# Introduction to Bioinformatics on Unity III

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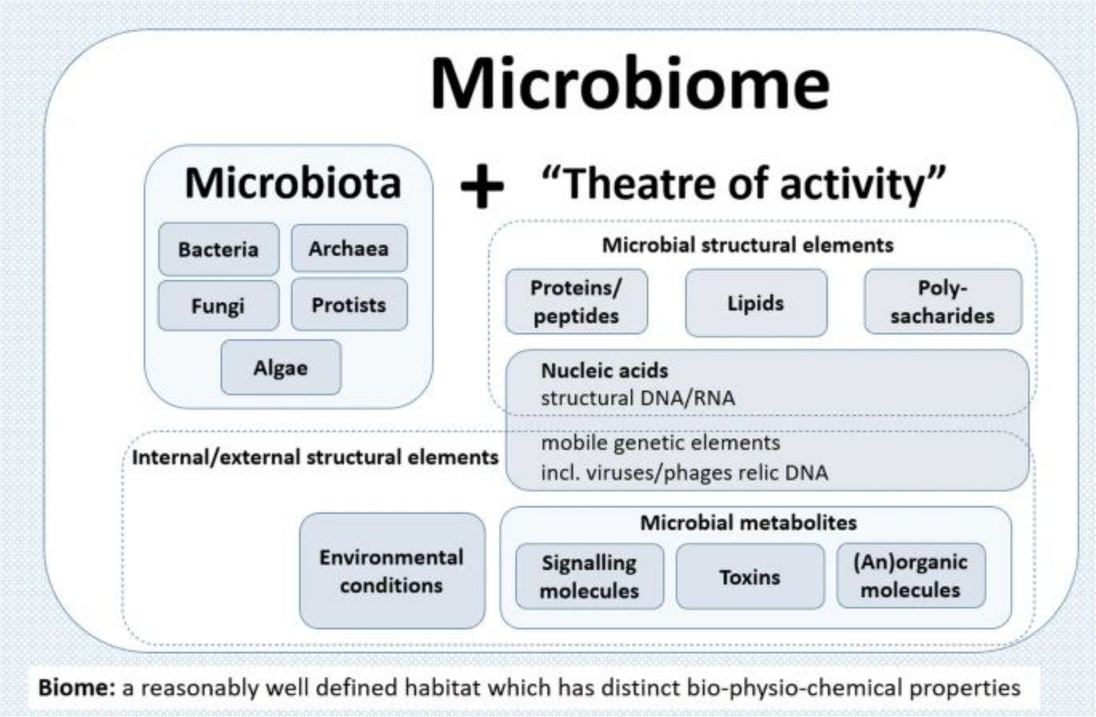
# Outline

- Microbiome research
- Amplicon sequencing overview
- Example of amplicon sequencing data analysis pipeline
  - Downloading Data
  - Data pre-processing (primer trimming)
  - Denoising
  - Taxonomic assignment
  - Phylogenetic placement & taxonomic assignment

# Microbiome research

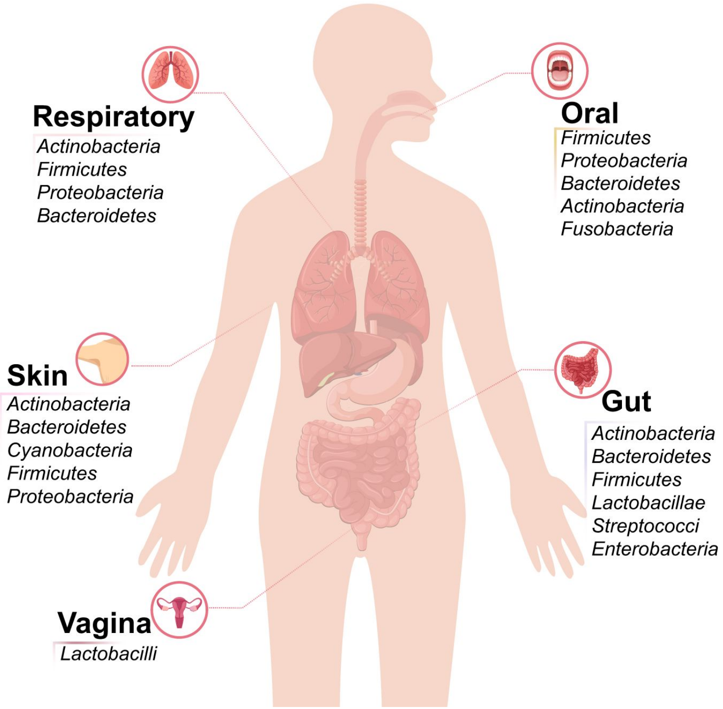
- A microbiome refers to the collection of genomes from all the microorganisms in a particular habitat as well as the structural elements, metabolites and environmental conditions
- The microbiota describes the microorganisms
- Example of microbiomes:
  - Human microbiome
  - Ocean microbiome
- What are the microbial species and what are their function?



