Module 6: Pathogen Variant Calling
Day2: Part3 Practical

Introduction

In this module we will be analyzing the case of four *Mycobacterium tuberculosis* isolates (3 multi-drug resistant and 1 susceptible) from TB patients to investigate following aspects from whole genome sequence data:

1. Genetic mechanisms of resistance
2. Identify resistance to other anti-Tb drugs
3. Determine the genetic relatedness (pairwise SNP difference)
4. Understand their phylogenetic relationship

To answer each of these questions we will first perform the analysis on one isolate and the steps would be repeated for others as part of the assignment.

Today we will try and answer the last two questions from above. Before we jump into the steps for analysis, we need to make sure we have all the required tools/softwares installed and a dataset in one place. Follow the following steps:

- Open the Terminal and determine the current working directory

  ```
  pwd
  cd
  ```

- Go to the folder "practical"

  ```
  cd course_data/variant_calling/pathogen/practical/MD001
  ```

- To ensure the tools are installed properly the following commands when typed in the terminal must not generate any error. The command figtree will open another window, please close it we will require this later in the practical.

  ```
  bcftools
  snp-sites -h
  snp-dists -h
  iqtree -h
  figtree
  ```

3: Inferring genetic relatedness

3.1 create a zipped file of the high-quality variants identified in step 2.7

```
bcftools view -O z -o MD001_filtered.vcf.gz MD001_SNPs_filtered.vcf
bcftools index MD001_filtered.vcf.gz
```

3.2 generating pseudogenome

```
bcftools consensus -f reference.fa MD001_filtered.vcf.gz > MD001_consensus.fa
```

3.4 renaming the pseudogenome

```
sed 's|^>.*|>MD001|' MD001_consensus.fa >MD001_pseudogenome.fa
```

3.5 Filter all high quality homozygous SNPs removing those in repetitive regions (file: MtbRepetitiveElemetsDrgenes.bed)

```
cp ../../dataset/MtbRepetitiveElemetsDrgenes.bed ./
```

```
bcftools filter -T MtbRepetitiveElemetsDrgenes.bed -i 'type="snp" && QUAL>=50 && FORMAT/DP>5 && MQ>=30 && DP4[2]/(DP4[2]+DP4[0])>=0.80 && DP4[3]/(DP4[3]+DP4[1])>=0.80' -g10 -G10 MD001_variants.vcf -o MD001_SNPs_repfiltered.vcf
```

3.6 create a zipped file of the high-quality variants

```
bcftools view -O z -o MD001_repfiltered.vcf.gz MD001_SNPs_repfiltered.vcf
```

```
bcftools index MD001_repfiltered.vcf.gz
```

3.7 generate pseudogenome

bcftools consensus -f reference.fa MD001_repfiltered.vcf.gz > MD001_repfiltered_consensus.fa

3.8 renaming the pseudogenome

sed 's|^>.*|>MD001_repfiltered|' MD001_repfiltered_consensus.fa >MD001_repfiltered_pseudogenome.fa

3.9 Now we have two pseudogenomes for MD001 isolates:

MD001_pseudogenome.fa : It has all the SNPs identified including those in repetitive regions

MD001_repfiltered_pseudogenome.fa : It has all the SNPs excluding those identified in repetitive regions

3.10 Concatenate the files

cat reference.fa MD001_pseudogenome.fa MD001_repfiltered_pseudogenome.fa >concatenated_alignment.fa

3.11 Identifying the variable sites (snp-sites) only

snp-sites -o snpsitesOut.fa concatenated_alignment.fa

You can determine the length of the alignment using the following bash command:

sed -n '2p' snpsitesOut.fa | wc

In the above command "-n" allows printing lines, '2p' states 2nd line to print and 'wc' counts the words in the line

Q3.1: What is the length of the alignment in "snpsitesOut.fa" file?

3.12 Identifying the pairwise genetic distance (pairwise SNPs) among the three pseudogenomes

snp-dists snpsitesOut.fa >matrix.tsv

Q3.2: What is the pairwise SNP difference between the following pairs: MD001--repMD001 and NC00962.3 – repMD001?

