Computational microbial genomics

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Chapter 1

Introduction

1.1 Microbes

Microbes are defined as whatever is not visible at the human eye: bacteria cells' dimensions are in the order of micrometre, while viruses in the order of nanometre. It is obvious how there is no visible part by eye. This is particularly true for viruses: their dimension make them almost impossible to perceive by any other method than genomics.

1.1.1 Prevalence of microbes

We are living in a microbial world: more than 90% of the biomass is composed of them and they are responsible for a great part of the biochemical cycle. Microbes can thrive in a variety of environment and according to some estimates they compose $10^{17}g$ of biomass. To put that in context the overall weight of the human species is three or four order of magnitude less. They also form the human microbiome, with important medical implication.

1.1.2 Difficulties in studying them

Of the predicted 30 million species to exist only thousands can be cultured in isolation in the lab. There is a need to create a way to directly study and characterized samples taken from the environment.

1.2 Genomics

Once the genetic material is isolated and sequenced a huge amount of information needs to be interpreted.

1.2.1 History of sequencing

- The first sequenced gene was one of a bacteriophage.
- Also the first complete genome wase one of a bacteriophage and is used by ILLUMINA as a control.
- The first bacterial genome was pub-

lished in 1995 and was that of Haemophilus influenzae.

- It has a dimension of $1.8Mb$ and sequencing took a year to complete.
- The first archea was sequenced in 1996.
- In 1996 the genome of S. cerevisiae has been sequenced and it was noticed that the genome shows a considerable amount of genetic redundancy.

After having sequenced the genomes there is a need to elucidate the biological functions of the genes contained in them.

1.2.2 Comparative genomics

Studying two different strains of the same organism allows to link difference in the genome to difference in phenotype.

1.2.3 Metagenomics

Metagenomics is the study of the DNA from all the genomes in an environment. By sampling all of the DNA from a given environment, it is possible to study the presence of bacterial ecosystems, independent of the ability to culture each bacterial strain in the laboratory. Large evolutionary radiation of bacterial lineages whose members are mostly uncultivated and only known through metagenomics and single cell sequencing have been described as nanobacterial. They have small genomes and lack several biosynthetic pathways and ribosomal proteins.

1.3 Leveraging computational power

Despite the advantages in DNA sequencing technology the sequencing of genomes has not progressed beyond clones on the order of the size of λ because of the lack of sufficient computational approaches that would enable the efficient assembly of a large number of independent, random sequences into a single assembly. When moving from low-throughput to high-throughput biology statistical power is needed: the genome of a bacterium must comprise all the DNA coding molecules present in the cell. With millions of reads from NGS of an environmental sample, it is possible to get a complete overview of any complex micro biome with thousands of species.

1.3.1 Comparing low-throughput and high-throughput pipeline

Let's consider the pipeline to find the pathogenic agent for a novel outbreak. In a low-throughput one a panel of reasonable putative causative agents is identified. Then one-by-one cultivation protocols to grow the agents from the infected tissue are performed. The pipeline ends when a pathogen grows in a sample. This is very time consuming. High-throughput instead sequences the full DNA repertoire of the sample and try to identify the pathogen by its genomic signature.

Chapter 2

Escherichia Coli general informations

2.1 *E. coli* genomics

Escherichia Coli is a Gram-negative, facultative anaerobic, rodshaped, coliform bacterium, it pertains to the phylum of proteobacteria and to the family of Enterobacteriaceae. It can be grown easily and inexpensively. Its genome has a length between 4.5-$4.7Mb$, including about 4000-5000 genes, and about seven ribosomal RNA operons. Only the 38% of the genes of K-12 (one of the most studied bacterial strains of *E. coli*) were experimentally identified, overall 40-50% of the genes are to date without a known function. The original *E. coli* strain K-12 was obtained from a stool sample of a diphtheria patient in Palo Alto, California in 1922.

2.1.1 *E. coli* long-term evolution experiment

The *E. coli* long-term evolution experiment led by Richard Lenski is one of the longest evolutionary experiments ever made. Starting from the 24th of Frbruary 1988 12 population of E. coli have been cultivated in parallel. After each day a portion of the population was introduced in a new flask, where it proliferated. Every 500 generations a sample from each flask is saved, so to track the evolutionary changes made. Today the $66000th$ generation have been reached. Long-term adaptation to a fixed environment can be characterized by a rich and dynamic set of population genetic processes, instead of the evolutionary desert expected near a fitness optima. In particular some bacteria developed the capacity to aerobically grow on citrate.

2.1.2 *E. coli* strains

E. coli could be found as commensal strains, pathogenic strains, or environmental strains. The pathogenic strains could pertain to these categories (which are not exclusive):

- enteropathogenic (EPEC),
- enteroinvasive (EIEC),
- enterotoxigenic (ETEC),
- diffusely adherent (DAEC),
- adherent invasive (AIEC),
- shiga-toxin producing (STEC),
- enteroaggregative(EAEC),
- extraintestinal pathogenic (ExPEC).

Resistances to antibiotics adds another layer of complexity in the categorization categorization of *E. coli*. Most of the genes are on plasmids, circular, additional to chromosome, and can be moved easily horizontally. Plasmids between different strains can be moved in enterobacteriacae, this doesn't happen normally in other families. Some *E. coli* strains are even capable of causing cancer in humans: for example, colibactin-positive *E. coli* can cause colon and rectal cancer, by creating mutations which are responsible of the of the cancer onset.

2.1.3 Stc-EAEC outbreak

In 2011 in Germany there was an outbreak of Stx-EAEC, An efficient counter-measure was found by sequencing the genome of those bacteria.

2.1.4 Shigella

Shigella is a strain of *E. coli* capable of producing the shiga toxin. It has been difficult to categorize for taxonomists. Several antigens can be used by taxonomists to cathegorize *E. coli* strains. In particular the O (171), H (56), K (80) antigens, respectively related to the somatic, the flagella and the capsule are widely used.

2.1.5 PanPhlAn - strain detection and characterization

Pangenome-based Phylogenomic Analysis (PanPhlAn) is a strain-level metagenomic profiling tool for identifying the gene composition and in-vivo transcriptional activity of individual strains in metagenomic samples. PanPhlAn's ability for strain-tracking and functional analysis of unknown pathogens makes it an efficient tool for culture-free infectious outbreak epidemiology and microbial population studies. This tool was for example used to study the strain responsible of an outbreak in Germany in 2011 and found a strain of shiga-toxigenic Escherichia coli (STEC). This method, due to its greater efficiency and accuracy has been used since instead of low-throughput, traditional pipelines.

2.2 Genomes of *E. coli*

2.2.1 Core and accessory genome

In the genome of *E. coli* strains, it is possible to distinguish between:

- Core genome: the set of all genes shared by all members of a bacterial species, it includes 1000 up to 3000 genes.
- Accessory or dispensable genome: the

set of genes present in some but not all genomes within the same bacterial species. It is found on a single strain or in a subset of strains.

2.2.2 Pangenome

The pangenome is the union of the core genome and the accessory genome. It is the set of all the set of all the genes that can be found in the species strains. The pangenome can be characterized regarding its size with respect to the number of genomes:

- Closed: the pangenome size tends to a maximum as number of genomes increases.
- Open: the pangenome size keeps increasing as you add new genomes.

Typically sequencing more organisms of the same species tends to lower the amount of genes in the core genome and increase the number of those in the pangenome, 2.1. Due to technical errors, the core genome should tend to a size of 0, but a more reasonable plateau can be predicted with a more accurate mathematical formulation.

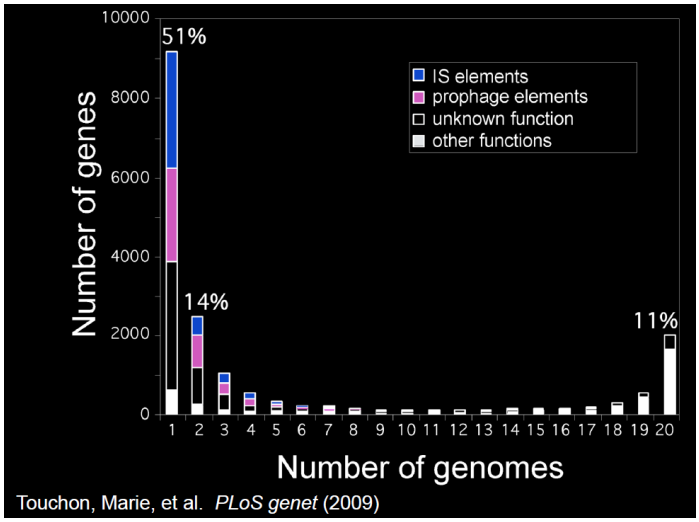


Figure 2.1: It can be seen that 51% of the genes are strain specific, and the other are shared between 2 to 20 strains of *E. coli*

Each *E. Coli* genome contains a balance genes of the core genome and of the pan genome, for a total amount of 4700 genes 2.2). Core genomes' genes are responsible of some basic cellular functionalities and utilities to survive the environment, while instead elements of the pangenome are quite usually specific to a single strain, like for example antibiotic resistance, and they are often not functionally well characterized.

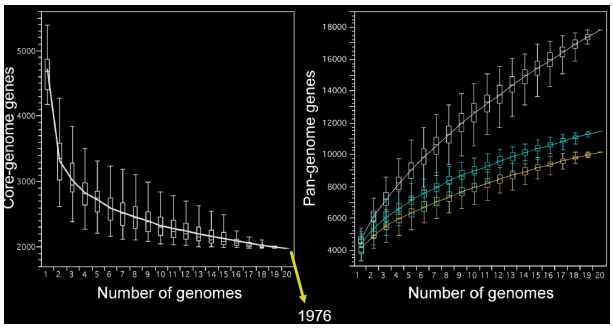


Figure 2.2: Balance between genes of the core- and of the pan-genome

Chapter 3

Next generation sequencing

3.1 Introduction

Next generation sequencing is the gold standard for sequencing nowadays. A series of discoveries allowed for the development of this technology:

- 1959: first homogeneous DNA purified.
- 1970: first discovery of type *II* restriction enzymes.
- 1972: first RNA gene sequence published.
- 1975: Sanger publishes his plus-minus method of sequencing, unable to distinguish homopolymers.
- 1977: Maxam and Gilbert publish their method that could distinguish homopolymers.
- 1977: Sanger publishes the dideoxy-sequencing method.

3.1.1 Progresses of sequencing

As can be seen in the graph 3.1 the cost of DNA sequencing is decreasing by a greater rate than the one predicted by Moore's law. This allows for greater number of samples and the sequencing of a different number of genomes.

3.1.2 Methods of sequencing

The methods of sequencing can be grouped in three groups:

- Chemical degradation of DNA: like the method of Maxam-Gilbert.
- Sequencing by synthesis ("SBS"): the most common approach and the first to be developed. It uses DNA polymerases in primer extension reactions. This technology is used by Illumina, Pacific Bioscences, Ion Torren and 454.
- Ligation-based: sequencing using short probes that hybridize to the template. This technology is used by SOLiD and Complete Genomics.
- Others like nanopores.

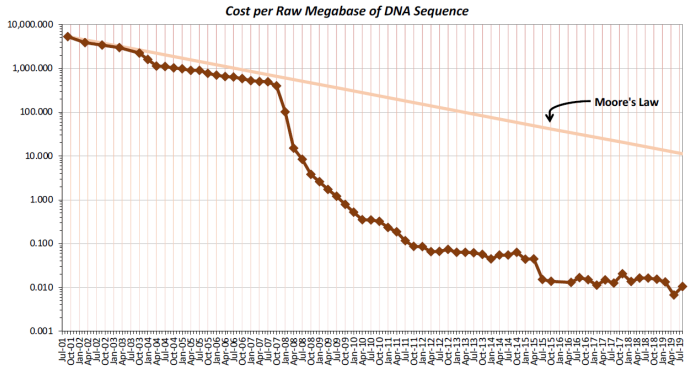
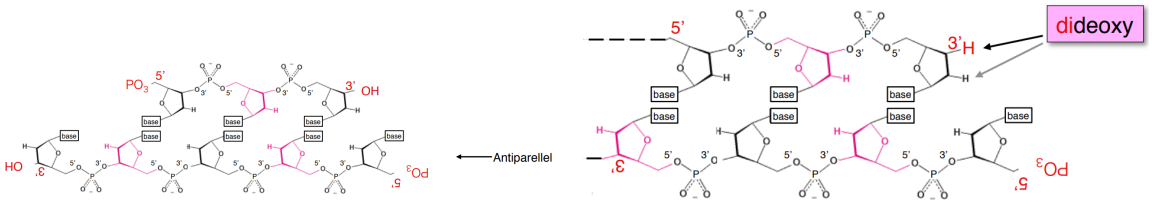


Figure 3.1

3.1.3 The Chain Terminators



(a) Normal DNA molecule, with oxydrilic group ligated to 3′-ends

(b) Figure representing the difference between a normal DNA chain and one with chain terminators

Figure 3.2: Normal DNA synthesys *vs* Chain terminators

Normally, the addition of new nucleotides to a generated molecule of DNA happens with the 3′-end of the nucleotide chain 3.2a. Chain terminators are dideoxy nucleotides, ddNTPs, that cannot be further extended. These nucleotides don't have the oxydrilic group at their 3′-end, so the DNA polymerase cannot further add other nucleotides to the chain 3.2b.

3.2 Sanger method

The first method ever used to sequence DNA was designed by Frederick Sanger. The Sanger manual sequencing system consists in an *in vitro* process described in figure 3.3. It is also named a primer extension method. It is performed over a single-filament DNA sample, and it uses chain terminators nucleotides, one for each type of nucleobase: ddATP, ddGTP, ddTTP, ddCTP. The reaction is done inside four different reactions tubes, each containing:

- The sample DNA to be reproduced.
- A DNA polymerase.
- The normal nucleotides.
- One of the four possible chain terminator marked with sulfur-35. In each tube, the corresponding dideoxynucleotide was used with a concentration 10 times lower than the other normal nucleotides.

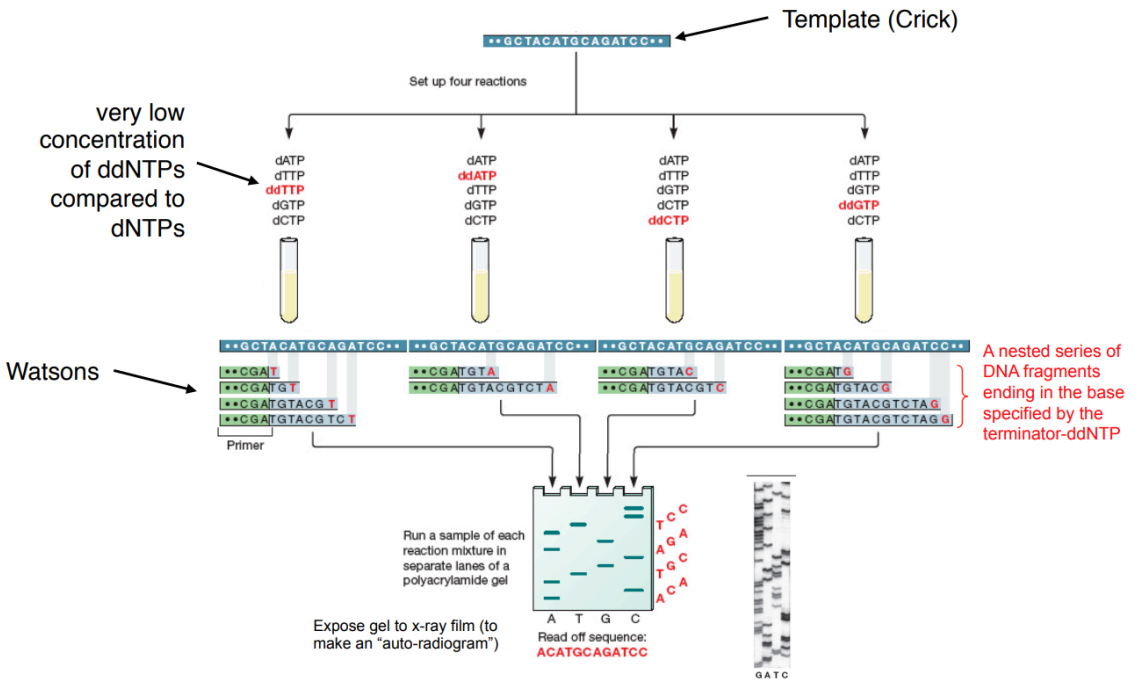


Figure 3.3: Sanger's method process

The polymerization reactions produces several molecules of DNA with different length: each replicative cycle is terminated after the addition of a chain terminator nucleotide. The initial DNA sequence is reconstructed using a long PAGE gel with high concentration of urea $(6-7M)$ to avoid the coiling of the DNA single-filaments. High voltages are required to achieve a highly risolutive run. This high resolution was needed as DNA's fragments are different only for a nucleotide. After having run it the bands were visualized through auto-radiography in order to evidentiate the phosphorescent signals.

The sequence is read starting from the shortest fragments at the end of the gel and going up along it, looking for the first presence of a band in one of the four runs.

