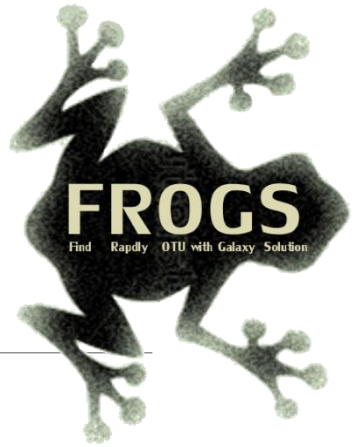


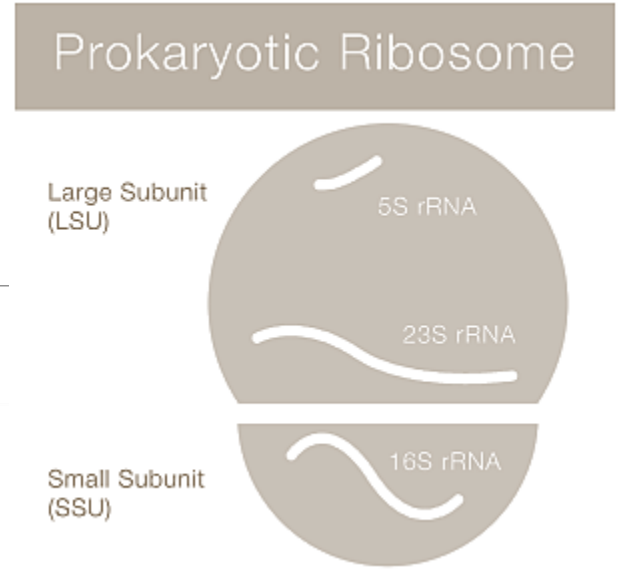

# ITS analysis in FROGS ?

## Training on Galaxy: Metagenomics

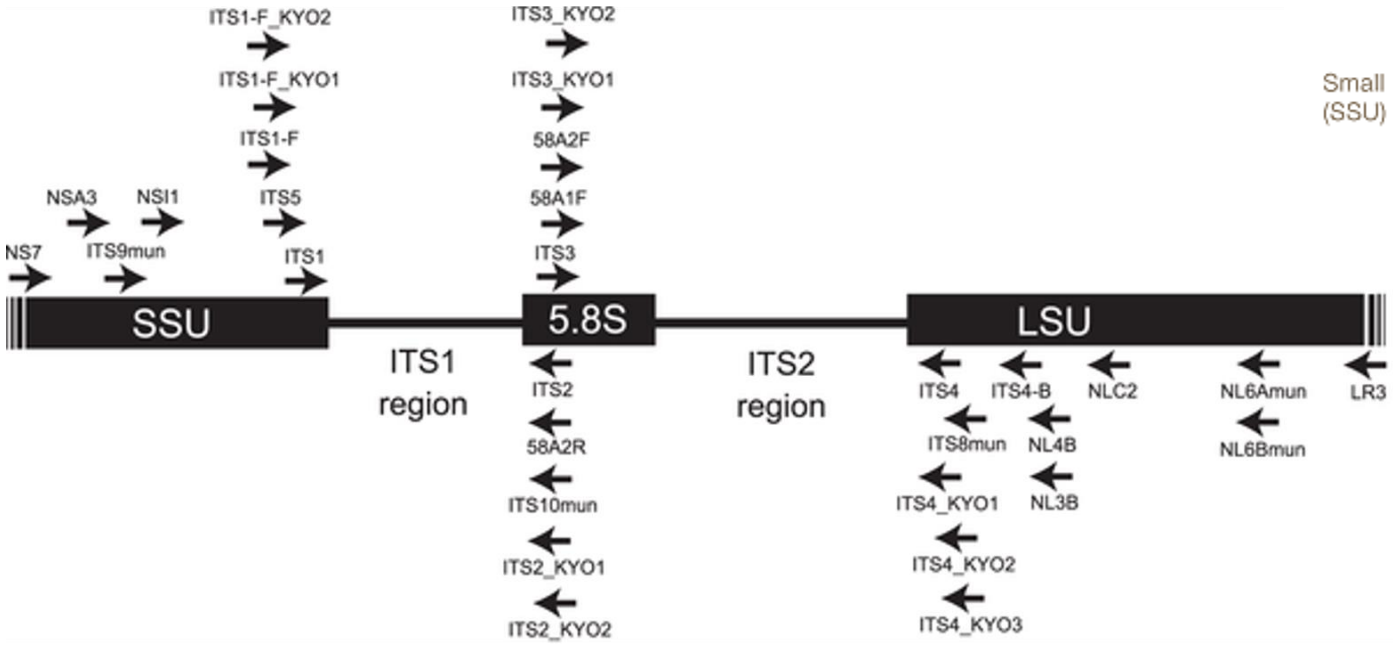
November 2018

MARIA Bernard, OLIVIER Rué, GÉRALDINE Pascal

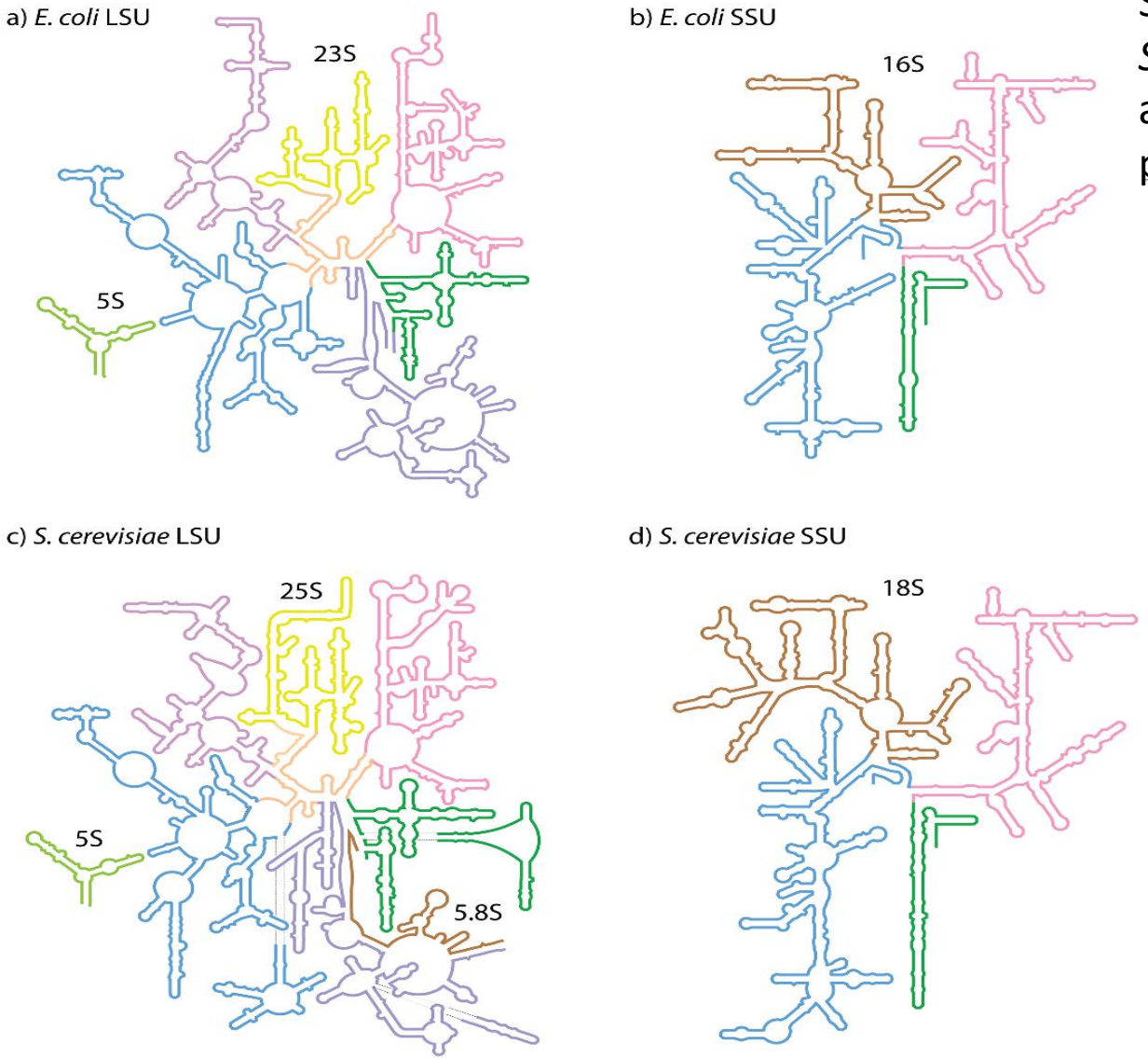
# What is a ITS ?




