Introduction To Metagenomics

Learning Objectives:

Describe the scope and basic principles of metagenomics.

Describe the common metagenomic sequencing strategies.

Define the levels of taxonomic classification.

Define the types of diversity examined in metagenomic analyses.

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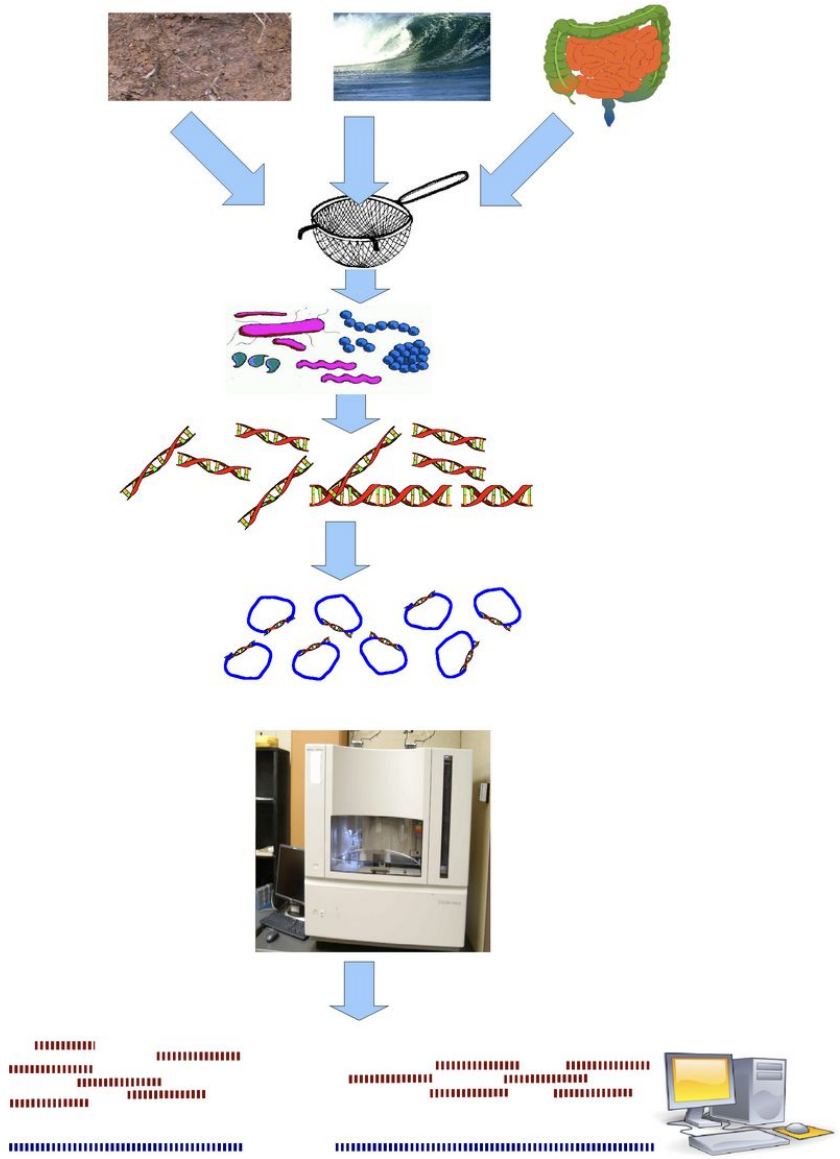
Introduction to Metagenomics

- Sequencing of communities of microorganisms
- No need for isolation and lab cultivation
- High depth short read sequencing (Next Generation Sequencing - NGS)
- Long read third generation sequencing
  - PacBio
  - Oxford Nanopore Technologies (ONT)



Wooley et al. 2010 - https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1000667

Sequencing Strategies

- Whole genome sequencing (WGS)
- Marker gene
  - 16S Ribosomal RNA (rRNA)
    - Bacteria
    - Archaea
  - 18S rRNA
    - Fungus
    - Eukaryotes

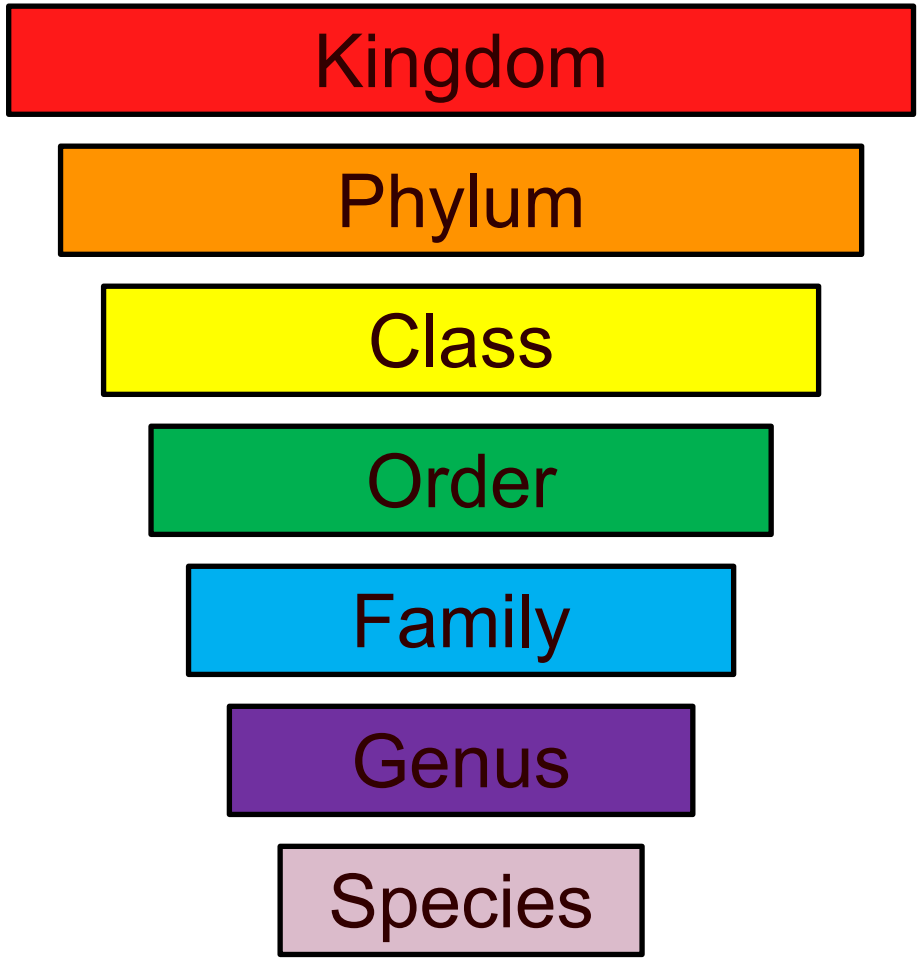
https://www.illumina.com/systems/sequencing-platforms/miseq.html

