Training on Galaxy: Metabarcoding

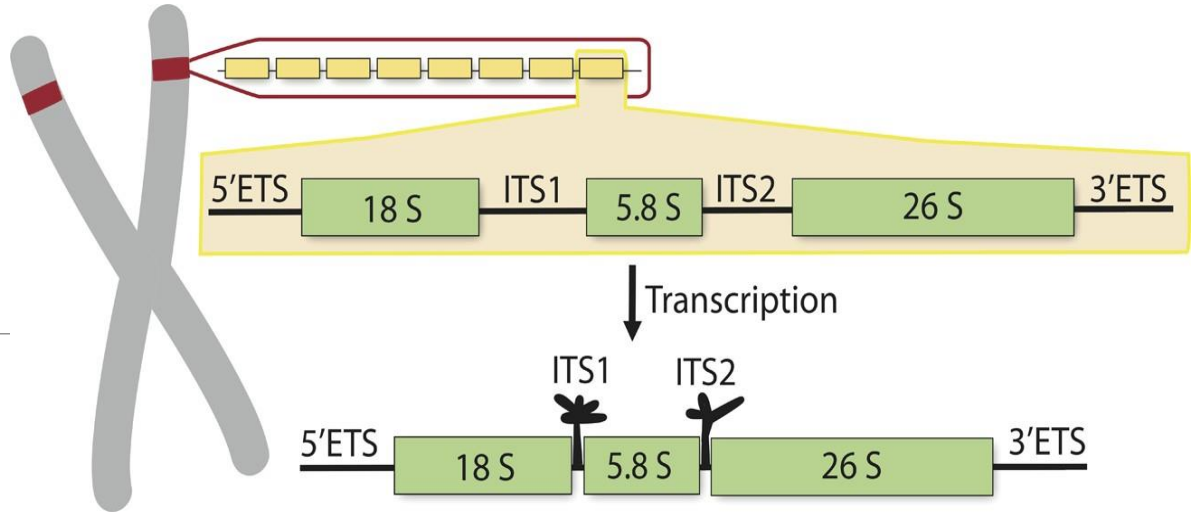
April 2023 - Webinar

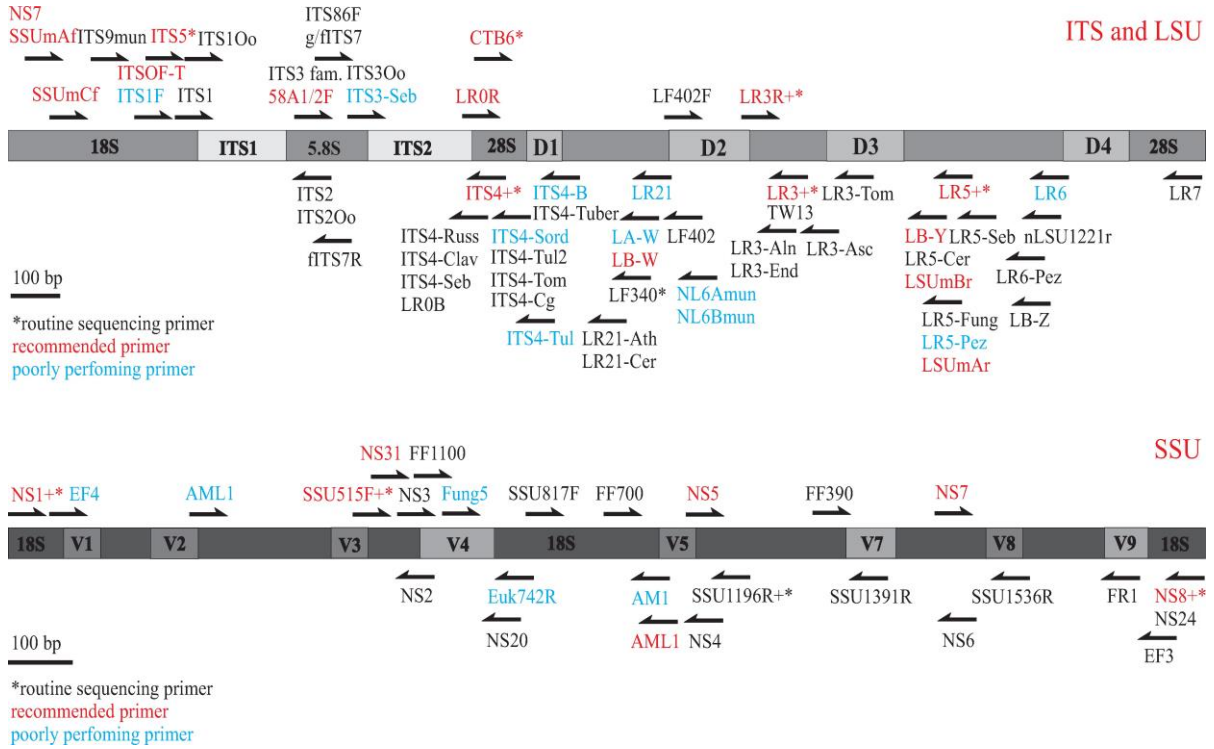
FROGS Practice on ITS data and Workflow creation

LUCAS AUER, MARIA BERNARD, LAURENT CAUQUIL, MAHENDRA MARIADASSOU, GÉRALDINE PASCAL & OLIVIER RUÉ

What is a ITS ?

ITS: Internal Transcribed Spacer





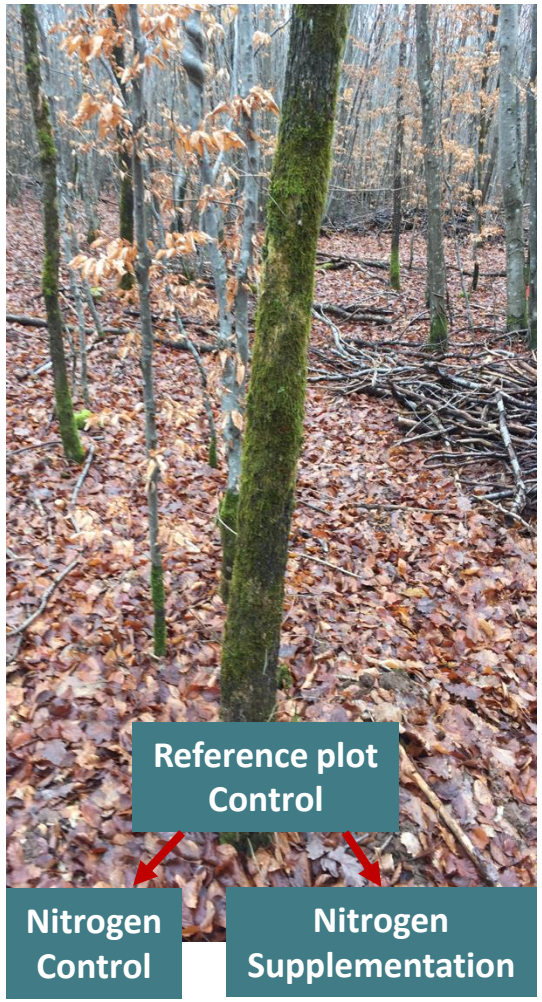
What is a ITS ?

- Size polymorphism of ITS (from 361 to 1475 bases in UNITE 7.1)
- Highly conserved regions of the neighboring of ITS1 and ITS2
- Lack of a generalist and abundant ITS databank (several small specialized databanks)
- Multiple copies* (14 to 1400 copies (mean at 113, median at 80))
- Do not target Glomeromycetes/Glomeromycota (→ alternative: 18S)

- If your sequencing platform preprocesses your data, it has to keep short and long sequences

* https://doi.org/10.1111/mec.14995

ITS data from manipulated organic soil (MOS network)



While in the past forest biomass exports concerned only trunks, these exports recently increased and now concern also the branches and smaller parts that were previously left on the ground (for pellet production).

The MOS network (18 sites in France) was designed to reveal the long-term effects of intense biomass exports on soil fertility and biodiversity. Different treatment of biomass export are applied with or without supplementation of nutrients.

The aim is to analyse the **impact** of these new forestry practices on **soil microbiota** and **tree health**.

The present dataset concerned one of the site (Champenoux) after 5 years of total Organic Matter removal (OMR treatment : all the organic matter on the ground including leaves was removed), **with** our **without nitrogen supplementation**.

