

# Training on Galaxy: Metagenomics

November 2017

## Find, Rapidly, OTUs with Galaxy Solution

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\*THESE AUTHORS HAVE CONTRIBUTED EQUALLY TO THE PRESENT WORK.

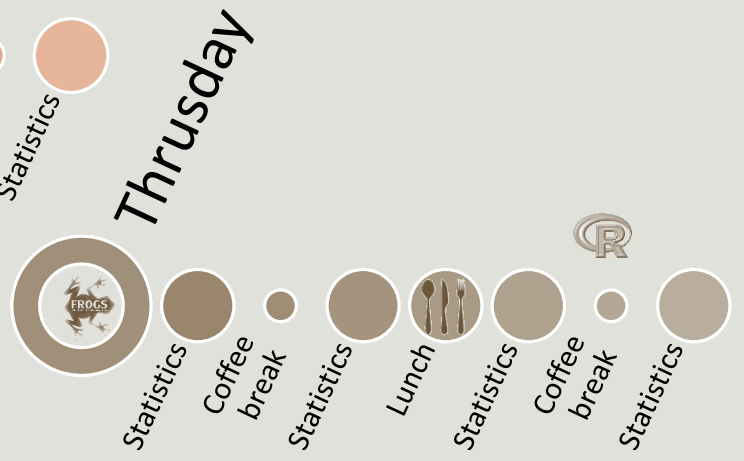
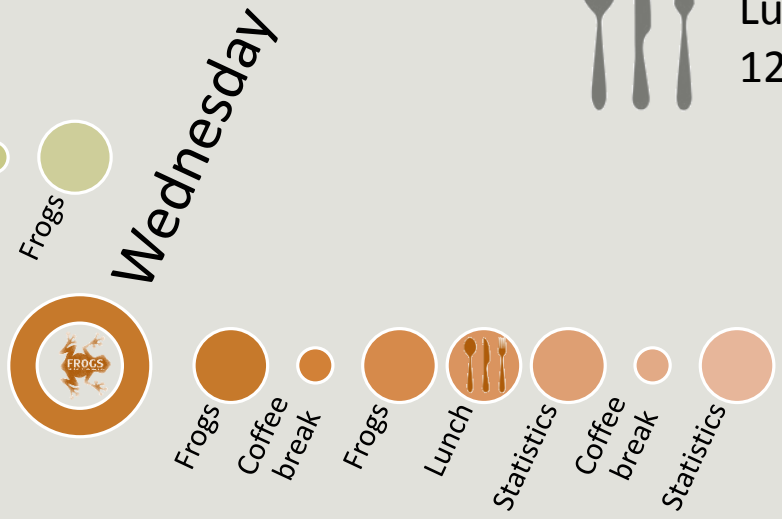
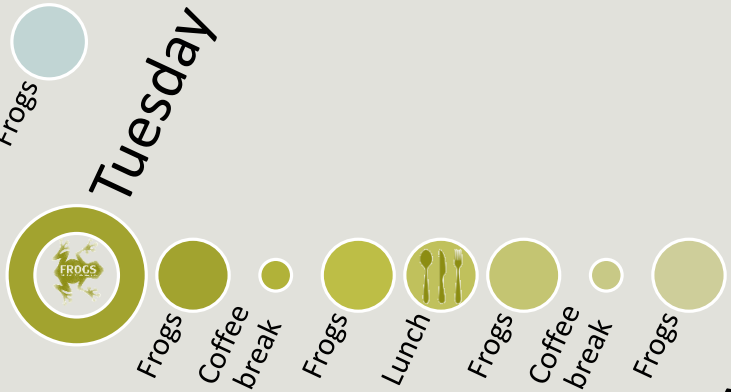
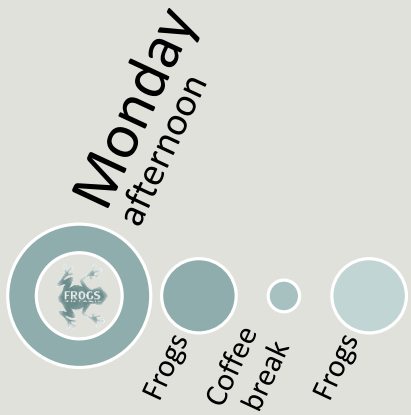
Feedback:

What are your needs in “metagenomics”?

454 / MiSeq ?

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Your background ?



9 am to 5 pm



2 short coffee breaks  
morning and afternoon



Lunch  
12.30 to 2.00 pm

# Overview

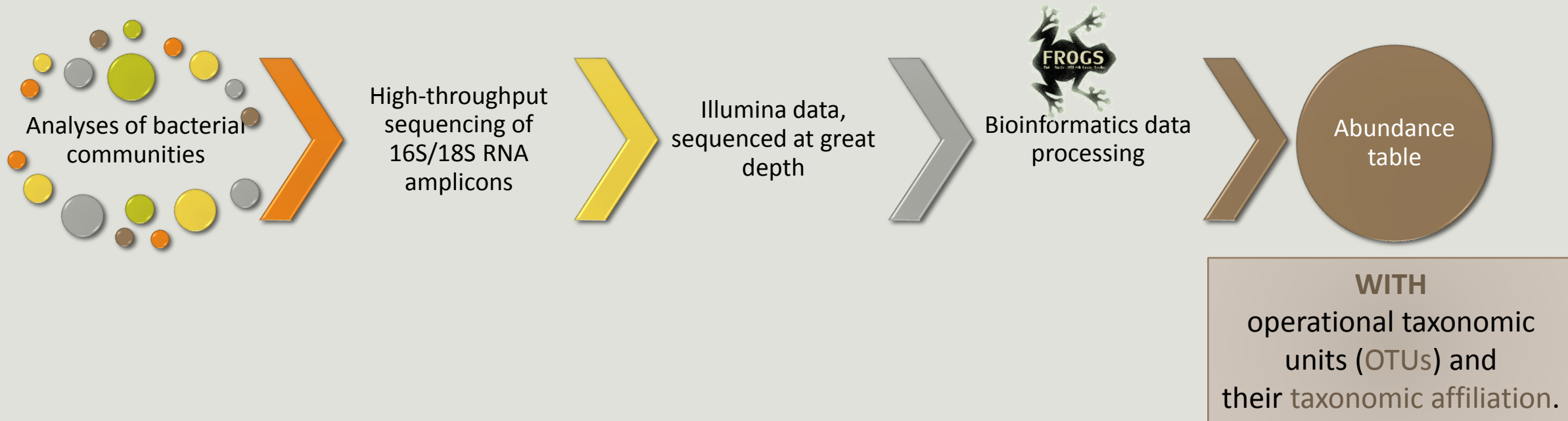


- 
- Objectives
  - Material: data + FROGS
  - Data upload into galaxy environment
  - Demultiplex tool
  - Preprocessing
  - Clustering + Cluster Statistics
  - Chimera removal
  - Filtering
  - Affiliation + Affiliation Statistics
  - Normalization
  - Tool descriptions
  - Format transformation
  - Workflow creation
  - Download data
  - Some figures



# Objectives

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# OTUs for ecology

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## Operational Taxonomy Unit:

a grouping of similar sequences that can be treated as a single « species »

## Strengths:

- Conceptually simple
- Mask effect of poor quality data
  - Sequencing error
  - In vitro recombination (chimera)

## Weaknesses:

- Limited resolution
- Logically inconsistent definition

# Objectives

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	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 ?

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The **current processing** pipelines **struggle** to run in a reasonable time.

The most effective solutions are often **designed for specialists** making access difficult for the whole community.

**In this context we developed the pipeline FROGS: « Find Rapidly OTU with Galaxy Solution ».**

# Who is in the FROGS group?



**Frédéric ESCUDIÉ**



**Maria BERNARD**



**Lucas AUER**



**Laurent  
CAUQUIL**



**Sylvie  
COMBES**



**Guillermina  
HERNANDEZ-RAQUET**



**Sarah MAMAN**

**Developers**

**Biology experts**

**Galaxy  
support**



**Mahendra  
MARIADASSOU**

**Statistical  
expert**



**Géraldine  
PASCAL**

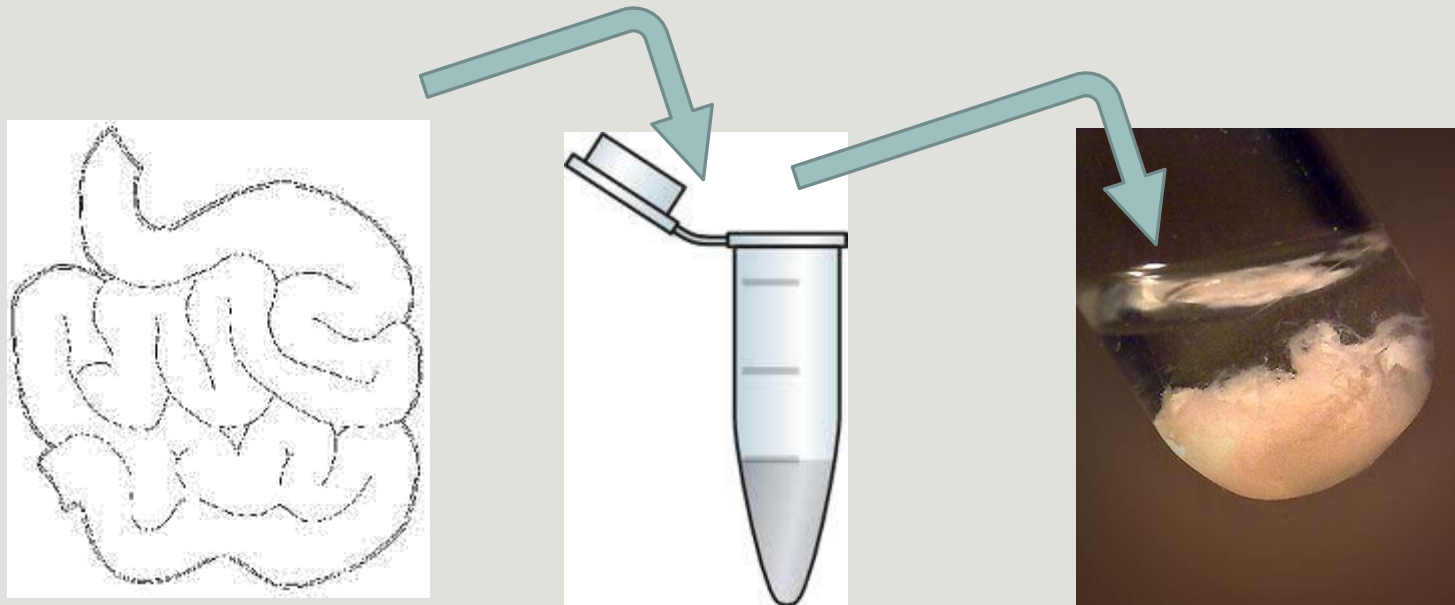
**Coordinator**

# Material

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# Sample collection and DNA extraction

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



DNA

RNA

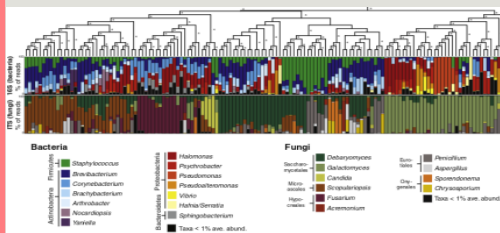
Metagenomics

Metatranscriptomics

Amplicon sequencing

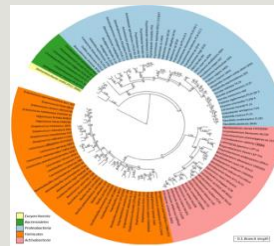
Shotgun sequencing

RNA sequencing



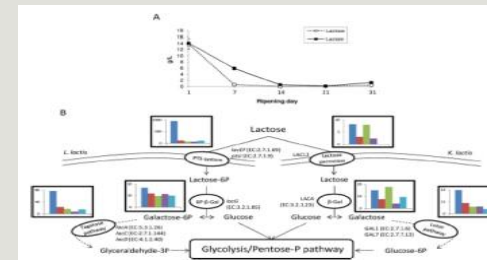
Wolfe et al., 2014

Who is here?



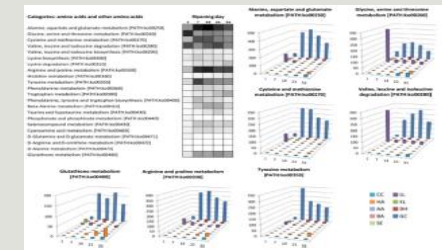
Almeida et al., 2014

What can they do?



Dugat-Bony et al., 2015

What are they doing?





# The gene encoding the small subunit of the ribosomal RNA

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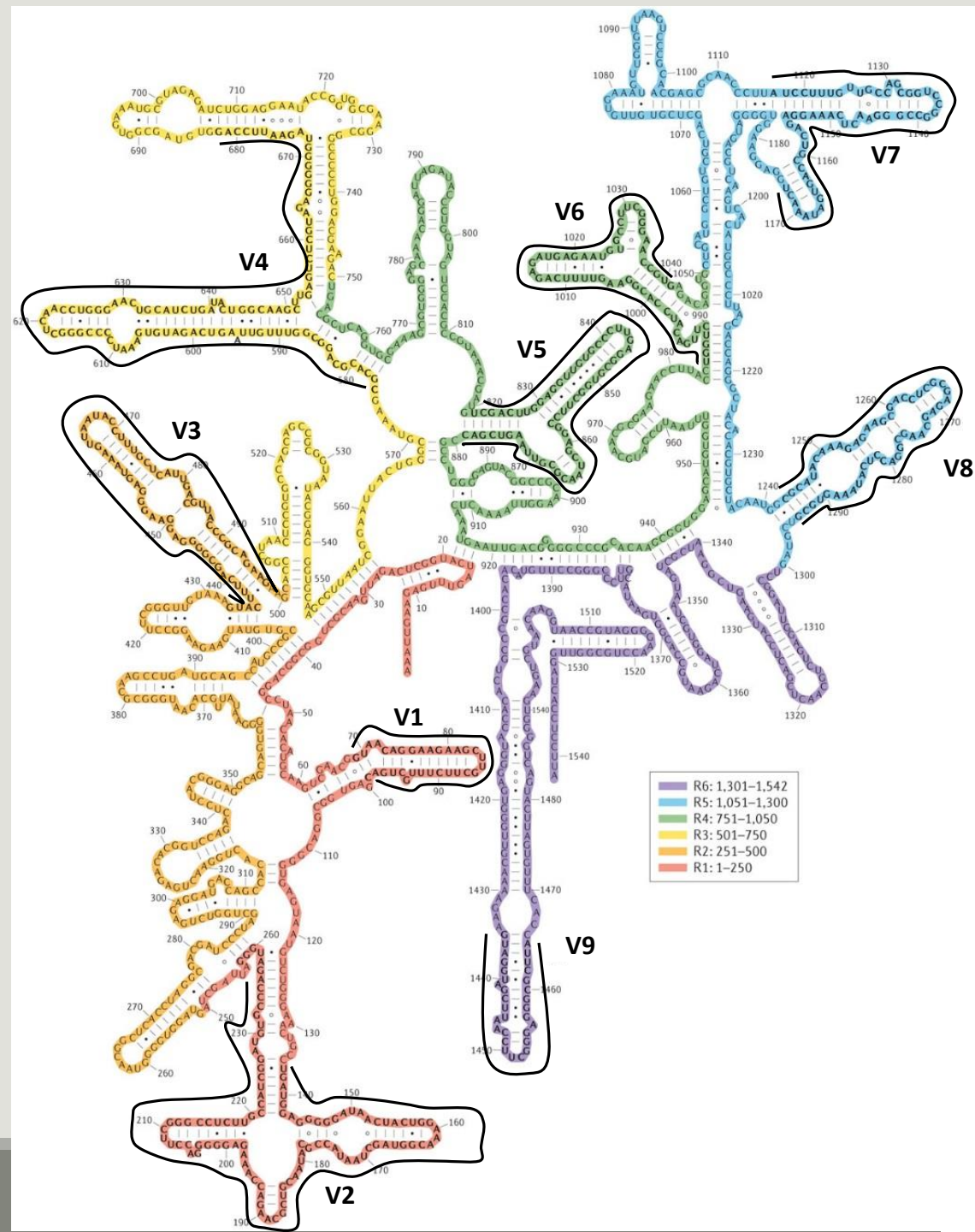
The most widely used gene in **molecular phylogenetic** studies

Ubiquist gene : **16S rDNA** in prokayotes ; **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 2015: >22000 type strains)



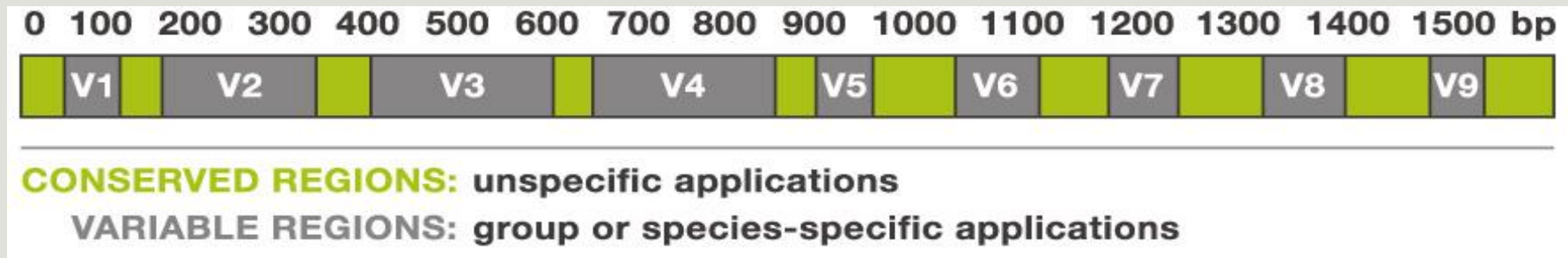
## Secondary structure of the 16S rRNA of *Escherichia coli*

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

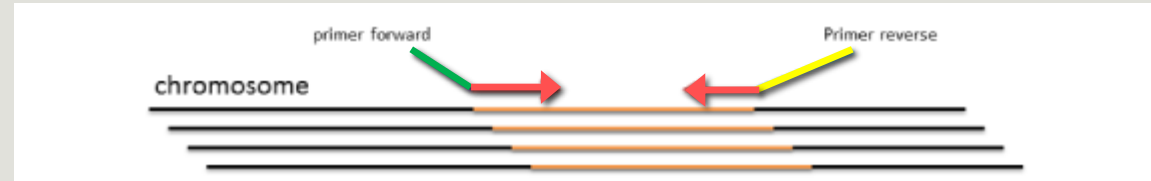
# The gene encoding the small subunit of the ribosomal RNA

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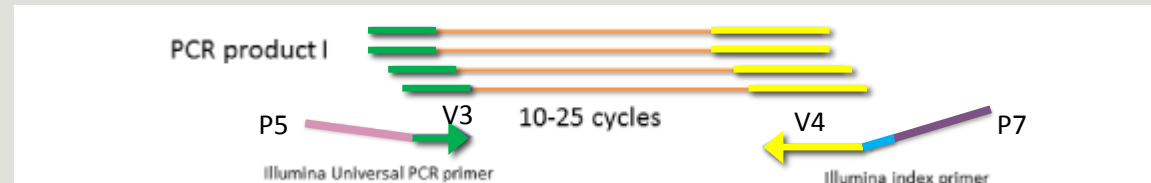


# Steps for Illumina sequencing

- 1<sup>st</sup> step : one PCR

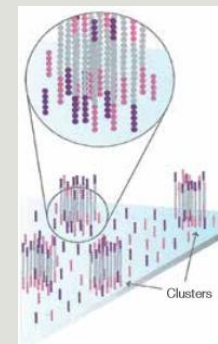
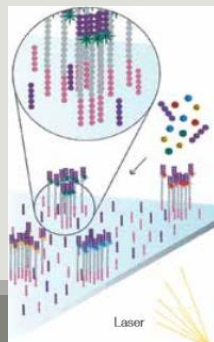


- 2<sup>nd</sup> step: one PCR



- 3<sup>rd</sup> step: on flow cell, the cluster generations

- 4<sup>th</sup> step: sequencing



# Amplification and sequencing

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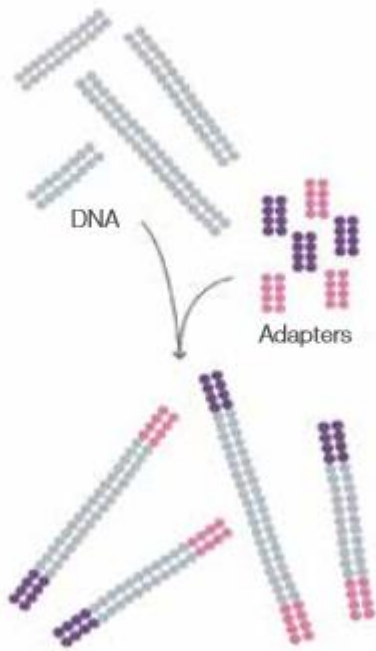
« **Universal** » primer sets are used for **PCR amplification** of the phylogenetic biomarker

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)



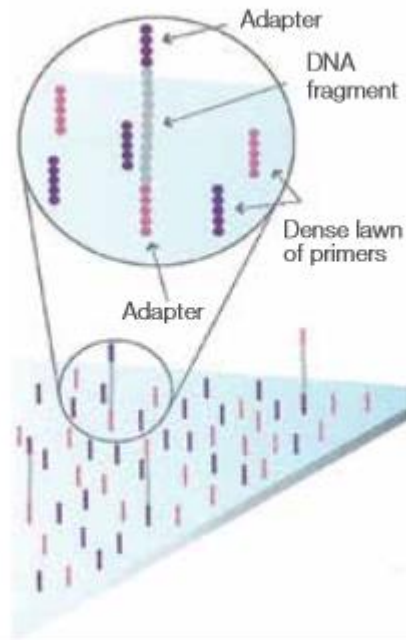
# Cluster generation

## Prepare Genomic DNA Sample



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

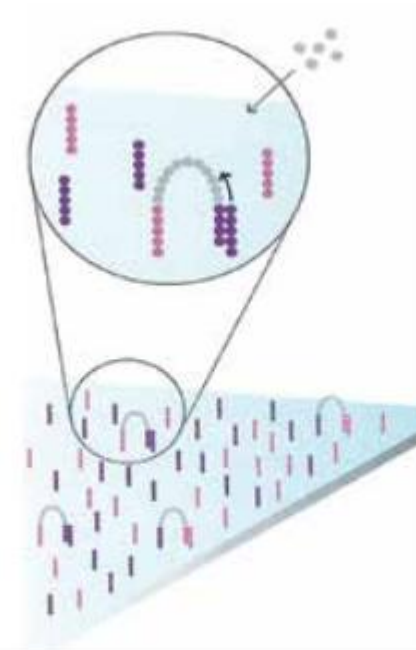
## Attach DNA to Surface



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

Attach DNA to surface

## Bridge Amplification

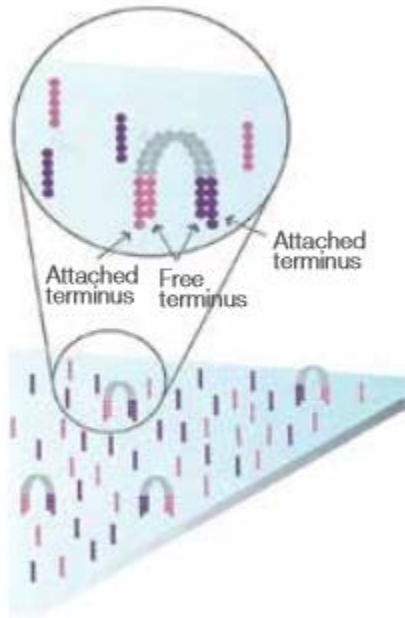


Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

Bridge amplification

# Cluster generation

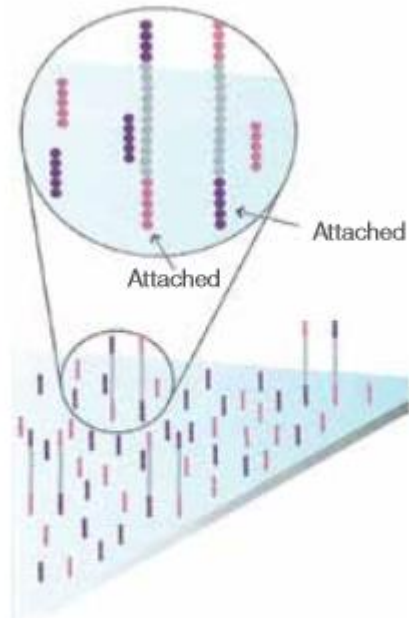
Fragments Become Double Stranded



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

Fragments become double stranded

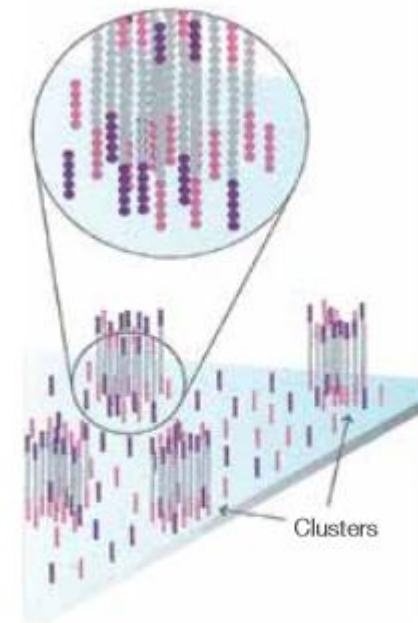
Denature the Double-Stranded Molecules



Denaturation leaves single-stranded templates anchored to the substrate.

Denature the double-stranded molecule

Complete Amplification



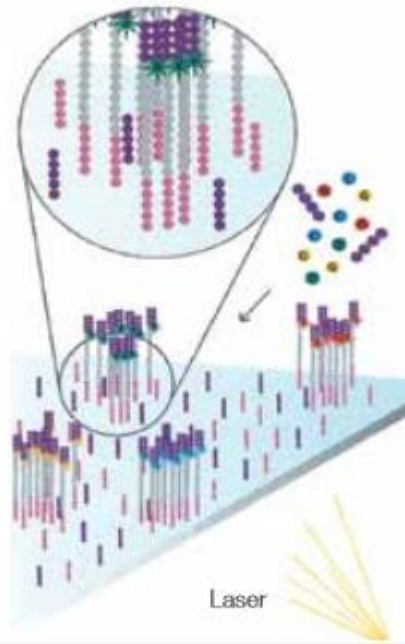
Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

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



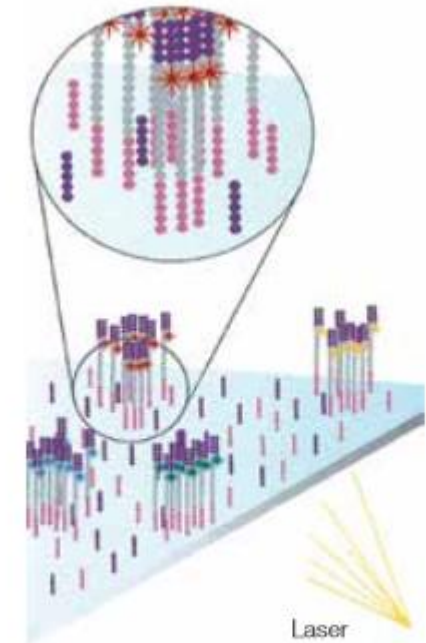
The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.  
Light signal is more strong in cluster

## 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.



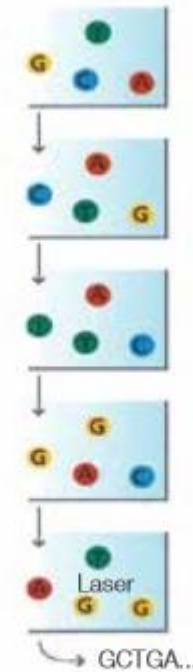
# Sequencing by synthesis

## Image Second Chemistry Cycle



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

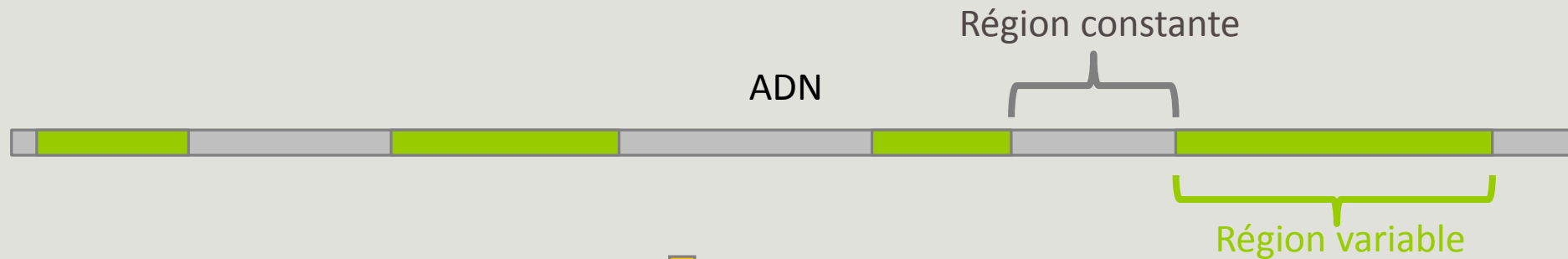
## Sequencing Over Multiple Chemistry Cycles



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

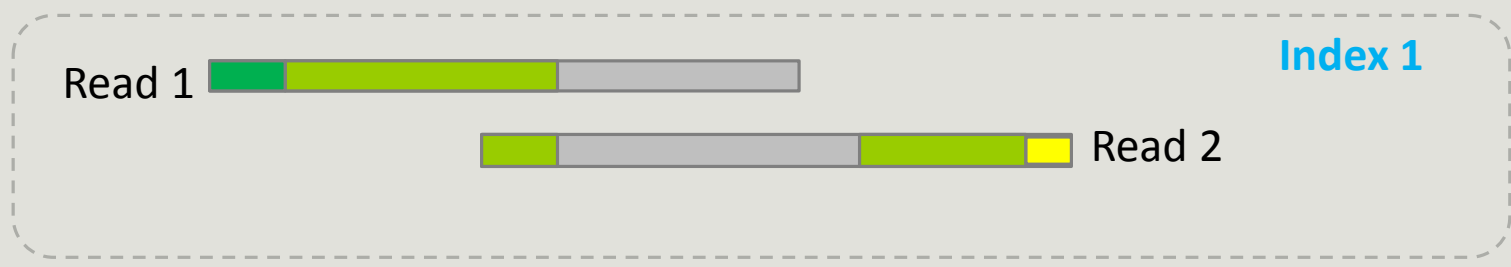
Index Illumina



Adaptateur Illumina

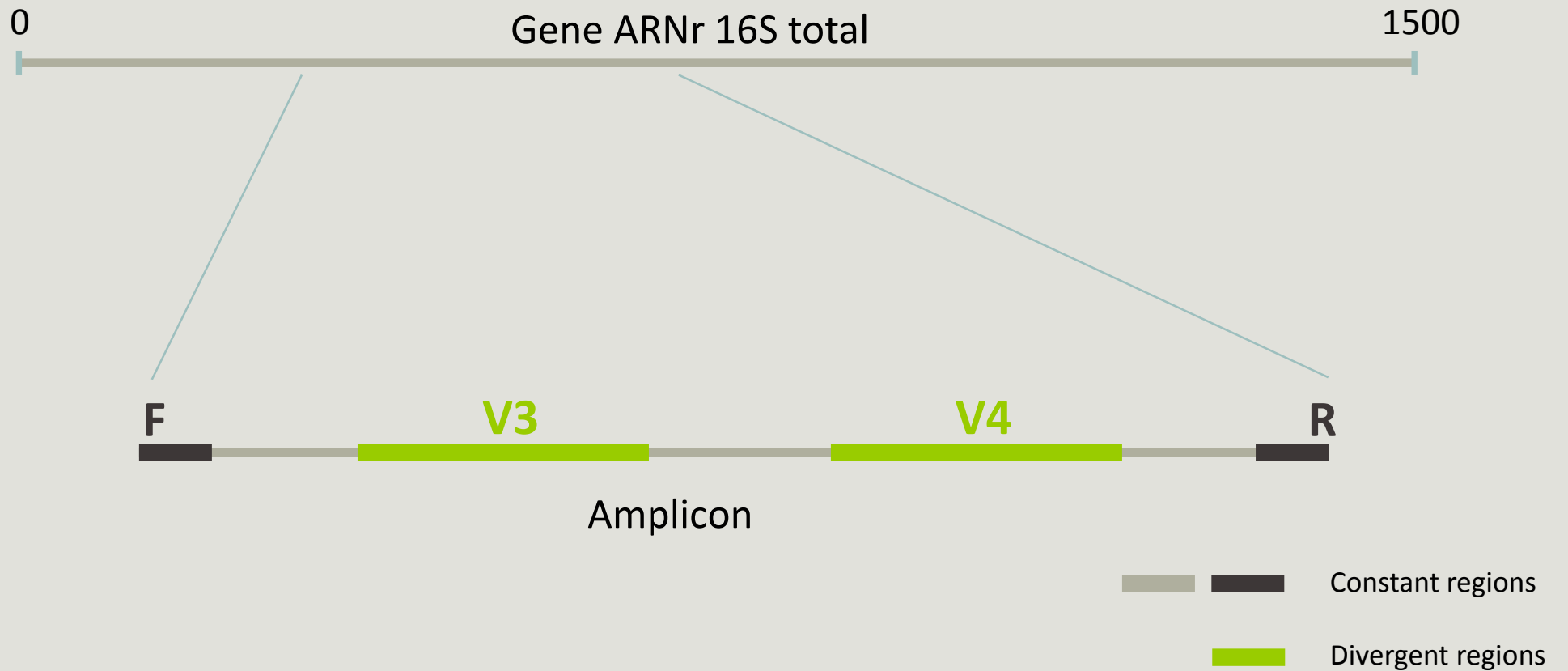
Adaptateur Illumina

↓ Séquençage



# Identification of bacterial populations may be not discriminating

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# Amplification and sequencing

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Sequencing is generally performed on **Roche-454** or **Illumina MiSeq** platforms.

Roche-454 generally produce ~ 10 000 reads per sample

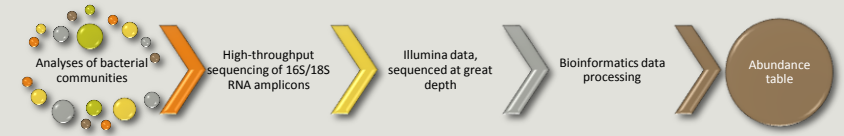
MiSeq ~ 30 000 reads per sample

Sequence length is **>650 bp** for pyrosequencing technology (Roche-454) and **2 x 300 bp** for the MiSeq technology in paired-end mode.



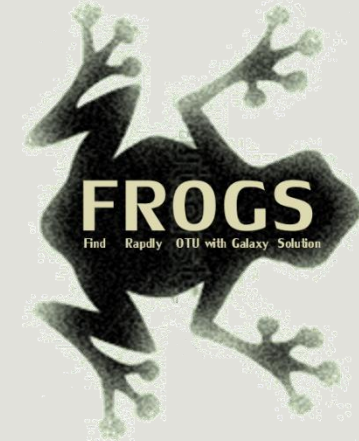
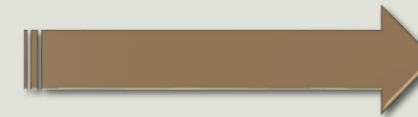
# Methods

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# Which bioinformatics solutions ?

	Disadvantages
QIIME	Installation problem Command lines
UPARSE	Global clustering command lines
MOTHUR	Not MiSeq data without normalization Global hierarchical clustering Command lines
MG-RAST	No modularity No transparency



**QIIME allows analysis of high-throughput community sequencing data**

J Gregory Caporaso et al, *Nature Methods*, 2010; doi:10.1038/nmeth.f.303

**Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities.**

Schloss, P.D., et al., *Appl Environ Microbiol*, 2009, doi: 10.1128/AEM.01541-09

**UPARSE: Highly accurate OTU sequences from microbial amplicon reads**

Edgar, R.C. et al, *Nature Methods*, 2013, dx.doi.org/10.1038/nmeth.2604

**The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes**

F Meyer et al, *BMC Bioinformatics*, 2008, doi:10.1186/1471-2105-9-386

# FROGS ?

Use platform **Galaxy**

Set of **modules** = Tools to analyze your “big” data

**Independent** modules

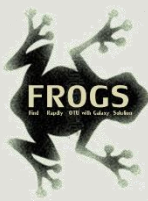
Run on Illumina/454 data **16S, 18S, and 23S**

**New clustering** method

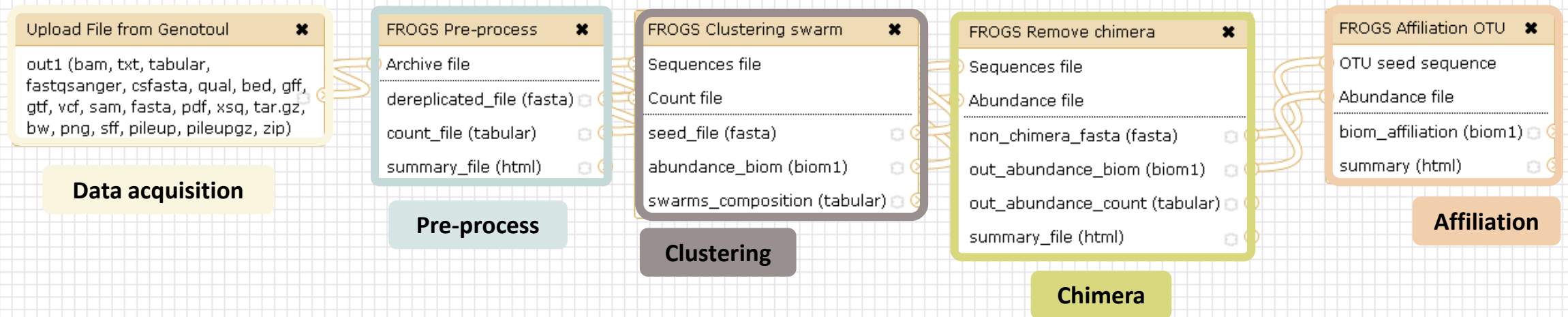
Many **graphics** for interpretation

**User friendly**, hiding bioinformatics infrastructure/complexity

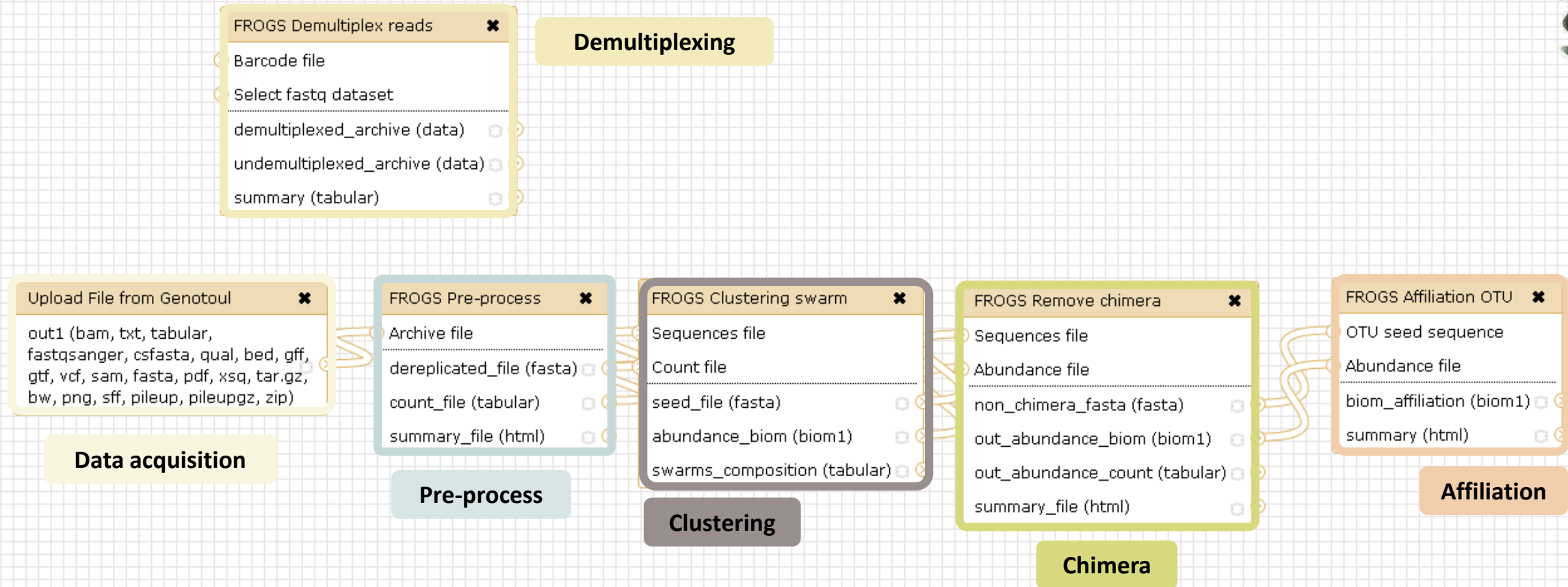
The screenshot displays the Galaxy Sigenae web interface. The main window shows the configuration for the 'FROGS Pre-process Illumina (version 1.0.0)' tool. The interface includes a 'Tools' sidebar on the left with a list of FROGS modules such as 'FROGS FIND RAPIDLY OTU WITH GALAXY SOLUTION', 'FROGS pipeline', 'FROGS Pre-process Illumina', 'FROGS Clustering swarm', 'FROGS Remove chimera', 'FROGS Affiliation otu 16S', 'FROGS abundance normalisation', 'FROGS Filters', 'FROGS Clusters stat', and 'FROGS BIOM to TSV'. The main configuration area contains fields for 'Input type' (Files by samples), 'Reads already contiged ?' (No), 'Samples' (Name), 'Reads 1' (R1 FASTQ file), 'Reads 2' (R2 FASTQ file), 'Expected amplicon size', 'Minimum amplicon size', and 'Maximum amplicon size'. A 'History' sidebar on the right shows a list of previous jobs, including 'FROGS Filters: abundance\_table.biom', 'FROGS Filters: summary.html', 'FROGS Filters: seed.fasta', 'FROGS Filters: summary.txt', 'FROGS Filters: abundance\_table.tsv', 'FROGS Clusters stat: summary.html', 'FROGS Clusters stat: summary.html', 'FROGS Affiliation otu 16S: excluded\_data\_report.html', 'FROGS Affiliation otu 16S: tax\_affiliation.biom', 'FROGS Remove chimera: excluded\_data\_report.html', 'FROGS Remove chimera: non\_chimera\_abundance.biom', 'FROGS Remove chimera: non\_chimera.fasta', and 'FROGS Clustering'.

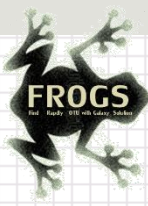


# FROGS Pipeline









**FROGS Abundance normalisation** ✕

- Sequences file
- Abundance file

---

output\_fasta (fasta)

output\_biom (biom1)

summary\_file (html)

**Normalization**

**Upload File from Genotoul** ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

**Data acquisition**

**FROGS Pre-process** ✕

- Archive file
- dereplicated\_file (fasta)
- count\_file (tabular)
- summary\_file (html)

**Pre-process**

**FROGS Clustering swarm** ✕

- Sequences file
- Count file
- seed\_file (fasta)
- abundance\_biom (biom1)
- swarms\_composition (tabular)

**Clustering**

**FROGS Remove chimera** ✕

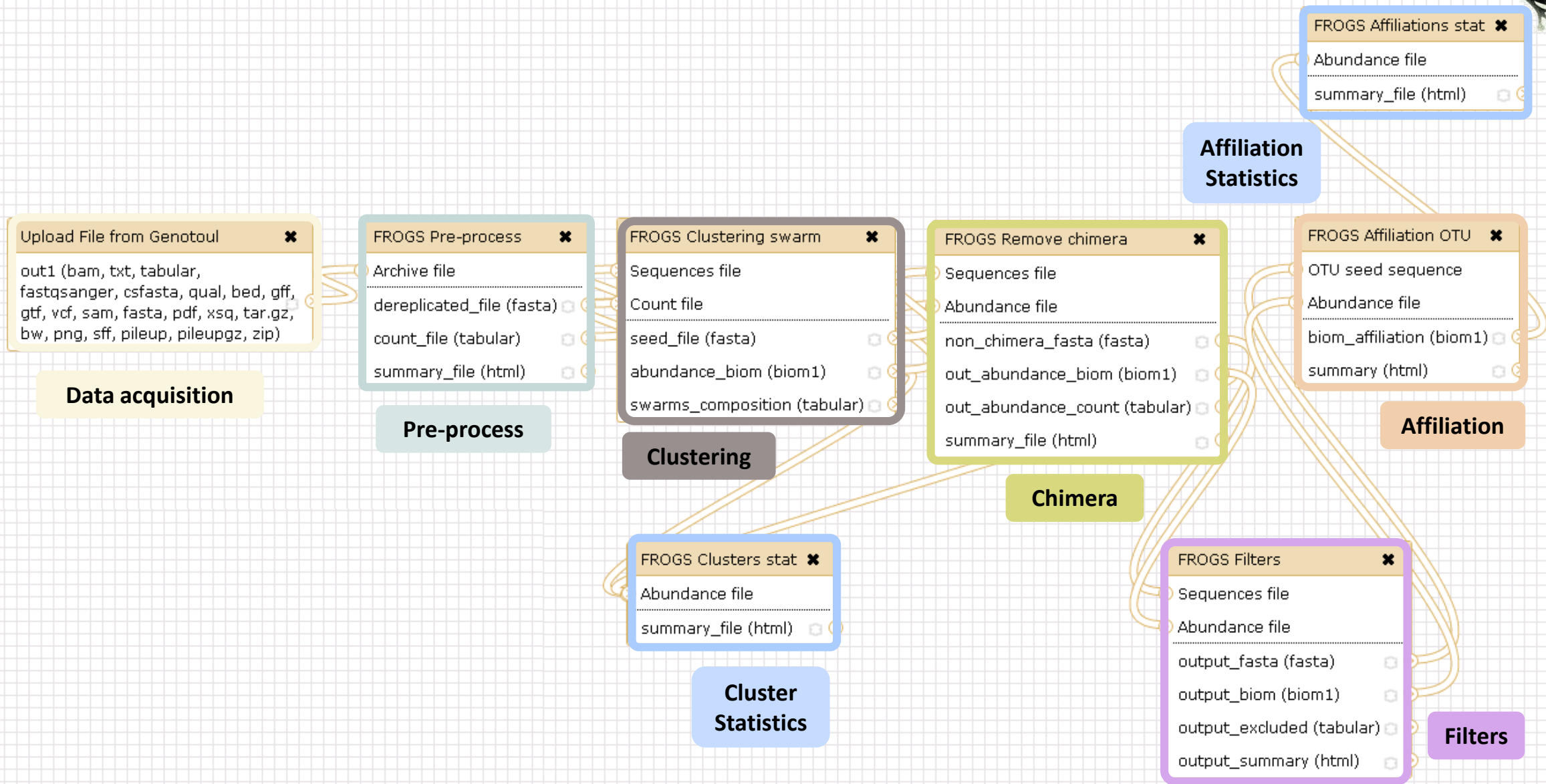
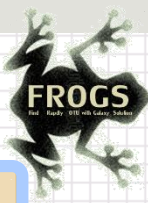
- Sequences file
- Abundance file
- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)

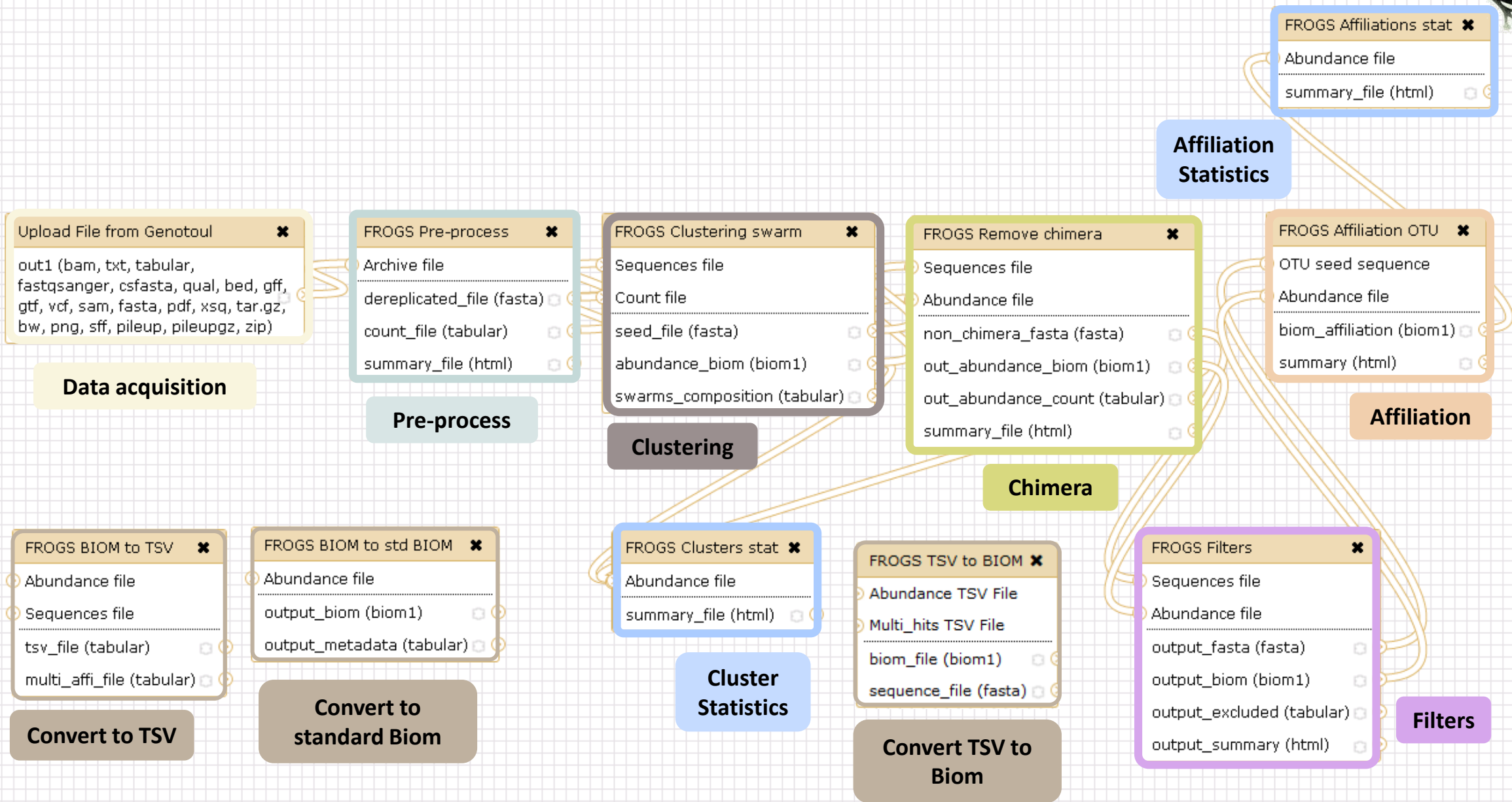
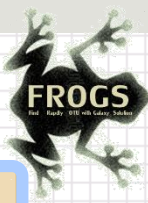
**Chimera**

**FROGS Affiliation OTU** ✕

- OTU seed sequence
- Abundance file
- biom\_affiliation (biom1)
- summary (html)

**Affiliation**







**FROGS Tree**

- OTUs sequence file
- Biom file

---

- out\_tree (nhx)
- html (html)

**Phylogenetics tree**

**FROGS Demultiplex reads**

- Barcode file
- Select fastq dataset

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- demultiplexed\_archive (data)
- undemultiplexed\_archive (data)
- summary (tabular)

**Demultiplexing**

**FROGS Abundance normalisation**

- Sequences file
- Abundance file

---

- output\_fasta (fasta)
- output\_biom (biom1)
- summary\_file (html)

**Normalization**

**Affiliation Statistics**

**FROGS Affiliations stat**

- Abundance file

---

- summary\_file (html)

**Upload File from Genotoul**

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

**Data acquisition**

**FROGS Pre-process**

- Archive file

---

- dereplicated\_file (fasta)
- count\_file (tabular)
- summary\_file (html)

**Pre-process**

**FROGS Clustering swarm**

- Sequences file
- Count file

---

- seed\_file (fasta)
- abundance\_biom (biom1)
- swarms\_composition (tabular)

**Clustering**

**FROGS Remove chimera**

- Sequences file
- Abundance file

---

- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)

**Chimera**

**FROGS Affiliation OTU**

- OTU seed sequence
- Abundance file

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- biom\_affiliation (biom1)
- summary (html)

**Affiliation**

**FROGS BIOM to TSV**

- Abundance file
- Sequences file

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- tsv\_file (tabular)
- multi\_affi\_file (tabular)

**Convert to TSV**

**FROGS BIOM to std BIOM**

- Abundance file

---

- output\_biom (biom1)
- output\_metadata (tabular)

**Convert to standard Biom**

**FROGS Clusters stat**

- Abundance file

---

- summary\_file (html)

**Cluster Statistics**

**FROGS TSV to BIOM**

- Abundance TSV File
- Multi\_hits TSV File

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- biom\_file (biom1)
- sequence\_file (fasta)

**Convert TSV to Biom**

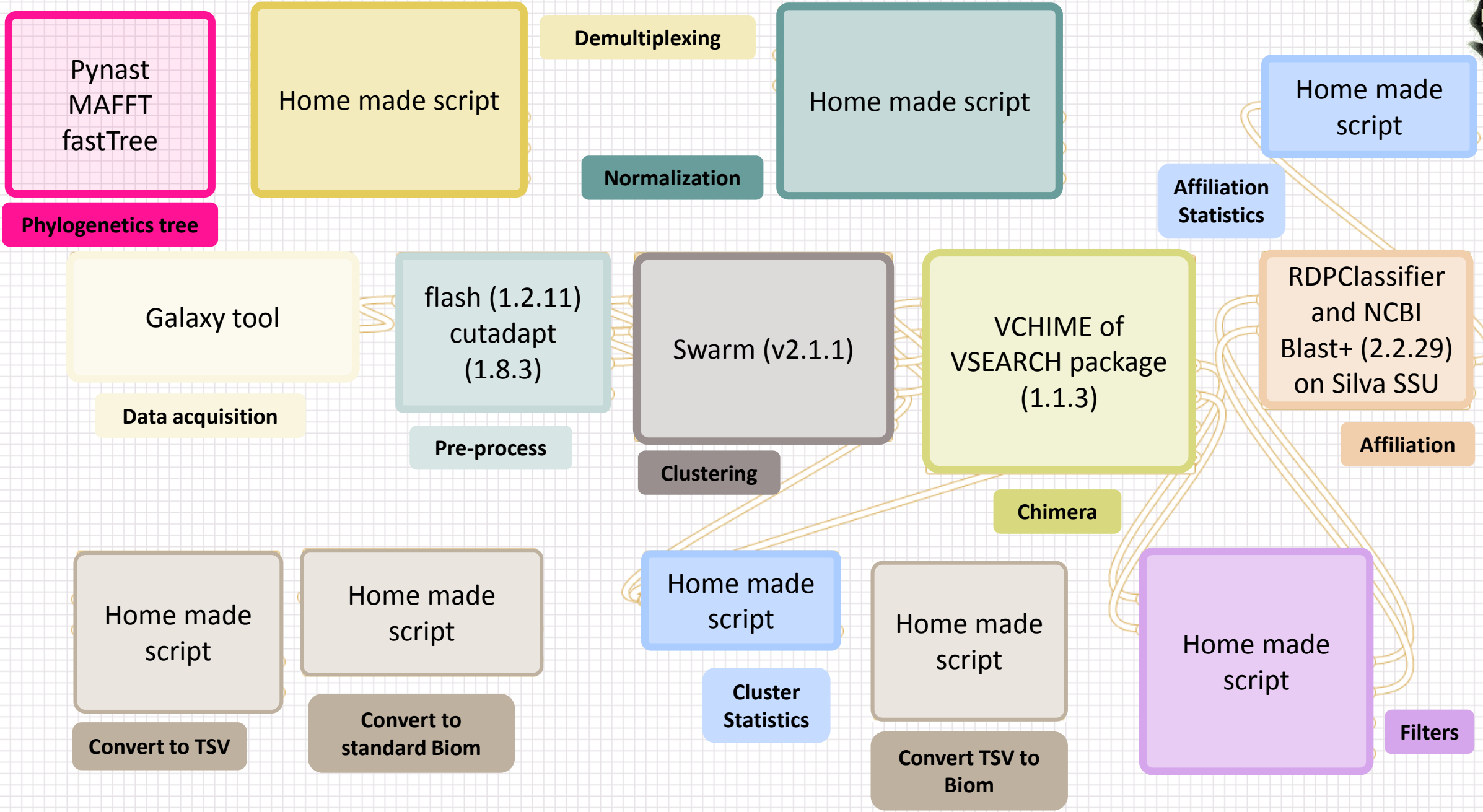
**FROGS Filters**

- Sequences file
- Abundance file

---

- output\_fasta (fasta)
- output\_biom (biom1)
- output\_excluded (tabular)
- output\_summary (html)

**Filters**



# Together go to visit FROGS

In your internet browser (Firefox, chrome, Internet explorer) :

<http://sigenae-workbench.toulouse.inra.fr/>

Enter your email address and password from GenoToul



The screenshot shows the top navigation bar of the Sigenae Galaxy Workbench. The 'User' menu is open, displaying the following options: 'Logged in as geraldine.pascal@inra.fr', 'Preferences', 'Custom Builds', 'Logout', 'Saved Histories', 'Saved Datasets', 'Saved Pages', and 'API Keys'. A red arrow points to the 'User' menu. Below the navigation bar, the main content area displays a welcome message: 'WELCOME TO YOUR GALAXY WORKBENCH. This instance is your toolbox. Sigenae and BioInfo Genotoul teams can add tools to make the best use of Galaxy'. At the bottom, there are logos for Sigenae, GenoToul BioInfo, and the European Union (Fonds Européen de Développement Régional). A banner for 'LA REGION OCCITANIE Pyrénées-Méditerranée' is also visible.



