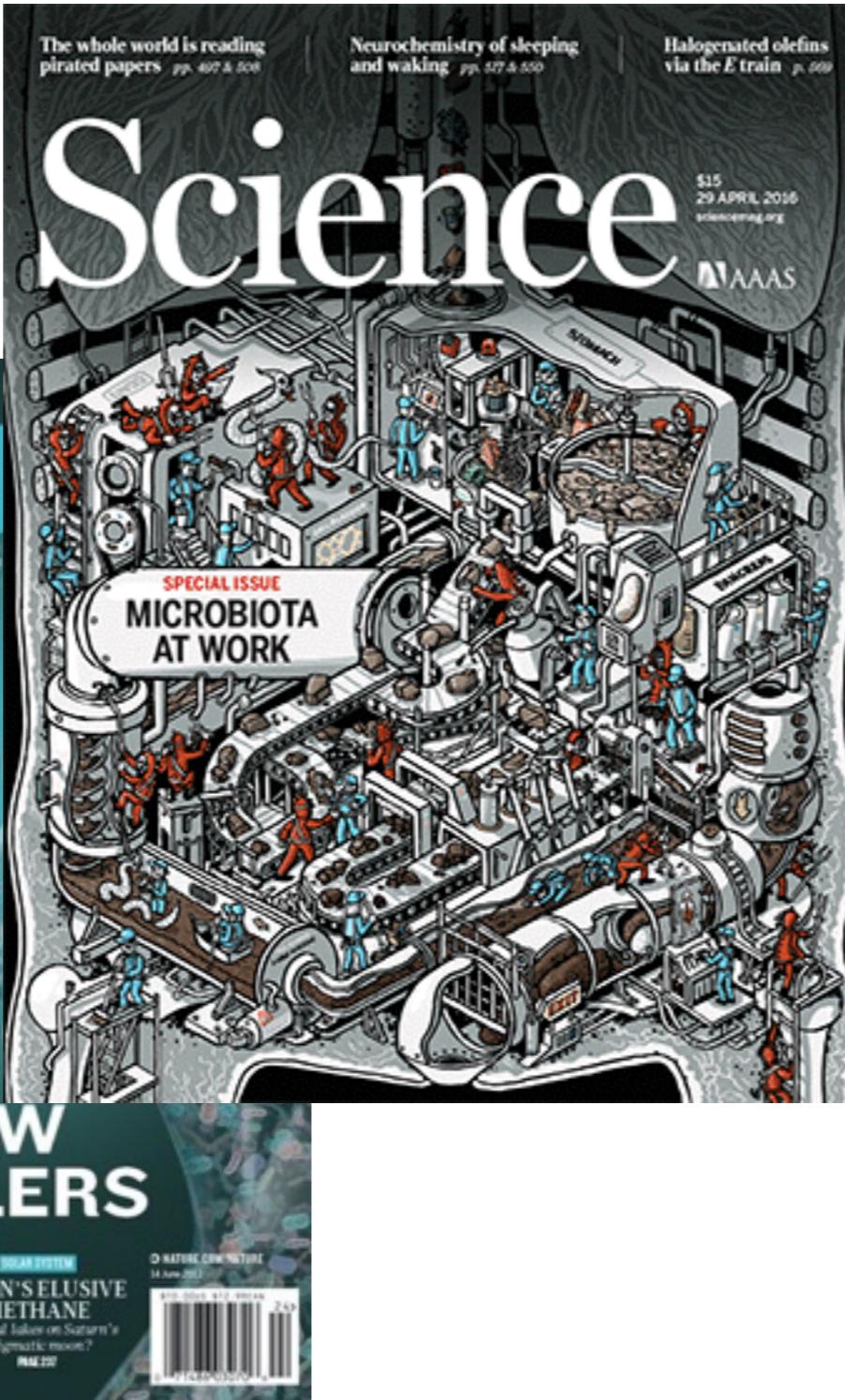


Microbial genomics

Charlotte Soneson
University of Zurich
Brixen 2016

What is the “microbiome”?



TARA
OCEANS



american
gut

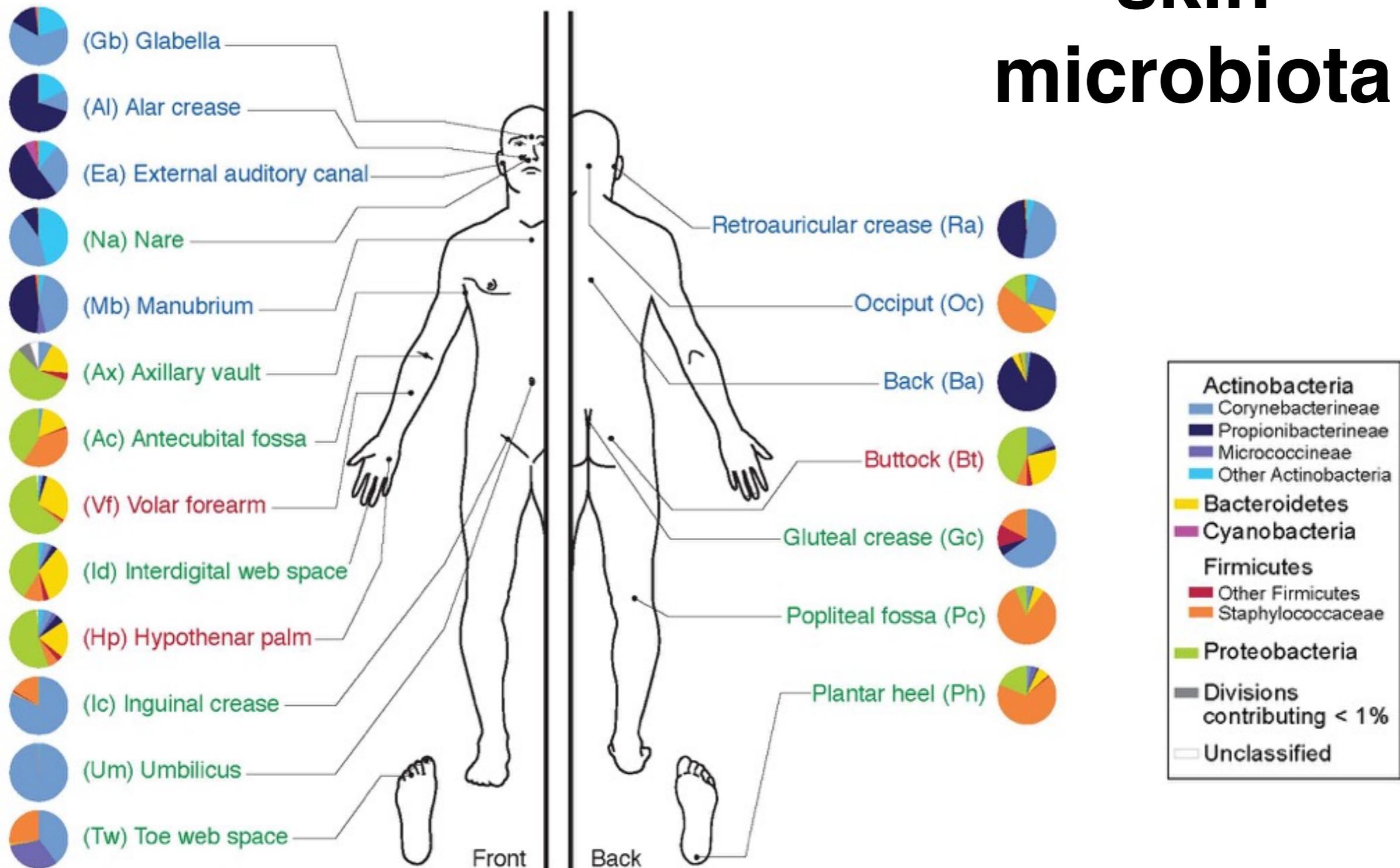
What is the “microbiome”?

- **microbiota** = the assemblage of microorganisms (e.g., bacteria, archaea, viruses, fungi)
- **microbiome** = the ecosystem comprising all microorganisms in an environment, as well as their genes and environmental interactions

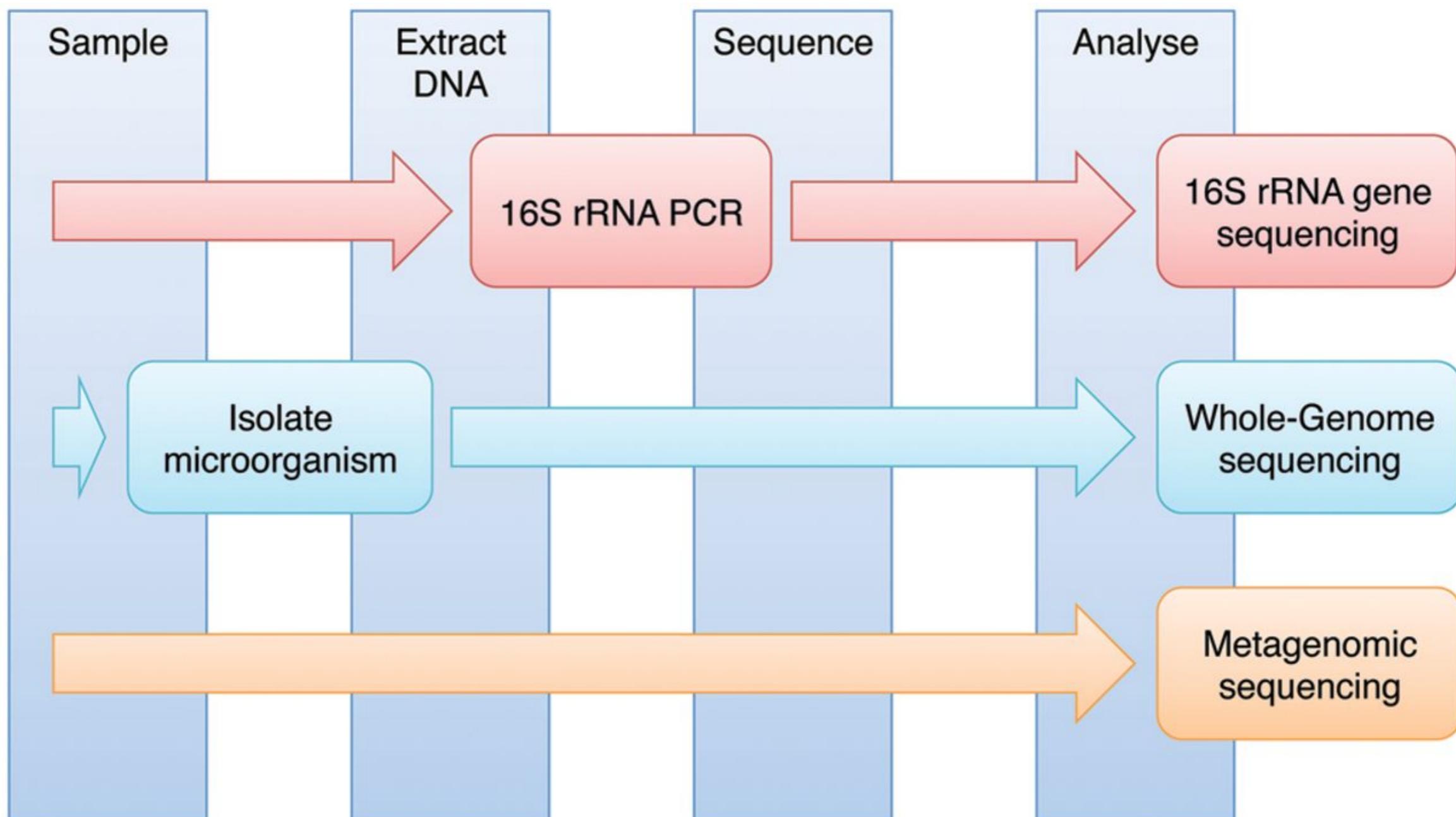
The human microbiota

- “microorganisms that exist upon, within or in close proximity to the human body”
- widely varying composition between body sites and individuals
- important for health: building vitamins, breaking down food etc.
- ratio of microbial to human genes in the body is estimated between 1:1 and 100:1

