Training on Galaxy: Metabarcoding

October 2022 - Webinar

FROGS Practice on ITS data

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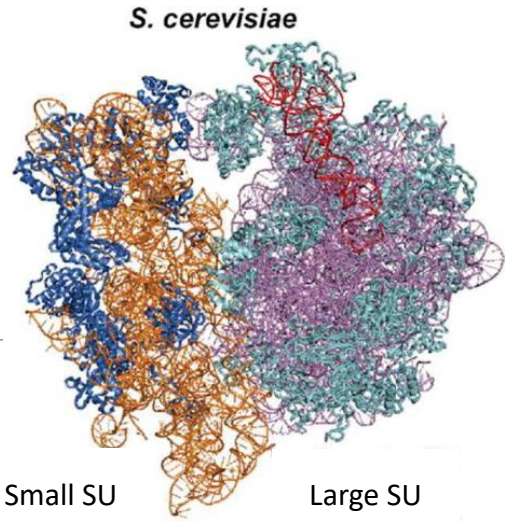
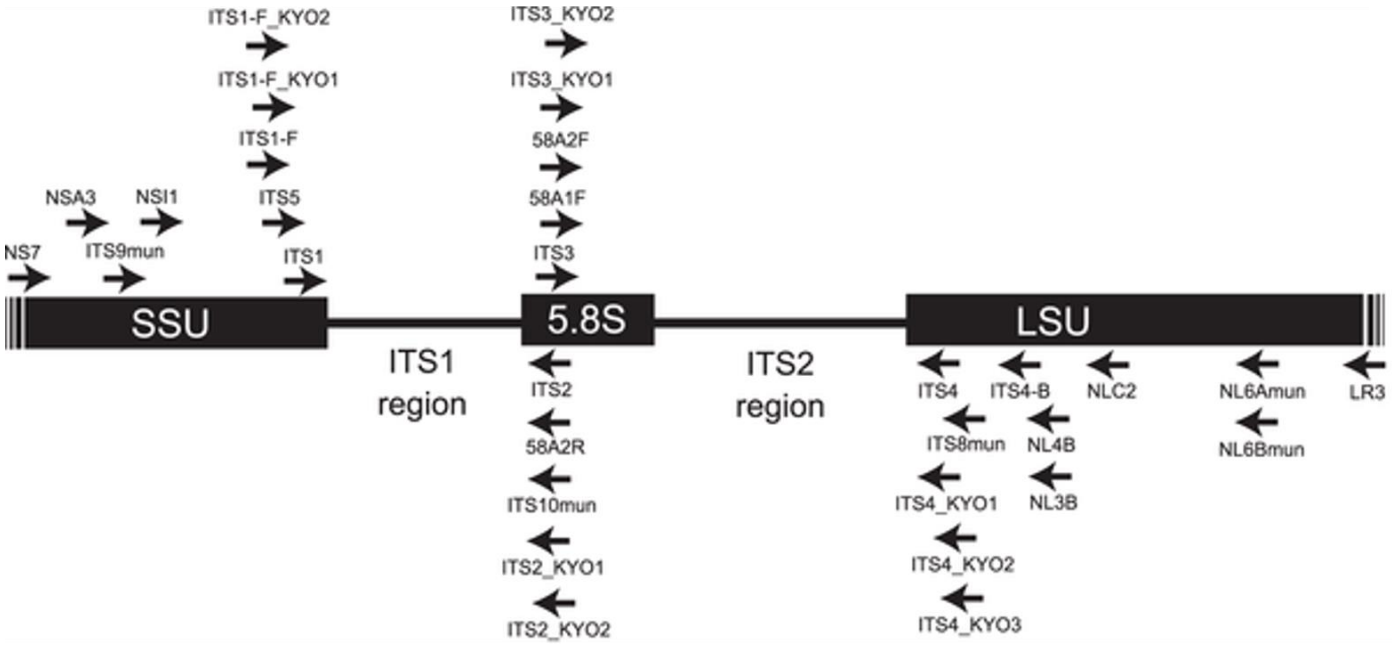
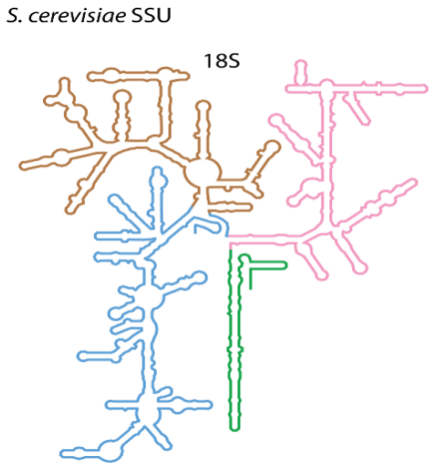
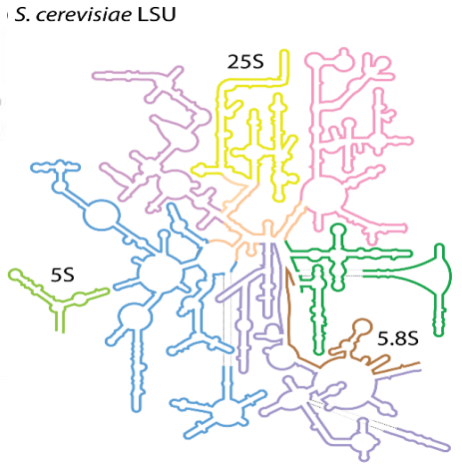
What is a ITS ?



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

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

What is a ITS ?

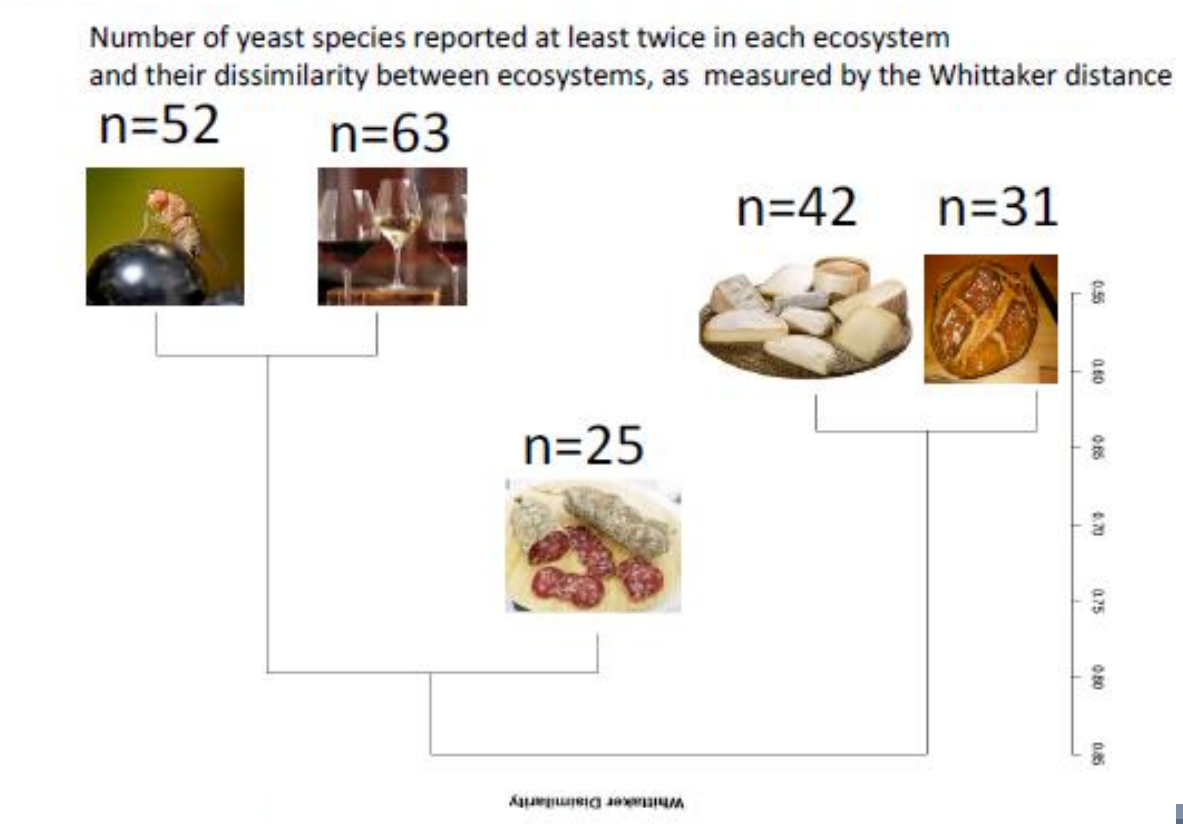
- Size polymorphism of ITS (from 361 to 1475 bases in UNITE 7.1)
- Highly conserved regions of the neighboring of ITS1 and ITS2
- Lack of a generalist and abundant ITS databank (several small specialized databanks)
- Multiple copies* (14 to 1400 copies (mean at 113, median at 80))
- Do not target Glomeromycetes/Glomeromycota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