WGS Metagenomics

- Sequencing whole genomes of the microorganisms present in the sample
- Facilitates discovering gene functions and genome structures
- Steps involved:
  - Genome assembly (special software/considerations)
  - Binning
  - Predicting and annotating genes

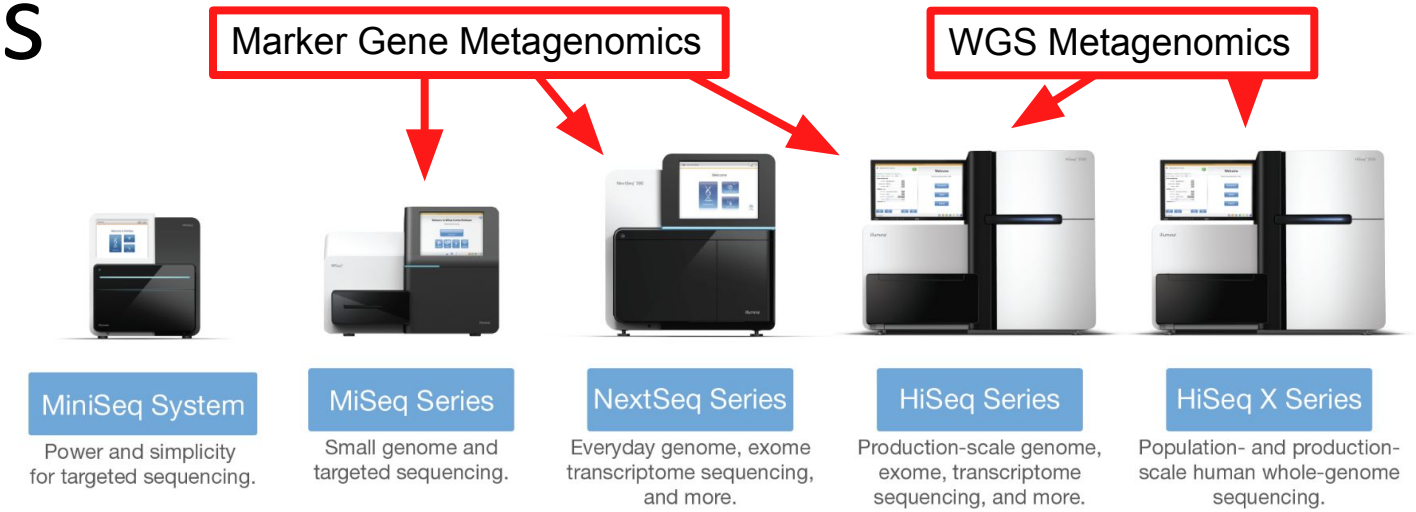
Marker Gene Metagenomics

- Usually based on 16S rRNA
  - Conserved within species
  - Varies greatly between species
  - Widely used for microbial ecology - many resources available
- Needs a reference database to match the Operational Taxonomic Units (OTUs)
  - Silva
  - Greengenes
- Steps
  - Preprocessing (removing noise, QC)
  - OUT clustering and taxonomic assignment
  - Alpha diversity analysis - within sample diversity
  - Beta diversity analysis - between sample diversity



Sequencing Platforms

- Illumina
  - Short reads (up to 300 bp)
  - Highly accurate
  - High depth sequencing
- PacBio
  - Long reads (10-25 kb)
  - Accurate (99.5%)
- Oxford Nanopore
  - Long reads (10-30 kb)
  - Less accurate (~95%)
  - Portable
  - Affordable



https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf

https://nanoporetech.com/

Metagenomics Software

Learning Objective:

Identify commonly used metagenomics software suites.

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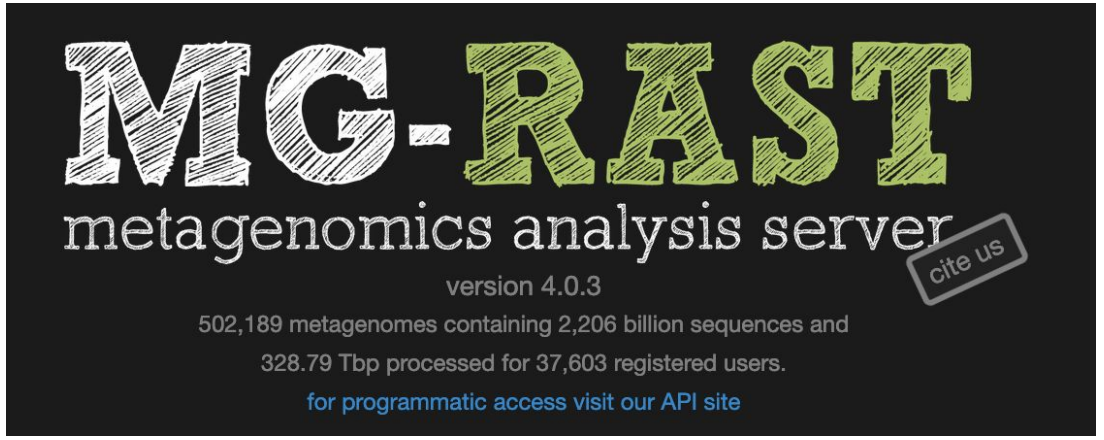
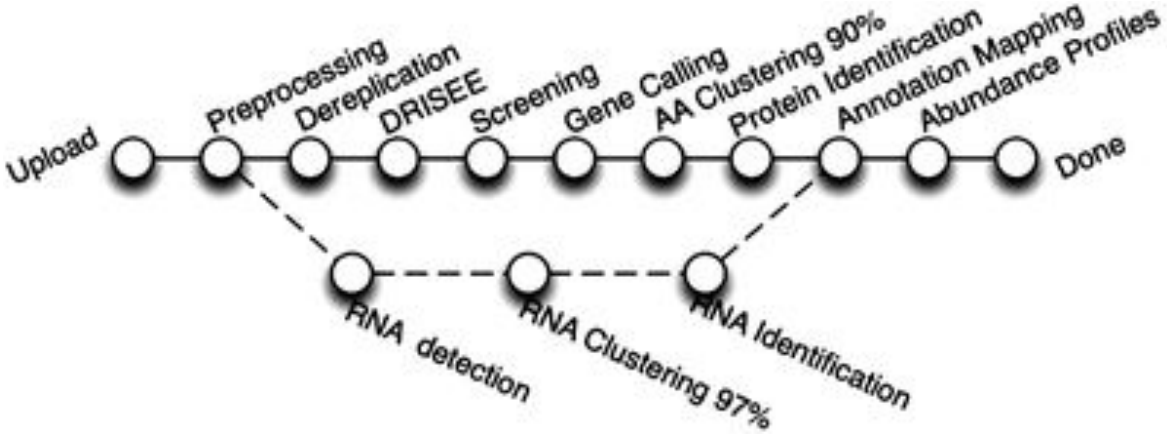
MG-RAST metagenomics analysis server