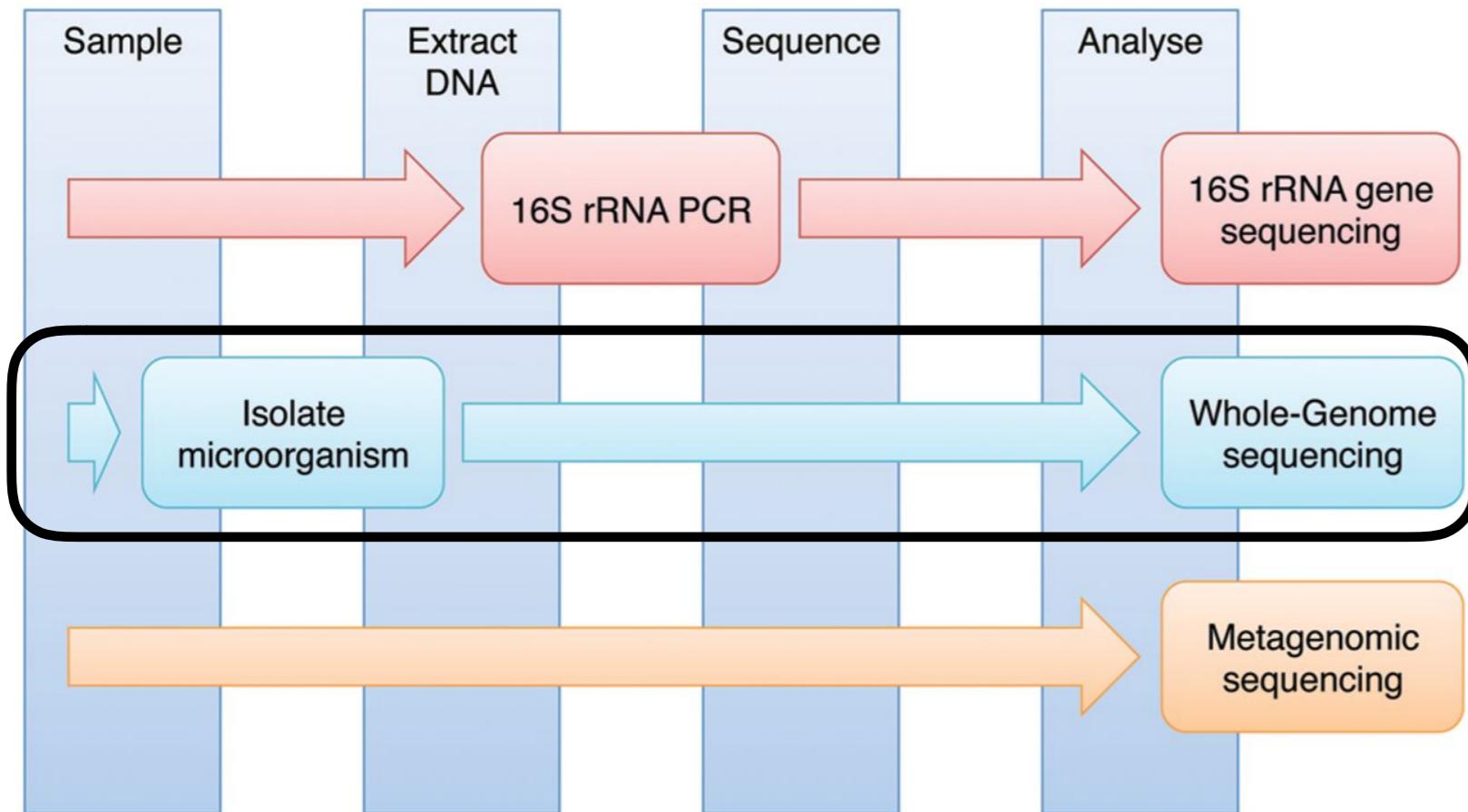
Human skin microbiota



How can we analyze the microbiome?

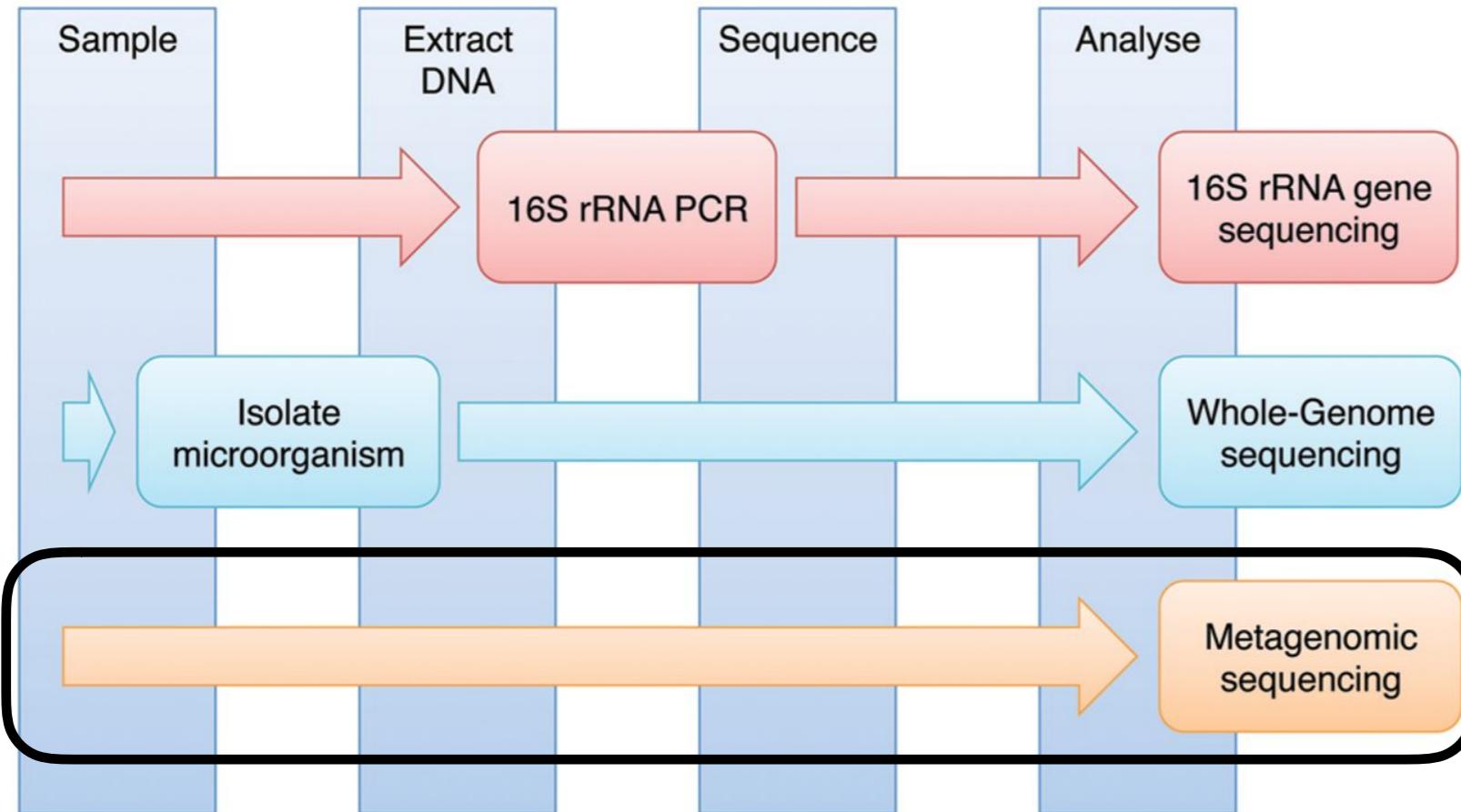


How can we analyze the microbiome?



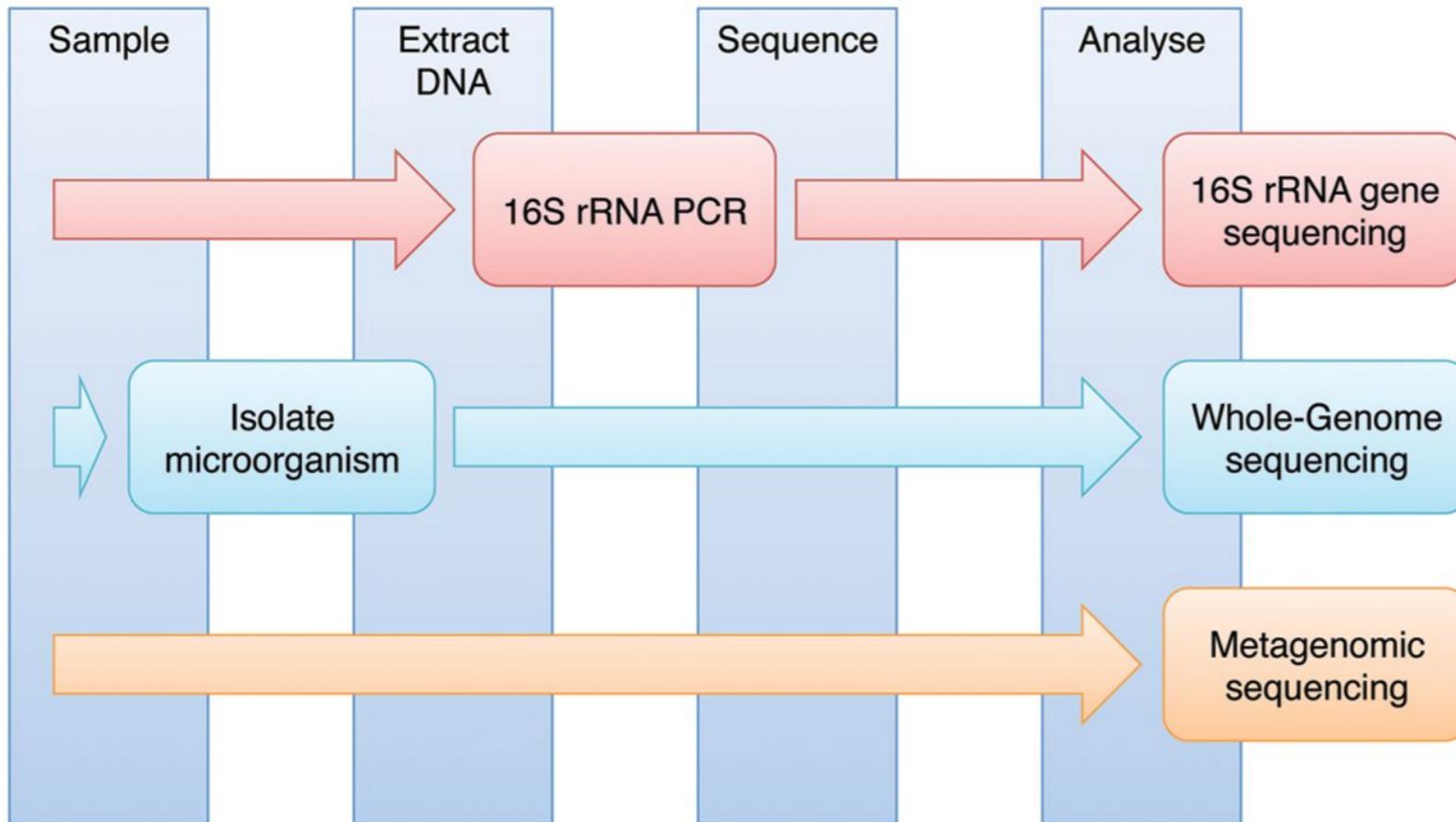
- Whole-genome sequencing: **characterize specific isolate**

How can we analyze the microbiome?



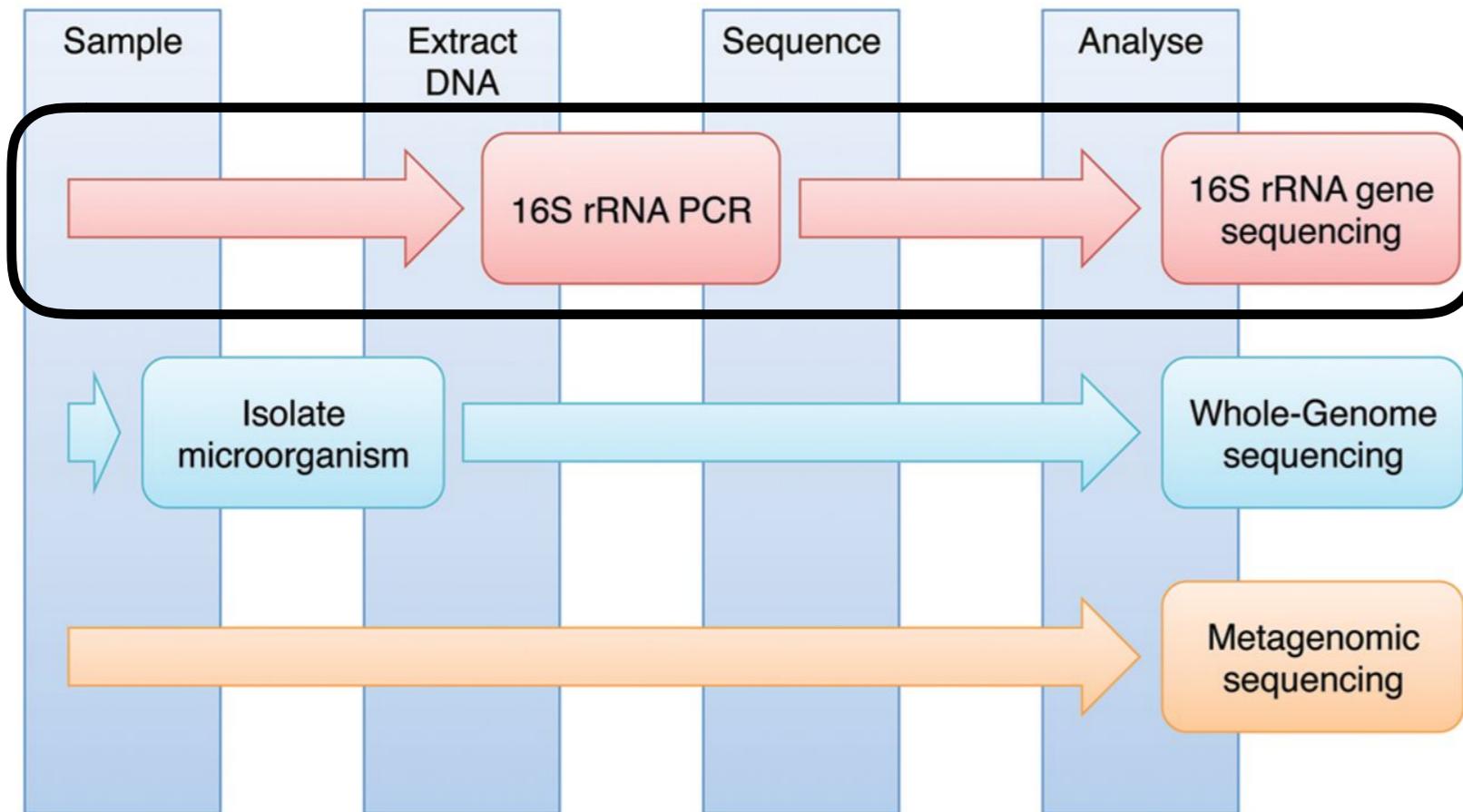
- Metagenomic (shotgun) sequencing: **sequence complete set of DNA in a sample**

How can we analyze the microbiome?



