

# Metagenomics: Characterization of Microbial Communities using NGS

16S



Brandi Cantarel, PhD  
10/19/2016

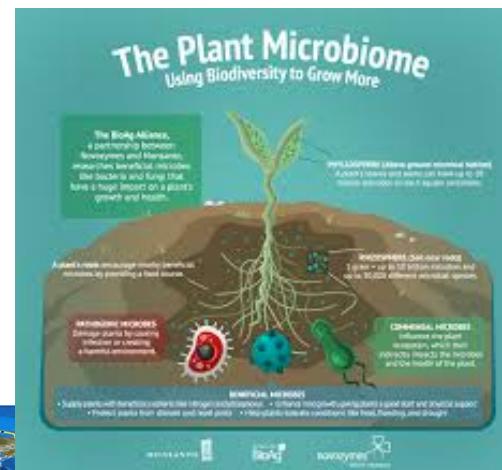
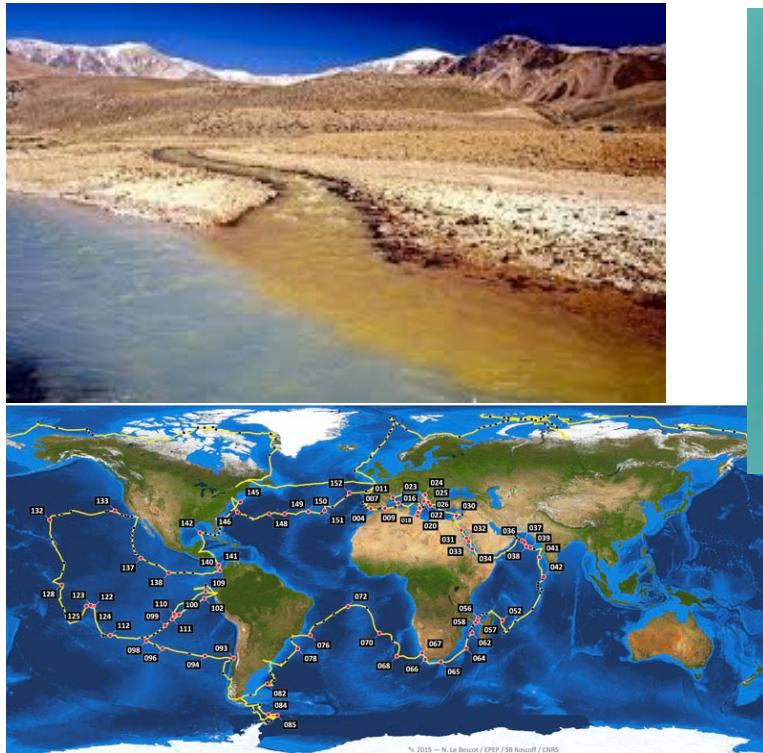
- *Introduction to Metagenomics*
  - What is a microbiome, metagenome, the relationship between the micro biome and it's environment?
  - Whole Community Sequencing Methods vs Traditional Culture Methods
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  - Taxonomy assignment and OTUs
  - Analysis platforms: Qiime and Mothur
  - Phylogenetic Trees, Rarefaction and Diversity and PCoA

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# What is a Microbiome?

- A term coined by Joshua Lederberg
- The ecological community of commensal, symbiotic and pathogenic microorganisms
- All plants and animals, from protists to humans, live in close association with microbial organisms.
- The hologenome theory proposes that the object of natural selection is not the individual organism, but the organism together with its associated microbial communities.

# Microbiomes Are Everywhere



## Metaorganisms (Superorganisms)

- Animal bodies (including humans) are superorganisms.
- Composed of microbial and animal cells
- Microbes are important for digestion, immune development and other functions essential for survival

Click on a label for more information

Mouth, Pharynx, Respiratory System

Streptococcus viridans  
Neisseria sicca  
Streptococcus salivarius

Gastric lumen

Stomach

Helicobacter pylori  
Bacteroides fragilis  
Sympatricus thermophilus

Intestines

Lactobacillus casei  
Lactobacillus gasseri  
Escherichia coli  
Bacteroides thetaiotaomicron

Intestines

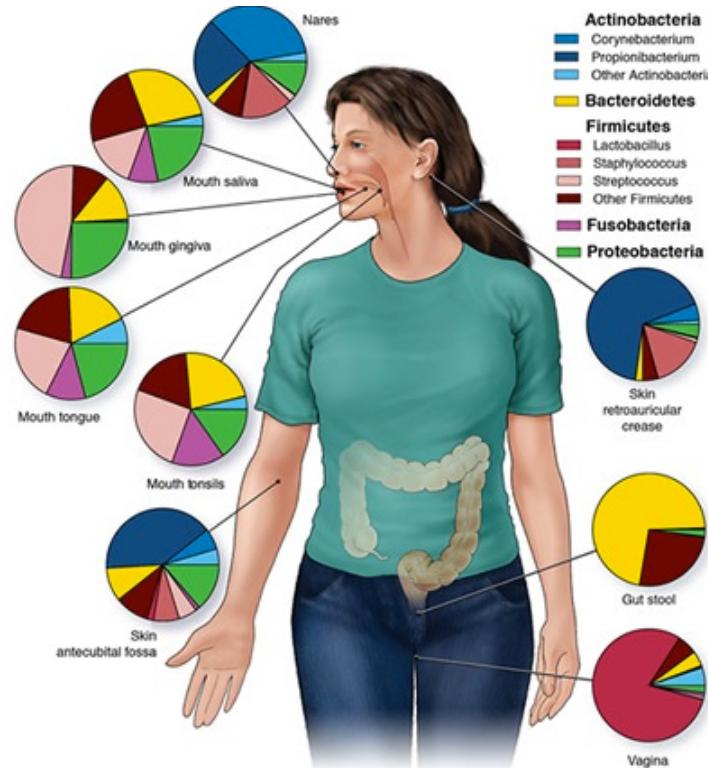
Urogenital tract

Uropathogens perineum  
Corynebacterium urealyticum

Skin

Corynebacterium phlegmatis  
Propionibacterium acnes  
Staphylococcus epidermidis  
Trichophyton