3.2.1 Automatic sequencing

To automatize the Sanger methods fluorescent proteins substituted the radioactive signal. Several versions were developed:

1. Fluorescent primers marked with a single fluorochrome.
2. Four aliquotes of the same primer were used marked with four different fluorochromes, able to emit different fluorescences.
3. Four different fluorochromes were used to mark the single ddNTPs

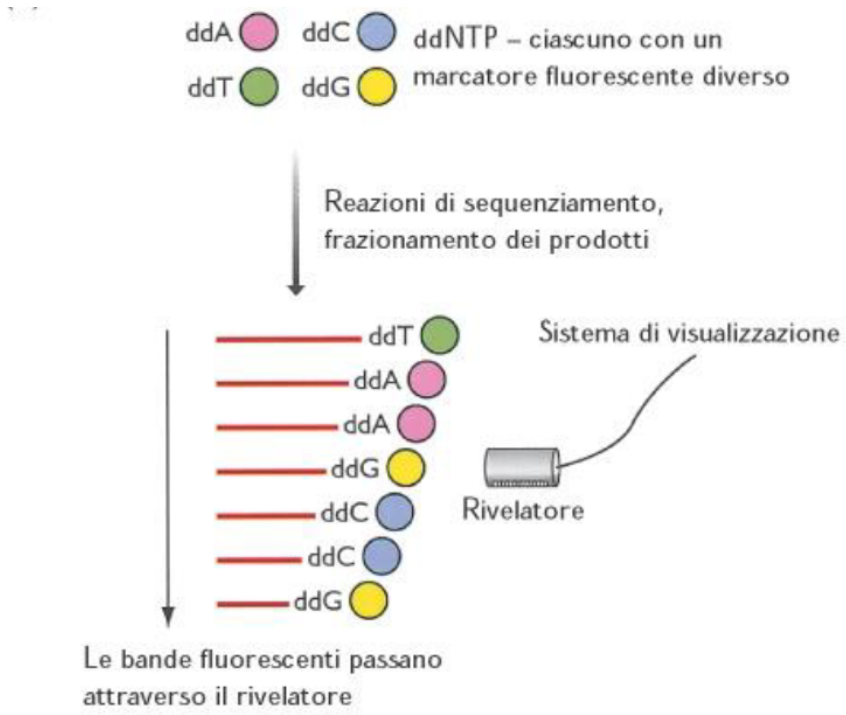
Thanks to the use of 4 different fluorochromes, it was possible to use a single electrophoretic lane to carry the sequencing reaction. Also a cyclic replicative reaction was

performed with this procedure using a thermal cycler:

1. Denaturation at 95°C of the DNA to be sequences
2. Annealing at $50 - 70$°C of the primer specific to one of the two filaments
3. Extension at 72°C by using a *Taq*-polymerase to avoid the formation of coiled structures in the DNA molecule to be sequenced.

The resulting molecules are run through a long PAGE gel and fluorescence is triggered irradiating the DNA molecules.

Figure 3.4



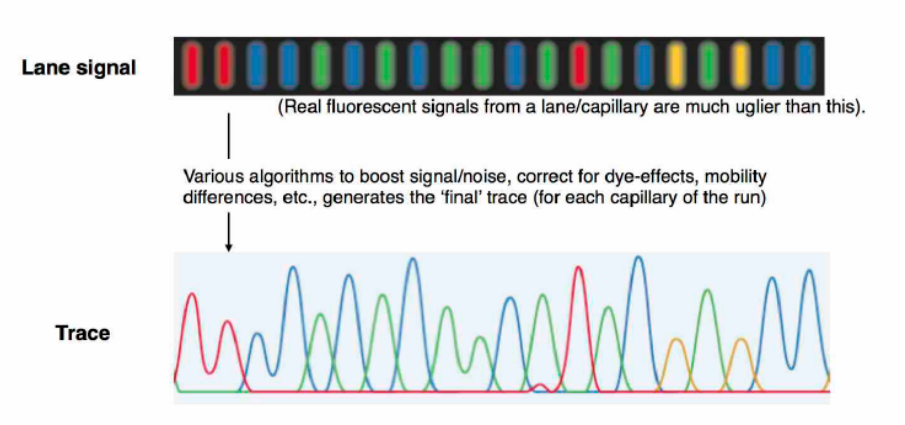
More usefully, this sequencing method is performed by using a capillar filled with a synthetic polymer with the same function of polyacrillamide. The analysis produces an electropherogram, with a color depicting the probability of each base being in each position. The electropherogram is refined through algorithms that can boost the signal to noise ration, correct the dye effect and reduce all systematic errors.

The Sanger automatic sequencing method was used extensively for the majority of the human project.

3.3 Development of Sequencing Machines

The method used for sequencing has to be chosen based on the wanted output, like the quality required and the type of input. Different technologies have different strengths and

Figure 3.5

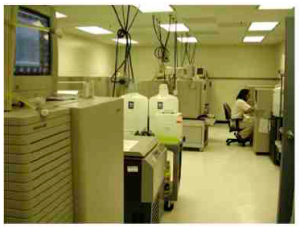


weaknesses:

- SOLID sequences reads long only 35-75 bases and it is not used anymore.
- Sanger sequencing, or the capillary, can read up to 1000 bases, but has a low throughput.
- MINION allowed to sequence an entire genome of *E. coli*.

A plethora of sequencing machines are available today. None of them is able to sequence DNA directly from a sample, requiring different preparation step. Today machines producing high-throughput output of short reads are preferred.

Radioactive
polyacrylamide
slab gel
Low throughput,
labor intensive



AB slab gel sequencers
(370, 373, 377)
Fluorescent sequencing
1990-1999
6 runs/day
96 reads/run
500 bp/read
288,000 bp/day

AB capillary sequencers
(3700, 3730)
1998-now
24 runs/day
96 reads/run
550 – 1,000 bp/read
1-2 million bp/day

Figure 3.6: The implementation of capillary sequencing machines gave the possibility to make more runs than with the others. $\tilde{1}000$ fold productivity increase was allowed

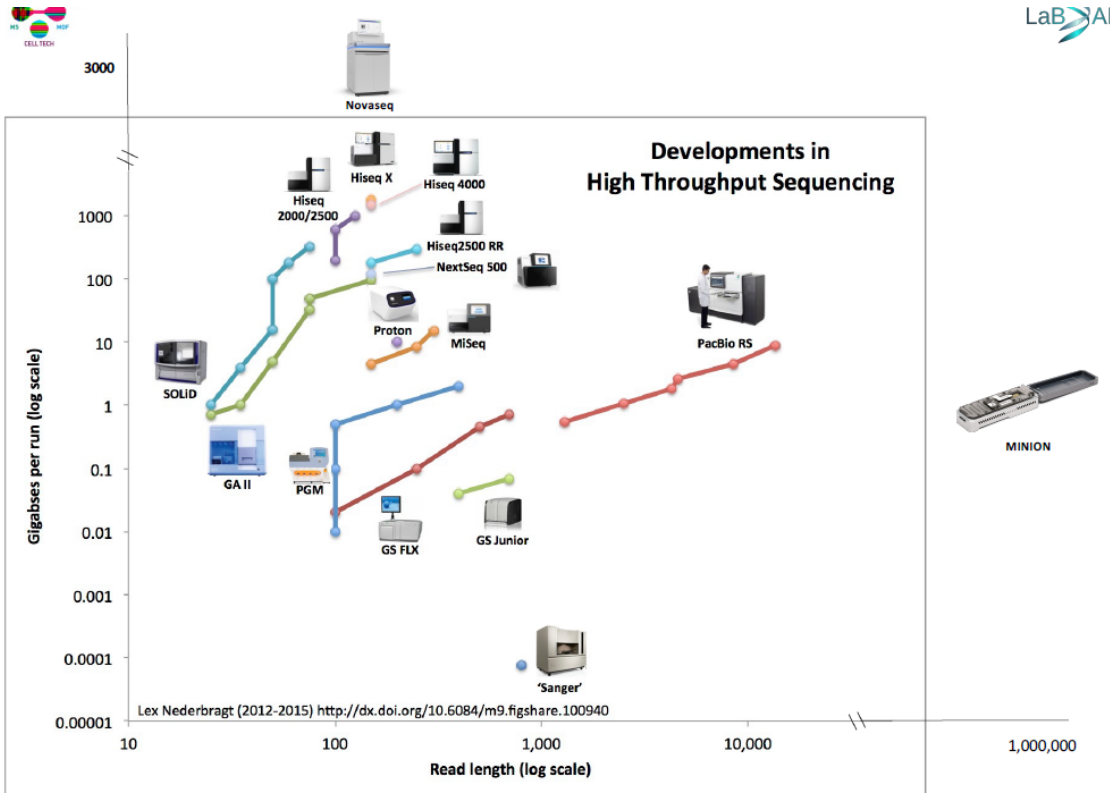


Figure 3.7: It can be noticed how recent developments had the scope of increasing the output data

The most wide-spread machines are from ILLUMINA like NovaSeq. They use sequencing

by synthesis and they amplify the signal through the use of clusters.

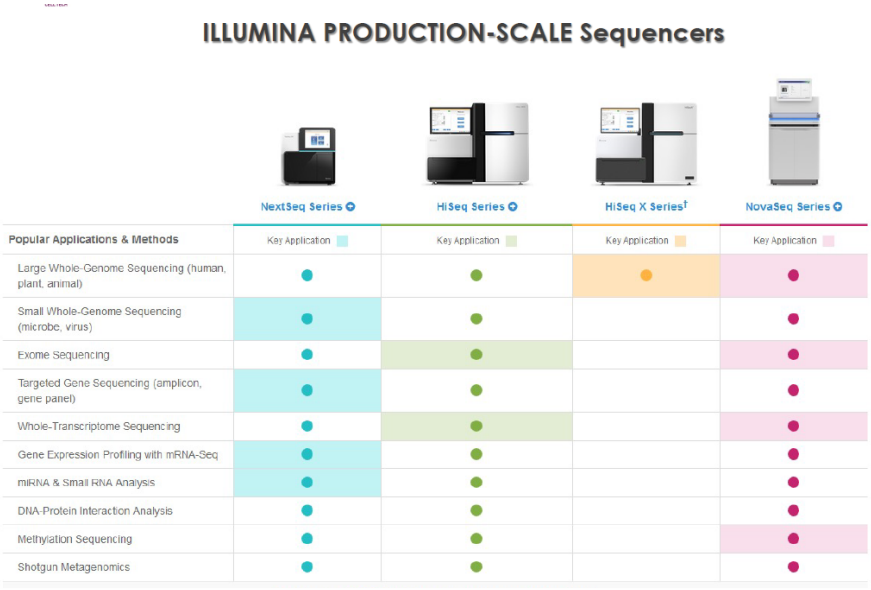


Figure 3.8

3.4 Next Generation Sequencing

The NGS protocol requires 3 steps:

1. Sample preparation: series of fragments added.
2. Clonal amplification: needed to replicate fragments attached to the solid surfaces, since machines are not sensible to single molecules.
3. Sequencing: ILLUMINA sequencing is one of the techniques used to obtain sequence data nowadays.

3^{rd} generation allow to read a molecule without replicating it.

3.4.1 ILLUMINA sequencing

3.4.1.1 Fragments and Library preparation

During fragmentation the DNA or RNA polymers that need to be sequenced are fragmented in short read sequences. This is because the machines are able to sequence only read with a length of a few hundred nucleotides. These fragments need to be tagmented: in this process one or two indexes, or barcodes are added to the fragment so that it has two sequencing primer binding sites and regions complementary to the oligonucleotides in the chamber. Indexes allow to distinguish between sample and, in the case of pair end sequencing, allow to distinguish between the forward and reverse read.

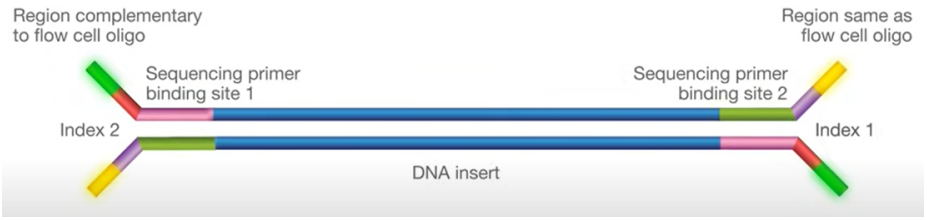


Figure 3.9: Figure representing the a good prepeared fragment, it has two indexes, two sequencing primer binding sites and regions complementary to the oligonucleotides present in the chamber

3.4.1.2 Clonal amplification and ILLUMINA sequencing procedure

Figure 3.10

Clonal amplification is necessary to amplify the signal from each fragment. ILLUMINA machines make use of clusters to sequence DNA: group of DNA strand positioned near each other that generate from a single fragment. Each cluter represents thousands of copies of the same DNA strand in a 1-2μm spot 3.10.

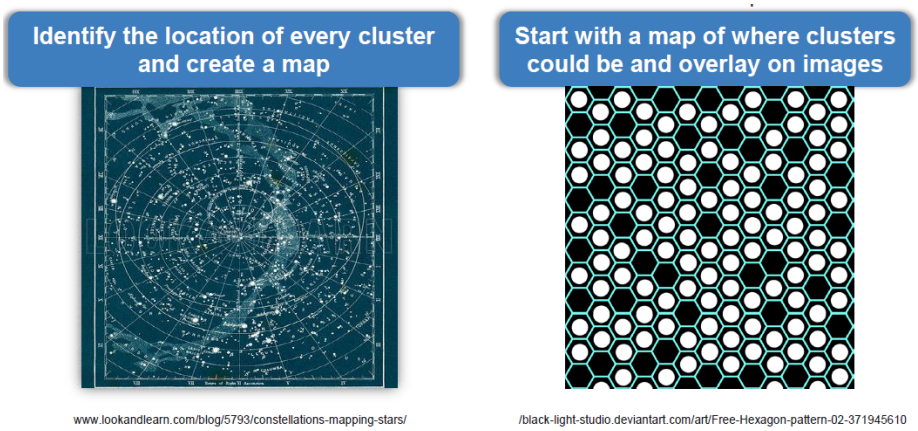


Figure 3.11

The clonal amplification process happens in flow cells, slides of glass in which fragments flow over channels. Changes in temperature allow for ligations and separations. The surface of the cells is functionalized with a series of oligos complementary to library adapters. These flow cell can be patterned (cluster in specific positions) or random (clus-

ter randomly positioned). In the former the location of the cluster can be known (rigid registration 3.11) or unknown.

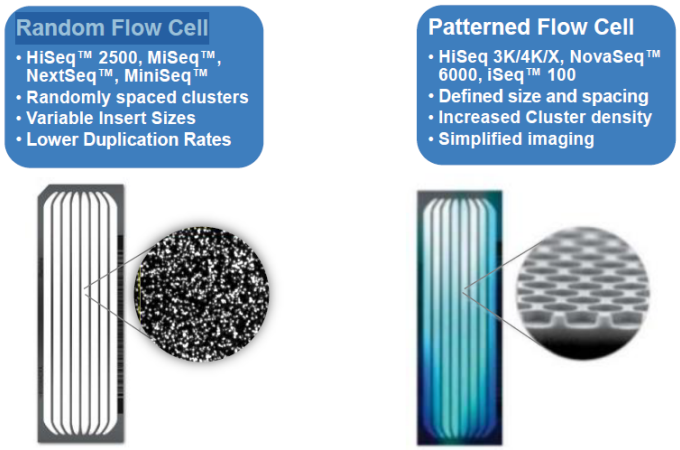
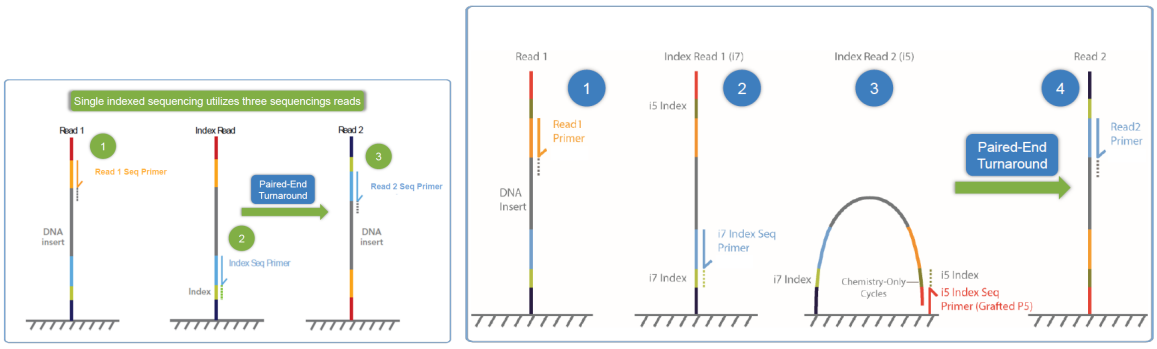


Figure 3.12

3.4.1.3 Fragment attachment to the clusters

Once the fragments start flowing over the chambers, they can bind only to one of the two oligos functionalizing the plate. Once they are attached to the surface the sequencing process is controlled through solvents and temperature. As shown in 3.13 the sequencing process differs between single end and paired end sequencing.

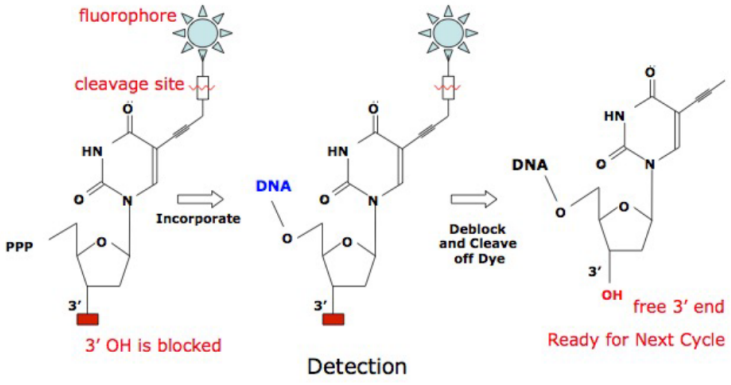


(a) single index **(b)** double index

Figure 3.13: Single/double index for ILLUMINA sequencing

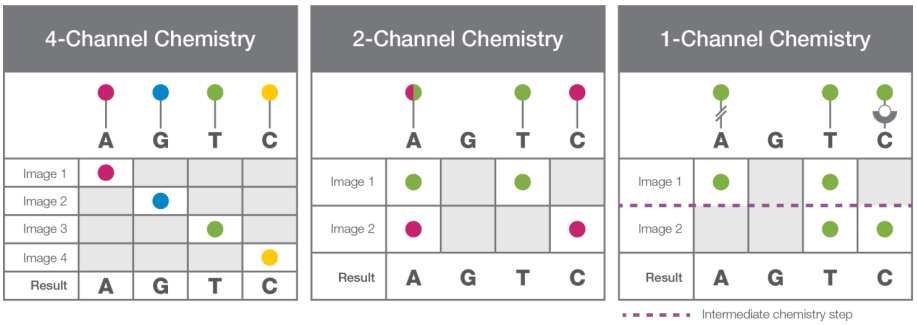
3.4.1.4 Sequencing

Figure 3.14



Reversible terminators 3.14 allow a real time analysis of the sequencing through the syntheses reaction. The fluorophore part of the terminator can be cleaved to eliminate the signal.

