

Metacoding / Metagenomics

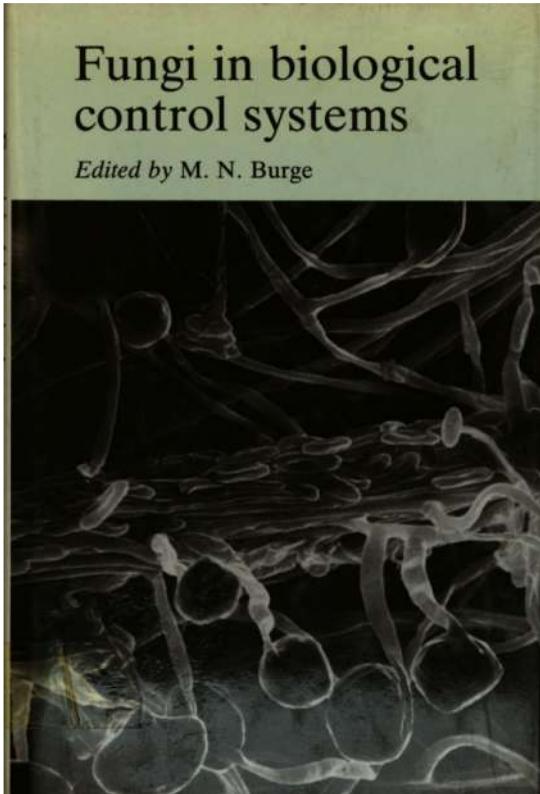
Isheng Jason Tsai

Introduction to NGS Data and Analysis
Lecture 10



What is the microbiome?

Fungi in Biological Control Systems (1988)



A convenient ecological framework in which to examine biocontrol systems is that of the microbiome. This may be defined as a characteristic microbial community occupying a reasonably well defined habitat which has distinct physico-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatres of activity. In relation to fungal diseases of crops and their control, major microbiomes are the phylloplane, spermosphere, rhizosphere and rhizoplane, and numerous kinds of plant residues persisting on or in the soil. Mention should also be made of the wood of standing or felled trees as microbiomes where biocontrol of forest diseases using fungi has been achieved. However, in most cases competitive interactions other than mycoparasitism seem to be of greater importance.

<http://microbe.net/2015/04/08/what-does-the-term-microbiome-mean-and-where-did-it-come-from-a-bit-of-a-surprise/>

And then what is the metagenome?

Crosstalk R245

Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products

Jo Handelsman¹, Michelle R Rondon¹, Sean F Brady², Jon Clardy² and Robert M Goodman¹

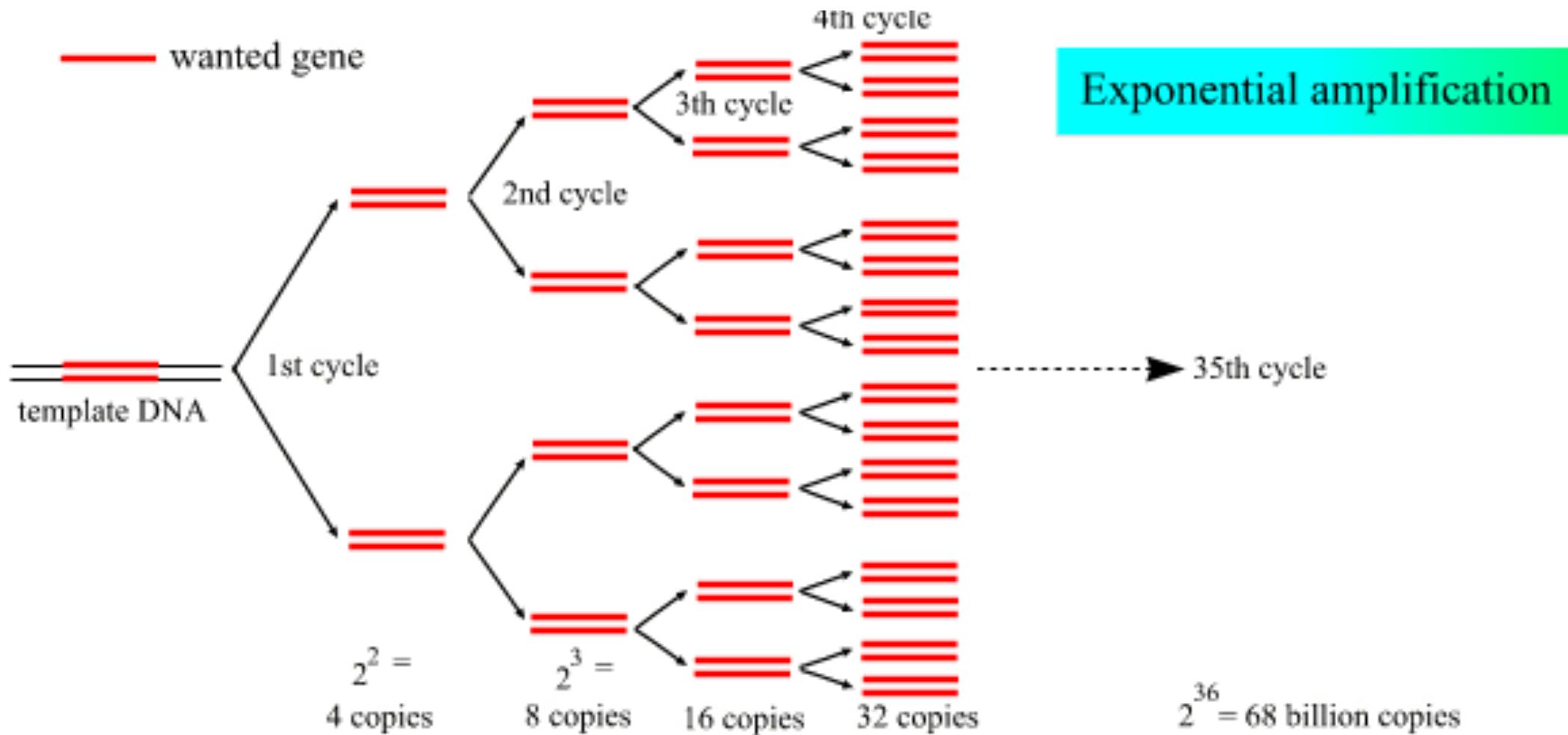


Chemistry & Biology October 1998, 5:R245–249
<http://biomednet.com/elecref/10745521005R0245>

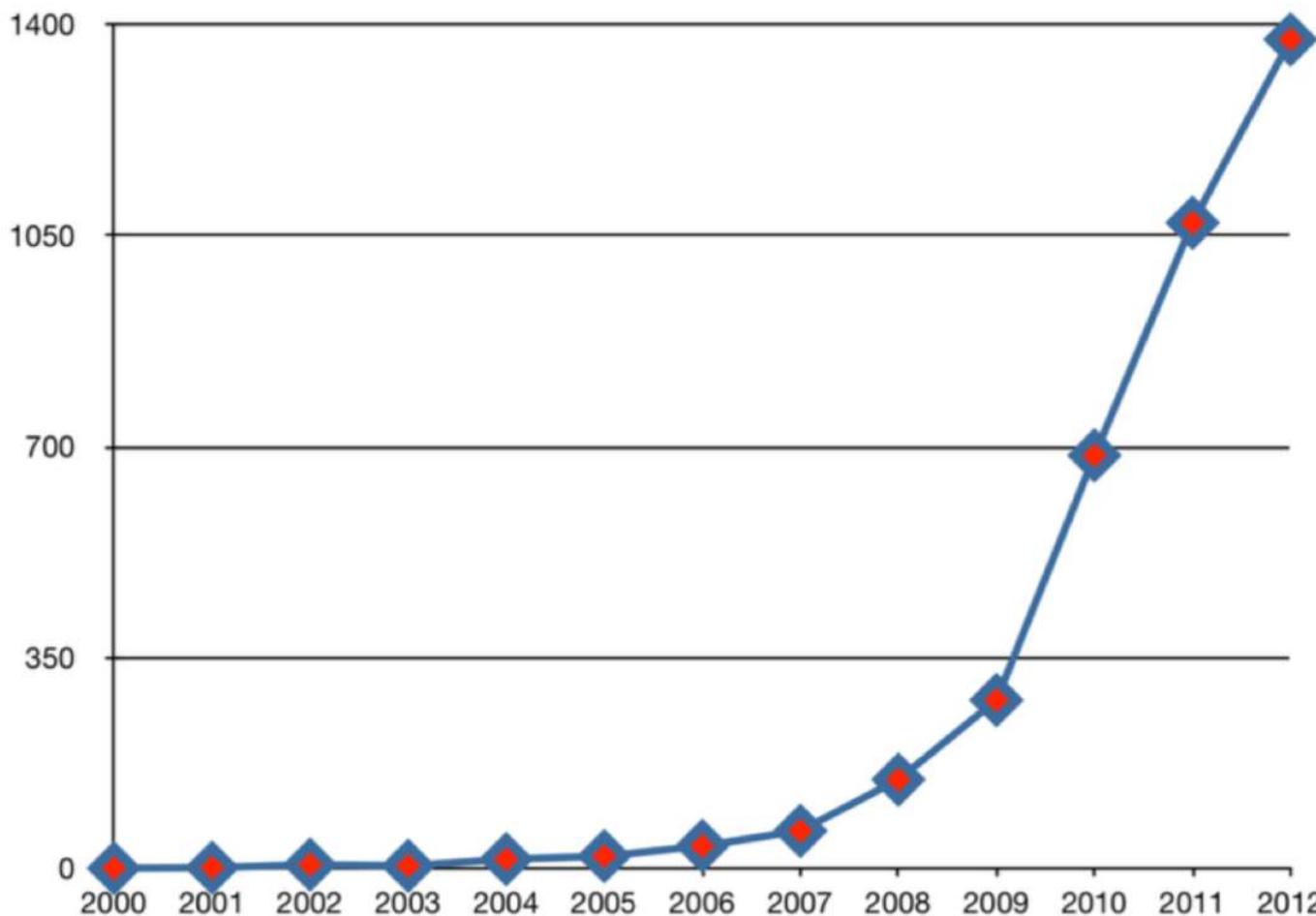
... This approach involves directly accessing the genomes of soil organisms that cannot be, or have not been, cultured by isolating their DNA

What is amplicon sequencing?

Anything that requires PCR-based amplification of a specific target gene (locus)



Pubmed hits for “Microbiome”



Metagenomics \neq Amplicon sequencing

Basic Purpose

Characteristics of (microbial) community

Who are they?

Where do they come from?

Are their similarities (at what level)

between communities

of different conditions

of similar conditions?

within a community?

What are they doing?

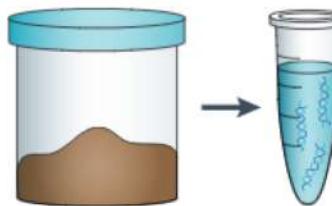
How are they doing?

Workflow

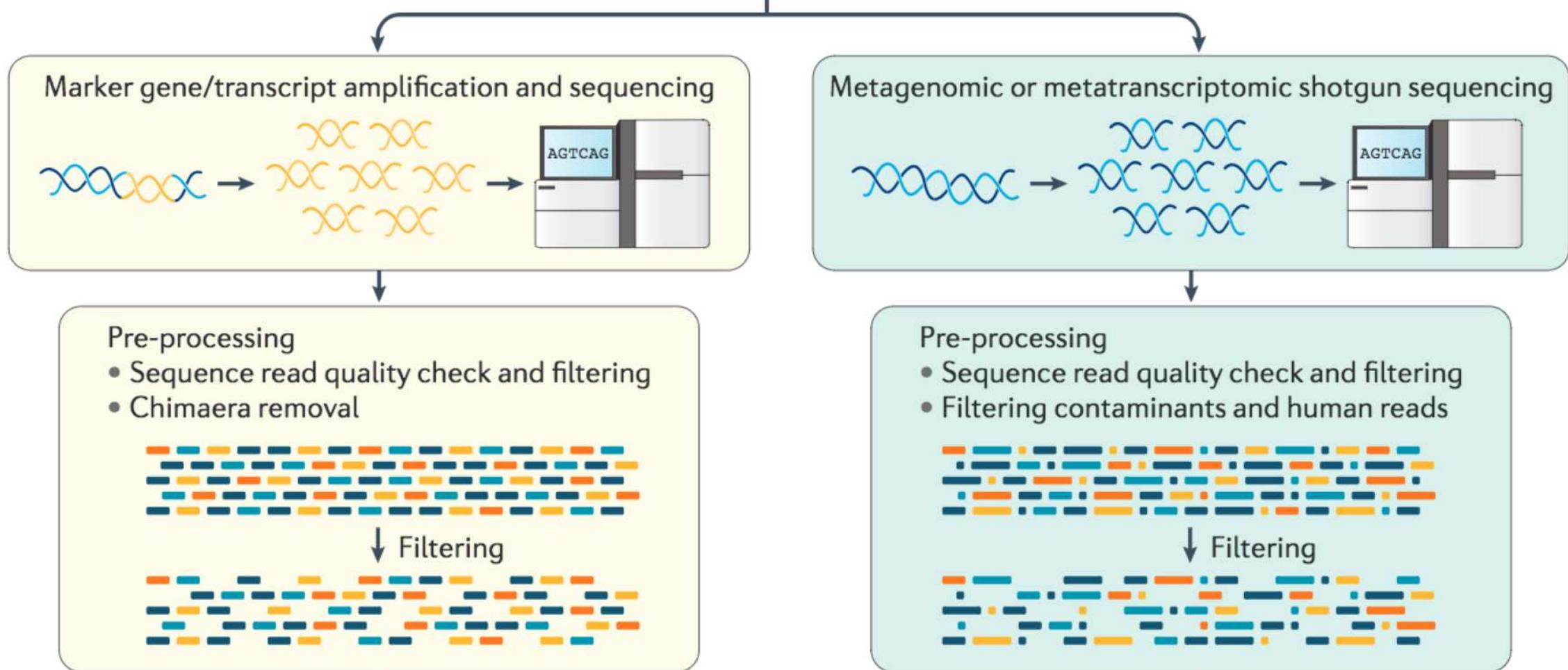
Study design	In the field	In the laboratory	At the keyboard
<p>Basic science or applied? (e.g., environmental biomonitoring)</p> <p>What is your study goal?</p> <ul style="list-style-type: none">• presence/absence• diversity assessment• absolute quantification <p>What taxa will you target?</p> <p>Is the scale of inference for your sample type appropriate to your question?</p> <p>Can you compare complementary data types? (e.g. traditional vs. eDNA)</p> <p>Does your sampling/replication scheme provide good statistical power?</p>	<p>What type of sample is needed? (water, soil, air)</p> <p>What metadata should you collect?</p> <p>How many replicates will you collect?</p> <p>Does your sampling protocol minimize/control for :</p> <ul style="list-style-type: none">• contamination (e.g., positive and negative controls)• any known biases (e.g., inhibitors, sample volume)	<p>Sample Handling Phase</p> <p>What extraction method? (physical vs. chemical)</p> <p>How much sample?</p> <p>What locus and primers?</p> <p>Do you need to generate reference sequence data?</p> <p>Are technical replicates needed?</p> <p>What library preparation method will you use?</p> <p>How many samples will you index and pool?</p> <p>What sequence depth is needed per sample ?</p> <p>What read length will you use?</p>	<p>DNA Processing Phase</p> <p>What sequencing platform will you use?</p> <p>Do you need paired-end sequencing?</p> <p>Have you included appropriate quality assurances? (e.g., mock community, qPCR, bioanalyser traces)</p> <p>Does your laboratory protocol minimize/control for:</p> <ul style="list-style-type: none">• contamination (e.g., positive and negative controls)• any known biases (e.g., primer bias, coverage, taxonomic resolution)

Useful terminology

Term	Definition	Examples	Reference
Biome	The world's major ecosystems, defined by temperature gradients in latitude and altitude, precipitation and seasonality.	Subtropical, Mediterranean, Polar	Walter and Box (1976)
Microbiome	An assemblage of microorganisms existing in or associated with a habitat; includes active and interacting member as well transient or inactive members.	Human microbiome, Earth microbiome, Lake Erie microbiome, Soil microbiome	Lederberg and McCray (2001)
Core microbiome	Requires qualifiers for locality and habitat of interest. Organisms common across microbiomes hypothesized to play a key role in ecosystem function within a habitat.	To be determined	Turnbaugh et al. (2007)
Habitat	The physical and chemical parameters of an environmental area that determine niche spaces.	Termite hindgut: physical structure, anaerobic conditions, acidity and cellulose availability permit Spirochaeta abundance. Siberian tundra: subarctic temperatures, short summers and low solar energy permit boreal forest predominance. Cornfield, Temperate forest, Permafrost, Tidal marsh, Mouse oral cavity New York City, Lake Michigan, Soil core from a pea field, Vagina of a human subject Algal mat, Biofilm, Dental plaque	Whittaker et al. (1973)
Ecosystem	The interactions and dynamics of physical, chemical and biological components of a locality.		Odum (1953)
Locality	A spatially defined environmental area.		Andrewartha and Birch (1984)
Microbial community	Microorganisms that are co-existing and interacting with flanking microbiome members and/or the environment.		Little et al. (2008)
Niche space	The activity range of a population along the physical and chemical dimensions of a habitat.		Hutchinson (1957)
Population	All the organisms belonging to the same species/operational taxonomic unit that live in a locality. For microbes, the species definition will vary by the genes of interest; a strain could be a population, as well as mutant and wild-type organisms.	Grizzly bear in Alaska Sulfolobus islandicus strain in a geothermal pool Wild-type Enterococcus faecalis	Waples and Gaggiotti (2006)
Connectivity	The amount or proportion of interactions within a system. Within a microbiome, this could include interactions among taxa (biotic interactions) or with environmental factors (abiotic) within the locality.	Quorum sensing, Predator-prey dynamics, Resource competition	Gardner and Ashby (1970)
Observation	The sampling unit, includes metadata of locality, habitat, time and/or experimental condition.	Meta-transcriptome of one location in an acid mine drainage site, collected at one time point. 16S tag-sequences from the right palm of one human subject.	
Persistent	Organisms that are consistently detected within a locality through time.	Pseudomonas aeruginosa in Cystic Fibrosis patients Firmicutes in infant guts	



Study design, sample collection, storage and DNA/cDNA/RNA extraction



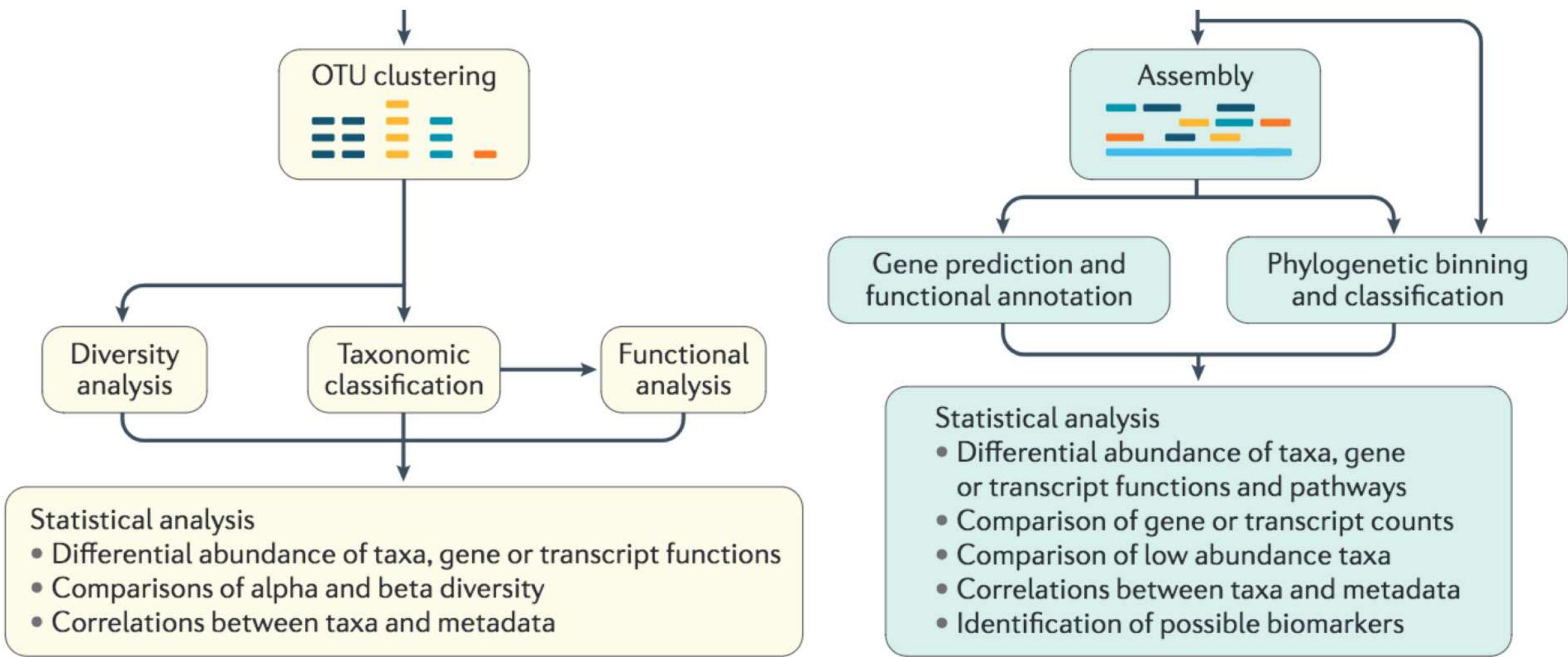


Figure 1 | Flowchart of the major steps involved in bioinformatic analysis of the microbiome. The analysis is divided into two sections depending on the type of sequencing. This schematic describes the basic steps and might vary depending on the aim of the analysis. OTU, operational taxonomic unit.

Applications

Exploration and categorisation



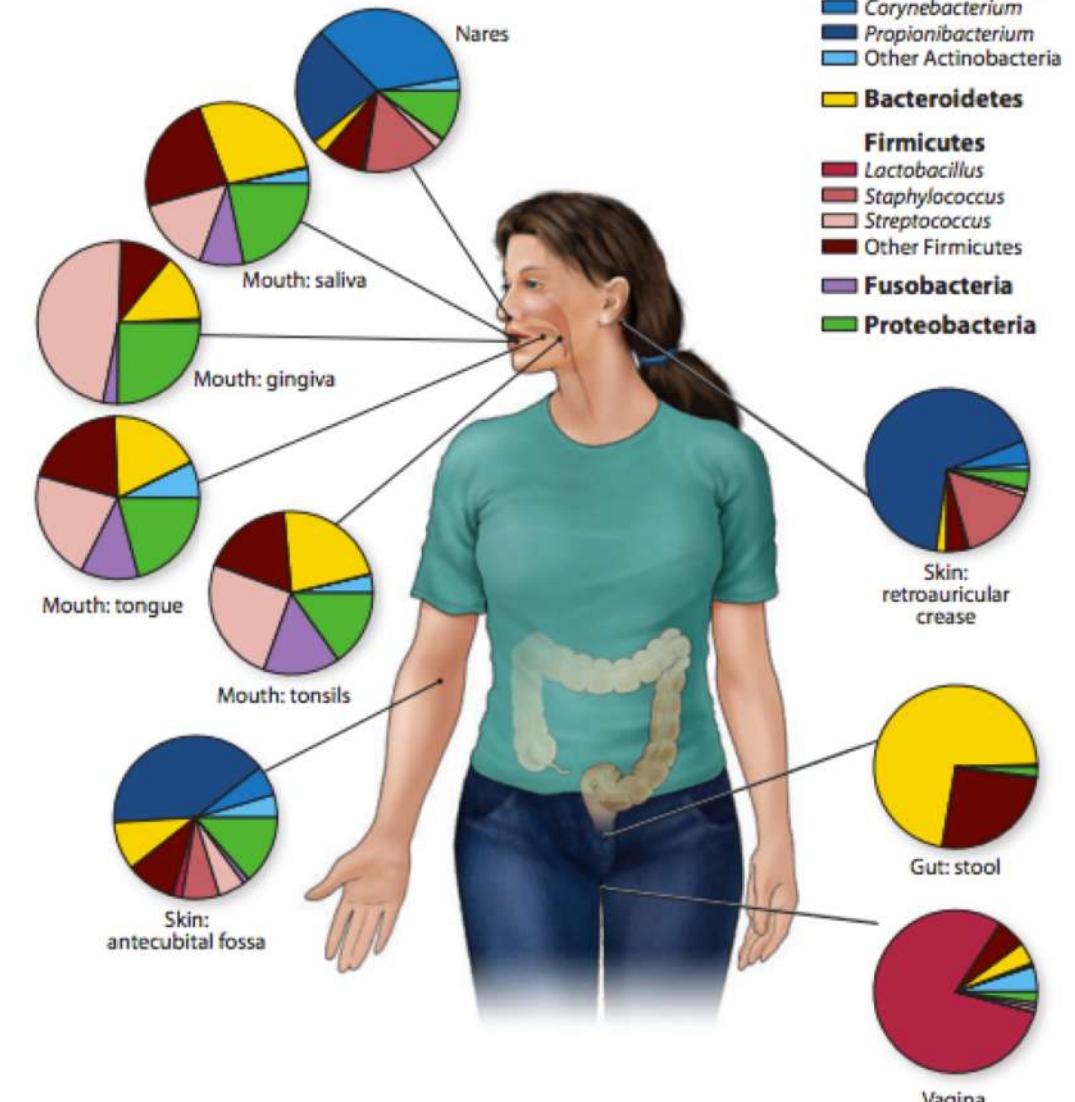
Rusch et al., 2007 Plos Biology

- 6.3 Gbp of sequence (2x Human genomes, 2000 x Bacterial genomes)
- Most sequences were novel compared to the databases



Qin et al., 2010 Nature

- 127 Human gut metagenomes
- 600 Gbp sequence (200 x Human genomes)
- 3.3 million genes identified
- Minimal gut metagenome defined



Grice and Segre (2012)

TARA STUDYING TAIWAN'S BIODIVERSITY

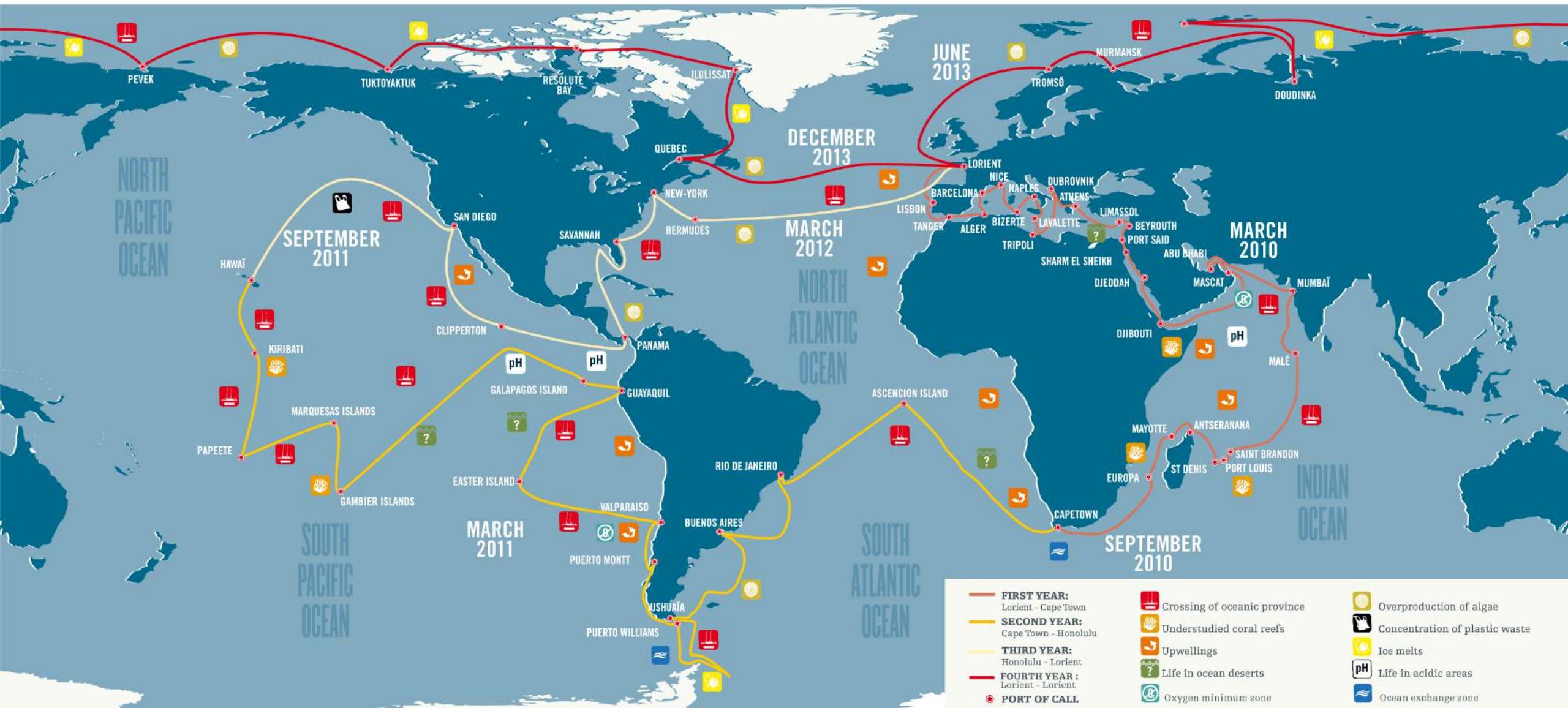


© Noémie Olive / Tara Expeditions Foundation

27 March 2018

For 4 days Tara successfully continued scientific research, then weighed anchor and left behind Orchid Island and Green Island, off the eastern coast of Taiwan.

Tara Pacific's 3 target species were found and collected on site. According to Emilie Boissin, scientific coordinator of this mission (CRIOBE), "*these sites are interesting because they represent the northern boundary of the distribution area for these tropical species*".



<https://oceans.taraexpeditions.org/wp-content/uploads/2014/05/TARAOCNEANS-CARTE.jpg>

TARA
length: 36 meters
width: 10 meters
height of mast: 27 meters

THE TARA OCEANS EXPEDITION 2009–2013

REVIVING THE TRADITION OF THE GREAT EXPEDITIONS OF THE 19TH CENTURY, TARA SAILED THE WORLD'S OCEANS FOR THREE AND A HALF YEARS.

FOR THE FIRST TIME, MARINE PLANKTON IN ITS ENTIRETY WAS COLLECTED AND STUDIED – FROM VIRUSES AND BACTERIA TO FISH LARVAE AND JELLYFISH.

WHY THIS EXPEDITION?

OXYGEN
CARBON

PLANKTON designates all the organisms drifting with the currents. These microscopic organisms are the foundation of the marine food chain, ensuring the survival of fish, marine mammals, and billions of humans being. They react quickly to climate changes and to ocean acidification. We must learn more about this complex, dynamic ecosystem and its role in global equilibrium.

CORAL REEFS are privileged places for aquatic biodiversity, but they are suffering from climate change, marine pollution, and over-fishing. Tara was the ideal platform for exploring 5 rarely-studied coral sites: Djibouti, Saint-Brandon, Mayotte, and the islands of Gambier and Kiribati.

A CONCENTRATION OF HIGH TECH

A unique space for microscopic imagery set up aboard Tara – the dry lab – where researchers characterize the organisms collected, their functional diversity and their complexity.

THE UNDERWATER VISION PROFILER observes plankton during sampling.

THE FLOWCAM is used to count and identify organisms as they pass through a laser beam at high speed.

VOYAGE OF THE SAMPLES

PORTS-OF-CALL: At stopovers every 6 to 8 weeks, the samples – conserved with liquid nitrogen, alcohol and fixatives – were sent to partner laboratories.

WORLD COURIER: This international specialist in shipping sensitive products expedited the precious samples collected aboard Tara to Heidelberg (Germany), then redistributed them to partner laboratories around the world.

FROM VIRUSES TO FISH LARVAE

TO SAMPLE ZOOPLANKTON: WE NEED 1 000 000 LITERS OF SEA WATER.

ZOOPLANKTON
1 TO 100,000 IN A LITER OF SEA WATER
Zooplankton consists of tiny animals, for example copepods, embryos and larvae, but also huge animals like jellyfish and siphonophores. They feed on living matter, bacteria, protists, or other multicellular organisms. Most zooplankton migrate either to the surface, or to great depths to feed and protect themselves from predators during the night.

BACTERIA
1 TO 10 BILLION IN A LITER OF SEA WATER
Bacteria are prokaryotes: cells without a nucleus. Certain species – the cyanobacteria – can perform photosynthesis. They are a food for protists and certain zooplankton. Bacteria are responsible for a wide array of metabolic functions in the ocean.

VIRUSES
10 TO 100 BILLION IN A LITER OF WATER
The marine virophere is immense, and includes the phages (viruses of bacteria) and giant viruses (giroviruses). Viruses play an essential role in recirculating living matter.
Size proportion of the micro-organisms are not reported in these drawings.

september 2009 – december 2013

60 STOPOVERS, 35 COUNTRIES
140,000 KILOMETERS AROUND THE WORLD

OCTOBER 2011
Tara crossed the "Plastic Continent" – a calm region where marine currents concentrate floating debris that accumulates in masses.

FEBRUARY 2010
Tara crossed the Gulf of Aden, a very dangerous region infested with pirates. Research was voluntarily interrupted for two weeks.

JANUARY 2011
Scientists collected samples during one month in Antarctic waters. This was the first Tara Oceans mission in a polar region.

PATNER LABORATORIES
23 LABS AND SCIENTIFIC INSTITUTIONS

- 8 in France
- 5 in the United States
- 2 in Germany
- 2 in Italy
- 1 in Belgium
- 1 in Ireland
- 1 in Spain
- 1 in Canada
- 1 in Saudi Arabia
- 1 in Russia

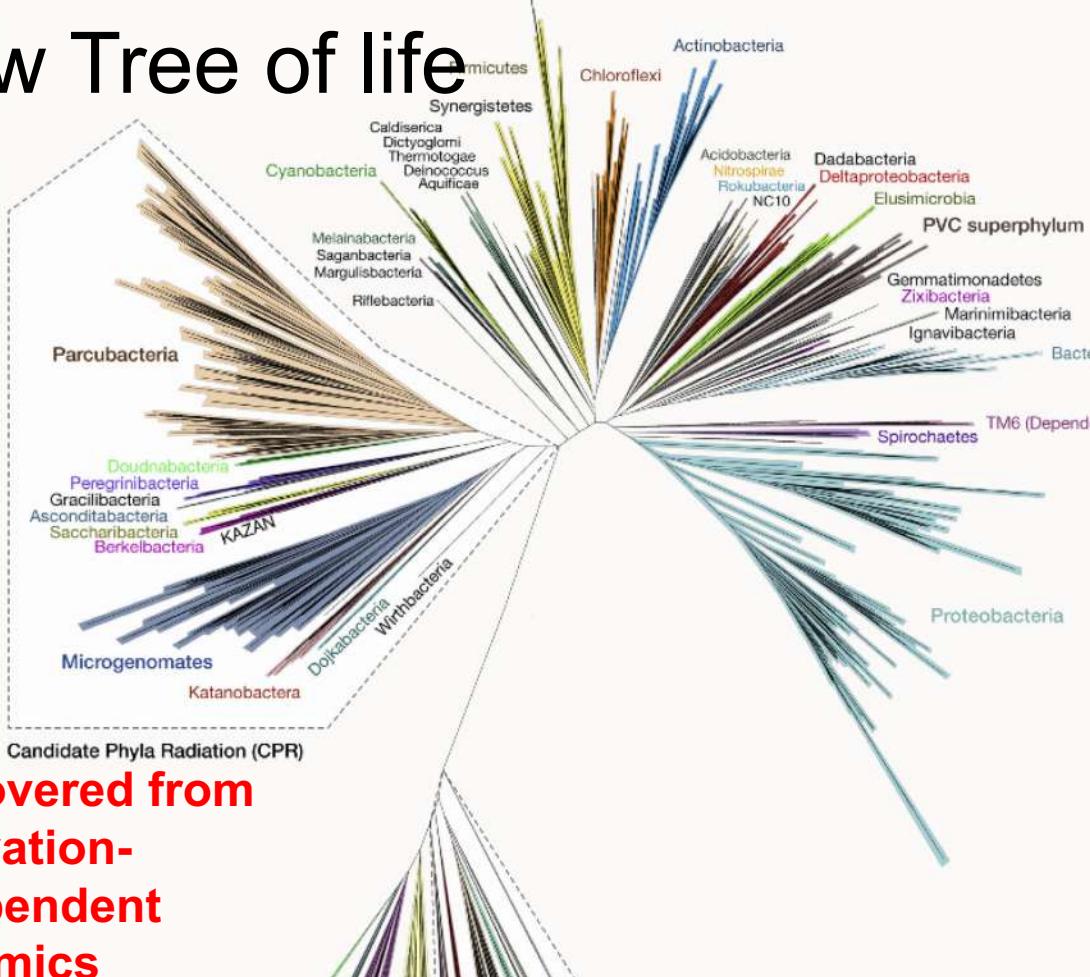
THE "TARANAUTES"
TAKING TURNS ON BOARD:

- 90 crew members, artists, and journalists
- 160 researchers
- 40 nationalities

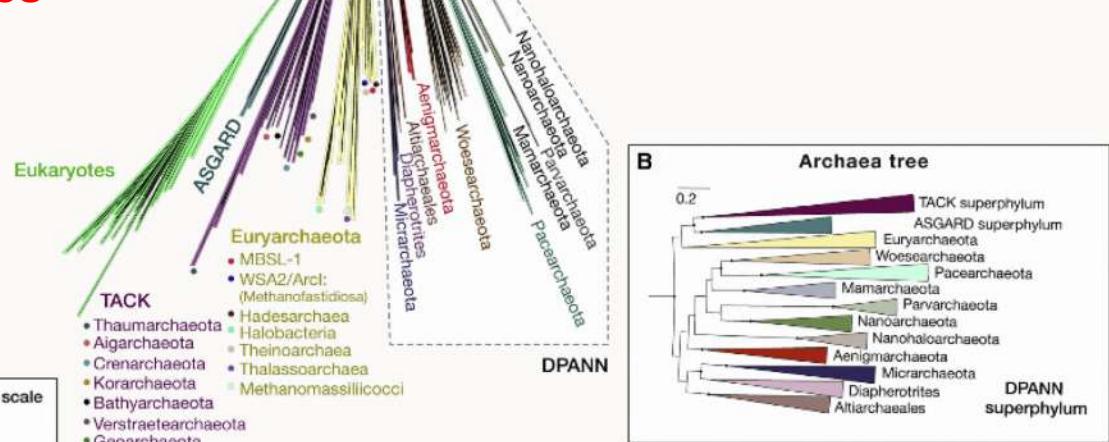
"Dynamars" (an investment of BNP Paribas project) aims to promote rational and sustainable use of marine plankton, one of the planet's most important reservoirs in terms of biodiversity, bio-resources, and global ecological change.

WEBSITE WWW.TARAEXPEDITIONS.ORG

New Tree of life



Discovered from
cultivation-
independent
genomics



Leading Edge Perspective

Major New Microbial Groups Expand Diversity and Alter our Understanding of the Tree of Life

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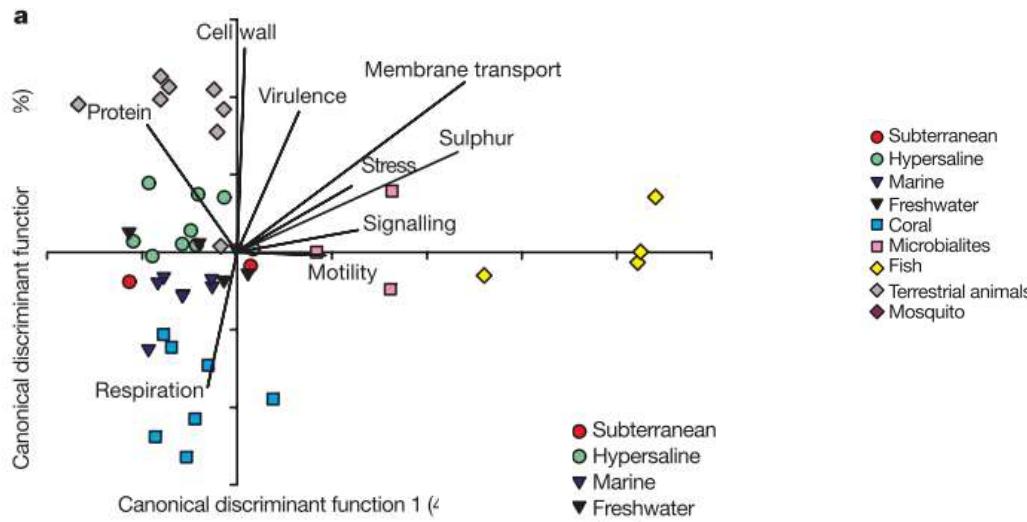
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<https://doi.org/10.1016/j.cell.2018.02.016>

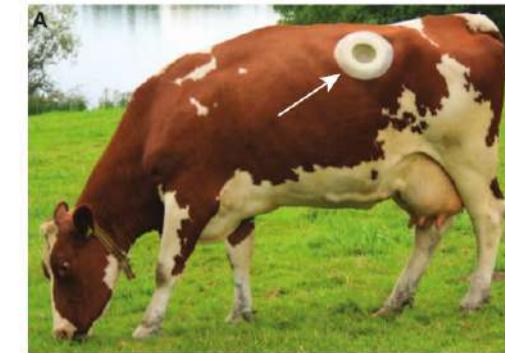
Comparative



Dinsdale *et al.*, 2008 **Nature**

- A characteristic microbial fingerprint for each of the nine different ecosystem types

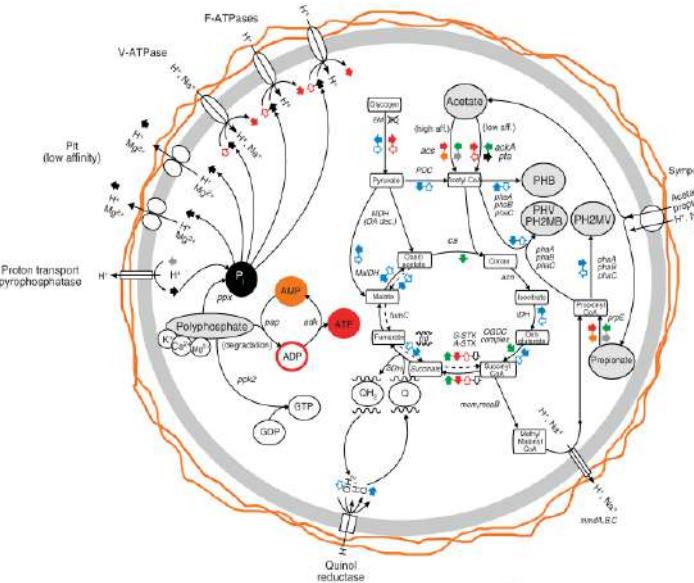
Specific functions



Hess *et al.*, 2011 **Science**

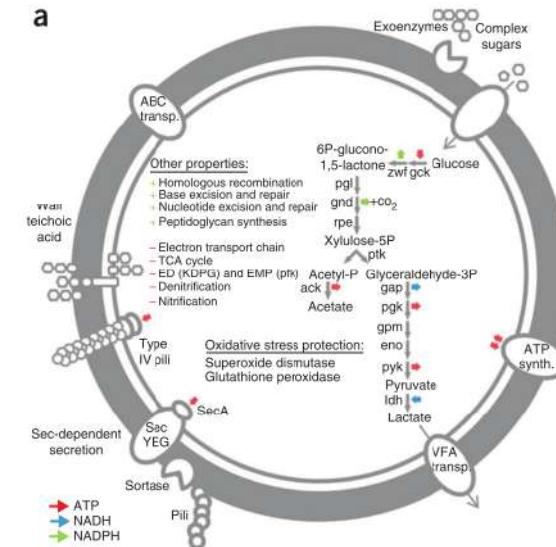
- Identified 27,755 putative carbohydrate-active genes from a cow rumen metagenome
- Expressed 90 candidates of which 57% had enzymatic activity against cellulosic substrates

Extracting genomes



Garcia Martin et al., 2006 **Nat. Biotechnol.** Albertsen et al., 2013 **Nat. Biotechnol.**

- Genome extraction from low complexity metagenome
- *Candidatus Accumulibacter phosphatis*
- The first genome of a polyphosphate accumulating organism (PAO) with a major role en enhanced biological phosphorus removal



- Genome extraction of low abundant species (< 0.1%) from metagenomes
- First complete TM7 genome
- Access to genomes of the "uncultured majority"



FIGURE 4 Opportunities and challenges of using environmental DNA as a tool for assessing community structure in different fields of study. The tool is reliant on a foundation (blue half circle) of continued research to improve technological aspects and continued development of DNA-based reference libraries for the identification of sequences found in the environment

Concept: OTU (Operational Taxonomic Unit)

OTU for Ecology

Operational Taxonomic Unit: a grouping of similar sequences that can be treated as a single “species”

Strengths

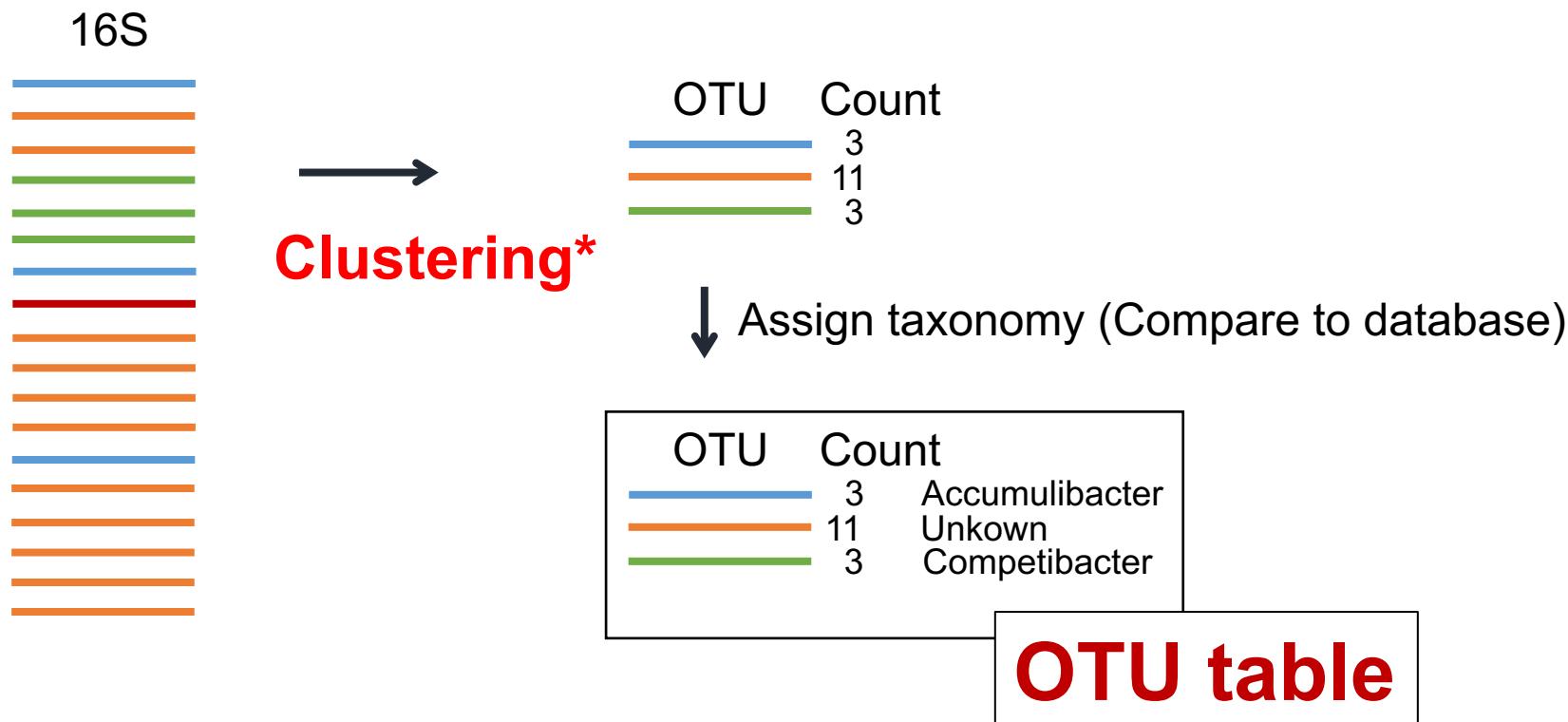
- Conceptually simple
- Mask effect of poor quality data
 - Sequencing error
 - in vitro recombination

Weaknesses

- Limited resolution
- Logically inconsistent definition

Assign OTU

- Cluster by their similarity to other sequences in the sample (operations taxonomic units → OTU)
- 95% genus level, **97% species level**, 99% strain level



OTU “picking”

The process of bin sequences into clusters of OTUs.