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

**Sequencer**  
 Illumina  
 Select the sequencer family 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 : Reads 1 and Reads 2 are already contiged by pair.

**Samples**

1: Samples

**Name**  
  
 The sample name.

**Reads 1**  
  
 R1 FASTQ file of paired-end reads.

**reads 2**  
  
 R2 FASTQ file of paired-end reads.

**+ Insert Samples**

**Reads 1 size**  
  
 The read1 size.

**Reads 2 size**  
  
 The read2 size.

**Expected amplicon size**

**TOOL CONFIGURATION AND EXECUTION**

**Tools**

**METAGENOMICS**

**FROGS - Find Rapidly Otu with Galaxy Solution**

**FROGS Demultiplex reads**  
 Split by samples the reads in function of inner barcode.

**FROGS Pre-process** Step 1 in metagenomics analysis: denoising and dereplication.

**FROGS Clustering swarm**  
 Step 2 in metagenomics analysis : clustering.

**FROGS Remove chimera** Step 3 in metagenomics analysis : Remove FROGS chimera from samples.

**FROGS Filter OTUs** on several criteria.

**FROGS Affiliation OTU** Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

**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 BIOM file.

**FROGS Abundance normalisation**

**AVAILABLE TOOLS**

**History**

search datasets

**Hantagulomic**  
 29 shown, 14 deleted  
 20.42 MB

**43: FROGS BIOM to std BIOM: blast\_metadata.tsv**

**42: FROGS BIOM to std BIOM: abundance.biom**

**41: FROGS Abundance normalisation: normalized.biom**

**40: FROGS Abundance normalisation: normalized.biom**

**39: FROGS Abundance normalisation: normalized.fasta**

**38: FROGS Abundance normalisation: report.html**

**37: FROGS Abundance normalisation: normalized.biom**

**36: FROGS Abundance normalisation: normalized.fasta**

**30: FROGS BIOM to TSV: multi\_hits.tsv**

**29: FROGS BIOM to TSV: abundance.tsv**

**23: FROGS Affiliation OTU: report.html**

**DATASETS HISTORY**



**Galaxy** Analyze Data Workflow Shared Data Visualization Help User Using 5%

**Tools**

**METAGENOMICS**

**FROGS**

**OTUS RECONSTRUCTION**

[FROGS Demultiplex reads](#)  
Attribute reads to samples in function of inner barcode.

[FROGS Pre-process](#) Step 1 in metagenomics analysis: denoising and dereplication.

[FROGS Clustering swarm](#)  
Step 2 in metagenomics analysis : clustering.

[FROGS Remove chimera](#) Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

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

[FROGS Affiliation OTU](#) Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

[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.

[FROGS Abundance normalisation](#)

[FROGS Tree](#) Reconstruction of phylogenetic tree

---

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

**Sequencer**  
Illumina  
Select the sequencer family 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 : Reads 1 and Reads 2 are already contiged by pair.

**Samples**

**1: Samples**

**Name**  
The sample name.

**Reads 1**  
No fastq dataset available.  
R1 FASTQ file of paired-end reads.

**reads 2**  
No fastq dataset available.  
R2 FASTQ file of paired-end reads.

**+ Insert Samples**

**Reads 1 size**  
The read1 size.

**Reads 2 size**  
The read2 size.

**Expected amplicon size**

---

**History**

**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

Demultiplexing

Pre-process

Clustering

Chimera

Filters

Affiliation

Cluster Stat

Affiliation Stat

Biom to std Biom

Biom to TSV

TSV to Biom

Normalization

Phylogenetics Tree

Waiting to run

Currently running

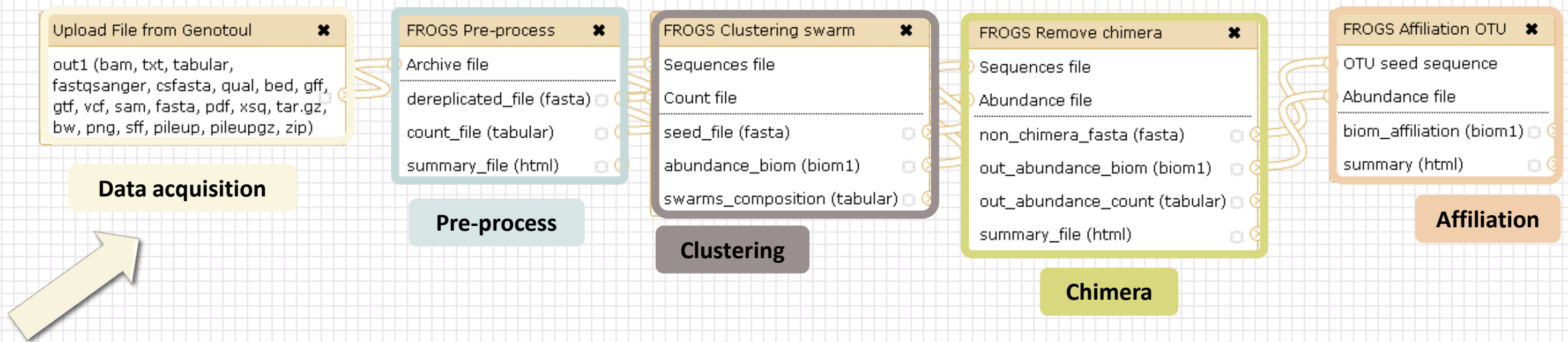
Result files

# Upload data

---



Go to demultiplexing tool



# What kind of data ?

4 Upload → 4 Histories

---

Multiplexed data

Pathobiomes  
rodents and ticks

`multiplex.fastq`

`barcode_forward.ta  
bular`

454 data

Freshwater sediment  
metagenome

`454.fastq.gz`

SRA number

- SRR443364

MiSeq

R1 fastq + R2 fastq

Farm animal feces  
metagenome

`sampleA_R1.fastq`

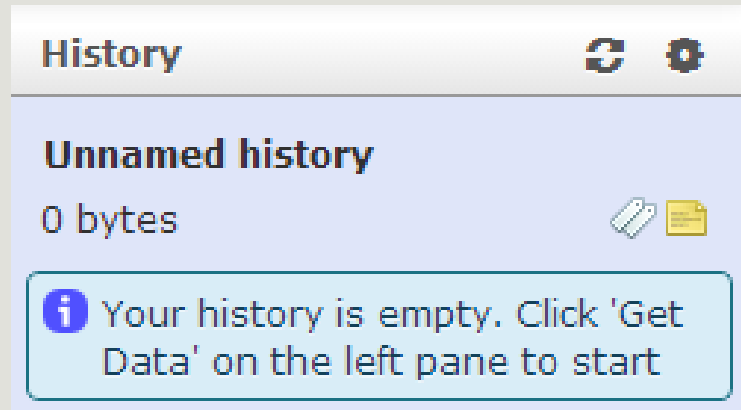
`sampleA_R2.fastq`

MiSeq merged fastq in  
archive tar.gz

Farm animal feces  
metagenome

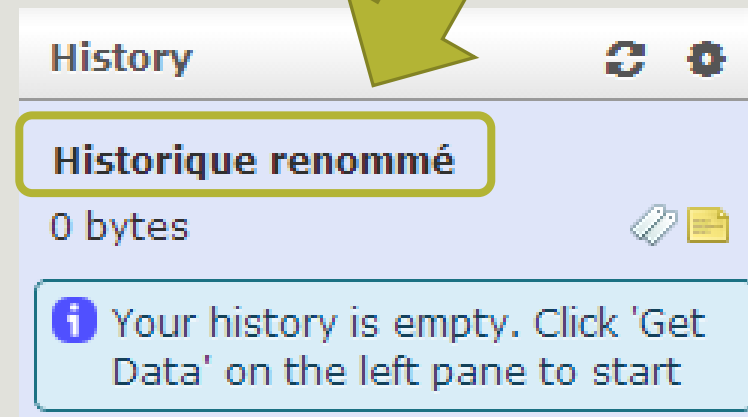
`100spec_90000seq_9s  
amples.tar.gz`

## 1<sup>ST</sup> CONNEXION



## RENAME HISTORY

- click on **Unnamed history**,
- Write your new name,
- Tap on Enter.



# History gestion

---

- Keep all steps of your analysis.
- Share your analyzes.
- At each run of a tool, a new dataset is created. The data are not overwritten.
- Repeat, as many times as necessary, an analysis.
- All your logs are automatically saved.
- Your published histories are accessible to all users connected to Galaxy (Shared Data / Published Histories).
- Shared histories are accessible only to a specific user (History / Option / Histories Shared With Me).
- To share or publish a history: User / Saved histories / Click the history name / Share or Publish

# Saved Histories

Logged in as mbernard@toulouse.inra.fr  
Logout  
**Saved Histories**  
Saved Datasets  
Saved Pages  
API Keys  
Public Name

### Saved Histories

search history names and tags  
[Advanced Search](#)

Name	Datasets	Tags	Sharing	Size on Disk	Created	Last Updated	Status
<input type="checkbox"/> Contiged	<span>20</span> <span>2</span> <span>5</span> <span>5</span>	<a href="#">0 Tags</a>		57.9 MB	~ 2 hours ago		current history
<input type="checkbox"/> MiSeq contiged	<span>11</span> <span>9</span> <span>12</span>	<a href="#">0 Tags</a> <a href="#">Shared</a>		175.9 MB	~ 7 hours ago	~ 3 hours ago	
<input type="checkbox"/> barcode_formation	<span>5</span>	<a href="#">0 Tags</a>		4.5 MB	~ 12 hours ago	~ 10 hours ago	

Analyse OK

Analyze in progress

Analyze in waiting

Analyze not OK

# Your turn! - 1

---

LAUNCH UPLOAD TOOLS



# Accounts:



- anemone
- arome
- aster
- bleuet
- camelia
- capucine
- chardon
- clematite
- cobee
- coquelicot
- cosmos
- Password: f1o2r3!

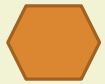
# Your turn: exo 1

---



Create the 1st history **multiplexed**

Import files « **multiplex.fastq** » and « **barcode\_forward.tabular** » present in the **Genotoul** folder /work/formation/FROGS/



Create the 2nd history **454**

Import file « **454.fastq.gz** » present in the **Genotoul** folder /work/formation/FROGS/  
(datatype fastq or fastq.gz is the same !)



Create the 3rd history **MiSeq R1 R2**

Import files « **sampleA\_R1.fastq** » and « **sampleA\_R2.fastq** » present in the **Genotoul** folder /work/formation/FROGS/

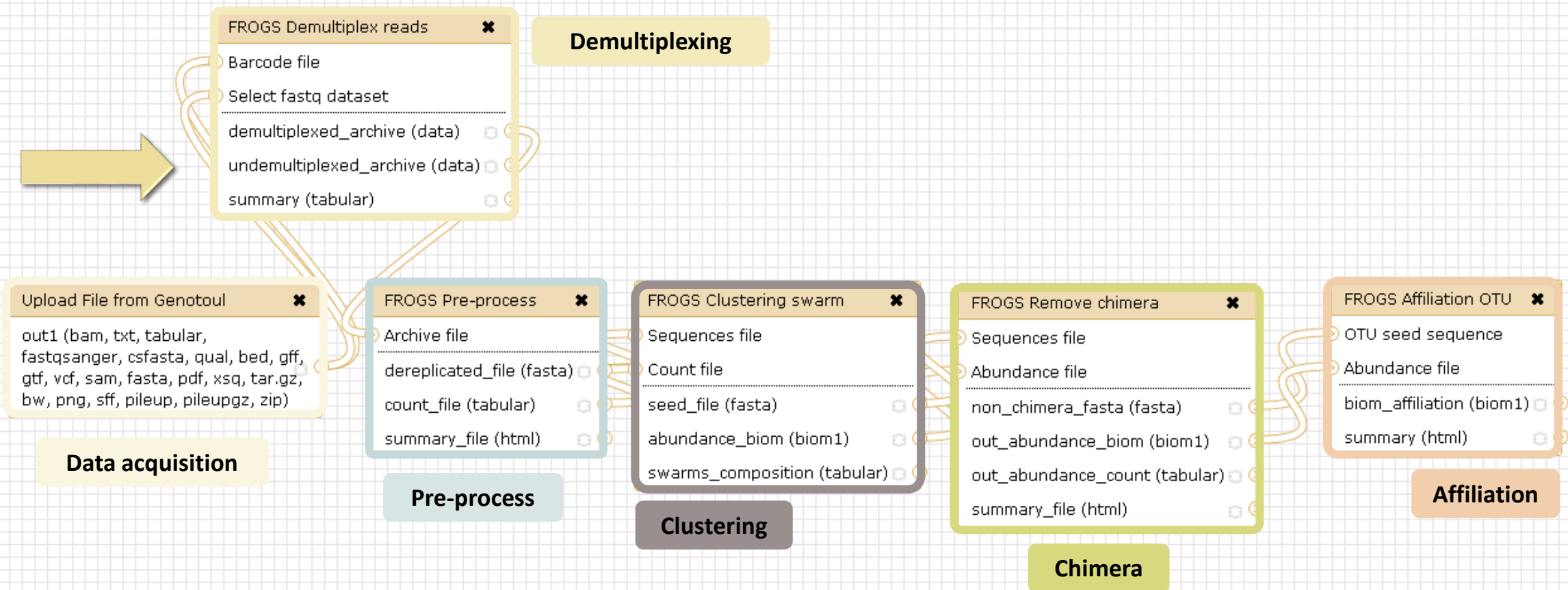


Create the 4th history **MiSeq merged**

Import archive file « **100spec\_90000seq\_9samples.tar.gz** » present in the **Genotoul** folder /work/formation/FROGS/

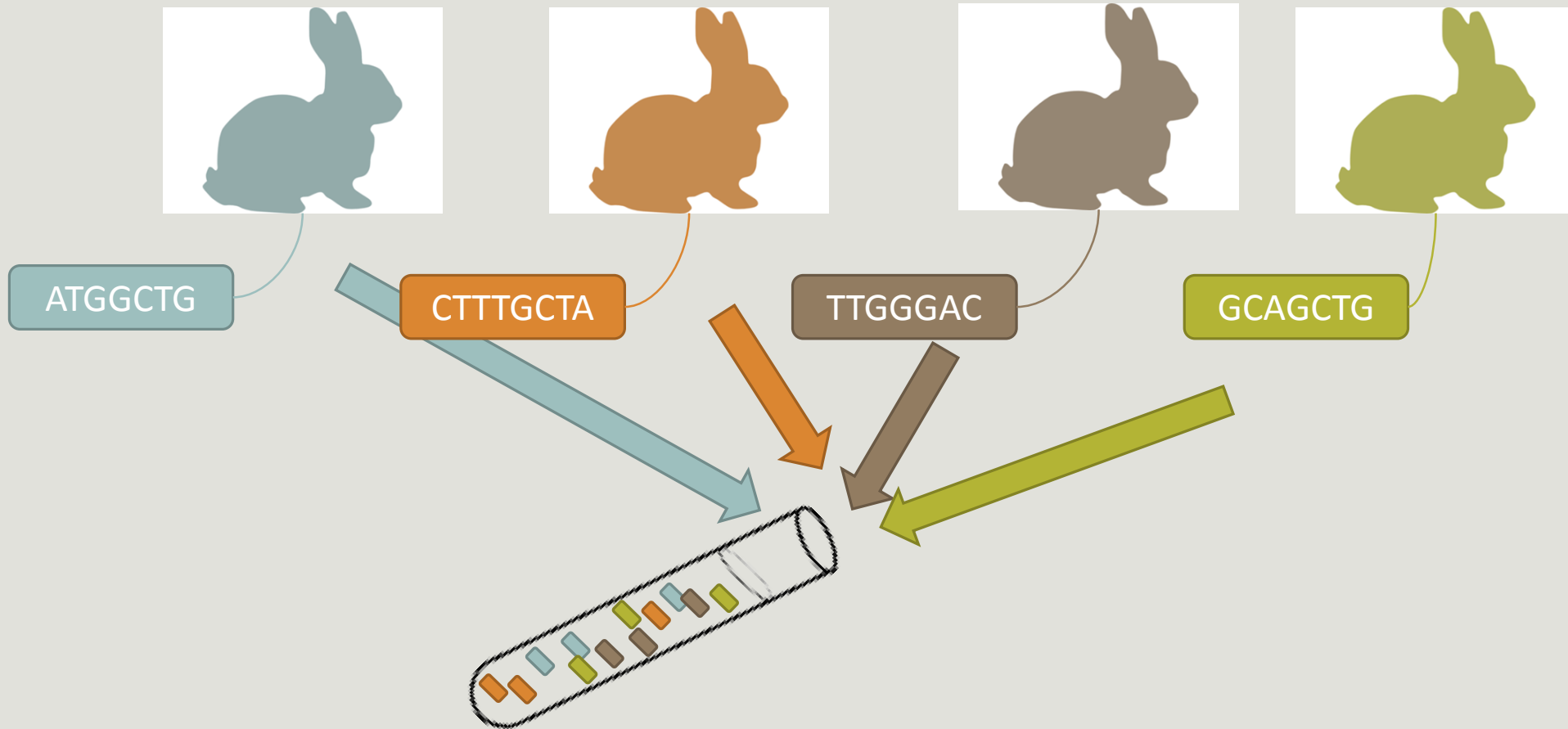
# Demultiplexing tool

---



# Barcoding ?

---



# Demultiplexing

---

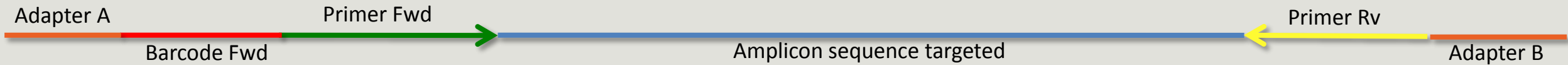
Sequence demultiplexing in function of barcode sequences :

- In forward
- In reverse
- In forward and reverse

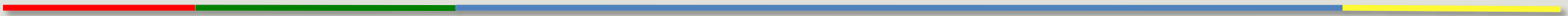
Remove unbarcoded or ambiguous sequences

# Demultiplexing forward

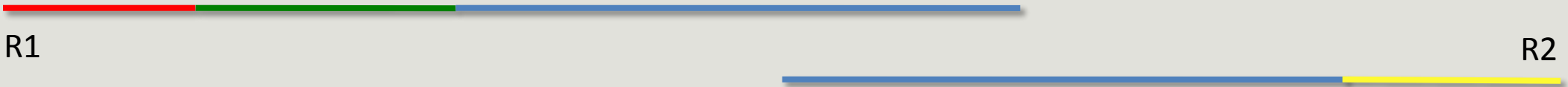
---



Single-end sequencing

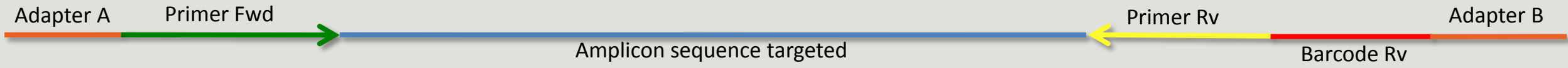


Paired-end sequencing

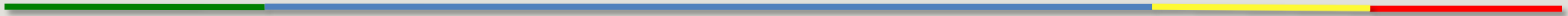


# Demultiplexing reverse

---



Single end sequencing



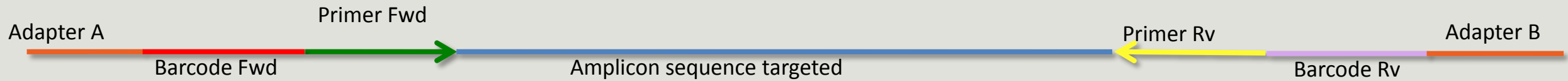
Paire end sequencing





# Demultiplexing forward and reverse

---

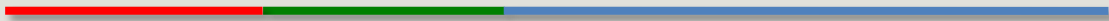


Single end sequencing

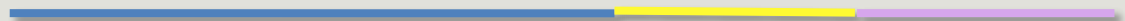


Paire end sequencing

R1



R2



# Your turn! - 2

---

LAUNCH DEMULTIPLEX READS TOOL

The tool parameters depend on the input data type

FROGS Demultiplex reads (version 1.1.0)

**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:**  

  
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 ?:**  

  
at the beginning of the forward end or of the reverse end or both?

Forward  
Reverse  
Both ends  
Execute

FROGS Demultiplex reads (version 1.1.0)

**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:**  

  
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:**  

  
Number of mismatches allowed in barcode

**barcode on which end ?:**  

  
at the beginning of the forward end or of the reverse end or both?

Forward  
Reverse  
Both ends  
Execute

FROGS Demultiplex reads ✕

Barcode file

Select fastq dataset

---

demultiplexed\_archive (data)

undemultiplexed\_archive (data)

summary (tabular)

## Exercise 2

---

In **multiplexed** history launch the demultiplex tool:

« The Patho-ID project, rodent and tick's pathobioms study, financed by the metaprogram INRA-MEM, studies zoonoses on rats and ticks from multiple places in the world, the co-infection systems and the interactions between pathogens. In this aim, they have extracted hundreds of or rats and ticks samples from which they have extracted 16S DNA and sequenced them first time on Roche 454 platform and in a second time on Illumina Miseq platform. For this courses, they authorized us to publicly shared some parts of these samples. »

Parasites & Vectors (2015) 8:172 DOI 10.1186/s13071-015-0784-7. **Detection of Orientia sp. DNA in rodents from Asia, West Africa and Europe.** Jean François Cosson, Maxime Galan, Emilie Bard, Maria Razzauti, Maria Bernard, Serge Morand, Carine Brouat, Ambroise Dalecky, Khalilou Bâ, Nathalie Charbonnel and Muriel Vaysier-Taussat

# Exercise 2

---

In **multiplexed** history launch the demultiplex tool:

Data are **single end** reads

→ only 1 fastq file

Samples are characterized by one barcode in forward strands

→ multiplexing « **forward** »

Inputs :

```
2: /work/frogs          
/multiplex.fastq
```










---

```
1: /work/frogs          
/barcode forward.tabular
```

# Exercise 2

---

Demultiplex tool asks for 2 files: one « fastq » and one « tabular »

1. Play with pictograms       
2. Observe how is built a fastq file. 
3. Look at the stdout, stderr when available (in the  pictogram )

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

**Barcode file**

24: barcode\_forward.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**

6: multiplex.fastq

Specify dataset of your single end reads

**Barcode mismatches**

0

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?

Execute




# 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](#)

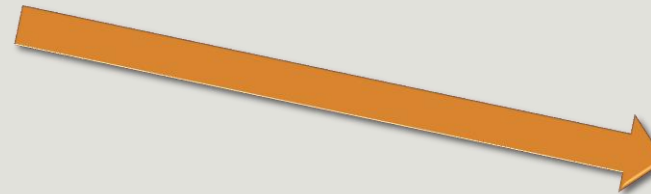


# Results

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. So these sequences are non-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

```
@HNHOSKD01ALD0H  
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 summary describes how many sequences are attributed for each sample.

# Pre-process tool

---

**FROGS Demultiplex reads** ✕

- Barcode file
- Select fastq dataset

---

demultiplexed\_archive (data) Ⓞ Ⓞ

undemultiplexed\_archive (data) Ⓞ Ⓞ

summary (tabular) Ⓞ

**Demultiplexing**

**Upload File from Genotoul** ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

**Data acquisition**



**FROGS Pre-process** ✕

- Archive file
- dereplicated\_file (fasta)
- count\_file (tabular)
- summary\_file (html)

**Pre-process**

**FROGS Clustering swarm** ✕

- Sequences file
- Count file
- seed\_file (fasta)
- abundance\_biom (biom1)
- swarms\_composition (tabular)

**Clustering**

**FROGS Remove chimera** ✕

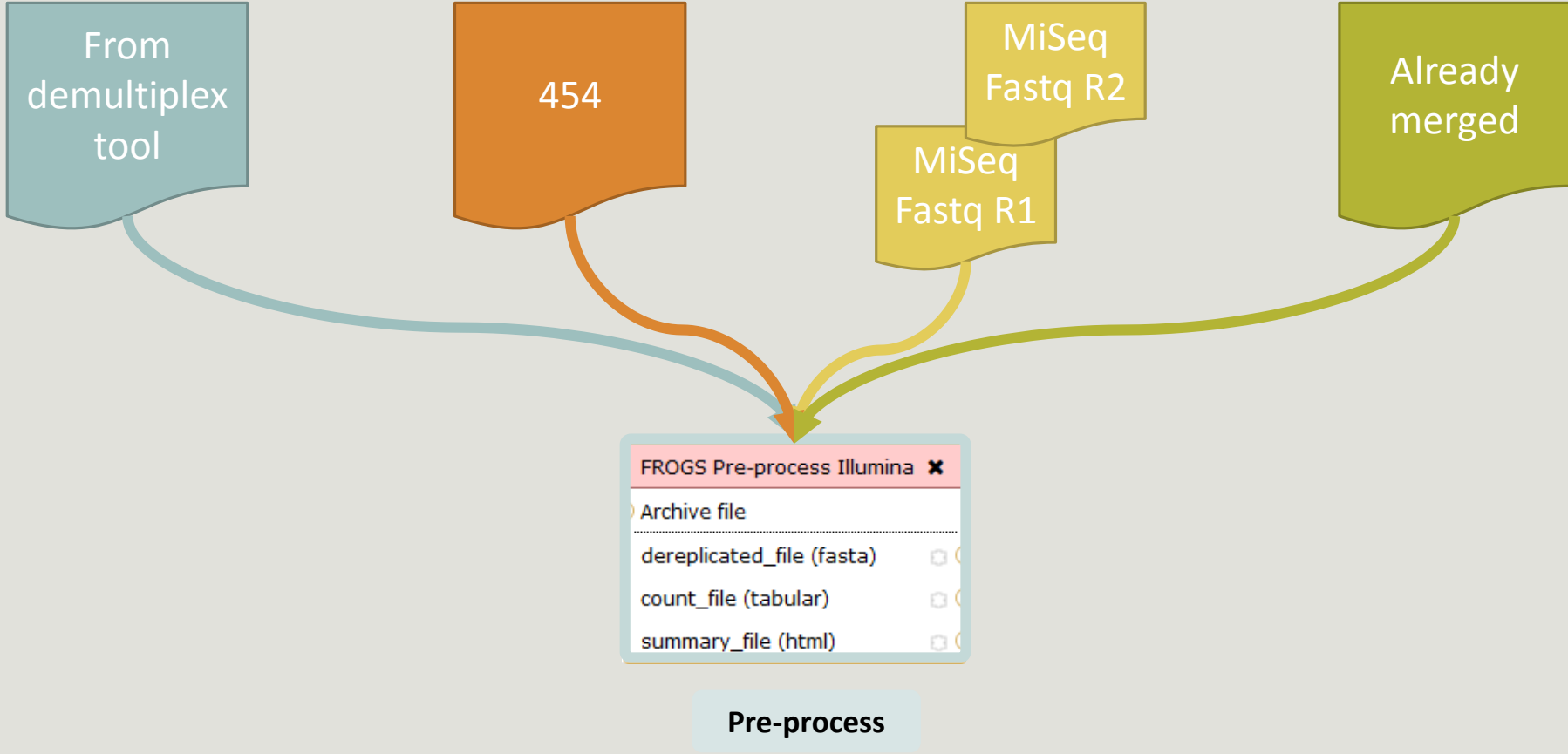
- Sequences file
- Abundance file
- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)

**Chimera**

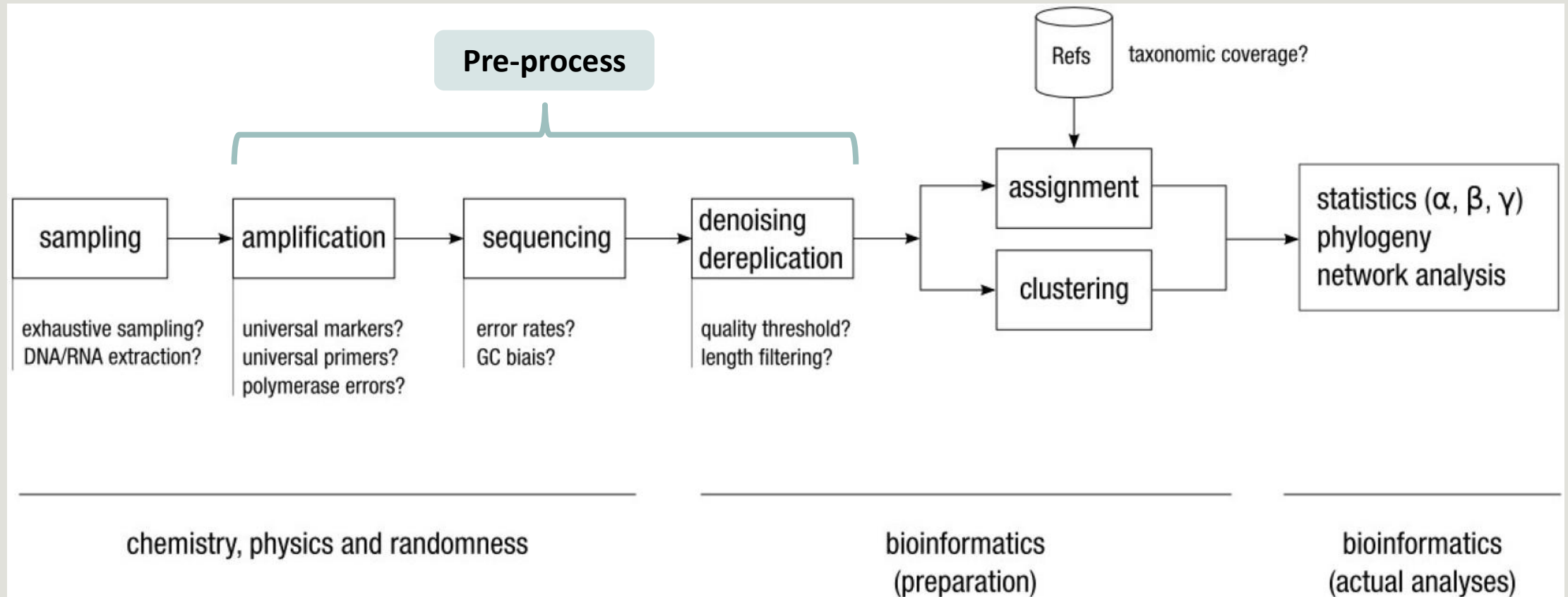
**FROGS Affiliation OTU** ✕

- OTU seed sequence
- Abundance file
- biom\_affiliation (biom1)
- summary (html)

**Affiliation**



# Amplicon-based studies general pipeline





# Pre-process

---

- Delete sequence with not expected lengths
- Delete sequences with ambiguous bases (N)
- Delete sequences do not contain good primers
- 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 Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 2.0.0)** 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**  
60: /work/formation/FROGS/sampleA\_R2.fastq  
R1 FASTQ file of paired-end reads.

**reads 2**  
60: /work/formation/FROGS/sampleA\_R2.fastq  
R2 FASTQ file of paired-end reads.

+ Insert Samples

**Reads 1 size**  
250  
The read1 size.

**Reads 2 size**  
250  
The read2 size.

**Expected amplicon size**  
410  
Maximum amplicon length expected in approximate.

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

Parameters for the merging

**Minimum amplicon size**

The minimum size for the amplicons.

**Maximum amplicon size**

The maximum size for the amplicons.

**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'.

**3' primer**

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

[V5] 16S variability

Primer sequences

Pre-process example 1

Example for:

- Sanger 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	Sequence file
my_sample The sample name.	1: /work/formation/FROGS/454.fastq.gz FASTQ file of sample.

+ Insert Samples

**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

# Your turn! - 3

---

GO TO EXERCISES 3

# Exercise 3.1

---

Go to « 454 » history

Launch the pre-process tool on that data set

→ objective : understand the parameters

1- Test different parameters for « minimum and maximum amplicon size »

2- Enter these primers: Forward: ACGGGAGGCAGCAG Reverse: AGGATTAGATACCCTGGTA

454

Size range of 16S V3-V4:  
[ 380 – 500 ]

**FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 2.0.0)** Options

**Sequencer**  
454  
Select the sequencing technology 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**  
70: /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 in the primer file.

**3' primer**  
AGGATTAGATACCCTGGTA  
The 3' primer sequence (wildcards are accepted). The orientation is detailed in the primer file.

Execute

Sample name is required

Primers used for sequencing V3-V4:  
Forward: ACGGGAGGCAGCAG  
Reverse: AGGATTAGATACCCTGGTA



# Exercise 3.1

---

What do you understand about amplicon size, which file can help you ?

What is the length of your reads before preprocessing ?

Do you understand how enter your primers ?



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



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

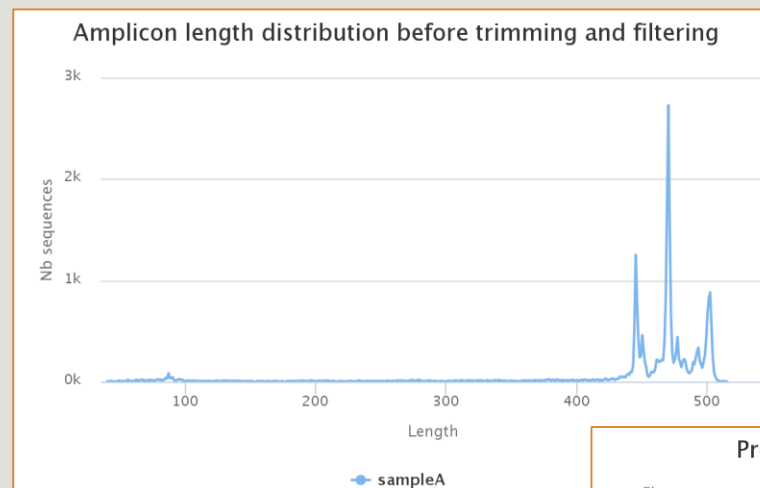
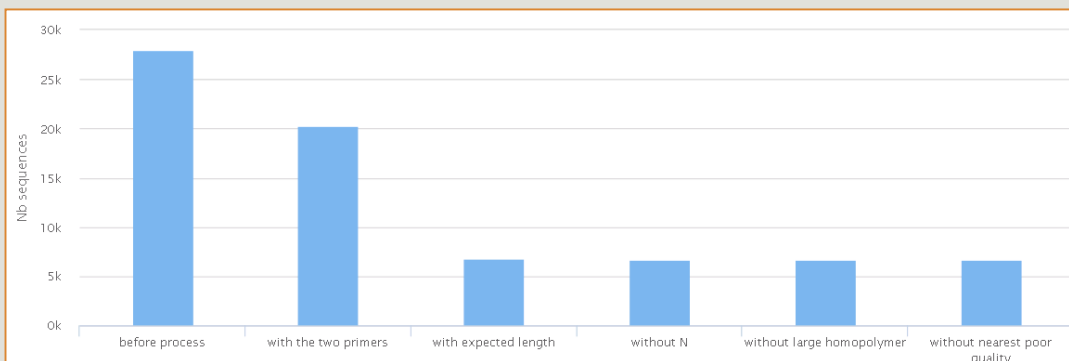


Explore the file « FROGS Pre-process: report.html »

*Who loose a lot of sequences ?*

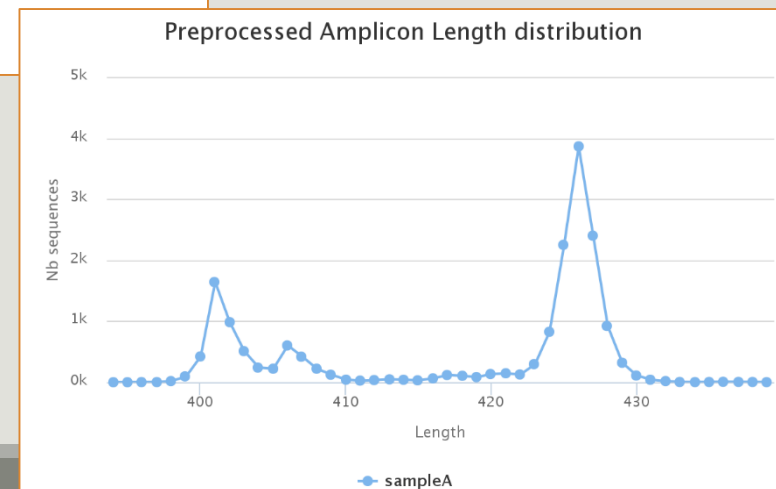
454

<input type="checkbox"/> Samples	before process	with the two primers	with expected length	without N	without large homopolymer	without nearest poor quality
<input type="checkbox"/> sample_454	28,009	20,227	6,806	6,677	6,675	6,672



To be kept, sequences must have the 2 primers

To adjust your filtering, check the distribution of sequence lengths.



# Cleaning, how it work ?

---

Filter contig sequence **on its length** which must be between min-amplicon-size and max-amplicon-size

use **cutadapt** to search and **trim primers** sequences with less than 10% differences

**Minimum amplicon size:**

The minimum size for the amplicons.

**Maximum amplicon size:**

The maximum size for the amplicons.

# Cleaning, how it work ?

---

**dereplicate** sequences and return one **uniq fasta file** for all sample and a **count table** to indicate **sequence abundances among sample**.

In the HTML report file, you will find for each filter the number of sequences passing it, and a table that details these filters for each sample.

# Exercise 3.2

---

Go to « [MiSeq R1 R2](#) » history

Launch the pre-process tool on that data set

→ objective: understand flash software

# The aim of Flash is to merge R1 with R2

---

1st case: Impossible to merge

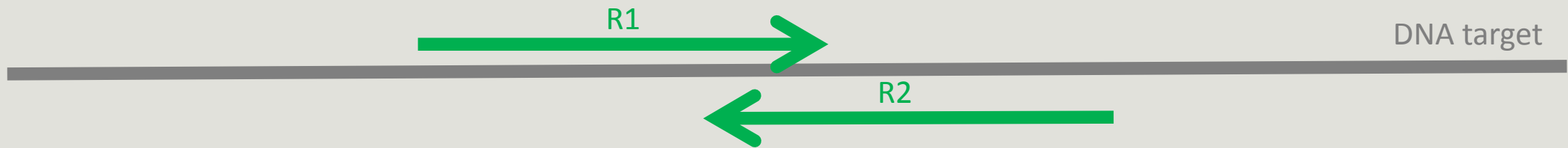


This case is not yet take in charge by FROGS, but soon available in FROGS V3.0

# The aim of Flash is to merge R1 with R2

---

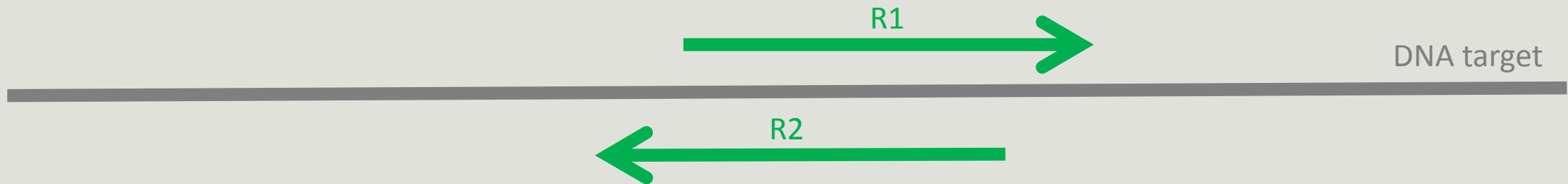
2<sup>nd</sup> case: flash have to find overlapping region between R1 and R2



# The aim of Flash is to merge R1 with R2

---

3rd case: R1 and R2 cover entirely the target region



Careful

This case is not treated by FROGS, you must trim your sequences before putting in FROGS.  
Ask to a bioinformatician to do it.

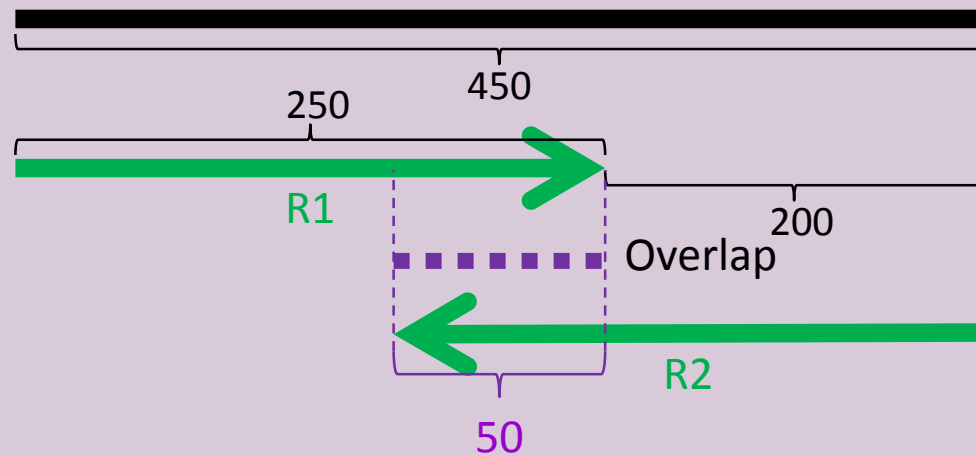


# Flash, have to determine the overlap size

## 1 - The Minimum overlap

### Representation

The longest amplicon = maximum\_amplicon\_size



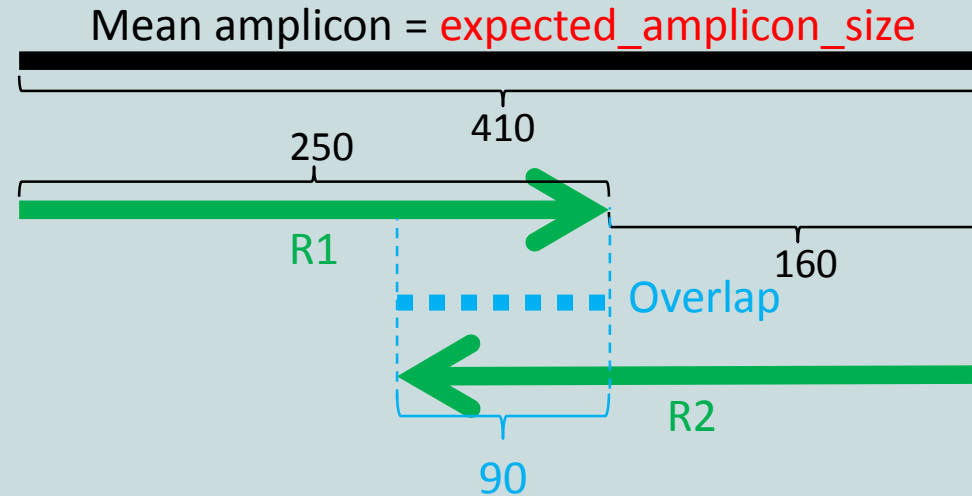
### Computation

$$\text{Min overlap} = R1\_size + R2\_size - \text{maximum\_amplicon\_size}$$

$$\text{Min overlap} = 250 + 250 - 450 = 50$$

## 2 - The Maximum overlap:

### Representation



### Computation

$$\text{Expected\_overlap} = R1\_size + R2\_size - \text{expected\_amplicon\_size} = 250 + 250 - 410 = 90$$

$$\text{Maximum\_overlap} = \text{Expected\_overlap} + \min(20, (\text{expected\_amplicon\_size} - \text{minimum\_amplicon\_size})/2)$$

$$\text{Maximum\_overlap} = 90 + \min(20, 410 - 340)$$

$$\text{Maximum\_overlap} = 90 + \min(20, 35) = 110$$

The flash **maximum\_overlap** is not the maximum overlap but the overlap for an amplicon size greater than 90% of the set of sizes. This is why we take the expected size (medium amplicon) and add a small correction factor. Anyway flash is not sensitive to the ten nucleotides.

# Waited data

---

- Reads 1 size → OK → 250
- Reads 2 size → OK → 250
- Expected amplicon size **More complex to understand** → **410**
- Minimum amplicon size → OK → 340
- Maximum amplicon size → OK → 450
- Sequencing protocol → OK → standard
- 5' primer → OK → CCGTCAATTC
- 3' primer → OK → CCGCNGCTGCT