Yeast catalog in food ecosystem

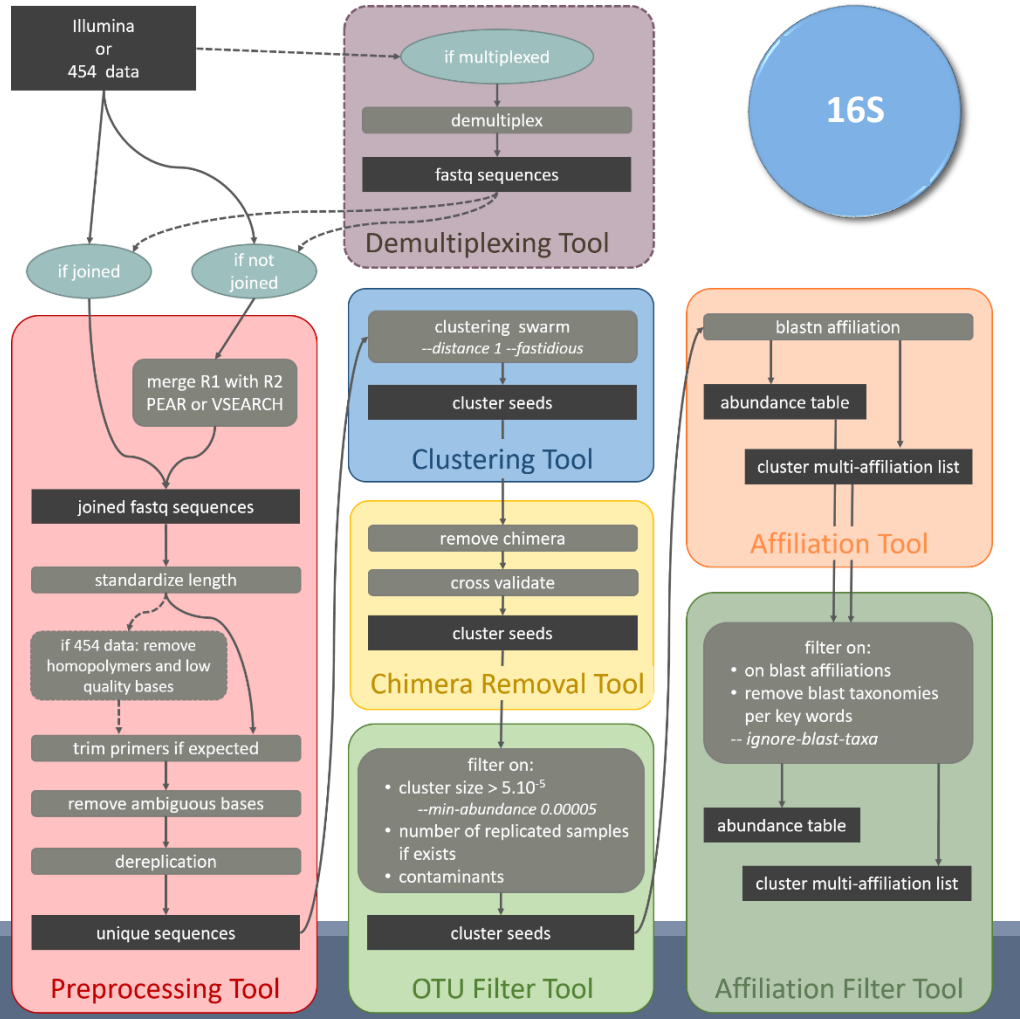


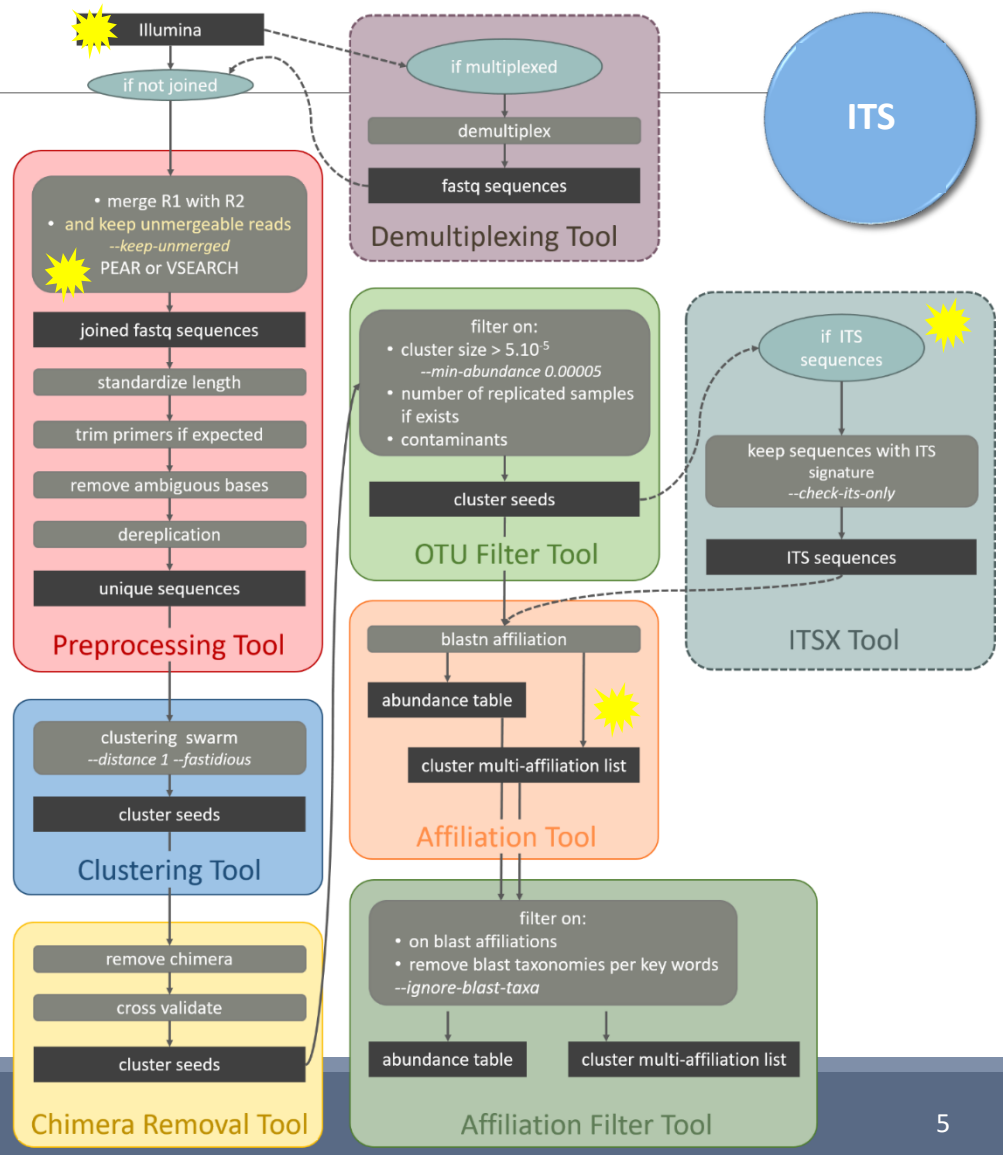
The universal fungal barcode, the Internal Transcribed Spacer (ITS) region, displays considerable size variation amongst yeasts and other micro-eukaryotes.

There are also several repeats leading to sequencing errors or termination.

: Differences between 16S and ITS pipelines

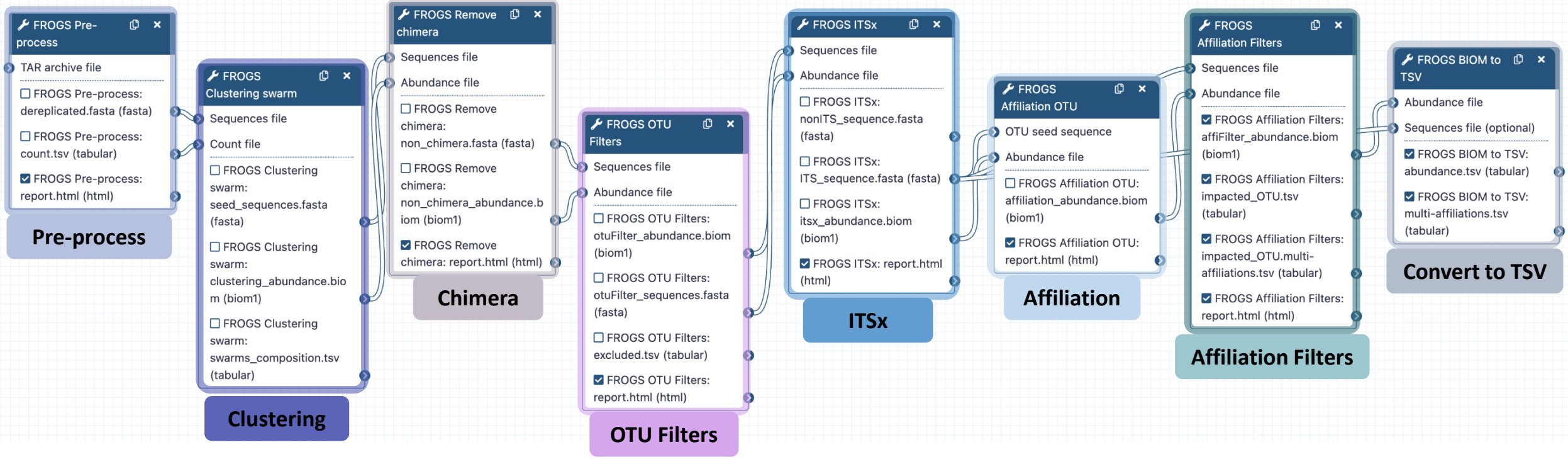
Overview



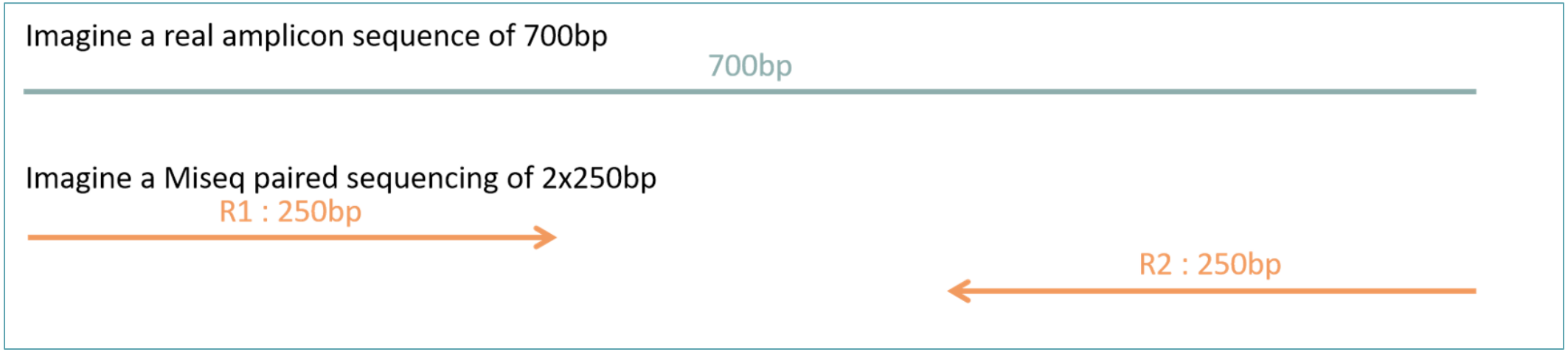


FROGS Pipeline

Minimal pipeline for ITS amplicon analyses



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

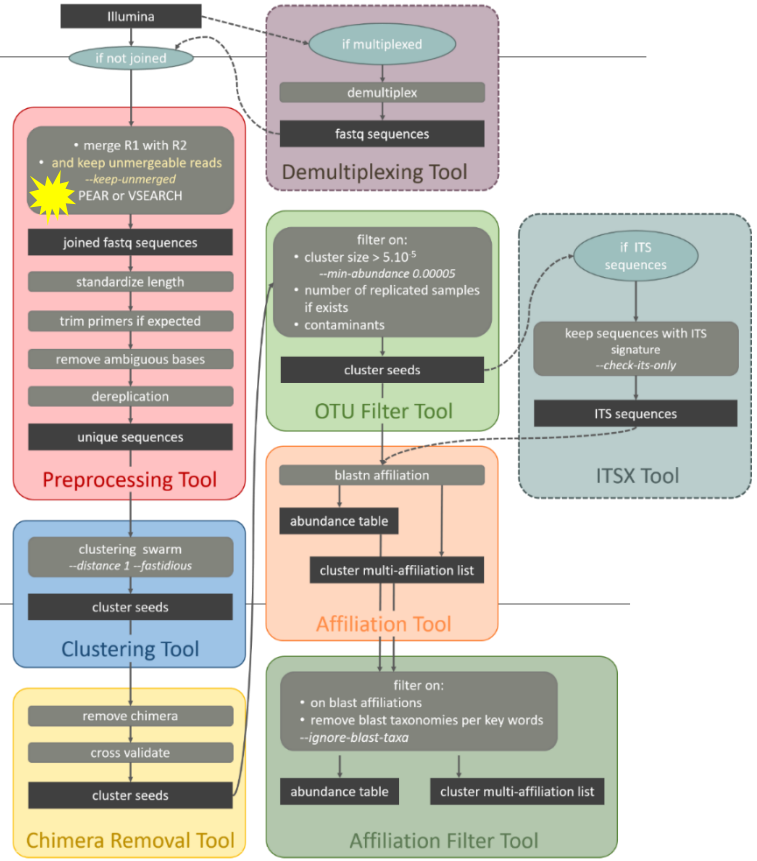
Imagine a real amplicon sequence of 700bp

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

Pre-process tool



ITS

FROGS Pre-process merging, denoising and dereplication. (Galaxy Version 3.2.3+galaxy2) ☆ Favorite ▾ Options

Sequencer

Illumina

Select the sequencing technology used to produce the sequences.

Input type

TAR Archive

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

TAR archive file

1: ITS1.tar.gz

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

Are reads already merged ?

No

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

Reads 1 size

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 : 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' help section.

3' primer

GCATCGATGAAGAACGCAGC

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

Email notification

No

Send an email notification when the job completes.