**Map of nuclear ribosomal RNA genes and their ITS regions.**



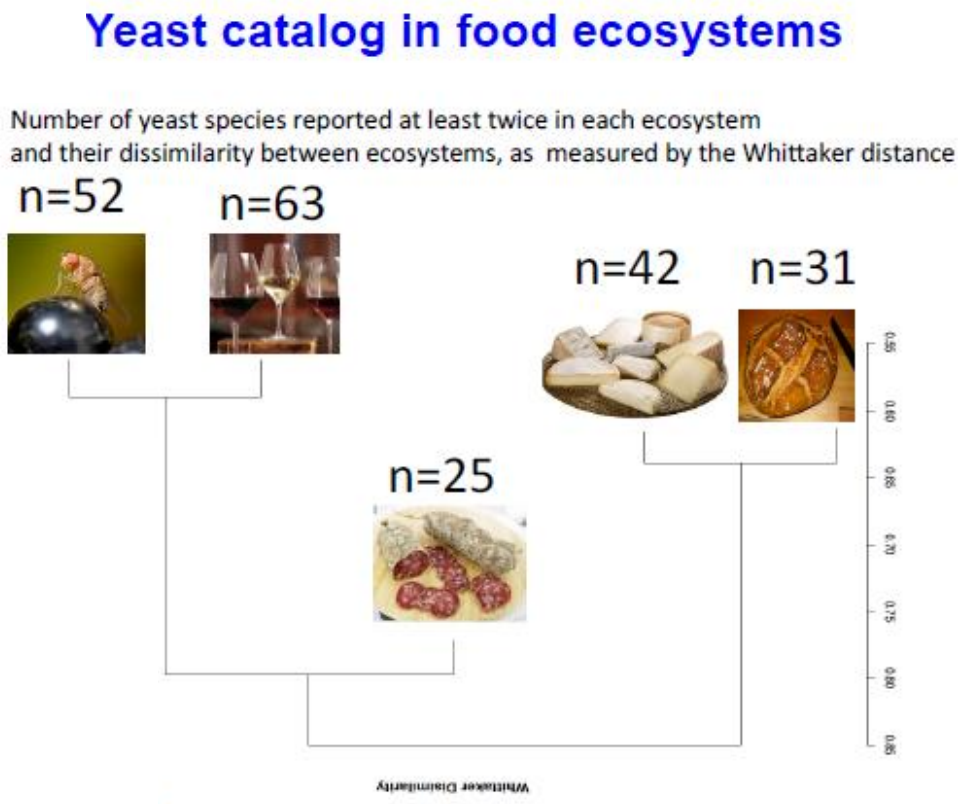

Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLOS ONE 7(7): e40863. https://doi.org/10.1371/journal.pone.0040863




Schematic rRNA 2° structures of a) *E. coli* LSU, b) *E. coli* SSU, c) *S. cerevisiae* LSU, and d) *S. cerevisiae* SSU. These 2° structures are derived from 3D structures, and include non-canonical base pairs.

**Secondary Structures of rRNAs from All Three Domains of Life**
Anton S. Petrov , Chad R. Bernier, Burak Gulen, Chris C. Waterbury, Eli Hershkovits, Chiaolong Hsiao, Stephen C. Harvey, Nicholas V. Hud, George E. Fox, Roger M. Wartell, Loren Dean Williams
February 5, 2014 https://doi.org/10.1371/journal.pone.0088222

# ITS data form METABARFOOD Project metaprogramme MEM




- While metabarcoding is commonly used to describe prokaryotes in the microbiome of many environments, methods for describing micro-eukaryote diversity is lacking and requires better methodology and standardisation.
- One reason is that the universal fungal barcode, the Internal Transcribed Spacer (ITS) region, displays considerable size variation amongst yeasts and other micro-eukaryotes.
- There are also several repeats leading to sequencing errors or termination.
- Additionally, the ITS databases are far from complete, especially for Ascomycota that are commonly found in food.
- Other rDNA barcodes have been used but often do not harbor enough polymorphism to detect taxa to the species level.
- In food, microbiota are usually composed of a reduced number of species compared to wild environments.
- Detecting micro-eukaryotes at the species level, and potentially strain level, is therefore necessary.

# Case of ITS1 amplicon MiSeq sequencing, a case of a sequencing of non-overlapping sequences

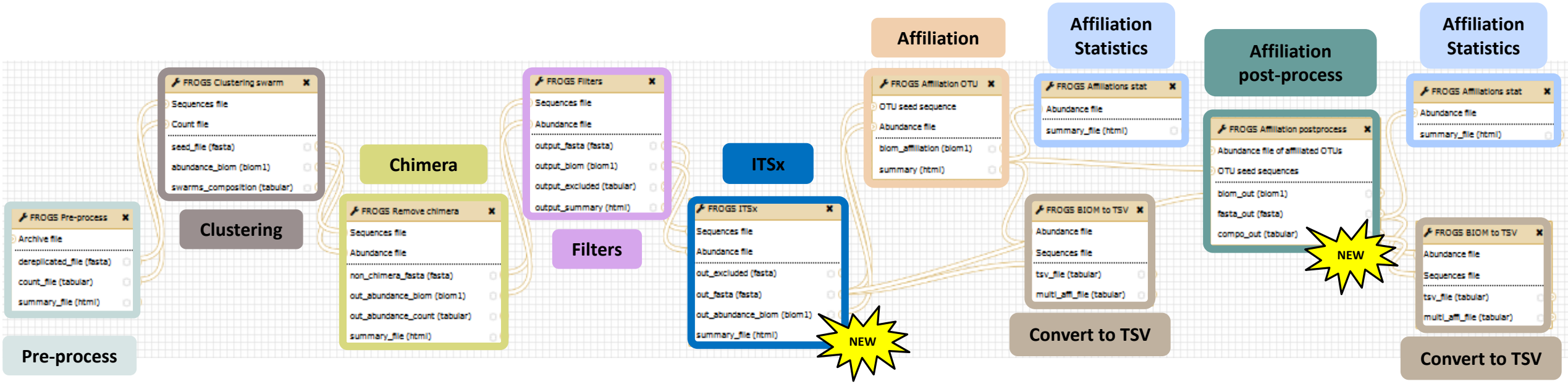
Imagine a real amplicon sequence of 700bp


Imagine a Miseq paired sequencing of 2x250bp


Reconstructing amplicon sequence is not possible with overlap, an arbitrary sequence of 100Ns is added. It is named « FROGS combined »


Combined sequence length : 600bp, with 100 Ns

# What workflow should we use to analyse ITS ?



# Pre-process tool

ITS

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

**Sequencer**

Illumina

Select the sequencing technology used to produce the sequences.

**Input type**

Archive

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

**Archive file**

1: /work/frogsfungi/ITS.tar.gz

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

**Reads already merged ?**

No

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

**Reads 1 size**

250

The maximum read1 size.

**Reads 2 size**

250

The maximum read2 size.

**mismatch rate.**

0.1

The maximum rate of mismatch in the overlap region

**Merge software**

Vsearch

Select the software to merge paired-end reads.

**Would you like to keep unmerged reads?**

Yes | No

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

To keep FROGS combined sequences, choose YES

ITS

**Minimum amplicon size**

50

The minimum size for the amplicons (with primers).

**Maximum amplicon size**

490

The maximum size for the amplicons (with primers).

**Sequencing protocol**

Illumina standard

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

**5' primer**

CTTGGTCATTTAGAGGAAGTAA

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

**3' primer**

GCATCGATGAAGAACGCAGC

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

✔ Execute

# Exercise 2.3

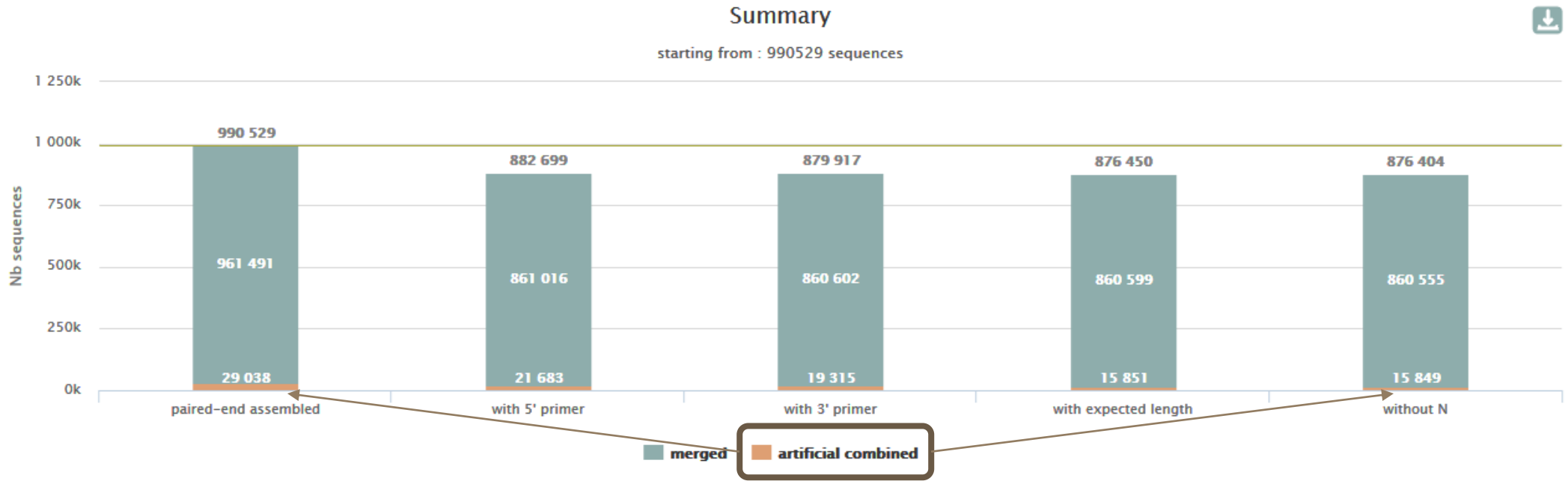
Go to « ITS » history

Launch the pre-process tool on this data set

→ objective: understand preprocess report and « FROGS combined sequences »

# Explore Preprocess report.html

## Preprocess summary



# Explore Preprocess report.html

2 tables:

## Details on merged sequences

CSV

Show 10 entries Search:

| Samples | % kept | paired-end assembled | with 5' primer | with 3' primer | with expected length | without N |
|---|---|---|---|---|---|---|
| complexe-ADN-1 | 91.09 | 54,121 | 49,322 | 49,303 | 49,303 | 49,299 |
| echantillon1-1 | 84.93 | 31,836 | 27,059 | 27,040 | 27,040 | 27,039 |
| echantillon1-2 | 94.73 | 54,774 | 51,938 | 51,895 | 51,895 | 51,890 |
| echantillon1-3 | 74.90 | 81,611 | 61,197 | 61,135 | 61,134 | 61,128 |
| echantillon2-1 | 90.17 | 51,984 | 46,886 | 46,875 | 46,874 | 46,873 |