- Open-source web application for metagenomic analysis
- Large repository for metagenomic data
- Hosted by The University of Chicago and Argonne National Laboratory
- Full analysis pipeline



https://www.mg-rast.org/



Keegan et al. 2016 - https://link.springer.com/protocol/10.1007/978-1-4939-3369-3_13#Fig1

Bacterial and Viral Bioinformatics Resource Center

- Formerly known as PATRIC
- Designed to support research on bacterial and viral diseases
- Among other tools, provides some metagenomic functions:
  - Metagenomic read mapping
  - Taxonomic classification
  - Metagenomic binning

https://www.bv-brc.org/

Mothur

- Open-source software for microbial ecology
- Installed on Grace and Terra
- Single piece of software - many functions/commands
- Example data and protocols available
- Contains accelerated versions of the DOTUR and SONS programs
- Highly cited (> 17700 citations)
- Schloss et al. 2009: https://journals.asm.org/doi/10.1128/aem.01541-09

https://mothur.org/

QIIME 2

- Open-source pipeline for microbiome analysis
- Installed on Grace and Terra
- Takes raw fastq data (multiplexed or demultiplexed)
- From demultiplexing to publication-ready figures
- Incorporates many other software packages (e.g. Mothur, FastTree)

http://qiime.org/

Accessing the Grace Cluster

Learning Objective:

Describe the different methods of accessing the Grace cluster.

Use one or more of the described methods to login to Grace.

