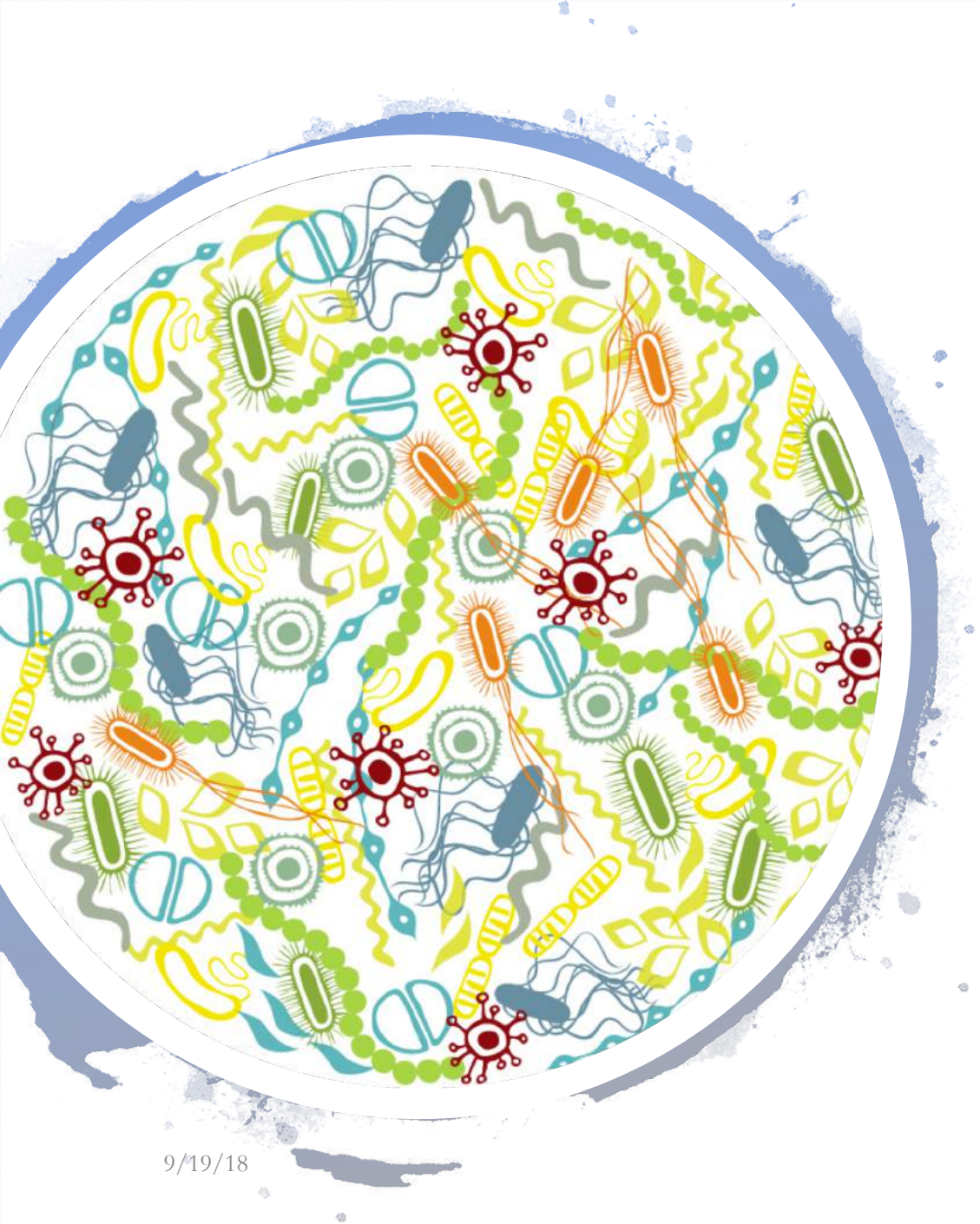
Microbiome bioinformatics

Not your usual DS!

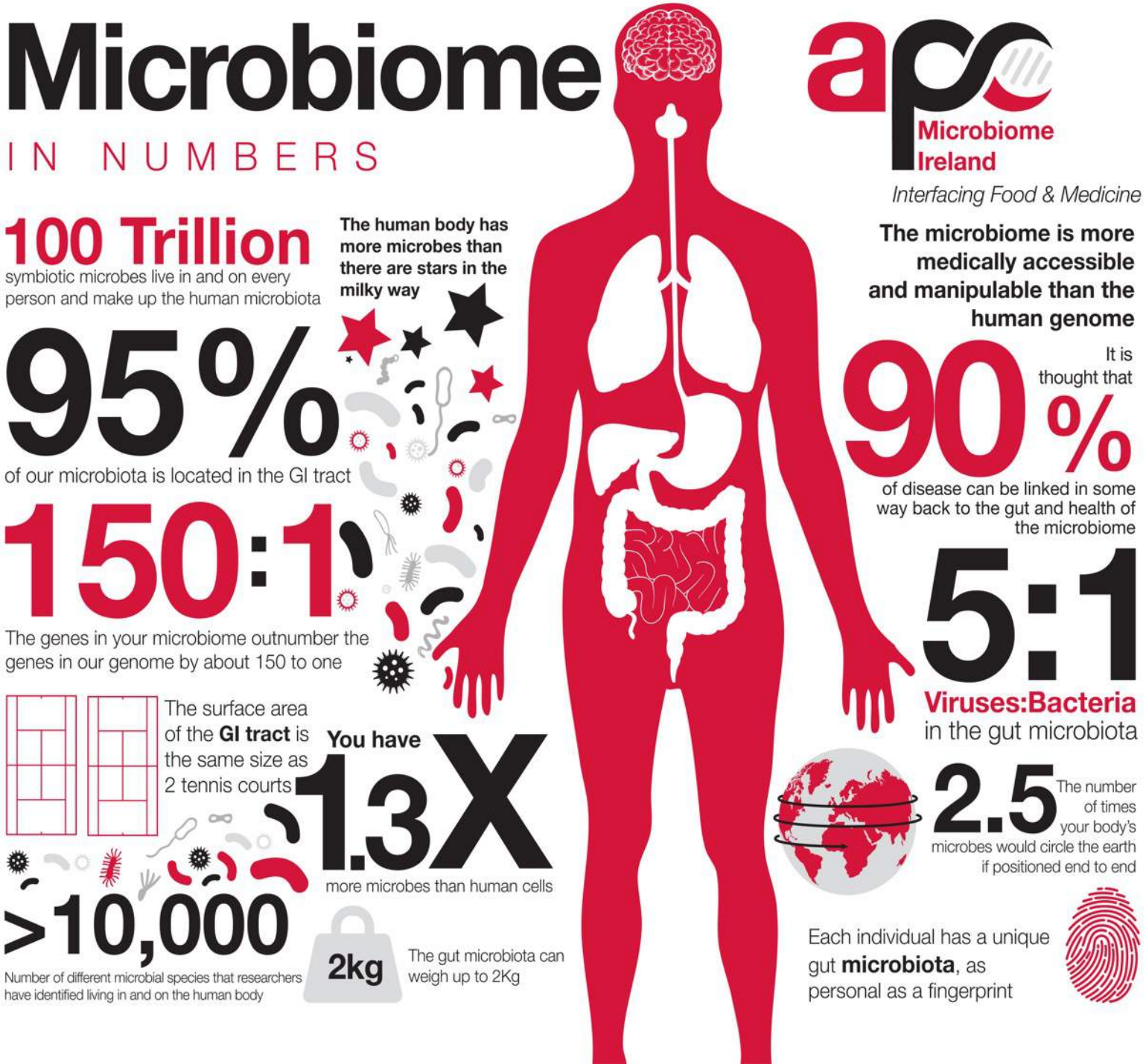


What's the microbiome

- A Microbial biome:
  - Community of microbes
  - Community of genes
- Microbiota → microbes
- Microbiome → microbial genes
- Includes: Bacteria, Archaea, Fungi, Viruses, Algae, Protozoa

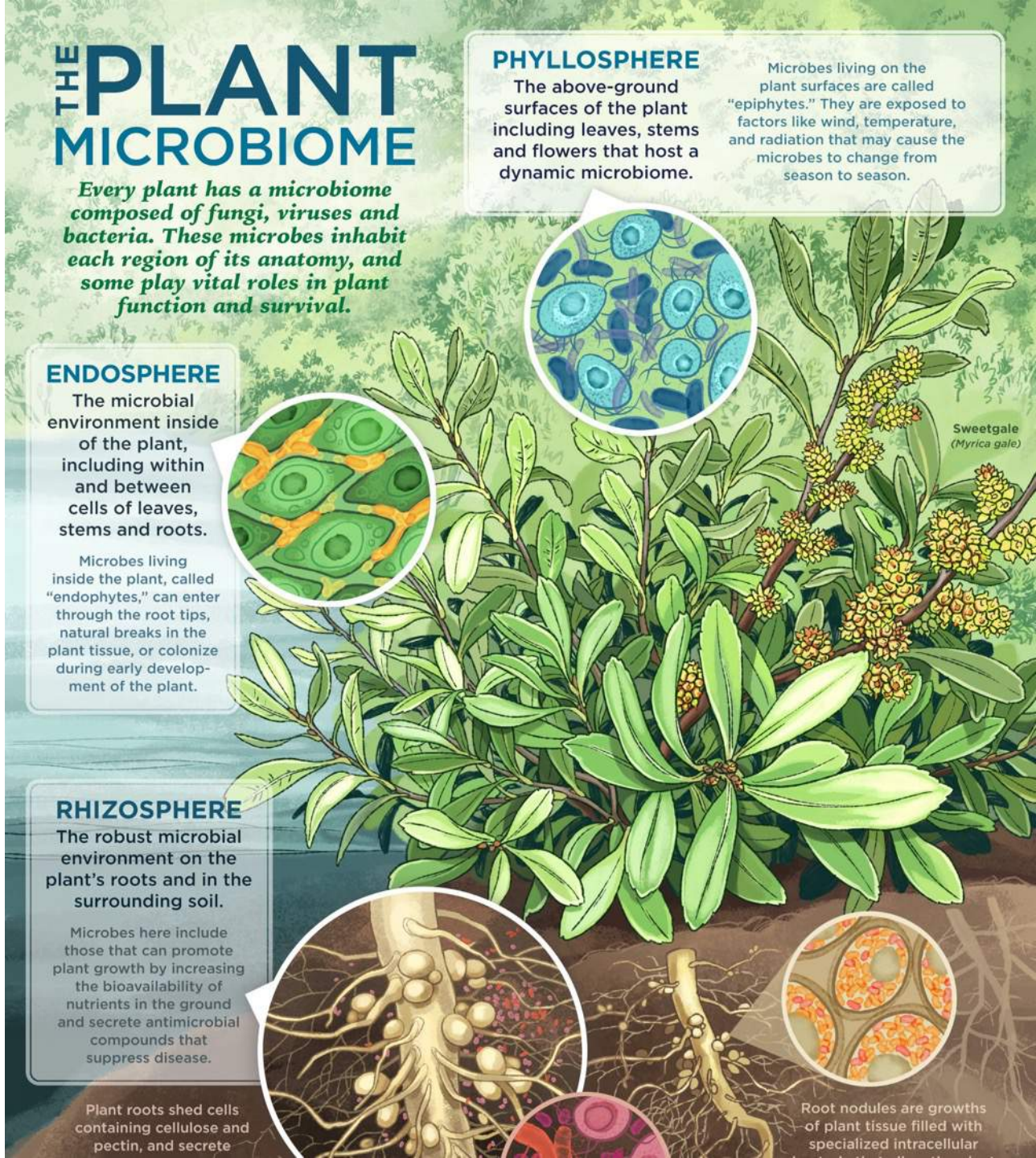
Why the Microbiome…

- In Humans?
  - 10% of body weight
  - Linked to good things:
    - Immune responses
    - Metabolism
    - Health
  - Linked to bad things:
    - Obesity
    - Diseases
    - Allergies
    - Depression
    - Autism (questionable)
    - Drug efficiency
  - Multi-organ influence!!!



Why the Microbiome…

- In Plants?
  - Linked to good things:
    - Yields
    - Protection from diseases
    - Provide nutrients
  - Linked to bad things:
    - Diseases
    - Loss of functions
    - Diversity
  - Multi-organ influence!!!



How do we approach the problem?

- Disentangle complexity:
  - Who is there?
  - What does he do?
  - Why?
  - When?
  - How?
  - Interactions between members
  - Interactions with environment
  - Interaction with host

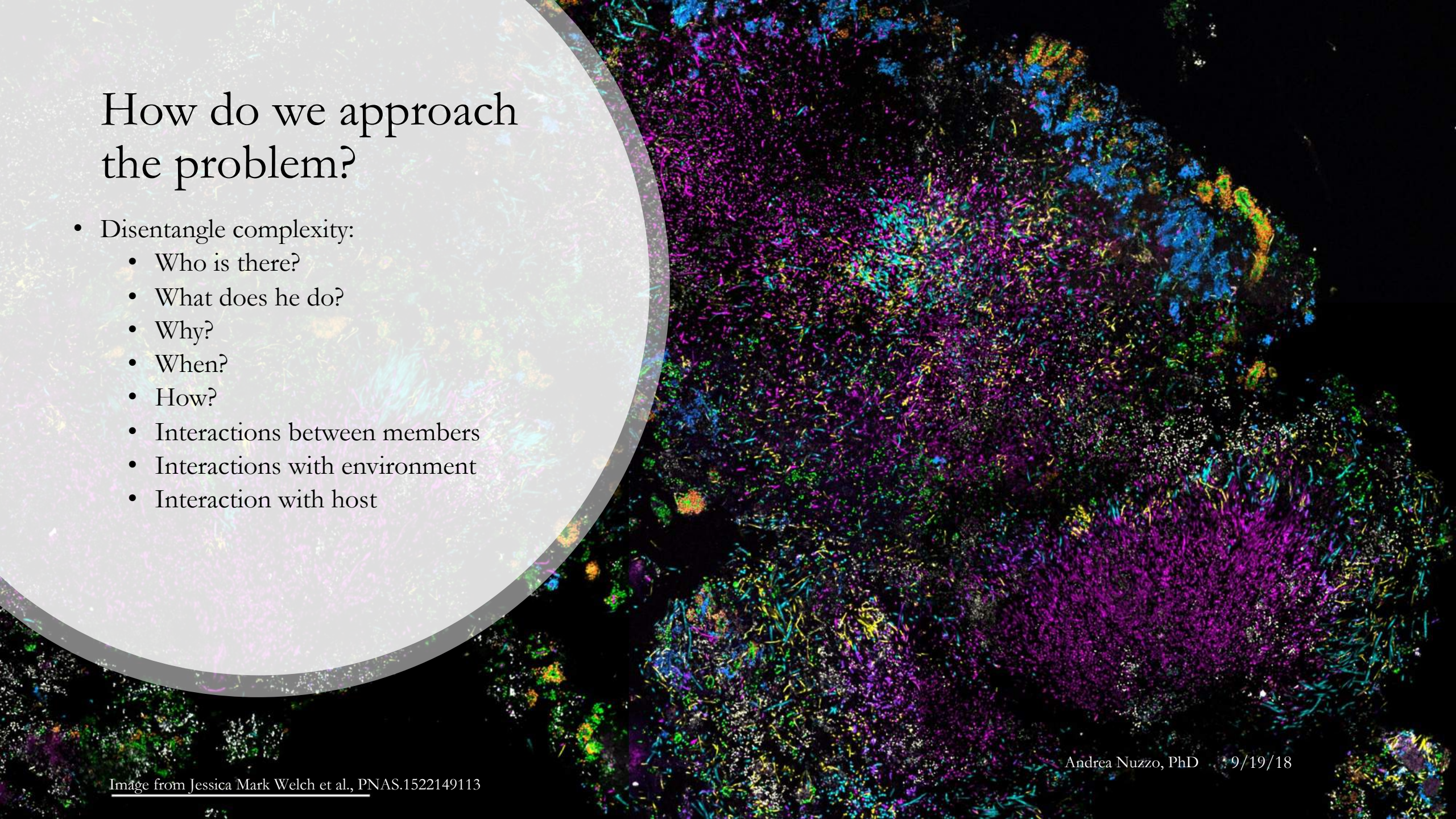
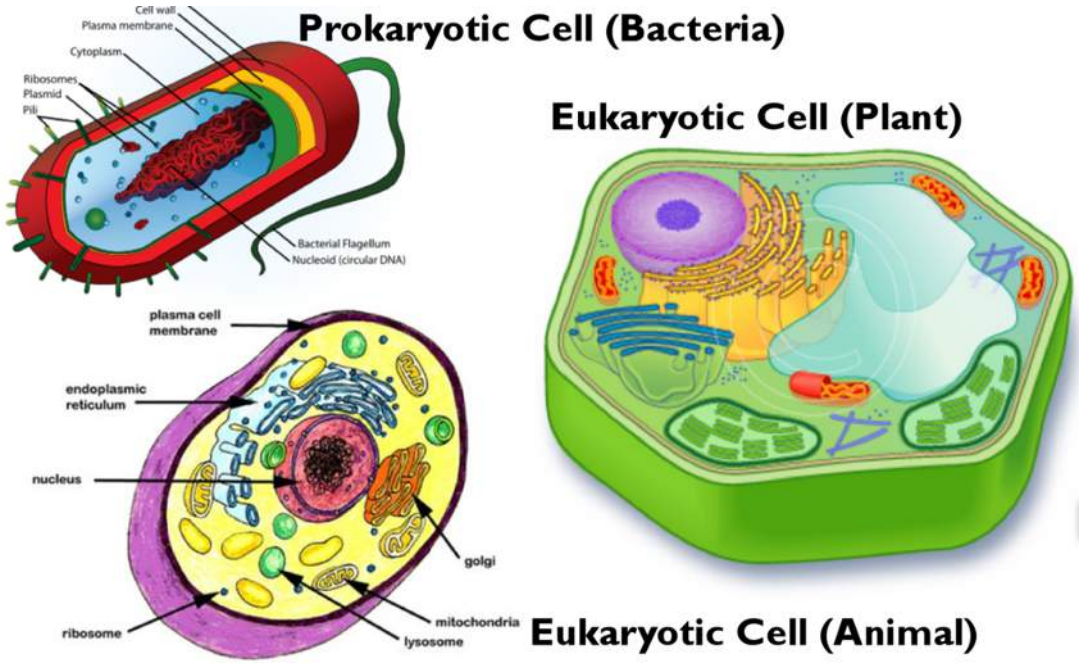