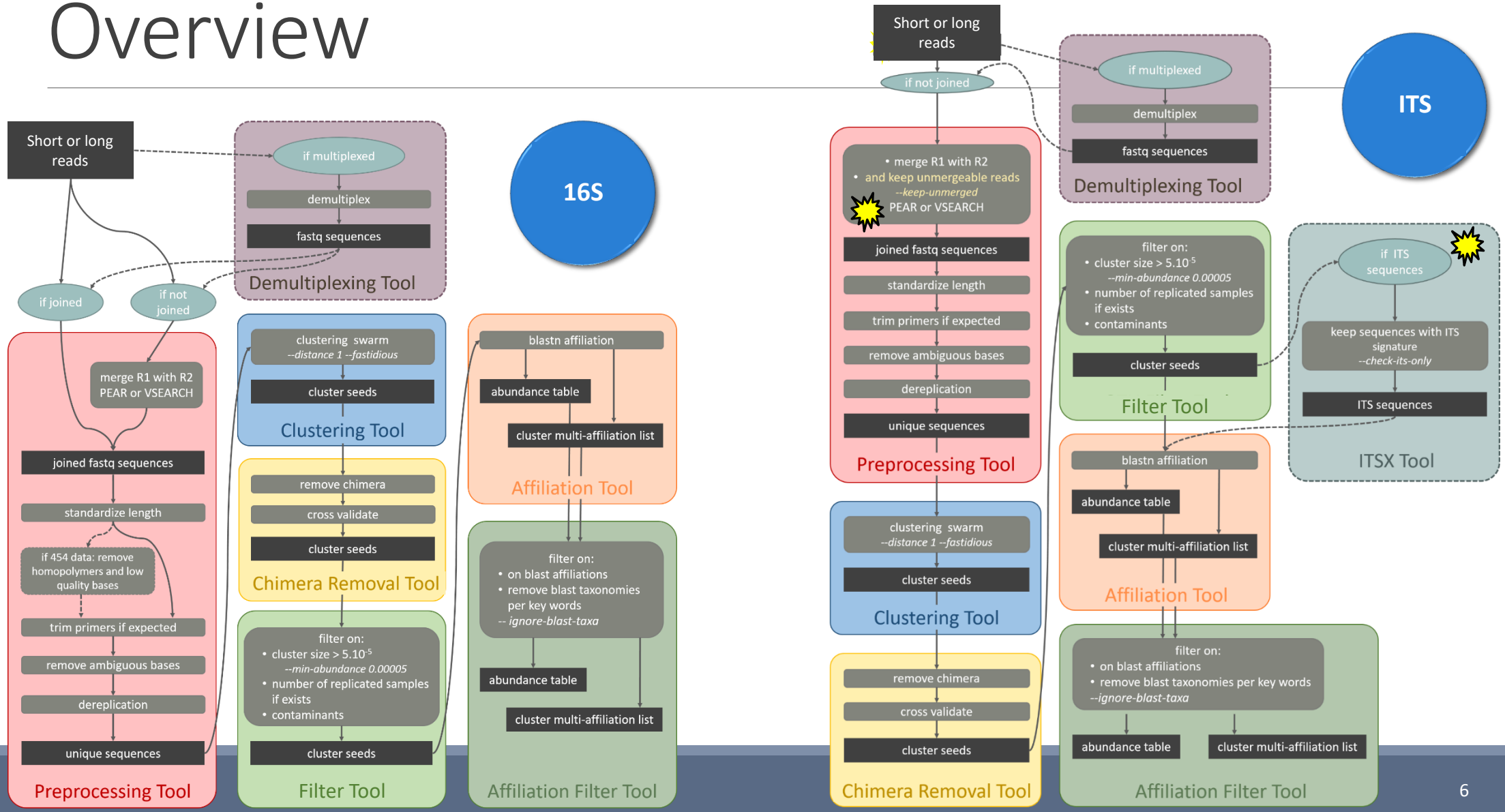
- 5 replicates Control x 2 treatments, 5 replicates OMR x 2 treatments
- DNA is extracted and **ITS1** is sequenced
- 2 x 250 bp Illumina MiSeq
- Primer 5’: CTTGGTCATTTAGAGGAAGTAA
- Primer 3’: GCATCGATGAAGAACGCAGC

Metadata for these samples

Samples	kept	Replicas	Incubation	Nitrogen	Forest_management	Quality	Treatment
Ph203	79.76	3	T4	Nitrogen_supplementation	Control	Low degradability	Control_with_N
Ph212	77.64	2	T4	Nitrogen_supplementation	Control	Low degradability	Control_with_N
Ph217	80.26	5	T4	Nitrogen_supplementation	Control	Low degradability	Control_with_N
Ph222	78.65	1	T4	Nitrogen_supplementation	Control	Low degradability	Control_with_N
Ph224	77.18	4	T4	Nitrogen_supplementation	Control	Low degradability	Control_with_N
Ph237	79.68	1	T4	Control	Control	Low degradability	Control
Ph241	78.7	2	T4	Control	Control	Low degradability	Control
Ph243	76.38	4	T4	Control	Control	Low degradability	Control
Ph246	76.37	5	T4	Control	Control	Low degradability	Control
Ph250	77.37	3	T4	Control	Control	Low degradability	Control
Ph407	72.52	3	T4	Nitrogen_supplementation	OMR	Low degradability	OMR_with_N
Ph414	64.98	4	T4	Nitrogen_supplementation	OMR	Low degradability	OMR_with_N
Ph415	78.13	2	T4	Nitrogen_supplementation	OMR	Low degradability	OMR_with_N
Ph417	71.17	1	T4	Nitrogen_supplementation	OMR	Low degradability	OMR_with_N
Ph423	75.2	5	T4	Nitrogen_supplementation	OMR	Low degradability	OMR_with_N
Ph428	73.48	2	T4	Control	OMR	Low degradability	OMR
Ph433	73.21	5	T4	Control	OMR	Low degradability	OMR
Ph434	74.01	3	T4	Control	OMR	Low degradability	OMR
Ph439	74.15	1	T4	Control	OMR	Low degradability	OMR
Ph449	73.77	4	T4	Control	OMR	Low degradability	OMR

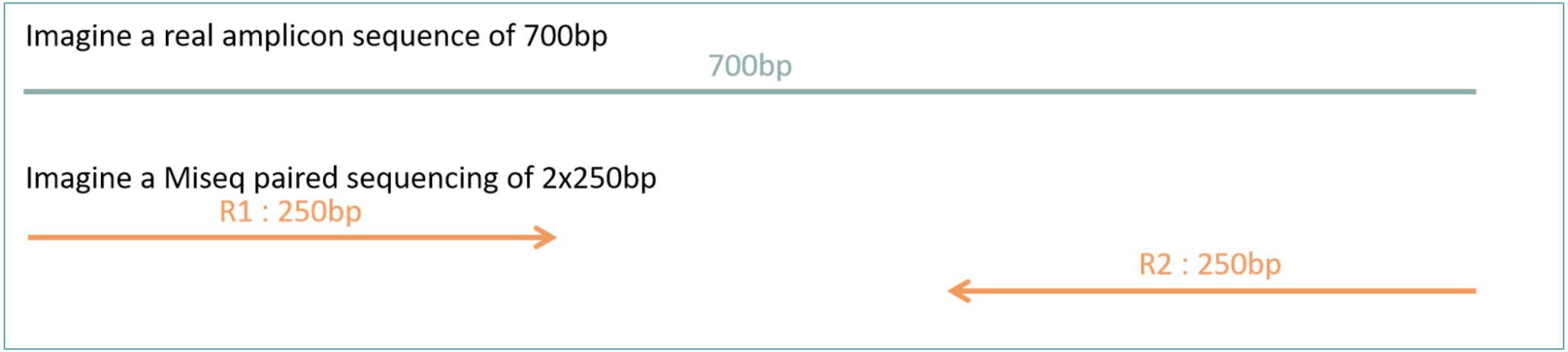
: Differences between 16S and ITS pipelines

Overview



FROGS Pipeline for ITS

Problematic:
some ITS reads (Miseq sequencing) are non-overlapping sequences



Consequence: during bioinformatics process, these reads are lost and underlying organisms will be never represented in the abundance table.

Solution: in preprocess step – creation of “FROGS combined” 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

FROGS_0 Demultiplex reads Attribute reads to samples in function of inner barcode

FROGS_1 Pre-process merging, denoising and dereplication

FROGS_2 Clustering swarm Single-linkage clustering on sequences

FROGS_Cluster_Stat Process some metrics on clusters

FROGS_3 Remove chimera Remove PCR chimera in each sample

FROGS_4 Cluster filters Filters clusters on several criteria.

FROGS ITSx Extract the highly variable ITS1 and ITS2 subregions from ITS sequences

FROGS_5 Taxonomic affiliation Taxonomic affiliation of each ASV's seed by RDPtools and BLAST

FROGS_6_Affiliation_Stat Process some metrics on taxonomies

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FROGSFUNC_2_functions Calculates functions abundances in each sample.

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Functional inference tools

FROGS BIOM to std BIOM Converts a FROGS BIOM in fully compatible BIOM

FROGS TSV_to_BIOM Converts a TSV file in a BIOM file 1

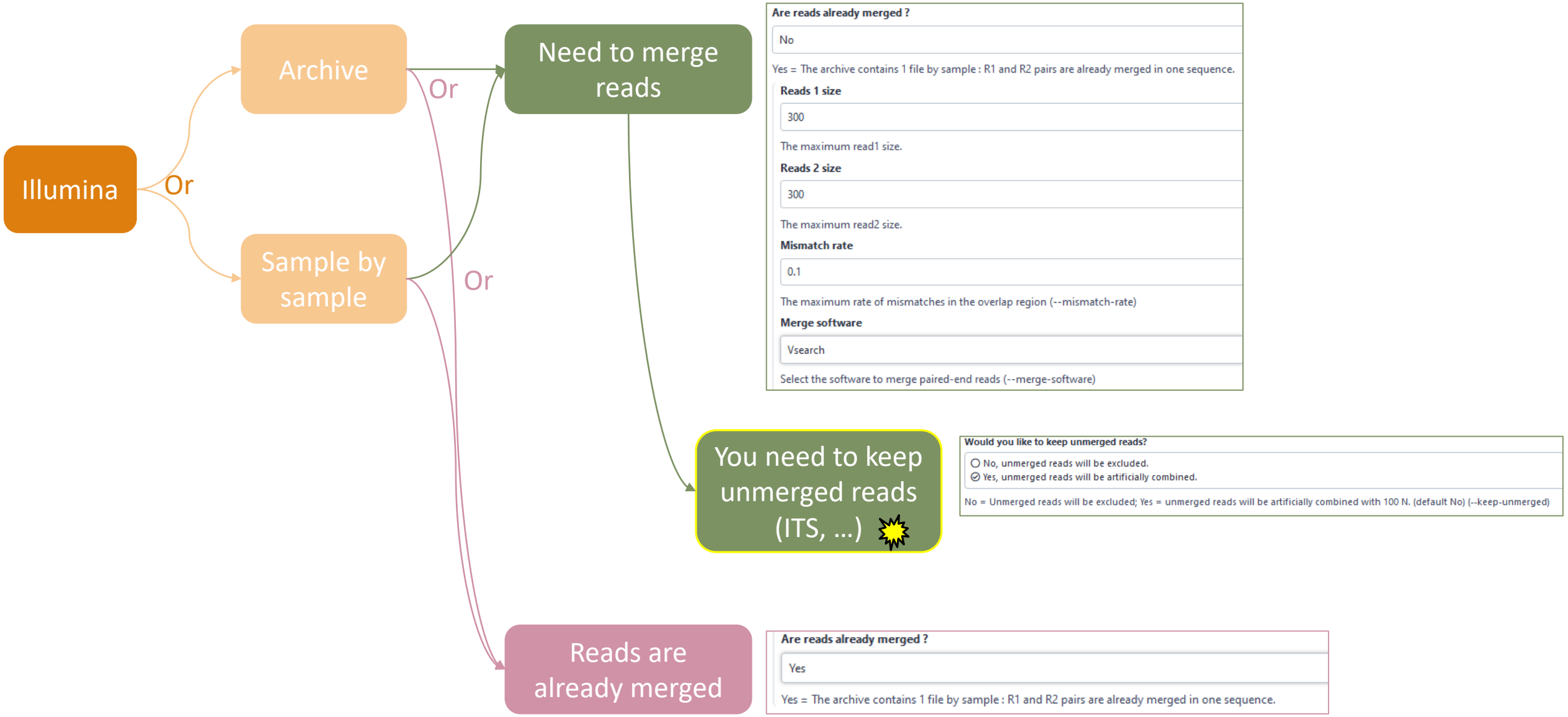
FROGS BIOM to TSV Converts a BIOM file in TSV file

Utilities tools

28 tools in total

Pre-process tool

For short reads from illumina



ITS

FROGS_1 Pre-process merging, denoising and dereplication (Galaxy Version 4.1.0+galaxy1)

Sequencer

Illumina

Select the sequencing technology used to produce the sequences.

