QIIME2 - Quantitative Insights Into Microbial Ecology

Command Line Hands On Tutorial

Logging on to the server

```
ssh -p 44111 <USERNAME>@gateway.training.ncgr.org

# Make it a practice to start a screen before you begin analysis

screen -S QIIME2
```

Setting up working directory for QIIME2 hands on analysis

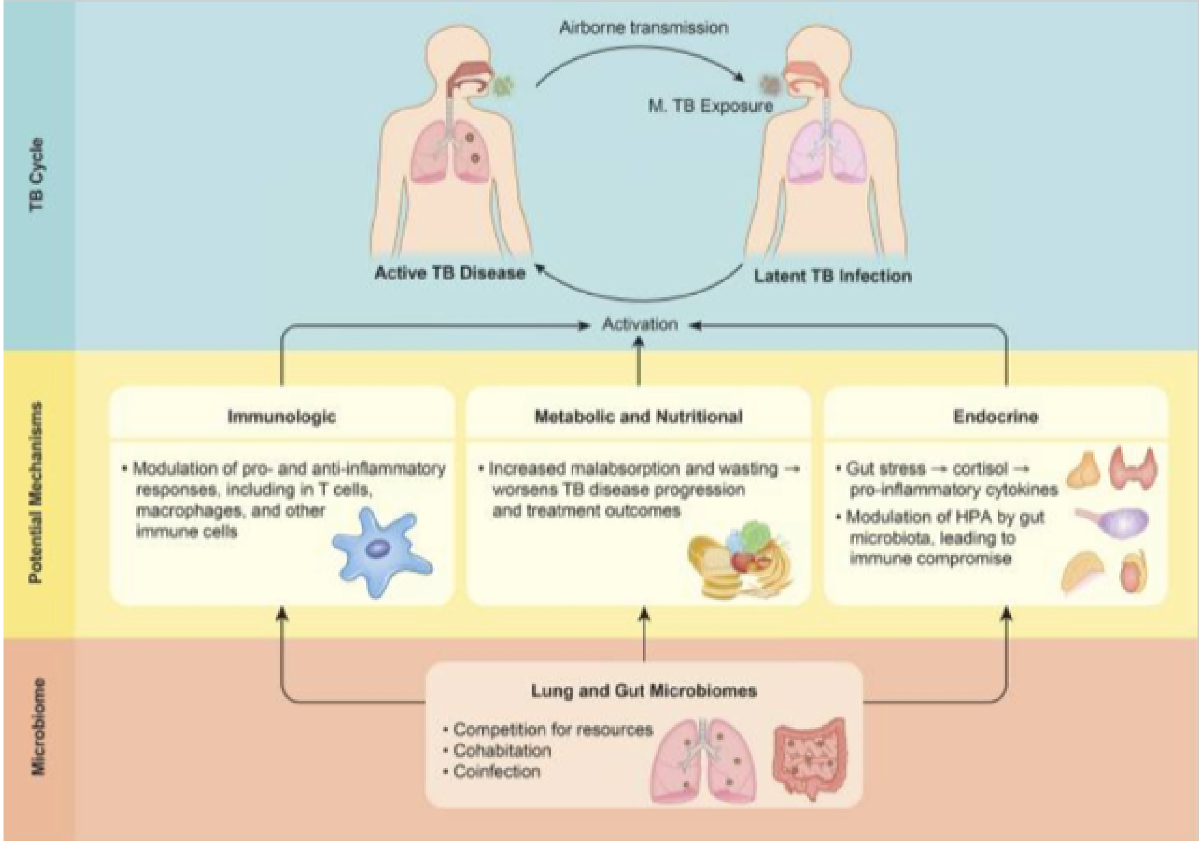
```
cd /home/<USERNAME>/

mkdir QIIME2_Analysis

cd QIIME2_Analysis
```

Data set for analysis

For this tutorial, we will use data from the following published manuscript, "Respiratory tract clinical sample selection for microbiota analysis in patients with pulmonary tuberculosis",

https://microbiomejournal.biomedcentral.com/articles/10.1186/2049-2618-2-29

Pulmonary Tuberculosis (The Human Microbiome in the Fight Against Tuberculosis, 2017)


TB Cycle
Airborne transmission
M. TB Exposure
Active TB Disease
Latent TB Infection
Activation
Potential Mechanisms
Immunologic
• Modulation of pro- and anti-inflammatory responses, including in T cells, macrophages, and other immune cells
Metabolic and Nutritional
• Increased malabsorption and wasting → worsens TB disease progression and treatment outcomes
Endocrine
• Gut stress → cortisol → pro-inflammatory cytokines
• Modulation of HPA by gut microbiota, leading to immune compromise
Microbiome
Lung and Gut Microbiomes
• Competition for resources
• Cohabitation
• Coinfection

Why study microbiome in relation to TB?

1. Microbiome composition is a potential risk factors for TB infection and disease susceptibility, disease progression, and treatment outcomes
2. TB infection and disease can affect microbiome, subsequently impacting health via alteration of immune responses

This pilot study was carried out using sputum, oropharynx, and nasal respiratory tract samples collected from patients with pulmonary tuberculosis and healthy control individuals, in order to compare sample types and their usefulness in assessing changes in bacterial and fungal communities.

