# Installation

## Step 1: Create conda environment with required packages and software

mamba create -n REPGENR -y python matplotlib drep checkm-genome ncbi-datasets-cli mashtree snippy iqtree ete3

## Step 2: Activate the environment

conda activate REPGENR

## Step 3: Download RepGenR and install using pip:

git clone <https://github.com/FOI-Bioinformatics/repgenr>

pip install repgenr

Alternatively:  
- Download in browser from <https://github.com/FOI-Bioinformatics/repgenr/archive/refs/heads/main.zip>)  
- Unpack the zipfile  
- Remove “-main” branch-suffix  
- Install using “pip install repgenr/”

## Step 4: Manual corrections needed

### Export data path for Checkm

conda env config vars set CHECKM\_DATA\_PATH=<path to checkm\_data folder inside the environment, e.g.: ../anaconda\_path/envs/REPGENR/checkm\_data>

### Library-file for libgsl.so.25 (use conda config --set channel\_priority strict before testing the solution below)

If you get error during dRep (fastANI step?) requiring libgsl.so.25, you can copy or softlink a higher version of the file, at ../anaconda\_path/envs/REPGENR/lib.

e.g. soft-link .27 as .25: ln -s libgsl.so.27 libgsl.so.25

This is no longer the case since changing procedure of installation.

# Inputs

* Name of species/Genus/Family
* Working directory

# Outputs

metadata\_level.txt 🡨 stores the specified output taxonomic level  
metadata\_selected.tsv 🡨 metadata for output samples  
metadata\_summary.tsv 🡨 summary of samples per taxonomic level  
metadata\_summary\_number\_in\_level.png 🡨 bar-plot of sample abundance at each taxonomic level  
metadata\_summary\_number\_per\_level.png 🡨 bar-plot of taxonomic level abundance  
outgroup\_accession.txt 🡨 NCBI accession number of outgroup  
genomes 🡨 folder of downloaded genomes, formatted as family\_genus\_species\_NCBIaccession  
outgroup 🡨 folder of downloaded outgroup, formatted as family\_genus\_species\_NCBIaccession  
ncbi\_acc\_download\_list.txt 🡨 list of downloaded genomes as NCBIaccession  
genomes\_derep\_representants 🡨 folder of dereplicated genome representatives  
derep\_parameters.txt 🡨 saves parameters used during dereplication (e.g., ANI thresholds)  
genomes\_derep\_representants.dnd 🡨 phylogenetic tree (newick) of cluster-representatives  
derep\_chunks\_clustered\_genomes.tsv 🡨 dereplication information, see next (if chunking dataset)  
derep\_clustered\_genomes.tsv 🡨 cluster-representing and cluster-contained datasets  
derep\_genomes\_tree2tax.tsv 🡨 parent-child relations of cluster-representatives phylogeny  
derep\_genomes\_map.tsv 🡨 accession-to-representative map

# Usage

The software is a workflow of modules that populates the “Work directory” folder.

Main script (wrapper): **repgenr**



## Modules

### metadata



The metadata module fetches the GTDB metadata table according to input criteria. It will output the NCBI accession numbers for all samples at requested taxonomic level. A random outgroup sample is selected at one taxonomic level above the specified taxonomic level. The outgroup sample is used in a later module for phylogeny to infer placement into the database. Optionally, the outgroup may be user-specified as an NCBI-accession number.

Metadata information can be found in the work-directory specified to the software:

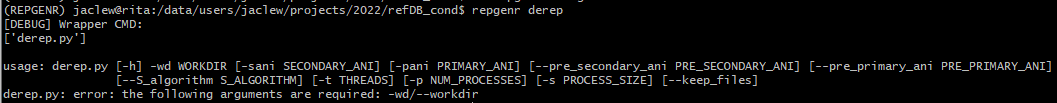
metadata\_level.txt 🡨 stores the specified output taxonomic level  
metadata\_selected.tsv 🡨 metadata for output samples  
metadata\_summary.tsv 🡨 summary of samples per taxonomic level  
metadata\_summary\_number\_in\_level.png 🡨 bar-plot of sample abundance at each taxonomic level  
metadata\_summary\_number\_per\_level.png 🡨 bar-plot of taxonomic level abundance  
outgroup\_accession.txt 🡨 NCBI accession number of outgroup