- *metatranscriptomics*
- *metaproteomics*
- *metabolomics*
- ...

How can we analyze the microbiome?



- [16S] rRNA amplicon/marker gene sequencing:
infer microbial composition

Amplicon sequencing - basic idea

- Amplify (part of) the 16S rRNA gene from all microbes - sequence amplified part
- Cluster sequences together in so called OTUs (= clusters of similar sequences ~ “species”)
- Get the number of sequences in each cluster/OTU for each sample
- Generate an abundance table (OTUs x samples)

Amplicon sequencing - basic idea

Which part?

We need primers!

- Amplify (part of) the 16S rRNA gene from all microbes - sequence amplified part

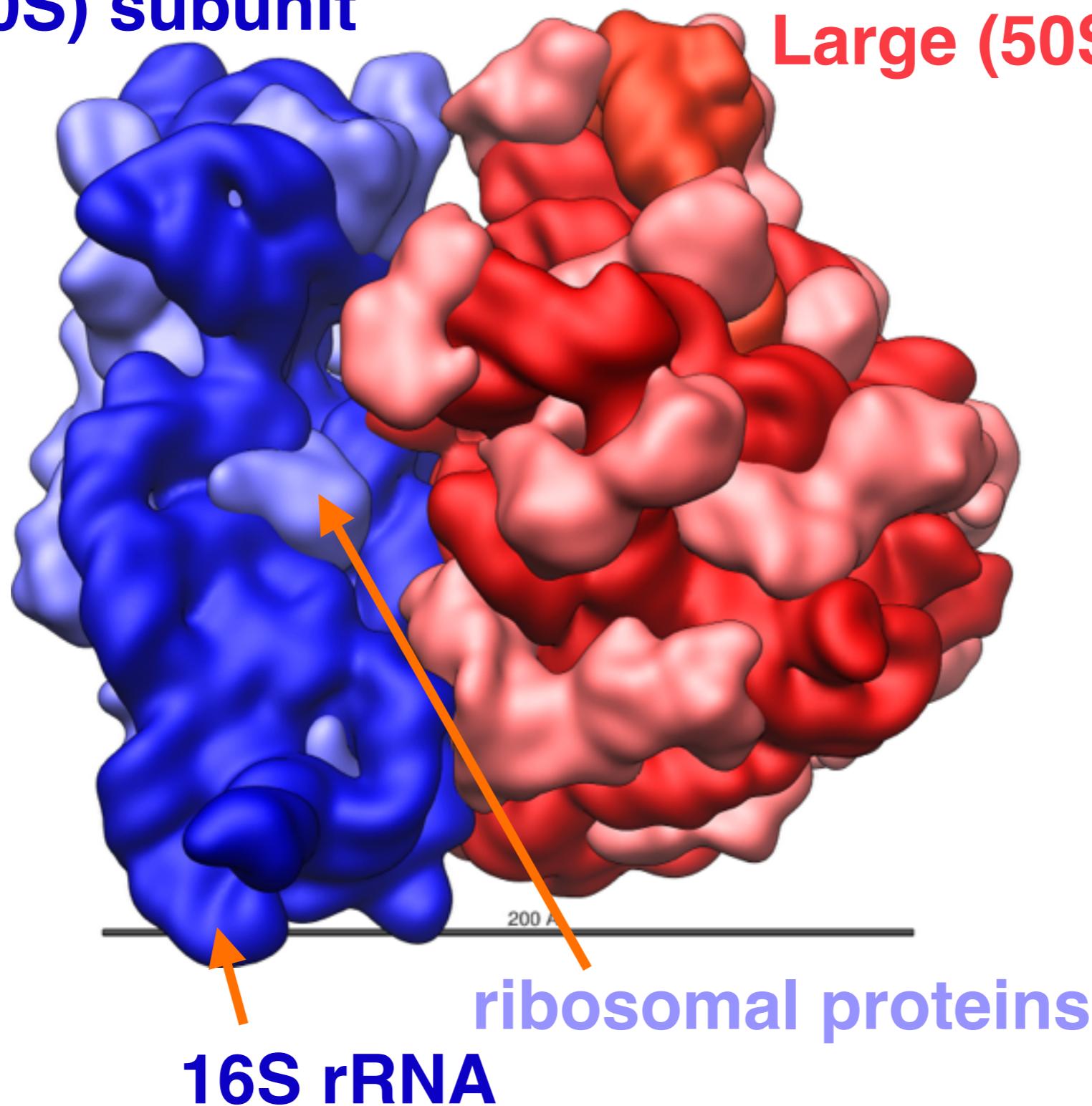
How to cluster?
- Cluster sequences together in so called OTUs (= clusters of similar sequences ~ “species”)
- Get the number of sequences in each cluster/OTU for each sample

How to analyze?
- Generate an abundance table (OTUs x samples)

An E.coli ribosome

Small (30S) subunit

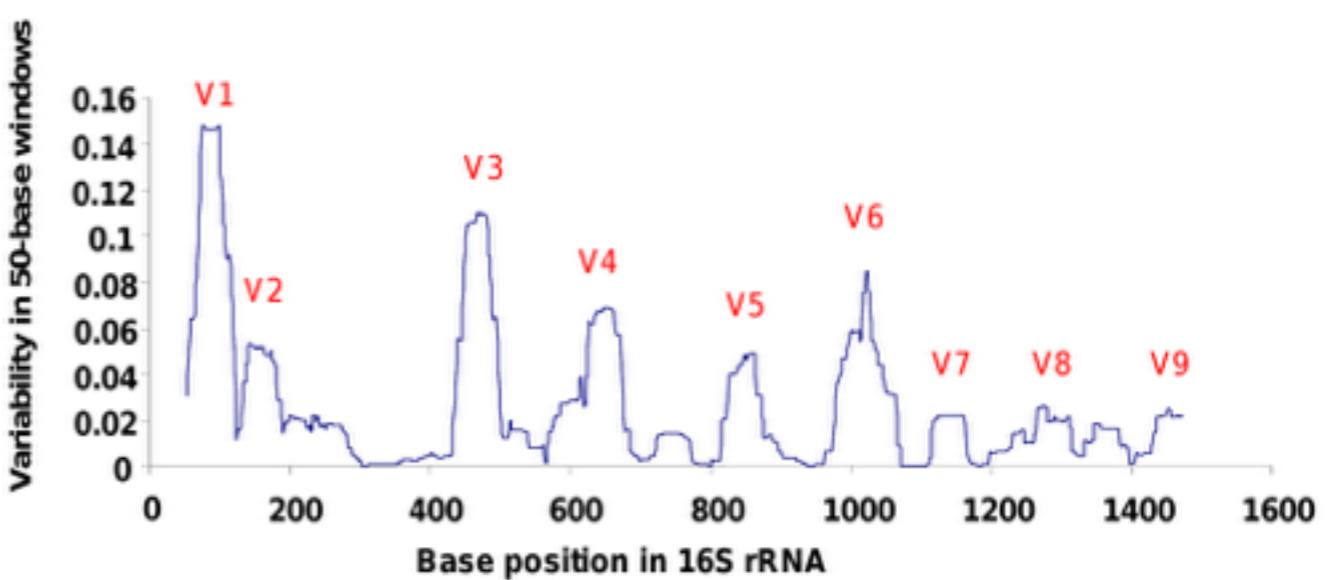
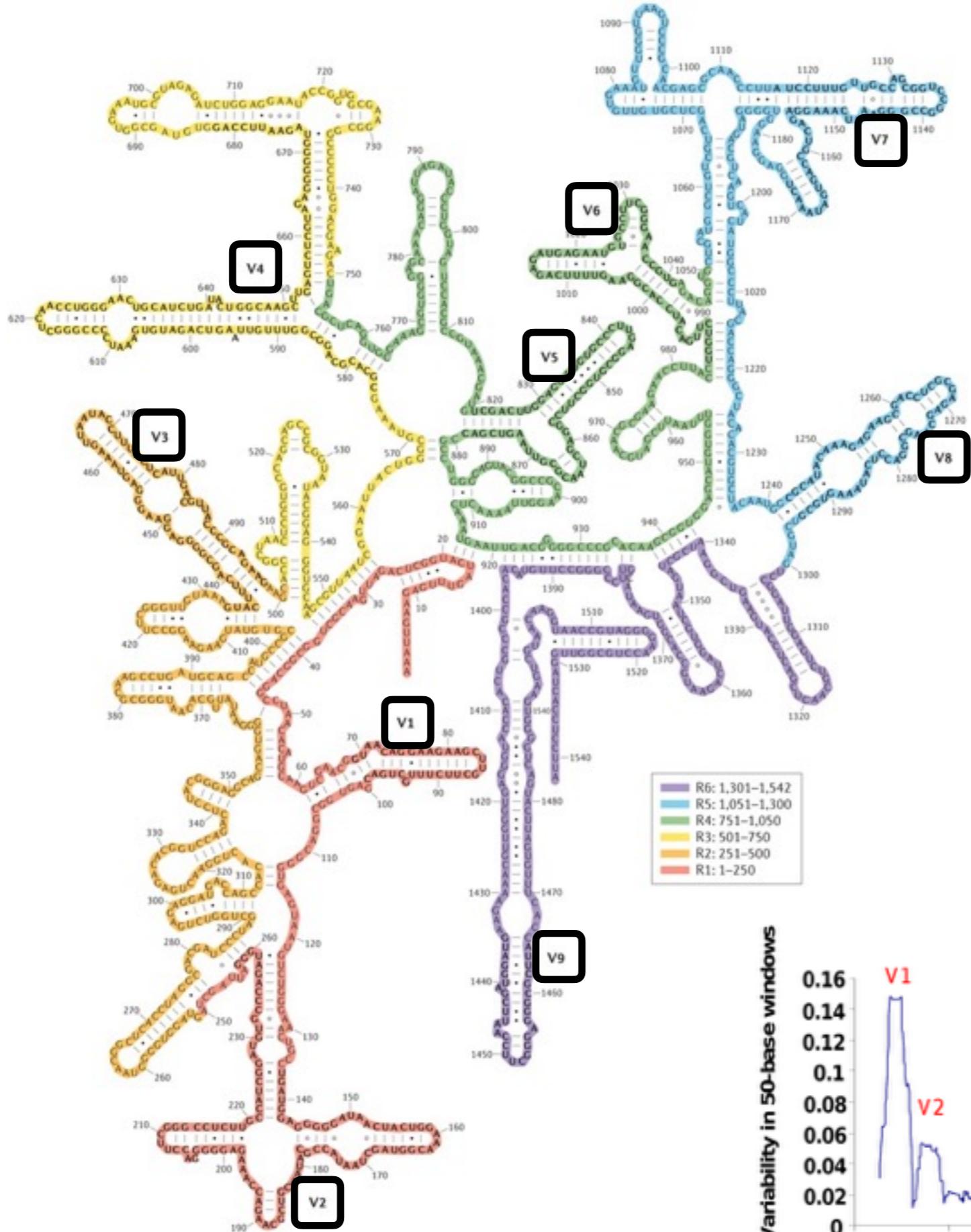
Large (50S) subunit



Why [16S] rRNA?