Figure 3.15



Depending on the number of fluorescent molecules used ILLUMINA sequencers are distinguished in the 4-channel, 2-channel or 1-channel type. In the case of the 4-channel 4 images are taken in each cycle and each cluster apperas in only one of the four images 3.15. The highest intensity base in a cluster is the called base for that cluster. In case no base is clearly related to a position the base calling returns N. This reading process can be done through single end reads on a single extreme of the fragment or through paired-ends reading, where each fragment is read in a forward and reverse way. This latter method gives structural information.

3.4.2 Pacific Bioscience

In the Pacific Bioscience PacBio sequencer the long DNA filament to be sequenced is attached to a polymerase, over the surface of a SMRT (Single Molecule Real Time) cell. This

cell is really small, and at each nucleation process a light signal is emitted. The produced light is not able to get out of the walls, and its duration is extremely restricted. The registration of the light signal correspond to a base calling. Its main advantage over ILLUMINA is the possibility of sequencing really long DNA molecules.

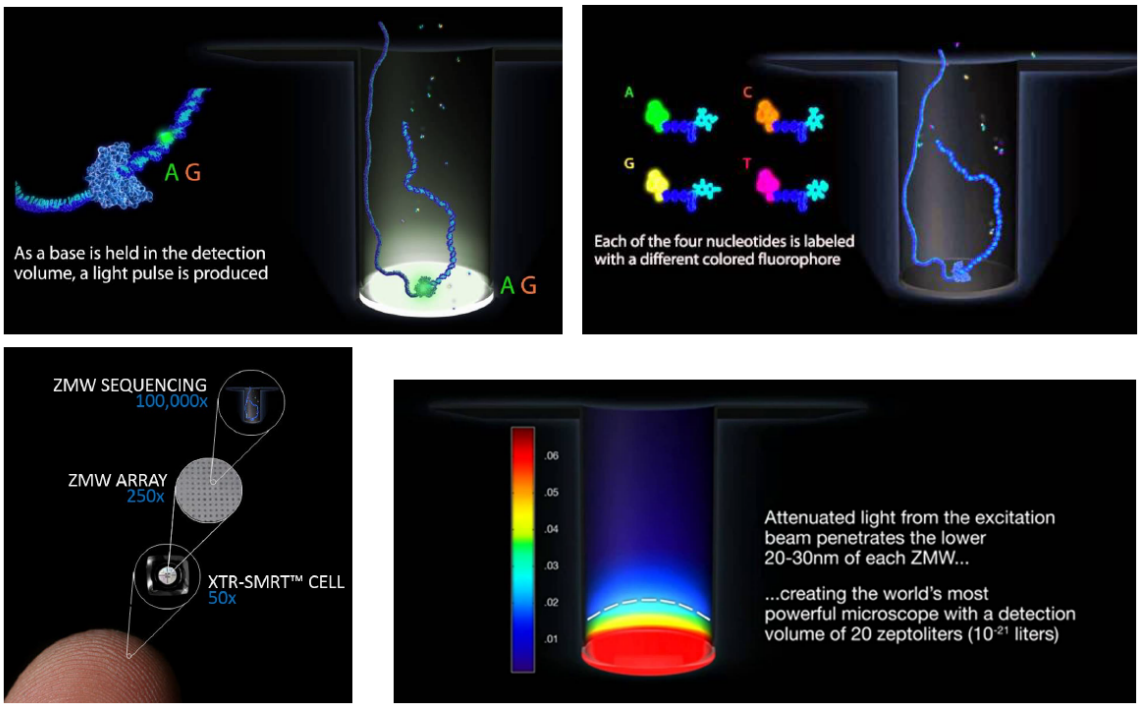


Figure 3.16

3.4.3 Nanopore sequencing

In nanopore sequencing the sequence is detected through the passage of DNA molecule into intramembrane protein. This produces a voltage changes that corresponds to a base calling. This technology is in constant development and is not widely used because of the, although improving, high error rate.

Chapter 4

Sequencing data

4.1 Choosing the optimal technology

When performing a genetics or genomics study it is best to be as hypothesis driven as possible and use already available data to guide the new analysis. Moreover to choose the optimal sequencer the parameters that need to be considered are:

- Throughput.
- Cost.
- Read lengths.
- Data output (reads per run).
- Coverage.
- Sequencing errors (indel, substitution, CG deletion, AT bias).
- Library preparation compatibility.
- Speed (run time).

4.1.1 Comparing different sequencing technologies

Illumina NovaSeq : optimal for sequencing a lot of DNA molecules at the same time like in the case of genomes or metagenomes. It can't go over $300bp$ readlines run, but it has the highest throughput so far. It is capable of multiplexing, differentiating the different samples with an unique barcode.

Illumina iSeq : optimal for sequencing shorter genomes.

NanoPore (minion) : it is a pocket-sized wet-lab free sequencer for DNA, RNA and (possibly) proteins, but the read lengths is smaller than Illumina's. The machine is cheap;, but the running flow is more expensive over time. It's a real-time sequencer.

PacBio : has very long reads but carries a high amount of error.

A solution to reduce the impact of error or weaknesses of one of the sequencers is to use more than one for the same project. Consider there is a need to sequence the genome of a bacterium: PachBio would give a lot of sequence errors, while Illumina wuild be unable to reconstruct the sample due to assembly ambiguities. In the end PacBio will construct the genome and Illumina will correct sequence errors. This solution doesn't work in complex

sample with more than one genome, because there is no way to a priori which reads are coming from one organism. Another widely adopted solution is to sequence multiple times one molecule so to reduce random errors. This is effective but does not resolve systematic error like the one of PacBio with homopolymers.

4.1.2 Sequencers' output

All sequencing platforms translate the physical read signal into files in FASTQ format. This files contain the sequencing reads and the quality of each base.

4.2 Base callers

A base caller is an algorithm that translates the analogical signal of the reading into numbers and nucleotides. The most popular algorithm is Phred. Phred tries to correct errors derived from the sequencing reaction and electrophoresis. It was tested on a huge dataset of gold standard sequences (finished human and C. elegans sequences generated by highly-redundant sequencing). Its results were compared with the traditional ABI base caller and Phred was considerably more accurate with 40-50% fewer errors. This algorithm need to be able to understand when it is impossible to recover an high quality sequencing and so it needs to be able to give up for low-quality reads. The confidence that the base caller has to call a certain nucleotide ATCG is annotated in the FASTQ file, allowing for quality control downstream.

4.2.1 Errors solved by Ilumina's base caller

Phasing noise ϕ : when a certain base is not seen frequently, the first time in which it will reappear there will be a spike in the graph, increasing the signal of the nearby bases. This will cause errors in estimating the real nucleotide that is occurring in the site. This problem can be solved by waiting more time between readings, but the sequencing will be less efficient and the throughput will be lower. The machine needs to find a trade-off between efficiency and clear reading.

Signal decay δ : after a while the sequencer has read the same base, or the same repeated couple of bases, the signal will go down and at some point will be indistinguishable. At some point you will need to cut the read since it will be not trustable after a while.

Mixed cluster μ : two different fragments can enter the same cluster and the sequencer will read the two signals simultaneously.

Boundary effects ω : in this case the machine needs to interpret an image and it cannot distinguish between the signal and the background.

Cross-talk $\mathcal{X}$

Fluophore accumulation τ

4.2.2 Density on the flow cell

The density of the flow cell is the number of cluster in it. Under clustering will reduce the sequencer throughput, while over clustering will cause errors due to the limited resolution

of the reading of the bases. There is a need to find the optimal number of cluster that maximises the throughput without introducing overlapping in the reading of clusters.

4.2.3 An ecology of base callers

Base callers need to find a satisfying trade-off between accuracy and computational efficiency. The quality is the estimation of the probability that the nucleotide in a certain position is correct. The PHRED score reported by the base caller and the PHRED score of mapping to a reference sequence are different. Base callers need to be calibrated with a standard to make sure that the estimation of the quality is accurate enough.

4.3 FASTQ format

4.3.1 Composition

The FASTQ format is composed by:

1. '@' followed by a sequence identifier. This identifier contains the unique instrument name, the flowcell and tile number, the x and y coordinates of the cluster within the tile, the index number for multiplexing and the pair number of paired-end sequencing. In Illumina MiSeq, each flowcell has 8 microfluidic channels (lanes), each lane contains three columns with 96 tiles, and can sequence up to 96 multiplexed samples.
2. The sequence. It could be mate paired for paired end sequencing.
3. '+', optionally followed by a sequence Identifier.
4. The quality scores. Quality is a number based on the estimated probability of error. p=probability of error, $Q = -10\dot{p}$. A base quality of at least 20 is needed to reach 1% of error. A base quality of 40 means the probability of error is 0.01%. The FASTQ quality score is the phred score +33, converted in CHAR code.

The FASTA file format is a FASTQ fomrat without the quality score reported and the seq ID is preceded by '>'.

4.3.2 Quality control: read length distribution

Quality scores are typically used to perform quality control and cleaning of the reads. This result in a FASTA output file to be used downstream. However there are algorithms that can use directly FASTQ files, performing autonomously the cleaning and quality control of the reads. The quality score is an indication of how well the sequencing run went. Typically the quality decreases when the read length increases due to the fact that sequencers have problem when are run in continuum. A solution to this problem is to cut the reads when the quality becomes too low. Another problem happens when the adapter is included into the read. This happens typically with short reads and a solution is to cut it and a part of the sequence. FastQC can be used to plot the quality distribution of the data. Another way to asses read quality is to consider the average quality of the entire read, discarding the low-quality ones.

4.3.3 Duplication artifacts

It is not frequent to see duplication, but it can be a problem especially when there is a need to quantify gene expression or copy number of genes in a bacterial genome. The distribution of the duplicates should be the same of the distribution of the reads. There should't be any bias along the length of the reads, but if there is it should be due to a repetition of sequencing of the same read or when the adapter and primer have been read.

4.3.4 GC content analysis

Each organism has a signature GC content, so when plotting it a normal distribution is expected. Multiple peaks are an indicator of the presence of two different organisms.

4.3.5 K-mers frequency plot

K-mer frequencies are a way to catch systematic sequencing error: when mapping a genome it is now the relative frequency of each K-mer. That can be compared with the K-mers frequencies to catch systematic errors in sequencing and to assess its quality. Frequent k-mers can be a signature, as the GC content. The expected coverage of a k-mer with reads of length L:

$$L_{cov} = \frac{L - k + 1}{L} \times Cov$$

Then, given a k-mer, it can be seen in how many reads it is present. For a typical K-mer its coverage should be around 40%. Coverage higher than 80% should indicate that this k-mer is located in more than one position. Other values arises from errors.

4.3.6 Low-complexity artefacts

Same nucleotide repeats (especially A) are in a lot of cases artefacts. This is due to systematic error and can be evidenced through quality control. To measure if these sequence are in fact artefacts parameters to take into consideration are:

- Low complexity.
- Low entropy.
- High compression (the artefacts increase the information inside the file).

But some low-complexity sequences are not artefacts:

- Hydrophobic transmembrane alpha-helical sequences in membrane proteins.
- CAG repeats in genes causing Huntington disease, spinal and bulbar muscular atrophy, dentatorubropalliodoluysian atrophy.
- Proline-rich regions in proteins.
- Poly-A tails in nucleotide sequence.
- Micro-satellites.

4.3.7 FASTQ quality control (QC)

FASTQ quality control is the first step in any NGS pipeline. It consists of:

Clipping/trimming : removing (low quality) parts of reads.

Masking : avoiding to consider parts of reads that can have low entropy for example.

Read removal : discard low quality reads or reads that are too short after clipping.

Additional features that can be exploited for QC are the GC content, clustering for contamination detection, TAG identification,ambiguous bases.

Chapter 5

Mapping

Mapping (or aligning) and assembly are the operations that allow us to make some sense out of fragments (input data) deriving from sequencing, produce assemblies.

- **Mapping** is a key step in a modern genomic analysis and consists in the process of aligning the reads on a reference genome, in order to assign them to a specific location. With mapping, insights like the expression level of genes can be gained.
- **Assembly** by contrast, is the process of aligning and merging overlapping sequences in longer consensus sequences in order to reconstruct the original sequence/genome.

A consensus sequence is the calculated order of most frequent residues, either nucleotide or amino acid, found at each position in a sequence alignment In many cases, someone may have already assembled the genome or part of the genome (available reference sequences), so we don't need to do sequence assembly, only mapping (like for the human genome). Assembly will be needed however when studying new organisms.

Problems related to mapping and assembly:

- absence of DNA fragments covering the gaps, makes it difficult to order the contigs (since there is no connection)
- presence of DNA artefacts (those must be discriminated with Quality Control)
- repeated sequences

5.1 Mapping

The coverage, or read depth, is the average number of reads representing a given nucleotide in the reconstructed sequence (Some points of the reference sequence will be aligned with more reads, some with less. The average number of reads for each nucleotide is the coverage). The coverage of a genome is defined as the average coverage of each single nucleotide across all nucleotides of the genome. A coverage of 1x does not mean that all reads are read once. This would be true if sampling were systematic, but sampling is not systematic, it is random and is biased. Hence, a coverage of 1x means that on average each nucleotide is covered once, but there will be some nucleotides covered more and some not covered (0

coverage). The coverage can be represented with a coverage map and can also be defined theoretically as:

$$\text{Pval} = \text{K } e^{-\text{lambda S}} = \text{Evalue/mn}$$

where G is the length of the genome; N is the number of reads; L is the average read length. Knowing the wanted coverage is important to set up the machine in order to obtain the right number of reads (to achieve a certain coverage). A higher coverage means indeed more reads to be obtained.

5.1.0.1 Exercise on coverage

How many reads do I need to cover the entire genome? Define the probability to cover the full genome (with a certain epsilon subtracted - so not 100%) or: define the number of reads N to cover the whole genome with a certain probability P = 1- epsilon.

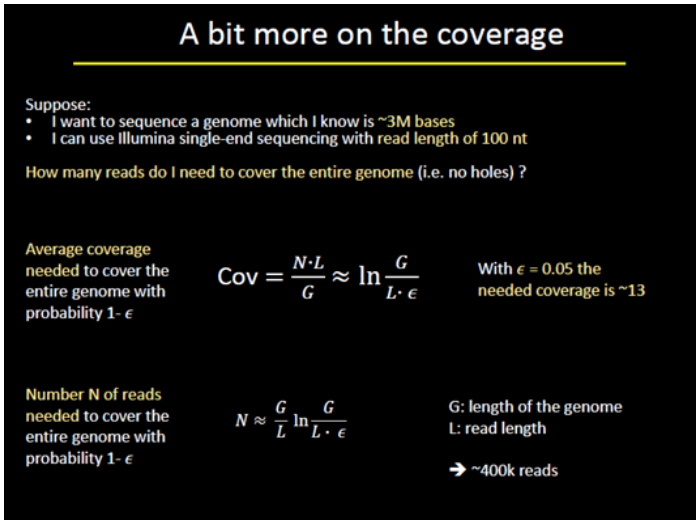


Figure 5.1

In general, the sequence mapping process consists in performing comparisons between experimental sequence data (reads obtained with sequencing) with some reference information, like reference genomes and known genes (eg. the human genome). The comparison can lead to obtaining new information, such as the presence of SNPs which can be linked to pathological conditions or whatsoever, based on the starting point.

5.2 Mapping algorithm

Over time many different mapping algorithms were implemented; some of them are not used anymore, while others are the base of current mapping and aligning genomic tools. Ideally, the **simplest aligning algorithm** could consists in: We have a smaller sequence that we want to align to a longer one. Start from the first position, align the query sequence against the subject, and look at how many nucleotides are correct (score: 4/10 = 40%),

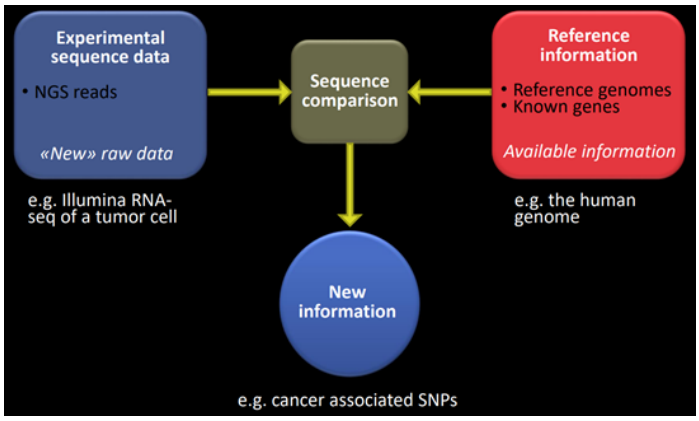


Figure 5.2

then repeat for all positions until a perfect match is found (if found). The problem with this algorithm is that it doesn't consider insertions and deletions.

5.2.1 Local vs Global alignment

Sequence alignment can follow two different approaches.

1. In **global alignment** an attempt is made to align completely the 2 sequences (end to end alignment). So global alignment finds the best alignment across the whole two sequences. This approach is suitable for comparing closely related sequences like homologous genes.

2. **Local alignment**, on the other hand, focuses on finding regions of similarity in parts of the sequences (so it aligns subsequences of the query sequence to a subsequence of the target sequence). This approach is suitable for aligning more divergent sequences or distantly related sequences. Used for example for finding out conserved patterns in DNA sequences of motifs in two proteins.

Sequence similarity is connected with **evolutionary distance** (also for cell populations in a tumour). Very high similarity implies a very low distance. But when the similarity goes down and reaches the twilight zone, it is more difficult to define the evolutionary distance and to give meaning to results (the zone depends on the experiments).