# The Human Body is Composed of Many Small Ecosystems (Microbiomes)



## Emerging Metagenomics

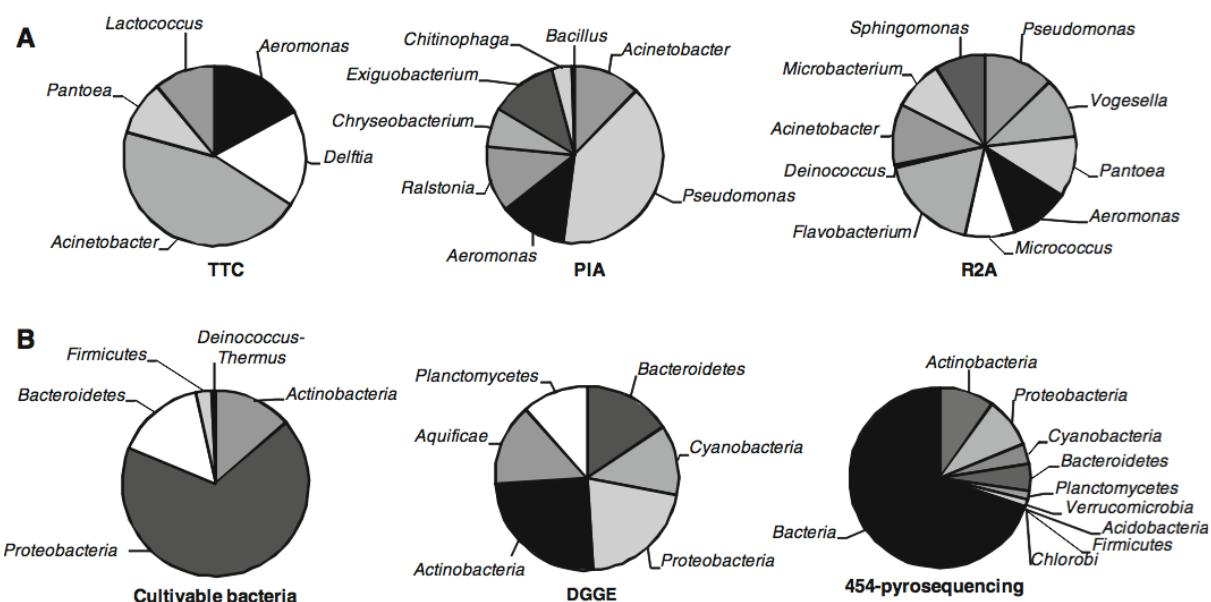
- Late 17th Century, Anton van Leeuwenhoek
  - First metagenomicist who directly studies organisms from pond water and his own teeth
- 1920s
  - Cell culture evolved, 16S rRNA sequencing of cultural microbes
  - If an organism could not be cultured, it could not be classified



# Traditional Culture Dependent Profiling

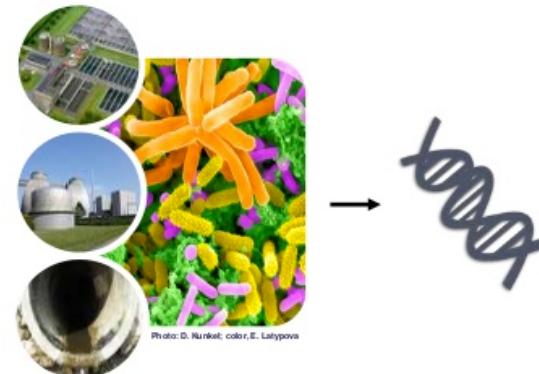
- It's estimated that only about <1% of microorganisms can be grown in culture
  - Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev*. 1995 Mar;59(1):143-69. Review. PubMed PMID: 7535888; PubMed Central PMCID: PMC239358.
- Discrepancies observed:
  - Number of organisms under microscope in conflict with amount on plates
  - Cellular activities in situ conflicted with activities in culture
  - Cells are viable but unculturable
- Even if all microbes could be grown in culture, it would be a daunting task to determine growth conditions for ALL microbes

## Culture Dependent Profiles Depends on Culture Methods



# What is a Metagenome?

- The term "metagenomics" was first used by Jo Handelsman, Jon Clardy, Robert M. Goodman, Sean F. Brady, and others, and first appeared in publication in 1998.
- A metagenome is the collection of genes in a microbial community.
- Metagenomics is the study of genetic material from an environmental sample
- Offers a culture independent methods



## “Modern” Metagenomics

- Norman Pace proposes the idea of cloning DNA directly from environmental samples in 1985
  - First report by Pace et al in 1991
- Ribosomal Database Project (RDP) is funded in 1989
- Metagenomics is defined 1998
- Metagenomic discovery of proteorhodopsin in 2000
- Term Microbiome is coined in 2001
- Sargasso and Acid Mine Drainage Projects published 2004
- Global Ocean Sampling Expedition in 2007
- Human Microbiome and MetaHit projects are launched in 2008



# Earth Microbiome Project



The Earth Microbiome Project is a proposed massively multidisciplinary effort to analyze microbial communities across the globe. The general premise is to examine microbial communities from their own perspective. A portal for visualization of all information.

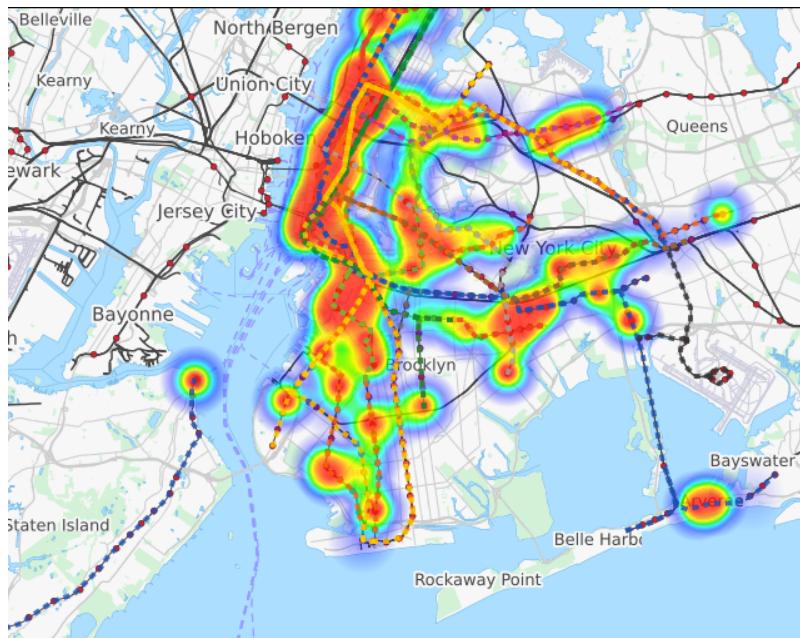
## Microbiomes in Extreme Environments



The Extreme Microbiome Project (XMP) is a scientific effort to characterize, discover, and develop new pipelines and protocols for extremophiles and novel organisms.