Input type

TAR Archive

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

TAR archive file

1: ITS_fast.tar.gz

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

Are reads already merged ?

No

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

Reads 1 size

250

The maximum read1 size.

Reads 2 size

250

The maximum read2 size.

Mismatch rate

0.1

The maximum rate of mismatches in the overlap region (--mismatch-rate)

Merge software

Vsearch

Select the software to merge paired-end reads (--merge-software)

Would you like to keep unmerged reads?

- ○ No, unmerged reads will be excluded.
- ⊘ Yes, unmerged reads will be artificially combined.

To keep FROGS combined sequences, choose YES

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

ITS

Minimum amplicon size

180

The minimum size of the amplicons (with primers) (--min-amplicon-size)

Maximum amplicon size

490

The maximum size of the amplicons (with primers) (--max-amplicon-size)

Do the sequences have PCR primers?

- (✓) Yes
- ( ) No

5' primer

CTTGGTCATTTAGAGGAAGTAA

The 5' primer sequence (wildcards are accepted). This primer must be written in 5' to 3' orientation (see details in 'Primers parameters' help section) (--five-prim-primer)

3' primer

GCATCGATGAAGAACGCAGC

The 3' primer sequence (wildcards are accepted). This primer must be written in 5' to 3' orientation (see details in 'Primers parameters' help section) (--three-prim-primer)

Primer 5’: CTTGGTCATTTAGAGGAAGTAA
Primer 3’: GCATCGATGAAGAACGCAGC

Exercise

Go to « ITS » history

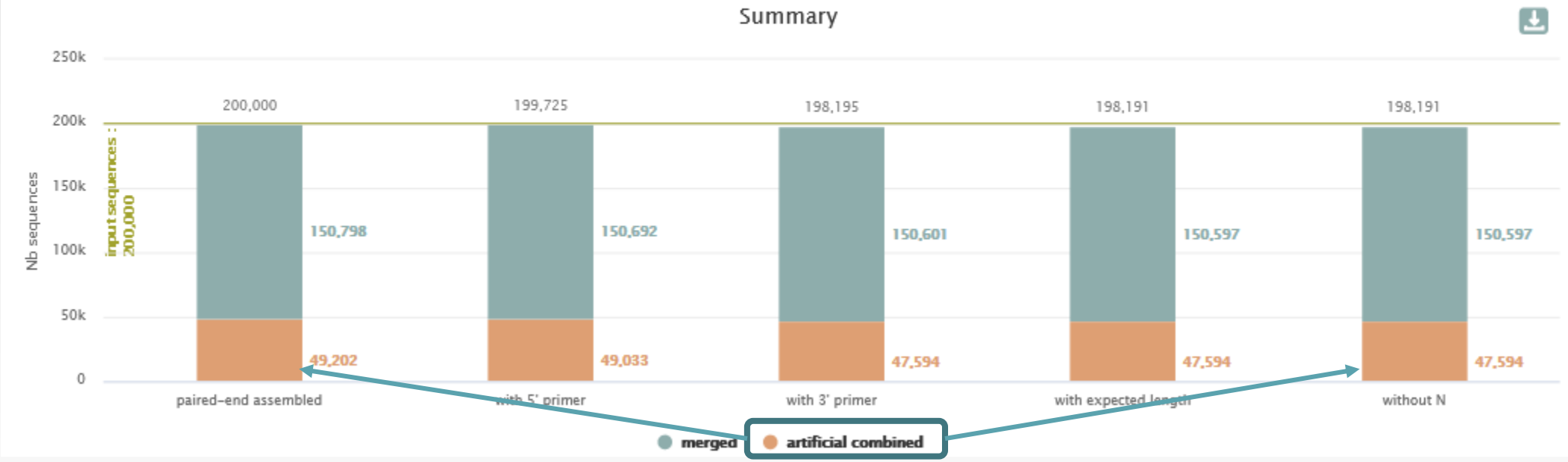
Launch the pre-process tool on this data set

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

ITS

Explore Preprocess report.html

Preprocess summary



ITS

2 tables:

Details on merged sequences

CSV

Show 10 entries Search:

Samples	before process	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
Ph203	10,000	79.42	7,954	7,948	7,942	7,942	7,942
Ph212	10,000	78.28	7,837	7,832	7,828	7,828	7,828
Ph217	10,000	80.48	8,061	8,052	8,048	8,048	8,048
Ph222	10,000	78.34	7,839	7,835	7,834	7,834	7,834

Details on artificial combined sequences

CSV

Show 10 entries Search:

Samples	before process	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
Ph203	10,000	19.68	2,046	2,038	1,968	1,968	1,968
Ph212	10,000	20.65	2,163	2,154	2,065	2,065	2,065
Ph217	10,000	18.63	1,939	1,928	1,863	1,863	1,863
Ph222	10,000	20.79	2,161	2,155	2,079	2,079	2,079

Own tag for combined sequences

```
>M01328:521:000000000-KRPTR:1:1103:15714:11240;size=6 1:N:0:238
AAGTCGTAACAAGGTAACCGTAGGTGAACCTGCGGTTGGATCATTAAAAATTTATGAGTTTCCGTTGAG
>M01328:521:000000000-KRPTR:1:2102:7650:15129;size=1 1:N:0:239
AAGTCGTAACAAGGTAACCGTAGGTGAACCTGCGGTTGGATCATTAAAAATTTATGAGTTTCCGTTGAG
>M01328:521:000000000-KRPTR:1:1112:8680:15899;size=1 1:N:0:202
>M01328:521:000000000-KRPTR:1:1111:21036:16514_FROGS_combined;size=1
>M01328:521:000000000-KRPTR:1:1106:19343:17084_FROGS_combined;size=1
```

Filter only on minimum length for « combined ».

Minimum length =
R1 + 100N + R2 – primers sizes

If the primers are very internal to the read, after trimming them, the combined sequence could be smaller than a read. FROGS rejects these cases.

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Utilities tools

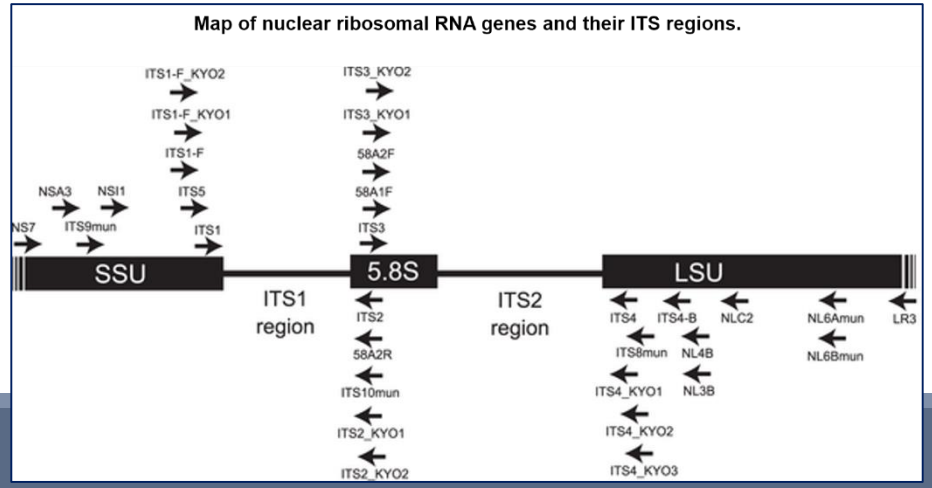
28 tools in total

ITSx tools

What is the purpose of the ITSx tool?

- ITSx is a tool to **filter** sequences.
- ITSx **identifies** and **trimms** ITS regions in sequences.
- It **excludes** the highly conserved neighboring sequences **SSU**, **5S** and **LSU** rRNA.
- 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).



