

# BIO390 - Introduction to Bioinformatics: Metagenomics

October 26th, 2021

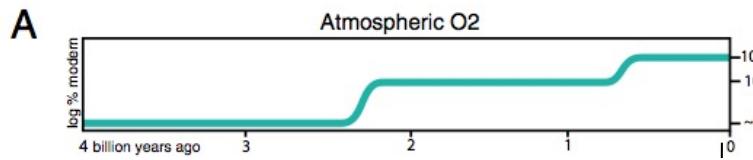
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# Evolution and significance of microbiomes

## From the origin of life to today



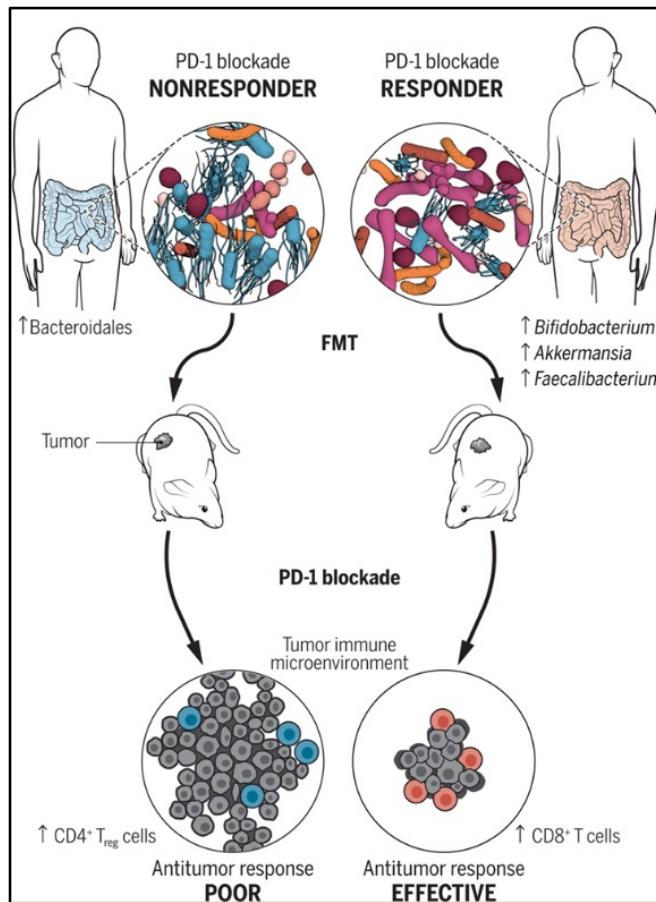
### Microorganisms

- originated some 3.8 billion years ago
- drive biogeochemical cycles of elements (C, N, P, S, etc.)
- transform energy and biomass

### Significance (examples):

- biogeochemistry: e.g., photosynthesis by microbes, carbon fixation/export, nitrogen fixation
- health: help us digest food, provide essential vitamins, train the immune system

# Describing microbial communities – Example 1



- Compositions of microbial communities are important to characterize, because many host-associated microbes are increasingly implicated in diseases (and personalized medicine)

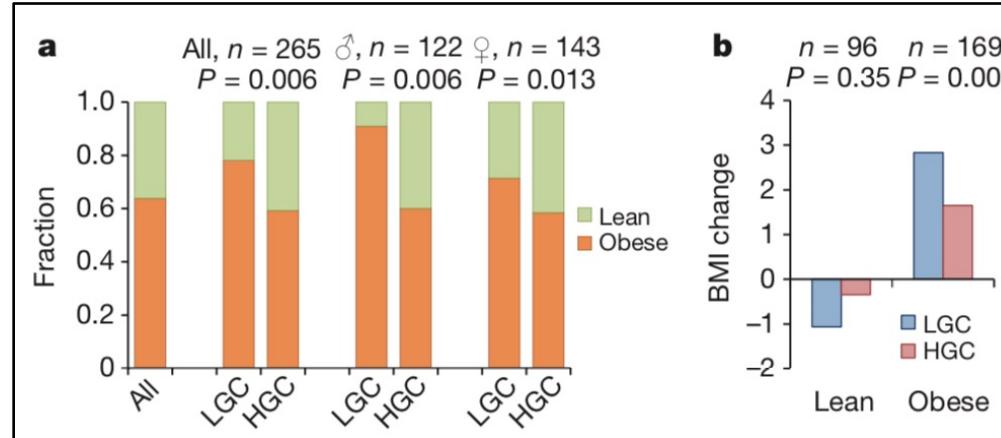
→ Enrichment of specific microbial taxa may influence the response to cancer immunotherapy

Routy et al., Gopalakrishnan et al., and Matson et al. Science 2018

GRAPHIC: V. ALTOUNIAN/SCIENCE

# Describing microbial communities – Example 2

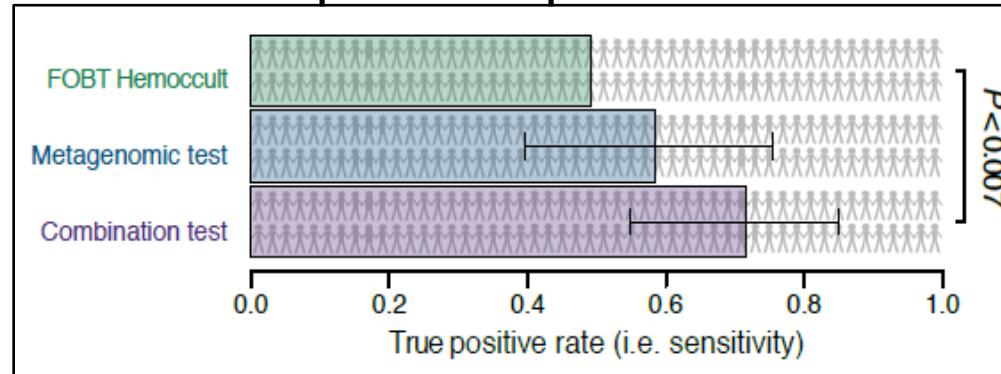
Less diverse microbiomes (LGC=low gene count) are associated with obesity (a) and higher weight gain (b)



- Many diseases are associated with low microbial diversity (e.g., obesity)
- Microbial community compositions can be indicative for disease (e.g., colorectal cancer)

Le Chatellier et al., Nature, 2014; Zeller et al., MSB, 2014

Microbiome composition can predict colorectal cancer



**LO 1: Today, you will learn how to formally describe the composition and diversity of, and differences between microbial communities**

# Why should I care about reconstructing, annotating and assessing the quality of microbial genomes?

- Discovery of many new microbial species, even entire new phyla
- Requirement to advance from: “Who is there?” to “What can they do?”
- New microbes may have health, ecologically and/or economically relevant functions
- Taxonomic information alone does not provide any information about the genomic capacity
- Genome-resolved analyses can explain many biologically relevant differences between very closely related organisms
  - Compare: differences between two human individuals: same 18S rRNA sequence, 99.6% genome similarity

**LO 2: Today, you will also learn about the process, value and challenges of reconstructing microbial genomes from metagenomes**

# Overview

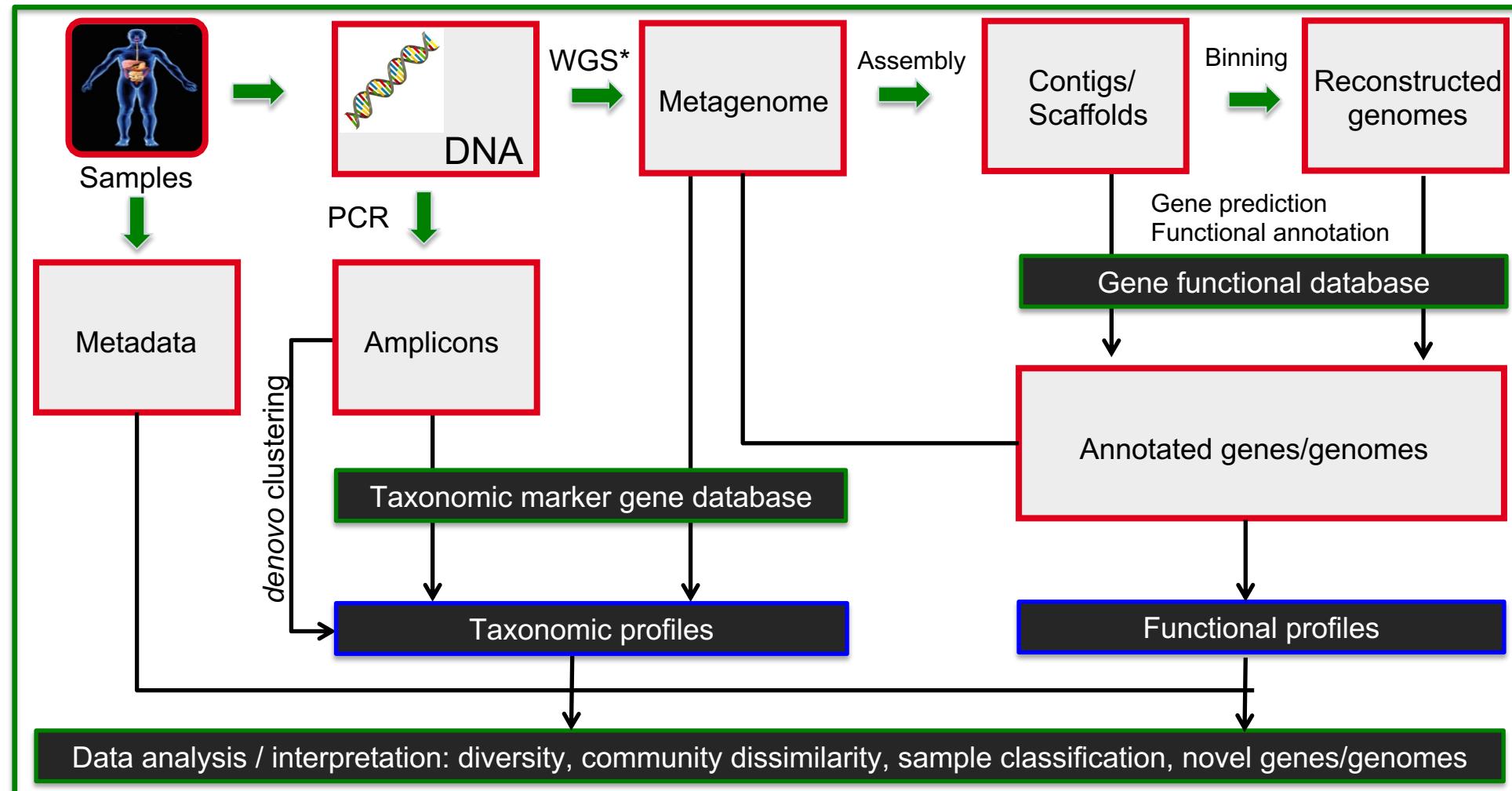
## **Part I - Microbial community structure**

- definition of genotype, taxonomic resolution and operational taxonomic units (OTUs)
- analysis of microbial diversity, richness, evenness
- comparison of microbial community compositions

## **Part II – Reconstruction and annotation of microbial community genomes**

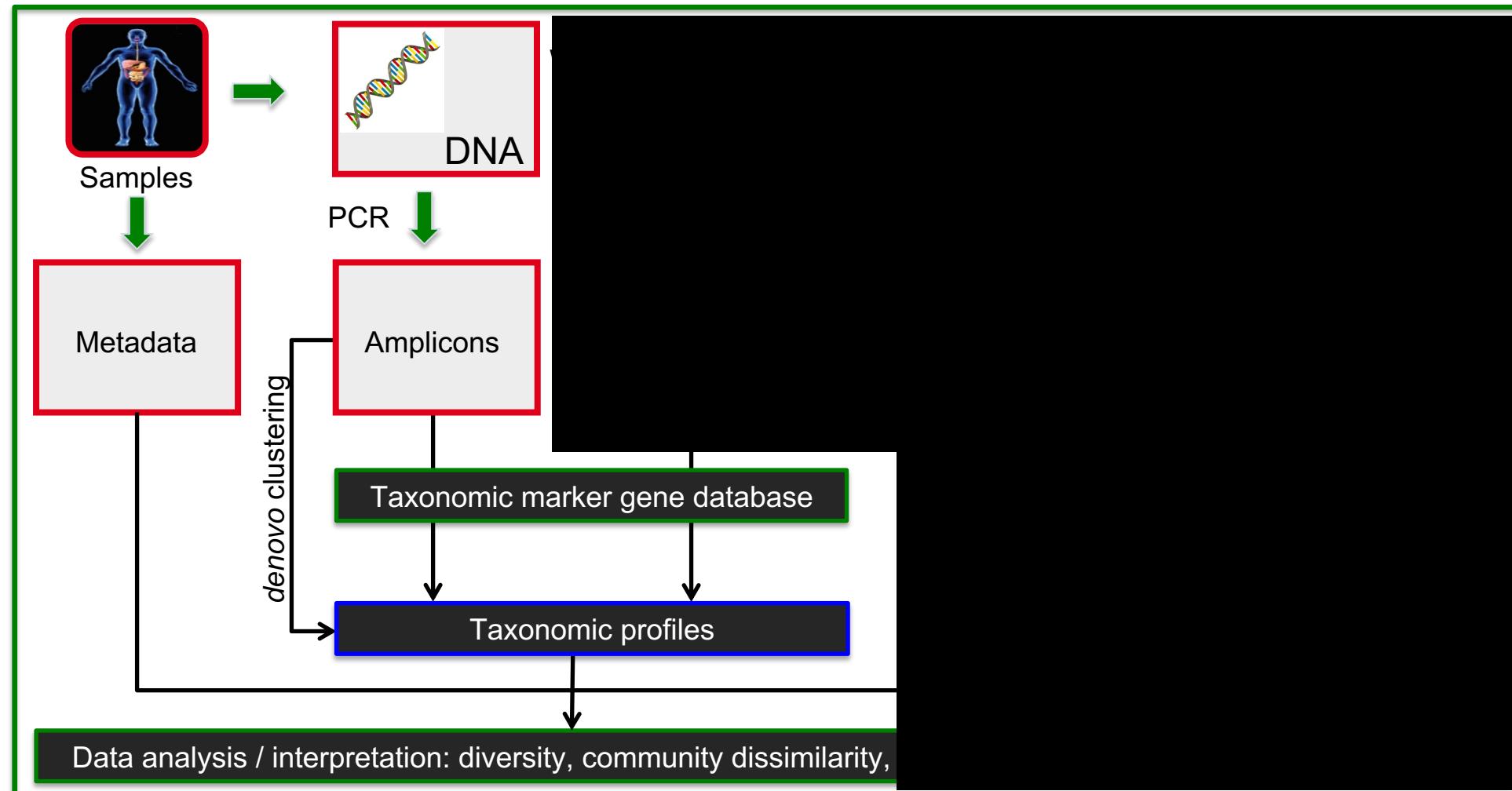
- assembly of individual genomes and metagenomes
- binning of metagenomic assemblies
- annotation of metagenomes and metagenome assembled genomes