```
>ERR619083.M00704
CCGTCAATTCATTGAGTTTCAACCTTGCGGCCGCTACTTCCCAGGCGGTACGTT
TATCGCGTTAGCTTCGCCAAGCACAGCATCCTGCGCTTAGCCAACGTACATCG
TTTAGGGTGTGGACTACCCGGGTATCTAATCCTGTTTCGCTACCCACGCTTTCG
AGCCTCAGCGTCAGTGACAGACCAGAGAGCCGCTTTCGCCACTGGTGTTCCTC
CATATATCTACGCATTTACCCGCTACACATGGAATTCCACTCTCCCCTTCTGC
ACTCAAGTCAGACAGTTTCCAGAGCACTCTATGGTTGAGCCATAGCCTTTTAC
TCCAGACTTTCCTGACCGACTGCACTCGCTTTACGCCAATAAATCCGGACAA
CGCTTGCCACCTACGTATTA CCGCNGCTGCT
```

Real 16S sequenced fragment

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

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

**Reads already contiged ?**  
  
 The inputs contain 1 file by sample : R1 and R2 are already merged by pair.

**Samples**  
 1: Samples

**Name**  
  
 The sample name.

**Reads 1**  
     
 R1 FASTQ file of paired-end reads.

**reads 2**  
     
 R2 FASTQ file of paired-end reads.

**Reads 1 size**  
  
 The read1 size.

**Reads 2 size**  
  
 The read2 size.

Size with primers

**Expected amplicon size**  
  
 Maximum amplicon length expected in approximately 90% of the amplicons.

**mismatch rate.**  
  
 The maximum rate of mismatches in the overlap region

**Minimum amplicon size**  
  
 The minimum size for the amplicons.

**Maximum amplicon size**  
  
 The maximum size for the amplicons.

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

**5' primer**  
  
 The 5' primer sequence (with parameters).

**3' primer**  
  
 The 3' primer sequence (with parameters).

Primers used for sequencing V5 region:  
Forward: CCGTCAATTC  
Reverse: CCGCNGCTGCT  
Lecture 5' → 3'

✓ Execute

# Exercise 3.2

---

Interpret « FROGS Pre-process: report.html » file.

**Expected amplicon size**

410

Maximum amplicon length expected in approximately 90% of the amplicons.

**mismatch rate.**

0.1

The maximum rate of mismatches in the overlap region

**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**

CCGTCAATTC

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

**3' primer**

CCGCNGCTGCT

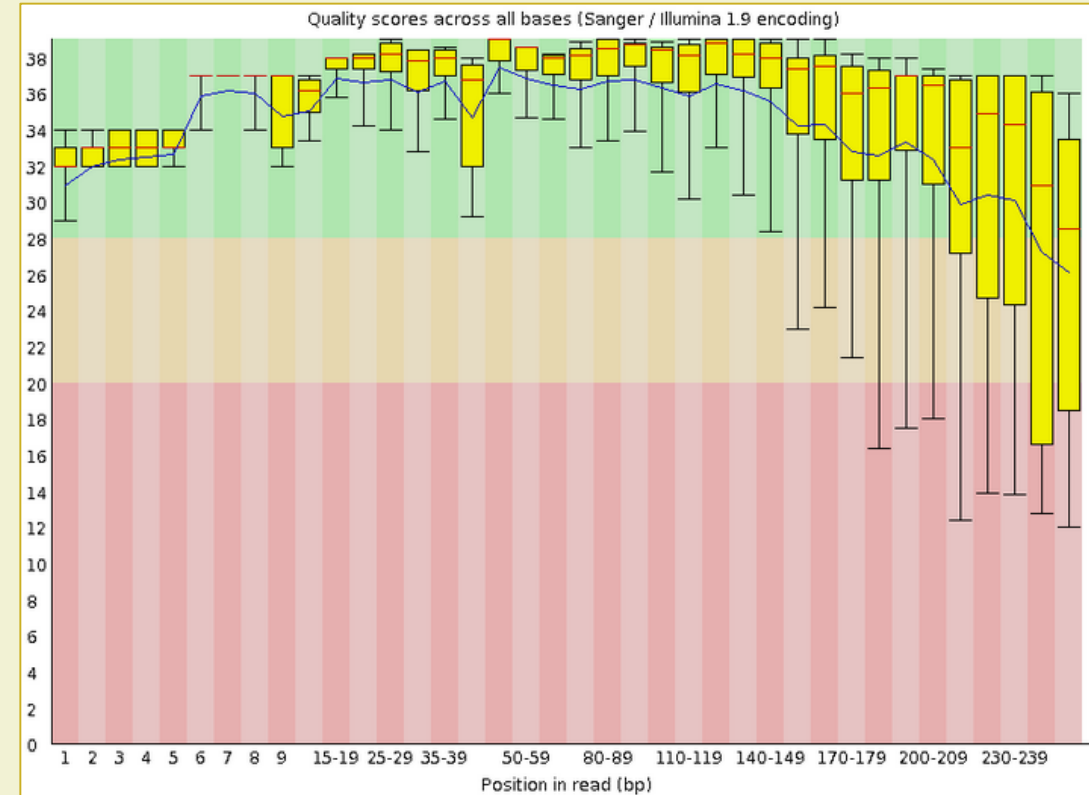
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Execute

To increase if your sequences have low qualities  
Use FASTQC to know it!

**FastQC: fastq/sam/bam**

FastQC:Read QC reports using FastQC



MiSeq  
merged

## Exercise 3.3

---

Go to« [MiSeq merged](#) » history

Launch the pre-process tool on that data set

→ objective: understand output files

# Exercise 3.3

---

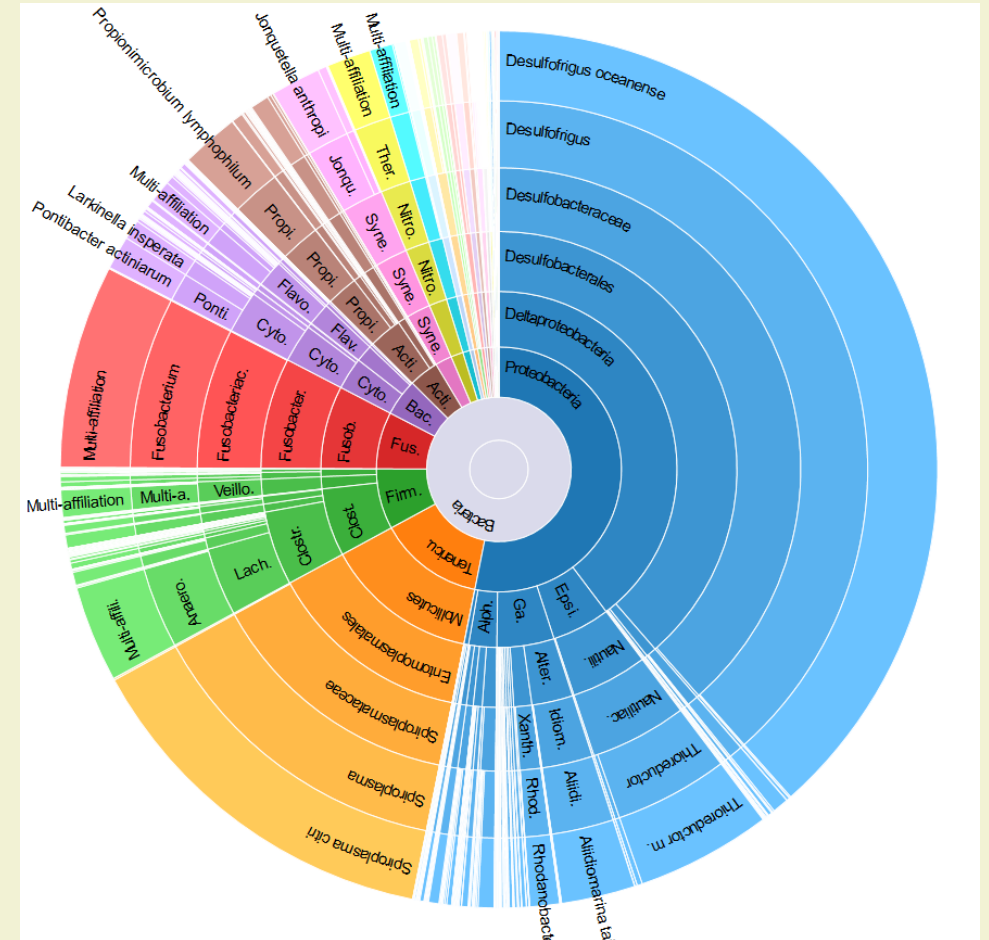
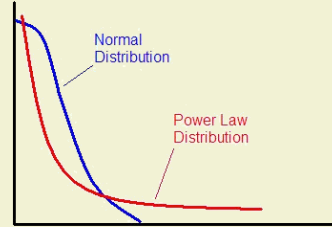
3 samples are **technically replicated** 3 times : 9 samples of 10 000 sequences each.

100_10000seq_sampleA1.fastq	100_10000seq_sampleB1.fastq	100_10000seq_sampleC1.fastq
100_10000seq_sampleA2.fastq	100_10000seq_sampleB2.fastq	100_10000seq_sampleC2.fastq
100_10000seq_sampleA3.fastq	100_10000seq_sampleB3.fastq	100_10000seq_sampleC3.fastq



# Exercise 3.3

- 100 species, covering all bacterial phyla
- Power Law distribution of the species abundances
- Error rate calibrated with real sequencing runs
- 10% chimeras
- 9 samples of 10 000 sequences each (90 000 sequences)



## Exercise 3.3

---

“Grinder (v 0.5.3) (Angly et al., 2012) was used to simulate the PCR amplification of full-length (V3-V4) sequences from reference databases. The reference database of size 100 were generated from the LTP SSU bank (version 115) (Yarza et al., 2008) by

- (1) filtering out sequences with a N,
- (2) keeping only type species
- (3) with a match for the forward (ACGGRAGGCAGCAG) and reverse (TACCAGGGTATCTAATCCTA) primers in the V3-V4 region and
- (4) maximizing the phylogenetic diversity (PD) for a given database size. The PD was computed from the NJ tree distributed with the LTP.”

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 2.0.0) Options

## Sequencer

Select the sequencing technology used to produce the sequences.

## Input type

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

## Archive file

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

## Reads already contiged ?

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

## Minimum amplicon size

The minimum size for the amplicons.

## Maximum amplicon size

The maximum size for the amplicons.

## Sequencing protocol

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

## 5' primer

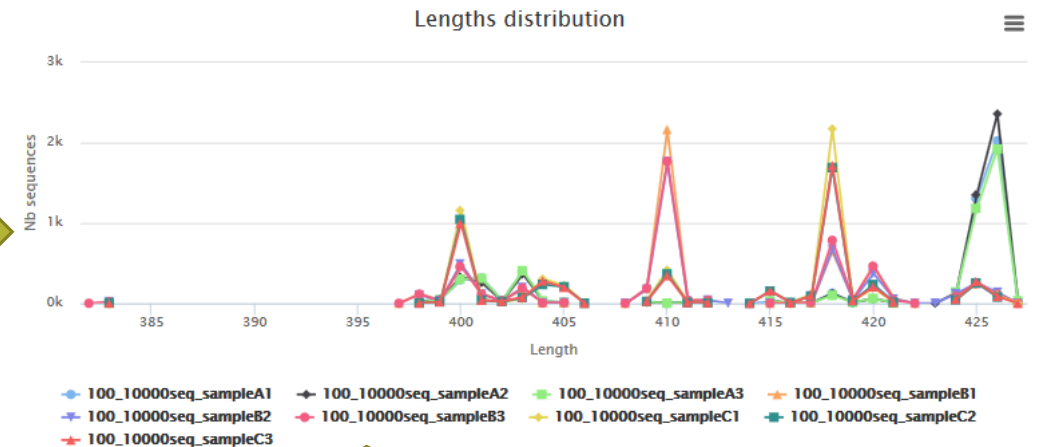
The 5' primer sequence (wildcards are accepted). The orientation is detailed in the primer information.

## 3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed in the primer information.

Execute

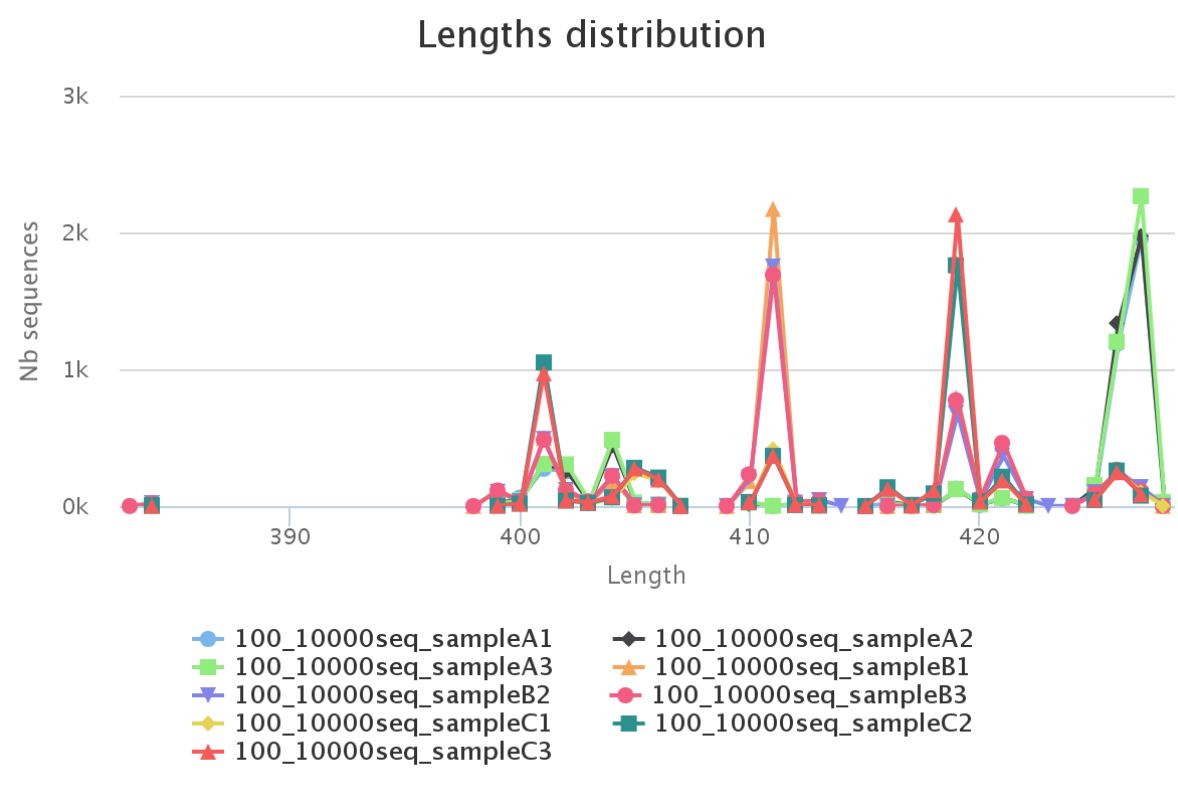
Amplicons lengths



Click on legend

Primers used for this sequencing :  
 5' primer: ACGGGAGGCAGCAG  
 3' primer: TAGGATTAGATACCCTGGTA  
 Lecture 5' → 3'

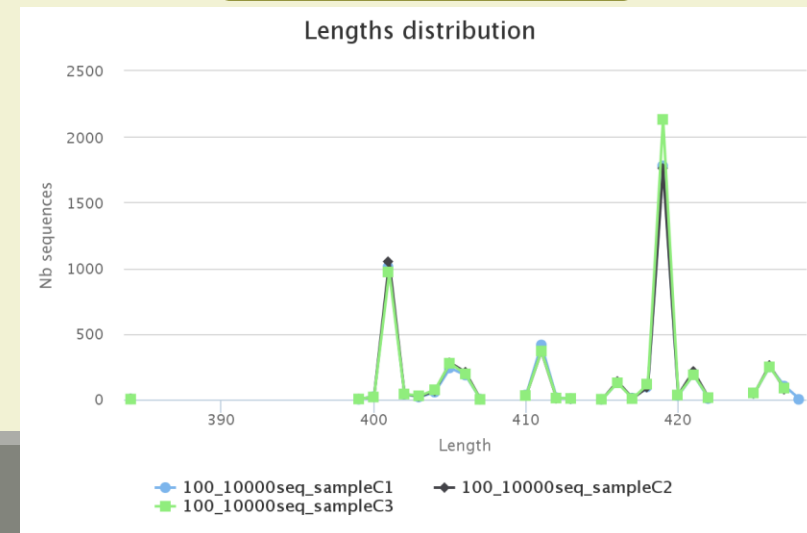
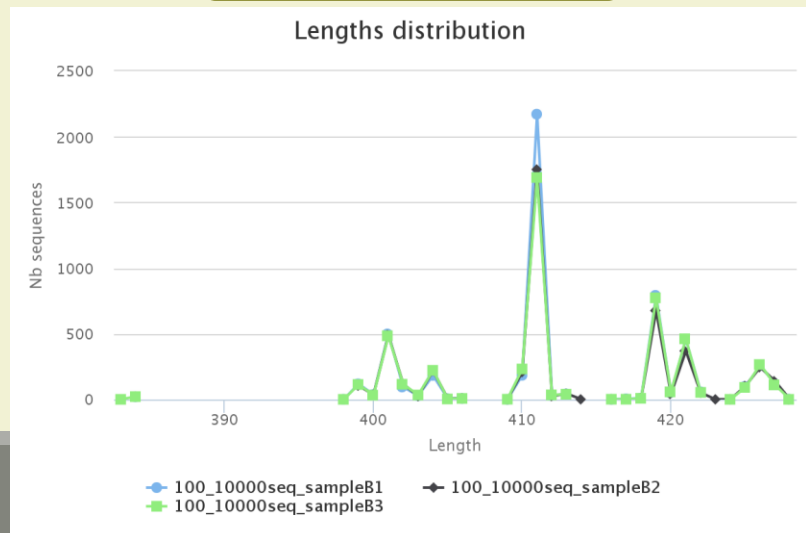
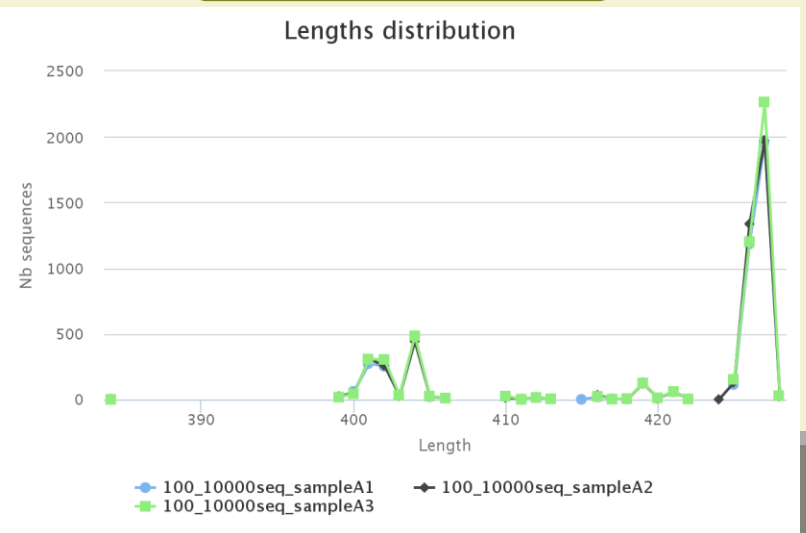
MiSeq merged



Samples A only

Samples B only

Samples C only



## Exercise 3.3 - Questions

---

1. How many sequences are there in the input file ?
2. How many sequences did not have the 5' primer?
3. How many sequences still are after pre-processing the data?
4. How much time did it take to pre-process the data ?
5. What can you tell about the sample based on sequence length distributions ?

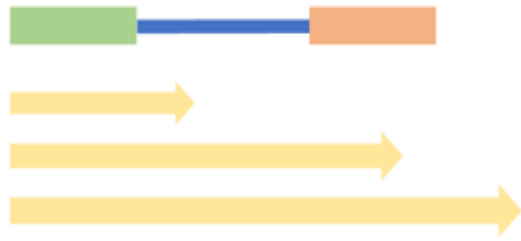
# Preprocess tool in bref

	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	soon
On-overlapping R1 R2	⊘

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

454

Single-end



Paired-end



**Legend**

- Primer Forward
- Primer Reverse
- DNA target
- Read
- Case not managed by FROGS
- Case where a pre-processing is necessary, outside FROGS

**Legend**

- Primer Forward (Green bar)
- Primer Reverse (Orange bar)
- DNA target (Blue bar)
- Read (Yellow arrow)
- Case not managed by FROGS (Red circle with slash)
- Case where a pre-processing is necessary, outside FROGS (Purple starburst)

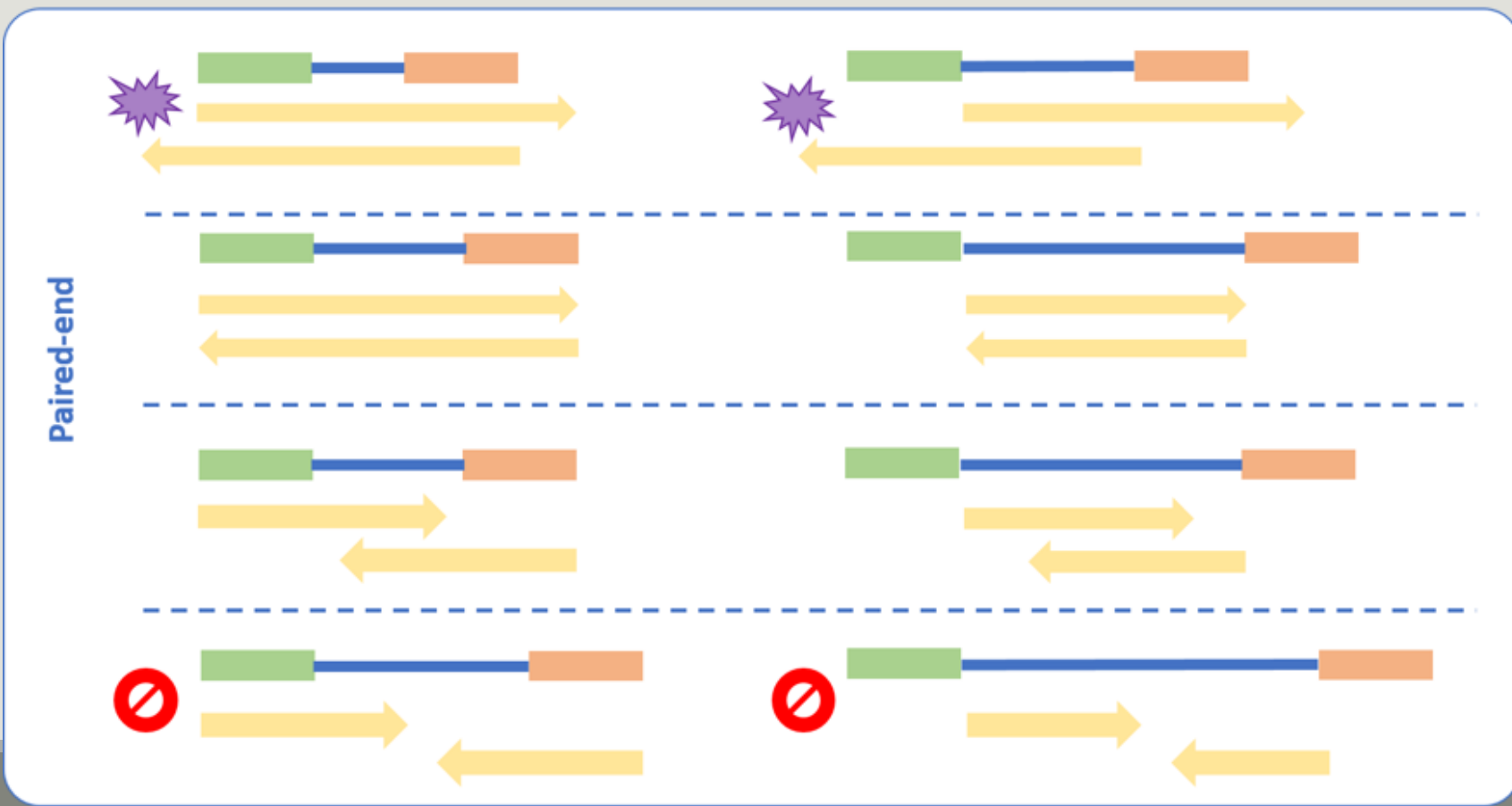
**Illumina**

Standard sequencing protocol

Kozich protocol : primers are not included in reads



→ Remove reverse primer before FROGS processing



Length of the sequenced target < length of one read

→ Remove ends of reads longer than DNA target

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



# Clustering tool

---

**FROGS Demultiplex reads** ✕

- Barcode file
- Select fastq dataset

---

demultiplexed\_archive (data) Ⓞ Ⓞ

undemultiplexed\_archive (data) Ⓞ Ⓞ

summary (tabular) Ⓞ

**Demultiplexing**

**Upload File from Genotoul** ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

**Data acquisition**

**FROGS Pre-process** ✕

- Archive file

---

dereplicated\_file (fasta) Ⓞ Ⓞ

count\_file (tabular) Ⓞ Ⓞ

summary\_file (html) Ⓞ Ⓞ

**Pre-process**

**FROGS Clustering swarm** ✕

- Sequences file
- Count file

---

seed\_file (fasta) Ⓞ Ⓞ

abundance\_biom (biom1) Ⓞ Ⓞ

swarms\_composition (tabular) Ⓞ Ⓞ

**Clustering**

**FROGS Remove chimera** ✕

- Sequences file
- Abundance file

---

non\_chimera\_fasta (fasta) Ⓞ Ⓞ

out\_abundance\_biom (biom1) Ⓞ Ⓞ

out\_abundance\_count (tabular) Ⓞ Ⓞ

summary\_file (html) Ⓞ Ⓞ

**Chimera**

**FROGS Affiliation OTU** ✕

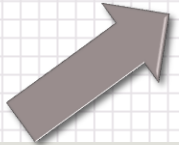
- OTU seed sequence
- Abundance file

---

biom\_affiliation (biom1) Ⓞ Ⓞ

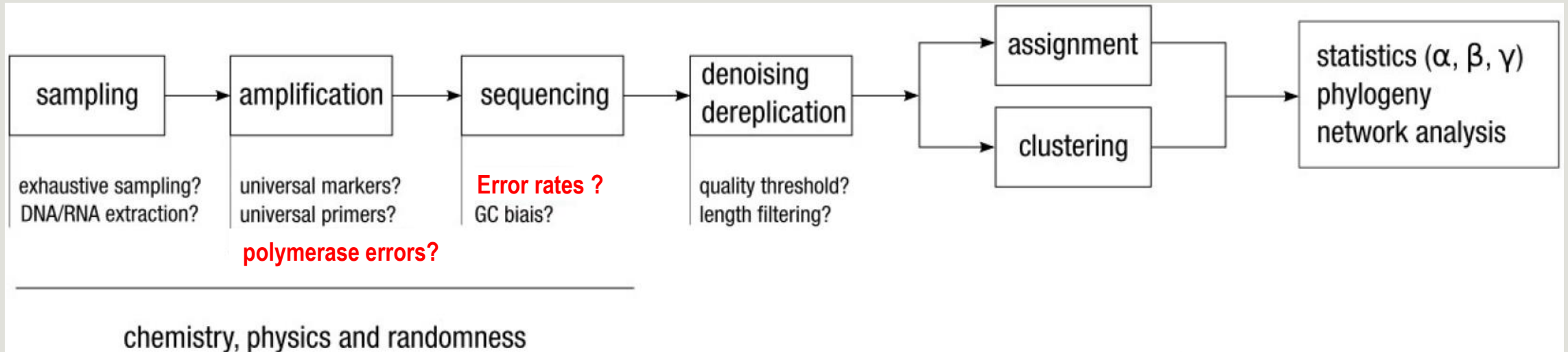
summary (html) Ⓞ Ⓞ

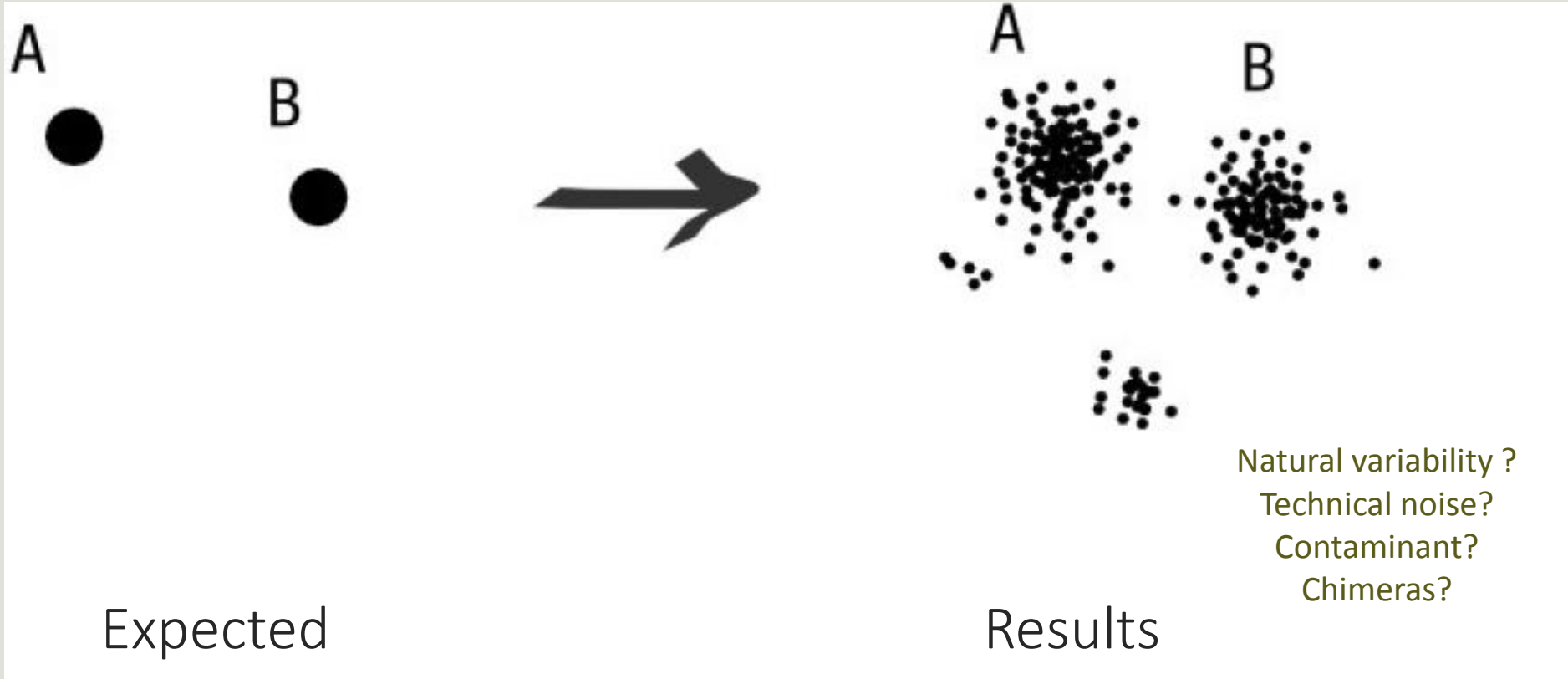
**Affiliation**

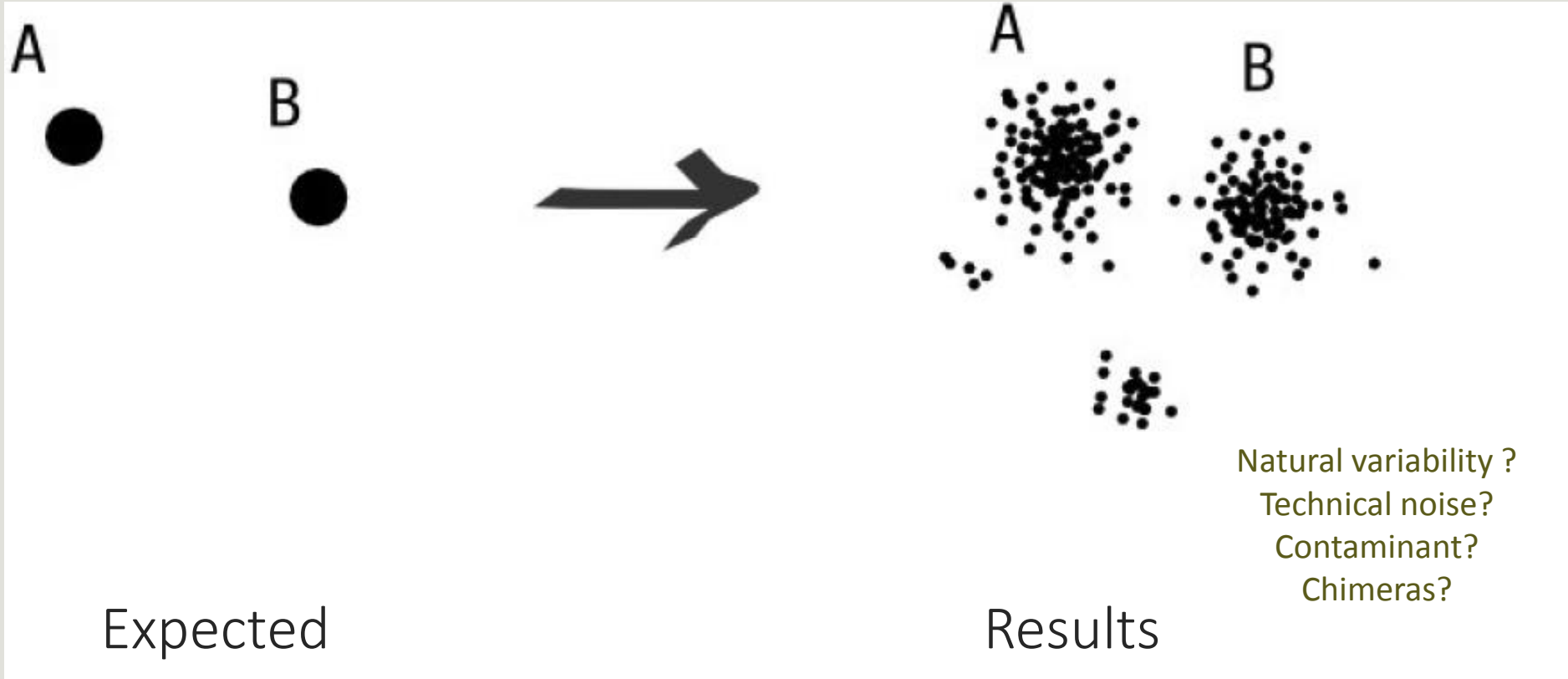


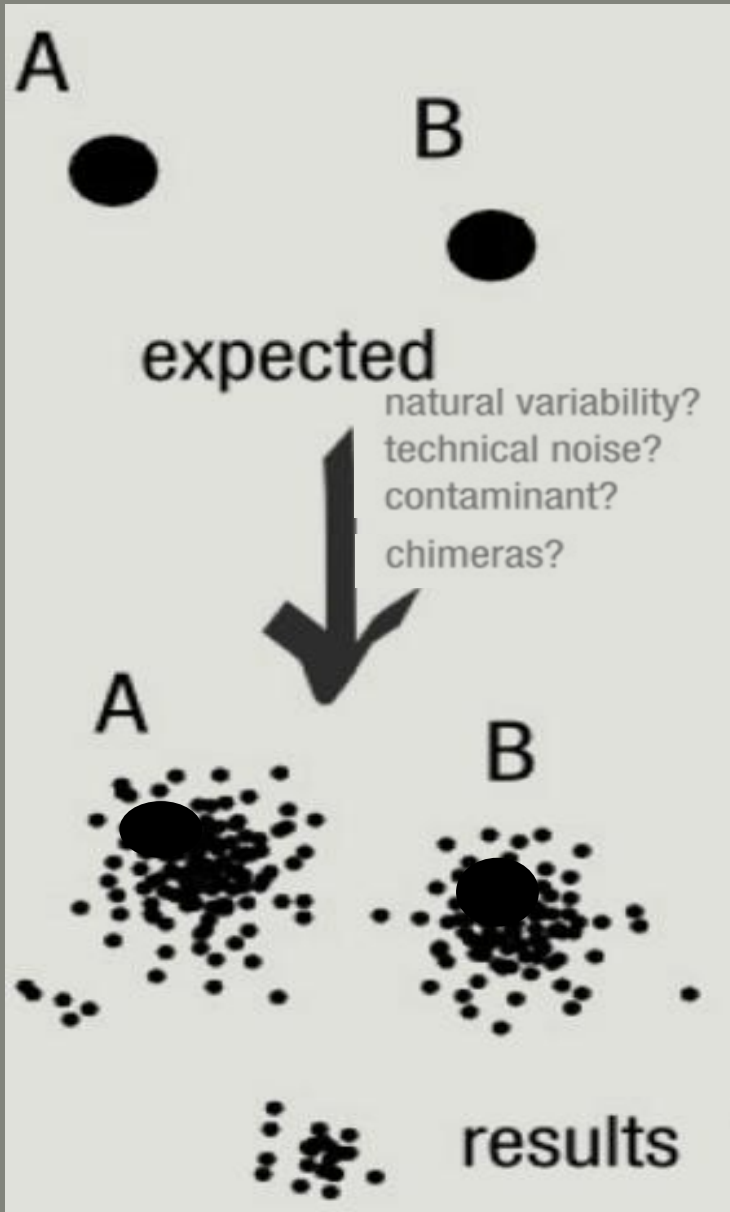
# Why do we need clustering ?

Amplification and sequencing and are not perfect processes









# To have the best accuracy:

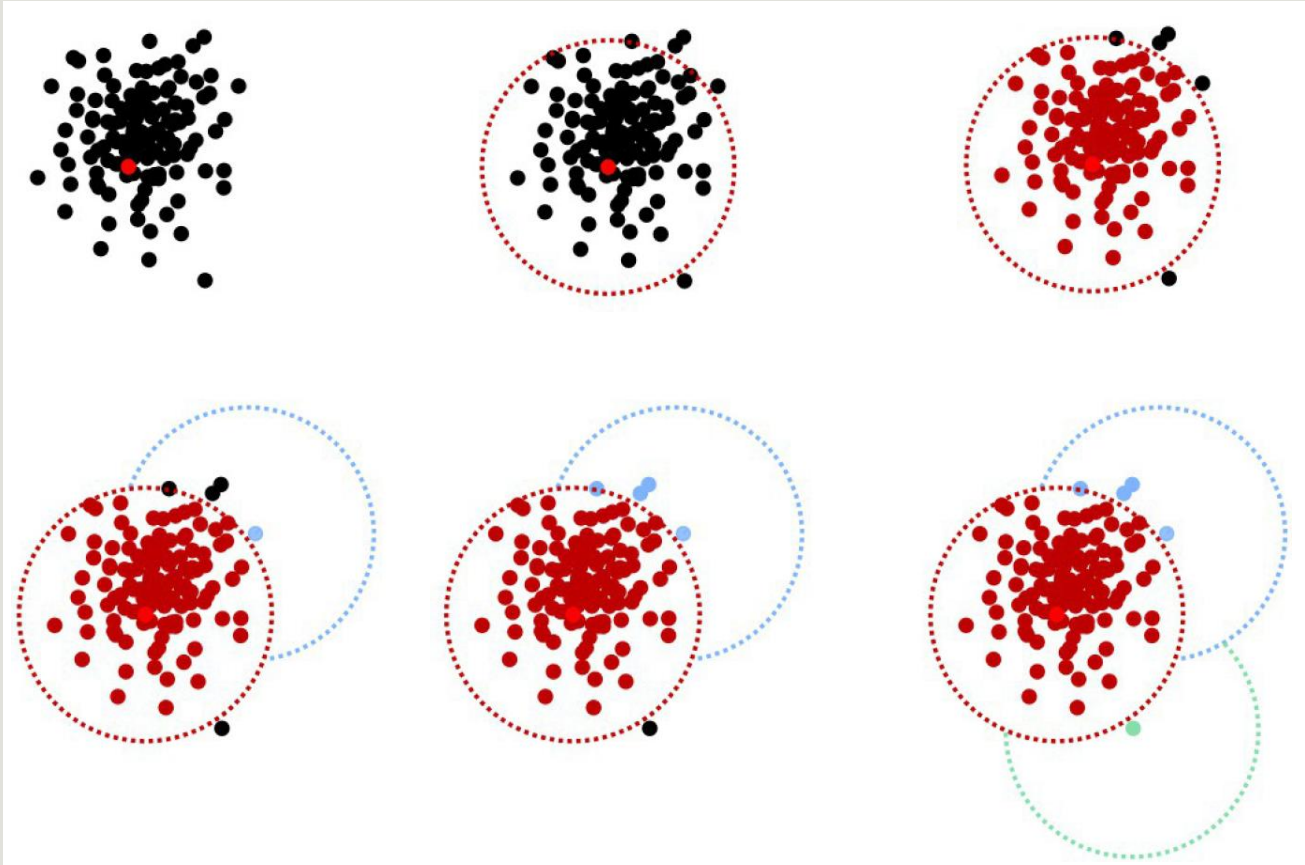
## Method: All against all

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

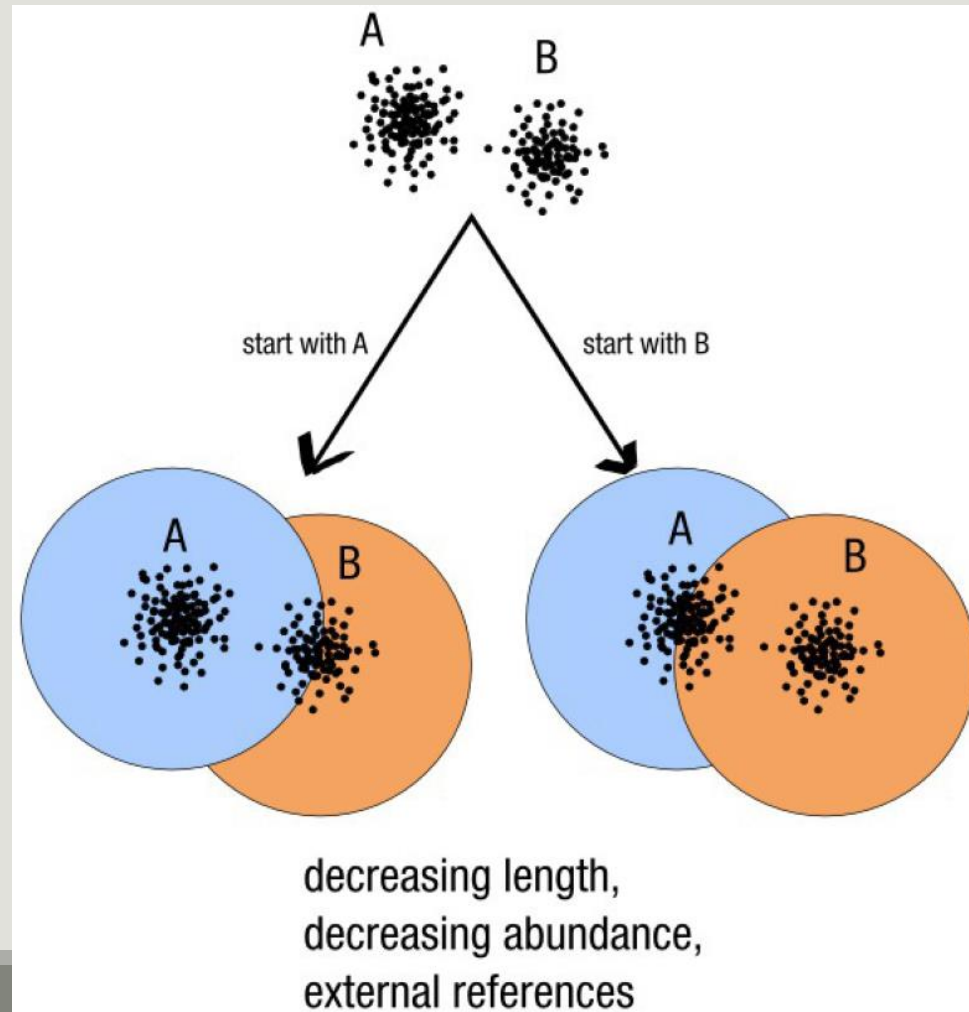
=> Impossible on very large datasets without strong filtering or sampling

# How traditional clustering works ?

---

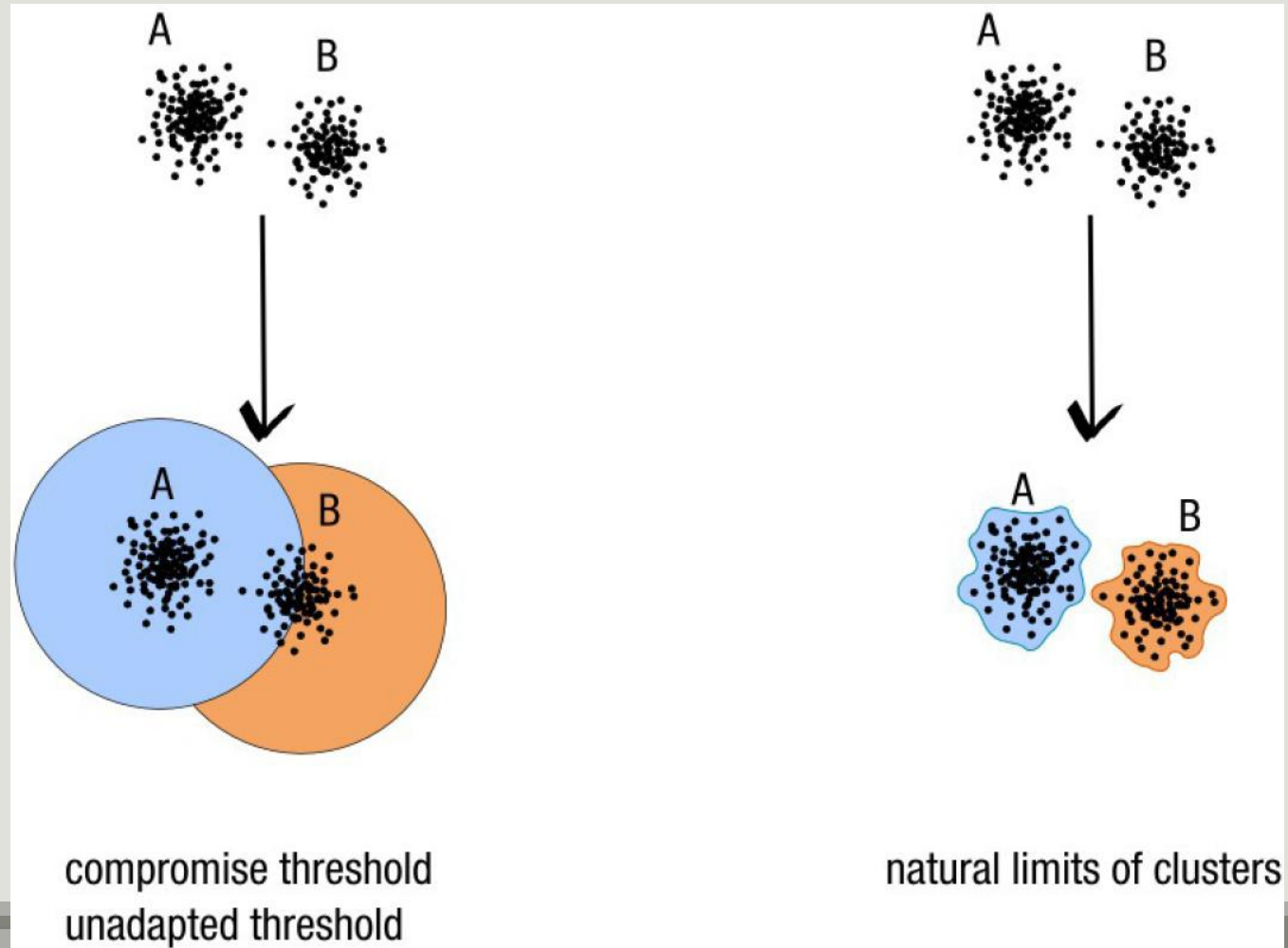


# Input order dependent results

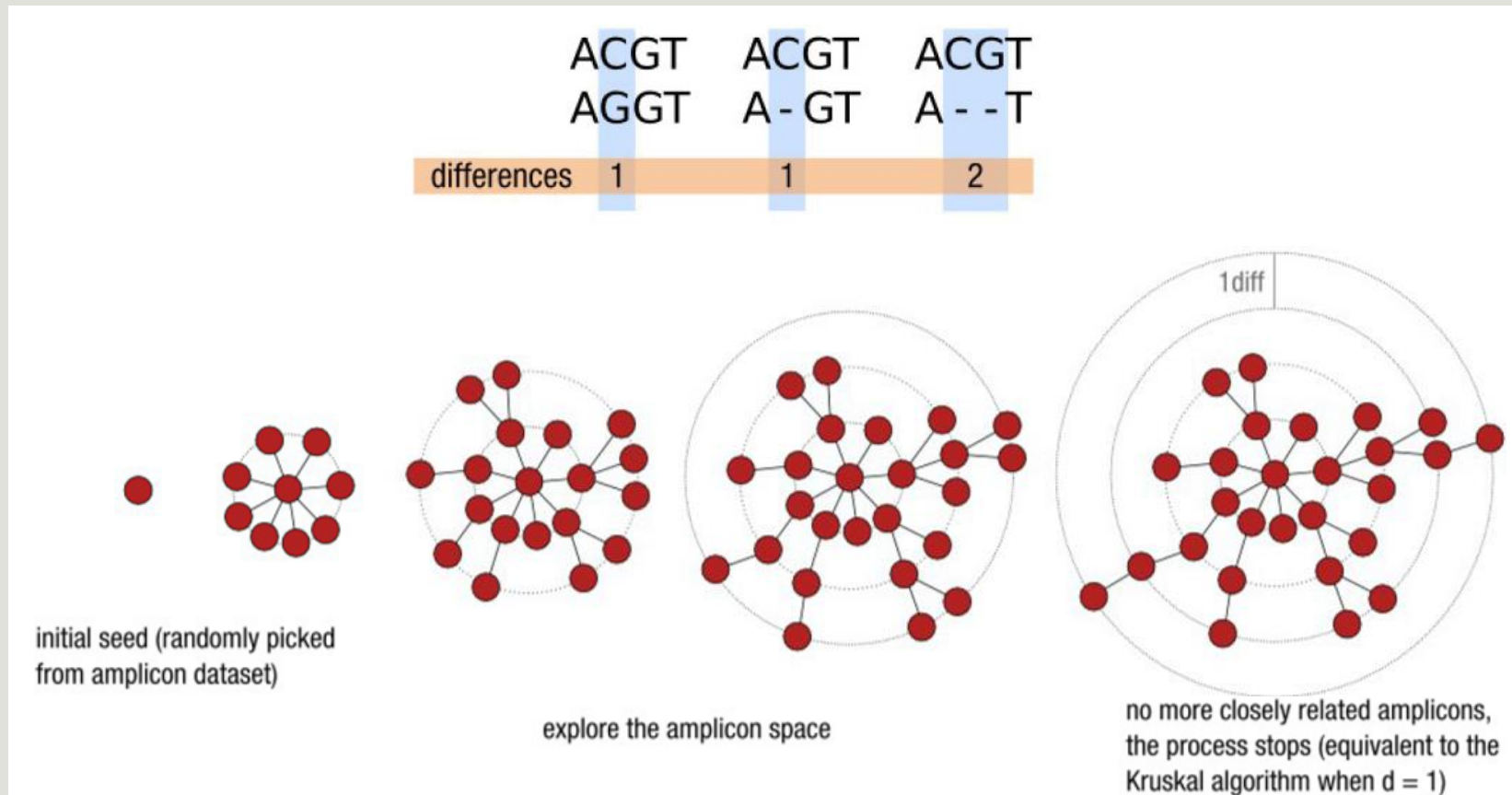




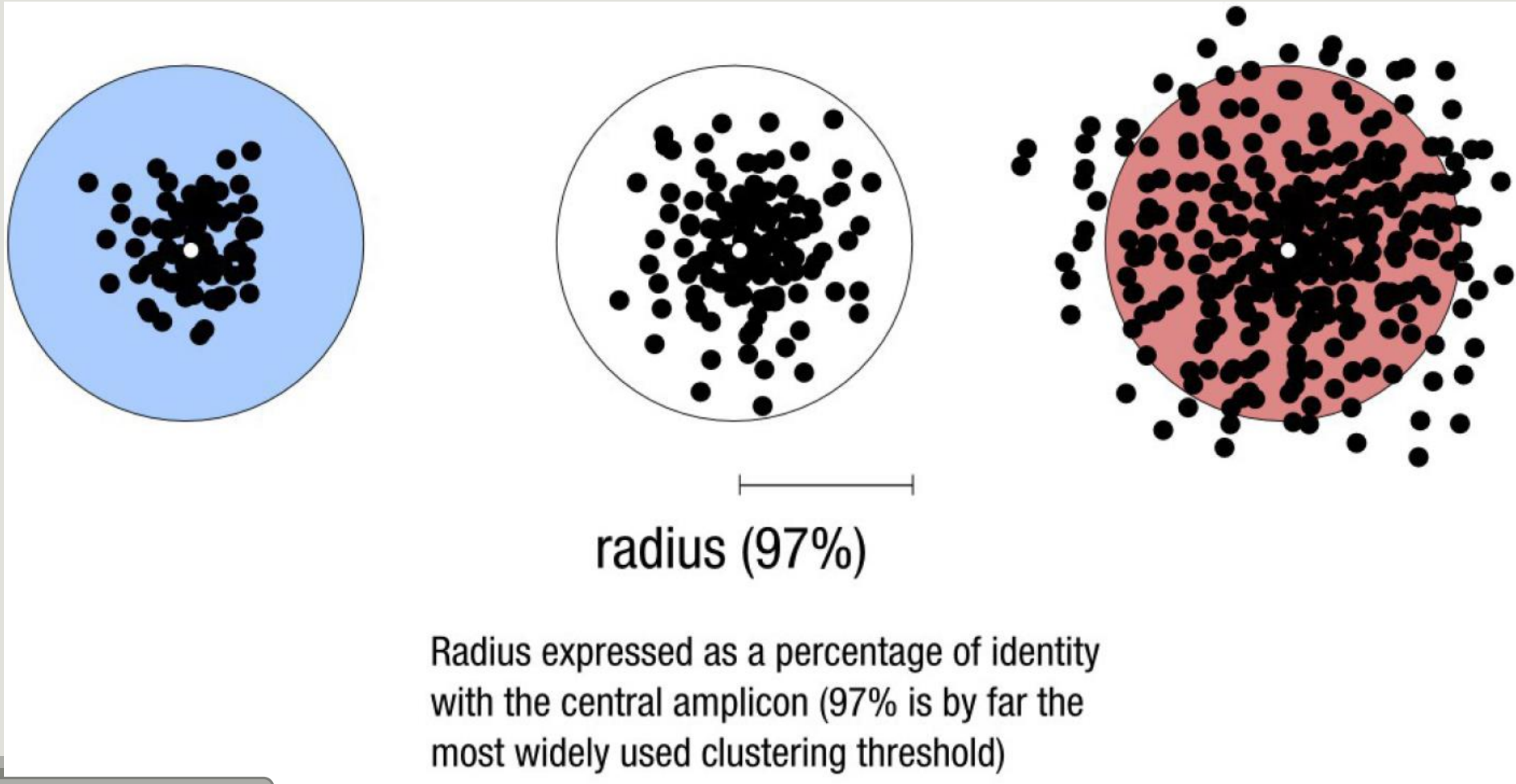
# Single a priori clustering threshold



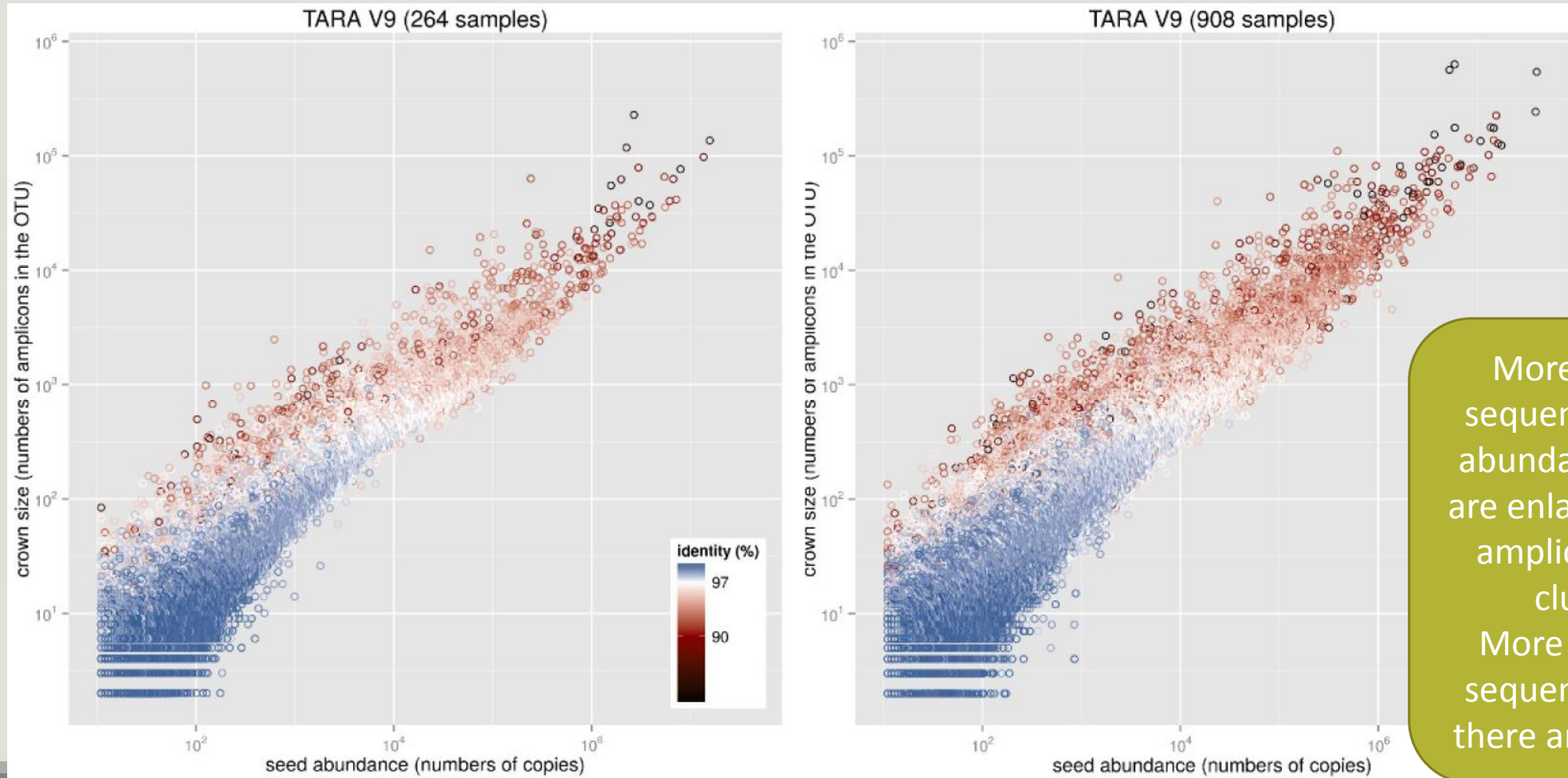
# Swarm clustering method



# Comparison Swarm and 3% clusterings



# Comparison Swarm and 3% clusterings



# 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 ✕

Sequences file

Count file

---

abundance\_biom (txt) ⊗

seed\_file (fasta) ⊗

swarms\_composition (tabular) ⊗

**Clustering**

**FROGS Clustering swarm** Step 2 in metagenomics analysis : clustering. (Galaxy Version 2.3.0) Options

**Sequences file**

2: FROGS Pre-process: dereplicated.fasta

The sequences file (format: fasta).

**Count file**

3: FROGS Pre-process: count.tsv

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

**Aggregation distance**

Maximum number of differences between sequences in each aggregation step.

**Performe denoising clustering step?**

If checked, clustering will be perform in two steps, first with distance = 1 and then with your input distance



1st run for denoising:

Swarm with  $d = 1$  -> high clusters definition  
linear complexity

2<sup>nd</sup> run for clustering:

Swarm with  $d = 3$  on the **seeds** of first Swarm  
quadratic complexity

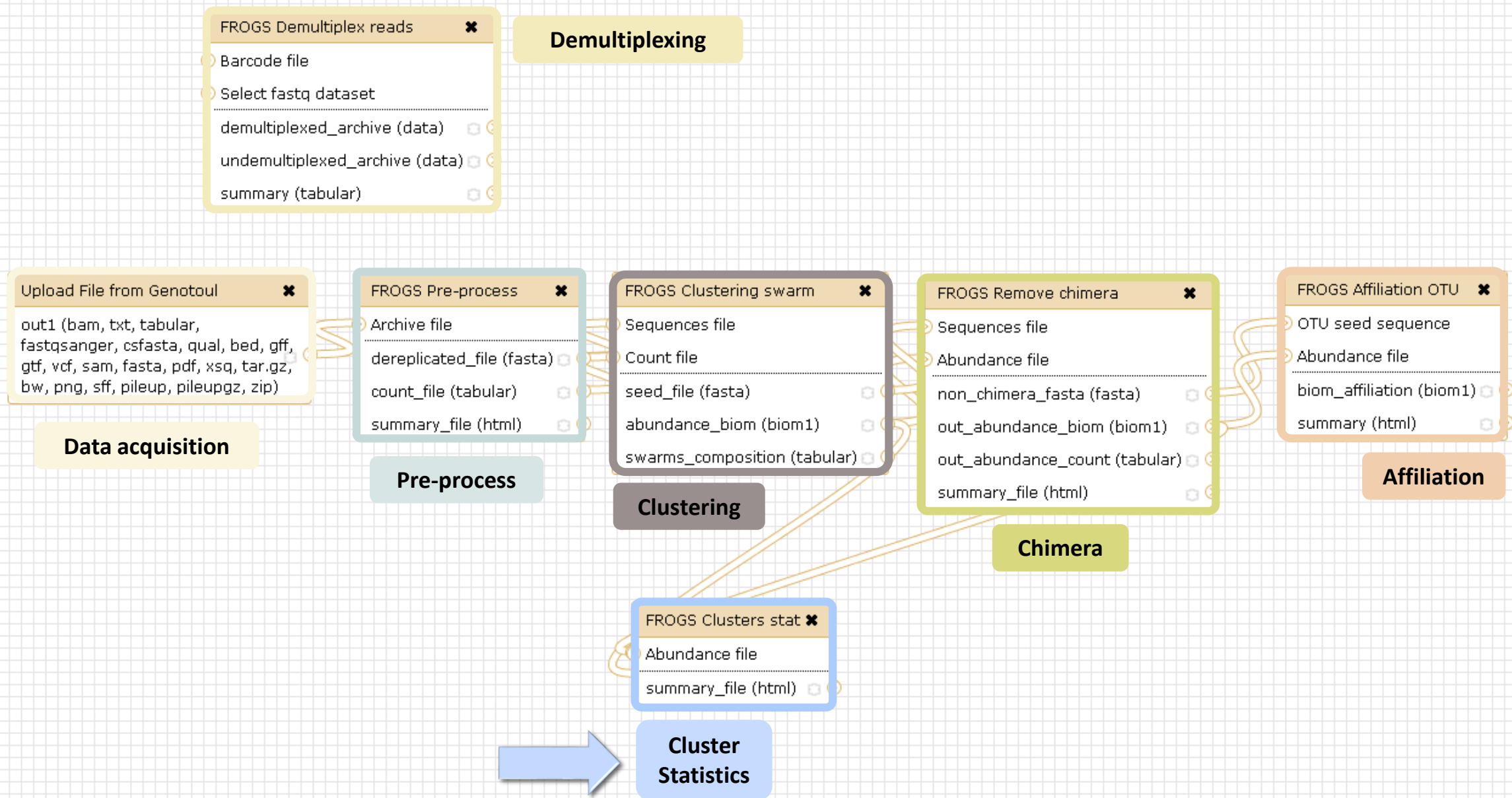
Gain time !

Remove false positives !

# Cluster stat tool

---







**FROGS Clusters stat** Process some metrics on clusters. (Galaxy Version 1.4.0)

Options

**Abundance file**



6: FROGS Clustering swarm: abundance.biom



Clusters abundance (format: BIOM).

Execute

# Your Turn! - 4

---

LAUNCH CLUSTERING AND CLUSTERSTAT TOOLS

# Exercise 4

---

Go to « [MiSeq merged](#) » history

Launch the Clustering SWARM tool on that data set with aggregation distance = 3 and the denoising

→ objectives :

- understand the denoising efficiency
- understand the ClusterStat utility

# Exercise 4

---

1. How much time does it take to finish?
2. How many clusters do you get ?

# Exercise 4

3. Edit the biom and fasta output dataset by adding **d1d3**



Attributes Convert Format Datatype Permissions

Edit Attributes

**Name:**  
warm: seed\_sequencesd1d3.fasta

**Info:**  
## Application  
Software :/usr/local/bioinfo  
/src/galaxy-test/galaxy-

**Annotation / Notes:**

FROGS Clusters stat Process  
some metrics on clusters.

4. Launch FROGS Cluster Stat tools on the previous abundance biom file

## Exercise 4

---

5. Interpret the boxplot: **Clusters size summary**
6. Interpret the table: **Clusters size details**
7. What can we say by observing the **sequence distribution**?
8. How many clusters share “sampleB3” with at least one other sample?
9. How many clusters could we expect to be shared ?
10. How many sequences represent the 550 specific clusters of “sampleC2”?
11. This represents what proportion of “sampleC2”?
12. What do you think about it?
13. 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).

- Tools**
- deepTools**
- FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION
  - FROGS pipeline**
  - FROGS Upload archive from your computer
  - FROGS Demultiplex reads Split by samples the reads in function of inner barcode.
  - FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication.
  - FROGS Clustering swarm Step 2 in metagenomics analysis : clustering.
  - FROGS Remove chimera Step 3 in metagenomics analysis : Remove PCR chimera in each sample.
  - FROGS Filters Filters OTUs on several criteria.
  - FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST
  - FROGS BIOM to TSV Converts a BIOM file in TSV file.
  - 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 Abundance normalisation

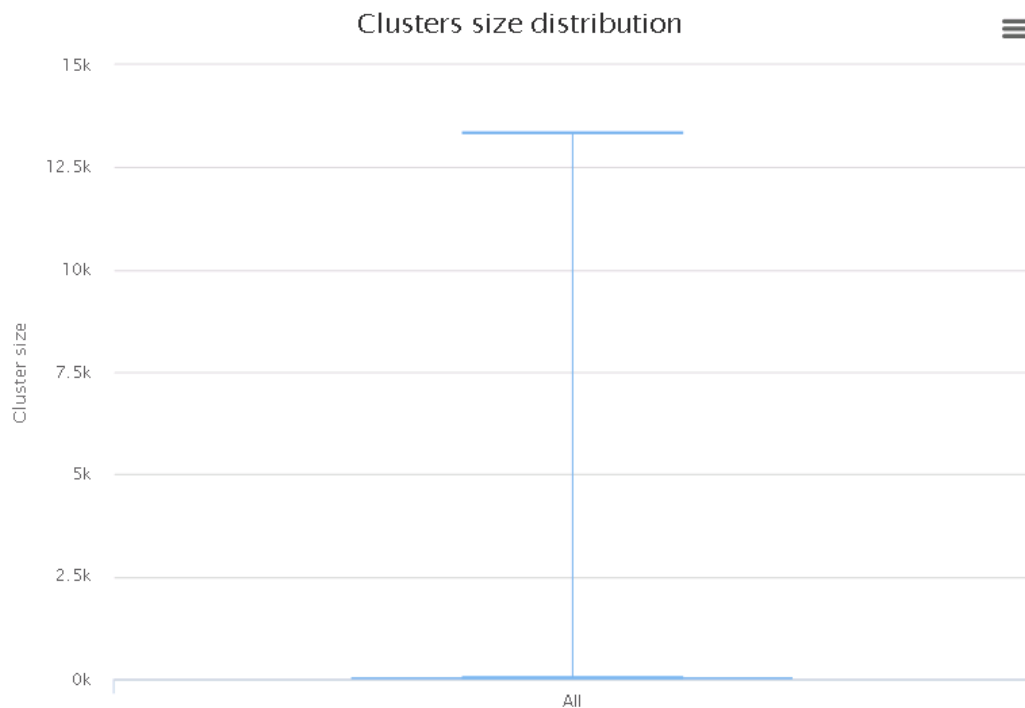
Clusters distribution Sequences distribution Samples distribution

Clusters  
5,945

Sequences  
89,721

Most of clusters are singletons

## Clusters size summary



Clusters size distribution (decile)

Decile	Value
Min	1
1	1
2	1
3	1
4	1
Median	1
6	1
7	1
8	2
9	2
Max	13,337

- History**
- 15: FROGS Filters: sequences.fasta
  - 14: FROGS Remove chimera: report.html
  - 13: FROGS Remove chimera: non\_chimera\_abundance.biom
  - 12: FROGS Remove chimera: non\_chimera.fasta
  - 11: FROGS Clusters stat: summary\_swarm\_d1d3.html**
  - 10: FROGS Clustering swarm: swarms\_composition\_d1d3.tsv
  - 9: FROGS Clustering swarm: abundance\_d1d3.biom
  - 8: FROGS Clustering swarm: seed\_sequences\_d1d3.fasta
  - 7: FROGS Pre-process: report.html

Clusters

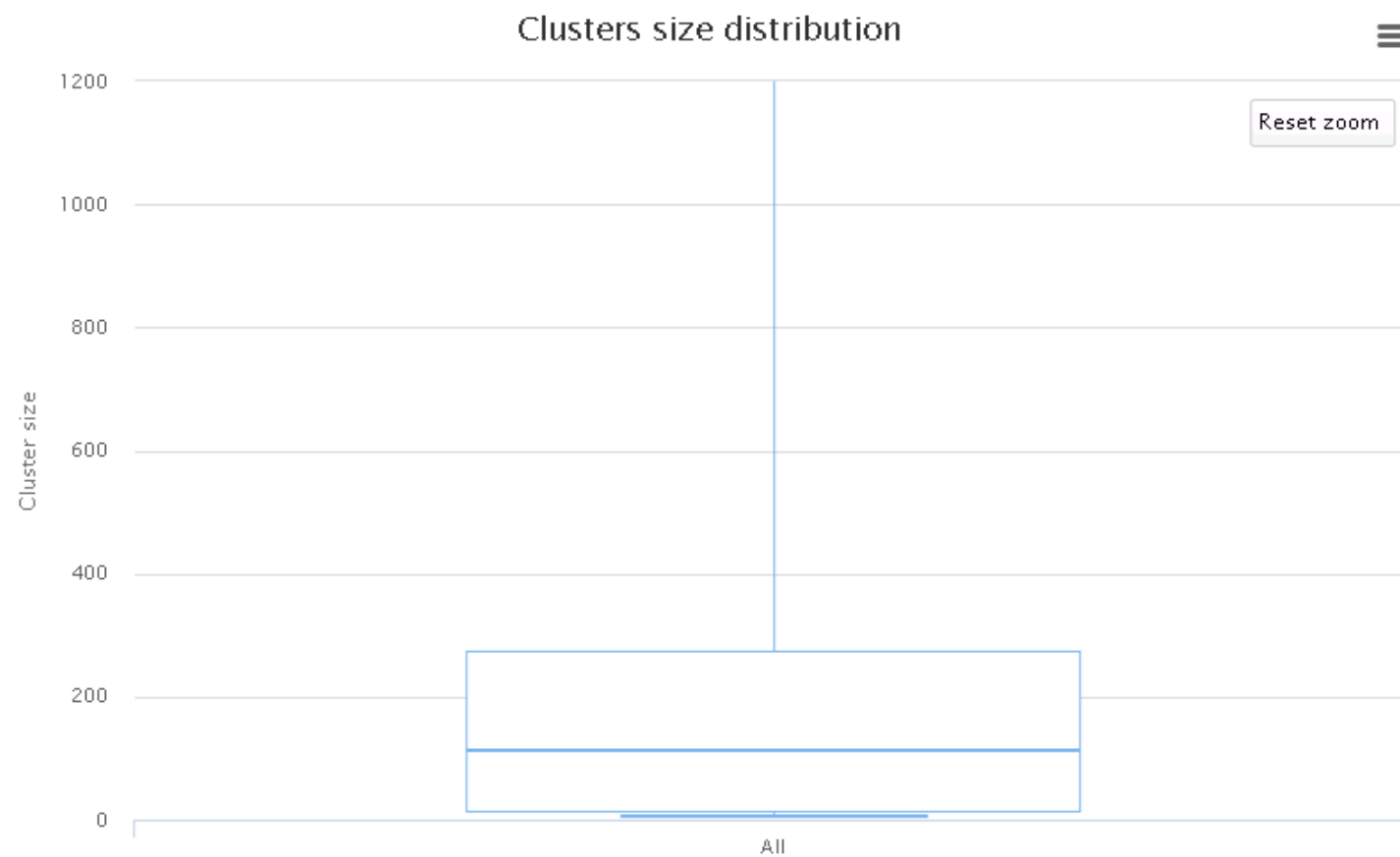
141

Sequences

81,838

## Clusters size summary

After filtering little clusters



Clusters size distribution (decile)

Decile	Value
Min	5
1	6
2	8
3	30
4	70
Median	112
6	145
7	225
8	412
9	994
Max	13,337



# Clusters size details

Most of clusters are singletons

CSV

Show 10 entries

Search:

## Clusters size

Cluster size	Number of cluster	% of all clusters
1	4,595	77.36
2	866	14.58
3	155	2.61
4	83	1.40
5	42	0.71
6	29	0.49
7	22	0.37
8	13	0.22
9	6	0.10
10	6	0.10

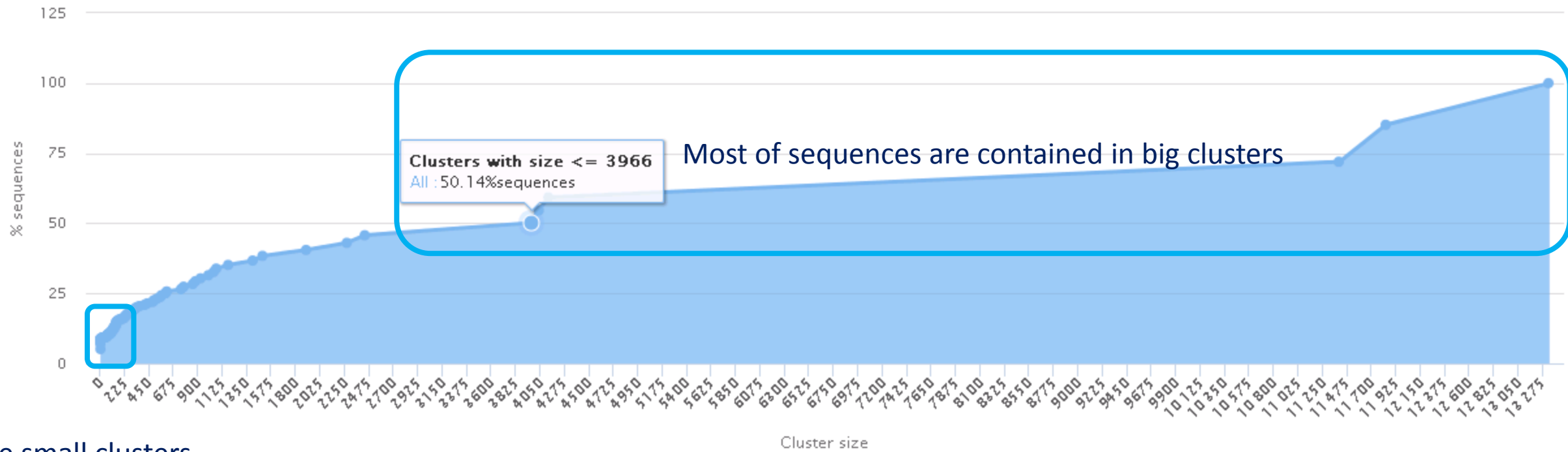
After clustering

Clusters distribution

Sequences distribution

Samples distribution

### Cumulative sequences proportion by cluster size



The small clusters represent few sequences

N.B.: Select area to zoom in.

# Sequences

367 clusters of sampleA1  
are common at least  
once with another  
sample

58 % of the specific clusters of sampleA1  
represent around 5% of sequences  
Could be interesting to remove if individual  
variability is not the concern of user



Show 10 entries

## Samples information

Sample	Shared clusters	Own clusters	Shared sequences	Own sequences
100_10000seq_sampleA1	367	513	9,447	528
100_10000seq_sampleA2	365	490	9,476	503
100_10000seq_sampleA3	384	483	9,478	494
100_10000seq_sampleB1	395	548	9,397	572
100_10000seq_sampleB2	375	508	9,455	515
100_10000seq_sampleB3	376	562	9,388	579
100_10000seq_sampleC1	372	539	9,413	552
100_10000seq_sampleC2	389	550	9,408	567
100_10000seq_sampleC3	361	516	9,442	525

Showing 1 to 9 of 9 entries

Previous

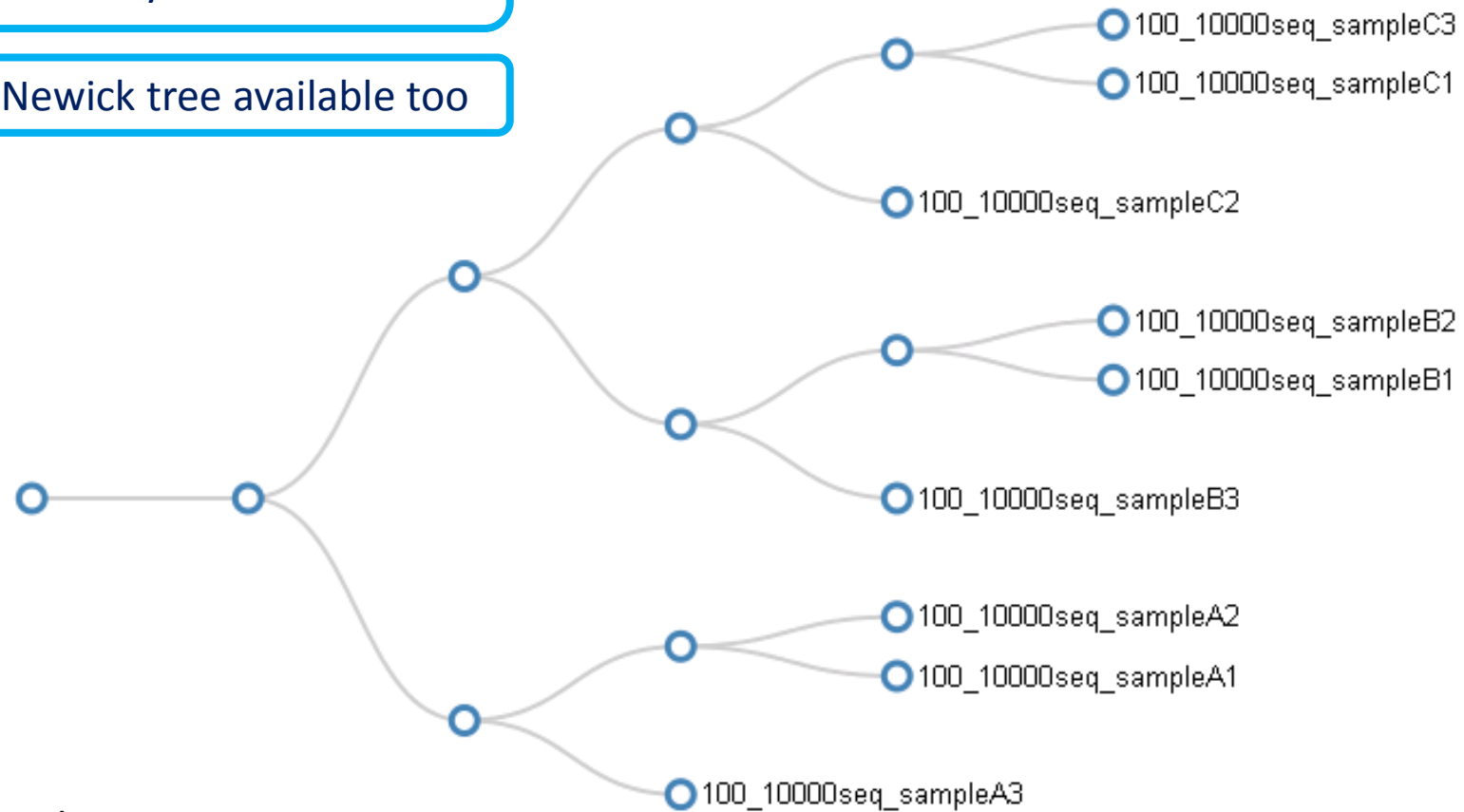
1

Next

# Hierarchical clustering

Hierarchical classification  
on Bray Curtis distance

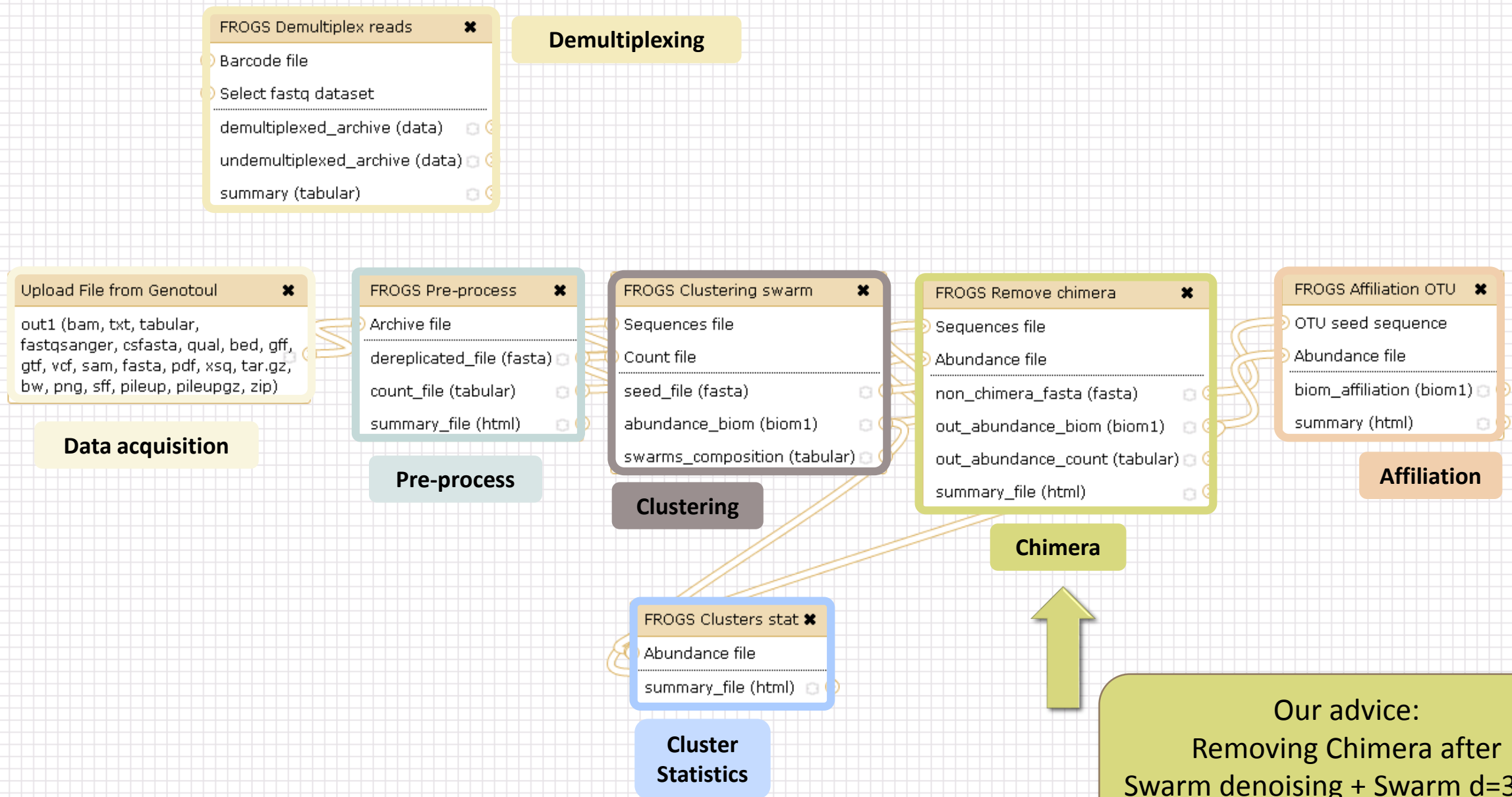
Newick tree available too



Samples distribution tab

# Chimera removal tool

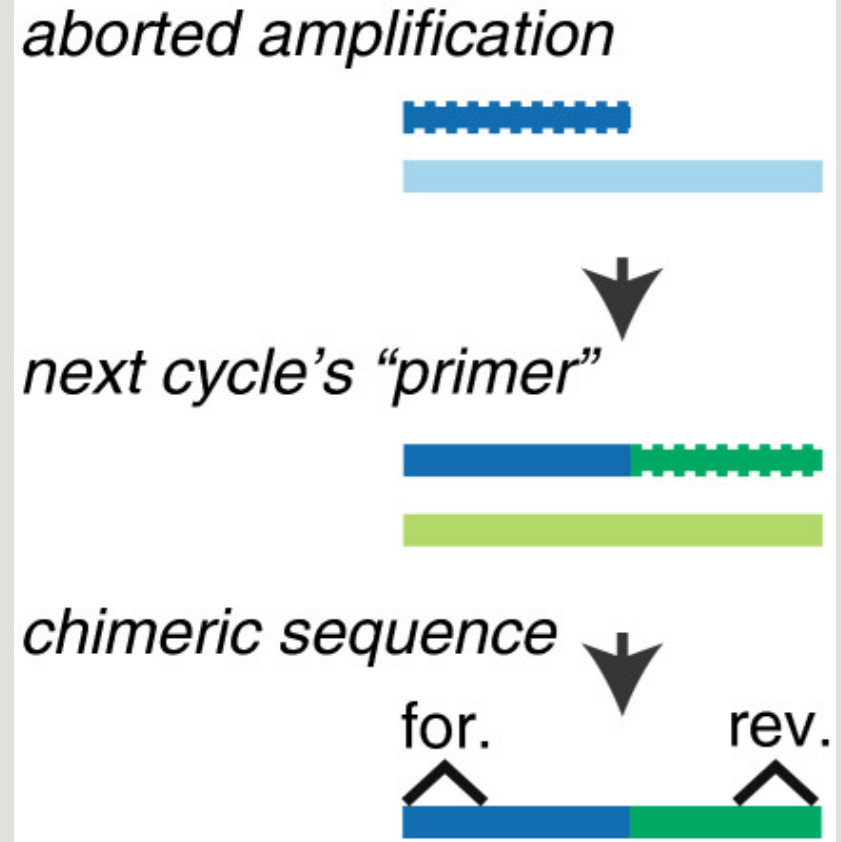
---



# 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.

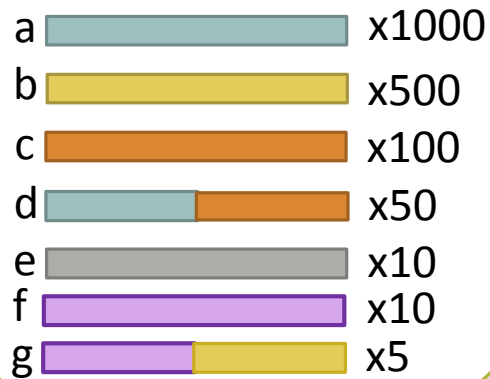