### genome



The genome module will read the accession numbers obtained through the metadata command and download them from NCBI. It produces a folder named “genomes” in where all downloaded genomes appear, formatted as “family”\_”genus”\_”species”\_”NCBI-accession-number”.fasta. A list of downloaded accessions is stored in “ncbi\_acc\_download\_list.txt.” The outgroup sample is downloaded to folder “outgroup.”

### derep



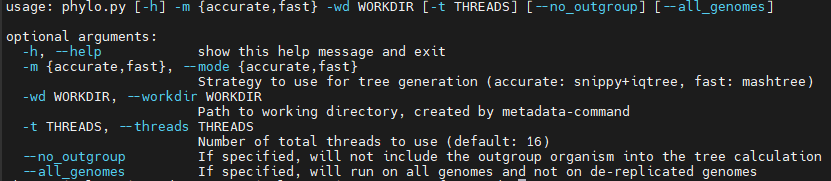
The derep module clusters the downloaded genomes based on average nucleotide identity (ANI) using the dRep software. The user may optionally change the ANI for final clustering (SECONDARY\_ANI, default=0.99) using a sensitive alignment strategy and for primary clustering (PRIMARY\_ANI, default=0.9) using a rough alignment-free strategy. The algorithm used to perform ANI-comparison can be controlled through S\_ALGORITHM (default: fastANI). The derep module requires great computation effort and may take multiple hours to finish. Large datasets can be split into chunks that are input to dRep as separate processes to reduce the number of all-vs-all comparisons. In a second step, dRep is executed on the output of the separate processes. This strategy reduces computation time for large datasets. The chunk size is controlled by PROCESS\_SIZE (a dataset of 2500 genomes can be divided to 5 chunks by setting PROCESS\_SIZE to 500). The number of chunks to compute in parallel is specified by NUM\_PROCESSES. The total number of threads used across processes is determined by THREADS. If desired, the ANI-thresholds for dRep primary and secondary clustering can be individually controlled for the chunk-processes (PRE\_SECONDARY\_ANI and PRE\_PRIMARY\_ANI – by default these parameters are SECONDARY\_ANI and PRIMARY\_ANI, respectively)

The derep module produces a folder named “genomes\_derep\_representants” where the representative genomes for each cluster is found. Contained genomes for each cluster is found in the file “derep\_clustered\_genomes.tsv” where column 1 holds the cluster-representative sequence and column 2 holds the cluster-contained sequences. If a large dataset was split into chunks an additional file “derep\_chunks\_clustered\_genomes.tsv” is produced that holds the cluster-representative sequence (column 1), cluster-contained sequence (column 2), and which chunk processed the data (column 3). The parameters used during dereplication is saved in the text-file named “derep\_parameters.txt” that includes ANI-thresholds and chunk size. The working-directory (“dereplication\_workdir”) of the derep module is removed by default but can be kept by specifying the KEEP\_FILES parameter.

#### New functions with preliminary documentation

* By default, when running “derep” in chunking-mode, the “derep” module will now attempt to re-use genome-quality data produced for the genomes in a previous step. This data is located in the main workdir under the folder “dereplication\_intermediary\_files”. This feature can be turned off using the “--skip\_intermediary”. If you copy a repgenr workdir to, e.g., run “derep” with different settings, you can include the “dereplication\_intermediary\_files” to save computation time.
  + Speed stats on Brucella (disclaimer: server load was >100% when testing so numbers are indicators but not accurate)
  + Params=('--primary\_ani', '0.97', '--secondary\_ani', '0.9995', '--pre\_primary\_ani', '0.95', '--pre\_secondary\_ani', '0.99', '-s', '50', '-p', '4', '-t', '20')
  + Without intermediate files: ~7h20min
  + With intermediate files: ~5h20min
    - Delta vs. not using intermediate files: 2h
  + Re-run with intermediate files: ~1h30min
    - Delta vs. first run: 3h50min
* If you want to control dereplication parameters after the first round of dRep that is run on chunks of your input dataset, you can specify the “--prompt” argument. This will let you review how many of your input genomes remain after running the first round of dRep, allowing you to re-run the software with higher or lower ANI settings, depending on how many genomes you wish to keep.