# Overview – Part 1



\*WGS: whole genome shotgun sequencing

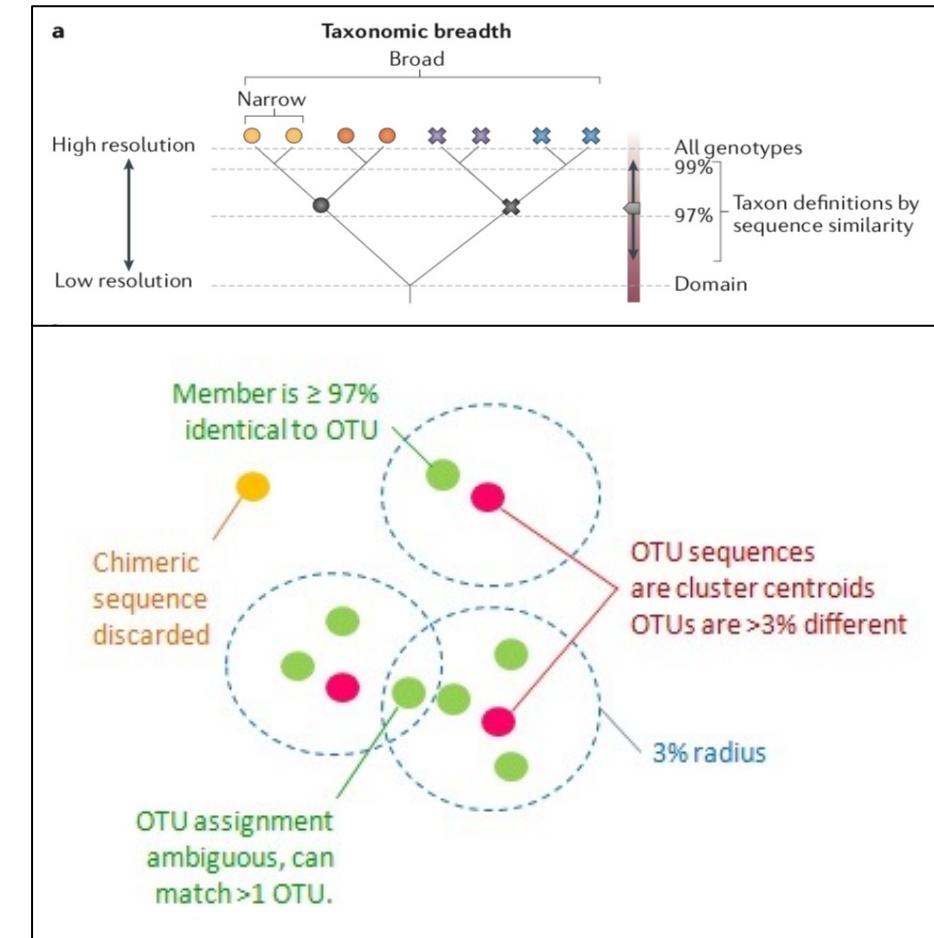
# Overview – Part 1



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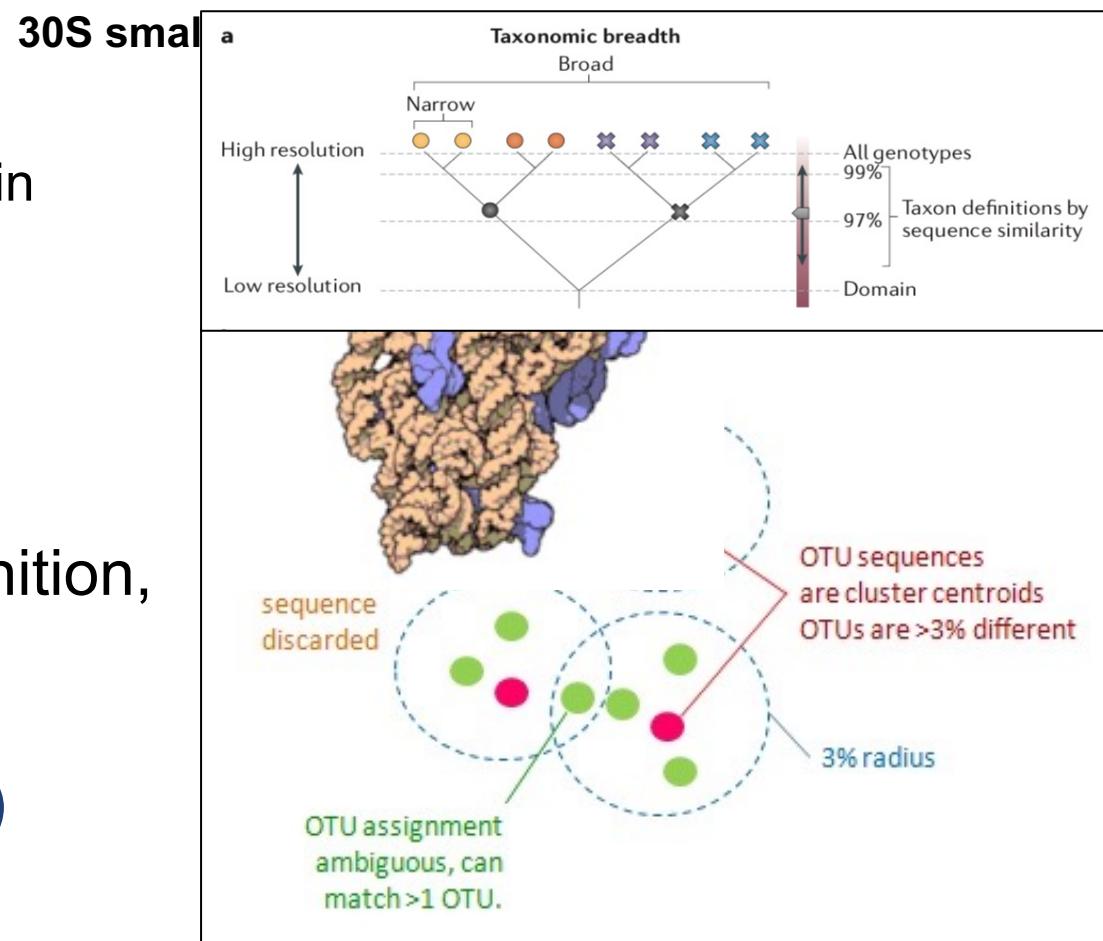
# Review: 16S rRNA-based Operational Taxonomic Units (OTUs)

- 16S rRNA
  - present in all prokaryotes
  - conserved function as integral part of the protein synthesis machinery
  - similar mutation rate: → molecular clock
- Proxy for phylogenetic relatedness of organisms
- Owing to lack of prokaryotic species definition, 97% sequence similarity is often used to define ‘species’-like:  
**“Operational Taxonomic Units” (OTUs)**



# Review: 16S rRNA-based Operational Taxonomic Units (OTUs)

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**“Operational Taxonomic Units” (OTUs)**



# Microbial community compositions

- Goal: determine ‘who’ is there at what abundance in one or more samples

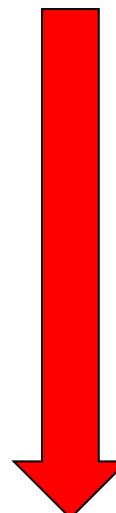
OTU count table

|     | OTU1 | OTU2 | OTU3 | OTU4 | OTU5 | ... | Sum |
|-----|------|------|------|------|------|-----|-----|
| S1  | 68   | 38   | 84   | 60   | 60   |     |     |
| S2  | 9    | 92   | 24   | 0    | 93   |     |     |
| S3  | 14   | 0    | 21   | 90   | 80   |     |     |
| S4  | 41   | 34   | 78   | 65   | 29   |     |     |
| S5  | 3    | 70   | 74   | 63   | 0    |     |     |
| ... |      |      |      |      |      |     |     |

Rows: S1 to Sn = samples  
Columns: OTUs

# Step 1: Generation of 16S rRNA amplicon reads by PCR

Community DNA extract



PCR

```
agtctcgctatgacgtcgtcgtcagactac  
gtcgtacgtcgatattctcgccggagc  
gtcgtacgtcgatattctcgccggagc  
agcctacgtcgatagtgcgttagtgtc
```

- Primers bind to conserved regions of constant regions.
- Variable regions are amplified by PCR



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

## Example

- “V4 primers” yield ca. 250 bp long amplicon reads
- After sequencing, amplicon reads are quality controlled, yielding high quality amplicon reads

→ Number of reads are proportional to number of gene copies in the community

## Step 2: De-replication of identical sequences

### High quality amplicon reads

ACGCTCTGAGCGGTAAGCACTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCACTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCACTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCACTAAGTCACACTG

ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG  
ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG

ACGCTCGGAGGGGTAAAGCACTAAGTCAGACTG  
ACGCTCGGAGGGGTAAAGCACTAAGTCAGACTG

### Unique high quality amplicon reads

{ ACGCTCTGAGCGGTAAGCACTAAGTCACACTG count = 4

{ ACGCTCTGAGCGGTAAGCTCTAAGTCACACTG count = 5

{ ACGCTCGGAGGGGTAAAGCACTAAGTCAGACTG count = 2

- All reads are aligned to each other to identify identical sequences
- Unique sequences are kept and the number of identical sequences is counted
- Output are unique sequences with records of identical sequences

# Step 3: Heuristic clustering of sequences into OTUs

Deterministic approach: calculate all pairwise similarities

→ too “expensive” (resource and time consuming)

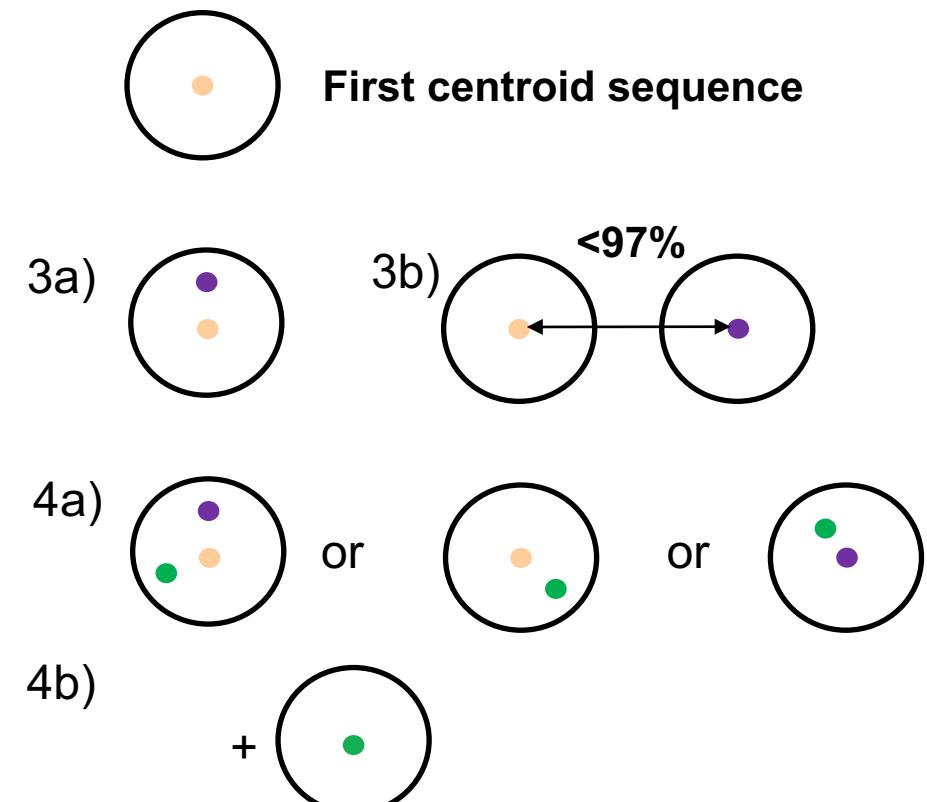
## Heuristic approach:

- 1) Unique high quality reads are sorted by counts (high to low)
- 2) Read with highest count is centroid of a new OTU (N=1)
- 3) Next read is compared to all OTU centroids

2 different possibilities:

- a) Centroid sequence and new read are  $\geq 97\%$  identical
    - read becomes new member of the OTU (N=1)
  - b) Centroid sequence and new read are  $< 97\%$  identical
    - read becomes centroid of a new OTU (N=2)
- 4) Next read is compared to all OTU centroids
- 2 different possibilities:
- a) Any centroid sequence and new read are  $\geq 97\%$  identical
    - read becomes new member of the OTU (N=N)
  - b) Any centroid sequence and new read are  $< 97\%$  identical
    - read becomes centroid of a new OTU (N=N+1)