Bengtsson-Palme, J., et al. (2013), Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol Evol, 4: 914-919. https://doi.org/10.1111/2041-210X.12073

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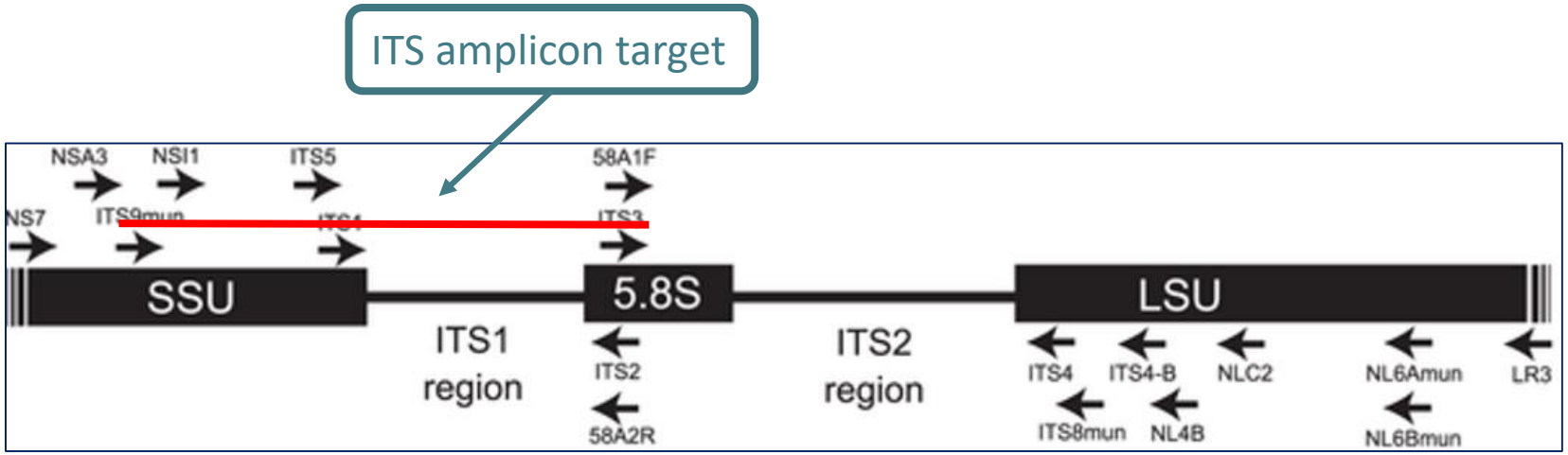
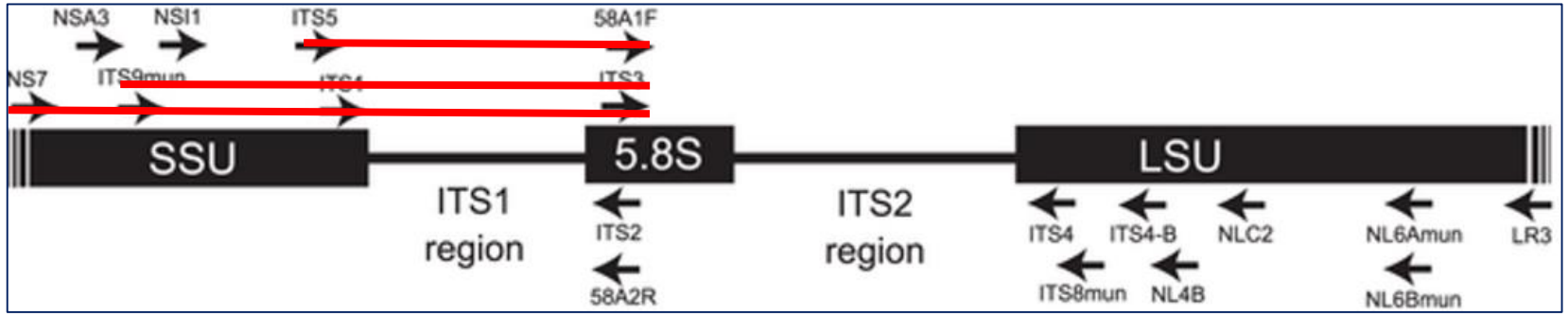


1st case: choose to trim
ITS1 is well detected
SSU part and 5.8S part are trimmed
Result: ——

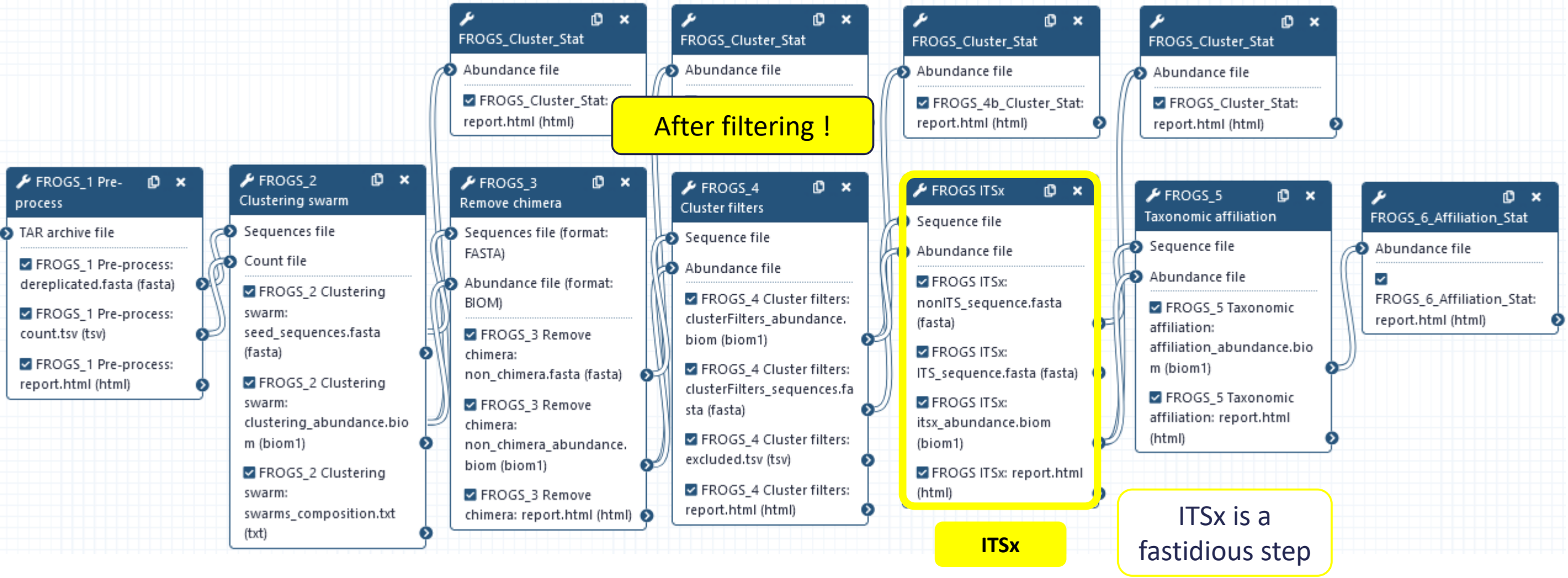
2nd case: choose to check only
ITS1 is well detected
SSU part and 5.8S part are not trimmed
Result: ——

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

- If not, only ITS1 or ITS2 part will be conserved
- This is interesting to keep only the ITS parts without the flanking sequences in case of :
  - comparison of sequenced amplicons with different primers targeting the same region to be amplified.
  - using a database with only ITS part



When should we use ITSx ?



FROGS ITSx Extract the highly variable ITS1 and ITS2 subregions from ITS sequences (Galaxy Version 4.1.0+galaxy1)

Sequence file

16: FROGS_4 Cluster filters: clusterFilters_sequences.fasta

The sequence file to filter (format: FASTA).

Abundance file

15: FROGS_4 Cluster filters: clusterFilters_abundance.biom

The abundance file to filter (format: BIOM)

Trim conserved sequence (SSU, 5.8S, LSU) ?

- (x) No, keep conserved regions
- ( ) Yes, trim conserved regions

If Yes, only part of the sequences with ITS signature will be kept, SSU, LSU or 5.8S regions will be trimmed (default : No) (--check-its-only)

By default, the ITSs are kept in their entirety.

Choose pertinent organisms to scan:

- [-] Select/Unselect all

- [x] Fungi
- [ ] Alveolata
- [ ] Bryophyta
- [ ] Bacillariophyta
- [ ] Amoebozoa
- [ ] Euglenozoa
- [ ] Chlorophyta
- [ ] Rhodophyta
- [ ] Phaeophyceae
- [ ] Marchantiophyta
- [ ] Metazoa
- [ ] Oomycota
- [ ] Haptophyceae
- [ ] Raphidophyceae
- [ ] Rhizaria
- [ ] Synurophyceae
- [ ] Tracheophyta
- [ ] Eustigmatophyceae

By defaut, sequences are considered as FUNGI sequences.
Change it, if it is not the case.

Save a lot of time by checking pertinent organism group model to scan (--organism-groups)

Email notification

No

Send an email notification when the job completes.

✔ Execute

Careful !

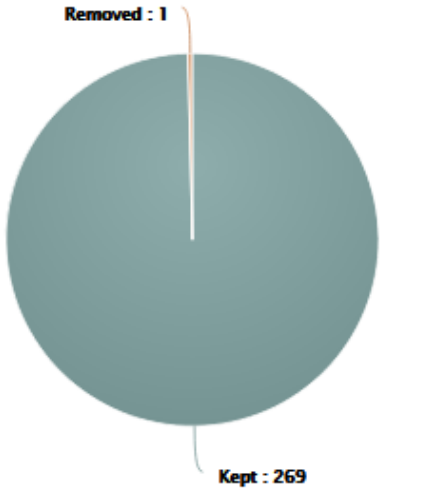
- The ITSx step is time consuming and has to be done on minimum of clusters.

1. Preprocess step,
2. Clustering step,
3. Chimera removing step,
4. Filter on ASVs abundances and replicates step,
5. ITSx

Report.html, ITSX output

Filters (ITSx) summary

ASVs



Abundance

Filters (ITSx) by samples

CSV

Show 10 entries

Search:

ASVs removed by sample

Sample name	Initial	Kept	Initial abundance	Kept abundance
Ph203	105	105	7,065	7,065
Ph212	65	65	7,474	7,474
Ph217	89	89	5,990	5,990

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Not specific for ITS but often useful

rts a FROGS BIOM in fully compatible BIOM

TSV file in a BIOM file 1

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Utilities tools

28 tools in total

Affiliation Post-process

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

This tool allows **grouping ASVs 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 ?

<u>In consequence:</u>

The different affiliations involved in multi-affiliation are merged.

The abundances are added together.

It is the most abundant ASV seed that is kept.