De Novo

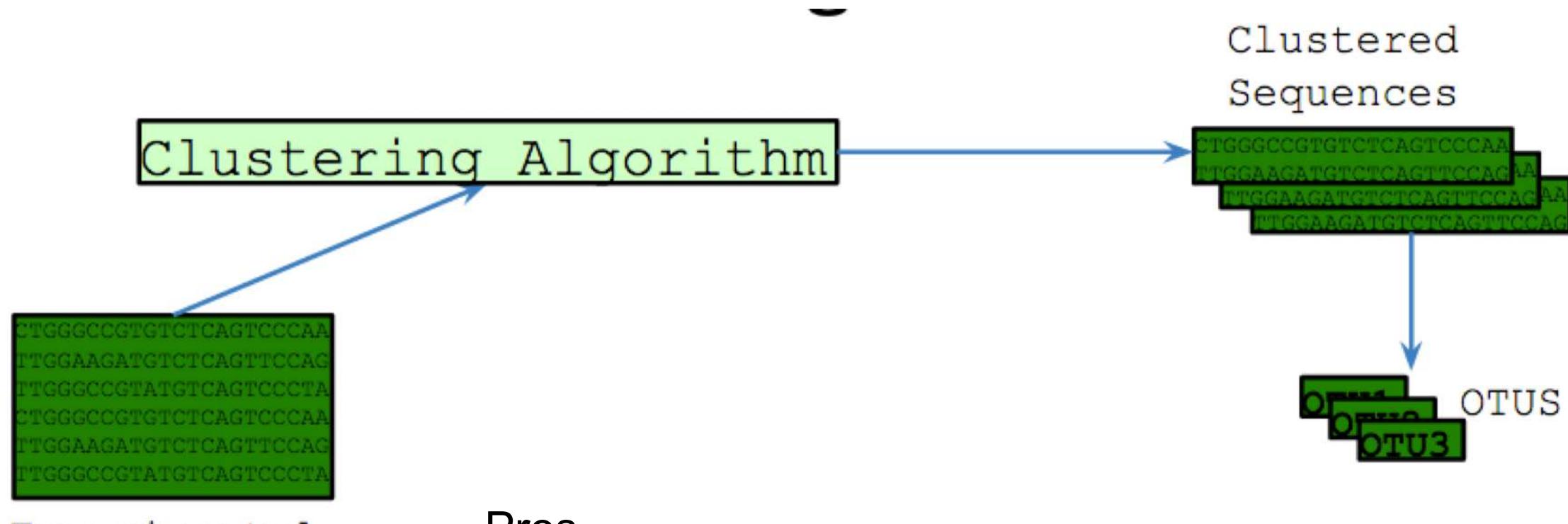
Reads are clustered based on similarity to one another.

Reference-based

Closed reference: any reads which don't hit a reference sequence are discarded

Open reference: any reads which don't hit a reference sequence are clustered de novo

De novo OTU picking



Experimental Sequences

Pros

All reads are clustered

Cons

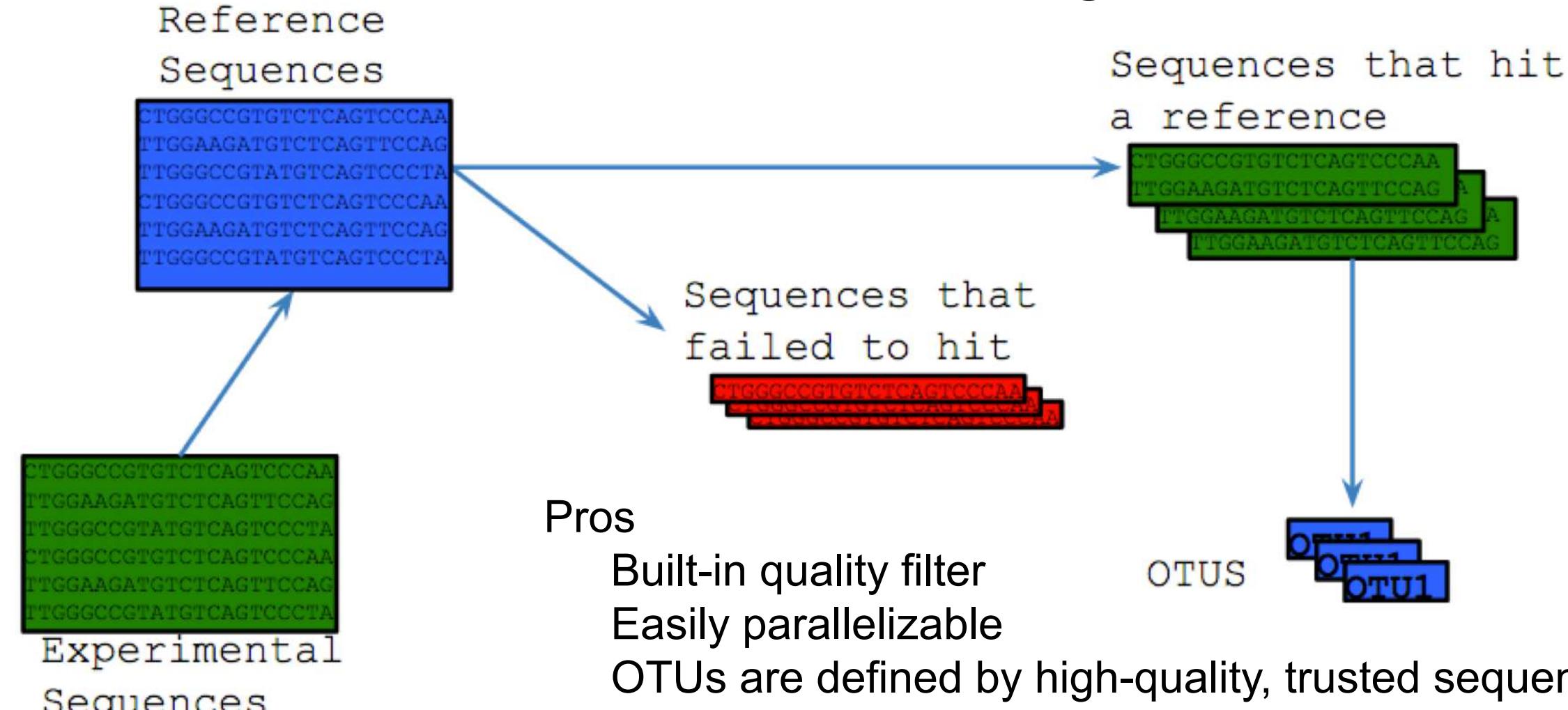
Not parallelizable

OTUs may be defined by erroneous reads

De novo OTU picking

- You **must** use if:
 - You do not have a reference sequence collection to cluster against, for example because you're working with an infrequently used marker gene.
- You **cannot** use if:
 - You are comparing non-overlapping amplicons, such as the V2 and the V4 regions of the 16S rRNA.
 - You are working with very large data sets, like a full HiSeq 2000 run.
(Technically you can, but it will be *really* slow.)

Closed-reference OTU picking



Pros

Built-in quality filter

Easily parallelizable

OTUs are defined by high-quality, trusted sequences

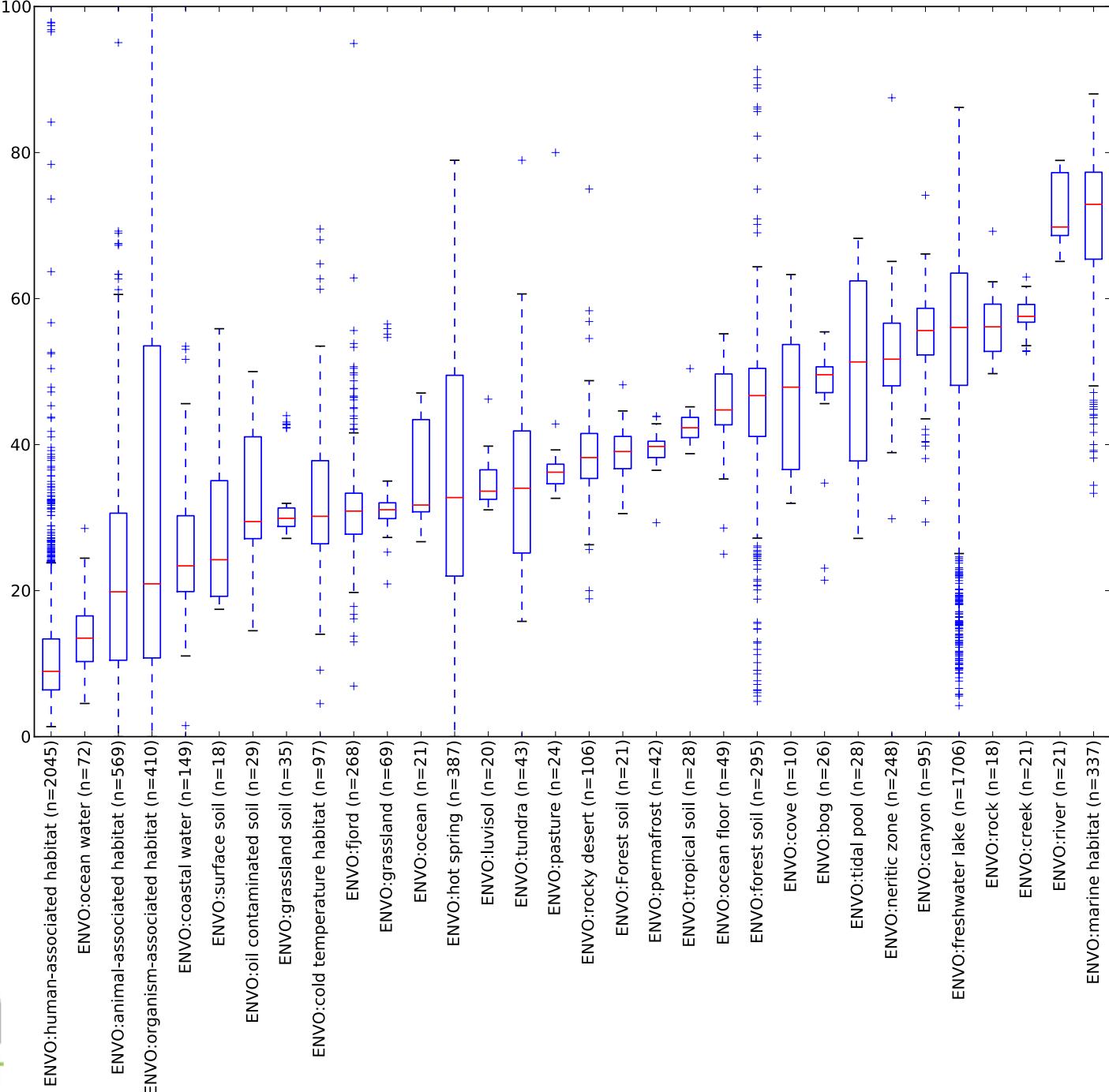
Cons

Reads that don't hit reference dataset are excluded, so you can never observe new OTUs

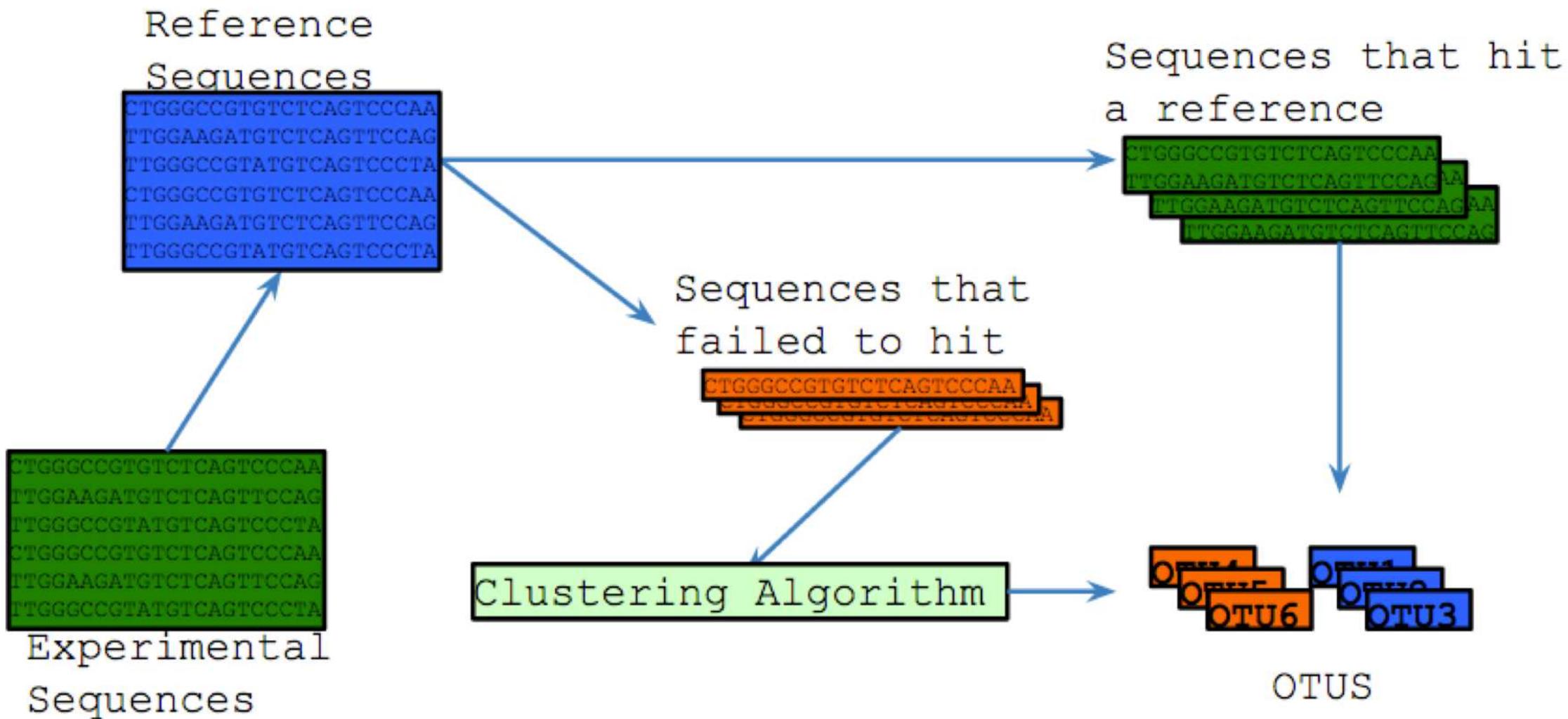
Closed-reference OTU picking

- You **must** use if:
 - You are comparing non-overlapping amplicons, such as the V2 and the V4 regions of the 16S rRNA. Your reference sequences must span both of the regions being sequenced.
- You **cannot** use if:
 - You do not have a reference sequence collection to cluster against, for example because you're working with an infrequently used marker gene.

Percentage of
reads that do not hit
the reference
collection, by
environment type.



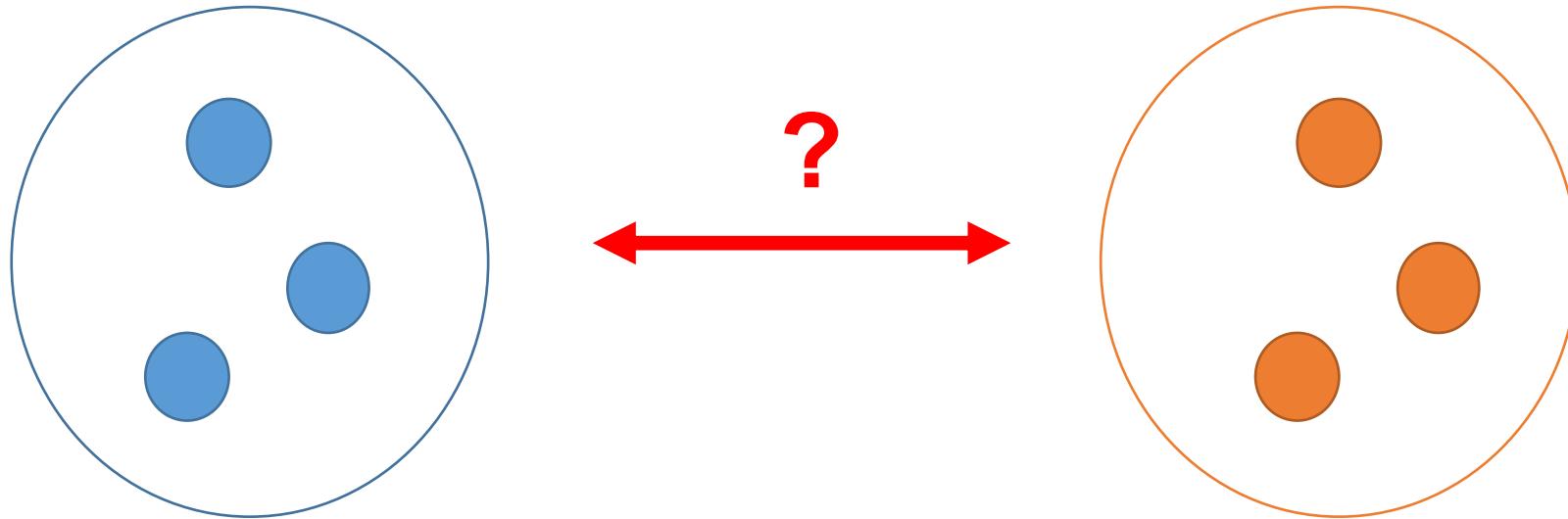
Open-reference OTU picking



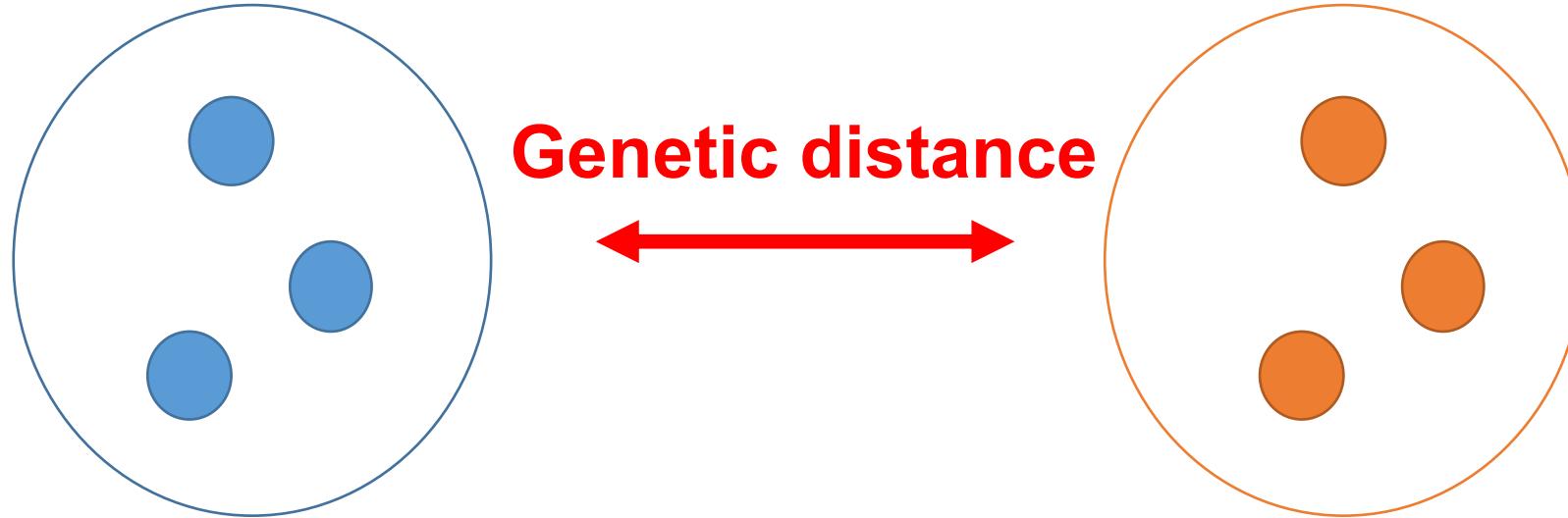
Open-reference OTU picking

- Pros
 - All reads are clustered
 - Partially parallelizable
- Cons
 - Only *partially* parallelizable
 - Mix of high quality sequences defining OTUs (i.e., the database sequences) and possible low quality sequences defining OTUs (i.e., the sequencing reads)

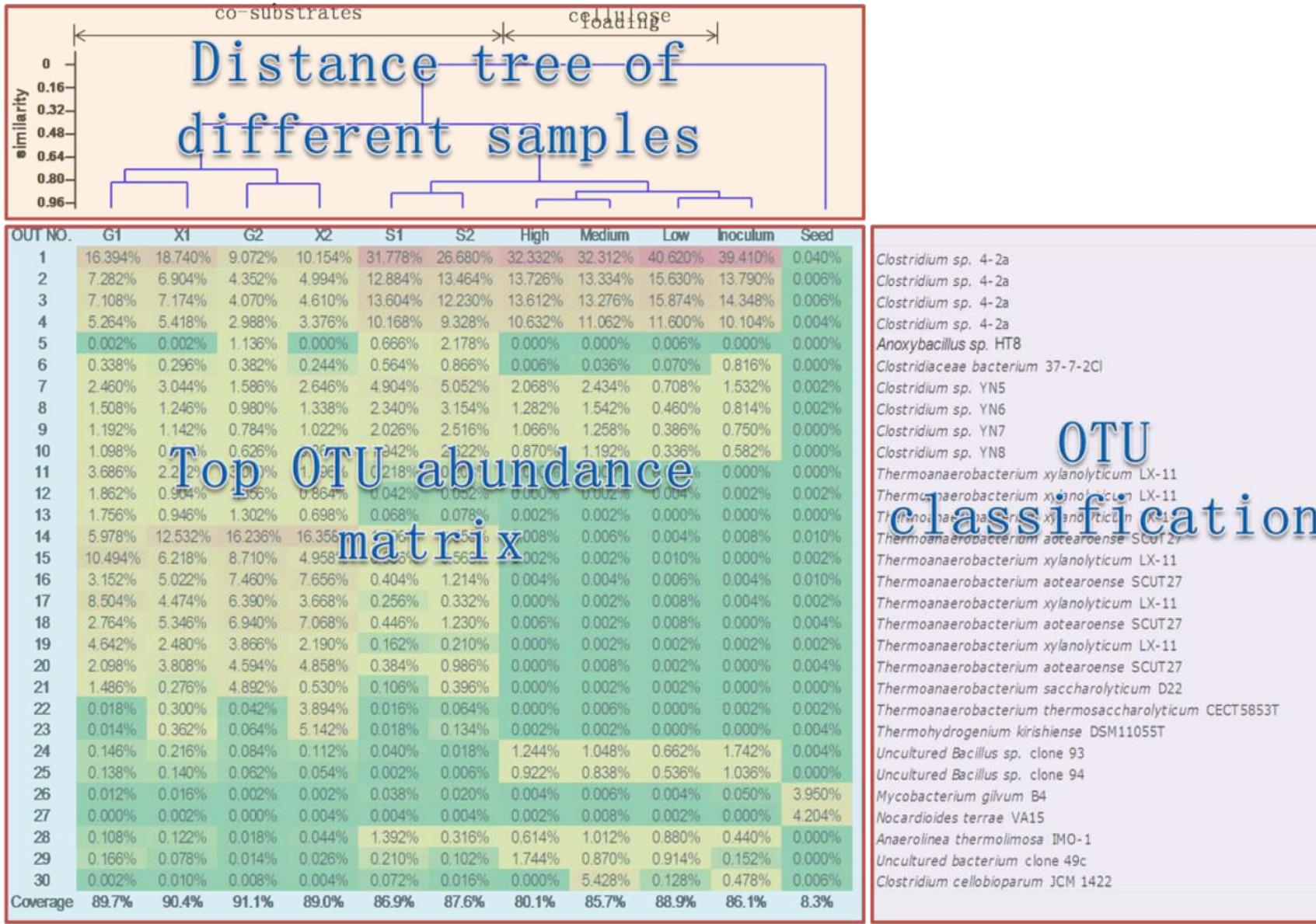
Assigned OTUs -> Loss of information



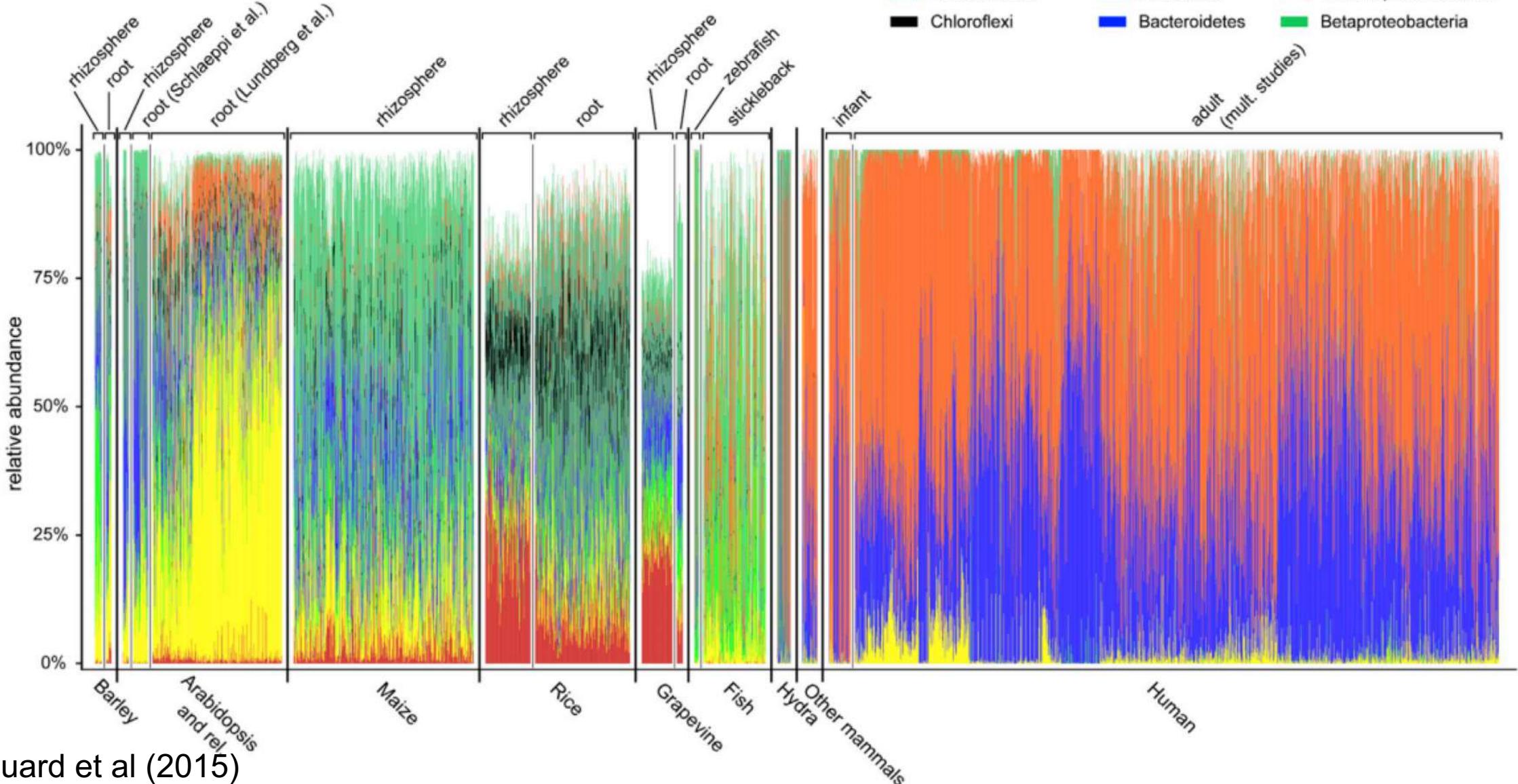
Assigned OTUs ; assigned distance using phylogeny



Tree way plot with top OTUs abundance and classification



Cumulative Abundance plots

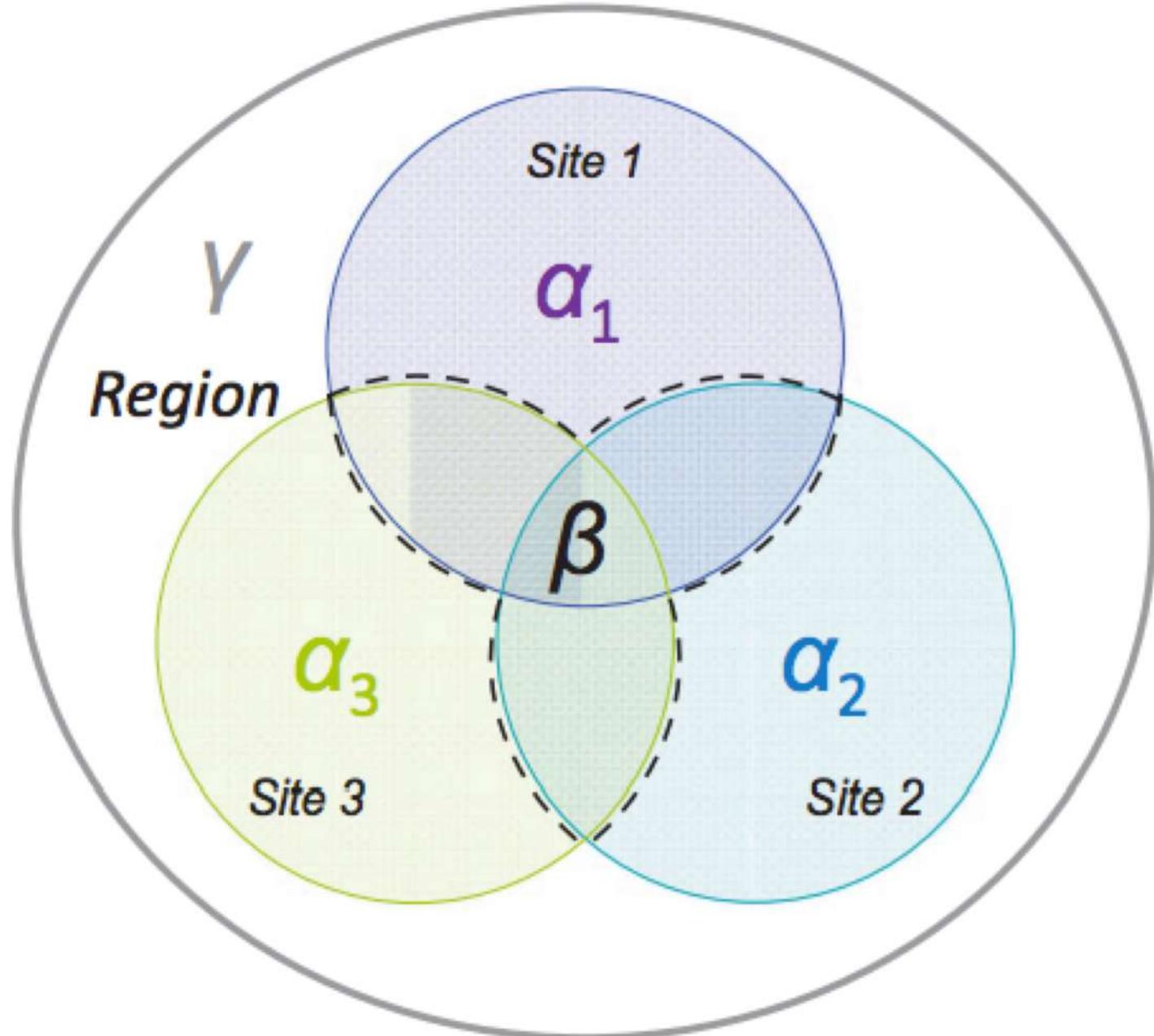


Concept: Diversity measures

Measures of biodiversity

Zinger et al (2012)

“... measuring biodiversity consists of characterizing the **number, composition** and **variation** in taxonomic or functional units (**OTU**) over a wide range of biological organizations”



Measures of biodiversity

Zinger et al (2012)

Alpha diversity refers to the diversity within one location or sample. It is often measured as species richness (i.e. number of species), seldom as species evenness (extent of species dominance). Species richness is strongly sensitive to sampling effort, and requires standardized samples, or the use of estimators that corrects undersampling biases, such as Chao1 or ACE. Evenness is less affected by undersampling biases and is usually assessed with Simpson's or Pielou's indices or rank abundance curves (review in Magurran 2004).

Beta diversity consists in determining the difference in diversity or community composition between two or more locations or samples (i) by considering species composition only, and use incidence data with associated metrics such as Jaccard or Sorensen similarity indices or (ii) by taking species relative abundances into account, and use Bray–Curtis or Morisita–Horn dissimilarity measures (Anderson *et al.* 2011). Using abundance data is, however, strongly discussed among microbiologists when dealing with rRNA gene data because of variations in gene copy number among strains (Acinas *et al.* 2004b; Zhu *et al.* 2005) as well as PCR artefacts.

Gamma diversity, or regional diversity, is similar to alpha diversity but applies for a larger area that encompasses the units under study.

Finally, the spatial scale of investigation can produce very different results and should be consistent in cross-study comparisons (Magurran 2004).

Alpha diversity

a measure of the diversity within a single sample

Types of alpha diversity

Total # of species = **richness**

How many OTUs?

Total # of genes = genetic richness

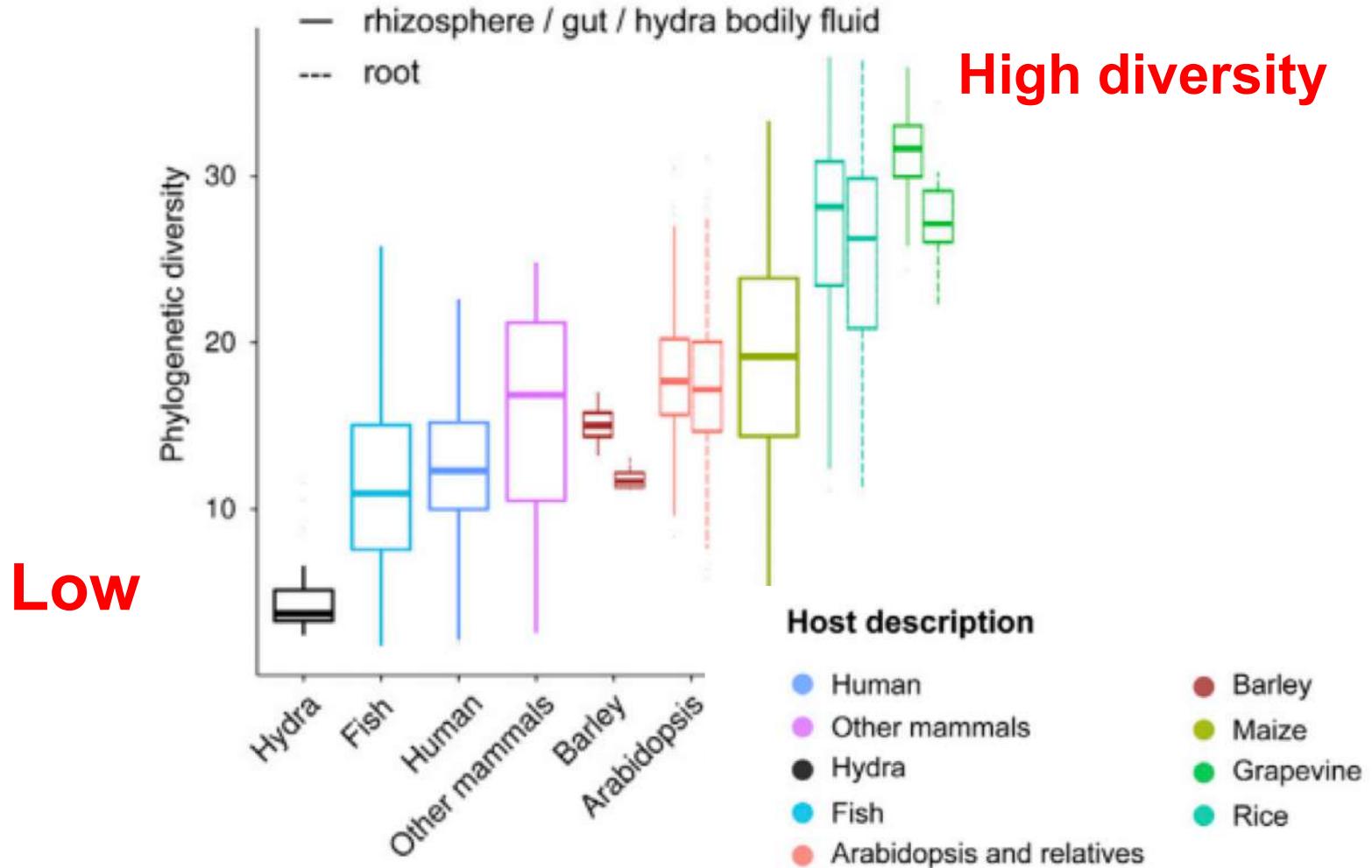
Phylogenetic diversity of genes = genetic PD

Evenness = What is the distribution of abundance in the community?

How many OTUs at high abundance and how many OTU at low abundance?