Image from Jessica Mark Welch et al., PNAS.1522149113

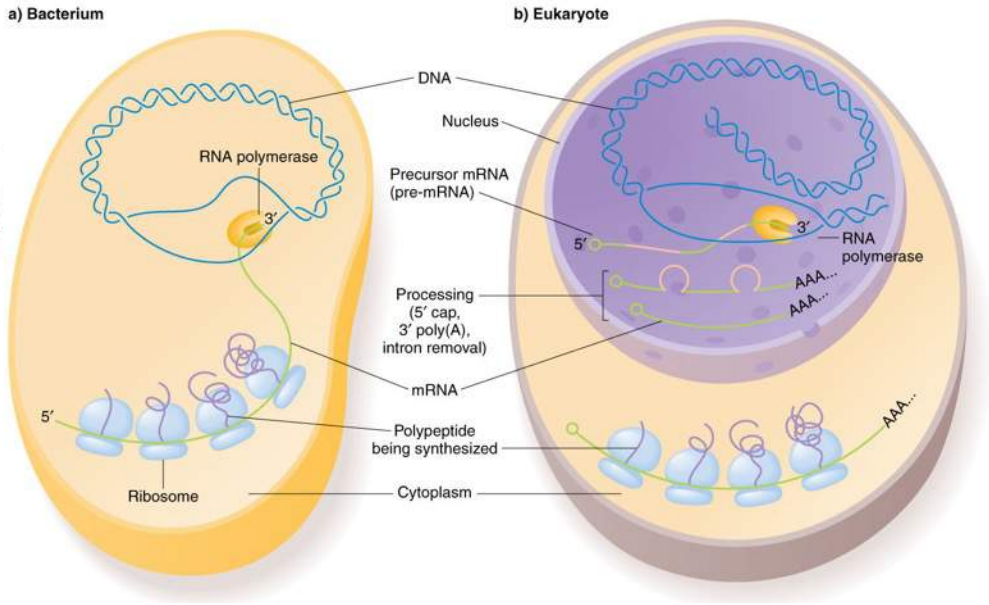
Quick recap of biology basics

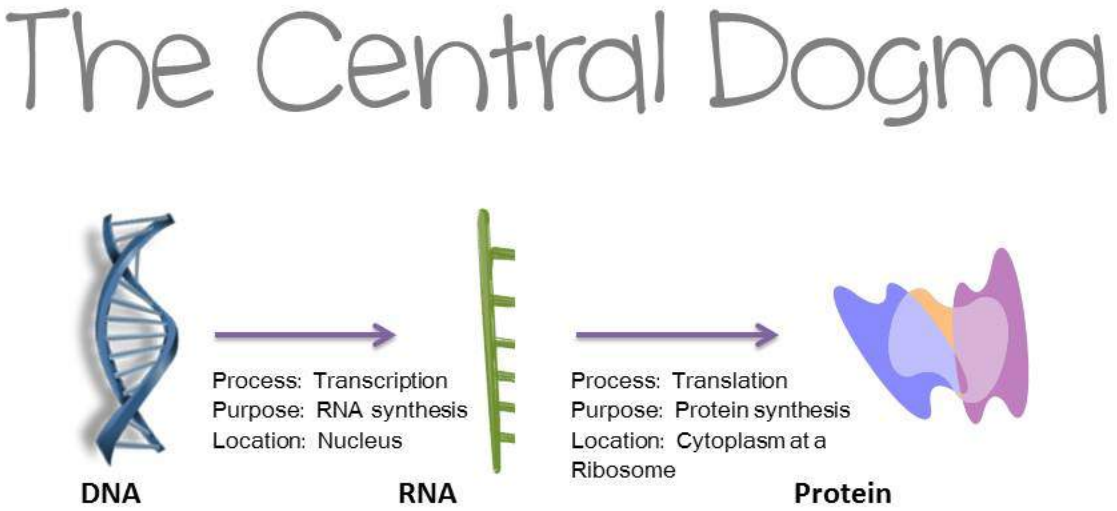
- Bacteria (Prokaryotes)
  - No nucleus, DNA is free inside cell space
- Plant, Fungi, Animals (Eukaryotes)
  - Several cell compartments including nucleus that contains DNA
- Archaea
  - cell compartments but no nucleus
- Viruses?
  - oh well… it's complicated



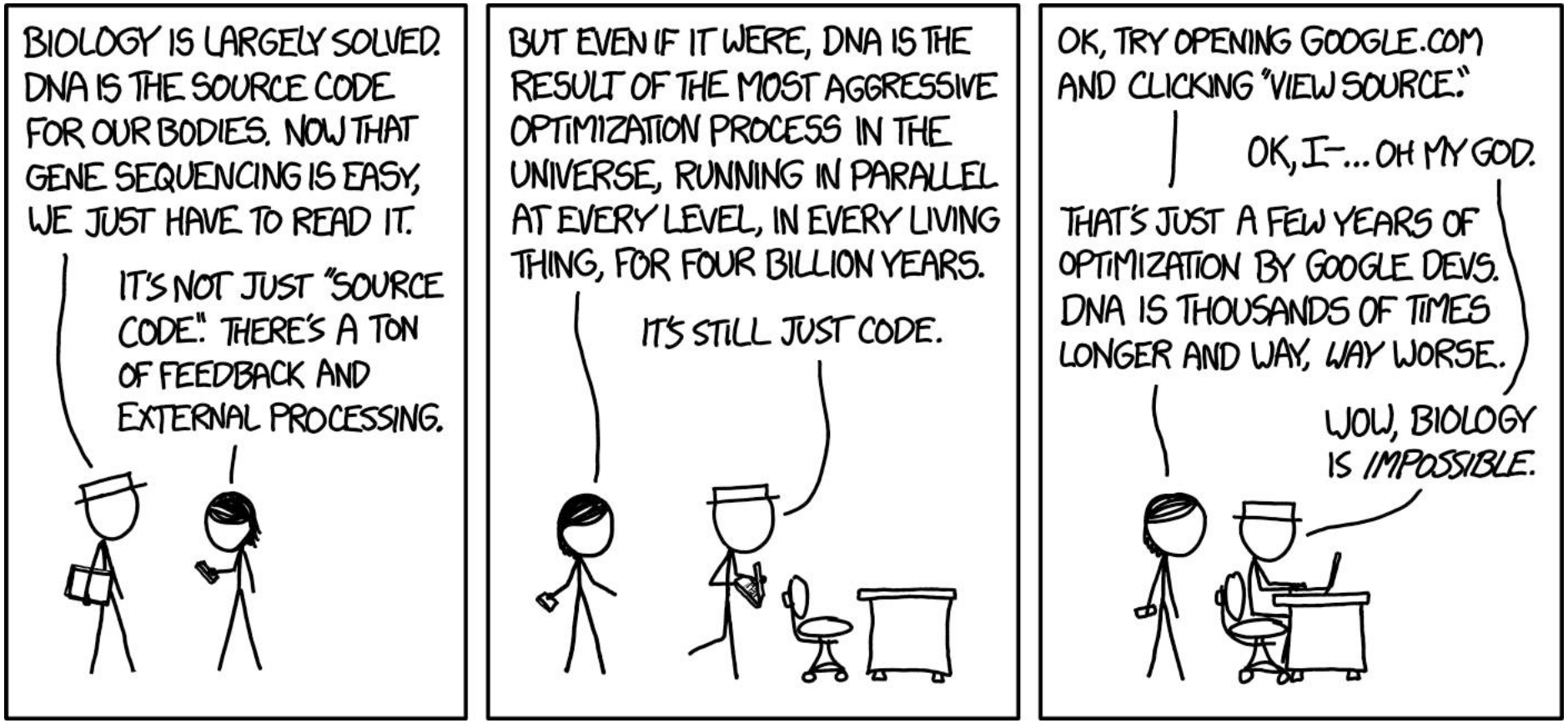
Quick recap of molecular biology basics

- Central dogma:
  - DNA is the code of life
    - objects, called loci, that contain
      - words written in characters (nucleotides) that can have
      - Meaning (genes)
      - Function (suppressors, activators, etc)
      - ?? (interspatial DNA)
  - GENES are transcribed into mRNA
    - processed (only in Eukaryotes)
  - mRNA is translated into proteins
    - Sequence of aminoacids that has several functions (metabolism, structure, etc.)

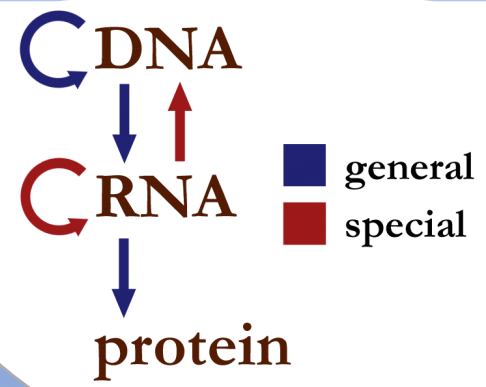




Well, that's easy!

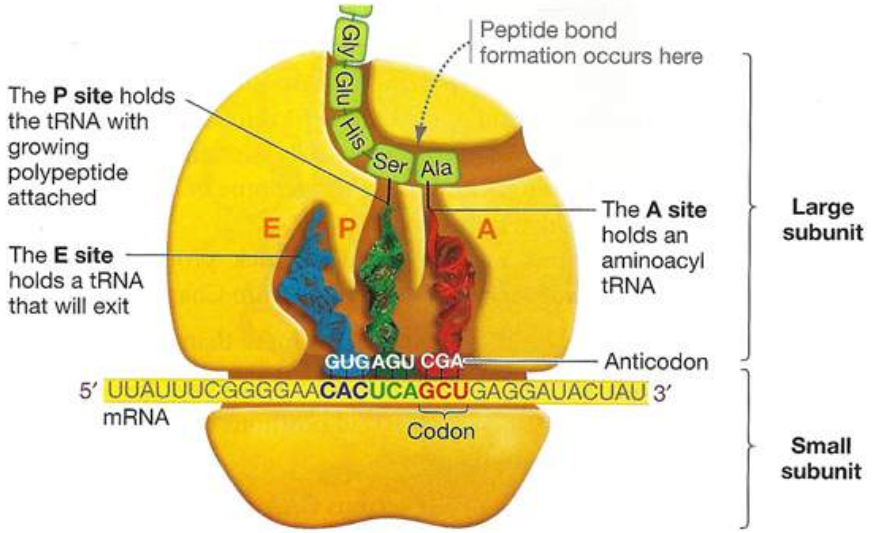


https://www.explainxkcd.com/wiki/index.php/1605:_DNA



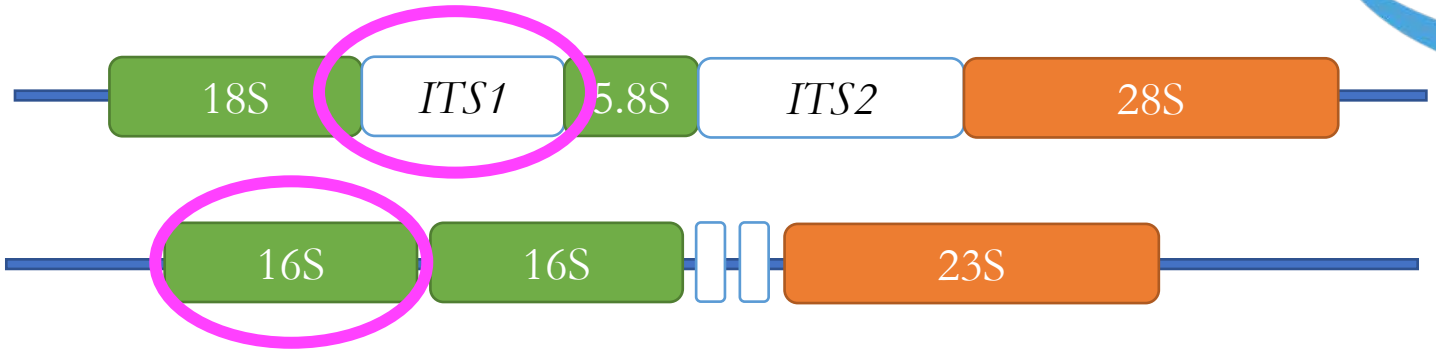
Example

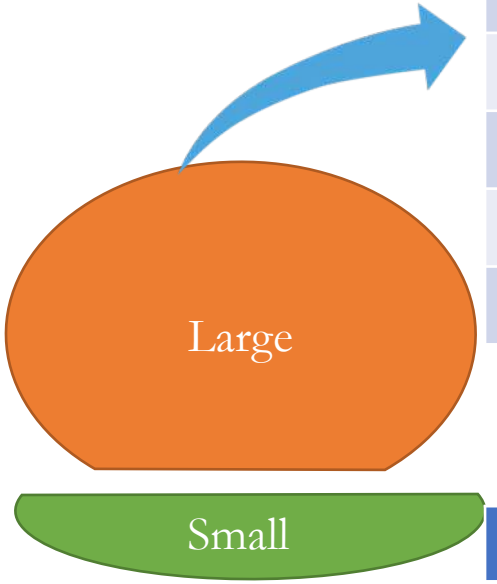
- Central dogma is not linear
- Some life forms are RNA-based (i.e. Viruses)
- RNA can function as a protein
  - RIBOSOMES!



Ribosomial genes

- Ribosomes are the basis of (mostly) all life
  - Made of rRNA and proteins
  - rRNA genes are EXTREMELY conserved
    - Can be used to ”name” life
  - Small unit and large unit



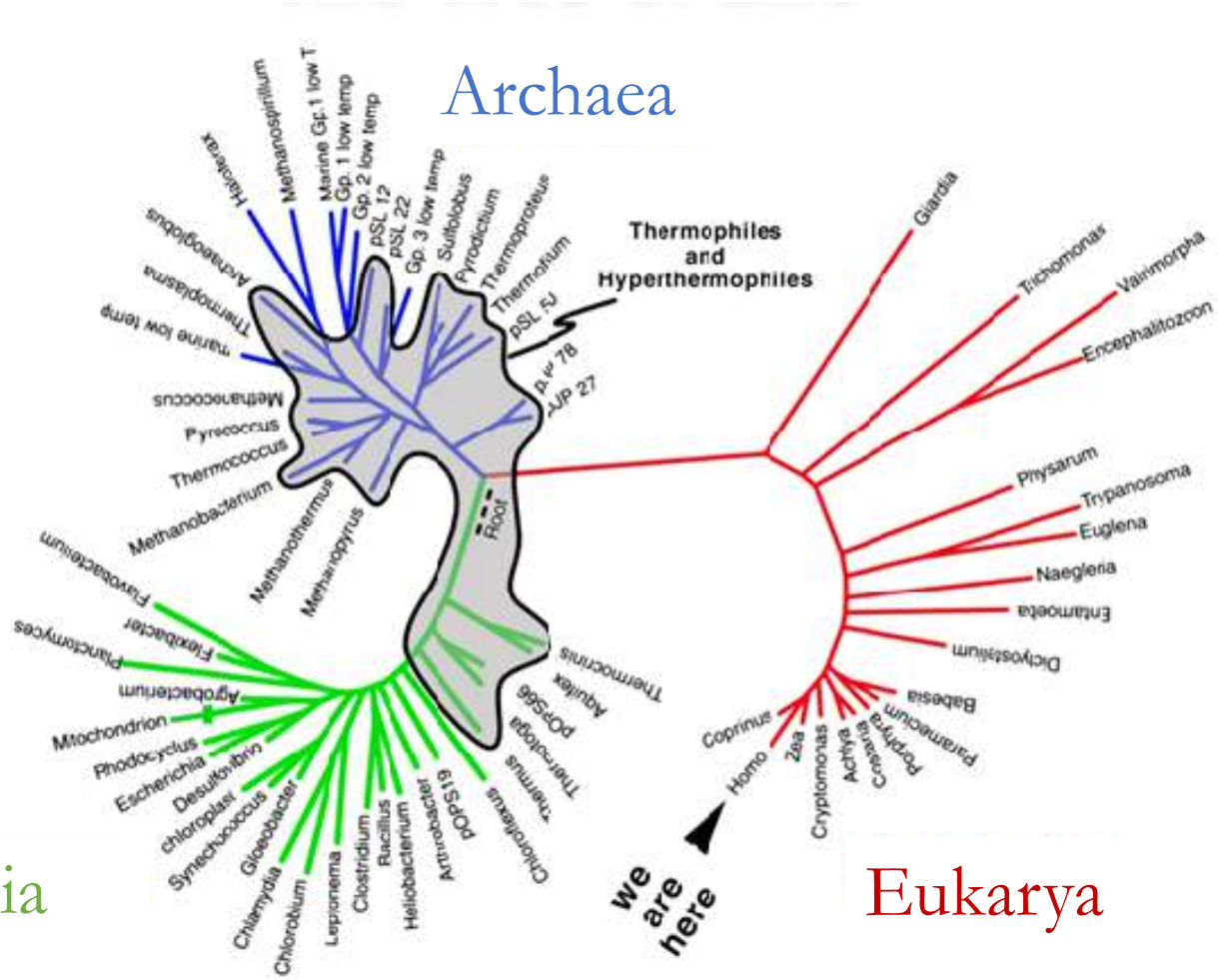


Eukaryotes	Prokaryotes
60S	50S
5.8S rRNA	5S rRNA
5S rRNA	23S rRNA
28S rRNA	-
49 proteins	34 proteins

Eukaryotes	Prokaryotes
40S	30S
18S rRNA	16S rRNA
33 proteins	21 proteins

What does it mean to "name" life?