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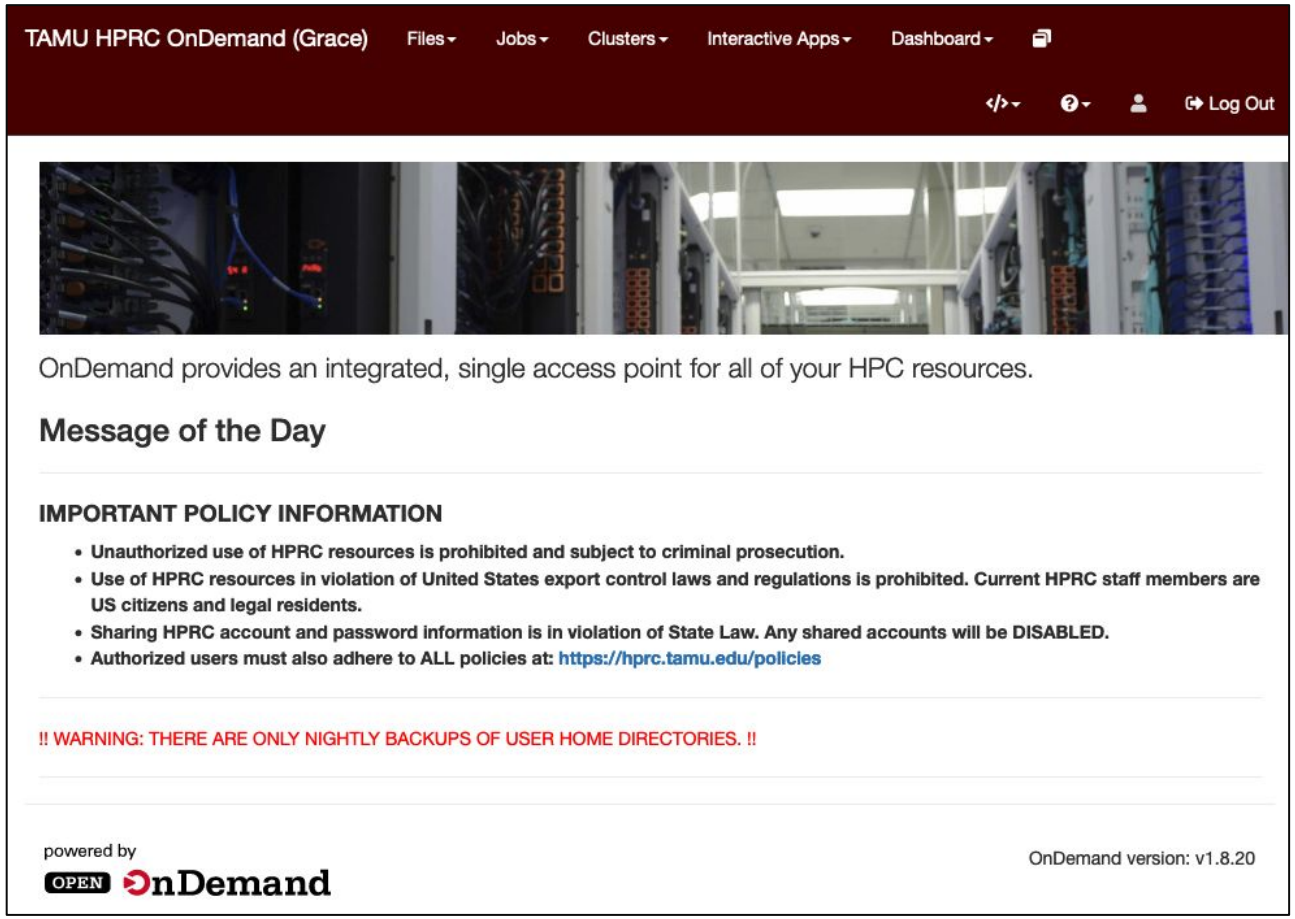
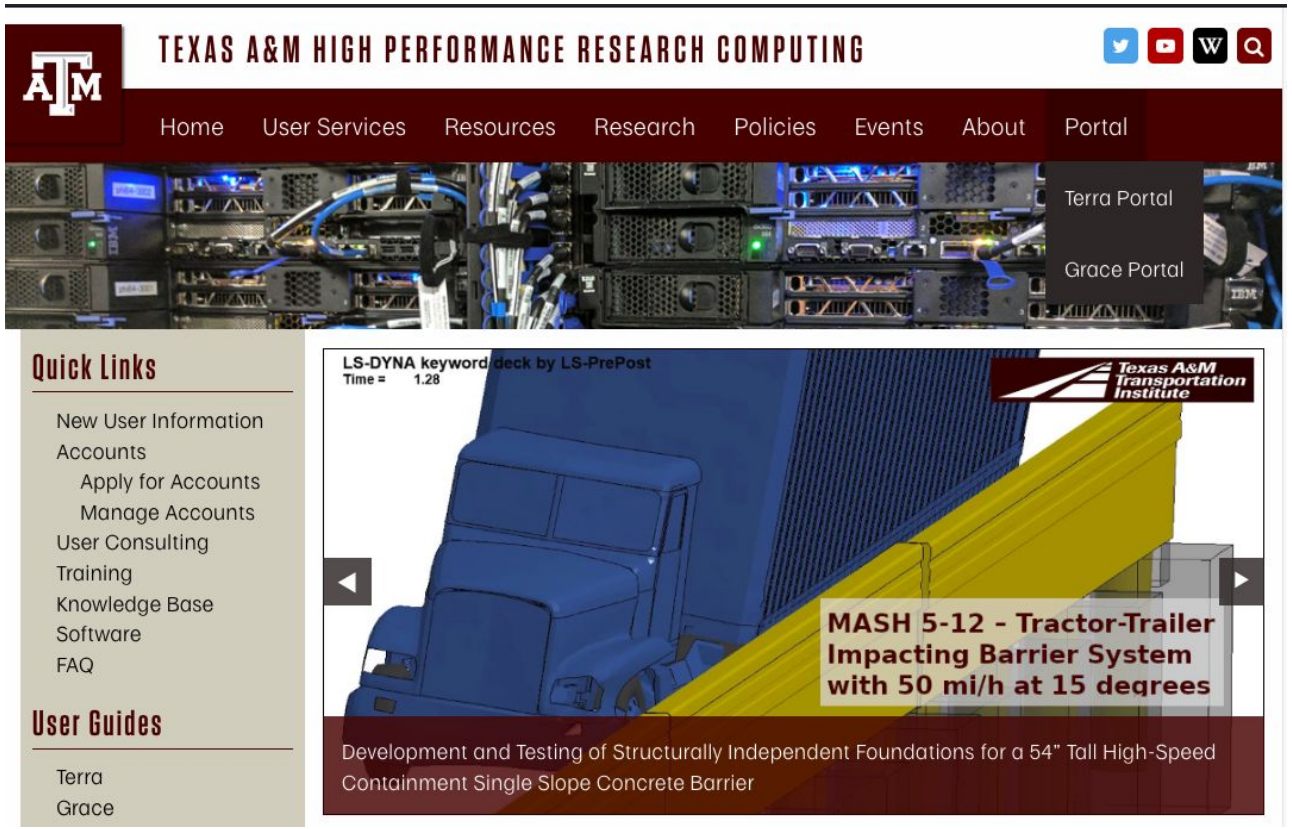
How to Access the Grace Cluster

- HPRC Portal
  - Access is web browser based
  - All software on Grace Cluster available as either GUI or UNIX command line
  - GUI File Browser
  - Best for GUI applications
    - RStudio
    - IGV



Demo

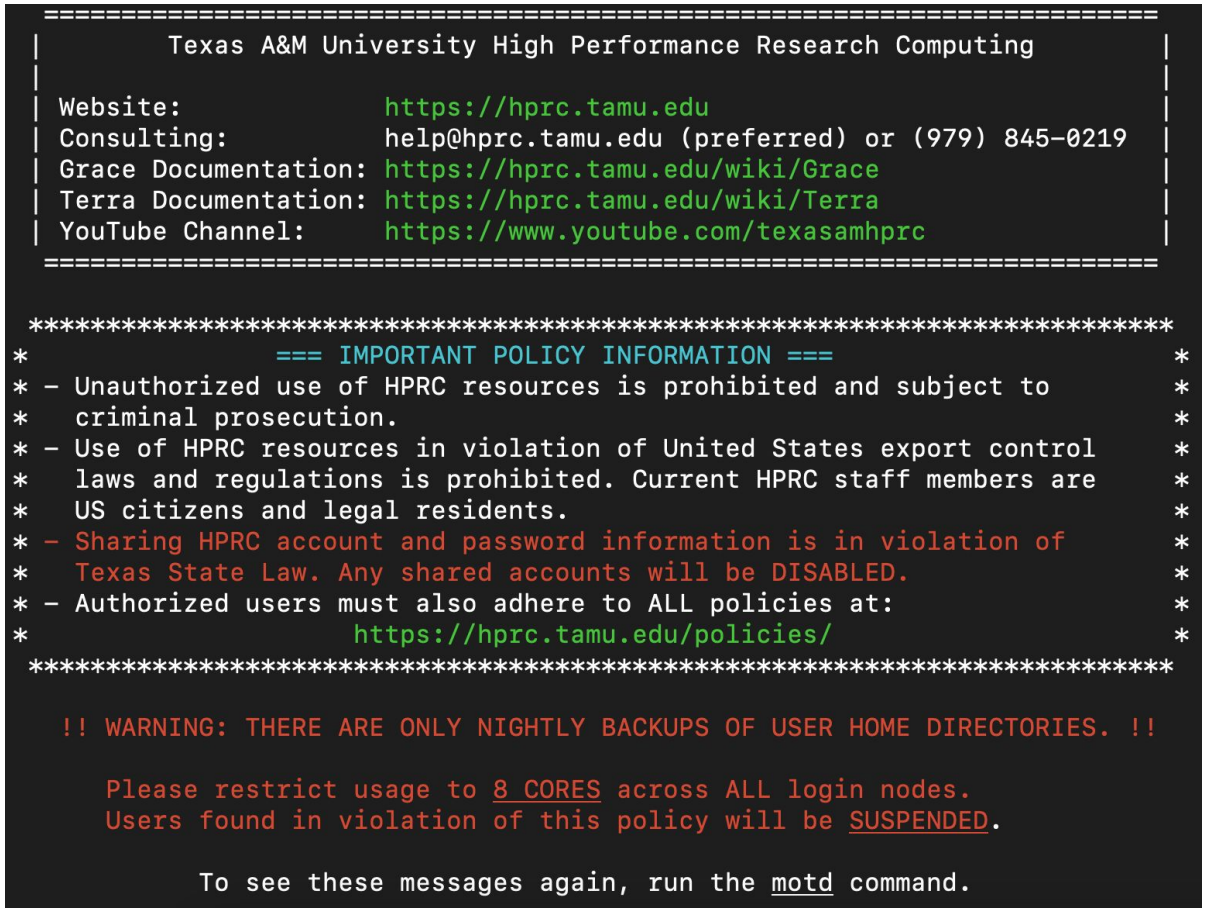
- Accessing the Grace Cluster through the HPRC Portal