- rRNA is one of the few gene products present in all cells
- 16S rRNA has 9 **hypervariable regions** allowing species identification, as well as **conserved regions** allowing primer construction
- conserved function
- sequence has been characterized for many species

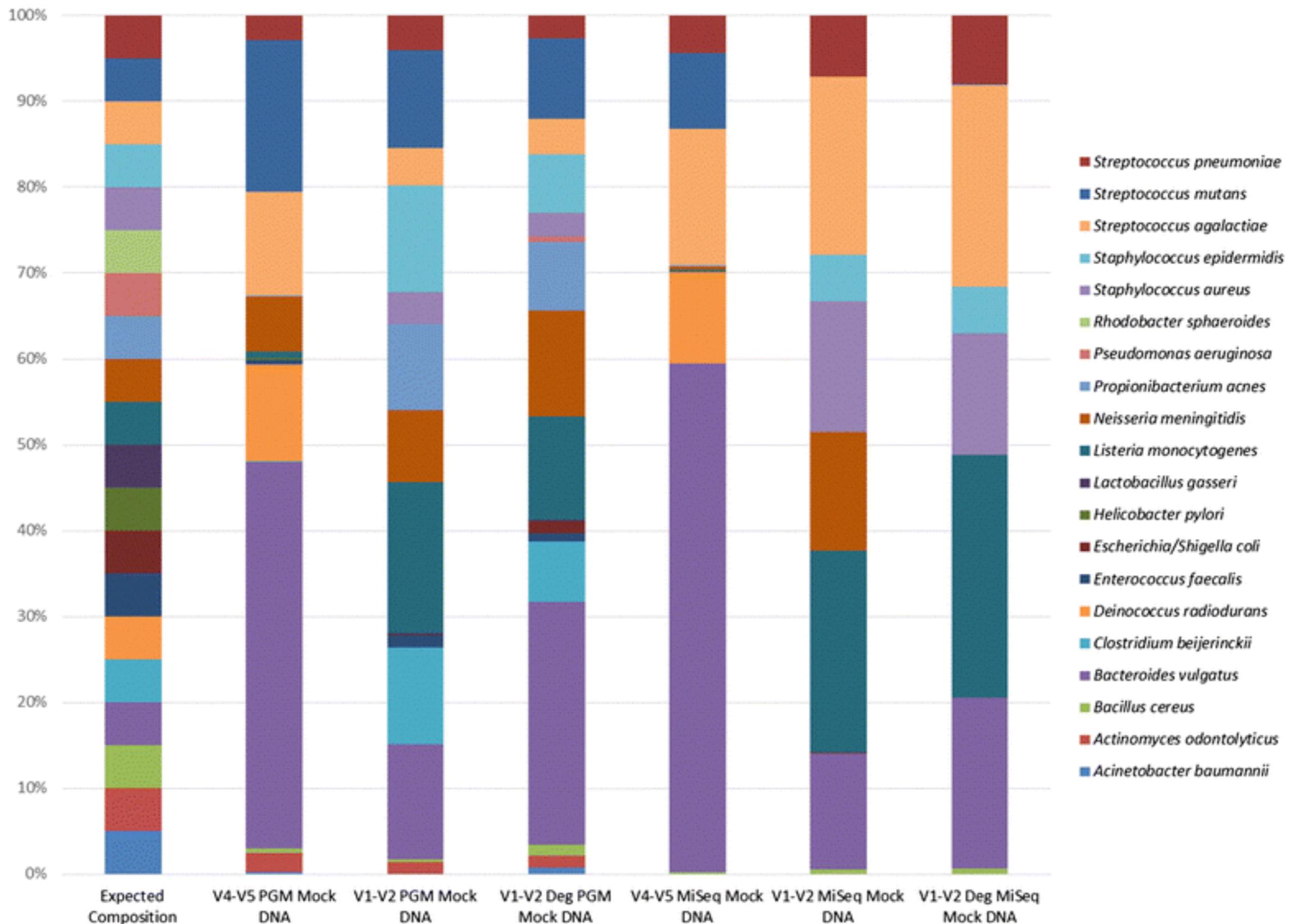
16S rRNA (*E. coli*)



16S is not perfect

- Different species can have similar 16S sequences
- A single species can have paralogs that are not identical
- Results can depend on which variable region is considered, and which sequencer is used

There are still challenges to overcome



Preprocessing of reads



Quantitative Insights Into Microbial Ecology



mothur

- Merge read pairs and remove low-quality reads
- Align reads to reference 16S sequence

16S sequence databases

- SILVA (<http://www.arb-silva.de/>)
- RDP (<https://rdp.cme.msu.edu/>)
- GreenGenes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>)

```
>AF515816.1
.....A-----T-G--A-C-G-C--T-G-G-C--G-G--C-A-T-G-----C---T-T--TA-C--AC-A----T-G--C----A
>AY688433.1
.....A---C---G---A-C-G-C--T-G-G-C--G-G--C-A-T-G-----C---T-T--A--C--AC-A----T-G--C----A
>Z22781.1
.....A---C---G---A-C-G-T--T---G-C--G-A--T-G-C-G-----T---C-T--TA-A--GC-A----T-G--C----A
>AJ582031.1
.....A---C---G-A---C-G-C--T-G-G-C--G-G--C-A-G-G-----C---C-T--AA-T--AC-A----T-G--C----A
>AB070566.1
.....A---C---G-A---C-G-C--T-G-G-C--G-G--C-A-G-G-----C---T-T--AA-C--AC-A----T-G--C----A
>AB070570.1
.....A---C---G-A---CAG-C--T-G-G-C--G-G--C-A-G-G-----C---T-T--AA-C--AC-A----T-G--C----A
>AY033301.1
.....A---C---G-A---A-C-G-C--T-G-G-C--G-G--C-A-G-G-----C---C-T--AA-C--AC-A----T-G--C----A
>AY035307.1
```

16S sequence databases

- SILVA (<http://www.arb-silva.de/>)
- RDP (<https://rdp.cme.msu.edu/>)
- GreenGenes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>)

AB000389.1 Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Pseudoalteromonadaceae;Pseudoalteromonas;
AB000699.1 Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Nitrosomonas;
AB000700.1 Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Nitrosomonas;
AB000701.1 Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Nitrosomonas;
AB000702.1 Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;Nitrosomonas;
AB001518.1 Bacteria;Proteobacteria;Alphaproteobacteria;Rickettsiales;Rickettsiaceae;
AB001724.1 Bacteria;Cyanobacteria;Chroococcales;Microcystis;
AB001774.1 Bacteria;Chlamydiae;Chlamydiales;Chlamydiaceae;Chlamydophila;
AB001775.1 Bacteria;Chlamydiae;Chlamydiales;Chlamydiaceae;Chlamydophila;

Preprocessing of reads



- Merge read pairs and remove low-quality reads
- Align reads to reference 16S sequence
- Denoise (cluster very similar sequences)
- Identify and remove chimeras and contaminants
- Cluster reads into **O**perational **T**axonomic **U**nits (OTUs)

OTU generation

- “**closed-reference** clustering”: compare sequences to a reference catalog, group together sequences that are similar to the same references.
- “**distance-based/*de novo*** clustering”: cluster based on pairwise distances among sequences.
- “**open-reference** clustering”: closed-reference clustering followed by *de novo* clustering of unclassified sequences

OTU generation

- “**closed-reference** clustering”: compare sequences to a reference catalog, group together sequences that are similar to the same references.

- 
- Fast, parallelizable
 - OTU assignment independent of other sequences
 - Comparable OTUs across studies
 - Relies on accuracy and completeness of reference catalog
 - Sequence can be similar to multiple reference sequences
 - Similarity among clustered sequences may be lower than the similarity between each of them and the reference

OTU generation

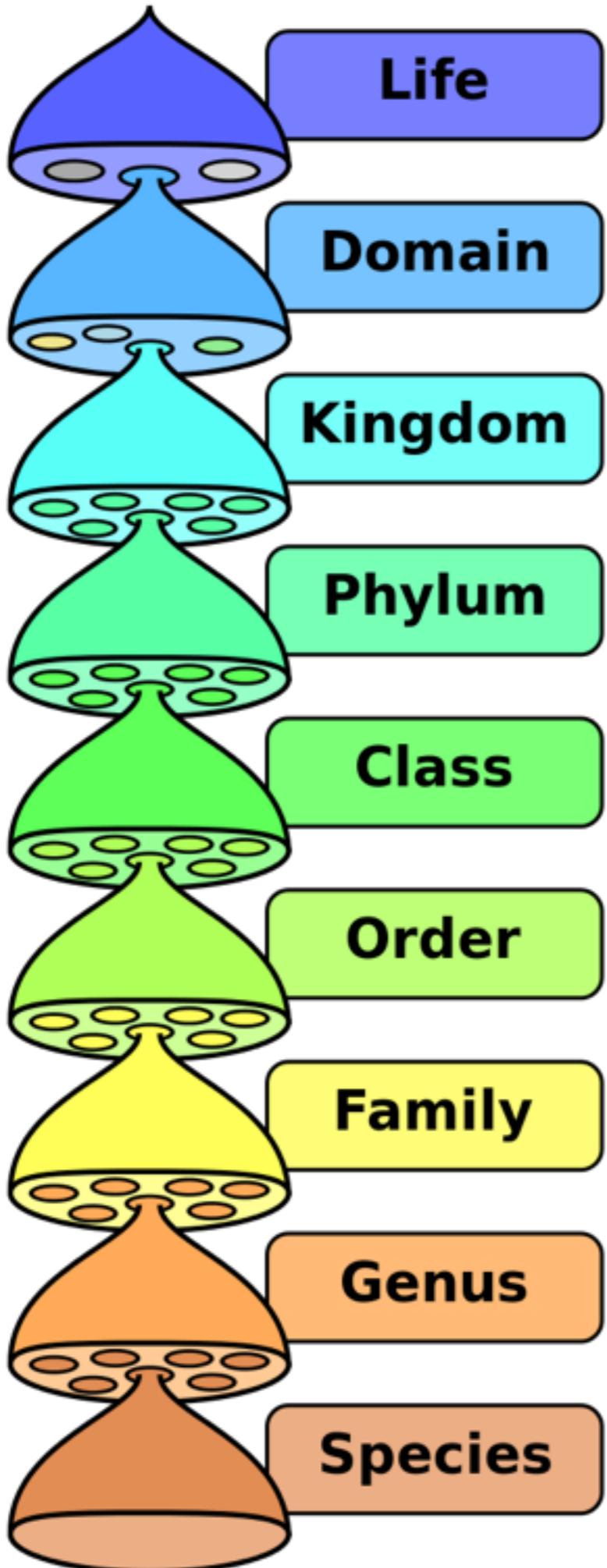
- “**closed-reference** clustering”: compare sequences to a reference catalog, group together sequences that are similar to the same references.
- “**distance-based/*de novo*** clustering”: cluster based on pairwise distances among sequences.

- Independent of reference catalog
- Scales quadratically with number of sequences
- OTU assignment depends on which other sequences are present

de novo OTU clustering

- results depend on clustering method
- single-linkage tends to be (too?) “inclusive” while average-linkage/complete-linkage are more “exclusive”

	700114607	700114380	700114716	700114798	Consensus	Lineage
OTU_97.4499	0	0	0	0		
OTU_97.44990	0	0	0	0		
OTU_97.44991	0	1	4	0		
OTU_97.44992	0	0	0	0		
OTU_97.44993	0	1	1	0		
OTU_97.4499					Root; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__	
OTU_97.44990					Root; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__	Odoribacter
OTU_97.44991					Root; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Propionibacteriaceae; g__	Propionibacterium
OTU_97.44992					Root; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Mycobacteriaceae; g__	Mycobacterium
OTU_97.44993					Root; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Campylobacteraceae; g__	Campylobacter



Which similarity threshold?

- Typical (but arbitrary) similarity threshold: 97% (for species level)
- This means different things depending on the clustering method that was used!