## Details on artificial combined sequences

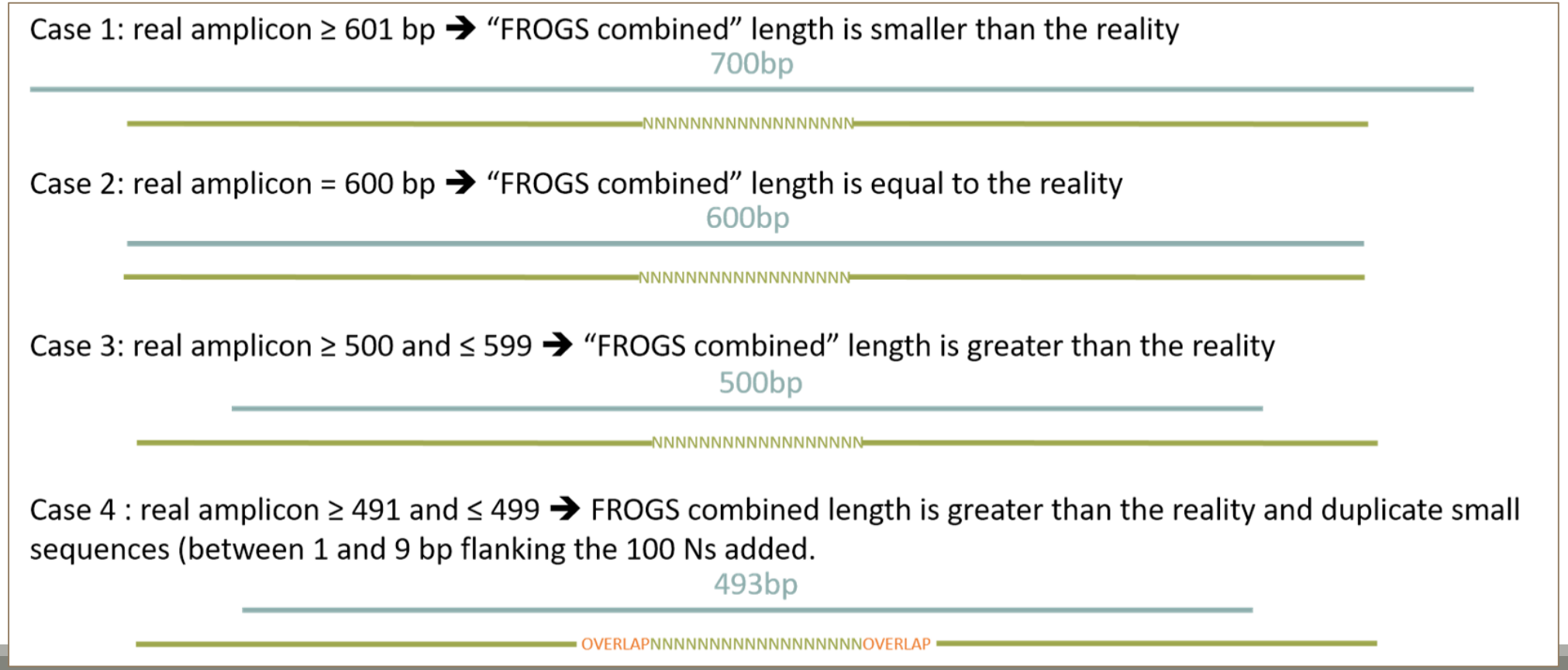
CSV

Show 10 entries Search:

| Samples | % kept | paired-end assembled | with 5' primer | with 3' primer | with expected length | without N |
|---|---|---|---|---|---|---|
| complexe-ADN-1 | 68.47 | 2,163 | 1,833 | 1,656 | 1,481 | 1,481 |
| echantillon1-1 | 54.92 | 1,047 | 751 | 620 | 575 | 575 |
| echantillon1-2 | 61.57 | 1,392 | 1,096 | 942 | 858 | 857 |
| echantillon1-3 | 49.54 | 2,491 | 1,617 | 1,334 | 1,234 | 1,234 |
| echantillon2-1 | 44.62 | 1,421 | 996 | 899 | 634 | 634 |

# FROGS "combined" sequences are artificial and present particular features especially on size.

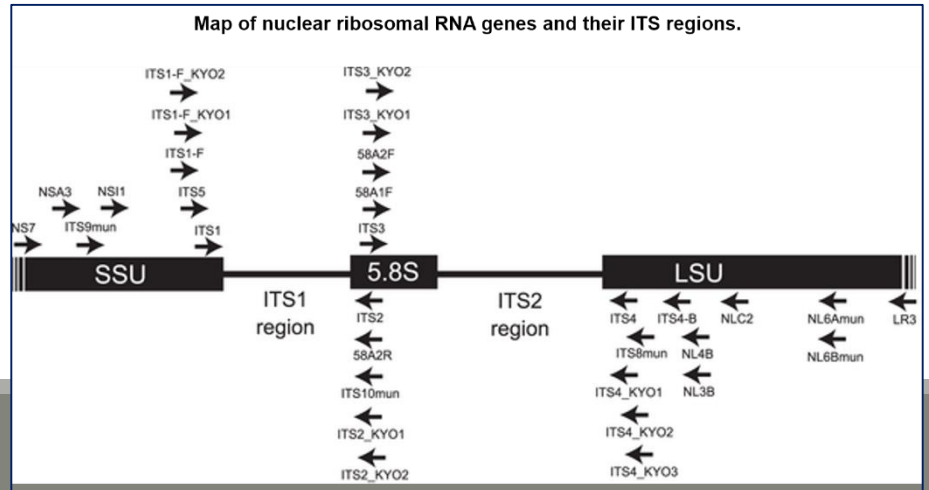
Imagine a MiSeq sequencing of 2x250pb with reads impossible to overlap. So FROGS "combined" length = 600 bp.



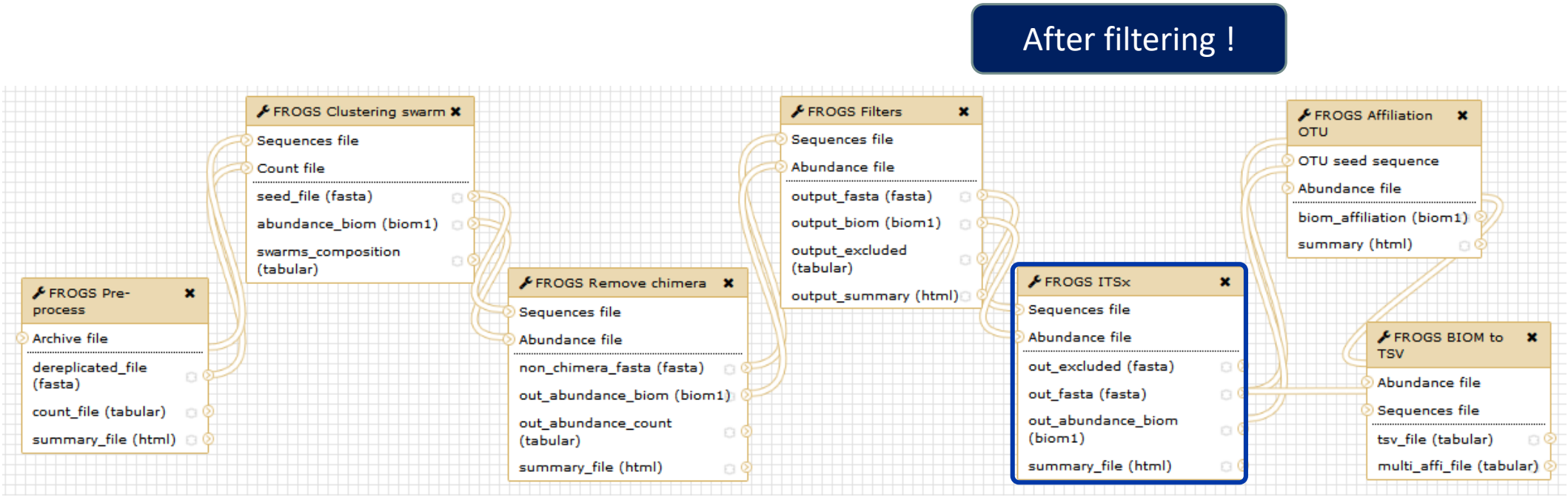
# ITSx tools

# What is the purpose of the ITSx tool?

- ITSx is a tool to filter sequences.
- ITSx identifies and trimms ITS regions in our sequences.
- It excludes the highly conserved neighbouring sequences SSU, 5S and ARNr LSU.
- If the ITS1 or ITS2 region is not detected, the sequence is discarded.
- You can choose to check only if the sequence is detected as an ITS.
- In this case, the sequence is not trimmed, only sequences not detected as ITS are rejected (*e.g.* contaminants).



# When should we use ITSx ?



**FROGS ITSx** Extract the highly variable ITS1 and ITS2 subregions from ITS sequences. (Galaxy Version r3.0-1.0) ▾ Options

**Sequences file**

13: FROGS Filters: sequences.fasta

The sequence file to filter (format: fasta).

**Abundance file**

14: FROGS Filters: abundance.biom

The abundance file to filter (format: BIOM).

**ITS region**

ITS1

Which fungal ITS region is targeted: either ITS1 or ITS2

**Check only if sequence detected as ITS ?**

Yes | No

If Yes, sequences with ITS signature will be kept without trimming SSU, LSU or 5.8S regions.

✔ Execute

# Check only if sequence is detected as ITS? Yes or not?

- It is interesting to keep only the ITS parts without the flanking sequences in case one would like to compare sequenced amplicons with different primers targeting the same region to be amplified.
- You can choose this option on configuration panel of ITSx Tool.
- Reply "No" to question "Check only if sequence is detected as ITS?".
- In opposite, if "Yes" is chosen, sequences with ITS signature will be kept without trimming SSU, LSU or 5.8S regions.