- Placing organisms on the tree of life: taxonomy
  - A hierarchical clustering of life characteristics
  - Based on similarities in the rRNA sequence (debated)
    - Species = 97%
    - Genus = 95%
    - Family…
    - Order…
    - Class…
    - Phylum…
    - Kingdom…



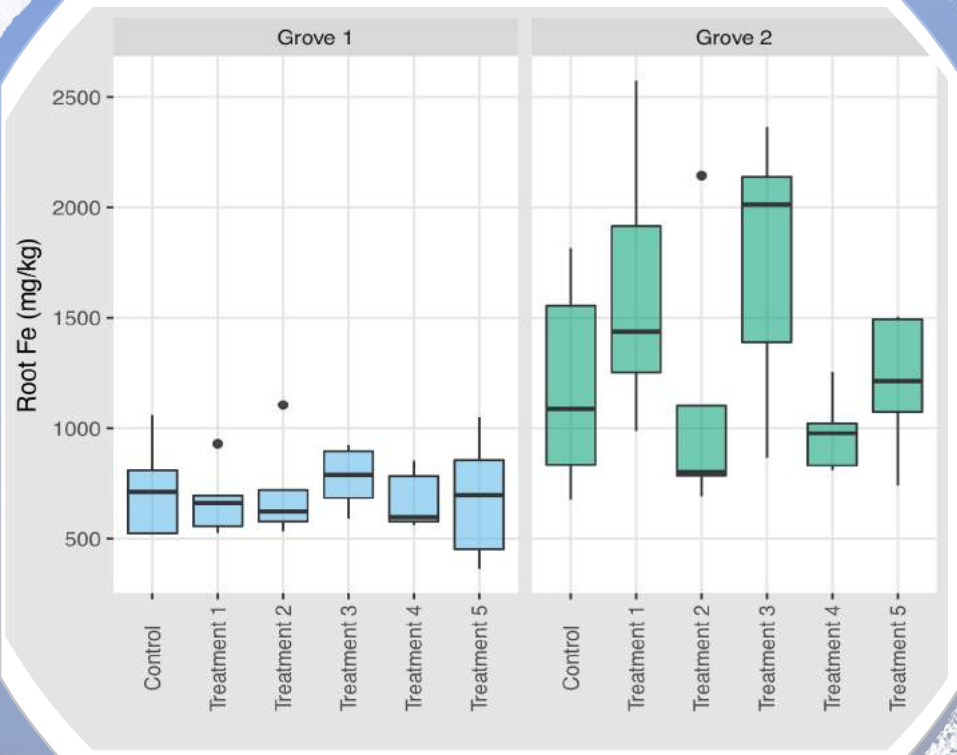
What's the matter?

UF | IFAS
UNIVERSITY of FLORIDA

Soil microbiome characterization and manipulation

- Protection from diseases
- Increase in plant yields and health
- Bio-fertilizers

Plant-microbiome interactions



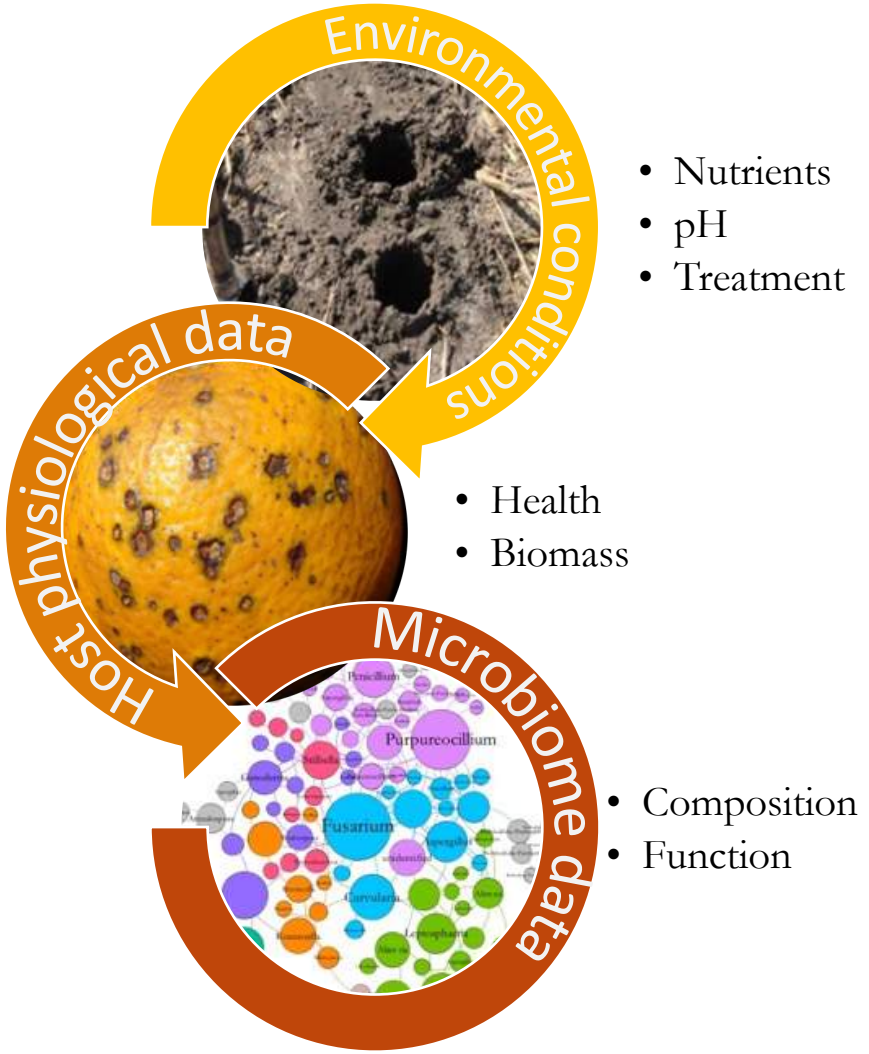
Case study: HLB

- Citrus greening (HLB) is an epidemic, lethal, incurable disease of citrus
  - Pathogen known but uncharacterized
  - Degrades roots first
- Two groves with different management strategy
  - Fertilizer, pesticides, etc.
- 4 treatments applied to try to alleviate roots
  - 2 of them include microbes
- Data show differences!

Unraveling soil microbiomes

- Soil data
- Plant data
- Metagenomic DNA

NGS of 16S and ITS rRNA genes

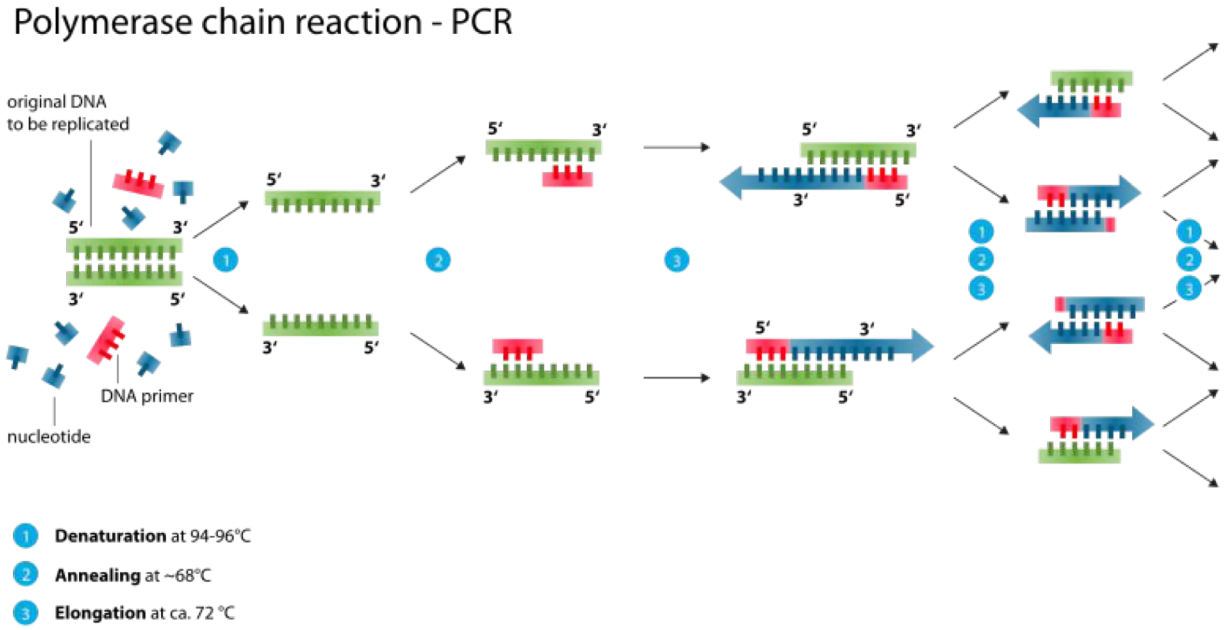


- Nutrients
- pH
- Treatment
- Health
- Biomass
- Composition
- Function

Host-microbiome interactions

Polymerase Chain Reaction (PCR)

- Replicates multiple copies of a DNA fragment
  - Requires primers, enzymes, nucleotides
  - We chose the primers which are our keys to select which region to amplify
  - Multiple cycles
  - After the first cycle we get 2^n copies of the *SELECTED* fragment every n cycle
- *PS: it's a biological way for data augmentation. With mistakes included.*



Next Generation Sequencing (NGS)

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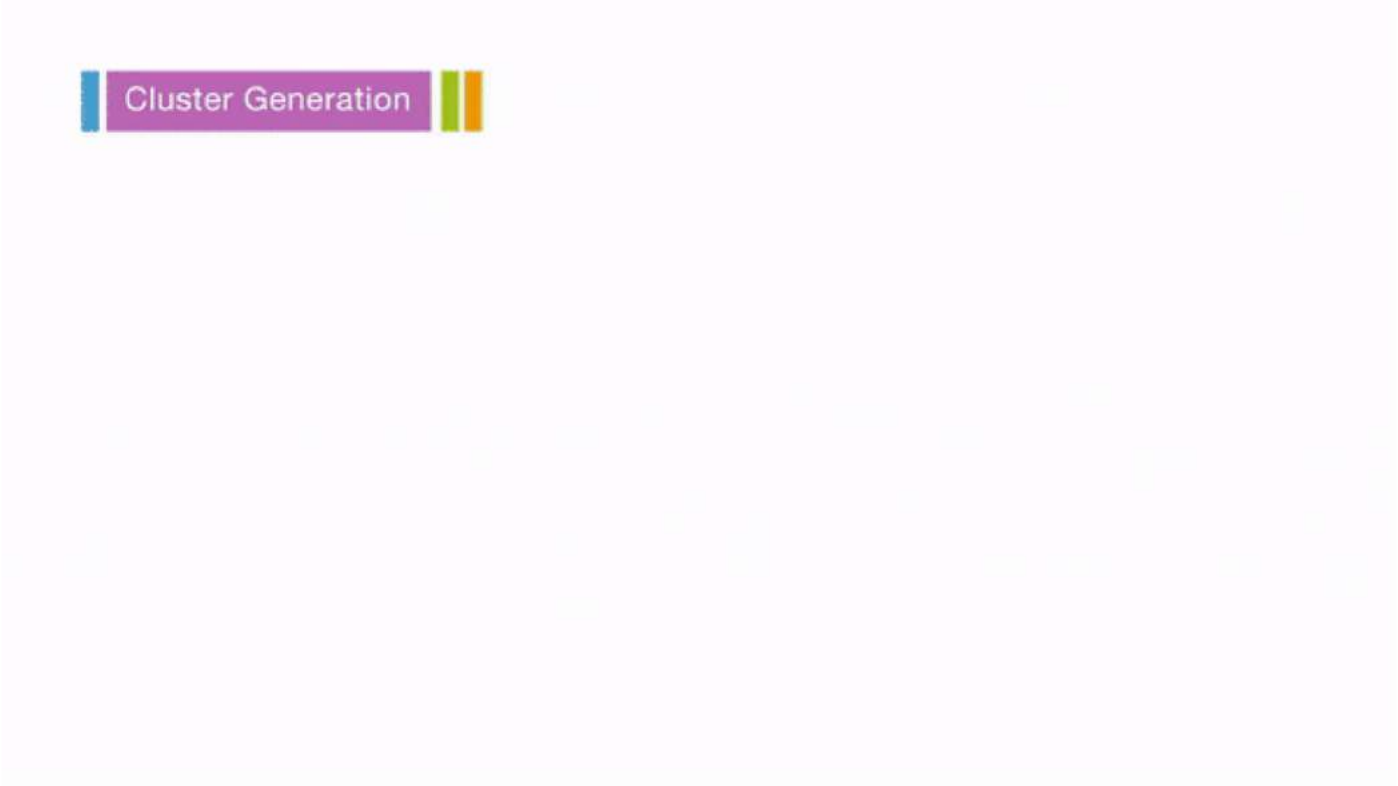
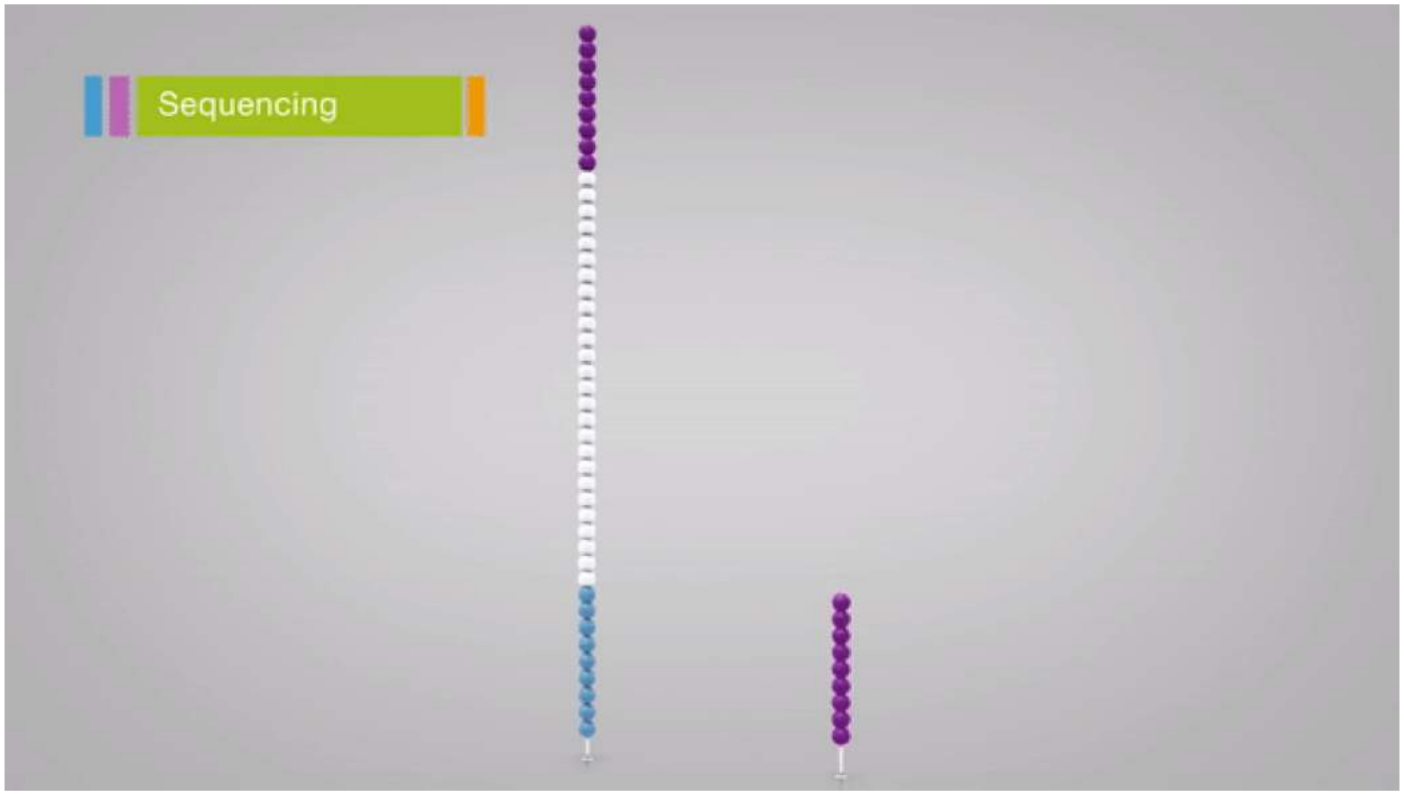
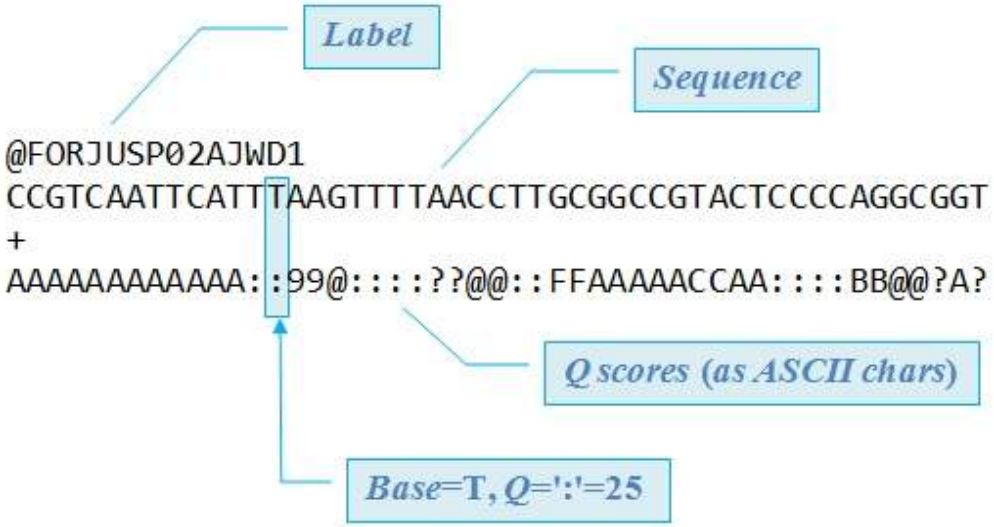
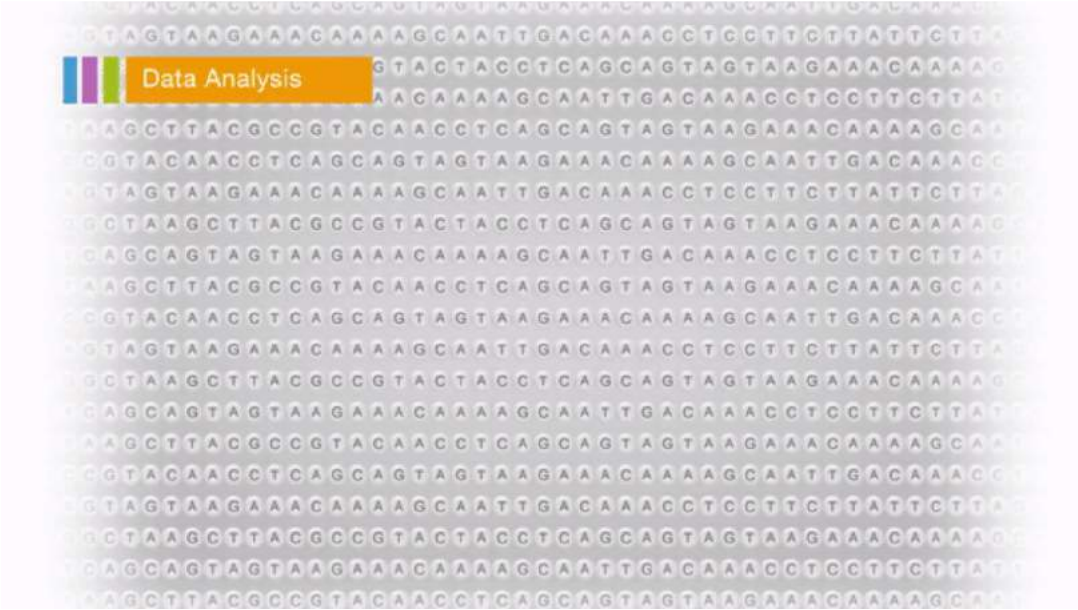