B

Alpha-diversity (phylogenetic diversity)



Beta diversity

a measure of **the similarity in diversity between samples**

Types of beta diversity

Species presence/absence

Shared phylogenetic diversity

Gene presence / absence

Shared phylogenetic diversity of genes

Frequently used as values for PCA of PCoA analysis

Beta diversity

A. Membership:

shared OTU occurrences across communities
1 = present, 0 = below detection

List of observed OTUs	Occurrences in community A	Occurrences in community B	Shared occurrences A & B
	OTU 1	0	X
OTU 2	0	1	
OTU 3	1	1	X
OTU 4	1	1	X
OTU 5	1	1	X

B. Composition:

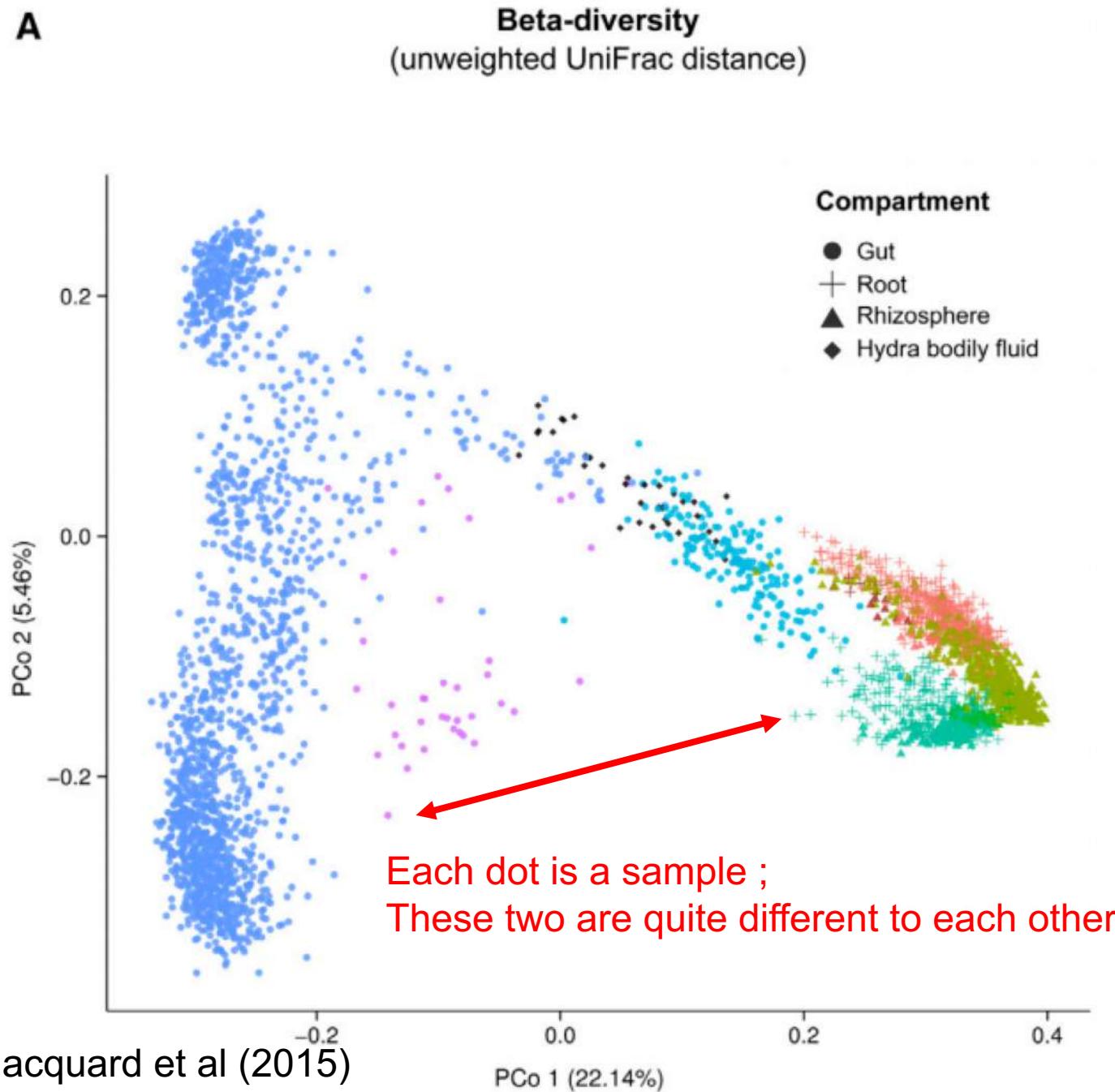
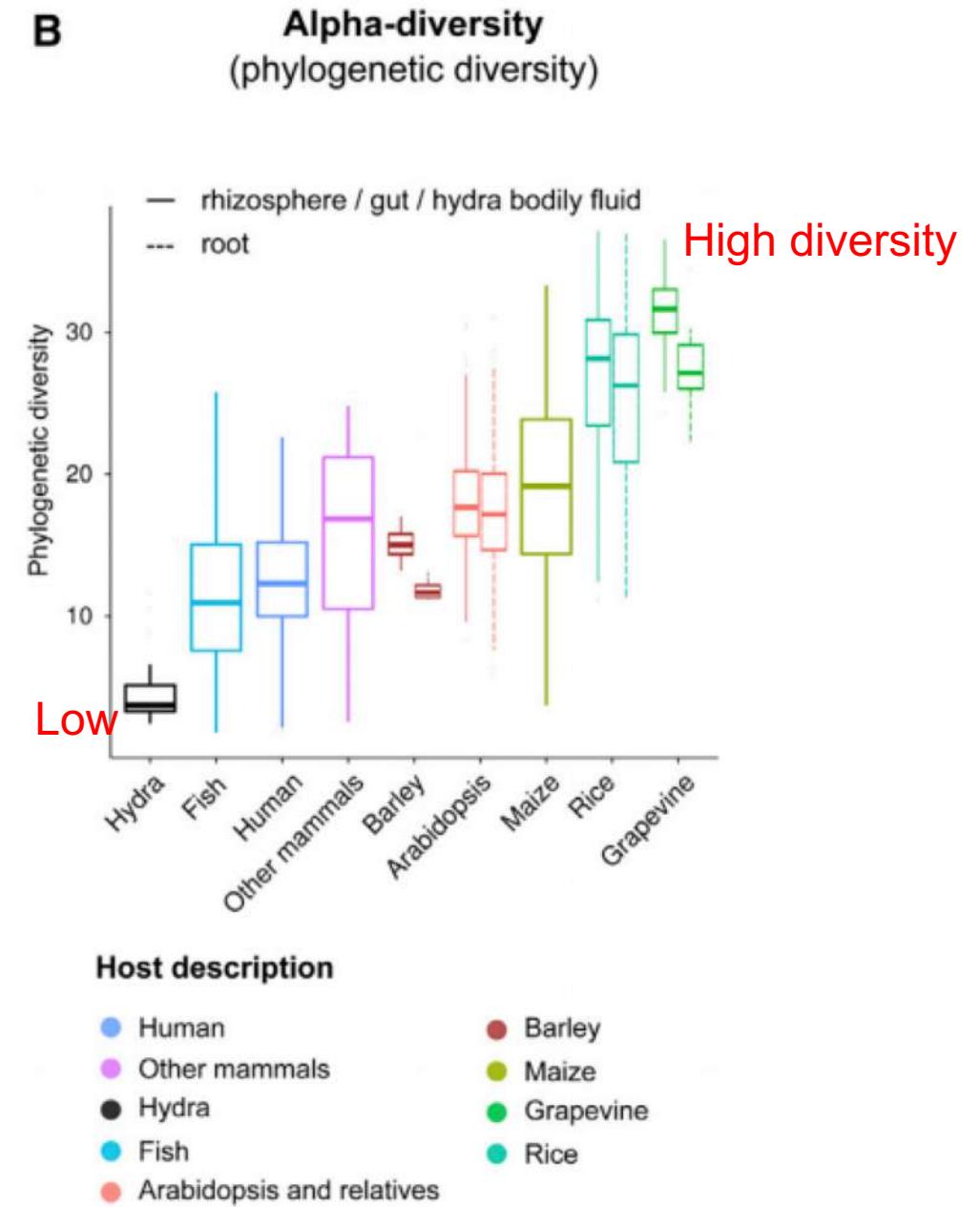
similar OTU abundances across communities

List of observed OTUs	Abundances community A	Abundances community B	Similar abundances A & B
	OTU 1	0.4	0
OTU 2	0	0.1	
OTU 3	0.1	0.1	X
OTU 4	0.2	0.5	
OTU 5	0.3	0.3	X

Phylogeny:

shared OTU lineages across communities

lineage	Abundances community A	Abundances community B	Similar abundances A & B
i.	OTU 1 0.4	0	X
ii.	OTU 2 0	0.1	
iii.	OTU 3 0.1	0.1	
	OTU 4 0	0.8	X
	OTU 5 0.5	0	

A**B**

Amplicon sequencing or metagenomes?

Workflow decision

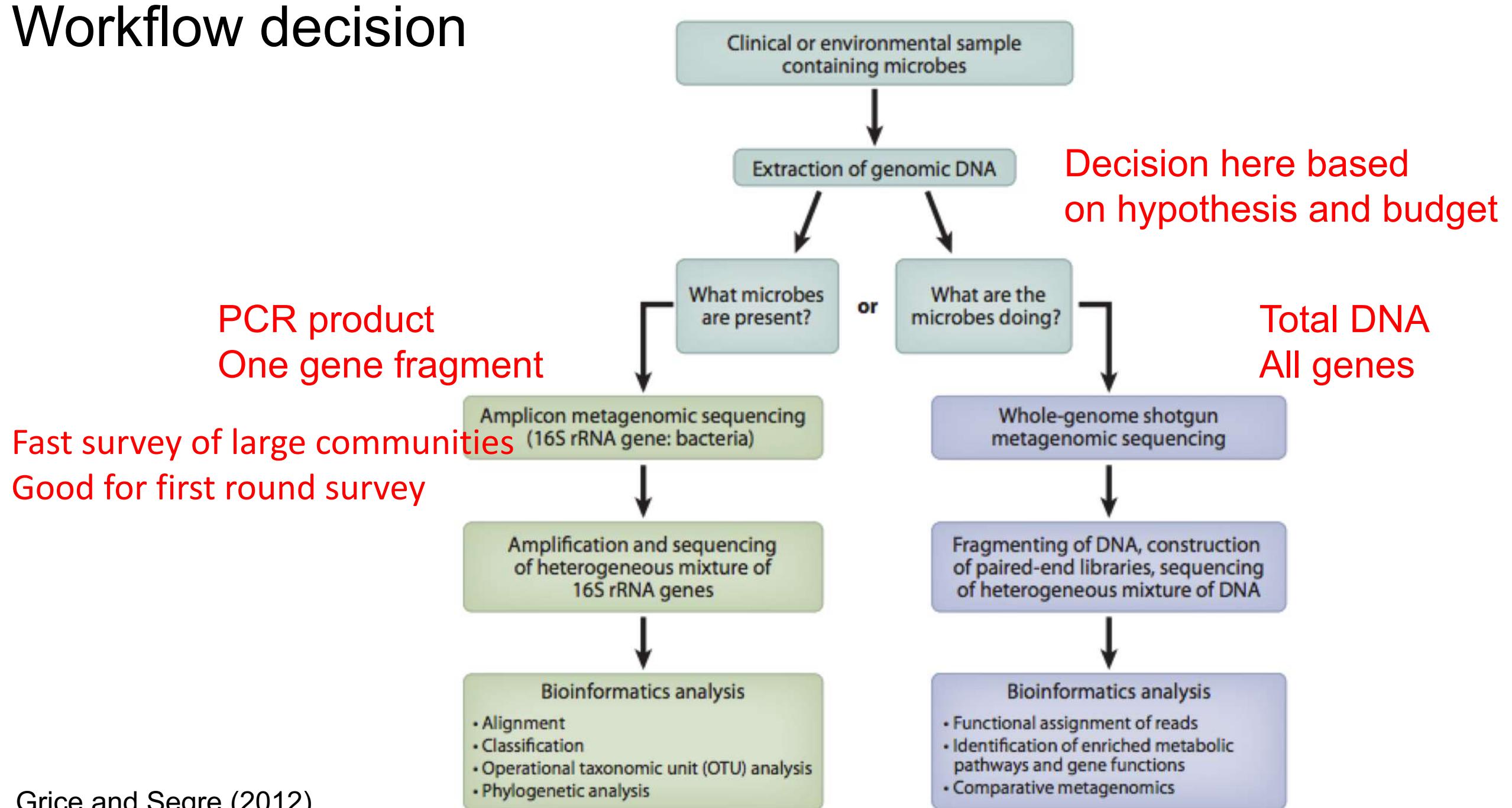
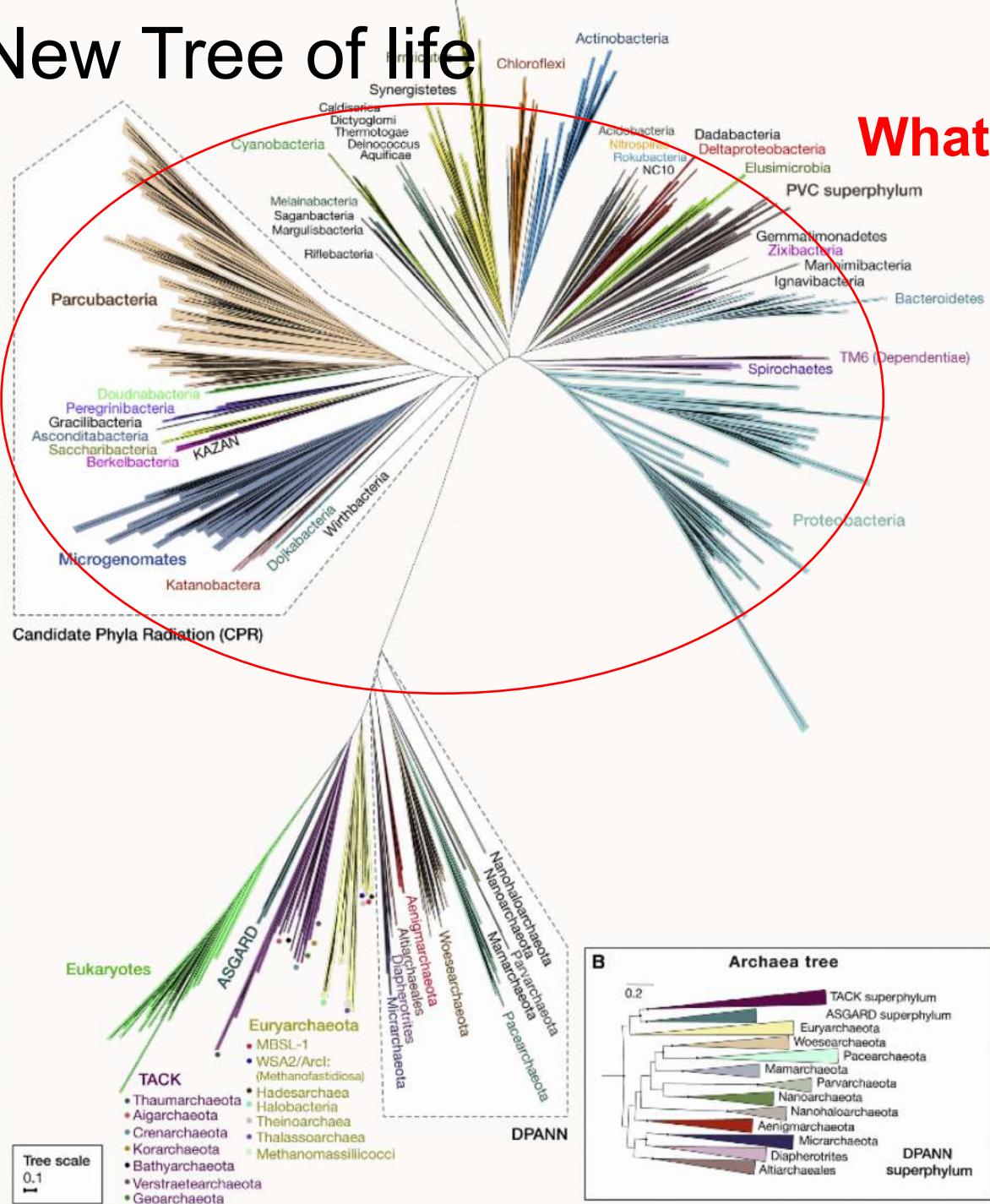


Table 1. Metataxonomics, metagenomics and meta-transcriptomics strategies

Technique	Advantages and challenges	Main applications
Metataxonomics using amplicon sequencing of the 16S or 18S rRNA gene or ITS	+ Fast and cost-effective identification of a wide variety of bacteria and eukaryotes – Does not capture gene content other than the targeted genes – Amplification bias – Viruses cannot be captured	* Profiling of what is present * Microbial ecology * rRNA-based phylogeny
Metagenomics using random shotgun sequencing of DNA or RNA	+ No amplification bias + Detects bacteria, archaea, viruses and eukaryotes + Enables <i>de novo</i> assembly of genomes – Requires high read count – Many reads may be from host – Requires reference genomes for classification	* Profiling of what is present across all domains * Functional genome analyses * Phylogeny * Detection of pathogens
Meta-transcriptomics using sequencing of mRNA	+ Identifies active genes and pathways – mRNA is unstable – Multiple purification and amplification steps can lead to more noise	* Transcriptional profiling of what is active

Amplicon sequencing

New Tree of life



What do they have in common?

Leading Edge **Perspective**

Major New Microbial Groups Expand Diversity and Alter our Understanding of the Tree of Life

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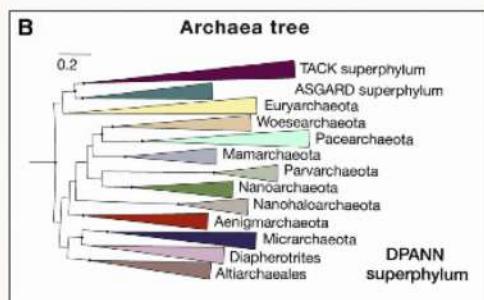
⁴University of Melbourne, Melbourne, VIC, Australia

⁵Lawrence Berkeley National Laboratory, Berkeley

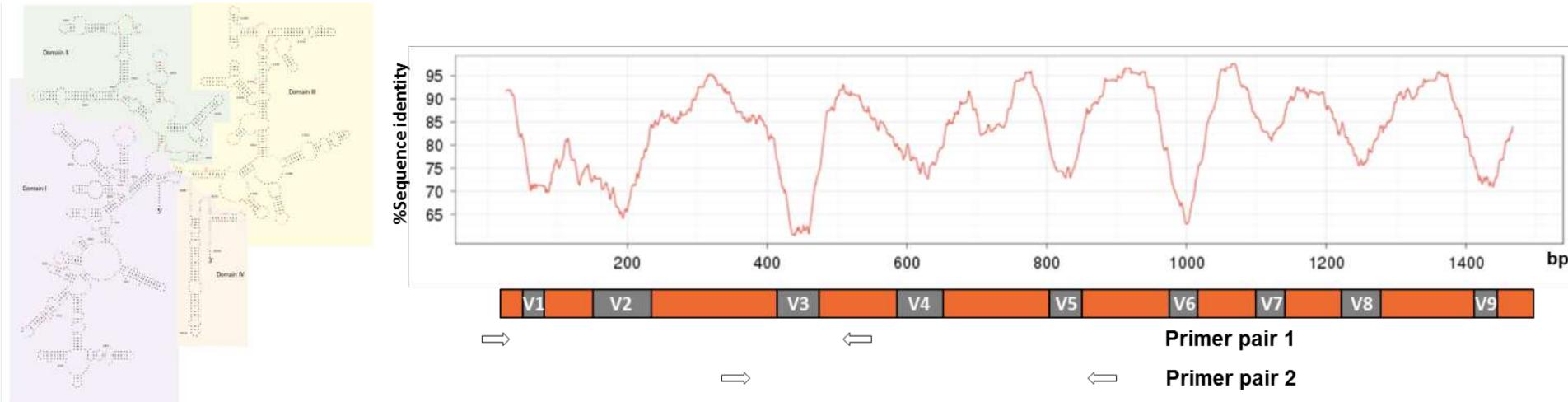
*Correspondence: ibenfield@berkeley.edu.

Correspondence: jbarneby@berkeley.edu
<https://doi.org/10.116/j.cell.2018.02.011>

<https://doi.org/10.1101/2018.02.01>



16S



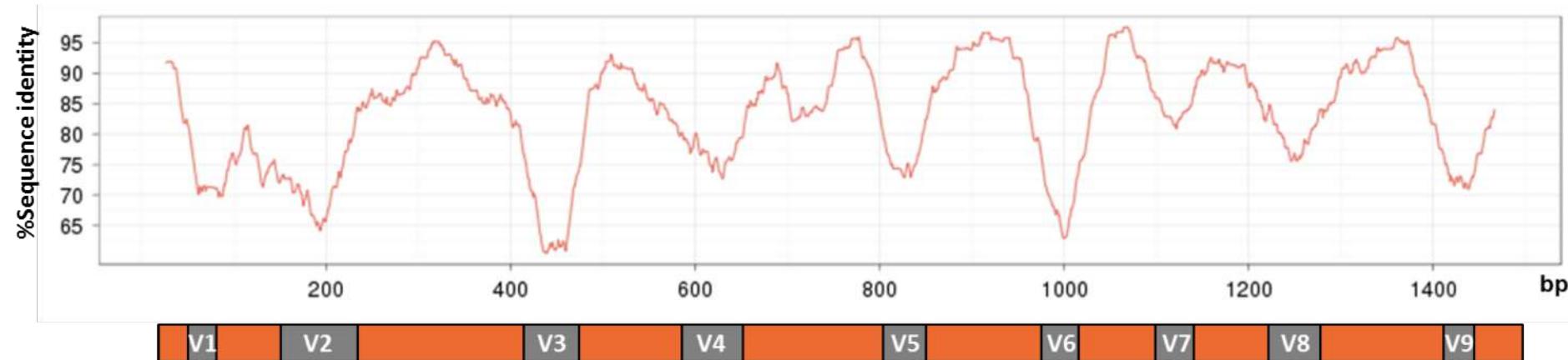
- **Advantages:**

- Universal: Every bacterial and archaea species has this gene
- Conserved regions (for primer design)
- **Variable regions (to distinguish different species)**
- Great databases and alignments (for human related species)
- Mainly used for taxonomical classification

- **Problems:**

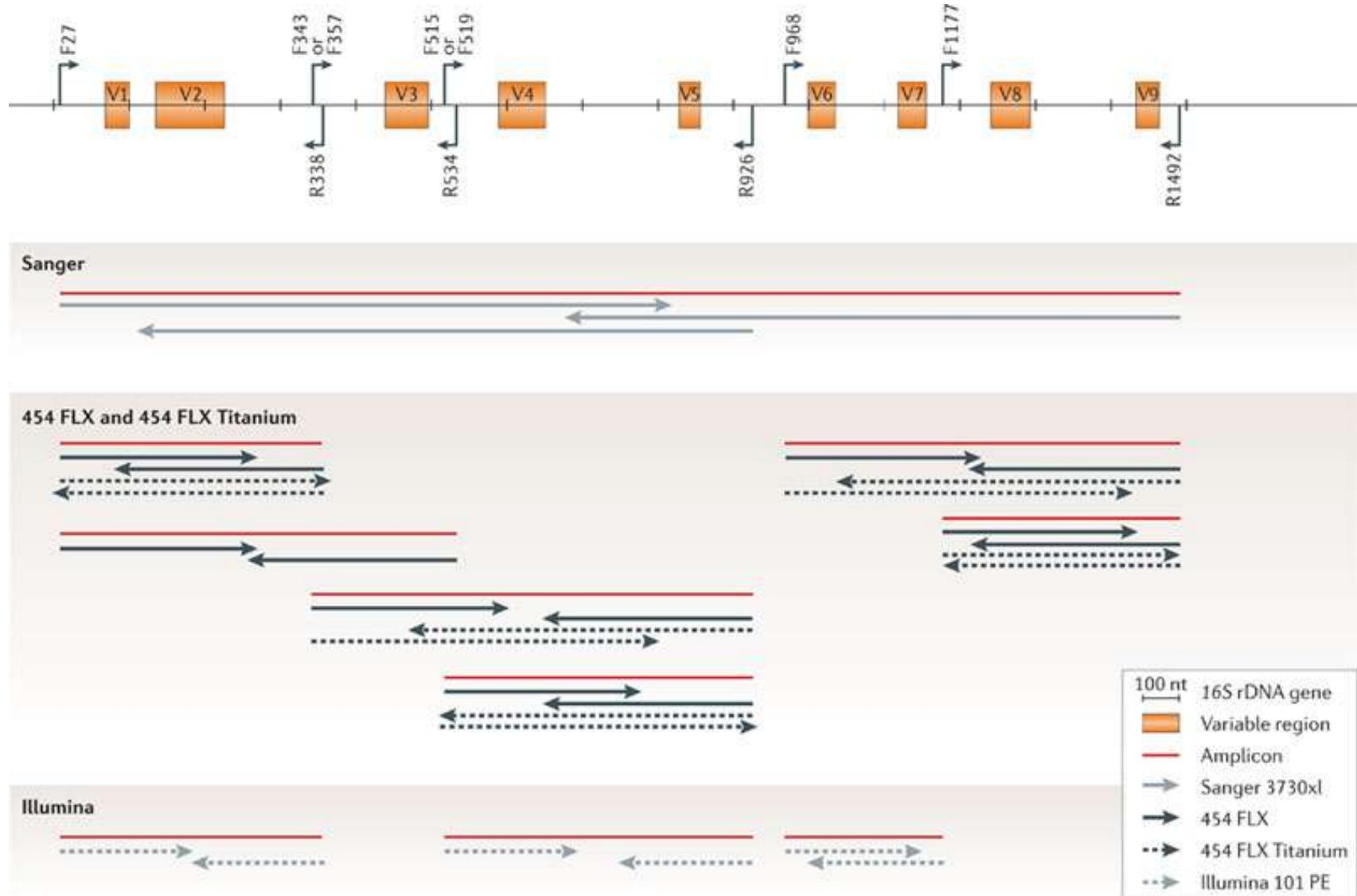
- Variable copy number in each species
- No universal (unbiased) primers
- (Not directly correlated with activity)
- (Lack of functional information)

Typical workflow

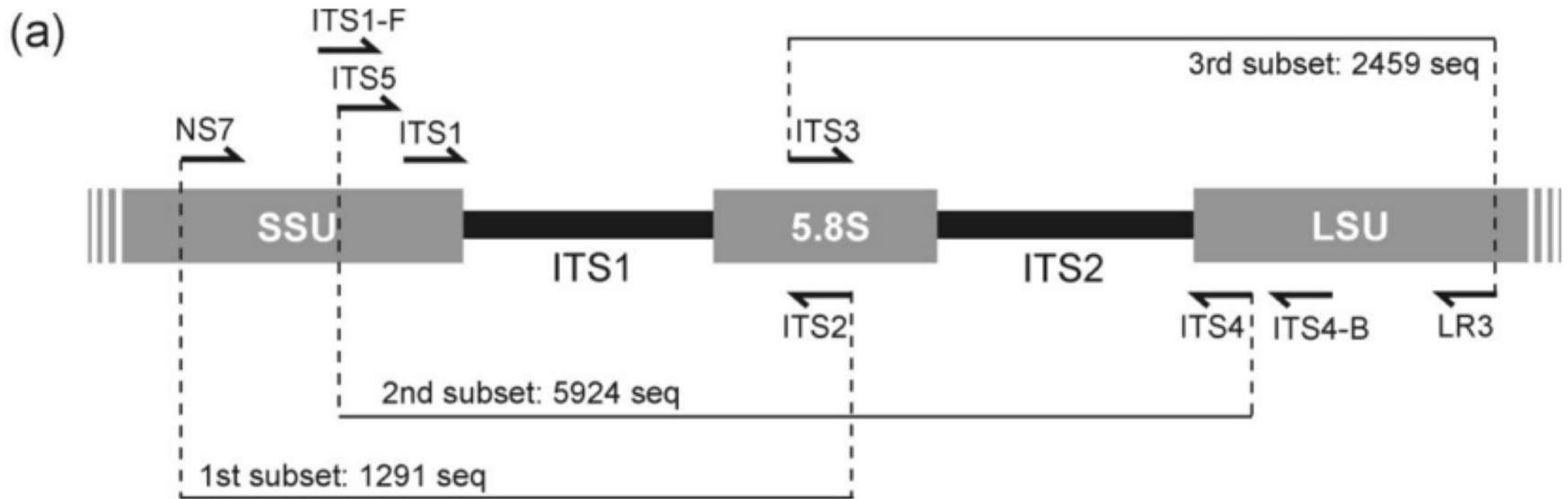


Which region to sequence?

16S amplified region



ITS for characterization of fungi species



Workflow

Filter for
contaminants and
low quality reads



Assemble
overlapping reads



Reduce datasets
(clustering)



Perform taxonomic
classification and
compute diversity
metrics

- Quality plots and read trimming
 - FastQC
<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
 - FASTX
http://hannonlab.cshl.edu/fastx_toolkit/
- Chimera removal
 - AmpliconNoise
<http://code.google.com/p/ampliconnoise/>
 - UCHIME
<http://www.drive5.com/uchime/>

Workflow

Filter for
contaminants and
low quality reads



**Assemble
overlapping reads**



Reduce datasets
(clustering)



Perform taxonomic
classification and
compute diversity
metrics

Because the read length (2x250 or 2x300) will be
longer than the actual DNA fragment

- Merge overlapping paired end reads

- FLASH

- <http://www.genomics.jhu.edu/software/FLASH/index.shtml>

- FastqJoin

- <http://code.google.com/p/ea-utils/wiki/FastqJoin>

- CD-HIT read-linker

- <http://weizhong-lab.ucsd.edu/cd-hit/wiki/doku.php?id=cd-hit-auxtools-manual>

Workflow

Filter for
contaminants and
low quality reads



Assemble
overlapping reads



**Reduce datasets
(clustering)**



Perform taxonomic
classification and
compute diversity
metrics

- **Clustering with high stringency**

- UCLUST/USEARCH (16S only)

<http://www.drive5.com/usearch/>

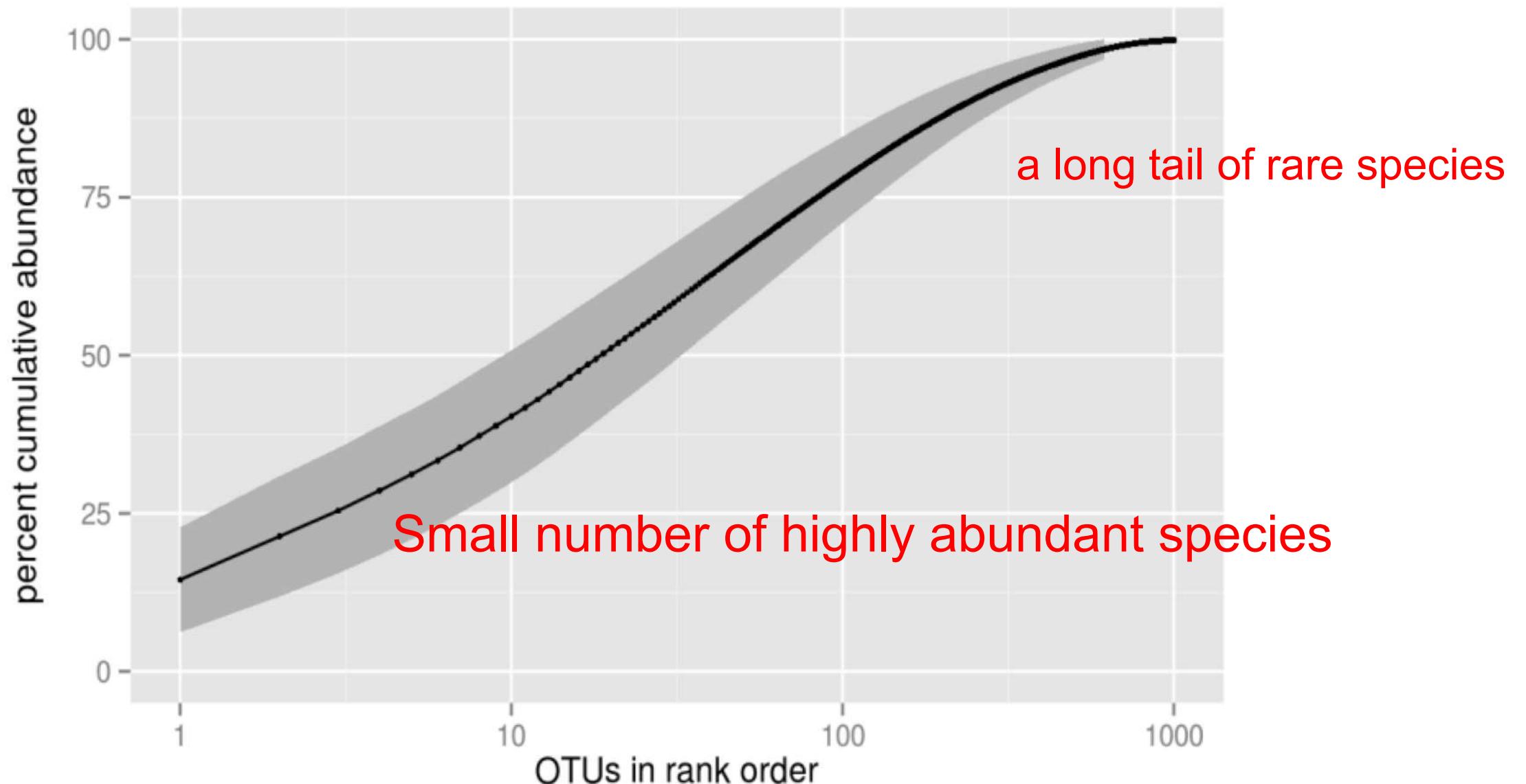
- CD-HIT-OTU (16S only)

<http://weizhong-lab.ucsd.edu/cd-hit-otu/>

- phylOTU (16S only)

<https://github.com/sharpton/PhyLOTU>

Typical number of OTUs



Workflow

Filter for
contaminants and
low quality reads



Assemble
overlapping reads



Reduce datasets
(clustering)



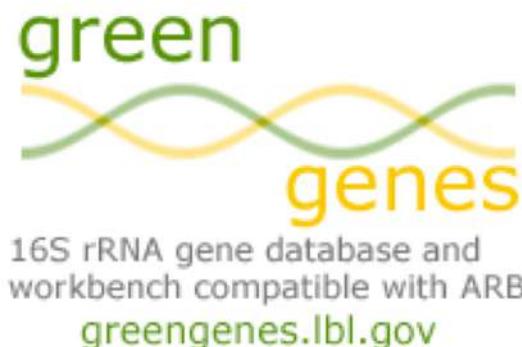
**Perform
taxonomic
classification and
compute diversity
metrics**

- **Composition based classifiers**
 - RDP database + classifier
<http://rdp.cme.msu.edu/classifier/classifier.jsp>
- **Homology based classifiers**
 - ARB + Silva database (16S only)
<http://www.arb-home.de/>
 - GreenGenes database (16S only)
<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>
 - UNITE database (ITS only)
<http://unite.ut.ee/>
 - FungalITS Pipeline (ITS only)
<http://www.emerencia.org/fungalitspipeline.html>

Using a “Classifier” to annotate OTUs

Uses an existing phylogeny

Find best unambiguous match to references



<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>



<http://www.arb-silva.de/>



<https://rdp.cme.msu.edu/>

Analysis Packages



Qiime2 (<https://qiime2.org/>)



Mothur (mothur.org)

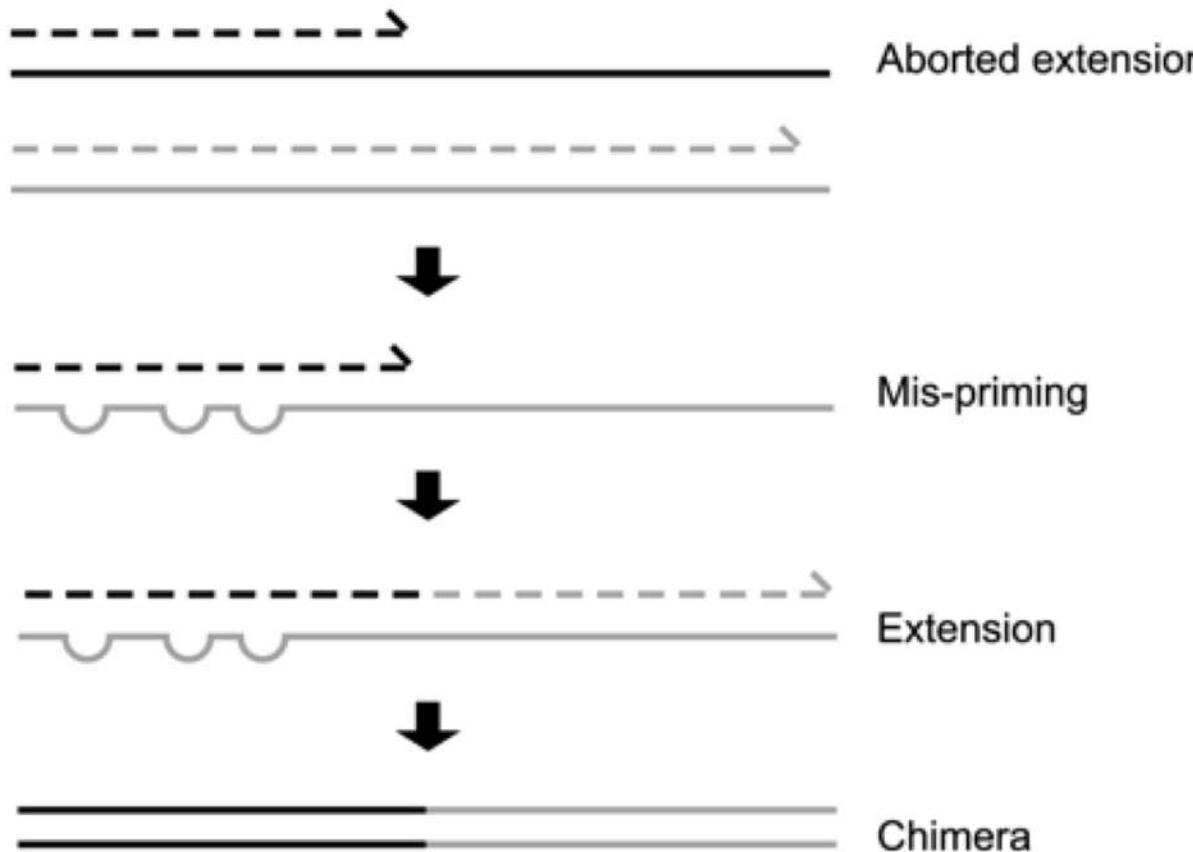
R

Phyloseq (<https://joey711.github.io/phyloseq/>)

Potential problem

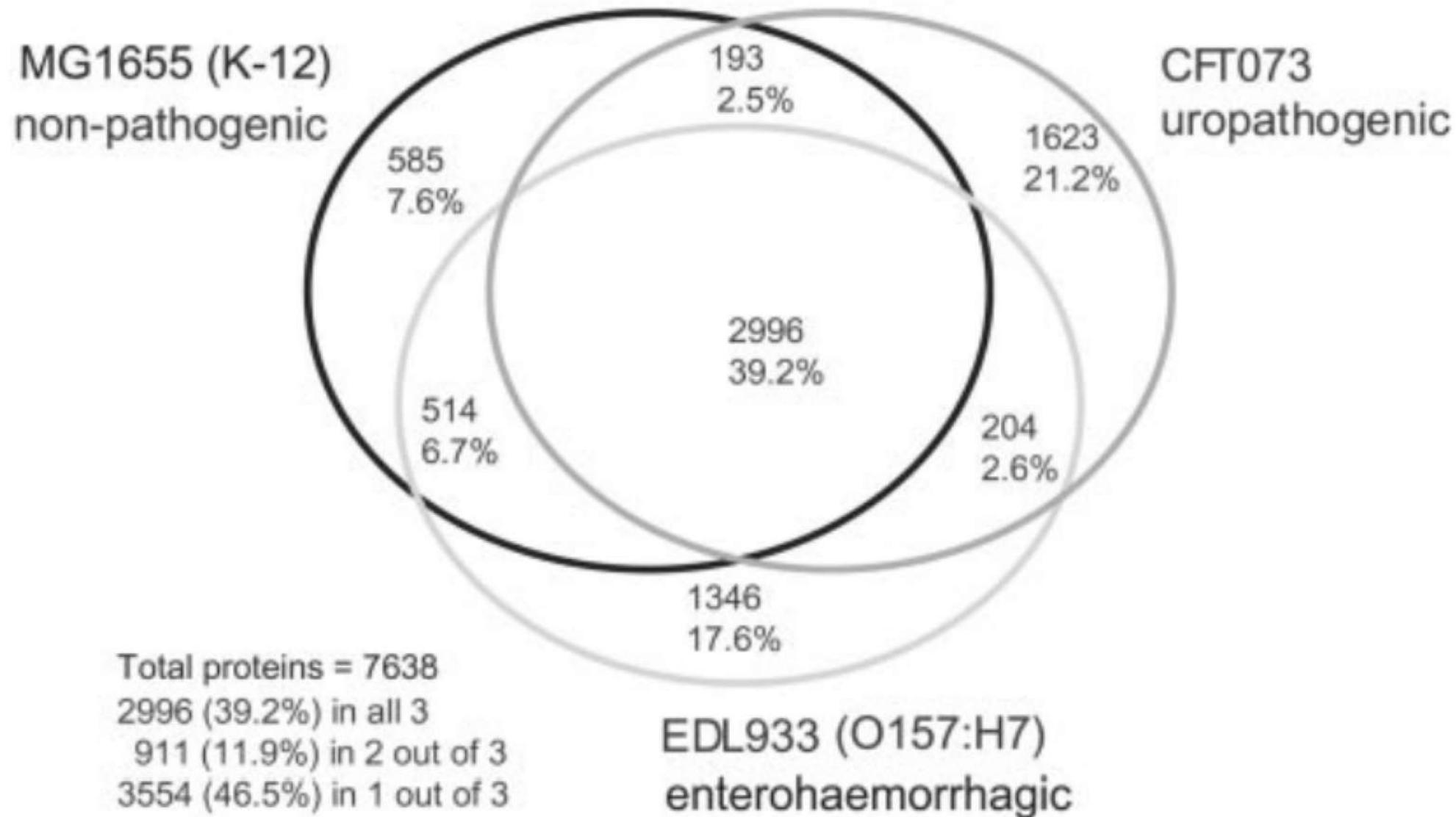
- Lack of tools for processing ITS/Fungal microbiome data sets
- Amplification bias effects accuracy and replication
- Use of short reads prevents disambiguation of similar strains
- 16S or ITS may not differentiate between similar strains –
 - Clustering is done at 97%
 - Regions may be >99% similar
- Sequencing error inflates number of OTUs
- Chloroplast 16S sequences can get amplified in plant metagenomes

Chimeric 16S (Artificial sequences formed during PCR amplification)



“Chimeras were found to reproducibly form among independent amplifications and contributed to false perceptions of sample diversity and the false identification of novel taxa, **with less-abundant species exhibiting chimera rates exceeding 70%**”

Same species (16S): Different genomes



Metagenomics

Advantage of metagenomics approach

Better classification with Increasing number of complete genomes

Focus on whole genome based phylogeny (whole genome phlyotyping)