3.13 creating a phylogenetic tree

iqtree -s snpsitesOut.fa >tree1.log

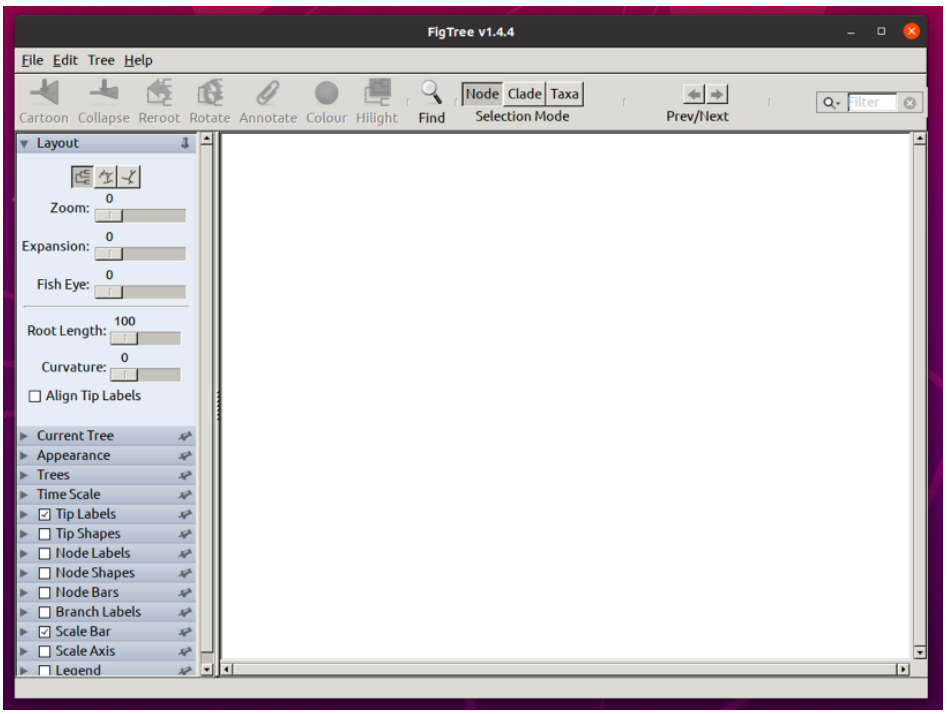
3.14 rename the file snpsitesOut.fa.treefile just created

mv snpsitesOut.fa.treefile MD001.treefile

Visualization of the tree

3.15 In the terminal type the command:
figtree

3.16 This will open a new window as shown



3.17 Open the tree file created in step 3.14 by clicking on the file tab on the top left corner and selecting the option “Open” (figure2). This will open another window, go to the “practical” folder where you have the tree file and load it in FigTree by clicking on “open” tab as show