**Chimera: from 5 to 45% of reads** (Schloss 2011)



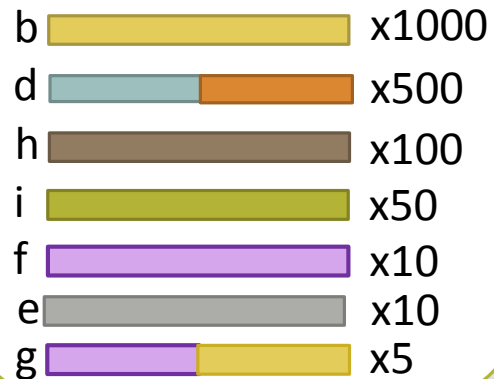
# A smart removal chimera to be accurate

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



# Your Turn! - 5

---

LAUNCH THE REMOVE CHIMERA TOOL

# Exercise 5

---

Go to « [MiSeq merged](#) » history

Launch the « [FROGS Remove Chimera](#) » tool

Follow by the « [FROGS ClusterStat](#) » tool on the swarm d1d3 non chimera abundance biom

→ objectives :

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

FROGS Remove chimera ✕

- Sequences file
- Abundance file

---

- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)

## Chimera

**FROGS Remove chimera** Step 3 in metagenomics analysis : Remove PCR chimera in each sample. (Galaxy Version 1.3.0) 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 5

---

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 ?

## Exercise 5

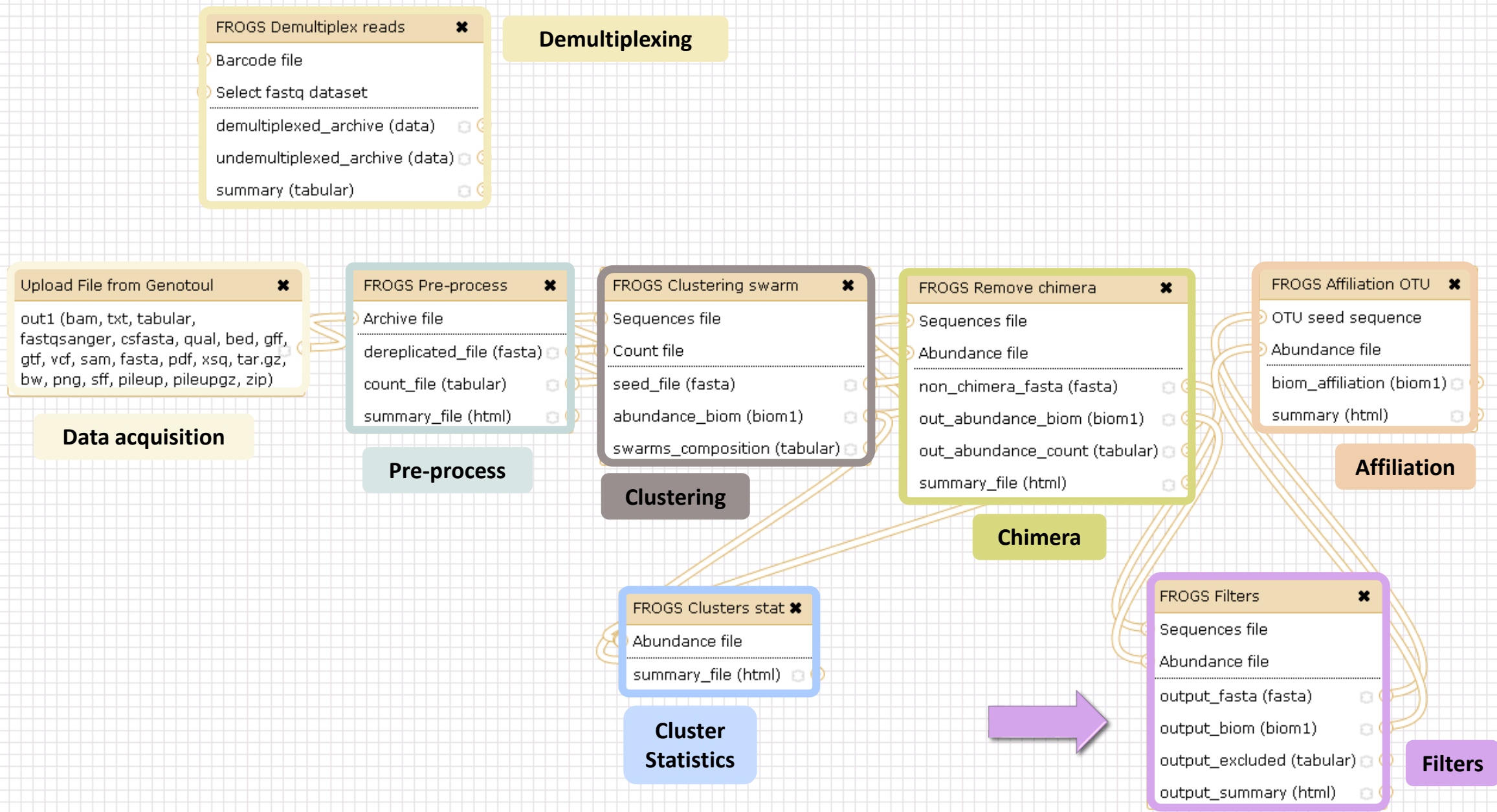
---

2. Launch « FROGS ClusterStat » tool on non\_chimera\_abundanced1d3.biom
3. Rename output in summary\_nonchimera\_d1d3.html
4. Compare the HTML files
  - a. Of what are mainly composed singleton ? (compare with precedent summary.html)
  - b. What are their abundance?
  - c. What do you conclude ?

The weakly abundant Clusters are mainly false positives, our data would be much more exact if we remove them

# Filters tool

---



Affiliation runs long time

Advise:

Apply filters between “Chimera Removal ” and “Affiliation”.  
Remove OTUs with weak abundance and non redundant before affiliation.

You will gain time !



# Filters

---

Filters allows to filter the result thanks to different criteria et may be used after different steps of pipeline :

- On the abundance
- On RDP affiliation
- On Blast affiliation
- On phix contaminant

After Affiliation tool

**FROGS Filters** ✕

- Sequences file
- Abundance file
- output\_fasta (fasta) 🗑️
- output\_biom (biom1) 🗑️
- output\_excluded (tabular) 🗑️
- output\_summary (html) 🗑️

Filters

4 filter sections

**FROGS Filters** Filters: OTUs on several criteria. (Galaxy Version 1.2.0) Options

**Sequences file**  
  
 The sequence file to filter (format: fasta).

**Abundance file**  
  
 The abundance file to filter (format: BIOM).

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters  
 If you want to filter OTUs on their abundance and occurrence.

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

**Minimum proportion/number of sequences to keep OTU**  
  
 Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

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

**\*\*\* THE FILTERS ON RDP**

Apply filters  
 If you want to filter OTUs on their taxonomic affiliation produced by RDP.

**Rank with the bootstrap filter**

**Minimum bootstrap % (between 0 and 1)**

**\*\*\* THE FILTERS ON BLAST**

Apply filters  
 If you want to filter OTUs on their taxonomic affiliation produced by Blast.

**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

**\*\*\* THE FILTERS ON CONTAMINATIONS**

Apply filters  
 If you want to filter OTUs on classical contaminations.

**Cotaminant databank**  
  
 The phiX databank (the phiX is a control added in Illumina sequencing technologies).

Abundance filters

RDP affiliation filters

BLAST affiliation filters

Contamination filter

## Input

**FROGS Filters** Filters OTUs on several criteria. (Galaxy Version 1.2.0) Options

**Sequences file**

The sequence file to filter (format: fasta).

**Abundance file**

The abundance file to filter (format: BIOM).

Fasta sequences and its corresponding abundance biom files

## Filter 1 : abundance

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters

If you want to filter OTUs on their abundance and occurrence.

**Minimum number of samples**

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

**Minimum proportion/number of sequences to keep OTU**

Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

**N biggest OTU**

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

### \*\*\* THE FILTERS ON RDP

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by RDP.

Rank with the bootstrap filter

Genus

Minimum bootstrap % (between 0 and 1)

0.8

### \*\*\* THE FILTERS ON BLAST

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by Blast.

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)

0.95

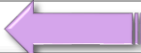
Fill the field only if you want this treatment

Minimum alignment length

Fill the field only if you want this treatment

Filter 2 & 3:  
affiliation

## Filter 4 : contamination

Contaminant databank
phix 
The phix databank (the phix is a control added in Illumina sequencing technologies).

Soon, several contaminant banks

# Your Turn! - 6

---

LAUNCH DE LA TOOL FILTERS

# Exercise 6

---

Go to history « **MiSeq merged** »

Launch « Filters » tool with non\_chimera\_abundanced1d3.biom, non\_chimerad1d3.fasta

Apply 2 filters :

- **Minimum proportion/number of sequences to keep OTU: 0.00005\***
- **Minimum number of samples: 3**

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

**FROGS Filters** ✕

- Sequences file
- Abundance file
- output\_fasta (fasta)
- output\_biom (biom1)
- output\_excluded (tabular)
- output\_summary (html)

**Filters**

**FROGS Filters** Filters OTUs on several criteria. (Galaxy Version 1.2.0) Options

**Sequences file**  
  
 The sequence file to filter (format: fasta).

**Abundance file**  
  
 The abundance file to filter (format: BIOM).

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

If you want to filter OTUs on their abundance and occurrence.

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

**Minimum proportion/number of sequences to keep OTU**  
  
 Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

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

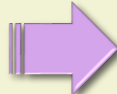
**\*\*\* THE FILTERS ON RDP**  
  
 If you want to filter OTUs on their taxonomic affiliation produced by RDP.

**\*\*\* THE FILTERS ON BLAST**  
  
 If you want to filter OTUs on their taxonomic affiliation produced by Blast.

**\*\*\* THE FILTERS ON CONTAMINATIONS**  
  
 If you want to filter OTUs on classical contaminations.

**Output**

- 92: FROGS Filters: report.html**
- 91: FROGS Filters: excluded.tsv**
- 90: FROGS Filters: abundance.biom**
- 89: FROGS Filters: sequences.fasta**



If Filters fields are « Apply » so you have to fill at one field. Otherwise, galaxy become red !



# Exercise 6

---

1. What are the output files of “Filters” ?
2. Explore “FROGS Filter : report.html” file.
3. How many OTUs have you removed ?
4. Build the Venn diagram on the two filters.
5. How many OTUs have you removed with each filter “abundance > 0.005%”, “Remove OTUs that are not present at least in 3 samples”?
6. How many OTUs do they remain ?
7. Is there a sample more impacted than the others ?
8. To characterize these new OTUs, do not forget to launch “FROGS Cluster Stat” tool, and rename the output HTML file.

Filters by OTUs

Filters by samples



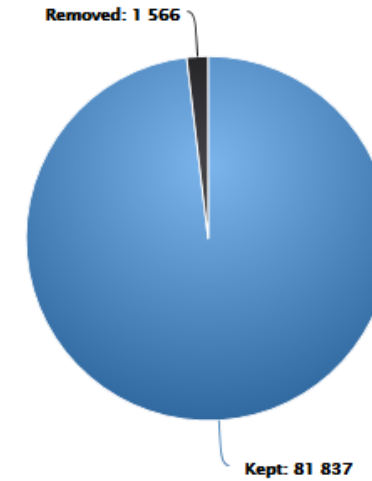
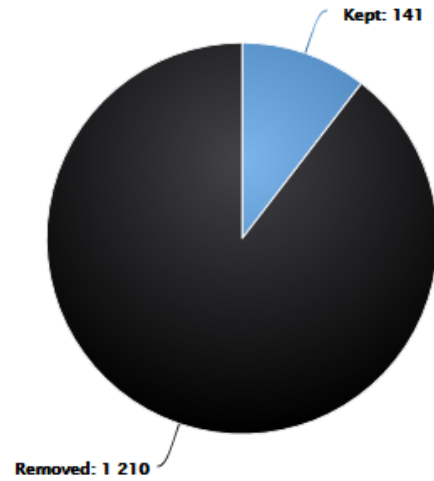
Configuration tabs

## Filters summary

OTUs



Abundance



## Filters intersections

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

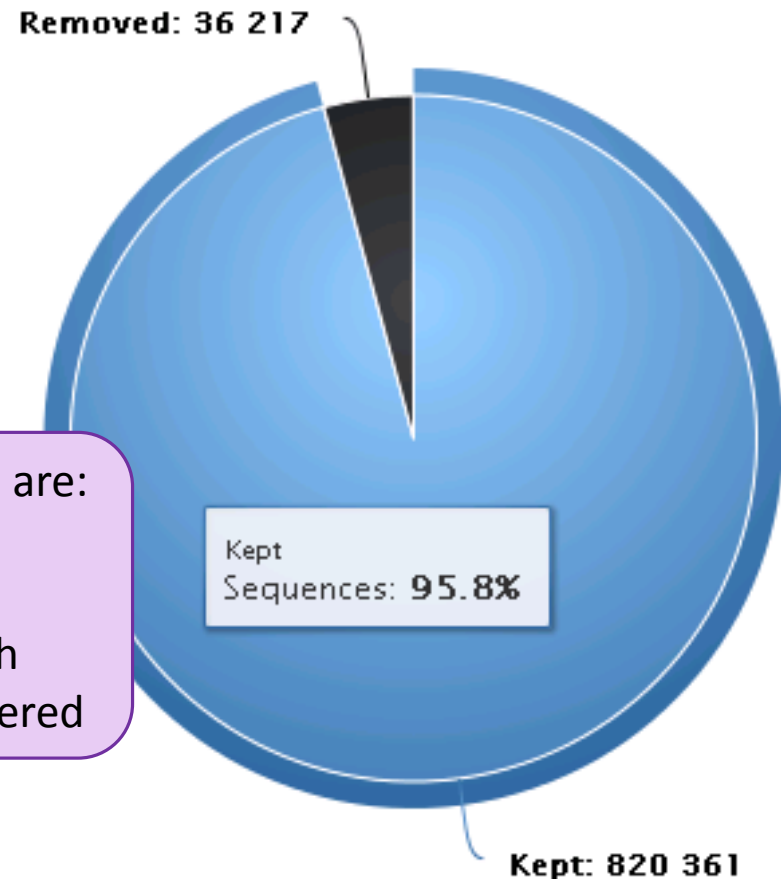
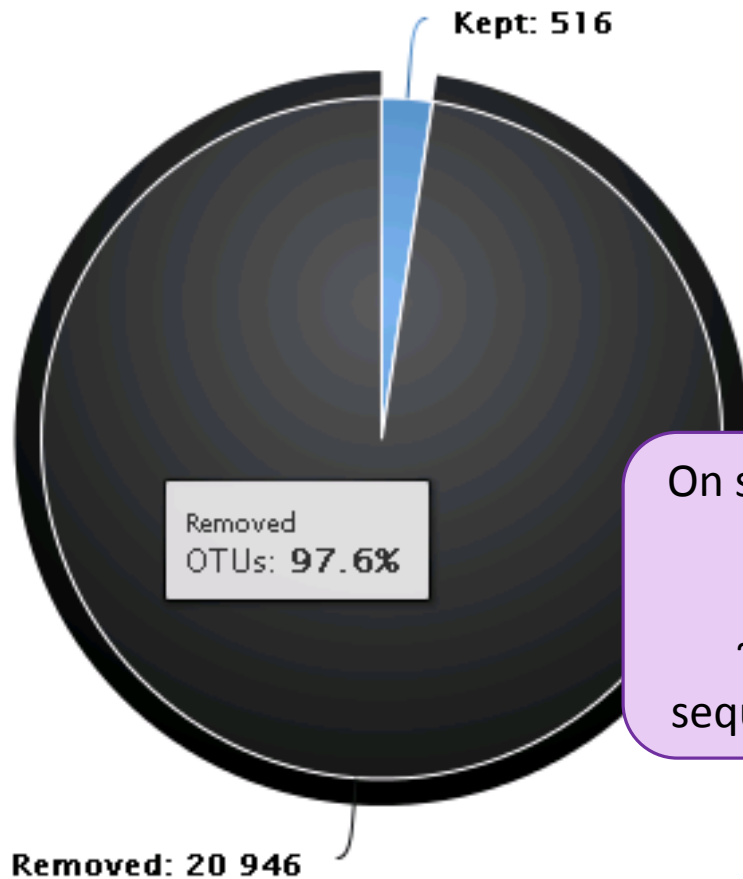
- Present in minus of 3 samples
- Abundance < 5e-05



OTUs



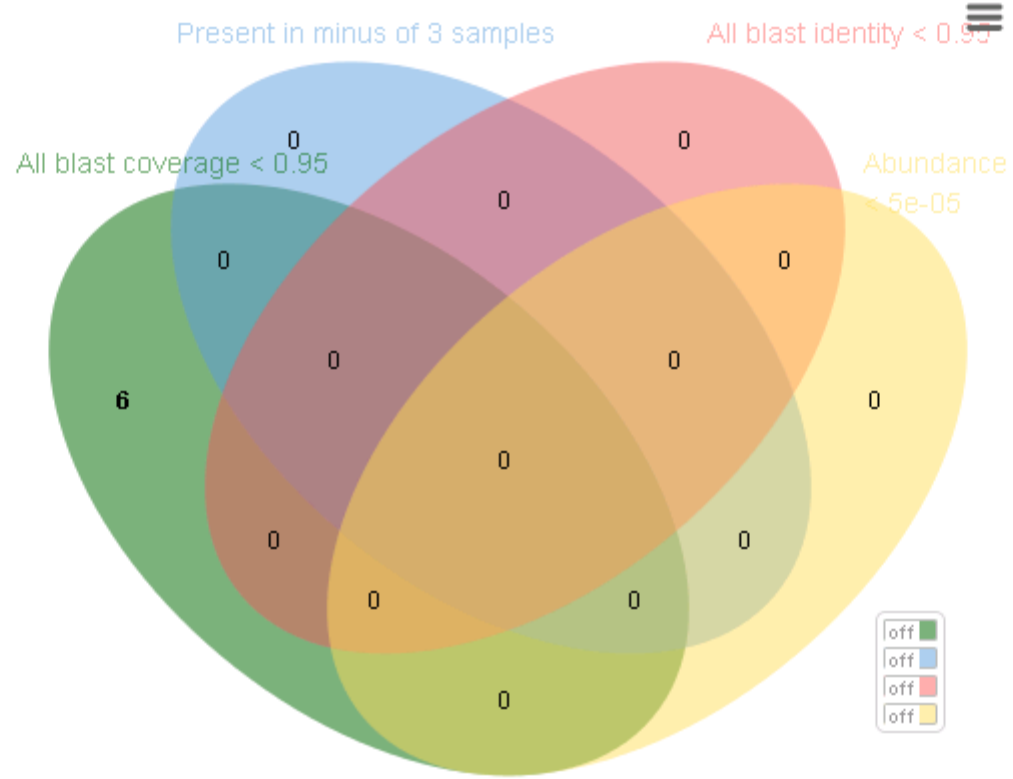
Abundance



On simulated data, singleton are:  
~99,9% are chimera  
and  
~0,1% are sequences with  
sequencing errors, non clustered

Removing little OTUs (conservation rate =0.005%)  
and non shared OTU (in less than 2 samples)

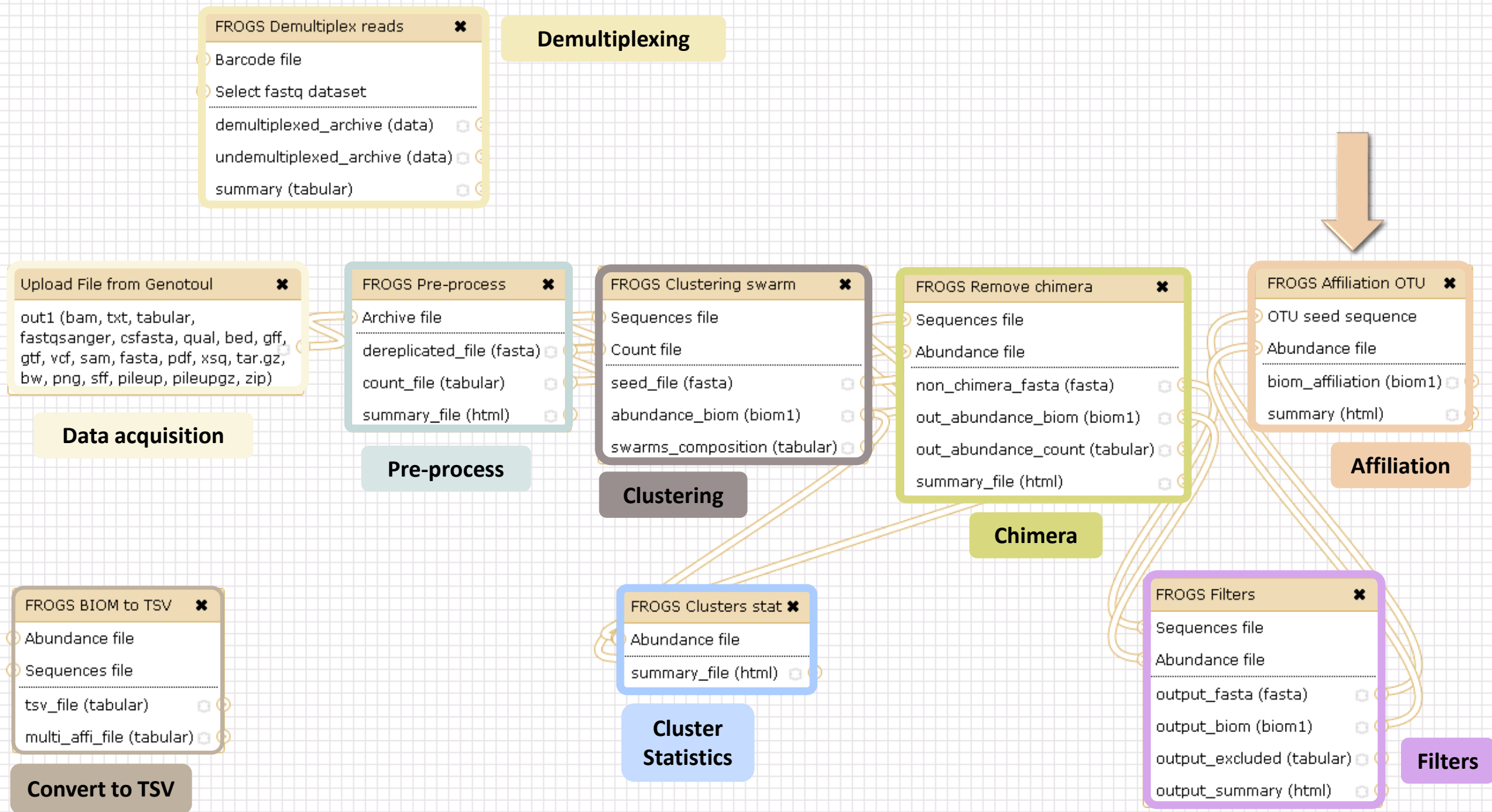
# Venn on removed OTUs



Close

# Affiliation tool

---



- OTU seed sequence
- Abundance file

---

- biom\_affiliation (biom1) 🗑
- summary (html) 🗑

**Affiliation**

**FROGS Affiliation OTU** Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST Options

(Galaxy Version 0.8.0)

**Using reference database**

silva123 16S OR silva128 16S

Select reference from the list

**Also perform RDP assignation?**

Yes No Optional

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

**OTU seed sequence**

17: FROGS Filters: sequences.fasta

OTU sequences (format: fasta).

**Abundance file**

18: FROGS Filters: abundance.biom

OTU abundances (format: BIOM).

Execute

# 1 Cluster = 2 affiliations

---

**Double Affiliation vs SILVA 123 (for 16S, 18S or 23S), SILVA 119 (for 18S) or Greengenes with :**

1. RDPClassifier\* (Ribosomal Database Project): one affiliation with bootstrap, on each taxonomic subdivision.

Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Pseudobutyrvibrio(80); Pseudobutyrvibrio xylanivorans (80)

2. NCBI Blastn+\*\* : all identical Best Hits with identity %, coverage %, e-value, alignment length and a special tag “**Multi-affiliation**”.

Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Pseudobutyrvibrio;Pseudobutyrvibrio ruminis; Pseudobutyrvibrio xylanivorans

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

V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S unknown species
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Butyrvibrio fibrisolvens
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S rumen bacterium 8 9293-9
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Pseudobutyrvibrio xylanivorans
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Pseudobutyrvibrio ruminis

5 identical blast best hits on SILVA 123 databank

# Affiliation Strategy of FROGS

---

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

V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S unknown species
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Butyrvibrio fibrisolvens
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S rumen bacterium 8   9293-9
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Pseudobutyrvibrio xylanivorans
V3 – V4	Bacteria   Firmicutes   Clostridia   Clostridiales   Lachnospiraceae   Pseudobutyrvibrio   16S Pseudobutyrvibrio ruminis



**FROGS Affiliation:** Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrvibrio | **Multi-affiliation**

# Your Turn! – 7

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LAUNCH THE « FROGS AFFILIATION » TOOL

# Exercise 7.1

---

Go to « **MiSeq merged** » history

Launch the « FROGS Affiliation » tool with

- **SILVA 123 or 128** 16S database
- FROGS Filters abundance biom and fasta files (after swarm d1d3, remove chimera and filter low abundances)



→ objectives :


- understand abundance tables columns
- understand the BLAST affiliation

## FROGS Affiliation OTU ✕

○ OTU seed sequence

○ Abundance file

biom\_affiliation (biom1)  

summary (html)  

## Affiliation

## FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST Options

(Galaxy Version 0.8.0)

### Using reference database

silva123 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

   17: FROGS Filters: sequences.fasta ▼

OTU sequences (format: fasta).



### Abundance file

   18: FROGS Filters: abundance.biom ▼

OTU abundances (format: BIOM).

Execute

# Exercise 7.1

1. What are the « FROGS Affiliation » output files ?
2. How many sequences are affiliated by BLAST ?
3. Click on the « eye » button on the BIOM output file, what do you understand ? 
4. Use the Biom\_to\_TSV tool on this last file and click again on the "eye" on the new output generated.   
 What do the columns ?  
 What is the difference if we click on case or not ? What consequence about weight of your file ?

**FROGS BIOM to TSV** Converts a BIOM file in TSV file. (Galaxy Version 2.1.0) Options

**Abundance file**  
  
 The BIOM file to convert (format: BIOM).

**Sequences file**  
  
 The sequences file (format: fasta). If you use this option the sequences will be add in TSV.

**Extract multi-alignments**  
   
 If you have used FROGS affiliation on your data, you can extract information about multiple alignements in a second TSV.

**Tools**

**FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION**

**FROGS pipeline**

[FROGS Upload archive from your computer](#)

[FROGS Demultiplex reads](#)  
Split by samples the reads in function of inner barcode.

[FROGS Pre-process Step 1](#) in metagenomics analysis: denoising and dereplication.

[FROGS Clustering swarm](#)  
Step 2 in metagenomics analysis : clustering.

[FROGS Remove chimera](#) Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

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

[FROGS Affiliation OTU](#) Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPTools and BLAST

**FROGS BIOM to TSV** Converts a BIOM file in TSV file.

[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 Abundance normalisation](#)

# Exercise 7.1

5. Understand Blast affiliations - Cluster\_2388 (affiliation from silva 123)

blast_subject	blast_evalue	blast_len	blast_perc_query_coverage	blast_perc_identity	blast_taxonomy
<a href="#">JN880417.1.1422</a>	0.0	360	88.88	99.44	Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Planctomycetaceae;Telmatocola;Telmatocola sphagniphila

# Blast JN880417.1.1422 vs our OTU

OTU length : 405

Excellent blast but no matches at the beginning of OTU.

Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence  
Sequence ID: [ref|NR\\_118328.1](#) Length: 1422 Number of Matches: 1

Range 1: 375 to 734 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
654 bits(354)	0.0	358/360(99%)	0/360(0%)	Plus/Plus
Query 46	CGCGTGCGCGATGAAGGCCTTCGGGTTGTAAGCGCGAAAGAGGTAATAAAGGGAAACCT	105		
Sbjct 375	CGCGTGCGCGATGAAGGCCTTCGGGTTGTAAGCGCGAAAGAGGSAATAAAGGGAAACTT	434		
Query 106	GATTGAACCTCAGTAAGCTCGGGCTAAGTTTGTGCCAGCAGCCGCGGTAAGACGAACCGA	165		
Sbjct 435	GATTGAACCTCAGTAAGCTCGGGCTAAGTTTGTGCCAGCAGCCGCGGTAAGACGAACCGA	494		
Query 166	GCGAACGTTGTTTCGGAATCACTGGGCATAAAGGGCGCGTAGGCGGGTTTCTAAGTCCGTG	225		
Sbjct 495	GCGAACGTTGTTTCGGAATCACTGGGCATAAAGGGCGCGTAGGCGGGTTTCTAAGTCCGTG	554		
Query 226	GTGAAATACTTCAGCTCAACTGGGAACTGCCTCGGATACTGGGAATCTCGAGTAATGTA	285		
Sbjct 555	GTGAAATACTTCAGCTCAACTGGGAACTGCCTCGGATACTGGGAATCTCGAGTAATGTA	614		
Query 286	GGGGCACGTGGAACGGCTGGTGGAGCGGTGAAATGCGTTGATATCAGTCGGAACCTCCGGT	345		
Sbjct 615	GGGGCACGTGGAACGGCTGGTGGAGCGGTGAAATGCGTTGATATCAGTCGGAACCTCCGGT	674		