✔ Execute

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 »

Explore Preprocess report.html

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
complexe-ADN-1	56,284	85.79	52,757	48,305	48,292	48,292	48,288
echantillon1-1	32,883	81.01	31,137	26,653	26,640	26,640	26,639
echantillon1-2	56,166	91.15	53,736	51,225	51,200	51,200	51,195
echantillon1-3	84,102	71.56	80,060	60,224	60,188	60,187	60,181
echantillon2-1	53,405	86.65	51,004	46,282	46,276	46,275	46,275

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
complexe-ADN-1	56,284	4.57	3,527	2,814	2,572	2,572	2,571
echantillon1-1	32,883	3.03	1,746	1,151	997	997	997
echantillon1-2	56,166	2.81	2,430	1,789	1,582	1,582	1,581
echantillon1-3	84,102	2.61	4,042	2,574	2,199	2,199	2,199
echantillon2-1	53,405	2.46	2,401	1,554	1,317	1,317	1,316

Own tag for combined sequences

```
>Cluster_20410 1:N:0:ATATAA
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
>Cluster_2881 1:N:0:ATATAA
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
>Cluster_10465 1:N:0:ATTACA
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
>Cluster_2714_FROGS_combined R1_desc:1:N:0
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
>Cluster_6993_FROGS_combined R1_desc:1:N:0
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
>Cluster_2580_FROGS_combined R1_desc:1:N:0
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
```

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.

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.

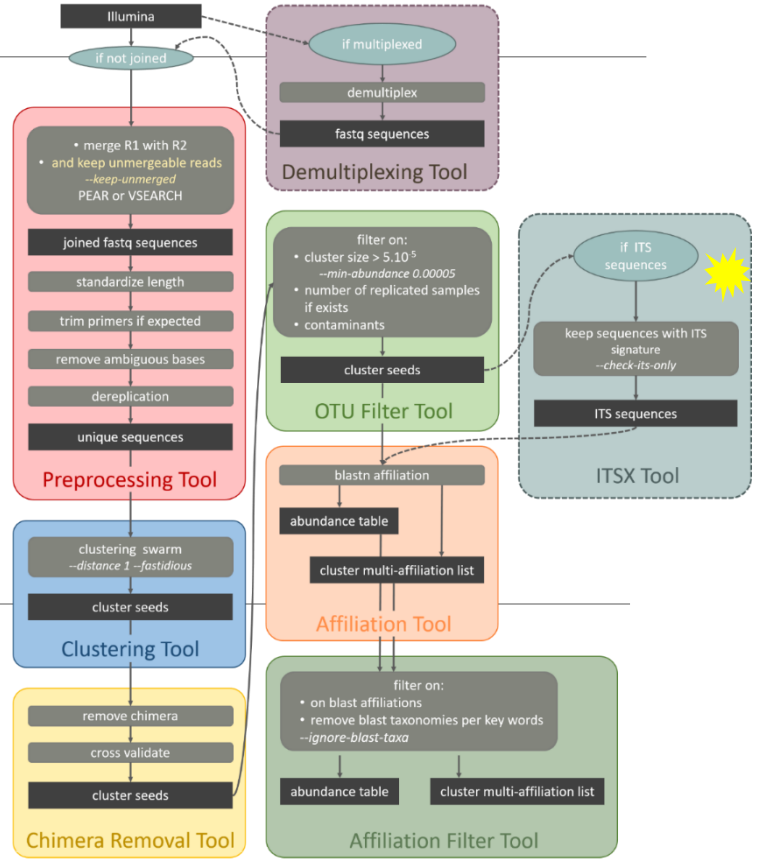
Case 1: real amplicon ≥ 601 bp ➔ "FROGS combined" length is smaller than the reality

Case 2: real amplicon = 600 bp ➔ "FROGS combined" length is equal to the reality

Case 3: real amplicon ≥ 500 and ≤ 599 ➔ "FROGS combined" length is greater than the reality

Case 4 : real amplicon ≥ 491 and ≤ 499 ➔ FROGS combined length is greater than the reality and duplicate small sequences (between 1 and 9 bp flanking the 100 Ns added).

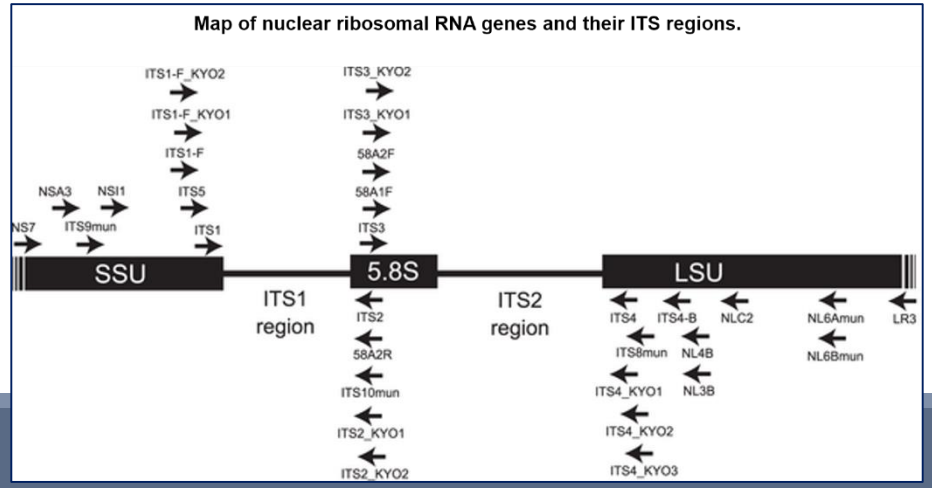
ITSx tools


Illumina
if not joined
if multiplexed
demultiplex
fastq sequences
Demultiplexing Tool
• merge R1 with R2
• and keep unmergeable reads
--keep-unmerged
PEAR or VSEARCH
joined fastq sequences
standardize length
trim primers if expected
remove ambiguous bases
dereplication
unique sequences
Preprocessing Tool
filter on:
• cluster size > 5.10-5
--min-abundance 0.00005
• number of replicated samples if exists
• contaminants
cluster seeds
OTU Filter Tool
if ITS sequences
keep sequences with ITS signature
--check-its-only
ITS sequences
ITSX Tool
clustering swarm
--distance 1 --fastidious
cluster seeds
Clustering Tool
blastn affiliation
abundance table
cluster multi-affiliation list
Affiliation Tool
remove chimera
cross validate
cluster seeds
Chimera Removal Tool
filter on:
• on blast affiliations
• remove blast taxonomies per key words
--ignore-blast-taxa
abundance table
cluster multi-affiliation list
Affiliation Filter Tool

What is the purpose of the ITSx tool?

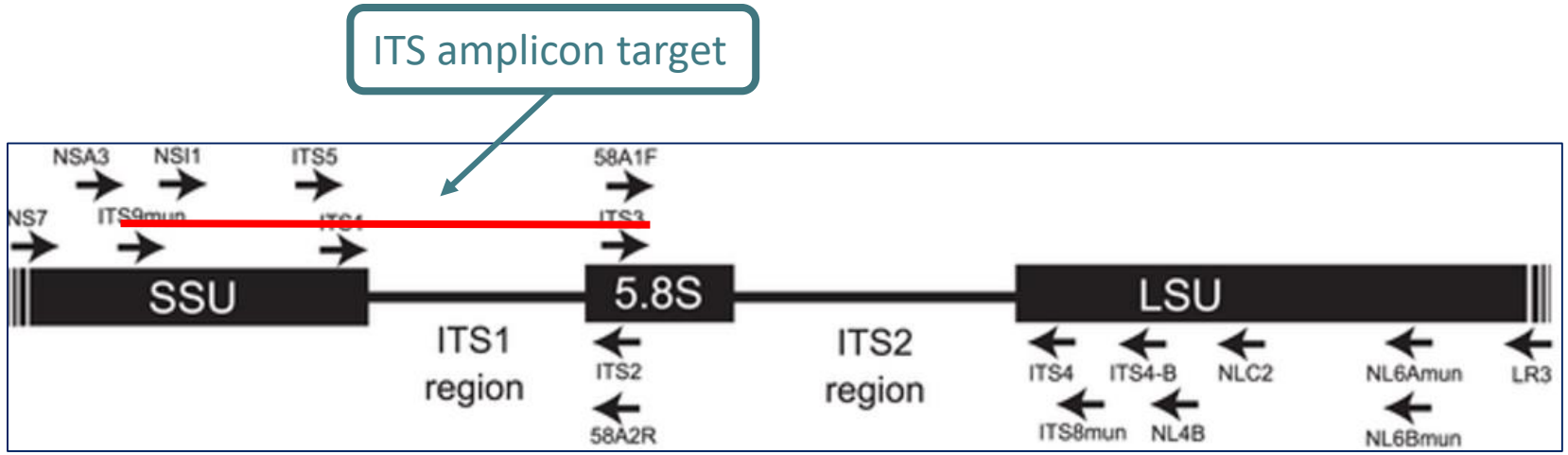
- ITSx is a tool to **filter** sequences.
- ITSx **identifies** and **trimms** ITS regions in sequences.
- It **excludes** the highly conserved neighboring sequences **SSU**, **5S** and **LSU** rRNA.
- If the ITS1 or ITS2 region is not detected, the sequence is discarded.
- You can choose to check only 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

What is the purpose of the ITSx tool?

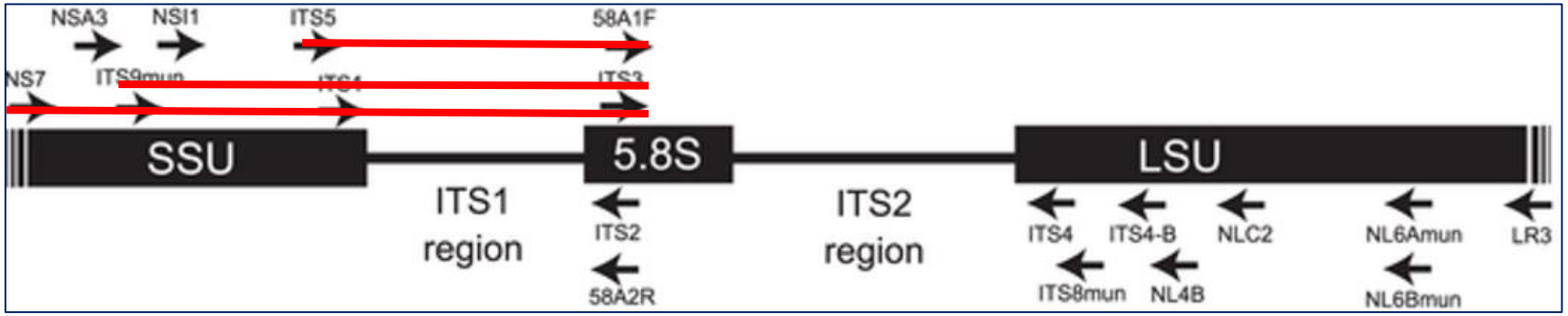


1st case: choose to trim
ITS1 is well detected
SSU part and 5.8S part are trimmed
Result: ——

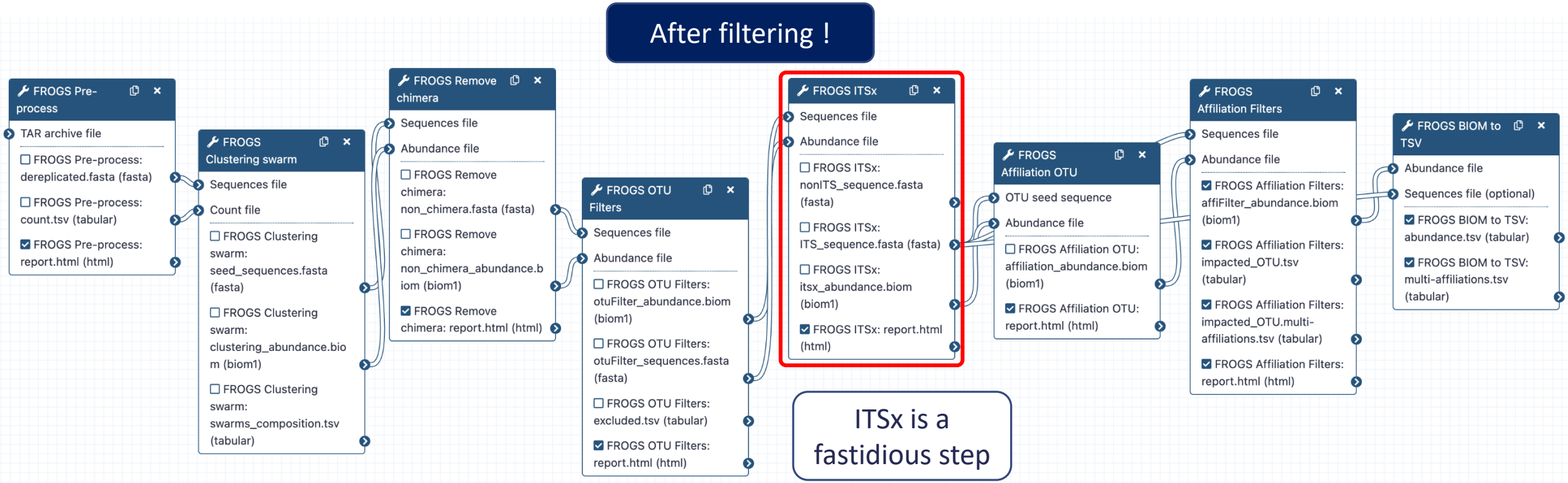
2nd case: choose to check only
ITS1 is well detected
SSU part and 5.8S part are not trimmed
Result: ——

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 3.2.3+galaxy2) ☆ Favorite ▾ Options

Sequences file

13: FROGS OTU Filters: otuFilter_sequences.fasta

The sequence file to filter (format: fasta).