Berg, G., Rybakova, D., Fischer, D. et al. Microbiome definition re-visited: old concepts and new challenges. Microbiome 8, 103 (2020). https://doi.org/10.1186/s40168-020-00875-0

# Human microbiome

- The composition of microbiota varies from one site to another
- Bacteria, archaea, fungi and viruses
- Goal: understand the relationship between microbiota and diseases
- Gut-brain axis: connection between brain physiology and gut microbial ecology

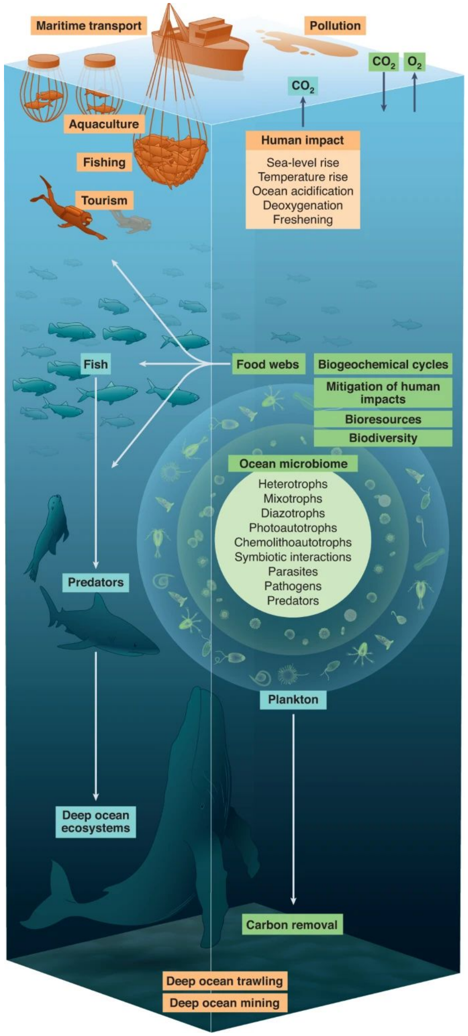


**Microbiota composition in different regions**

Hou, K., Wu, ZX., Chen, XY. et al. Microbiota in health and diseases. Sig Transduct Target Ther 7, 135 (2022). https://doi.org/10.1038/s41392-022-00974-4

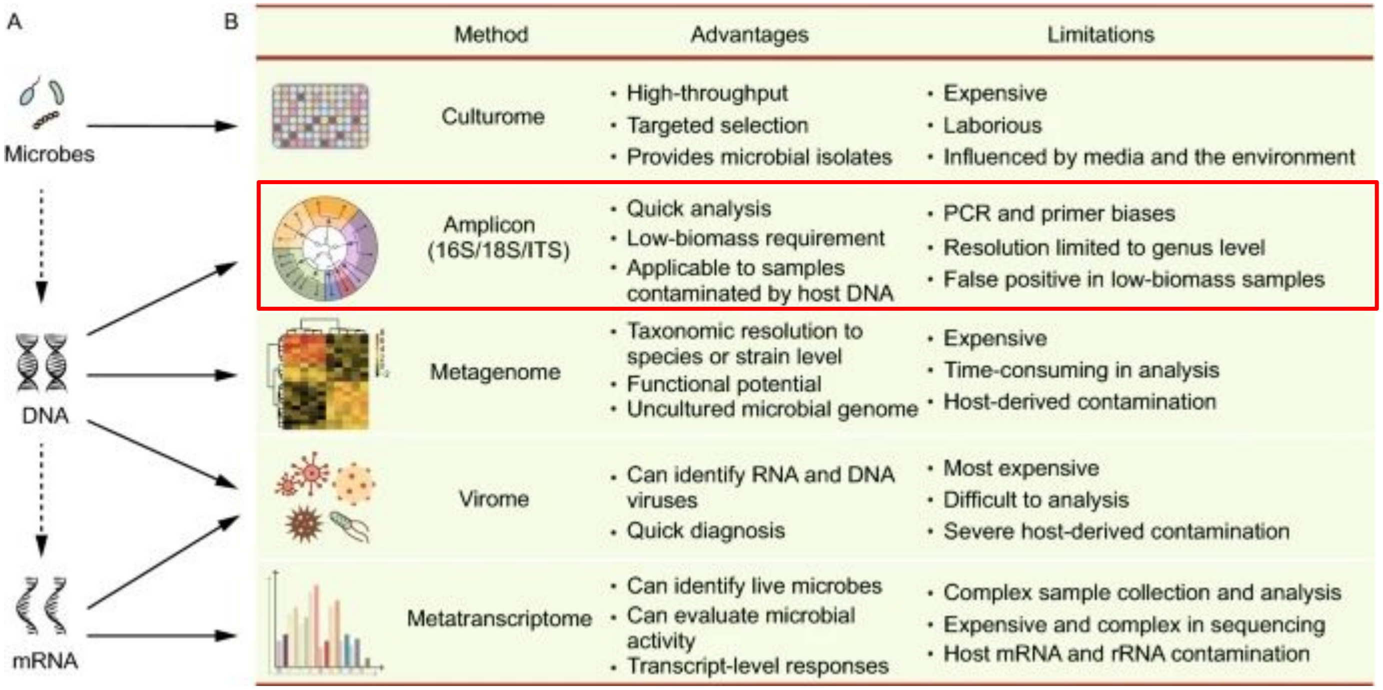
# Ocean microbiome

- Prokaryotes, eukaryotic microbes and viruses
  - Biogeochemical cycling (CO2 capture, O2 generation and carbon removal)
- Marine water, sediments, coral reefs, hydrothermal vents
- Goal: improve our understanding of microorganisms and their roles in the ocean



Tara Ocean Foundation., Tara Oceans., European Molecular Biology Laboratory (EMBL). et al. Priorities for ocean microbiome research. Nat Microbiol 7, 937–947 (2022). https://doi.org/10.1038/s41564-022-01145-5