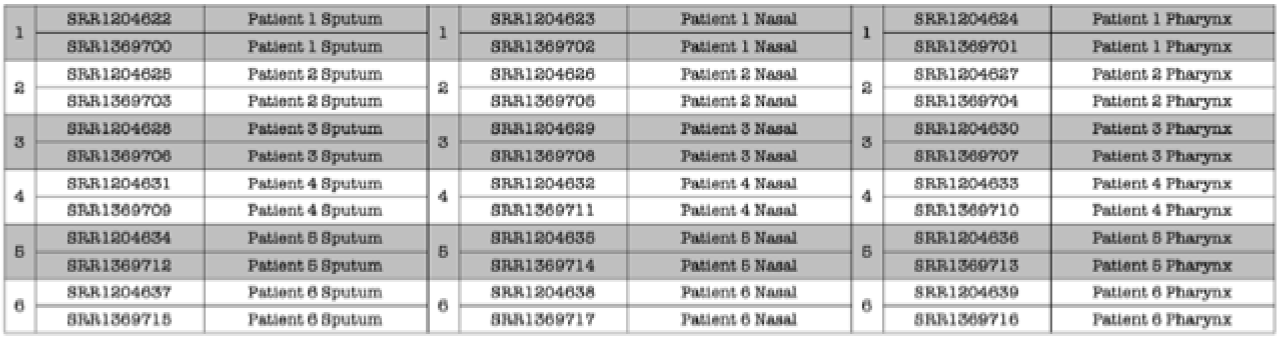
Data was downloaded from the National Center for Biotechnology Information (NCBI) based on the SRA accession numbers reported in the manuscript - PRJNA242354.

A program called fastq-dump version 2.10.0 was used to download the data directly from NCBI SRA database. Tbe following exercise will help you use fastq-dump for your own projects too.

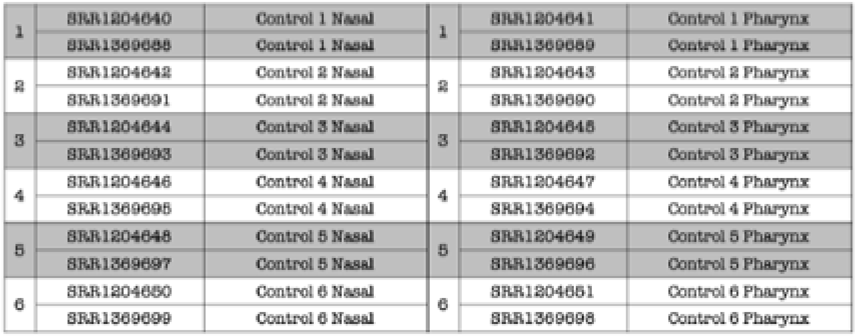
```
source activate fastqdump

# MAKE SURE YOU ARE IN THE FOLLOWING FOLDER

/home/$USER/QIIME2_Analysis

# CREATE A FOLDER FOR DOWNLOADING FASTQ FILES TO ANALYZE
```

```
mkdir input_data

cd input_data

fastq-dump --gzip --outdir ./  \
SRR1204628 SRR1204630 SRR1204627 SRR1204629 SRR1204626 SRR1204623 \
SRR1204625 SRR1204624 SRR1204622 SRR1204631 SRR1204638 SRR1204642 \
SRR1204633 SRR1204632 SRR1204634 SRR1204639 SRR1204646 SRR1204647 \
SRR1204641 SRR1204640 SRR1204636 SRR1204637 SRR1369696 SRR1369694 \
SRR1204635 SRR1204643 SRR1204644 SRR1204645 SRR1369709 SRR1369707 \
SRR1369690 SRR1369691 SRR1369692 SRR1369693 SRR1369710 SRR1369713 \
SRR1204650 SRR1204651 SRR1369688 SRR1369689 SRR1369695 SRR1204649 \
SRR1204648 SRR1369697 SRR1369704 SRR1369703 SRR1369706 SRR1369705 \
SRR1369708 SRR1369698 SRR1369699 SRR1369701 SRR1369700 SRR1369702 \
SRR1369714 SRR1369717 SRR1369716 SRR1369715 SRR1369711 SRR1369712

# --outdir -> Path to Output directory
# --gzip -> Compress output files using gzip


# After running this step, you will have 60 files in your input_data folder
# To check, cd into folder that has fastq files, type this command

ls -l | wc -l

# Optional exercise:
# Count the number of sequences in the two fastq files pertaining to one sample and sum them.

conda deactivate

# Filter poor quality reads
source activate fastp

for file in *.fastq; do fastp -i ${file} -o ${file}.filtered; done

conda deactivate
```

SRR FILE TO SAMPLE CORRESPONDENCE TABLE



1	SRR1204622	Patient 1 Sputum	1	SRR1204623	Patient 1 Nasal	1	SRR1204624	Patient 1 Pharynx
	SRR1369700	Patient 1 Sputum		SRR1369702	Patient 1 Nasal		SRR1369701	Patient 1 Pharynx
2	SRR1204625	Patient 2 Sputum	2	SRR1204626	Patient 2 Nasal	2	SRR1204627	Patient 2 Pharynx
	SRR1369703	Patient 2 Sputum		SRR1369705	Patient 2 Nasal		SRR1369704	Patient 2 Pharynx
3	SRR1204628	Patient 3 Sputum	3	SRR1204629	Patient 3 Nasal	3	SRR1204630	Patient 3 Pharynx
	SRR1369706	Patient 3 Sputum		SRR1369708	Patient 3 Nasal		SRR1369707	Patient 3 Pharynx
4	SRR1204631	Patient 4 Sputum	4	SRR1204632	Patient 4 Nasal	4	SRR1204633	Patient 4 Pharynx
	SRR1369709	Patient 4 Sputum		SRR1369711	Patient 4 Nasal		SRR1369710	Patient 4 Pharynx
5	SRR1204634	Patient 5 Sputum	5	SRR1204635	Patient 5 Nasal	5	SRR1204636	Patient 5 Pharynx
	SRR1369712	Patient 5 Sputum		SRR1369714	Patient 5 Nasal		SRR1369713	Patient 5 Pharynx
6	SRR1204637	Patient 6 Sputum	6	SRR1204638	Patient 6 Nasal	6	SRR1204639	Patient 6 Pharynx
	SRR1369715	Patient 6 Sputum		SRR1369717	Patient 6 Nasal		SRR1369716	Patient 6 Pharynx