Abundance file

12: FROGS OTU Filters: otuFilter_abundance.biom

The abundance file to filter (format: BIOM).

ITS region

ITS1

Which fungal ITS region is targeted: either ITS1 or ITS2

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

Yes

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

By defaut, only part of the sequence with ITS signature is kept (SSU, LSU or 5.8S are trimmed)

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.

Email notification

No

Send an email notification when the job completes.

✔ Execute

Careful !

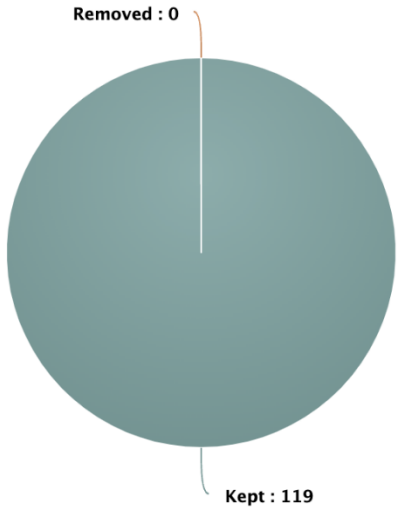
- The ITSx step is time consuming and has to be done on clusters. We advise our users to apply ITSx in 5th step:

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

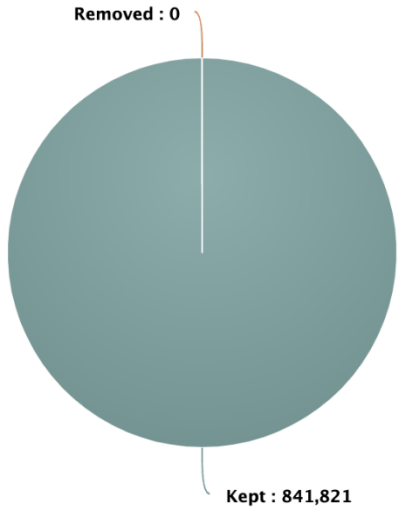
Report.html, ITSX output

Filters (ITSx) summary

OTUs



Abundance



Filters (ITSx) by samples

CSV

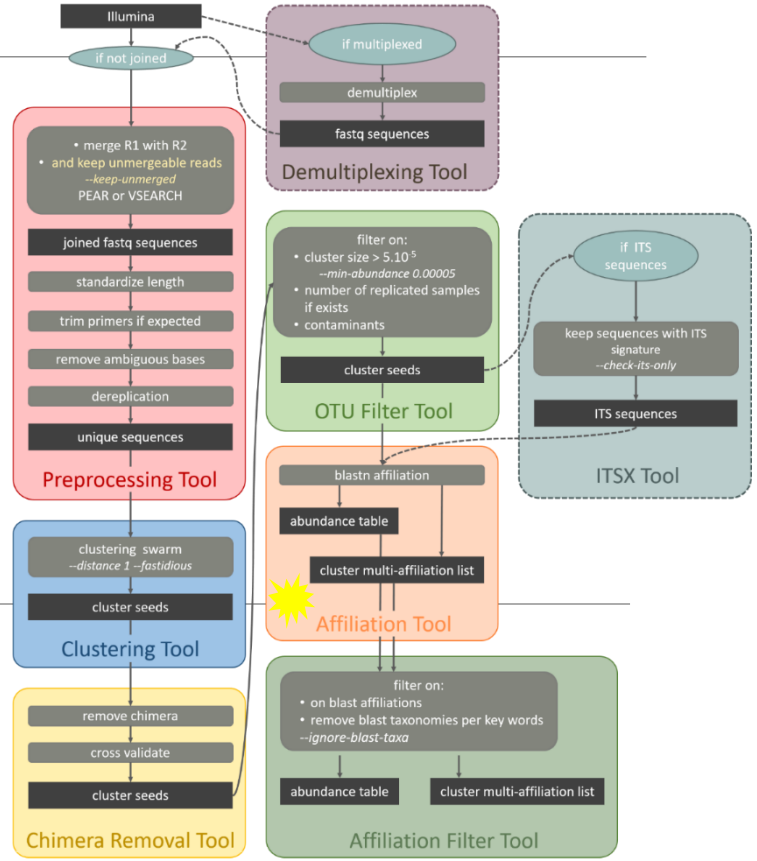
Show 10 entries

Search:

OTUs removed by sample

Sample name	Initial	Kept	Initial abundance	Kept abundance
complexe-ADN-1	92	92	46,438	46,438
echantillon1-1	71	71	26,497	26,497
echantillon1-2	71	71	50,940	50,940

ITS Affiliation

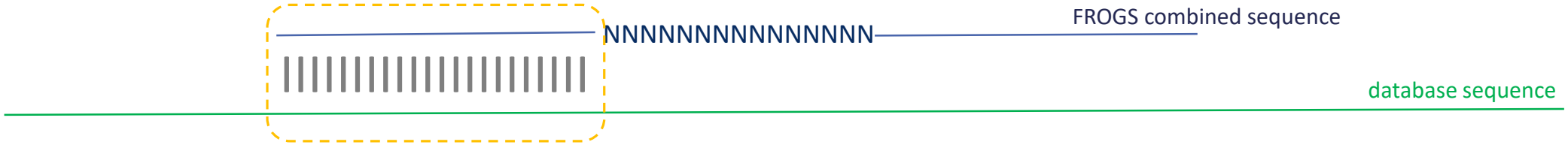


What is special about the affiliation of ITS/FROGS combined sequences?

- 2 alignment tools - blastn+ or needleall - are used to find alignments between each OTU and the database.
- Only the bests hits with the same score are reported.
- blastn+ is used for classical **merged read pair**, and blastn+ then needleall are used for **artificially combined sequence**.
- For each alignment, several metrics are computed: %identity, %coverage and alignment length.

What is special about the affiliation of ITS/FROGS combined sequences?

- blastn+ *i.e.* a local aligner, is not usable for "**combined**" sequences



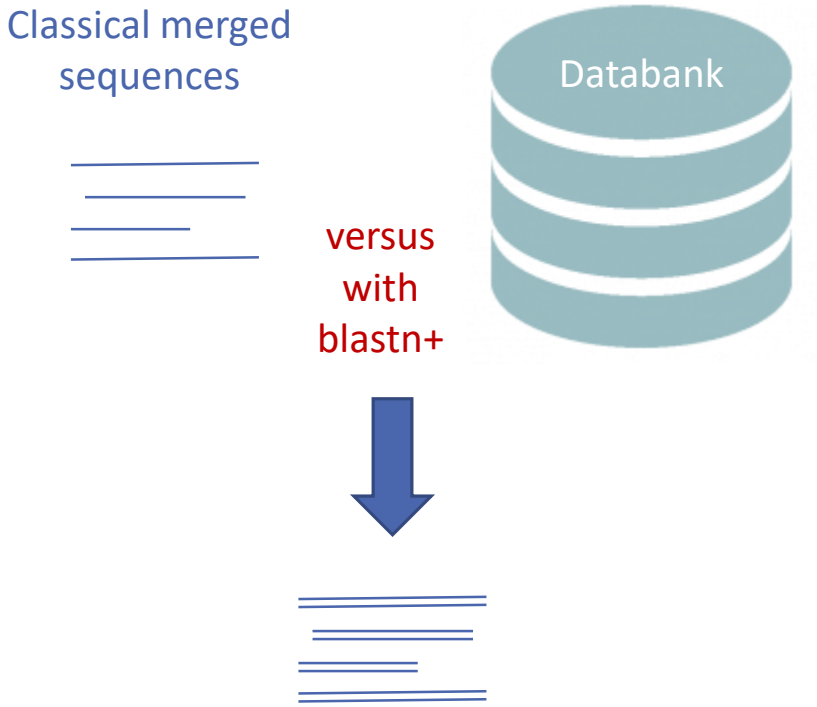
Between combined and the database sequence, alignment is perfect until N stretch with blastn+. Information about the 2nd part of sequence are not explored !

- It is necessary to use a global aligner *i.e.* Needleall (the sequence must be aligned in its entirety), but it is computationally too hard.

What is special about the affiliation of ITS/FROGS combined sequences?

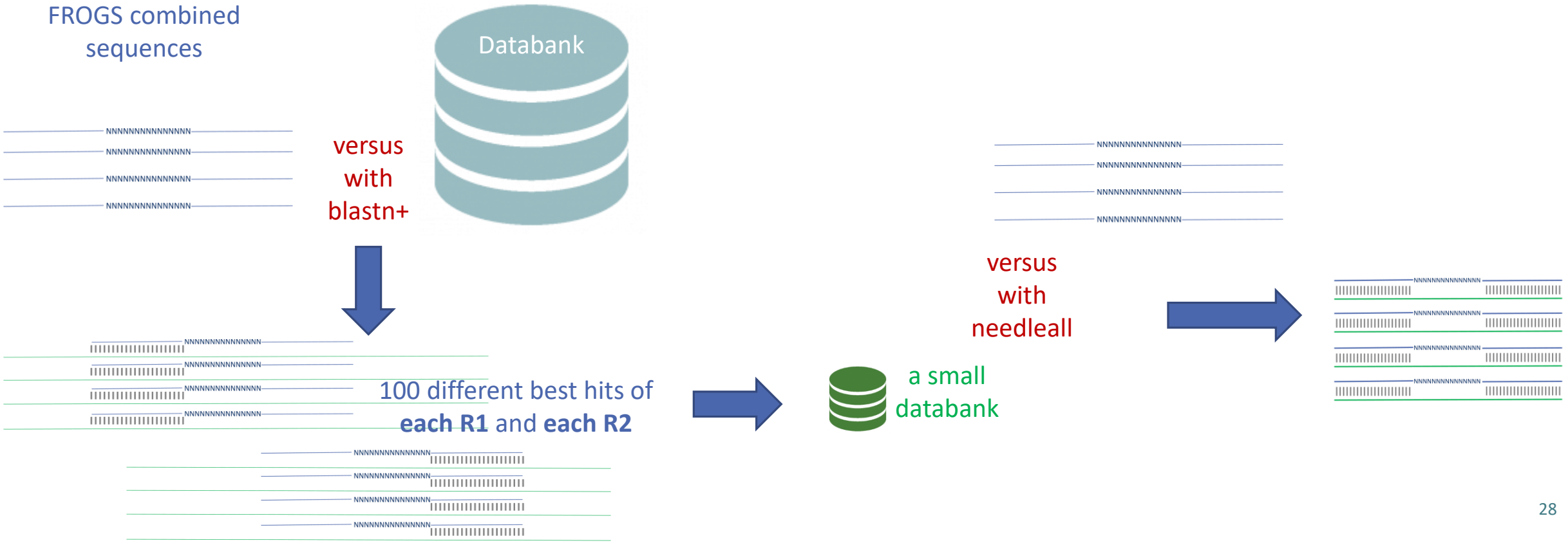
Solution:

- 1st step treat classical merged sequences with blastn+



What is special about the affiliation of ITS/FROGS combined sequences?