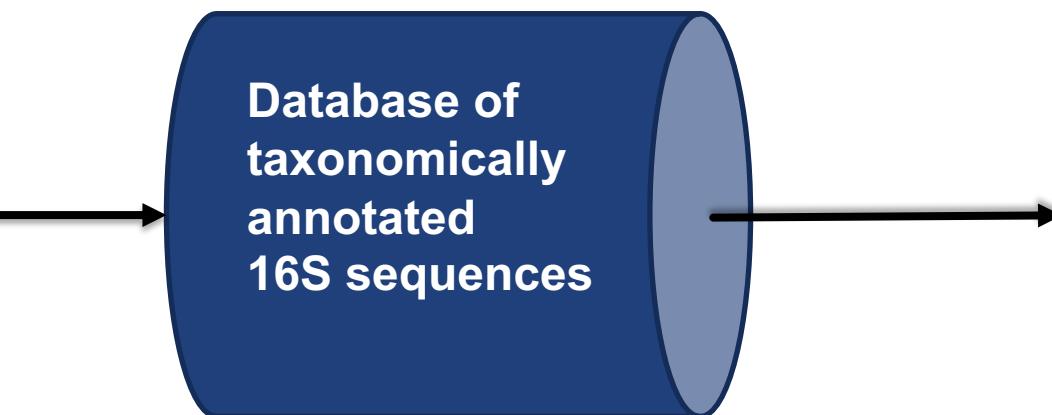
● ACGCTCTGAGCGGTAAAGCTTAAGTCACACTG count = 5  
● ACGCTCTGAGCGGTAAAGCCTTAAGTCACACTG count = 4  
● ACGCTCGGAGGGTAAGCCTTAAGTCAGACTG count = 2



## Step 4: Taxonomic annotation of OTUs

Sequence of OTU 1

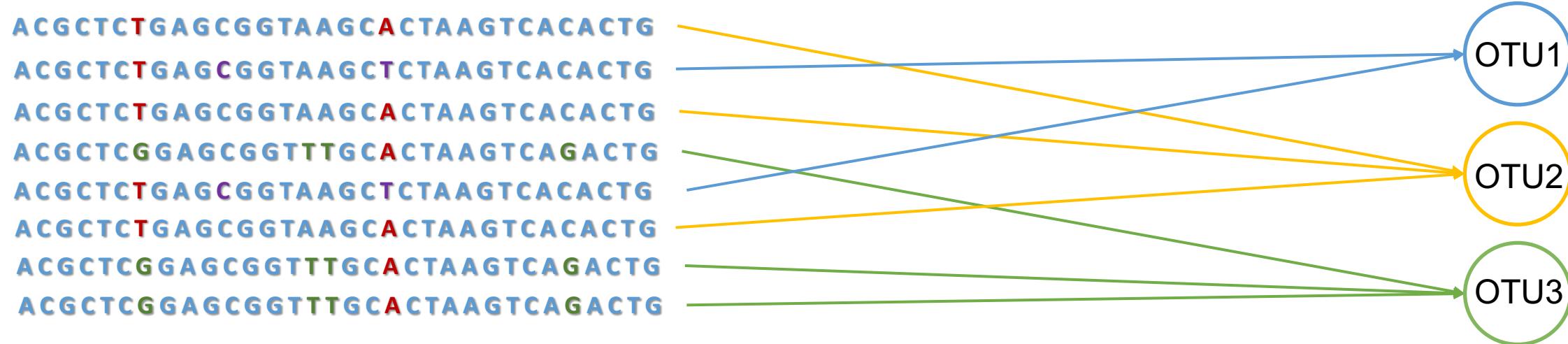
**ACGCTCAGAGCGGTAAAGCACTAA**



- Identification of taxon to which an OTU belongs
    - The centroid sequence of each OTU is compared to a database of annotated 16S rRNA gene sequences
- sequences are assigned to taxonomic ranks: phylum, class, family etc.

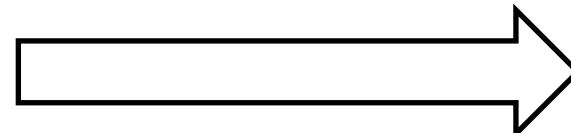
## Step 5: Quantification of OTU abundances

All reads are aligned to best matching OTU centroid sequence (and counted)



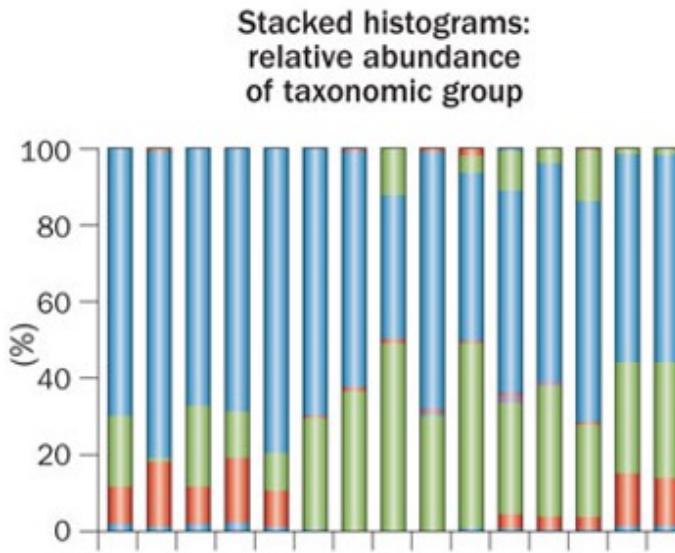
The result is an OTU count table, summarizing read counts for each OTU for each sample:

| OTU  | S1  | S2  | S3  |
|------|-----|-----|-----|
| OTU1 | 234 | 87  | 166 |
| OTU2 | 23  | 0   | 93  |
| OTU3 | 2   | 137 | 191 |
| OTU4 | 455 | 0   | 112 |
| OTU5 | 23  | 229 | 66  |

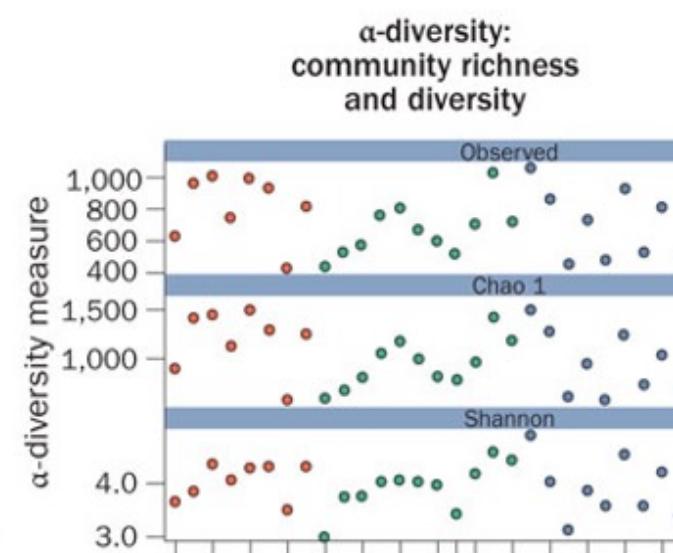


Data analysis / interpretation: diversity, community dissimilarity, sample classification

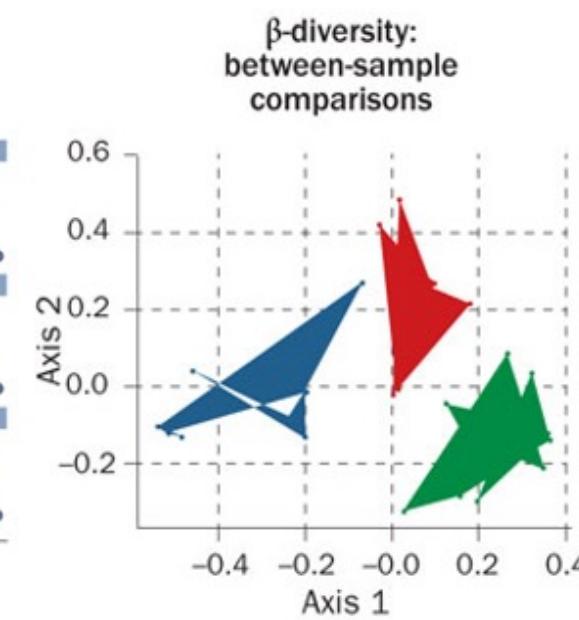
# Typical analyses using OTU count tables



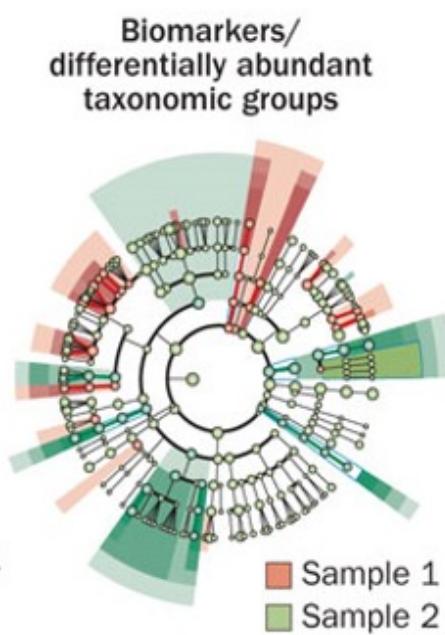
Determine the **relative abundance** of different taxa in microbial communities



Determine the **taxonomic diversity** of microbial communities  
→ alpha diversity



Compare **differences between** microbial community compositions  
→ beta diversity



Determine **differential abundance** of taxa between samples

## In-class task 1: alpha diversity

- Assume 4 different samples (A-D), each with 100 reads sequenced

| OTUs | Sample A | Sample B | Sample C | Sample D |
|------|----------|----------|----------|----------|
| 1    | 20       | 1        | 25       | 0        |
| 2    | 20       | 10       | 25       | 0        |
| 3    | 20       | 20       | 0        | 0        |
| 4    | 20       | 30       | 25       | 0        |
| 5    | 20       | 39       | 25       | 100      |
| Sum  | 100      | 100      | 100      | 100      |

- In pairs, discuss how the diversity of one sample could be formally described (i.e., measured in quantitative terms):
  - How are the 100 reads distributed among the 5 OTUs?
  - Are all OTUs present in a given sample?

→ What are the factors that influence the differences between samples?

# In-class task 1: alpha diversity

Shannon's diversity index ( $H'$ )

$$H' = - \sum_{i=1}^R p_i \ln p_i$$

R = richness

$p_i$  = the proportion of the  $i$ -th OTU

$n_i$  = the number individuals of the  $i$ -th OTU

n = total number of individuals

Pielou's evenness ( $J'$ )

$$J' = \frac{H'}{\ln R}$$

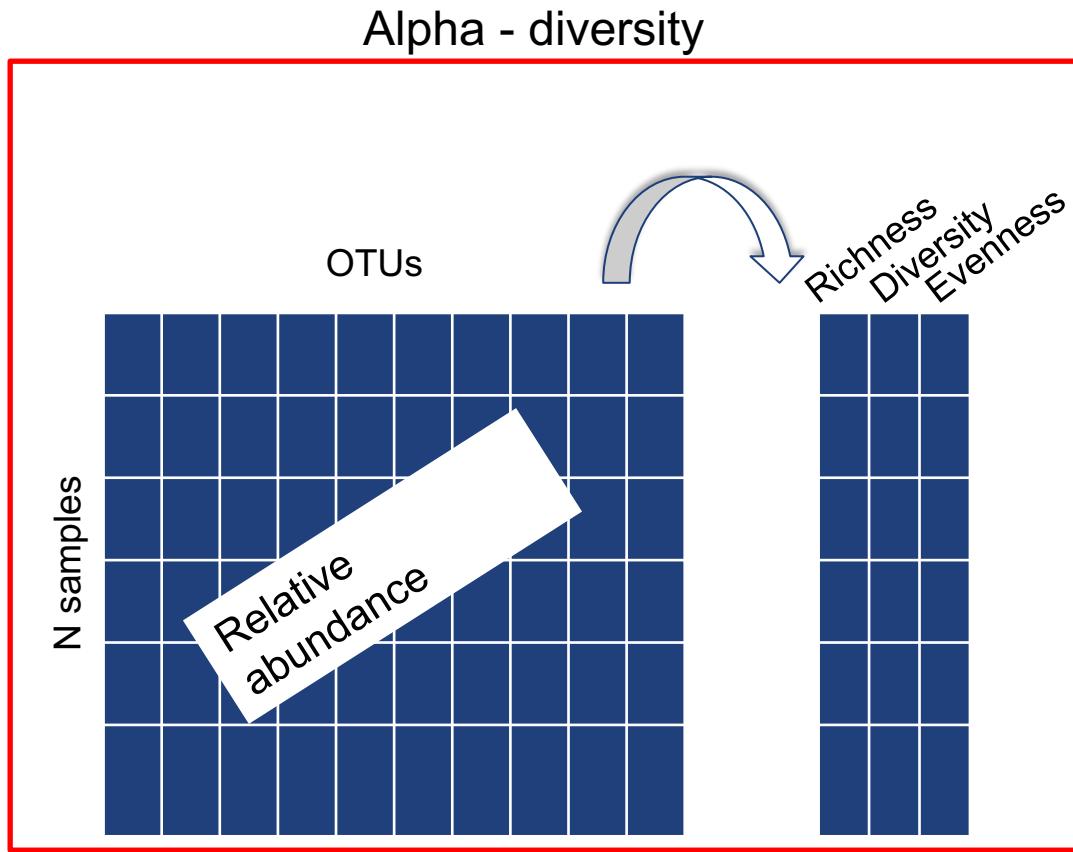
# Test your newly acquired knowledge

| OTUs | Sample A | Sample B | Sample C | Sample D |
|------|----------|----------|----------|----------|
| 1    | 1        | 1        | 4        | 1        |
| 2    | 1        | 2        | 2        | 2        |
| 3    | 1        | 3        | 1        | 3        |
| 4    | 0        | 4        | 0        | 4        |
| 5    | 0        | 5        | 0        | 0        |