Next Generation Sequencing (NGS)



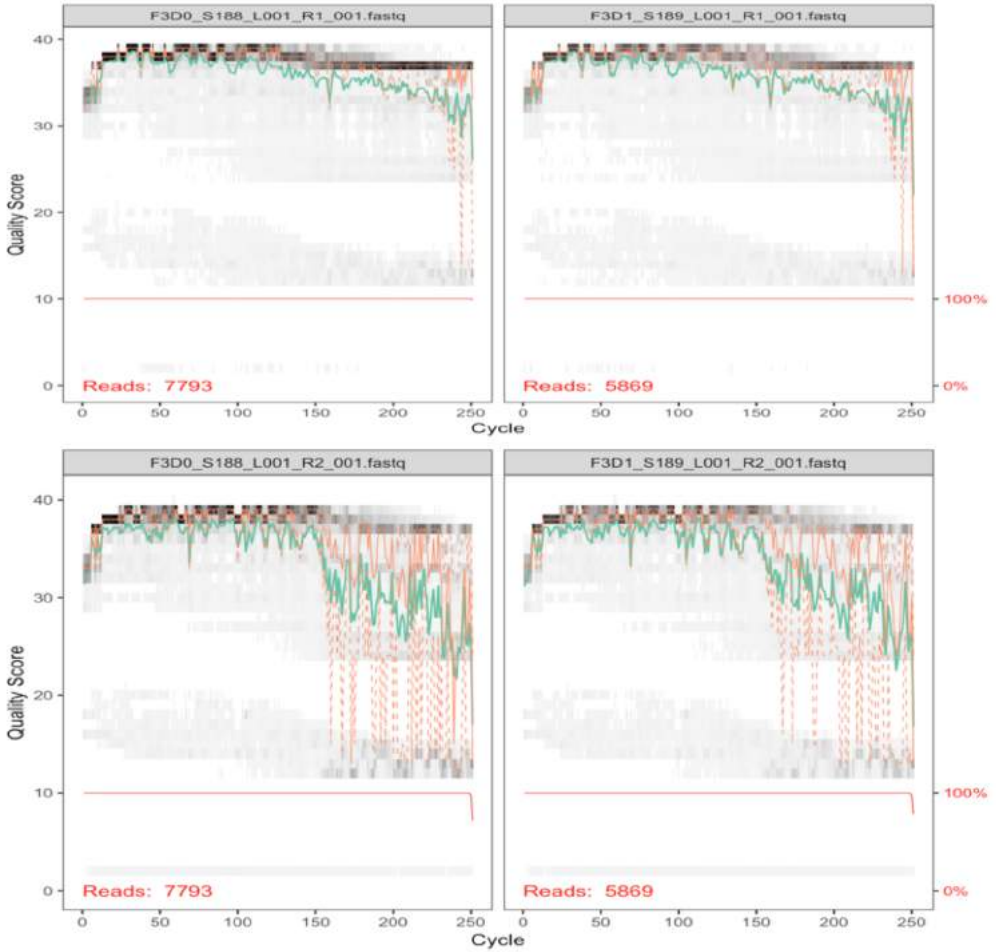
FASTQ files


Label
Sequence
@FORJUSP02AJWD1
CCGTCAATTCATTTAAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGT
+
AAAAAAAAAAAA::99@::::??@@::FFAAAAACCAA::::BB@@?A?
Q scores (as ASCII chars)
Base=T, Q=':'=25


Data Analysis

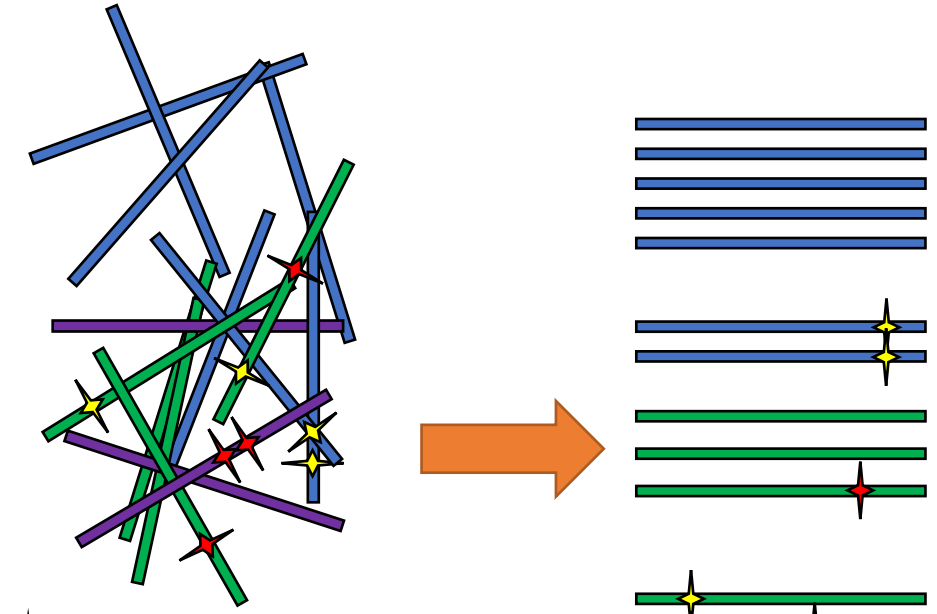
First challenge: how to get good quality paired sequences?

- Forward reads generally better than reverse reads
- How do I know that the base in a certain position is good or a misreading?
- How can I avoid PCR mistakes?
- How can I avoid merging amplicons from different sequences just because they share a common region? (chimeras)



Generating amplicon sequence variants with DADA2*

- Detect error rates
- Inferring a parametrized model of substitution
- Discriminate biological mutations from PCR errors
- Merge overlapping regions
- Infer chimeras by eliminating sequences with more than one other sequence in common
- More biologically relevant than clustering by x% similarity



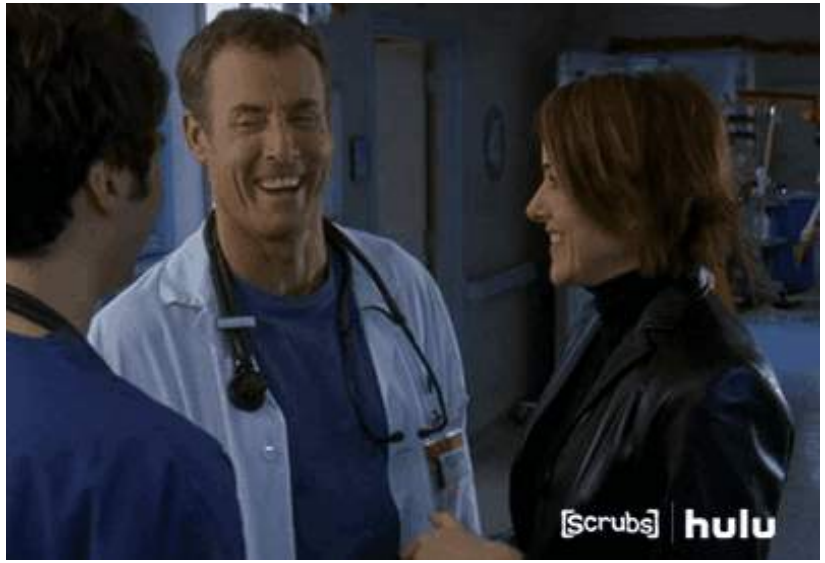
Actual biological mutation (i.e. high frequency)

PCR error (i.e. low frequency)

$$p_A(j \to i) = \frac{1}{1 - \rho_{pois}(n_j \lambda_{ji}, 0)} \sum_{a=a_i}^{\infty} \rho_{pois}(n_j \lambda_{ji}, a)$$

*Callahan, B.J. et al. (2016). Nat. Methods, 13, 581

That was just the first step…

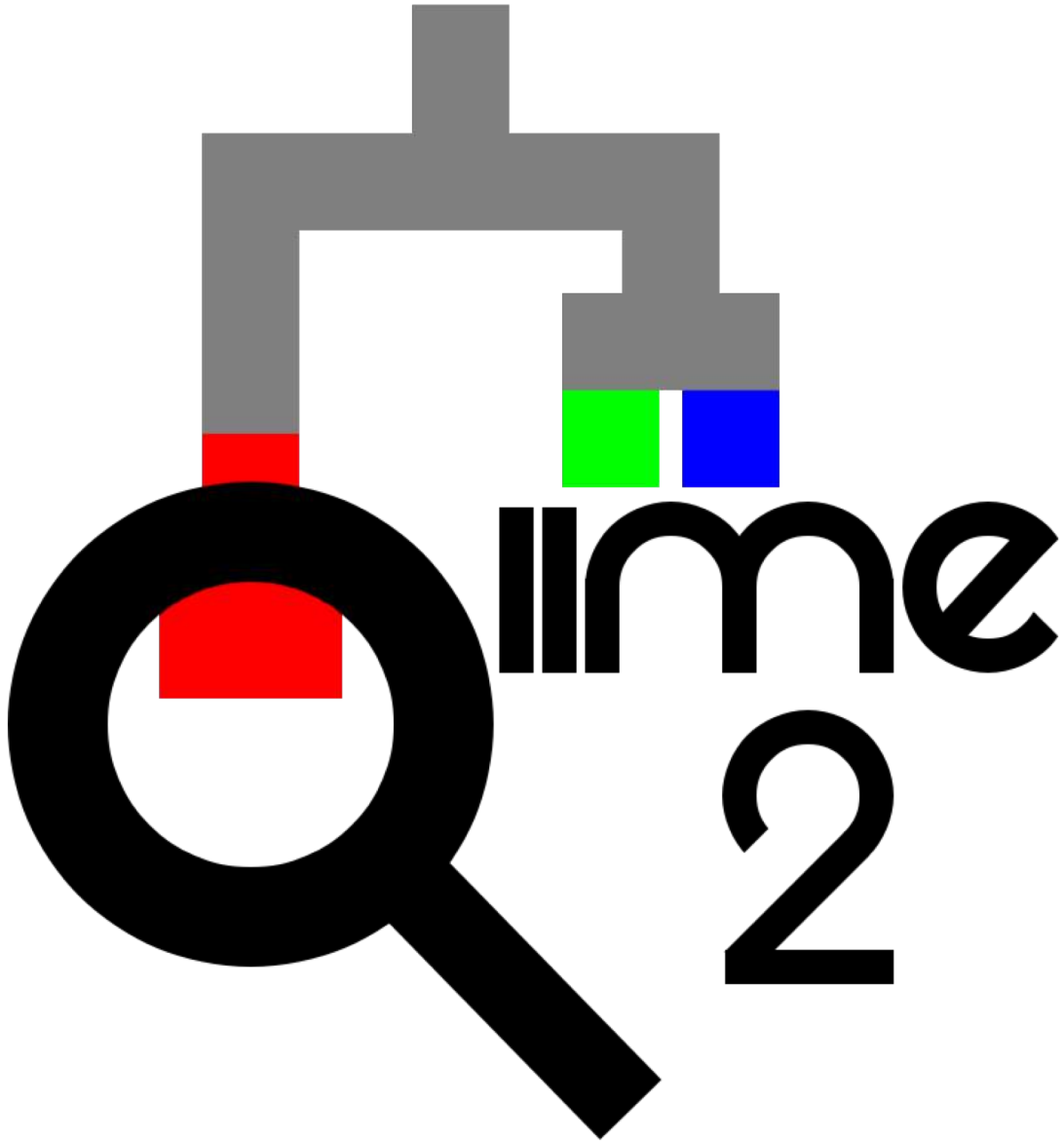


	16S	ITS
Paired-end FASTQ		
Dereplication, quality filtering	DADA2 (calling Amplicon sequence variants)	DADA2 (calling Amplicon sequence variants)
Phylogenetic Affiliation	Database: SILVA 128 Naïve Bayes classifier	Database: UNITE 7.2 Naïve Bayes classifier
Metabolic predictions	PICRUSt	FunGuild

Diversity	Phyloseq (NMDS, PCoA, CAP, PERMANOVA, DESeq2, etc.)
Network	SPIEC-EASI
Modeling	OLS, LME, Random Forests, GBoost

Introducing: Qiime2

- Modular platform for Quantitative insights in microbial ecology
- Written in Python3
  - Calling scripts from bash
  - API in Jupyter
- Plugin-structure
- Performs: DADA2, diversity analyses, machine learning, time series analyses and much more
- Standard for microbiome characterization

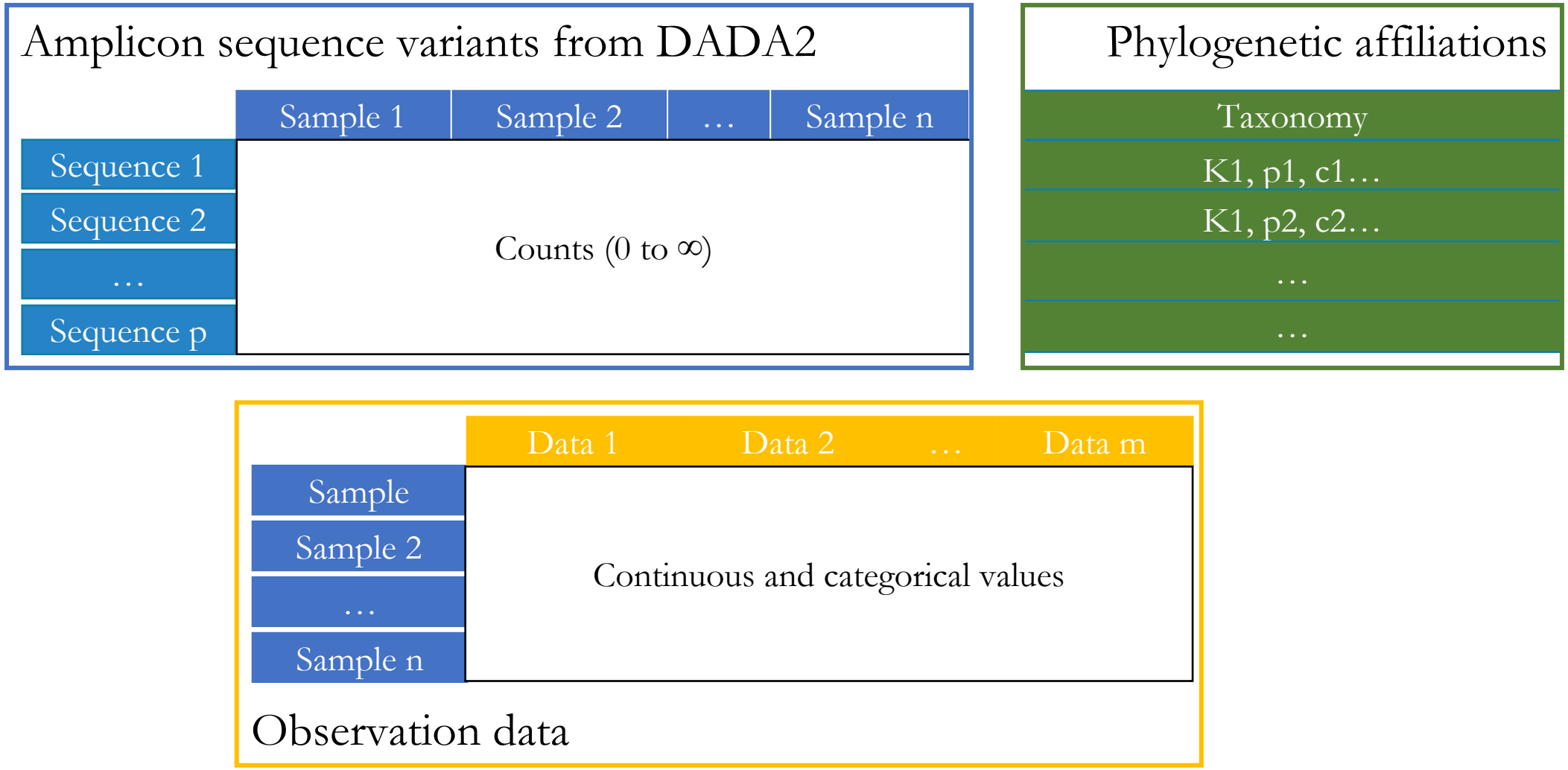


Phylogenetic affiliation

- Uses a pretrained Naïve Bayes Multinomial classifier where:
  - Sequence of ATCG are the x
  - Taxonomical levels are the multiple classes
- Predict classification based on the sequence
- Can be improved by assigning weights (expected taxonomy)