- 2nd step for FROGS combined sequences: use blastn+ to create a small databank and align with needleall this small databank versus FROGS combined sequences



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%

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

Filtering on %id will systematically removed “FROGS combined” OTUs.
So, we replaced 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 ?

<u>In consequence:</u>

The different affiliations involved in multi-affiliation are merged.

The abundances are added together.

It is the most abundant OTU seed that is kept.

FROGS Affiliation postprocess Optionnal step to resolve inclusive amplicon ambiguities and to aggregate OTUs based on alignment metrics (Galaxy Version 3.2.3+galaxy2)

☆ Favorite ▾ Options

Sequences file

17: FROGS ITSx: ITS_sequence.fasta

The sequence file to filter (format: fasta).

Abundance file

20: FROGS Affiliation OTU: affiliation_abundance.biom

The abundance file to filter (format: BIOM).

Is this an amplicon hyper variable in length?

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 targetted amplicon.

Using reference database

ITS UNITE Fungi 8.3

same database used for affiliation

Select reference from the list

minimum identity for aggregation

100

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

minimum coverage for aggregation

100

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

Email notification

No

Send an email notification when the job completes.

✔ Execute

Here, we wanted to aggregate OTUs only if they are identical

Exemple

After Preprocessing + Clustering + OTU Filter:

>Cluster_3
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGT
ATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTC

>Cluster_54
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGT
ATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGTTCCG

>Cluster_414_FROGS_combined
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGT
ATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCAGATCGGAANNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTCCGATCTCTTGGT
CATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTAC
TTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTC

Exemple

After Preprocessing + Clustering + OTU Filter + **ITSX** :

>Cluster_3
GTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATA

>Cluster_54
GTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATA

>Cluster_414_FROGS_combined
GTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTTCTACTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTATTATAACAAAATA

These 3 sequences have become **strictly identical** !

Exemple

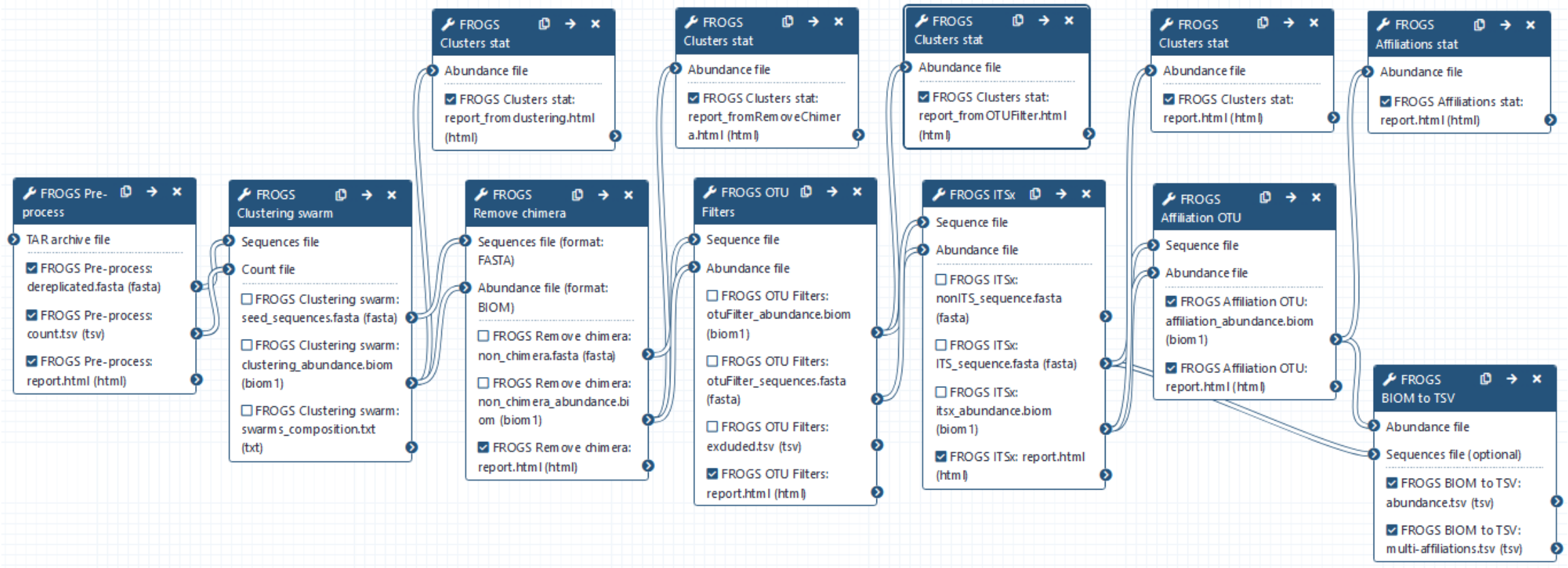
After Preprocessing + Clustering + OTU Filter + ITSX + **Affiliation Post-process** :

Cluster_3, Cluster_54 and Cluster_414_FROGS_combined are **aggregated** in a same OTU

FROGS Affiliation postprocess: aggregation_composition.txt

Cluster_1 Cluster_244 Cluster_448_FROGS_combined Cluster_471_FROGS_combined
Cluster_2 Cluster_320 Cluster_357 Cluster_435 Cluster_468 Cluster_312 Cluster_364 Cluster_477 Cluster_466 Cluster_480
Cluster_3 Cluster_54 Cluster_414_FROGS_combined
Cluster_4 Cluster_15 Cluster_27 Cluster_42 Cluster_67 Cluster_77 Cluster_137 Cluster_209 Cluster_422
Cluster_5 Cluster_5171
Cluster_6 Cluster_53
Cluster_9 Cluster_71
Cluster_7

Workflow creation



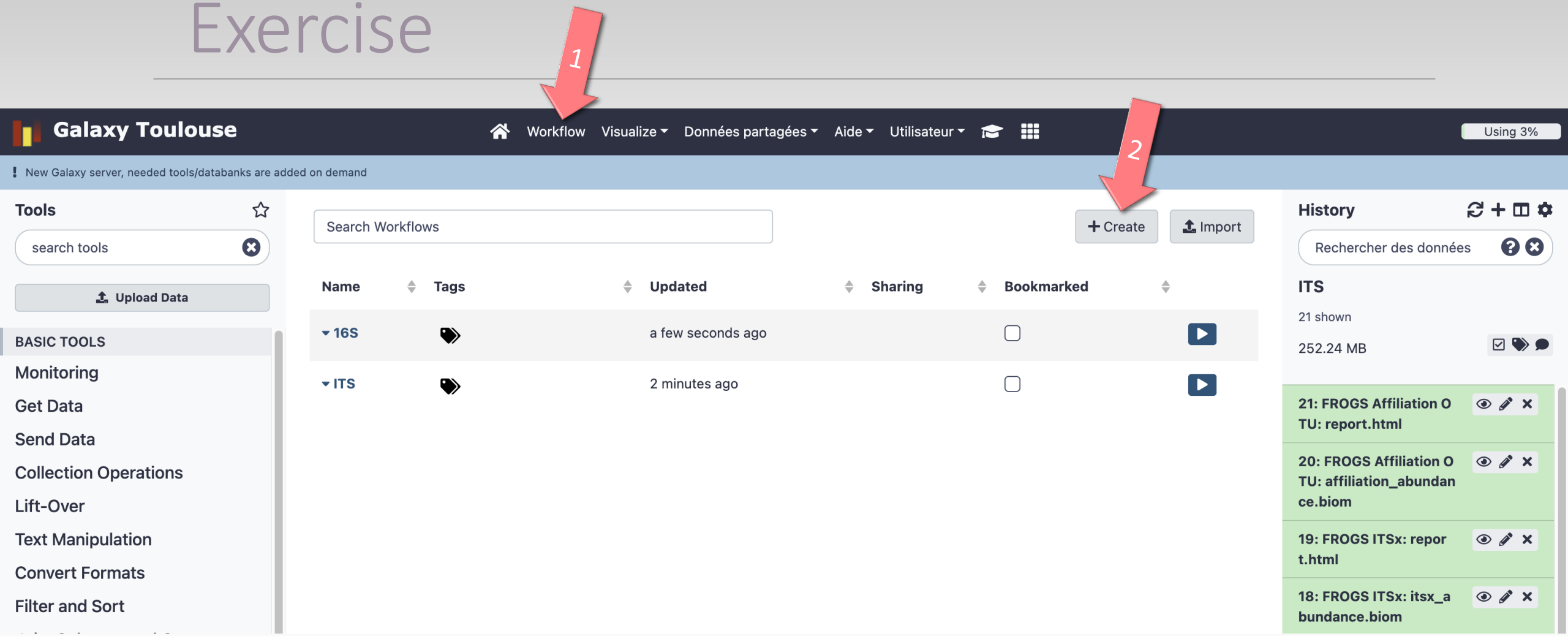
Workflow are useful for routine analyses

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

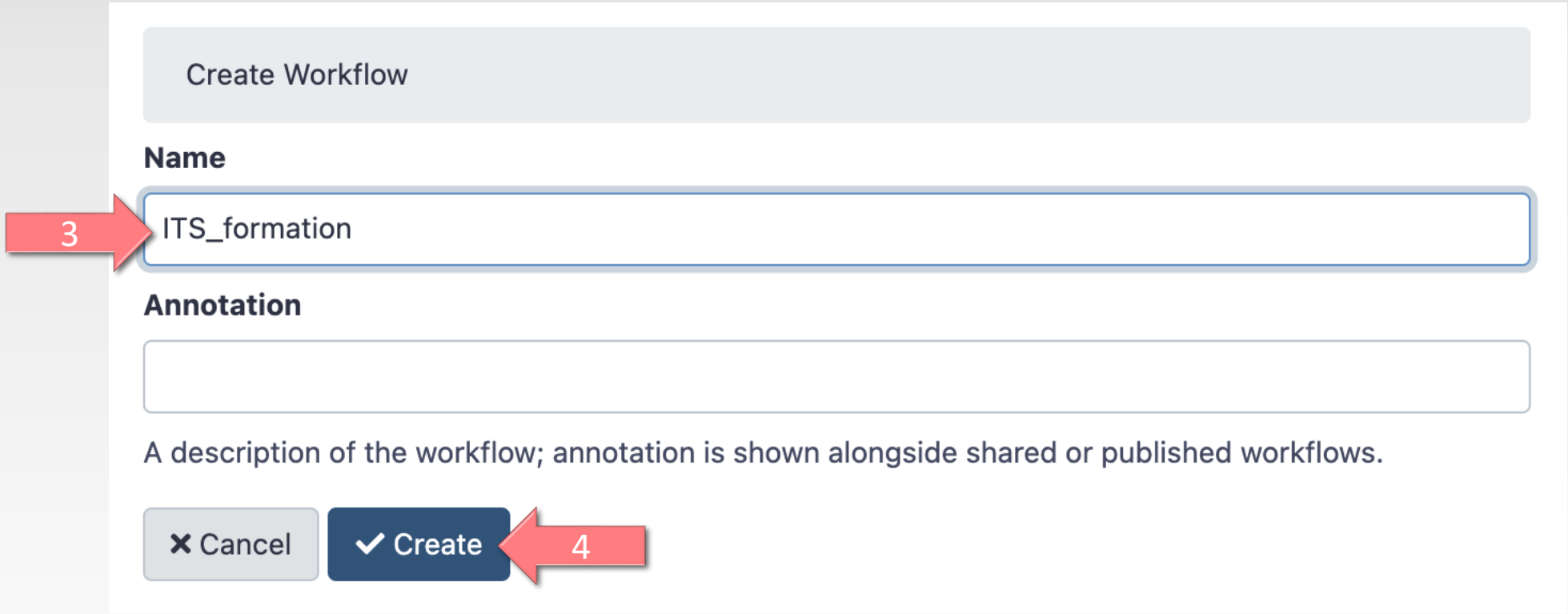
Practice

CREATE YOUR OWN WORKFLOW !