- Advantages

No amplification bias like in 16S/ITS

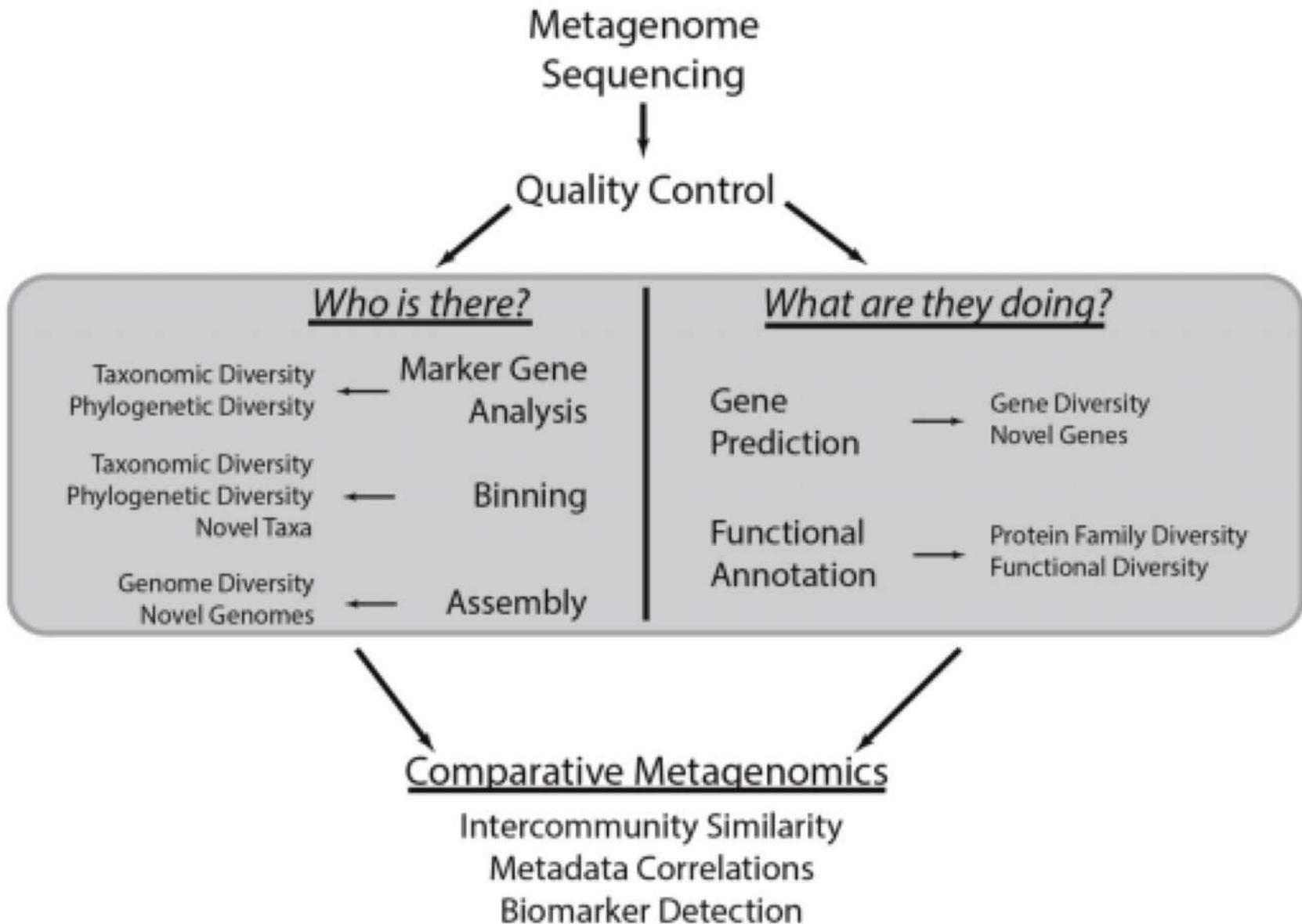
Issues

Poor sampling beyond eukaryotic diversity

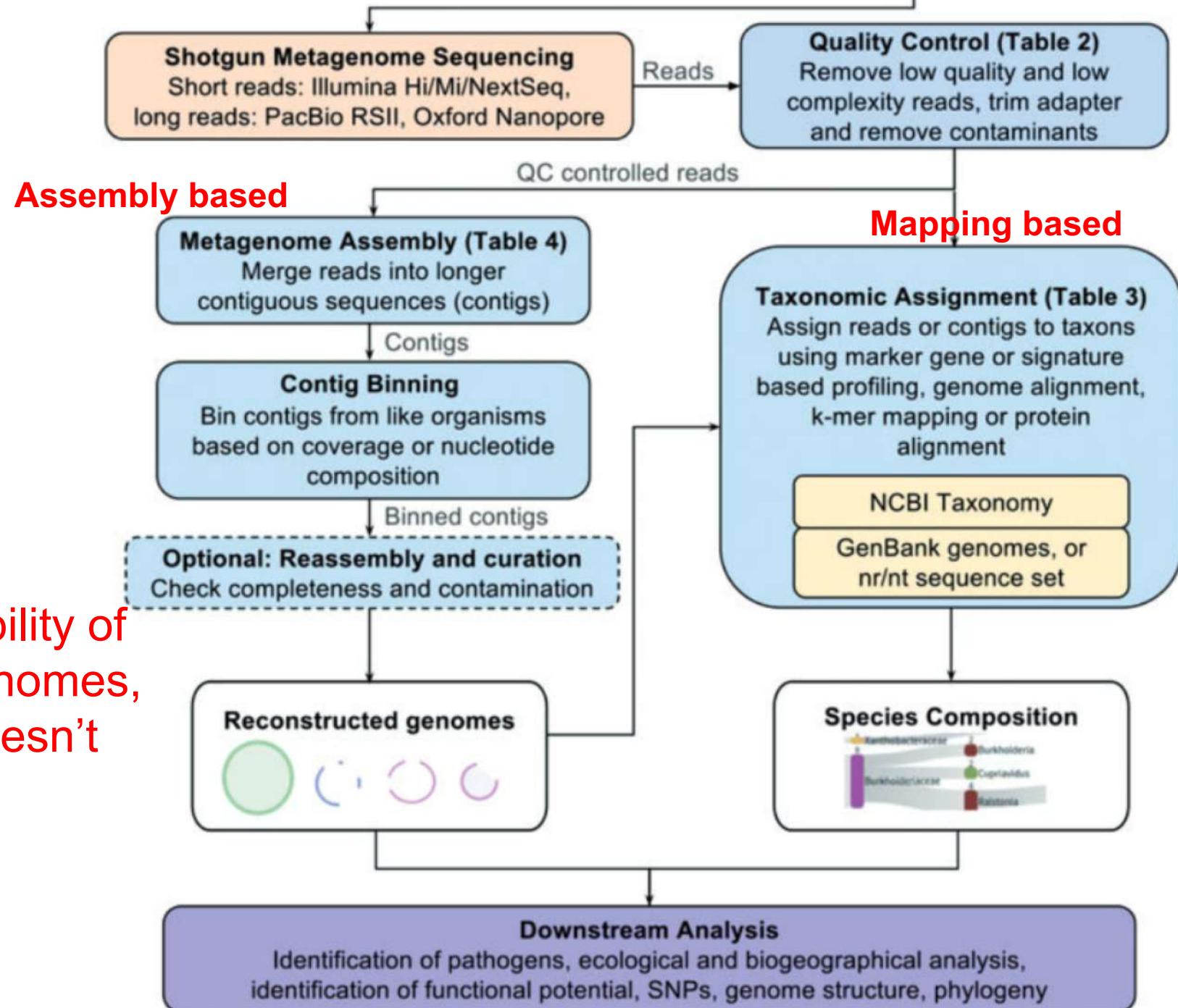
Assembly of metagenomes is **challenging** due to uneven coverage

Requires **high** depth of coverage

Overall workflow

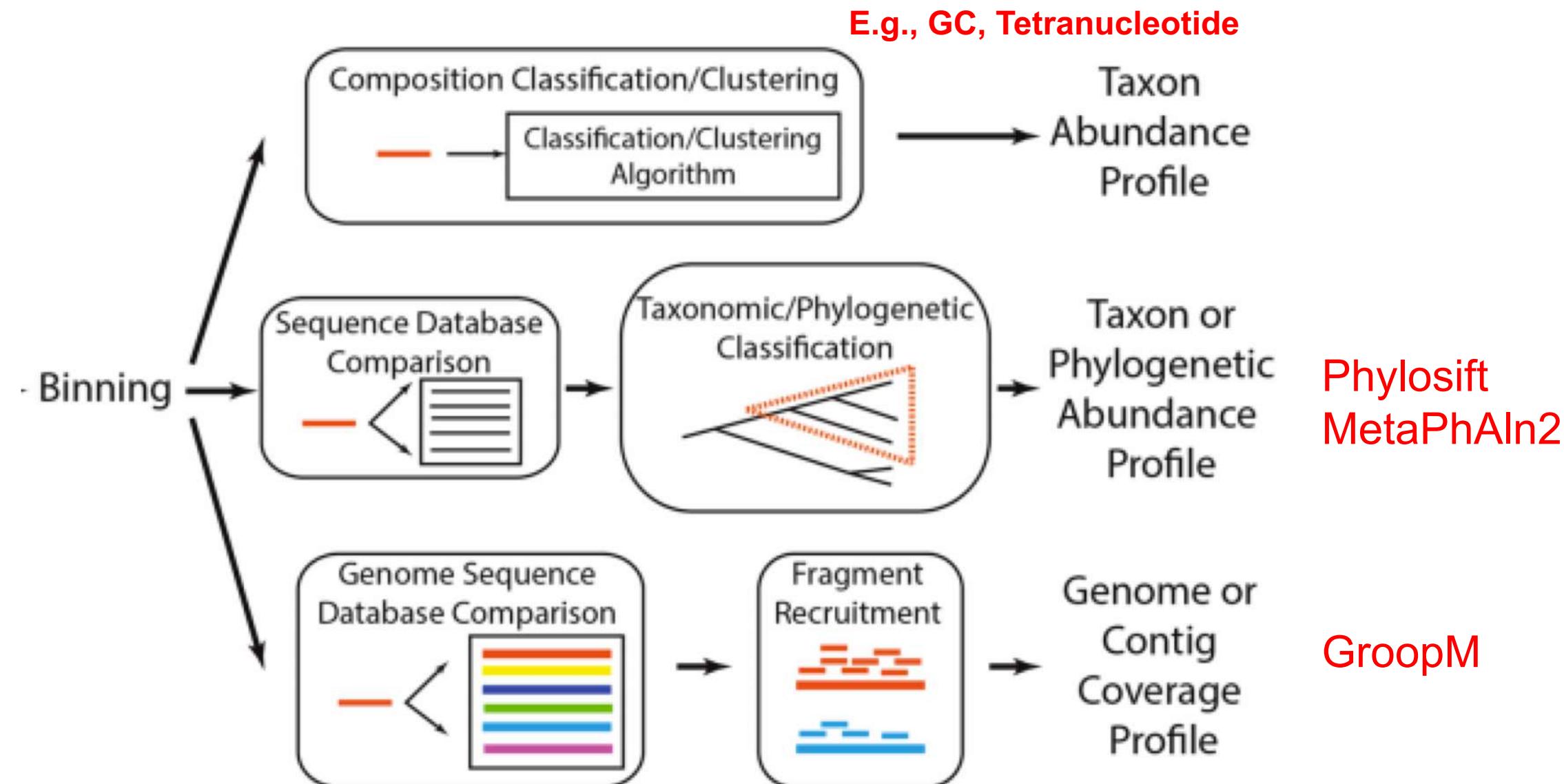


Overall workflow

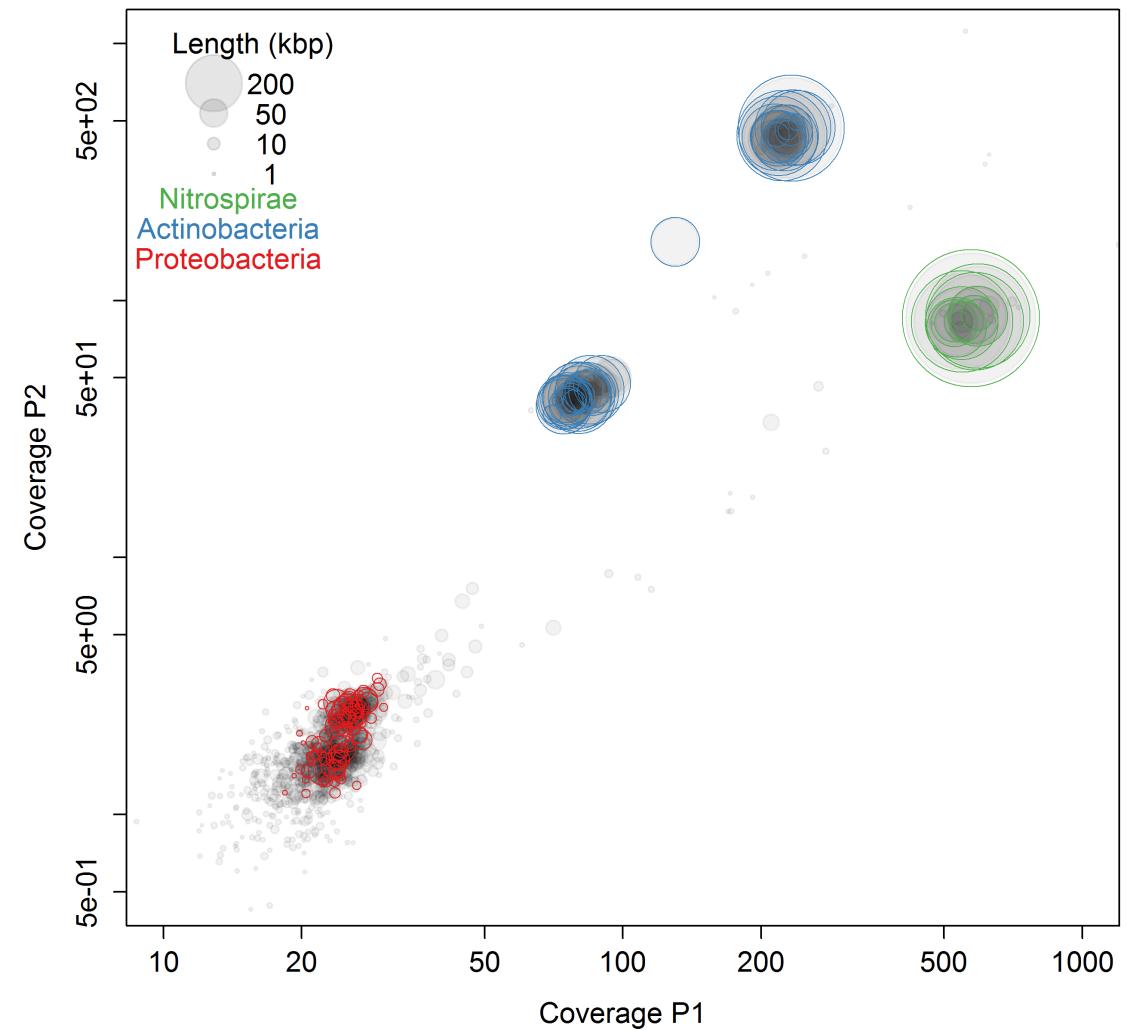
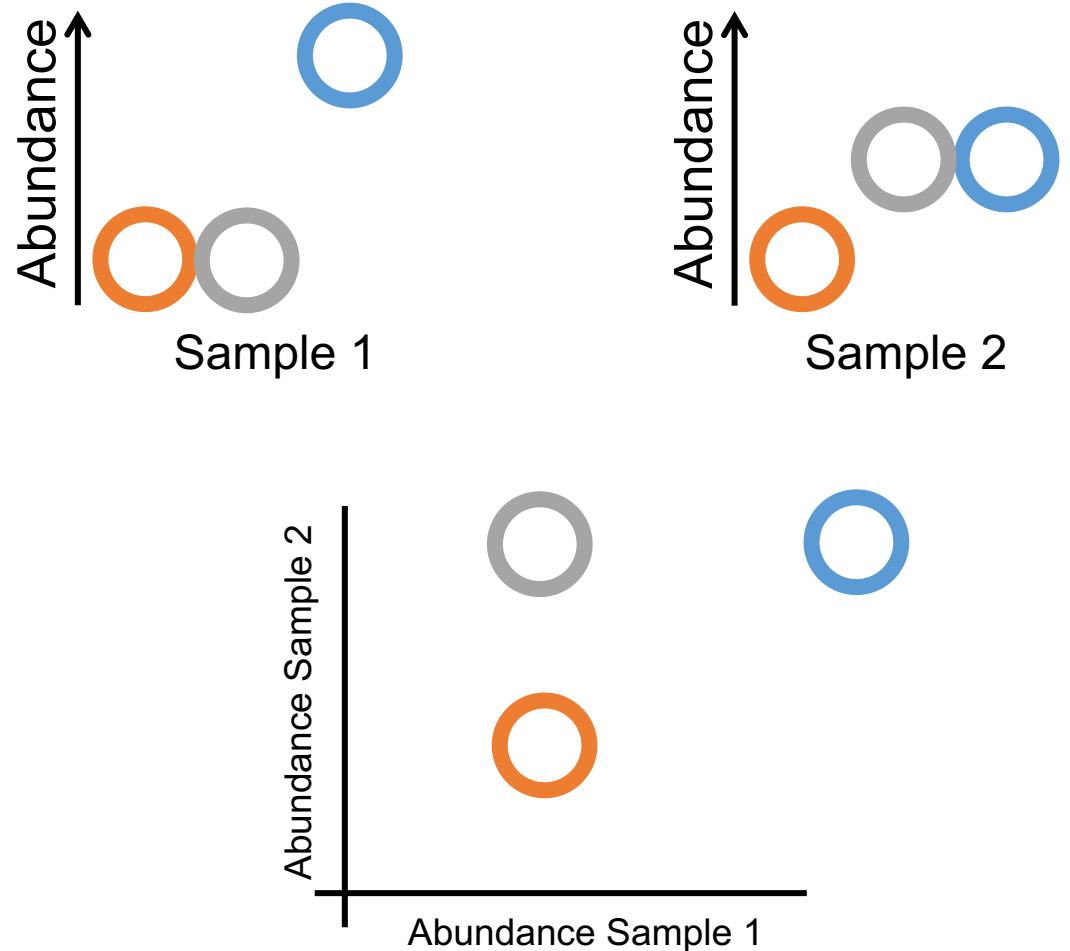


With the increase availability of reference sequenced genomes, probably one day one doesn't require assembly of metagenomes

Binning methods



Example of binning based on differential coverage



Binning methods: A combination of

Classification based on **sequence composition**:

Advantage : all reads can be categorised into bins

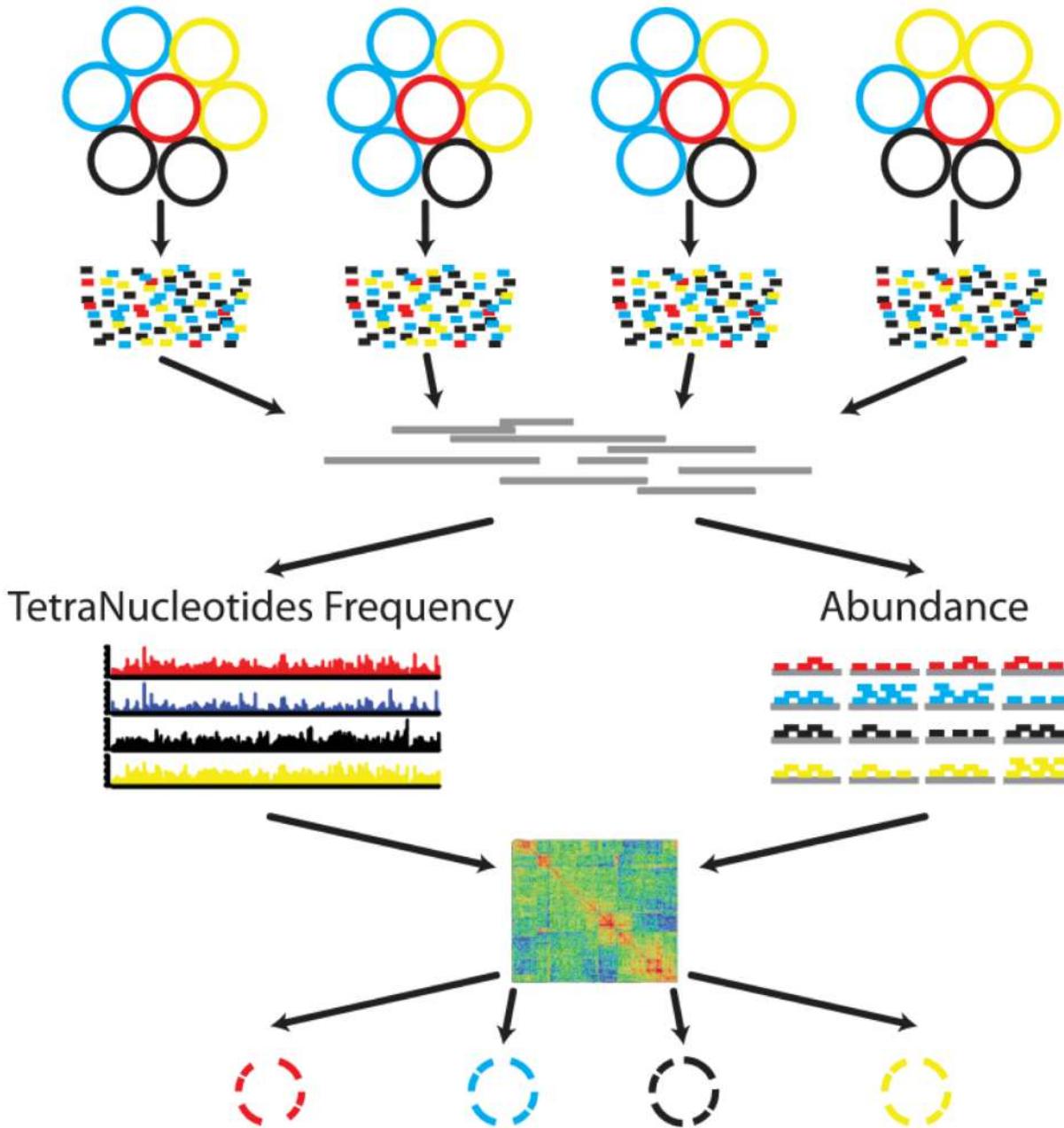
Disadvantage: no taxonomy / function of the bins.

Classification based on **sequence similarity (of known genes)**

Advantage: One can determine taxonomy and function of reads.

Disadvantage: reads with similarity can not be classified .

Metabat



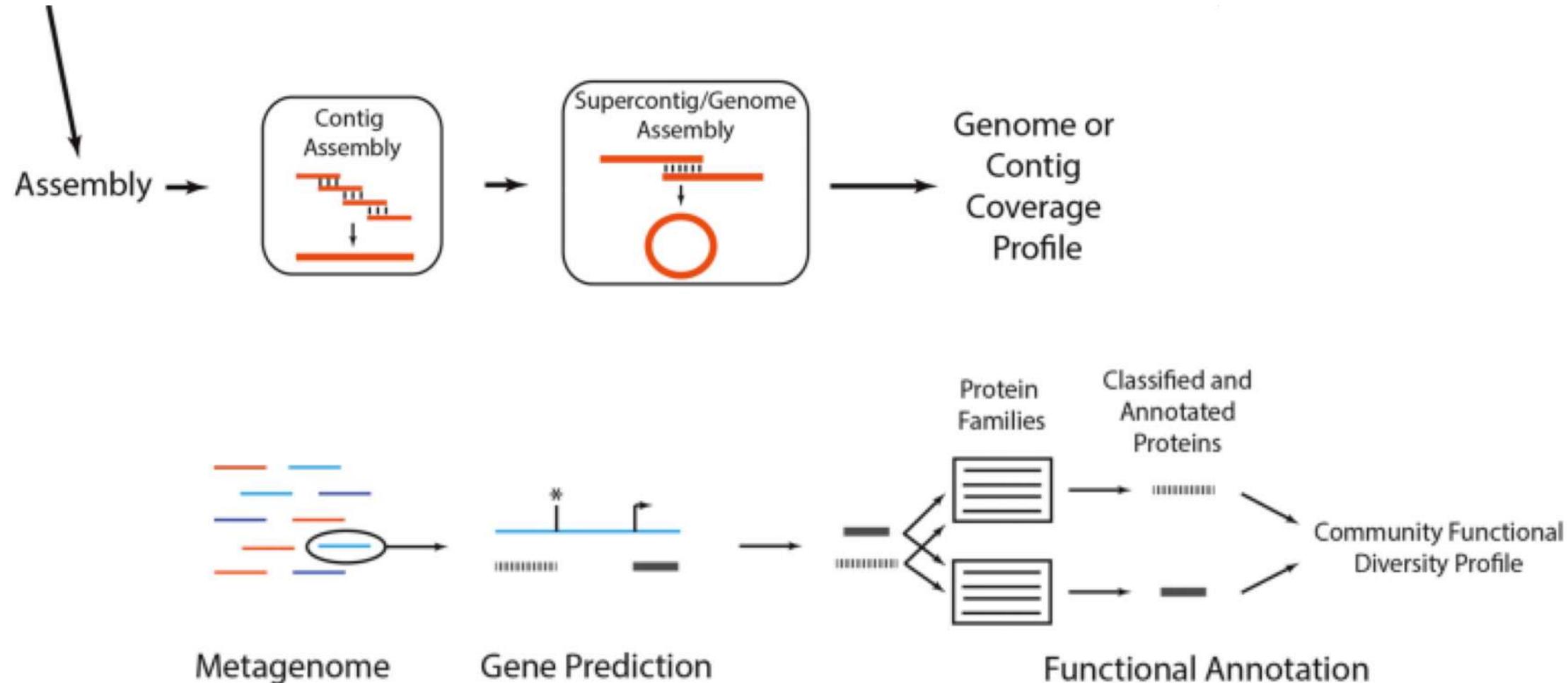
Preprocessing

- ① Samples from multiple sites or times
- ② Metagenome libraries
- ③ Initial de-novo assembly using the combined library

MetaBAT

- ④ Calculate TNF for each contig
- ⑤ Calculate Abundance per library for each contig
- ⑥ Calculate the pairwise distance matrix using pre-trained probabilistic models
- ⑦ Forming genome bins iteratively

Actual assembly



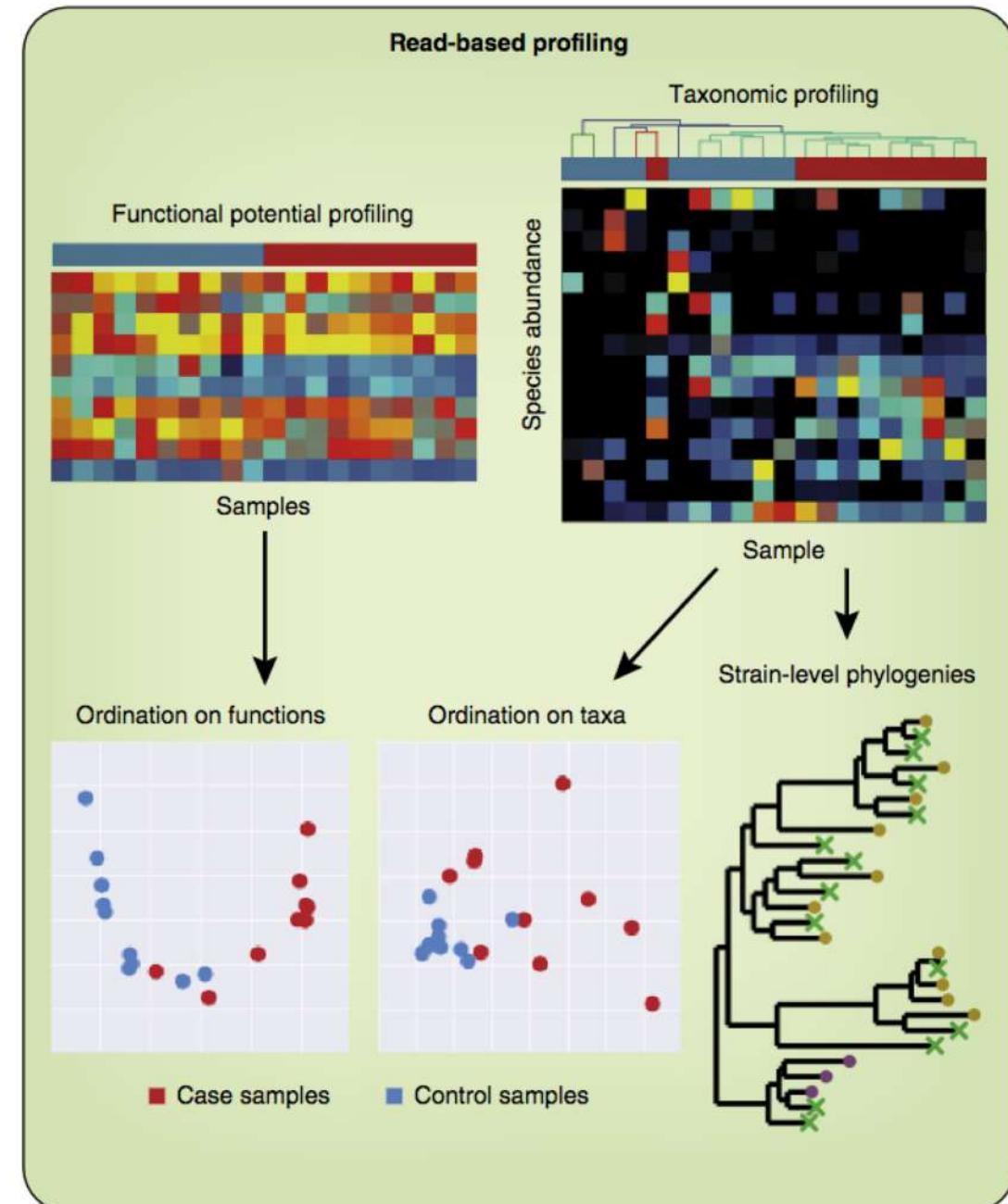
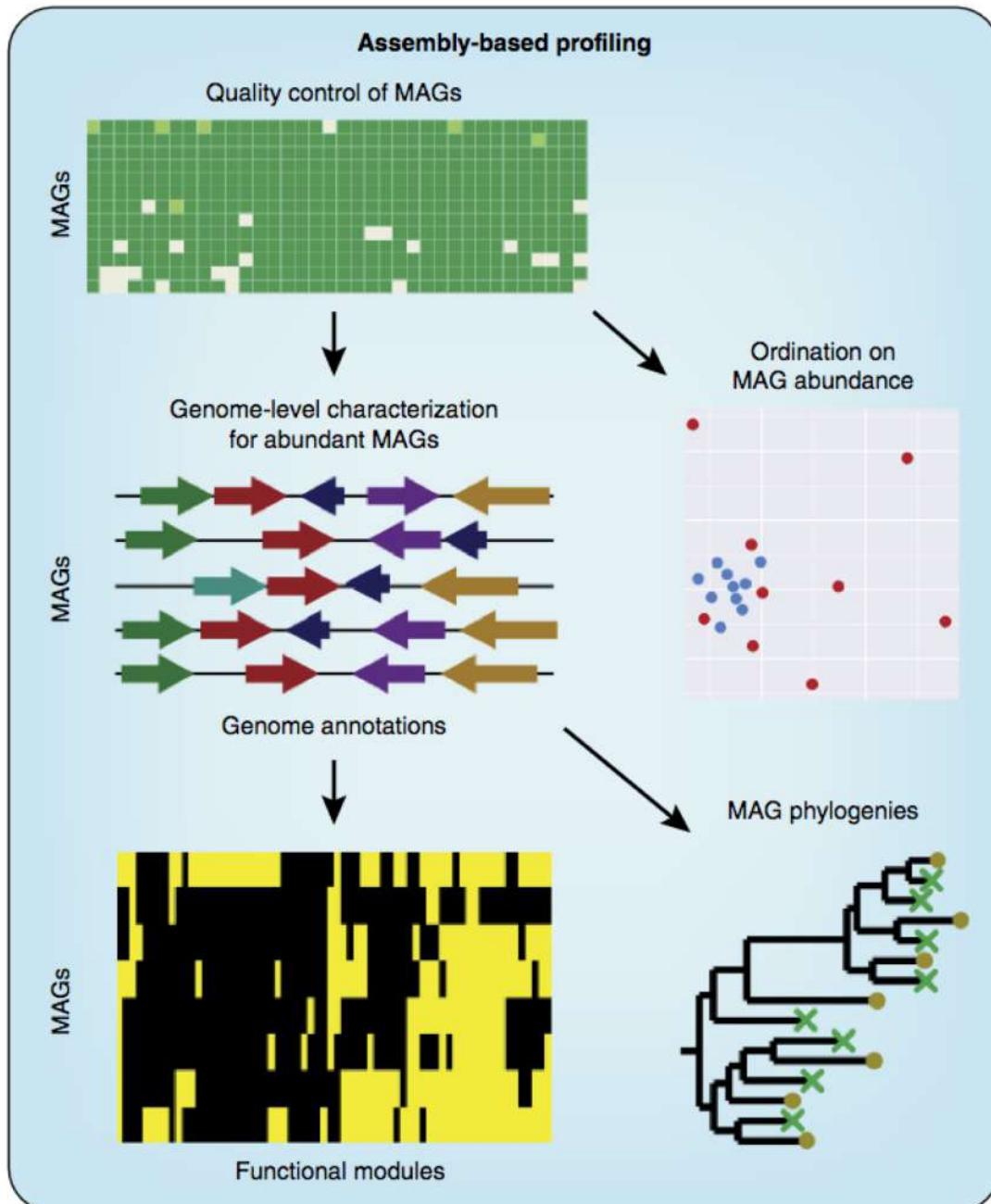


Table 4 Strengths and weaknesses of assembly-based and read-based analyses for primary analysis of metagenomics data

	Assembly-based analysis	Read-based analysis ('mapping')
Comprehensiveness	Can construct multiple whole genomes, but only for organisms with enough coverage to be assembled and binned.	Can provide an aggregate picture of community function or structure, but is based only on the fraction of reads that map effectively to reference databases.
Community complexity	In complex communities, only a fraction of the genomes can be resolved by assembly.	Can deal with communities of arbitrary complexity given sufficient sequencing depth and satisfactory reference database coverage
Novelty	Can resolve genomes of entirely novel organisms with no sequenced relatives.	Cannot resolve organisms for which genomes of close relatives are unknown.
Computational burden	Requires computationally costly assembly, mapping and binning.	Can be performed efficiently, enabling large meta-analyses.
Genome-resolved metabolism	Can link metabolism to phylogeny through completely assembled genomes, even for novel diversity.	Can typically resolve only the aggregate metabolism of the community, and links with phylogeny are only possible in the context of known reference genomes.
Expert manual supervision	Manual curation required for accurate binning and scaffolding and for misassembly detection.	Usually does not require manual curation, but selection of reference genomes to use could involve human supervision.
Integration with microbial genomics	Assemblies can be fed into microbial genomic pipelines designed for analysis of genomes from pure cultured isolates.	Obtained profiles cannot be directly put into the context of genomes derived from pure cultured isolates.

Every step counts



The Madness of Microbiome: Attempting To Find Consensus “Best Practice” for 16S Microbiome Studies

Jolinda Pollock,^{a,b} Laura Glendinning,^b Trong Wisedchanwet,^b Mick Watson^b

- SAMPLE COLLECTION
- SAMPLE STORAGE
- FRESH VERSUS FROZEN SAMPLES
- USE OF CRYOPROTECTANT
- DNA EXTRACTION
- SEQUENCING STRATEGY
- MOCK BACTERIAL COMMUNITIES
- ANALYSIS STRATEGY
- OPERATIONAL TAXONOMIC UNIT PICKING METHODS
- CORRECTING FOR GENE COPY NUMBER
- CONTAMINATION ISSUES

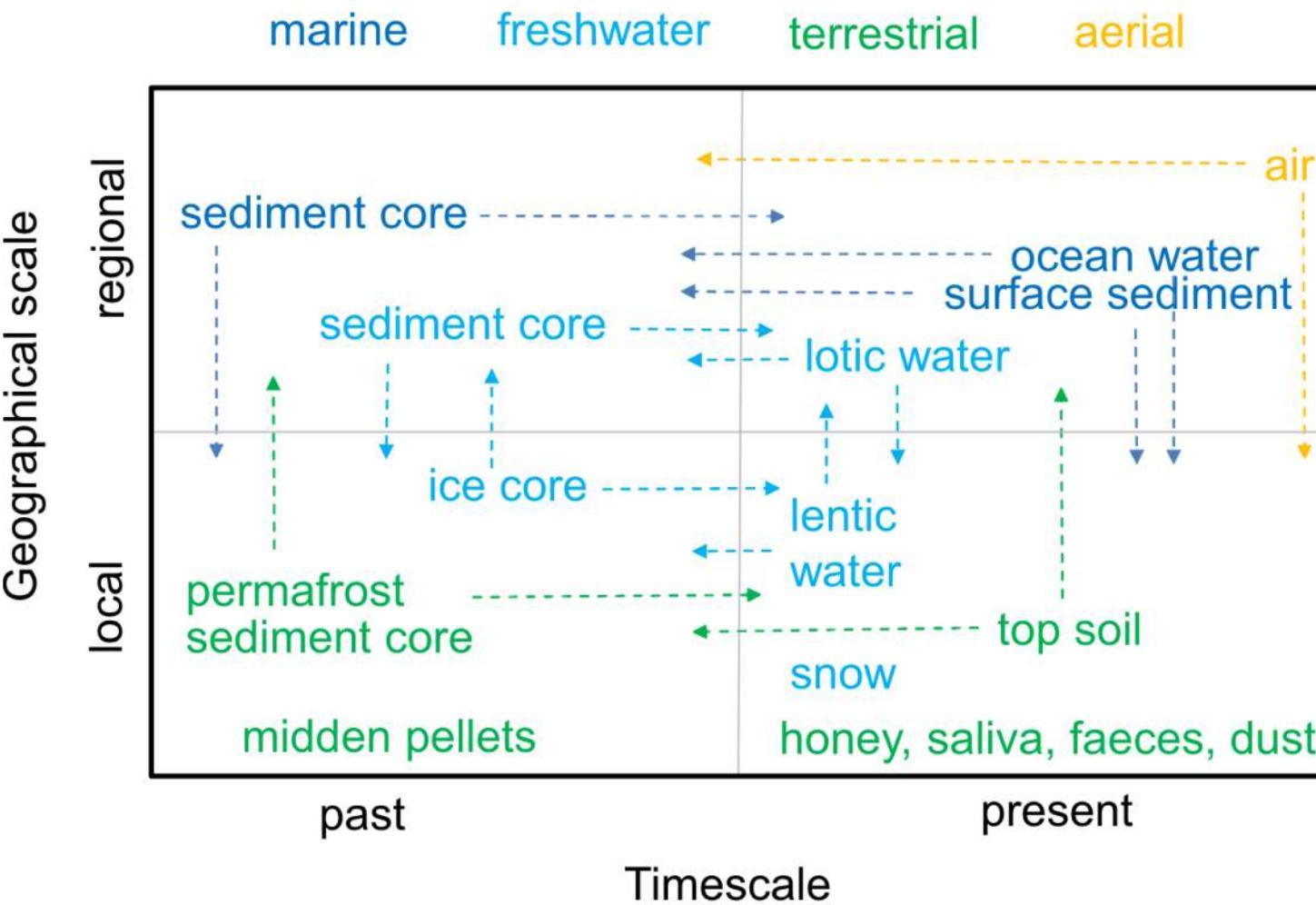
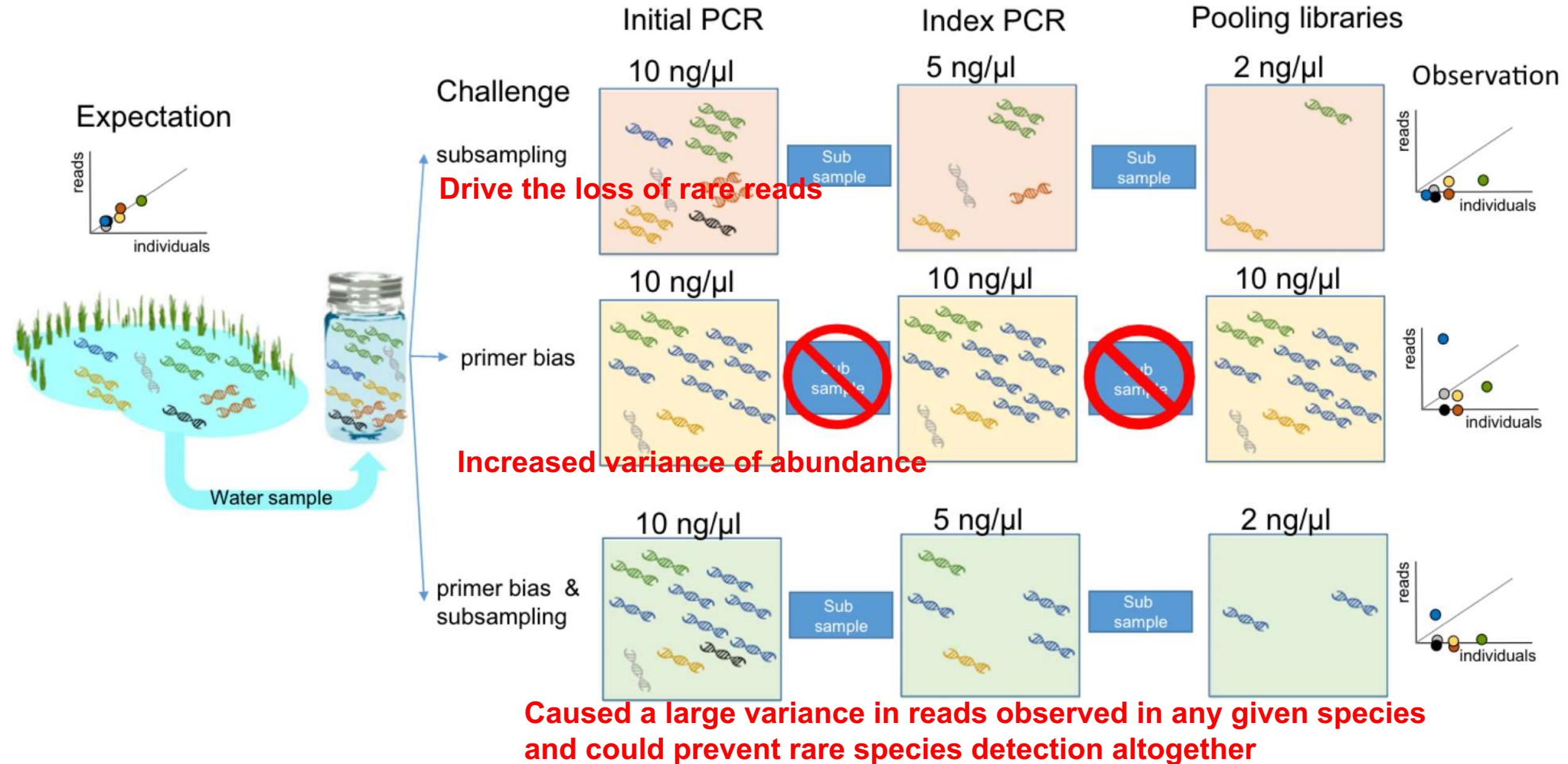


FIGURE 1 Environmental DNA sample types have different spatial and temporal scopes of inference from different habitats. Consider each sample type as a single sample from that environment. Placement of a sample type in a quadrant is not quantitative, but represents a common scale at which it has been used. Dashed arrows indicate the potential for a sample type to confer information at multiple scales of inference, but additional research to quantify these possibilities is needed



Database error

At Least 1 in 20 16S rRNA Sequence Records Currently Held in Public Repositories Is Estimated To Contain Substantial Anomalies

Kevin E. Ashelford,^{1*} Nadia A. Chuzhanova,³ John C. Fry,¹ Antonia J. Jones,² and Andrew J. Weightman¹

Cardiff School of Biosciences, Cardiff University, Main Building, Park Place, P.O. Box 915, Cardiff CF10 3TL, United Kingdom¹; Cardiff School of Computer Science, Cardiff University, Queen's Buildings, 5 The Parade, Roath, Cardiff CF24 3AA, United Kingdom²; and Biostatistics and Bioinformatics Unit and Institute of Medical Genetics, Cardiff School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom³

Received 7 April 2005/Accepted 28 July 2005

A new method for detecting chimeras and other anomalies within 16S rRNA sequence records is presented. Using this method, we screened 1,399 sequences from 19 phyla, as defined by the Ribosomal Database Project, release 9, update 22, and found 5.0% to harbor substantial errors. Of these, 64.3% were obvious chimeras, 14.3% were unidentified sequencing errors, and 21.4% were highly degenerate. In all, 11 phyla contained obvious chimeras, accounting for 0.8 to 11% of the records for these phyla. Many chimeras (43.1%) were formed from parental sequences belonging to different phyla. While most comprised two fragments, 13.7% were composed of at least three fragments, often from three different sources. A separate analysis of the *Bacteroidetes* phylum (2,739 sequences) also revealed 5.8% records to be anomalous, of which 65.4% were apparently chimeric. Overall, we conclude that, as a conservative estimate, 1 in every 20 public database records is likely to be corrupt. Our results support concerns recently expressed over the quality of the public repositories. With 16S rRNA sequence data increasingly playing a dominant role in bacterial systematics and environmental biodiversity studies, it is vital that steps be taken to improve screening of sequences prior to submission. To this end, we have implemented our method as a program with a simple-to-use graphic user interface that is capable of running on a range of computer platforms. The program is called Pintail, is released under the terms of the GNU General Public License open source license, and is freely available from our website at <http://www.cardiff.ac.uk/biosi/research/biosoft/>.