5.2.2 Smith-Waterman algorithm (local alignment) - 1981

Take the algorithm for string recognition and apply it to find common molecular sequences. Not good for indels and substitutions. The S-W Algorithm is a local-alignment algorithm based on dynamic programming, whose aim is to find the best match among all possible (optimal local alignment) with respect to the scoring system used . Firstly, we need to define a **score** for matches and penalties for mismatches and gaps (defined by the formula). The algorithm starts by putting zeros in the first row/column (**INITIALIZATION**). Then, starting from the first position H(1,1), at each iteration a number, based on the formula,

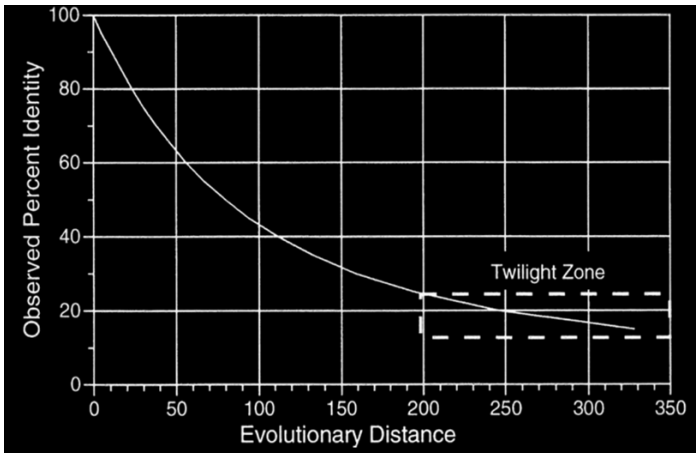


Figure 5.3

is added (**ITERATION**). The number to be added is the greater between the 4 numbers defined by the formula, where d represents the penalty for gaps, and s(x,y) = score when 2 NTs are the same (This gives a score of 5 if the 2 bases are equal, -3 if they are different) (PARAMETER SETTING).

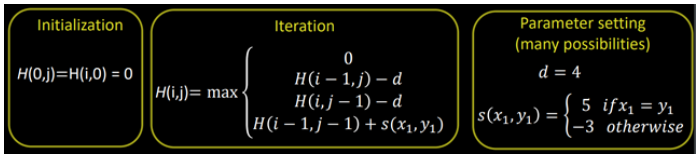


Figure 5.4

Example
First position H(1,1) = (G,C), max between:

- 0
- H(i-1, j) = 0-4 = -4
- H(i, j-1) = 0-4 = -4
- H(diag) + s(x, y) = 0 – 3 = -3
- → We put 0

Second position H(2,1) = (A, G):

- - 0
- -4
- -4
- -3

- → We put 0

Third position H(3,1) = (C e C):

- - 0
- -4
- -4
- 0 + 5 (since C=C)
- → We put 5

Here we also put an arrow indicating the nucleotide in the diagonal, to indicate that the 5 score derived from that point (hence from that path). The arrows are needed to reconstruct the alignment at the end of the process. The iteration is performed for each cell of the table (column wise). The parameters chosen for d (gap penalty) and s(x,y) (match or mismatch) can change the results. Final Solution:

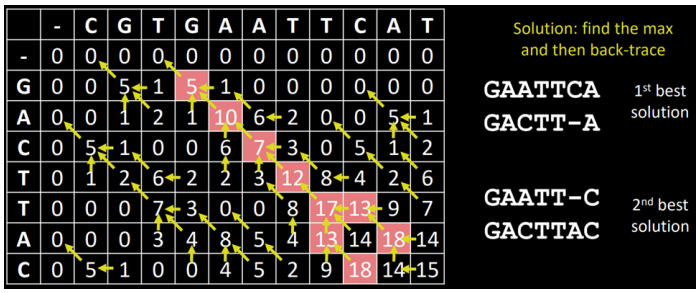


Figure 5.5: Aligning CGTGAATTCAT and GACTTAC

Find the **maximum number** (18, here we have 2 of them) and go back following the arrows. In case of multiple highest scores, traceback should be done starting with each highest score. From this path, the sequence is constructed by these rules:

- A diagonal arrow represents a match or mismatch, so the letter of the column and the letter of the row of the origin cell will align.
- A horizontal or vertical arrow represents an indel. Vertical arrows will align a gap ("-") to the letter of the row (the "side" sequence), horizontal arrows will align a gap to the letter of the column (the "top" sequence).
- If there are multiple arrows to choose from, they represent a branching of the alignments. If two or more branches all belong to paths from the bottom right to the top left cell, they are equally viable alignments. In this case, note the paths as separate alignment candidates.

This algorithm is not used anymore due to a too high computational expense (huge number of comparisons for real genomes) and storage (memory and speed). In fact comparing two Eukaryotic genomes will result in a excessively great aligning matrix (∼3Gb x ∼3Gb), and also using small reads against the human genome is unfeasible: 100 x ∼3Gb for each read!

5.2.3 Needleman-Wunsch algorithm (global alignment)

This global alignment algorithm was developed in 1970 and is also based on dynamic programming. The purpose of the algorithm is to find all possible alignments having the highest score. Again, a scoring system must be defined, then the algorithm proceeds in a similar way as the SW.

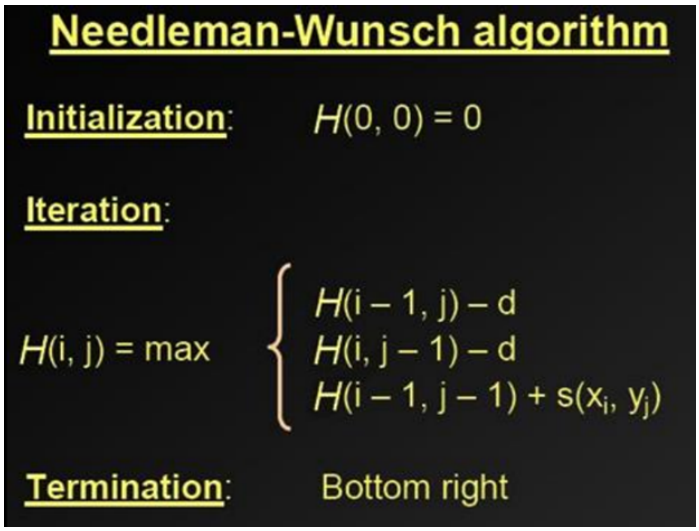


Figure 5.6

The differences are:

- Initialization: we start from only one value, one zero, putted in H(0,0) (need to start from the first nucleotide - global).
- The formula contains no 0 anymore, it adds the maximum score between 3 possible scores, we are always comparing with the first nucleotide of the sequence.

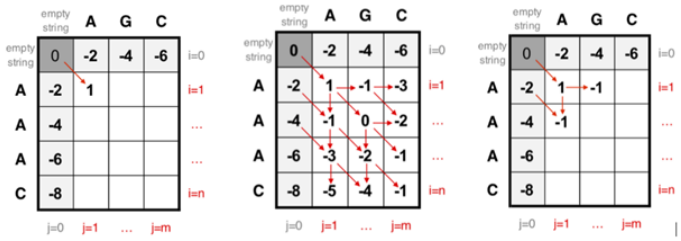


Figure 5.7

New algorithms were implemented later. BWA and BowTie2 are the best ones available right now for short reads. Blast could go too but it would take a lot of time.

The methods going from FastA to BowTie2 are **heuristic methods**: the solution found does not need to be the best one, but the best approximation given certain parameters

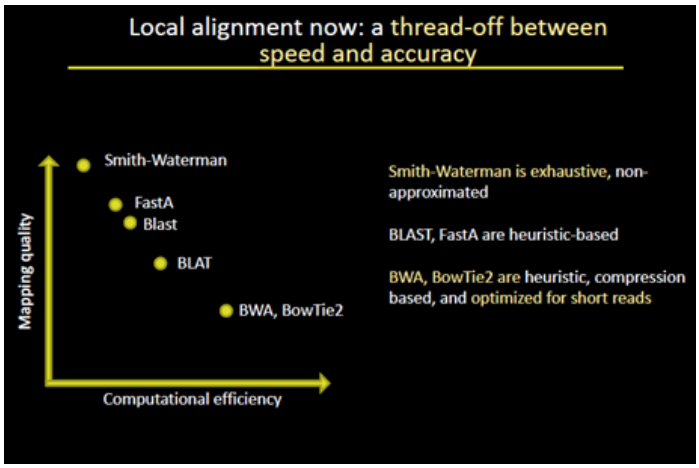


Figure 5.8

(like time). Also, Blast gives an answer based on a fixed level of sequence similarity (in a reasonable amount of time) and gives no result under that level. So Blast online is not very sensitive, it is trained to provide only very good results to not overload the servers. A **heuristic**, or heuristic technique, is any approach to problem solving or self-discovery that employs a practical method that is not guaranteed to be optimal, perfect, or rational, but is nevertheless sufficient for reaching an immediate, short-term goal or approximation. Where finding an optimal solution is impossible or impractical, heuristic methods can be used to speed up the process of finding a satisfactory solution. Heuristics can be mental shortcuts that ease the cognitive load of making a decision. BWA and BowTie2 are also 'compression based' algorithms, meaning that they exploit data compression techniques in order to compress reference genome files to reduce the number of bits used to encode the document (better explained below).

5.2.4 BLAST (Basic Local Alignment Search Tool)

Despite its limitations, Blast is still used a lot and when it was first released it revolutionised the field making sequence alignment available for anyone and for any computer (it can be run online). How does it work? Steps:

1. **Seeding**: find perfect or almost exact k-mer matches (series of sequences with defined length). The idea is to look for identical short matches and try to expand from that.
2. **Extension**: extend the seeds at point one 1 with possibly some non-exact but high-score matches, that permit to obtain better alignments.
3. **Evaluation**: create alignments for the regions of high-scoring extended seeds. Every time the statistical significance of the match is evaluated with methods inspired on the NW and SW approaches.

In online Blast tools the seed is set to 25/29. If a sequence of that length does not match perfectly another sequence we will have no results. One solution would be to lower

the seed, but this cannot be done online because it would take too much time. Another option is to reduce the dataset in which we search. There are many types of blast available, shown in figure:

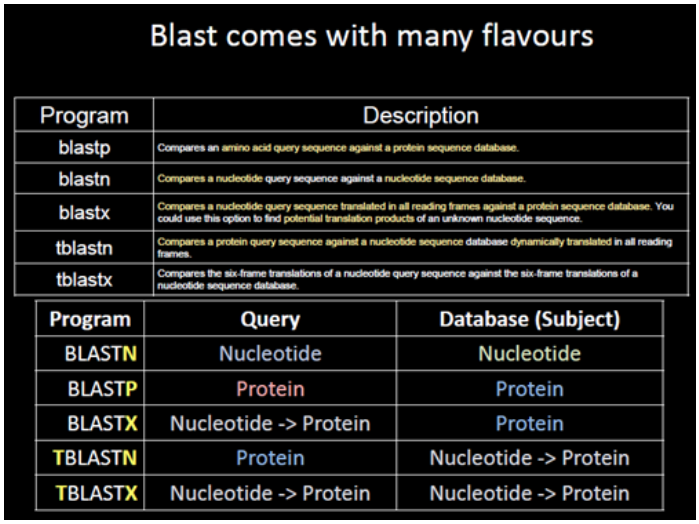


Figure 5.9

Scoring matrices are very important especially for amino acids, they are used to score alignments between protein sequences. Blosum 62 (BLOcks SUbstitution Matrix) is one of the most used. They put non simple penalties in substitutions of different amino acids, for example based on the functional properties of the substitutions. A scoring matrix contains values proportional to the probability that amino acid i mutates into amino acid j for all pairs of amino acids. Such matrices are constructed by assembling a large and diverse sample of verified pairwise alignments of protein sequences. Other **parameters** can be set in online BLAST:

- Max target sequences → number of reported sequences
- Expected threshold → e-value
- Words size → seed
- Gap cost → cost for adding multiple gaps. Linear = twice the gap penalty (if I have to add a second gap after another one).
- Filter low complexity regions to avoid getting stuck

5.2.4.1 BLAST E-value

If we have a match and the database is random, how many other matches I will likely find?

The *e-value* represents the number of distinct alignments with a score equivalent to or higher than S, that are expected to occur in a database search by chance. An e-value of 10 means that up to 10 alignmentscan be expected to be found just by chance, given the same size of a random database. E-value can be used as a first quality filter for the BLAST

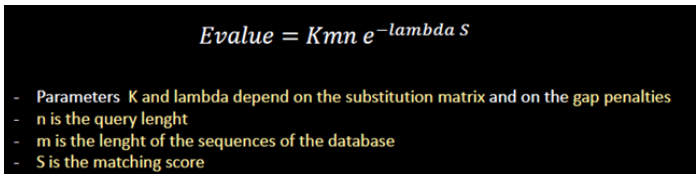


Figure 5.10

search result, to obtain only results equal to or better than the number given by the e-value option. Blast results are sorted by E-value by default (best hit in first line). The smaller the e-value, the better is the match. A small e-value means a low number of hits, but of high quality, whereas a high e-value indicates many hits, partly of low quality (if the number of better possible alignments is high, it means that it is particularly probable to find by chance an alignment which is better the the one found, and so it is more probable that those alignments are bad). There is a relationship between the E-value and the p-value.

- The E-value is the number of sequences that we would find by chance;
- The p-value measures the probability of finding by chance another sequence with an equal or better score.

$$\text{Pval} = K\, e^{-\text{lambda } S} = \text{Evalue}/mn$$

5.2.5 Speed seed alignment

There are algorithms implemented 10/15 years ago that focus on seeds -> not used anymore. Those methods cut both the reads and the reference sequence (read-sized pieces of reference sequence) into small seeds. The reference seeds are then stored in an index (hash look-up table). The idea is to do that for all possible k-mers. Eg. we choose a seed of 10: the first starts at position 1, the second at position 2, ecc. And then to look up seeds for each read and identify the positions in genome where spaced seed pair is found.

- 1 SNPs means that at most 1 seed is not-matching.
- X SNPs means that at most X seeds are not-matching.

Algorithms based on this approach are: Maq, SOAP, MOSAIK. Problem: the lookup table (the reference) for the human genome is too big: 50Gb $\rightarrow$ problem in RAM.

5.2.6 Burrow-Wheeler alignment

This algorithm is based on a very efficient way to store the reference genome, based on the BW transformation, that allows to have a reference index which is way lighter (with respect to the spaced seed approach); in fact, at the end of the transformations, equal letters are next to each other. It is successful because the database is compressed and does not need to be decompressed. However, when we compress a file, the compression must be reversible. There must be a way to go from the BW compressed/transformed index to the original one, to find reads in the genome. The search is based on finding suffixes of the reads in the BW structure. With this approach the index of the human genome is around

2GB. The algorithms BW-based are the fastest currently available. BWT is also used in text compression. Example: We want to compress the word BANANA

- - we take all the rotations of the word
- we sort them based on lexigographic order (Lex order - lexicographical order is a generalization of the alphabetical order of the dictionaries to sequences of ordered symbols)
- we take the last column

We end up with a string which is more compressible, there are sequences of the same letters that can be described at a higher level (Eg. there is 1 B, 2 NN, ecc) In the method shown, in order to **reverse the transformation** and go back to the original word, the input sequence (output of the compression) is added as a column and then sorted (based in lex order), and this two passages are repeated as many times as are the characters of the sequence (8 in this case), obtaining at the end a matrix with equal number of columns and rows. As a result we go back to the ordered sequence of the word's rotations, in which the last one (last line) is the original input string. It takes a lot of time but it makes it reversible; plus, there are actually smarter ways to go back, such as ones base on the LF property.

5.2.7 LF (Last-First) property

As in the previous method, the first step consists in sorting all word's rotations and taking the last column which will be the compressible one. The LF property is then applied to the list of all rotations, to get back to the input sequence, by following this principle: the ith occurrence of character X in the last column corresponds to the same text character as the ith occurrence of X in the last column. Example: We want to reverse the transformation of the string "gc$aaac".

Figure 5.11

- *g* is the first occurrence of g in the last column and corresponds to (arrow) the first occurrence of *g* in the first column. Tracing a horizontal arrow gives the next nucleotide.
- *c* is the second occurrence in the second column and corresponds to (arrow) the second occurrence of *c* in the first column. Tracing an horizontal arrow gives the next nucleotide.

Figure 5.12

- a is the third occurrence of a in the second column and corresponds (arrow) to the third occurrence of a in the first column. Tracing an horizontal arrow gives the next nucleotide.

And so on. Each time we keep track of the character that we are looking at and we end up by constructing the original word. With this method we reconstruct the word from its end to its beginning (backward).

5.2.8 Exact mapping using LF property

In mapping, we can look for sequence matches using the compressed database. Backward exact mapping works by calculating the range of matrix rows beginning with successively longer suffixes of the query. Example: we want to match the string 'aac' with the database.

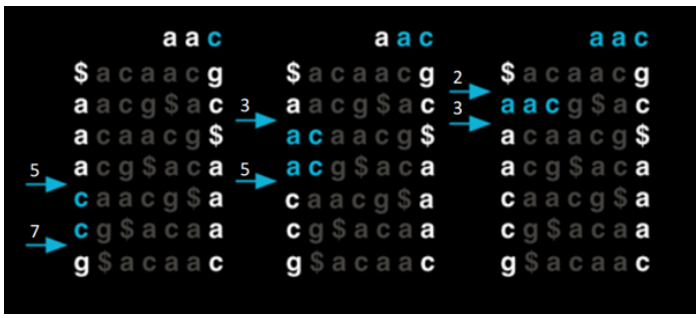


Figure 5.13

- we take the 'first suffix' **c** of the query string and we look for it in the first column. We find 2 matches in lines 5 and 6, so in the range 5-7.
- we pass to a 'second longer suffix' **ac** and see if we can find it in the matrix → lines 3 and 4, range 3-5.
- we look at the suffix **aac** and find it in the 2-3 range.

Doing the backward matching we exploit the characteristics of the BWT transform. This same approach could be used to find a match between a query read and a reference genome which has been compressed using the BW alignment. The problem is that it does not take into accounts indels, mismatches. A possible solution to this problem could be the **inexact mapping**. Perform exact mapping and, if the query is not found, go back and perform backtracking by hypothesising mismatches. For example we want to find a

match for the query GGTA. As always, we start looking for a match starting from the last character.

- we find a match for a, t, g but not for the last g.
- so we go back and hypothesize a mismatch for the first letter a. We then look for all the matches that we could find if we replace a with another nucleotide (we do the same for the other NTs in the query?).
- we find than by replacing a with a g, we find a match (ggtg) (not exact match).