FROGS Affiliation postprocess Aggregates ASVs based on alignment metrics (Galaxy Version 4.1.0+galaxy1)

☆ Favorite | Versions | ▾ Options

Sequence file

21: FROGS ITSx: ITS_sequence.fasta

The sequence file to filter (format: FASTA).

Abundance file

25: FROGS_5 Taxonomic affiliation: affiliation_abundance.biom

The abundance file to filter (format: BIOM)

Is this an amplicon hyper variable in length?

○ No
⊘ Yes

Yes, we have combined sequences

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

Using reference database

UNITE 8.2 ITS1

same database used for taxonomic affiliation

Select reference from the list (--reference)

Minimum identity for aggregation

99

ASVs will be aggregated if they share the same taxonomy with at least X% identity (--identity)

Minimum coverage for aggregation

99

ASVs will be aggregated if they share the same taxonomy with at least X% alignment coverage (--coverage)

Here, we wanted to aggregate ASVs only if they are very closed

Email notification

No

Send an email notification when the job completes.

✔ Execute

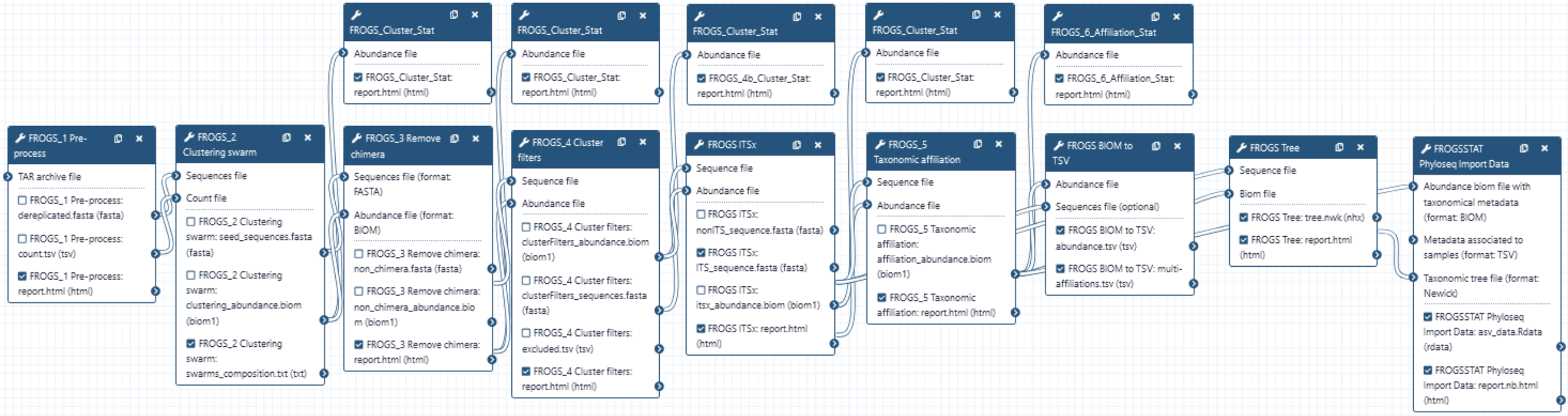
FROGS Affiliation postprocess: OTU_aggregation_composition.txt

FROGS Affiliation postprocess: sequences.fasta

FROGS Affiliation postprocess: affiliation_abundance.biom

Cluster_1
Cluster_2
Cluster_8
Cluster_3
Cluster_5
Cluster_4
Cluster_6
Cluster_7
Cluster_9
Cluster_13
Cluster_10
Cluster_11
Cluster_16
Cluster_17
Cluster_14
Cluster_12
Cluster_15
Cluster_22
Cluster_18
Cluster_23
Cluster_25
Cluster_19
Cluster_21
Cluster_26
Cluster_29
Cluster_34
Cluster_35
Cluster_28
Cluster_31
Cluster_32
Cluster_42
Cluster_33
Cluster_75_FROGS_combined Cluster_121_FROGS_combined Cluster_137_FROGS_combined Cluster_144_FROGS_combi

Workflow creation



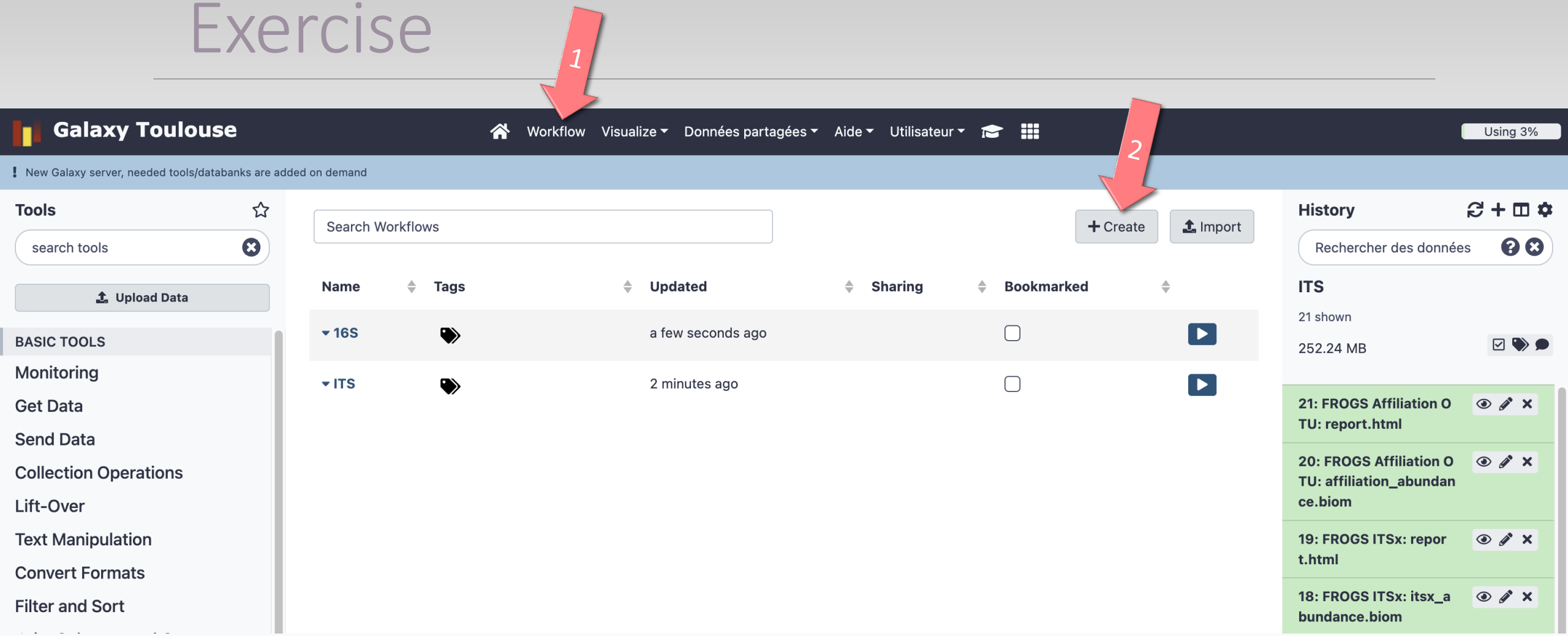
Workflow are useful for routine analyses

A workflow links FROGS steps together and when it is launched, all the steps run automatically.

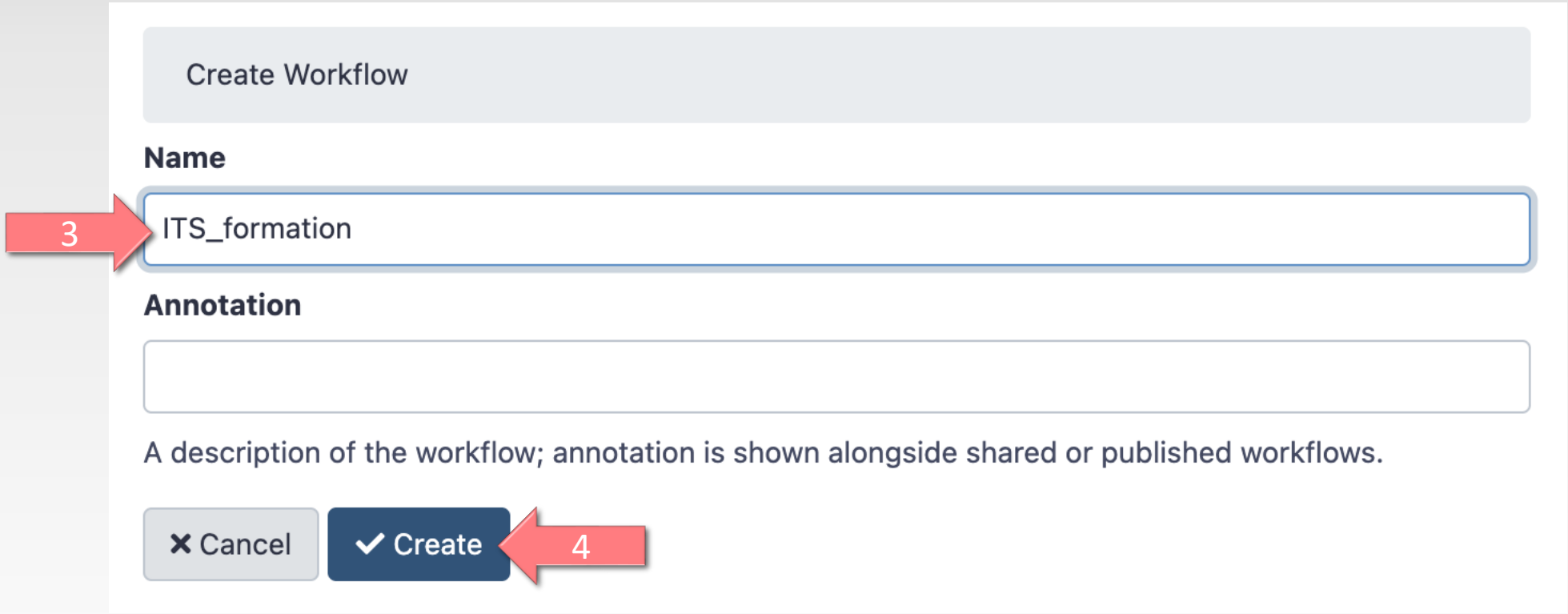
Practice

CREATE YOUR OWN WORKFLOW !