### phylo



The phylo module computes a phylogenetic tree based on the representative genome sequences generated by the derep module. It can be run either in a fast and rough mode or a slow but accurate mode, specified by parameter --mode. Optionally, the outgroup can be ignored when creating the tree. The module outputs the tree in newick format to the file “genomes\_derep\_representants.dnd.”

### tree2tax



The tree2tax module produces modification-files that can be input to FlexTaxD to modify an existing database. Node basenames that will be enumerate may be specified by the user, resulting in node naming like so: <basename>\_1,…,<basename>\_N. If unspecified, each node receives a MD5 hash based on all its’ descending leaves and thus produces unique node names in any non-redundant database. If the output is going to replace a branch in the database, the parent node for that branch should be specified as the root using the –r parameter. If the –remove\_outgroup parameter is specified the outgroup is removed from the output.

The parent-child representation of phylogeny is found in file “derep\_genomes\_tree2tax.tsv” and the path to genome files is found in file “derep\_genomes\_map.tsv.”

# Example: Francisellaceae

Suppose we want to create a database for *Francisellaceae* using representative-genomes in GTDB but we want to increase the resolution of *Francisella tularensis* species. We will use RepGenR to select and download genomes, and FlexTaxD to create a database and later modify it. Finally, we will make a Kraken2-database using the FlexTaxD-database.

## Initiating the Francisellaceae database

Download metadata for *Francisellaceae* representative species using repgenr:  
>> repgenr metadata -r 207.0 -v bac120 -d rep -l family -tf Francisellaceae -wd francisellaceae\_repset

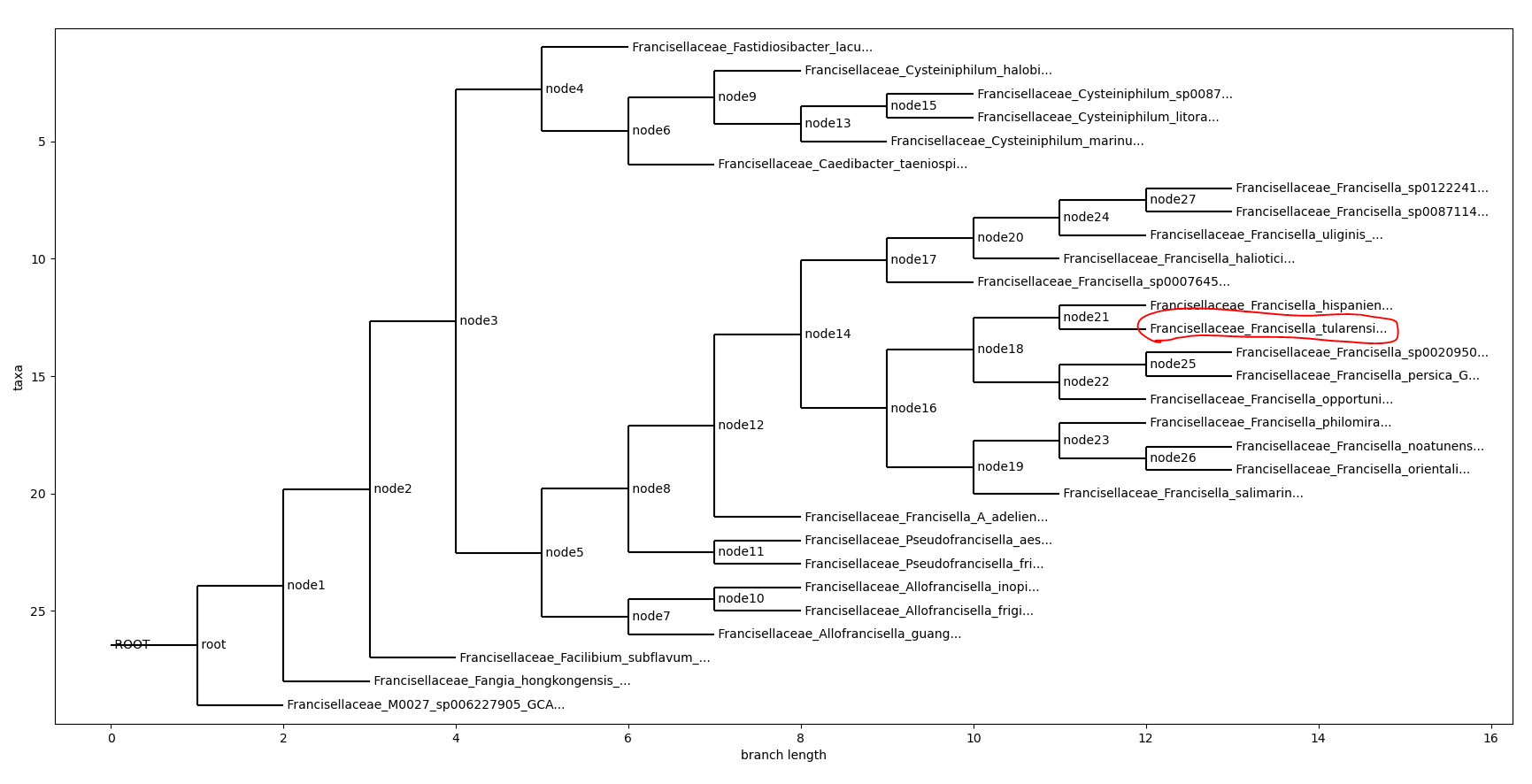
Download the genomes:  
>> repgenr genome -wd francisellaceae\_repset