# Methods used in microbiome research


A
Microbes
DNA
mRNA
B

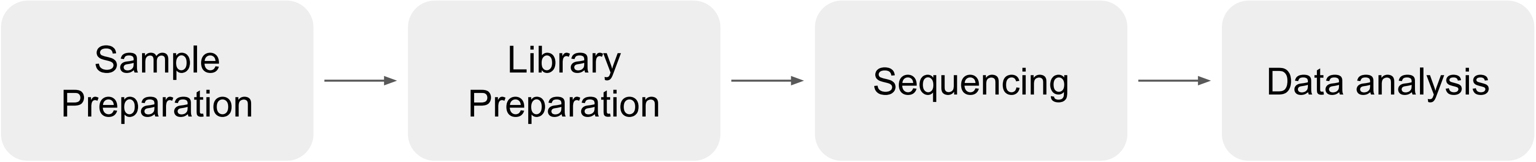
| Method | Advantages | Limitations |
|---|---|---|
| Culturome | • High-throughput<br>• Targeted selection<br>• Provides microbial isolates | • Expensive<br>• Laborious<br>• Influenced by media and the environment |
| Amplicon (16S/18S/ITS) | • Quick analysis<br>• Low-biomass requirement<br>• Applicable to samples contaminated by host DNA | • PCR and primer biases<br>• Resolution limited to genus level<br>• False positive in low-biomass samples |
| Metagenome | • Taxonomic resolution to species or strain level<br>• Functional potential<br>• Uncultured microbial genome | • Expensive<br>• Time-consuming in analysis<br>• Host-derived contamination |
| Virome | • Can identify RNA and DNA viruses<br>• Quick diagnosis | • Most expensive<br>• Difficult to analysis<br>• Severe host-derived contamination |
| Metatranscriptome | • Can identify live microbes<br>• Can evaluate microbial activity<br>• Transcript-level responses | • Complex sample collection and analysis<br>• Expensive and complex in sequencing<br>• Host mRNA and rRNA contamination |

Liu, YX., Qin, Y., Chen, T. et al. A practical guide to amplicon and metagenomic analysis of microbiome data.
Protein Cell 12, 315–330 (2021). https://doi.org/10.1007/s13238-020-00724-8

# Amplicon sequencing

- Amplicon: the resulting sequence of a targeted amplification of genetic material
- Useful for detection of hotspot mutations, gene fusions and single-nucleotide polymorphisms (SNPs), taxonomic classification of microorganisms
- Targeted (use of primers) sequencing of marker genes
  - 16S ribosomal DNA in prokaryotes
  - 18S ribosomal DNA in eukaryotes

Workflow of amplicon sequencing:

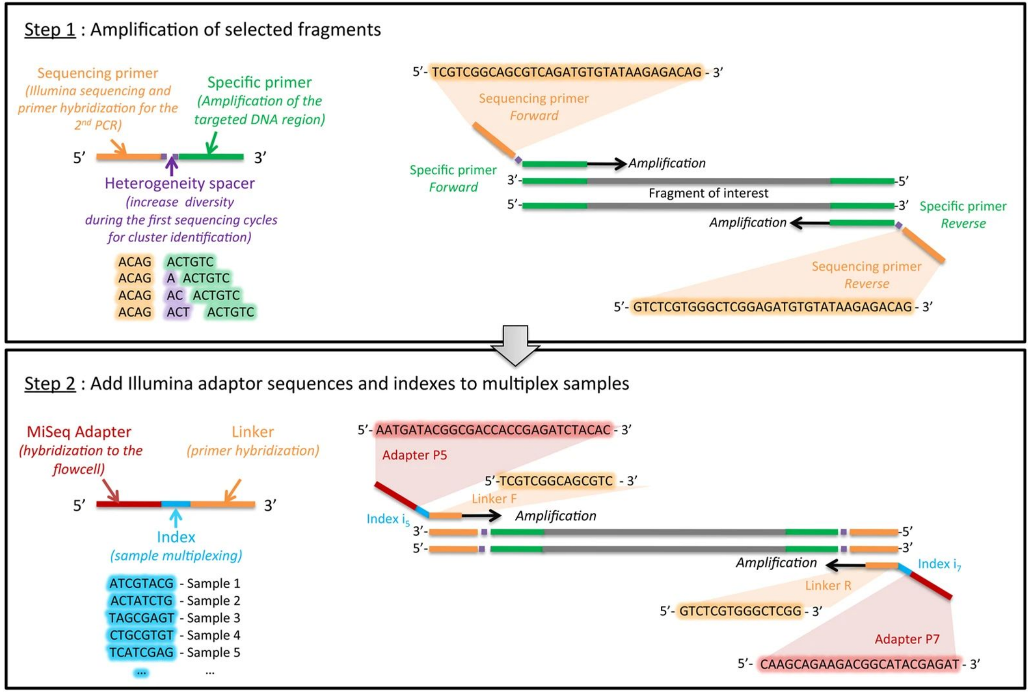


Sample Preparation → Library Preparation → Sequencing → Data analysis

# Amplicon sequencing

## Library preparation

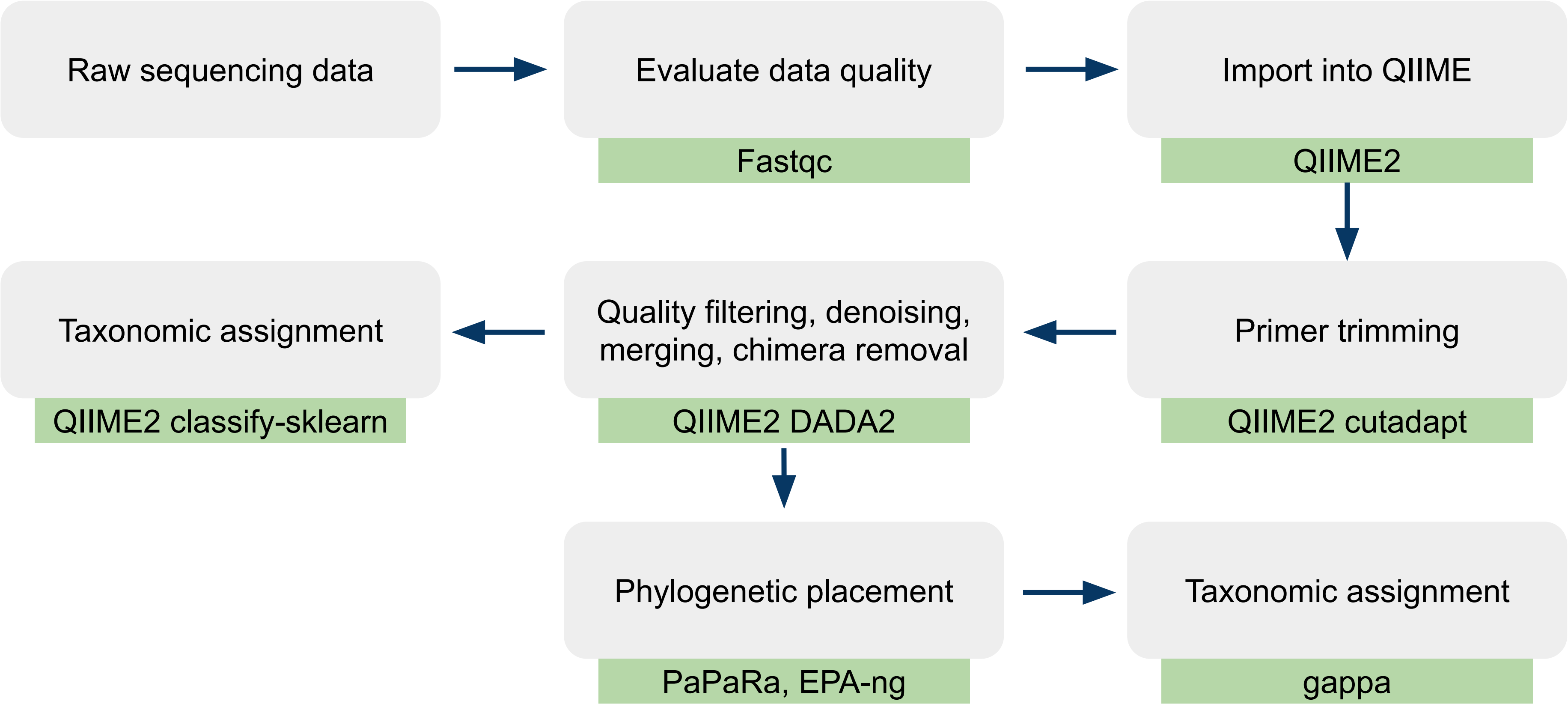
- Two-step polymerase chain reactions (PCR):
  - First PCR reaction: the targeted DNA region is amplified using specific primers flanked by sequencing primers
  - Second PCR reaction: the sequencing primers allow for a second PCR reaction to add adapter sequences and indexes for sample multiplexing



Cruaud, P., Rasplus, JY., Rodriguez, L. et al. High-throughput sequencing of multiple amplicons for barcoding and integrative taxonomy. Sci Rep 7, 41948 (2017). https://doi.org/10.1038/srep41948

Demultiplexing: step in high-throughput sequencing data analysis where sequences are sorted based on their sample of origin