1	SRR1204640	Control 1 Nasal	1	SRR1204641	Control 1 Pharynx
	SRR1369688	Control 1 Nasal		SRR1369689	Control 1 Pharynx
2	SRR1204642	Control 2 Nasal	2	SRR1204643	Control 2 Pharynx
	SRR1369691	Control 2 Nasal		SRR1369690	Control 2 Pharynx
3	SRR1204644	Control 3 Nasal	3	SRR1204645	Control 3 Pharynx
	SRR1369693	Control 3 Nasal		SRR1369692	Control 3 Pharynx
4	SRR1204646	Control 4 Nasal	4	SRR1204647	Control 4 Pharynx
	SRR1369695	Control 4 Nasal		SRR1369694	Control 4 Pharynx
5	SRR1204648	Control 5 Nasal	5	SRR1204649	Control 5 Pharynx
	SRR1369697	Control 5 Nasal		SRR1369696	Control 5 Pharynx
6	SRR1204650	Control 6 Nasal	6	SRR1204651	Control 6 Pharynx
	SRR1369699	Control 6 Nasal		SRR1369698	Control 6 Pharynx

Data was collected from 30 Individuals.

- 6 TB Patients - Nasal
- 6 TB Patients - oropharynx
- 6 TB Patients - Sputum
- 6 Healthy Controls - Nasal
- 6 Healthy Controls - oropharynx

DATA TABLE:

SAMPLE FASTA FILE	TOTAL NUMBER OF READS
Control_1_Nasal_L001_R1_001.fasta	41,056
Control_1_Pharynx_L001_R1_001.fasta	119,972
Control_2_Nasal_L001_R1_001.fasta	32,142
Control_2_Pharynx_L001_R1_001.fasta	42,973
Control_3_Nasal_L001_R1_001.fasta	32,953
Control_3_Pharynx_L001_R1_001.fasta	39,148
Control_4_Nasal_L001_R1_001.fasta	83,174
Control_4_Pharynx_L001_R1_001.fasta	17,475
Control_5_Nasal_L001_R1_001.fasta	29,146
Control_5_Pharynx_L001_R1_001.fasta	32,728
Control_6_Nasal_L001_R1_001.fasta	32,738
Control_6_Pharynx_L001_R1_001.fasta	15,320
Patient_1_Nasal_L001_R1_001.fasta	69,995
Patient_1_Pharynx_L001_R1_001.fasta	59,646
Patient_1_Sputum_L001_R1_001.fasta	66,725
Patient_2_Nasal_L001_R1_001.fasta	61,070
Patient_2_Pharynx_L001_R1_001.fasta	27,106
Patient_2_Sputum_L001_R1_001.fasta	71,434
Patient_3_Nasal_L001_R1_001.fasta	91,920
Patient_3_Pharynx_L001_R1_001.fasta	36,224
Patient_3_Sputum_L001_R1_001.fasta	27,533
Patient_4_Nasal_L001_R1_001.fasta	28,996
Patient_4_Pharynx_L001_R1_001.fasta	30,713
Patient_4_Sputum_L001_R1_001.fasta	59,433
Patient_5_Nasal_L001_R1_001.fasta	70,843
Patient_5_Pharynx_L001_R1_001.fasta	39,674
Patient_5_Sputum_L001_R1_001.fasta	29,576
Patient_6_Nasal_L001_R1_001.fasta	106,178
Patient_6_Pharynx_L001_R1_001.fasta	58,956
Patient_6_Sputum_L001_R1_001.fasta	35,703

QIIME2

What is QIIME2?- https://docs.qiime2.org/2020.11/about/

QIIME 2 is a powerful microbiome analysis package with a focus on data and analysis transparency. QIIME2 enables researchers to start an analysis with raw DNA sequence data and finish with publication-quality figures and statistical results.

Key features:

- Integrated and automatic tracking of data provenance
- Plugin system for extending microbiome analysis functionality
- Support for multiple types of user interfaces (e.g. API, command line, graphical)

Plugin availability - https://docs.qiime2.org/2020.11/plugins/available/

STEP 1: Setting up your metadata file

Metadata file that we will be using for our data set (mappingFile.txt on logrus)

Here is how you will make a metadata file on your own: https://docs.qiime2.org/2017.6/tutorials/metadata/

#SampleID	Treatment	TreatmentSite	TreatmentNumber	Sex	BodySite	Description
C_1_N	C	CN	1	F	Nasal	Control 1 Nasal
C_1_P	C	CP	2	F	Oropharynx	Control 1 Pharynx
C_2_N	C	CN	1	M	Nasal	Control 2 Nasal
C_2_P	C	CP	2	M	Oropharynx	Control 2 Pharynx
C_3_N	C	CN	1	M	Nasal	Control 3 Nasal
C_3_P	C	CP	2	M	Oropharynx	Control 3 Pharynx
C_4_N	C	CN	1	M	Nasal	Control 4 Nasal
C_4_P	C	CP	2	M	Oropharynx	Control 4 Pharynx
C_5_N	C	CN	1	M	Nasal	Control 5 Nasal
C_5_P	C	CP	2	M	Oropharynx	Control 5 Pharynx
C_6_N	C	CN	1	M	Nasal	Control 6 Nasal
C_6_P	C	CP	2	M	Oropharynx	Control 6 Pharynx
P_1_N	P	PN	3	F	Nasal	Patient 1 Nasal
P_1_P	P	PP	4	F	Oropharynx	Patient 1 Pharynx
P_1_S	P	PS	5	F	Sputum	Patient 1 Sputum
P_2_N	P	PN	3	M	Nasal	Patient 2 Nasal
P_2_P	P	PP	4	M	Oropharynx	Patient 2 Pharynx
P_2_S	P	PS	5	M	Sputum	Patient 2 Sputum
P_3_N	P	PN	3	M	Nasal	Patient 3 Nasal
P_3_P	P	PP	4	M	Oropharynx	Patient 3 Pharynx
P_3_S	P	PS	5	M	Sputum	Patient 3 Sputum
P_4_N	P	PN	3	M	Nasal	Patient 4 Nasal
P_4_P	P	PP	4	M	Oropharynx	Patient 4 Pharynx
P_4_S	P	PS	5	M	Sputum	Patient 4 Sputum
P_5_N	P	PN	3	M	Nasal	Patient 5 Nasal
P_5_P	P	PP	4	M	Oropharynx	Patient 5 Pharynx
P_5_S	P	PS	5	M	Sputum	Patient 5 Sputum
P_6_N	P	PN	3	M	Nasal	Patient 6 Nasal
P_6_P	P	PP	4	M	Oropharynx	Patient 6 Pharynx
P_6_S	P	PS	5	M	Sputum	Patient 6 Sputum

```
# Move out of the input_data directory

cd ../

# Copy metadata file from my workspace to your workspace,

cd /home/$USER/QIIME2_Analysis

cp /home/elavelle/QIIME2_Analysis/mappingFile.txt ./

less mappingFile.txt
```