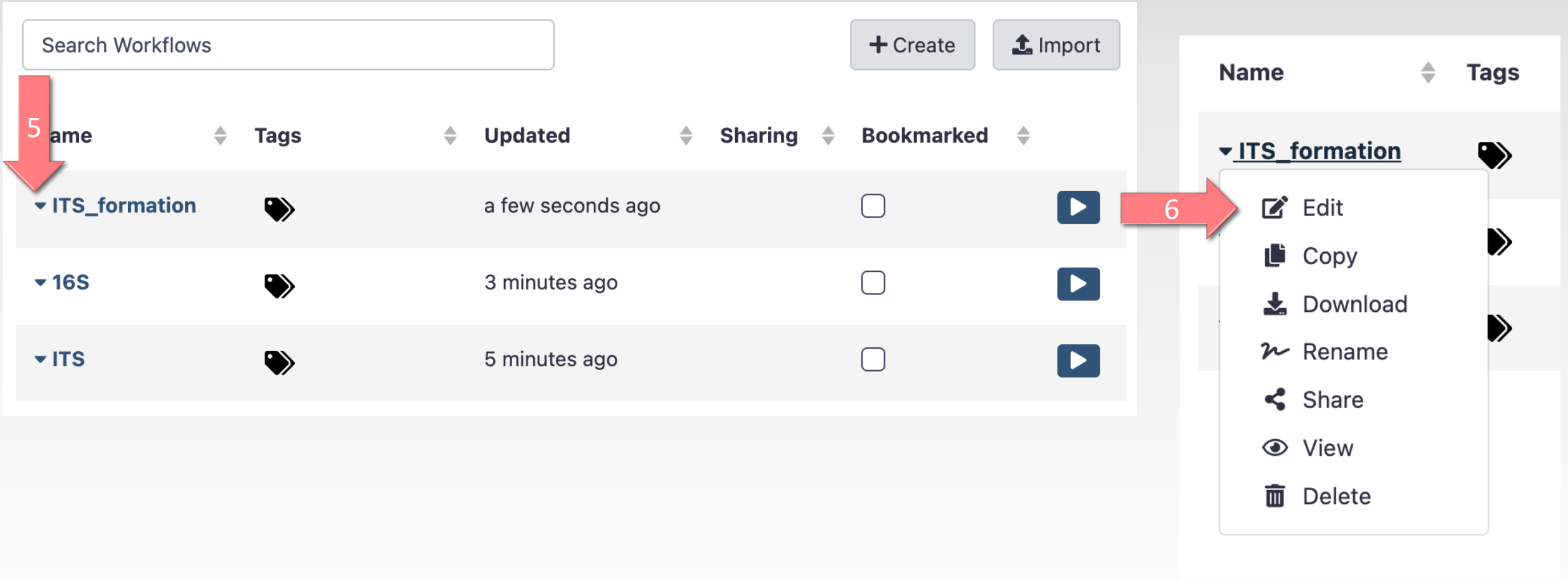
Exercise


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

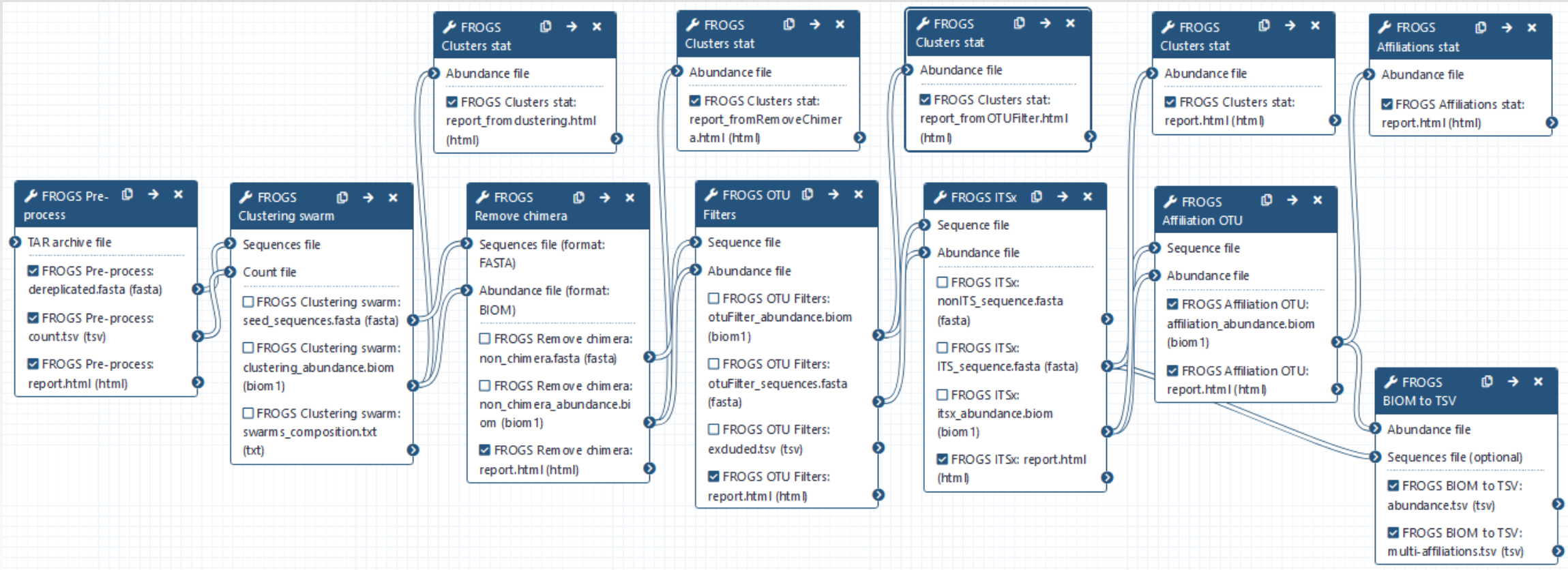
Exercise

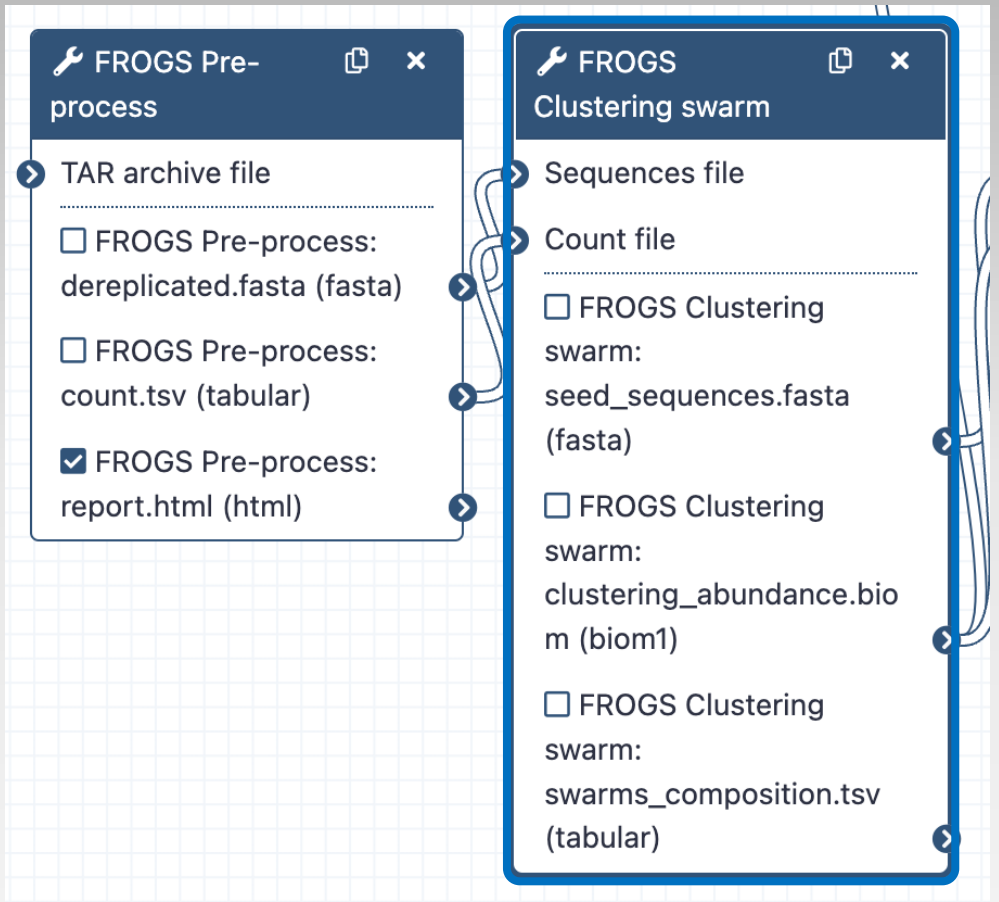

Create Workflow
Name
3
ITS_formation
Annotation
A description of the workflow; annotation is shown alongside shared or published workflows.
Cancel
Create
4

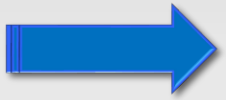
Exercise

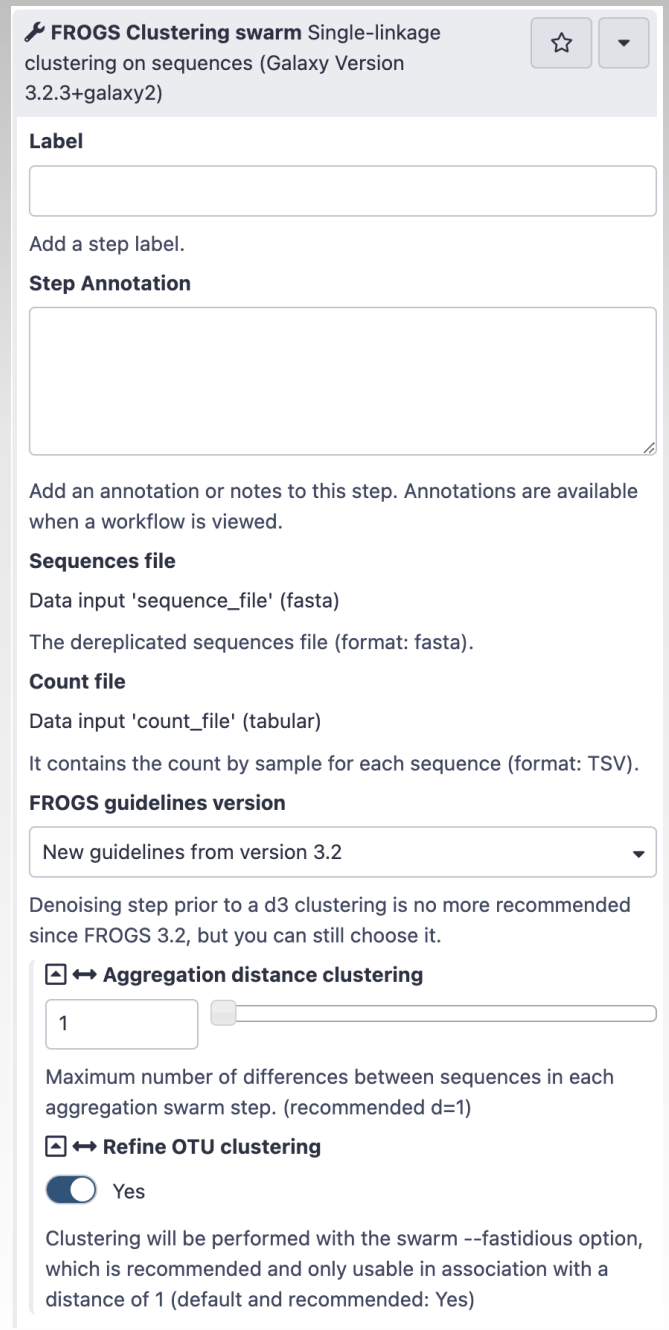

Search Workflows
+ Create
Import
5
ame
Tags
Updated
Sharing
Bookmarked
ITS_formation
a few seconds ago
16S
3 minutes ago
ITS
5 minutes ago
6
Name
Tags
ITS_formation
Edit
Copy
Download
Rename
Share
View
Delete