https://www.youtube.com/watch?v=dqa2ZzsEmQs&list=PLHR4HLly3i4aJJDxKTZIpxyJG6uSqgAgd

How to Access the Grace Cluster

- Unix Command Line
  - Working knowledge of Unix and Slurm
  - Bioinformatics template scripts available
  - Not well-suited for GUI applications
  - Use Terminal Application on Max or Linux machines
  - Windows machines require and SSH client



Demo

- Accessing the Grace Cluster through the Unix command line
- Using the Terminal application on Mac/Linux machines
  - https://www.youtube.com/watch?v=KjHwfZI_ej4&list=PLHR4HLly3i4YrkNWcUE77t8i-AkwN5AN8&index=4
- Using MobaXTerm on Windows machines
  - https://www.youtube.com/watch?v=PXIGhqLJP3g&list=PLHR4HLly3i4YrkNWcUE77t8i-AkwN5AN8&index=3

Getting Started with QIIME 2

Learning Objectives:

Define the terminology used by QIIME for the input and output file formats.

Learn how to visualize QIIME artifacts.

Learn how to use QIIME2 on the Grace cluster.

Use the QIIME 2 plugin tools to import example data.

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QIIME 2 Terminology

- Artifacts (QIIME 2 data)
  - Contain data and metadata
  - .qza file extension
  - Allows QIIME to track type, format, and provenance of the data

- Visualizations
  - Terminal output of an analysis (e.g. tables, graphs)
  - Also contain data and metadata
  - Can be viewed at https://view.qiime2.org/

QIIME 2 Terminology

- Semantic types
  - Essentially a classification of a given artifact
  - Helps users avoid using incorrect artifacts for analysis

Common semantic types

Unless otherwise noted the following semantic types are defined by, and importable from, the q2-types plugin. It is also possible to define semantic types in any plugin, so the available semantic types are not limited to those defined in q2-types. Instructions will be added soon for how to accomplish this. In the meantime, you can refer to the q2-dummy-types repository for annotated examples.

`FeatureTable[Frequency]` : A feature table (e.g., samples by OTUs) where each value indicates the frequency of an OTU in the corresponding sample expressed as raw counts.

`FeatureTable[RelativeFrequency]` : A feature table (e.g., samples by OTUs) where each value indicates the relative abundance of an OTU in the corresponding sample such that the values for each sample will sum to 1.0.

`FeatureTable[PresenceAbsence]` : a feature table (e.g., samples by OTUs) where each value indicates whether an OTU is present or absent in the corresponding sample.

`FeatureTable[Composition]` : A feature table (e.g., samples by OTUs) where each value indicates the frequency of an OTU in the corresponding sample, and all frequencies are greater than zero.

`Phylogeny[Rooted]` : A rooted phylogenetic tree.