STEP 2: Import sequences into QIIME artifact- https://docs.qiime2.org/2020.11/tutorials/importing/

We will be beginning our analysis with demultiplexed, quality controlled sequences.

For the purpose of this workshop we will not use the entire dataset per sample. We have created 1000 sequences per sample as subset. This will be your input dataset.

```
cd /home/$USER/QIIME2_Analysis

# Copy the subset file of sequences:

cp /home/metag/data/seqs.1000.fna ./

conda deactivate

source activate qiime2
```

```
# This command will vary depending input data type and format
# Here, we are importing .fasta reads (no quality scores)

qiime tools import \
--input-path seqs.1000.fna \
--output-path seqs.1000.qza \
--type 'SampleData[Sequences]'
```

STEP 3: After importing data, dereplicate and/or denoise

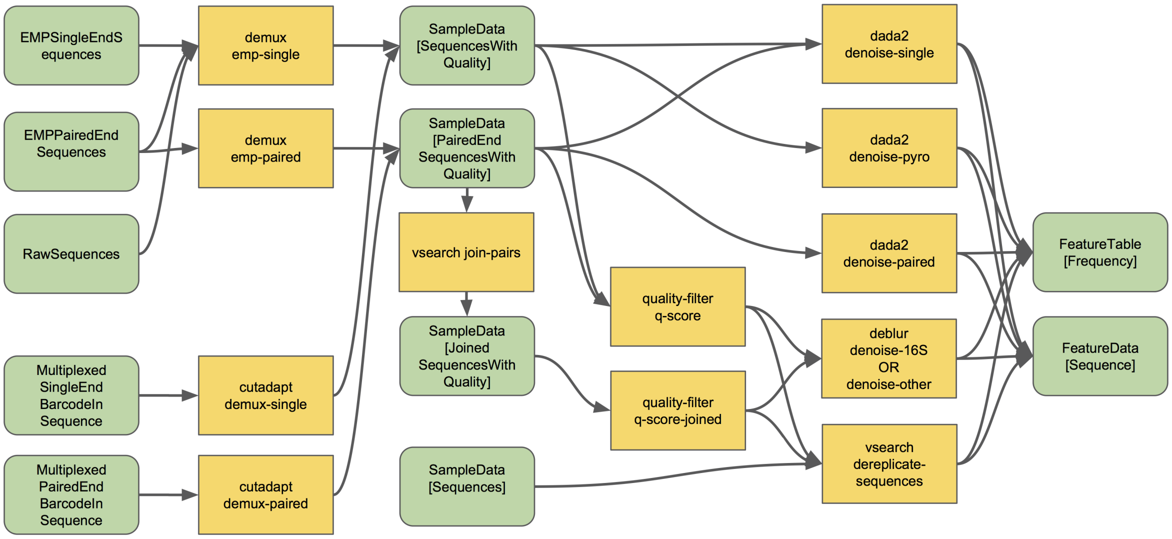
From https://docs.qiime2.org/2021.2/tutorials/overview/#derep-denoise

- We denoise our sequences to remove and/or correct noisy reads.
- We dereplicate our sequences to reduce repetition and file size/memory requirements in downstream steps (don't worry! we keep count of each replicate).
- We cluster sequences to collapse similar sequences (e.g., those that are $\geq$ 97% similar to each other) into single replicate sequences. This process, also known as OTU picking, was once a common procedure, used to simultaneously dereplicate but also perform a sort of quick-and-dirty denoising procedure (to capture stochastic sequencing and PCR errors, which should be rare and similar to more abundant centroid sequences). Use denoising methods instead if you can. Times have changed. Welcome to the future.

```
qiime vsearch dereplicate-sequences \
--i-sequences seqs.1000.qza \
--o-dereplicated-table table.qza \
--o-dereplicated-sequences rep-seqs.qza
```

STEP 4: Generating a Feature table

The final products of all denoising and clustering methods/workflows are a FeatureTable[Frequency] (feature table) artifact and a FeatureData[Sequence] (representative sequences) artifact. These are two of the most important artifacts in an amplicon sequencing workflow, and are used for many downstream analyses, as discussed below. Indeed, feature tables are crucial to any QIIME 2 analysis, as the central record of all observations per sample.



The feature-table summarize command will give you information on how many sequences are associated with each sample and with each feature, histograms of those distributions, and some related summary statistics. The feature-table and tabulate-seqs commands will provide a mapping of feature IDs to sequences, and provide links to easily BLAST each sequence against the NCBI nt database.

```
qiime feature-table summarize \
--i-table table.qza \
--o-visualization table.qzv


qiime feature-table tabulate-seqs \
--i-data rep-seqs.qza \
--o-visualization rep-seqs.qzv
```

Open an example and look at the format of a feature table from mock dataset as well as other tutorials.