The Burrows-Wheeler algorithm is used in BowTie and Bowtie2. Right now there are many algorithms to choose between and many review papers compare their characteristics and performance. No tool outperforms the others in all the test, hence the decision of which algorithm to choose must depends on the specific needs.

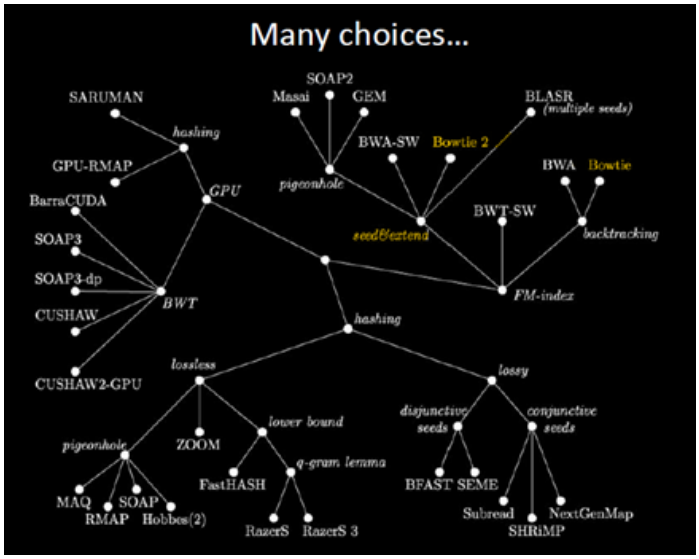


Figure 5.14

Chapter 6

Assembly

Assembly is the process of **aligning and merging overlapping sequences** in longer consensus sequences to reconstruct an original sequence or genome. No reference databases are used to perform assembly, unlike in mapping. Assembly is the process of aligning and merging overlapping sequences in longer consensus sequences to reconstruct an original sequence or genome. No reference databases are used to perform assembly, unlike in mapping.

- sequencing a genome for the first time (eg. the genome of a new bacterium);
- when the reference genome is not complete or very distant phylogenetically and hence not usable as a reference;
- in case of new genes, which cannot be discovered just by mapping.

With human genetics assembly is rarely needed, since the human genome is already available. By contrast, in microbial genomics it is particularly important. Different strains of *E. coli* for example could have new genes, that cannot be discovered by mapping against a reference genome. Applied also for viruses or yeasts. The mechanism of assembly is quite complicated. In theory, all assembly algorithms could be based on this basic framework

1. They start from the reads obtained through sequencing and identify overlaps between them (this is performed by all algorithms).
2. Once found, the order of the reads is defined by connecting reads by overlaps.

In practice this is not always possible, due to many issues that occur, like:

- multiple overlapping: there will be multiple overlapping of the same reads. Sometimes this is just due to a high coverage, sometimes (frequently) there may be different locations in the genomes that are matching, at least partially, our reads.
- partial matches, ecc.

One possible solution would be to **keep track of all possible matches** (overlaps) and then try to reconstruct the sequence. One way to represent the possible overlaps between reads is by using a **graph** in which the nodes represent the reads and the edges

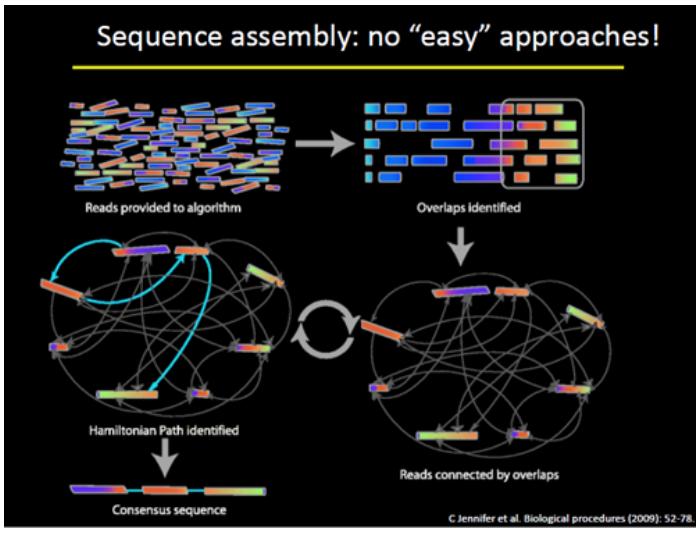


Figure 6.1

represent the connections between the reads that are overlapping in some way. Then, a **mathematical algorithm** is needed to find a solution in this very intricate network. Many assembly algorithms are available, and they are all based on this idea. For this reason, we always end up with multiple copies of genomes, not just one. Also, the output is almost never the full genome, but pieces of the genome, **multiple contigs**. Ideally, we could close the genome, but this is not possible just with short-read sequencing; other experiments are needed. By default, assembly is done in this way and gives a good representation of the genome, but not complete. Some quality control is also needed, especially to detect contamination.

The **general pipeline** for sequence assembly consists in a few steps:

1. Find overlapping reads.
2. Merge the 'good pairs' of reads into longer **contigs**. A contig is a contiguous sequence formed by several overlapping reads with no gaps.
3. Link contigs to form **scaffolds** (or supercontigs), which are ordered and oriented sets of contigs. Scaffold assembly usually exploits mate pairs (which allows to link different contigs together, even if the information in the middle is not completely known). Mate pairs, also called long-insert paired-end reads (LIPERs), are a kind of read obtained from paired-end sequencing which can pair reads across greater distances. Scaffolds do contain gaps, but at least the order of the contigs is known and helps to reconstruct the whole genome.
4. Derive consensus sequence. Sometimes we have multiple scaffolds and the consensus sequence is the set of all scaffolds representing the genome or the chromosome that is being reconstructed.

Finding the order of contigs is not easy and different approaches are used, hence scaffolding is usually a quite time-consuming operation. The aim would be to try to close the

genome; for this purpose, some additional experiments could be needed. For example, one could use PCR to try to clone pieces between two contigs. In microbial genomics, assembly usually finishes at the contig step. Contigs contained in the output files allow to define the genes present in the genome (for this goal, the order is not fundamental), whereas the position of genes in the genome is more difficult to study. **What are mate pairs?** - Mate pair library preparation process Following DNA fragmentation, the DNA fragments are end-repaired with labeled dNTPs. The DNA fragments are **circularized**, and non-circularized DNA is removed by digestion. Circular DNA is fragmented, and the labeled fragments (corresponding to the ends of the original DNA ligated together) are affinity-purified. Purified fragments are end-repaired and ligated to Illumina paired-end sequencing adapters. Additional sequences complementary to the flow cell oligonucleotides are added to the adapter sequence with tailed PCR primers. The final prepared libraries consist of short fragments made up of two DNA segments that were originally separated by several kilobases. These libraries are ready for paired-end cluster generation, followed by sequencing utilizing an Illumina next-generation sequencing (NGS) system. Combining data generated from mate pair library sequencing with that from short-insert paired-end reads provides a powerful combination of read lengths for maximal sequencing coverage across the genome.

6.1 Feasibility of sequence assembly

Doing a full assembly is considered to be impossible from a computational point of view. It is completely infeasible (NP hard), cannot be finished in a reasonable time, regardless the computational resources available. So technically assembly is impossible, differently from mapping, which is feasible. Read length $\rightarrow$ important for reconstructing the whole sequence Coverage $\rightarrow$ many reads but impossible to assemble.

6.1.1 Last year exercise

As an example of the assembly process and of the aspects that might be important, students were asked to reconstruct a piece of English text which was previously fragmented.

	Group 1	Group 2	Group 3
Coverage	5	5	4
Read length	10 PE	10	14
Mut. Rate (every 1k)	20	20	50
Number of corr. words	20/120	21/120	73/120
Number of sentences	2	3	7
Reconstruction perc.	16.6	17.5	60.8
Time (mins)	45	45	45

Figure 6.2

Group 3 was the winner (highest percentage of reconstruction), despite having a lower coverage and a higher error rate, indicating that the length of the reads was very important. Without long-enough reads it was impossible to assemble sentences, going out of the repetitive regions of the text. Knowing the characteristics of the sequence reads ('the language') might also help assembly. Eg. knowing that some sequences are just artifacts.

6.1.2 Merging overlapping reads

Basic principle: the strongest the similarity between the end of one read and the beginning of another the highest the likelihood the reads are coming from the same overlapping sequence region. When overlapping reads, we must maximize the length of the overlap but also accuracy. Of course, since sequencing is not perfect, errors will happen. So again, we must find a score for the overlap. The fact that two reads are not perfectly overlapping can be due to different reasons:

- they are not really contiguous (just similar sequences inside the genome). We should be able to distinguish between the two (not easy).
- Sequencing error/noise
- Diploid genome or multiple copy genomes: sometimes sequences coming from 2 different chromosomes; they are similar but not contiguous and this increases the difficulty.

6.1.3 Overlap graphs

As said, one way to represent reads overlaps is by using graphs. In an overlap graph, each read is a node, each overlap is an edge between two nodes, representing the two overlapping reads. Edges are directed: indicating that the final part of the first sequence matches the initial part of the second sequence. Directed graphs are very common in biology, for example to describe binding between proteins, or other biological functions. Different overlaps can have different "strengths", based on the score of the overlap, so to each node we can also associate a weight. Graphs are ideally cyclic, since in theory most organisms' DNAs are circular. So we could have the last read matching the first one. However, sometimes cycles are just due to random overlapping or to the presence of repeats. Those problems must be resolved in the mathematical object. **The problem of the repeats** is a key aspect in bacterial genomics since bacterial genomes have many regions with repeats. Also, as we previously saw, short reads are the default sequencing technology for bacteria, and this increases the difficulty in assembly.

If the read length is not long at least the length of the repeat, we will never be able to resolve this. The read that matches a part of the repeat and a part of the non-repeated region will be known, but for all reads that are inside the repeated region it will be impossible to define how long these regions are. There will be a lot of cycles and connecting the 2 non repeated parts won't be possible. By knowing the **coverage** we can try to solve at least partially the problem. If we know that all the genes have a coverage of 10x for example, we could assume that the repeated part has a 10x coverage too and we could define a length for this region proportional to the expected coverage. This is however a bit risky: repeated regions are also problematic for the sequencing machine, so there could be a skew in coverage for these regions. The **safest idea** would be to **stop the contig** before the repetitive region (A) and start it again after it. Some assemblers will try to solve these repetitive regions and might produce a wrong contig. Hence, if we try to minimize the number of contigs produced (to obtain longer genome sequences) we might end up having wrong contigs (without even knowing it). **Long reads** are the ultimate solution for this problem (they have many errors but represent a backbone on which to do the assembly). With overlap graphs, which are not what assemblers are using right now, we can

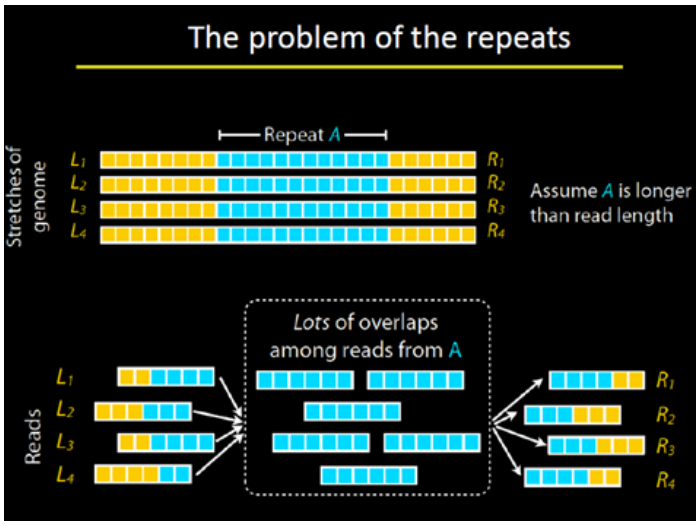


Figure 6.3: Example of genome with repeats

lead to having wrong contigs. The sides of regions containing repeats (mix of yellow and light-blue seen before) will overlap pretty good and will be considered as consequential in the sequence, discarding the repeated sequence in the middle. This will produce a wrong contig, in which fragments are considered to be **closer** than they are in reality. A solution could be to put many Ns in between and to keep track of the fact that the sequence is not reliable. Some hints of the presence of repeated regions can be given by:

- Coverage: we could see a region with a much higher coverage, which might contain actually repeats that were wrongly assembled.
- Paired-end: we could notice that the pairs over repeated regions have a shorter insert size than they should have. With long paired end sequencing we could also try to estimate the length of the regions with repeats.

6.2 How to solve an overlapping graph?

In this graph we have five known reads. Overlaps of length 1 are also present, usually not considered. A possible ideal solution would be to use **Hamiltonian paths**. Hamiltonian paths are the paths touching all connected nodes once, so they provide all possible solutions. Then, how do we define which is the best one? We choose the one that maximizes the length of the overlaps. This problem however will explode, it implies too many possibilities. It is equivalent to the shortest common superstring (SCS) problem, and it is NP-hard = not feasible. Another solution what be to use a **greedy approach**, which maximizes each choice, by following these steps:

1. Randomly select a starting point/node.
2. Select the connected node with maximum overlap as the next visitor.

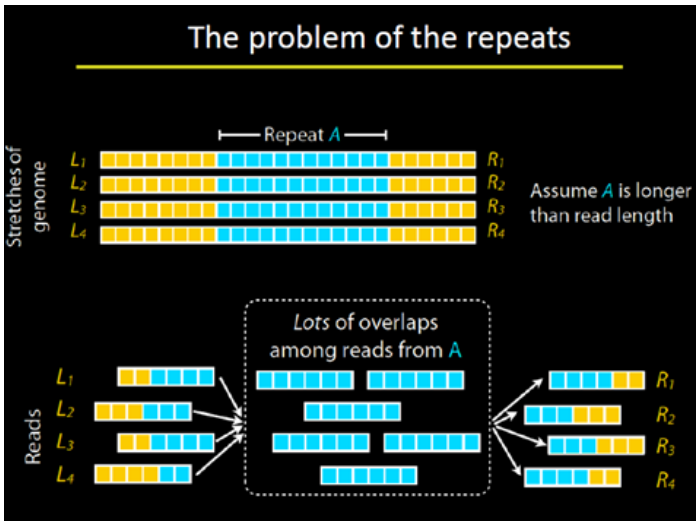


Figure 6.4

As always, the greedy approach will provide the best local solution, which might not be the best global solution.

6.3 Graph simplification operations

A practical trick that could be used to solve the graph, and to minimize the number of operations which must be performed (in an attempt to make it feasible), is to simplify it. Simplification operations include:

- Merging consecutive nodes: if among more reads there is only one possible path, we can just merge those reads, without losing information. So, nodes that are sequentially connected only with each other are linked together.
- Remove dead ends: if some reads are branching from a path toward a dead-end, we can remove them.

Here we may lose some information since the removed path could be the right one. Assembly by overlap graphs has several problems:

- It is problematic when dealing with repeats: maximizing the overall weight will produce wrong assemblies.
- It is not tractable: finding the optimal solution is not computationally feasible.

Overlap graphs can work to some extent, but now other algorithms are used in assemblers, like the de Bruijn Grpahs (DBG) and the Overlap Layout Consensus (OLC).

As a simple example, in the case of the DBG, this problem would be splitted into 2 contigs, which is actually the best solution: better to have 2 right contigs, instead of 1 wrong contig.

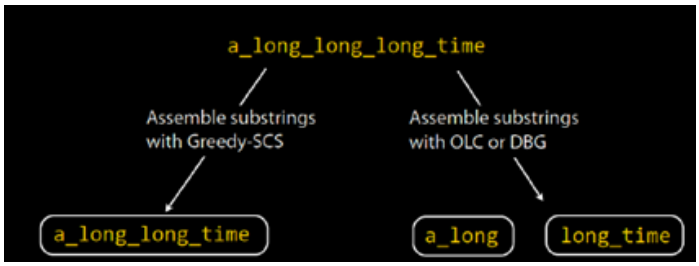


Figure 6.5

6.3.1 De Brujin graph assembly

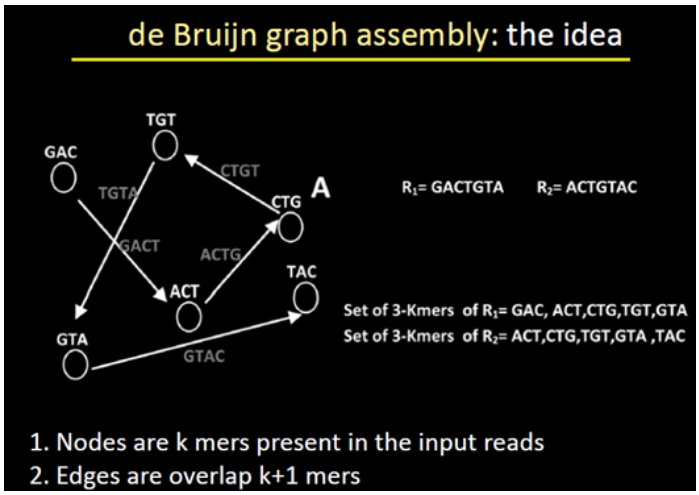


Figure 6.6

On this graph the nodes are not the reads but the k-mers present in the input reads (with k smaller that the read-length). We define the length of k-mers length in advance and create all combinations of k-mers for each read, for example for reads R1 and R2. Clearly there will be some overlap, some k-mers will be present in both sequences, but in the graph there will be only one copy of them. Hence, the nodes will be the **unique k-mers** present in the reads, the edges are the **overlaps** of the k-mers of length k+1 from the 2 sequences. Then, instead of using Hamiltonian paths touching the nodes, we use **Eulerian paths**. Those are paths in which all edges are visited, each edge exactly once. From a computationally point of view, they can be found much more efficiently than Hamiltonian paths (no explosion of search space). Other advantages:

- if we have a k-mer of length 3, we cannot have a huge amount of nodes, but 4^3 possibilities. So the set of nodes will not be too big compared to the number of reads. With k = 3 $\rightarrow$ huge amount of overlaps hence edges. But with longer k-mers (eg. 15) there will be a good trade off between the number of nodes and the number of edges.
- Cleaning reads: we can avoid putting in the k-mers that appear only once in a genome

with a lot of coverage and are therefore the result of sequencing errors (otherwise the number of nodes would increase a lot).