**Which of the samples on the left (A-D) is:**

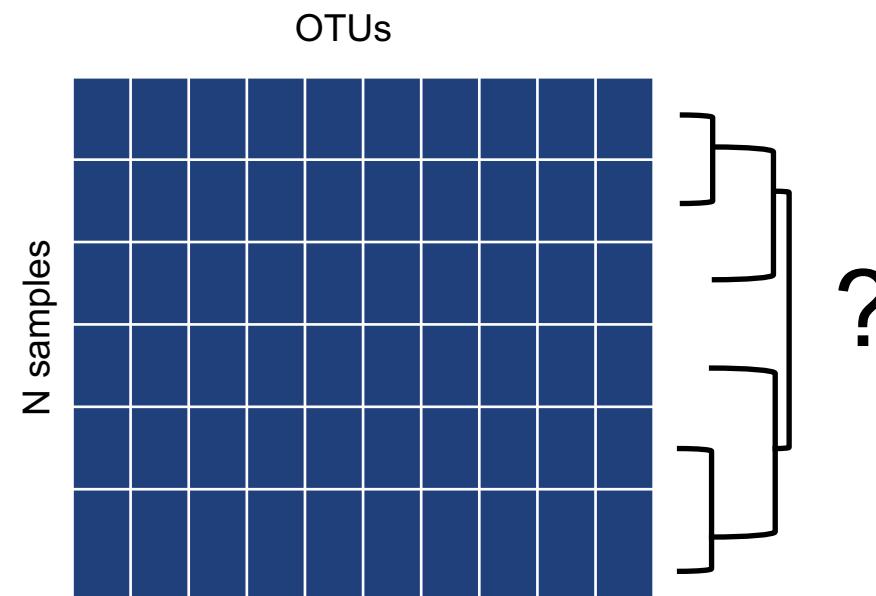
- **the richest?**
- **the most even?**
- **the most diverse?**

# Concept of alpha diversity - summary



# Beta diversity: between sample dissimilarity

- Now that we learned how to describe the diversity of an individual sample, how do we compare different communities to each other?



## In-class task 2: beta diversity

| OTUs | Sample A |
|------|----------|
| 1    | 1        |
| 2    | 1        |
| 3    | 1        |
| 4    | 0        |
| 5    | 0        |

| OTUs | Sample B |
|------|----------|
| 1    | 1        |
| 2    | 1        |
| 3    | 1        |
| 4    | 1        |
| 5    | 1        |

| OTUs | Sample C |
|------|----------|
| 1    | 4        |
| 2    | 1        |
| 3    | 1        |
| 4    | 0        |
| 5    | 0        |

| OTUs | Sample D |
|------|----------|
| 1    | 2        |
| 2    | 2        |
| 3    | 2        |
| 4    | 0        |
| 5    | 0        |

→ In pairs, please discuss how pairwise similarities of samples A, B, C, and D could be quantified?

→ Both qualitative differences vs quantitative differences can be taken into account.

## In-class task 2: beta diversity

| OTUs | Sample A |
|------|----------|
| 1    | 1        |
| 2    | 1        |
| 3    | 1        |
| 4    | 0        |
| 5    | 0        |

| OTUs | Sample B |
|------|----------|
| 1    | 1        |
| 2    | 1        |
| 3    | 1        |
| 4    | 1        |
| 5    | 1        |

| OTUs | Sample C |
|------|----------|
| 1    | 4        |
| 2    | 1        |
| 3    | 1        |
| 4    | 0        |
| 5    | 0        |

| OTUs | Sample D |
|------|----------|
| 1    | 2        |
| 2    | 2        |
| 3    | 2        |
| 4    | 0        |
| 5    | 0        |

### Example: Jaccard index/dissimilarity

Jaccard index:  $J = a / (a + b + c)$

where

a = # of species shared

b= # of species unique to sample 1

c= # of species unique to sample 2

Jaccard distance / dissimilarity:  $D = 1 - J$

→ Note: For Jaccard distance, only presence/absence of species are considered!

# Other distance (dissimilarity) measures

The formulae for calculating the ecological distances are:

$$\text{Bray-Curtis: } D = 1 - 2 \frac{\sum_{i=1}^S \min(a_i, c_i)}{\sum_{i=1}^S (a_i + c_i)}$$

$$\text{Kulczynski: } D = 1 - \frac{1}{2} \left( \frac{\sum_{i=1}^S \min(a_i, c_i)}{\sum_{i=1}^S a_i} + \frac{\sum_{i=1}^S \min(a_i, c_i)}{\sum_{i=1}^S c_i} \right)$$

$$\text{Euclidean: } D = \sqrt{\sum_{i=1}^S (a_i - c_i)^2}$$

$$\text{Chi-square: } D = \sqrt{\sum_{i=1}^S \frac{(a_+ + c_+)}{(a_i + c_i)} \left( \frac{a_i}{a_+} - \frac{c_i}{c_+} \right)^2} \text{ with } a_+ = \sum_{i=1}^S a_i$$

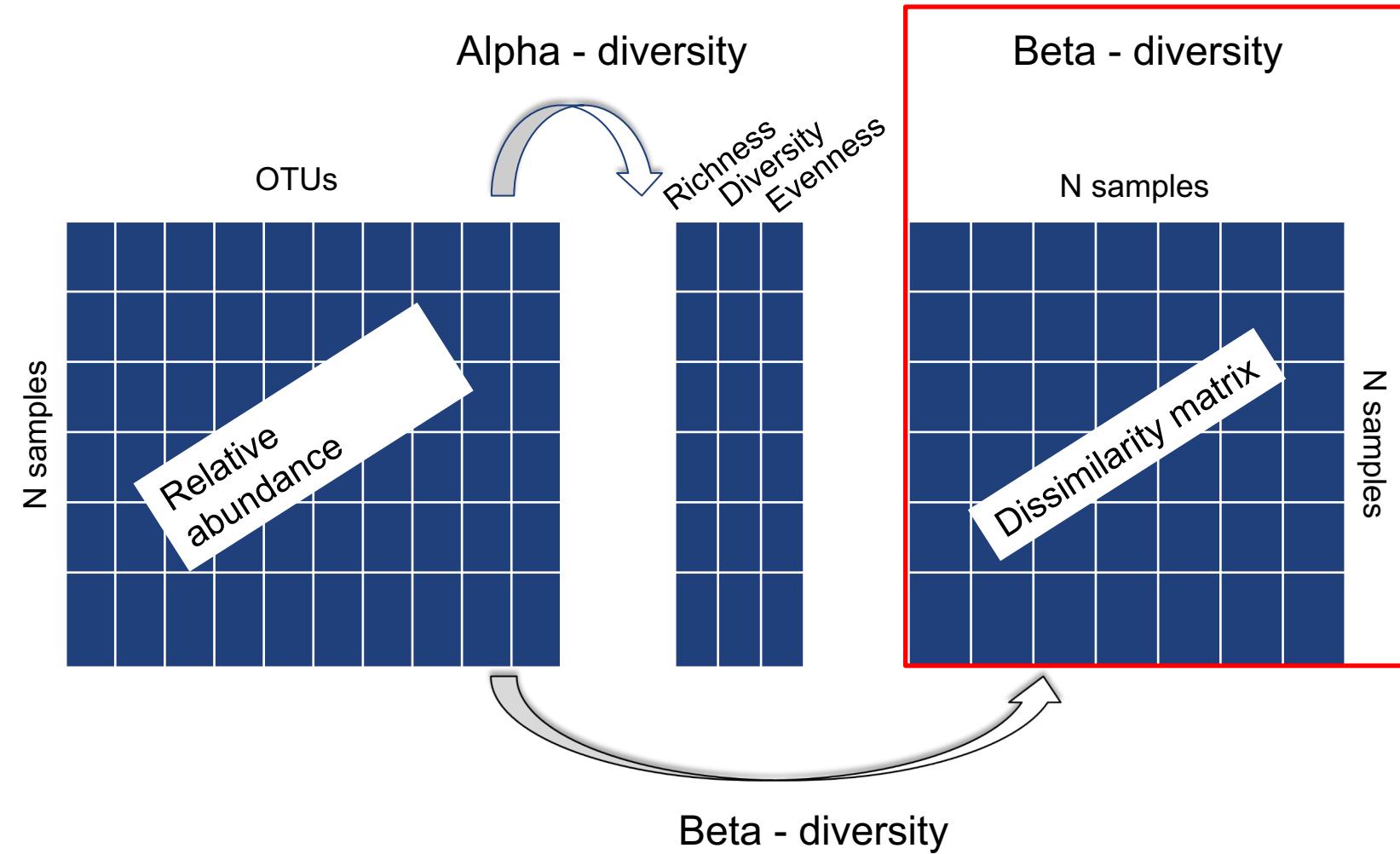
$$\text{Hellinger: } D = \sqrt{\sum_{i=1}^S \left( \sqrt{\frac{a_i}{a_+}} - \sqrt{\frac{c_i}{c_+}} \right)^2} \text{ with } a_+ = \sum_{i=1}^S a_i$$

**UniFrac distance** = phylogenetically weighted distance

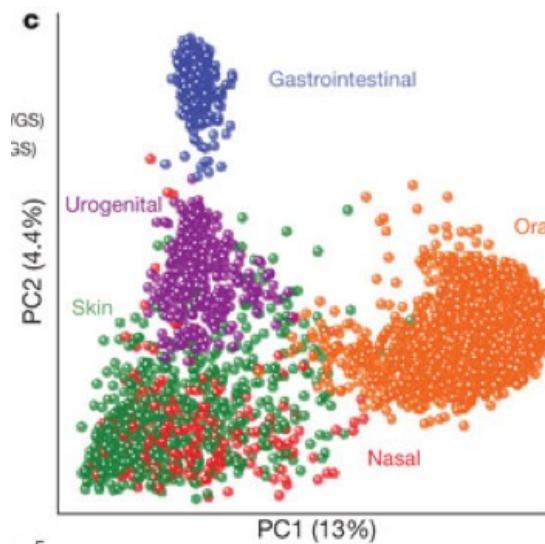
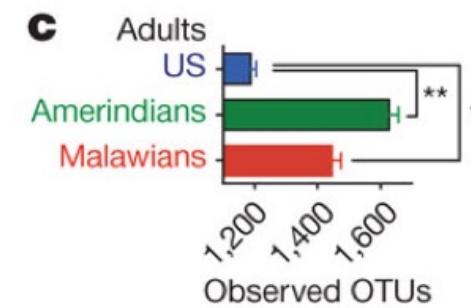
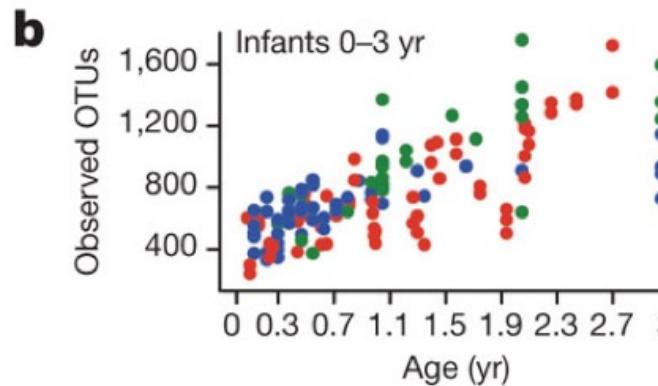
Lozupone et al., 2005

$a_i$  = abundance of taxon  $i$  in sample  $a$ , and  
 $c_i$  = abundance of taxon  $i$  in sample  $c$