<http://extrememicrobiome.org/>

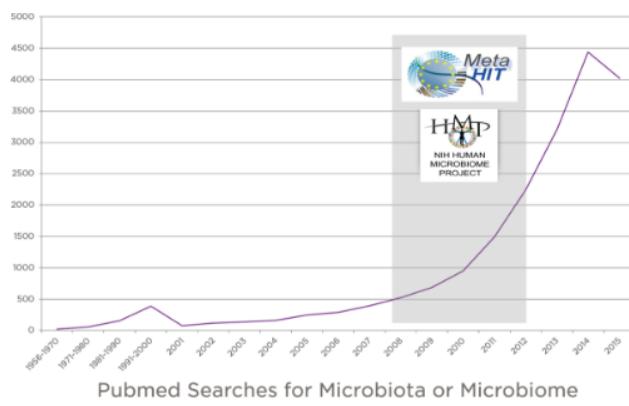
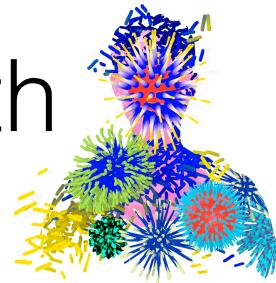
# Urban Microbiomes



<http://www.pathomap.org/>

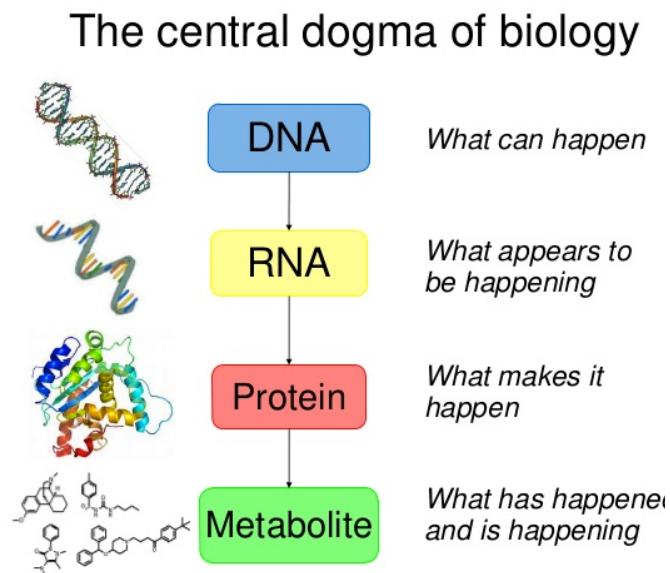
## Microbiomes in Health

- Acne
- Antibiotic-associated diarrhea
- Asthma/allergies
- Autism
- Autoimmune diseases
- Cancer
- Dental cavities
- Depression and anxiety
- Diabetes
- Eczema
- Gastric ulcers
- Hardening of the arteries
- Inflammatory bowel diseases
- Malnutrition
- Obesity



# Understanding Interactions between Microbial Communities and Environment

- Experimental and computational techniques are necessary to make inferences about the community:
  - Community Structure
  - Gene Content
  - Expression
  - Translation
  - Metabolites



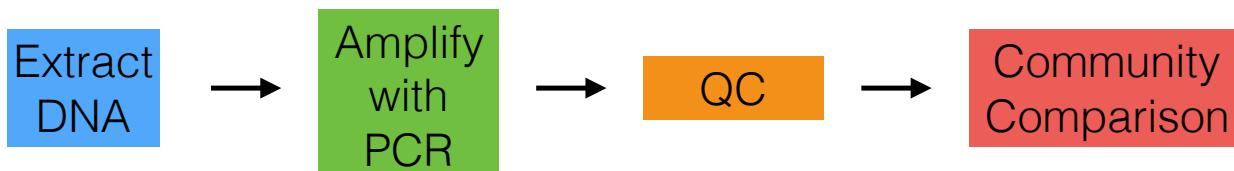
Much of the Early Studies Focused on DNA



- Community Structure
- Gene Content

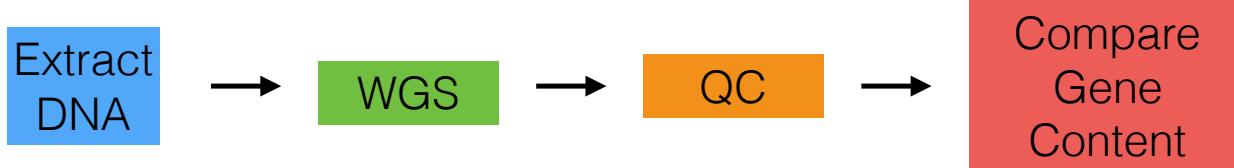
# Using Marker Genes to Examine Community Structure

- Asking the questions: what organisms are there?
- A good marker gene needs to be:
  - Present in all organisms compared
  - Vertically and slowly evolving
  - Amplify-able with small set of “universal primers”
  - Have an established database of reference



# Whole Genome Shotgun

- Asking the questions: what is the functional capability?
- WGS can
  - Identify organisms present — if closely related to organisms with sequenced genomes
  - Identify gene families present — if homologs have been functionally characterized
  - Identify functional pathways present — if homologs have been annotated to gene pathways
  - Identify new species/strains — if assemblies are of sufficient depth



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## Historic Sequencing Technologies

- Sanger
  - DNA Sequencing with chain-terminating inhibitors (1977)
  - Used in Early Microbiome Studies
  - Considered Gold Standard for Accuracy
- Roche 454
  - Pyrosequencing (2004)
  - Used for many 16S, and early WGS Sequencing studies
  - Inaccuracies accumulated in homopolymer regions



# Commonly Used Sequencing Technologies

- Ion Torrent
  - 400bp reads
  - Inaccuracies accumulated in homopolymer regions
  - ~ \$0.63/Mbp — Hardware ~\$70K/machine
  - Low upfront and maintenance costs makes it attractive to independent labs
- Illumina HiSeq
  - 150/200 bp reads
  - \$0.04 Mbp — Hardware ~ \$1M
  - Used for WGS projects
- Illumina MiSeq
  - 250/300 bp reads
  - \$0.05 Mbp — Hardware ~ \$125K
  - Used for 16S projects — 384 samples/run
  - Desktop Sequencer



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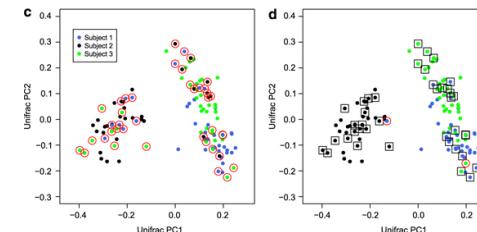
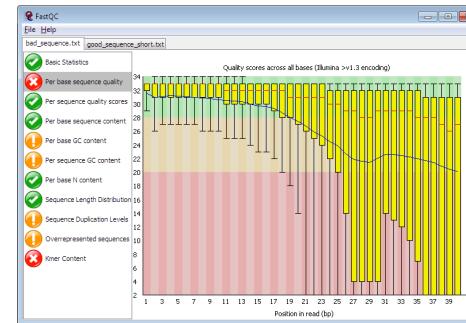
## Third Generation Long-Read Sequence Technologies

- Pacific Biosciences
  - Single Molecule Real Time (SMRT) Sequencing
  - Very High Error Rate
  - Average read length > 1kb
  - Great for Finishing Genomes by Illumina/PacBio Hybrid Assembly
  - ~\$2/Mbp
- Oxford Nanopore MinION
  - A “laptop powered” sequencing
  - Average Read Length 5.4kb
  - Light weight and low power usage makes it interesting for “in the field” applications
  - Potential for pathogen identification in ~ 4 hours in the clinic