- The de Bruijn graphs seem to be bigger sometimes, but they are not. With the de Bruijn graph the paths are more linear, there are less possibilities of wrong cycles.

6.3.2 Scaffolding

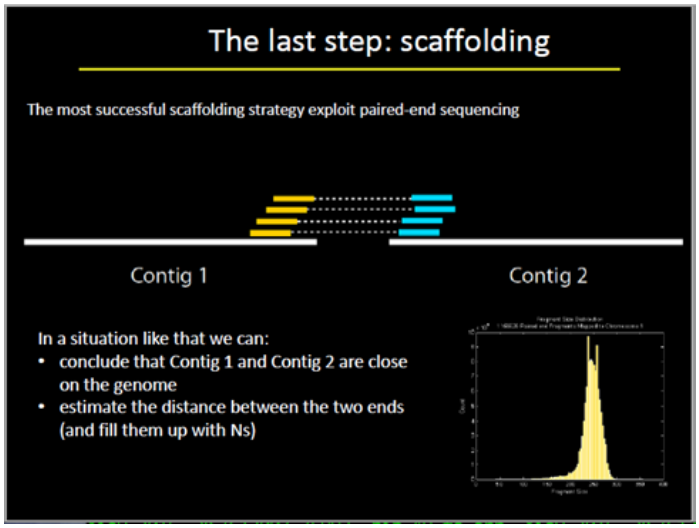


Figure 6.7

Scaffolding can be done with paired-end sequencing or with the estimation of the coverage. The fragment size in this case was 250 bp on average and with a Gaussian distribution like that one we can put an optimized number of Ns between the 2 contigs (not perfect, but connected). Doing it for all contigs leads again to a very complex situation. Problems: If the end of a contig matches the middle of another one in paired-end sequencing, there might be a mistake. Either the contig must be splitted because the overlaps are wrong, or the two matches are wrong.

6.3.3 Evaluating assemblies

The **N50** is the size of a set of entities (e.g., contigs or scaffolds) which represents the largest entity E such that at least half of the total size of the entities is contained in entities larger than E. Kind of a weighted median. For example, if we have a collection of contigs with sizes 7, 4, 3, 2, 2, the N50 is 4 because. (4 or more must be equal to what is 4 or less: 7+4 = 11, 4+2+2+3 = 11. Value in the middle for which the total length above and below is equal). Long contigs help to have a higher N50. Other definition: N50 length is the length 'x' such that 50% of the sequence is contained in contigs of length x or greater. X must be the length of a real contigs, not just a number. One long read = perfect N50 but might be wrong, we might check other characteristics. Eg. we can look at what the genes are coding for (is there at least a gene that codes for ribosomes? if not, wrong).

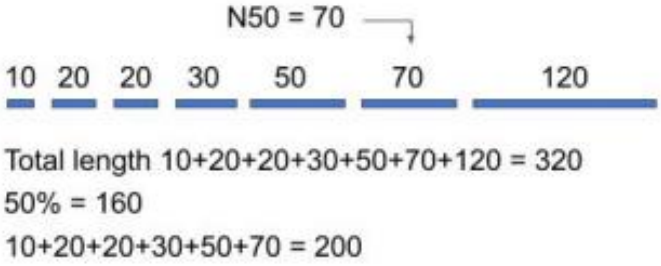

N50 = 70
10 20 20 30 50 70 120
Total length 10+20+20+30+50+70+120 = 320
50% = 160
10+20+20+30+50+70 = 200

Figure 6.8

Chapter 7

16S-rRNA sequencing

7.1 Introduction to metagenomics

7.1.1 Definition of metagenomics

The term **metagenomics** refers to the "*study of* ***uncultured microorganisms*** *from the environment, which can include humans or other living hosts*" whith "*focus on taxonomic and functional characteristics of the* ***total collection of microorganisms*** *within a community*". The main way to analyze the entire microbial population of an environment is through **high-throughput sequencing** of nucleic acids isolated from the sample; we can further distinguish two approaches, namely **16S rRNA gene sequencing** and **shotgun metagenomics**.

7.1.2 Why studying the metagenome

Microbes are basically everywhere, in and outside of our bodies, in oceans, glaciers, hot springs and rocks. Given how widespread and abundant microbes are, studying the metagenome provides us plenty of information (both on human and non-human microbiome and environment). For instance, it has been shown that the microbiome correlates to several diseases, therefore it can be used as a non-invasive **biomarker** (colorectal cancer, immunotherapy efficacy, autoimmune diseases...); the list of activities microbes are involved in is evergrowing.

7.1.3 Differences with older microbiome studies

The microbiome was discovered many years ago but there were no tools to analyze it properly; the only way was to culture and isolate each bacterium, which is an unfeasible approach to study the entire community, since only some bacteria can be grown in lab and it still would take an unreasonably long amount of time. The advent of high-throughput technologies is what made possible to study the microbiome of a sample, reducing significantly times, costs and increasing substantially the fraction of the microbiome that can be known.

7.1.4 Example: skin microbiome

Some studies were performed on skin microbiome (Segata et al, Nature Methods 2012, Truong et al, Nature Methods, 2015); only about 60% of the contigs (of various size) were mapped to known microbes while 40% belonged to unknown species. When separating these sequences based on GC content and abundance, many clusters formed, some with higher abundance while others with lower abundance, probably due to the low GC content that makes more difficult for the machine to sequence them, therefore causing them to be underestimated. Studying this 40% of unknown sequences is one of the main tasks of metagenomics.

7.2 16S rRNA sequencing

16S rRNA sequencing is one of the first techniques developed to study the microbiome, since it does not require a huge amount of sequences nor excessive costs; for these reason the technique became popular.

7.2.1 Simplified 16S rRNA analysis workflow

The general workflow for a 16S rRNA analysis is the following:

- **DNA extraction** from the entire community present in the sample; some bacteria will be over-represented while other will be under-represented.
- **Selective PCR amplification of 16S rRNA gene** (due to the characteristics explained below)
- **High-throughput sequencing**
- **Sequence mapping** against genomes in databases; this allows to define which bacteria (and which variants of those) are present in the sample and to find new and unknown bacteria.

7.2.2 16S rRNA gene

The ribosome is one of the most conserved, if not the most conserved, structure in all living organisms, making it one of the best phylogenetic markers. In prokaryotes, the ribosome is composed of several elements, both proteic and RNA based. Of the RNA based ones, 3 of them are **ribosomial RNAs** (rRNAs), namely 5S, 16S, 23S. Since these components are fundamental for any bacterium, all bacteria present the genes codifying for these rRNAs; most of the sequences are highly conserved but some regions have some variability which, since this variability is species-specific, can be used as a *barcode* to find and classify species (it also allows to distinguish between Archea and Bacteria). The most conserved of the rRNAs is 23S but the one used for microbiome analysis is 16S (which corresponds to the human 18S). The 16S rRNA gene is a few thousands nucleotides long, most of which are highly conserved; the bulk of the differences among species is in the hypervariable regions (named V1 to V9), which are terminal loops of the structure (basically regions far away from the catalityc site, therefore more free to mutate). Despite the high degree

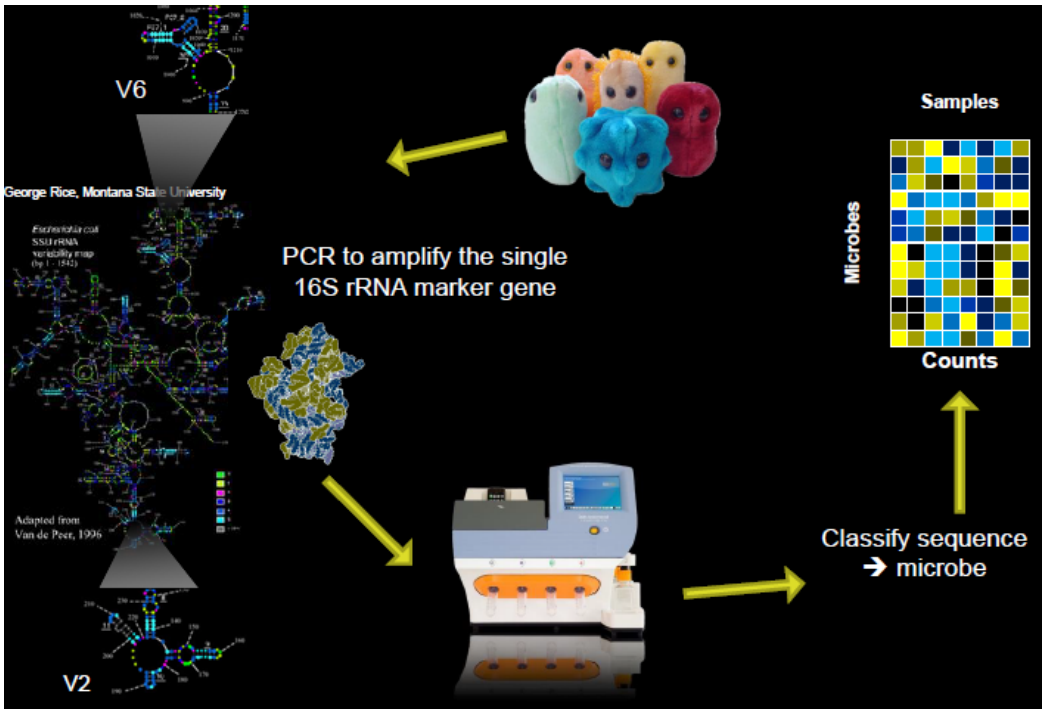


Figure 7.1: General 16S gene analysis workflow

of conservation, some variability can be found outside the hypervariable regions too (eg. 530 loop structure). The annotation of which portions of the 16S rRNA gene are conserved has been performed using *E. coli* as a reference; for a few hundred organisms the gene has been compared to the reference one to define the degree of conservation of each stretch of nucleotides. Some totally conserved regions (meaning pretty much identical in all species) are present but they are not very big.

7.2.3 Primer and high-throughput machine choice

One could sequence the entirety of the 16S rRNA gene, for example using NanoPore seq, but this would introduce many errors that could lead to mapping the sequence to the wrong organism; for this reason it is preferred to amplify only certain specific regions of the gene. To study the microbiome in a high-thoughput way you need primers which can bind to all species, but since the sequences conserved in all species are too short, you use primers that bind highly conserved regions; for this reason, regardless of which primers you choose there will be bias in your results (some species will not be identifiable using those primers). This bias can be somewhat minimized using *in silico* primer validation, which means testing your primers against databases of 16S tRNA genes (silva and green genes), to test and decide the best pair of primers for your experiment.

Still, two experiments conducted with different primers will always have some differences. Moreover the binding regions must flank some variable region, in order to include it in the amplicon; finally you need pair end amplification (both primer back and forward)

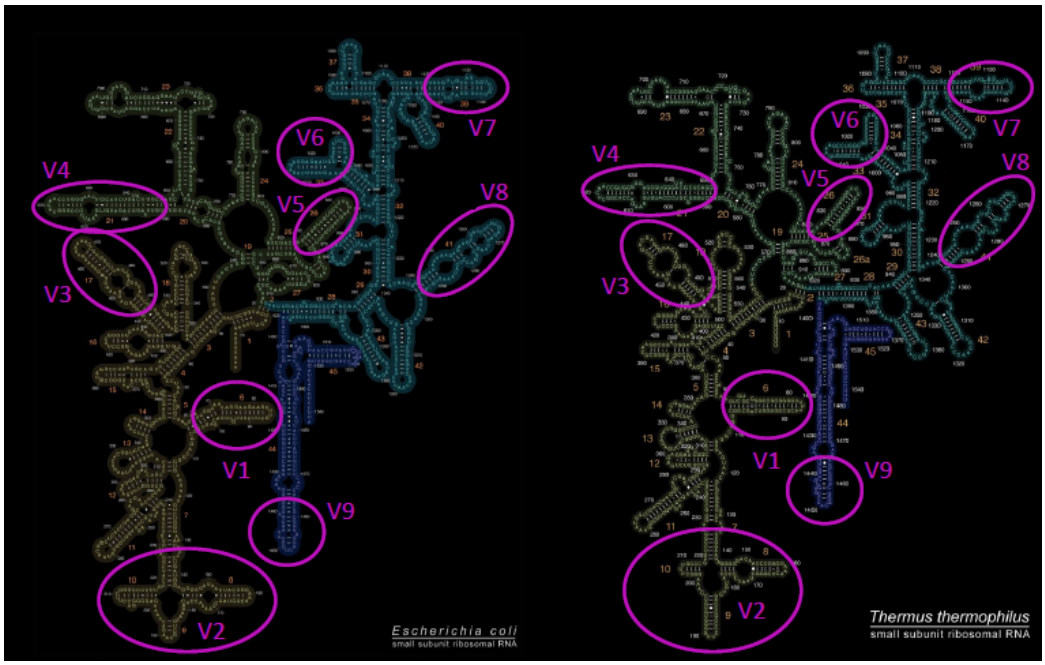


Figure 7.2: Structure of the 16S rRNA in *E. coli* and *T. termophilus*

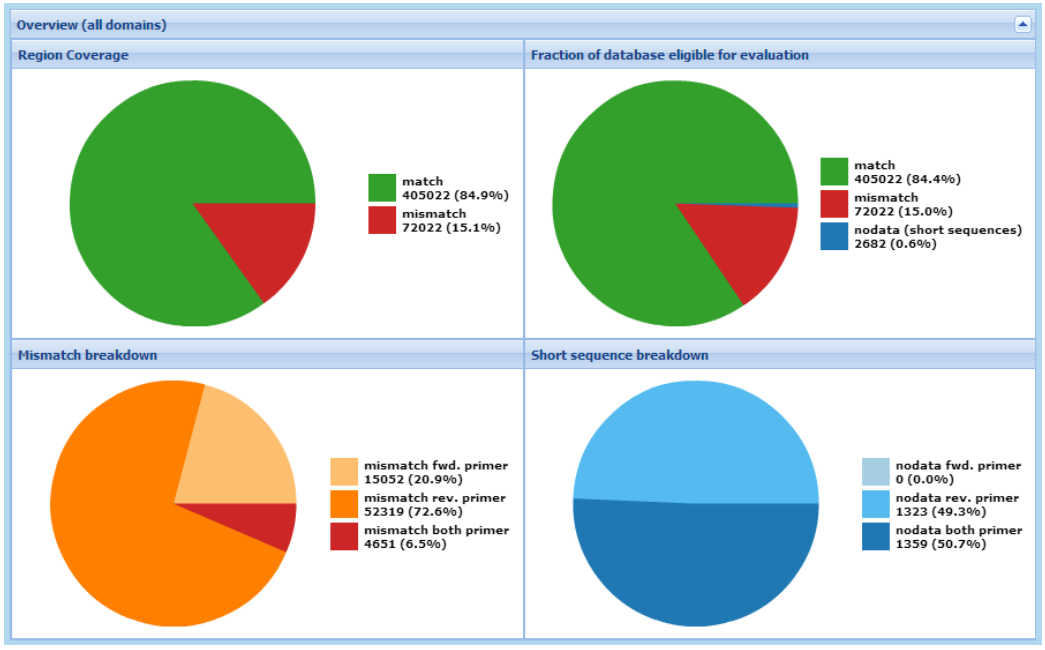


Figure 7.3: Example of *in silico* primer validation using silva; you can notice the different efficiency of the primers relative to different parameters.

in order to have the complete amplicon (to make comparison easier). Given these characteristics there are multiple possible priming sites, based on the sequence and on chemical properties of the primers. Moreover, primers can be used as forward or reverse to obtain different combinations and sequences.

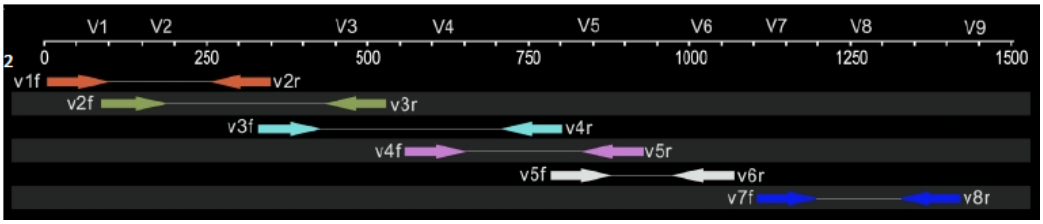


Figure 7.4: Examples of common primer placements relative to hypervariable regions

As an example of the importance of the choice of primers, in some skin microbiome analyses, researchers could not find two bacteria always present on human skin due to the choice of primers. Moreover *S. aureus* seemed over-represented due to the non-amplification of other important species. Despite the biases, this technique is still extremely useful even today.

There are different protocols to target conserved regions also based on the machine used. In general:

- Sanger machines are not very good for this application since they have low throughput and they are more suited for longer sequencing tasks (full genomes for instance)
- Roche 454 machines have historically been well suited, since it was possible to sequence three hypervariable regions toghether using 400 nucleotides reads, providing a good cost/throughput trade-off.
- Illumina HiSeq is not the optimal choice since it has shorter reads and unnecessarely high througput; Illumina MiSeq and IonTorrent can be a decent compromise.

7.2.4 In depth 16S rRNA analysis workflow

A more in depth 16S rRNA analysis worflow is the following:

- **DNA extraction** from each of your samples
- **Selective PCR amplification of 16S rRNA gene**, introducing a barcode in the sequences using tagged primers.
- **High-throughput sequencing** of all the samples in a single run (to reduce costs); the result is a set of amplicons belonging to different samples and with a barcode attached.
- **Demultiplexing**, which means removing the barcodes and assigning each sequence to the corresponding sample. Sequencing noise must be taken into account, therefore low quality reads must be removed.
- **Multiple sequence alignment** against reference sequences. Some reads will probably not map to any reference sequence.