Extraction protocol matters



Soil Biology & Biochemistry 36 (2004) 1607–1614

Soil Biology &
Biochemistry

www.elsevier.com/locate/soilbio

Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis

Julia R. de Liphay^{a,b}, Christiane Enzinger^{b,1}, Kaare Johnsen^{a,2}, Jens Aamand^a,
Søren J. Sørensen^{b,*}

^aDepartment of Geochemistry, Geological Survey of Denmark and Greenland, Øster Voldgade 10, DK-1350 Copenhagen K, Denmark

^bDepartment of Microbiology, University of Copenhagen, Sølygade 83H, DK-1307 Copenhagen K, Denmark

Received 1 September 2003; received in revised form 6 March 2004; accepted 15 March 2004

Abstract

The impact of DNA extraction protocol on soil DNA yield and bacterial community composition was evaluated. Three different procedures to physically disrupt cells were compared: sonication, grinding-freezing-thawing, and bead beating. The three protocols were applied to three different topsoils. For all soils, we found that each DNA extraction method resulted in unique community patterns as measured by denaturing gradient gel electrophoresis. This indicates the importance of the DNA extraction protocol on data for evaluating soil bacterial diversity. Consistently, the bead-beating procedure gave rise to the highest number of DNA bands, indicating the highest number of bacterial species. Supplementing the bead-beating procedure with additional cell-rupture steps generally did not change the bacterial community profile. The same consistency was not observed when evaluating the efficiency of the different methods on soil DNA yield. This parameter depended on soil type. The DNA size was of highest molecular weight with the sonication and grinding-freezing-thawing procedures (approx. 20 kb). In contrast, the inclusion of bead beating resulted in more sheared DNA (approx. 6–20 kb), and the longer the bead-beating time, the higher the fraction of low-molecular weight DNA. Clearly, the choice of DNA extraction protocol depends on soil type. We found, however, that for the analysis of indigenous soil bacterial communities the bead-beating procedure was appropriate because it is fast, reproducible, and gives very pure DNA of relatively high molecular weight. And very importantly, with this protocol the highest soil bacterial diversity was obtained. We believe that the choice of DNA extraction protocol will influence not only the determined phylogenetic diversity of indigenous microbial communities, but also the obtained functional diversity. This means that the detected presence of a functional gene... and thus the indication of enzyme activity... may depend on the nature of the applied DNA extraction procedure.

“we found that each DNA extraction method resulted in unique community patterns”

Wesolowska-Andersen et al. *Microbiome* 2014, 2:19
<http://www.microbiomjournal.com/content/2/1/19>



Microbiome

Open Access

RESEARCH

Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis

Agata Wesolowska-Andersen¹, Martin Iain Bah², Vera Carvalho², Karsten Kristiansen³, Thomas Sicheritz-Pontén¹, Ramneek Gupta^{1*} and Tine Rask Licht^{2*}

Abstract

Background: In recent years, studies on the human intestinal microbiota have attracted tremendous attention. Application of next generation sequencing for mapping of bacterial phylogeny and function has opened new doors to this field of research. However, little attention has been given to the effects of choice of methodology on the output resulting from such studies.

Results: In this study we conducted a systematic comparison of the DNA extraction methods used by the two major collaborative efforts: The European MetaHIT and the American Human Microbiome Project (HMP). Additionally, effects of homogenizing the samples before extraction were addressed. We observed significant differences in distribution of bacterial taxa depending on the method. While eukaryotic DNA was most efficiently extracted by the MetaHIT protocol, DNA from bacteria within the Bacteroidetes phylum was most efficiently extracted by the HMP protocol.

Conclusions: Whereas it is comforting that the inter-individual variation clearly exceeded the variation resulting from choice of extraction method, our data highlight the challenge of comparing data across studies applying different methodologies.

“We observed significant differences in distribution of bacterial taxa depending on the method.”

Alpha diversity is always overestimated

Table 1. Effect of quality filtering and clustering on diversity estimates (OTU number), error rate and data loss of pyrotags amplified from two regions of *E. coli* MG1655 16S rRNA genes.

Read filtering	Number of OTUs at percentage identity thresholds						% errorless reads	% reads used
	100	99	98	97	95	90		
5' forward (V1 and V2)								
Theoretical number	5	4	3	1	1	1		
No quality filtering	643	95	31	16	5	3	68.7	77.9
Reads with N's removed	600	85	29	14	4	3	69.8	76.7
Quality score-based filtering (% per-base error probability)								
3	638	92	31	13	3	3	68.9	77.7
2	632	90	30	14	3	3	69.0	77.6
1	609	79	24	9	3	3	69.1	77.3
0.5	562	66	15	7	3	3	70.7	75.3
0.2	469	30	6	3	3	3	73.2	70.8
0.1	372	26	5	3	3	3	77.8	57.8
3' reverse (V8)								
Theoretical number	1	1	1	1	1	1		
No quality filtering	385	43	13	7	5	4	84.6	94.4
Reads with N's removed	361	40	12	6	4	3	85.3	93.6
Quality score-based filtering (% per-base error probability)								
3	378	40	12	7	5	4	84.8	94.2
2	368	32	10	6	5	4	85.1	93.8
1	342	25	9	6	5	4	85.3	93.3
0.5	310	20	8	6	5	4	87.5	89.5
0.2	236	7	2	2	2	2	89.6	82.1
0.1	196	4	2	2	2	2	90.7	70.6

Diversity estimates should be considered relative to the theoretical number of OTUs from *E. coli*.

Kunin et al (2010)

Reagent and laboratory contamination

RESEARCH ARTICLE

Open Access

Reagent and laboratory contamination can critically impact sequence-based microbiome analyses

Susannah J Salter^{1*}, Michael J Cox², Elena M Turek², Szymon T Calus³, William O Cookson², Miriam F Moffatt², Paul Turner^{4,5}, Julian Parkhill¹, Nicholas J Loman³ and Alan W Walker^{1,6*}

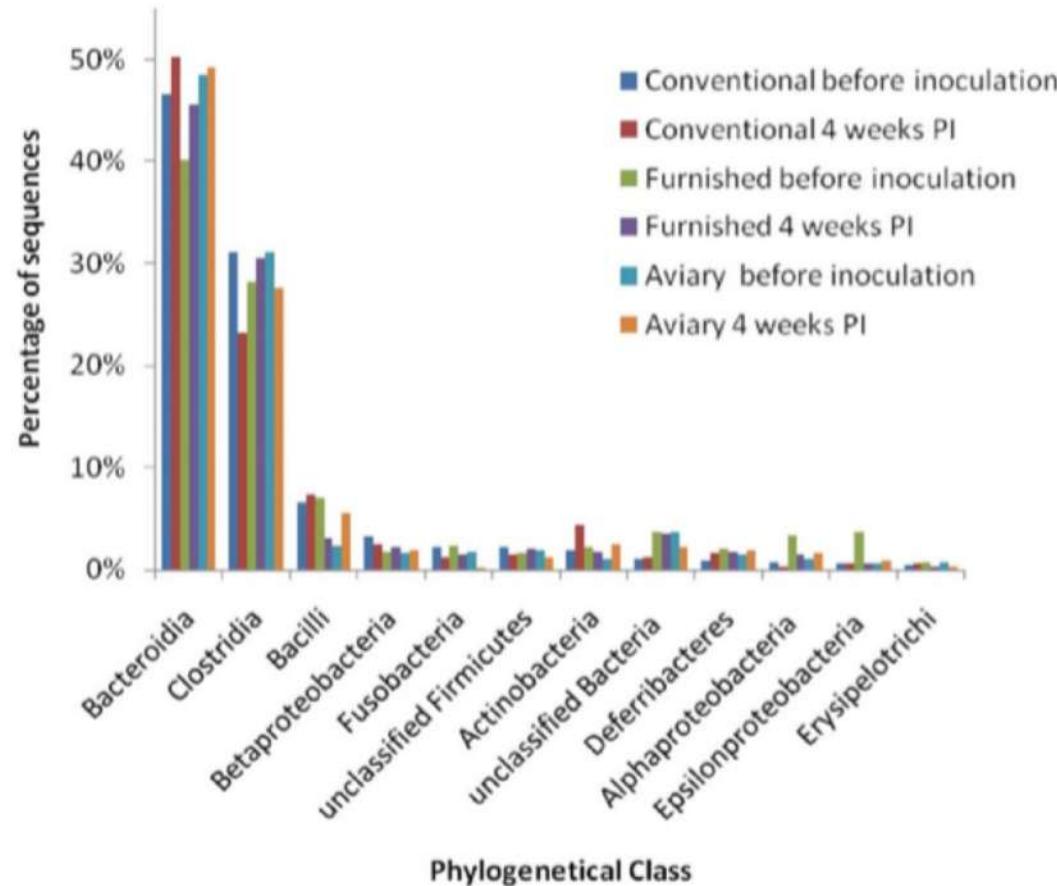
RESEARCH HIGHLIGHT

Tracking down the sources of experimental contamination in microbiome studies

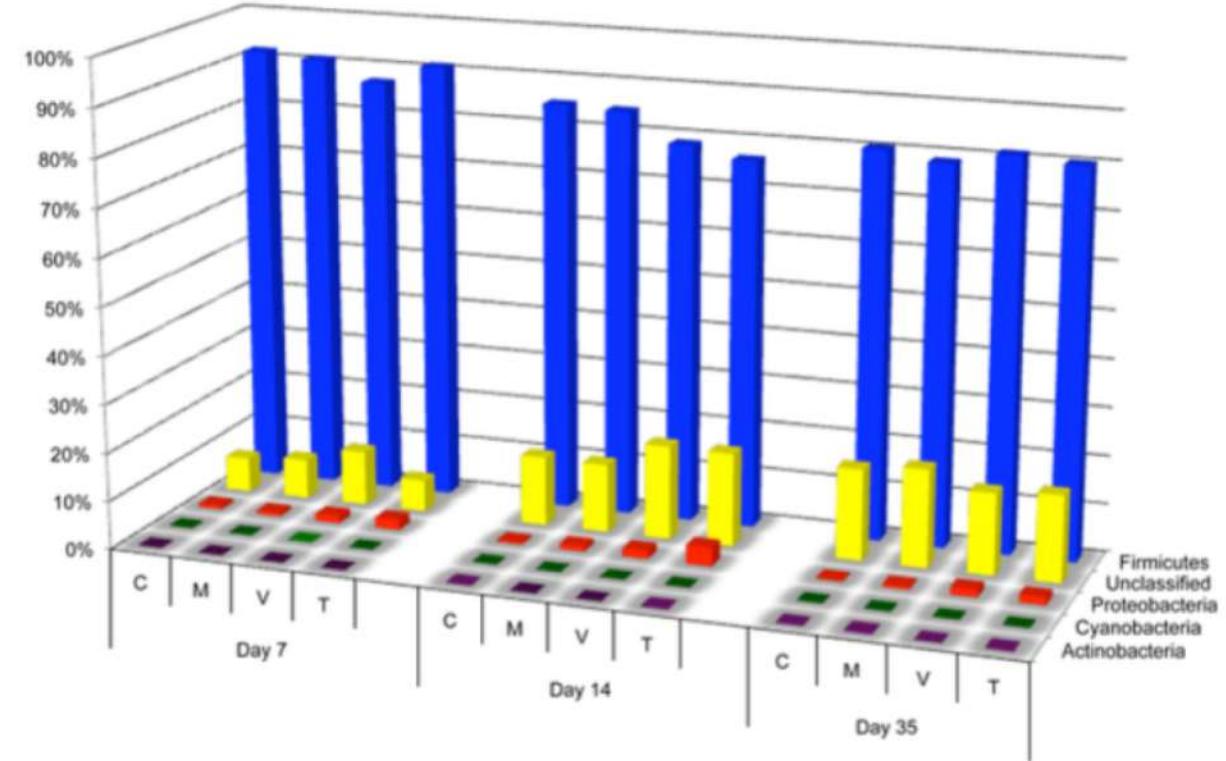
Sophie Weiss¹, Amnon Amir², Embriette R Hyde², Jessica L Metcalf², Se Jin Song² and Rob Knight^{2,3,4*}

2 papers with different results at the same year

Bacteroidetes >>> rest

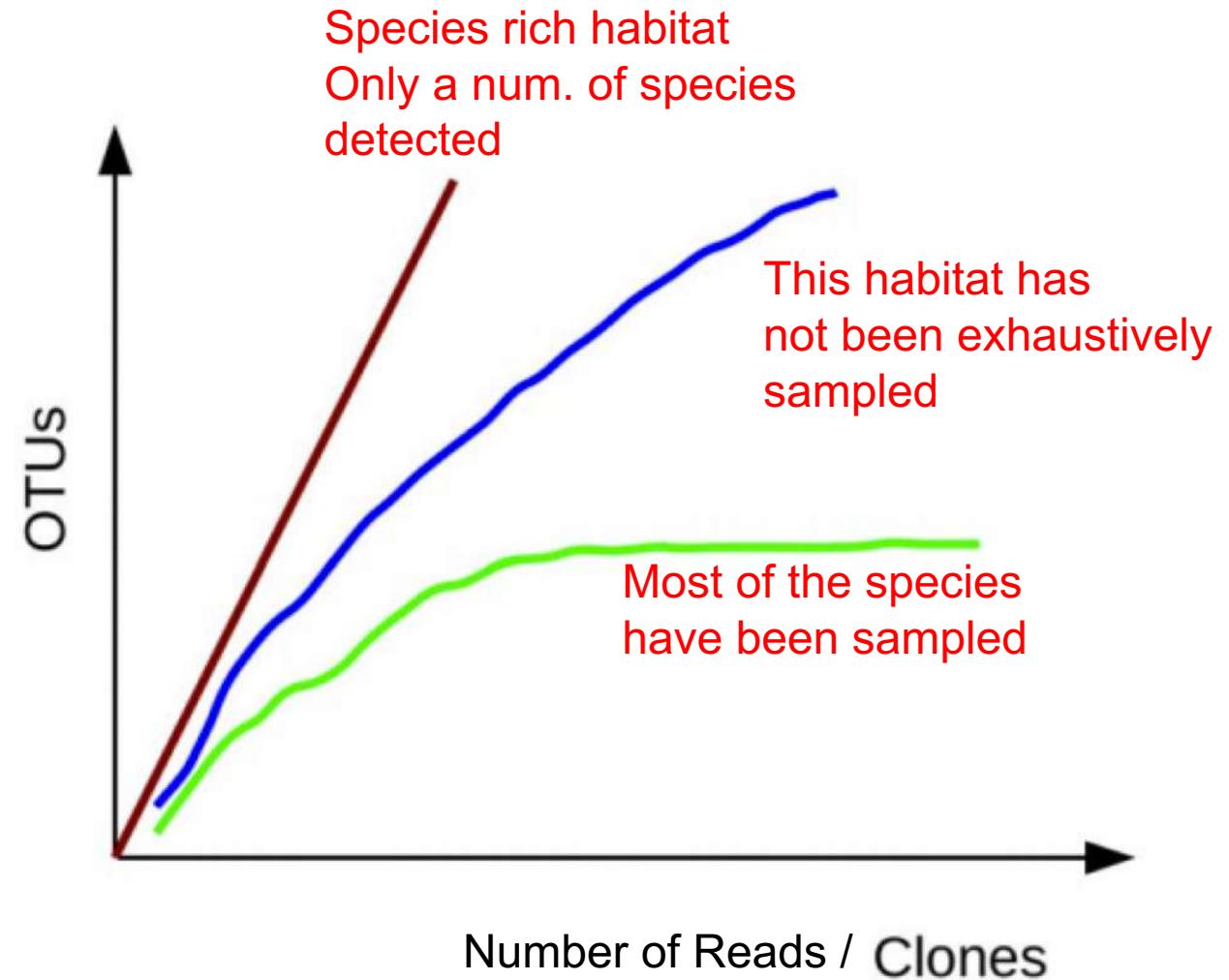


firmicutes >>> rest > bacteroidetes



Species sampling and Rarefaction

Rarefaction allows the calculation of **species richness** for a given number of individual samples, based on the construction of so-called **rarefaction curves**. This curve is a plot of the number of species as a function of the number of samples



But rarefying microbiome data is wrong

OPEN  ACCESS Freely available online

 PLOS COMPUTATIONAL BIOLOGY

Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible

Paul J. McMurdie, Susan Holmes*

Statistics Department, Stanford University, Stanford, California, United States of America

Current practice in the normalization of microbiome count data is inefficient in the statistical sense. Moreover, specific implementations for DNA sequencing read count data (based on a Negative Binomial model for instance) are already available in RNA-Seq focused R packages such as edgeR and DESeq.... We show how both proportions and rarefied counts result in a high rate of false positives in tests for species that are differentially abundant across sample classes. Regarding microbiome sample-wise clustering, we also show that the rarefying procedure often discards samples that can be accurately clustered by alternative methods. Based on these results and well-established statistical theory, **we advocate that investigators avoid rarefying altogether**. We have provided microbiome-specific extensions to these tools in the R package, phyloseq.

Original Abundance			Rarefied Abundance		
	A	B		A	B
OTU1	62	500	OTU1	62	50
OTU2	38	500	OTU2	38	50
Total	100	1000		100	100

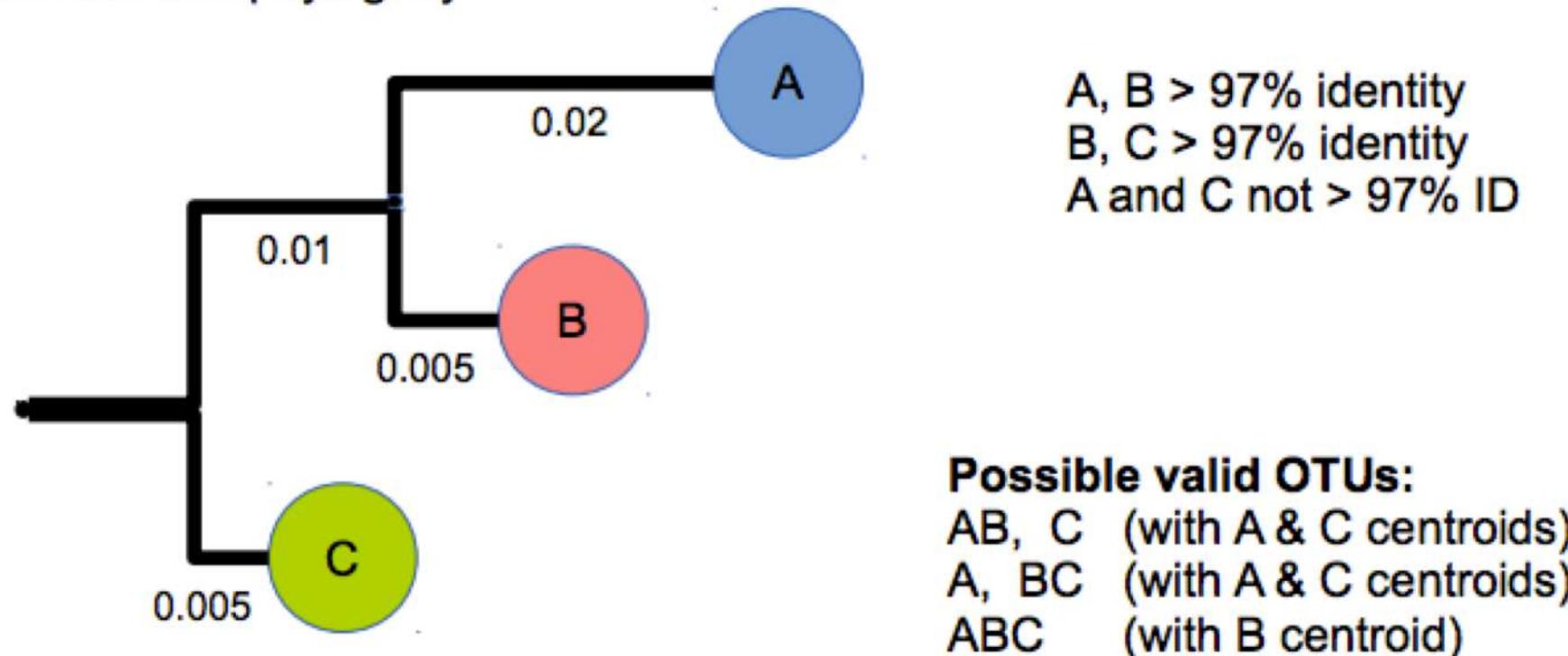
Standard Tests for Difference

P-value	chi-2	Prop	Fisher
Original	0.0290	0.0290	0.0272
Rarefied	0.1171	0.1171	0.1169

Figure 1. A minimal example of the effect of rarefying on statistical power. Hypothetical abundance data in its original (Top-Left) and rarefied (Top-Right) form, with corresponding formal test results for differentiation (Bottom).
doi:10.1371/journal.pcbi.1003531.g001

Logical inconsistency: OTUs at 97% ID

Assume the true phylogeny:



OTU pipelines will arbitrarily pick one of the three solutions.
Is this actually a problem??

PERSPECTIVE

Exact sequence variants should replace operational taxonomic units in marker-gene data analysis

Benjamin J Callahan¹, Paul J McMurdie² and Susan P Holmes³

¹Department of Population Health and Pathobiology, NC State University, Raleigh NC, USA; ²Whole Biome Inc, San Francisco CA, USA and ³Department of Statistics, Stanford University, Stanford CA, USA

DADA2: High-resolution sample inference from Illumina amplicon data

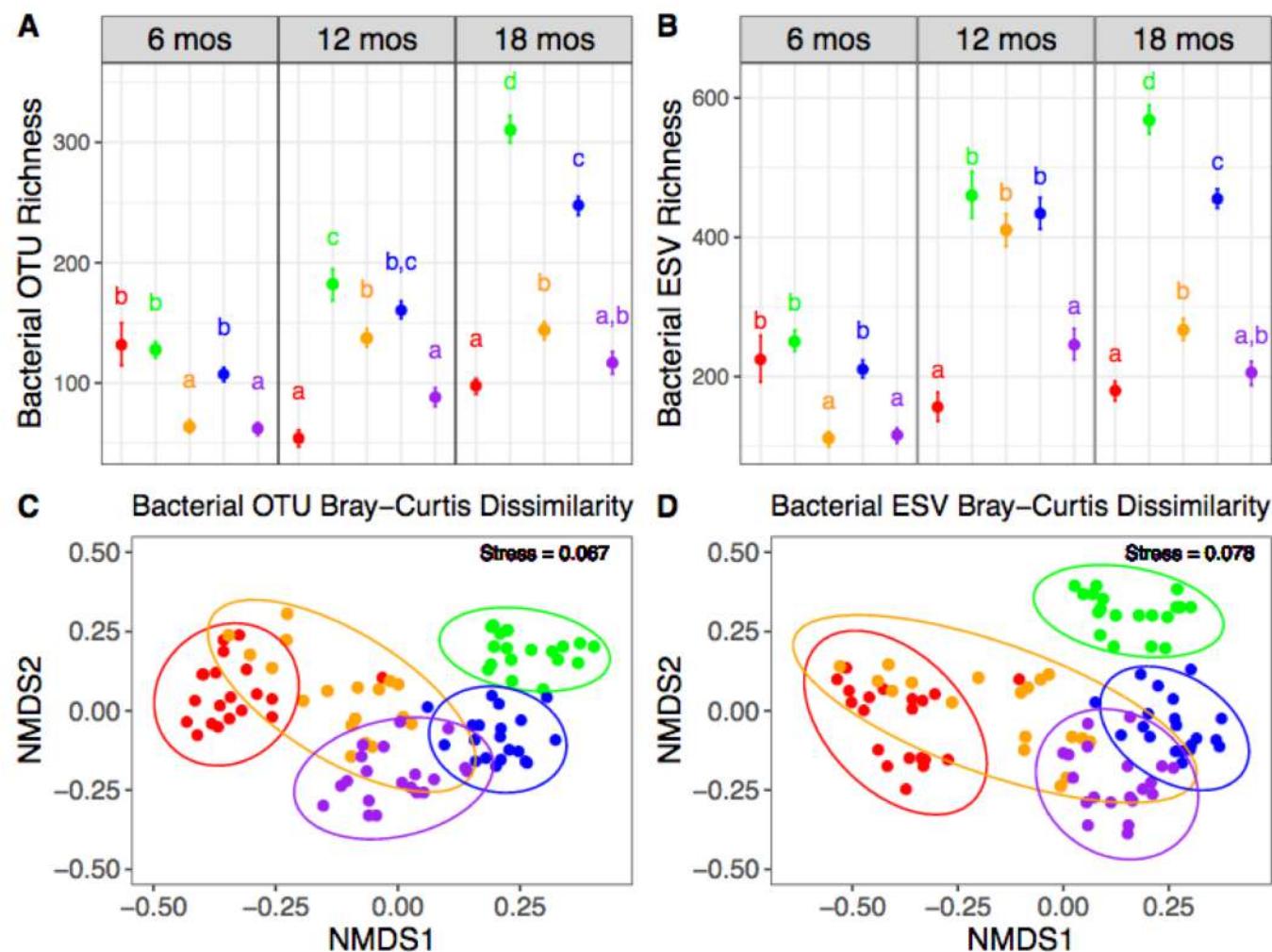
Benjamin J Callahan¹, Paul J McMurdie², Michael J Rosen³, Andrew W Han², Amy Jo A Johnson² & Susan P Holmes¹

We present the open-source software package DADA2 for modeling and correcting Illumina-sequenced amplicon errors (<https://github.com/benjjneb/dada2>). DADA2 infers sample sequences exactly and resolves differences of as little as 1 nucleotide. In several mock communities, DADA2 identified more real variants and output fewer spurious sequences than other methods. We applied DADA2 to vaginal samples from a cohort of pregnant women, revealing a diversity of previously undetected *Lactobacillus crispatus* variants.

Title: Ecological patterns are robust to use of exact sequence variants versus operational taxonomic units

Authors: Sydney I. Glassman and Jennifer B.H. Martiny

Despite quantitative differences in microbial richness, we found that all alpha- and beta-diversity metrics were highly positively correlated ($r > 0.90$) between samples analyzed with both approaches

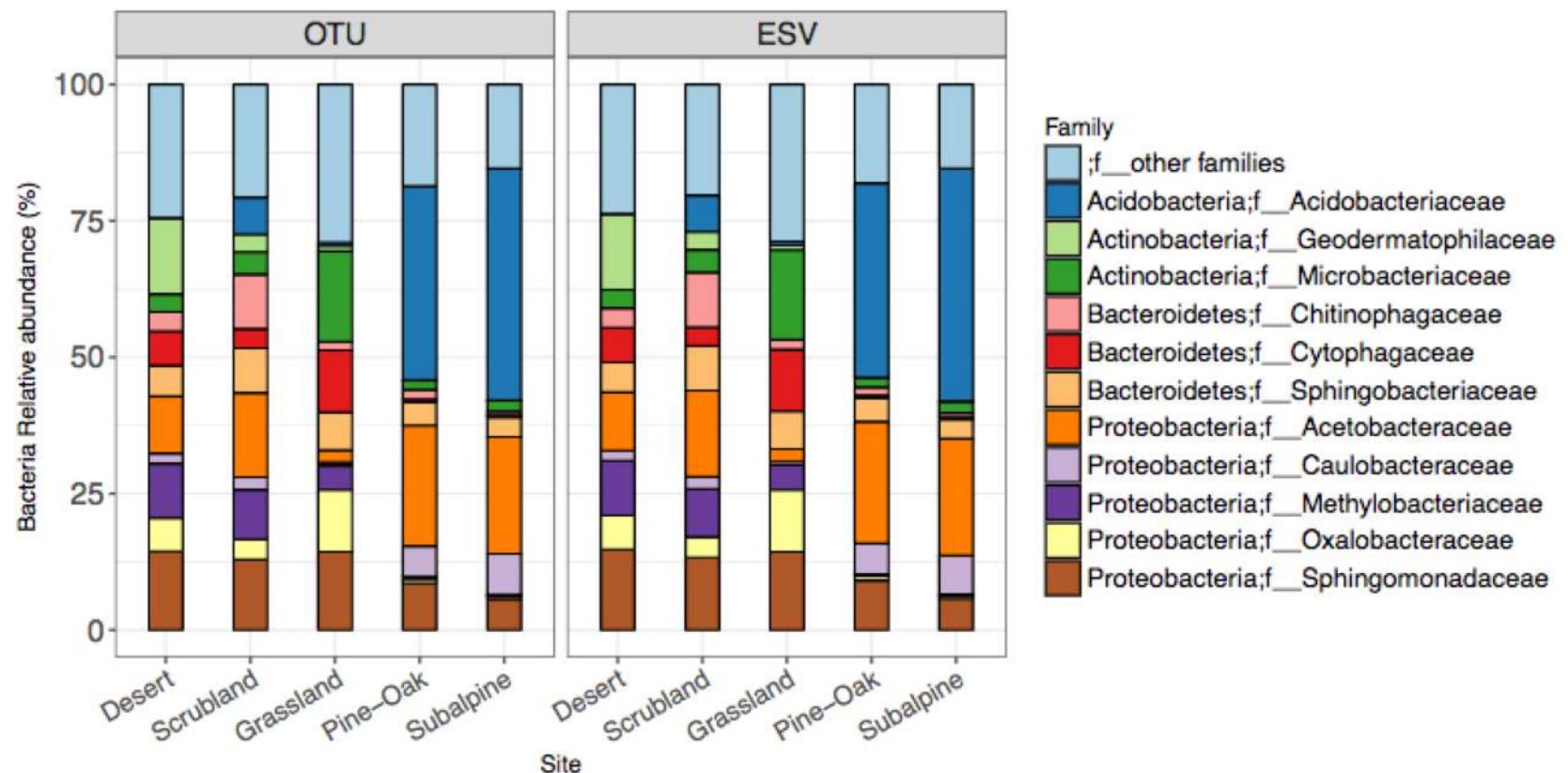


Title: Ecological patterns are robust to use of exact sequence variants versus operational taxonomic units

Authors: Sydney I. Glassman and Jennifer B.H. Martiny

Figure S2. Relative abundance of bacterial sequences per sample of the inoculum leaf litter from each site ($n=20$) for OTUs versus ESVs summarized by A) the 12 most abundant families or B) the 12 most abundant genera.

A)



Is it time to revisit bacterial taxonomy?

A proposal for a standardized bacterial taxonomy based on genome phylogeny

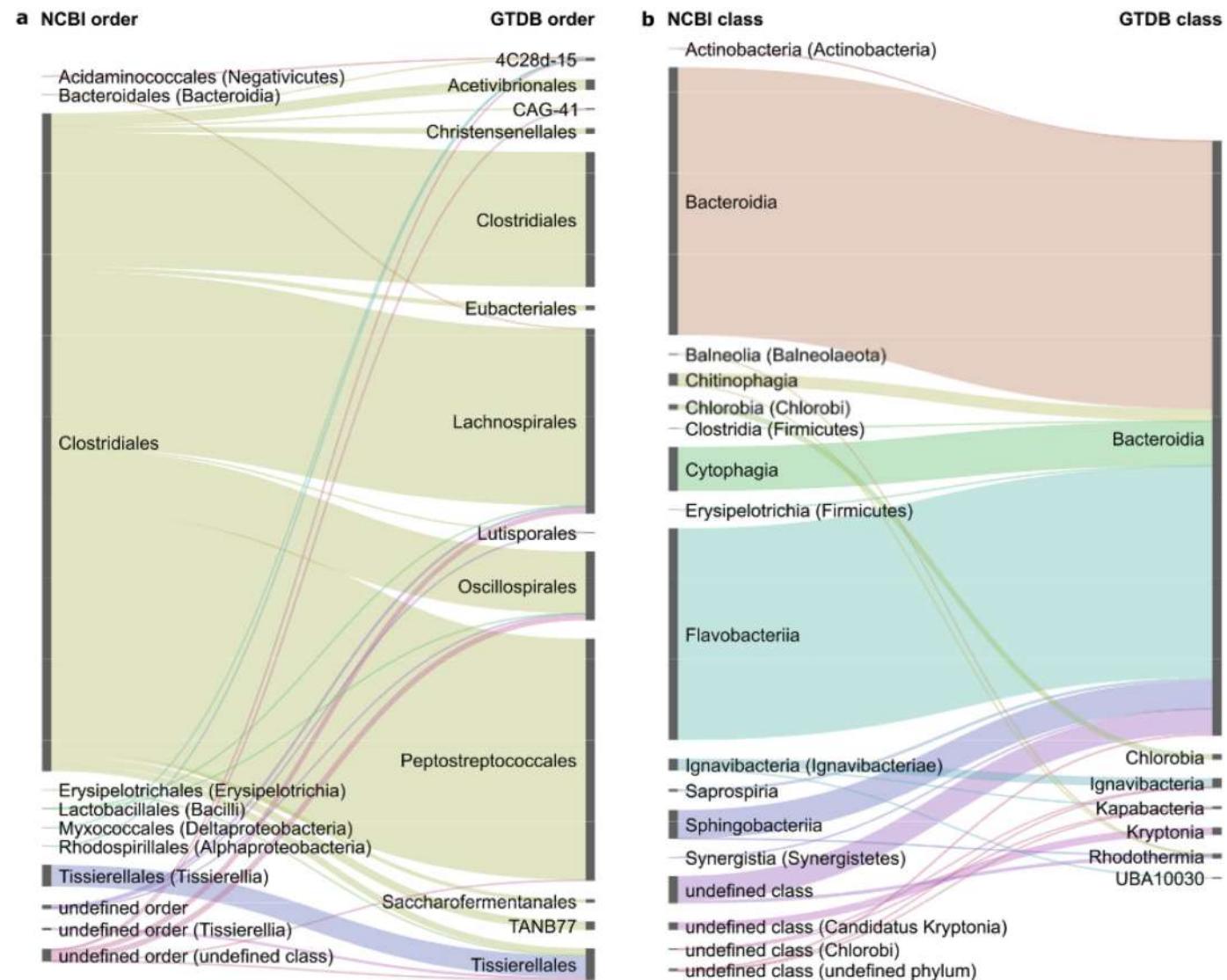
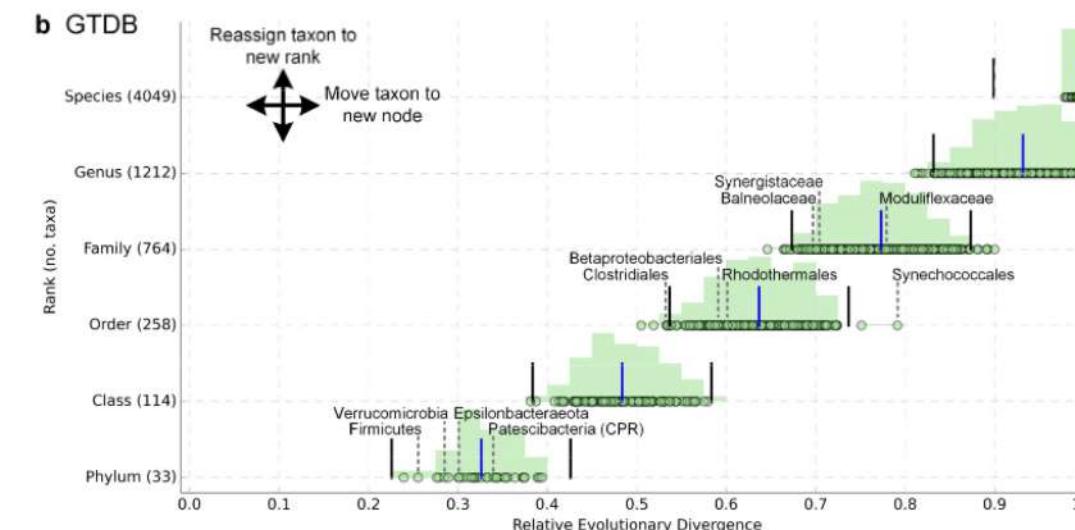
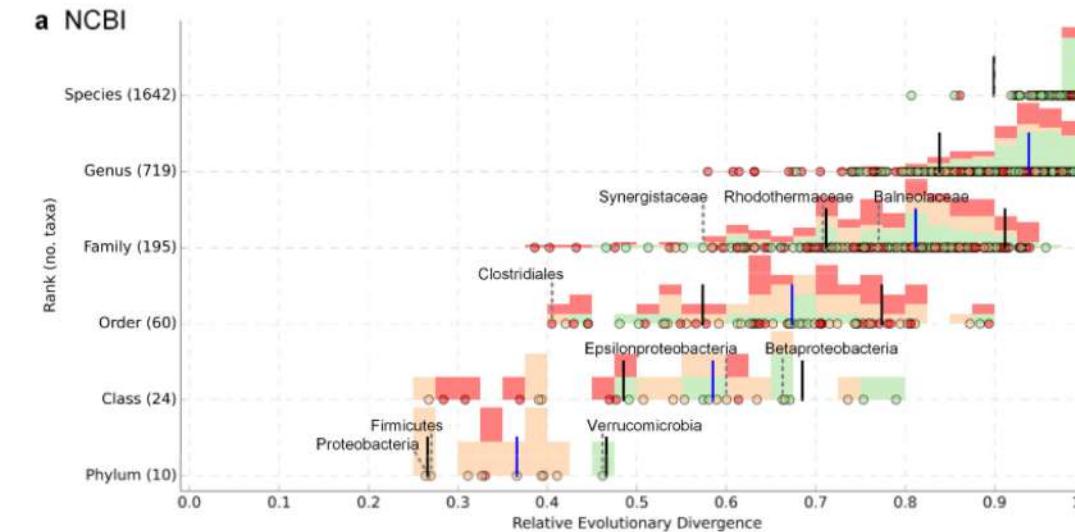
Donovan H. Parks¹, Maria Chuvochina¹, David W. Waite¹, Christian Rinke¹, Adam Skarszewski¹, Pierre-Alain Chaumeil¹, Philip Hugenholtz^{1*}

¹ Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, The University of Queensland, QLD 4072, Australia

* Correspondence to: p.hugenholtz@uq.edu.au

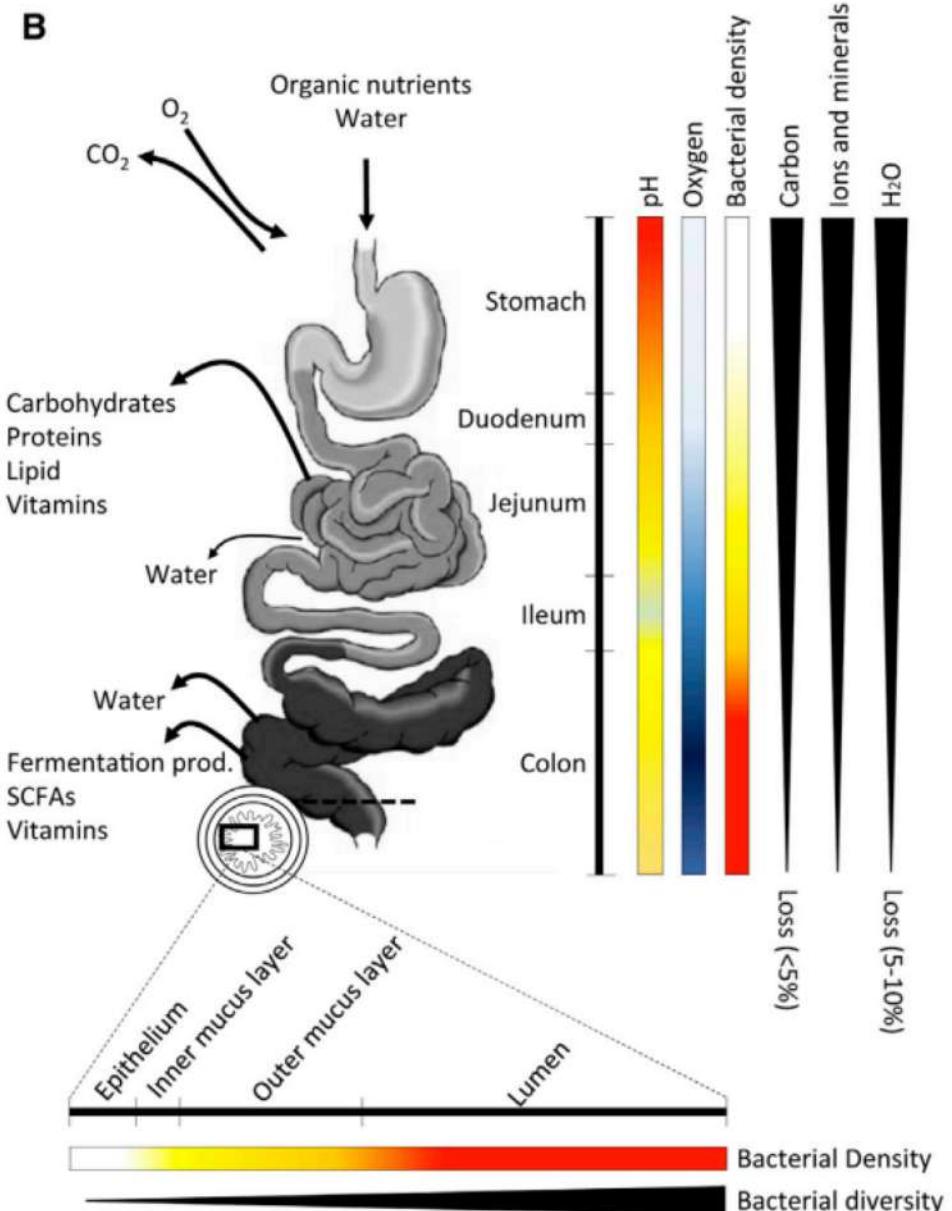
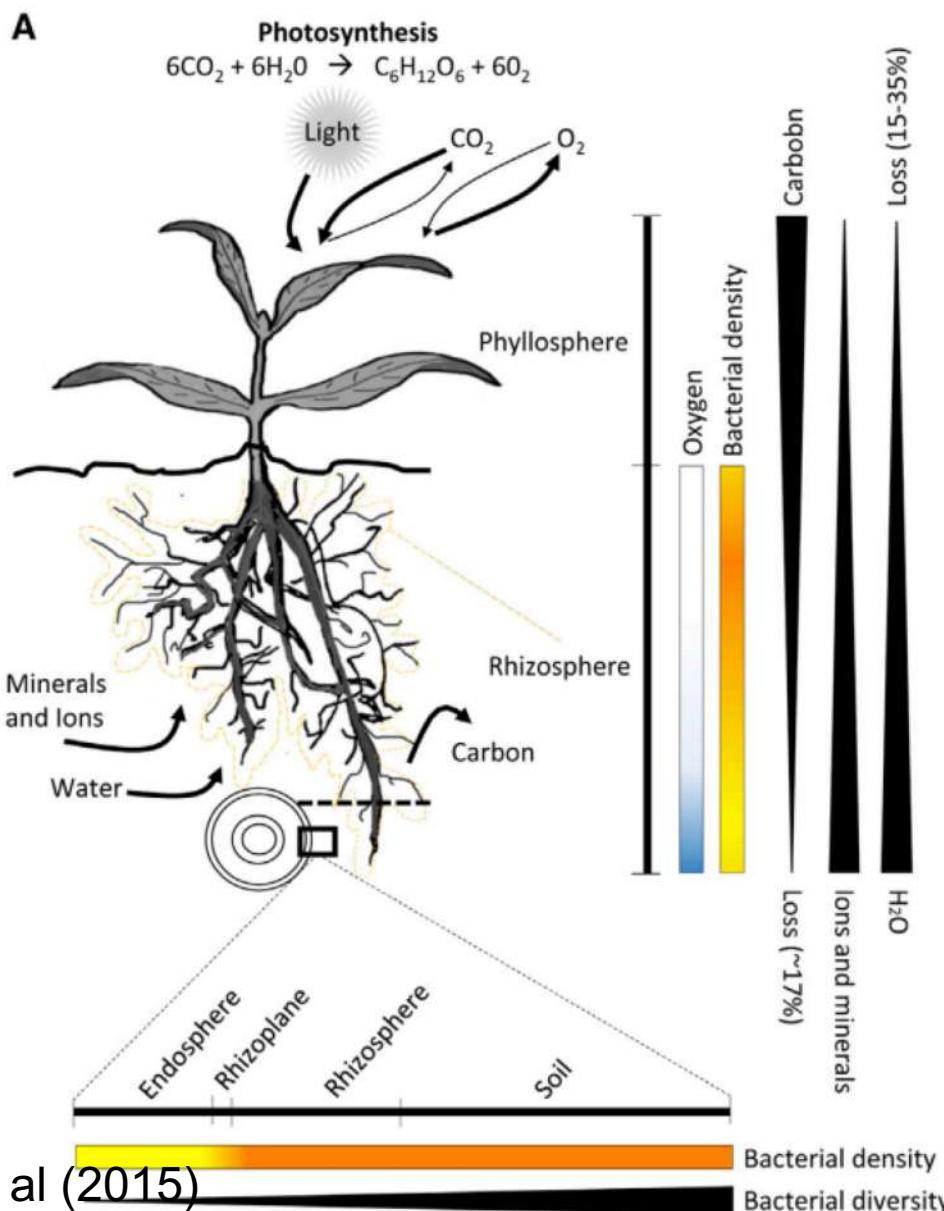
From 94,759 bacterial genomes, 99 phyla are described including six major normalized monophyletic units from the subdivision of the Proteobacteria, and amalgamation of the Candidate Phyla Radiation into the single phylum Patescibacteria. **In total, 73% of taxa had one or more changes to their existing taxonomy**

Is it time to revisit bacterial taxonomy?