# Quality Control

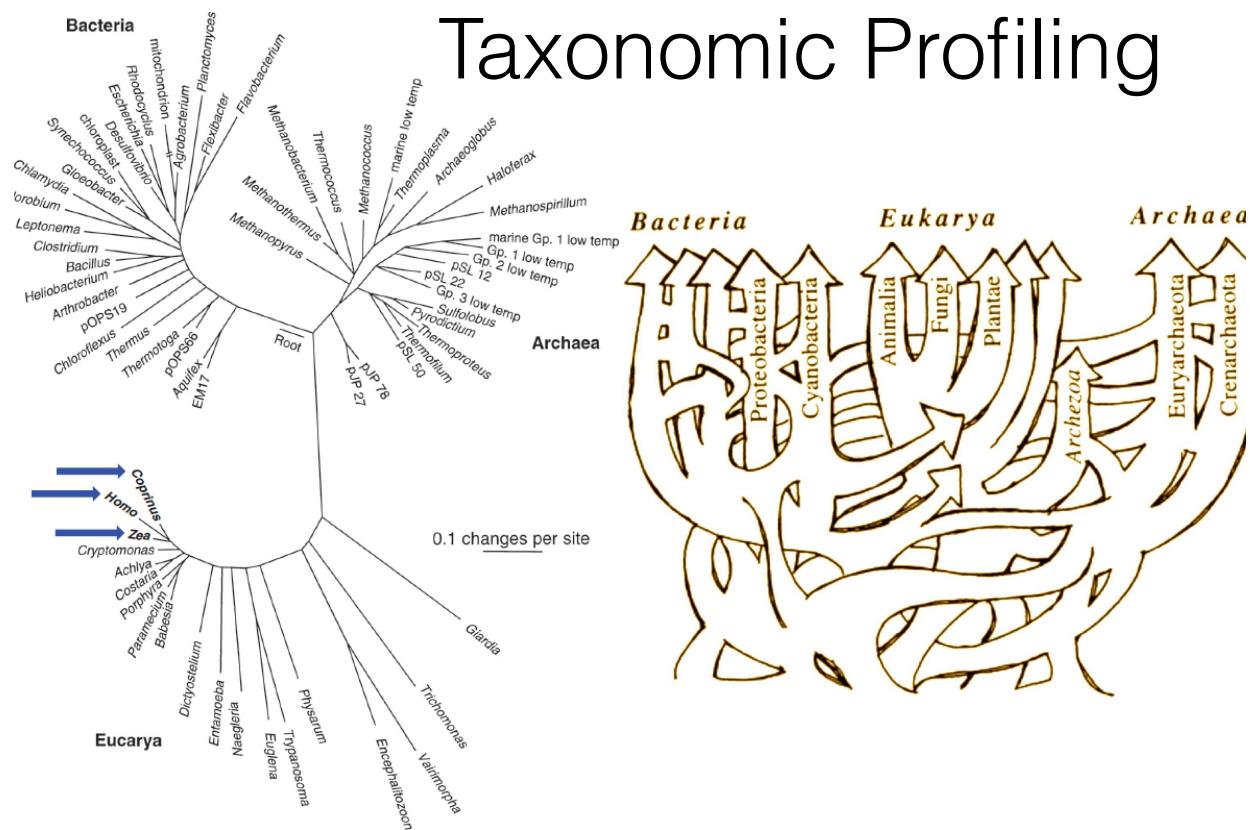
- Sequencing Errors
  - Low Quality Bases
  - Homopolymer Strings
  - Too short trimmed reads
- Contamination, Mislabeled and Sampling Error
  - Negative Controls are the best way to identify lab contamination
  - Biological and Technical Replicates



Knights D, Kuczynski J, Koren O, Ley RE, Field D, Knight R, DeSantis TZ, Kelley ST. Supervised classification of microbiota mitigates mislabeling errors. ISME J. 2011 Apr;5(4):570-3. doi: 10.1038/ismej.2010.148. Epub 2010 Oct 7. PubMed PMID: 20927137; PubMed Central PMCID: PMC3105748.

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# Marker Genes Allow For Taxonomic Profiling



# Marker Genes Allow For Taxonomic Profiling

- Should be present in all prokaryotic organisms compared
  - Vertically and slowly evolving
  - Amplify-able with small set of “universal primers”
  - Has an established database of reference sequences

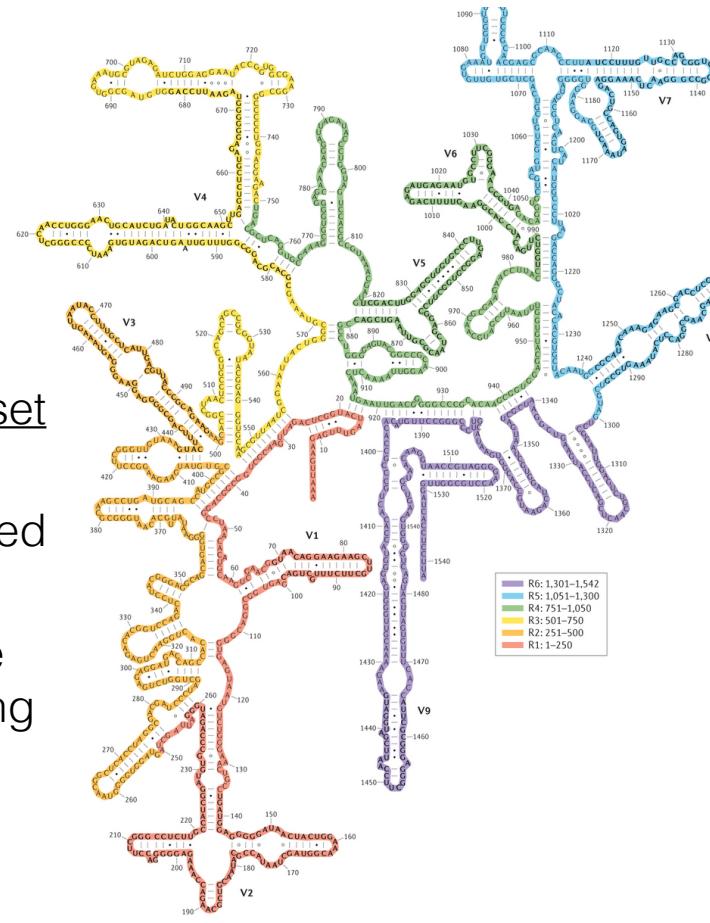
# rRNAs as phylogenetic markers

- Ribosomal RNAs are present in all living organisms
    - 16S present in all prokaryotes
    - 18S present in all eukaryotes
  - rRNAs are vertically and slowly evolving
    - Play a critical role in protein translation
    - rRNAs are relatively conserved and rarely acquired horizontally
    - rRNAs are amplify-able with small set of “universal primers”
  - rRNAs has an established reference database