Compute phylogeny on downloaded genomes (without dereplication) by supplying --all\_genomes:  
>> repgenr phylo -wd francisellaceae\_repset --all\_genomes --mode fast

Produce parent-child and genome files of phylogeny for FlexTaxD (removing outgroup and specifying root name):  
>> repgenr tree2tax -wd francisellaceae\_repset --all\_genomes --node\_basename node –-root\_name root --remove\_outgroup

Compile database using FlexTaxD:  
>> flextaxd -db francisellaceae.db --taxonomy\_file francisellaceae\_repset/genomes\_tree2tax.tsv --genomeid2taxid francisellaceae\_repset/genomes\_map.tsv

Visualize database:  
>> flextaxd -db francisellaceae.db --vis\_type tree --visualise\_node root --vis\_depth 0

  
Phylogenetic tree of *Francisellaceae* with *tularensis*-species indicated in red. Below we will replace the *tularensis*-species to expand the genetic resolution using an average nucleotide identity-based strategy.

## Modifying the database to increase the resolution of the *tularensis*-branch

To increase tree resolution of *tularensis* species, we download metadata using repgenr:  
>> repgenr metadata -r 207.0 -v bac120 -d all -l species -tg Francisella -ts tularensis -wd tularensis

Download genomes:  
>> repgenr genome -wd tularensis

Perform dereplication at 0.99 average nucleotide identity (ANI) and splitting the dataset (N=860 genomes) into three chunks (300+300+260) that are run in parallel (all three) at a total of 70 threads – to increase performance (possibly at a slightly reduced accuracy):  
>> repgenr derep -wd tularensis -sani 0.99 --process\_size 300 --num\_processes 3 --threads 70