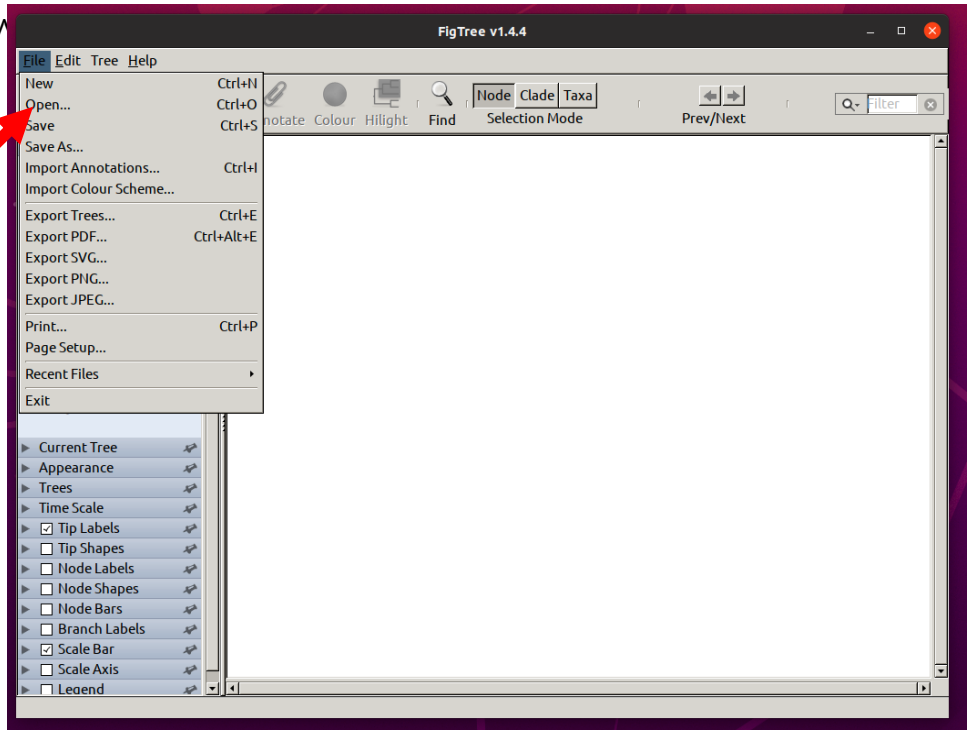


Figure 2

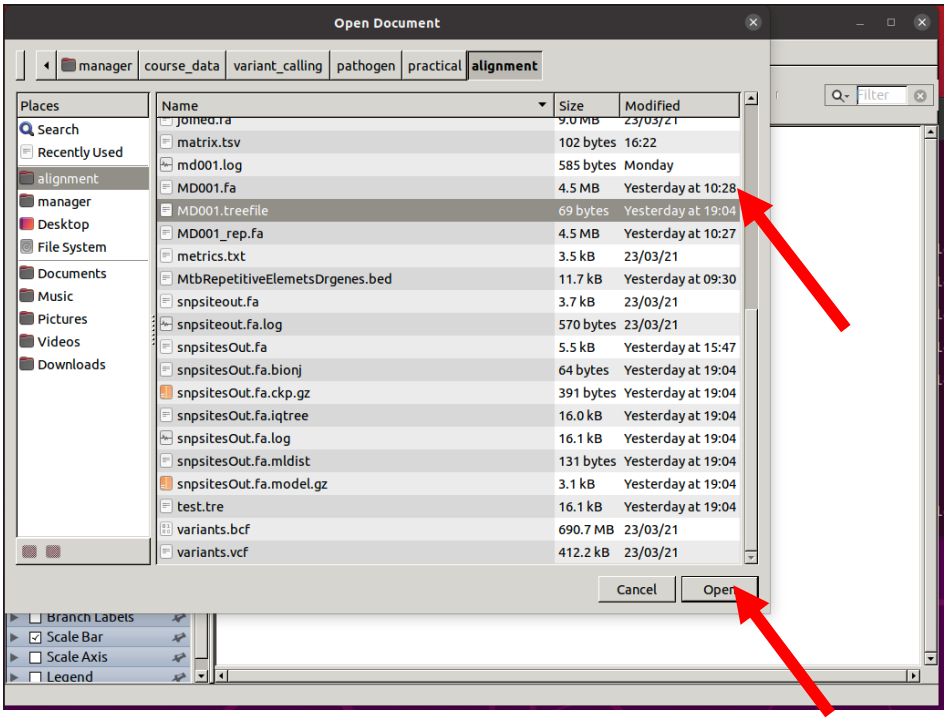
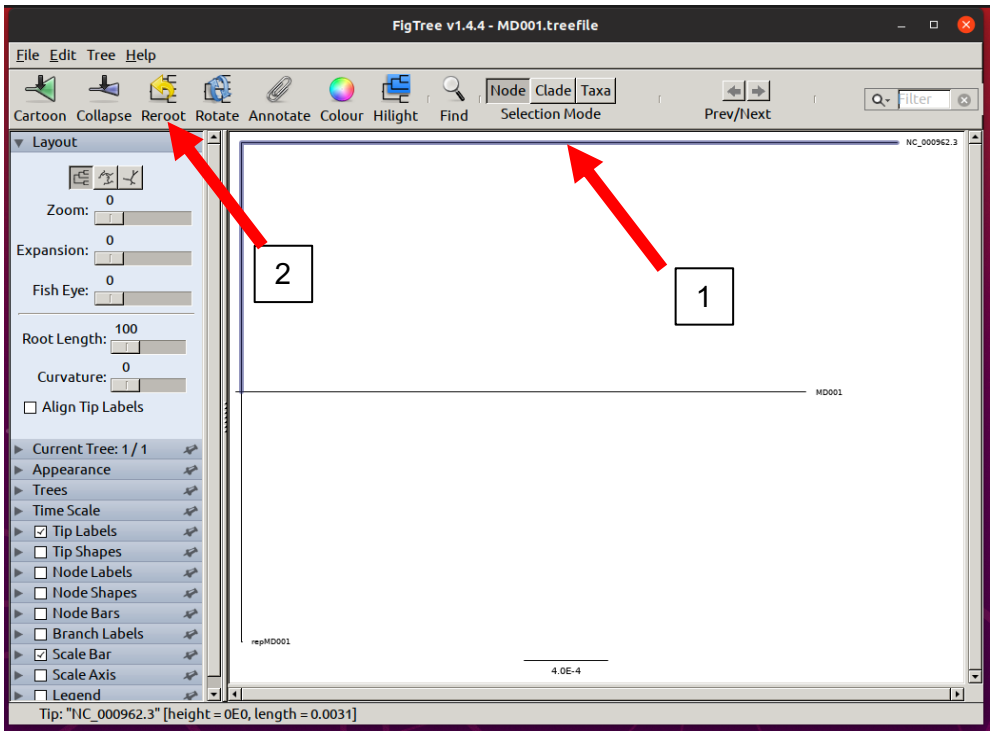
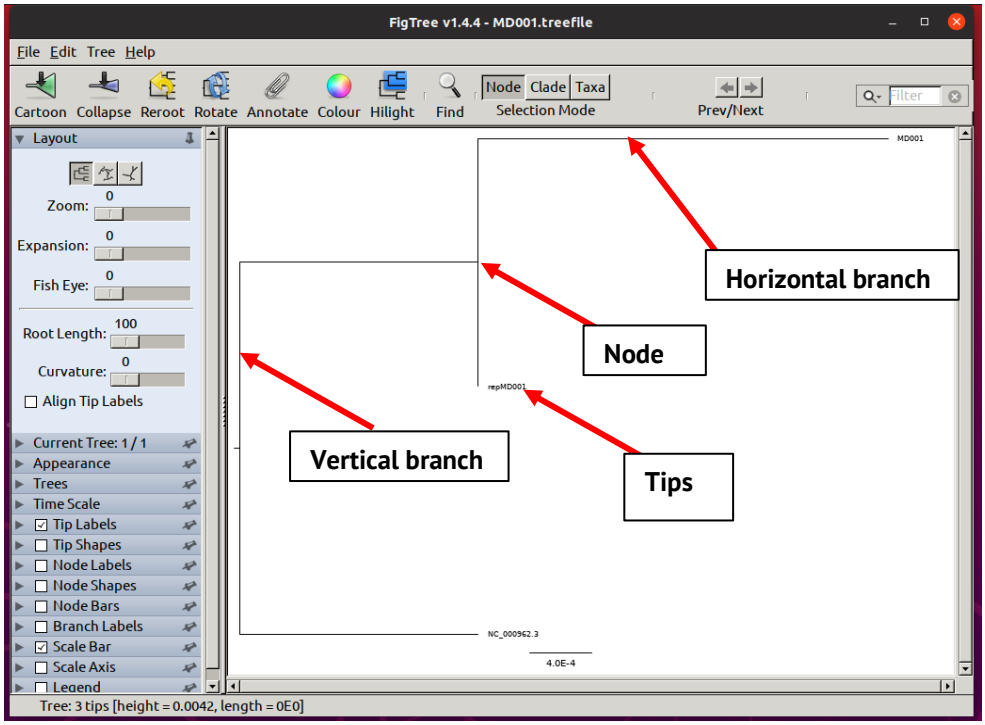