# Carreful !

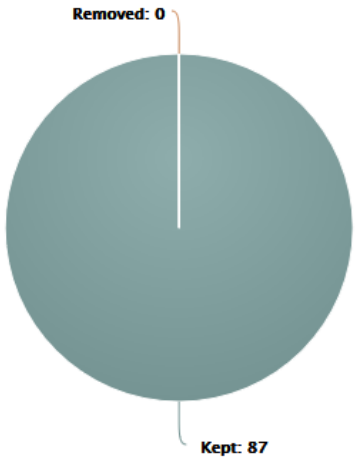
- The ITSx step is time consuming and has to be done on clusters. We advise our users to apply ITSx in 5th step:

1. Preprocess step,
2. Clustering step,
3. Chimera removing step,
4. Filter on OTUs abundances and replicats step,
5. ITSx if Fungi ITS amplicons.

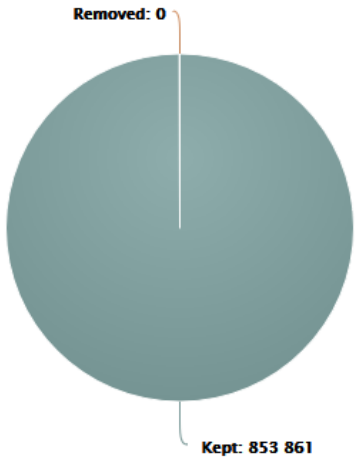
- Carreful, ITSx is currently usable for the detection of fungi ITS neither plants nor other eukaryotes.

# Filters (ITSx) summary

OTUs




Abundance




# Filters (ITSx) by samples

CSV

Show 10 entries

Search:

**OTUs removed by sample**

| Sample name | Initial | Kept | Initial abundance | Kept abundance |
|---|---|---|---|---|
| complexe-ADN-1 | 65 | 65 | 47,980 | 47,980 |
| echantillon1-1 | 63 | 63 | 26,797 | 26,797 |
| echantillon1-2 | 64 | 64 | 51,499 | 51,499 |

# ITS Affiliation

# What is special about the affiliation of ITS (with combined sequences more broadly)?

- blastn+ or needlall is used to find alignment between each OTU and the database.
- Only the bests hits with the same score are reported.
- blastn+ is used for merged read pair, and needall is used for **artificially combined sequence**.
- For each alignment returned, several metrics are computed: identity percentage, coverage percentage, and alignment length.
- If "combined" sequences are stayed presents in OTUs, blastn+ is not usable as for classical merged sequences.

# What is special about the affiliation of ITS (with combined sequences more broadly)?

- So, sequences are affiliated in 3 steps: Alignment of classical "merged" sequences with blastn+ *versus* chosen database (*e.g.* UNITE),
- Alignment of "combined" sequences with blastn+ *versus* chosen database, best hits are collected and a very small new databank (at most 200 references per blast hit) is created composed exclusively of "subject" sequences from these best hits,
- Alignment of "combined" sequences with needlall (global alignment: very time consuming) *versus* these small new databank.

# Careful, with "combined" sequences, we introduced some modification on identity percentage

# Case 1: a sequencing of overlapping sequences i.e. 16S V3-V4 amplicon MiSeq sequencing

Imagine a real amplicon sequence of 400bp


Reconstructing amplicon sequence is a merged sequence (length : 400bp, with 100bp overlap)

Affiliation is notably made by a local alignment with NCBI Blast+

Imagine a perfect sequencing without error:

classical %id = number of matches / alignment length = 400 matches / 400 positions = 100% identity

# Case2: a sequencing of non-overlapping sequences: case of ITS1 amplicon MiSeq sequencing

Imagine a real amplicon sequence of 700bp


Reconstructing a FROGS combined sequence (length : 600bp, with 100Ns)


Affiliation could not be made by a local alignment but with a global alignment with Emboss needleall


Imagine a perfect sequencing without error:

classical %id = number of matches / alignment length = (250+250 matches) / 700 positions = 71%

# Conclusion on identity percentage for ITS

Filtering on %id will systematically removed “FROGS combined” OTUs.

So, we proposed to replace the classical %id by a %id computed on the sequenced bases only.

% sequenced bases identity = number of matches / (seed length – artificial added N)

Case 1 : 16S V3V4 ➔ overlapped sequence

% sequenced bases identity = 400 matches / 400 bp = **100 %**

Case 2 : very large ITS1 ➔ “FROGS combined” shorter than the real sequence

% sequenced bases identity = (250 + 250 ) / (600 - 100) = **100%**

This calculation allows the 100% identity score to be returned on FROGS "combined" shorter or longer than reality in case of perfect sequencing. And returns a lower percentage of identity in the case of repeated small overlaps kept in the FROGS "combined".

# Affiliation Post-process

# What is the purpose of the *Affiliation post-process* tool ?

This tool allows grouping OTUs together in accordance with the %id and %cov chosen by the user and according to the following criteria:

1. They must have the same affiliation

Or

2. If they have "multi-affiliation" tag in FROGS taxonomy, they must have in common in their list of possible affiliations at least one identical affiliation.

# What is the purpose of the *Affiliation post-process* tool ?

In consequence:

The different affiliations involved in multi-affiliation are merged.

The abundances are added together.

It is the most abundant OTU seed that is kept.

# What is the purpose of the *Affiliation post-process* tool ?

In case of ITS amplicon analyses,

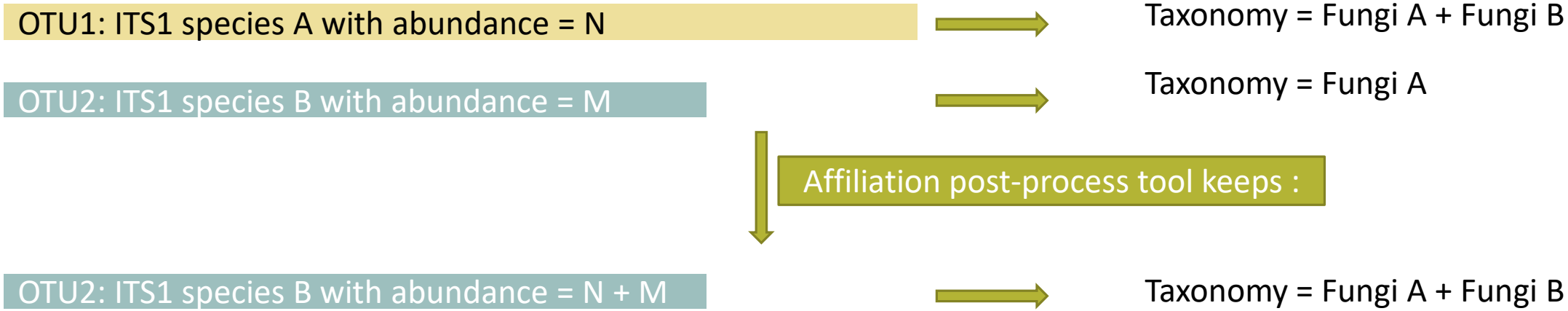
you may have ambiguities due to inclusive ITS sequence coming from different species.

The tool will keep affiliation of the shortest sequence in case of multi-affilition tag.

This "Affiliation post-process" tool helps to resolve ambiguities due to potentially inclusive sequences such as ITS.

# What is the purpose of the *Affiliation post-process* tool ?

ITS1 blue is completly included (with 100% identity) in ITS1 yellow



**FROGS Affiliation postprocess** Optionnal step to resolve inclusive amplicon ambiguities and to aggregate OTUs based on alignment metrics (Galaxy Version r3.0-1.0) ▾ Options

**Abundance file of affiliated OTUs**

23: FROGS ITSx: itsx.biom

Abundances of affiliated OTUs (format: BIOM).

**OTU seed sequences**

22: FROGS ITSx: itsx.fasta

OTU sequences (format: fasta).

**Is this an hyper variable in length amplicon ?**

Yes | No

Multi-affiliation tag may be resolved by selecting the shortest amplicon reference. For this you need the reference fasta file of your kind of amplicon.

**Using reference database**

UNITE_7.1_ITS1

you which ITS 1 or 2 you want to analyse,

UNITE_7.1_ITS1

UNITE_7.1_ITS2

**m**

9

OTUs will be aggregated if they share the same taxonomy with at least X% identity.

**minimum coverage for aggregation**

99

OTUs will be aggregated if they share the same taxonomy with at least X% alignment coverage.