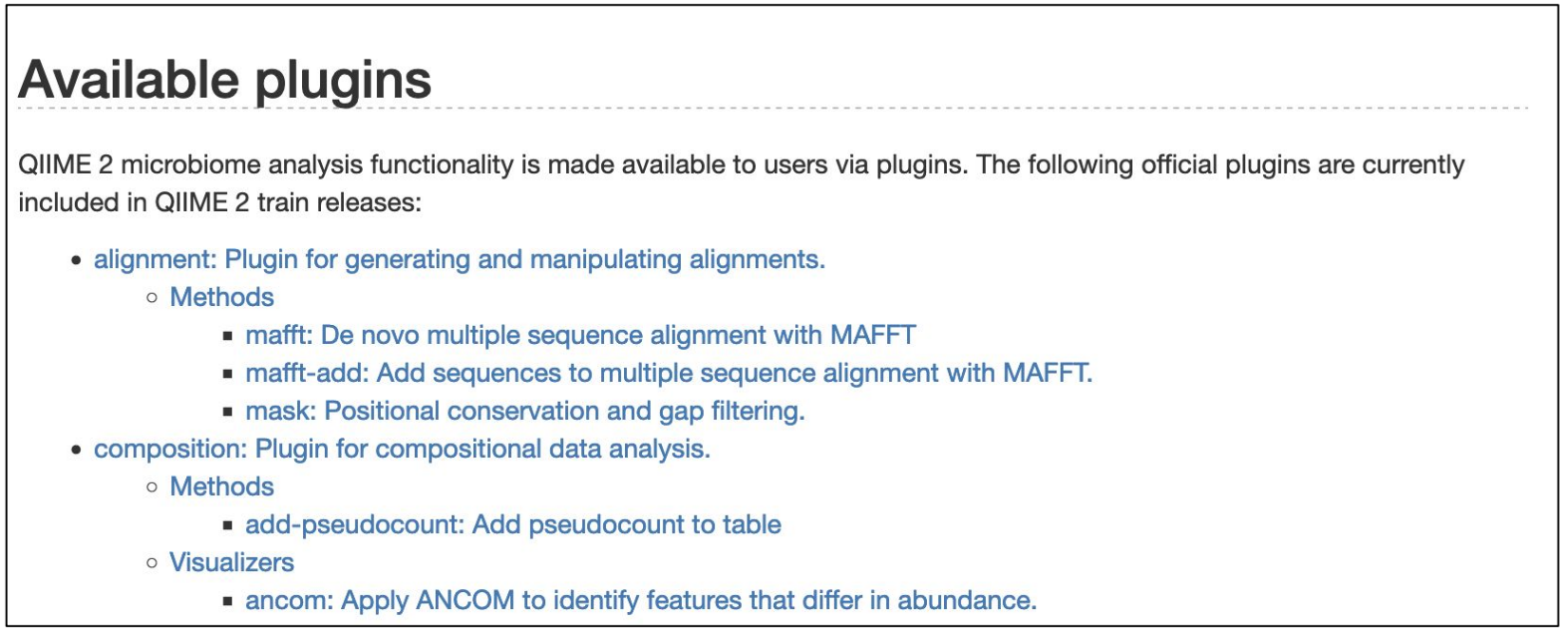
`Phylogeny[Unrooted]` : An unrooted phylogenetic tree.

`DistanceMatrix` : A distance matrix.

https://docs.qiime2.org/2022.2/semantic-types/

QIIME 2 Terminology

- Plugins
  - Software packages that perform specific analyses (e.g. q2-demux, q2-diversity)
  - Can be written by third-party developers
  - Plugins available for all steps necessary for complete pipeline



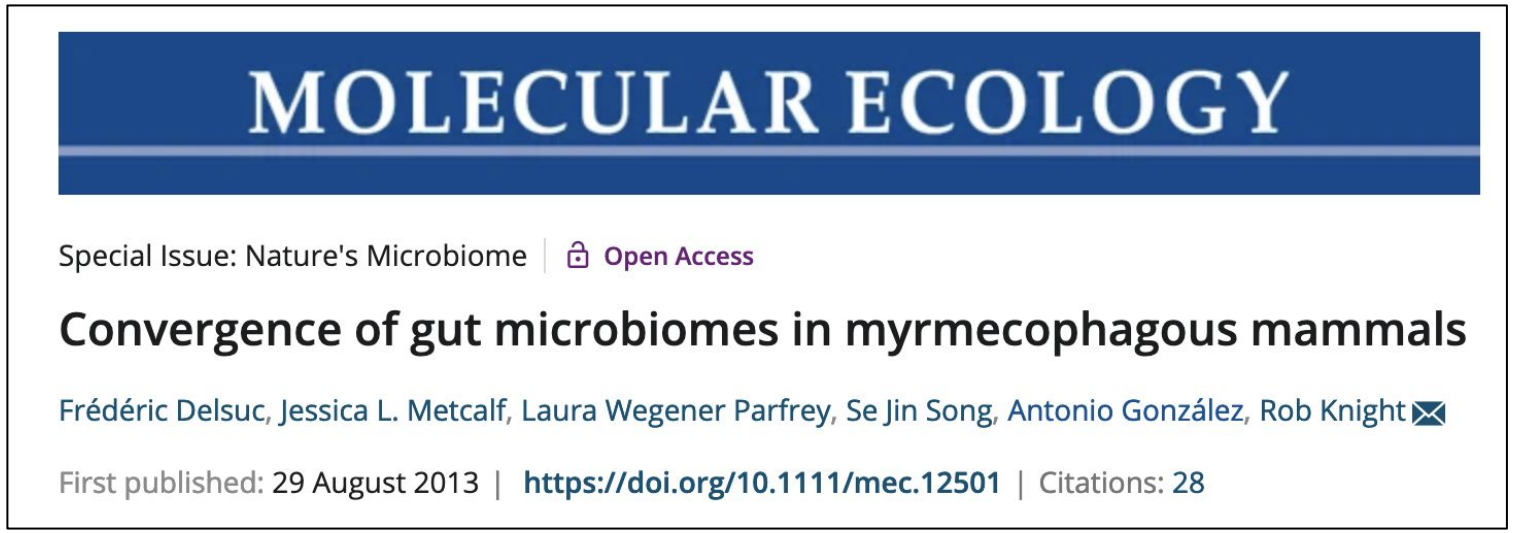
Available plugins

QIIME 2 microbiome analysis functionality is made available to users via plugins. The following official plugins are currently included in QIIME 2 train releases:

- alignment: Plugin for generating and manipulating alignments.
  - Methods
    - mafft: De novo multiple sequence alignment with MAFFT
    - mafft-add: Add sequences to multiple sequence alignment with MAFFT.
    - mask: Positional conservation and gap filtering.
- composition: Plugin for compositional data analysis.
  - Methods
    - add-pseudocount: Add pseudocount to table
  - Visualizers
    - ancom: Apply ANCOM to identify features that differ in abundance.

https://docs.qiime2.org/2022.2/plugins/available/

Getting Started with QIIME 2 on Grace



MOLECULAR ECOLOGY

Special Issue: Nature's Microbiome | Open Access

Convergence of gut microbiomes in myrmecophagous mammals

Frédéric Delsuc, Jessica L. Metcalf, Laura Wegener Parfrey, Se Jin Song, Antonio González, Rob Knight