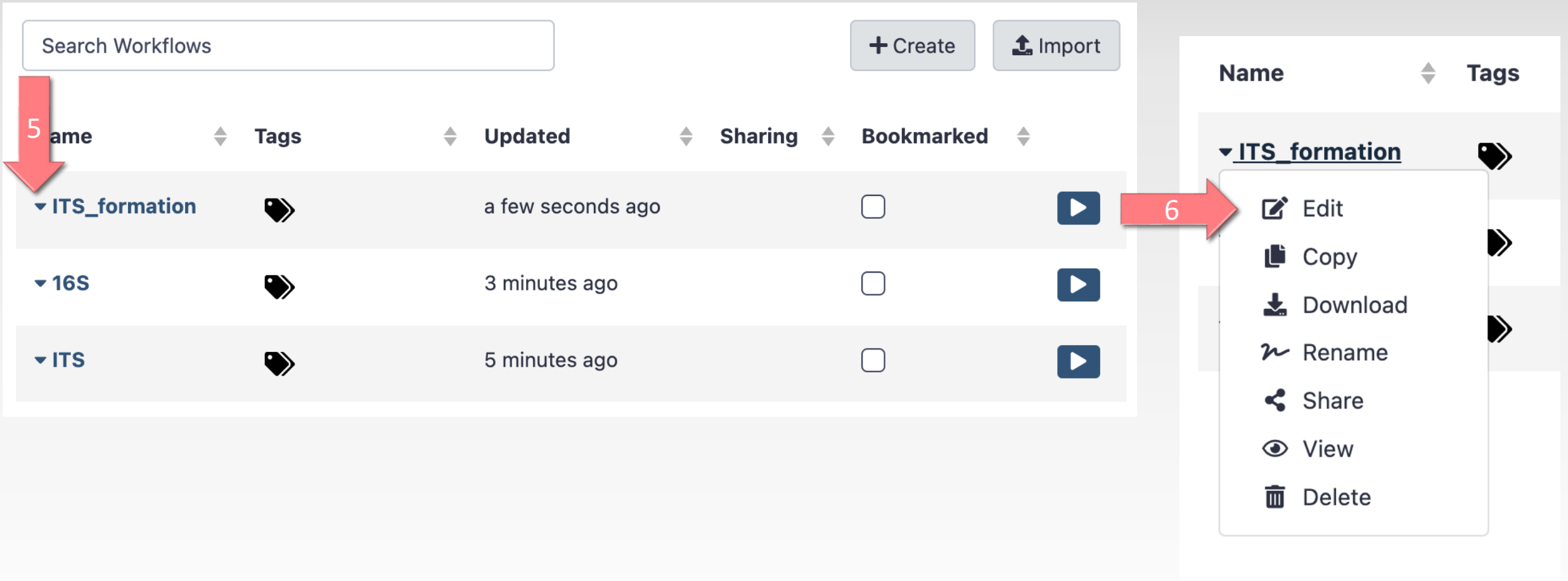
Exercise


1
2
Galaxy Toulouse
Workflow
Visualize
Données partagées
Aide
Utilisateur
Using 3%
New Galaxy server, needed tools/databanks are added on demand
Tools
search tools
Upload Data
BASIC TOOLS
Monitoring
Get Data
Send Data
Collection Operations
Lift-Over
Text Manipulation
Convert Formats
Filter and Sort
Search Workflows
+ Create
Import
Name
Tags
Updated
Sharing
Bookmarked
16S
a few seconds ago
ITS
2 minutes ago
History
Rechercher des données
ITS
21 shown
252.24 MB
21: FROGS Affiliation OTU: report.html
20: FROGS Affiliation OTU: affiliation_abundance.biom
19: FROGS ITSx: report.html
18: FROGS ITSx: itsx_abundance.biom

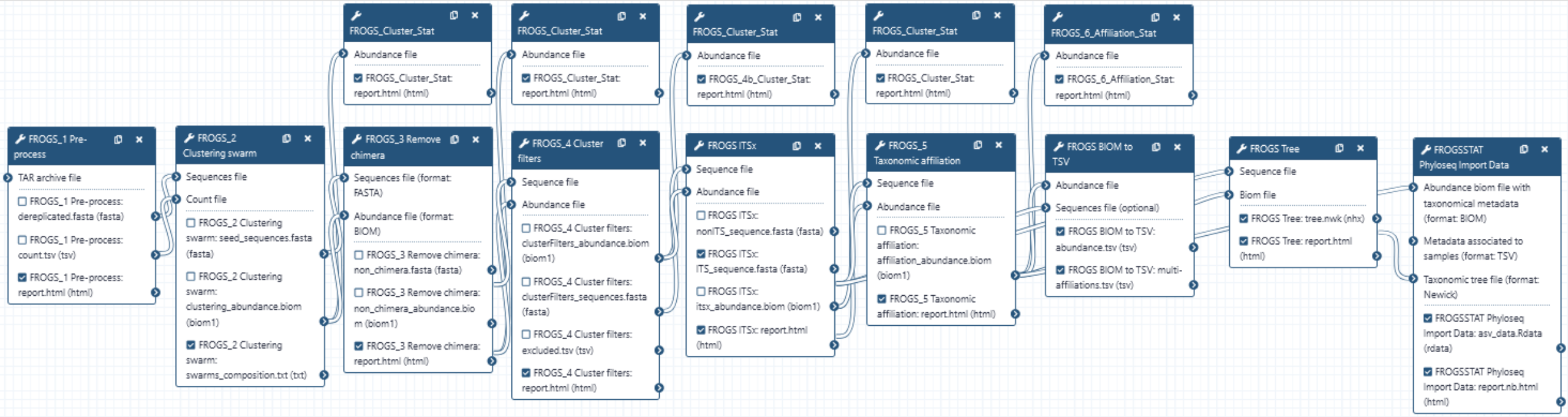
Exercise

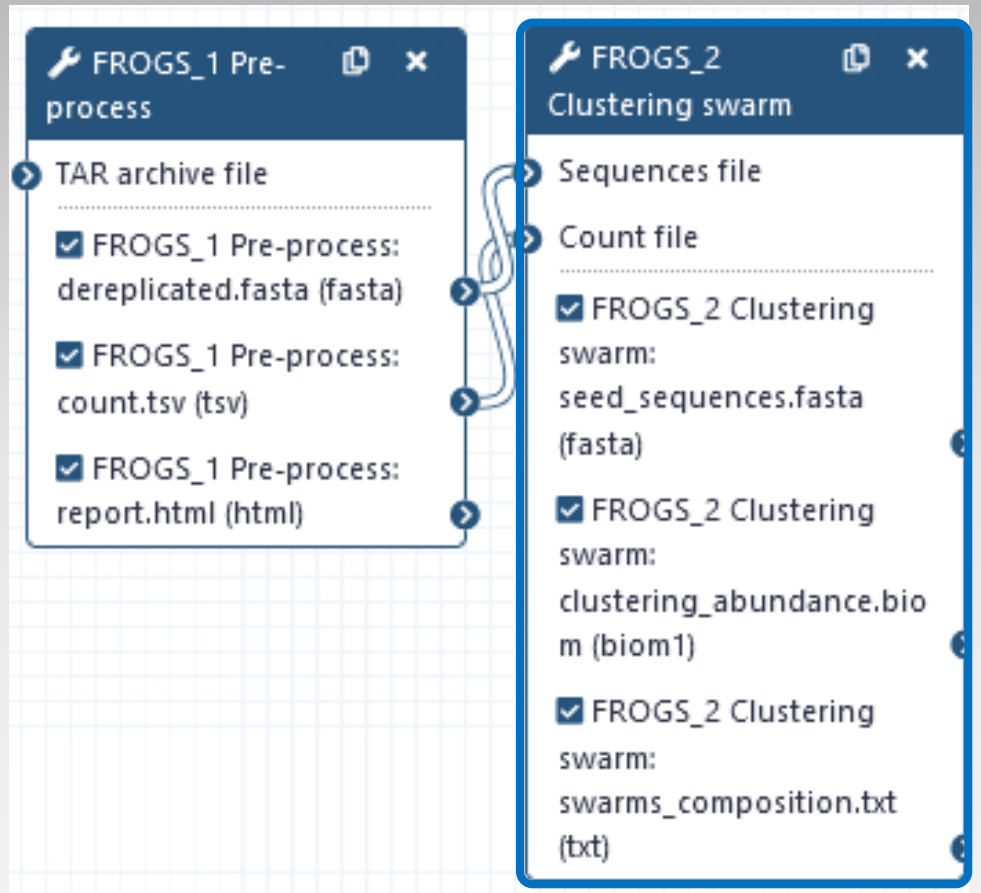

Create Workflow
Name
3
ITS_formation
Annotation
A description of the workflow; annotation is shown alongside shared or published workflows.
Cancel
Create
4