Query 346	GGCGAAGGCGATGTGCTGGACATTTACTGACGCTGAGGCCGCGAAAGCCAGGGGAGCAAAC	405		
Sbjct 675	GGCGAAGGCGATGTGCTGGACATTTACTGACGCTGAGGCCGCGAAAGCCAGGGGAGCAAAC	734		

Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence  
NCBI Reference Sequence: NR\_118328.1  
[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS NR\_118328 1422 bp rRNA linear BCT 03-FEB-2015  
DEFINITION Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence  
ACCESSION [NR\\_118328](#)  
VERSION [NR\\_118328.1](#) GI:645321338  
DBLINK Project: [33175](#)  
BioProject: [PRJNA33175](#)  
KEYWORDS RefSeq.  
SOURCE Telmatocola sphagniphila  
ORGANISM [Telmatocola sphagniphila](#)  
Bacteria; Planctomycetes; Planctomycetia; Planctomycetales;  
Planctomycetaceae.  
REFERENCE 1 (bases 1 to 1422)  
AUTHORS Kulichevskaya,I.S., Serkebaeva,Y.M., Kim,Y., Rijpstra,W.I.,  
Damste,J.S., Liesack,W. and Dedysh,S.N.  
TITLE Telmatocola sphagniphila gen. nov., sp. nov., a novel dendriform  
planctomycete from northern wetlands  
JOURNAL Front Microbiol 3, 146 (2012)  
PUBMED [22529844](#)  
REMARK Publication Status: Online-Only  
REFERENCE 2 (bases 1 to 1422)  
CONSTRM NCBI RefSeq Targeted Loci Project  
TITLE Direct Submission  
JOURNAL Submitted (28-APR-2014) National Center for Biotechnology  
Information, NIH, Bethesda, MD 20894, USA  
REFERENCE 3 (bases 1 to 1422)  
AUTHORS Dedysh,S.N.  
TITLE Direct Submission  
JOURNAL Submitted (20-OCT-2011) Winogradsky Institute of Microbiology RAS,  
Prospect 60-Letya Otyabrya 7/2, Moscow 117312, Russia  
COMMENT REVIEWED [REFSEQ](#): This record has been curated by NCBI staff. The  
reference sequence is identical to [JN880417:1-1422](#).



# Blast columns

OTU\_2 seed has a best BLAST hit with the reference sequence AJ496032.1.1410

The reference sequence taxonomic affiliation is this one.

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404

Convert to TSV

FROGS BIOM to TSV ✕

Abundance file

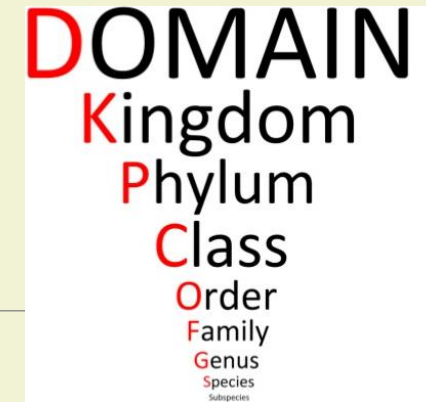
Sequences file

tsv\_file (tabular)

multi\_affi\_file (tabular)

Evaluation variables of BLAST

# Focus on “Multi-”



(affiliation from silva 123)

Observe line of Cluster 1 inside abundance.tsv and multi\_hit.tsv files, what do you conclude ?

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404



Cluster\_1 has 5 identical blast hits, with different taxonomies as the species level

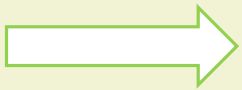
# Focus on “Multi-”

---

*(affiliation from silva 123)*

Observe line of Cluster 11 inside abundance.tsv and multi\_hit.tsv files, what do you conclude ?

Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae;Henriciella;Henriciella marina	multi-subject	100.0	100.0
--	---------------	-------	-------



Cluster\_11 has 2 identical blast hits, with identical species but with different strains (strains are not written in our data)

# Focus on “Multi-”

(affiliation from silva 123)

Observe line of Cluster 43 inside abundance.tsv and multi\_hit.tsv files, what do you conclude ?

Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Multi-affiliation;Multi-affiliation		multi-subject	99.3	100.0
Cluster_43	Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Selenomonas 3;unknown species			JQ447821.1.1420
Cluster_43	Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Centipeda;Centipeda periodontii			AJ010963.1.1494



Cluster\_43 has 2 identical blast hits, with different taxonomies at the genus level

# Back on Blast parameters

---

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404

Evaluation variables of BLAST

# Blast variables : e-value

---

The Expect value (E) 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.

# Blast variables : blast\_perc\_identity

Identity percentage between the Query (OTU) and the subject in the alignment  
(length subject = 1455 bases)

Score	Expect	Identities	Gaps	Strand
760 bits(411)	0.0	411/411(100%)	0/411(0%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 331	TGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	390		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTTACTGTGAGTGTACTTTT	120		
Sbjct 391	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTTACTGTGAGTGTACTTTT	450		
Query 121	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 451	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGGTGCAAGCGTT	510		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	240		
Sbjct 511	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	570		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 571	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGCAGGCTAGAGTGTAGTAGGGGAGACT	630		
Query 301	GGAATCCCGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	360		
Sbjct 631	GGAATCCCGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	690		
Query 361	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 691	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	741		

Query length = 411  
Alignment length = 411  
0 mismatch  
-> 100% identity

# Blast variables :

## blast\_perc\_identity

Identity percentage between the Query (OTU) and the subject in the alignment  
(length subject = 1455 bases)

Score	Expect	Identities	Gaps	Strand
614 bits(332)	5e-172	385/411(94%)	5/411(1%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 140728	TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	140787		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATTGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	120		
Sbjct 140788	CCTTCGGGTTGTAAACCGCTTTTGAATTGGGAGCAAGC-G----AGAGTGTGTACTTTT	140842		
Query 121	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 140843	CGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	140902		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTTCGCGTCTGGTGTGAAAGTC	240		
Sbjct 140903	ATCCGGAATTATTGGGCGTAAAGRGCTCGTAGGCGGTTTGTTCGCGTCTGGTGTGAAAGTC	140962		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 140963	CATCGCTTAACGGTGGATCTGCGCCGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	141022		
Query 301	GGAATCCCAGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACCACCAATGGCGAAGGC	360		
Sbjct 141023	GGAATCCCAGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACCACCAATGGCGAAGGC	141082		
Query 361	AGGTCCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 141083	AGGTCCTGGGCCGTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	141133		

Query length = 411  
Alignment length = 411  
26 mismatches (gaps included)  
-> 94% identity



# Blast variables :

## blast\_perc\_query\_coverage

Coverage percentage of alignment on query (OTU)

Score	Expect	Identities	Gaps	Strand
760 bits(411)	0.0	411/411(100%)	0/411(0%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 331	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	390		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	120		
Sbjct 391	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	450		
Query 121	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 451	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	510		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	240		
Sbjct 511	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	570		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 571	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	630		
Query 301	GGAATCCCAGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	360		
Sbjct 631	GGAATCCCAGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	690		
Query 361	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 691	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	741		

Query length = 411  
100% coverage

# Blast variables : blast-length

---

Length of alignment between the OTUs = “Query” and “subject” sequence of database

	Coverage %	Identity %	Length alignment
OTU1	100	98	400
OTU2	100	98	500



More mismatches/gaps

FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file
- biom\_affiliation (biom1) 🔄
- summary (html) 🔄

**Affiliation**

**FROGS Affiliation OTU** Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST Options  
 (Galaxy Version 0.8.0)

**Using reference database**  
 silva123 16S  
 Select reference from the list

**Also perform RDP assignation?**  
  Optional and not in our guideline

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

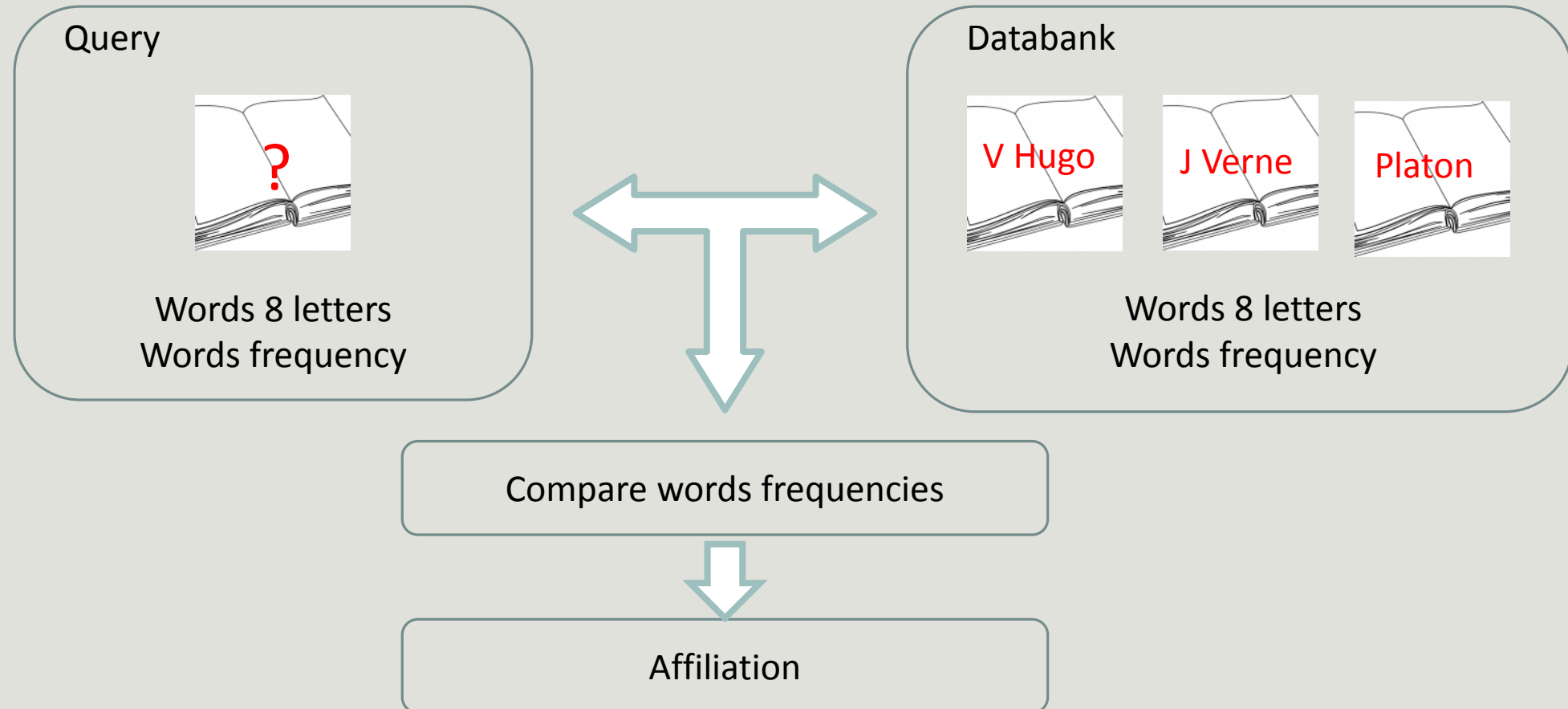
**OTU seed sequence**  
   17: FROGS Filters: sequences.fasta  
 OTU sequences (format: fasta).

**Abundance file**  
   18: FROGS Filters: abundance.biom  
 OTU abundances (format: BIOM).

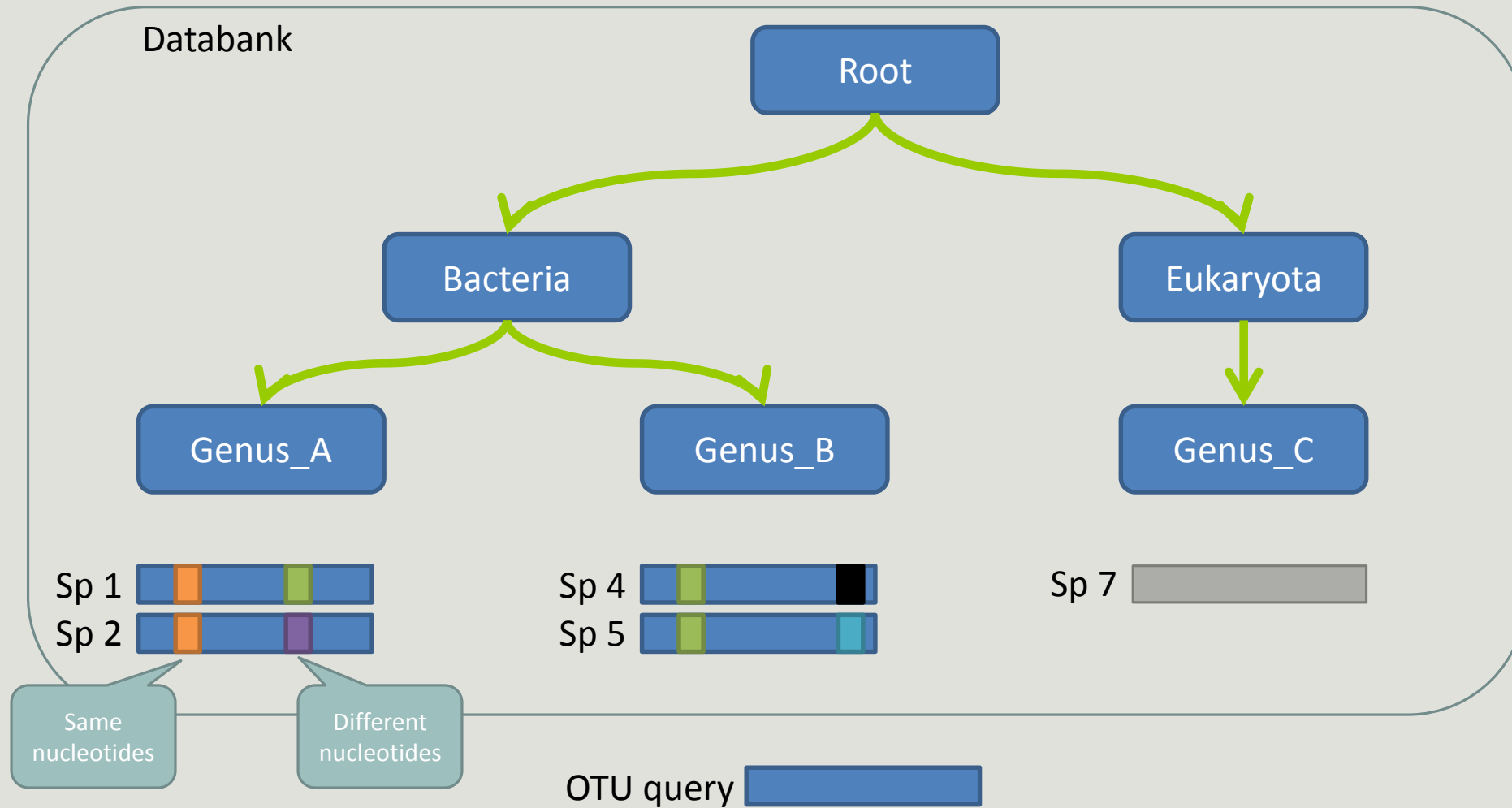
Who have already used RDP previously ?



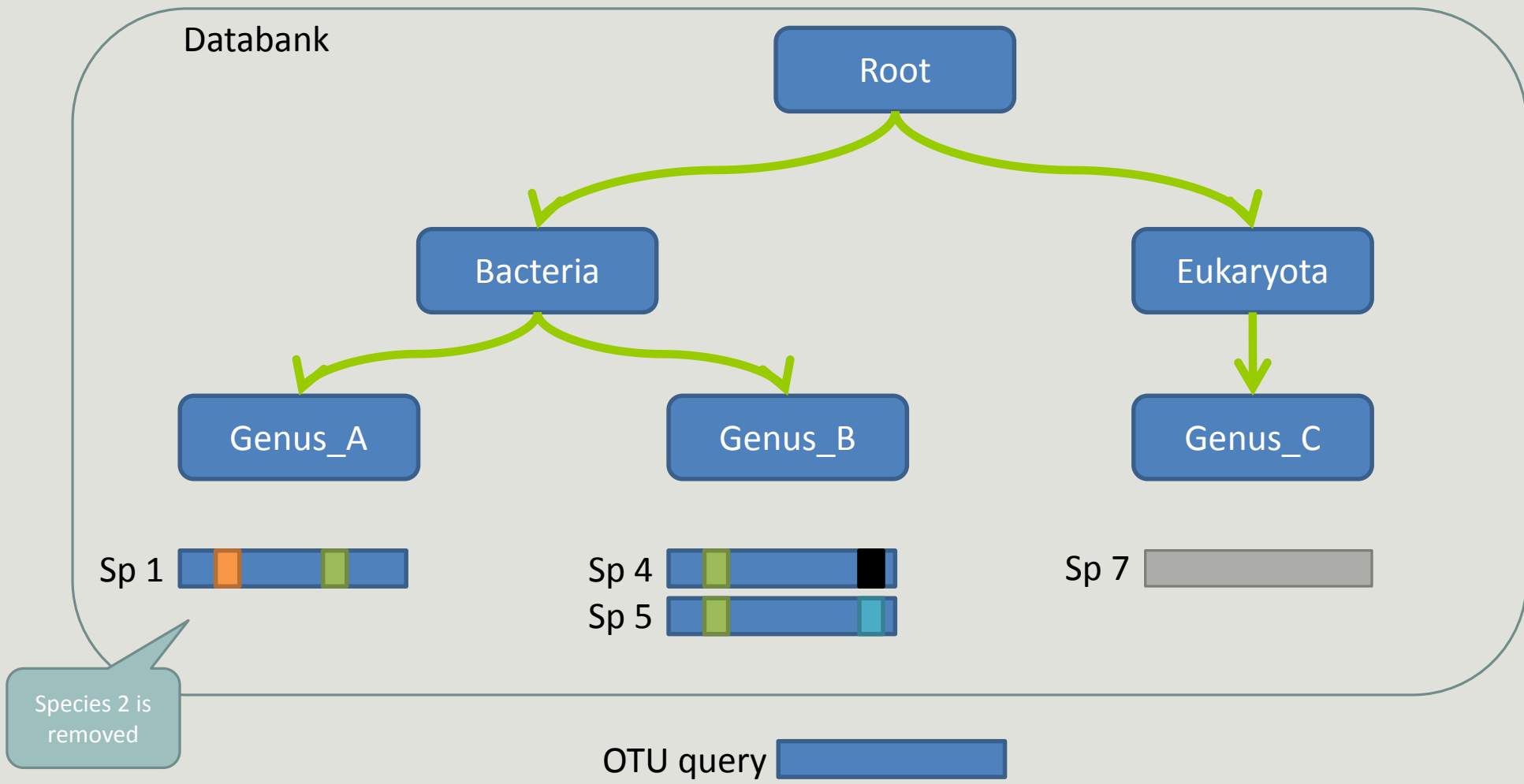
# How works RDP ?



# How works RDP ?

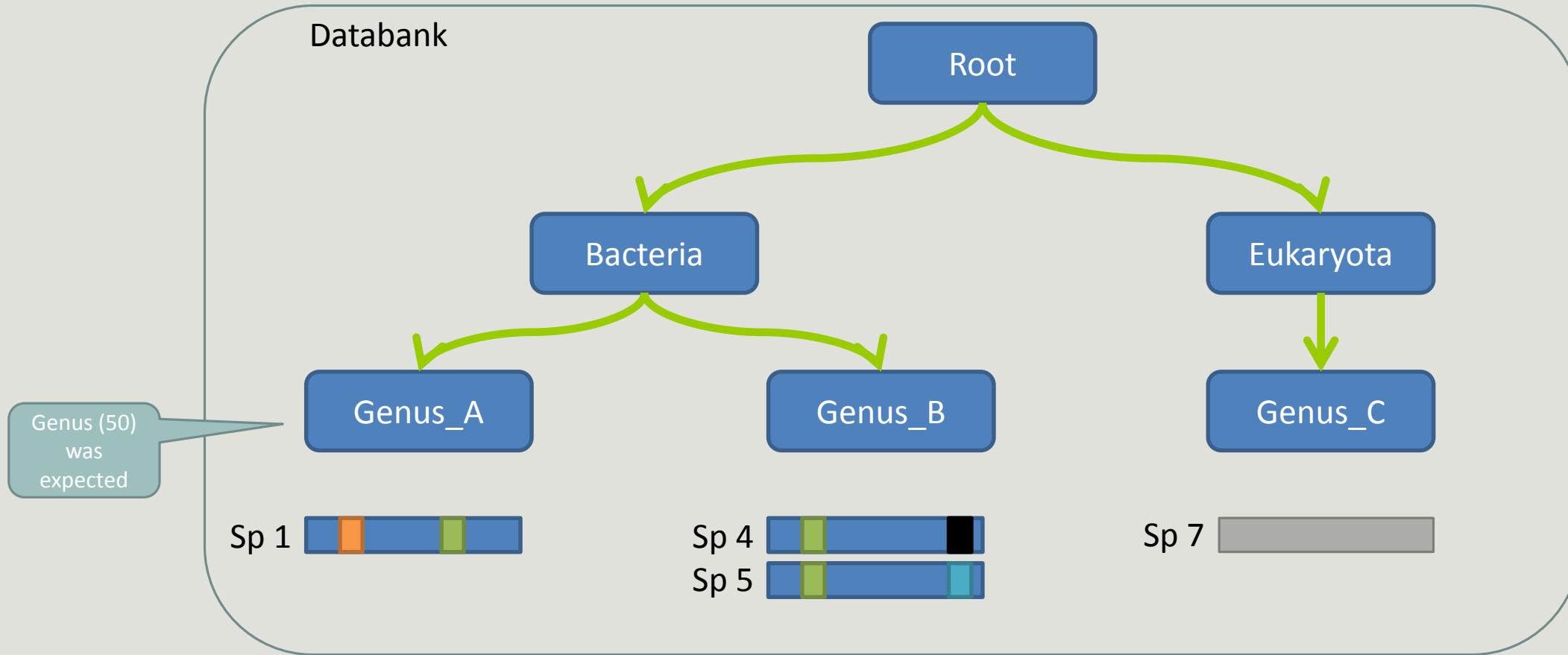


# The dysfunctions of RDP ?



**Result:**  
?

# The dysfunctions of RDP n°1 ?



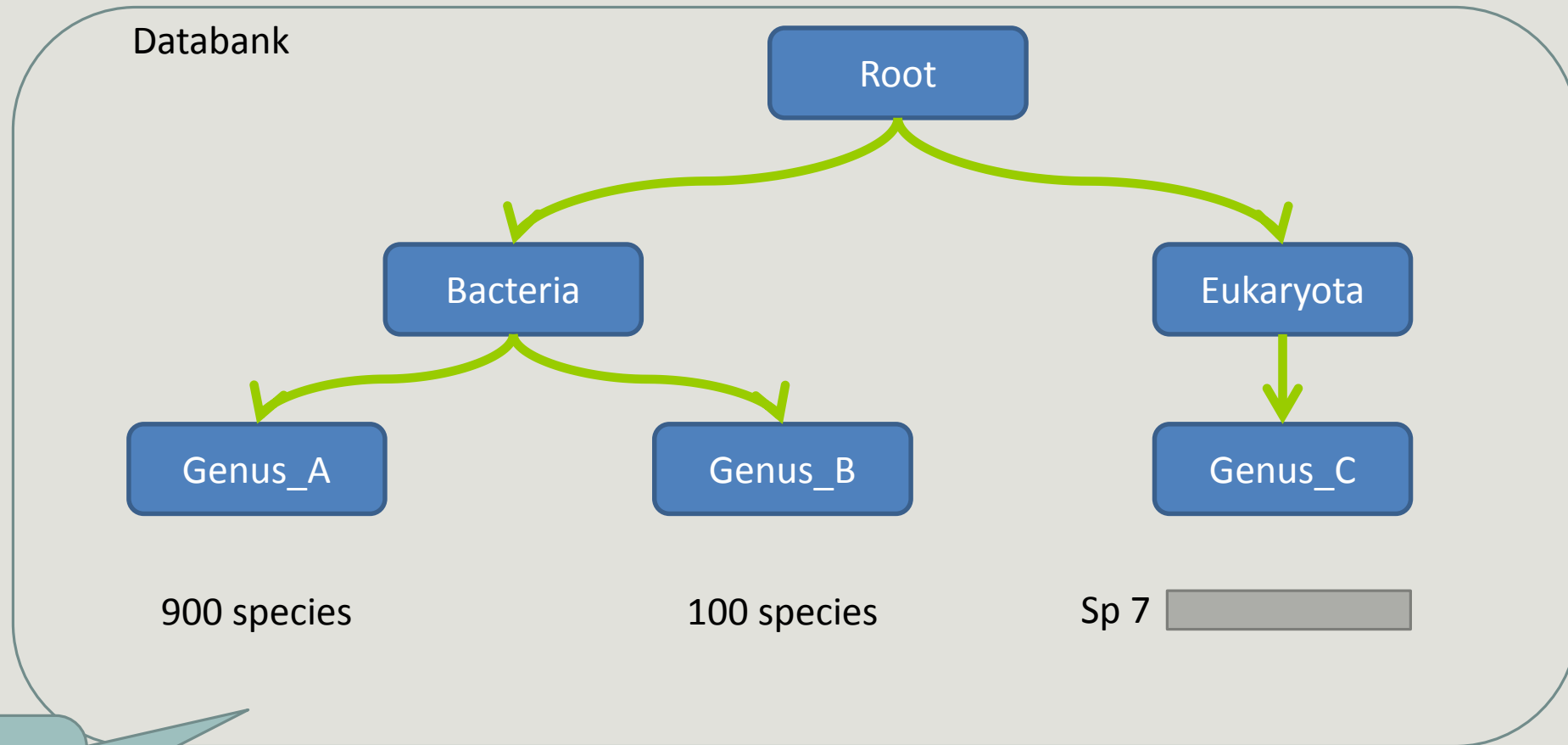
OTU query 

Genus (50) was expected.  
If seq order was inverted in banks, so we got the second statistics.

**Result:**  
Bacteria(100); Genus\_A(33); sp1(33) **OR** Bacteria(100); Genus\_B(66); sp5(33)

Order dependent

# The dysfunctions of RDP n°2 ?



Many species in one genus and little in the other:  
So, RDP can give very different results

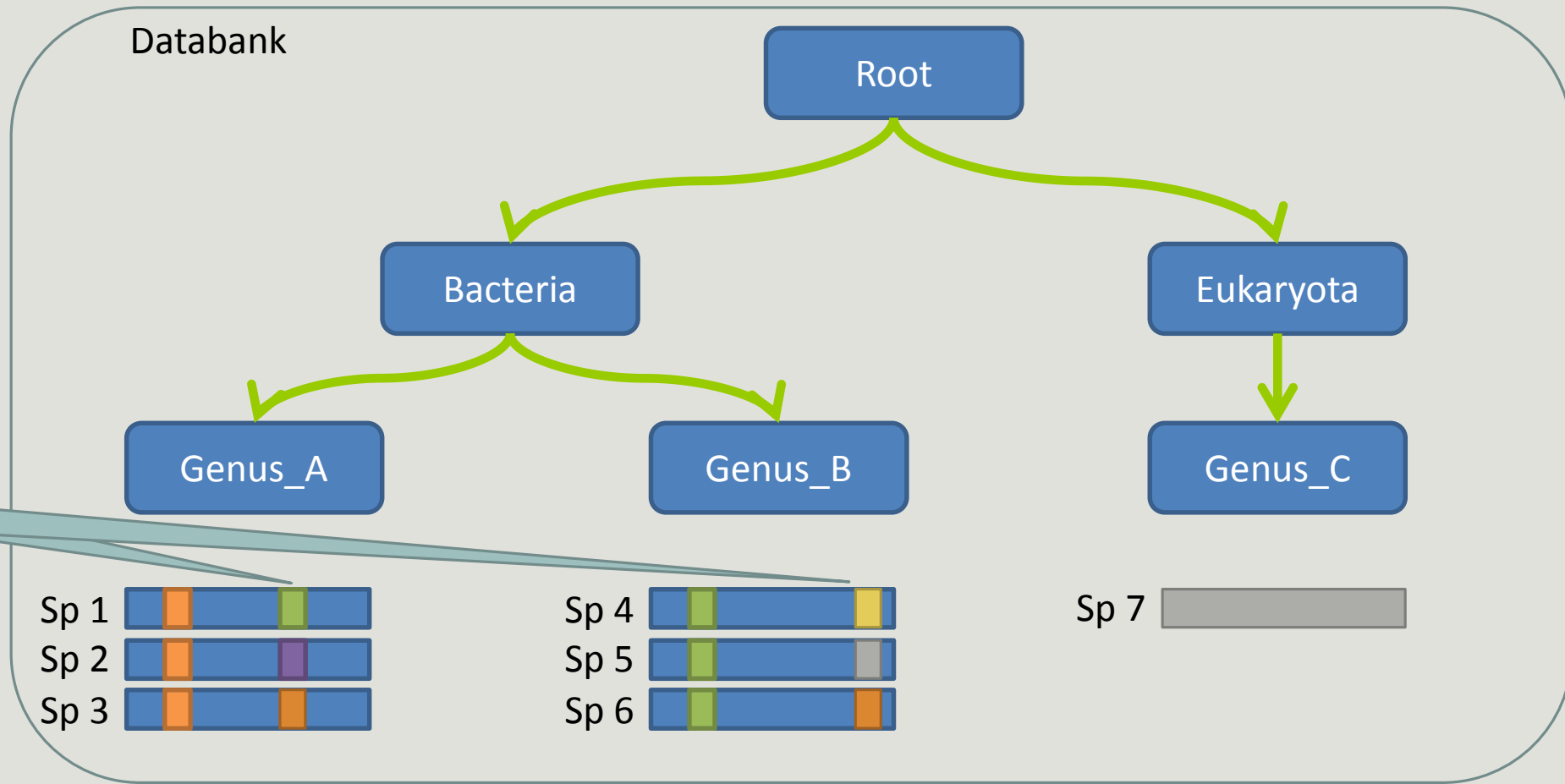
OTU query

Influenced by heterogeneity in last ranks

**Result:**  
Bacteria(100); Genus\_A(90); spX(0.1) **OR** Bacteria(100); Genus\_B(10); spX(0.1)



# The dysfunctions of RDP n°3 ?

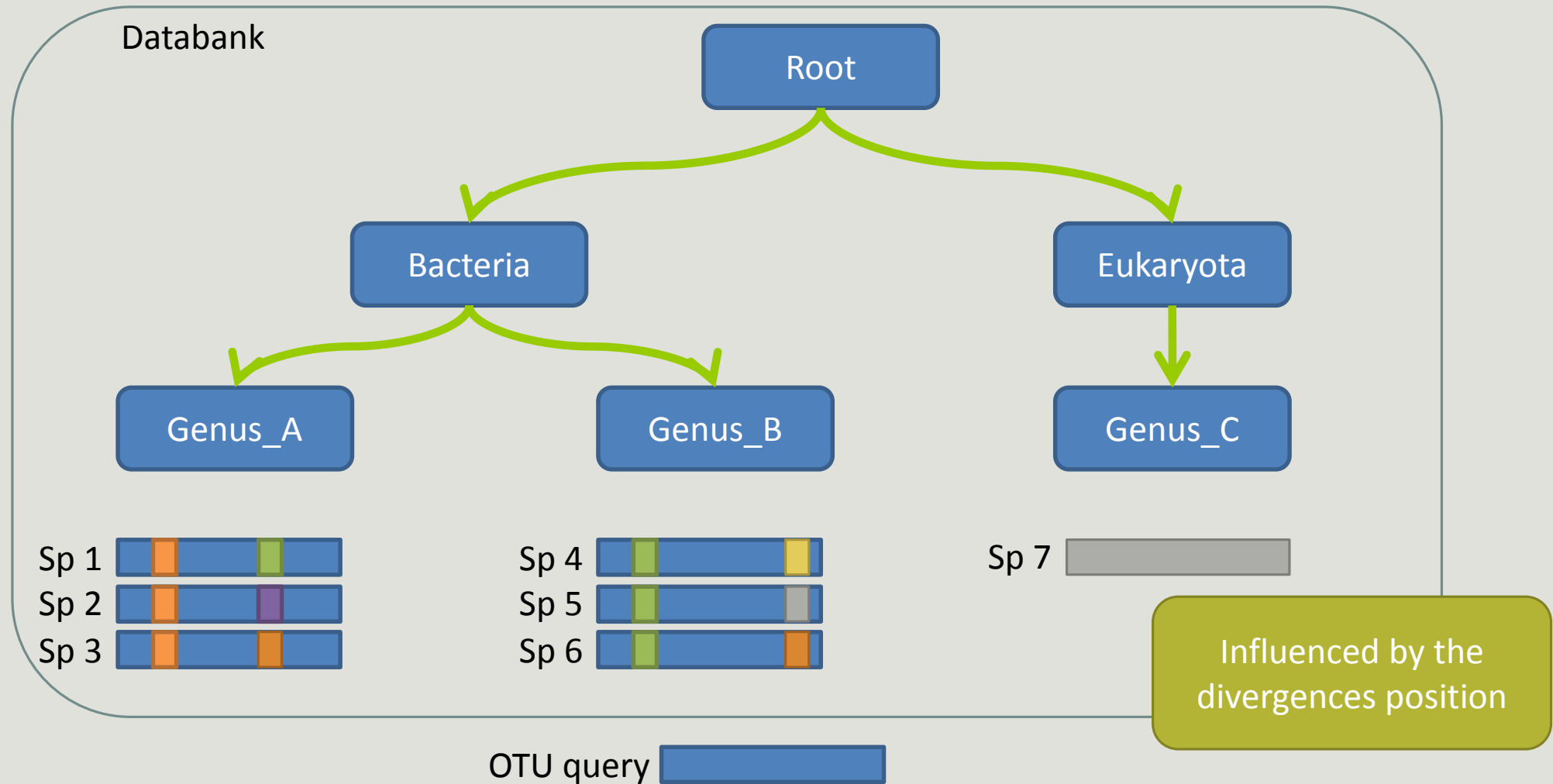


Mismatches are at different places

OTU query

**Result:**  
?

# The dysfunctions of RDP n°3 ?



Si le mismatch se fait sur un mot très "significatif" dans le profil de k-mers, RDP ne tombera que rarement sur l'espèce lors du bootstrap. Avec une même distance d'édition (2 mismatches) on peut donc avoir une grande différence de bootstrap pour peu que le mot affecté soit important dans le profil.

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

RDPClassifier  
NCBI blastn+

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

Identical  
V3-V4

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

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

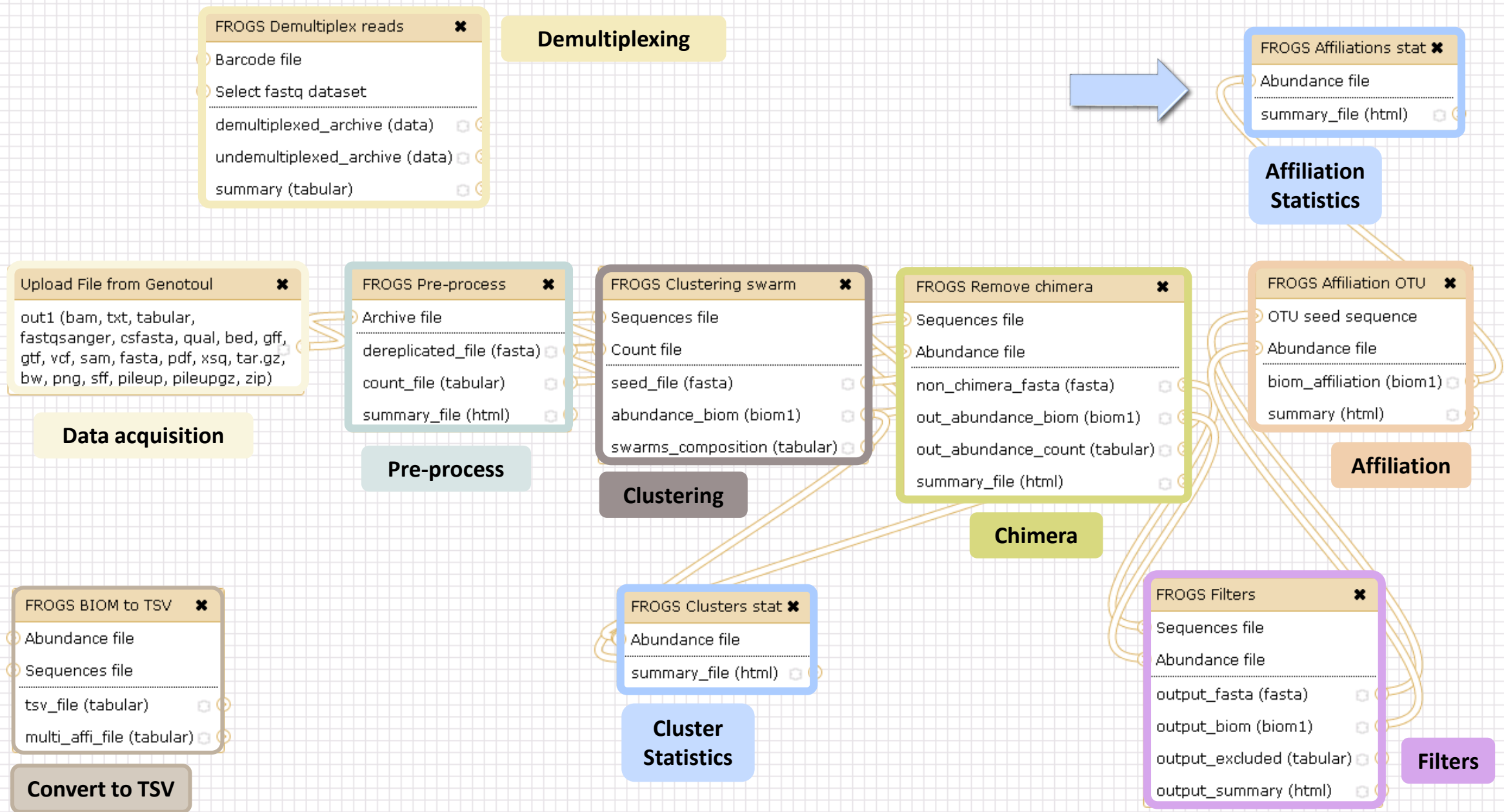
# Careful: Multi hit blast table is non exhaustive !

---

- Chimera (multiple affiliation)
- V3V4 included in others
- Missed primers on some 16S during database building

# Affiliation Stat

---



**FROGS Affiliations stat** Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

**Abundance file**  
  
 OTUs abundances and affiliations (format: BIOM).

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

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



**FROGS Affiliations stat** Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

**Abundance file**  
  
 OTUs abundances and affiliations (format: BIOM).

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

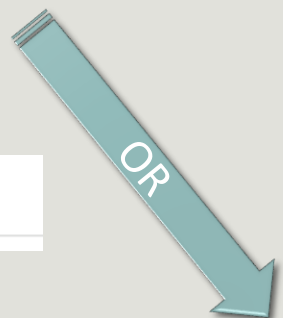
**Affiliation processed**  
  
 Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.



Taxonomy distribution | Alignment distribution



Taxonomy distribution | Bootstrap distribution



**FROGS Affiliations stat** Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

**Abundance file**  
  
 OTUs abundances and affiliations (format: BIOM).

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

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

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

**Taxonomy tag**  
  
 The metadata title in BIOM for the taxonomy.

**Bootstrap tag**  
  
 The metadata title in BIOM for the taxonomy bootstrap.

**Identity tag**  
  
 The metadata tag used in BIOM file to store the alignment identity.

**Coverage tag**  
  
 The metadata tag used in BIOM file to store the alignment OTUs coverage.



# Exercise 7.2

FROGS Affiliations stat (version 1.1.0)

**Abundance file:**  
17: FROGS Affiliation OTU: affiliation.biom  
OTUs abundances and affiliations (format: BIOM).

**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

FROGS Affiliations stat (version 1.1.0)

**Abundance file:**  
17: FROGS Affiliation OTU: affiliation.biom  
OTUs abundances and affiliations (format: BIOM).

**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 rdp  
**Is it adequate on our data ? Why ?**  
Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute

23: FROGS Affiliations stat: summary.html

# Exercise 7.2

---

→ objectives :

understand rarefaction curve and sunburst

1. Explore the [Affiliation stat](#) results on FROGS blast affiliation.
2. What kind of graphs can you generate? What do they mean?

**Tools**

**RADseq STACKS**

**RADseq STACKS**

**METHYLATION - BISULFITE**

**Bisulfite BISMARK**

**DEEPTOOLS**

**deepTools**

**FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION**

**FROGS pipeline**

**FROGS Upload archive** from your computer

**FROGS Demultiplex reads** Split by samples the reads in function of inner barcode.

**FROGS Pre-process** Step 1 in metagenomics analysis: denoising and dereplication.

**FROGS Clustering swarm** Step 2 in metagenomics analysis : clustering.

**FROGS Remove chimera** Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

**FROGS Filters** Filters OTUs on several criteria.

**FROGS Affiliation OTU** Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

**FROGS BIOM to TSV** Converts a BIOM file in TSV file.

**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

Taxonomy distribution Alignment distribution

Display global distribution

CSV

Show 10 entries

Search:

Taxonomies by sample

<input type="checkbox"/> Samples	Nb domain	Nb phylum	Nb class	Nb order	Nb family	Nb genus	Nb species	Nb sequences
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-01-reads	1	29	59	129	243	491	492	81,572
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-02-reads	1	29	59	130	243	491	492	82,466
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-03-reads	1	29	59	130	243	491	493	82,159
<input type="checkbox"/> 500taxas_With_Error_Power_Law-04-reads	1	29	59	130	243	491	492	81,985
<input type="checkbox"/> 500taxas_With_Error_Power_Law-05-reads	1	29	59	130	241	487	488	82,039
<input type="checkbox"/> 500taxas_With_Error_Power_Law-06-reads	1	29	59	130	244	493	494	81,758
<input type="checkbox"/> 500taxas_With_Error_Power_Law-07-reads	1	29	59	130	244	491	492	81,714
<input type="checkbox"/> 500taxas_With_Error_Power_Law-08-reads	1	29	58	129	243	493	494	82,255