✔ Execute

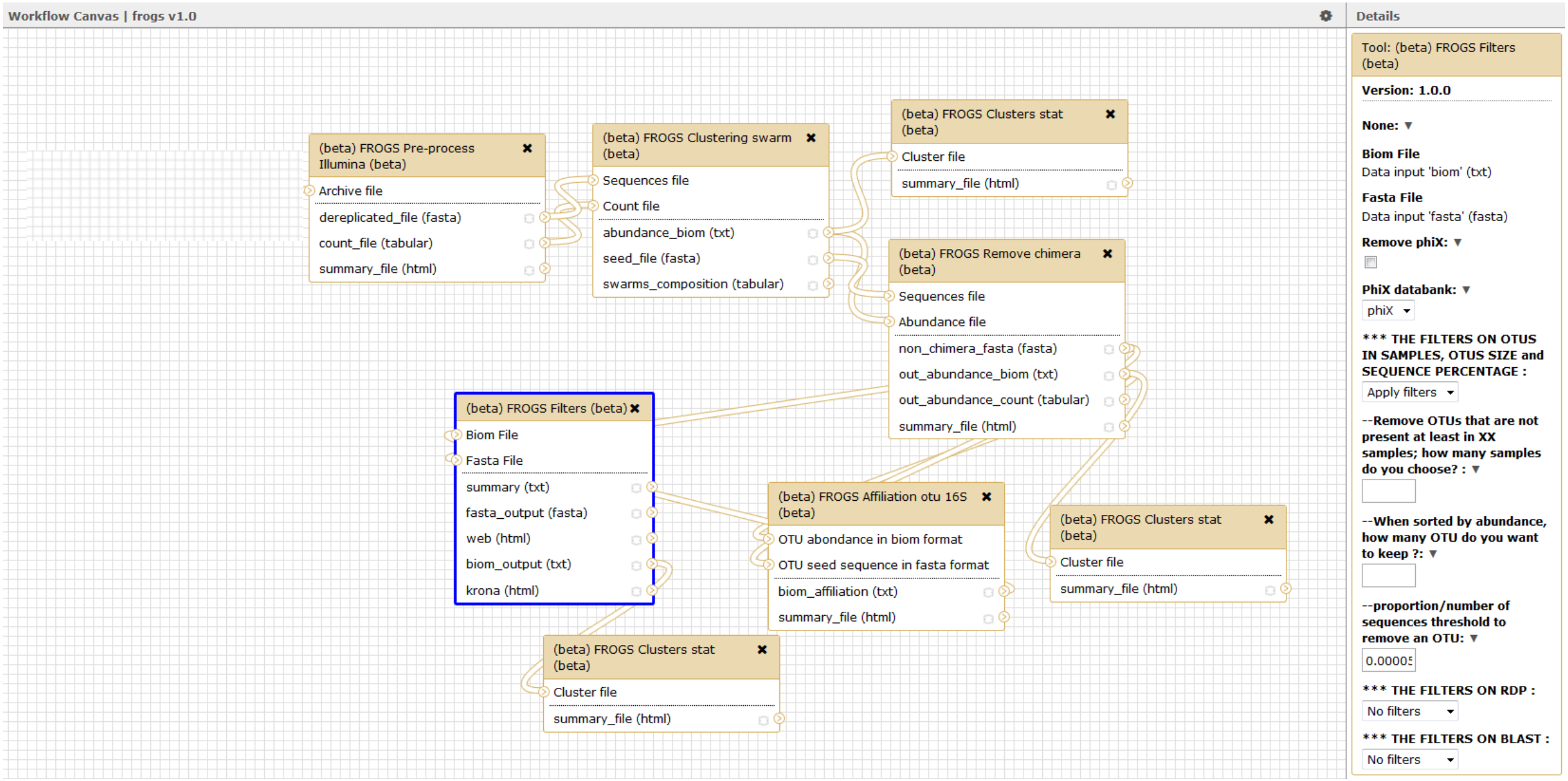
# Post-affiliation Tool - output

```
Cluster_1 Cluster_781 Cluster_922 Cluster_930 Cluster_3573 Cluster_1298 Cluster_798 Cluster_738 Cluster_918
Cluster_2 Cluster_313 Cluster_469 Cluster_445 Cluster_105 Cluster_912 Cluster_471 Cluster_1152 Cluster_1145
Cluster_3 Cluster_599 Cluster_114
Cluster_4 Cluster_109
Cluster_5 Cluster_140 Cluster_3850
Cluster_6 Cluster_195 Cluster_905 Cluster_388 Cluster_275
Cluster_7
Cluster_8
Cluster_9
Cluster_10
Cluster_11
Cluster_12
Cluster_13
Cluster_14
Cluster_15
Cluster_16
Cluster_17
Cluster_18
Cluster_20
Cluster_19
Cluster_21
Cluster_22
Cluster_23
Cluster_25
Cluster_24
```

Cluster 1 encapsulate also clusters 781, 922, 930, 3573, 1298, 798 and 918

```
>Cluster_1 reference=AB241105 amplicon=1..444 position=1..444 errors=440%A
TGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTTAATTGGGAGCAAGCAGTTTTA
>Cluster_2 reference=AJ496032 amplicon=1..452 position=1..452 errors=440%G
TAGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCACGTGTGGGAAGAAGCATTTCGGTGTGTAAACCACTGTCATGAGGGAATAAGGCCCGCCT
>Cluster_3 reference=EU240886 amplicon=1..460 position=1..460
TAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGTGCGAAGAAGGTCTTCGGATCGTAAAGCACTGTTGTTAAGGAAGAACGACAGTAA
>Cluster_4 reference=U39399 amplicon=1..459 position=1..459
TGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCAGTGAAGAAGACTCCGTG
>Cluster_5 reference=FR733705 amplicon=1..452 position=1..452 errors=436%C
TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCGACGCCGCGTGAAGGAAGAAGTCCTTCGGGATGTAAACTTCTGAACTAATCGAATAAGAGGGTAGT
>Cluster_6 reference=GU575117 amplicon=1..434 position=1..434
TGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGATGAAGGCCCTAGGGTTGTAAAGCACTTTCAACGGTGAAGATAATGACGGT
>Cluster_7 reference=AB272165 amplicon=1..454 position=1..454 errors=441%A
TAGGGAATATTGGGCAATGGGTGAGAGCCTGACCCAGCCATGCCGCGTGCCGGACGAAGGCCCTCAGGGTCGTAAACGGCTTTTGCCGGGGAAGAAGAGGTTCC
>Cluster_8 reference=AJ292759 amplicon=1..437 position=1..437
TAGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCGACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCGCAGGGGAATAACACAATGAA
>Cluster_9 reference=CP000027 amplicon=1..435 position=1..435
CAAGGAATCTTGGGCAATGGGCGAAAGCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCTTTCGGGTTGTAAACCTCTTTTCACAGGGAAGAATAATGACGG
>Cluster_10 reference=JN880417 amplicon=1..438 position=1..438 errors=11%A
TCGAGGATCTTCGTCAATGGGCGAAAGCCTGAACGAGCGATTAGCCGCGTGCGCGATGAAGGCCTTCGGGTTGTAAAGCGCGAAAGAGGTAATAAAGGGAAACT
>Cluster_11 reference=EF660760 amplicon=1..434 position=1..434 errors=424%C
TGGGGAATCTTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGAATGATGAAGGCCTTAGGGTTGTAAAATTCTTTCGCCAGGGATGATAATGACAGT
>Cluster_12 reference=AB594446 amplicon=1..443 position=1..443 errors=438%G
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACTCCTTTCGCCATTGACGAAGCCTTTTTG
>Cluster_13 reference=U93332 amplicon=1..439 position=1..439 errors=426%C
```

# Workflow creation


Workflow Canvas | frogs v1.0
Details
(beta) FROGS Pre-process Illumina (beta)
Archive file
dereplicated_file (fasta)
count_file (tabular)
summary_file (html)
(beta) FROGS Clustering swarm (beta)
Sequences file
Count file
abundance_biom (txt)
seed_file (fasta)
swarms_composition (tabular)
(beta) FROGS Clusters stat (beta)
Cluster file
summary_file (html)
(beta) FROGS Remove chimera (beta)
Sequences file
Abundance file
non_chimera_fasta (fasta)
out_abundance_biom (txt)
out_abundance_count (tabular)
summary_file (html)
(beta) FROGS Filters (beta)
Biom File
Fasta File
summary (txt)
fasta_output (fasta)
web (html)
biom_output (txt)
krona (html)
(beta) FROGS Affiliation otu 16S (beta)
OTU abondance in biom format
OTU seed sequence in fasta format
biom_affiliation (txt)
summary_file (html)
(beta) FROGS Clusters stat (beta)
Cluster file
summary_file (html)
(beta) FROGS Clusters stat (beta)
Cluster file
summary_file (html)
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:
0.00005
*** THE FILTERS ON RDP :
No filters
*** THE FILTERS ON BLAST :
No filters

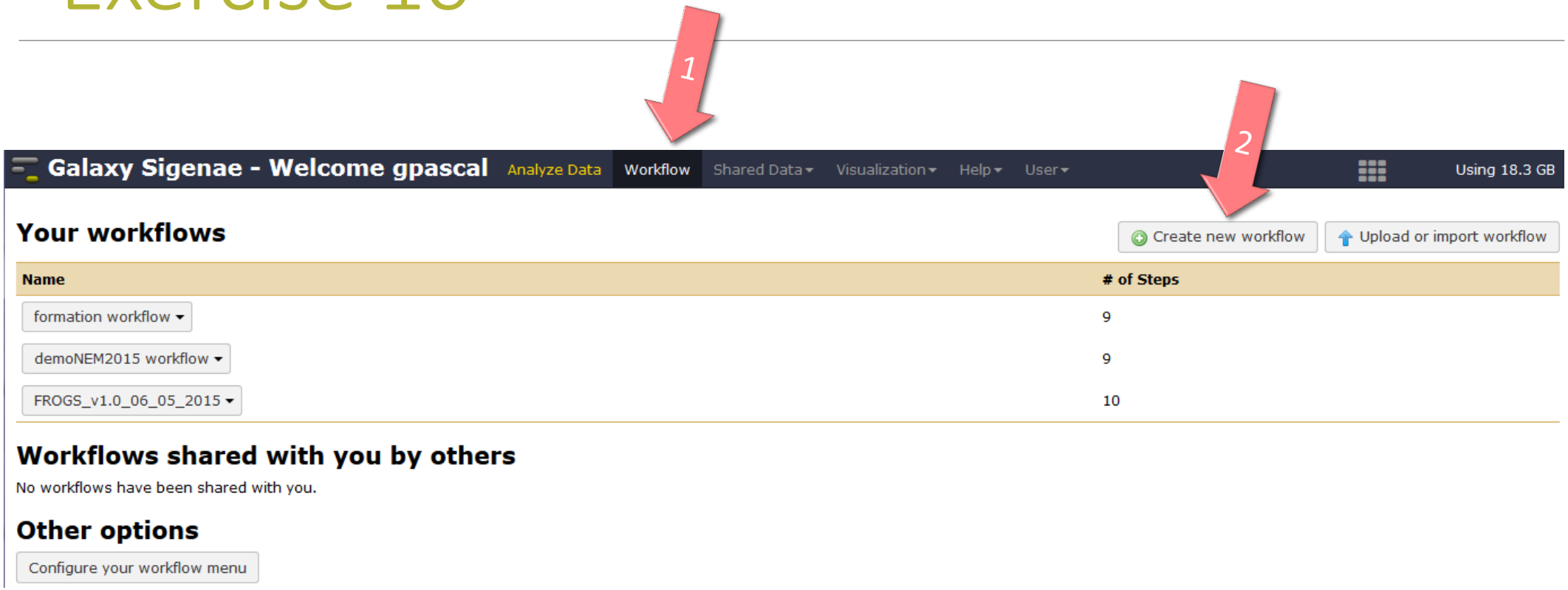
# Your Turn! – 10

CREATE YOUR OWN WORKFLOW !