First published: 29 August 2013 | https://doi.org/10.1111/mec.12501 | Citations: 28

https://onlinelibrary.wiley.com/doi/10.1111/mec.12501

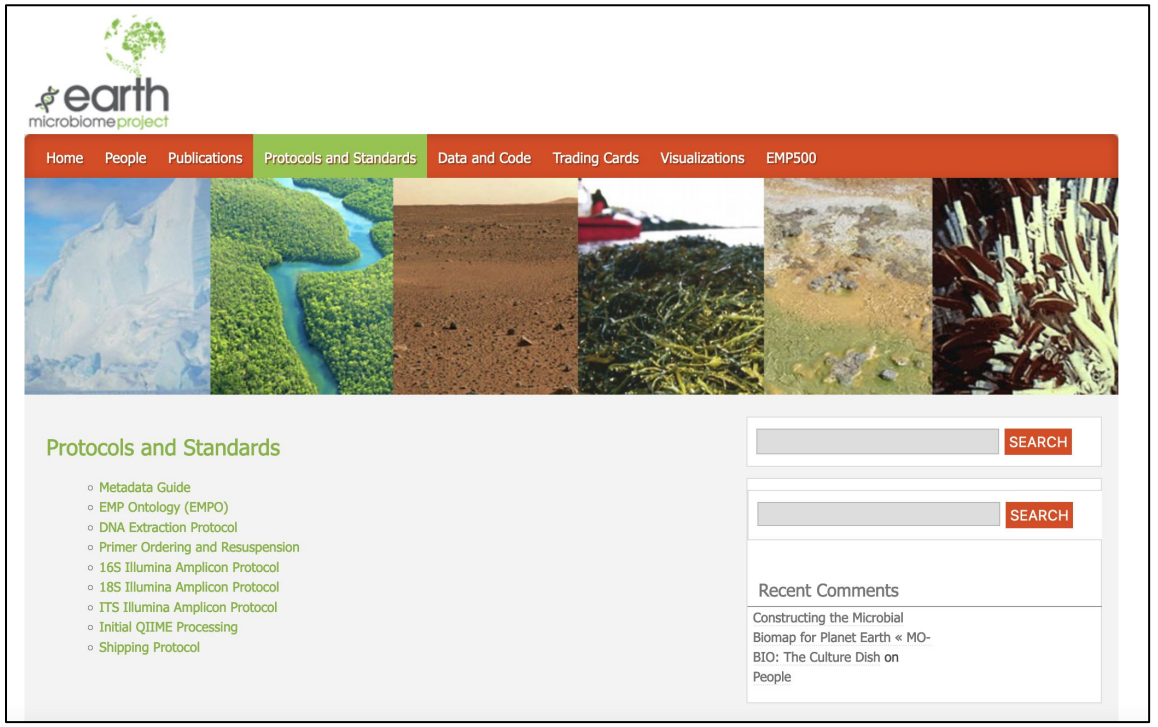
Importing Data

- QIIME 2 can import many types of data:
  - Fastq (single and paired-end)
  - Fasta
  - Feature tables
  - Phylogenetic trees
- Importing data creates QIIME 2 artifacts (with specific semantic types)
- Semantic types for raw fastq files:
  - EMPSingleEndSequences
  - EMPPairedEndSequences
  - MultiplexedSingleEndBarcodeInSequence
  - MultiplexedPairedEndBarcodeInSequence …

https://docs.qiime2.org/2022.2/tutorials/importing/

Importing Data

- Example data is in the EMP single end format
- Data is still multiplexed (single fastq file)
- Directory with two fastq files
  - Sequences
  - Barcodes



Getting Started with QIIME 2 on Grace

- Log on to the Grace Cluster (through the HPRC portal or command line) and use the following commands to load QIIME 2 and access the example data

```
module purge
```

```
module load Anaconda3/2021.11
```

```
source activate /sw/hprc/sw/Anaconda3/2021.11/envs/qiime2-2021.11
```

- Set up working directory:

```
mkdir $SCRATCH/metagenomics
```

```
cd $SCRATCH/metagenomics
```

```
cp -r /scratch/training/bio/metagenomics/* .
```

Getting Started with QIIME 2 on Grace

- Use the following command to import the example data and create a QIIME 2 artifact (this will take several minutes)

```
qiime tools import
   --type EMPSingleEndSequences \
   --input-path fastqs \
   --output-path reads.qza
```

Demultiplexing and Quality Control

Learning Objective:

Use the QIIME 2 demux and dada2 plugins to demultiplex and filter the example data.

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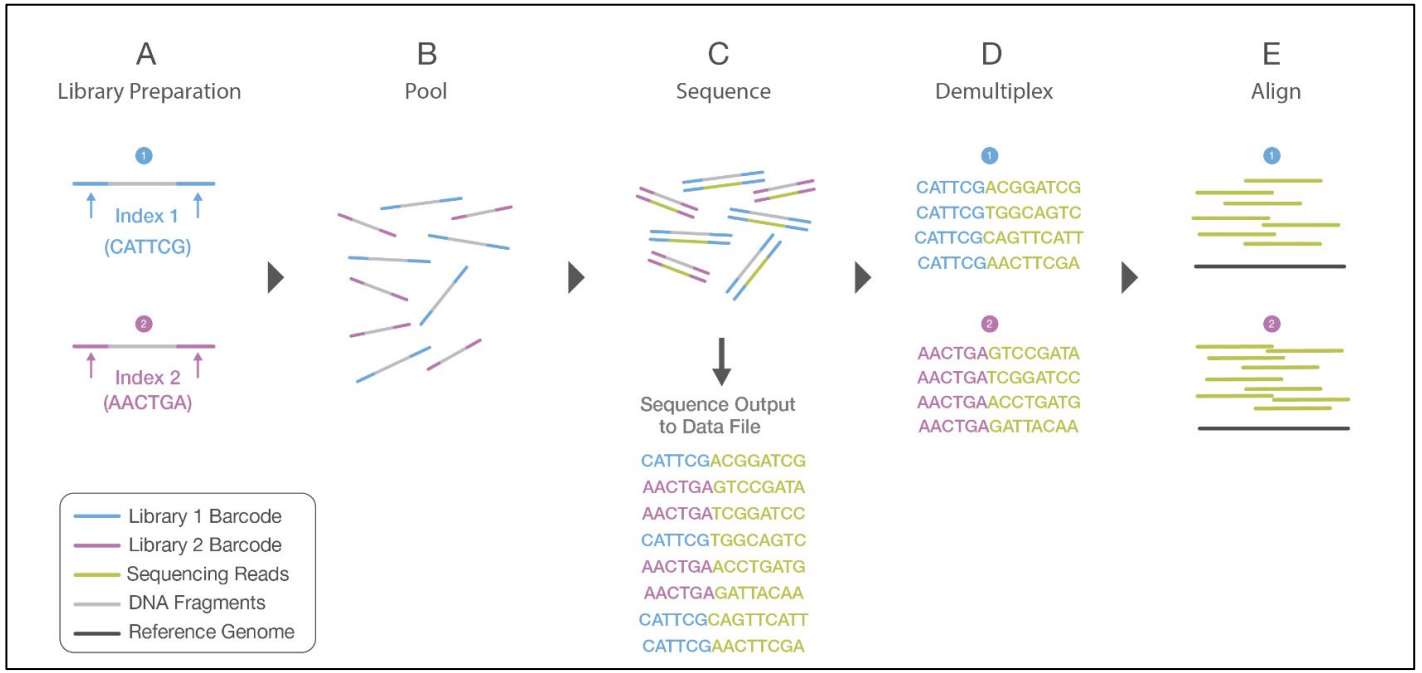
Demultiplexing