Representation in R - phyloseq object

```

> library(phyloseq)
> data(GlobalPatterns)
> GlobalPatterns
phyloseq-class experiment-level object
otu_table() OTU Table: [ 19216 taxa and 26 samples ]
sample_data() Sample Data: [ 26 samples by 7 sample variables ]
tax_table() Taxonomy Table: [ 19216 taxa by 7 taxonomic ranks ]
phy_tree() Phylogenetic Tree: [ 19216 tips and 19215 internal nodes ]
> head(otu_table(GlobalPatterns))
OTU Table: [6 taxa and 26 samples]
taxa are rows
CL3 CC1 SV1 M31FcsW M11FcsW M31Plmr M11Plmr F21Plmr M31Tong M11Tong LMEpi24M SLEpi20M AQC1cm AQC4cm
549322 0 0 0 0 0 0 0 0 0 0 0 0 1 27 100
522457 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2
951 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0
244423 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 22
586076 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2
246140 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1
AQC7cm NP2 NP3 NP5 TRRsed1 TRRsed2 TRRsed3 TS28 TS29 Even1 Even2 Even3
549322 130 1 0 0 0 0 0 0 0 0 0 0 0 0 0
522457 6 0 0 0 0 0 0 0 0 0 0 0 0 0 0
951 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
244423 29 0 0 0 0 0 0 0 0 0 0 0 0 0 0
586076 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0
246140 3 0 0 0 0 0 0 0 0 0 0 0 0 0 0

```

Representation in R - phyloseq object

```
> head(sample_data(GlobalPatterns))
```

Sample Data: [6 samples by 7 sample variables]:

	X.SampleID	Primer	Final_Barcodes	Barcode_truncated_plus_T	Barcode_full_length	SampleType
CL3	CL3	ILBC_01	AACGCA	TGCGTT	CTAGCGTGC GT	Soil
CC1	CC1	ILBC_02	AACTCG	CGAGTT	CATCGACGAGT	Soil
SV1	SV1	ILBC_03	AACTGT	ACAGTT	GTACGCACAGT	Soil
M31FcsW	M31FcsW	ILBC_04	AAGAGA	TCTCTT	TCGACATCTCT	Feces
M11FcsW	M11FcsW	ILBC_05	AAGCTG	CAGCTT	CGACTGCAGCT	Feces
M31Plmr	M31Plmr	ILBC_07	AATCGT	ACGATT	CGAGTCACGAT	Skin

Description

CL3	Calhoun South Carolina Pine soil, pH 4.9
CC1	Cedar Creek Minnesota, grassland, pH 6.1
SV1	Sevilleta new Mexico, desert scrub, pH 8.3
M31FcsW	M3, Day 1, fecal swab, whole body study
M11FcsW	M1, Day 1, fecal swab, whole body study
M31Plmr	M3, Day 1, right palm, whole body study

```
> head(tax_table(GlobalPatterns))
```

Taxonomy Table: [6 taxa by 7 taxonomic ranks]:

	Kingdom	Phylum	Class	Order	Family	Genus	Species
549322	"Archaea"	"Crenarchaeota"	"Thermoprotei"	NA	NA	NA	NA
522457	"Archaea"	"Crenarchaeota"	"Thermoprotei"	NA	NA	NA	NA
951	"Archaea"	"Crenarchaeota"	"Thermoprotei"	"Sulfolobales"	"Sulfolobaceae"	"Sulfolobus"	"Sulfolobusacidocaldarius"
244423	"Archaea"	"Crenarchaeota"	"Sd-NA"	NA	NA	NA	NA
586076	"Archaea"	"Crenarchaeota"	"Sd-NA"	NA	NA	NA	NA
246140	"Archaea"	"Crenarchaeota"	"Sd-NA"	NA	NA	NA	NA

Construct phyloseq object

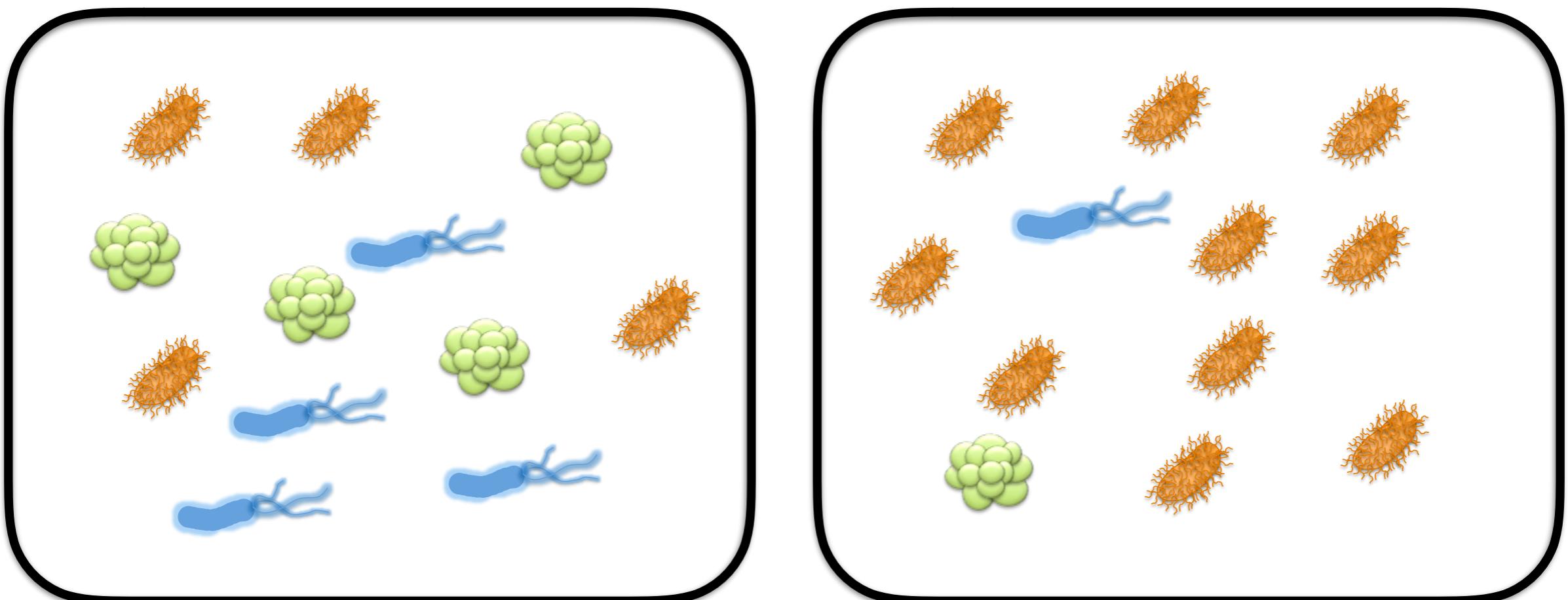
```
> otu_table[1:3, 1:5]
  700013549 700014386 700014403 700014409 700014412
OTU_97.1      0      0      0      0      0
OTU_97.10     0      0      0      0      0
OTU_97.100    0      0      0      0      0
>
> sample_info[1:3, ]
  RSID visitno sex RUNCENTER HMPbodyssite Mislabeled Contaminated
700013549 158013734 1 female BCM Stool NA NA
700014386 158398106 1 male   BCM,BI Stool NA NA
700014403 158398106 1 male   BCM,BI Saliva NA NA
                                         Description HMPbodyssite
700013549 HMP_Human_metagenome_sample_700013549_from_subject_158013734__sex_female_ Gastrointestinal tract
700014386 HMP_Human_metagenome_sample_700014386_from_subject_158398106__sex_male_ Gastrointestinal tract
700014403 HMP_Human_metagenome_sample_700014403_from_subject_158398106__sex_male_ Oral
>
> lineage2[1:3, ]
  Phylum           Class          Order          Family          Genus
OTU_97.1 "Firmicutes" "Bacilli" "Lactobacillales" "Streptococcaceae" "Streptococcus"
OTU_97.10 "Proteobacteria" "Betaproteobacteria" "Neisseriales" "Neisseriaceae" "Neisseria"
OTU_97.100 "Bacteroidetes" "Bacteroidia" "Bacteroidales" "Bacteroidaceae" "Bacteroides"
>
> phylo <- phyloseq(otu_table = otu_table(otu_table, taxa_are_rows = TRUE),
+                     sample_data = sample_data(sample_info),
+                     tax_table = tax_table(lineage2))
>
> phylo
phyloseq-class experiment-level object
otu_table() OTU Table: [ 45383 taxa and 4743 samples ]
sample_data() Sample Data: [ 4743 samples by 9 sample variables ]
tax_table() Taxonomy Table: [ 45383 taxa by 5 taxonomic ranks ]
```

Filtering and subsetting

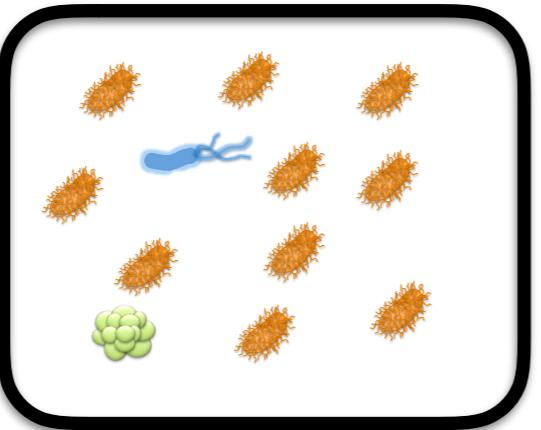
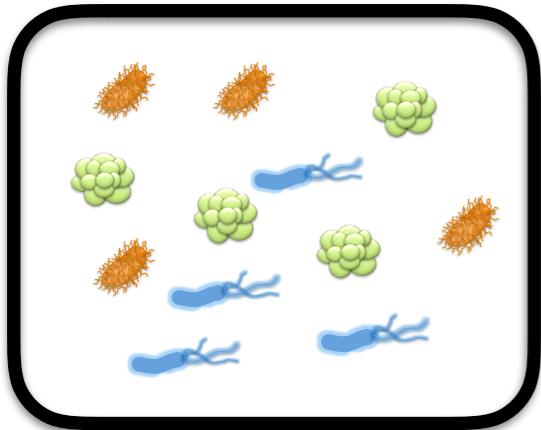
```
> phylo
phyloseq-class experiment-level object
otu_table() OTU Table: [ 45383 taxa and 4743 samples ]
sample_data() Sample Data: [ 4743 samples by 9 sample variables ]
tax_table() Taxonomy Table: [ 45383 taxa by 5 taxonomic ranks ]
>
> phylo <- prune_taxa(taxa_sums(phylo) > 0, phylo)
> phylo <- prune_samples(sample_sums(phylo) > 100, phylo)
>
> phylo
phyloseq-class experiment-level object
otu_table() OTU Table: [ 45369 taxa and 4586 samples ]
sample_data() Sample Data: [ 4586 samples by 9 sample variables ]
tax_table() Taxonomy Table: [ 45369 taxa by 5 taxonomic ranks ]
>
> phylosub <- subset_taxa(phylo, Phylum == "Acidobacteria")
>
> phylosub
phyloseq-class experiment-level object
otu_table() OTU Table: [ 26 taxa and 4586 samples ]
sample_data() Sample Data: [ 4586 samples by 9 sample variables ]
tax_table() Taxonomy Table: [ 26 taxa by 5 taxonomic ranks ]
```

Richness and alpha diversity

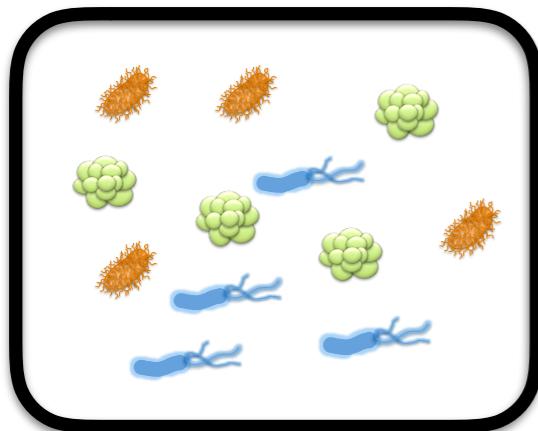
- richness = number of species observed in a sample
- alpha diversity ~ diversity (“unevenness”) of species abundances within a sample



Richness and alpha diversity



Richness and alpha diversity



> phx

OTU Table:

[3 taxa and 2 samples]

taxa are rows

	sample1	sample2
OTU1	4	10
OTU2	4	1
OTU3	4	1

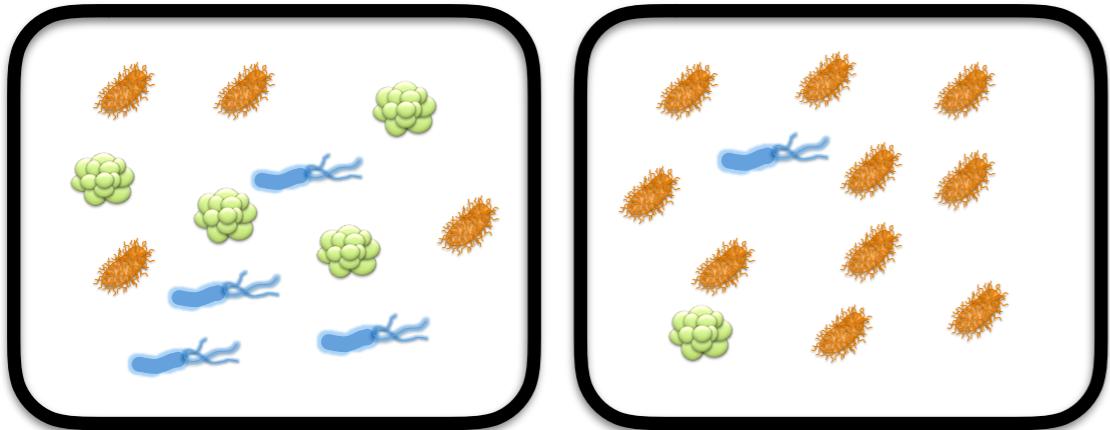
>

> estimate_richness(phx, measures = "Observed")