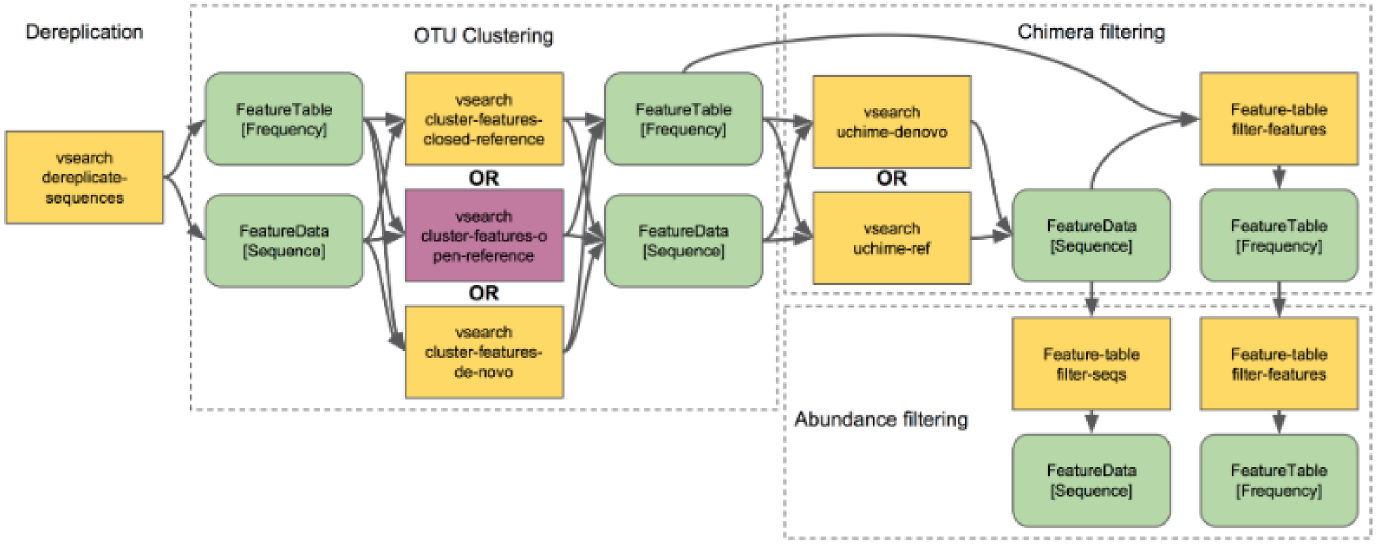
https://view.qiime2.org/visualization/?type=html&src=https%3A%2F%2Fdocs.qiime2.org%2F2020.11%2Fdata%2Ftutorials%2F
pictures%2Ftable.qzv

STEP 5: Setting up reference.

Greengenes (16S rRNA), Silva (16S/18S rRNA) databases has to be imported as QIIME2 artifacts before using them.

```
qiime tools import \
--input-path /home/metag/QIIME2_greengenes/Greengenes_16S_rRNA/\
gg_13_8_otus/rep_set/99_otus.fasta \
--output-path 99_otus.qza \
--type FeatureData[Sequence]
```

STEP 6: Clustering -Clustering - For this dataset, you will only run open -reference OTU Clustering.



There are three types of clustering,

I. De novo Operational Taxonomic Unit (OTU) clustering

De novo OTU picking is used when we dont have a reference sequence collection to cluster against, for example, working with an infrequently used marker gene.

De novo OTU picking cannot be used in the following two scenarios,

1. Comparing non-overlapping amplicons, such as V2 and V4 regions of 16S rRNA
2. Working with very large data sets, e.g., HiSeq

Advantage: No reference is required.

Disadvantage: Run time can be very expensive depending on the volume of data (parallel processing not possible)

```
# EXAMPLE- DO NOT RUN THE ABOVE COMMAND TO COMPLETION.

qiime vsearch cluster-features-de-novo \
--i-table table.qza \
--i-sequences rep-seqs.qza \
--p-perc-identity 0.99 \
--o-clustered-table table-dn-99.qza \
--o-clustered-sequences rep-seqs-dn-99.qza
```

II. Closed-reference OTU clustering

Reads are clustered against a reference sequence collection. Any reads which do not hit a sequence in the reference sequence collection are excluded from downstream analyses.

Closed-reference OTU picking is used:

1. When comparing non-overlapping amplicons, such as V2 and V4 regions of the 16S rRNA.
2. When reference sequences span the sequenced regions.

Closed-reference OTU picking is not used when reference sequence collection is not available

Advantage: Fast, useful for extremely large data sets. Better trees and taxonomy. OTUs are defined in the reference and have a reliable tree/taxonomy.

Disadvantage: Inability to detect novel diversity with respect to your reference sequence collection

```
# EXAMPLE- DO NOT RUN THE ABOVE COMMAND TO COMPLETION

qiime vsearch cluster-features-closed-reference \
--i-table table.qza \
--i-sequences rep-seqs.qza \
--i-reference-sequences 99_otus.qza \
--p-perc-identity 0.99 \
--o-clustered-table table-cr-99.qza \
--o-clustered-sequences rep-seqs-cr-99.qza \
--o-unmatched-sequences unmatched-cr-99.qza
```

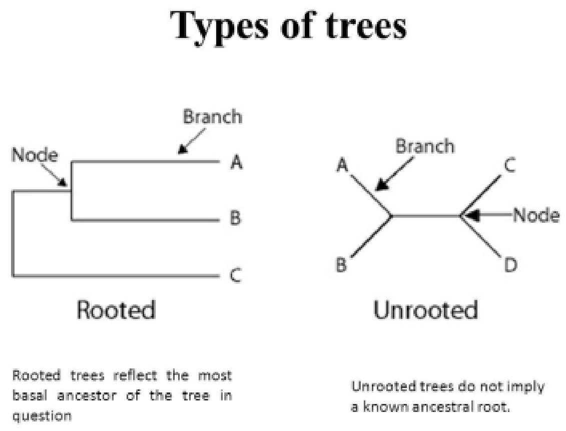
III. Open-reference OTU clustering

In open-reference OTU clustering, sequence reads are clustered against a reference sequence collection and any reads which do not hit the reference sequence collection are subsequently clustered de novo.

```
qiime vsearch cluster-features-open-reference \
--i-table table.qza \
--i-sequences rep-seqs.qza \
--i-reference-sequences 99_otus.qza \
--p-perc-identity 0.99 \
--o-clustered-table table-or-99.qza \
--o-clustered-sequences rep-seqs-or-99.qza \
--o-new-reference-sequences new-ref-seqs-or-99.qza
```