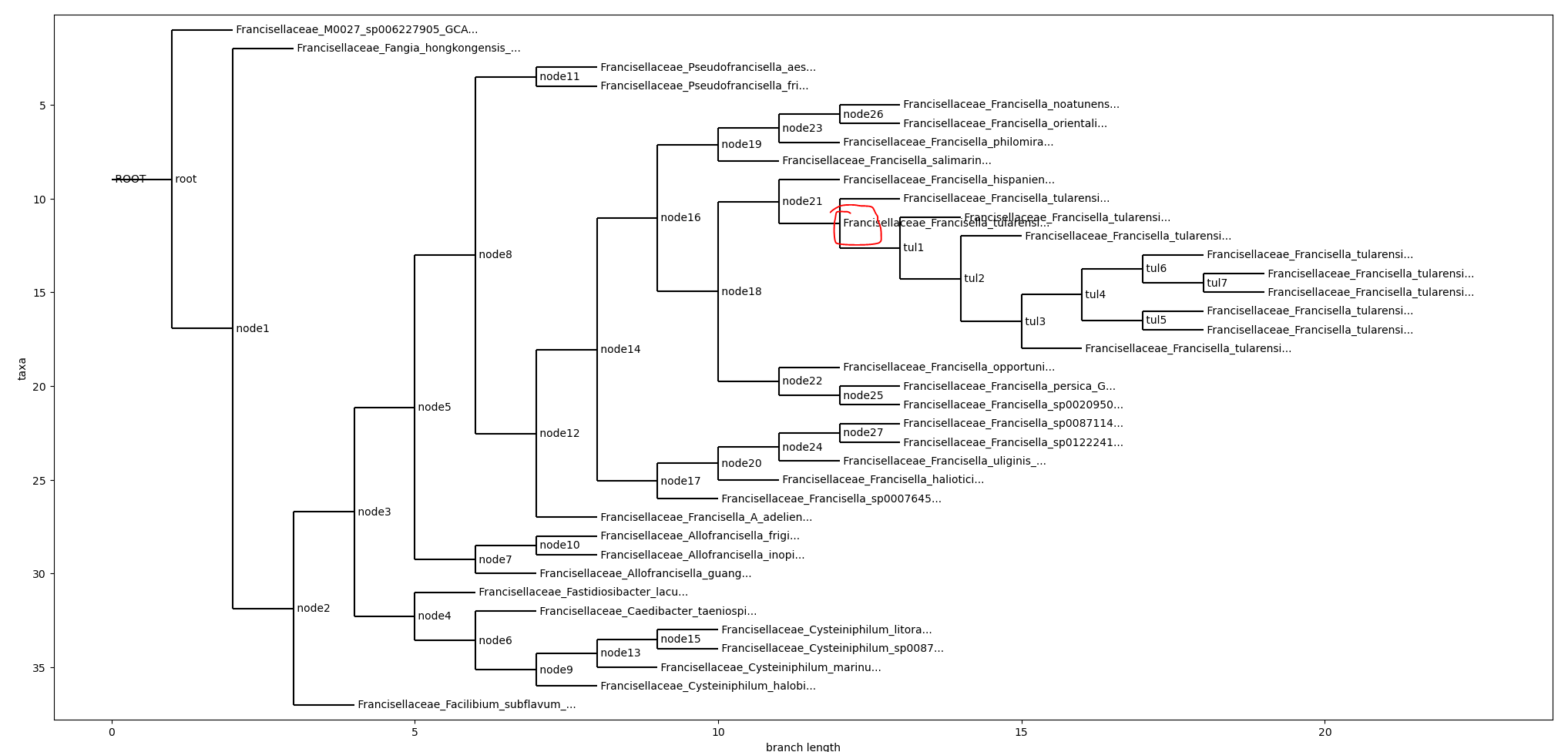
* Alternative single-process command:  
  >> repgenr derep -wd tularensis -sani 0.99 --threads 70
* Runtime comparison: 2h03m (parallel processes) vs. 3h10m (single processes)

Compute phylogeny on dereplicated genomes:  
>> repgenr phylo –wd tularensis --mode fast

Produce parent-child and genome files of phylogeny for FlexTaxD (defining root as the node in our *Francisellaceae* database to expand – in this case the *tularensis* species):  
>> repgenr tree2tax -wd tularensis --node\_basename tul --root\_name Francisellaceae\_Francisella\_tularensis\_GCF\_000008985.1 --remove\_outgroup