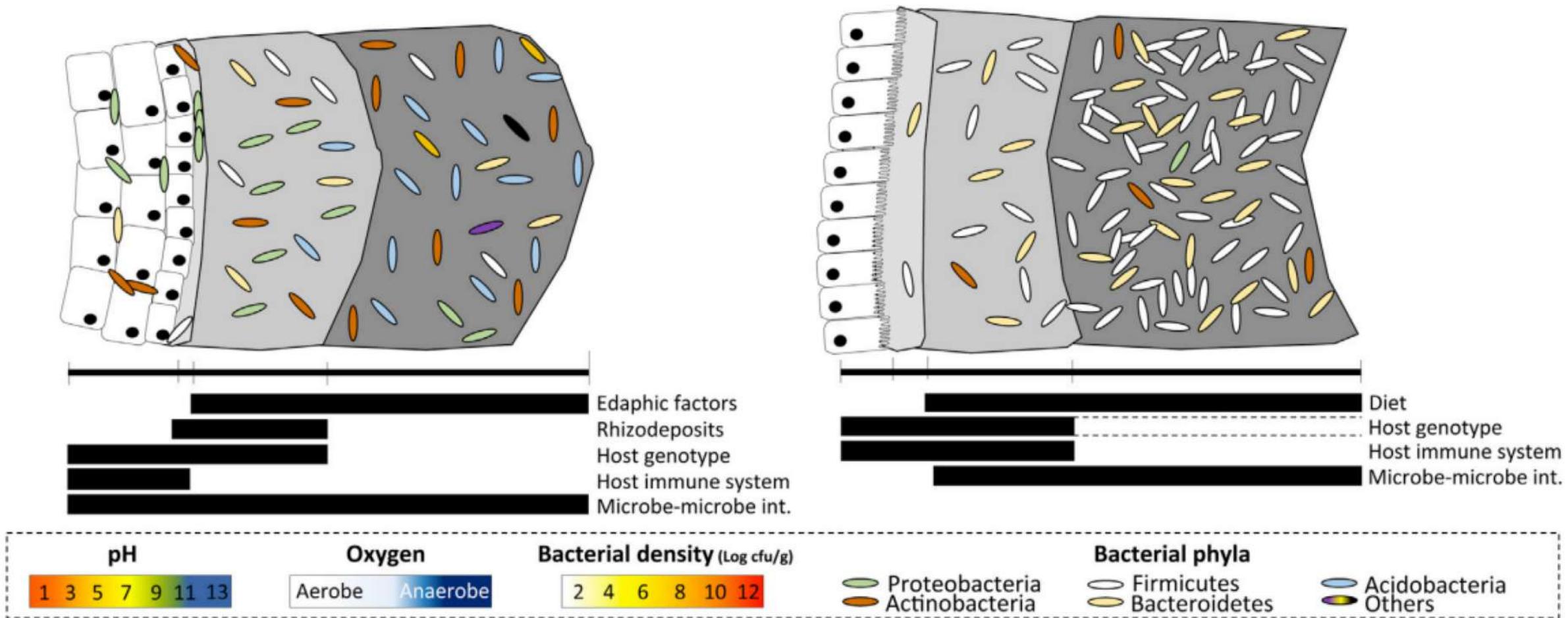


Case studies

Two most common systems



Two most common systems



Two most common systems

Table 1. Percentage of Shotgun Metagenome Reads Assigned to Each Kingdom of Life across Metagenome Studies

	Cucumber ^a	Wheat ^a	Soybean ^b	Wheat ^c	Oat ^c	Pea ^c	Barley ^d	Gut ^e
Bacteria	99.36	99.45	96	88.5	77.3	73.7	94.04	99.1
Archaea	0.02	0.02	<1	<0.5	<0.5	<0.5	0.054	
Eukaryotes	0.54	0.48	3	3.3	16.6	20.7	5.90	<0.1

^aOfek-Lalzar et al. (2014) (metagenomics of rhizoplane samples).

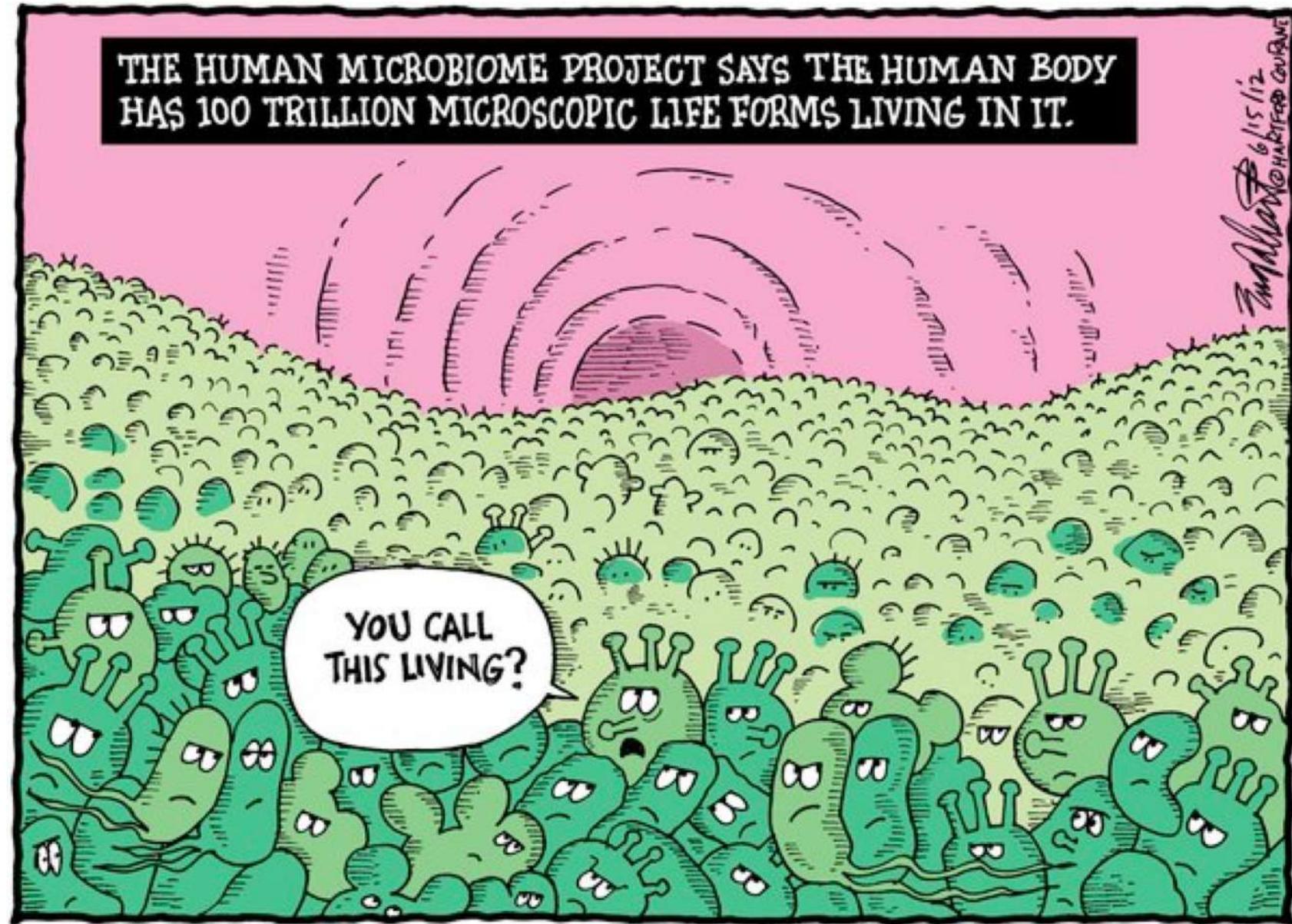
^bMendes et al. (2014) (metagenomics of rhizosphere samples).

^cTurner et al. (2013) (metatranscriptomics of rhizosphere samples).

^dBulgarelli et al. (2015) (metagenomics of rhizosphere samples).

^eQin et al. (2010) (metagenomics of gut samples).

Human gut microbiome



Human gut microbiome

Vol 464 | 4 March 2010 | doi:10.1038/nature08821

nature

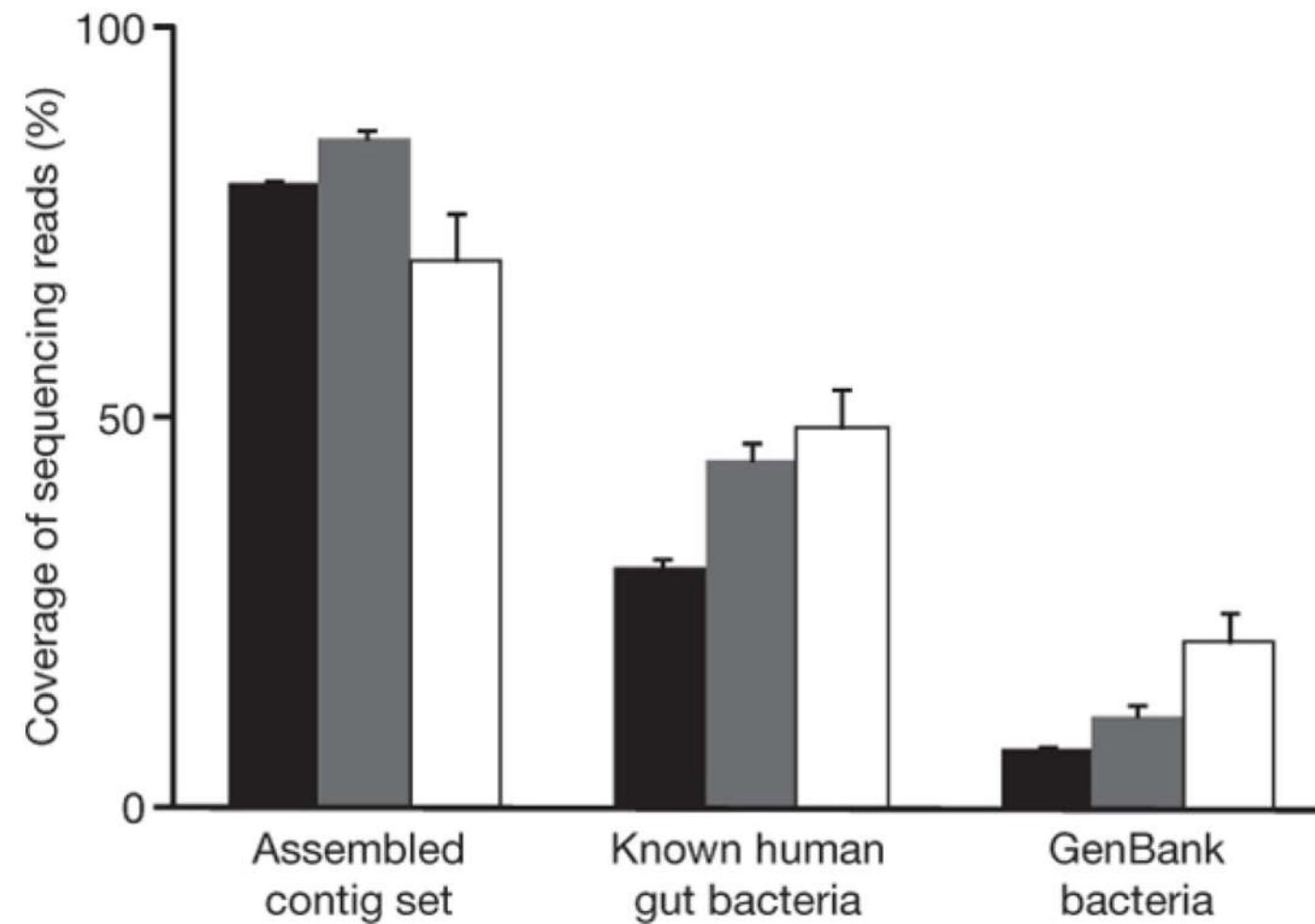
ARTICLES

A human gut microbial gene catalogue established by metagenomic sequencing

Junjie Qin^{1*}, Ruiqiang Li^{1*}, Jeroen Raes^{2,3}, Manimozhiyan Arumugam², Kristoffer Solvsten Burgdorf⁴, Chaysavanh Manichanh⁵, Trine Nielsen⁴, Nicolas Pons⁶, Florence Levenez⁶, Takuji Yamada², Daniel R. Mende², Junhua Li^{1,7}, Junming Xu¹, Shaochuan Li¹, Dongfang Li^{1,8}, Jianjun Cao¹, Bo Wang¹, Huiqing Liang¹, Huisong Zheng¹, Yinlong Xie^{1,7}, Julien Tap⁶, Patricia Lepage⁶, Marcelo Bertalan⁹, Jean-Michel Batto⁶, Torben Hansen⁴, Denis Le Paslier¹⁰, Allan Linneberg¹¹, H. Bjørn Nielsen⁹, Eric Pelletier¹⁰, Pierre Renault⁶, Thomas Sicheritz-Ponten⁹, Keith Turner¹², Hongmei Zhu¹, Chang Yu¹, Shengting Li¹, Min Jian¹, Yan Zhou¹, Yingrui Li¹, Xiuqing Zhang¹, Songgang Li¹, Nan Qin¹, Huanming Yang¹, Jian Wang¹, Søren Brunak⁹, Joel Doré⁶, Francisco Guarner⁵, Karsten Kristiansen¹³, Oluf Pedersen^{4,14}, Julian Parkhill¹², Jean Weissenbach¹⁰, MetaHIT Consortium†, Peer Bork², S. Dusko Ehrlich⁶ & Jun Wang^{1,13}

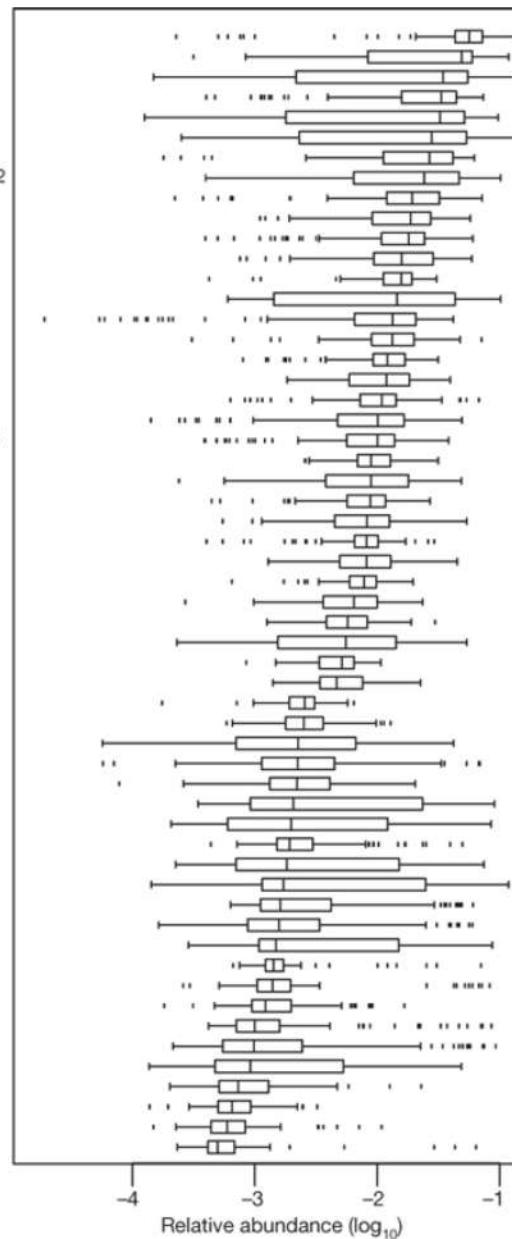
doi:10.1038/nature08821

Human gut microbiome

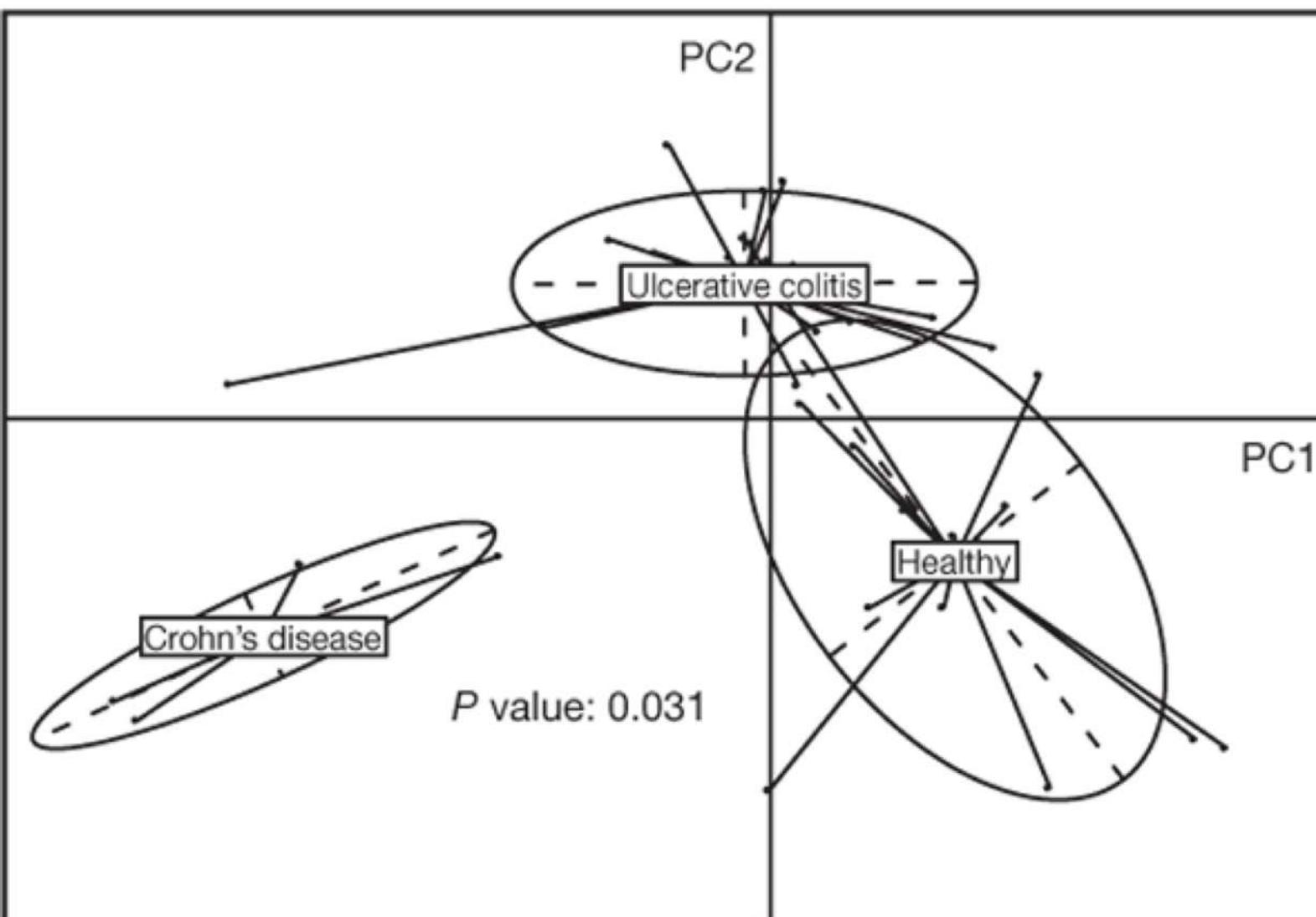


Human gut microbiome

Bacteroides uniformis
Alistipes putredinis
Parabacteroides merdae
Dorea longicatena
Ruminococcus bromii L2-63
Bacteroides caccae
Clostridium sp. SS2-1
Bacteroides thetaiotaomicron VPI-5482
Eubacterium hallii
Ruminococcus torques L2-14
Unknown sp. SS3 4
Ruminococcus sp. SR1 5
Faecalibacterium prausnitzii SL3 3
Ruminococcus lactaris
Collinsella aerofaciens
Dorea formicigenerans
Bacteroides vulgatus ATCC 8482
Roseburia intestinalis M50 1
Bacteroides sp. 2_1_7
Eubacterium siraeum 70 3
Parabacteroides distasonis ATCC 8503
Bacteroides sp. 9_1_42FAA
Bacteroides ovatus
Bacteroides sp. 4_3_47FAA
Bacteroides sp. 2_2_4
Eubacterium rectale M104 1
Bacteroides xylanisolvens XB1A
Coprococcus comes SL7 1
Bacteroides sp. D1
Bacteroides sp. D4
Eubacterium ventriosum
Bacteroides dorei
Ruminococcus obeum A2-162
Subdoligranulum variabile
Bacteroides capillosus
Streptococcus thermophilus LMD-9
Clostridium leptum
Holdemania filiformis
Bacteroides stercoris
Coprococcus eutactus
Clostridium sp. M62 1
Bacteroides eggertthii
Butyrivibrio crossotus
Bacteroides finegoldii
Parabacteroides johnsonii
Clostridium sp. L2-50
Clostridium nexile
Bacteroides pectinophilus
Anaerotruncus colihominis
Ruminococcus gnavus
Bacteroides intestinalis
Bacteroides fragilis 3_1_12
Clostridium asparagiforme
Enterococcus faecalis TX0104
Clostridium scindens
Blautia hansenii



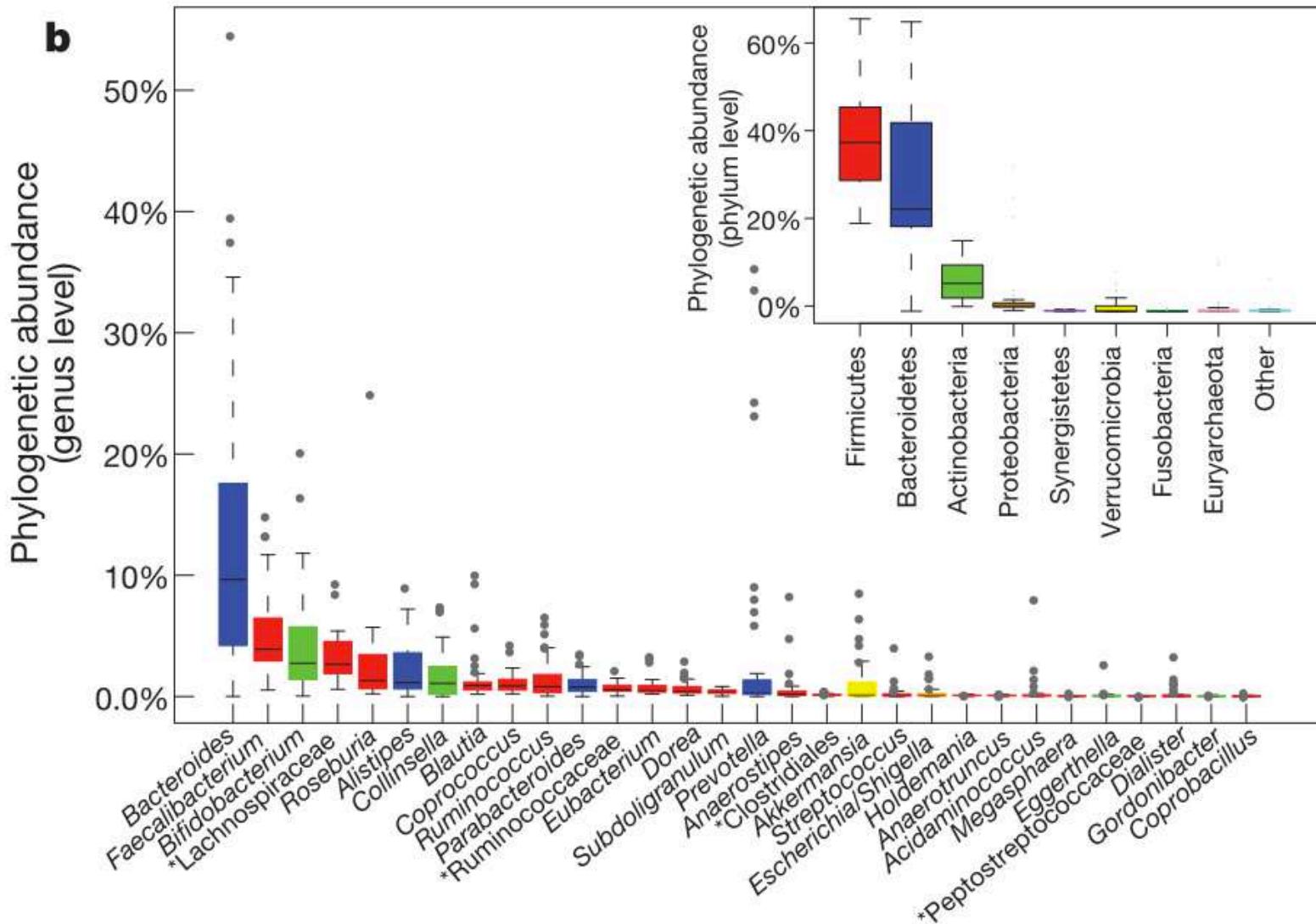
Human gut microbiome



We can check which OTUs constitute the clustering (and separation) patterns

- > Biology
- > Biomarkers

Human gut microbiome



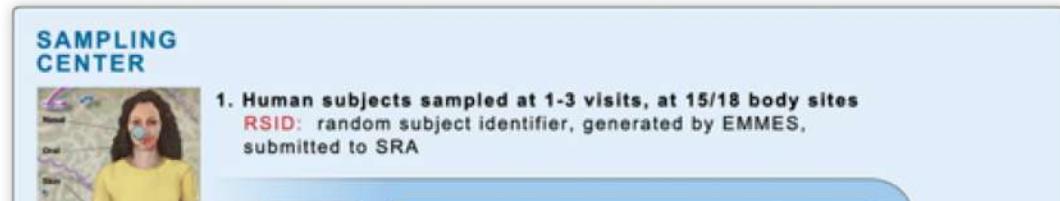


Table 1 | HMP donor samples examined by 16S and WGS

Body region	Body site	Total samples	Total 16S samples	V13 samples	V13 read depth (M)*	V35 samples	V35 read depth (M)*	Samples V13 and V35	Total WGS samples	Total read depth (G)†	Filtered reads (%)‡	Human reads (%)§	Remaining read depth (G)†	Samples 16S and WGS
Gut	Stool	352	337	193	1.4	328	2.4	184	136	1,720.7	15	1	1,450.6	124
Oral cavity	Buccal mucosa	346	330	184	1.3	314	1.7	168	107	1,438.0	9	82	136.7	91
	Hard palate	325	325	179	1.2	310	1.7	164	1	10.9	20	25	5.9	1
	Keratinized gingiva	335	329	183	1.3	319	1.7	173	6	72.3	5	47	34.4	0
	Palatine tonsils	337	332	189	1.2	315	1.9	172	6	74.8	2	80	13.5	1
	Saliva	315	310	166	0.9	292	1.5	148	5	55.7	1	91	4.2	0
	Subgingival plaque	334	328	186	1.2	314	1.8	172	7	92.1	5	79	15.3	1
	Supragingival plaque	345	331	192	1.3	316	1.9	177	115	1,500.7	15	40	674.8	101
	Throat	331	325	176	1.0	312	1.7	163	7	78.8	4	79	13.6	1
Airway	Tongue dorsum	348	332	193	1.3	320	2.0	181	122	1,620.1	15	19	1,084.3	106
Airway	Anterior nares	316	302	169	1.0	283	1.2	150	84	1,129.9	3	96	14.3	70
Skin	Left antecubital fossa	269	269	158	0.7	221	0.5	110	0	NA	NA	NA	0	NA
	Left retroauricular crease	313	312	188	1.6	295	1.5	171	9	126.3	9	73	22.1	8
	Right antecubital fossa	274	274	158	0.7	229	0.5	113	0	NA	NA	NA	0	NA
Vagina	Right retroauricular crease	319	316	190	1.4	304	1.6	178	15	181.9	18	59	42.4	12
	Mid-vagina	145	143	91	0.6	140	1.0	88	2	22.6	0	99	0.2	0
	Posterior fornix	152	142	89	0.6	136	1.0	83	53	702.1	6	90	25.2	43
	Vaginal introitus	142	140	87	0.6	131	0.9	78	3	36.5	1	98	0.6	1
Total		5,298	5,177	2,971	19	4,879	26.3	2,673	681	8,863.3	11	49	3,538.1	560

NCBI

6. Data submitted to NCBI Sequence Read Archives (SRA)

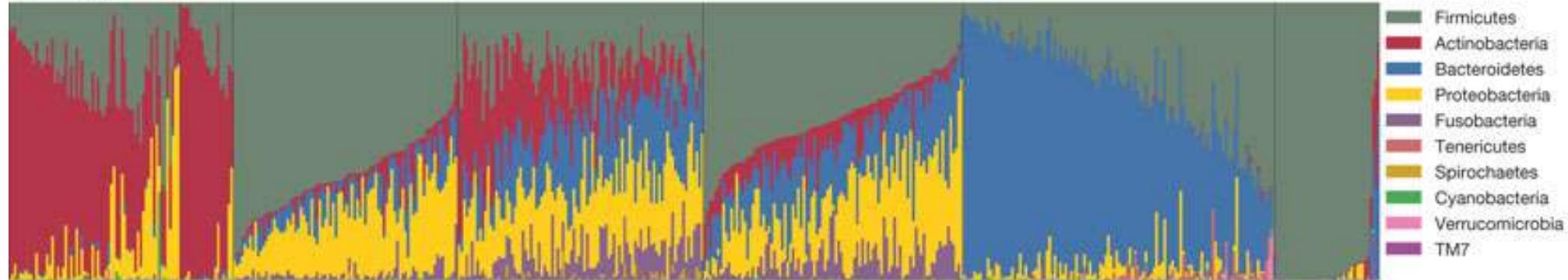
SRX: sequencing experiment

SRR: sequence run

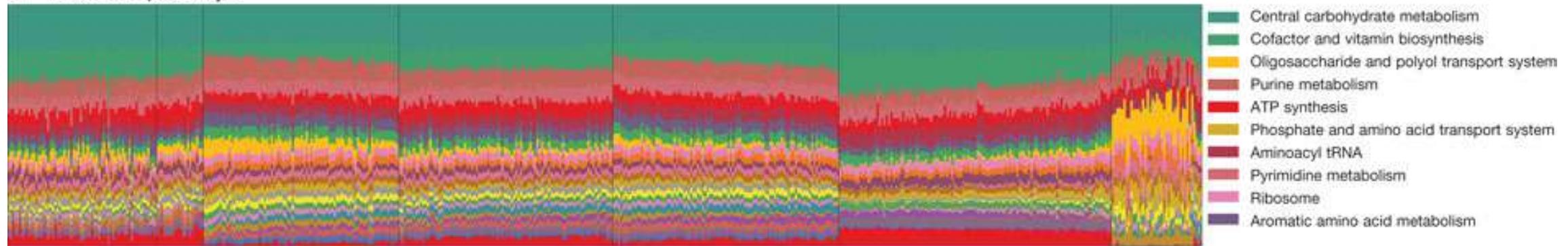
SRS: sequencing sample (maps to SN)

Human microbiome

a Phyla



b Metabolic pathways



Anterior nares

RC

Buccal mucosa

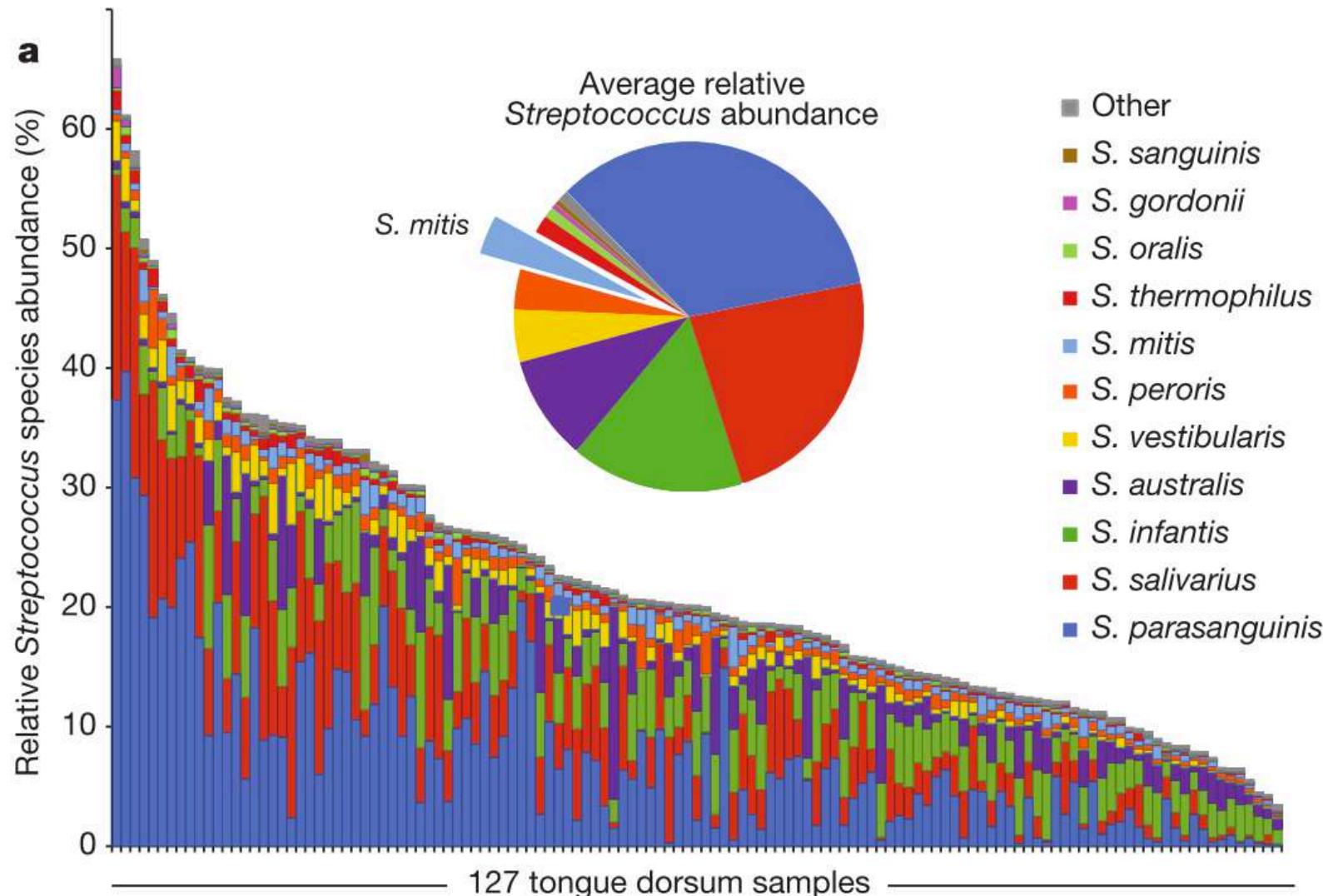
Supragingival plaque

Tongue dorsum

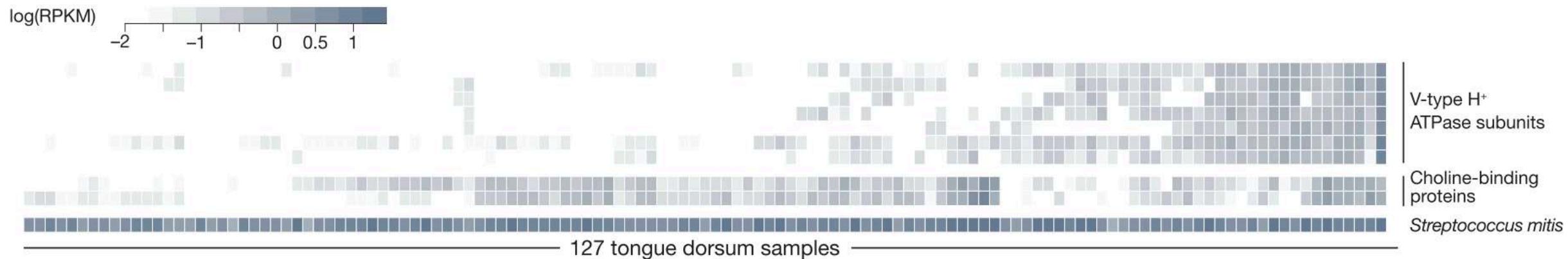
Stool

Posterior fornix

Inter-individual variation in the microbiome proved to be specific, functionally relevant and personalized



Gene loss & Structural variants are common



Skins



Genus level

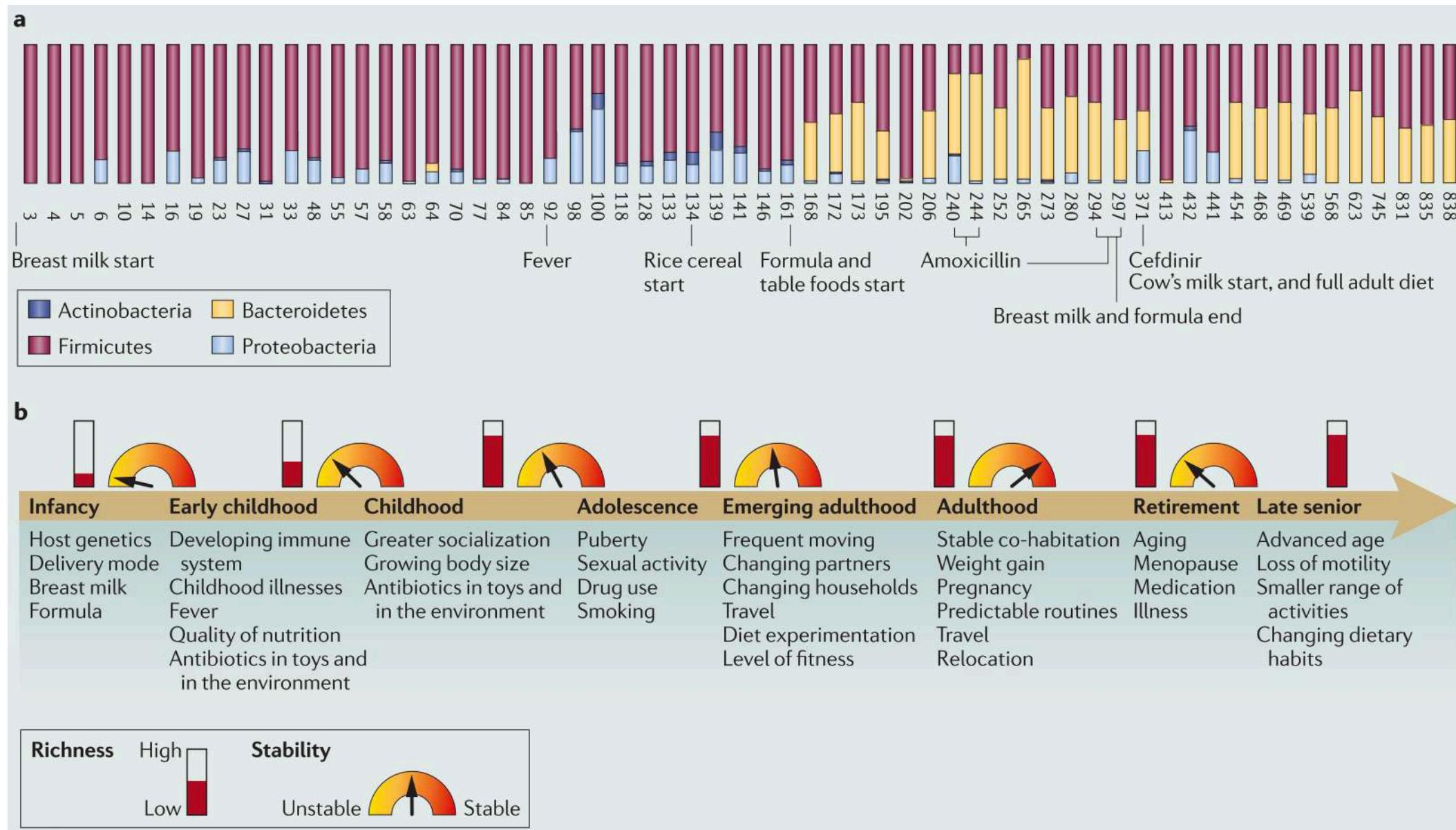
Ascomycetes

- Arthrodermataceae
- Aspergillus
- Candida
- Chrysosporium
- Epicoccum
- Leptosphaerulina
- Penicillium
- Phoma
- Saccharomyces

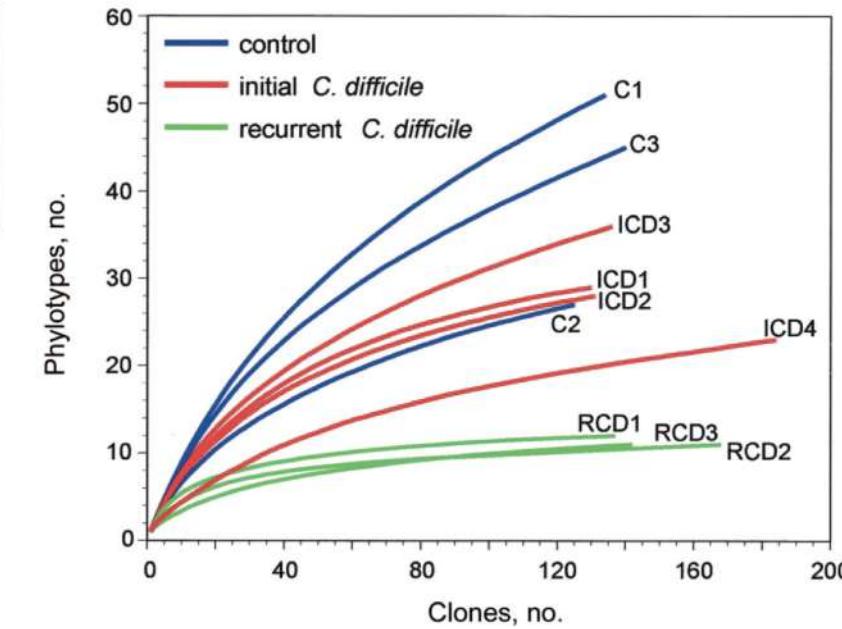
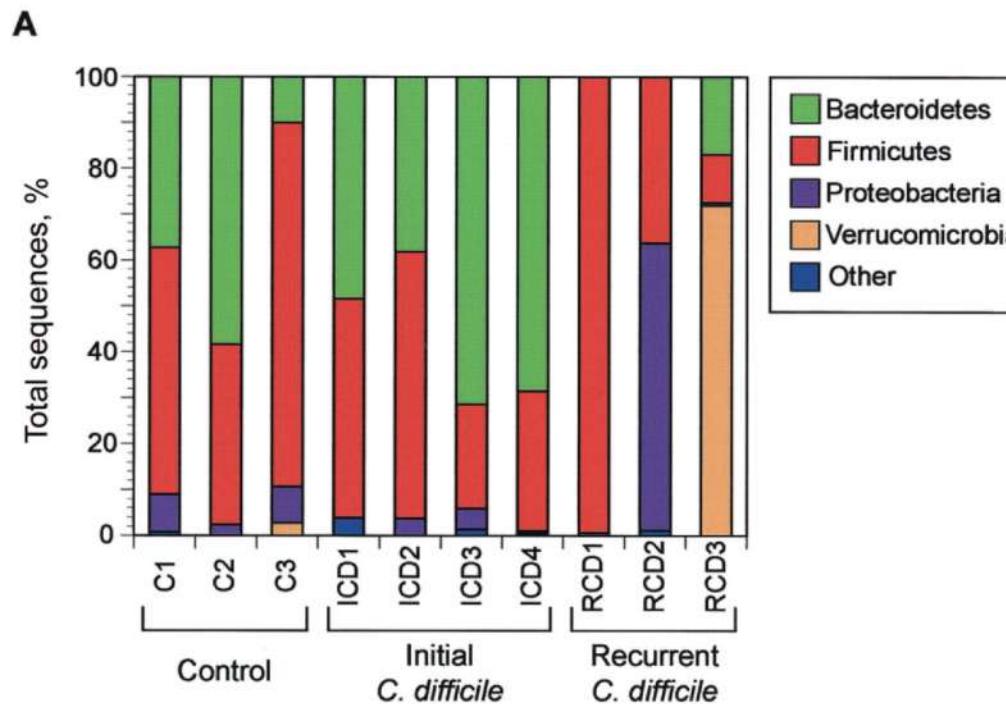
Basidiomycetes