# 16S as a Marker Gene

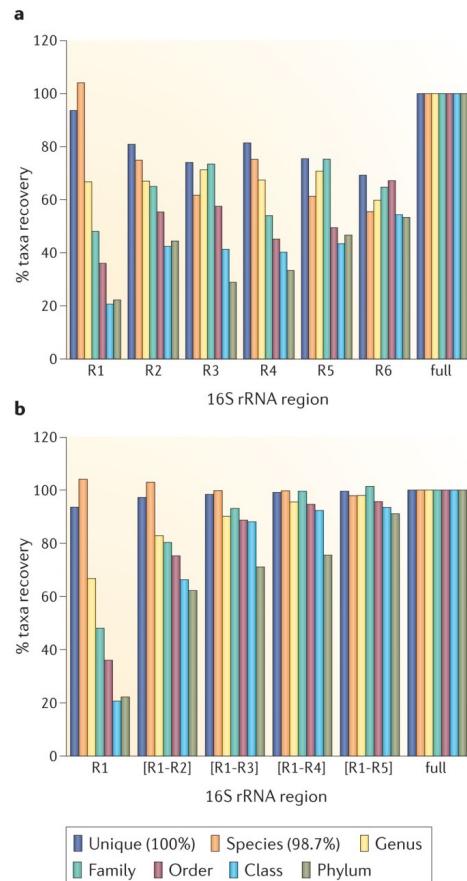
Amplify-able with small set  
of “universal primers”

- Better to have specialized primers for archaea
  - Primers for 16S variable regions can give differing results



# 16S as a Marker Gene

- 16S: Different V regions generates different community profiles
- Strain-level diversity will be missed by amplicon



Nature Reviews | Microbiology

## rRNA Reference Databases



Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, Y. Sun, C. T. Brown, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucl. Acids Res. 42(Database issue):D633-D642; doi: 10.1093/nar/gkt1244 [PMID: 24288368]



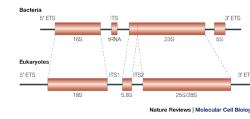
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucl. Acids Res. 41 (D1): D590-D596.



DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol 72:5069-72.

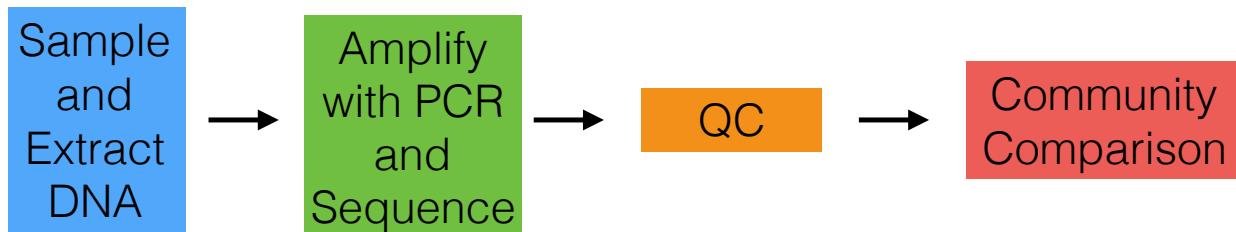
# Other Marker Genes

- Intergenic Transcribed Spacer (ITS)
  - RecA: Response to DNA Stress in Bacteria
  - Cpn60: Chaperonin Database



# 10 Minute Break

# Workflow for Marker Gene Analysis



Sample and Extract DNA

## Sampling

- Sampling Must be Standardized
- Samples should be
  - collected with sterile instrumentation or swabs
  - transported into a sterile tube without too much interaction with the environment
  - stabilized depending on molecule of interest
  - “frozen in time”
- [http://www.hmpdacc.org/doc/  
HMP\\_Protocol\\_Version\\_9\\_032210.pdf](http://www.hmpdacc.org/doc/HMP_Protocol_Version_9_032210.pdf)

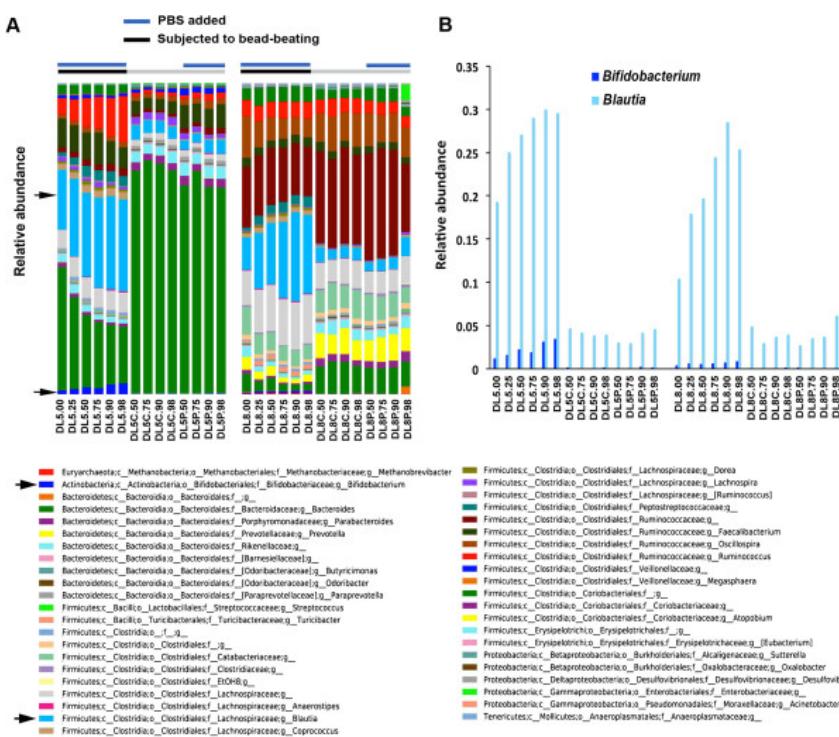


# Sources of Contamination

- At Collection — use sampling protocol
    - Host DNA
    - Environmental
  - In the lab
    - Use a negative control (water or stabilization buffer) sample to determine likely lab contamination
    - Your microbiome covers is a cloud around your body



# DNA Extraction



Santiago A, Panda S, Mengels G, Martinez X, Azpiroz F, Dore J, Guarner F, Manichanh C. Processing faecal samples: a step forward for standards in microbial community analysis. *BMC Microbiol.* 2014 May 1;14:112. doi: 10.1186/1471-2180-14-112. PubMed PMID: 24884524; PubMed Central PMCID: PMC4021188.

Extraction Methods that Include a Bead Beating Step shows greater diversity

# DNA Extraction

- Many protocols/kits available but kit-bias exists
  - Santiago A, Panda S, Mengels G, Martinez X, Azpiroz F, Dore J, Guarner F, Manichanh C. Processing faecal samples: a step forward for standards in microbial community analysis. *BMC Microbiol.* 2014 May 1;14:112. doi: 10.1186/1471-2180-14-112. PubMed PMID:24884524; PubMed Central PMCID: PMC4021188.
    - *Bead-beating is important for breaking down the cell-wall of some organisms*
- HMP and other major projects have standardized DNA extraction protocols
  - <http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>
  - [http://www.hmpdacc.org/doc/  
HMP\\_MOP\\_Version12\\_0\\_072910.pdf](http://www.hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf)