- **Group related sequences into OTUs** (operational taxonomic units), which means grouping sequences that share some common variants; since there are some SNPs in the microbial genome, the similarity threshold between sequences cannot be too restrictive. OTUs can be used to define the relative abundance of each species in the sample, but in order to do so it is necessary to normalize for the copy number of the 16S gene sequence; this is very difficult since an accurate estimate can be made only if long reach sequencing has been performed on the organism, which is almost never the case since for microbes that basically corresponds for full genome mapping (needless to say it is therefore non applicable on unknown microbes).
- **Build phylogenetic tree** using one representative for each OTU.
- **Annotate** the OTUs using 16S gene databases.
- **Downstream analysis** is performed, such as clustering to visualize similarities among samples.

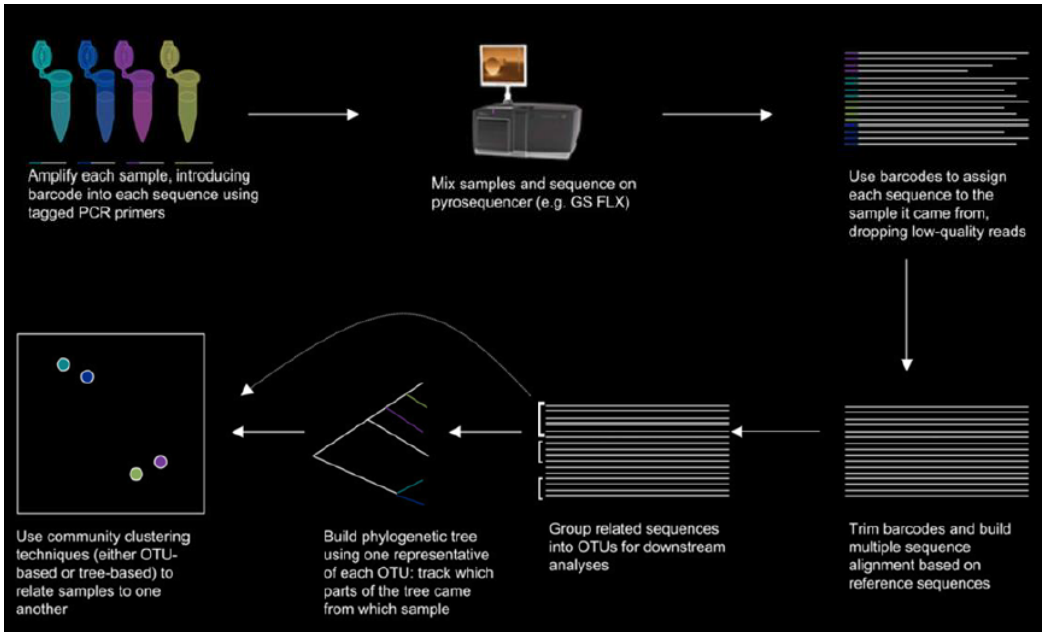


Figure 7.5: Expanded 16S gene analysis workflow

7.2.5 OTU clustering

Defining OTUs requires using multiple sequence alignment; since this approach is a generalization of the mapping algorithm (meaning you have to compare every sequence with every sequence) it is quite complex in terms of speed, but still feasible. Generally, greedy algorithms, which add the lowest possible amount of gaps, are used to perform multiple sequence alignment. After the alignment, sequences are split into OTUs (operational taxonomic units), which are basically groups of 16S sequences very similar to each other.

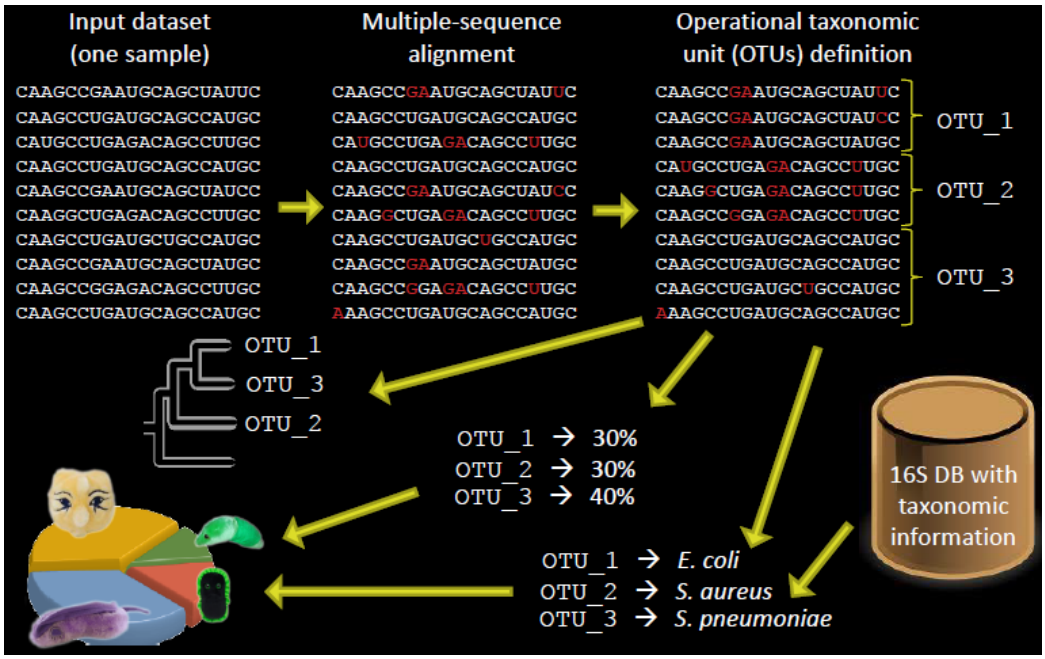


Figure 7.6: Zoom in on 16S gene analysis workflow

Generally a sequence is defined as the representative of the OTU, meaning that it has a certain threshold of identity with all other sequences in the OTU (usually 97% when considering species) and that minimizes the differences of all other sequences of the OTU with itself. Some OTUs can be assigned univocally to a species, some others may be associated to more species, some others cannot be mapped to know species. The fact that a species may map to multiple OTUs is often a negative factor (confusion in the analysis) but it may sometimes allow to find subspecies.

After sequence alignment, OTU clustering (= splitting the sequences into OTUs), can be done through several supervised or unsupervised learning methods. Each methods has pros and cons, therefore there is not an always optimal method. The most common unsupervised clustering methods are:

- **Single linkage clustering** (nearest neighbour): assign the sequence to a cluster if that OTU already contains **at least a sequence** similar enough (97%). However two distant sequences in the OTU network could share a similarity which is way lower than 97% (because of a series of connections above 97%); this could result in **underclustering** (defining too few clusters).
- **Complete linkage clustering** (furthest neighbour): assign the sequence to a cluster only if **all the sequences** of the OTU are similar enough (97%). However two sequences may be similar enough (97%), yet belong to different OTUs, because the overall cluster width, or **divergence**, is at most 3%; this approach could then generate different solutions, based on the order the points are added in. Moreover, if the clustering conditions are too stringent, sequencing errors and SNPs in the microbial genome may result in **overclustering** (defining too many clusters).

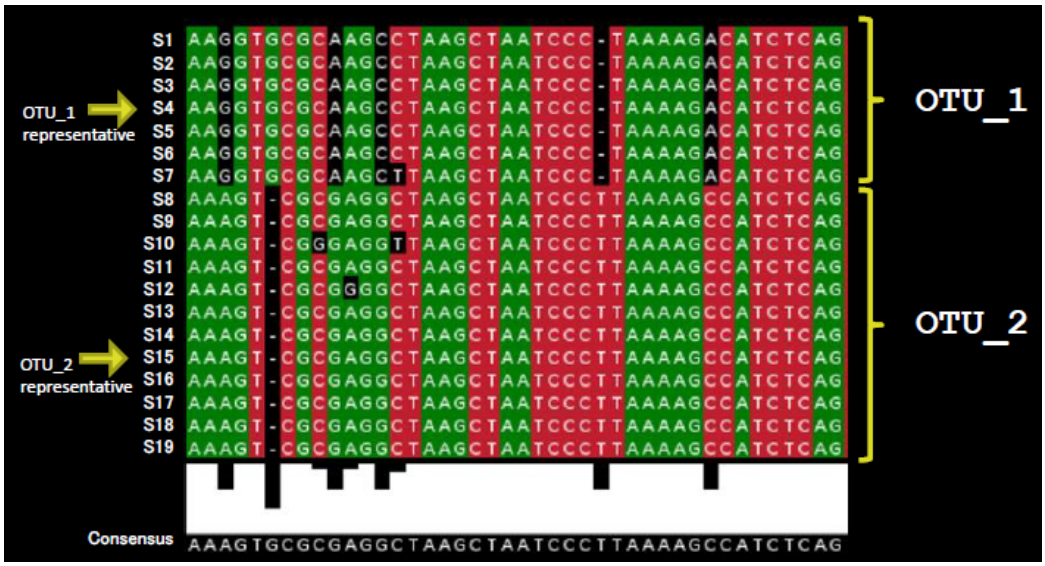


Figure 7.7: Example of multiple sequence alignment for OTUs

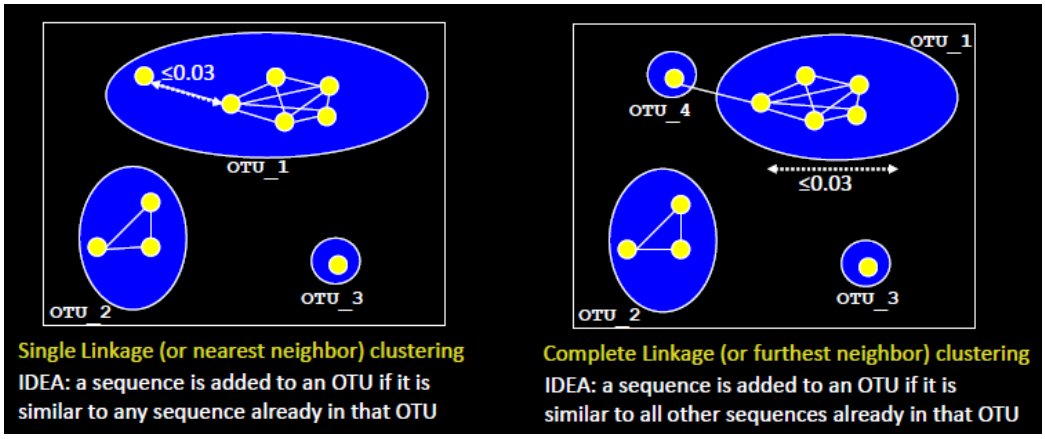


Figure 7.8: Visualization of single linkage analysis and complete linkage analysis

7.2.6 OTU taxonomic annotation

NOTE: this topic continues in the next lecture; when done merge together Assigning a taxonomic annotation to an OTU cannot be done simply using BLAST to get the best matching sequence; this is because there is too much noise in the sequences and because it is difficult to classify new strains. A better way is using some other algorithm that assigns the terms of the taxonomic notation (since it is more than just one label) and provides some degree of confidence in the prediction. For instance the algorithm may be able to correctly assign the first taxonomical terms, up until *Enterobacteriaceae*, but then it provides a prediction of the OTU belonging to *E. coli* with some confidence interval, say 85%, and some alternative like *S. dysenteriae*, say with 15% confidence.

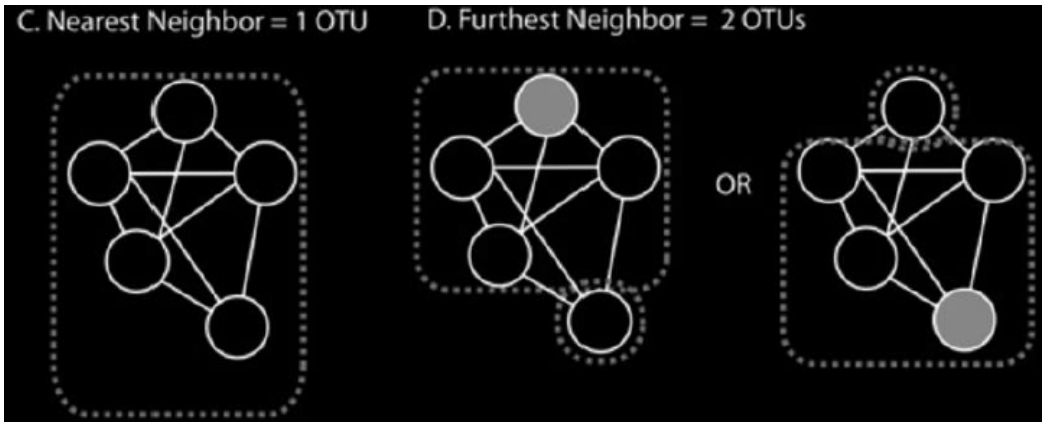


Figure 7.9: Example of overclustering and result multiplicity due to complete linkage analysis

7.2.7 RDP classifier (Naive Bayes Model)

P(S|G) is computed by RDP using a 8-mer strategy basically comparing the 8-mers in S with the 8-mers in all the training sequences available for genus G. The confidence of each prediction is computed by bootstrapping:

- Select a (random) subsequence of S, Say S'
- Compute the G that maximizes P(S'|G)
- Repeat the procedure 100 times
- The number of time G is selected by the bootstrapping procedure is the confidence

Leave one-out-cross validation:

- Take out one data point from the training set
- Apply the classifier on the left-out point (without using it in the training set)
- Check the accuracy of the prediction
- Repeat the procedure for each training data point

The RDP classification accuracy is evaluated with the leave-one-out cross validation on the training set of hundreds thousands of 16S references:

- Accuracy $\rightarrow$ percentage of correct classification (over the all leave-one-out runs)
- Varying levels of the taxonomic resolution (from phylum to genus)
- Varying sequence lengths
- In real applications accuracies are probably smaller:
  - Sequencing errors
  - "unknown bacteria"

7.3 Diversity analysis

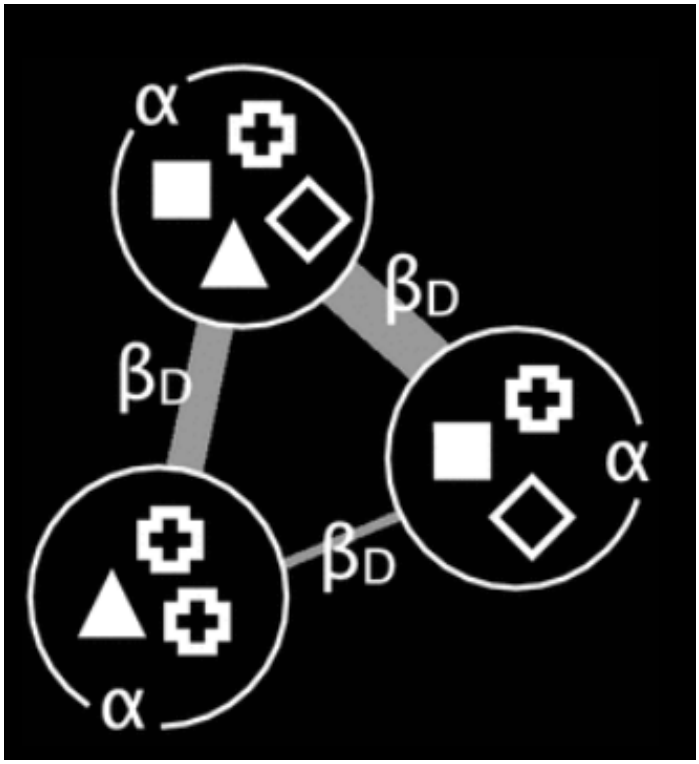


Figure 7.10

7.3.1 Alpha diversity analysis

Alpha diversity analysis is a measure of how diverse, so complex, is a microbial community. "Within sample" diversity. Species richness is a widely use alpha diversity index. All individuals considered have non-zero abundance, some will have high abundance ($\sim 99\%$) or low abundance (1%). High alpha diversity are usually associated with populations that are more robust and resilient to changes. For examples gut microbiome with a high richness is usually associated with healthy state, instead of disease.

7.3.2 Beta diversity analysis

Beta diversity analysis is a measure of how different two microbial communities are. "Between sample" diversity. It is possible to measure the beta-diversity using the inverse of number of shared species. An example of beta-diversity is **UniFrac**. In UniFrac the distance is equal to the fraction of the total branch length that is unique to any particular environment. UniFrac can be also weighted in order to include abundances for each OTU.

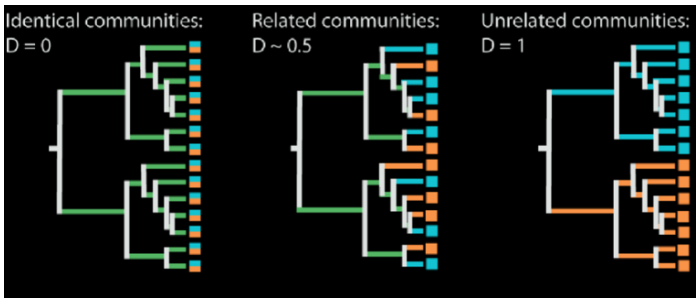


Figure 7.11: D=0. Blue and orange samples always have the same OTUs. Each 16S (each ramo) is present in both samples. **D=∼0.5.**In reality we usually have a mix of the 2 situations. Some OTUs are present only in one of the samples and are either quite distant from the others or close (based on upstream the branch goes). **D=1.** Completely distinct OTUs . The difference is also in the upstream branches, which have different colors.

7.4 Principal Coordinate Analysis (PCoA

PCoA is also known as multidimensional scaling. It is one of the most powerful approaches for exploratory analysis. The idea is to represent the multidimensional relationship between samples in a two or three dimensional space. It is possible to use any similarity function as Euclidean distance, UniFrac, bray-Curtis distance. We find frequently hierarchical clustering plots.

7.5 Study: *Enhanced microbial diversity in the saliva microbiome induced by short-term probiotic intake revealed by 16S rRNA sequencing on the IonTorrent PGM platform*

The pool was made of 14 samples and some of them with probiotic administration, so a very small number of high-quality reads. The samples number 1 and 2 are of the same student that repeat the sampling twice in order to see the reproducibility of the technique. All the students had firmicutes, proteobacteria and Bacteroidetes, plus a little bit of actinobacteria.