# Amplicon sequencing data analysis pipeline



Raw sequencing data

Evaluate data quality

Fastqc

Import into QIIME

QIIME2

Primer trimming

QIIME2 cutadapt

Quality filtering, denoising, merging, chimera removal

QIIME2 DADA2

Taxonomic assignment

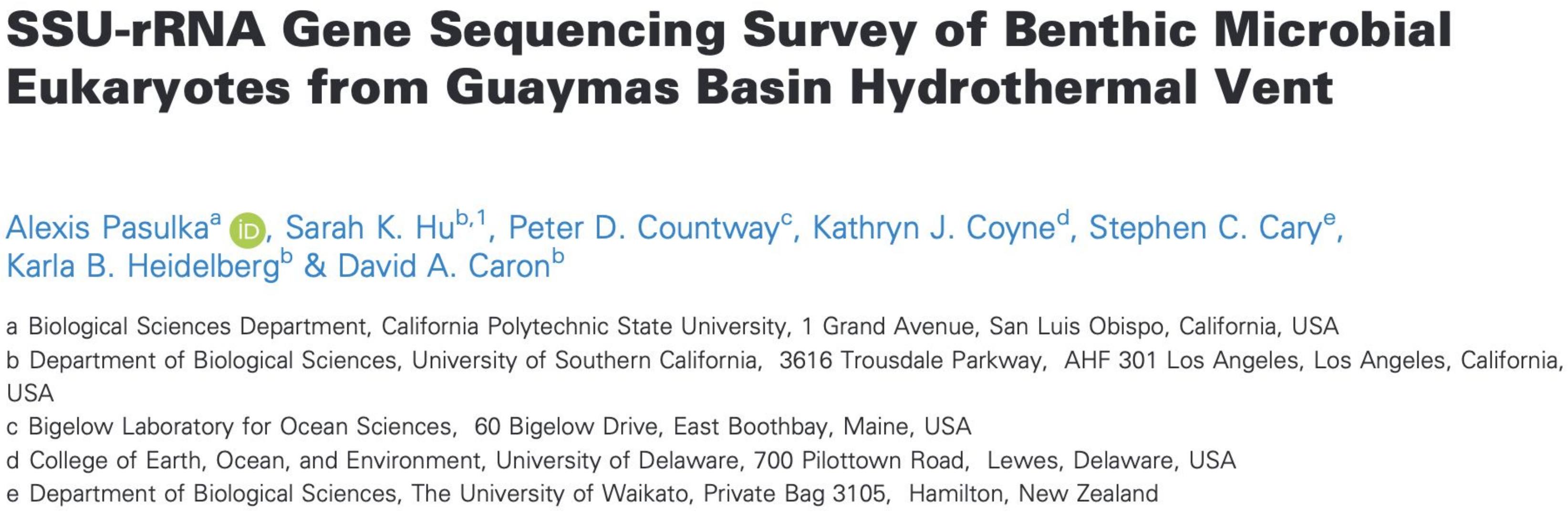
QIIME2 classify-sklearn

Phylogenetic placement

PaPaRa, EPA-ng

Taxonomic assignment

gappa



# SSU-rRNA Gene Sequencing Survey of Benthic Microbial Eukaryotes from Guaymas Basin Hydrothermal Vent

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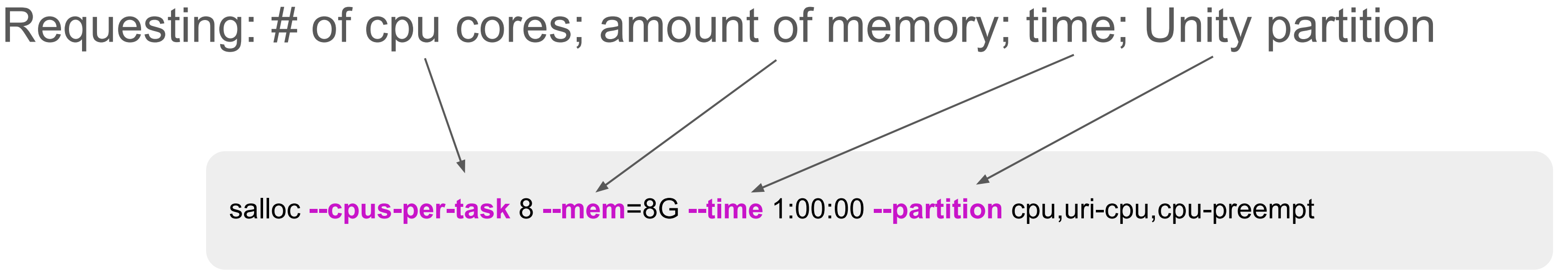
c Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive, East Boothbay, Maine, USA
d College of Earth, Ocean, and Environment, University of Delaware, 700 Pilottown Road, Lewes, Delaware, USA
e Department of Biological Sciences, The University of Waikato, Private Bag 3105, Hamilton, New Zealand

- 18S rRNA gene high-throughput sequencing of the V4 region (expected amplicon size: ~300 bp)
- Raw sequence data (Illumina MiSeq) available in the Sequence Read Archive (SRA) repository (BioProject accession ID: PRJNA391741)
- The following example for amplicon sequencing data analysis is done one one sample

# Start interactive session on Unity command line



Requesting: # of cpu cores; amount of memory; time; Unity partition

```
salloc --cpus-per-task 8 --mem=8G --time 1:00:00 --partition cpu,uri-cpu,cpu-preempt
```

# Download from NCBI Sequence Read Archive (SRA)

Need to use NCBI's SRA toolkit to download data from the SRA

```
module load sratoolkit/3.0.7
module load entrezdirect/10.7.20190114
module load parallel/20210922

project='PRJNA391741'
esearch -db sra -query $project | efetch -format runinfo > runinfo.csv
cat runinfo.csv | cut -d "," -f 1 > SRR.numbers
sed -i '1d' SRR.numbers
cat SRR.number | parallel fastq-dump --split-files --origfmt --gzip
```

Fetches information about sequencing runs based on accession ID

Pulls out sample IDs

Downloads fastq files from all samples in parallel

```
mkdir fastq/
cd fastq/
fastq-dump SRR5753741 --split-files --origfmt --gzip
```

Download only one sample based on sample ID on SRA

# Visualize quality of reads with fastqc

```
module load fastqc/0.11.9
module load MultiQC/1.12-foss-2021b

mkdir fastqc/
fastqc fastq/*_1* --outdir fastqc/
fastqc fastq/*_2* --outdir fastqc/

multiqc fastqc/*_1_fastqc.zip --filename forward_multiqc.html --outdir multiqc/
multiqc fastqc/*_2_fastqc.zip --filename reverse_multiqc.html --outdir multiqc/
```

Summarizes sequence quality for each fastq file (forward and reverse)

If you have many samples, summarizes the fastqc results into one file per read direction

# Import into QIIME2

```
module load uri/main QIIME2/2021.8

cd fastq/
rename 's/_/_00_L001_/g' *
rename 's/.fastq.gz/_001.fastq.gz/g' *
rename 's/_1/_R1/g' *
rename 's/_2/_R2/g' *

qiime tools import \
        --type 'SampleData[PairedEndSequencesWithQuality]' \
        --input-path fastq/ \
        --output-path work/demux_PE.qza \
        --input-format CasavaOneEightSingleLanePerSampleDirFmt
```