# Within sample descriptions → between sample comparisons



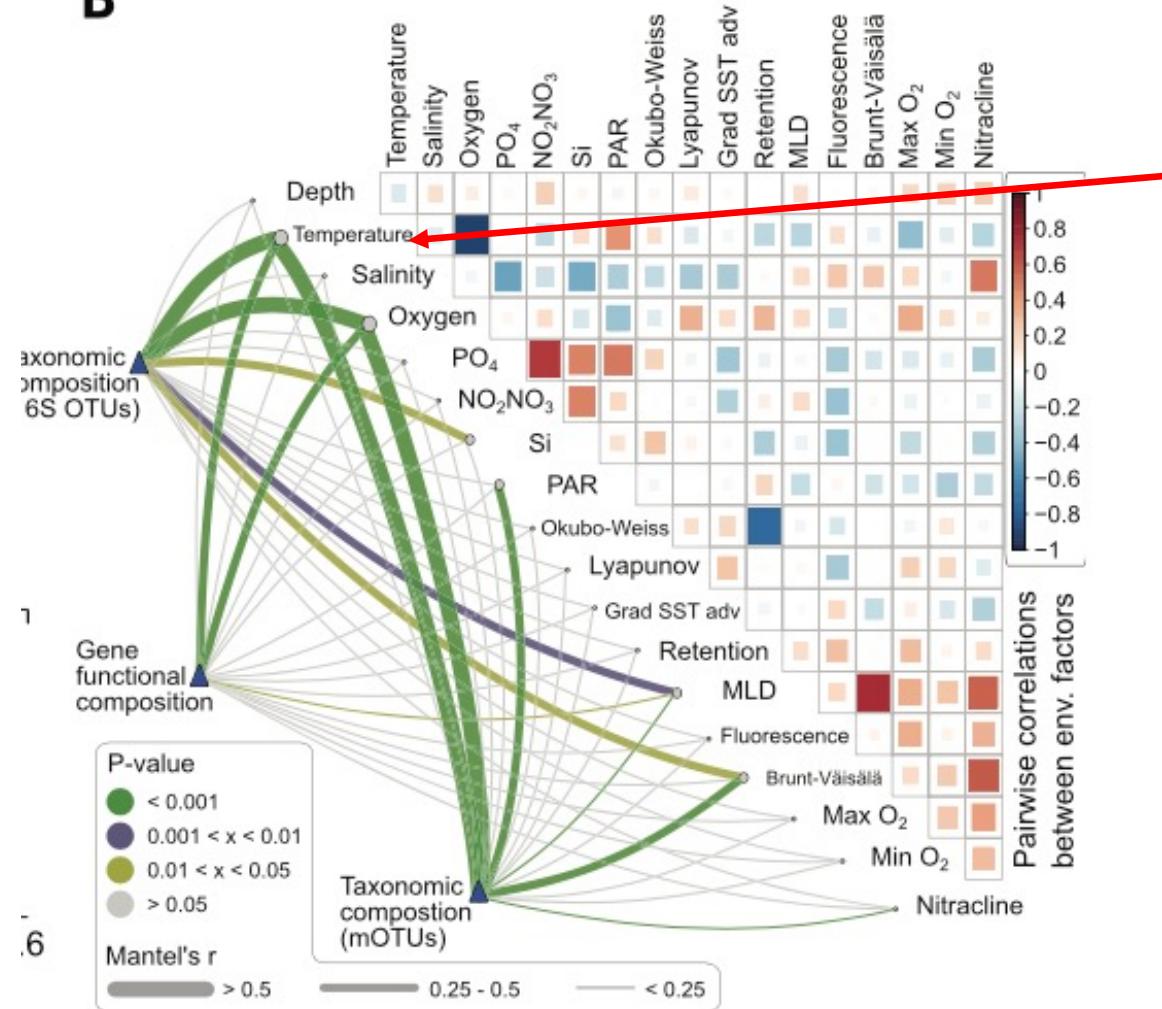
# Applied examples I



- Microbial diversity in human gut increases with age
- US citizens have harbor less diverse gut microbiota relative to other populations
- Microbial communities cluster by human body site rather than by individual

# Applied examples II

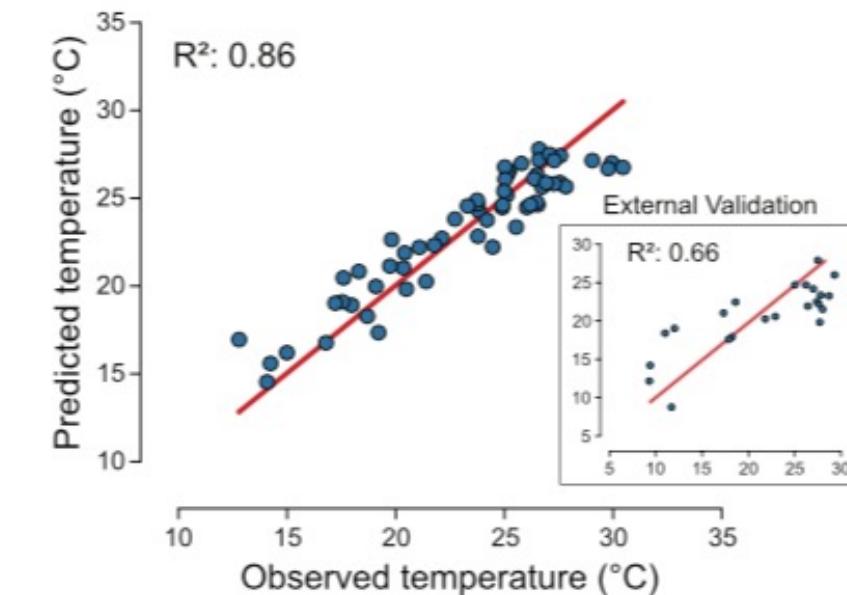
B



Temperature has highest correlation with surface microbial community composition in the open ocean

B

Cross-validation Tara Oceans samples



# Summary – Part I

- Metagenomics provides information about microorganisms that are often difficult or impossible to be cultivated in their natural environment
- Due to the lack of species concept for prokaryotes, researchers use sequence identity cutoffs of marker genes to define operational taxonomic units
- Microbial community structure describes the richness (number of species) of taxa and their evenness (the distribution of their abundances)
  - Alpha diversity (within sample diversity) is a function of richness and evenness
  - Alpha diversity can be quantified by diversity indices (e.g., Shannon, Simpson)
- Beta diversity describes differences in microbial community structures
  - Differences are quantified by dissimilarity indices (e.g., Jaccard, Bray-Curtis)

# Overview of the Metagenomics lecture

## Part II – Reconstruction and annotation of microbial community genomes

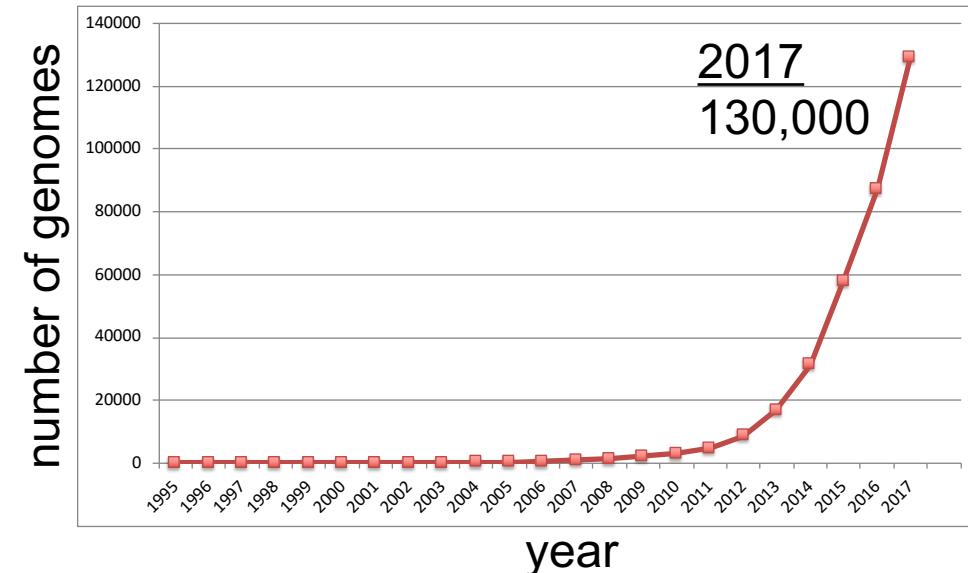
- assembly of individual genomes and metagenomes
- binning of metagenomic assemblies into metagenome-assembled genomes
- taxonomic and functional annotation of metagenomes

# Background: from taxonomic composition to genomic content

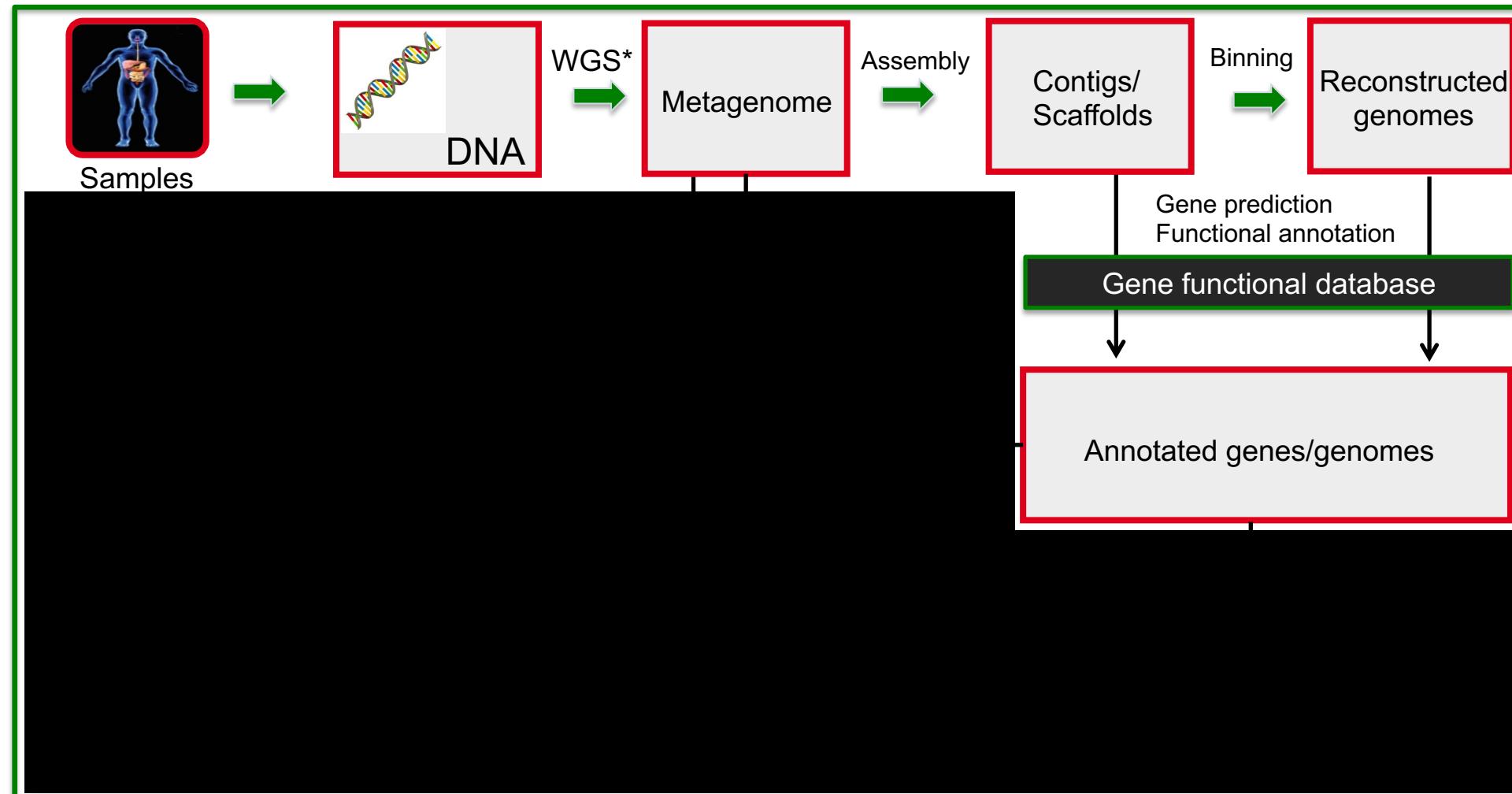
- First microbial genome (1995): *Haemophilus influenzae* *Fleischmann et al. 1995*
- Followed by many isolated pathogens of diseases (plague, anthrax, tuberculosis, Lyme disease, malaria, and sleeping sickness)
- Many isolates of important non-pathogenic species: e.g., *Prochlorococcus*, *Lactobacillus*, *Bradyrhizobium*
- Bacteria and archaea have ca. 500–10,000 genes, usually arrayed on circular DNA molecules (e.g., chromosomes and plasmids)
- Protein coding genes are on average ca. 1,000 base pairs long
- Their genomes are ca. 600,000–12 million bp in size (human 2 x 3 billion bp)

# Background: added value of metagenomics

- Most bacteria and archaea cannot be isolated, as they live in communities and depend on other organisms
  - Metagenomics provides access, in principle, to all genomic resources of a microbial community. This allows us to ask “what can they do?” (in addition to “who is there?”).
  - Sequencing costs human genome: \$1,000
  - Microbial genomes: ~\$1
- Costs for data analysis are now much higher than for data generation