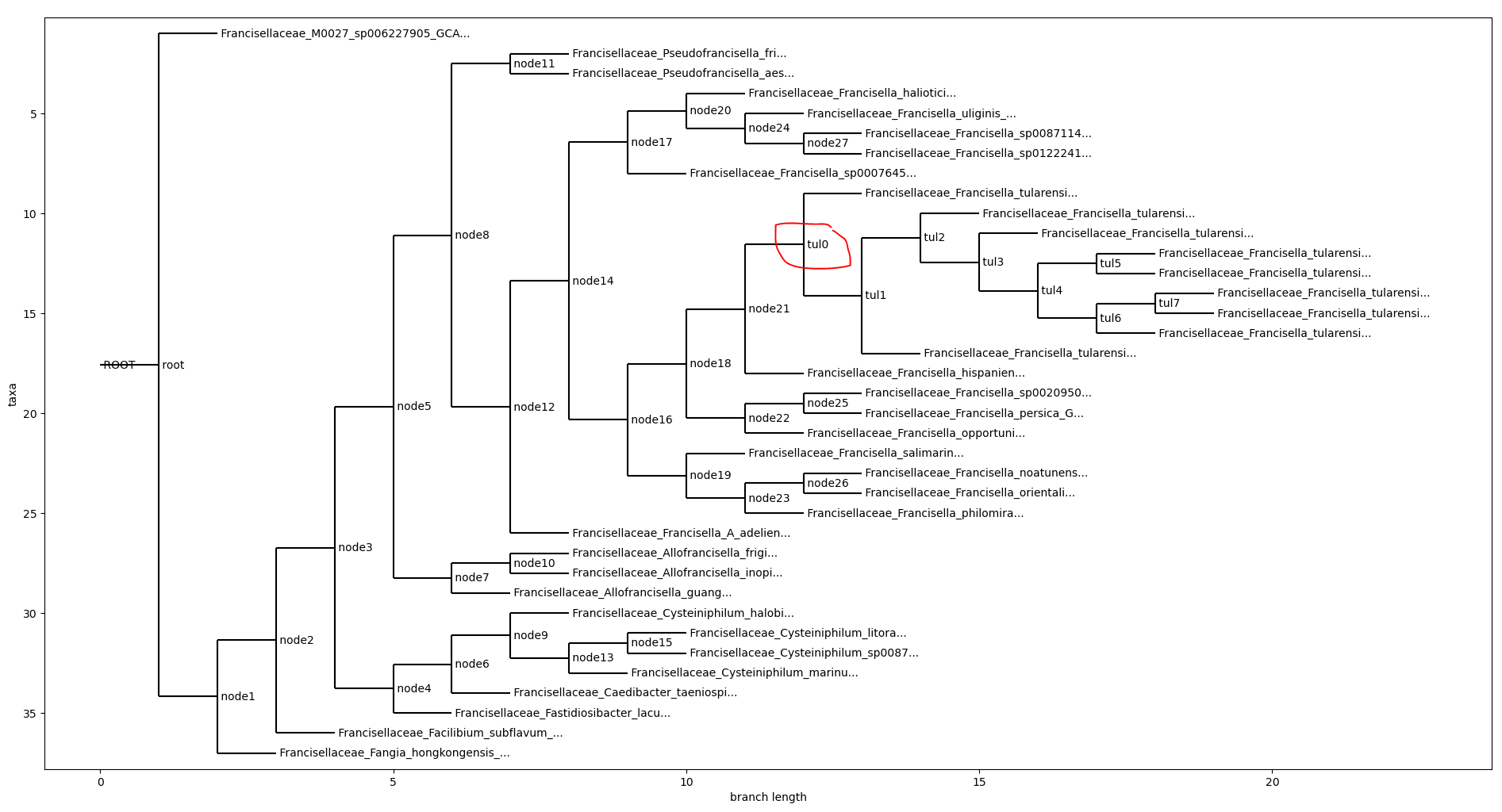
Modify *Francisellaceae* database using FlexTaxD, specifying the node to replace:  
>> flextaxd -db francisellaceae.db --mod\_file tularensis/derep\_genomes\_tree2tax.tsv --genomeid2taxid tularensis/derep\_genomes\_map.tsv --replace --parent Francisellaceae\_Francisella\_tularensis\_GCF\_000008985.1

Visualize database:  
>> flextaxd -db francisellaceae.db --vis\_type tree --visualise\_node root --vis\_depth 0

  
Phylogenetic tree of *Francisellaceae* with *tularensis*-branch expanded. The red marking indicates where the representative *tularensis*-dataset has been replaced by our ANI-based dereplication method (nodes with basename “tul”) to increase the resolution of this species.