Solution of exercise:



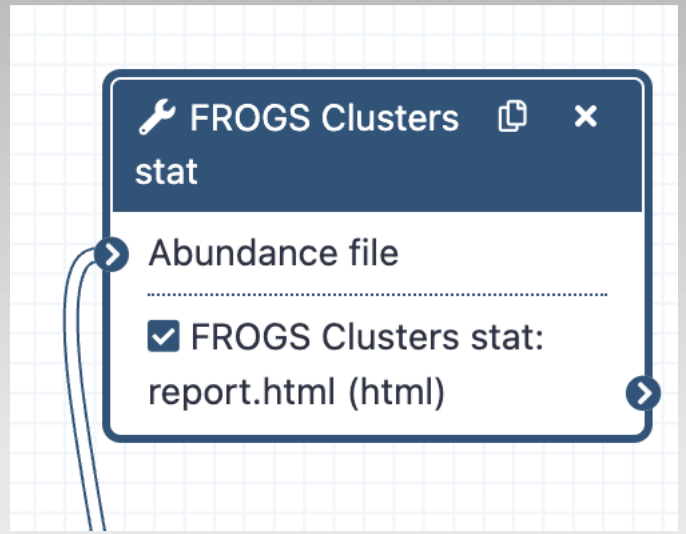






For each tool, think to:

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.

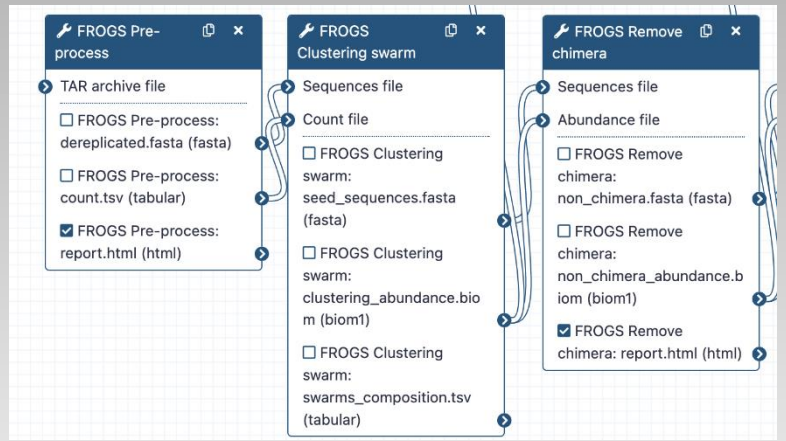
Remove Tags

This action will remove tags for the dataset.

Assign columns

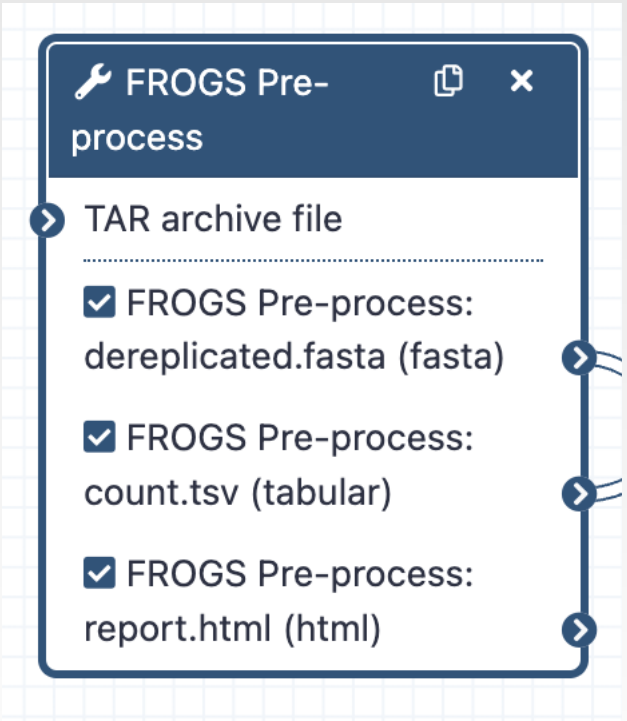
For each tool, think to:

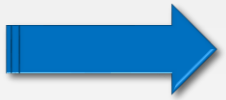
1. Set parameters
2. Rename output files

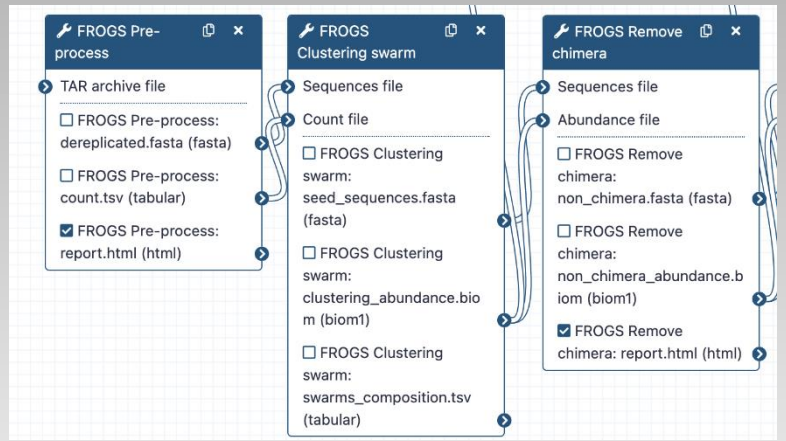


For each tool, think to:

1. Set parameters
2. Rename output files
3. Hide intermediate files

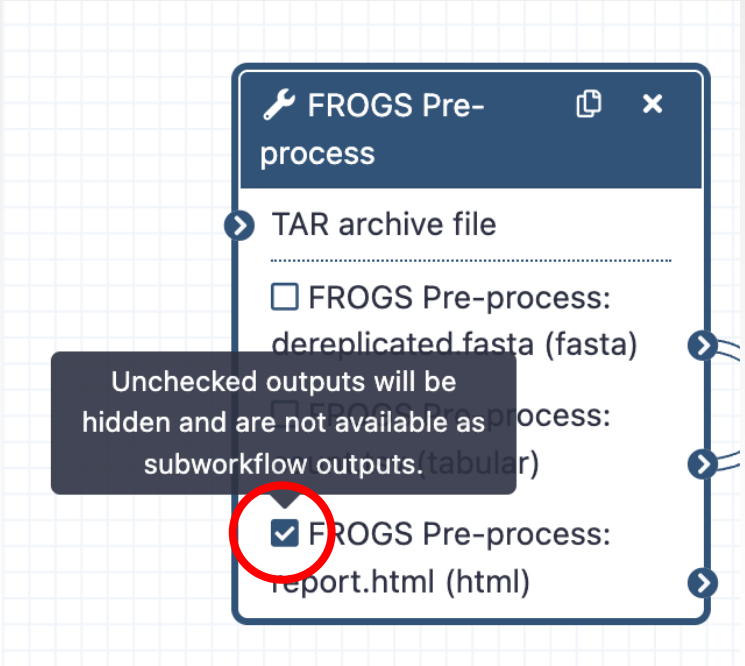


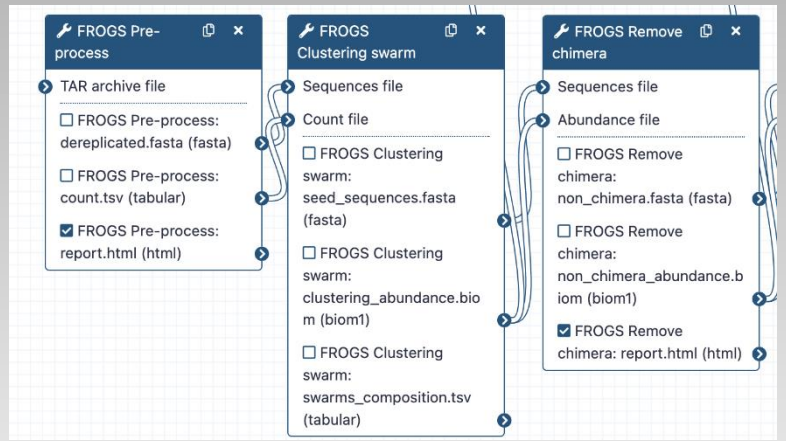




For each tool, think to:

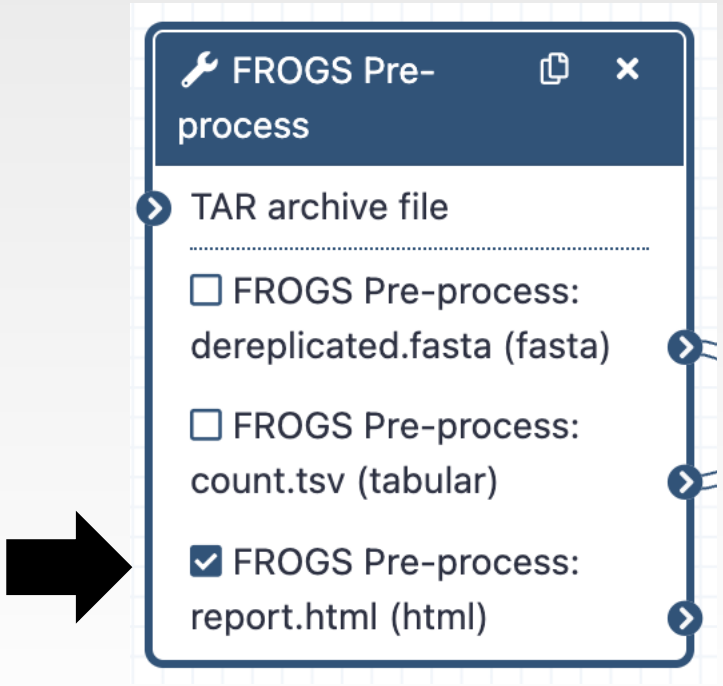
1. Set parameters
2. Rename output files
3. Hide intermediate files