## Target Amplification



**CONSERVED REGIONS:** unspecific applications

**VARIABLE REGIONS:** group or species-specific applications



Read 1



Read 2



overlapping region: sequence comparison



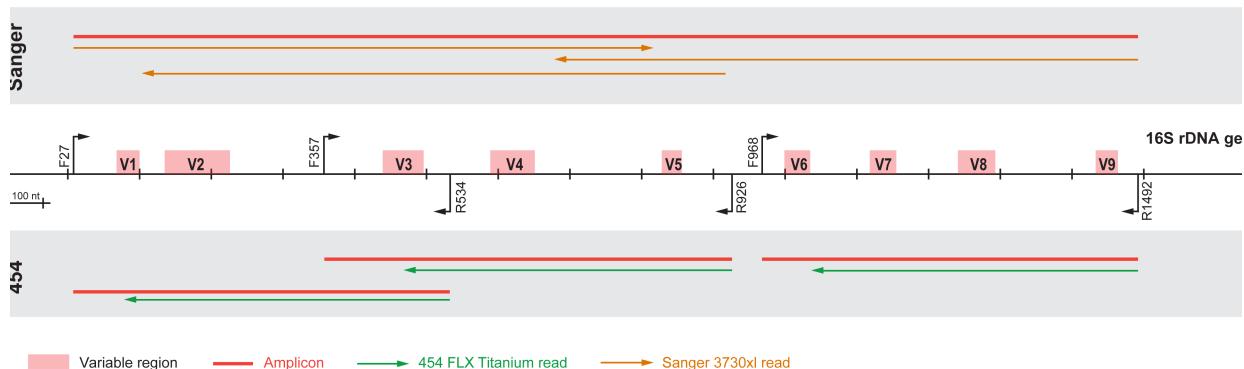
Amplify  
with PCR  
and  
Sequence

# Target Amplification

- PCR primers are designed to amplify specific regions of the genome
  - Primer sets should be able to amplify that regions from a diverse set of taxa
  - For 16S we “prime” off of the conserved regions and use the variable regions to sample diversity
- “Dirty” samples could contain PCR inhibitors
- QC products using BioAnalyzer or gel

Amplify  
with PCR  
and  
Sequence

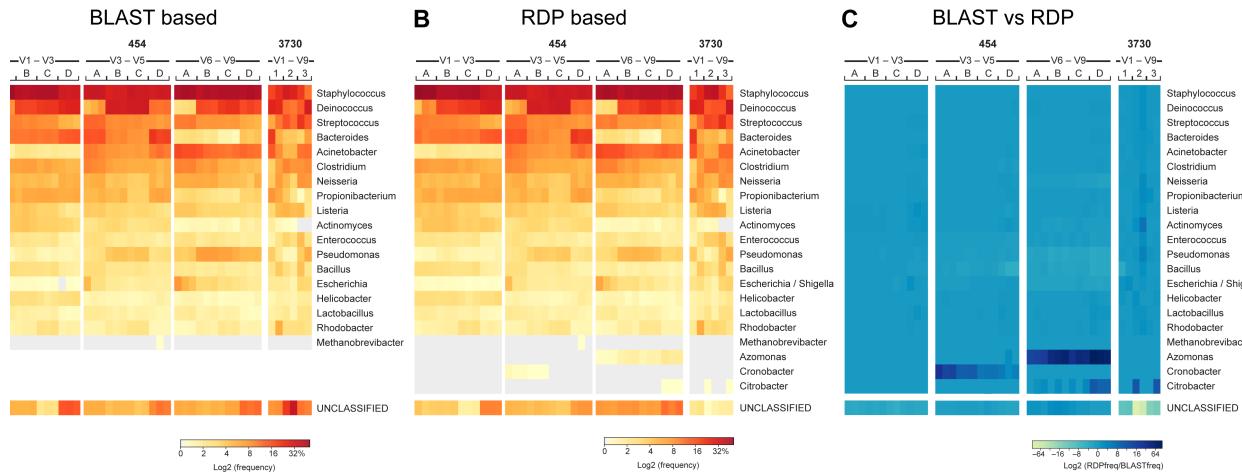
# HMP Target Selection



Jumpstart Consortium Human Microbiome Project Data Generation Working Group.  
Evaluation of 16S rDNA-based community profiling for human microbiome research.  
PLoS One. 2012;7(6):e39315. doi: 10.1371/journal.pone.0039315. Epub 2012 Jun 13.  
PubMed PMID: 22720093; PubMed Central PMCID: PMC3374619.

Amplify  
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and  
Sequence

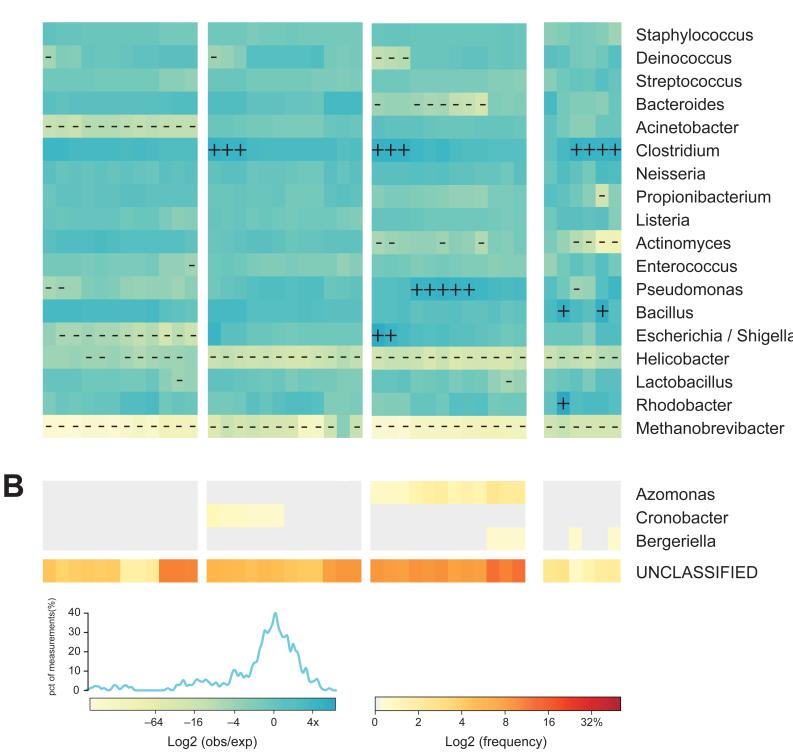
# HMP Target Selection



Comparable results when using BLAST and RDP.