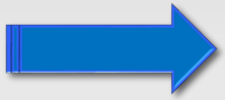
Exercise

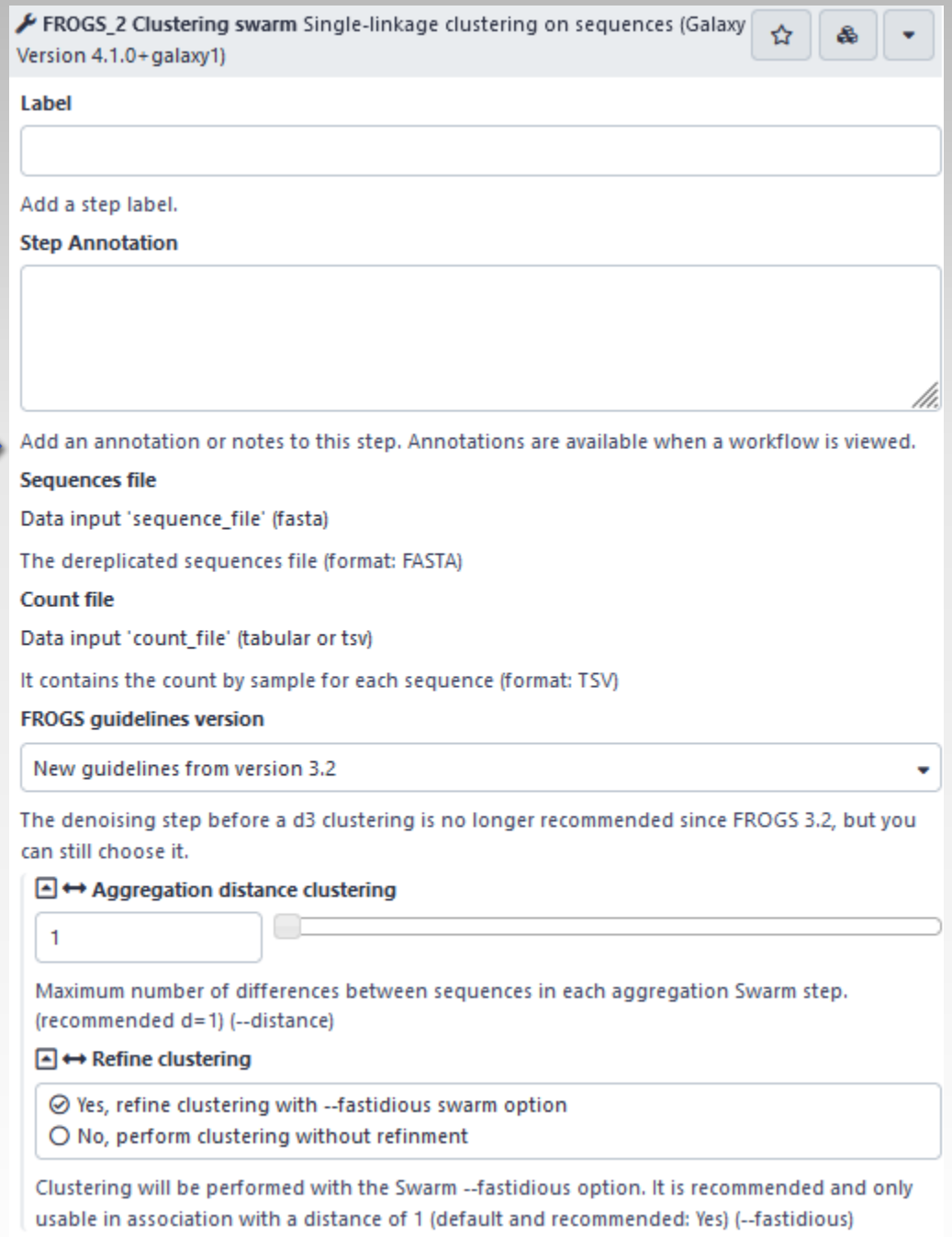

Search Workflows
+ Create
Import
5
ame
Tags
Updated
Sharing
Bookmarked
ITS_formation
a few seconds ago
16S
3 minutes ago
ITS
5 minutes ago
6
Name
Tags
ITS_formation
Edit
Copy
Download
Rename
Share
View
Delete

Solution of exercise:



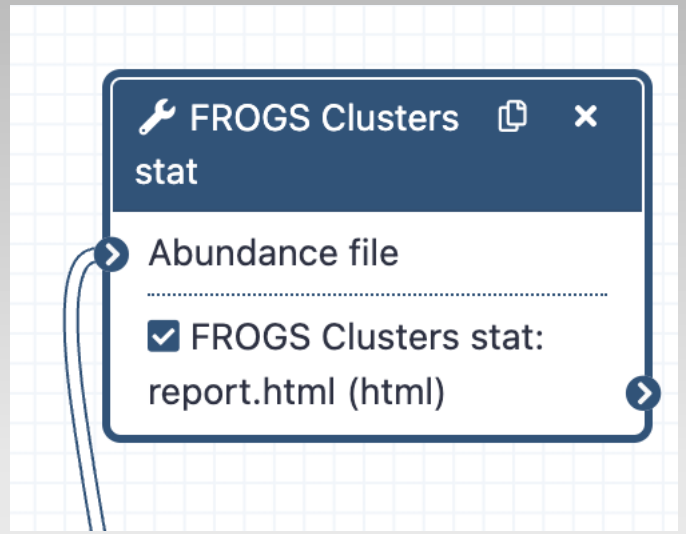






For each tool, think to:

1. Set parameters



Configure Output: 'FROGS Clusters stat: report.html'

Configure Output: 'FROGS Clusters stat: report.html'

Label

Don't use label

This will provide a short name to describe the output - this must be unique across workflows.

Rename dataset

This action will rename the output dataset. Click here for more information. Valid input variables are:

- **biom** (Abundance file)

Change datatype

Leave unchanged

This action will change the datatype of the output to the indicated datatype.

Add Tags

This action will set tags for the dataset.

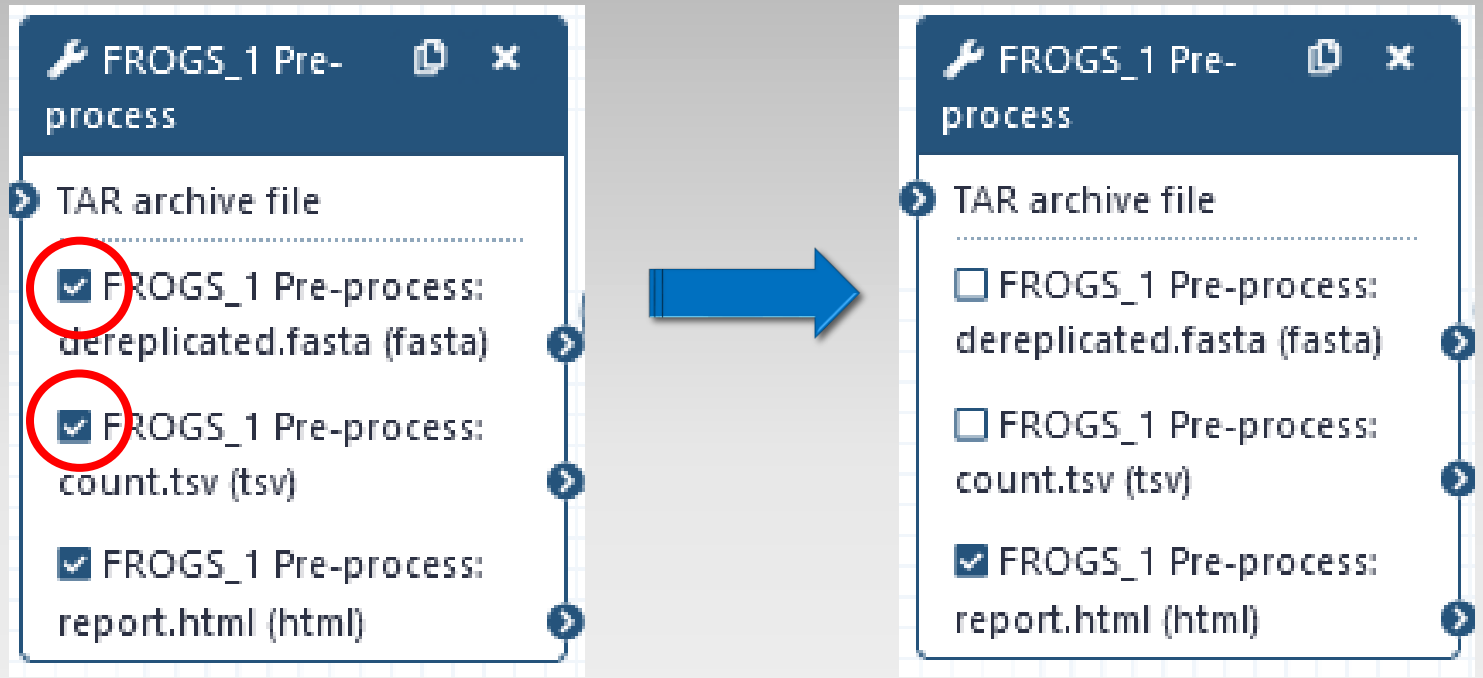
Remove Tags

This action will remove tags for the dataset.

Assign columns

For each tool, think to:

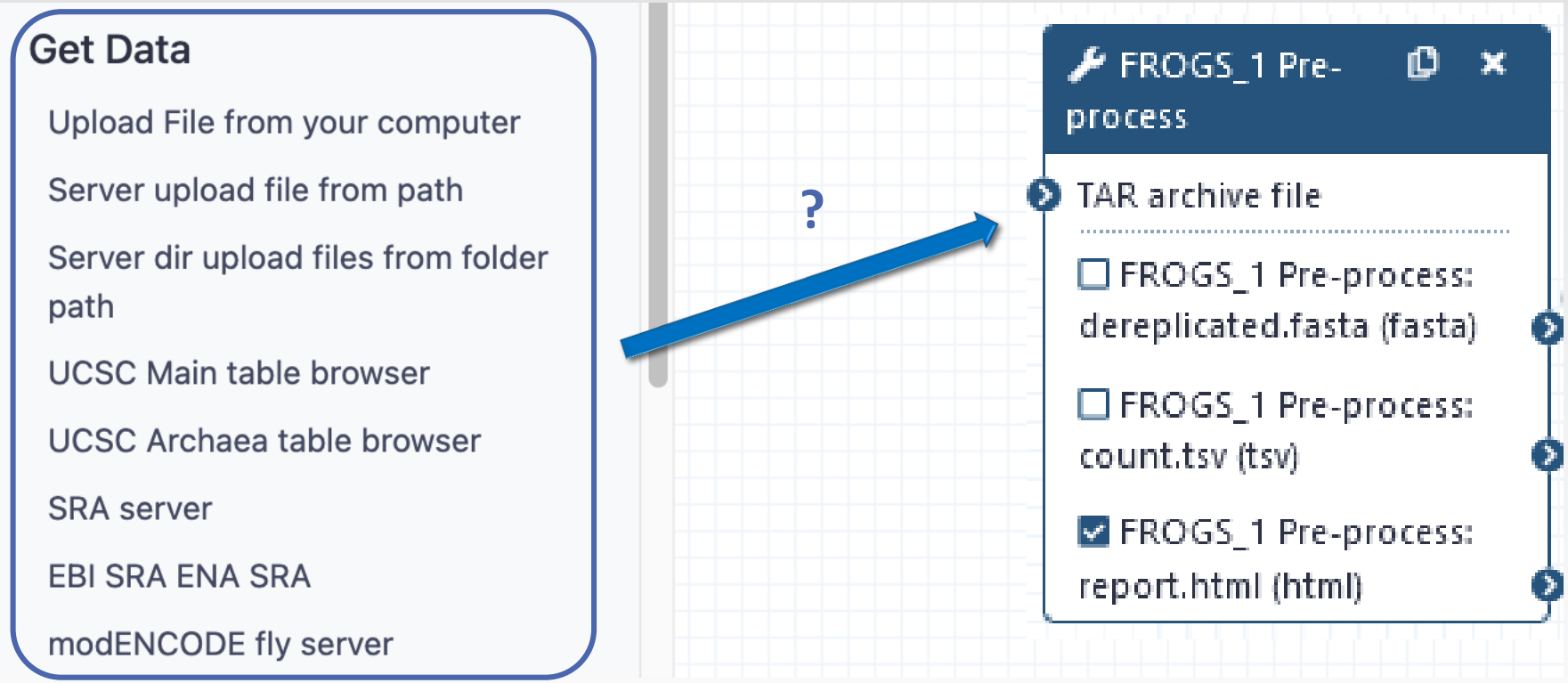
1. Set parameters
2. Rename output files



For each tool, think to:

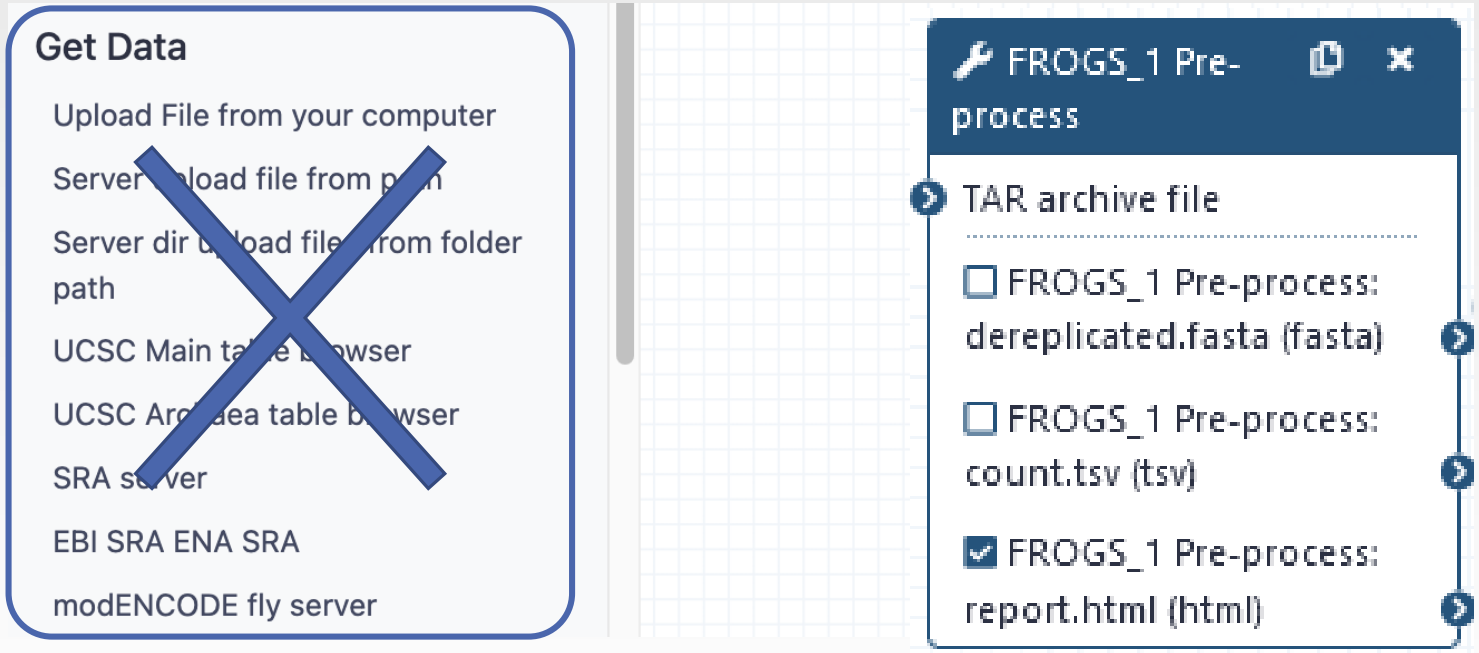
1. Set parameters
2. Rename output files
3. Hide intermediate files to simplify your history

Could you integrate « upload file » in the workflow ?

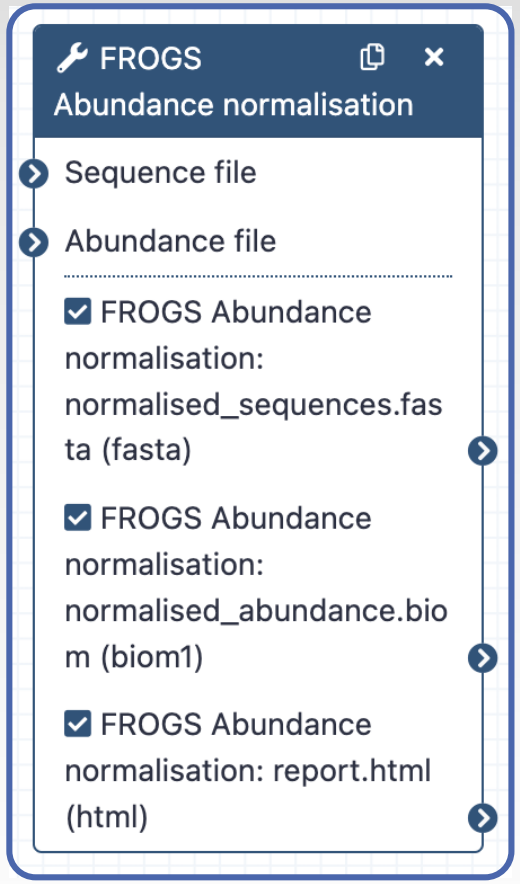


Could you integrate « upload file » in the workflow ?

Upload file cannot be automitized because the workflow, at each run, will be processed with different input data



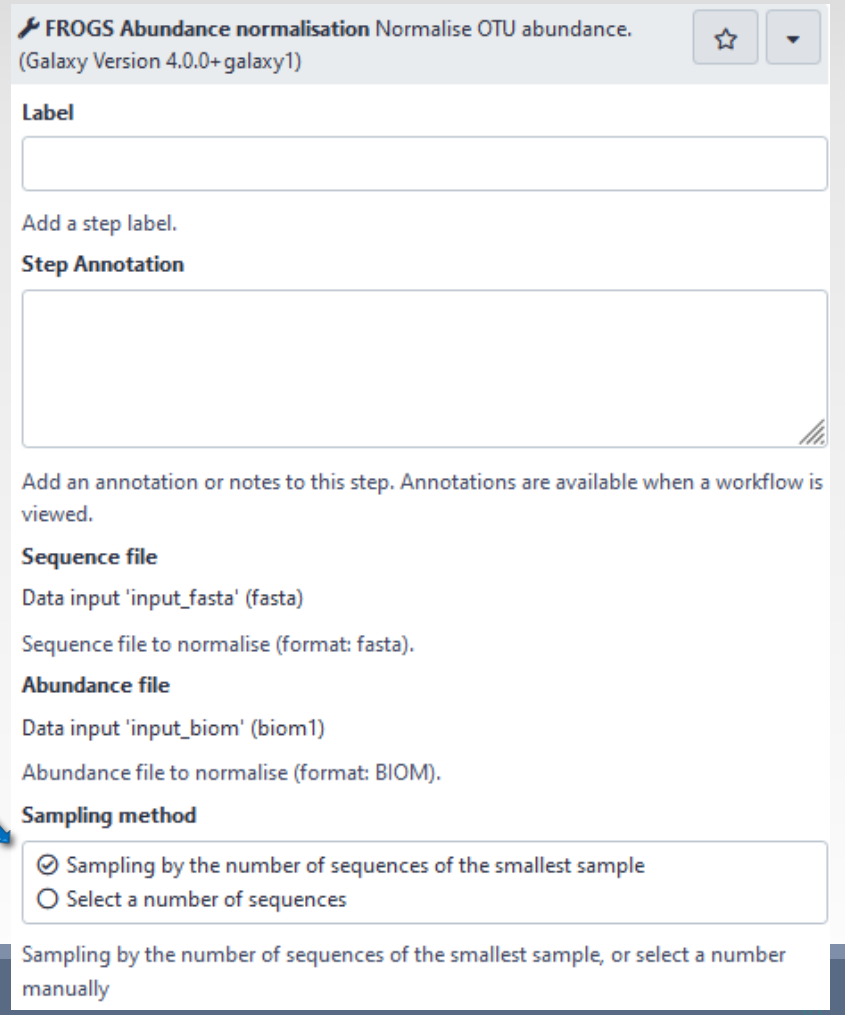
Could you integrate « Normalisation tool » in the workflow ?



?

Could you integrate « Normalisation tool » in the workflow ?

Yes, but **only** if you select
« **sampling by the number** of sequence of the smallest sample »



Exercise

When your workflow is built

1. Run your own workflow with ITS data with :

ITS_fast.tar.gz ITS_fast_metadata.tsv ITS_fast_replicates.tsv

2. Run FROGSSTAT tools