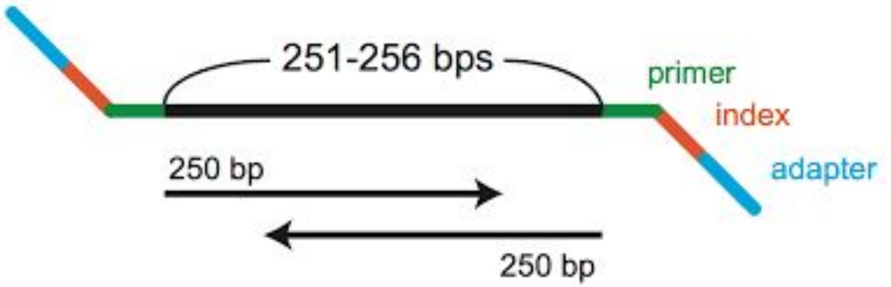
Note: this is an older version of qiime, you should install the latest version in a conda environment

We are importing into QIIME using a specific file name format (Casava), so we need to rename our files first. You can also use a manifest file to import fastq files, instructions here

Import fastq files into QIIME

# Pre-processing - trim primers

- Primers don't always bind perfectly to the target sequence (sequence not identical to the target DNA sequence).
- Use Cutadapt to remove primers and any preceding bases



- Primer: short nucleotide sequence complementary to the target sequence
- Index: short nucleotide sequence that serves as a unique identifier associated with a sample
- Adapter: short nucleotide sequence that allows the library to bind to the sequencing flow cell

# Pre-processing - trim primers

```
qiime cutadapt trim-paired \
        --i-demultiplexed-sequences work/demux_PE.qza \
        --p-cores 8 \
        --p-front-f CCAGCASCYGCGGTAATTCC \
        --p-front-r ACTTTCGTTCTTGATYRA \
        --p-match-adapter-wildcards \
        --p-match-read-wildcards \
        --p-minimum-length 10 \
        --p-discard-untrimmed \
        --verbose \
        --o-trimmed-sequences work/demux_PE_trimmed.qza

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

Forward primer sequence
Reverse primer sequence

Discard reads that were not trimmed/did not contain primers

# Denoising - DADA2

DADA2: Filters based on quality score of bases, denoises sequences (models and corrects sequencing errors from Illumina sequencer), merges forward and reverse reads, and then filters out chimeras

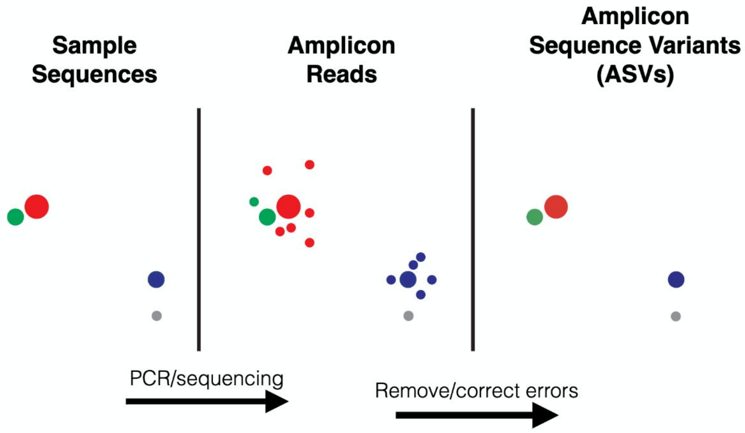
```
qiime dada2 denoise-paired \
        --i-demultiplexed-seqs work/demux_PE_trimmed.qza \
        --p-trunc-len-f 220 \
        --p-trunc-len-r 210 \
        --p-n-threads 8 \
        --verbose \
        --o-table work/table.qza \
        --o-representative-sequences work/rep-seqs.qza \
        --o-denoising-stats work/DADA2-stats.qza

qiime metadata tabulate --m-input-file work/DADA2-stats.qza --o-visualization
work/DADA2-stats.qzv
```

Truncate sequences when they start to drop off in quality

# Amplicon denoising

- Sequence quality control step to remove sequence errors from amplicon reads and obtain Amplicon Sequence Variants (ASVs)
- Used to improve taxonomic assignment of amplicon reads
- Use DADA2 to perform denoising
  - DADA2 implements a ‘quality-aware model’ of sequencing errors and corrects the reads by removing noise related to the sequencing methodology



# Assign taxonomy

Download premade classifier from QIIME2 website

Can also create your own (e.g. with another database like PR2 - instructions to train your own)

```
wget https://data.qiime2.org/2021.8/common/silva-138-99-nb-classifier.qza

qiime feature-classifier classify-sklearn \
        --i-classifier work/silva-138-99-nb-classifier.qza  \
        --i-reads work/rep-seqs.qza \
        --o-classification work/taxonomy.qza
```

# Export QIIME artifacts