STEP 7: Building a phylogenetic tree

Evolution is the core concept of biology. There is only so much you can learn from microbes while ignoring evolution. Evolution-aware analyses of a dataset needs a phylogenetic tree of its sequences. These trees can be built de-novo or they can be reference based. Phylogeny-based analyses in QIIME2 generally need a rooted tree.



Rooted trees reflect the most basal ancestor of the tree in question

Unrooted trees do not imply a known ancestral root.

```
# Carry out a multiple sequence alignment using Mafft

qiime alignment mafft \
--i-sequences rep-seqs-or-99.qza \
--o-alignment aligned-rep-seqs.qza

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 4 MINUTES TO RUN TO COMPLETION

# Mask (or filter) the alignment to remove positions that are highly variable.
# These positions are generally considered to add noise to a
# resulting phylogenetic tree.

qiime alignment mask \
--i-alignment aligned-rep-seqs.qza \
--o-masked-alignment masked-aligned-rep-seqs.qza

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 4 MINUTES TO RUN TO COMPLETION

# Create the tree using the Fasttree program

qiime phylogeny fasttree \
--i-alignment masked-aligned-rep-seqs.qza \
--o-tree unrooted-tree.qza

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 9 MINUTES TO RUN TO COMPLETION

# root the tree using the longest root

qiime phylogeny midpoint-root \
--i-tree unrooted-tree.qza \
--o-rooted-tree rooted-tree.qza

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 6 SECONDS TO RUN TO COMPLETION
```

STEP 8: Alpha rarefaction

What is rarefaction?

Species richness is the number of species present in a system. However, the deeper you sequence, the more species you will find. That can be problematic if you gathered 500 reads from one sample and only 100 reads from another sample- you would expect to find more species if you sequenced 5x as many reads! To account for this, we perform an in-silico (i.e., on your computer) experiment called rarefaction. A rarefaction is a random collection of sequences from a sample, with a specified depth (number of sequences). For example, a rarefaction with a depth of 75 reads per sample is a simulation of what your sequencing results would look like if you sequenced exactly 75 reads from each sample. To look at alpha diversity systematically, we can perform many rarefactions: at multiple depths and repeat many times at each depth. In QIIME, this task is performed on your OTU table.

- Concerns:
  - Too low: ignore a lot of samples' information
  - Too high: ignore a lot of samples

```
cp /home/elavelle/mappingFile.txt ./

qiime diversity alpha-rarefaction \
--i-table table-or-99.qza \
--i-phylogeny rooted-tree.qza \
--p-max-depth 1000 \
--m-metadata-file mappingFile.txt \
--o-visualization alpha-rarefaction.qzv

# Why specify a maximum depth of 1000?
```

Look at the following example and decide what sampling-depth you would use:

https://view.qiime2.org/visualization/?type=html&src=https%3A%2F%2Fdocs.qiime2.org%2F2020.11%2Fdata%2Ftutorials%2F
pictures%2Falpha-rarefaction.qzv

STEP 9: Calculating and exploring diversity metrics

Species diversity is a valuable tool for describing the ecological complexity of a single sample (alpha diversity) or between samples (beta diversity).

However, diversity is not a physical quantity that can be measured directly, and many different metrics have been proposed to quantify diversity

Species diversity consists of three components: species richness, taxonomic or phylogenetic diversity and species evenness.

1. Species richness = the number of different species in a community.
2. Species evenness = how close the species abundencies in a community are.
3. Phylogenetic diversity = how closely related the species in a community are.