Observed

sample1	3
sample2	3

Richness and alpha diversity

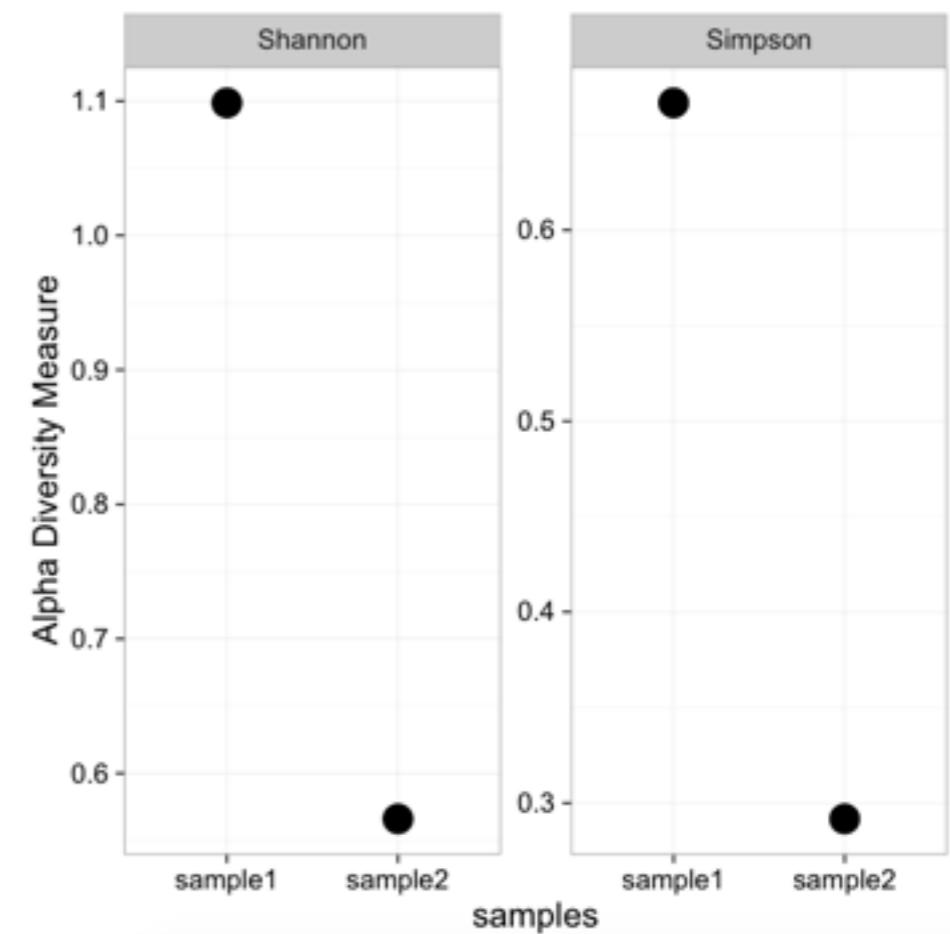


```
> estimate_richness(phx, measures = c("Shannon", "Simpson"))
  Shannon  Simpson
sample1 1.0986123 0.6666667
sample2 0.5660857 0.2916667
>
> plot_richness(phx, measures = c("Shannon", "Simpson")) +
+   theme_bw(base_size = 18) + geom_point(size = 7)
```

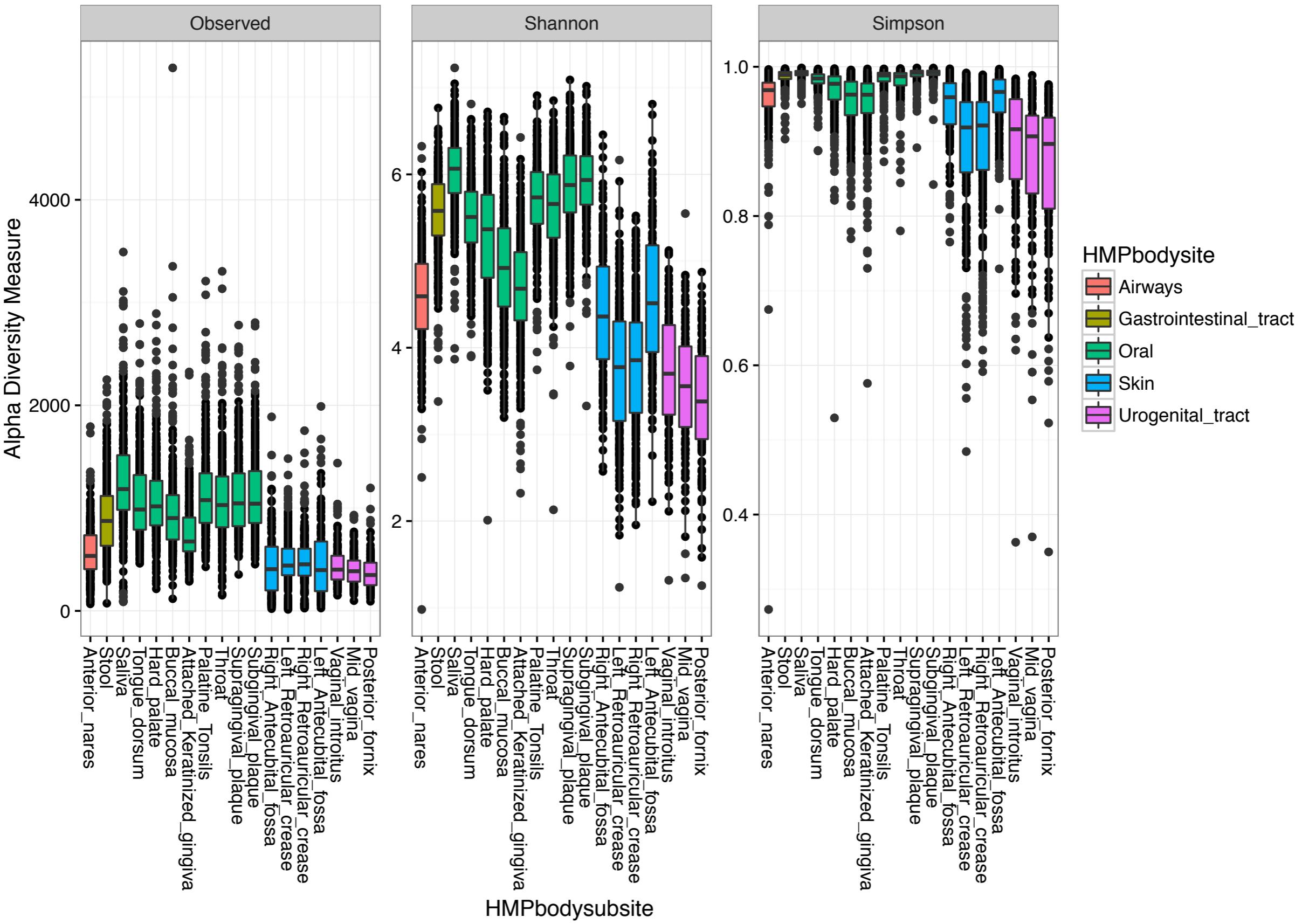
$$\text{Shannon: } H = - \sum_i p_i \log p_i$$

$$\text{Simpson: } D = 1 - \sum_i p_i^2$$

relative abundance of species i

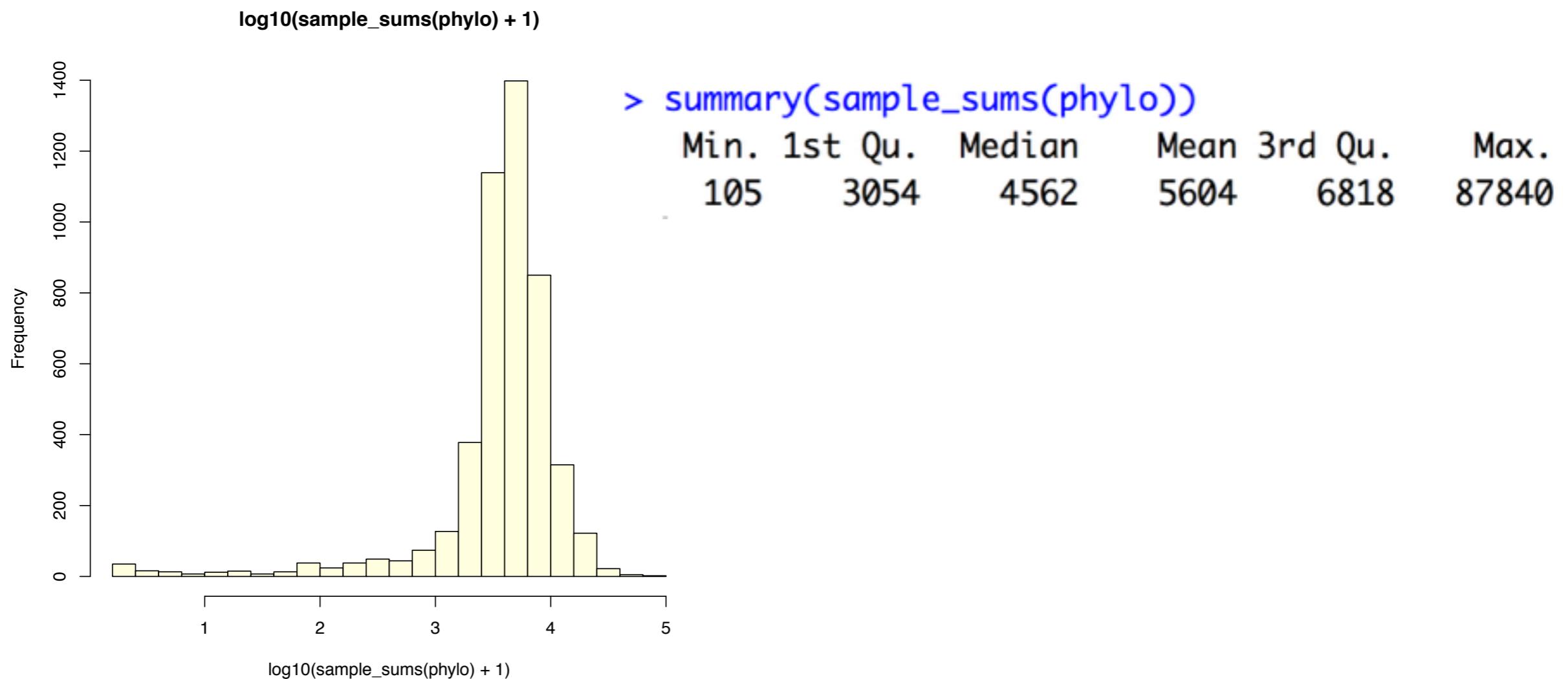


Richness and alpha diversity - HMP data



Normalization

- Library sizes vary greatly between samples

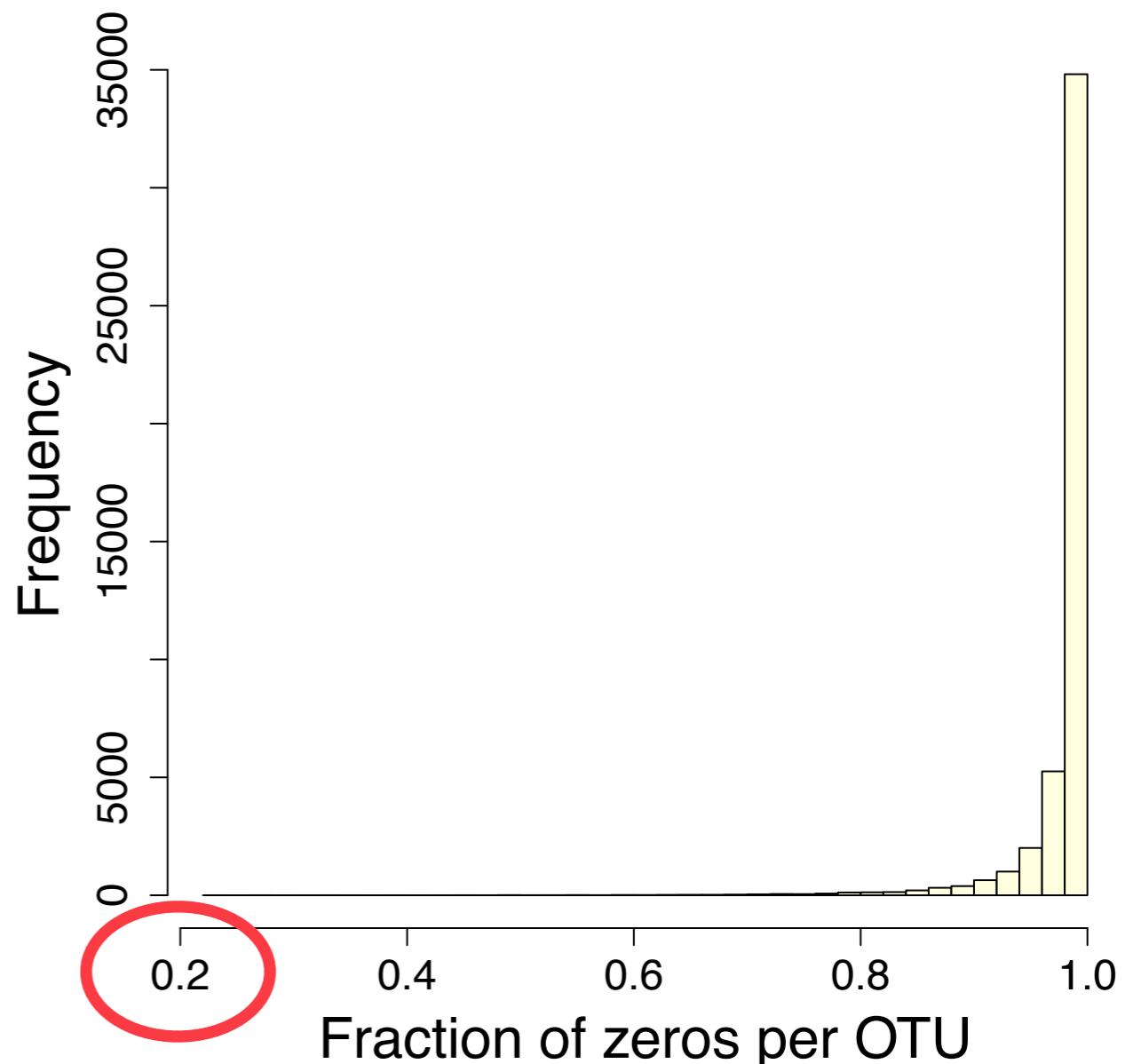


Normalization

- Library sizes vary greatly between samples
- OTU abundances are often normalized by **rarefying** (subsampling to equal sequencing depth across samples) or by representing them as **relative abundances**.
- Recent studies have suggested using scaling normalization (similar to RNA-seq).