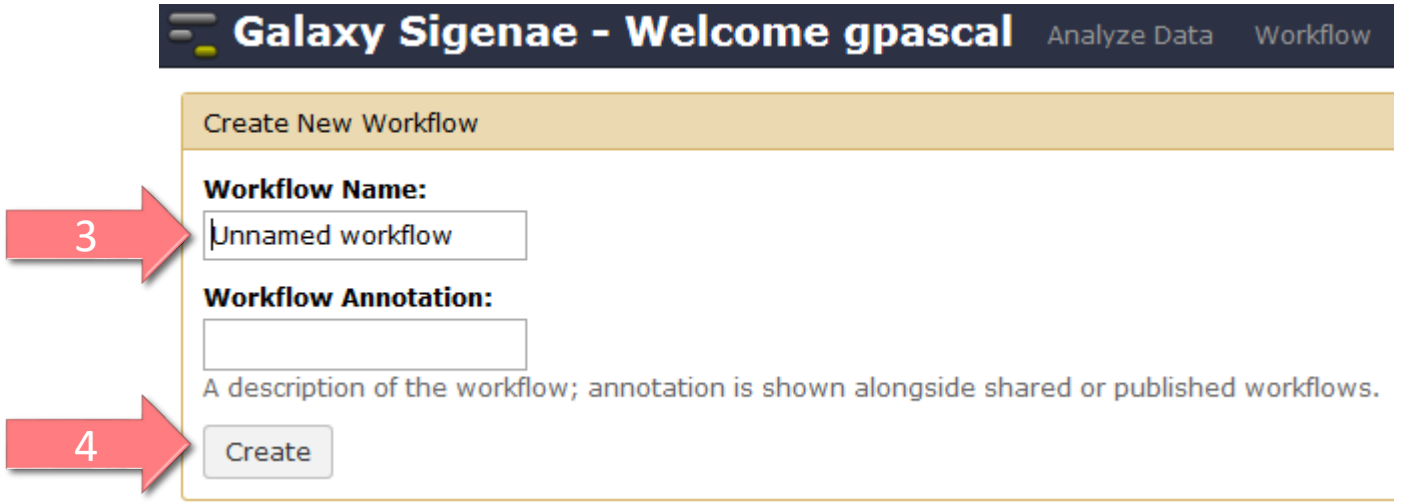
# Exercise 10

1. Create your own workflow with ITS data

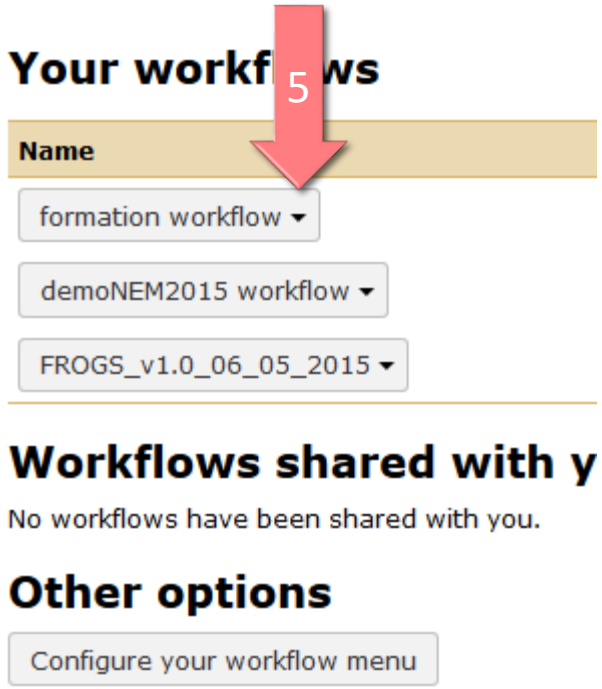
# Exercise 10

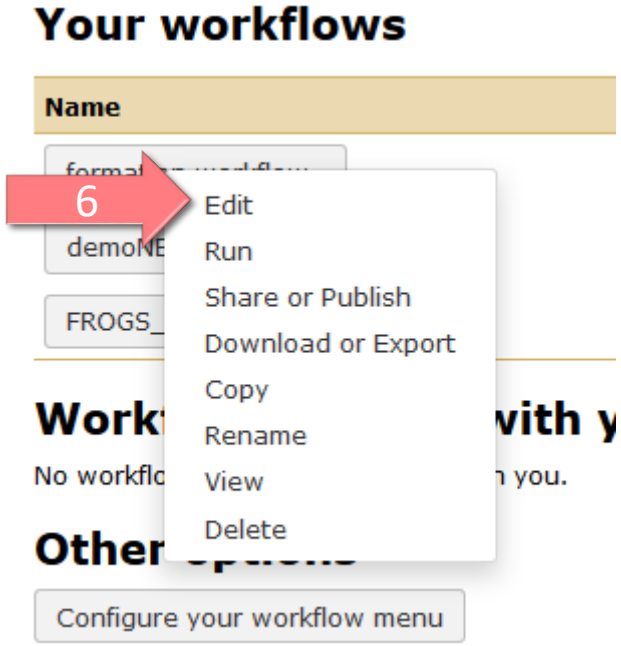

1
2
Galaxy Sigenae - Welcome gpascal
Analyze Data
Workflow
Shared Data
Visualization
Help
User
Using 18.3 GB
Your workflows
Create new workflow
Upload or import workflow
Name
# of Steps
formation workflow
9
demoNEM2015 workflow
9
FROGS_v1.0_06_05_2015
10
Workflows shared with you by others
No workflows have been shared with you.
Other options
Configure your workflow menu

# Exercise 10

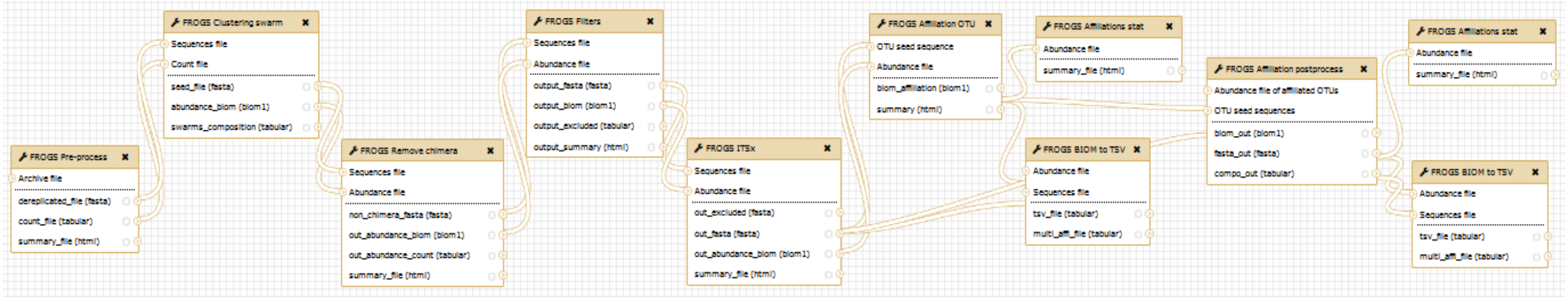

Galaxy Sigenae - Welcome gpascal
Analyze Data
Workflow
Create New Workflow
Workflow Name:
Unnamed workflow
Workflow Annotation:
A description of the workflow; annotation is shown alongside shared or published workflows.
Create
3
4

# Exercise 10

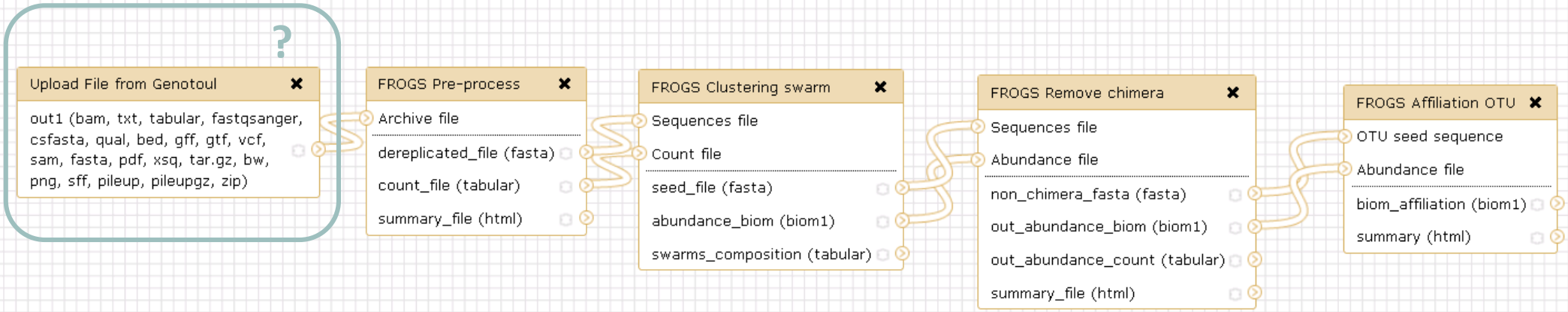

Your workf ws
5
Name
formation workflow
demoNEM2015 workflow
FROGS_v1.0_06_05_2015
Workflows shared with y
No workflows have been shared with you.
Other options
Configure your workflow menu