<input type="checkbox"/> 500taxas_With_Error_Power_Law-09-reads	1	29	59	130	244	493	494	82,113
<input type="checkbox"/> 500taxas_With_Error_Power_Law-10-reads	1	29	58	128	240	487	489	82,300



With selection: Class

Showing 1 to 10 of 10 entries

Previous 1 Next

**History**

imported: 500WEPL\_setA  
451.3 MB

**106: FROGS Clusters stat summary.html**

**105: report\_download**

**103: Vsearch Clusters stat**

**102: FROGS Affiliations stat summary.html**  
299.1 KB  
format: html, database: ?  
## Application Software:  
affiliations\_stat.py (version: 1.1.0)  
Command: /usr/local/bioinfo  
/src/galaxy-dev/galaxy-dist/tools  
/FROGS/tools/affiliations\_stat.py  
--input-biom /galaxydata/database  
/files/054/dataset\_54829.dat  
--output-file /work/galaxy-dev/data

HTML file

**101: swarm cluster stat**

**100: FROGS BIOM to std BIOM: blast metadata.tsv**

**99: FROGS BIOM to std BIOM: abundance.biom**

**98: FROGS BIOM to TSV: multi\_hits.tsv**

**97: FROGS BIOM to TSV: abundance.tsv**

**96: FROGS Affiliations stat summary.html**  
295.0 KB  
format: html, database: ?  
## Application Software:  
affiliations\_stat.py (version: 1.1.0)  
Command: /usr/local/bioinfo

Tools

[FROGS Demultiplex reads](#)  
Split by samples the reads in function of inner barcode.

[FROGS Pre-process](#) Step 1 in metagenomics analysis: denoising and dereplication.

[FROGS Clustering swarm](#)  
Step 2 in metagenomics analysis : clustering.

[FROGS Remove chimera](#) Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

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

[FROGS Affiliation OTU](#) Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

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

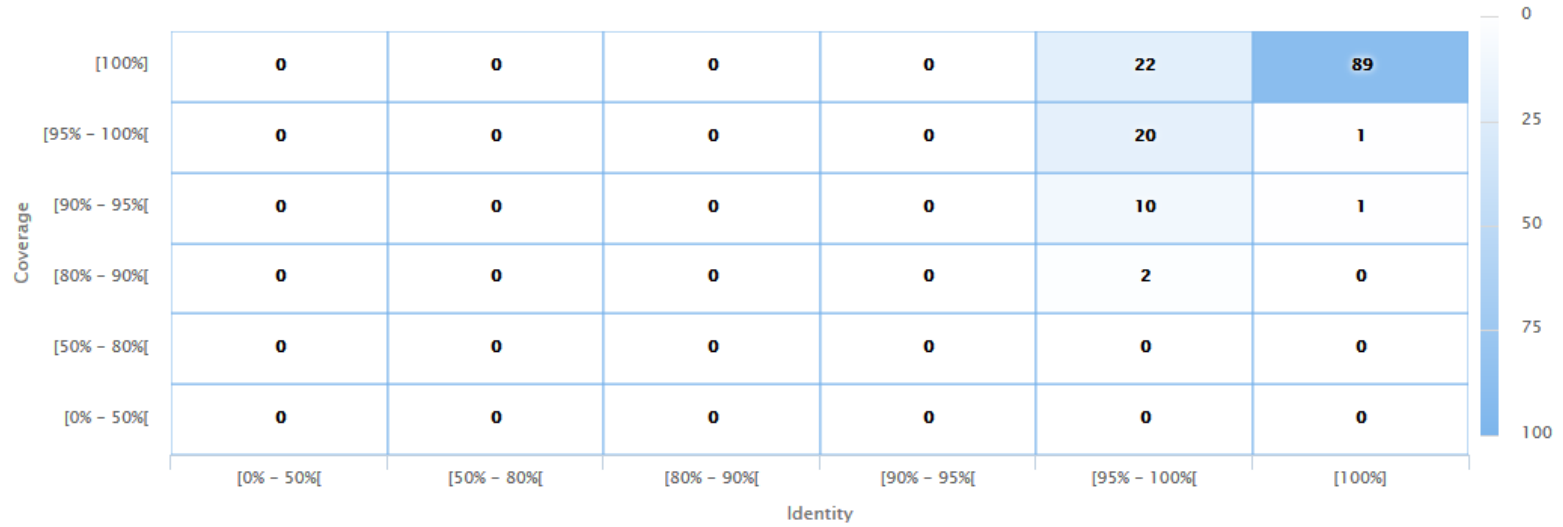
[FROGS Clusters stat](#) Process some metrics on clusters.

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

Taxonomy distribution

Alignment distribution

Number of OTUs among their alignment results



by OTUs

by sequences

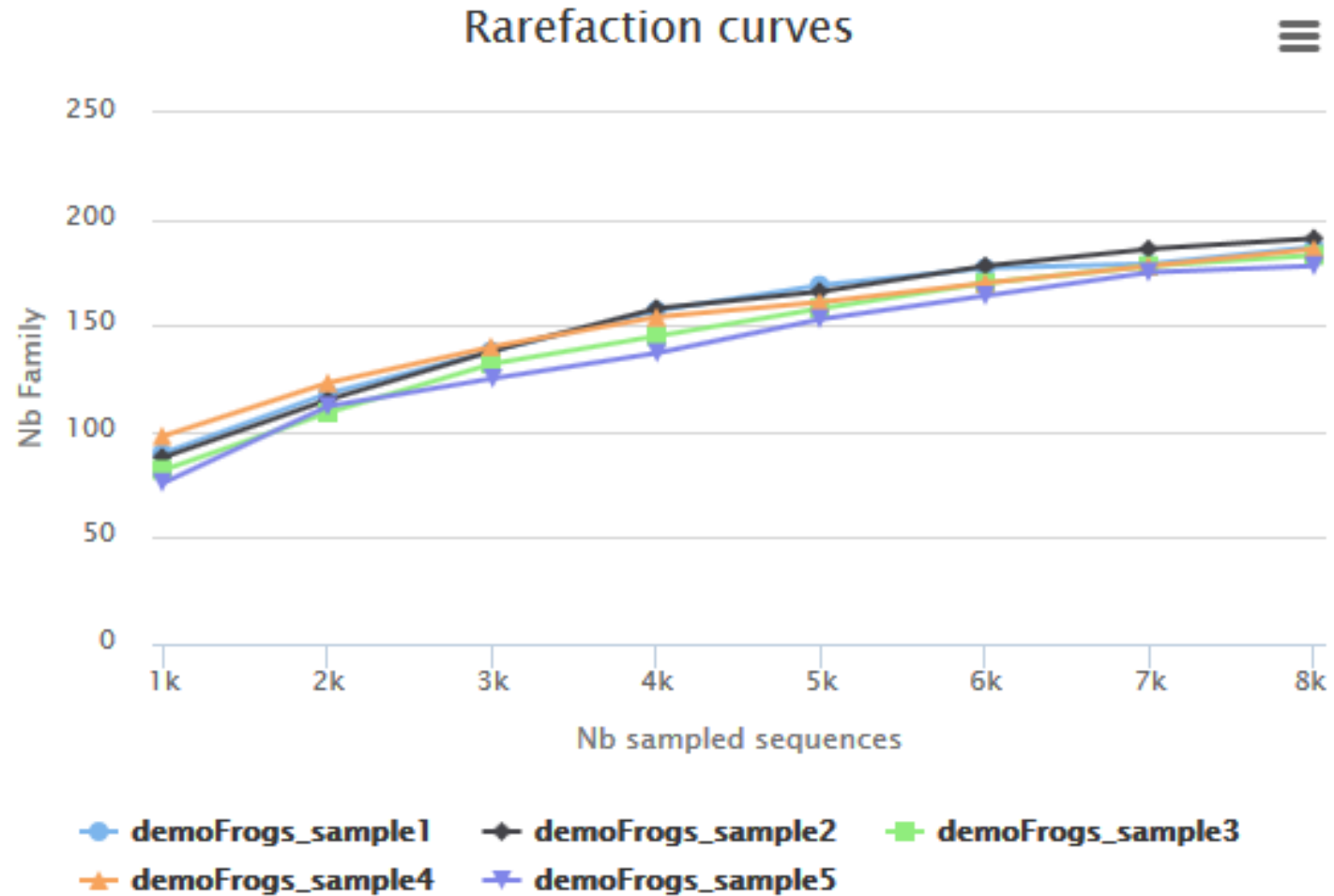
History

- Formation 9samples**  
20.3 MB
- 21: FROGS BIOM to TSV: multi\_hits.tsv**
- 20: FROGS BIOM to TSV: abundance.tsv**
- 19: FROGS Affiliations stat: summary.html**  
230.0 KB  
format: html, database: ?  
## Application Software: affiliations\_stat.py (version: 1.1.0) Command: /usr/local/bioinfo/src/galaxy-dev/galaxy-dist/tools/FROGS/tools/affiliations\_stat.py --input-biom /galaxydata/database/files/060/dataset\_60522.dat --output-file /work/galaxy-dev/data
- 18: FROGS Affiliation OTU: report.html**

Available only after  
AFFILIATION TOOL

Samples size ~8500  
sequences

## Rarefaction



The curve continues  
to rise

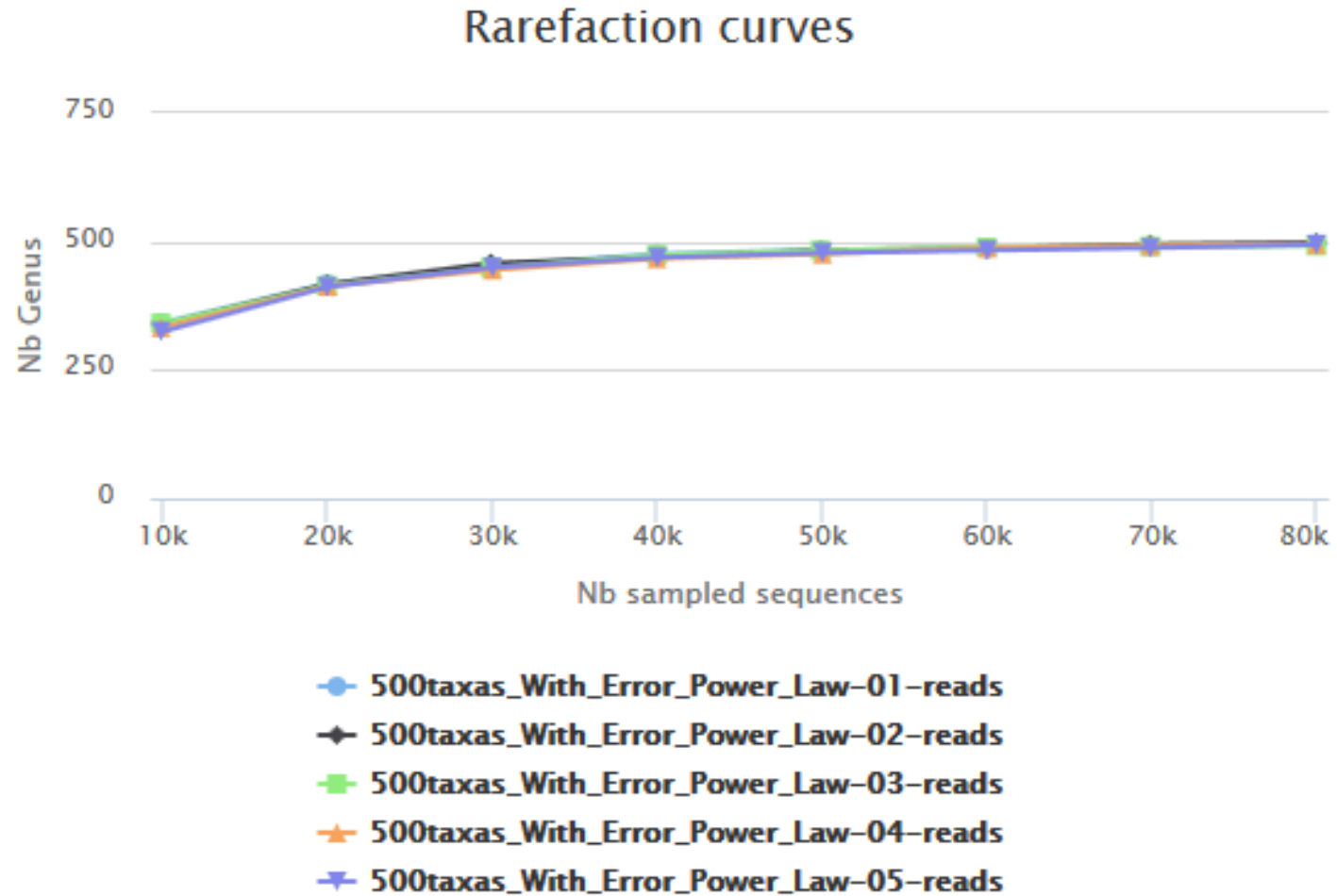
The number of  
sequences per  
sample is not large  
enough to cover all  
of the bacterial  
families

Rarefaction tab

Available only after  
AFFILIATION TOOL

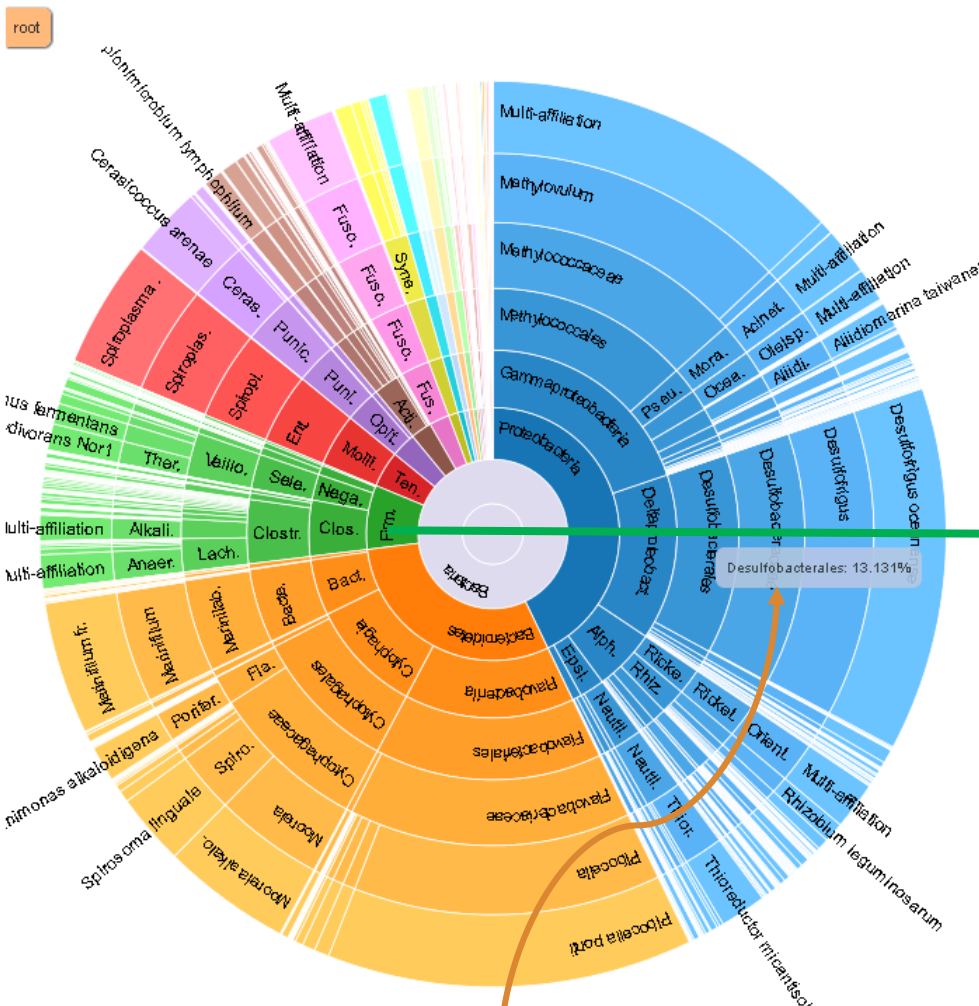
Samples size ~85 000  
sequences

## Rarefaction



The curve slows to  
rise with ~50 000  
sequences

With 60 000  
sequences, we catch  
almost all genus of  
bacteria

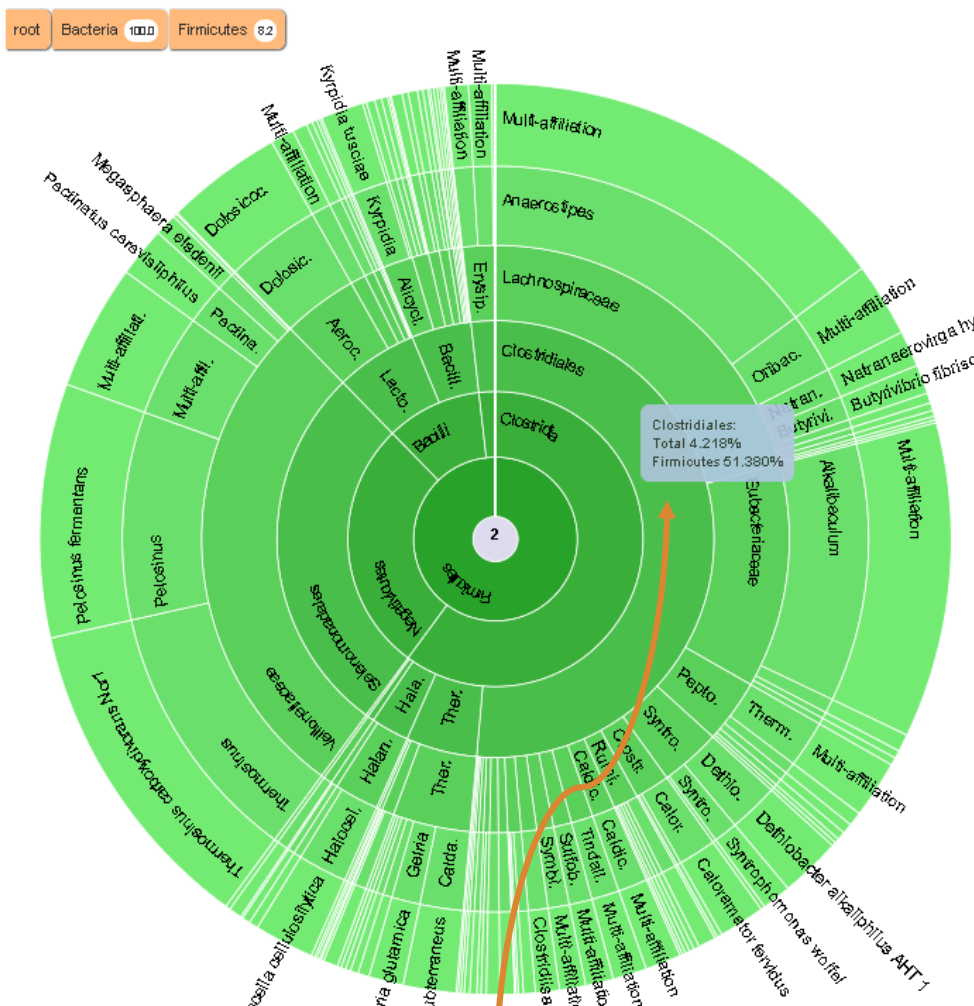


Zoom in on firmicutes

Detail on selected:

Name	Size	Global %	Parent %
root	246197		
Bacteria	246197	100.000	100.000
Proteobacteria	105524	42.862	42.862
Deltaproteobacteria	35987	14.617	34.103
Desulfobacterales	32328	13.131	89.832

Desulfobacterales nb children: 2



Detail on selected:

Name	Size	Global %	Parent %
root	246197		
Bacteria	246197	100.000	100.000
Firmicutes	20212	8.210	8.210
Clostridia	12142	4.932	60.073
Clostridiales	10385	4.218	85.530

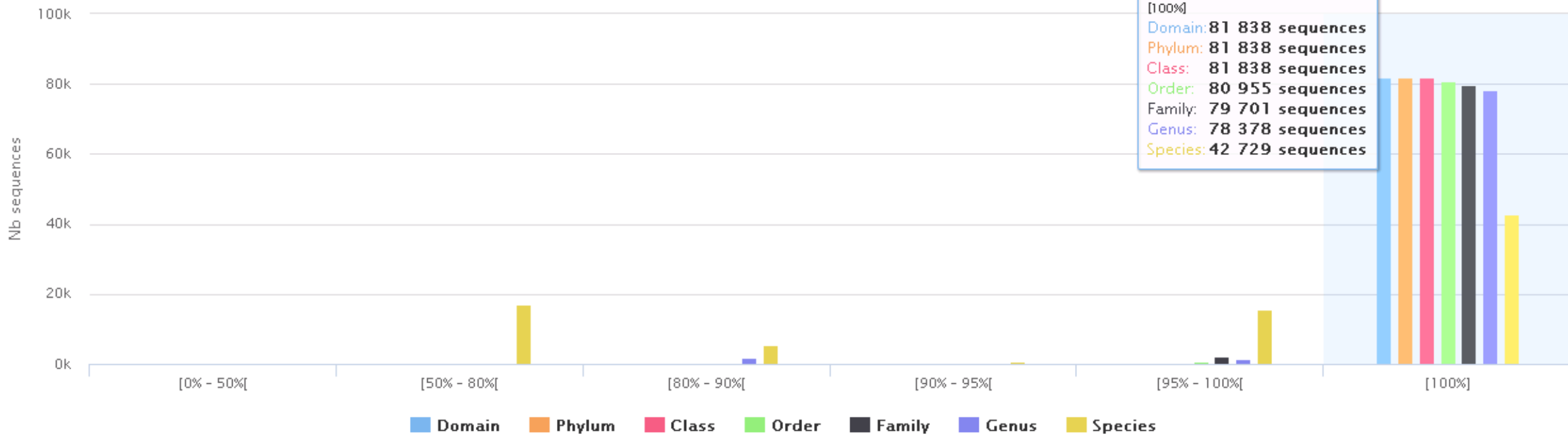
Clostridiales nb children: 20



Taxonomy distribution

Bootstrap distribution

### Number of sequences by bootstrap on affiliation

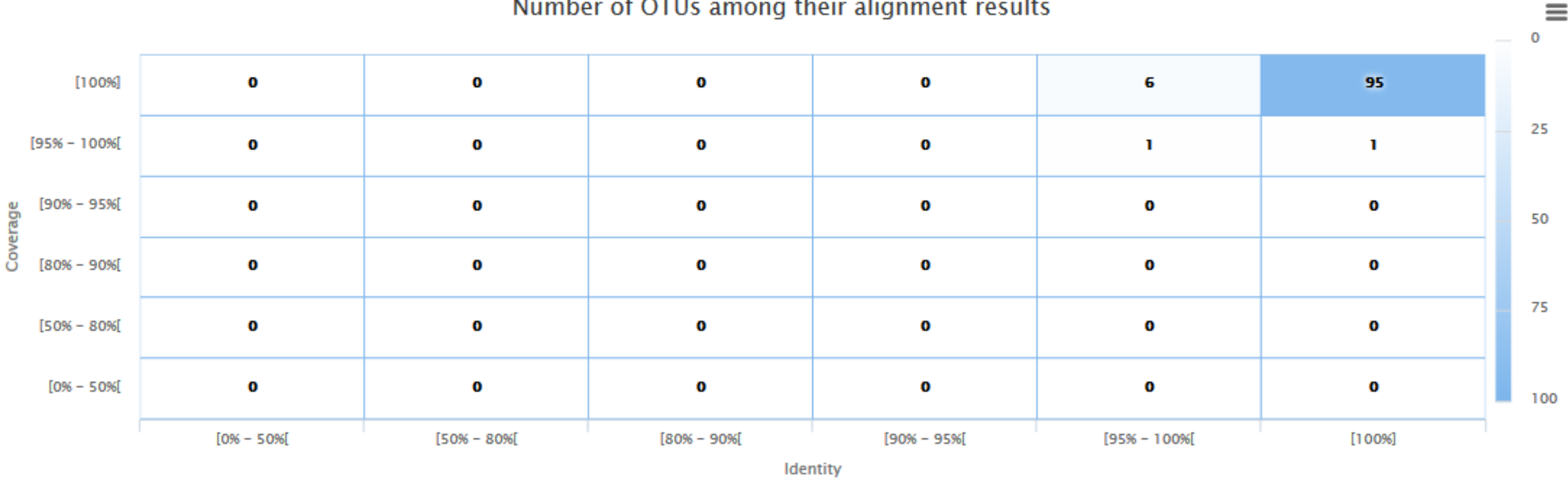


by OTUs

by sequences



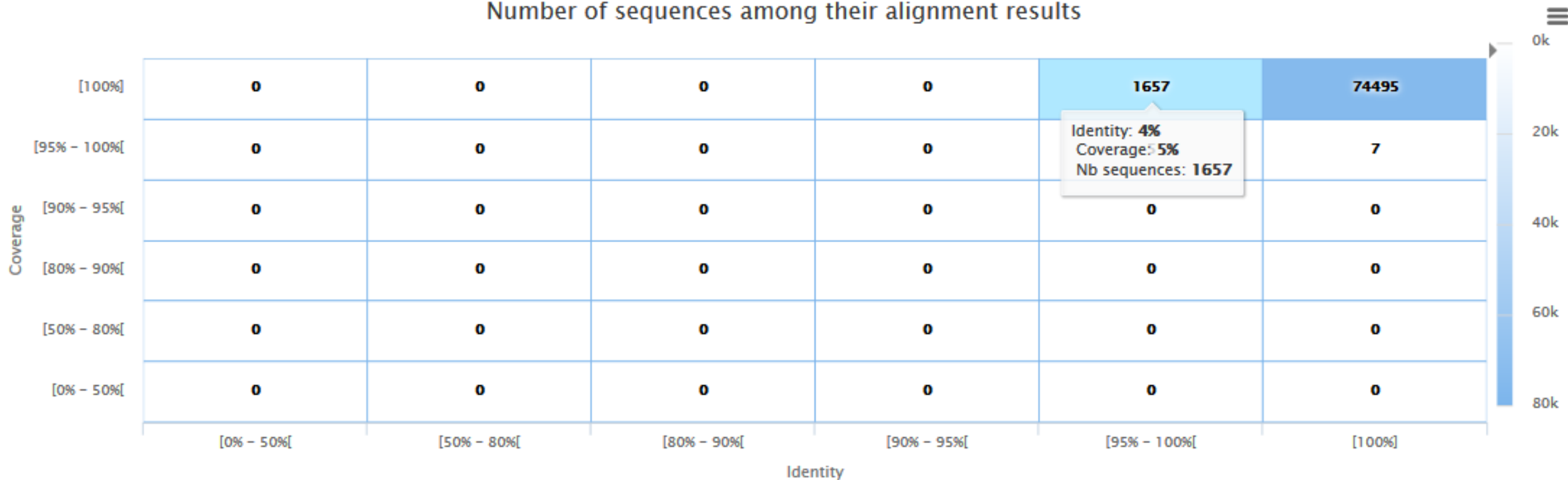
### Number of OTUs among their alignment results



by OTUs

by sequences

## Number of sequences among their alignment results

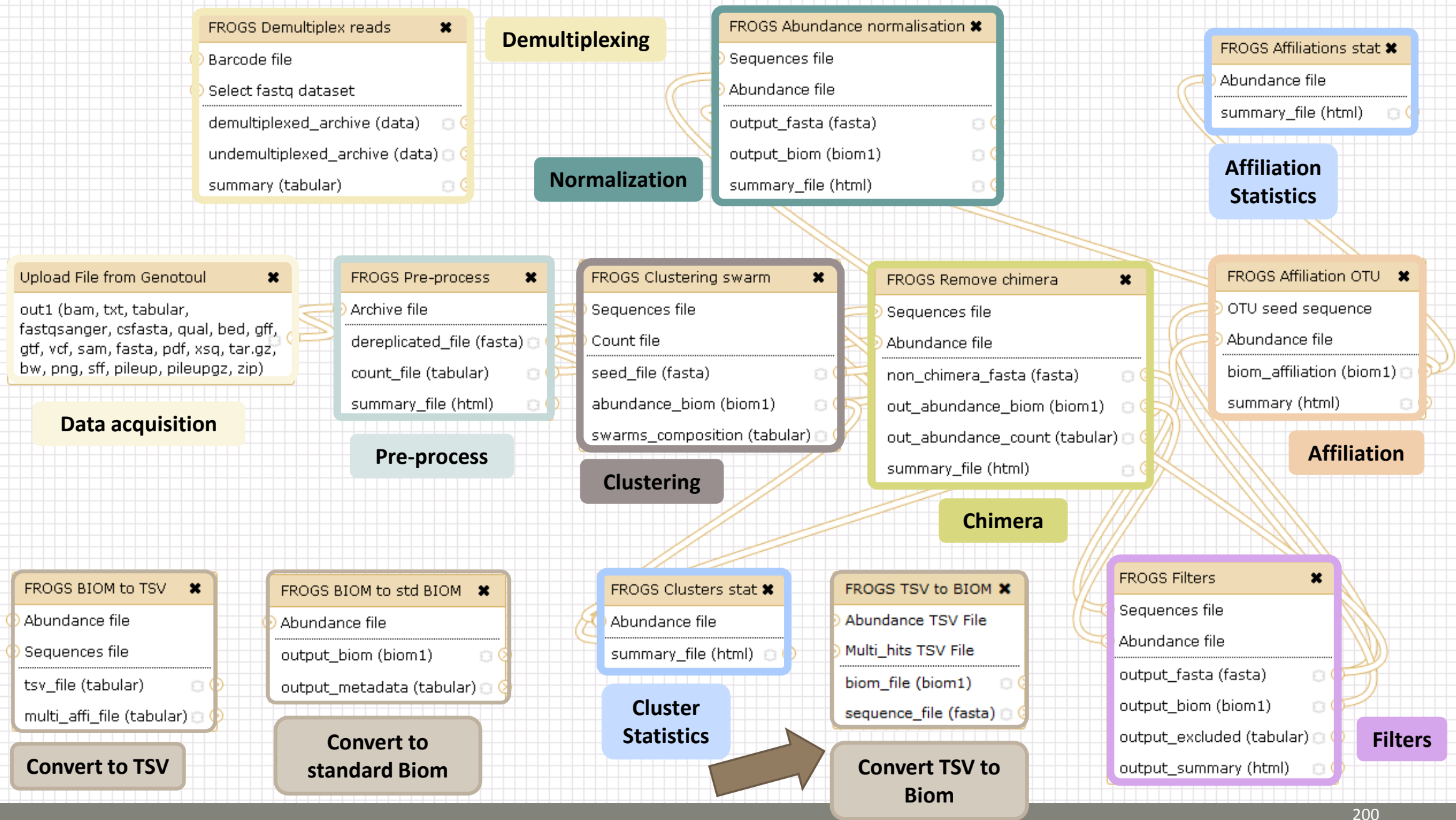


by OTUs

by sequences

# TSV to BIOM

---



# TSV to BIOM

---

After modifying your abundance TSV file you can again:

- generate rarefaction curve
- sunburst



Careful :

- do not modify column name
- do not remove column
- take care to choose a taxonomy available in your multi\_hit TSV file
- if deleting line from multi\_hit, take care to not remove a complete cluster without removing all "multi tags" in you abundance TSV file.
- if you want to rename a taxon level (ex : genus "Ruminiclostridium 5;" to genus "Ruminiclostridium;"), do not forget to modify also your multi\_hit TSV file.

# TSV to BIOM

**FROGS TSV\_to\_BIOM** Converts a TSV file in a BIOM file. (Galaxy Version 2.0.0) Options

**Abundance TSV File**  
21: FROGS BIOM to TSV: abundance.tsv  
Your FROGS abundance TSV file. Take care to keep original column names.

**Multi\_hits TSV File**  
25: multihit\_renamed.txt  
TSV file describing multi\_hit blast results.

**Extract seeds in FASTA file**  
Yes No  
If there is a 'seed\_sequence' column in your TSV table, you can extract seed sequences in a separated FASTA file.

Execute

# Your Turn! – 8

---

PLAY WITH TSV\_TO\_BIOM

# Exercise 8

→ objectives : Play with multi-affiliation and TSV\_to\_BIOM

## 1. Observe in Multi\_hit.tsv and abundance.tsv cluster\_8 annotation

#blast_taxonomy	blast_subject	observation_name	observation_sum
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	Cluster_1	13337
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	Cluster_2	11830
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	Cluster_3	11405
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	Cluster_4	4125
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	Cluster_5	4034
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	Cluster_6	3966
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	Cluster_7	2433
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	Cluster_8	2268

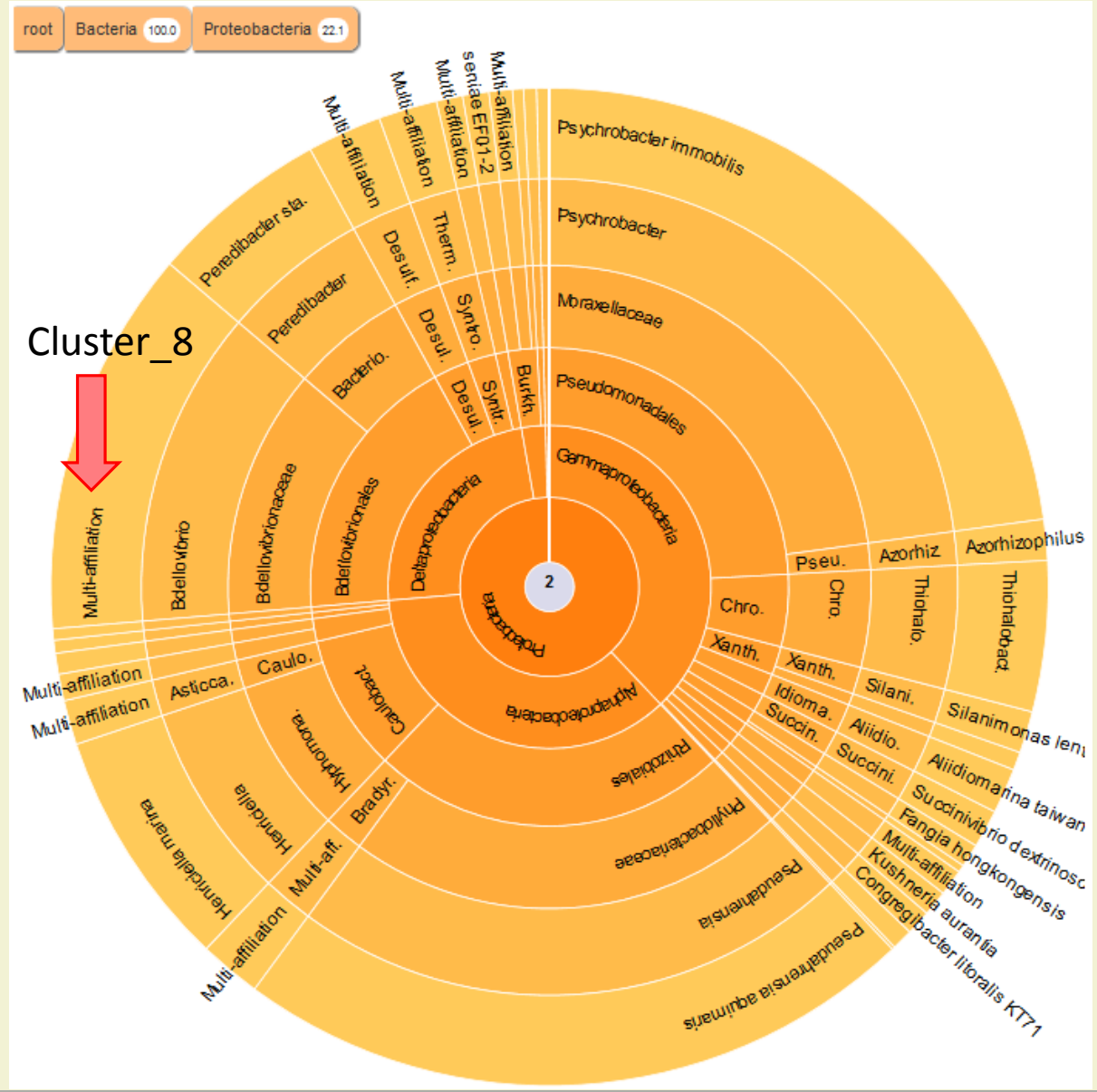
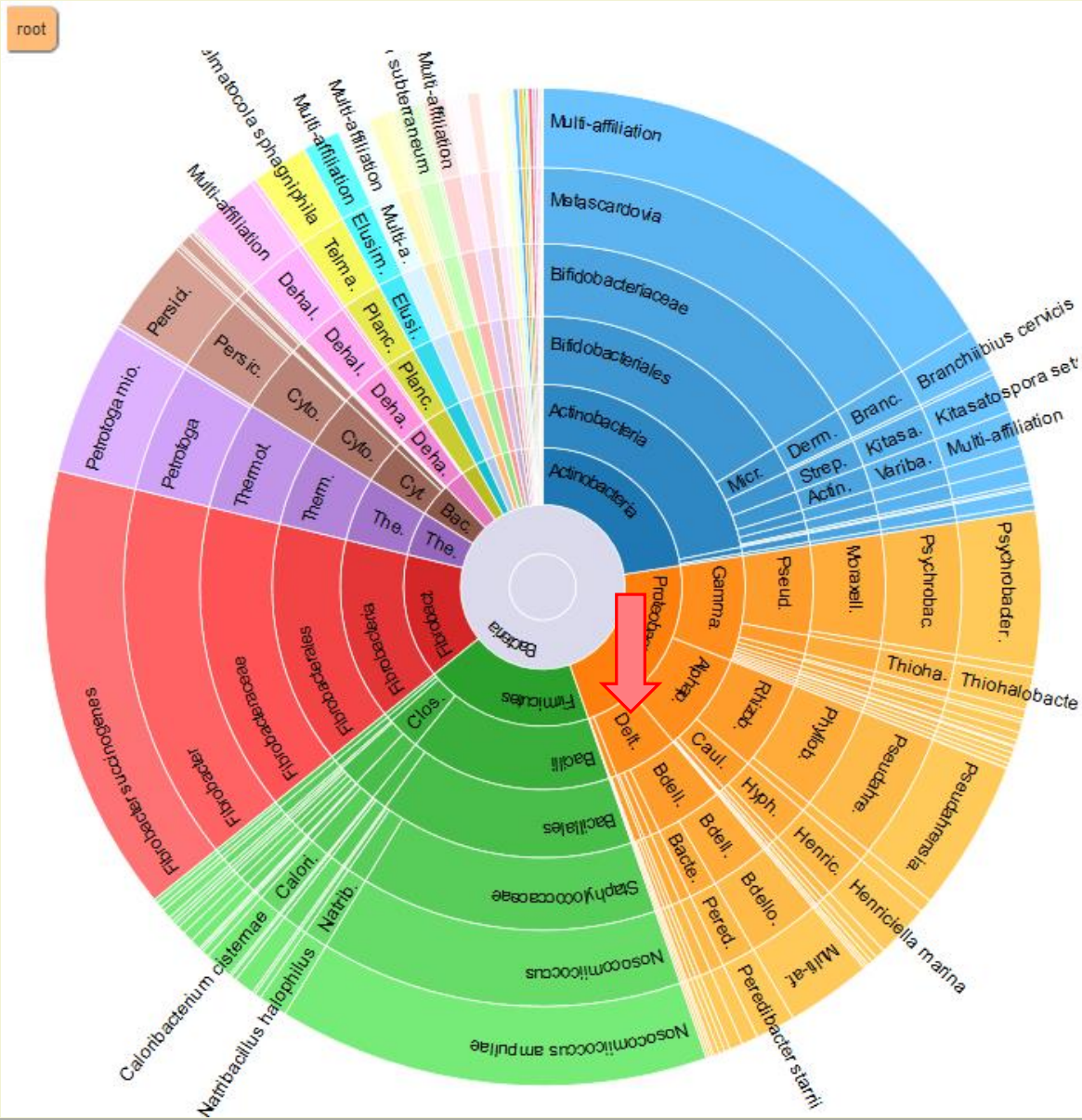
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	CP007656.1036900.1038415
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus str. Tiberius	CP002930.1837665.1839157
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus str. Tiberius	CP002930.842397.843889
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	AJ292760.1.1334
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	AF084850.1.1436
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus HD100	BX842648.123565.125058
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus HD100	BX842650.295616.297109



**Bdellovibrio bacteriovorus**



## 2. Observe le diversity diagramm



# Exercise 8

---

3. How to change affiliation of cluster 8 ????

# Exercise 8

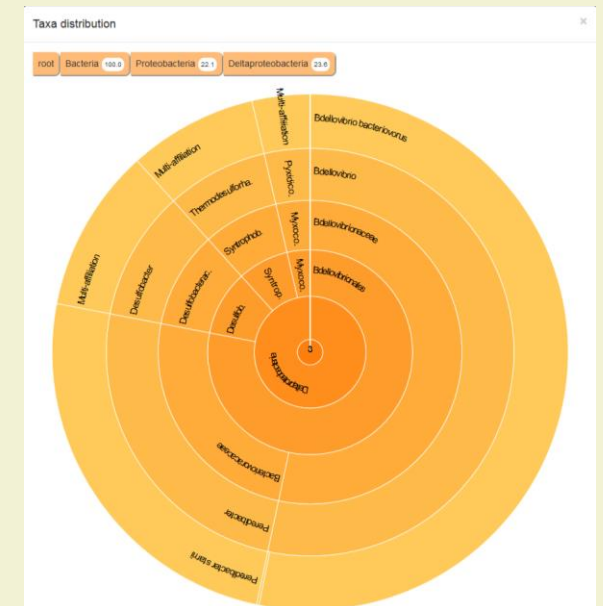
---

4. Modify multi\_hit.tsv and keep only :

Cluster\_8 Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus CP007656.103690.1038415

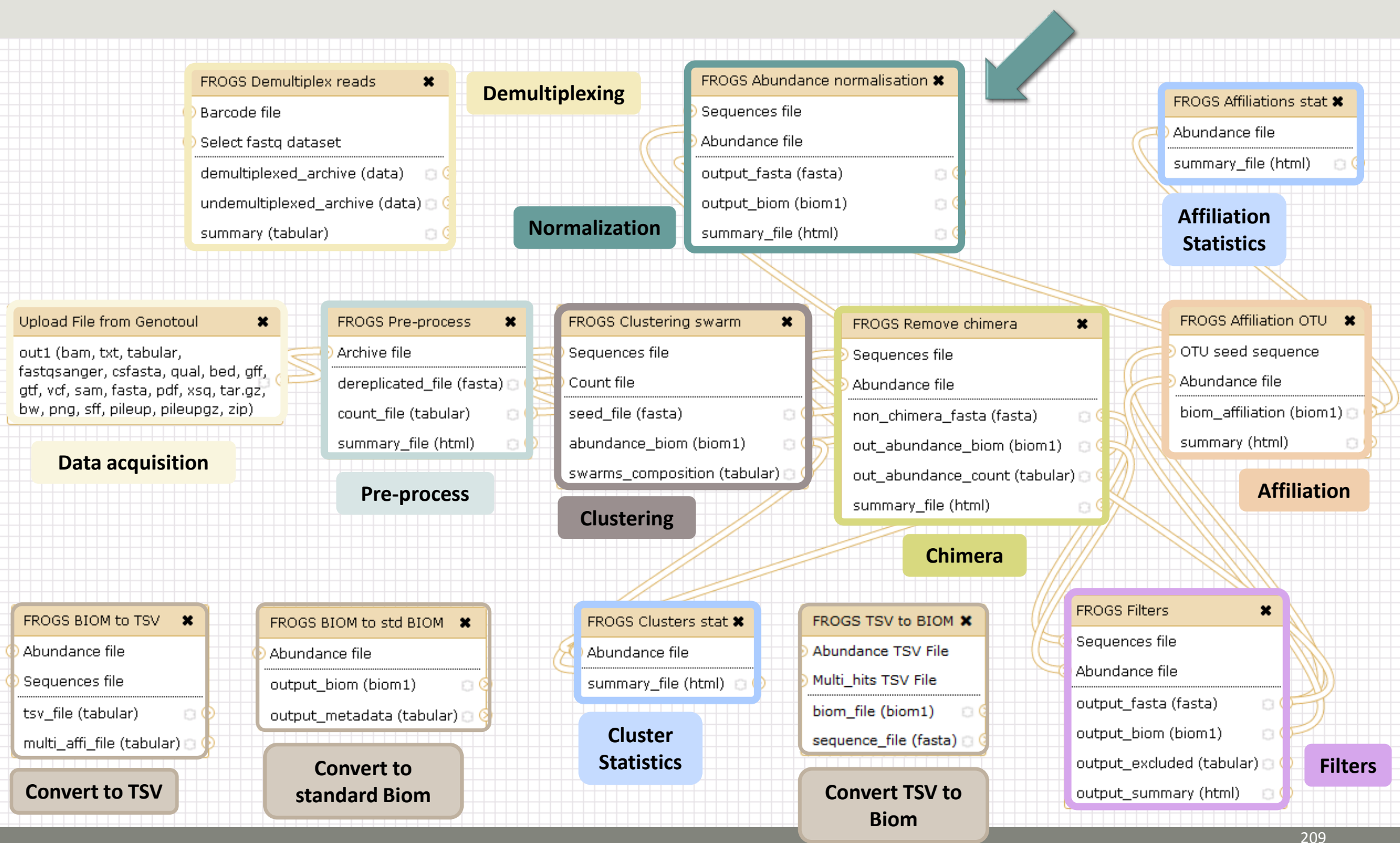
Careful, no quotes around text !!!

5. Upload the new multihit file.
6. Create a new biom with a TSV\_to\_BIOM tool
7. Launch again the affiliation\_stat tool on this new biom
8. Observe the diversity diagram



# 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

# Your Turn! – 9

---

LAUNCH NORMALIZATION TOOL

# Exercise 9

---

## Launch Normalization Tool

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.
4. Try other threshold and explore the report HTML result  
What do you remark ?



**FROGS Abundance normalisation** (Galaxy Version 1.1.1)

Options

**Sequences file**

   17: FROGS Filters: sequences.fasta

Sequences file to normalize (format: fasta).

**Abundance file**

   22: FROGS Affiliation OTU: affiliation.biom

Abundances file to normalize (format: BIOM).

**Number of reads**

9088

The final number of reads per sample.

Execute

**FROGS Abundance normalisation** (Galaxy Version 1.1.1) Options

**Sequences file**

17: FROGS Filters: sequences.fasta

Sequences file to normalize (format: fasta).

**Abundance file**

22: FROGS Affiliation OTU: affiliation.biom

Abundances file to normalize (format: BIOM).

**Number of reads**

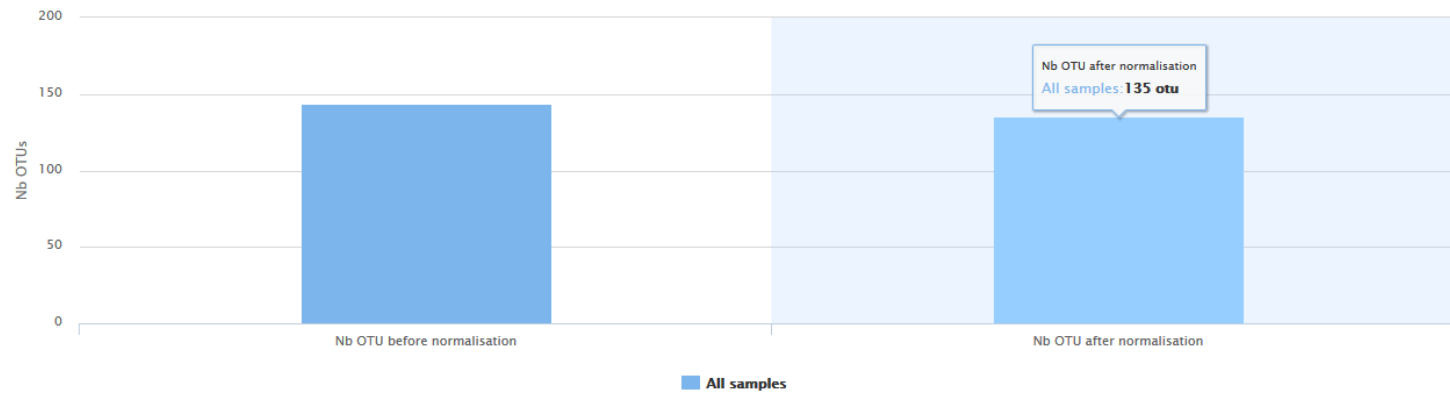
2000

The final number of reads per sample.

Execute

Or, this number can be chosen according to the rarefaction curve. For example, we can choose the smallest number of sequences that still retain all the genus.

## Composition summary

[CSV](#)Show **10** entriesSearch: 

### Composition by sample

Sample	▲ Nb OTU before normalisation	◆ Nb OTU after normalisation
100_10000seq_sampleA1_cutadapt	144	135
100_10000seq_sampleA2_cutadapt	144	135
100_10000seq_sampleA3_cutadapt	144	135
100_10000seq_sampleB1_cutadapt	144	135
100_10000seq_sampleB2_cutadapt	144	135
100_10000seq_sampleB3_cutadapt	144	135
100_10000seq_sampleC1_cutadapt	144	135
100_10000seq_sampleC2_cutadapt	144	135
100_10000seq_sampleC3_cutadapt	144	135

Showing 1 to 9 of 9 entries

Previous

1

Next

# Filters on affiliations

---

Do not forget, with filter tool we can filter the data based on their affiliation

**FROGS Filters** Filters OTUs on several criteria. (Galaxy Version 1.2.0) 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).

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters  
If you want to filter OTUs on their abundance and occurrence.

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

**Minimum proportion/number of sequences to keep OTU**  
Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

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

**\*\*\* THE FILTERS ON RDP**

Apply filters  
If you want to filter OTUs on their taxonomic affiliation produced by RDP.

**Rank with the bootstrap filter**  
Nothing selected

**Minimum bootstrap % (between 0 and 1)**

**\*\*\* THE FILTERS ON BLAST**

Apply filters  
If you want to filter OTUs on their taxonomic affiliation produced by Blast.

**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

**\*\*\* THE FILTERS ON CONTAMINATIONS**

Apply filters  
If you want to filter OTUs on classical contaminations.

**Cotaminant databank**  
phix  
The phix databank (the phix is a control added in Illumina sequencing technologies).

Execute

Abundance filters

RDP affiliation filters

BLAST affiliation filters

Contamination filter

# Exercise 10

---

1. Apply filters to keep only data with perfect alignment.
2. How many clusters have you keep ?

**Sequences file**

17: FROGS Filters: sequences.fasta

The sequence file to filter (format: fasta).

**Abundance file**

22: FROGS Affiliation OTU: affiliation.biom

The abundance file to filter (format: BIOM).

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

No filters

If you want to filter OTUs on their abundance and occurrence.

**\*\*\* THE FILTERS ON RDP**

No filters

If you want to filter OTUs on their taxonomic affiliation produced by RDP.

**\*\*\* THE FILTERS ON BLAST**

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by Blast.

**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

# FROGS Tree

---

CREATE A PHYLOGENETICS TREE OF OTUS



**FROGS Tree** ✕

- OTUs sequence file
- Biom file

---

out\_tree (nhx)

html (html)

**Phylogenetic Tree**

**FROGS Demultiplex reads** ✕

- Barcode file
- Select fastq dataset

---

demultiplexed\_archive (data)

undemultiplexed\_archive (data)

summary (tabular)

**Demultiplexing**

**FROGS Abundance normalisation** ✕

- Sequences file
- Abundance file

---

output\_fasta (fasta)

output\_biom (biom1)

summary\_file (html)

**Normalization**

**FROGS Affiliations stat** ✕

- Abundance file

---

summary\_file (html)

**Affiliation Statistics**

**Upload File from Genotoul** ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

**Data acquisition**

**FROGS Pre-process** ✕

- Archive file

---

dereplicated\_file (fasta)

count\_file (tabular)

summary\_file (html)

**Pre-process**

**FROGS Clustering swarm** ✕

- Sequences file
- Count file

---

seed\_file (fasta)

abundance\_biom (biom1)

swarms\_composition (tabular)

**Clustering**

**FROGS Remove chimera** ✕

- Sequences file
- Abundance file

---

non\_chimera\_fasta (fasta)

out\_abundance\_biom (biom1)

out\_abundance\_count (tabular)

summary\_file (html)

**Chimera**

**FROGS Affiliation OTU** ✕

- OTU seed sequence
- Abundance file

---

biom\_affiliation (biom1)

summary (html)

**Affiliation**

**FROGS BIOM to TSV** ✕

- Abundance file
- Sequences file

---

tsv\_file (tabular)

multi\_affi\_file (tabular)

**Convert to TSV**

**FROGS BIOM to std BIOM** ✕

- Abundance file

---

output\_biom (biom1)

output\_metadata (tabular)

**Convert to standard Biom**

**FROGS Clusters stat** ✕

- Abundance file

---

summary\_file (html)

**Cluster Statistics**

**FROGS TSV to BIOM** ✕

- Abundance TSV File
- Multi\_hits TSV File

---

biom\_file (biom1)

sequence\_file (fasta)

**Convert TSV to Biom**

**FROGS Filters** ✕

- Sequences file
- Abundance file

---

output\_fasta (fasta)

output\_biom (biom1)

output\_excluded (tabular)

output\_summary (html)

**Filters**

2 choices to do your phylogenetics tree

**FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 1.0.0)** Options

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

**Do you have the template alignment file ?**  
Yes **No**  
If yes, precise the template multi-alignment file.

**Biom file**  
16: FROGS Affiliation OTU: affiliation.biom  
The abundance table of OTUs (format: biom).

Execute

**FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 1.0.0)** Options

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

**Do you have the template alignment file ?**  
**Yes** No  
If yes, precise the template multi-alignment file.

**Template alignment file**  
22: otus\_pynast.fasta  
Template multi-alignment file (format: fasta).

**Biom file**  
16: FROGS Affiliation OTU: affiliation.biom  
The abundance table of OTUs (format: biom).

Execute

# Exercise 10

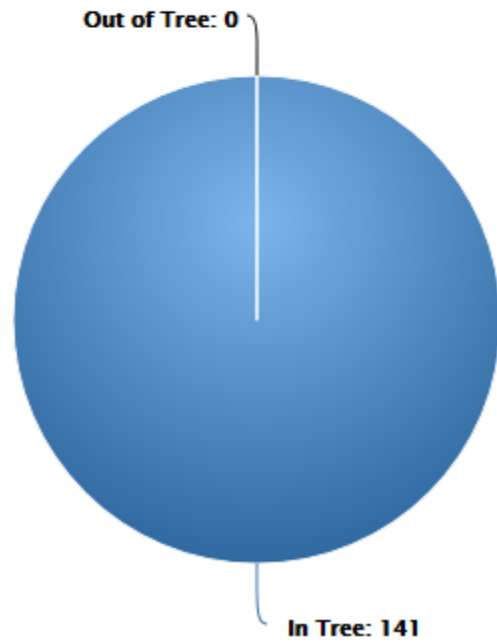
---

1. Create a tree with the filtered OTUs without template
2. Explore the HTML file
3. Look tree.nwk

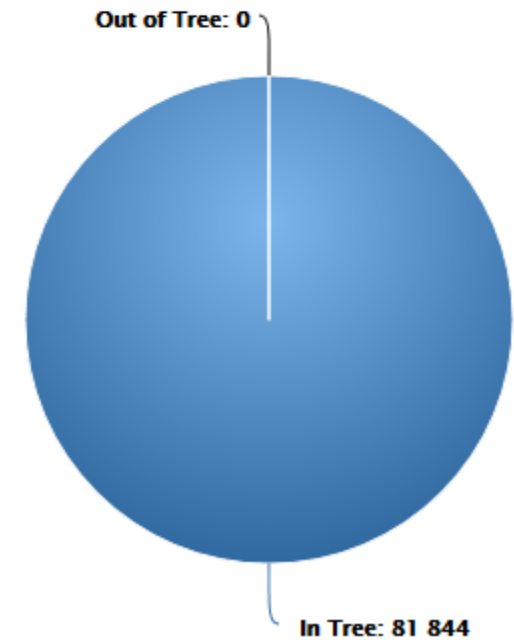


# Summary

OTUs



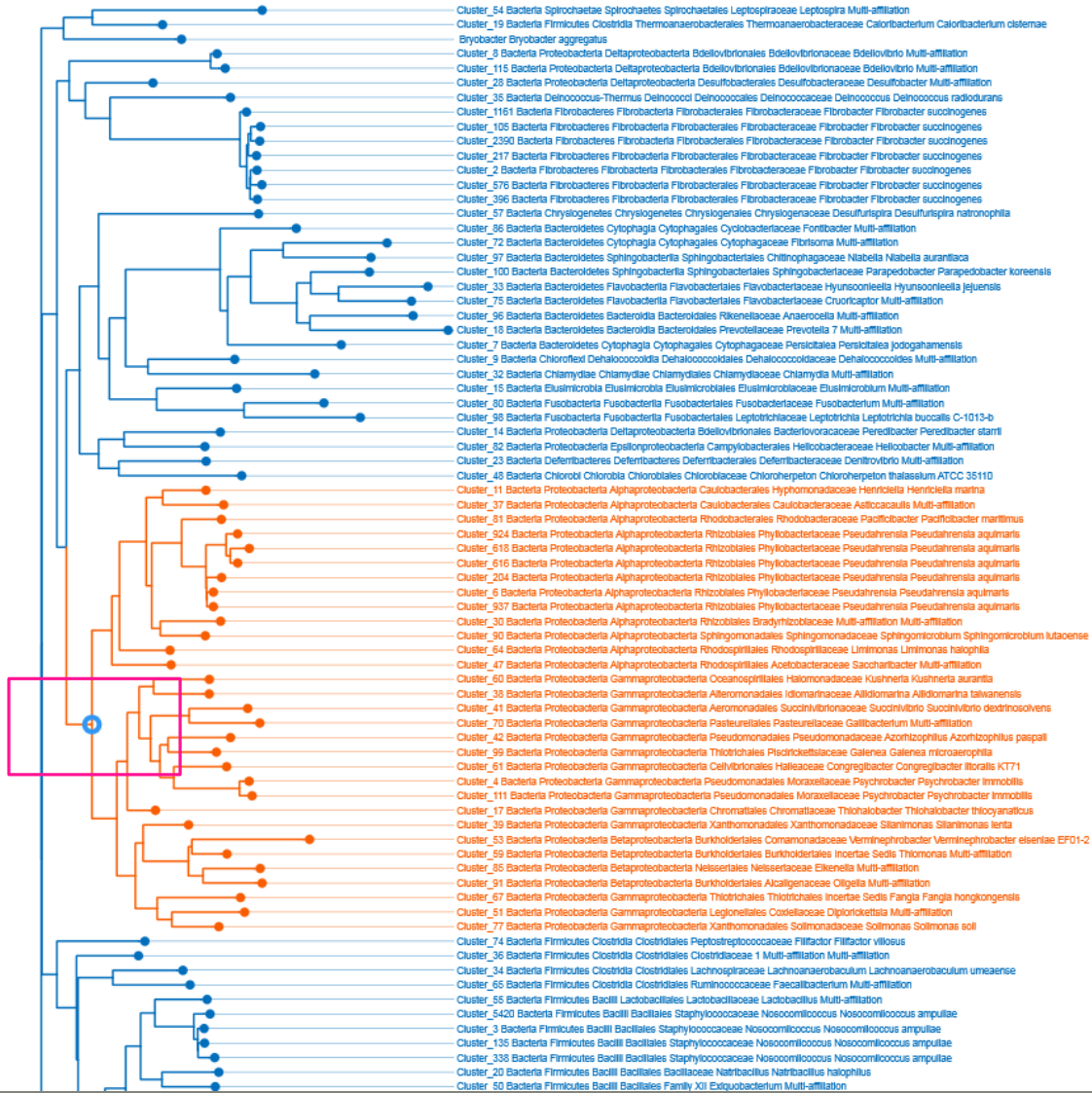
Abundance



# Tree View

Enabling zoom:

ON



Tree.nwk:

```
((((Cluster_54:0.19489,Cluster_19:0.07629)0.892:0.03423,Cluster_58:0.13306)0.853:0.02661,(((Cluster_8:0.00054,Cluster_115:0.01025)1.000:0.16828,(Cluster_28:0.07332, .....))))
```

# How works FROGS TREE ?

Pynast needs alignment template to go fast

But if your species is not similar at 75% with a sequence in the template, your species will be not in the tree !

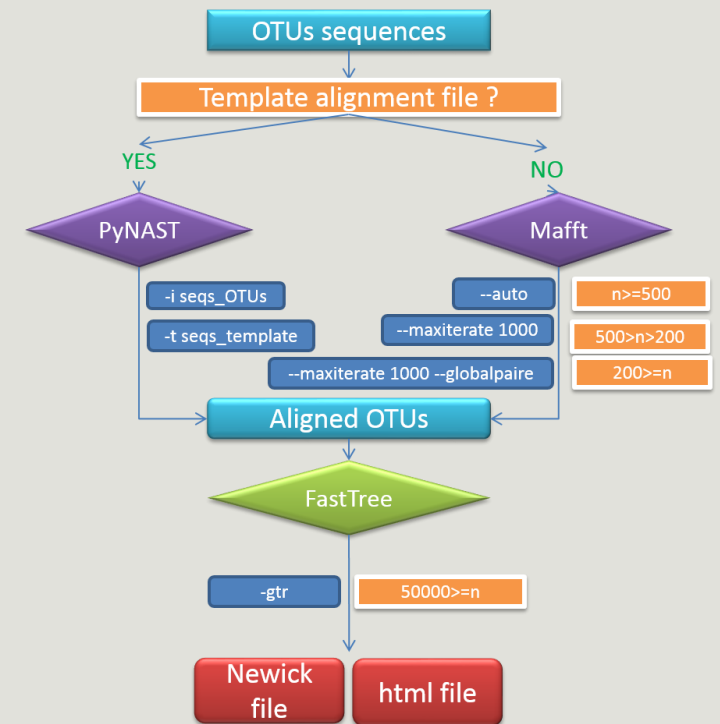
To find templates:

Based on 16S GreenGenes databank

[https://github.com/biocore/qiime-default-reference/blob/master/qiime\\_default\\_reference/gg\\_13\\_8\\_otus/rep\\_set\\_aligned/85\\_otus.pynast.fasta.gz](https://github.com/biocore/qiime-default-reference/blob/master/qiime_default_reference/gg_13_8_otus/rep_set_aligned/85_otus.pynast.fasta.gz)

Based on 16S SILVA databank

[https://www.arb-silva.de/fileadmin/silva\\_databases/qiime/Silva\\_128\\_release.tgz](https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_128_release.tgz)



# Tool descriptions

---



## **i** What it does

FROGS Pre-process filters and dereplicates amplicons for use in diversity analysis.

## **i** Inputs/Outputs

### Inputs

By sample your sequences and their qualities.

#### Illumina inputs

**Usage:** The amplicons have been sequenced in paired-end. The amplicon expected length is inferior than the R1 and R2 length. R1 and R2 can be merge by the common region.

**Files:** One R1 and R2 by sample (format [FASTQ](#))

**Example:** splA\_R1.fastq.gz, splA\_R2.fastq.gz, splB\_R1.fastq.gz, splB\_R2.fastq.gz

OR

**Usage:** The single end sequencing cover all the amplicons or the R1 and R2 have already been overlaped.

**Files:** One sequence file by sample (format [FASTQ](#)).

**Example:** splA.fastq.gz, splB.fastq.gz

#### 454 inputs

**Files:** One sequence file by sample (format [FASTQ](#))

**Example:** splA.fastq.gz, splB.fastq.gz

These files must be added sample by sample or provide in an archive file (tar.gz).

Remark: In an archive if you use R1 and R2 files they names must end with `_R1` and `_R2`.



## Outputs

### Sequence file (dereplicated.fasta):

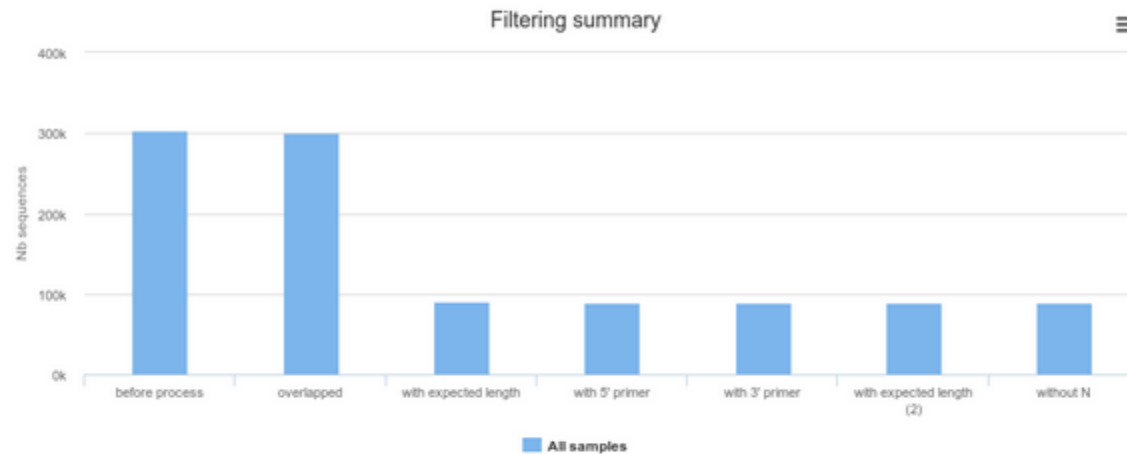
Only one file with all samples sequences (format [FASTA](#)). These sequences are dereplicated: strictly identical sequence are represented only one and the initial count is kept in count file.

### Count file (count.tsv):

This file contains the count of all uniq sequences in each sample (format [TSV](#)).

### Summary file (excluded\_data.html):

This file presents the ordered filters and the number of sequences passing these (format [HTML](#)).



Show  entries

Search:

#### Filtering by sample

Sample	before process	overlapped	with expected length	with 5' primer	with 3' primer	with expected length (2)	without N
sampleA	90,126	90,126	90,126	89,697	89,697	89,697	89,697
sampleB	213,043	209,801	0	0	0	0	0

Showing 1 to 2 of 2 entries

Previous  Next

## **i** How it works

<b>Steps</b>	<b>Illumina</b>	<b>454</b>
1	For uncontiged data: contig read1 and read2 with a maximum of 10% mismatch in the overlaped region ( <a href="#">FLASH</a> )	/
2	Filter contig sequence on its length which must be between "Minimum amplicon size" and "Maximum amplicon size"	/
3	Remove sequences where the two primers are not present and remove primers sequence ( <a href="#">cutadapt</a> ). The primer search accept 10% of differences	Remove sequence where the two primers are not present, remove primers sequence and reverse complement the sequences with strand - ( <a href="#">cutadapt</a> ). The primer search accept 10% of differences
4	Filter sequences on its length and with ambiguous nucleotids	filter sequences on its length, with ambiguous nucleotids, with at least one homopolymer with size >7nt and with distance between two poor qualities (< 10) of <= 10 nt
5	Dereplicate sequences	Dereplicate sequences

## **i** Advices/details on parameters

### **Primers parameters**

The primers must be provided in 5' to 3' orientation.

Example:

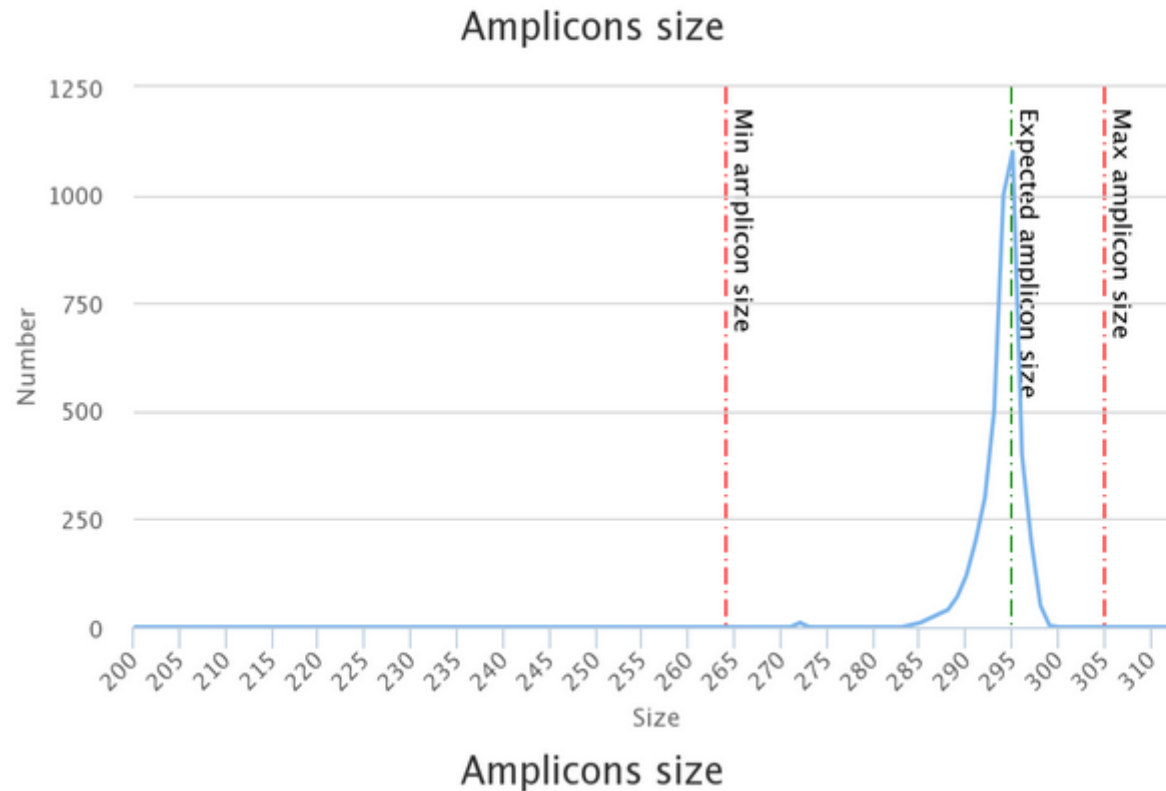
5' **ATGCC** GTCGTCGTAAAATGC **ATTTCAG** 3'

Value for parameter 5' primer: ATGCC

Value for parameter 3' primer: ATTTCAG

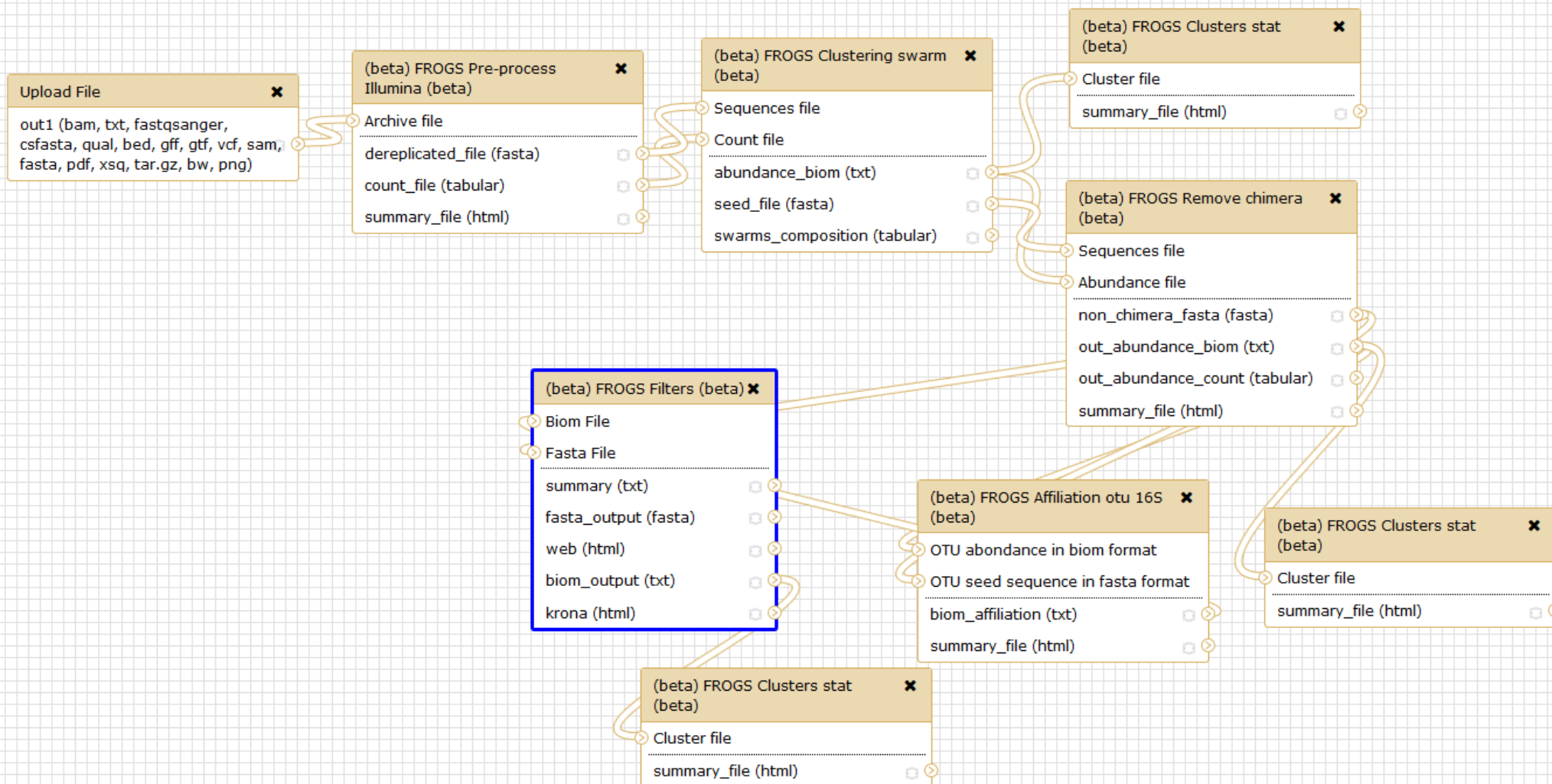
### **Amplicons sizes parameters**

The two following images shown two examples of perfect values for sizes parameters.



# Workflow creation

---



Tool: (beta) FROGS Filters (beta)

Version: 1.0.0

None: ▾

**Biom File**

Data input 'biom' (txt)

**Fasta File**

Data input 'fasta' (fasta)

**Remove phiX:** ▾



**PhiX databank:** ▾

phiX ▾

**\*\*\* THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE :**

Apply filters ▾

--Remove OTUs that are not present at least in **XX** samples; how many samples do you choose? : ▾

--When sorted by abundance, how many OTU do you want to keep?: ▾

--proportion/number of sequences threshold to remove an OTU: ▾

**\*\*\* THE FILTERS ON RDP :**

No filters ▾

**\*\*\* THE FILTERS ON BLAST :**

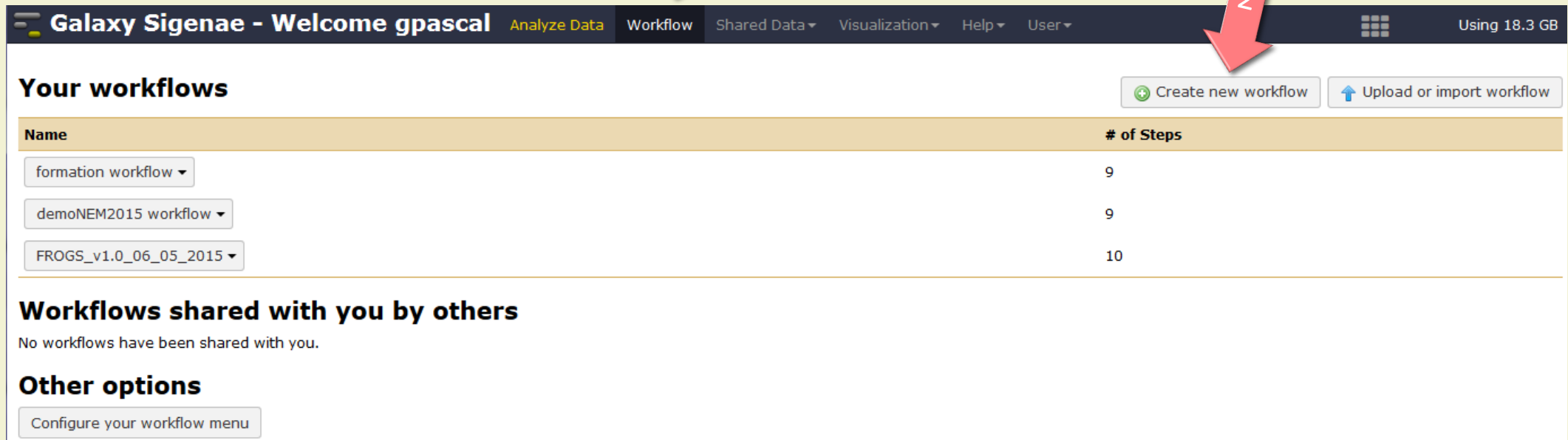
No filters ▾

# Your Turn! – 11

---

CREATE YOUR OWN WORKFLOW !

# Exercise 11



Galaxy Sigenae - Welcome gpascal Analyze Data Workflow Shared Data Visualization Help User Using 18.3 GB

### Your workflows

[+ Create new workflow](#) [↑ Upload or import workflow](#)

Name	# of Steps
formation workflow ▾	9
demoNEM2015 workflow ▾	9
FROGS_v1.0_06_05_2015 ▾	10

### Workflows shared with you by others

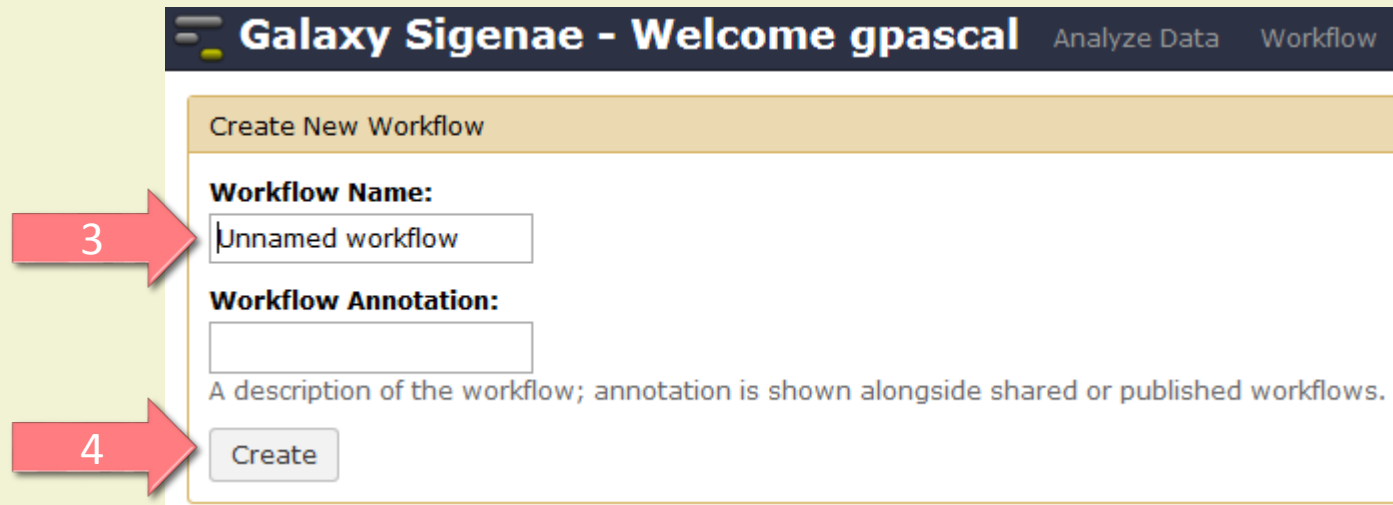
No workflows have been shared with you.

### Other options

[Configure your workflow menu](#)

# Exercise 11

---



**Galaxy Sigenae - Welcome gpascal** Analyze Data Workflow

Create New Workflow

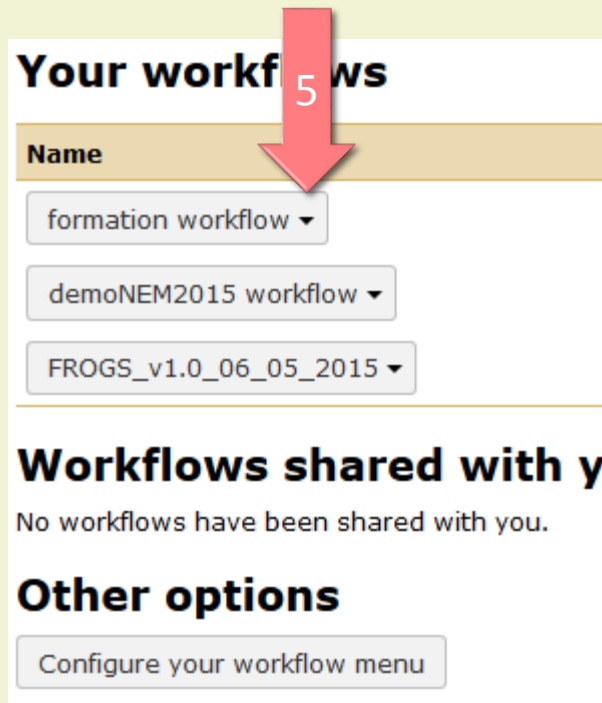
**Workflow Name:**

**Workflow Annotation:**

A description of the workflow; annotation is shown alongside shared or published workflows.



# Exercise 11



**Your workflows**

**Name**

formation workflow ▾

demoNEM2015 workflow ▾

FROGS\_v1.0\_06\_05\_2015 ▾

---

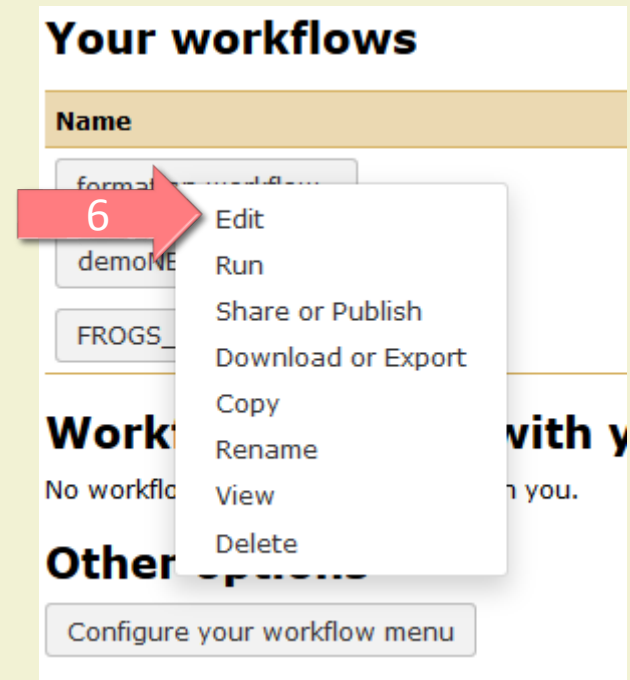
**Workflows shared with you**

No workflows have been shared with you.

**Other options**

Configure your workflow menu

A red arrow with the number 5 points to the 'Name' header of the workflow list.



**Your workflows**

**Name**

formation workflow ▾

demoNEM2015 workflow ▾

FROGS\_v1.0\_06\_05\_2015 ▾

---

**Workflows shared with you**

No workflows have been shared with you.

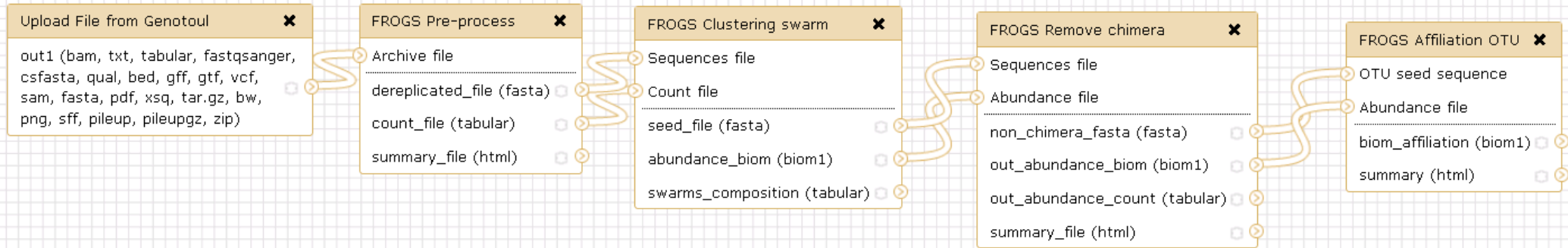
**Other options**

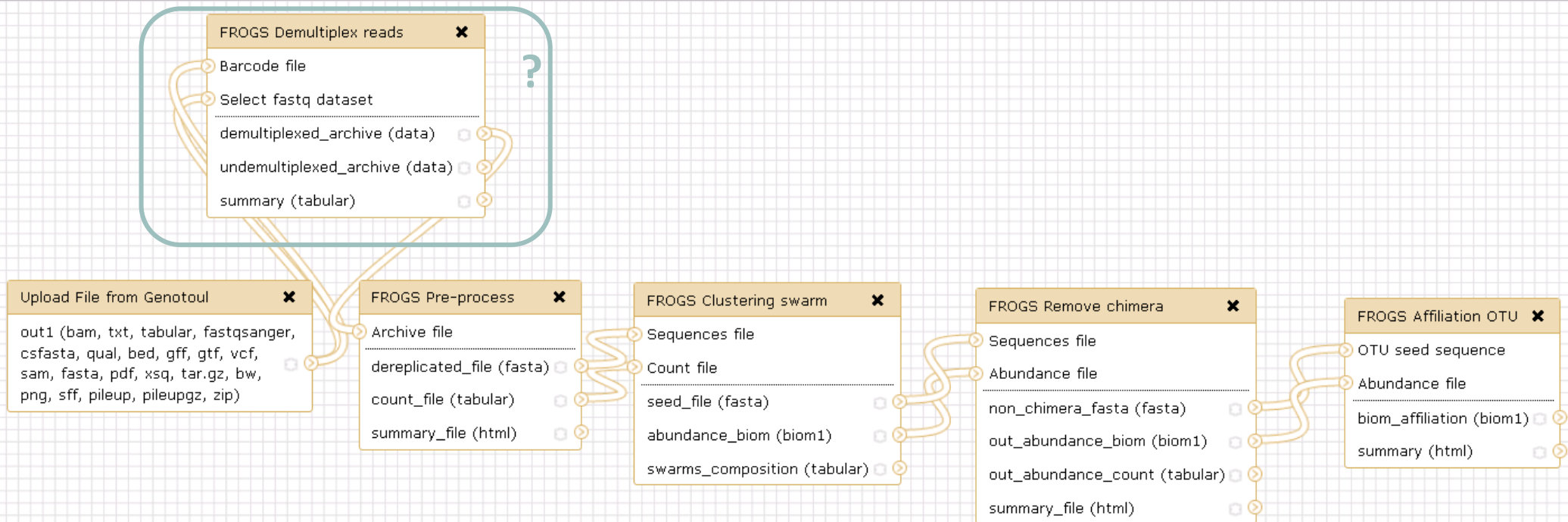
Configure your workflow menu

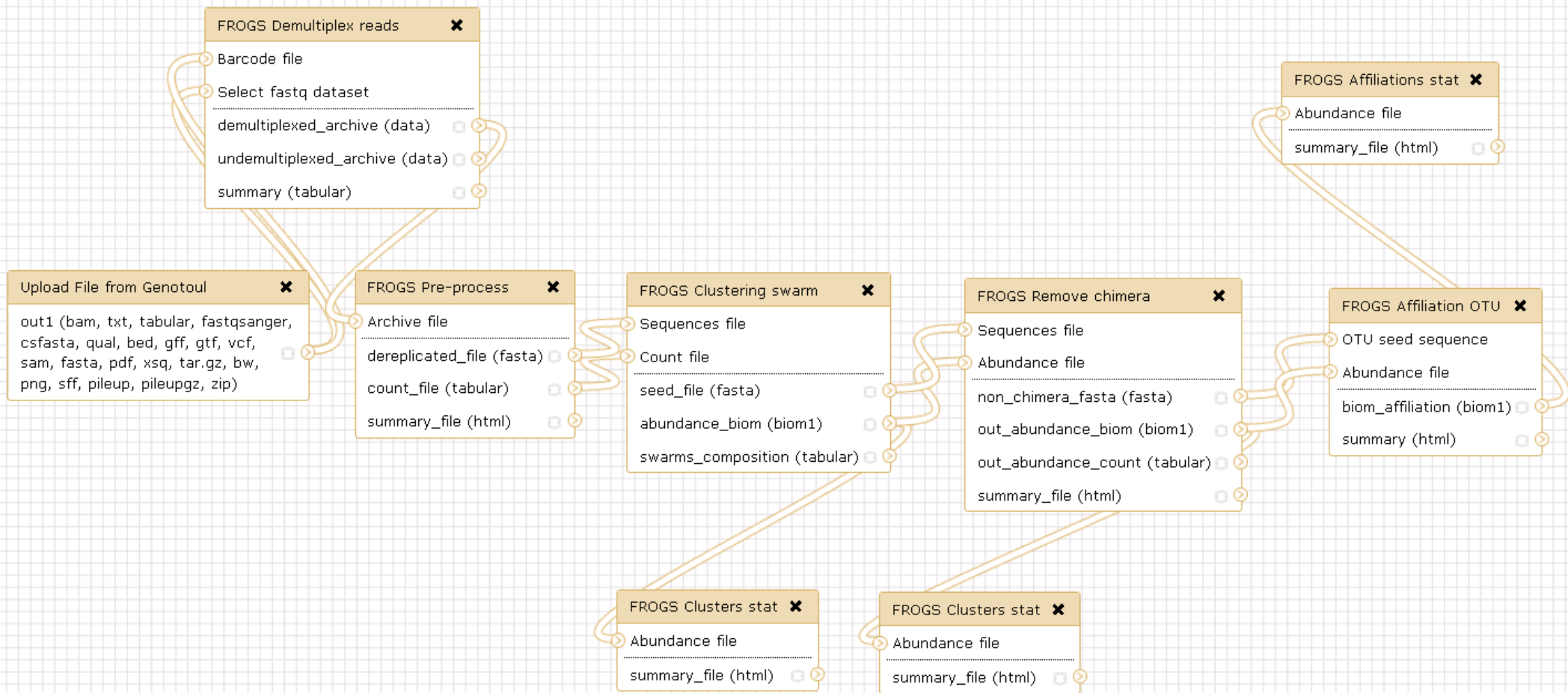
Context menu options:

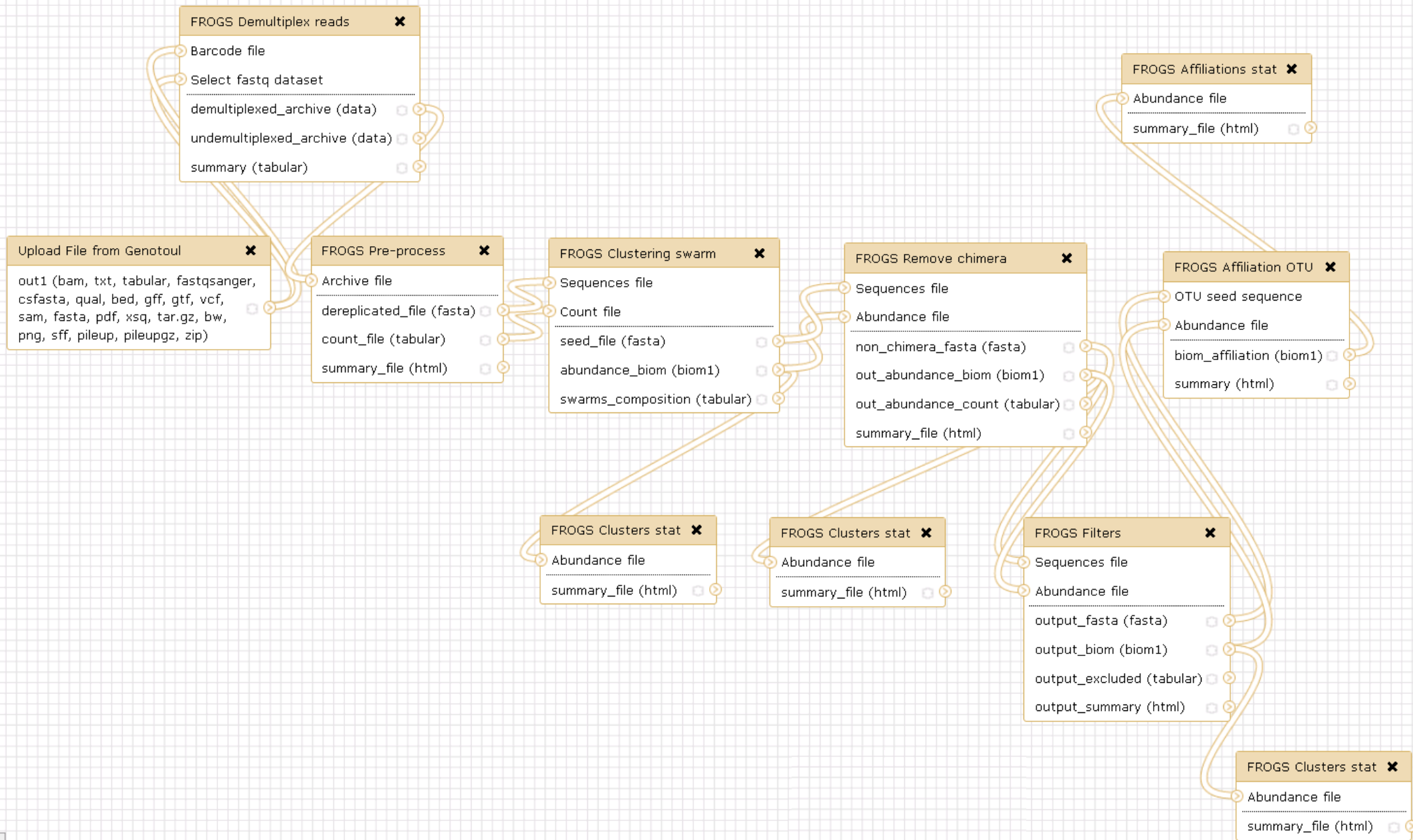
- Edit
- Run
- Share or Publish
- Download or Export
- Copy
- Rename
- View
- Delete

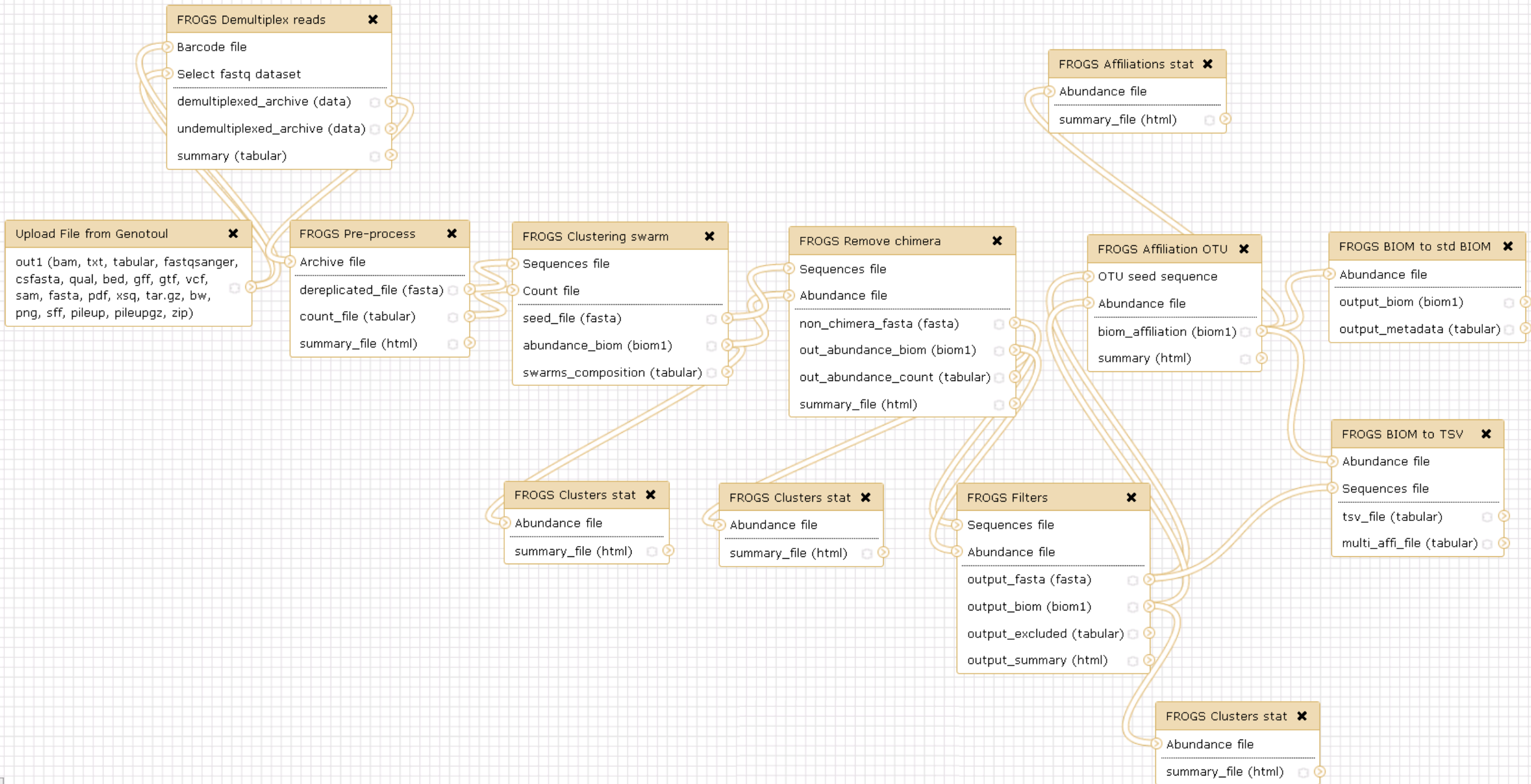
A red arrow with the number 6 points to the 'demoNEM2015 workflow' item, which has a context menu open over it.



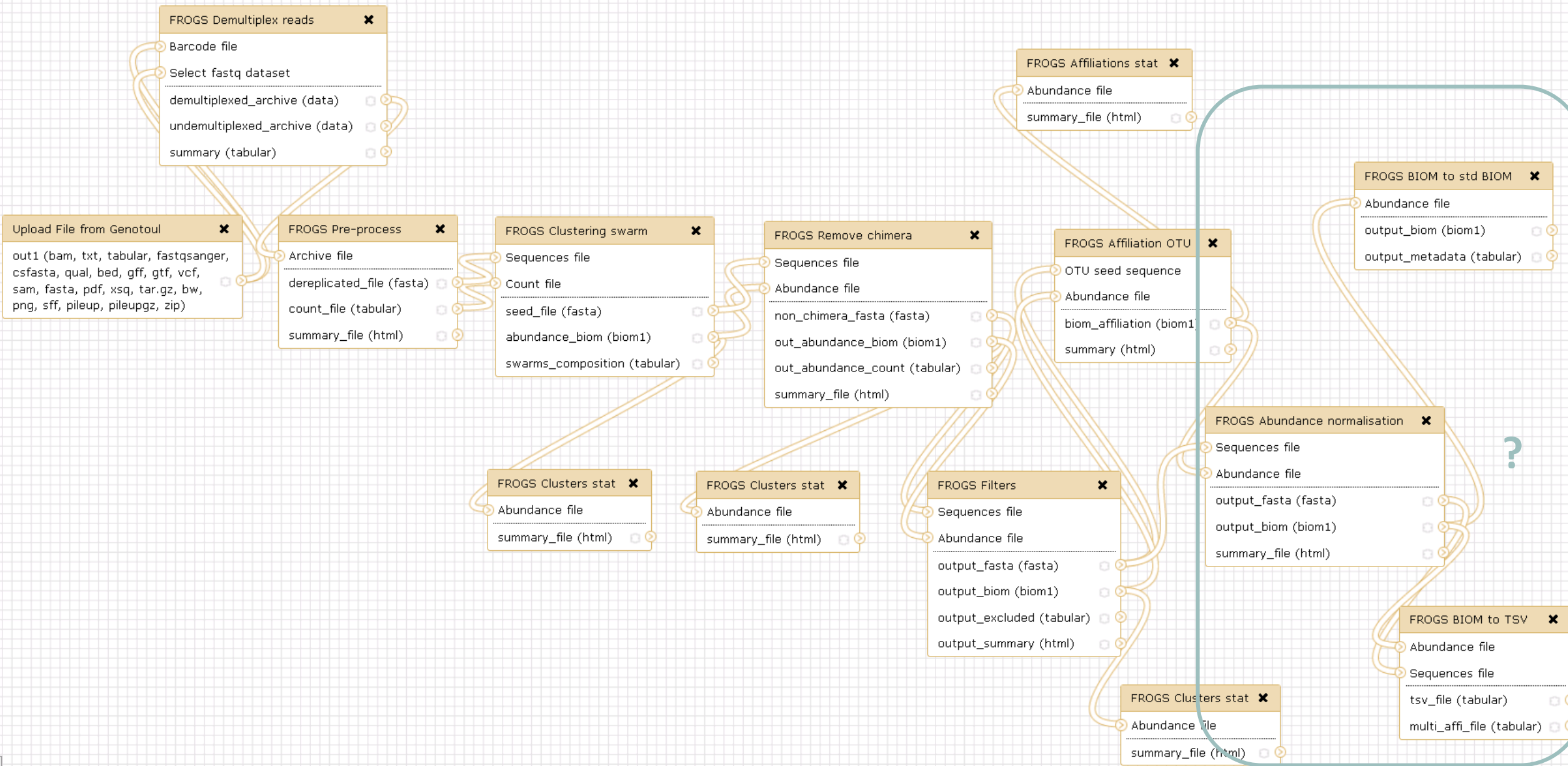


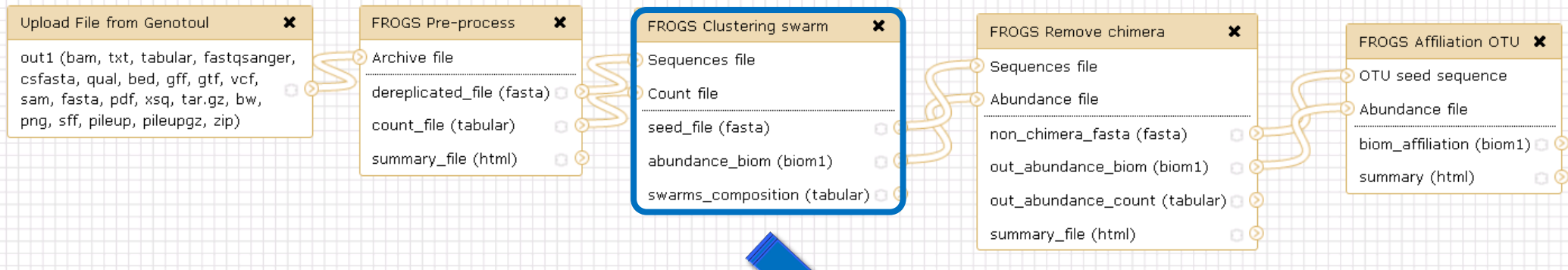












For each tool, think to:

- Fixe parameter ?

?

**FROGS Clustering swarm** ▼  
 Step 2 in metagenomics  
 analysis : clustering. (Galaxy  
 Version 2.3.0)

**Sequences file**  
 Data input 'sequence\_file' (fasta)  
 The sequences file (format: fasta).

**Count file**  
 Data input 'count\_file' (tabular)  
 It contains the count by sample for  
 each sequence (format: TSV).

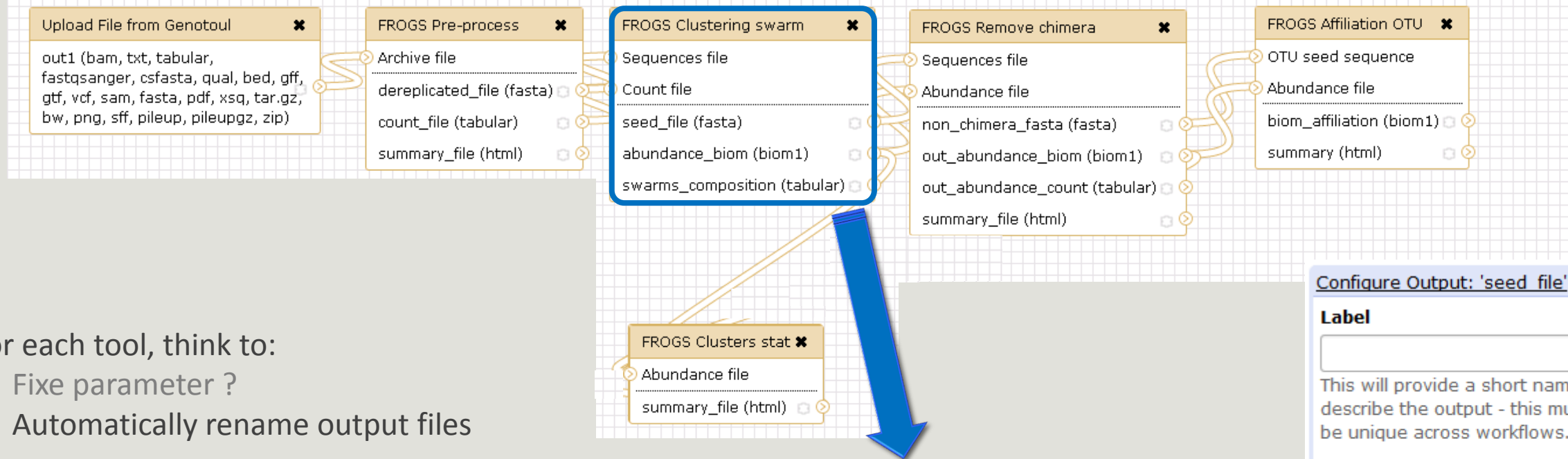
**Aggregation distance**  
 Set at Runtime

Maximum number of differences  
 between sequences in each  
 aggregation step.

**Performe denoising clustering  
 step?**

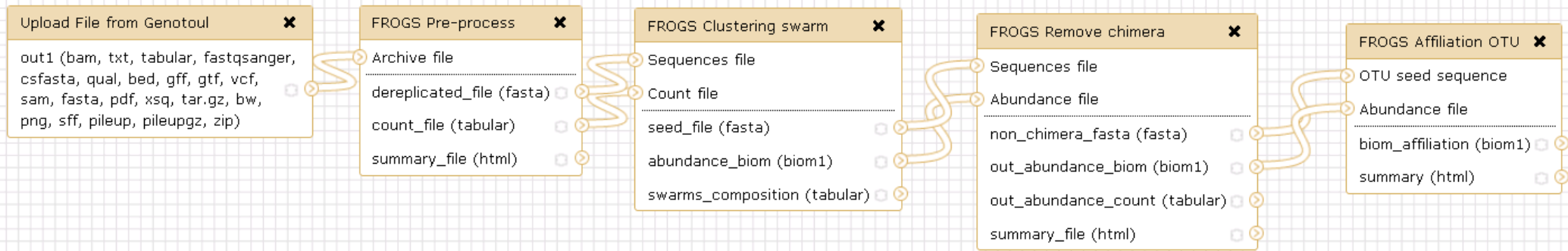
If checked, clustering will be  
 perform in two steps. first with





For each tool, think to:

- Fixe parameter ?
- Automatically rename output files

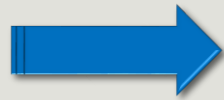


For each tool, think to:

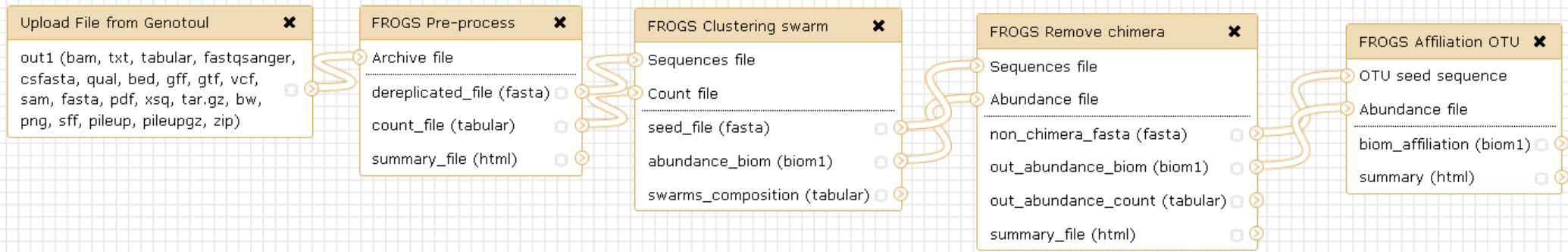
- Fixe parameter ?
- Automatically rename output files
- Hide intermediate files ?

**FROGS Remove chimera** ✕

- Sequences file
- Abundance file
- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)



<b>11: FROGS Remove chimera: report.html</b>	👁️ ✎ ✕
<b>10: FROGS Remove chimera: non chimera abundance.biom</b>	👁️ ✎ ✕
<b>9: FROGS Remove chimera: non chimera.fasta</b>	👁️ ✎ ✕

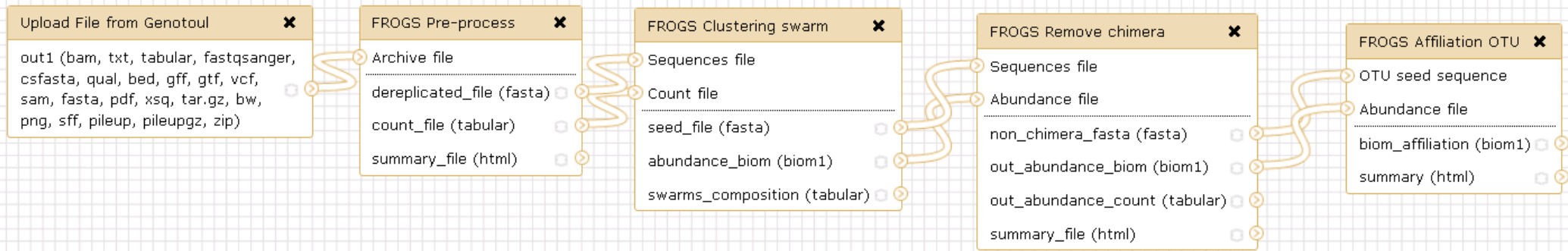


For each tool, think to:

- Fixe parameter ?
- Automatically rename output files
- Hide intermediate files ?

**FROGS Remove chimera**

- Sequences file
- Abundance file
- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1) *Mark dataset as a workflow output. All unmarked datasets will be hidden.*
- out\_abundance\_count (tabular)
- summary\_file (html)

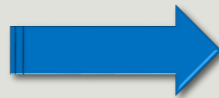


For each tool, think to:

- Fixe parameter ?
- Automatically rename output files
- Hide intermediate files ?

**FROGS Remove chimera**

- Sequences file
- Abundance file
- non\_chimera\_fasta (fasta)
- out\_abundance\_biom (biom1)
- out\_abundance\_count (tabular)
- summary\_file (html)



**11: FROGS Remove chimera: report.html**

# Download your data

---

You have to download one per one your files