Rename node using FlexTaxD (requires update of FlexTaxD, this is a new function):  
>> flextaxd -db francisellaceae.db --rename\_from Francisellaceae\_Francisella\_tularensis\_GCF\_000008985.1 --rename\_to tul0

Visualize database after renaming:  
>> flextaxd -db francisellaceae.db --vis\_type tree --visualise\_node root --vis\_depth 0

  
Showing the renaming of *tularensis* local root.

## Creating the kraken2 database using FlexTaxD

### Setting up genome directory

Make directory for genome-files:  
>> mkdir genomes\_for\_db

Soft-link *Francisellaceae-*genomes:  
>> find francisellaceae\_repset/genomes/ -name "\*.fasta" -exec ln -s $PWD/{} genomes\_for\_db/ \;

Soft-link *tularensis*-genomes:  
>> find francisellaceae\_repset/genomes/ -name "\*.fasta" -exec ln -s $PWD/{} genomes\_for\_db/ \;

Rename genomes so they are found by FlexTaxD (expected format: “GCx\_0000000.0”):   
>> find genomes\_for\_db/ -name "\*.fasta" | rev | cut -d '\_' -f1,2 | rev | xargs -I {} sh -c 'mv genomes\_for\_db/\*{} genomes\_for\_db/{}'

### Creating database

Dump FlexTaxD-database (make sure to specify kraken2-format):  
>> flextaxd -db francisellaceae.db --dump –-dbprogram kraken2 -o fdb

Create kraken2 database:  
>> flextaxd-create -db francisellaceae.db --create\_db --dbprogram kraken2 -o fdb --db\_name kraken2.francisellaceae\_tularensisExpand --genomes\_path genomes\_for\_db

#### Developer comment

The flextaxd-create command (lower) should include the database-dump command (upper). The lower command requires the –db argument to be specified. If the users does not input –dbprogram to the upper command, the resulting kraken2 database lacks information. The current setup is not intuitive and is redundant.

## Testing the kraken2 database

To test the database we run kraken2 using a mock dataset. The mock dataset will contain a known amount of randomly generated reads from the genomes that were downloaded. We will generate a similar amount of reads for the representative species genomes that was obtained when constructing the *Francisellaceae* database. Then, we will select three *tularensis* species and generate reads for these genomes. Finally, the mock dataset is input to kraken2 to validate assignment.