- Cryptococcus
- Malassezia
- Rhodotorula
- Ustilago
- Others (<1%)

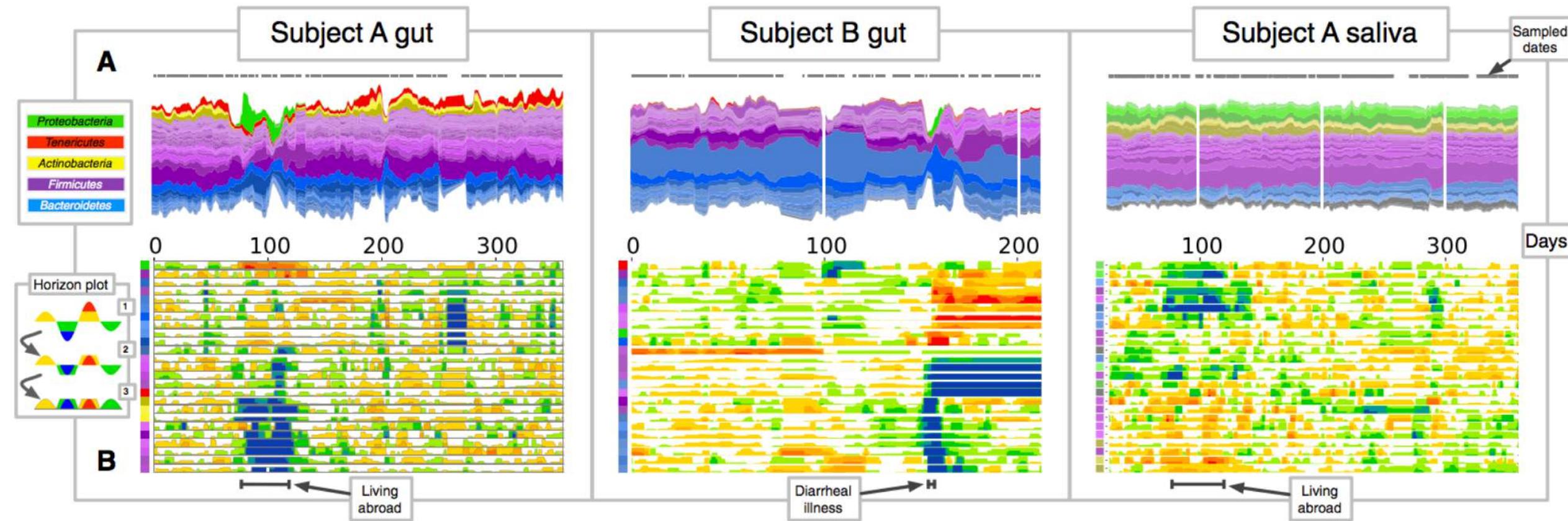
The gut microbiome during life



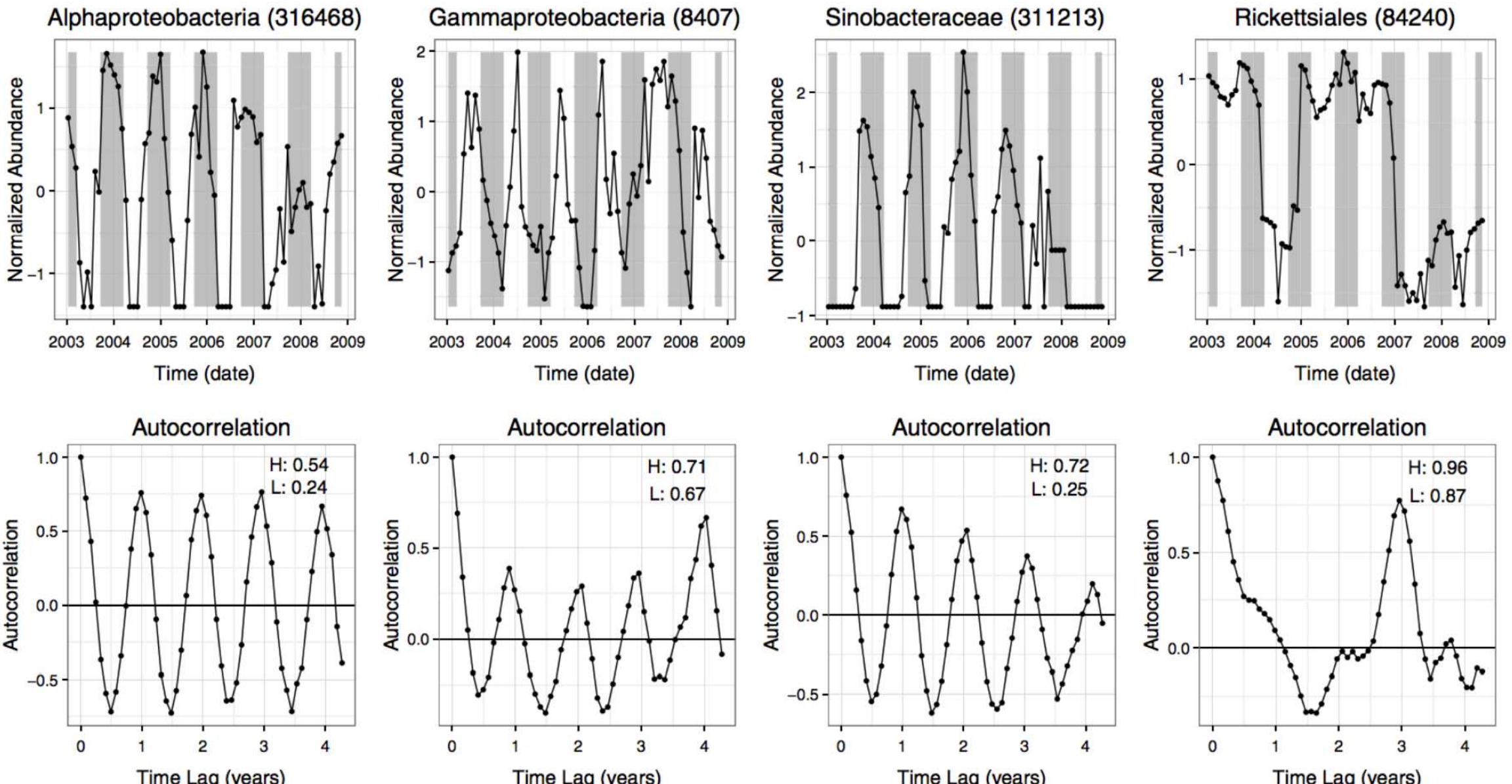
Decreased diversity with *Clostridium difficile* – associated diarrhea



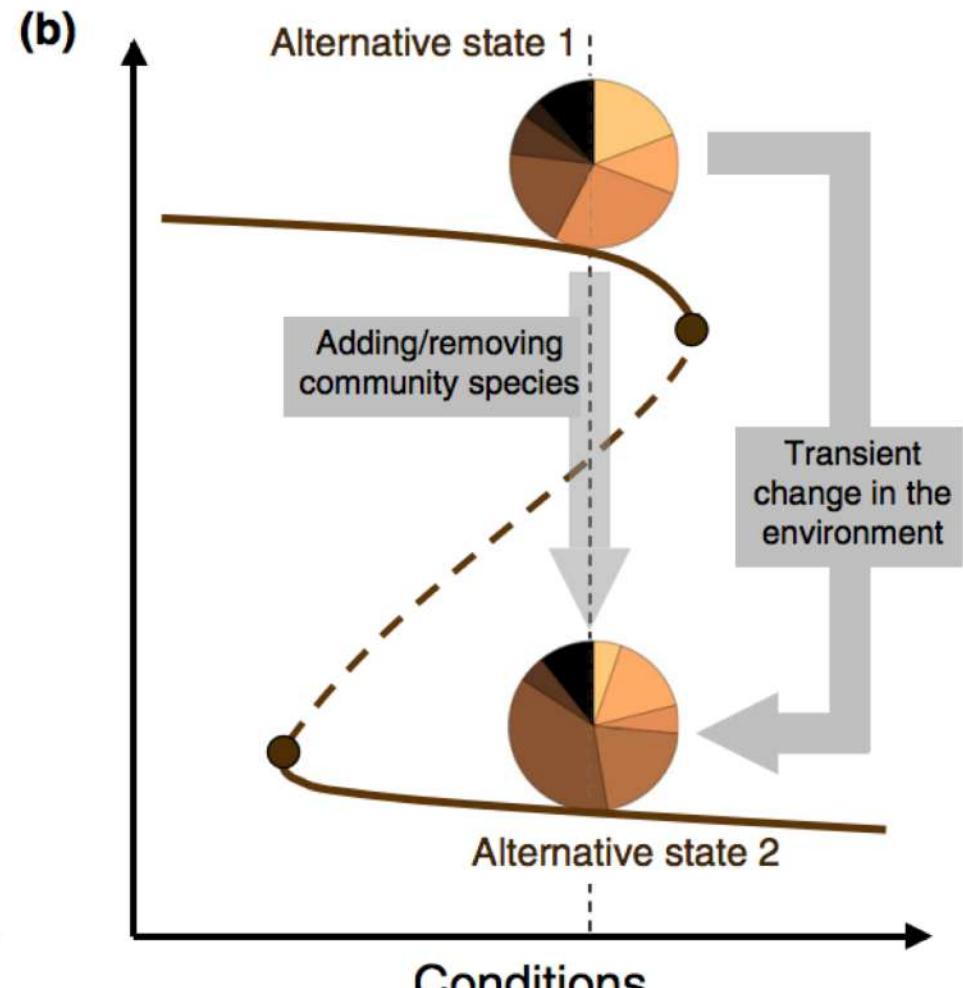
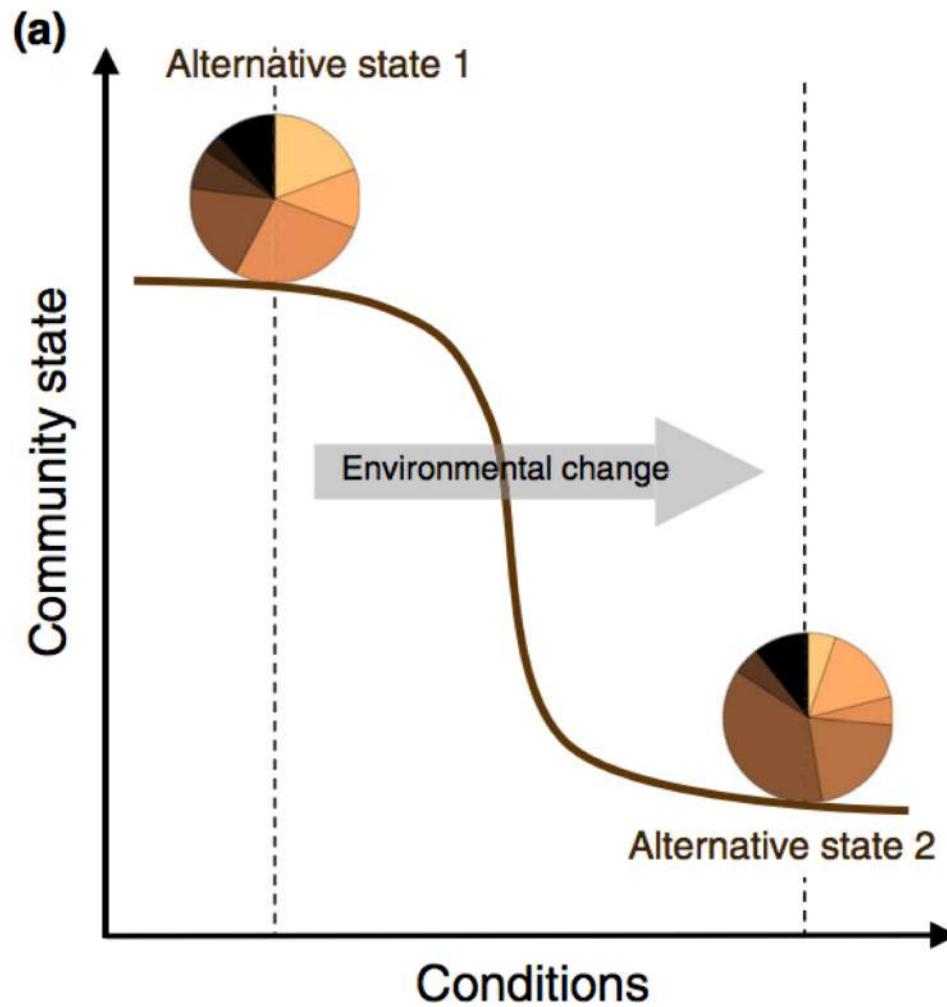
Tracking microbiome on a daily scale



Tracking microbiome spanning 6 years



Tracking microbiome on a daily scale



Current Opinion in Microbiology

Question: What community gets reset and what don't?

A. Shade, J.S. Read, N.D. Youngblut, N. Fierer, R. Knight, T.K. Kratz, N.R. Lottig, E.E. Roden, E.H. Stanley, J. Stombaugh, et al.

Lake microbial communities are resilient after a whole-ecosystem disturbance **Yes**
ISME J, 6 (2012), pp. 2153–2167

L. Dethlefsen, D.A. Relman

Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation

Proc Natl Acad Sci U S A, 108 (2011), pp. 4554–4561

No

L.A. David, A.C. Materna, J. Friedman, M.I. Campos-Baptista, M.C. Blackburn, A. Perrotta, S.E. Erdman, E.J. Alm

Host lifestyle affects human microbiota on daily timescales
Genome Biol, 15 (2014), p. R89

Yes and No

Priority effect

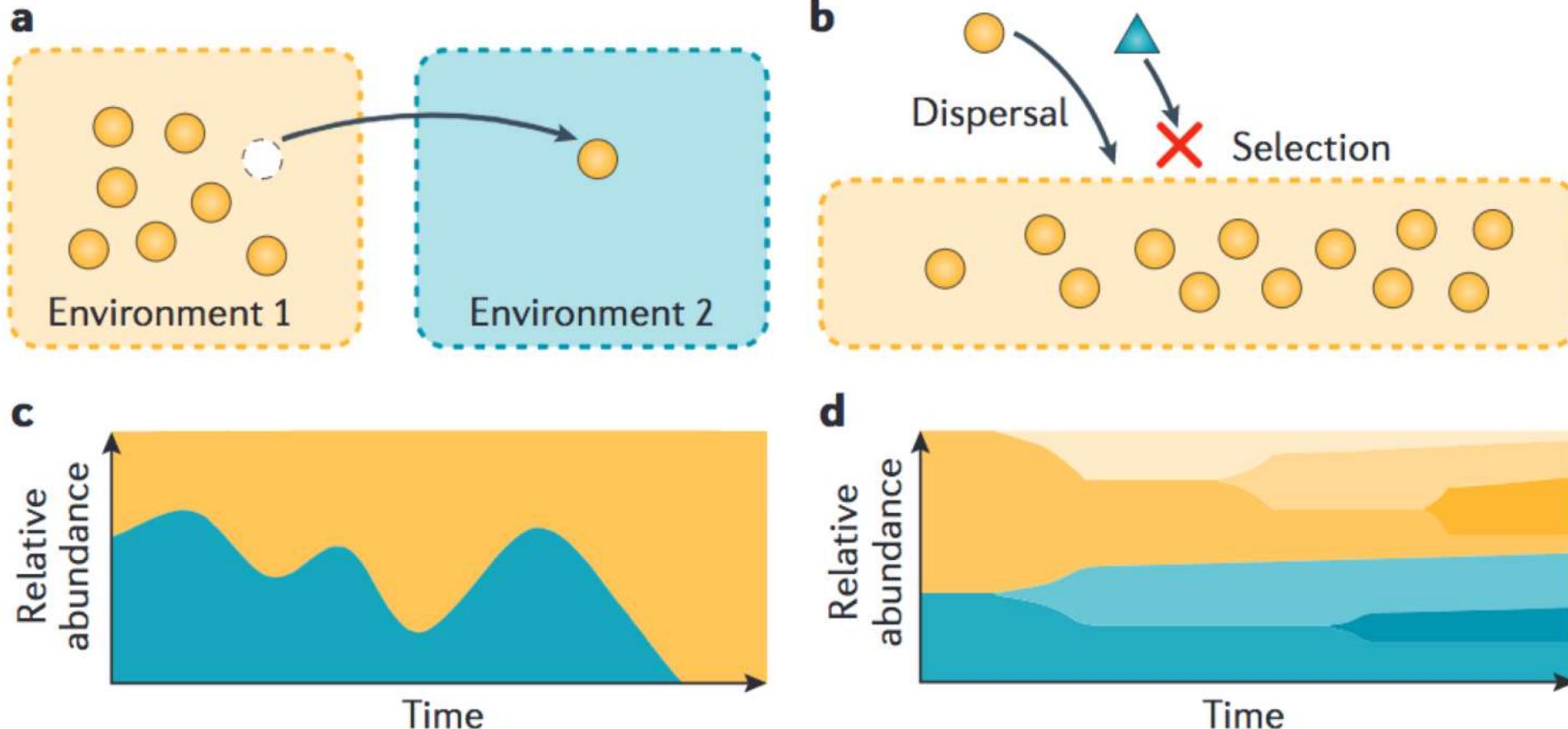
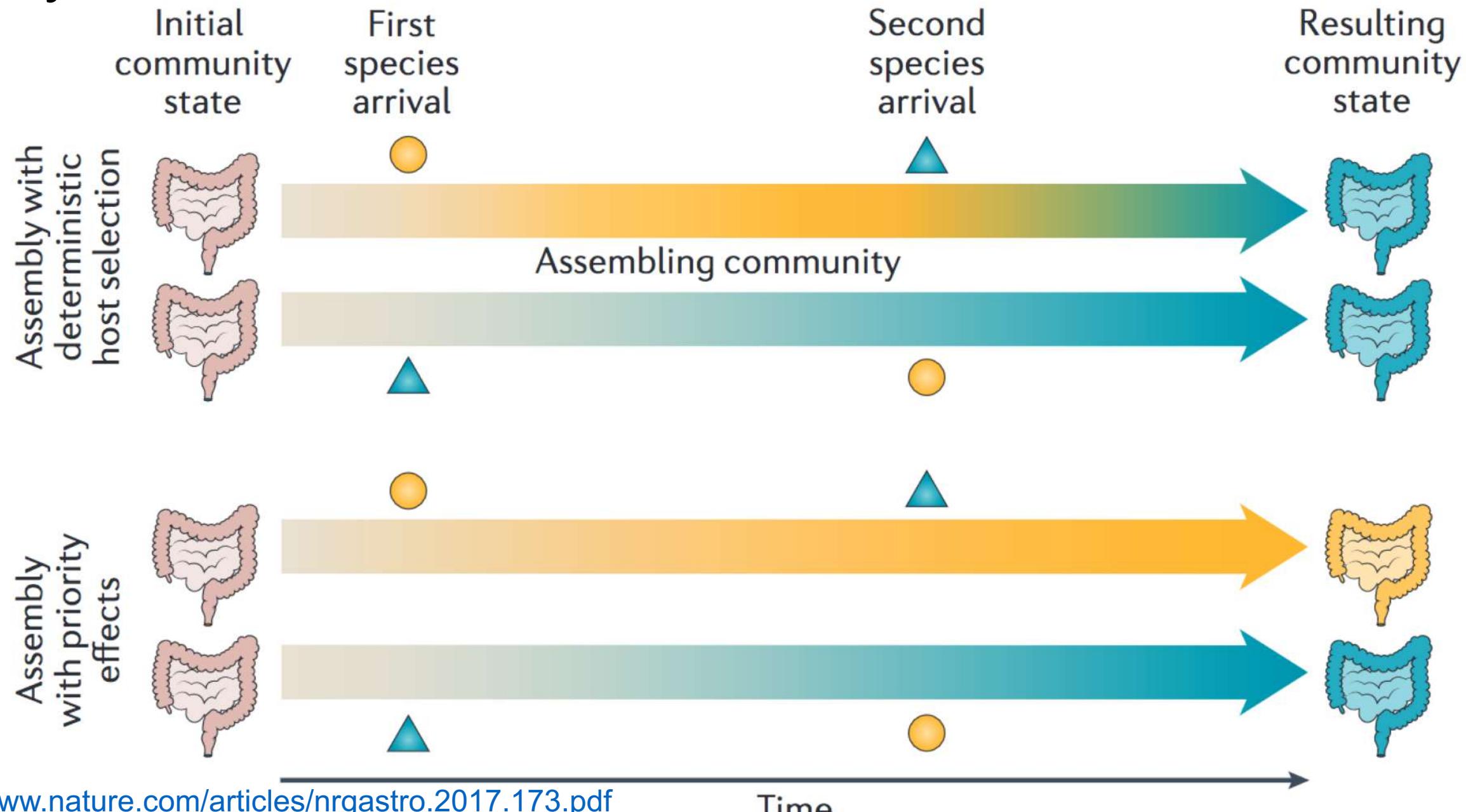


Figure 1 | Four processes that affect ecological communities. **a** | The arrow represents dispersal of an organism (orange circle) from Environment 1 (orange shading) to Environment 2 (blue shading). **b** | Deterministic fitness differences between two species (orange circle, blue triangle) cause the orange environment to select for one (orange circle) and against the other (blue triangle). **c** | Stochastic changes in the relative abundances of two species (orange area and blue area) result in changes in community structure within one environment through time. As a result, one population (blue) has gone locally extinct by the end of the time period. **d** | Mutation and/or recombination within a population (blue and orange areas) results in new genetic variation through time, leading to new strains (as denoted by different shades).

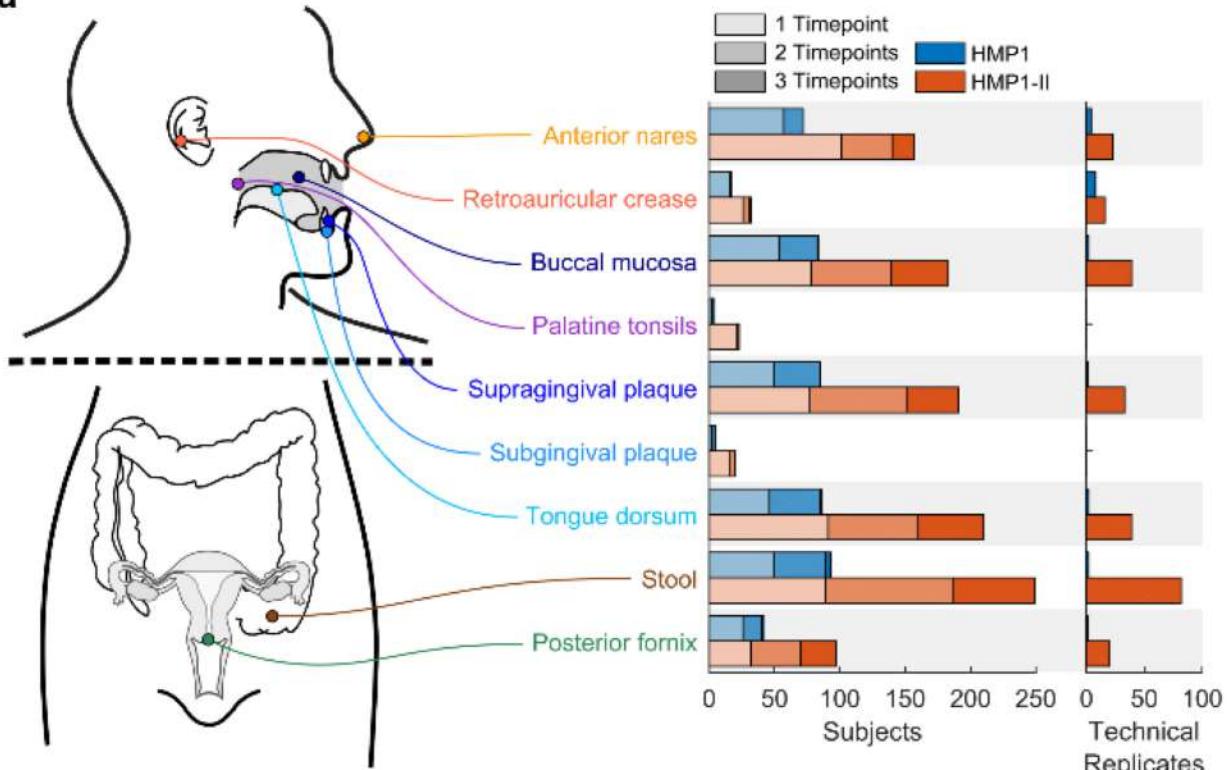
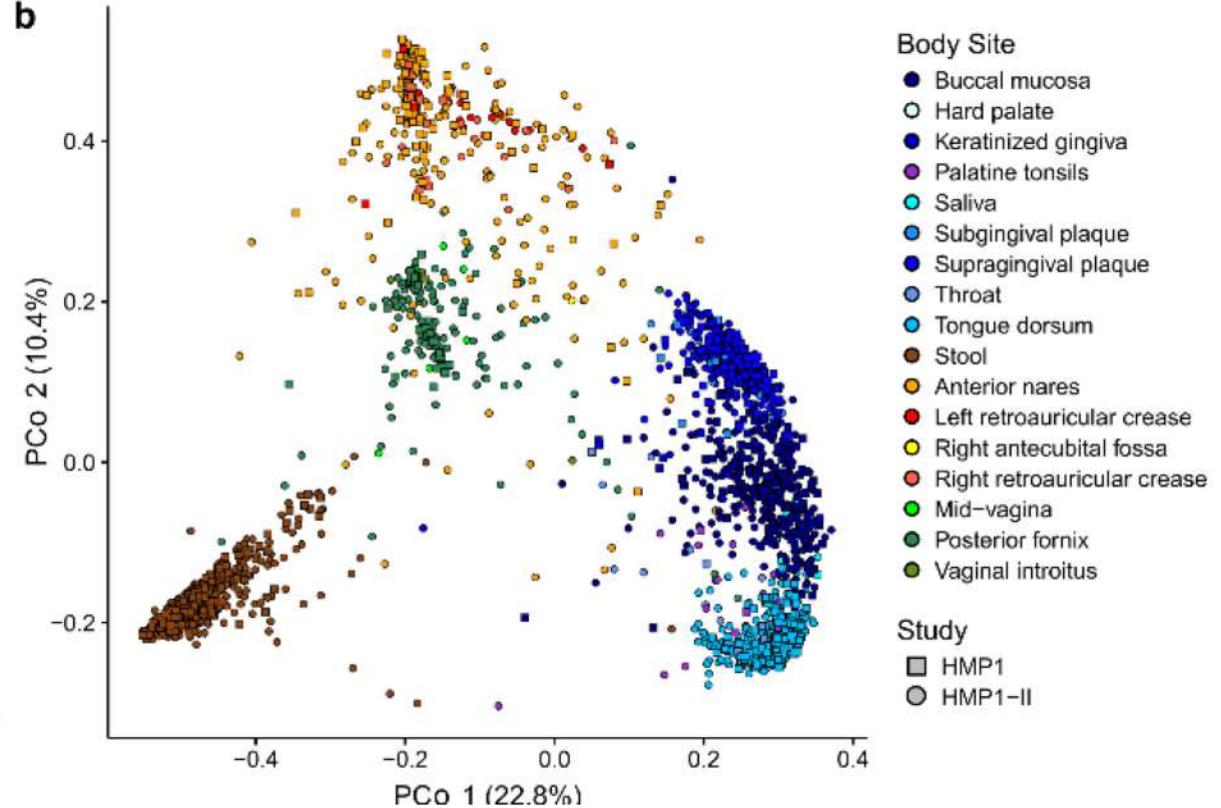
Priority effect



Strains, functions and dynamics in the expanded Human Microbiome Project

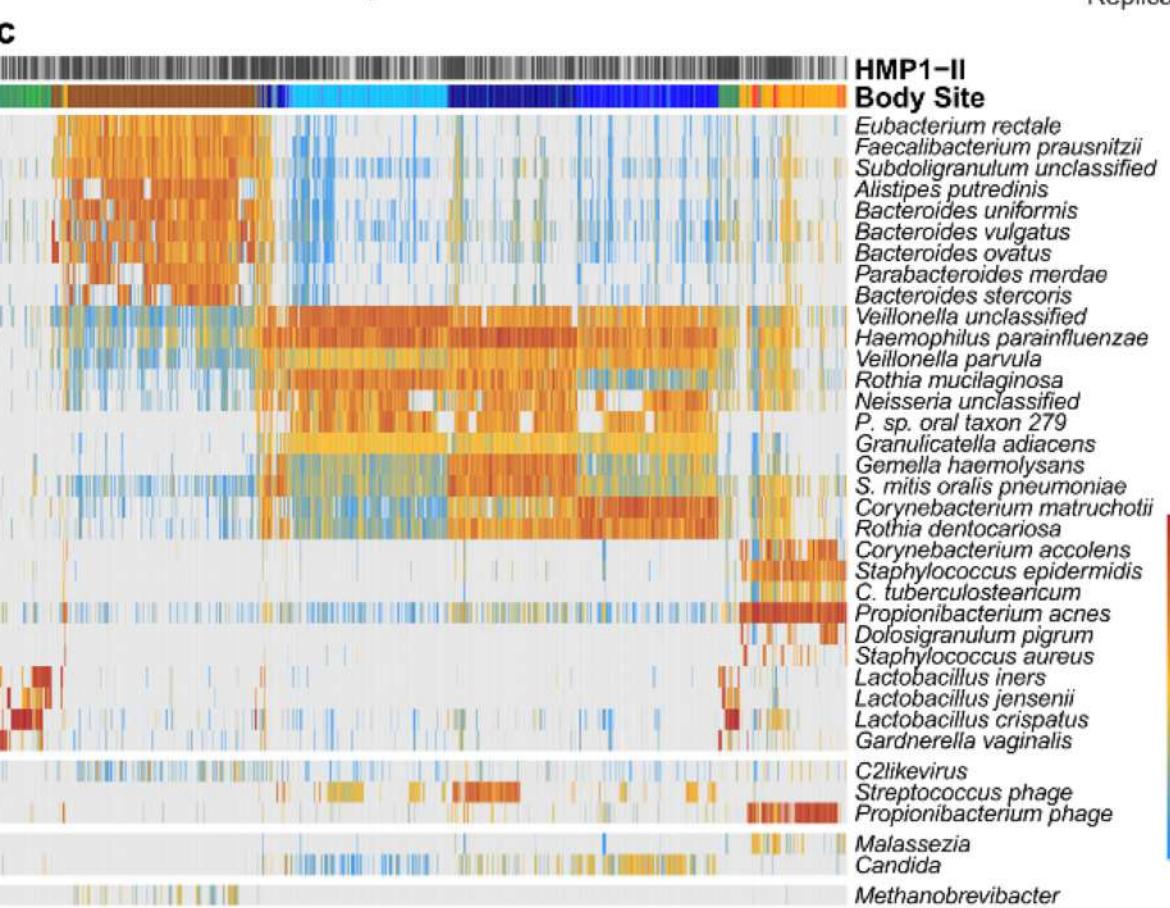
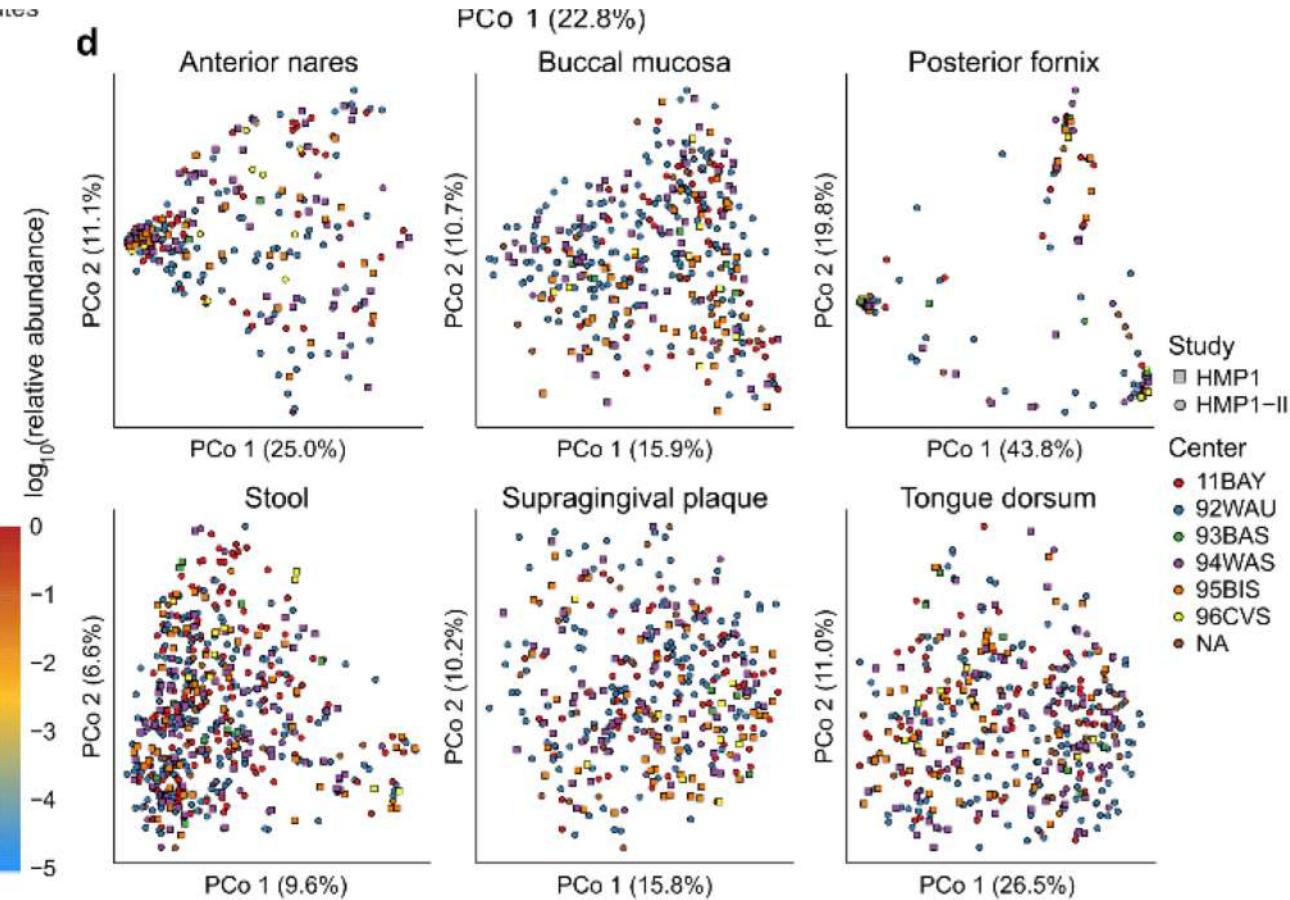
Jason Lloyd-Price^{1,2*}, Anup Mahurkar^{3*}, Gholamali Rahnavard^{1,2}, Jonathan Crabtree³, Joshua Orvis³, A. Brantley Hall², Arthur Brady³, Heather H. Creasy³, Carrie McCracken³, Michelle G. Giglio³, Daniel McDonald⁴, Eric A. Franzosa^{1,2}, Rob Knight^{4,5}, Owen White³ & Curtis Huttenhower^{1,2}

The characterization of baseline microbial and functional diversity in the human microbiome has enabled studies of microbiome-related disease, diversity, biogeography, and molecular function. The National Institutes of Health Human Microbiome Project has provided one of the broadest such characterizations so far. Here we introduce a second wave of data from the study, comprising 1,631 new metagenomes (2,355 total) targeting diverse body sites with multiple time points in 265 individuals. We applied updated profiling and assembly methods to provide new characterizations of microbiome personalization. Strain identification revealed subspecies clades specific to body sites; it also quantified species with phylogenetic diversity under-represented in isolate genomes. Body-wide functional profiling classified pathways into universal, human-enriched, and body site-enriched subsets. Finally, temporal analysis decomposed microbial variation into rapidly variable, moderately variable, and stable subsets. This study furthers our knowledge of baseline human microbial diversity and enables an understanding of personalized microbiome function and dynamics.

a**b**

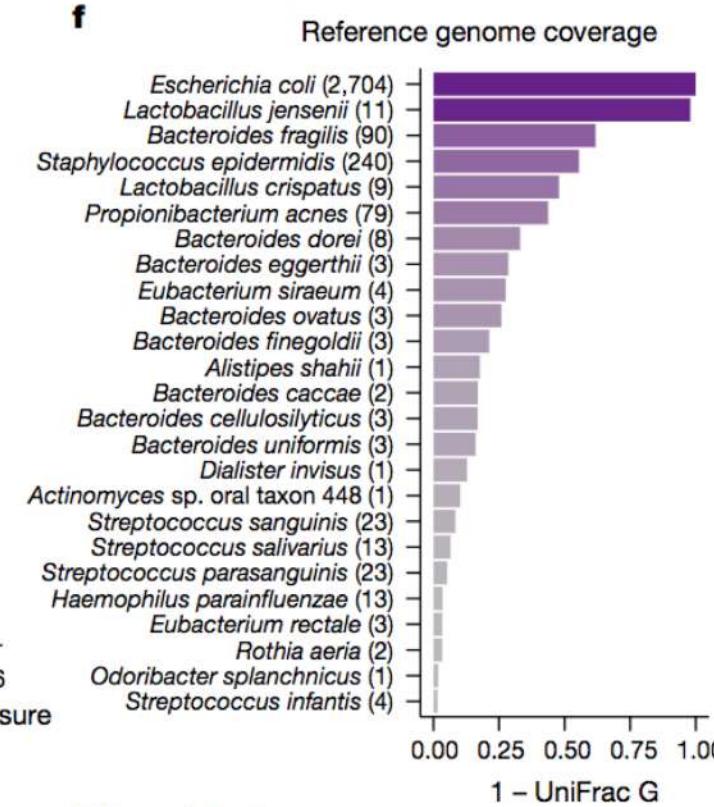
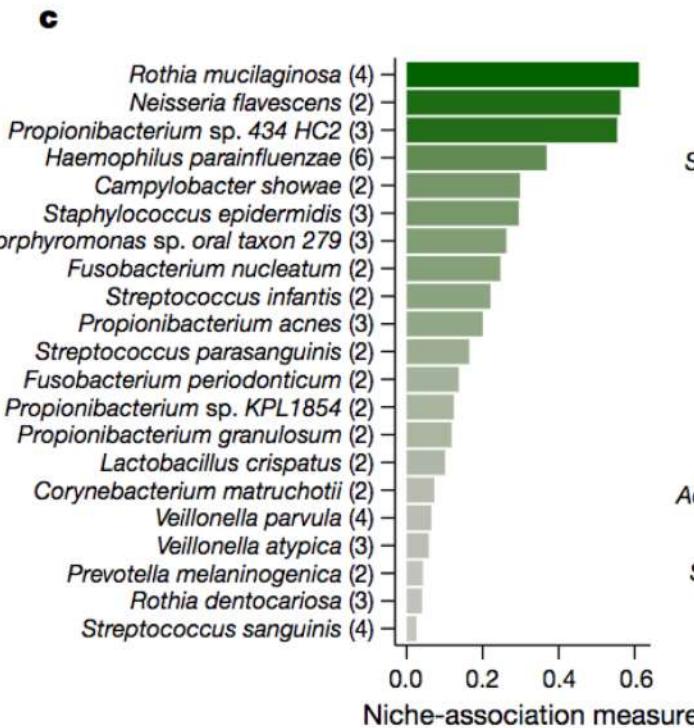
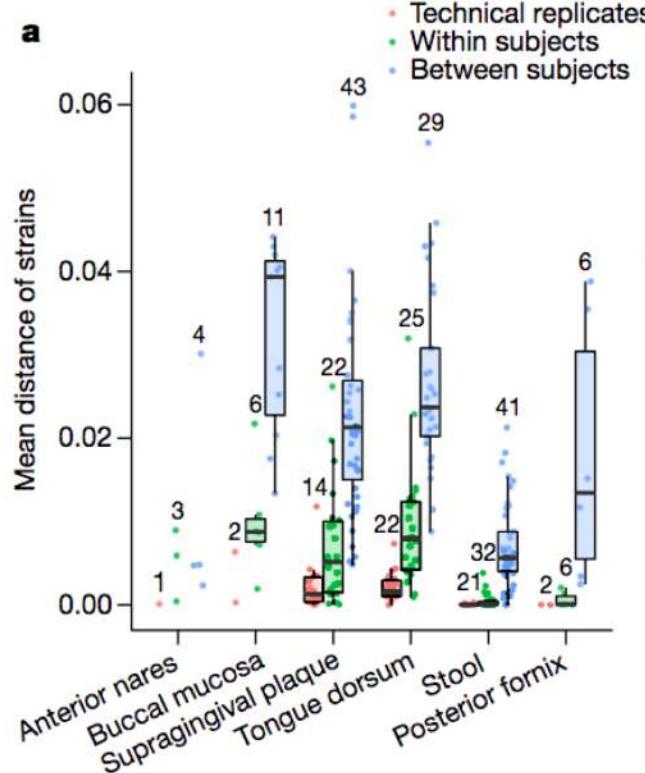
Extended Data Figure 1 | Extended body-wide metagenomic taxonomic profiles in HMP1-II. **a**, The combined HMP1-II datasets include a total of 2,355 metagenomes (724 previously published and 1,631 new, including 252 technical replicates). These span the project's six targeted body sites (anterior nares, buccal mucosa, supragingival plaque, tongue dorsum, stool, and posterior fornix) in addition to at least 20 samples each from 3 additional sites, of the 18 total sampled sites: retroauricular crease, palatine tonsils, and subgingival plaque. Metagenomes are now available for at least one body site for a total of 265 individuals. **b**, PCoA using Bray–Curtis

distances among all microbes at the species level. **c**, Relative abundances of the most prevalent and abundant microbes (bacterial, viral, eukaryotic, and archaeal) among all body sites, as profiled by MetaPhlAn2²⁰. Prevalent eukaryotic microbes are shown at the genus level. **d**, Taxonomic profiles do not vary more between sequencing centres, batches, or clinical centres than they do among individuals within body sites. Ordinations show Bray–Curtis principal coordinates of species-level abundances at each body site. Within-site ecological structure is as expected¹, with no divergence associated with technical variables along the first two ordination axes.