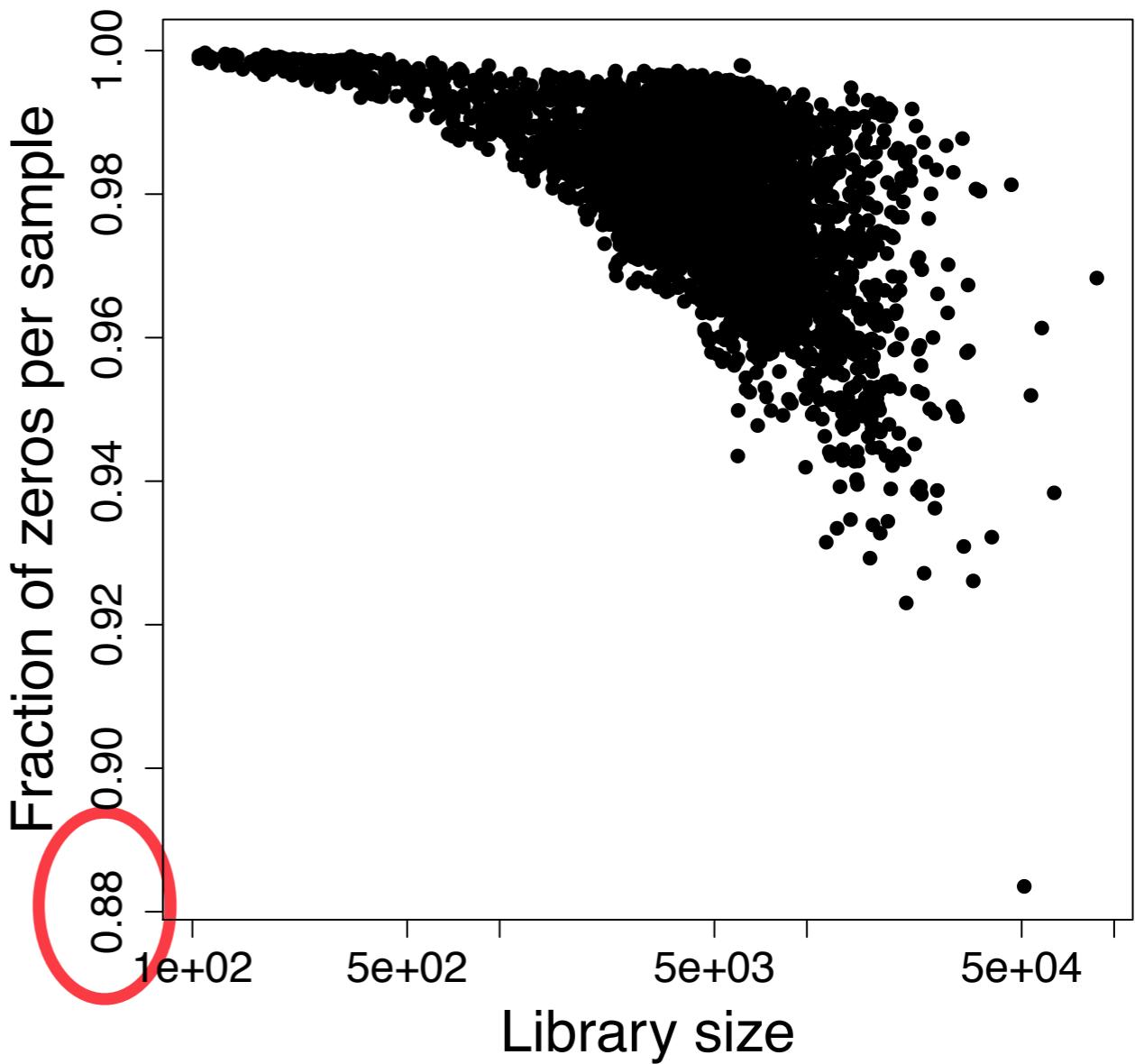
Scaling normalization - challenges

- Lots of zero counts!
- Assumption that “most things don’t change” across samples may not be valid.
- RNA-seq normalization methods require (e.g.) at least one OTU which is observed in all samples.



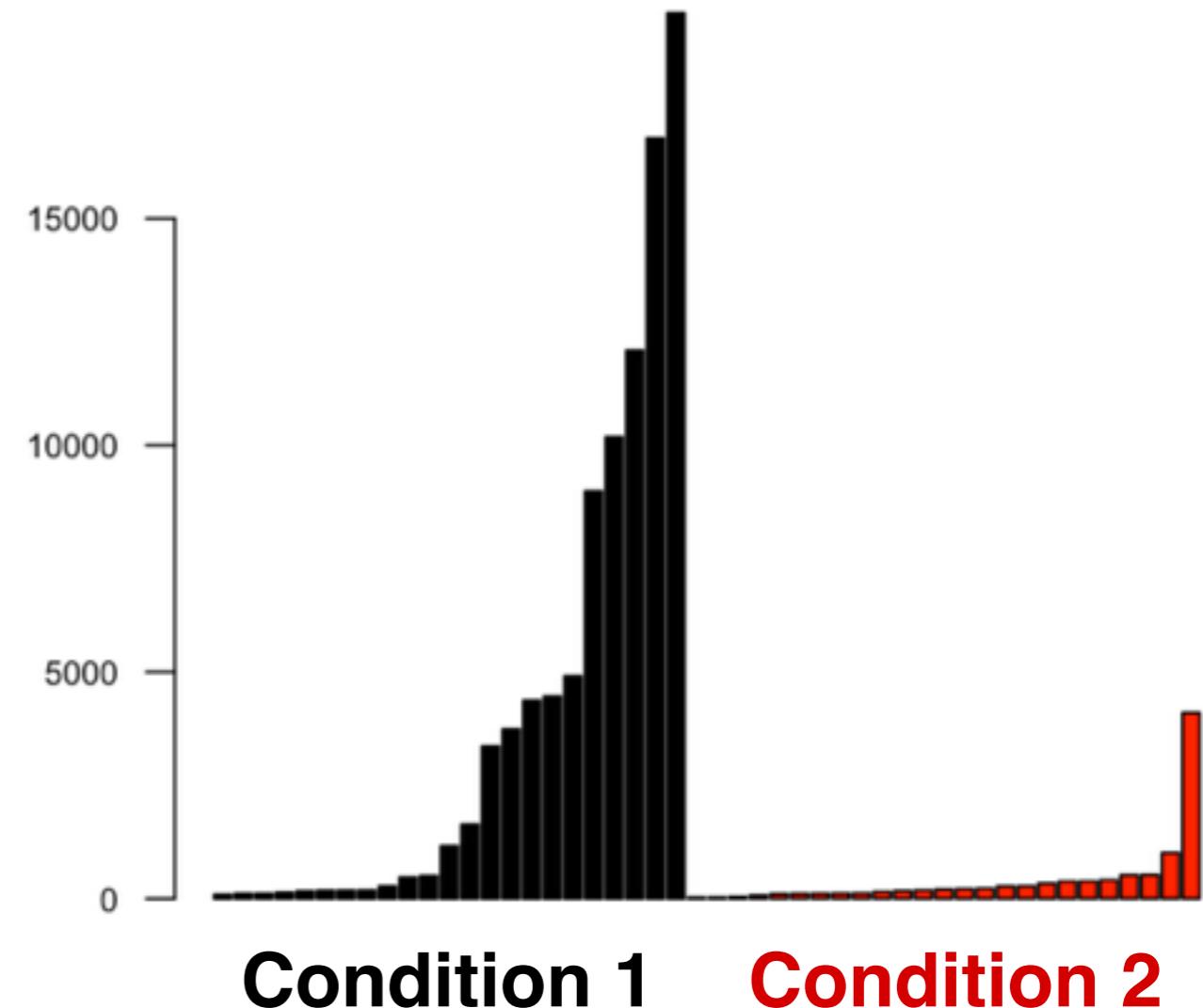
Scaling normalization - challenges

- Lots of zero counts!
- Many of them are likely due to undersampling - would be nonzero if the library size increased



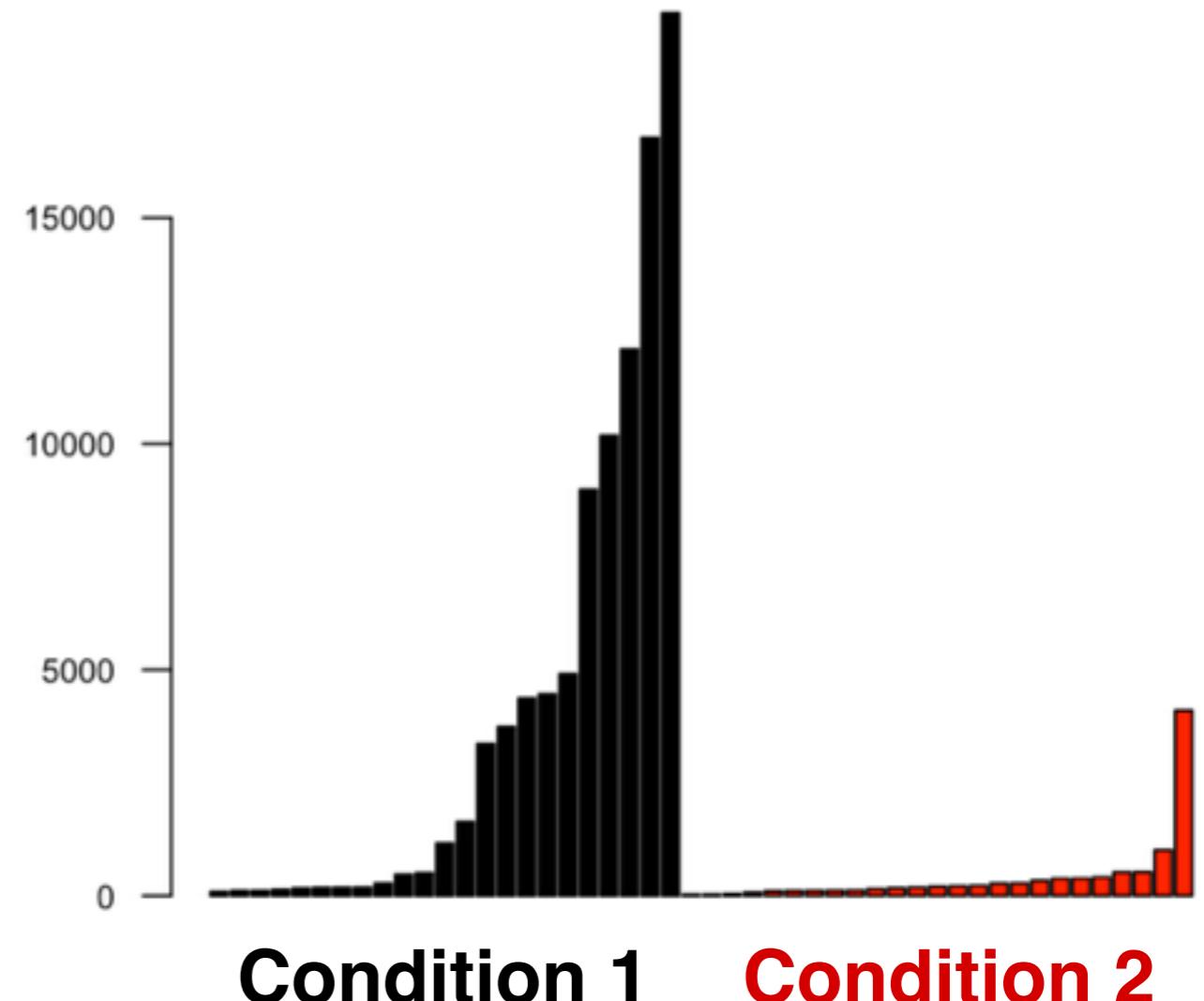
Differential abundance testing

- What do we want to test?
 - Difference between mean abundance
 - Difference in fraction of zeros
 - Difference between mean abundance conditioning on being present
 - Overall difference in OTU composition