Your workflows
Name
6
Edit
Run
Share or Publish
Download or Export
Copy
Rename
View
Delete
Configure your workflow menu

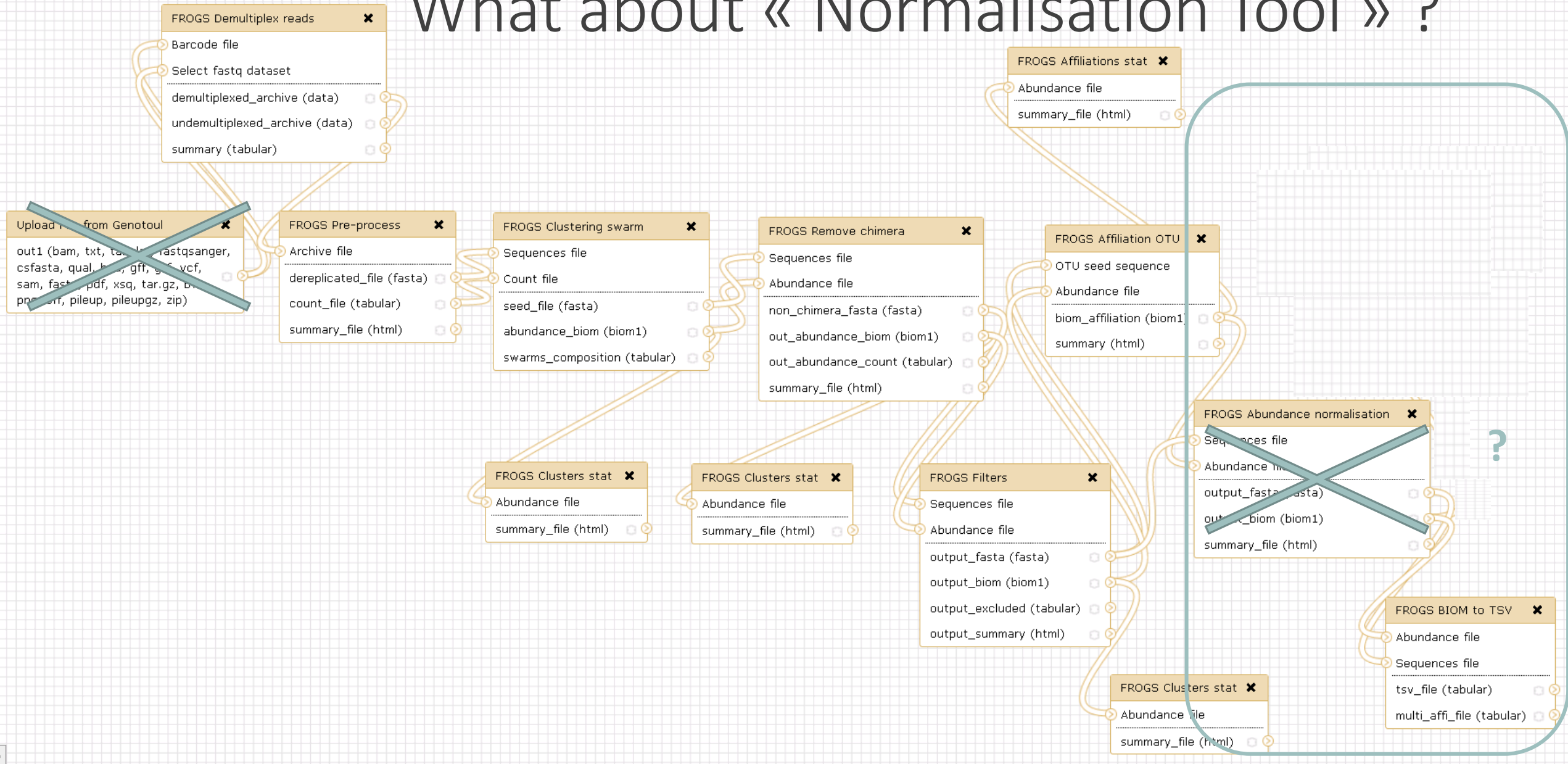
# Solution of exercise:

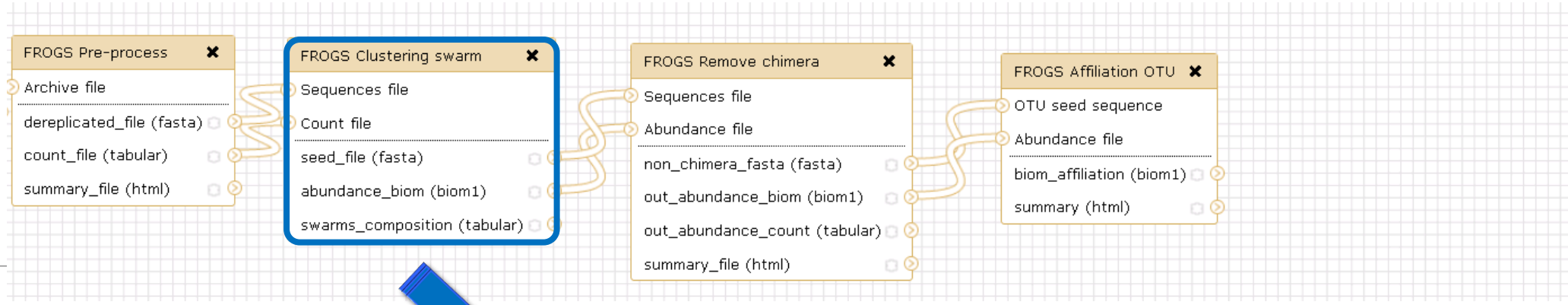


# What about « upload file » ?



# What about « Normalisation Tool » ?

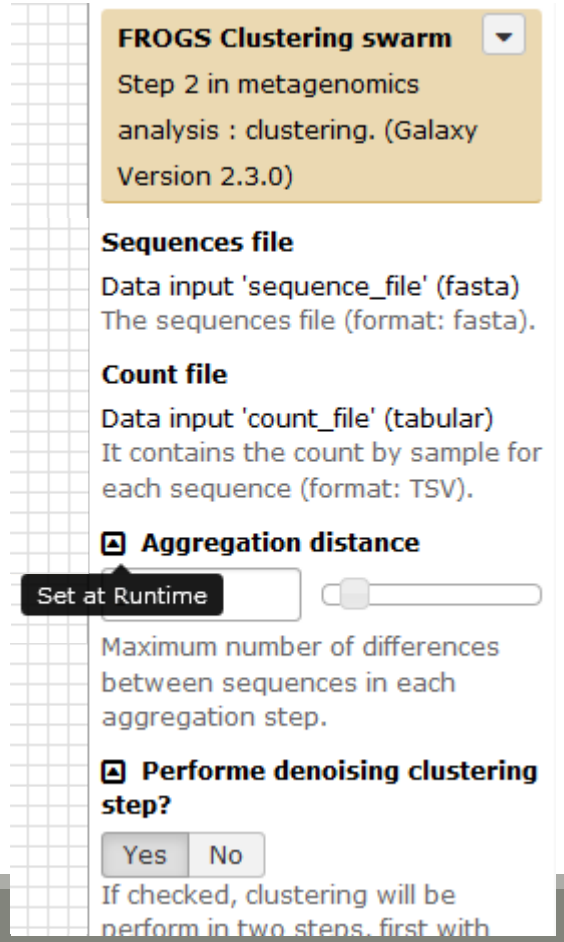





For each tool, think to:

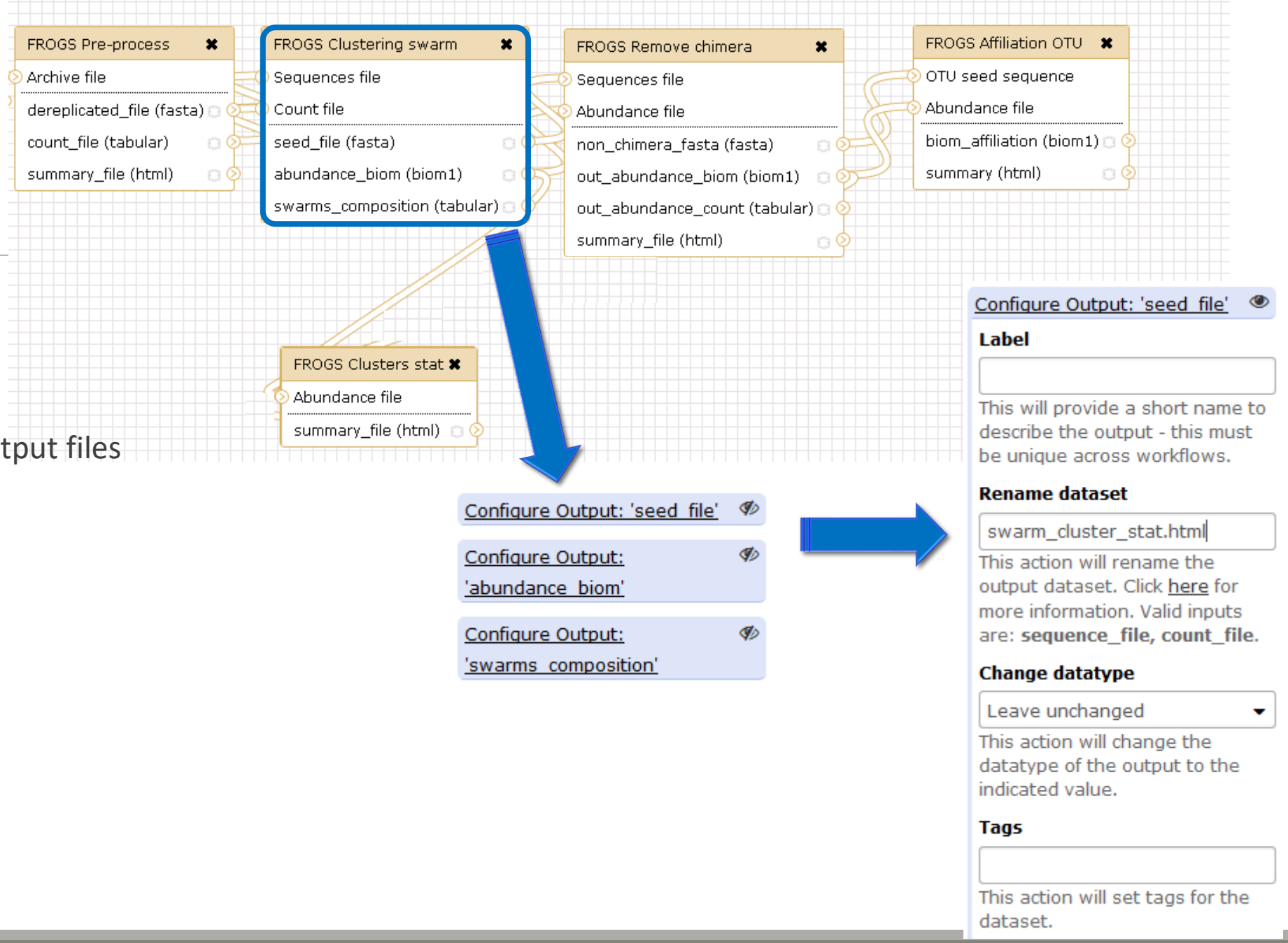
- Fixe parameter ?

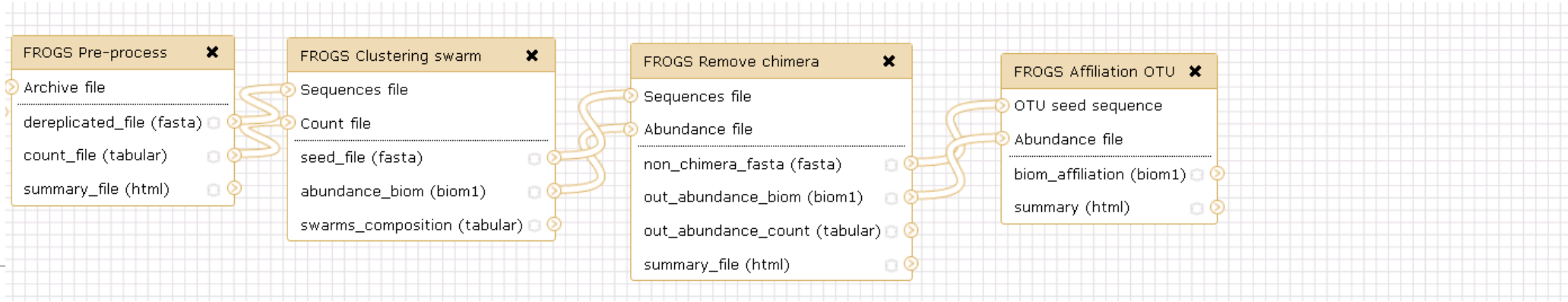
?



For each tool, think to:

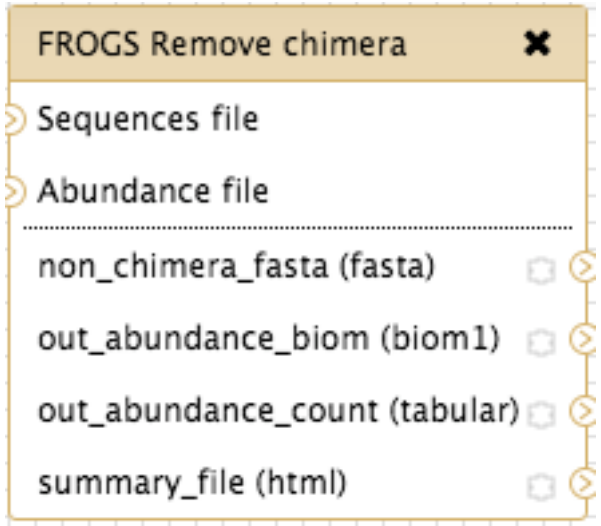
- Fixe parameter ?
- Automatically rename output files

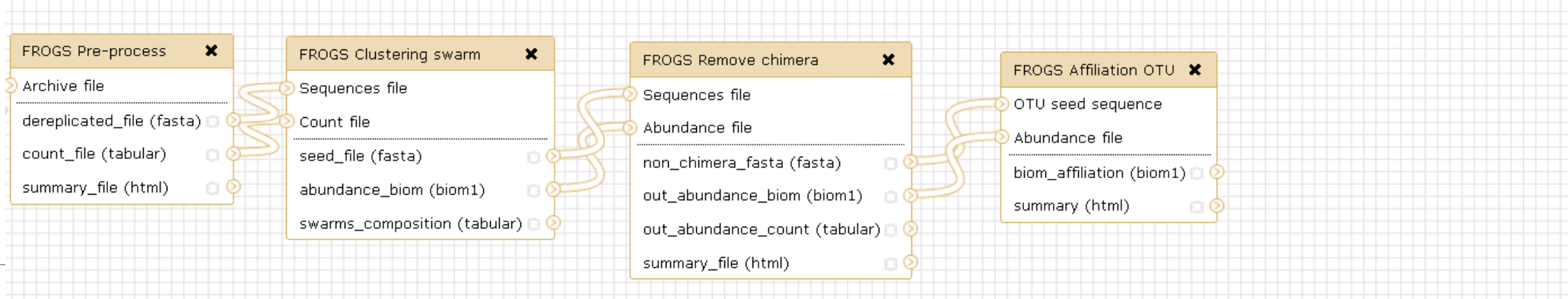





For each tool, think to:

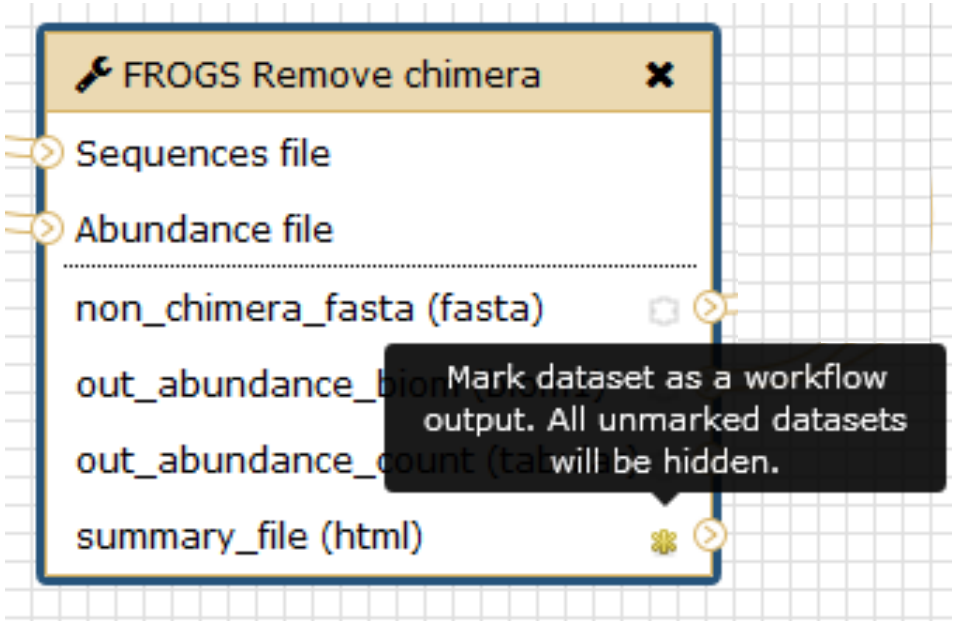
- Fixe parameter ?
- Automatically rename output files
- Hide intermediate files ?

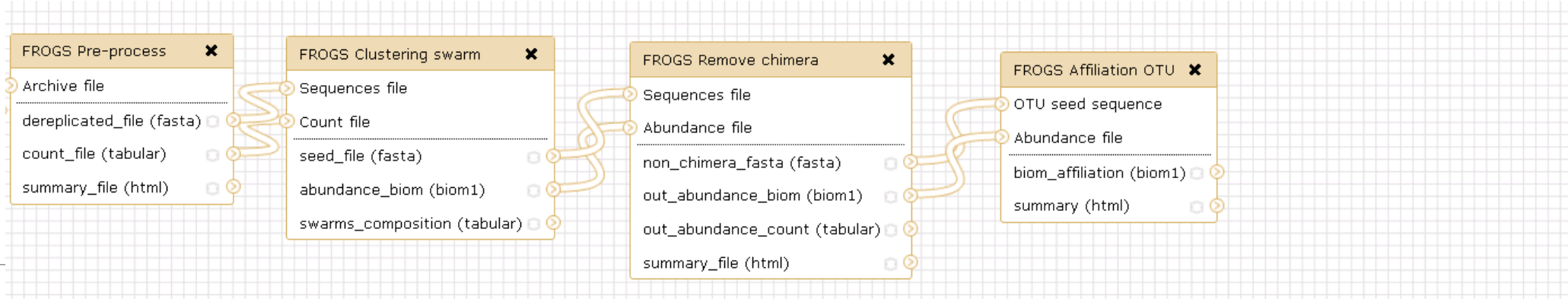



For each tool, think to:

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






For each tool, think to:

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