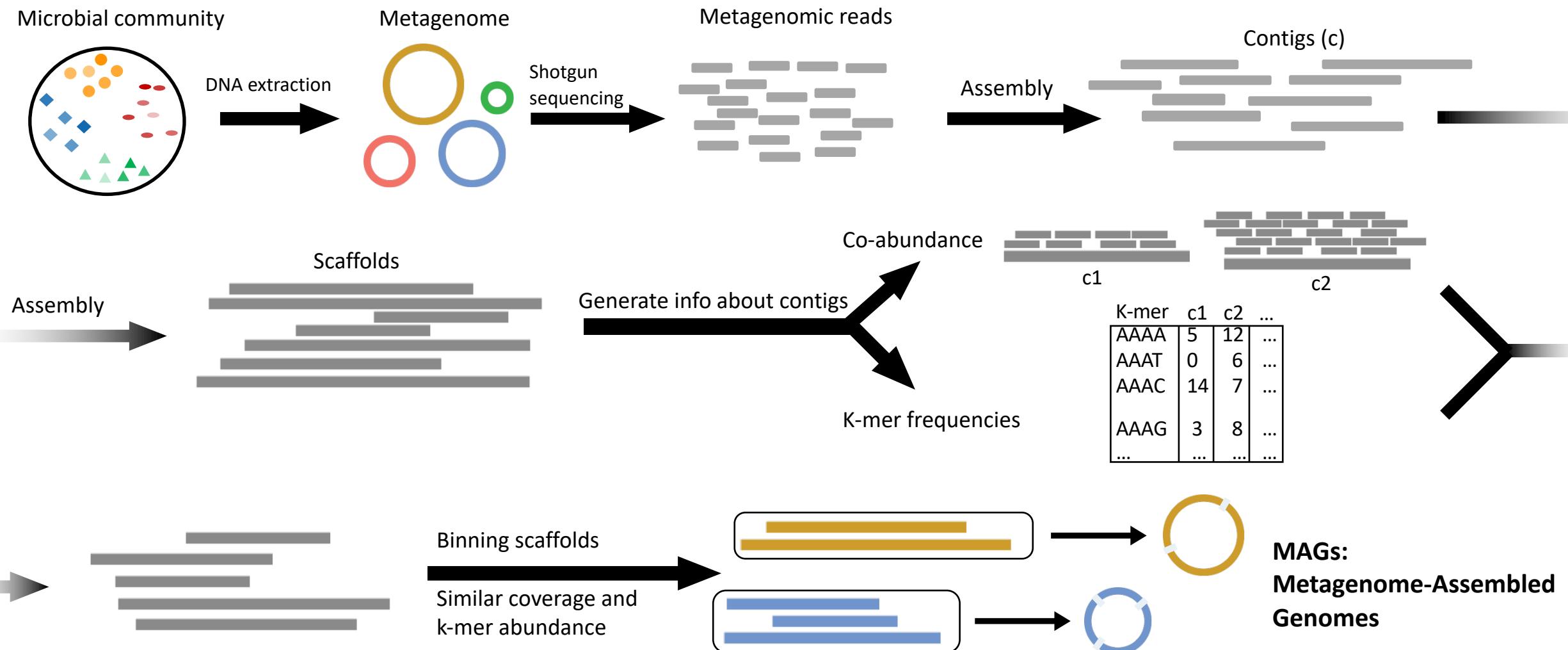


# Overview – Part 2



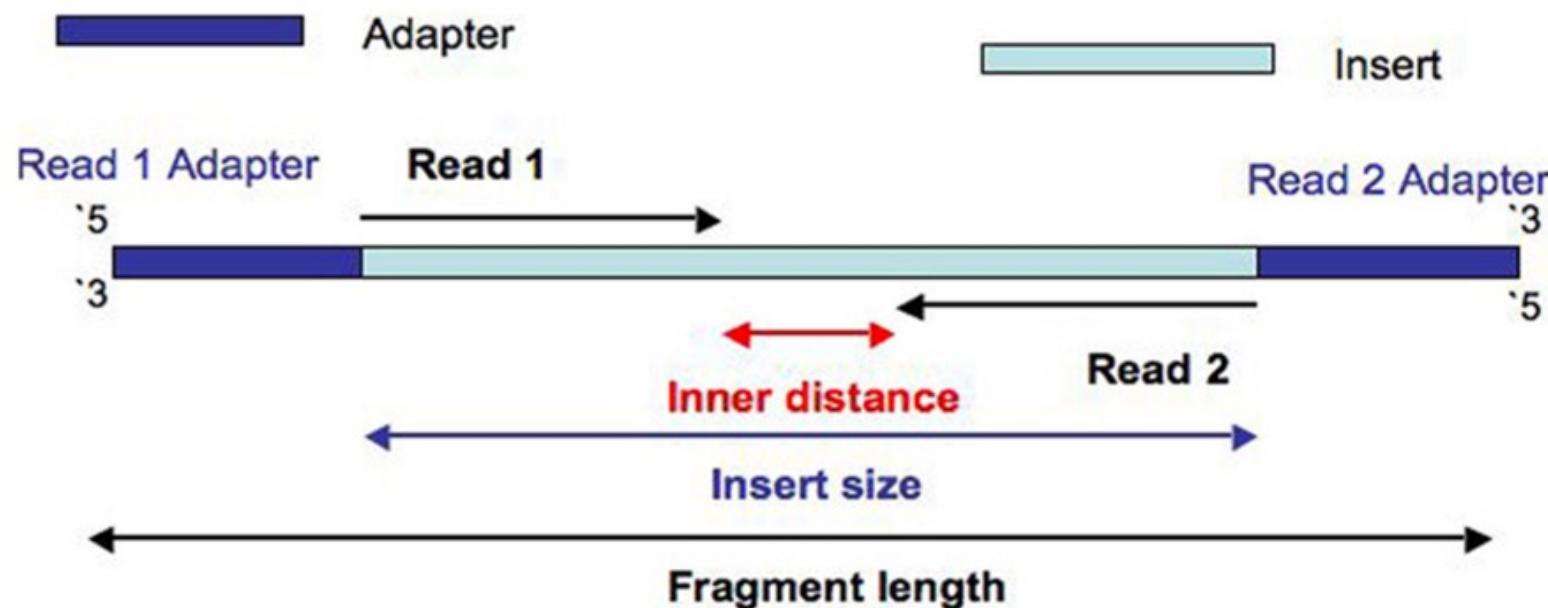
\*WGS: whole genome shotgun sequencing

# Reconstruction of (community) genomes: overview



# Background: DNA sequencing libraries

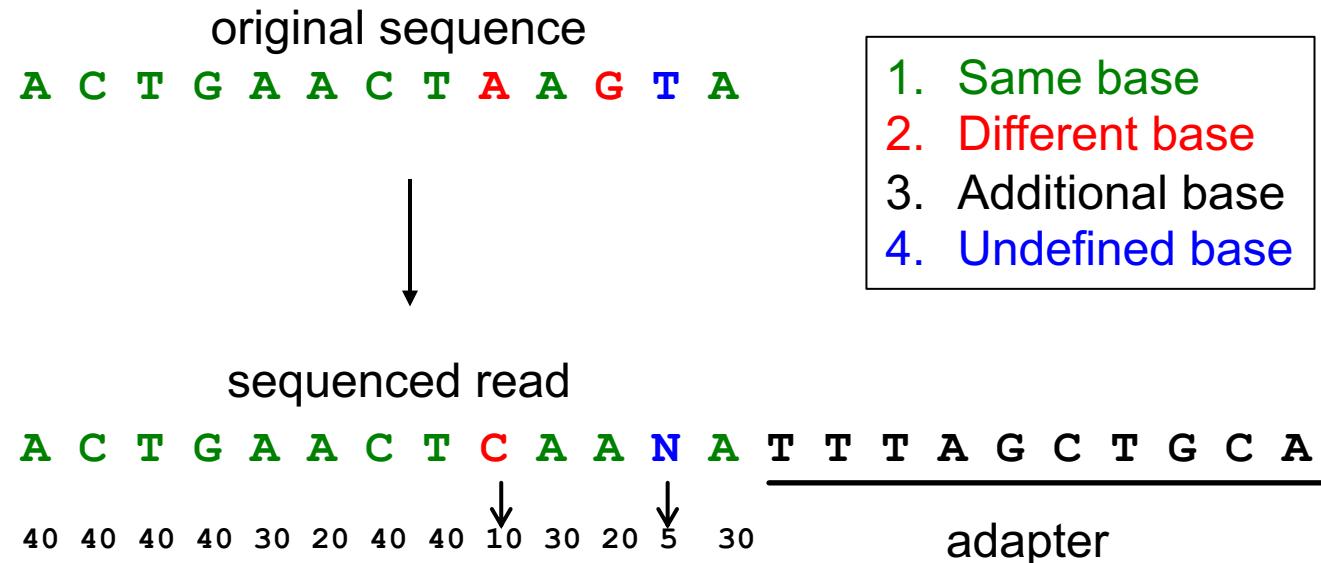
- DNA extracted from a metagenomic sample is randomly sheared into inserts of known size distribution (i.e., min, max, mean)
- Adapters are added to facilitate the sequencing of these inserts



Note: Paired end reads may be overlapping providing the possibility to “merge” reads into one or not. In the latter case, the sequence between the paired reads remains unknown, while the length can be estimated due to the known insert size distribution

# Step 1: Data quality control - sources of errors

## 1. Low base calling quality scores



## Other sources of error

2. Residual adapter sequences
3. Residual control DNA sequences (e.g., “PhiX spike-ins”)
4. Contamination from non-target organisms

## Base calling quality (*phred*) scores

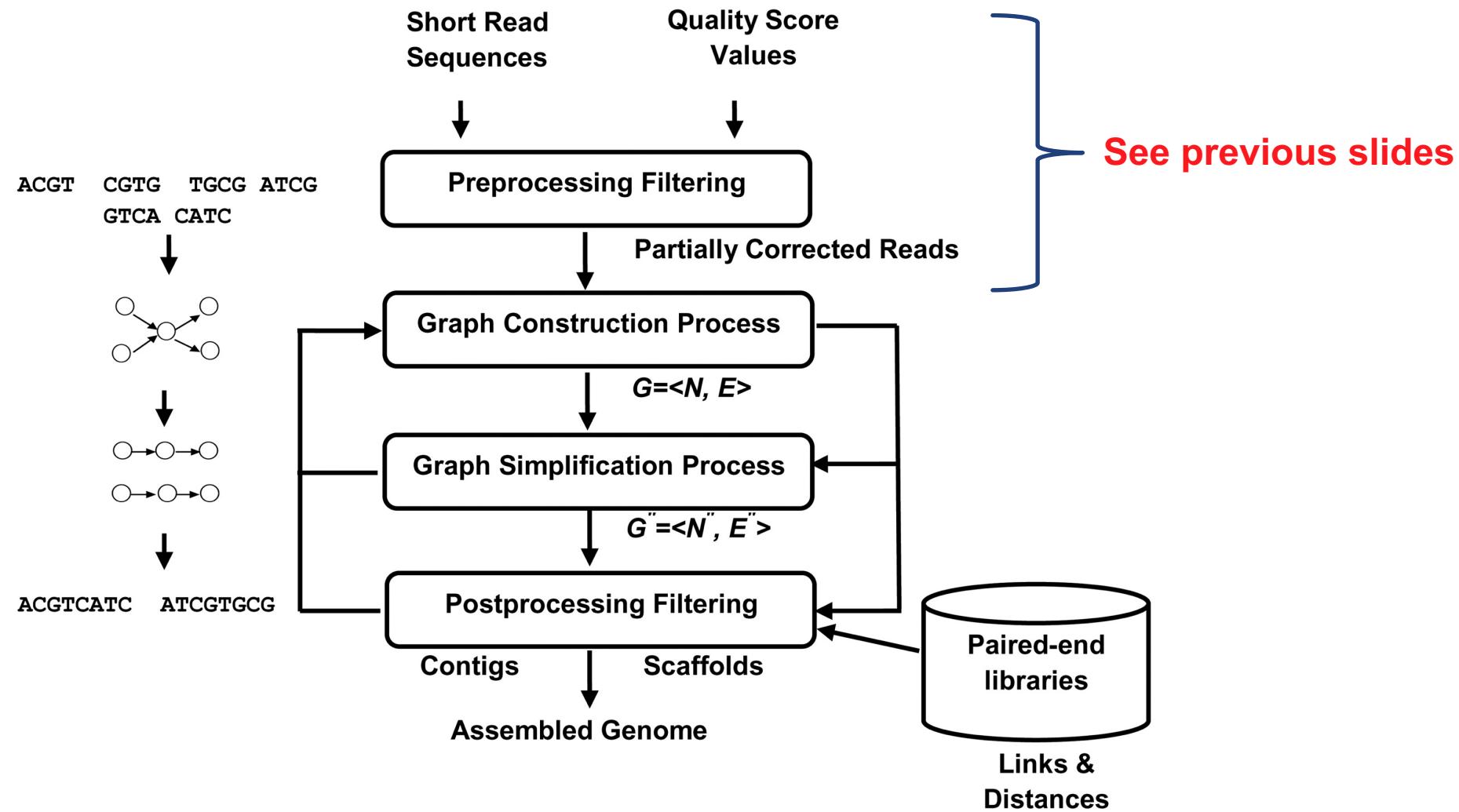
$$Q = -10 \log_{10} P$$

Probability of error:  $P = 10^{-Q/10}$

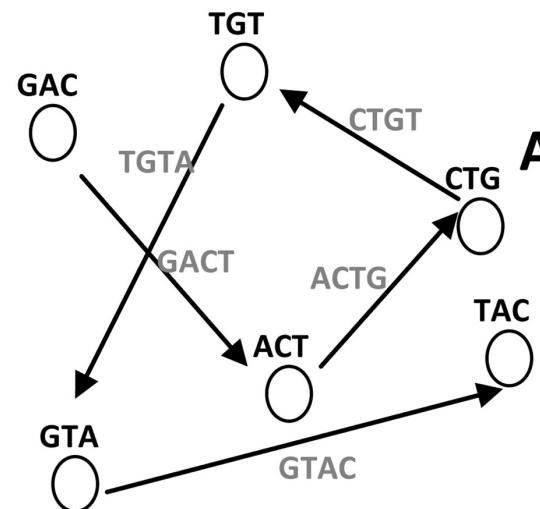
Probability of truth:  $1 - P$

| Quality score | % Correct Base |
|---------------|----------------|
| 40            | 99.99          |
| 30            | 99.9           |
| 20            | 99             |
| 10            | 90             |

## Step 2: Assembly and scaffolding: overview

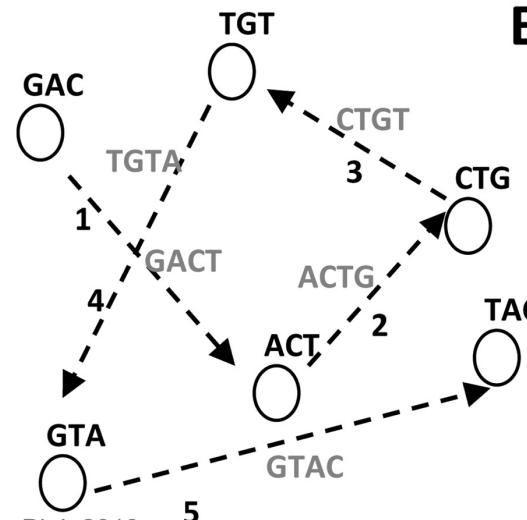


# Graph construction: k-mer based assembly



$$R_1 = \text{GACTGTA} \quad R_2 = \text{ACTGTAC}$$

Set of 3-Kmers of  $R_1$  = GAC, ACT, CTG, TGT, GTA  
Set of 3-Kmers of  $R_2$  = ACT, CTG, TGT, GTA, TAC