```
qiime tools export \
        --input-path work/table.qza \
        --output-path work/export/table
```

Export count table (BIOM format)

- Convert to readable format (.csv, .tsv) with tool like biom-format

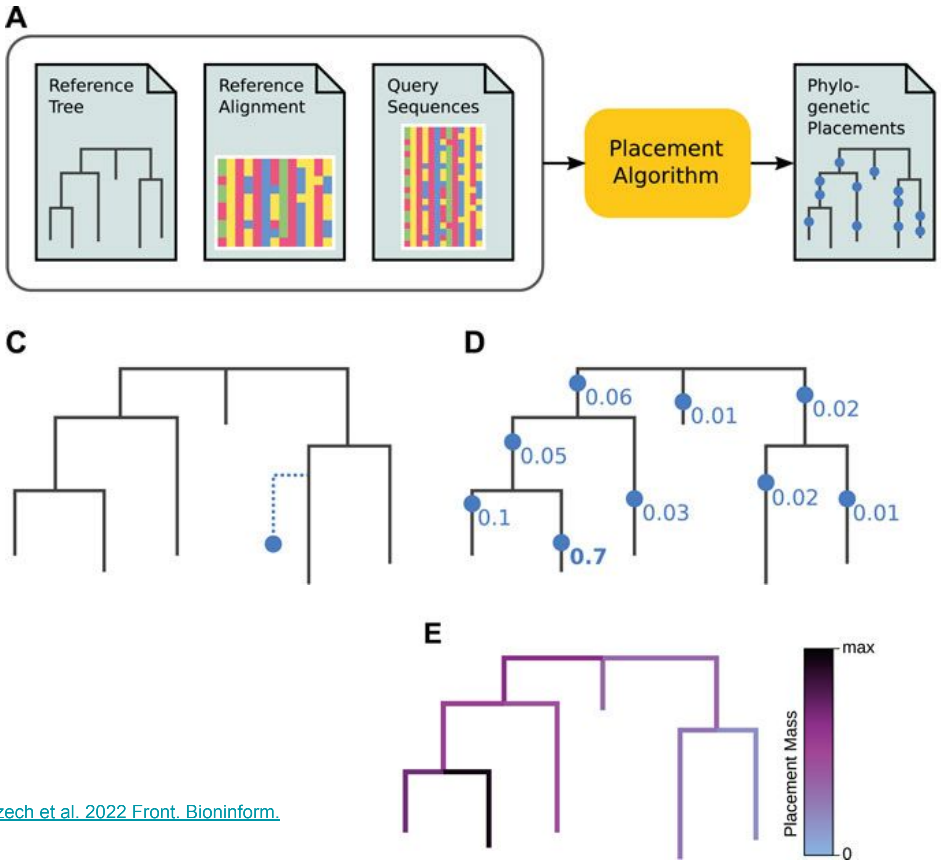
```
qiime tools export \
        --input-path work/rep-seqs.qza \
        --output-path work/export/rep-seqs
```

Export representative sequences of ASVs (.fasta file)

```
qiime tools export \
        --input-path work/taxonomy.qza \
        --output-path work/export/taxonomy
```

Export taxonomic assignments of ASVs (.tsv file)

# Phylogenetic placement



Czech et al. 2022 Front. Bioninform.

Place query sequences (ASVs) onto a reference phylogenetic tree in order to get a deeper understanding of the phylogenetic composition of your samples

- Capture diversity which is underrepresented in reference databases - do not need exact match, takes evolutionary history into account
- More accurate way to analyze phylogeny of your samples and conduct phylogenetically aware diversity analyses (versus *de novo* tree-building methods)
- Can use for taxonomic assignment, diversity quantification, sample comparison, correlation with environmental variables

# Phylogenetic placement

Need a reference phylogenetic tree to place sequences onto:

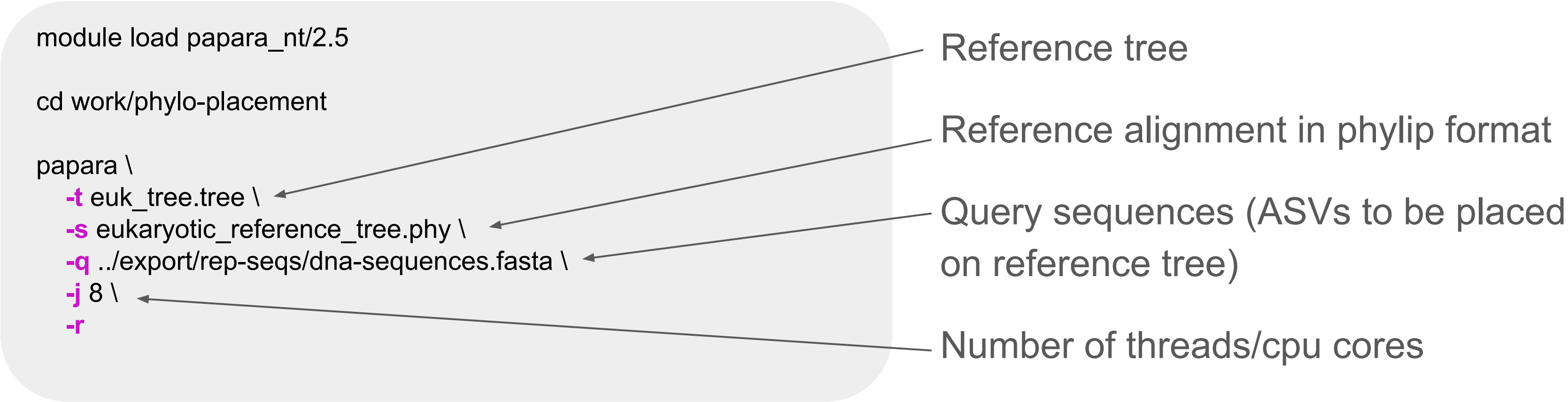
1. Reference phylogenetic tree
2. Reference alignment
3. File describing taxonomy of each tip of phylogenetic tree

This reference tree should span the diversity of sequences that will be placed on the tree, and should contain (nearly) full-length, high quality, curated sequences from relevant gene (here, 18S rRNA)

Here, using eukaryotic tree of life from this publication

# Phylogenetic placement - PaPaRa

Aligns ASVs to reference sequences so that they can be placed onto reference phylogenetic tree