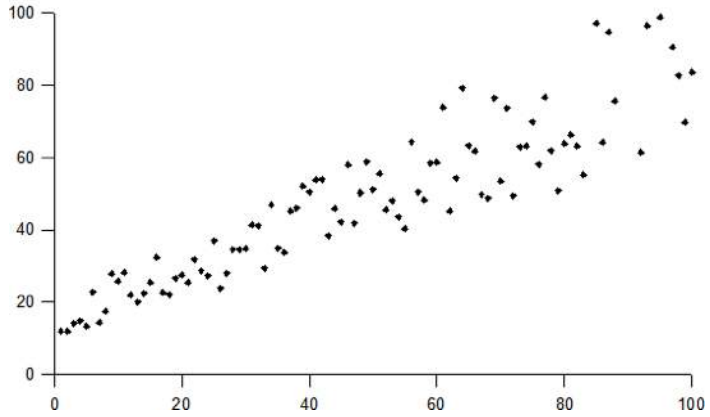
- Depends on the database on which it's trained!!!
  - Considering that ~90% of microbes in the world are estimated to be unknown…
  - And ~99% (some say 60%) of them are not culturable (so you can't easily obtain new sequences to update your database…)

OUR DATA

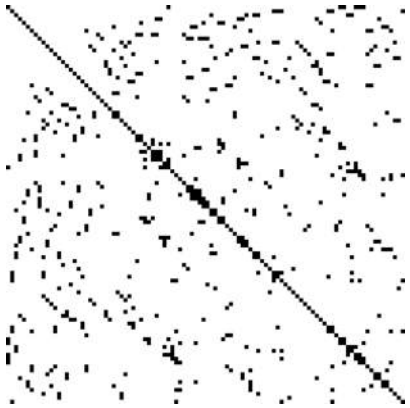

Amplicon sequence variants from DADA2
Sample 1
Sample 2
…
Sample n
Sequence 1
Sequence 2
…
Sequence p
Counts (0 to ∞)
Phylogenetic affiliations
Taxonomy
K1, p1, c1…
K1, p2, c2…
…
…
Data 1
Data 2
…
Data m
Sample
Sample 2
…
Sample n
Continuous and categorical values
Observation data

Challenges

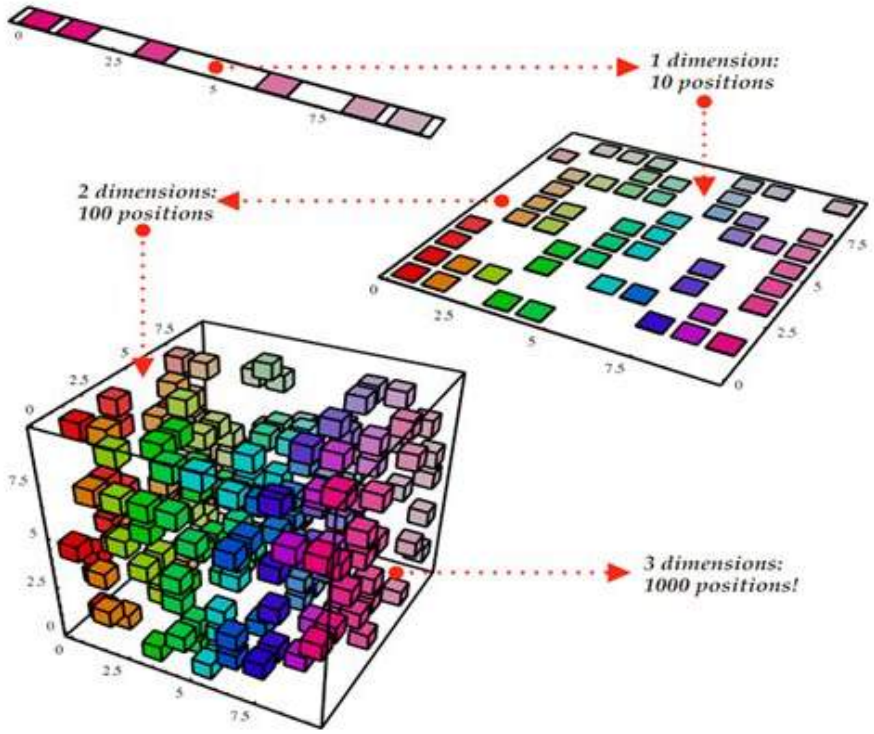
Heteroscedasticity



Sparsity



Dimensionality



How can I combine this to find information?

Can do

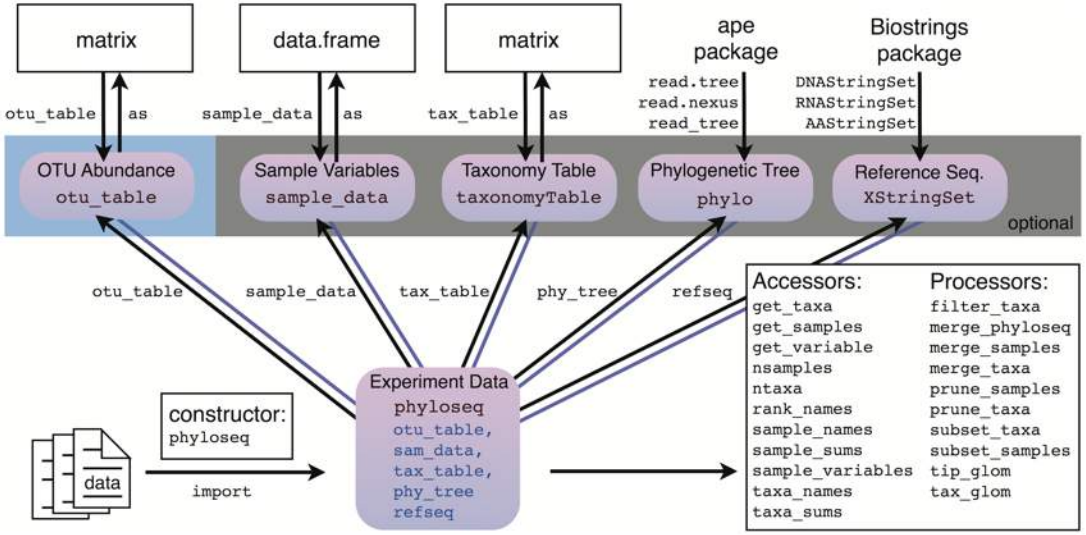
- Dimensionality reduction
- Feature engineering
- Multivariate analysis
- Infer correlations
- Understand magnitude and significance
- Time-series analyses

No can do

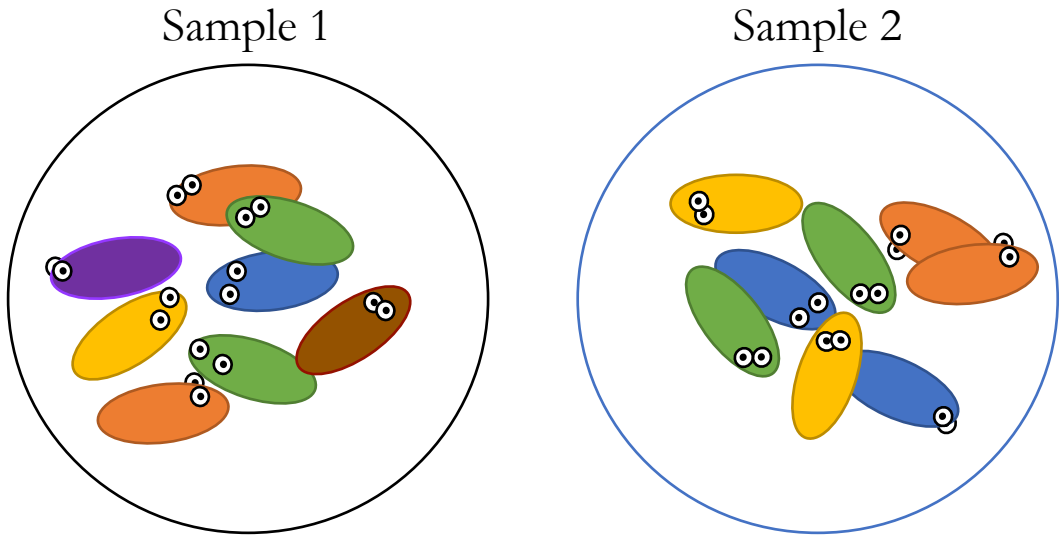
- Scaling
  - PCR and classification flaws
  - Your total of sequences does not necessary represent the total in the sample
- Subsampling
  - Increase FDR, Type I and Type II errors!
- Filter outliers
  - Often the important microbe is the less abundant

Introducing Phyloseq

- Package in R
- Made by biostatisticians
  - Much more complete and accurate than qiime
- Integrates with ggplot2
  - *Nice graphs*!
- Performs: DADA2, in-depth diversity analyses, DeSeq2, network analysis, and much more



Diversity Analysis



α_1

Alpha diversity of sample 1: 6 species with different abundances

α_2

Alpha diversity of sample 1: 4 species with similar abundance

$\beta_{1\text{-}2}$

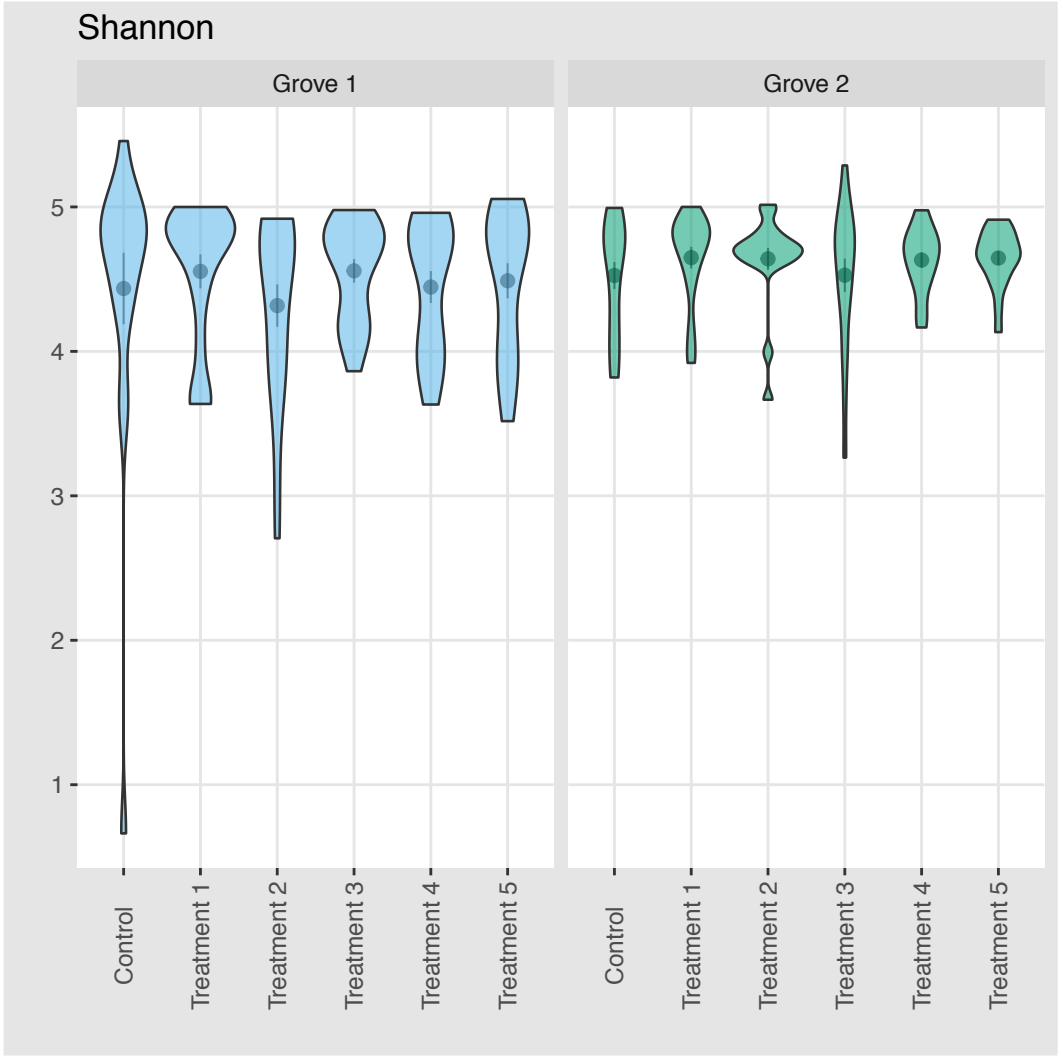
Beta diversity: differences between the two samples (i.e. distances)

ALPHA DIVERSITY

- DOES NOT NEED distribution assumptions:
  - DO NOT normalize your data
  - DO NOT filter your data
- Several indexes are possible (and often different names mean the same index), but they do different things, with different power, and measure different aspects:
  - Shannon, chao1, observed: measures *Richness*
  - Simpson: measures *Dominance*
  - Inverse Simpson: measures *Evenness*
- Rarefaction curves are a good proxy for data quality check!
  - Split the dataset into bins and plot the cumulative sums of the selected α diversity index
  - Look for plateau

Does it help?

```
library(phyloseq)
library(ggplot2)
library(tidyverse)

biomfile < file.path(”dada2_output.biom")
treefile <- file.path("tree.nwk")
mapfile <- file.path(”plant_data.txt")

#Import into Phyloseq
tree <- read_tree(treefile)
table <- import_biom(BIOMfilename = biomfile,
          parseFunction = parse_taxonomy_default,
          parallel = T)
metadata <- import_qiime_sample_data(mapfile)

#Create Phyloseq object
phylo <- merge_phyloseq(table, metadata)

#Plot alpha diversity
plot_richness(phylo, x = "Treatment", measure=“Shannon”,
          color = ”Management") + geom_violin()
```

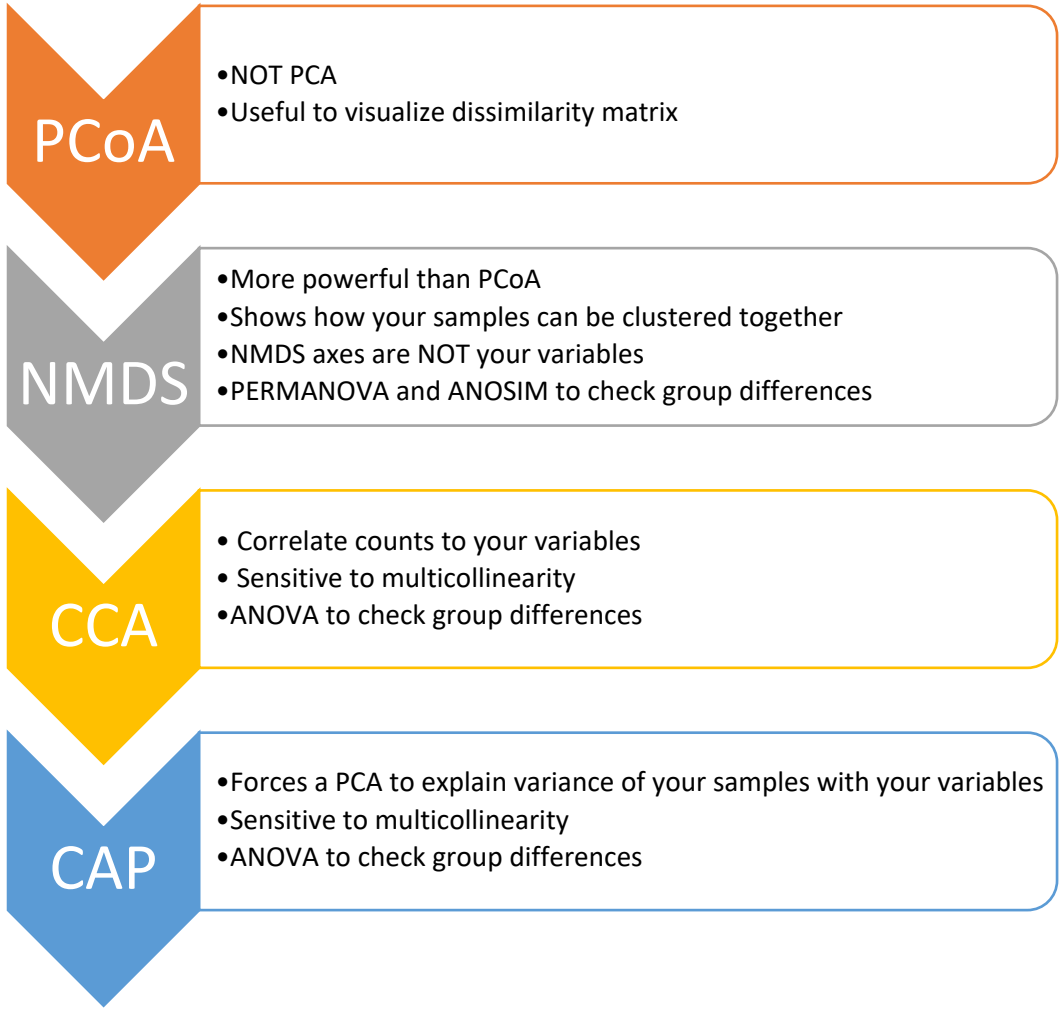


Beta diversity

- Due to the nature of the data, making comparisons between samples is pretty difficult
- To compare samples diversities, you NEED to make them comparable, which means:
  - Normalize (i.e. fitting counts in a Gaussian curve within same range for all samples)
  - Reduce number of outliers
  - Etc.
- Introducing DISTANCES:
  - Indexes representing "dissimilarity" between samples
  - Make lots of assumptions
- Several indexes existing, including Bray-Curtis (works better on log- or root-transformed data), Jaccard (intersection over union), Manhattan (Euclidean distances), UniFrac (includes phylogeny)
- Overall, when plotting beta diversity, samples close together are more similar with each other