- Example data (and most amplicon/targeted metagenomics datasets) are pooled for sequencing
- Unique barcodes (short oligos) applied to each sample
- Barcodes used to sort reads after sequencing



https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf

Preprocessing Sequence Data

- Use the following commands to demultiplex the sequence data (will take 4-5 minutes to complete)

```
qiime demux emp-single --i-seqs reads.qza \
   --m-barcodes-file myrme-sample-data.txt \
   --m-barcodes-column barcode-sequence \
   --o-per-sample-sequences demux.qza \
   --o-error-correction-details demux-details.qza \
   --p-rev-comp-mapping-barcodes
```

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

- Upload and qzv files here: https://view.qiime2.org

Denoising and Filtering

- Identify and correct sequenced amplicons
- Filter chimeric reads
- Filter phiX reads
- Multiple options for this denoising and filtering in QIIME 2
  - DADA2
  - Deblur

Preprocessing Sequence Data

- Use the following commands to denoise and filter the example data (first command should take 8-10 minutes to complete).
- Remember the interactive quality plot to choose the right values for the trimming options

```
qiime dada2 denoise-single \
   --i-demultiplexed-seqs demux.qza \
   --p-trim-left 8 --p-trunc-len 148 \
   --o-representative-sequences rep-seqs.qza \
   --o-table table.qza --o-denoising-stats stats.qza
```

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

- Upload and qzv files here: https://view.qiime2.org

Preprocessing Sequence Data

Generate feature/sequence tables for visualization

```
qiime feature-table summarize --i-table table.qza \
   --o-visualization table.qzv \
   --m-sample-metadata-file myrme-sample-data.txt
```

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

Diversity Analysis

Learning Objectives:

Define the standard statistical methods used for metagenomic diversity analysis.

Use QIIME 2 to conduct diversity analyses on the example data.

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Diversity Analysis

- Alpha Diversity - within sample
  - Shannon’s Diversity Index
  - Observed Features
  - Faith’s Phylogenetic Diversity
  - Evenness
- Beta Diversity - between samples
  - Jaccard Distance
  - Bray-Curtis Distance
  - Unweighted UniFrac Distance
  - Weighted UniFrac Distance

Diversity Analysis

- Use the following command to generate a phylogenetic tree for the diversity analyses

```
qiime phylogeny align-to-tree-mafft-fasttree \
   --i-sequences rep-seqs.qza \
   --o-alignment aligned-rep-seqs.qza \
   --o-masked-alignment masked-aligned-rep-seqs.qza \
   --o-tree unrooted-tree.qza \
   --o-rooted-tree rooted-tree.qza
```

Diversity Analysis

- Use the following command to generate the alpha and beta diversity metrics, as well as PCA plots for the beta diversity metrics

```
qiime diversity core-metrics-phylogenetic \
   --i-phylogeny rooted-tree.qza \
   --i-table table.qza \
   --p-sampling-depth 10590 \
   --m-metadata-file myrme-sample-data.txt \
   --output-dir diversity-analysis
```

- Upload and view the beta diversity qzv files here: https://view.qiime2.org

Diversity Analysis

- Use the following commands to test for significance of alpha-level diversity

```
qiime diversity alpha-group-significance \
   --i-alpha-diversity diversity-analysis/faith_pd_vector.qza \
   --m-metadata-file myrme-sample-data.txt \
   --o-visualization diversity-analysis/faith_pd_vector-significance.qzv
```

```
qiime diversity alpha-group-significance \
   --i-alpha-diversity diversity-analysis/evenness_vector.qza \
   --m-metadata-file myrme-sample-data.txt \
   --o-visualization diversity-analysis/evenness_vector-significance.qzv
```

- Upload and view the alpha diversity qzv files here: https://view.qiime2.org

Diversity Analysis

- Use the following commands to test for significance of beta-level diversity

```
qiime diversity beta-group-significance \
    --i-distance-matrix diversity-analysis/unweighted_unifrac_distance_matrix.qza \
    --m-metadata-file myrme-sample-data.txt --m-metadata-column diet \
    --o-visualization diversity-analysis/unweighted_unifrac_diet-significance.qzv \
    --p-pairwise
```

```
qiime feature-table filter-samples --i-table table.qza \
    --m-metadata-file myrme-sample-data.txt \
    --p-where "[species]!='panda' AND [species]!='pink-fairy-armadillo'" \
    --o-filtered-table only-multi-species.qza
```

```
qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza \
    --i-table only-multi-species.qza --p-sampling-depth 10590 \
    --m-metadata-file myrme-sample-data.txt \
    --output-dir diversity-analysis-only-multi-species
```

Diversity Analysis

- Use the following commands to test for significance of beta-level diversity

```
qiime diversity beta-group-significance \
    --i-distance-matrix \
    diversity-analysis-only-multi-species/unweighted_unifrac_distance_matrix.qza \
    --m-metadata-file myrme-sample-data.txt --m-metadata-column species \
    --o-visualization \
    diversity-analysis-only-multi-species/unweighted_unifrac_species-significance.qzv \
    --p-pairwise
```

- Upload and view the beta diversity qzv files here: https://view.qiime2.org