```
module load papara_nt/2.5

cd work/phylo-placement

papara \
   -t euk_tree.tree \
   -s eukaryotic_reference_tree.phy \
   -q ../export/rep-seqs/dna-sequences.fasta \
   -j 8 \
   -r
```

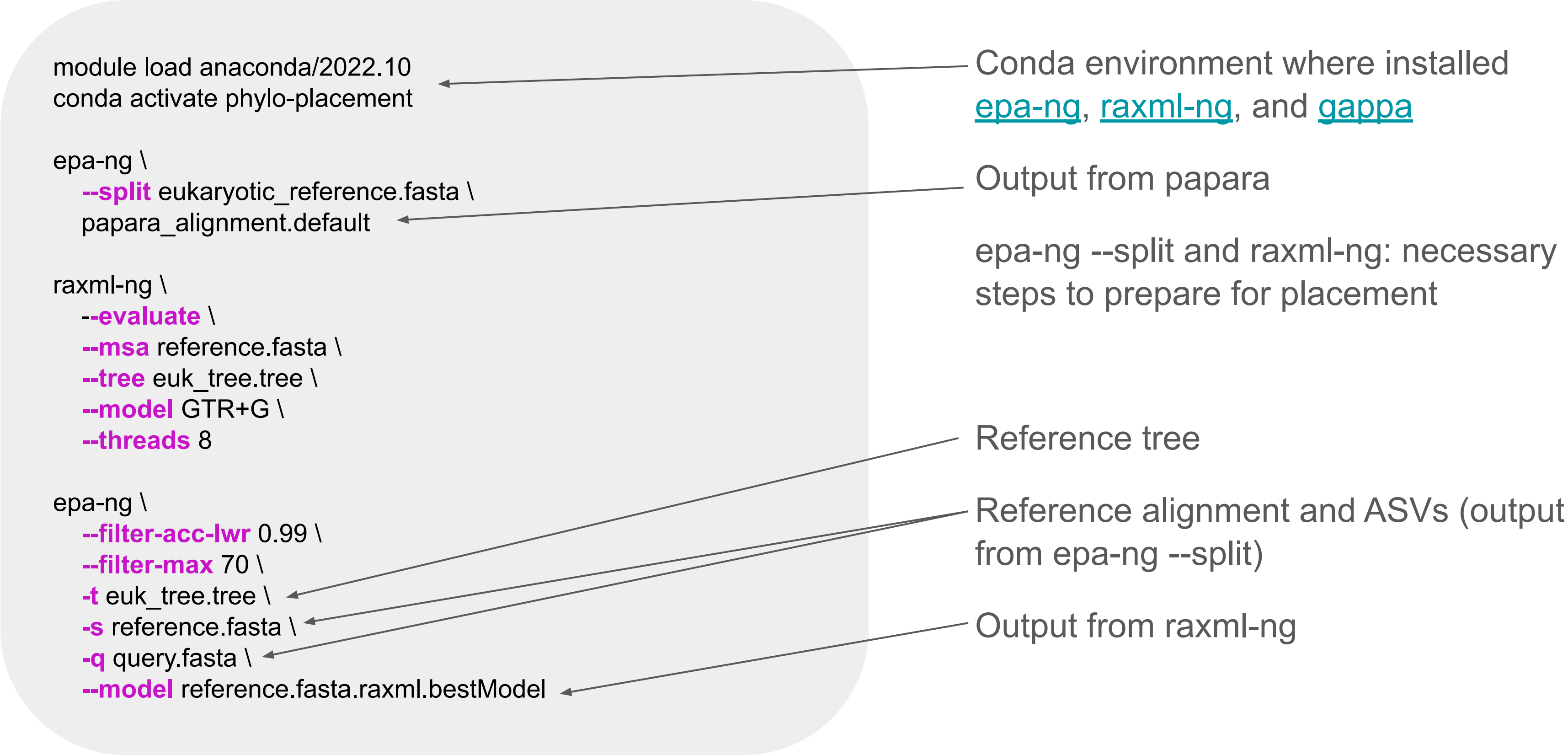
Reference tree

Reference alignment in phylip format

Query sequences (ASVs to be placed on reference tree)

Number of threads/cpu cores

# Phylogenetic placement - EPA-ng



```
module load anaconda/2022.10
conda activate phylo-placement

epa-ng \
    --split eukaryotic_reference.fasta \
    papara_alignment.default

raxml-ng \
    --evaluate \
    --msa reference.fasta \
    --tree euk_tree.tree \
    --model GTR+G \
    --threads 8

epa-ng \
    --filter-acc-lwr 0.99 \
    --filter-max 70 \
    -t euk_tree.tree \
    -s reference.fasta \
    -q query.fasta \
    --model reference.fasta.raxml.bestModel
```

Conda environment where installed epa-ng, raxml-ng, and gappa

Output from papara

epa-ng --split and raxml-ng: necessary steps to prepare for placement

Reference tree

Reference alignment and ASVs (output from epa-ng --split)

Output from raxml-ng

# Phylogenetic placement - gappa

Tools to visualize and analyze results from phylogenetic placement

```
gappa \
   examine heat-tree \
   --jplace-path epa_result.jplace \
   --mass-norm absolute \
   --write-svg-tree \
   --write-newick-tree \
   --write-nexus-tree

gappa \
   examine assign \
   --jplace-path epa_result.jplace \
   --taxon-file eukaryotic_reference_tax.txt \
   --per-query-results

gappa \
   examine lwr-list \
   --jplace-path epa_result.jplace
```

Provides you with reference tree annotated with ASV placements

Output from placement

Figures out the best taxonomic assignment based on all the placements

File describing the taxonomy of tips of tree

Summary of likelihood ratios for all placements

# Additional Resources

- Unity Onboarding video (Spring 2024)
- QIIME2 snakemake pipeline
- Snakemake workshop

- Unity community Slack
- More contact information