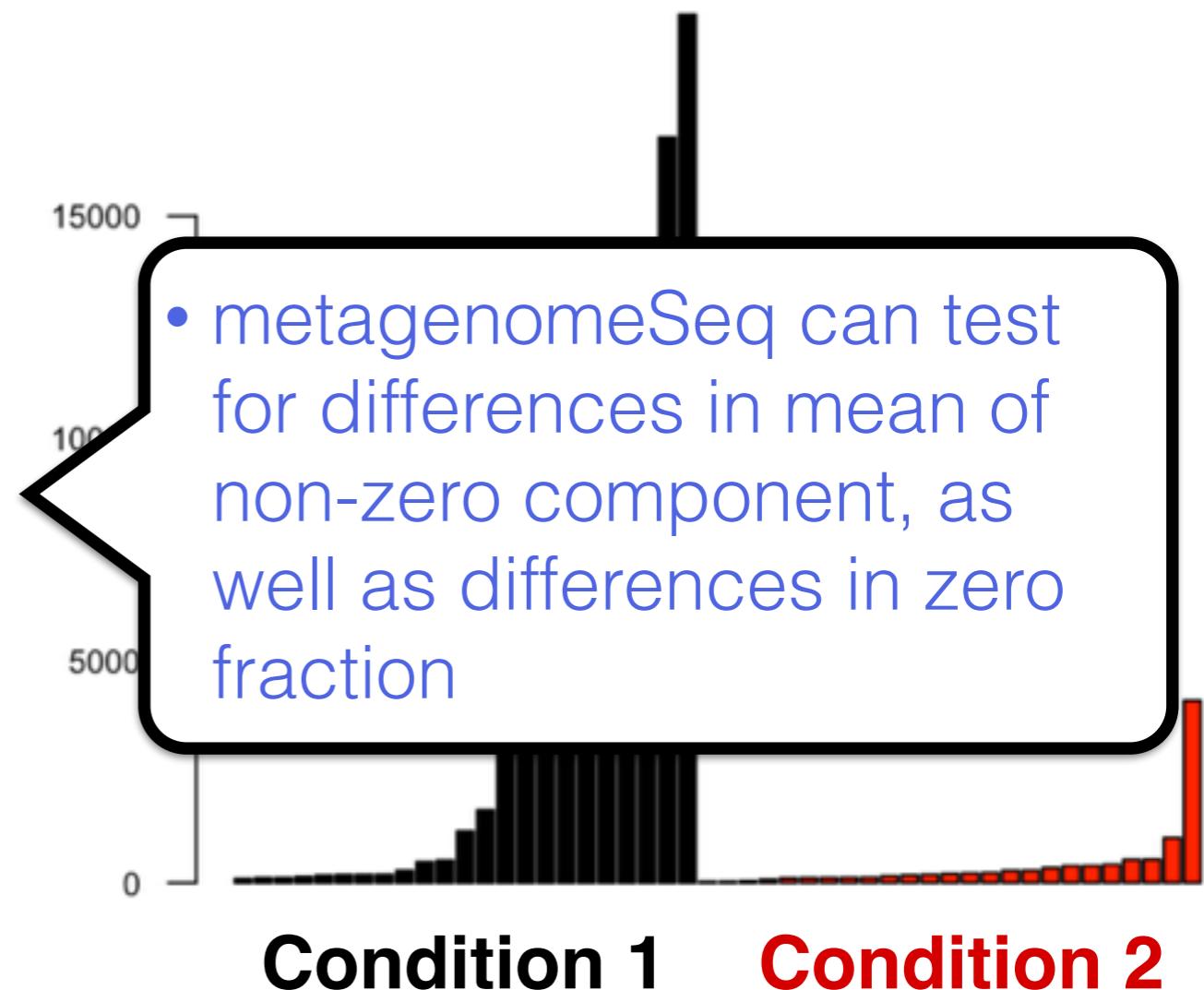
Differential abundance testing

- What do we want to test?
 - Difference between mean abundance
- RNA-seq methods like edgeR and DESeq2 can be used, but
 - be careful when comparing very different habitats
 - “outliers” may influence the results



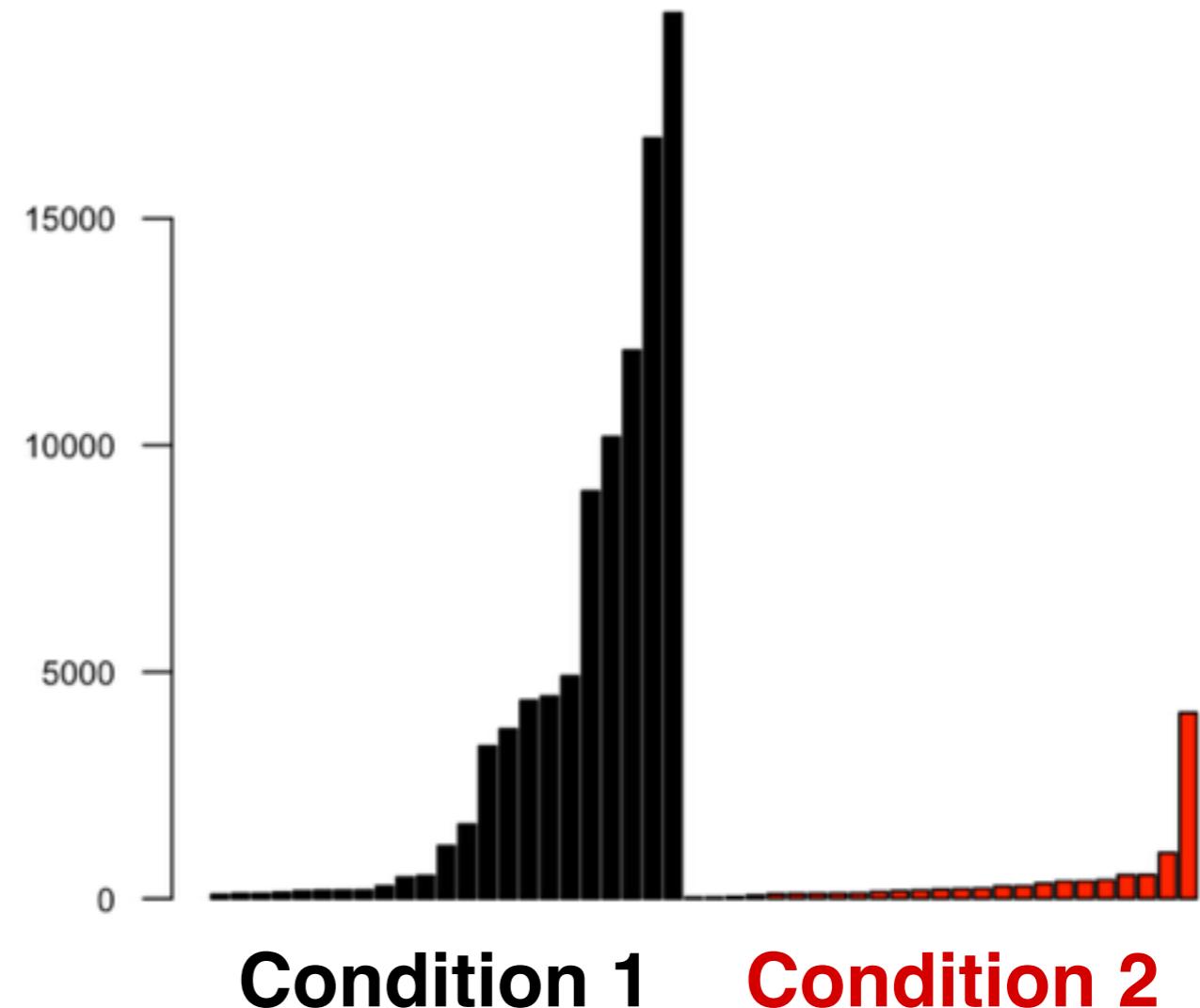
Differential abundance testing

- What do we want to test?
 - Difference between mean abundance
 - Difference in fraction of zeros
 - Difference between mean abundance conditioning on being present
 - Overall difference in OTU composition



Differential abundance testing

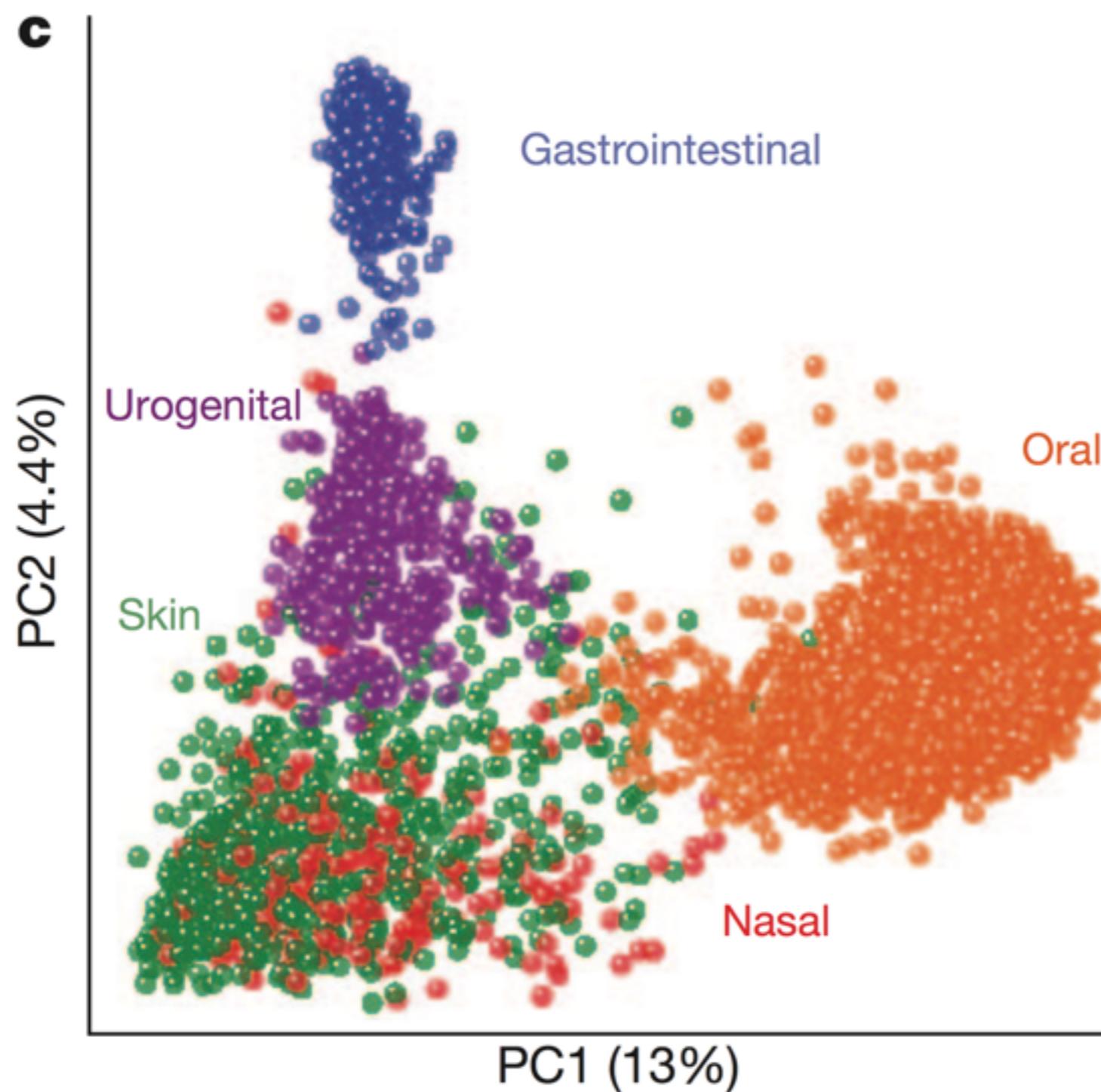
- What do we want to test?
 - Difference between mean abundance
 - ANCOM is a recent method for comparing composition of OTUs between conditions
 - Overall difference in OTU composition



Visualization/ordination

- Calculate pairwise distances between samples (a.k.a. **beta diversity**)
 - Based on presence/absence of OTUs
 - Incorporating abundances of OTUs
- Common distance measures: UniFrac, Bray-Curtis
- Generate low-dimensional representation that preserves these distances

Visualization/ordination



References

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