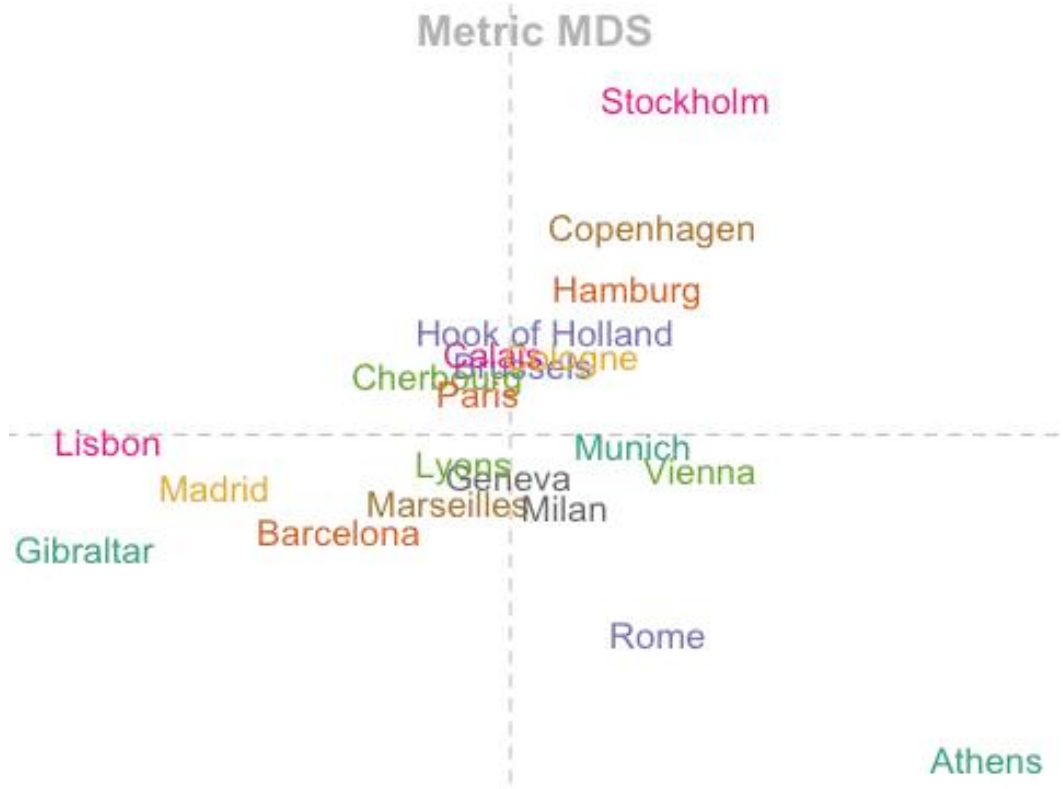
Multivariate analyses

- You want to find out how the WHOLE microbiome is related to your conditions or plant data
  - Possibly, which part of the microbiome is the most important
- AVOID SUBSAMPLING!
  - To make samples comparable it's better to log-transform



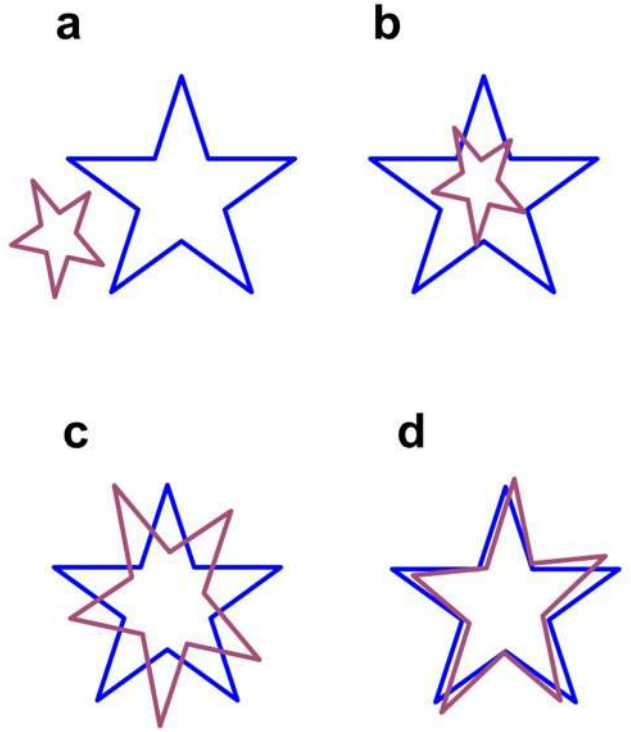
PCoA: Principal coordinate analysis (or MDS)

- NOT PCA
- If I know distance between cities, but not their coordinates, how can I draw a map?
- Your count table is converted into a matrix of dissimilarity (using the diversity index chosen)
- May be impacted by high variance (so, you need to normalize)
- If some results in the dissimilarity matrices are negative, it leads to imaginary numbers in the eigenvectors
- The eigenvectors are not your variables, but are correlated with different percentages



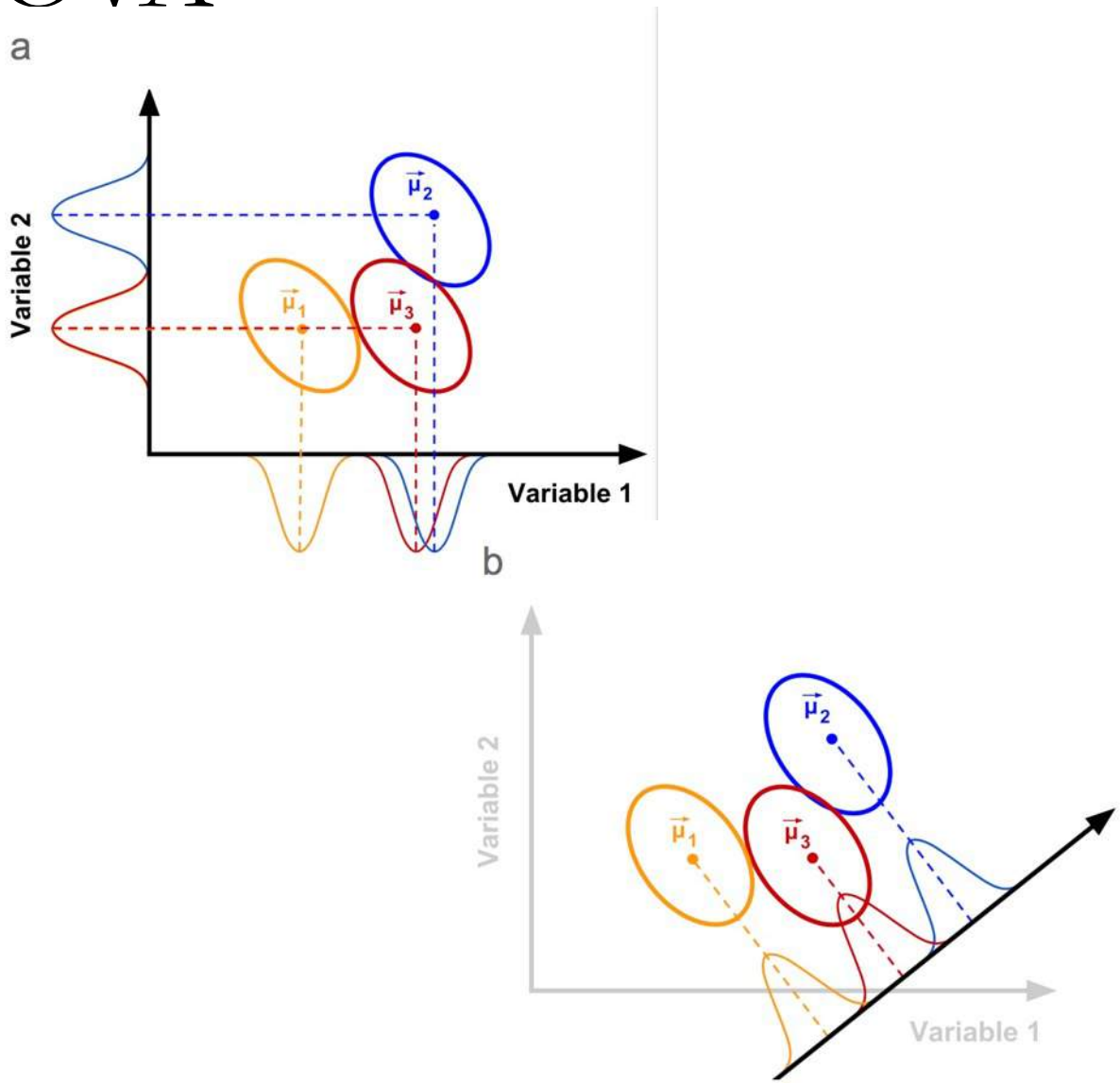
NMDS: NON-metric multidimensional scaling

- Starts from a dissimilarity matrix
  - Ranking
- More robust than PCoA
- Applies Procrustes analysis to modify the matrix so that the eigenvectors are close to the original dimensions
  - Generate a ”stress value”
    - <0.1 good model
    - 0.1 < stress < 0.2 meh model
    - 0.2 < stress < 0.3 bad model
    - >0.3 random
- Eingenvectors are still NOT the original dimensions



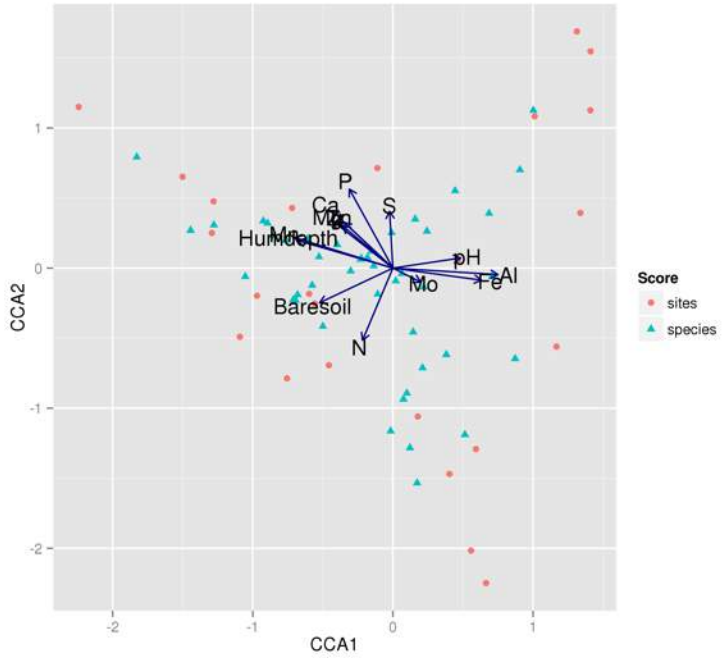
ANOSIM & PERMANOVA

- Both similar to ANOVA
- ANOSIM uses a dissimilarity matrix instead of the raw data
  - Finds differences between groups
  - Highly sensitive to dispersity
- PERMANOVA is a
  - Multivariate ANOVA (i.e. multiple factors influence multiple responses)
  - With PERmutations (solves the problem of limited number of samples)
  - Sensitive to dispersity



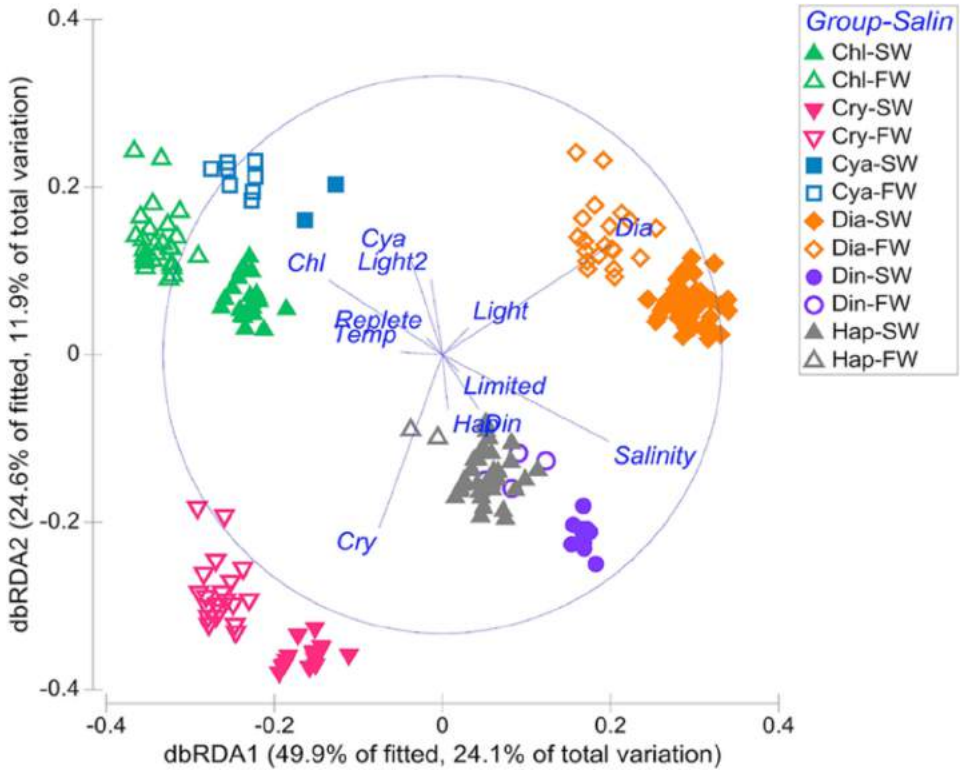
CCA: Canonical Correspondence analysis

- Analyzes correspondences between a matrix of frequencies and a matrix of variables
  - How much the frequencies deviate from random per each variable?
- Correlate counts to variables (finally!)
- Does not try to maximize coverage of variance (unlike PCA)
- You can use ANOVA on CCA
- BEWARE of using only significant variables
  - Avoid collinearity (VIF)



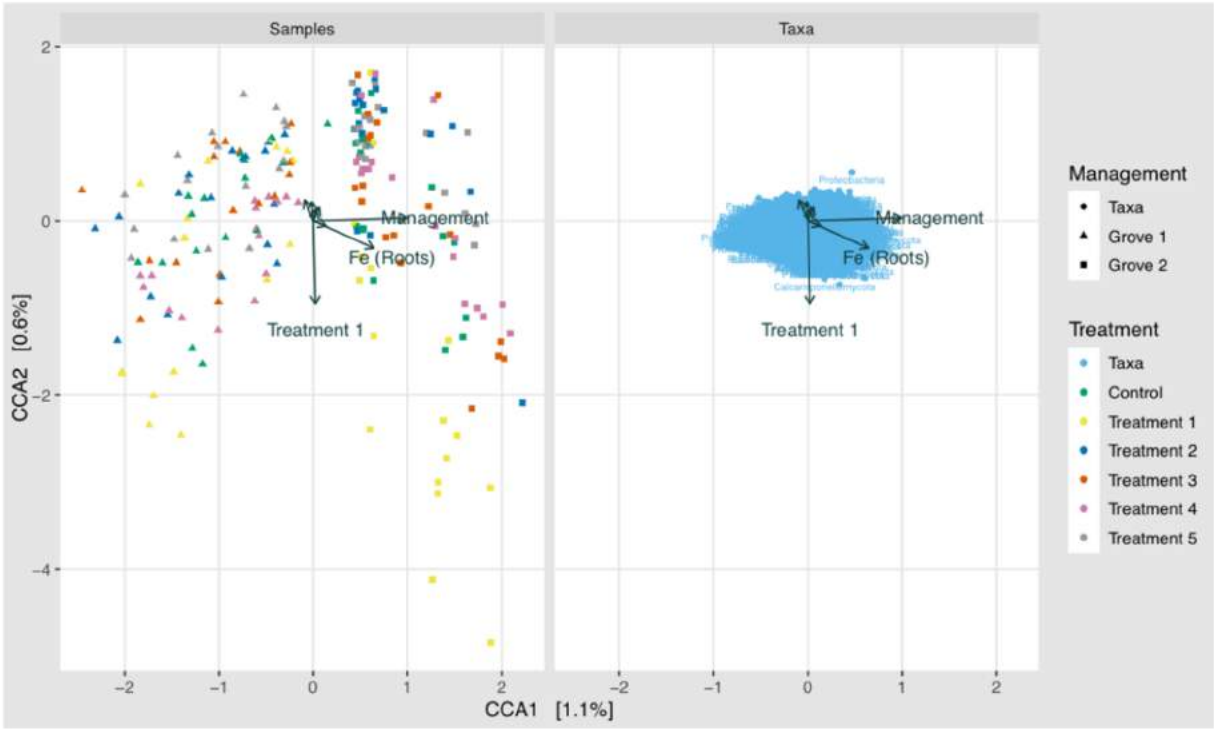
CAP (or db-rda): Constrained analysis of principal coordinates

- Basically a PCA where you constrain your components to your variables
  - Maximizes explanation of variance
- Similar (but different) to CCA
  - Still sensitive to collinearity
- BEWARE of using only significant variables