QIIME 2 calculates comprehensive set of metrics for you with one command

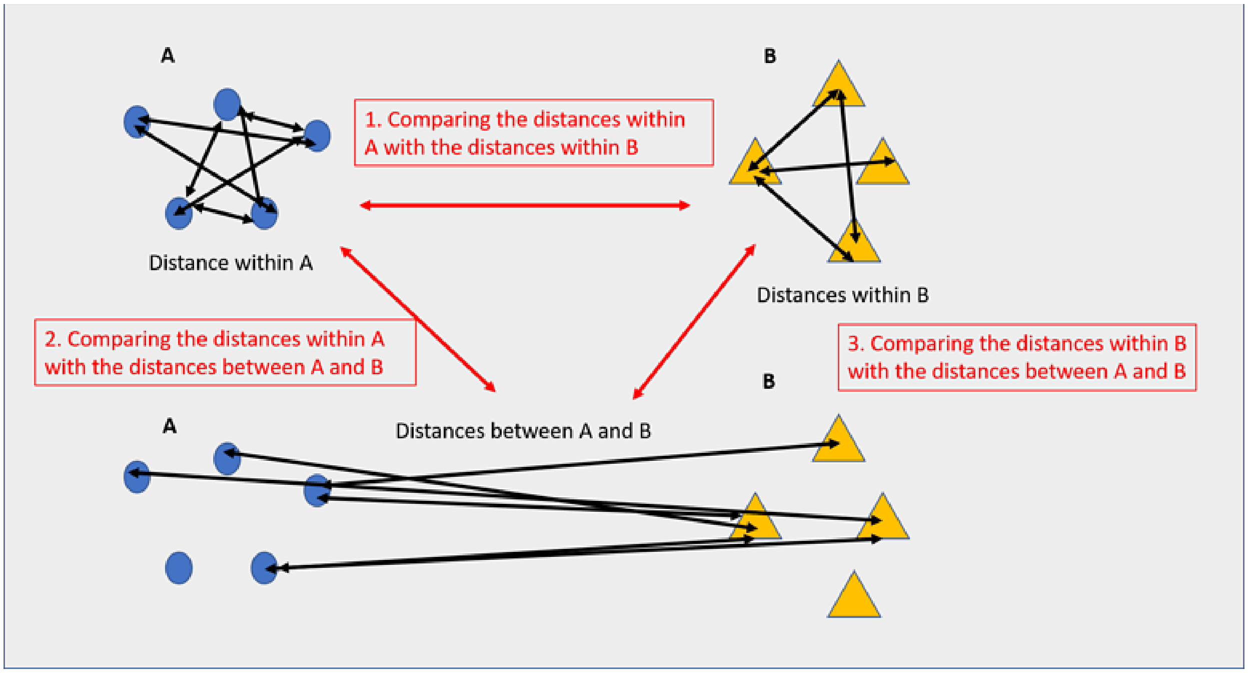
Alpha diversity metrics

- Observed Features (a measure of community richness, called "observed OTUs" here for historical reasons)
- Evenness (or Pielou's Evenness; a measure of community evenness; Pielou, 1966)
- Shannon Diversity Index (a measure of community diversity; Shannon & Weaver, 1949)
- Faith's Phylogenetic Diversity (a measure of diversity that incorporates phylogenetic relationships between the features; Faith, 1992); this metric is sometimes referred to as PD_whole_tree, but we discourage the use of that name in favor of Faith's Phylogenetic Diversity or Faith's PD

Beta diversity metrics

- Jaccard distance (qualitative comparison: # shared OTUs / # union OTUs *100 ; (Jaccard, 1908)
- Bray-Curtis distance (a quantitative measure of community dissimilarity- includes abundance; Sørensen, 1948)
- unweighted UniFrac distance (a qualitative measure of community dissimilarity- analogous to Jaccard- that incorporates phylogenetic relationships between the features; Lozupone & Knight, 2005); implementation based on Striped UniFrac (McDonald et al., 2018) method
- weighted UniFrac distance (a quantitative measure of community dissimilarity- analogous to Bray-Curtis- that incorporates phylogenetic relationships between the features; Lozupone, Hamady, Kelley, & Knight, 2007); implementation based on Striped UniFrac (McDonald et al., 2018) method.

```
# Generate diversity metrics

qiime diversity core-metrics-phylogenetic \
--i-phylogeny rooted-tree.qza \
--i-table table-or-99.qza \
--p-sampling-depth 1000 \
--m-metadata-file mappingFile.txt \
--output-dir core-metrics-results

# SEVERAL FILES WILL BE GENERATED IN A FOLDER/DIRECTORY TITLED "core-metrics-results"
# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 17 SECONDS TO RUN TO COMPLETION

qiime diversity alpha-group-significance \
--i-alpha-diversity core-metrics-results/faith_pd_vector.qza \
--m-metadata-file mappingFile.txt \
--o-visualization core-metrics-results/faith-pd-group-significance.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime diversity alpha-group-significance \
--i-alpha-diversity core-metrics-results/evenness_vector.qza \
--m-metadata-file mappingFile.txt \
--o-visualization core-metrics-results/evenness-group-significance.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime diversity alpha-group-significance  \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file mappingFile.txt \
--o-visualization core-metrics-results/shannon_group-significance.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime diversity beta-group-significance \
--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
--m-metadata-file mappingFile.txt \
--m-metadata-column BodySite \
--o-visualization core-metrics-results/unweighted-unifrac-body-site-significance.qzv \
--p-pairwise

#THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime diversity beta-group-significance \
--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \
--m-metadata-file mappingFile.txt \
--m-metadata-column Sex \
--o-visualization core-metrics-results/unweighted-unifrac-sex-significance.qzv \
--p-pairwise

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 6 SECONDS TO RUN TO COMPLETION
```


A
B
1. Comparing the distances within A with the distances within B
Distance within A
Distances within B
2. Comparing the distances within A with the distances between A and B
3. Comparing the distances within B with the distances between A and B
A
B
Distances between A and B

Significance for a beta diversity metric indicates lesser ingroup distances for one or more groups compared to the inter-group distances.

STEP 10: Creating PCoA plot to explore beta diversity metrics (Optional. Run any one metric for beta diversity, you do not have to run all the methods. Pick the one that most defines your dataset)

```
qiime emperor plot \
--i-pcoa core-metrics-results/unweighted_unifrac_pcoa_results.qza \
--m-metadata-file mappingFile.txt \
--p-custom-axes TreatmentNumber  \
--o-visualization core-metrics-results/unweighted-unifrac-emperor-TreatmentNumber.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime emperor plot \
--i-pcoa core-metrics-results/bray_curtis_pcoa_results.qza \
--m-metadata-file mappingFile.txt \
--p-custom-axes TreatmentNumber \
--o-visualization core-metrics-results/bray-curtis-emperor-TreatmentNumber.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

# Download the files to examine in the Qiime viewer

scp -P 44111 -r <username>@gateway.training.ncgr.org:~/QIIME2_Analysis/core-metrics-results Desktop/
```