Amplify  
with PCR  
and  
Sequence

# HMP Target Selection



# DIY Marker Gene Analysis



Quantitative Insights Into Microbial Ecology

<http://qiime.org/>



<http://www.mothur.org/>

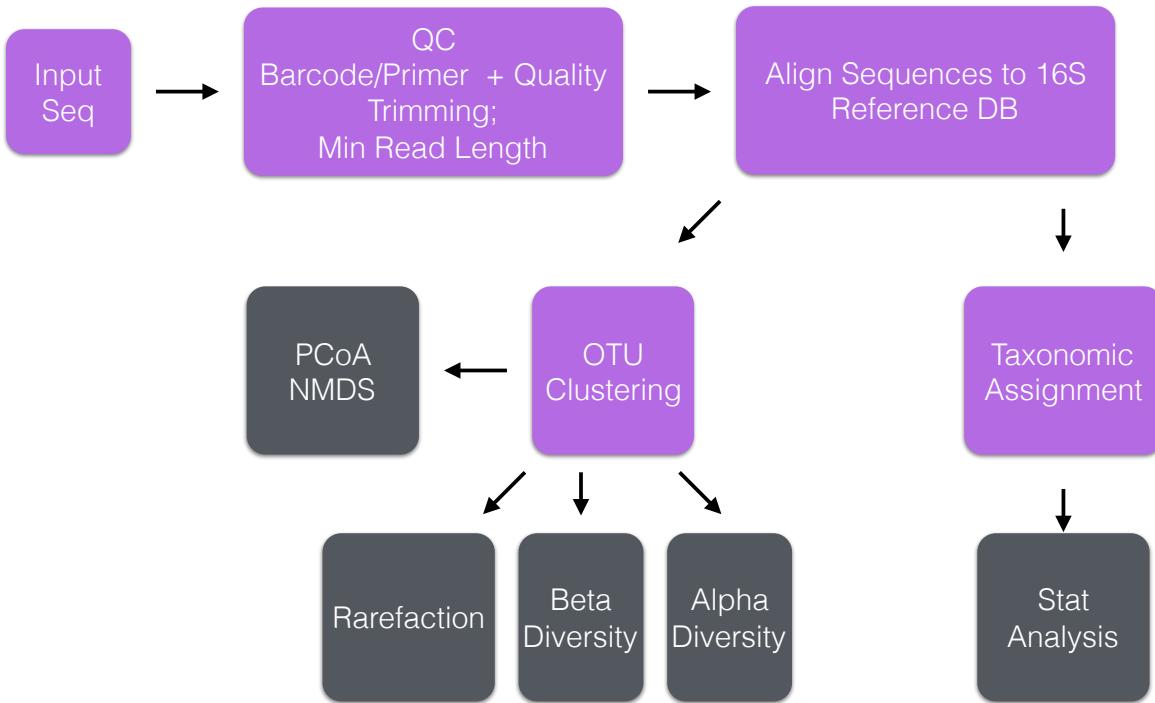


## Mothur or Qiime?



- Mothur
  - Single program with all dependancies built in
  - Reimplementation/Optimization of popular algorithms
  - Easy to install, use
  - Less scalable and hard to add “your own tools”
- Qiime
  - A collection of python scripts, which are wrappers for popular algorithms
  - Large number of dependencies, VM available (for small datasets)
  - More scalable and easy to add “your own tools”

# Overall Analysis Pipeline



QC

## Sequence Processing

- Primer and Barcode Trimming
- Very large (pyrosequencing) and very small sequence trimming
- Low Quality Trimming
- Remove Reads with high likelihood of errors
  - High Number of Homopolymers (pyrosequencing)
  - Ambiguous bases

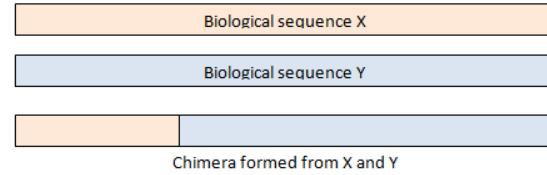
# Sequence Analysis

- Removal of sequences not aligned to the targeted region of the 16S Gene
- Removal of chimera
- Removal of contamination (negative control)

## Alignment

- Choose a reference for the alignment
  - SILVA \*
  - Greengenes
  - RDP
- Choose a search algorithm (Identify the best hit)
  - blast
  - kmer \*
  - suffix
- Choose an alignment algorithm (Align query to best hit)
  - Needleman (Global Aligner) \*
  - Blastn
  - Gotoh
- Penalties for gaps and mismatches (Score the alignment)

# Chimeras

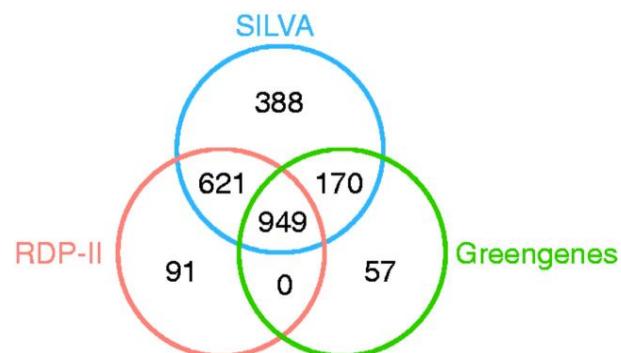


- The chimera was a beast in greek mythology made up of parts from four different creatures
- DNA sequence chimera are artifacts created by PCR
- Chimeras can be detected by comparison to a reference database
  - ChimeraSlayer
  - Uchime

Community  
Comparison

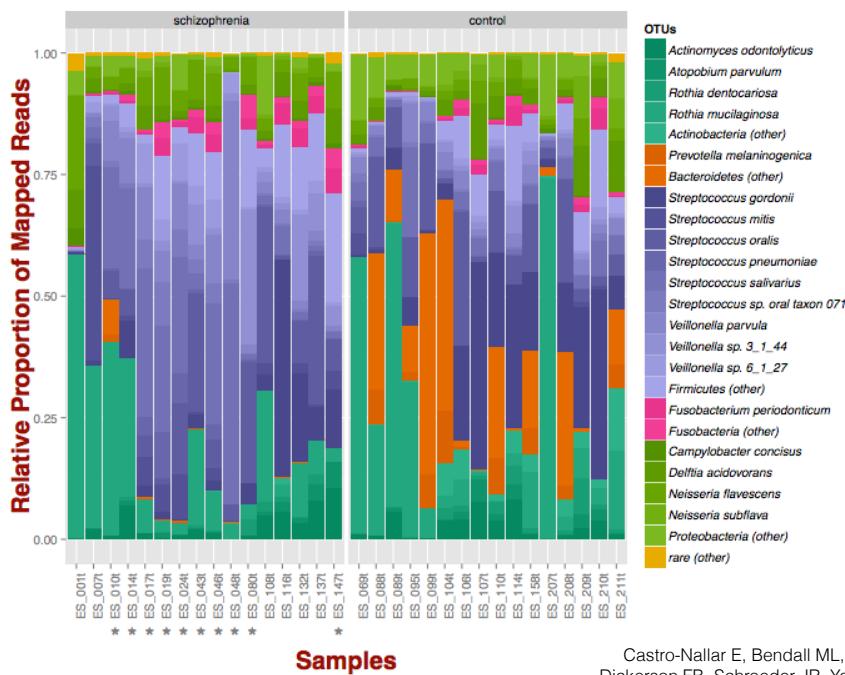
## Taxonomic Classification

- Choose a method
  - Wang — Implemented at the RDP classifier
  - K nearest neighbor
- Choose a hierarchy
  - RDP-II
  - Greengenes
  - Silva



Community  
Comparison

# Taxonomic Comparisons



Castro-Nallar E, Bendall ML, Pérez-Losada M, Sabuncyan S, Severance EG, Dickerson FB, Schroeder JR, Yolken RH, Crandall KA. Composition, taxonomy and functional diversity of the oropharynx microbiome in individuals with schizophrenia and controls. PeerJ. 2015 Aug 25;3:e1140. doi: 10.7717/peerj.1140 eCollection 2015. PubMed PMID: 26336637; PubMed Central PMCID: PMC455614.