Example of an Eulerian path :

GACT  
ACTG  
CTGT  
TGTA  
GTAC

C Assembled Reads :  
GACTGTAC

A) k-mer-based graph  
Nodes = k-mers  
Edges = k-1 overlaps

B) Layout shortest Eulerian path  
Visit each edge once

C) Combine into consensus

# Post processing: scaffolding

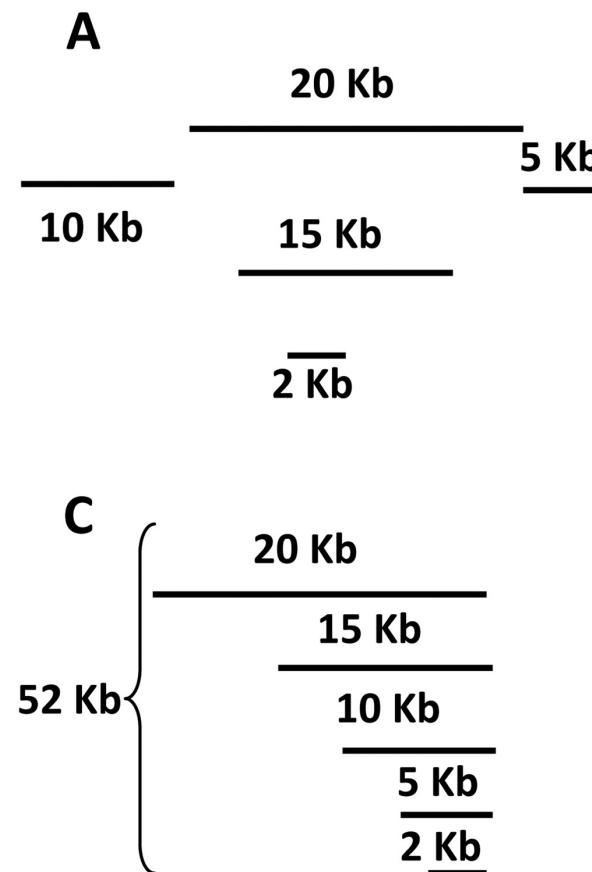


**A) Align paired-end reads**

**B) Orientation of contigs  
→ according to read orientation**

**C) Scaffolding  
→ use information on insert size distribution and fill ‘gaps’ with ‘Ns’**

# Assembly quality: N50 and L50



A) Set of contigs

B) Sort contigs by size in descending order

C) Calculate total length of all contigs

D) Add length in descending order until sum equals or exceeds 50% of the total length.

→ N50 = size of last contig added (15kb)

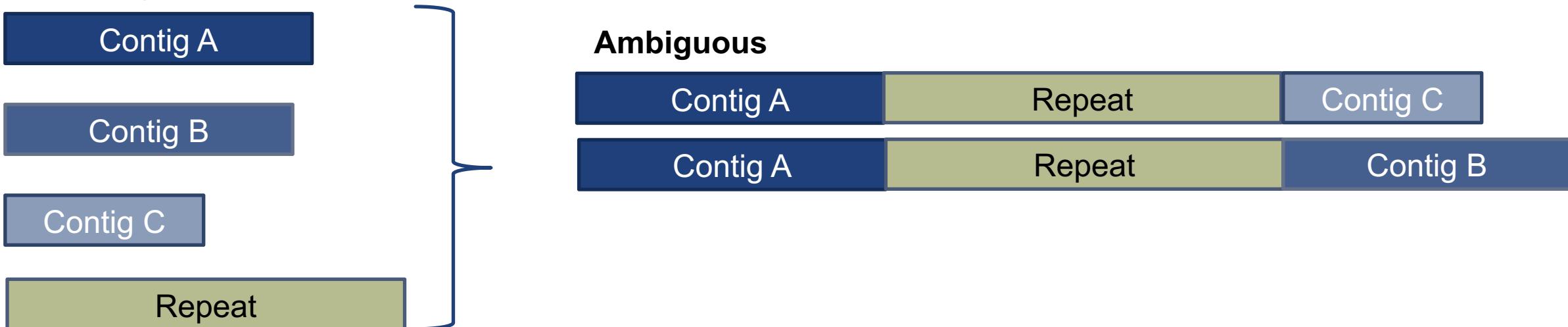
→ L50 is the number of contigs required to equal or exceed N50

# Repetitive sequences in genomes prevent full assembly

Genome



Assembly



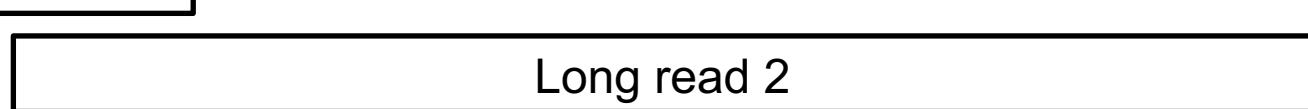
# Repetitive sequences in genomes prevent full assembly

Genome

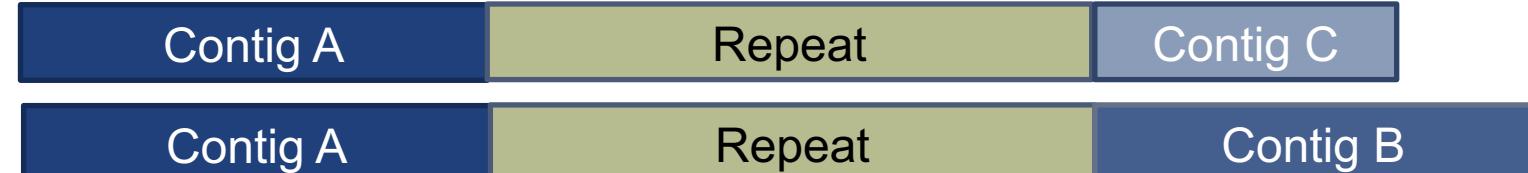


Long read 1

Assembly



**Ambiguous**

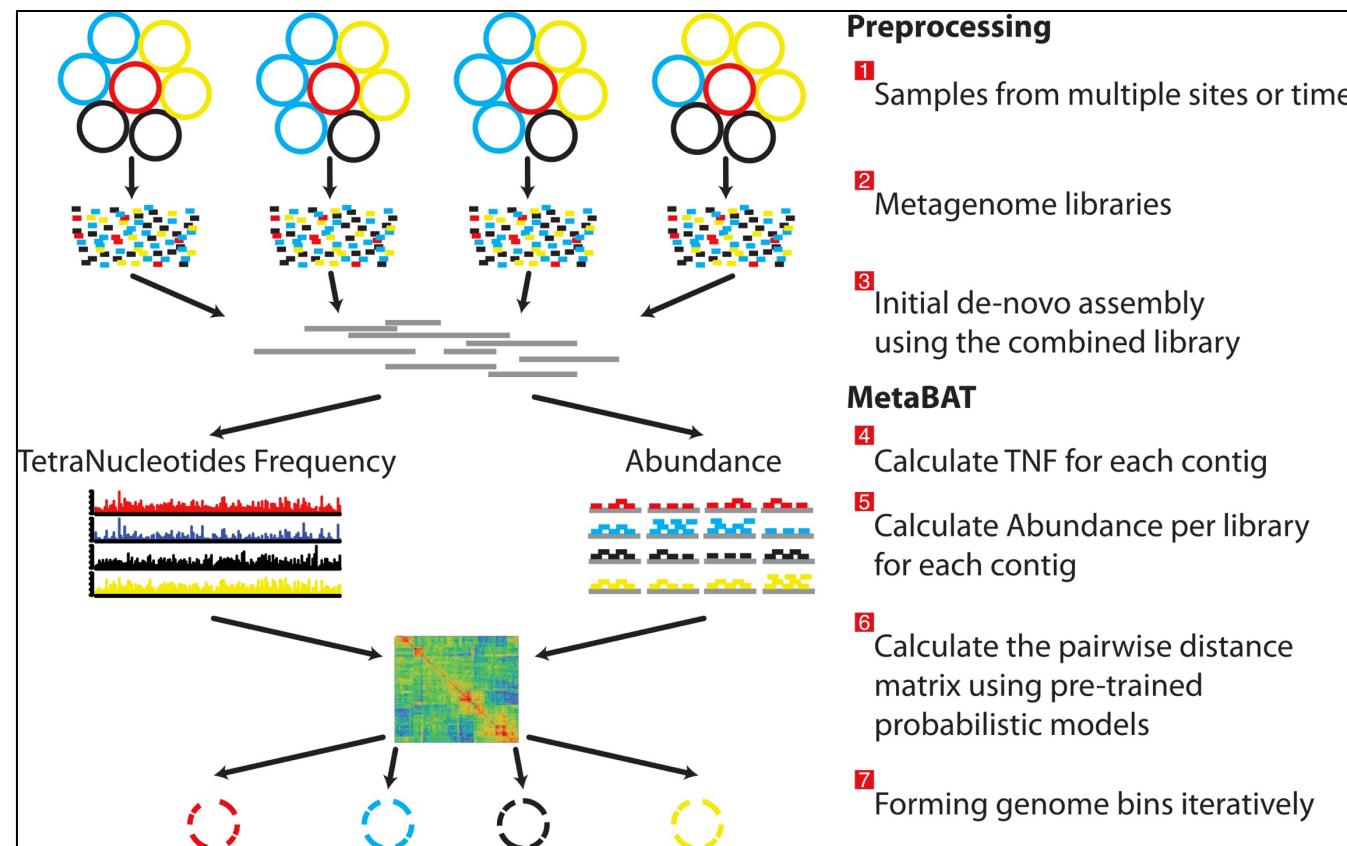


→ Long sequencing reads can be used to resolve repeats

# Step 3: Binning contigs/scaffolds – composition guided

Composition-guided binning (independent from external information):

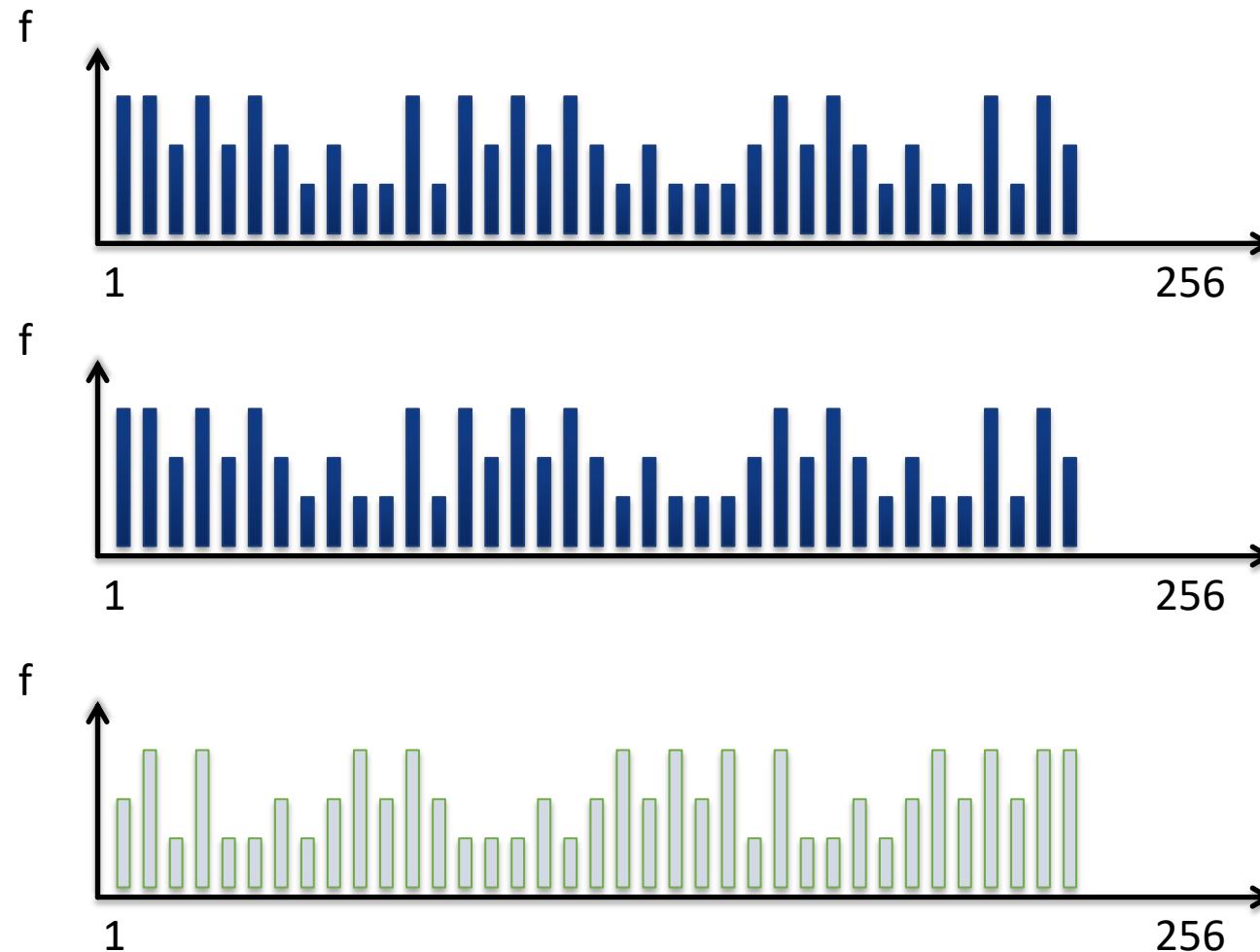
Tetranucleotide (or other k-mer) frequencies (e.g., GGAG vs. GGAC) + co-abundance