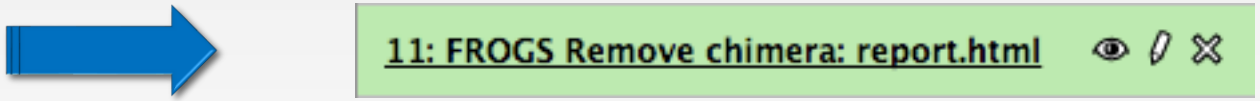




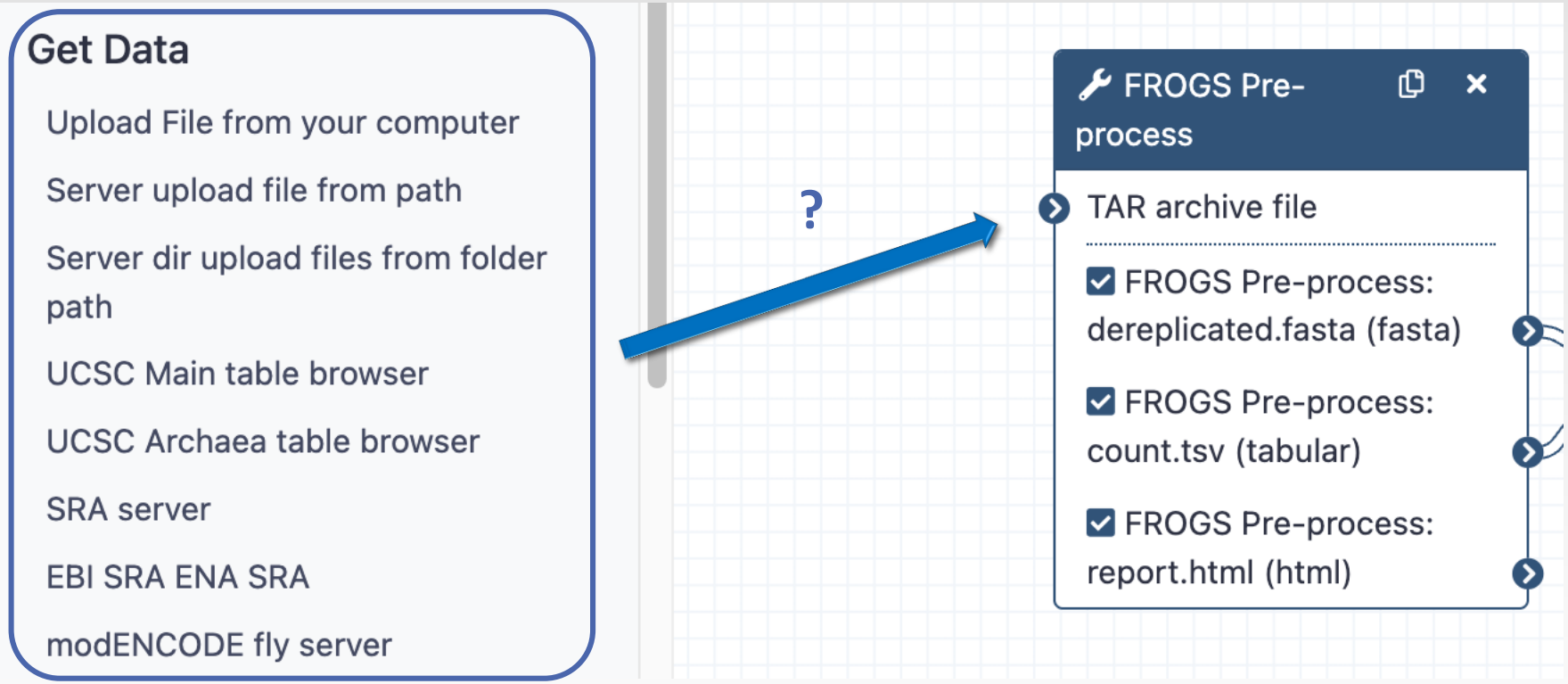
For each tool, think to:

1. Set parameters
2. Rename output files
3. Hide intermediate files



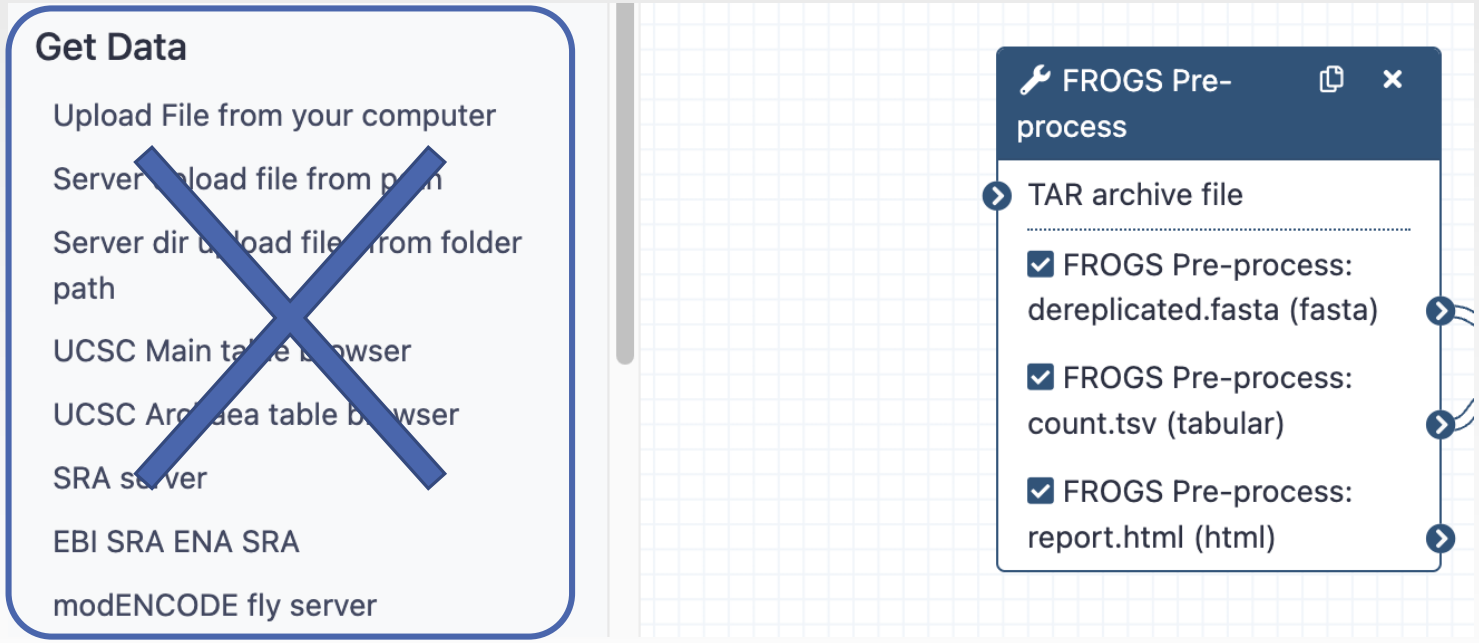


Could you integrate « upload file » in the workflow ?

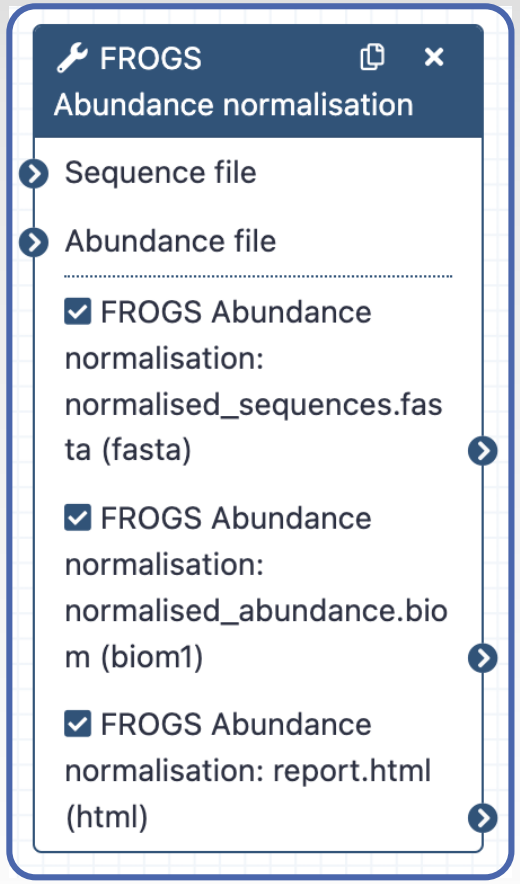


Could you integrate « upload file » in the workflow ?

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



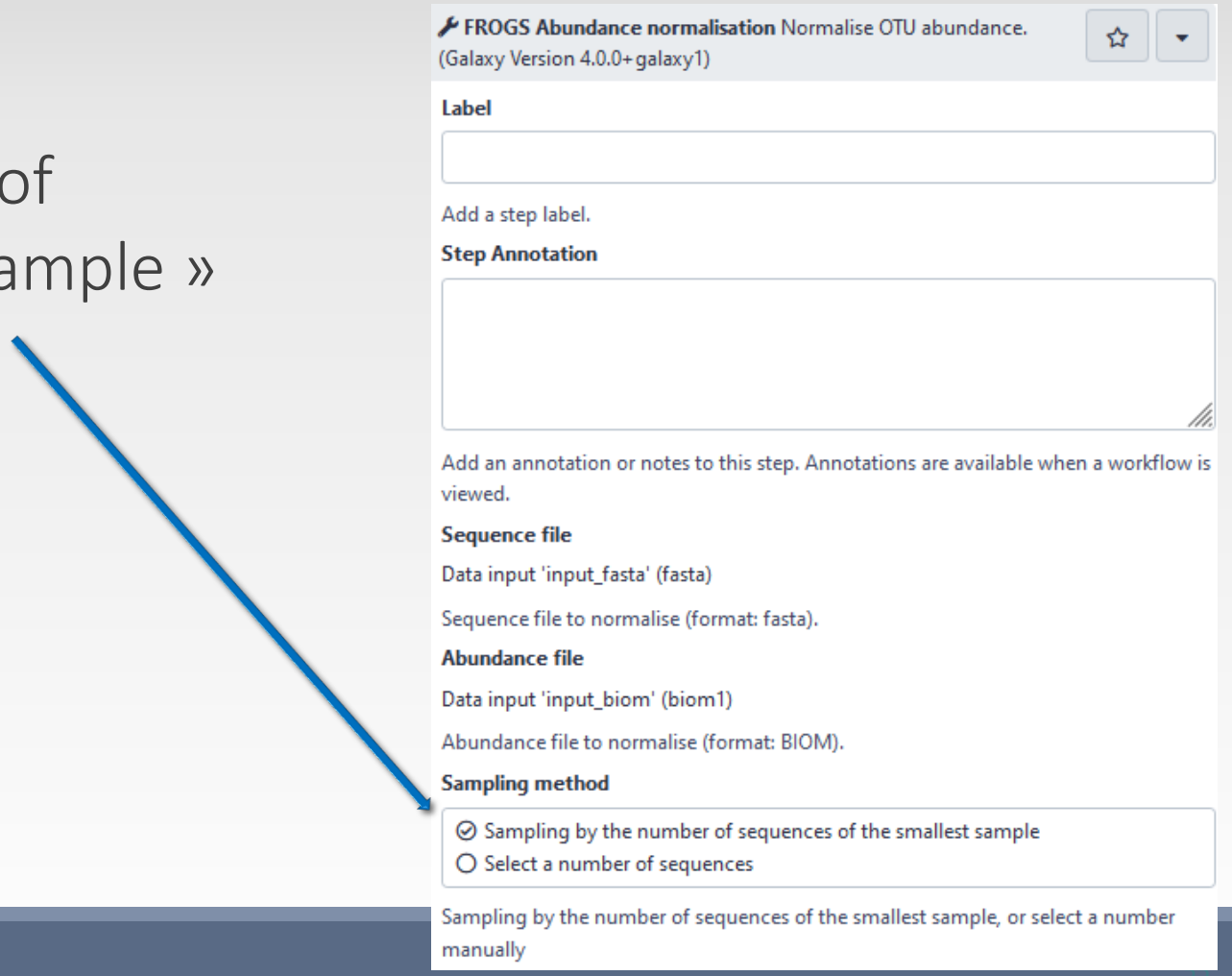
Could you integrate « Normalisation tool » in the workflow ?



?

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 :

http://genoweb.toulouse.inra.fr/~formation/15_FROGS/Webinar_data/ITS1.tar.gz

2. Import metadata for statistics analyses

http://genoweb.toulouse.inra.fr/~formation/15_FROGS/Webinar_data/metadata_ITS.tsv

3. Run FROGS_stat tools