### Generating mock dataset

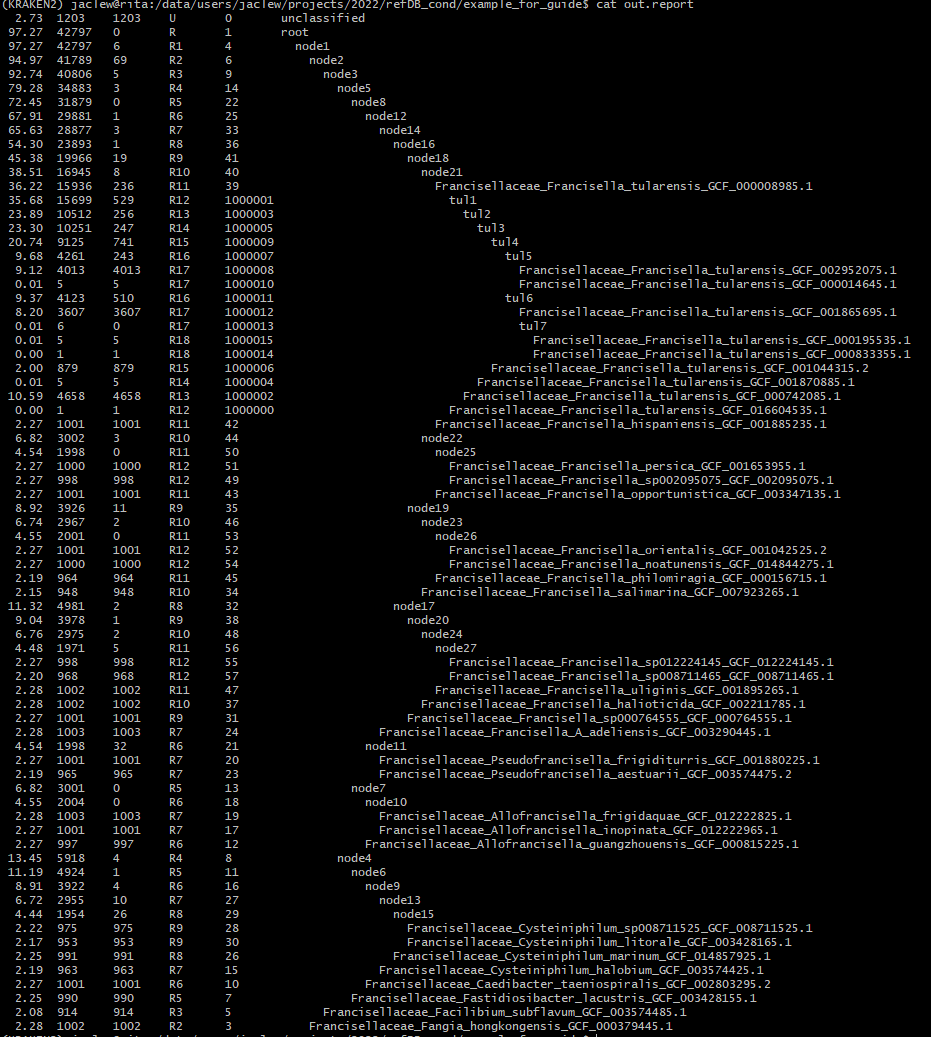
This file may exist for download on github: “mock\_dataset.fasta.gz”.

Generate 1000 reads from all representative genomes excluding *tularensis* datasets:  
>> find francisellaceae\_repset/genomes/ -exec basename {} \; | cut -d '.' -f1,2 | xargs -I {} sh -c 'fasta\_simulate\_sequences.py francisellaceae\_repset/genomes/\*{}\*fasta --num\_seqs 1000 --basename {}' >> mock\_dataset.fasta

Generate 5000 reads from three *tularensis* species, closest to tul1, tul5, and tul6 branches:  
>> fasta\_simulate\_sequences.py tularensis/genomes\_derep\_representants/Francisellaceae\_Francisella\_tularensis\_GCF\_000742085.1.fasta --num\_seqs 5000 --basename tul1 >> mock\_dataset.fasta  
>> fasta\_simulate\_sequences.py tularensis/genomes\_derep\_representants/Francisellaceae\_Francisella\_tularensis\_GCF\_002952075.1.fasta --num\_seqs 5000 --basename tul5 >> mock\_dataset.fasta  
>> fasta\_simulate\_sequences.py tularensis/genomes\_derep\_representants/Francisellaceae\_Francisella\_tularensis\_GCF\_001865695.1.fasta --num\_seqs 5000 --basename tul6 >> mock\_dataset.fasta

### Running kraken2 classification

Classify mock dataset:  
>> kraken2 --db kraken2.francisellaceae\_tularensisExpand/ --threads 30 mock\_dataset.fasta.gz --report kraken2.report --output kraken2.reads\_classified

Shown below is the output from kraken2.report. The expected number of reads at each reference is 1000 for non-*tularensis* species and 5000 for the listed *tularensis* species.  


In the output summary below, the number of assigned reads are in the first column to the left. The *tularensis* species are underlined with red marker. The expected number of reads at each reference is 1000 for non-*tularensis* species and 5000 for the listed *tularensis* species.  