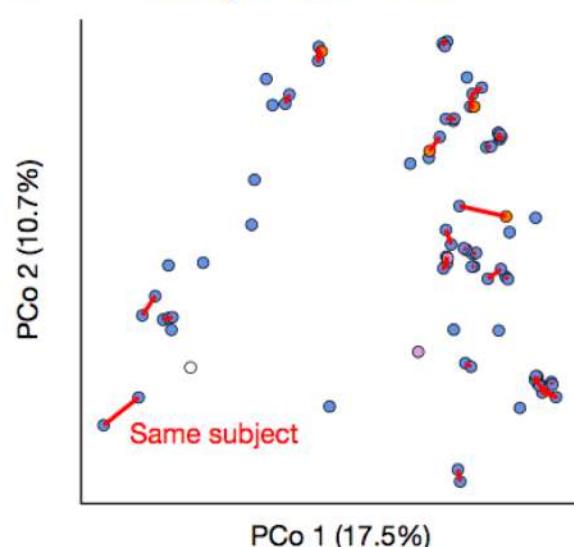
C**d**

Extended Data Figure 1 | Extended body-wide metagenomic taxonomic profiles in HMP1-II. **a**, The combined HMP1-II datasets include a total of 2,355 metagenomes (724 previously published and 1,631 new, including 252 technical replicates). These span the project's six targeted body sites (anterior nares, buccal mucosa, supragingival plaque, tongue dorsum, stool, and posterior fornix) in addition to at least 20 samples each from 3 additional sites, of the 18 total sampled sites: retroauricular crease, palatine tonsils, and subgingival plaque. Metagenomes are now available for at least one body site for a total of 265 individuals. **b**, PCoA using Bray–Curtis

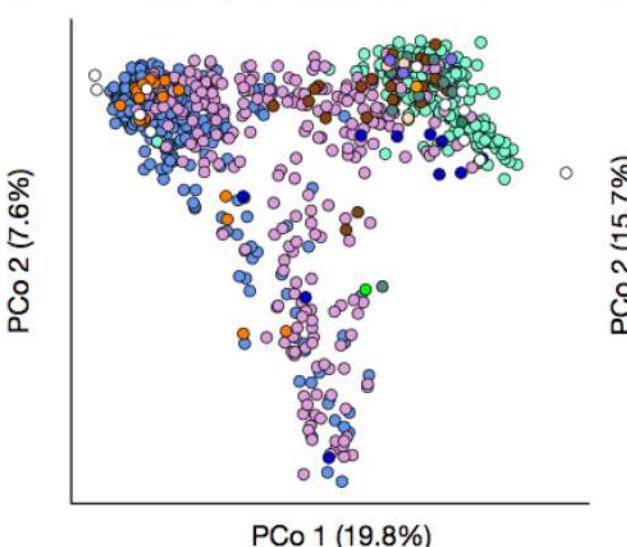
distances among all microbes at the species level. **c**, Relative abundances of the most prevalent and abundant microbes (bacterial, viral, eukaryotic, and archaeal) among all body sites, as profiled by MetaPhlAn2²⁰. Prevalent eukaryotic microbes are shown at the genus level. **d**, Taxonomic profiles do not vary more between sequencing centres, batches, or clinical centres than they do among individuals within body sites. Ordinations show Bray–Curtis principal coordinates of species-level abundances at each body site. Within-site ecological structure is as expected¹, with no divergence associated with technical variables along the first two ordination axes.



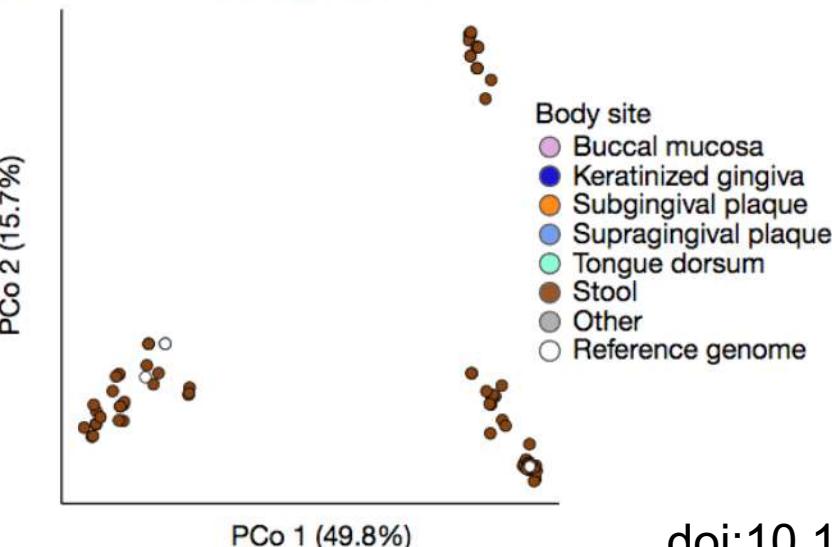
b *Actinomyces* sp. oral taxon 448

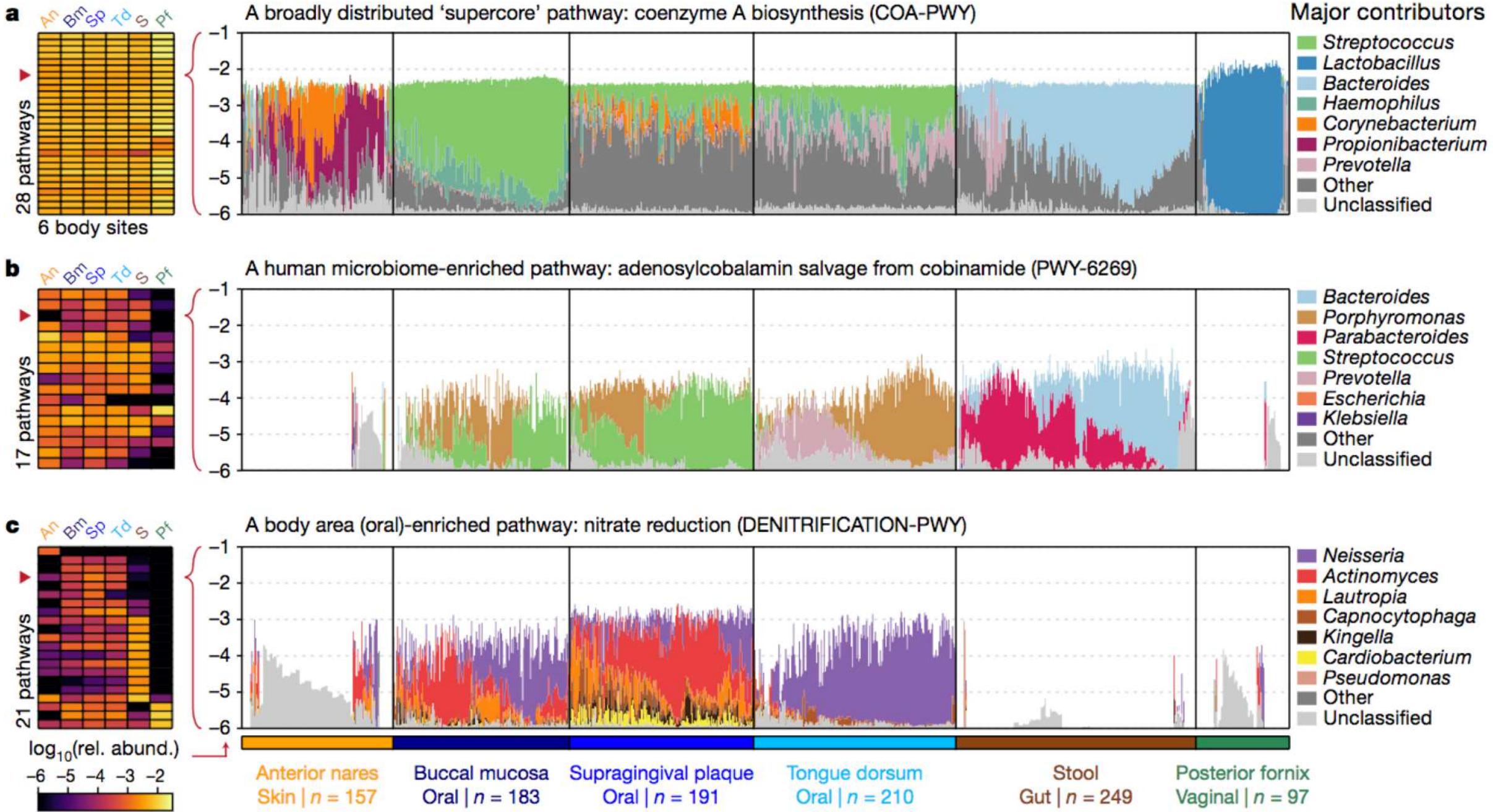


d *Haemophilus parainfluenzae*



e *Eubacterium siraeum*





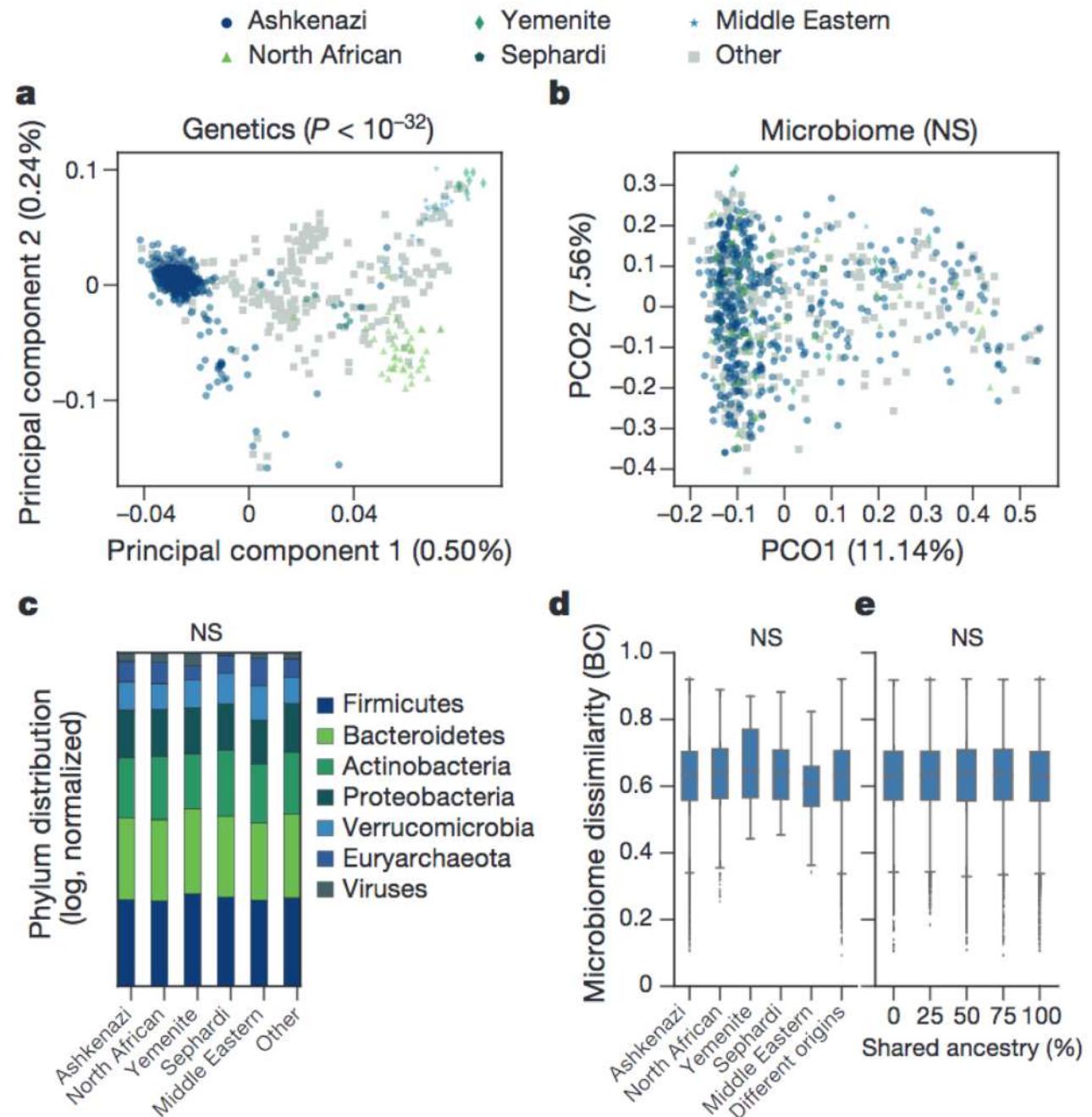
Environment dominates over host genetics in shaping human gut microbiota

Daphna Rothschild^{1,2*}, Omer Weissbrod^{1,2*}, Elad Barkan^{1,2*}, Alexander Kurilshikov³, Tal Korem^{1,2}, David Zeevi^{1,2}, Paul I. Costea^{1,2}, Anastasia Godneva^{1,2}, Iris N. Kalka^{1,2}, Noam Bar^{1,2}, Smadar Shilo^{1,2}, Dar Lador^{1,2}, Arnau Vich Vila^{3,4}, Niv Zmora^{5,6,7}, Meirav Pevsner-Fischer⁵, David Israeli⁸, Noa Kosower^{1,2}, Gal Malka^{1,2}, Bat Chen Wolf^{1,2}, Tali Avnit-Sagi^{1,2}, Maya Lotan-Pompan^{1,2}, Adina Weinberger^{1,2}, Zamir Halpern^{7,9}, Shai Carmi¹⁰, Jingyuan Fu^{3,11}, Cisca Wijmenga^{3,12}, Alexandra Zhernakova³, Eran Elinav⁵§ & Eran Segal^{1,2}§

Human gut microbiome composition is shaped by multiple factors but the relative contribution of host genetics remains elusive. Here we examine genotype and microbiome data from 1,046 healthy individuals with several distinct ancestral origins who share a relatively common environment, and demonstrate that the gut microbiome is not significantly associated with genetic ancestry, and that host genetics have a minor role in determining microbiome composition. We show that, by contrast, there are significant similarities in the compositions of the microbiomes of genetically unrelated individuals who share a household, and that over 20% of the inter-person microbiome variability is associated with factors related to diet, drugs and anthropometric measurements. We further demonstrate that microbiome data significantly improve the prediction accuracy for many human traits, such as glucose and obesity measures, compared to models that use only host genetic and environmental data. These results suggest that microbiome alterations aimed at improving clinical outcomes may be carried out across diverse genetic backgrounds.

- 1,046 healthy Israeli adults
- 16S rRNA + metagenomics
- Genotyping 712,540 SNPs
- Questionnaires

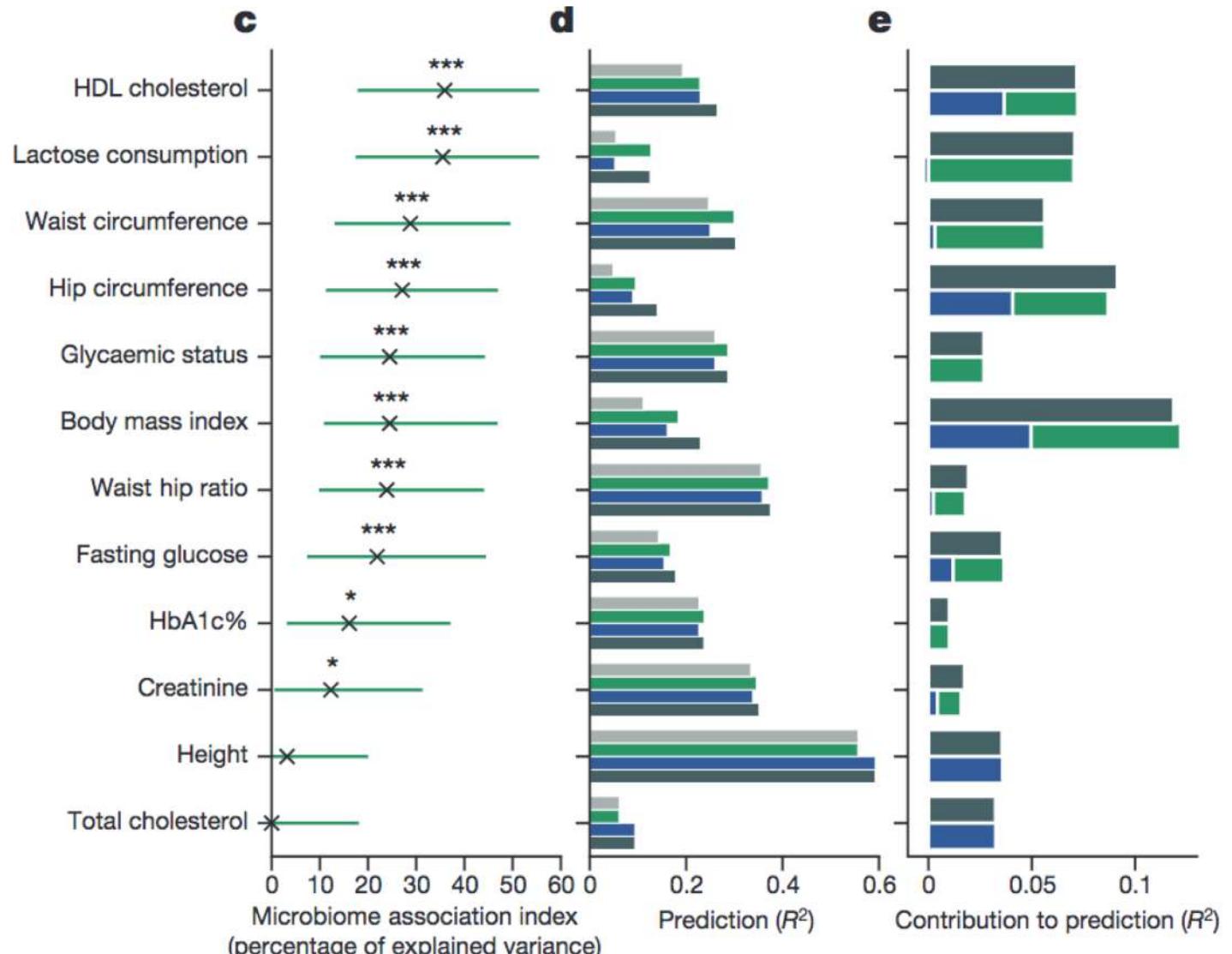
Figure 1 | Genetic ancestry is not significantly associated with microbiome composition. **a**, Genetic principal components are strongly associated with self-reported ancestry, with Ashkenazi ($n = 345$), North African ($n = 42$), Middle Eastern ($n = 24$), Sephardi ($n = 10$), Yemenite ($n = 8$) and admixed/other (other) ($n = 286$) ancestries ($P < 10^{-32}$; Kruskal–Wallis). **b**, As in **a**, but for microbiome principal coordinate analysis ($P > 0.08$; Kruskal–Wallis). **c**, The distribution of average phylum abundance among 582 non-admixed individuals (in log scale, normalized to sum to 1.0) is not associated with ancestry ($P > 0.05$; Kruskal–Wallis). NS, not significant. **d**, Box plots of Bray–Curtis (BC) dissimilarities across all pairs of 737 individuals for whom the ancestries of all grandparents are known, demonstrating that microbiome composition is not associated with ancestry ($P > 0.06$; Kruskal–Wallis test for the top five Bray–Curtis PCOs). $n = 105,570$ (Ashkenazi), 1,711 (North African), 528 (Middle Eastern), 136 (Sephardi) and 78 (Yemenite) same ancestry pairs; $n = 61,048$ different ancestry pairs. The lower and upper limits of the boxes represent the 25% and 75% percentiles, respectively, and the top and bottom whiskers represent the 5% and 95% percentiles, respectively. **e**, Box plots of Bray–Curtis dissimilarity across pairs of 946 individuals (including admixed individuals), organized according to shared ancestry fraction (the fraction of grandparents of the same ancestry), for pairs with 0% ($n = 167,618$), 25% ($n = 33,119$), 50% ($n = 100,163$), 75% ($n = 34,187$) and 100% ($n = 111,898$) shared ancestry fractions. The lower and upper limits of the boxes represent the 25% and 75% percentiles, respectively, and the top and bottom whiskers represent the 5% and 95% percentiles, respectively. The figure demonstrates that microbiome similarity is not associated with ancestral similarity ($P = 0.73$; Mantel test).



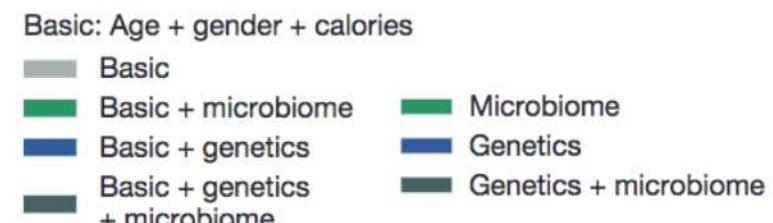
b

Phenotype	Microbiome association index		Genetic heritability (literature)
	Israeli cohort	LLD cohort	
HDL	35.9%***	27.9%***	23.9%–48%
Lactose cons.	35.5%***	N/A	N/A
Waist circ.	28.8%***	26%***	15%–24%
Hip circ.	27.1%***	28%***	10.6%–27%
Glycaemic status	24.5%***	N/A	N/A
BMI	24.5%***	27.8%***	14%–32%
WHR	23.9%***	6.9%*	12%–14%
Fasting glucose	21.9%***	8%**	9%–33%
HbA1c%	16.1%*	8.4%	21%–32%
Creatinine	12.3%*	6.7%	19%–25%
Height	3.2%	25.9%***	33%–68%
Total cholesterol	0%	13.5%	14%–53%

indicate a greater confidence in the estimation. **b**, b^2 estimates from the analysis of 715 individuals with measured genotyped and gut microbiomes from the Israeli cohort (left column) and of 836 individuals from the LLD cohort (middle column) are comparable to previous genetic heritability estimates^{27–34} (right column). *FDR < 0.05, **FDR < 0.01 and ***FDR < 0.001. Cons., consumption, circ., circumference. **c**, b^2 estimates



***FDR < 0.001. Cons., consumption, circ., circumference. **c**, b^2 estimates of several human phenotypes and their 95% confidence intervals, evaluated using 715 individuals. *FDR < 0.05, **FDR < 0.01 and ***FDR < 0.001. **d**, Phenotype prediction accuracy for 715 individuals, evaluated using a LMM under different sets of predictive features (measured using coefficient of determination (R^2)), using four different models for each phenotype: (i) 'Basic', age, gender and diet features; (ii) 'Basic + microbiome', basic features and relative abundances of bacterial genes; (iii) 'Basic + genetics', basic features and host genotypes; and (iv) 'Basic + genetics + microbiome': basic features, relative abundances of bacterial genes and host genotypes. **e**, The additive contribution of microbiome and genetics to prediction performance evaluated using a LMM across 715 individuals, over a model that includes only basic features. The joint contribution of microbiome and genetics is similar to the sum of the individual contributions, suggesting these are independent contributions.



ARTICLE

OPEN

doi:10.1038/nature24621

A communal catalogue reveals Earth's multiscale microbial diversity

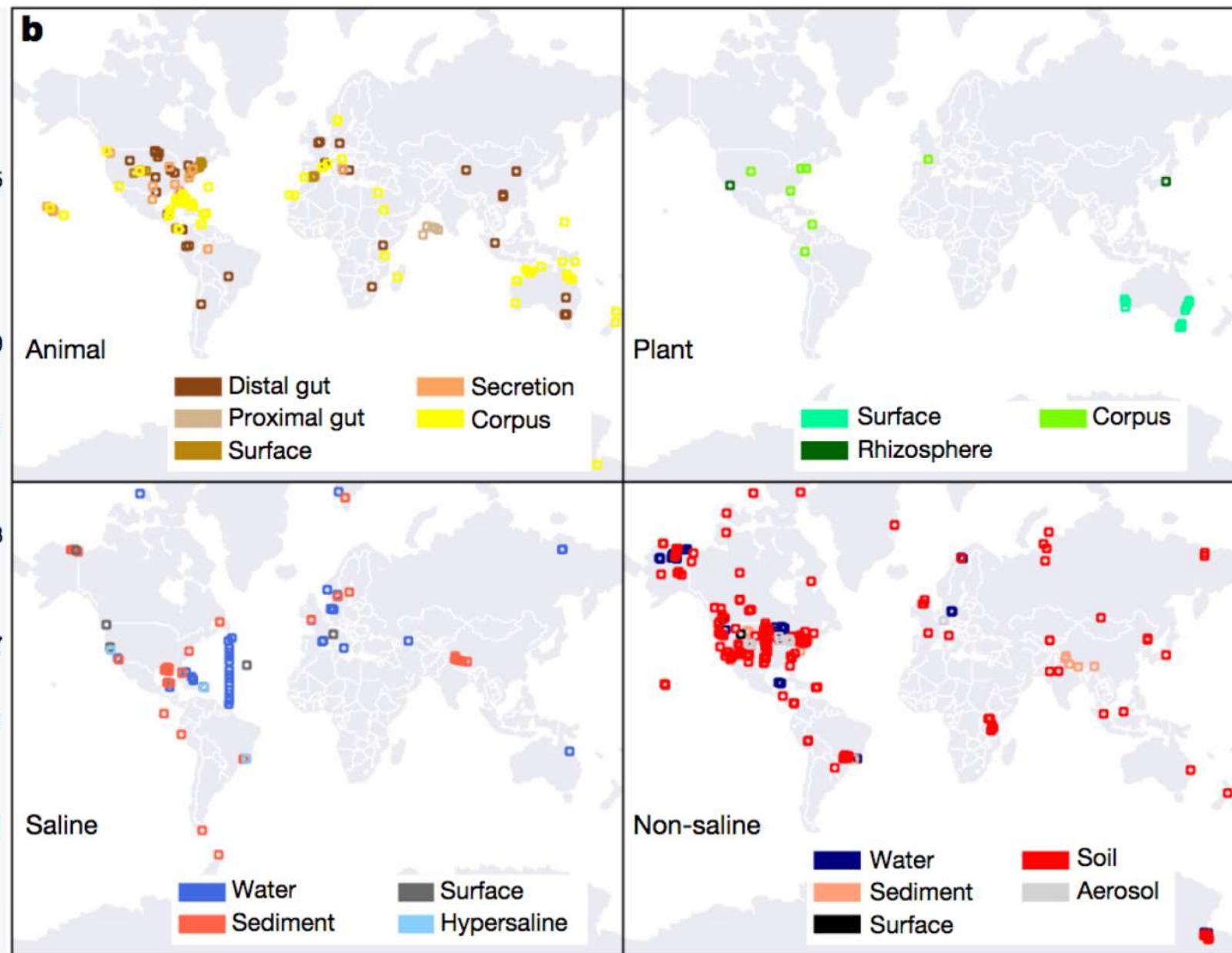
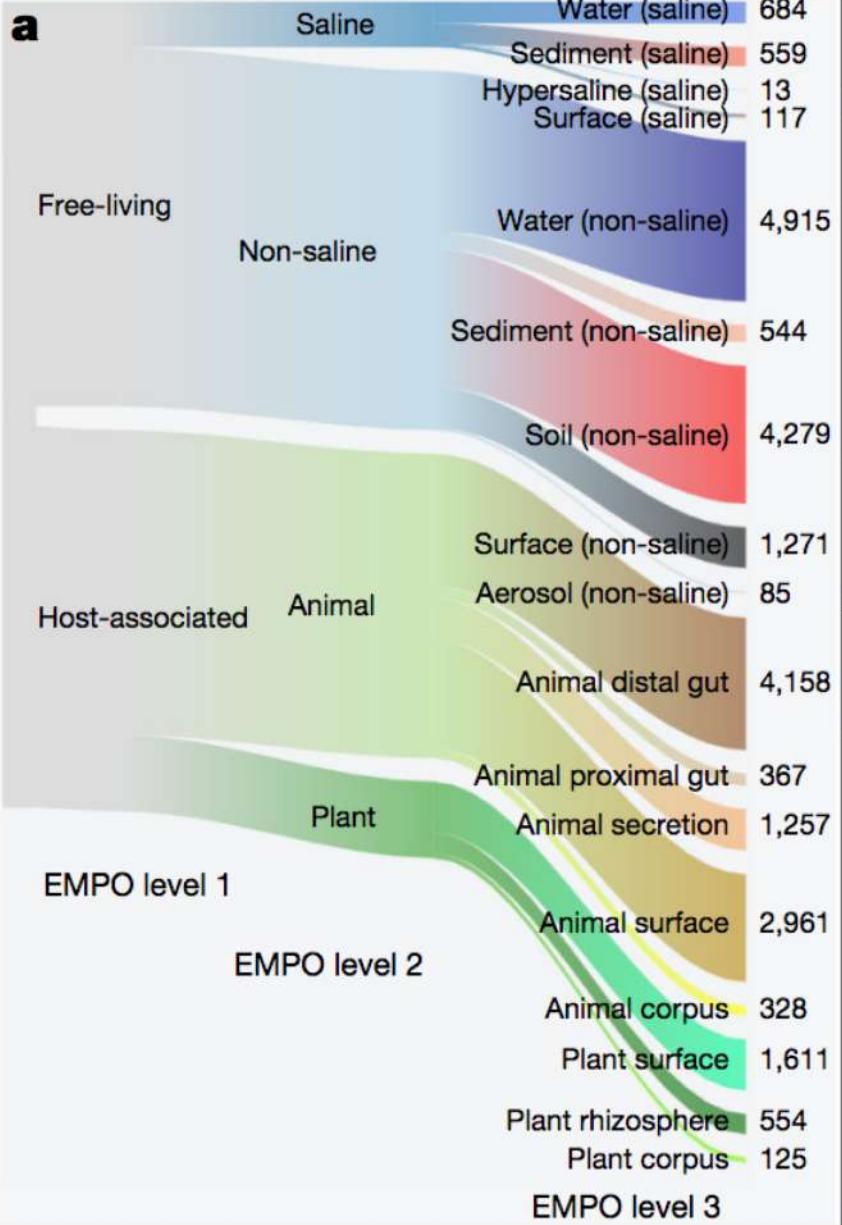
Luke R. Thompson^{1,2,3}, Jon G. Sanders¹, Daniel McDonald¹, Amnon Amir¹, Joshua Ladau⁴, Kenneth J. Locey⁵, Robert J. Prill⁶, Anupriya Tripathi^{1,7,8}, Sean M. Gibbons^{9,10}, Gail Ackermann¹, Jose A. Navas-Molina^{1,11}, Stefan Janssen¹, Evguenia Kopylova¹, Yoshiaki Vázquez-Baeza^{1,11}, Antonio González¹, James T. Morton^{1,11}, Siavash Mirarab¹², Zhenjiang Zech Xu¹, Lingjing Jiang^{1,13}, Mohamed F. Haroon¹⁴, Jad Kanbar¹, Qiyun Zhu¹, Se Jin Song¹, Tomasz Kosciolek¹, Nicholas A. Bokulich¹⁵, Joshua Lefler¹, Colin J. Brislawn¹⁶, Gregory Humphrey¹, Sarah M. Owens¹⁷, Jarrad Hampton-Marcell^{17,18}, Donna Berg-Lyons¹⁹, Valerie McKenzie²⁰, Noah Fierer^{20,21}, Jed A. Fuhrman²², Aaron Clauset^{19,23}, Rick L. Stevens^{24,25}, Ashley Shade^{26,27,28}, Katherine S. Pollard⁴, Kelly D. Goodwin³, Janet K. Jansson¹⁶, Jack A. Gilbert^{17,29}, Rob Knight^{1,11,30} & The Earth Microbiome Project Consortium*

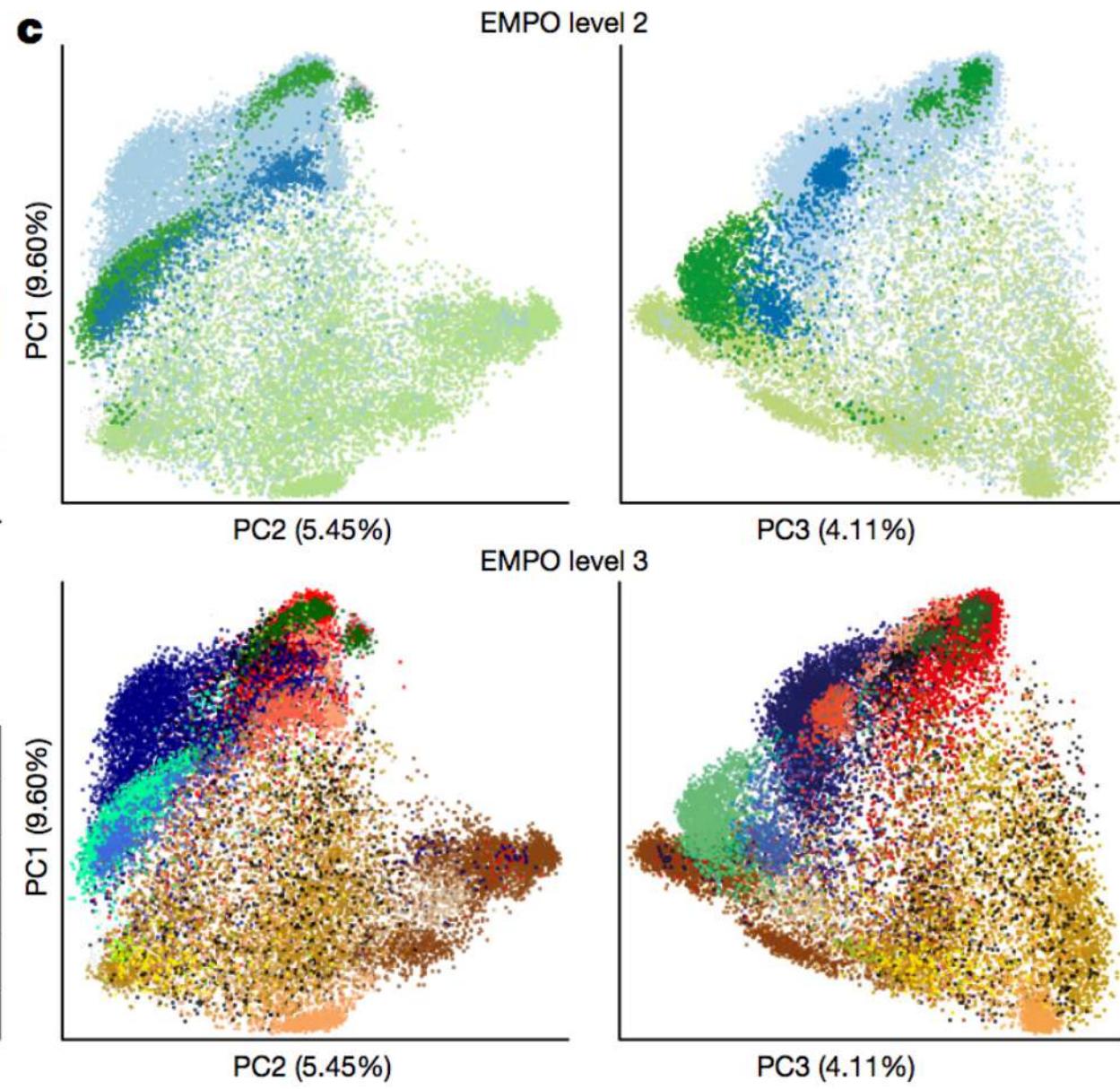
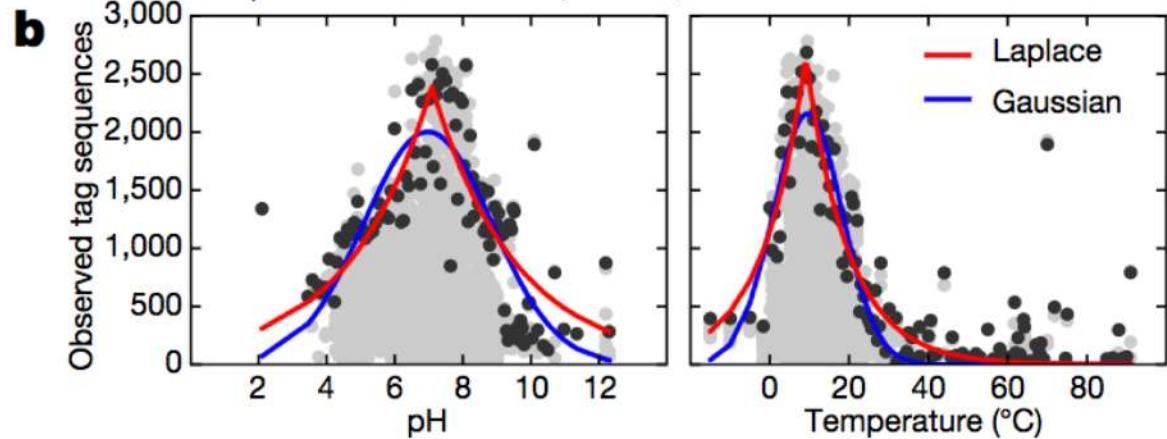
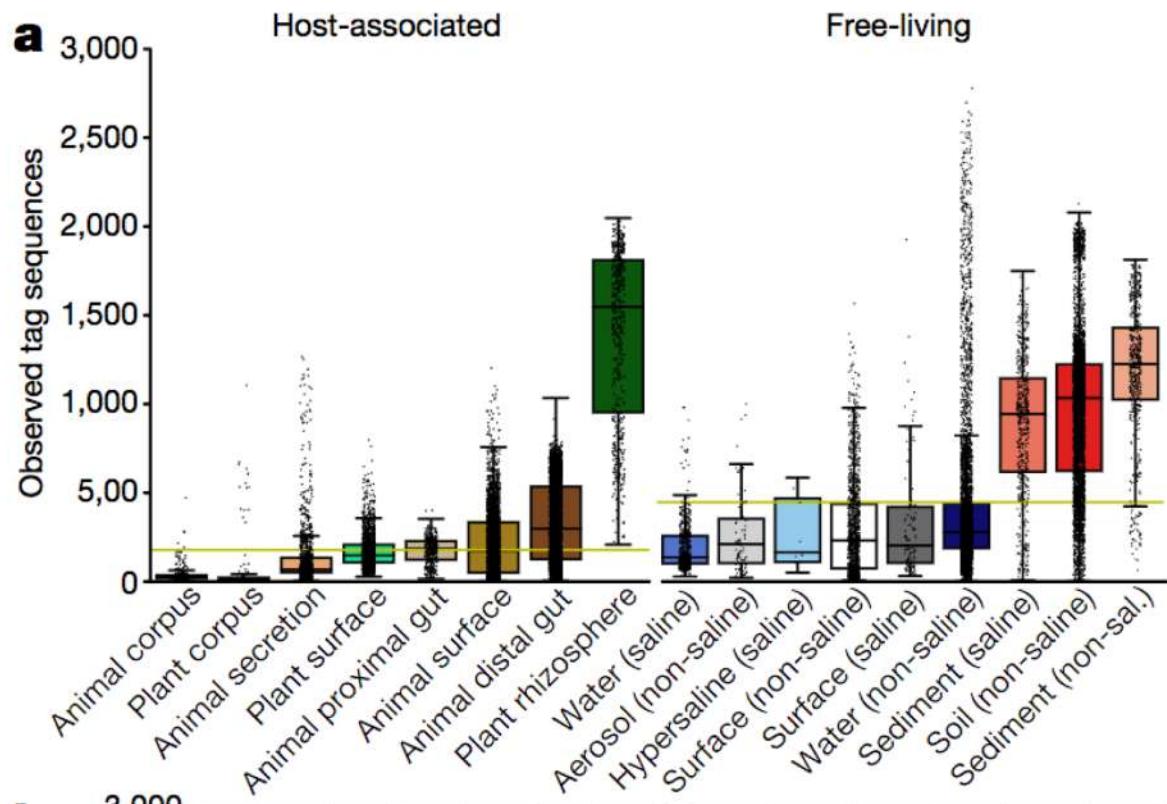
Our growing awareness of the microbial world's importance and diversity contrasts starkly with our limited understanding of its fundamental structure. Despite recent advances in DNA sequencing, a lack of standardized protocols and common analytical frameworks impedes comparisons among studies, hindering the development of global inferences about microbial life on Earth. Here we present a meta-analysis of microbial community samples collected by hundreds of researchers for the Earth Microbiome Project. Coordinated protocols and new analytical methods, particularly the use of exact sequences instead of clustered operational taxonomic units, enable bacterial and archaeal ribosomal RNA gene sequences to be followed across multiple studies and allow us to explore patterns of diversity at an unprecedented scale. The result is both a reference database giving global context to DNA sequence data and a framework for incorporating data from future studies, fostering increasingly complete characterization of Earth's microbial diversity.



BY THE NUMBERS

27,751	samples	7 continents
2,212,796,183	total DNA sequences	
307,572	unique DNA sequences (approx. species)	
50+	peer-reviewed publications	500+ scientists
92	environmental features	2 – 12 pH range (stomach acid to household ammonia)
66	animal host species	1 reference database of bacteria that reside on Planet Earth
78.9 °N – 78.2 °S	latitude range (Arctic Circle to Antarctica)	





Algorithm advancements lead to recovery of genomes

nature
microbiology

ARTICLES

DOI: 10.1038/s41564-017-0012-7

OPEN

Corrected: Author correction

Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life

Donovan H. Parks , Christian Rinke , Maria Chuvochina, Pierre-Alain Chaumeil, Ben J. Woodcroft, Paul N. Evans, Philip Hugenholtz * and Gene W. Tyson*

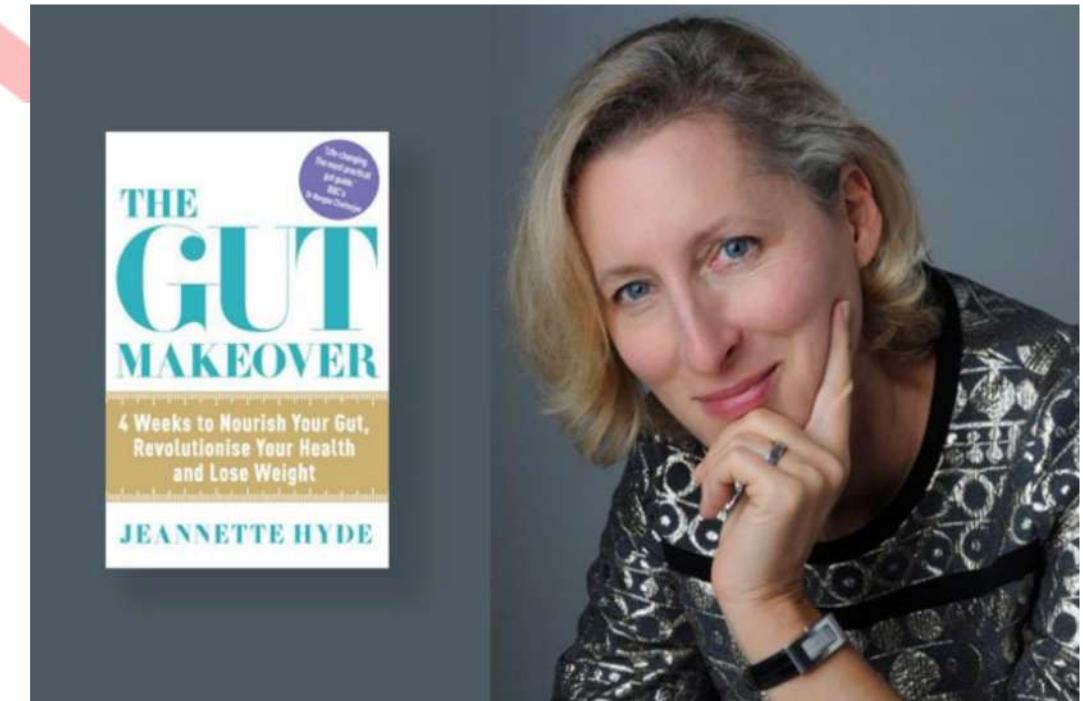
RESEARCH ARTICLE

Microbiome restoration diet improves digestion, cognition and physical and emotional wellbeing

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https://www.buzzfeed.com/stephaniemlee/gut-makeover-microbiome-diet-retraction?utm_term=.haVvkqdo1Xx#.gaGqjrQPRw

Therefore in summary, this "research article" has the following attributes:

1. **No objective data on health outcomes was collected; the study presents only participant-reported subjective data,**
2. **No objective data on treatment compliance was collected; we do not know if the participants followed the diet nor to what extent,**
3. **No objective data on treatment effect/mechanisms: The authors claim that the intervention changes the gut microbiome but failed to measure even a single parameter, microbe, molecule, or metabolite.**
4. **Participants were a positively self-selected "convenience sample" ripe and ready for a placebo response given their demonstrated positive expectations.**
5. **Impossible attribution, especially to the gut microbiome:** With no control group, no-one knows if the supposed "improvements" were due to the psychosocial intervention, the diet, the season, the natural history the non-disease being non-studied, chance; the attribution of supposed benefit to a mechanism involving the gut microbiome is not supported by any data in this publication.
6. **Short duration with no durability of effect:** No demonstrated durability to the supposed benefits; the study was of notably short duration (4 weeks),
7. **Wild attribution without any shred of evidence:** The treatment included 1) diet intervention and 2) psychosocial support and then **the authors attributed (without any supporting data whatsoever) the subjective/undocumented/purported benefits to 3) changes in the gut microbial composition.**

Summary

- Amplicon sequencing moving away from OTU to Exact Sequence Variants (ESV)
- Every step from sample collection to data deposit matters
- Amplicon sequencing : inexpensive but effective
- Metagenomics: expensive but has all the information you want (or not want)
- Tremendous potential in this field ; but please do not oversell it

Additional references

<https://www.nature.com/subjects/metagenomics>