Figure 3

3.18 Now you will be able to see the midpoint rooted phylogeny with three tips. Select the branch of NC_000962.3 (arrow 1) and then click on “reroot” (arrow 2) option on the top.



3.19 This is the final phylogenetic tree. The horizontal branches show the number of differences (longer the branch more are the SNPs for a particular isolate/strain). The two pseudogenomes that we created in this practical for isolates MD001 are closely related compared to reference. The branch of MD001 is longer than repMD001, guess why?



Assignment

Now that we have learnt the process of mapping to a reference, variant calling, variant filtering, pseudogenome creation, determination of pairwise SNP difference and creating a phylogenetic tree. Your next exercise would be to run the same series of steps for the other three isolates (MD003, MD012, MD024) to create pseudogenomes and then create a phylogenetic tree of the isolates (reference, MD001, MD003, MD012 and MD024) along with reference. Please follow the following steps:
The sequence reads for the three isolates are in
"course_data/variant_calling/pathogen/dataset" folder.
Step 1: Create separate folders for each of the three isolates

Step 2: Follow the steps until 3.9 (both day1 and Day2 practicals) in order to create pseudogenomes (using the repfiltered SNPs: step: 3.5) for each of the three isolates in the manual

Step 3: Concatenate the pseudogenomes of all 4 isolates together with the reference as per step 3.10, for example:

cat reference.fa MD001_ repfiltered_pseudogenome.fa MD003_ repfiltered_pseudogenome.fa MD012_ repfiltered_pseudogenome.fa MD024_ repfiltered_pseudogenome.fa > combined_aln.fa

Step 4: Follow the steps 3.11 to 3.19 to create the phylogenetic tree.

Once you have completed the steps above answer the following questions and submit it to vula.

Q3.3 : What is the pairwise SNP difference for following pairs:
a) MD001-MD003:
b) MD012-MD024:
c) MD003-MD012:
d) MD001-MD024:

Q3.4 : Report the resistance conferring variant identified for the following isolate drug combination: (record the position/coordinate and the mutation identified in the cases where isolates are resistant)

Isolate	Drug	Gene	Position	Genotype (R/S)
MD003	Isoniazid	*katG*		
MD003	Streptomycin	*rpsL*		
MD012	Rifampicin	*rpoB*		
MD012	Fluoroquinolone	*gyrA*		
MD024	Isoniazid	*katG*		
MD024	Streptomycin	*rpsL*		
MD024	Rifampicin	*rpoB*		
MD024	Fluoroquinolone	*gyrA*		