- For each contig:
  - a) calculate k-mer frequencies
  - b) calculate read abundance
- Combine a) and b) into a distance matrix
- Resolve distance matrix into clusters of highly correlated contigs

# Example for tetranucleotide frequency (TNF) distances

$[ATGC]^4 = 256$  possible combinations



Contig 1

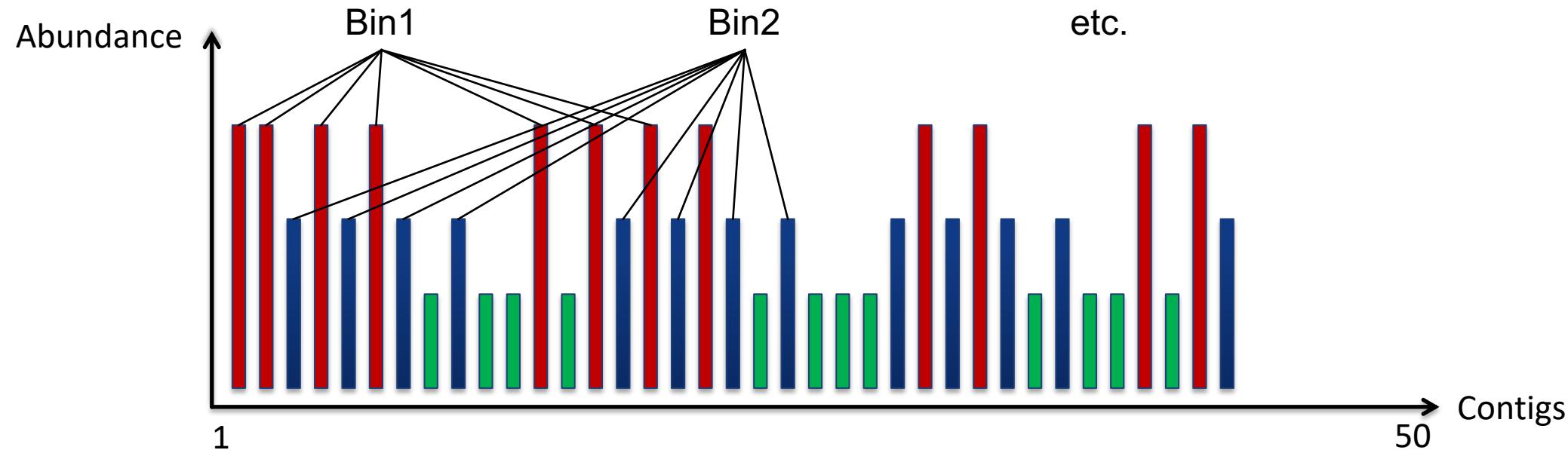
Contig 2

Contig 3

small distance –  
high likelihood for  
same bin

large distance

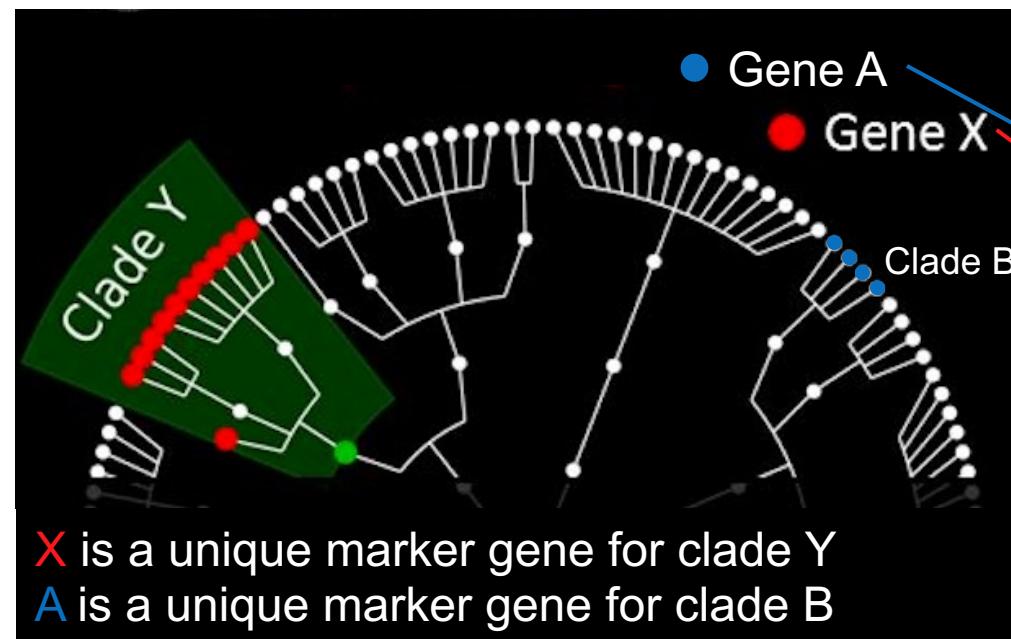
# Example for abundance-based distances



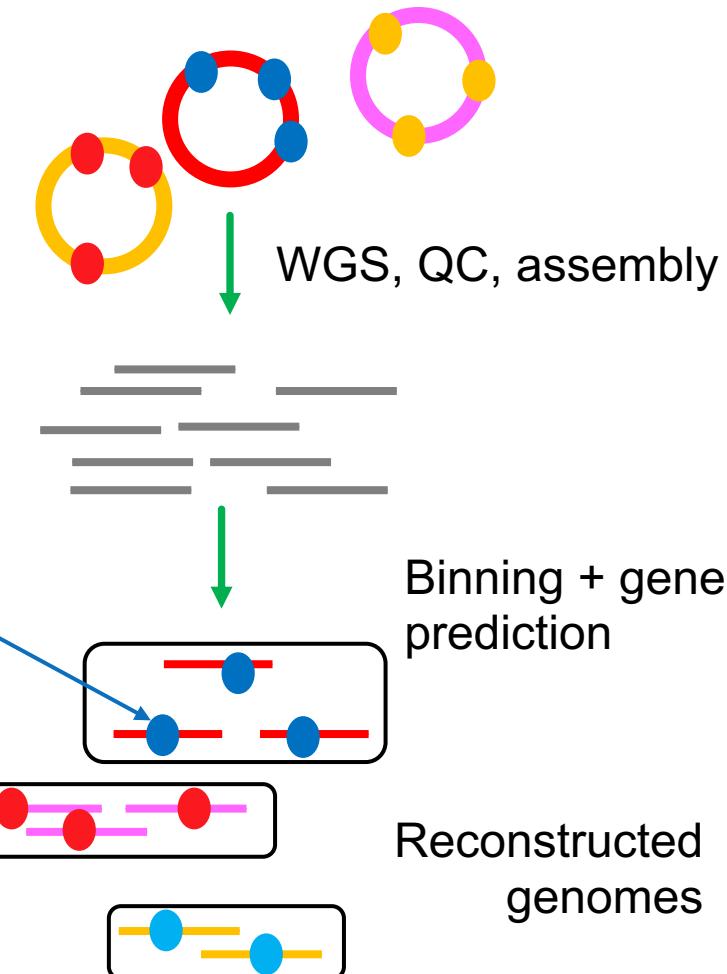
→ TNF and abundance-based distances can be combined and used for iterative binning

## Step 3: Binning contigs/scaffolds – taxonomy guided

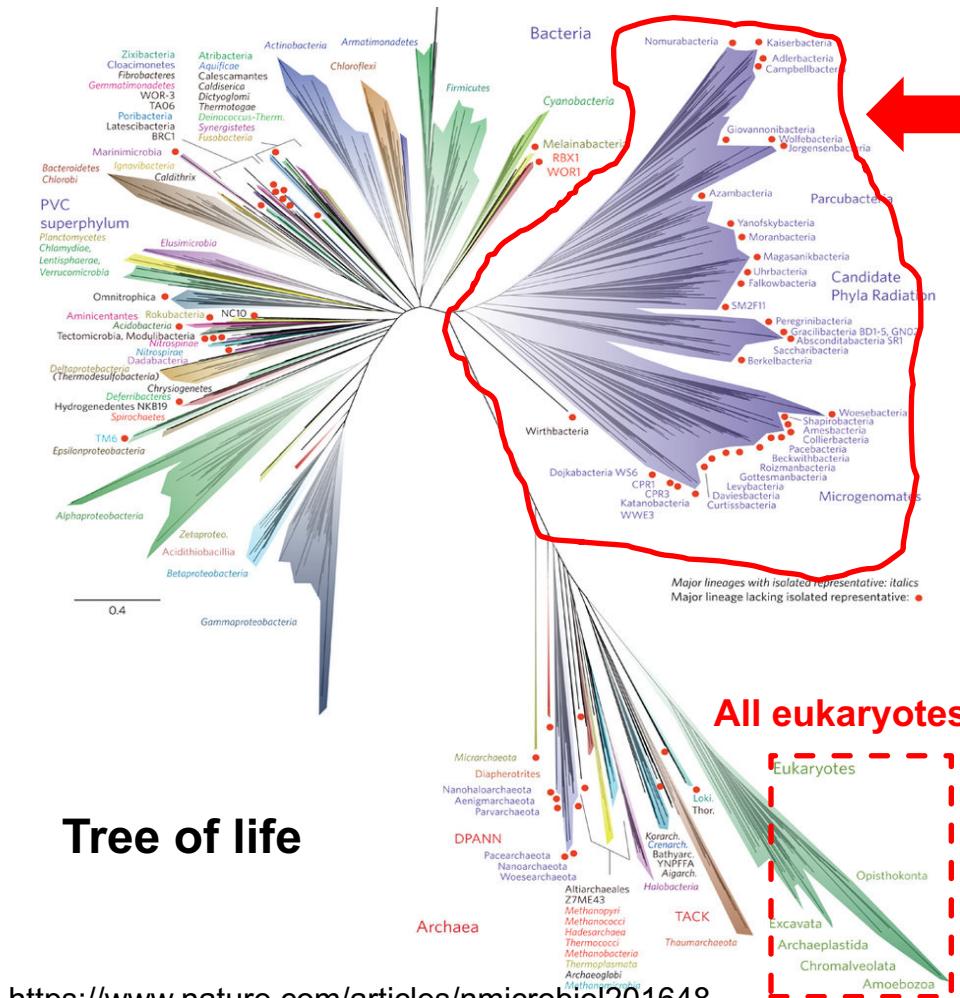
Taxonomy-guided (based on reference genomes) use of clade specific marker genes for binning metagenomic assemblies into draft genomes



→ Note that clade specific marker genes can also be used to identify contaminated bins



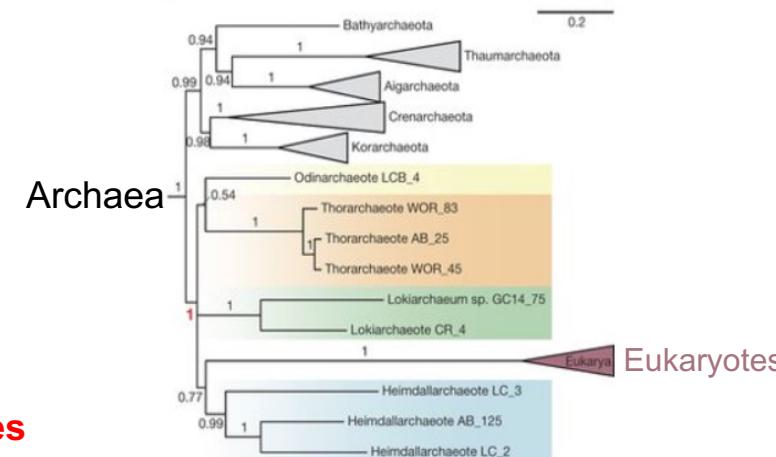
# Applied examples III



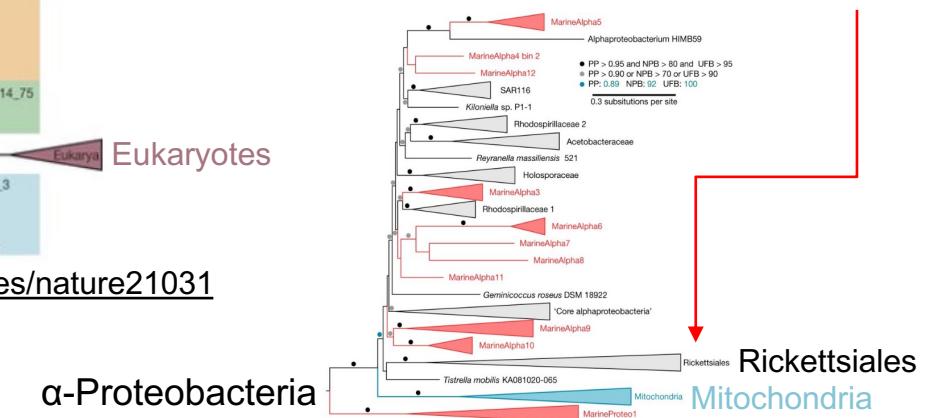
Tree of life

Candidate phyla radiation discovered by metagenomics

Only two domains of life?



Mitochondrial origin not within Rickettsiales?



$\alpha$ -Proteobacteria

## Summary – Part 2

- Reconstruction of microbial genomes from metagenomes is challenging as natural microbial communities are complex (many co-existing strains, uneven distribution of abundance)
- Assembled contigs/scaffolds can be binned by composition and/or taxonomy-based approaches
- Assembly metrics provide means of quality control and lineage-specific genes can reveal contaminations in genomic bins
- Functional annotation of genes/genomes can involve several search strategies against many different databases
- MAGs (metagenome assembled genomes) have revealed unexpected diversity and new implications on evolutionary origin of eukaryotes and mitochondria