STEP 11: Assigning Taxonomy

For many experiments, investigators aim to identify the organisms that are present in a sample. E.g., what genera or species are present in my samples? Are there any human pathogens in this patient's sample? We can do this by comparing our query sequences (i.e., our features, be they ASVs or OTUs) to a reference database of sequences with known taxonomic composition. Simply finding the closest alignment is not really good enough — because other sequences that are equally close matches or nearly as close may have different taxonomic annotations. So we use taxonomy classifiers to determine the closest taxonomic affiliation with some degree of confidence or consensus (which may not be a species name if one cannot be predicted with certainty!), based on alignment, k-mer frequencies, etc.

```
wget "https://data.qiime2.org/2020.6/common/gg-13-8-99-nb-classifier.qza"

qiime feature-classifier classify-sklearn \
--i-classifier gg-13-8-99-nb-classifier.qza \
--i-reads rep-seqs-or-99.qza \
--o-classification taxonomy.qza

#THE ABOVE STEP SHOULD APPROXIMATELY TAKE 6 MINUTES TO RUN TO COMPLETION

qiime metadata tabulate \
--m-input-file taxonomy.qza \
--o-visualization taxonomy.qzv

#THE ABOVE STEP SHOULD APPROXIMATELY TAKE 5 SECONDS TO RUN TO COMPLETION

qiime taxa barplot \
--i-table table-or-99.qza \
--i-taxonomy taxonomy.qza \
--m-metadata-file mappingFile.txt \
--o-visualization taxa-bar-plots.qzv

# THE ABOVE STEP SHOULD APPROXIMATELY TAKE 7 SECONDS TO RUN TO COMPLETION
```

Visualizing QIIME2 .qzv files.

```
# Copy the visualization files

scp -P 44111 <USERNAME>@gateway.training.ncgr.org:/home/<USERNAME>/QIIME2_1000_Analysis\
/core-metrics-results/*.qzv ./

# OPEN YOUR WEB BROWSER AND GO TO THE FOLLOWING LINK

https://view.qiime2.org/
```

STEP 12: Differential Abundance - ANCOM - Analysis of Composition of Microbes (Optional)

- Identifies taxa that are present in different abundances across sample groups
- Compares log ratio of the abundance of each taxon to the abundance of all remaining taxa one at a time
- Assumes small percentage (<25%) of features are changing between groups

```
# Limit ANCOM analysis to samples from a single body site (as many features
# are changing in abundance across body sites)

qiime feature-table filter-samples \
--i-table table.qza \
--m-metadata-file mappingFile.txt \
--p-where "BodySite='Nasal'" \
--o-filtered-table nasal-table.qza

=================================================

# You should run the following command/plugin on your own dataset where you collapse
# features to a chosen taxonomic level before ANCOM
# (levels are 1-7, but choose something like 5 or 6)

qiime taxa collapse \
--i-table nasal-table.qza \
--i-taxonomy taxonomy.qza \
--p-level 6 \
--o-collapsed-table nasal-table-level6.qza

==================================================

# In this step, ANCOM takes logs and adds one count
# to every value (pseudocount)

qiime composition add-pseudocount \
--i-table nasal-table.qza \
--o-composition-table comp-nasal-table.qza

#run ancom

qiime composition ancom \
--i-table comp-nasal-table.qza \
--m-metadata-file mappingFile.txt \
--m-metadata-column Treatment \
--o-visualization ancom-Treatment-nasal.qzv
```

EXERCISE: Preparing your raw sequence data and importing -https://docs.qiime2.org/2020.11/tutorials/imp

When using Earth Microbiom Project (EMP) protocol, you should have access to forward reads, reverse reads (if applicable),

and a barcode list, i.e.:

1. forward.fastq.gz
2. reverse.fastq.gz
3. barcodes.fastq.gz

```
# Exercise; download the example EMP files from the above site and run the following commands:
source activate qiime2

mkdir emp_example
```

```
cd emp_example

qiime tools import \
--type EMPPairedEndSequences \
--input-path ./ \
--output-path emp-paired-end-sequences.qza
```

Demultiplexing

To demultiplex sequences we need to know which barcode sequence is associated with each sample. This information is contained in the sample metadata file.

Note: Our TB data was is already demultiplexed.

```
# Exercise; download the metadata file and run the following command:

wget https://data.qiime2.org/2019.1/tutorials/atacama-soils/sample_metadata.tsv

qiime demux emp-paired \
--i-seqs emp-paired-end-sequences.qza \
--m-barcodes-file sample_metadata.tsv \
--m-barcodes-column BarcodeSequence \
--o-per-sample-sequences demux.qza \
--p-rev-comp-mapping-barcodes \
--o-error-correction-details demux-details.qza

# convert the .qza artifact to a .qzv file for visualization

qiime demux summarize \
--i-data demux.qza \
--o-visualization demux.qzv

# scp the output file to your local machine and view in a web browser.
```

Denoising

https://docs.qiime2.org/2021.2/tutorials/atacama-soils/

```
qiime dada2 denoise-paired \
--i-demultiplexed-seqs demux.qza \
--p-trim-left-f 13 \
--p-trim-left-r 13 \
--p-trunc-len-f 150 \
--p-trunc-len-r 150 \
--o-table table.qza \
--o-representative-sequences rep-seqs.qza \
--o-denoising-stats denoising-stats.qza
```

Chimeric feature filtering

https://docs.qiime2.org/2021.2/tutorials/chimera/

```
qiime vsearch uchime-denovo \
--i-table table.qza \
--i-sequences rep-seqs.qza \
--output-dir uchime-dn-out

# tabulate for visualization

qiime metadata tabulate \
--m-input-file uchime-dn-out/stats.qza \
--o-visualization uchime-dn-out/stats.qzv

# creates filtered files

qiime feature-table filter-features \
--i-table table.qza \
--m-metadata-file uchime-dn-out/nonchimeras.qza \
--o-filtered-table uchime-dn-out/table-nonchimeric-wo-borderline.qza

qiime feature-table filter-seqs \
--i-data rep-seqs.qza \
--m-metadata-file uchime-dn-out/nonchimeras.qza \
--o-filtered-data uchime-dn-out/rep-seqs-nonchimeric-wo-borderline.qza

qiime feature-table summarize \
--i-table uchime-dn-out/table-nonchimeric-wo-borderline.qza \
--o-visualization uchime-dn-out/table-nonchimeric-wo-borderline.qzv
```