Community  
Comparison

# OTU Classification

- Calculate a distance between sequences
- Cluster Sequences by similarity
  - Nearest Neighbor
    - Each of the sequences within an OTU are at most X% distant from the most similar sequence in the OTU.
  - Average Neighbor
    - All of the sequences within an OTU are at average X% distant from all of the other sequences within the OTU.
  - Furthest Neighbor
    - All of the sequences within an OTU are at most X% distant from all of the other sequences within the OTU.

# Alpha Diversity

- Species richness is a survey of the number of distinct organism in a community
- Rarefaction is a method to assess species richness
- Species evenness measures how equal the community ie 2 taxa each at 50% abundance vs 9 to 1 ratio.
- Alpha diversity is a measurement composed of richness and evenness.

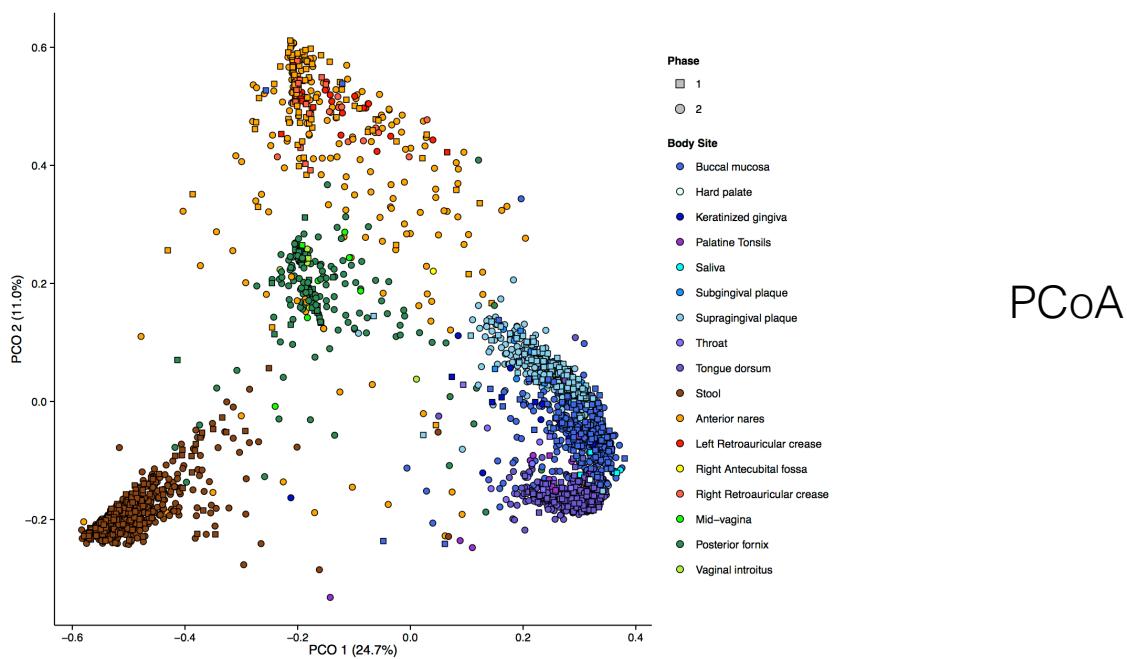
# Beta-Diversity

- Beta-diversity measures including absolute or relative overlap describe how many taxa are shared between habitats
- Beta diversity acts like a similarity score between populations, allowing analysis by sample clustering or, again, by dimensionality reductions such as PCA
- Beta diversity can be measured by simple taxa overlap such as Bray-Curtis dissimilarity

# Unifrac

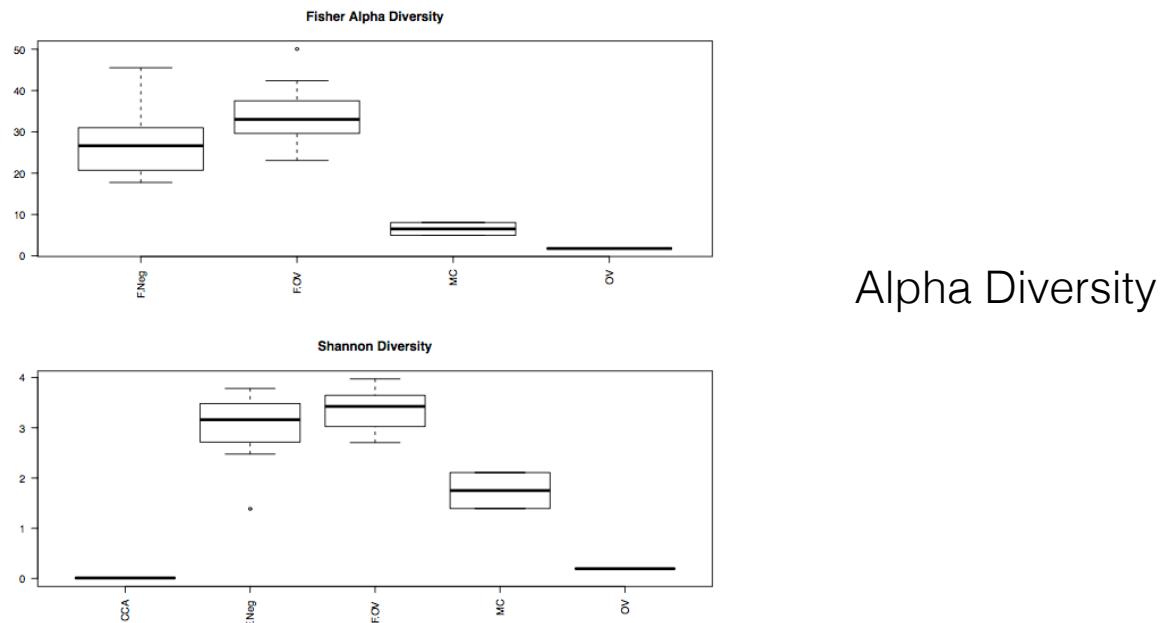
- A distance metric used for comparing biological communities
- It differs from distance metrics (Bray Curtis) as it incorporates phylogenetic distances (tree based) between observed organisms in the computation
- Weighted Unifrac also incorporates taxonomic abundances

## Sample Comparison based on OTU Composition



Community  
Comparison

# Sample Comparison based on OTU Composition



Community  
Comparison

# Statistics

- MetagenomeSeq is a software used for detecting differentially abundant features (ie phyla or genera)
  - Employs the false discovery rate to improve specificity in high-complexity environments, and separately handles sparsely-sampled features using Fisher's exact test
- LEfSe is an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features (genes, pathways, or taxa) characterizing the differences between two or more biological conditions (or classes)

# Time for Workshop