologists need user friendly tools to analyze their data on their own. **Results:** This Galaxy-supported pipeline, called FROGS, is designed to analyze large sets of amplicon sequences and produce abundance tables of Operational Taxonomic Units (OTUs) and their taxonomic affiliation. The clustering uses Swarm. This chimera removal uses VSEARCH combined with original cross-sample validation. The affiliation output to highlight databases contains graphical illustrations are produced along for the detection and quantification of OTUs, robust and highly sensitive. It compares taxonomic affiliation.

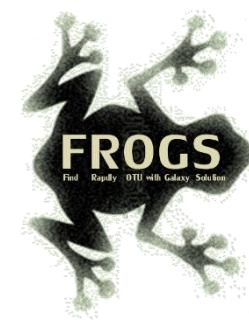
Availability and implementation: Source code: geraldinepascal/FROGS.git. A companion web: Contact: geraldine.pascal@inra.fr **Supplementary information:** Supplementary

1 Introduction

The expansion of high-throughput sequencing of rDNA has opened new horizons for the study of microbial diversity by making it possible to study all micro-organisms in an environment without the need to cultivate them, leading to major advances in many fields of microbial ecology: study of the impact of microbiota on human and animal

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Advance Access Publication Date: 7 December 2017
Original Paper



Briefings in Bioinformatics, 22(6), 2021, 1–6

<https://doi.org/10.1093/bib/bbab318>
Problem Solving Protocol

FROGS: a powerful tool to analyse the diversity of fungi with special management of internal transcribed spacers

Maria Bernard¹, Olivier Rué¹, Mahendra Mariadassou² and Géraldine Pascal²

Corresponding author: Géraldine Pascal, GenPhySE, Université de Toulouse, INRAE, INPT, ENVT, F-31326, Castanet Tolosan, France. Tel.: +33 (0)5 63 28 51 05; E-mail: geraldine.pascal@inrae.fr

¹Maria Bernard and Olivier Rué are joint first authors.

Abstract

Fungi are present in all environments. They fulfill important ecological functions and play a crucial role in the food industry. Their accurate characterization is thus indispensable, particularly through metabarcoding. The most frequently used markers to monitor fungi are ITSs. These markers are the best documented in public databases but have one main weakness: polymerase chain reaction amplification may produce non-overlapping reads in a significant fraction of the fungi. When these reads are filtered out, traditional metabarcoding pipelines lose part of the information and consequently produce biased pictures of the composition and structure of the environment under study. We developed a solution that enables processing of the entire set of reads including both overlapping and non-overlapping, thus providing a more accurate picture of fungal communities. Our comparative tests using simulated and real data demonstrated the effectiveness of our solution, which can be used by both experts and non-specialists on a command line or through the Galaxy-based web interface.

Key words: fungi; ITS; metabarcoding; workflow; amplicon; metagenomics

Introduction

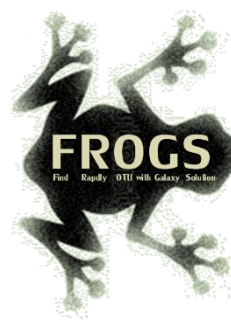
Using amplicon sequencing to describe the microbial composition of an environment is a time saving and cost-effective strategy and can be used even for very large-scale surveys [1]. Most studies currently focus on the bacterial fraction of microbial communities but the fungal fraction is equally important, as fungi are ubiquitous and provide several ecosystem services [2]. Unfortunately, studying the fungal fraction using metabarcoding has its own challenges. Indeed, in fungi, there is no equivalent of the 16S rDNA gene, which is widely used and highly suitable

for bacteria. The best candidates are internal transcribed spacers (ITS), but these are more difficult to manipulate. The main problem with ITS is size polymorphism, with a size range of 361–1475 bases in UNITE 7.1 [3] (unlike 16S where 95% of the sequences have a length between 1205 and 1556 bases). Most studies describing ITS data analyses process either (i) paired-end reads but filter out non-overlapping, non-mergeable reads, thus systematically discarding taxa with longer ITS, or (ii) single-end reads, thus limiting taxonomic resolution and losing the benefit of information contained in longer sequences [4, 5].