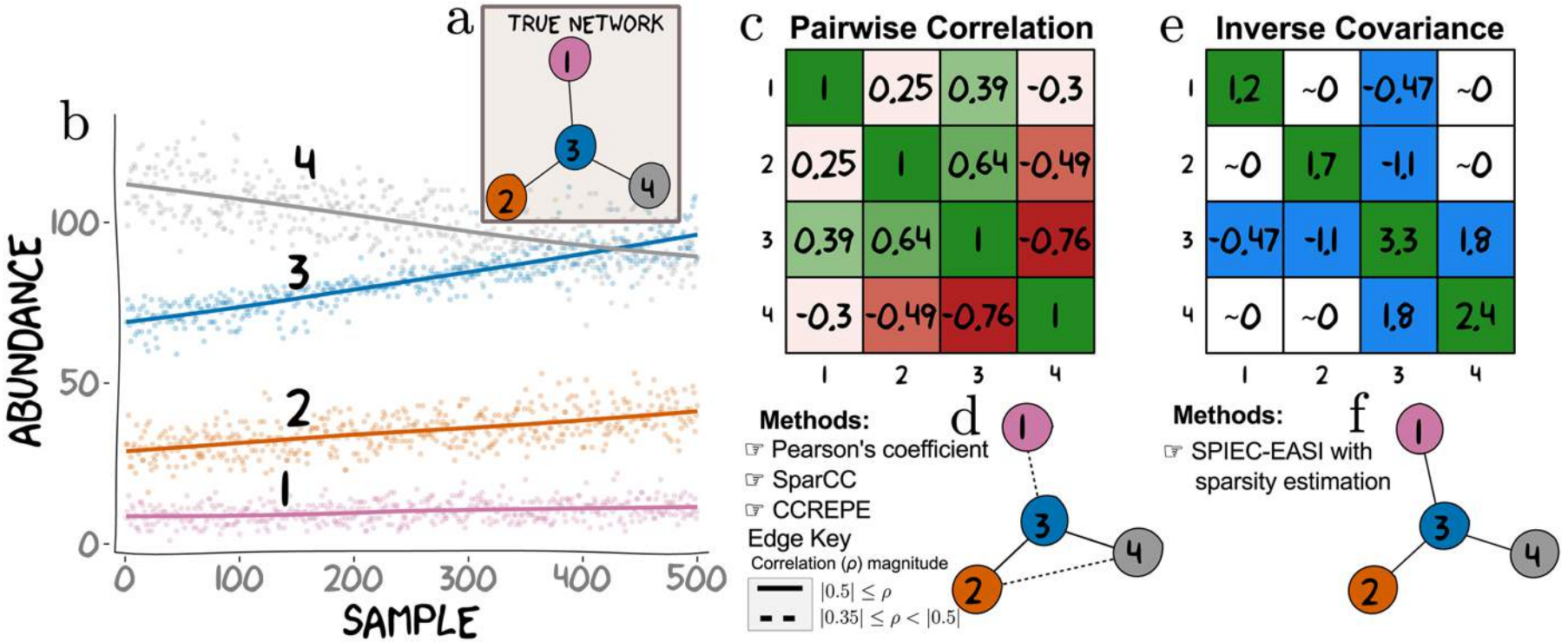
Does it help?

```
source('vif.cca.bw_sel.R')

pslog <- transform_sample_counts(phylo,function(x){log(1 + x)})

cca_vif <- vif.cca.bw_sel(pslog, vifvariables,
                          threshold = 5)

cca_plot <- plot_ordination(physeq = pslog,
                        ordination = cca_vif,
                        type= 'split', color = "Treatment",
                        label = 'Phylum' ) +
            aes(shape = TimePoint) +
            geom_point(aes(colour = Treatment))

cca_arrowmat <- scores(cca_vif, display = "bp")
cca_arrowdf <- data.frame(labels = rownames(cca_arrowmat),
                        cca_arrowmat)

cca_arrow_map <- aes(xend = CCA1, yend = CCA2,
                     x = 0, y = 0, color = NULL, shape = NULL)

cca_label_map <- aes(x = 1.3 * CCA1, y = 1.3 * CCA2,
                     color = NULL, label = labels, shape = NULL)

cca_arrowhead = arrow(length = unit(0.02, "npc"))

cca_plot +
geom_segment(mapping = cca_arrow_map, size = .5, data =
            cca_arrowdf, color = "black", arrow = cca_arrowhead) +
geom_text(mapping = cca_label_map, size = 2, data = cca_arrowdf)+
scale_color_pander()
```



Network analyses with SPIEC-Easi

- Assume interdependencies of OTUs
- Draws samples from negative binomial distributions
- Sparse data → inverse covariance matrix depends on the conditional states of all available nodes
- Avoids weak or false positive associations



*Kurtz, Z.D. et al. (2015). PLoS Comput. Biol., 11, 1–25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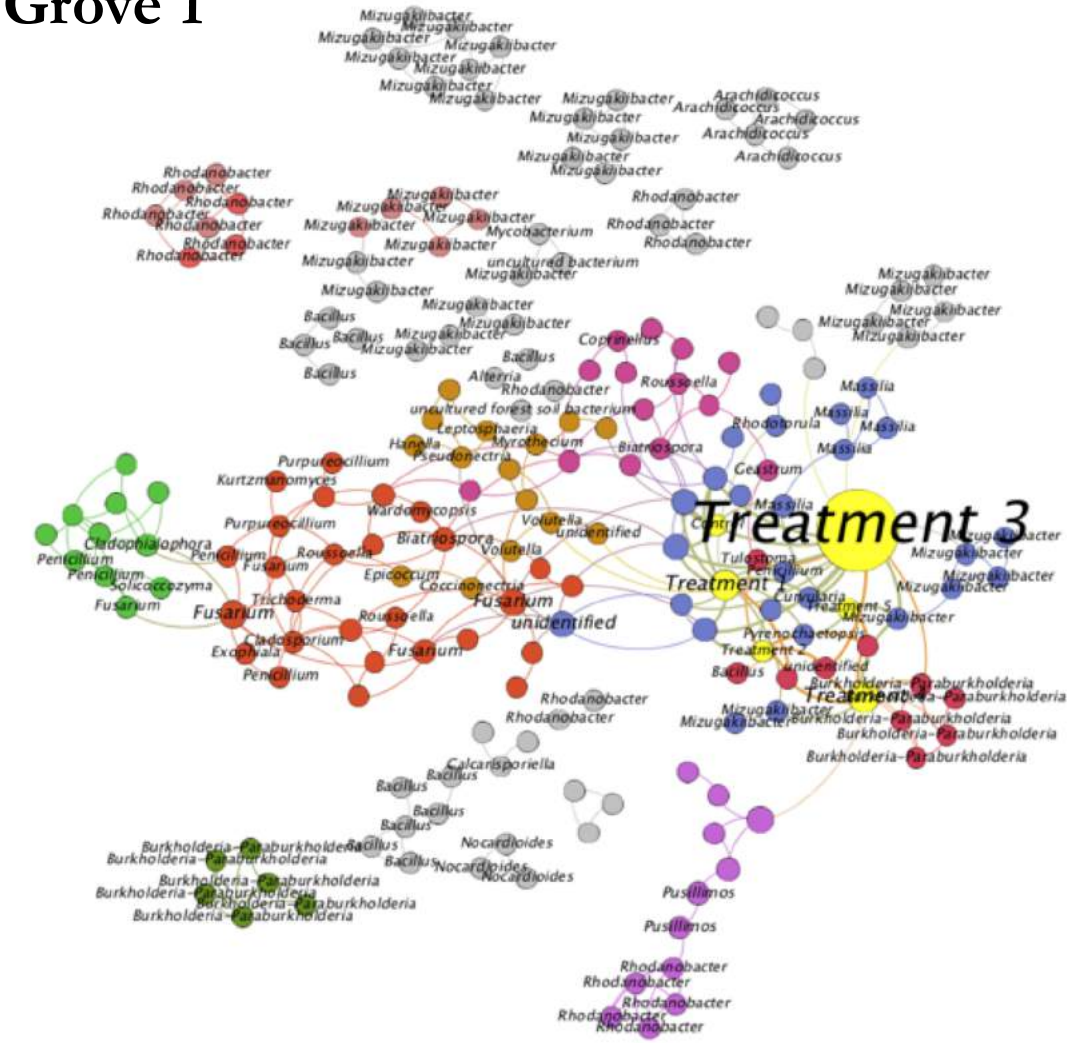
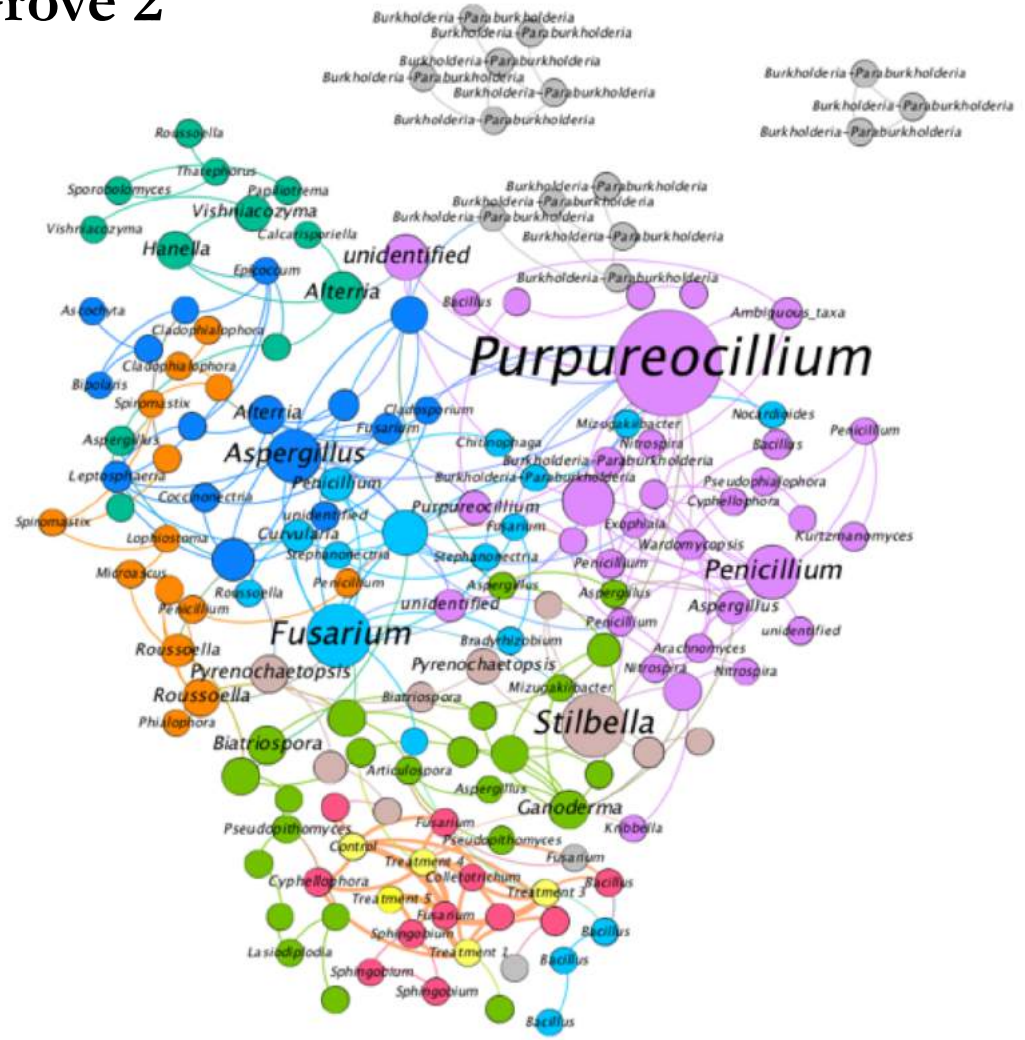
Does it help?

Grove 1



Grove 2

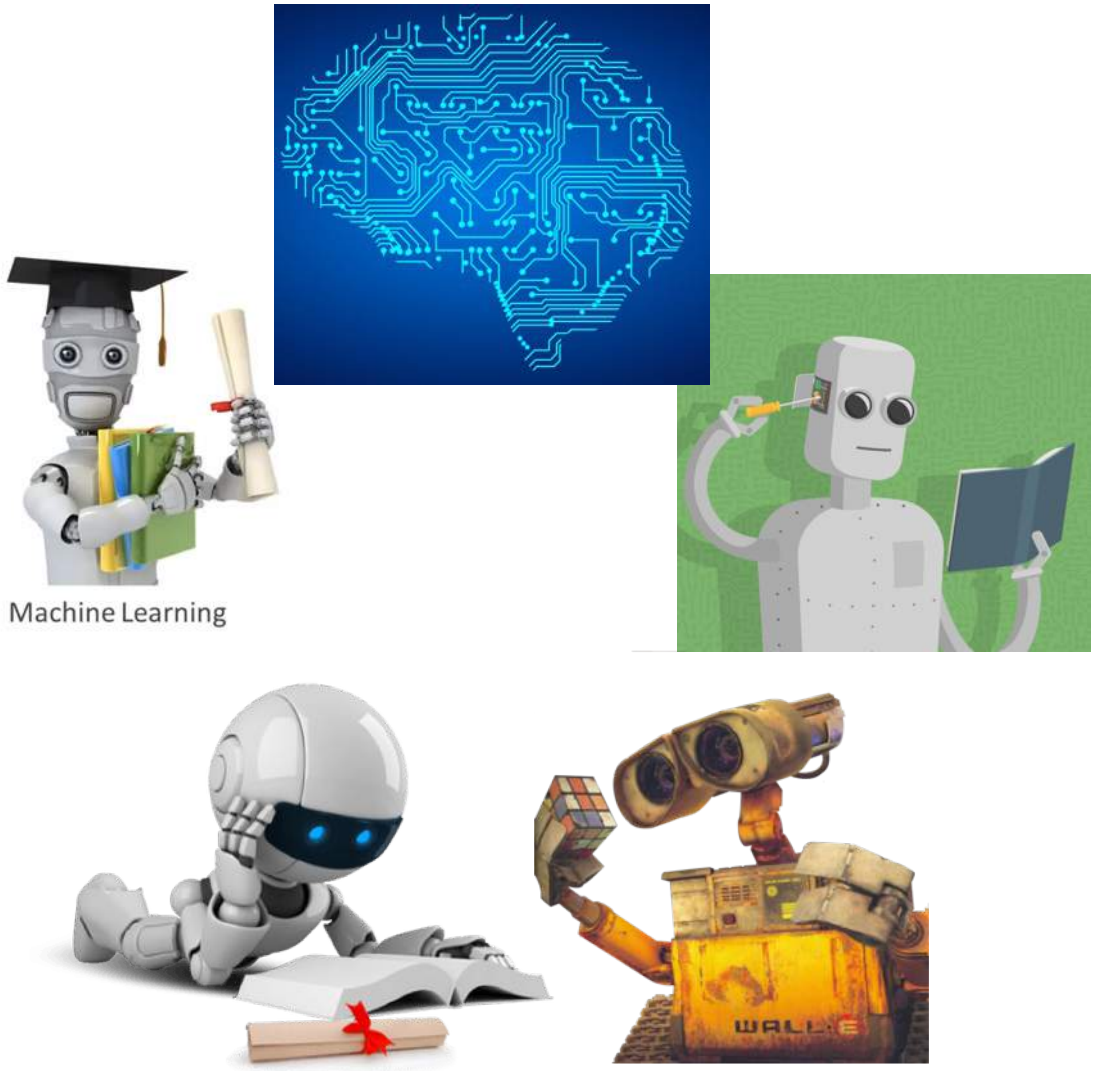


AN ALTERNATIVE METHOD

- Generalized
- Powerful
- Advanced
- Sensitive

Guess what I'm talking about

- An excuse for fantasy android/brain images that don't really explain anything
  - (please use Wall-E).
- Not statistics!
  - Statistics focuses on describing observations (mean, variance, etc)
  - ML focuses on predicting NEW observations BY generalizing observations into a model
  - Like all good things, this is just a simplification
- Do we care about predictions? No, but we care about understanding which are the best predictors (variables) for our model, i.e. understanding relationships in our data



Gneiss (Qiime2)

- Method to transform count tables
  - Applies logarithmic transformation on species ratio
  - Reduces heteroscedasticity
  - Infers relationships
  - Clusters those log-ratio using hierarchical clustering
  - Results in a weighted tree of balances
- Weighted log-ratios change between pair of species BUT NOT in the whole dataset
  - Avoids the problem of relative abundances
  - Breaks time-dependence