```
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
[download] [info] [refresh] [share] [print]
HTML file
```



# FROGS BIOM to Standard BIOM

---




# FROGS biom to standard Biom

---

This step is required to run R

**FROGS BIOM to std BIOM** Converts a FROGS BIOM in fully compatible BIOM. (Galaxy Version 1.1.0) Options

**Abundance file**

   22: FROGS Affiliation OTU: affiliation.biom

The FROGS BIOM file to convert (format: BIOM).

Execute



**43: FROGS BIOM to std BIOM: blast\_metadata.tsv**   

**42: FROGS BIOM to std BIOM: abundance.biom**   



# Some figures

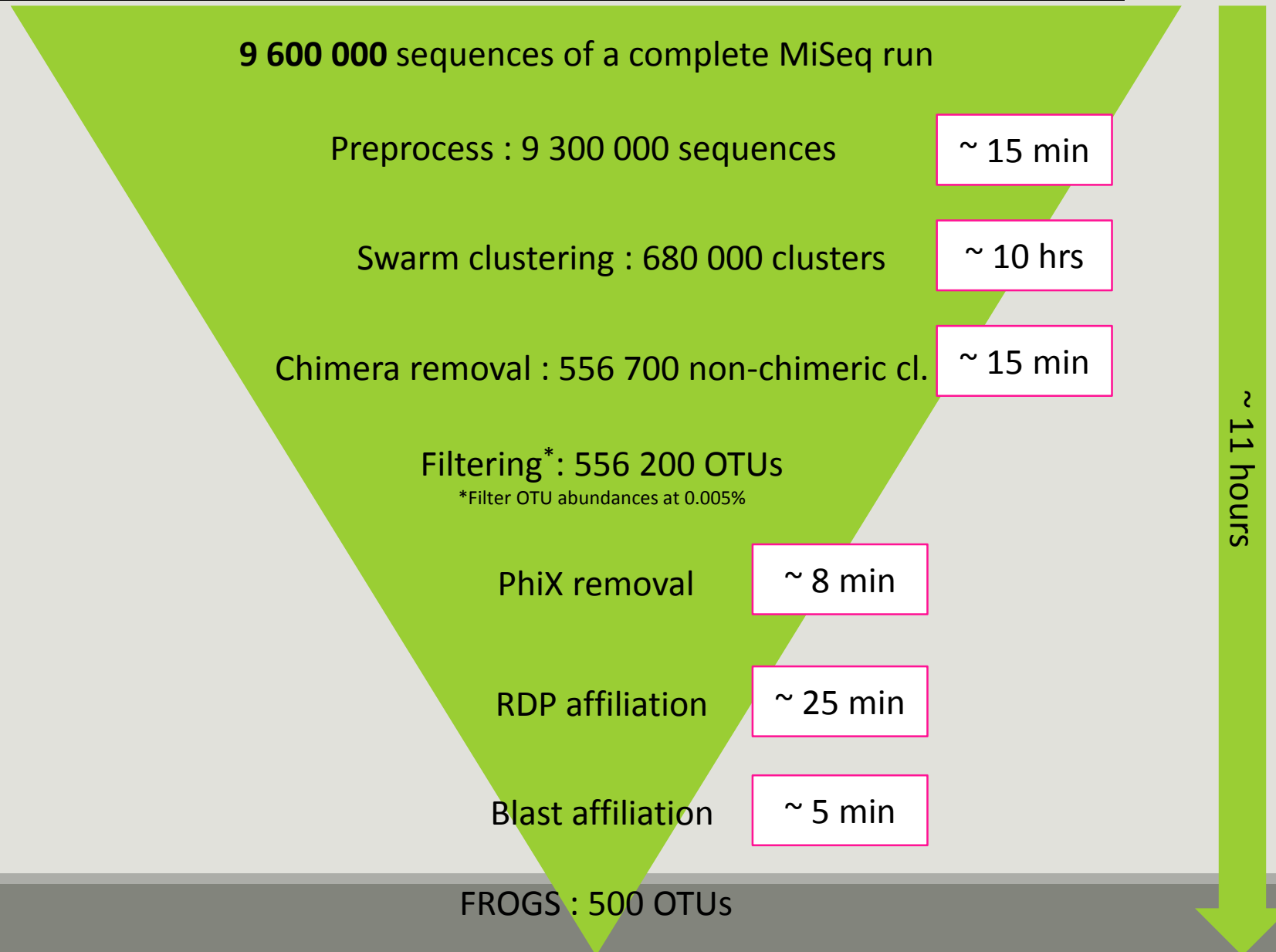
---

# Some figures - Fast

---

NB SEQ	TIME with complete pipeline <b>without Filters</b>
50 000	40 min
400 000	4 hrs
3 500 000	2 days
10 000 000	5 days

# Speed on real datasets



# Simulated datasets, for testing FROGS' Accuracy

- 500 species, covering all bacterial phyla
- Power Law distribution of the species abundances
- Error rate calibrated with real sequencing runs
- 20% chimeras
- 10 samples of 100 000 sequences each (1M sequences)

**Simulated dataset : 1M sequences**



**SWARM : 109 000 clusters**

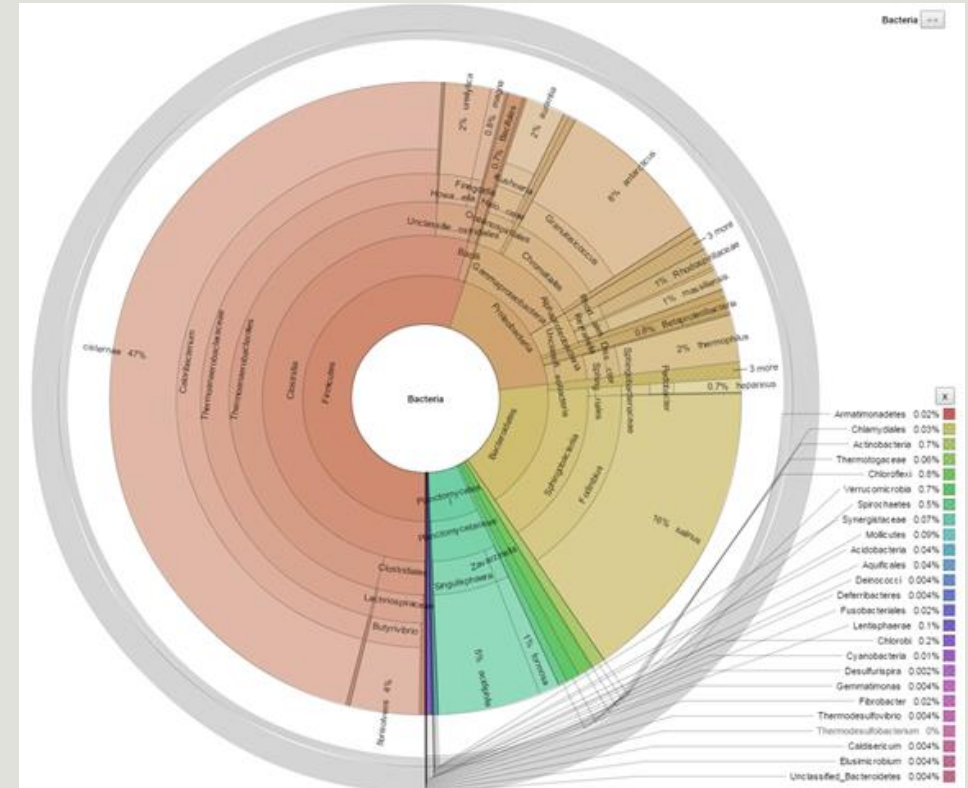


**VSEARCH: 21 000 clusters**



**filters : 0.005%**

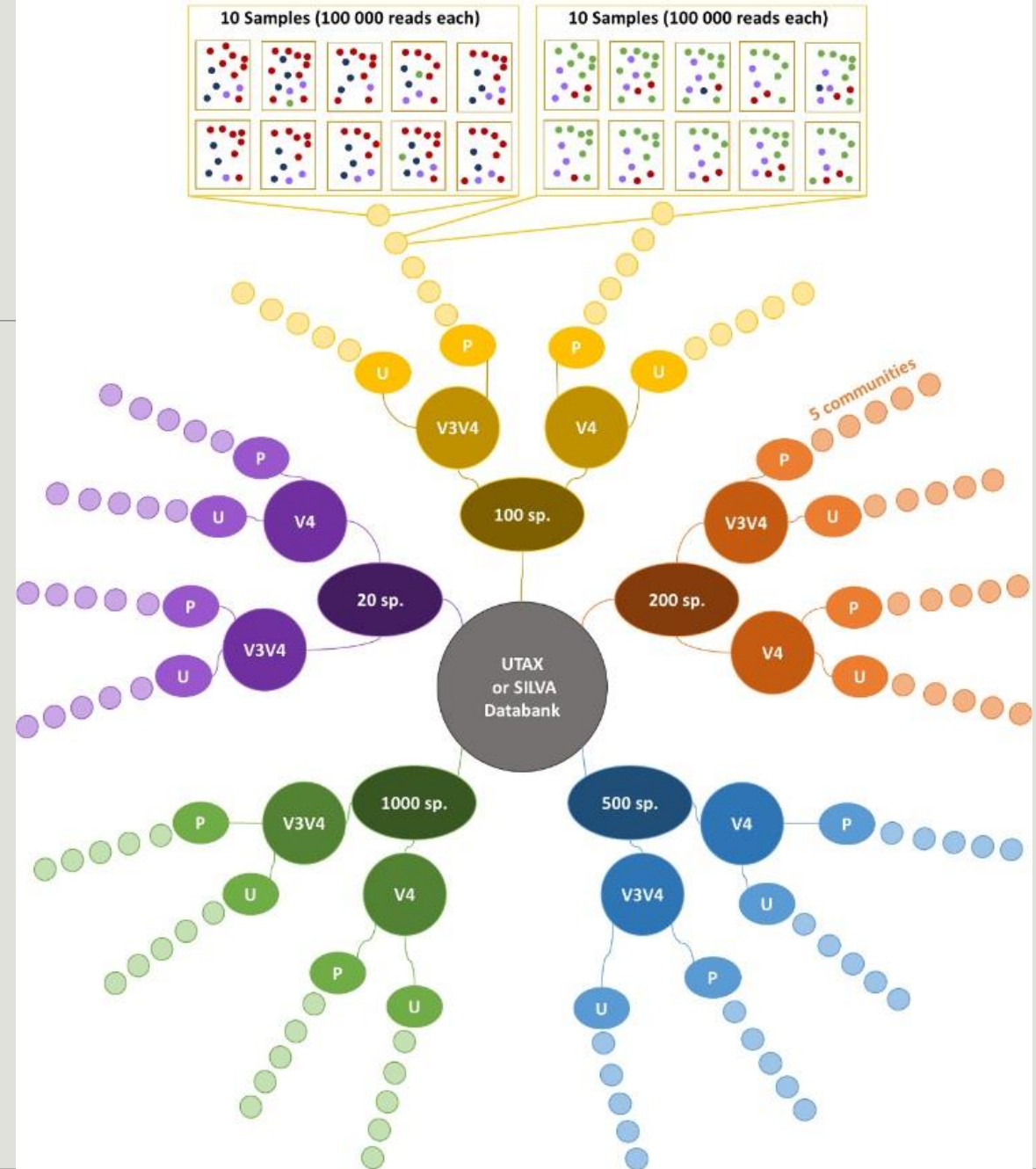
**505 OTUs**



# FROGS' Accuracy






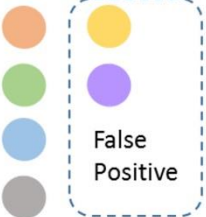
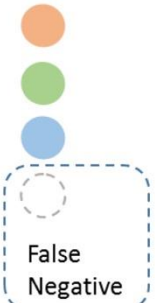
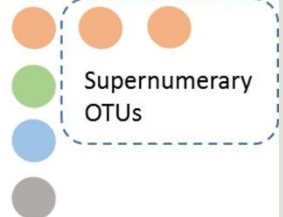
- $1.10^{+8}$  synthetic sequences were treated with **FROGS**, **UPARSE** and **MOTHUR**, **QIIME**, with their guidelines, to compare their performances
- 20, 100, 200, 500 or 1000 different species
- power law or a uniform distribution
- 5 to 20% of chimera

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



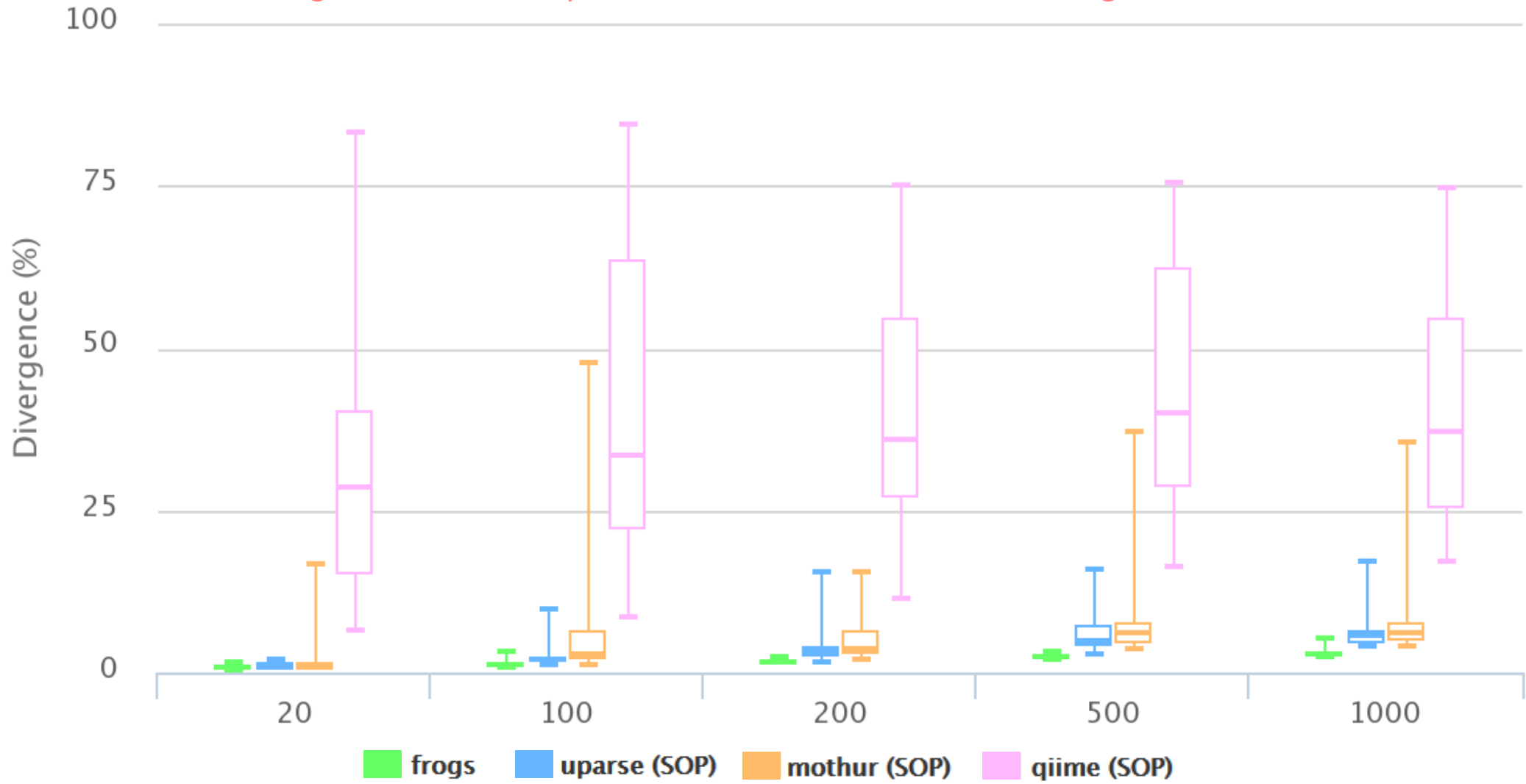
# FROGS' Accuracy

The four metrics used to compare results of FROGS, UPARSE, QIIME and MOTHUR are :

<p><b>Expected</b></p>  <p>1 species with abundance of 30 reads</p>	 <p>4 species</p>	 <p>4 species</p>	 <p>4 species</p>
<p><b>Observed</b></p>  <p>divergence rate = +50% of abundance</p>	 <p>2 FPs = 2 not real OTUs are kept</p>	 <p>1 FN = a real OTU is lost</p>	 <p>2 SOs = 2 additional OTUs with same origin as the expected OTU</p>

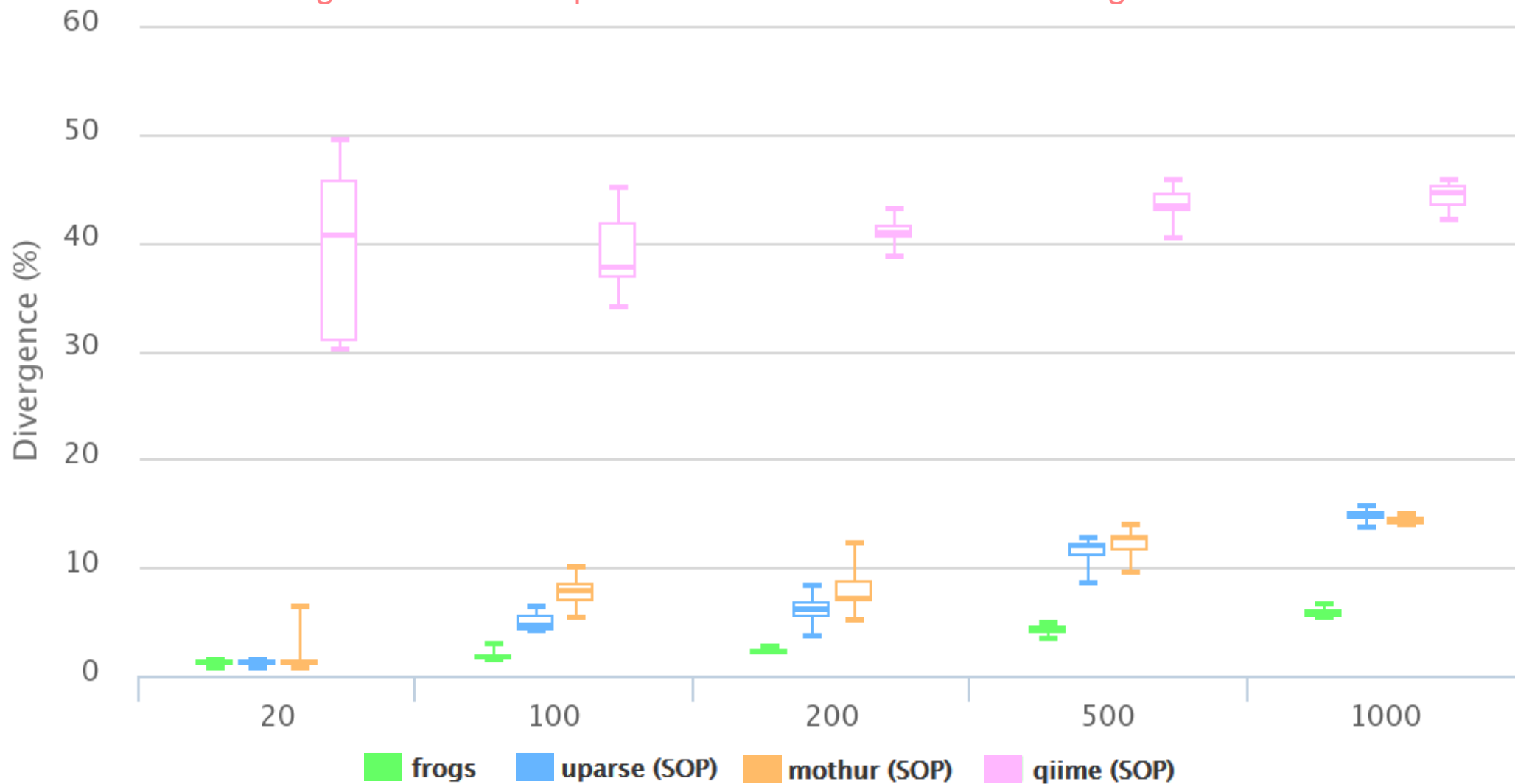
# Affiliations divergence

Divergence on the composition of microbial communities at genus rank



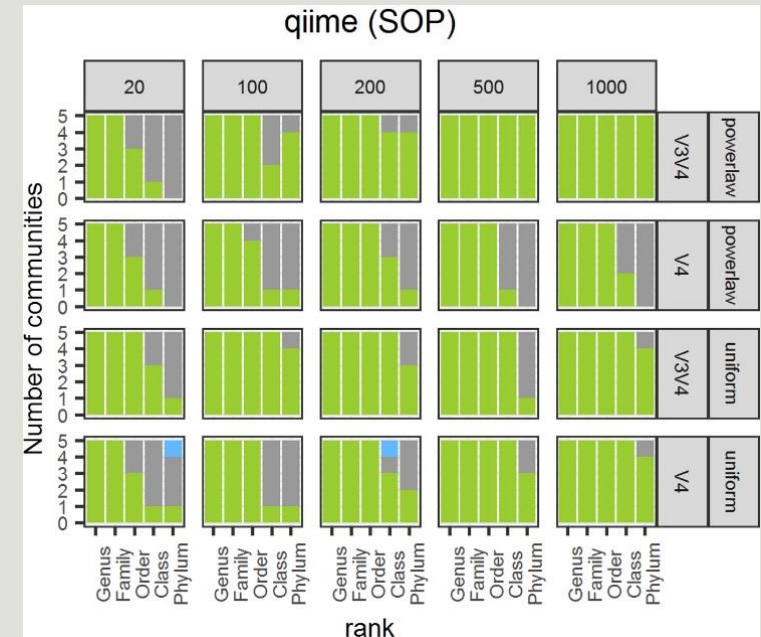
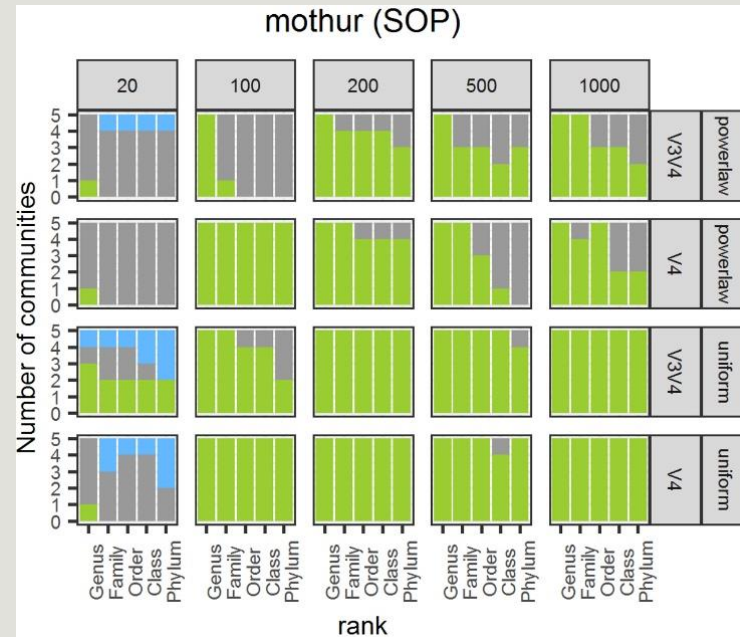
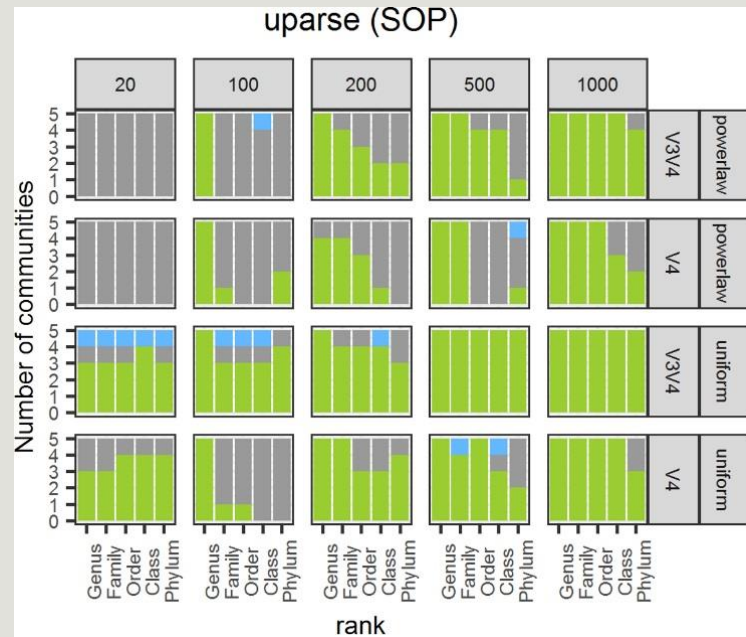
# Affiliations divergence

Divergence on the composition of microbial communities at genus rank





# The results of non-parametric paired tests (signed rank test) of Affiliation divergence on simulated data from UTAX

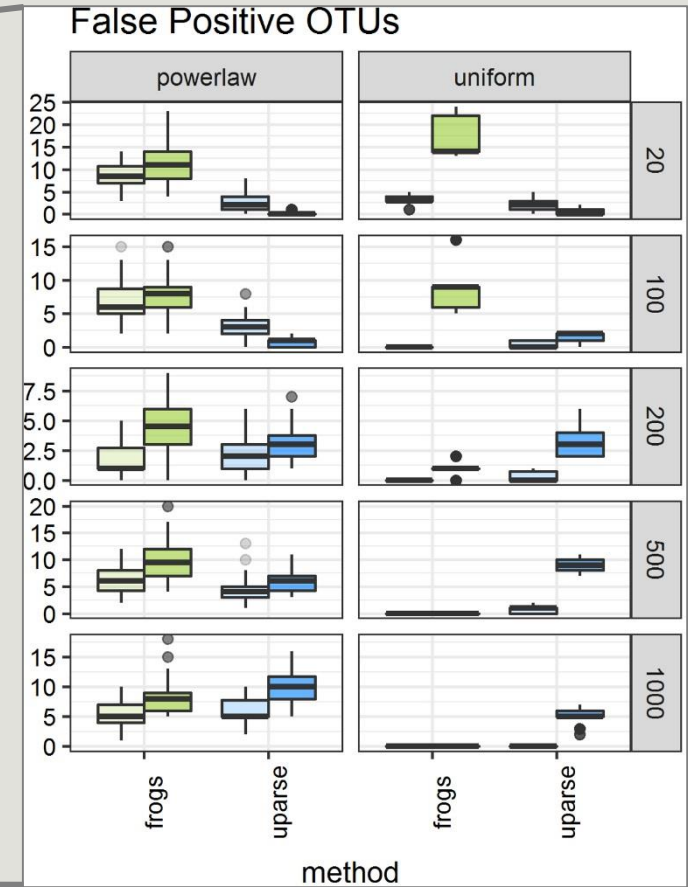
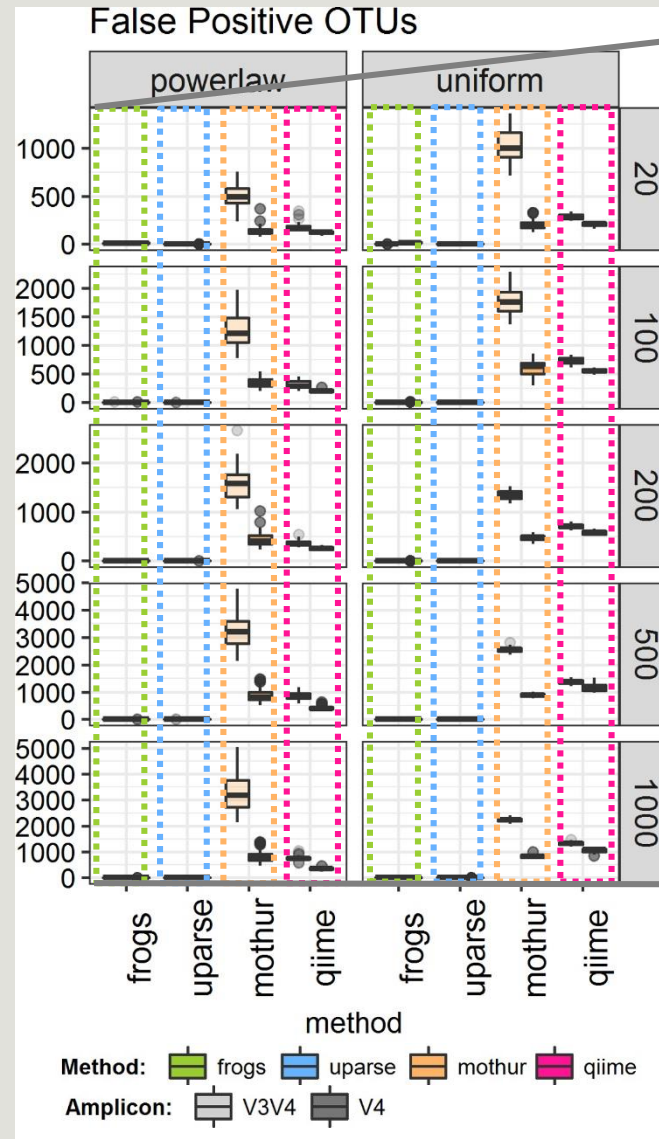


FROGS performed as well as or better than UPARSE and mothur in most settings. The infrequent condition in which FROGS performed worse than UPARSE and mothur was for small community sizes (20 species), except at genus level. It performed better than QIIME in all settings.

Huge number of FP inferred by mothur (up to 20 times more than the expected community size).

a few more FPs under power law abundance distributions and a few less under uniform abundance distributions (except for size < 100 species)

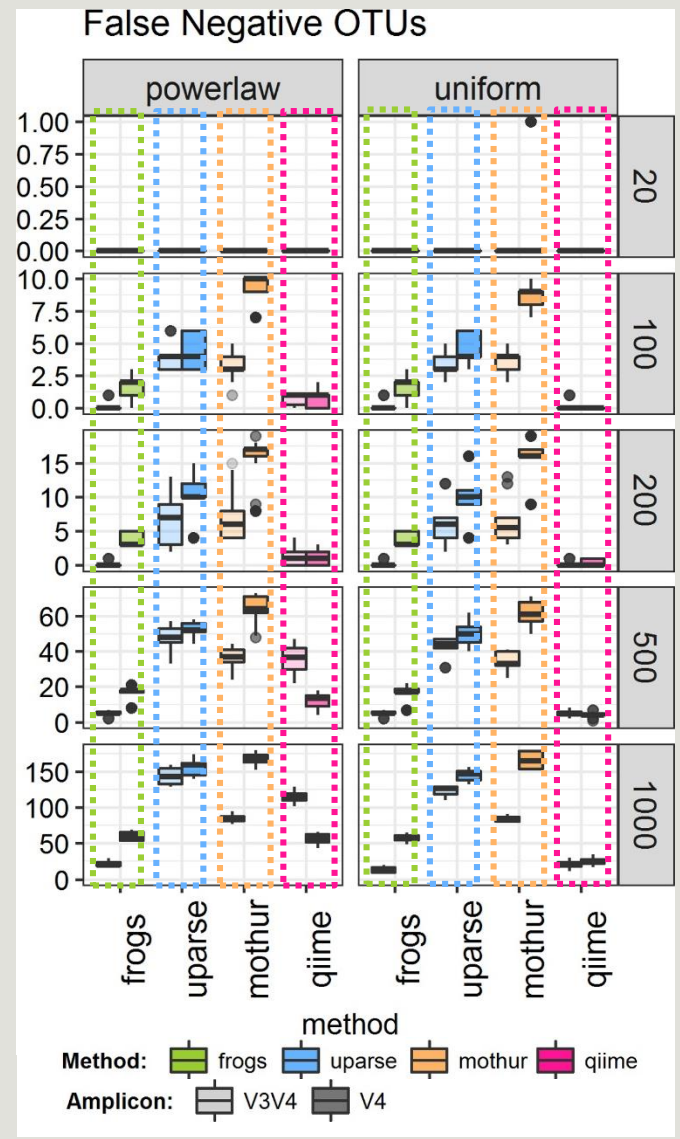
FROGS performed better than QIIME in all settings



FROGS truly outperformed mothur in terms of FN taxa

FROGS always produced fewer FNs than UPARSE.

FROGS sometimes produced more FNs than QIIME, especially on the V4 region.



# Conclusions on assessments

---

FROGS performed much better than mothur in all settings

FROGS is less conservative than UPARSE for small size communities and better (for both FPs and FNs) for large size communities

FROGS is more conservative than QIIME on the V4 region and better (for both FPs and FNs) on V3V4 regions.

FROGS maintained both the number of FP and FN OTUs low, especially in complex communities.

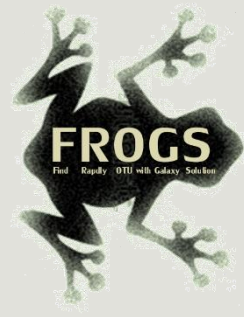
→ cross-validation of chimeras, only used in FROGS, which avoids confusing real OTUs with chimeras.

→ 3 step strategy (clustering by Swarm + chimera removal with cross-validation + filtering) = a low FP rate and the high probability of detecting a species that is really present in the dataset *i.e.* a high recall rate.

→ unlike QIIME or mothur, FROGS never produced Supernumerary OTUs, which further validates the FROGS OTU picking strategy.

# Conclusions

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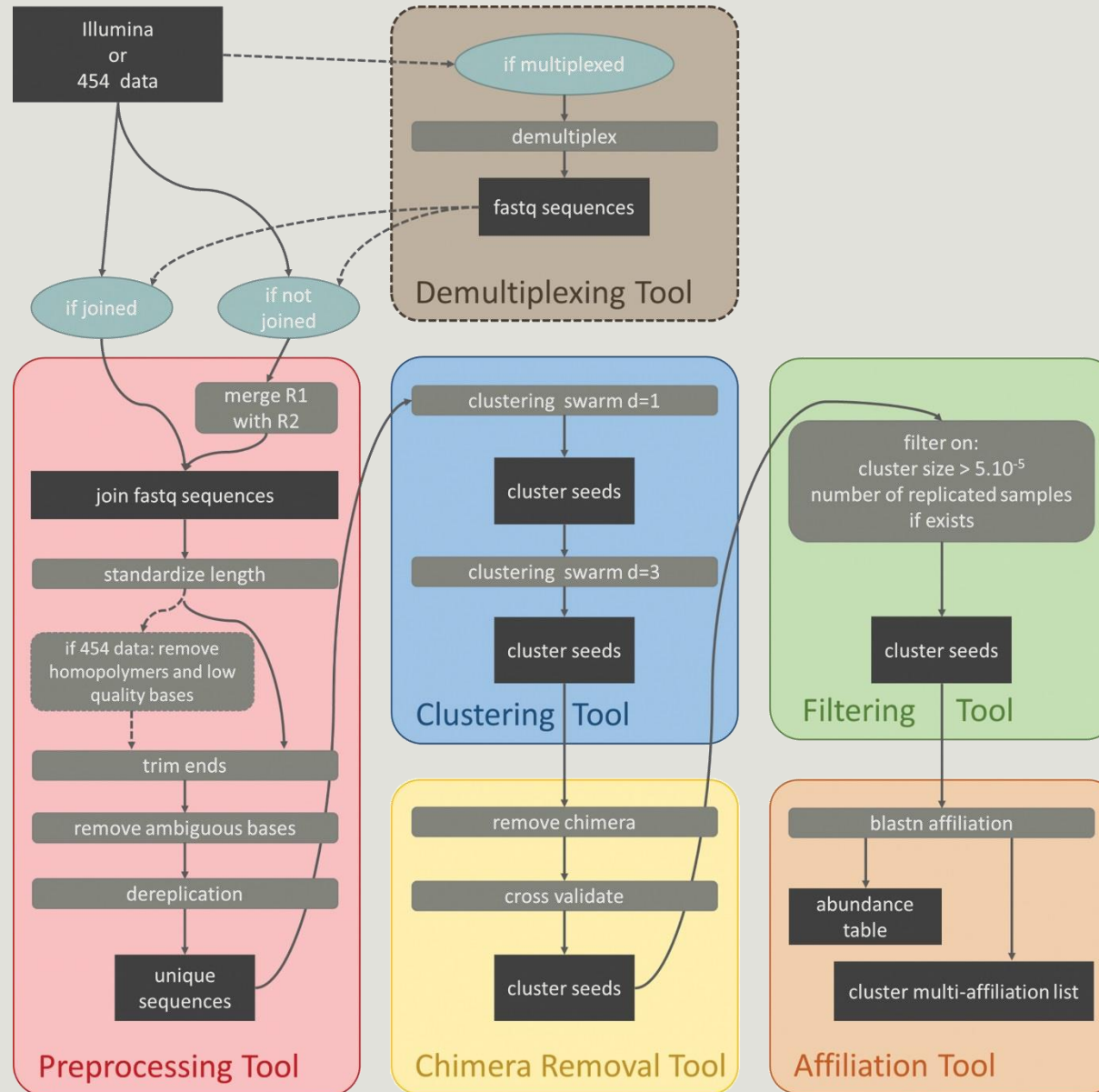


# Why Use FROGS ?

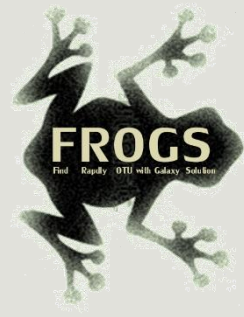
---

- User-friendly
- Fast
- 454 data and Illumina data
  - sequencing methods change but same tool
  - easier for comparisons
- Clustering without global threshold and independent of sequence order
- New chimera removal method (Vsearch + cross-validation)
- Filters tool
- Multi-affiliation with 2 taxonomy affiliation procedures
- Cluster Stat and Affiliation Stat tools
- A lot of graphics
- Independant tools
- Few FPs and few FNs

# Our recommended guideline:







# How to cite FROGS

---

In waiting for the publication:

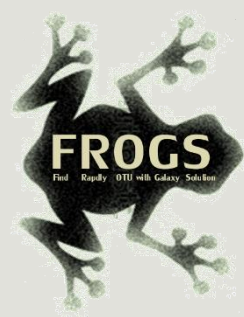
Pipeline FROGS on <http://sigenae-workbench.toulouse.inra.fr/>

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

Poster FROGS: Escudie F., Auer L., Bernard M., Cauquil L., Vidal K., Maman S., Mariadassou M., Combes S., Hernandez-Raquet G., Pascal G., 2016. FROGS: Find Rapidly OTU with Galaxy Solution. In: ISME-2016 Montreal, CANADA,

[http://bioinfo.genotoul.fr/wp-content/uploads/FROGS\\_ISME2016\\_poster.pdf](http://bioinfo.genotoul.fr/wp-content/uploads/FROGS_ISME2016_poster.pdf)





# To contact

---

FROGS:

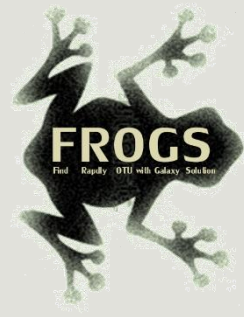
[frogs@inra.fr](mailto:frogs@inra.fr)

Galaxy:

[support.sigenae@inra.fr](mailto:support.sigenae@inra.fr)

Newsletter – demande d’abonnement:

[frogs@inra.fr](mailto:frogs@inra.fr)



# Next training sessions

---

9<sup>th</sup> to 12<sup>th</sup> April 2018 - 4 days

0.5 Galaxy day

2 FROGS days

1.5 Statistics phyloseq days