Maria Bernard is a bioinformatics engineer. She is a member of a platform team conducting NGS sequence analysis and designing software. She specializes in workflow development in particular for metabarcoding analysis. Olivier Rué is a bioinformatics engineer. He is in charge of data analysis at the Migale bioinformatics facility. He specializes in the analysis of metabarcoding and metagenomics data. Mahendra Mariadassou has a PhD in statistics. He is involved in the development of new statistical methods and tools for metabarcoding analysis. Géraldine Pascal has a PhD in bioinformatics and coordinates the FROGS project. She is currently involved in designing solutions for long read problems, workflow development and metagenomics analysis. Submitted: 19 April 2021. Received (in revised form): 19 July 2021

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1



FROGS'docs

Website: <http://frogs.toulouse.inrae.fr>

Tuto: <https://youtu.be/Kh6ZrlmKGoY>

The user-friendly and Galaxy-supported pipeline FROGS analyses large sets of DNA amplicons sequences accurately and rapidly, essential for microbe community studies.

- FROGS was designed to support multiplexed and demultiplexed sequences.
- FROGS supports 16S, 15S, 23S, 11S and other amplicon reads.
- FROGS supports short or long reads.
- The preprocessing tool is optimized to paired sequences merging, cleaning and denoising.
- The clustering tool uses Swarm with a local clustering (invariant), not a global clustering (between the other software etc).
- Chimera removal tool uses VSEARCH combined with an innovative chimera cross-validation.
- A filtering tool allows to remove noisy data.
- Attribution tool returns taxonomic affiliation for each OTU using two methods with a unique multi-affiliation output.
- FROGS offers numerous banks for affiliation step (cf. ...).
- A set of statistical results and numerous graphical visualizations are also produced.
- FROGS is designed for non-specialists thanks to its Galaxy interface, but it is also available with command line: `phius`.
- Its tools can be used independently, or as a workflow.
- To install FROGS, check or galaxy: `conda`.

FROGS was tested on many datasets

- The "FROGS 16S Benchmark lab" and "FROGS ITS Benchmark lab" show comparisons between FROGS and other popular pipelines.

Standard Operation Procedure for amplicons
(A, 16S, 18S, etc., 150...)

To test FROGS

Play with FROGS on the Galaxy server of Toulouse

- Register an account to generate data on the platform via a form `Genotool Platform`.
- Enter your credentials (once for apache server connection and a second time for galaxy platform connection). `Genotool Server` (on the top) lower `login`.
- Get data. In `also simulated data` (4th history of the documentation below) from `version 3.0.0`.
- Play with the workflow: `Workflow Test` and export it on your account (green cross on the top).
- To learn more: `Formation documentation`.

Help FROGS measurements with command lines for analysis `16S` `18S` `ITS`

Get data built with sequences from `16S` and `18S` datasets. doi.org/10.13044/GENEVOJ

Help FROGS measurements with command lines for analysis `11S` `23S` `ITS`

Get data built with sequences from `UNITES`. doi.org/10.13044/GENEVOJ

License

GNU GPL3 (open)

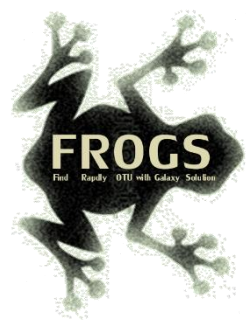
REPUBLIQUE FRANÇAISE

Pipeline FROGS on

<http://sigenae-workbench.toulouse.inra.fr/galaxy/u/gpascal/w/to-test-frogs>

All scripts on Github: <https://github.com/geraldinepascal/FROGS.git>





To contact

FROGS:

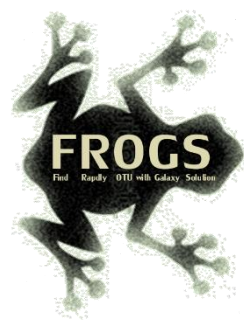
frogs-support@inrae.fr

Galaxy:

support.sigenae@inrae.fr

Newsletter – subscription request:

frogs@inrae.fr



Play list FROGS:

https://www.deezer.com/fr/playlist/5233843102?utm_source=deezer&utm_content=playlist-5233843102&utm_term=18632989_1545296531&utm_medium=web