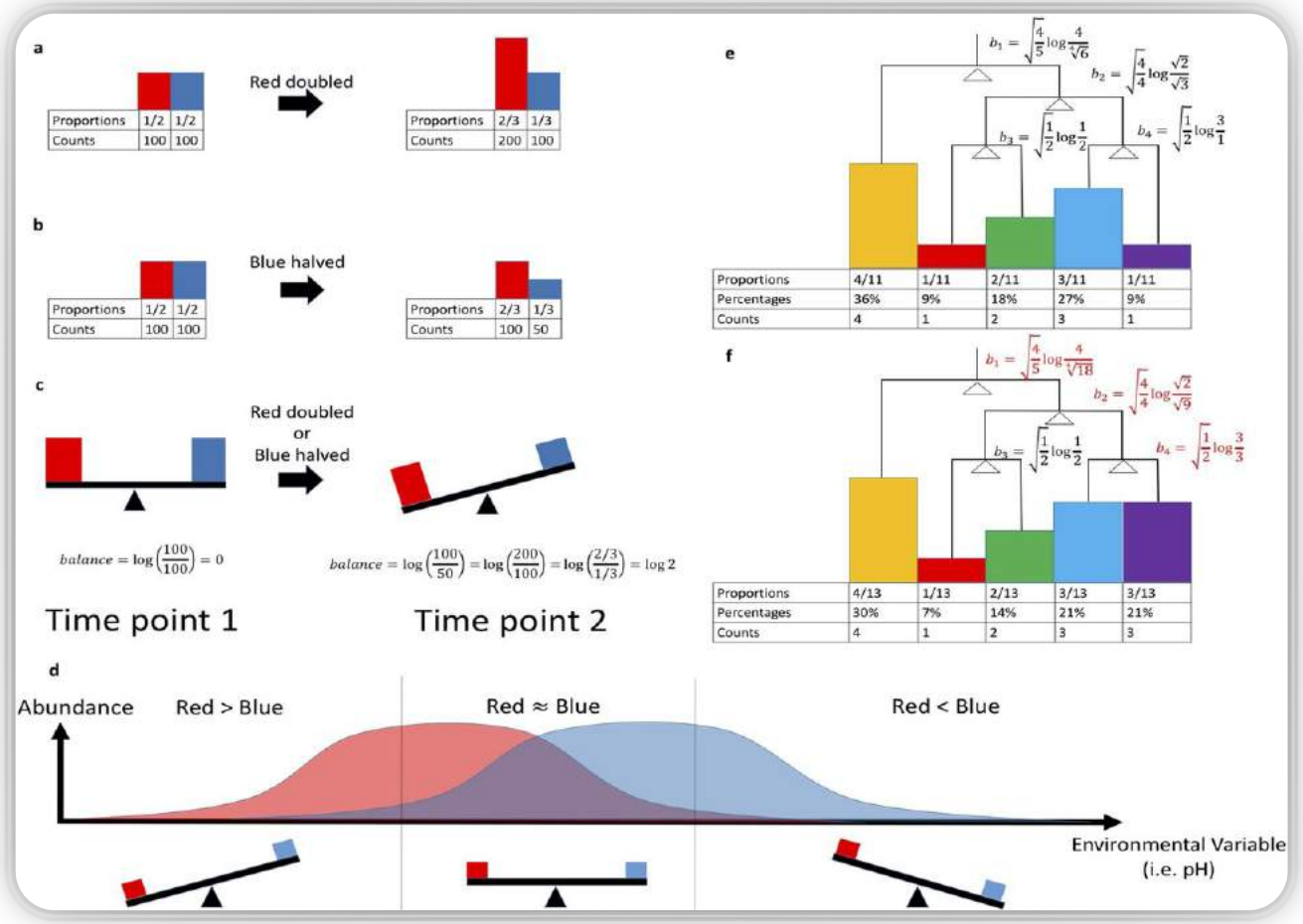


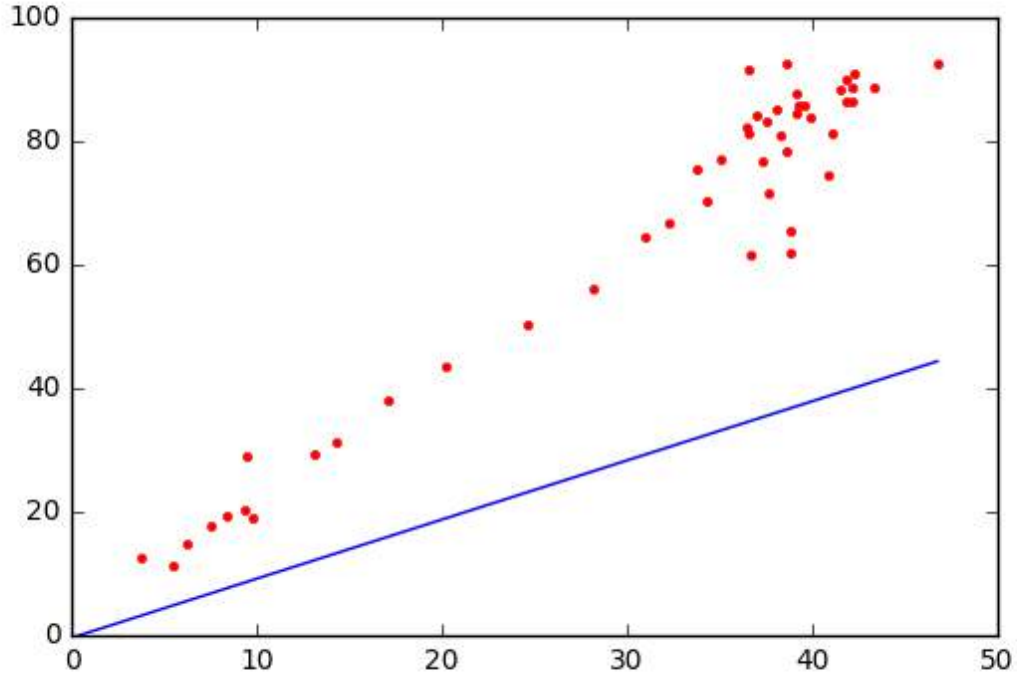
Image from James T. Morton et al. mSystems 2017; 2:e00162-16

Ordinary Least Squares (OLS)

- Finds linear fit to the variables
  - In our case equation is

$$balance_i \sim var_1 + var_2 + etc$$

- We use balances to solve heteroscedasticity and magnitude issues
- Results: tells us which balance is significantly correlated with each variables
- Balances must be exported



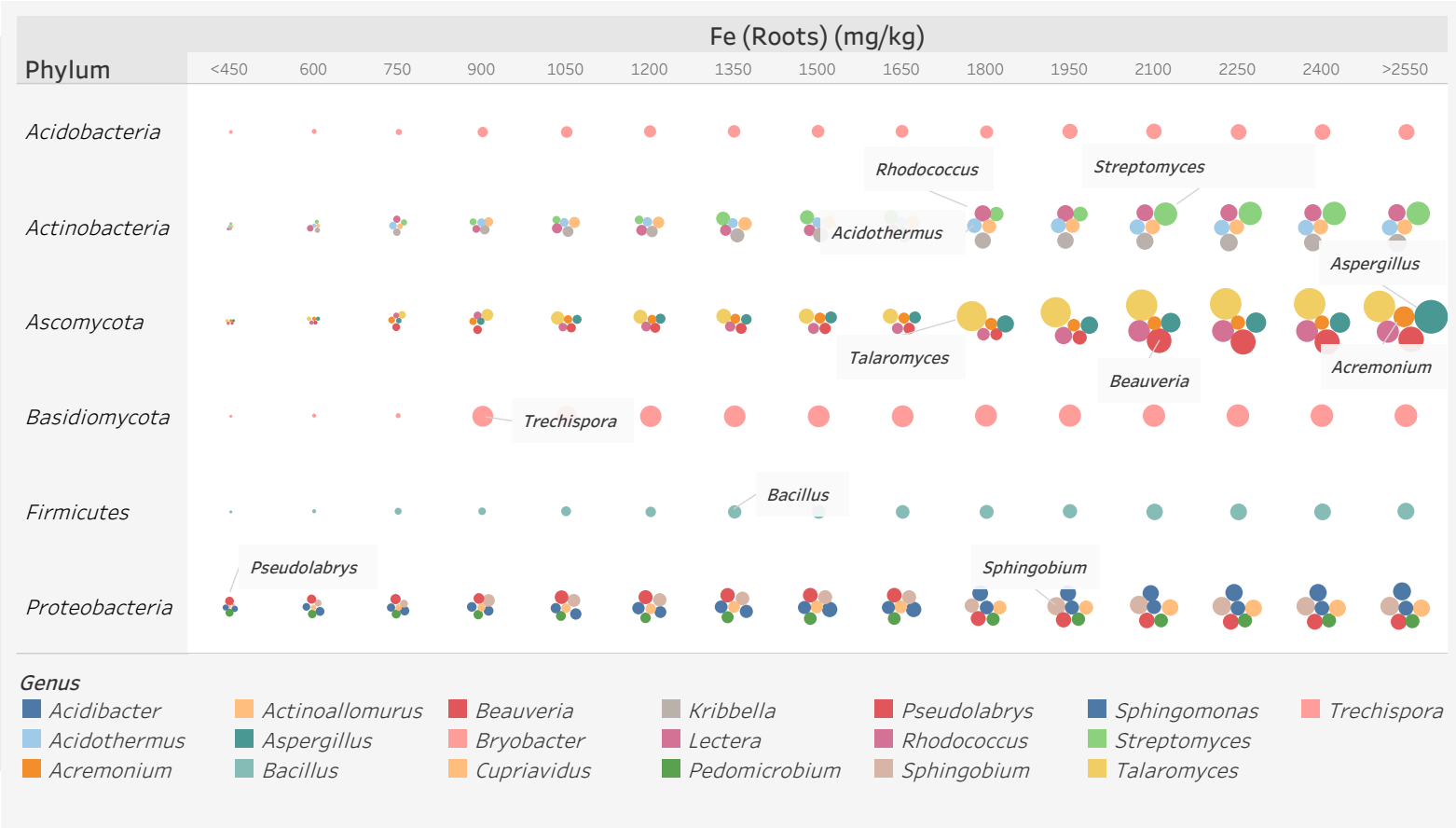
Does it help?

```
qiime feature-table filter-features \
  --i-table dada2_output.qza \
  --o-filtered-table filtered-table.qza \
  --p-min-samples 5

qiime gneiss correlation-clustering \
  --i-table filtered-table.qza \
  --o-clustering hierarchy.qza

qiime gneiss ilr-hierarchical \
  --i-table filtered-table.qza \
  --i-tree hierarchy.qza \
  --o-balances balances.qza

qiime gneiss ols-regression \
  --p-formula
"TimePoint+Treatment+Location+Root_Fe+...”\
  --i-table balances.qza \
  --i-tree hierarchy.qza \
  --m-metadata-file plant_data.txt \
  --o-visualization regression_summary.qzv
```

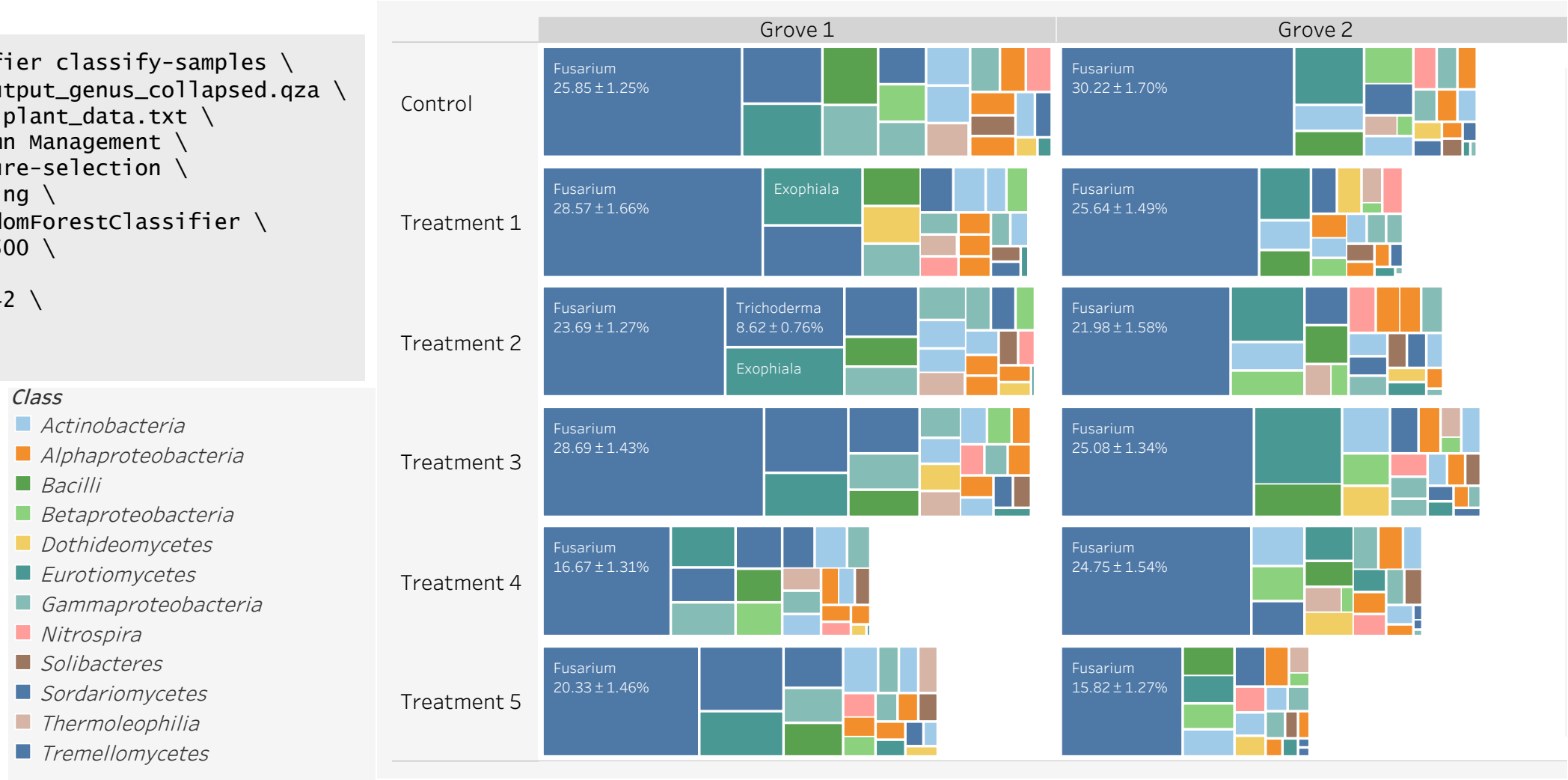


Random Forest Analysis

- Does not require log-transform
- Can optimally discriminate rare species
- Produces feature importances
- Can be optimized by collapsing the taxa at different levels

Does it help?

```
qiime sample-classifier classify-samples \
  --i-table dada2_output_genus_collapsed.qza \
  --m-metadata-file plant_data.txt \
  --m-metadata-column Management \
  --p-optimize-feature-selection \
  --p-parameter-tuning \
  --p-estimator RandomForestClassifier \
  --p-n-estimators 500 \
  --p-cv 5 \
  --p-random-state 42 \
  --p-n-jobs -1 \
  --output-dir RFC
```



Did we find the needle?

- Multivariate analyses (beta diversity) can tell us if there are significant differences correlated to our host or treatments
- Network analyses can help us determine microbial association that might reveal metabolic chains
- When these do not work, Machine Learning can help overcome sensitivity and accuracy problems.
- Fe concentration in the root correlated to higher abundance of potentially beneficial species in Grove 2
- Random forest helped determining *correlations* between treatments and specific taxa (at the genus level) that *might* explain Fe differences
- Work in progress!
- Requires *VALIDATION*!
- We only know the (alleged) *name* of those species. What about their *function*?

Adding complexity

Dimensionality reduction

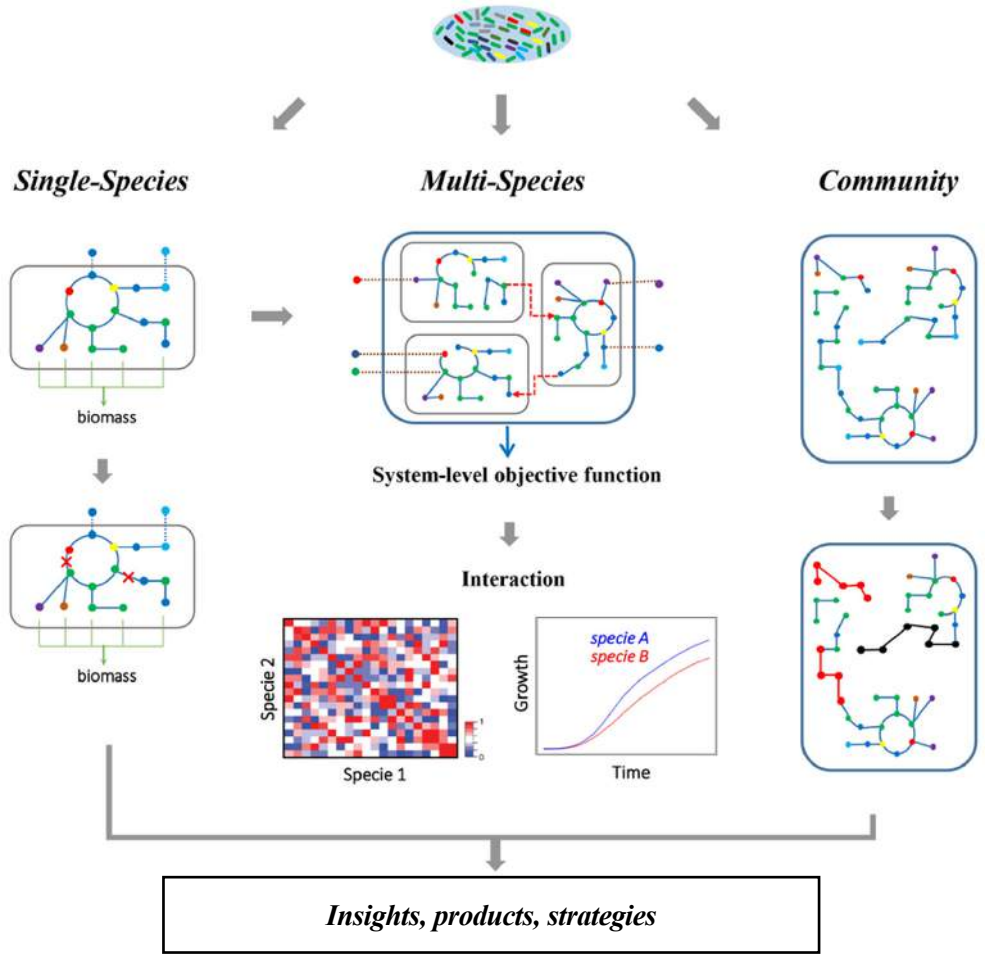
- LASSO
- ElasticNet
- t-SNE (?)

Microbiome-wide association

- Whole shotgun metagenome
  - (not limited to rRNA)
- Integration with plant gene expression

Time-series analysis

- Dynamic networks
- Metabolites
- State prediction



So what?

- Microbes are everywhere
- Microbes potentially impact anything
- Lot of unsolved questions
  - Correlation vs Causation
  - Functions
  - Metabolites
  - Interactions
  - Manipulation
- From 2011 to 2015:
  - Venture funding up 485% ($114.5 mln)
  - More than $600 mln invested in 2016
  - Includes GSK, Novartis, Indigo, Syngenta. etc
- Huge challenges ahead!!
  - Diseases, Obesity, Depression, Cancer, Longevity
  - Climate change, Food shortage, Loss of biodiversity
- Bioinformatics *accelerates* discovery
- You have to know what you're talking about!!
  - Biochemistry
  - Biology
  - Physiology
  - Pharmacokinetics

OTHER RESOURCES

- GUSTA.ME website for multivariate statistics (https://mb3is.megx.net/gustame)
- Phyloseq website with tutorials (https://joey711.github.io/phyloseq/index.html)
- Bioconductor workflow (https://f1000research.com/articles/5-1492/v2)
- Qiime2 tutorials (https://docs.qiime2.org/2018.8/tutorials/)
- Slides()


THANK YOU FOR LISTENING
HERE'S A POTATO