You cannot compare alpha-diversity of different student at different sequencing depth, but at the same. When you do PCR, multiplexing and you put multiple samples on the same sequencing run the sequencing machine will not get the exact n° of reads for each sample, you have a quite high variability. Since you cannot compare alpha-diversities at multiple sequencing depths, usually you have to decide a sequencing depth cut-off at least for alpha-diversity analysis. The greater the sequencing depth, the greater the alpha-diversity.

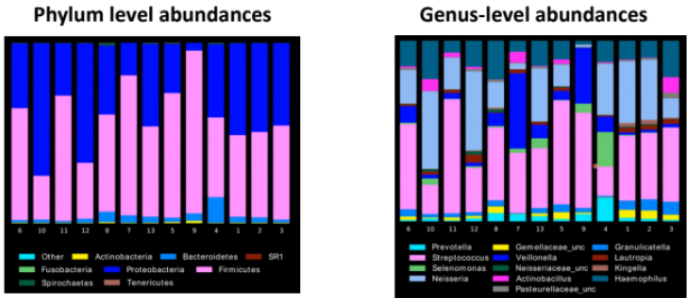


Figure 7.12

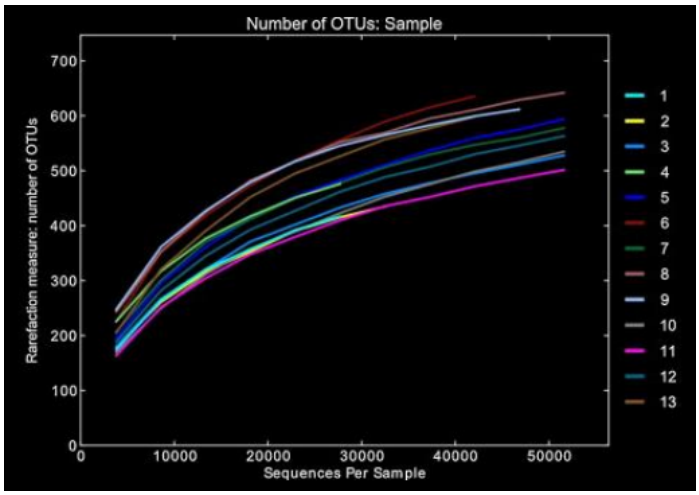


Figure 7.13: Alpha diversity and rarefaction plots. The cut-off should not be too high, otherwise some samples will not be included in the further analysis. (If they do not reach depth, like the student 4 in light green.)

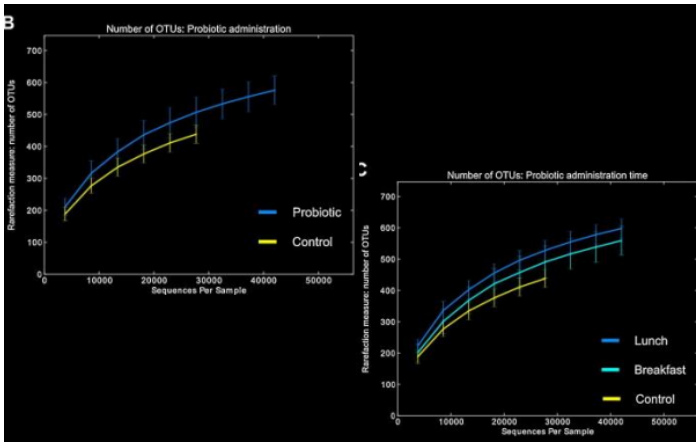


Figure 7.14: Probiotic effect on saliva microbiome? Students that took the probiotic had a more diverse microbiome, and the maximum diversity was reached by taking probiotics at lunch.

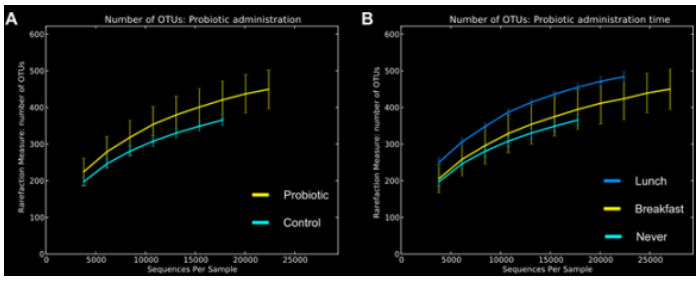


Figure 7.15: Is the effect due to the probiotic Streptococcus? The probiotic product contained Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus and *Lactobacillus paracasei*. What if we remove OTUs from these genera from the dataset?The analysis is still significant. This means that the probiotics yogurt induces a variation in the microbiome that make it more diverse.

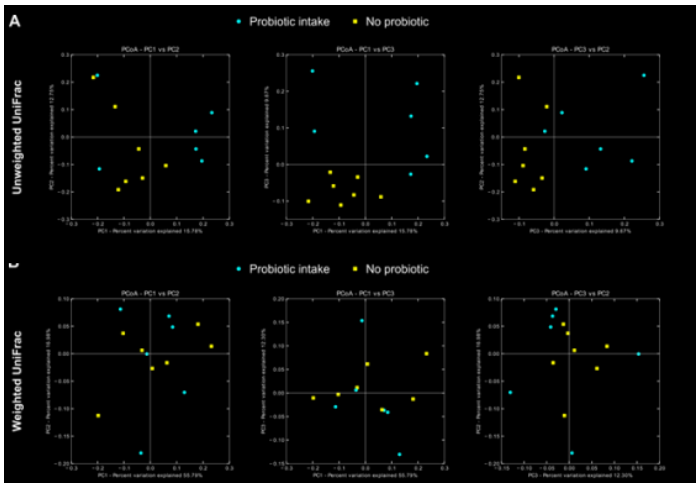


Figure 7.16: Looking at beta diversity There is no clusters.

Chapter 8

Shotgun Metagenomics

8.1 Workflow

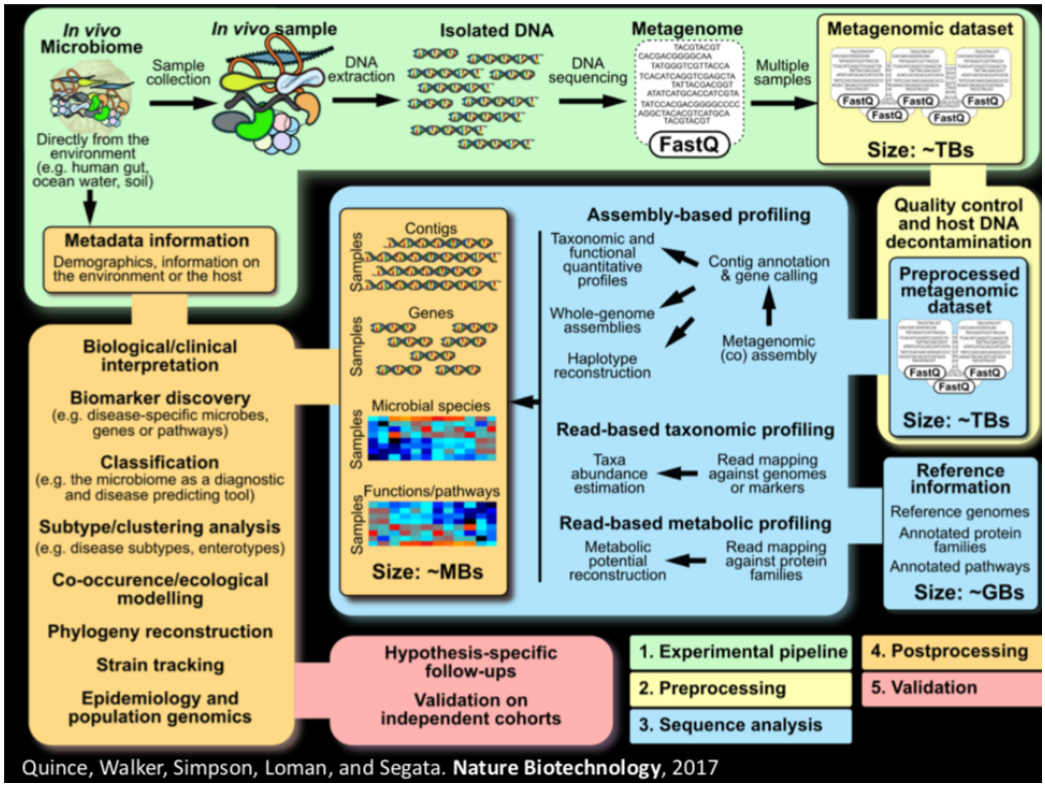


Figure 8.1: Shotgun metagenomics workflow

- **Experimental pipeline** → from sample collection to DNA sequencing.
- **Preprocessing** → decontamination + quality control

- **Mapping** or **assembling** (or both)
- **Sequence analysis** → identification of microbial species + identification of present pathways/functions
- **Post Processing** → integrate data with other information (where do the sample come from, healthy vs disease and other metadata)
- **Validation** → follow-up experiments + independent replicates

8.2 Comparison with the 16s sequencing

16S sequencing	
Pros	**Cons**
Cost-effective	Non genome-wide
Avoids non-bacterial contamination	Limited taxonomic resolution
Can catch low-abundance bacteria	Not useful for pathogens profiling
The output has reasonable size and complexity	Does not detect viruses or eukaryotes
Mature software available to perform the computations	Several biases
	Cross studies are difficult: the comparison is not possible due to biases

Shotgun sequencing	
Pros	**Cons**
Genome-wide: it is possible to retrieve information about all the genes present in the metagenome	Expensive (but costs are decreasing: right now the cost for the sequencing of one sample is 100$)
High taxonomic resolution	DNA contamination are hard to remove
Easy cross-study comparison thanks to the lack of biases	Low-abundance bacteria could be missed
All domains of life can potentially be observed in the same study	Large dataset as output, that can be difficult to process (TBs of data)

8.3 Latest technology

The cost of 100$ for the sequencing of one sample refers to a sample size of 5Gb, that should be enough for shotgun metagenomics. More sequencing depth could be needed if you want to detect microbes with very low abundances, if you want to assemble as many genomes as possible and if there is a lot of contamination.

Shotgun metagenomics is possible with **Illumina Hiseq** technology, but the latest and most used technology nowadays is **Illumina NovaSeq**.

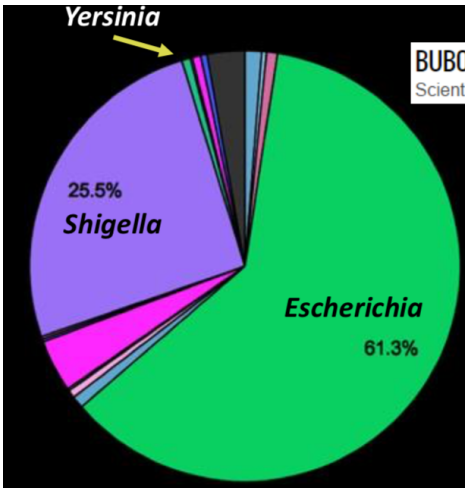


Figure 8.2

8.4 Identification of microbes from Shotgun Metagenomics data: do we really need something fancy?

N.Loman studied why a simple alignment (with BowTie because with BLAST it is not feasible) is not enough to determine which microbial species are present in our samples. He took one *E. coli* strain (K12), shredded its genome into 100 nts reads and classified these "reads" based on the best hit given by an alignment tool. Only 61% of the reads were assigned to *E. coli*. Even some Y.pestis resulted, probably because of some plasmid it has in common with *E. coli* (8.2).

With a similar approach, a study identified genes belonging to all sorts of organisms in samples taken in the New York City subway.

So yes, we need some fancy technique to solve this problem. The main **challenges** are:

- How to obtain specie-specific resolution
- Computational feasibility
- Being able to detect both bacteria and archaea. Phages are also a problem since there is little reference
- Obtain relative abundances of organisms with different genome sizes: the problem is to obtain the organism abundance and not the DNA abundance
- Consistent detection confidence for all clades
- How to handle reads as short as 50 nts
- Detect organisms without a sequenced genome or still unknown species.

8.4.1 MetaPhlAn: unique marker genes for taxonomic profiling

Solution brought to you by Segata with C. Huttenhower (reference)

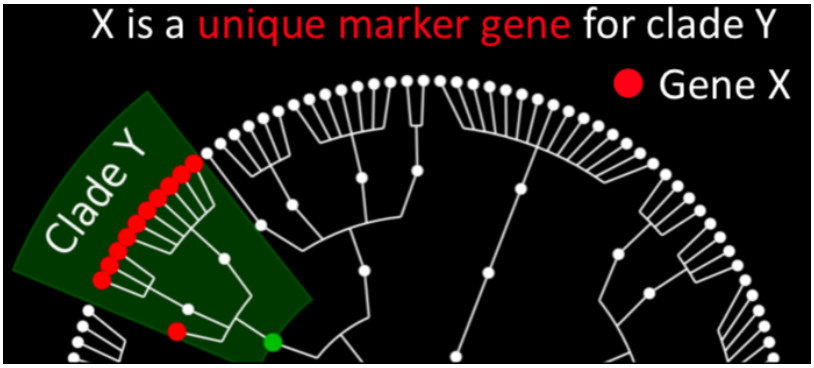


Figure 8.3

The idea is to find a marker gene that uniquely characterizes a species(8.3): it has to be present in all strains of a species and in no other species. These markers are then used to form taxonomic clades. The tool that generates the database is named **ChocoPhlAn**.

From a number of genomes, with ChocoPhlAn they created a database of marker genes of about 2 millions (400k are considered "most representative") markers: the **MetaPhlAn** database. It contains about 200 markers per species. With the reduced size of this database compared with the whole genomes, it is possible to align the reads (first with BLAST, now they do it with BowTie2) directly on the markers database.

When they first created the marker genes database, they used 2887 genomes (and half of them were drafts) that belonged to 1222 species. The number of markers per species decreases with more sequenced genomes: the core genome tends to become smaller in size and noise is eliminated as more information is available. In the current version of BioBakery (the suite containing these tools) 1 million genomes are considered, of which about 200 000 are genome isolates and 800 000 are **MAGs** (Metagenomics-Assembled Genomes → putative genomes obtained from metagenomes).

MetaPhlAn computational performance can deal with about 200 000 reads per second and can profile thousands of microbiomes in some hours.

Validation with synthetic metagenomes: 10 well-known metagenomes were created and evaluated with MetaPhlAn to check if the results correspond to the actual species of microbes used to assemble the synthetic metagenome. Errors were also added in order to simulate sequencing noise.

Validation with biological methods: comparison of the results with the ones obtained with 16S-based abundance estimation.

8.4.1.1 The problem of the unknowns

Microbial species that were never observed and do not have a marker in the database cannot be detected with this method.

The solution is to cluster together contigs obtained from the metagenome based on coverage, GC content, codon bias, and other possible features. Strict quality controls are then performed on these putative genomes: on the number of genes and the number of

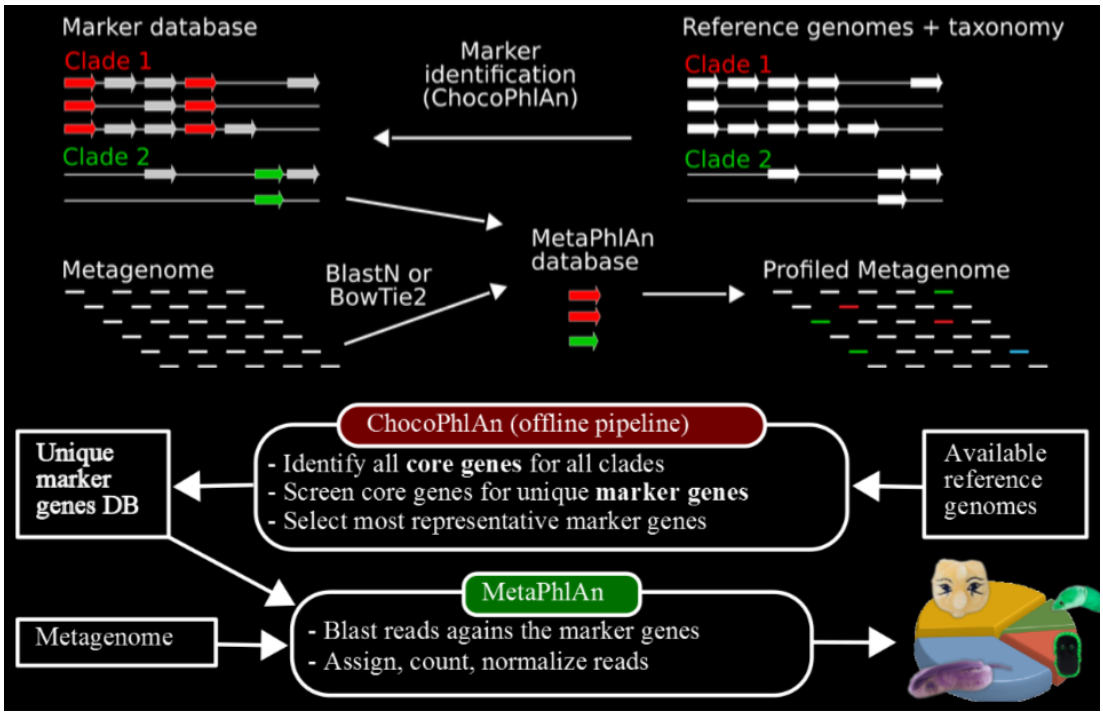


Figure 8.4: MetaPhlAn overview

known single-copy genes in order to be sure that what has been found is not a mixture of genomes. High quality putative genomes are considered MAGs and version 4 of MetaPhlAn can include them in the creation of the marker database. This way, these species can be detected in metagenomes even though they do not have a name yet.

8.4.2 Other approaches

Reference-free approaches:

- **Sequence-based clustering** of contigs to create putative genomes. The output are unlabelled bins with relative abundances. The main problem is that high coverage is needed to obtain valid results.
- **Machine learning algorithms** that exploit GC content (and possibly other features) to give as output clades with relative abundances. This method is not completely reference-free as it uses reference genomes to extract the features used for the classification. The main problem is that many species could have really similar features.

On the opposite side: **read-to-genome sequence mapping**. There is no processing of reference genome. The problems were discussed before: many reads map more than one genome but only one species is detected for that read.

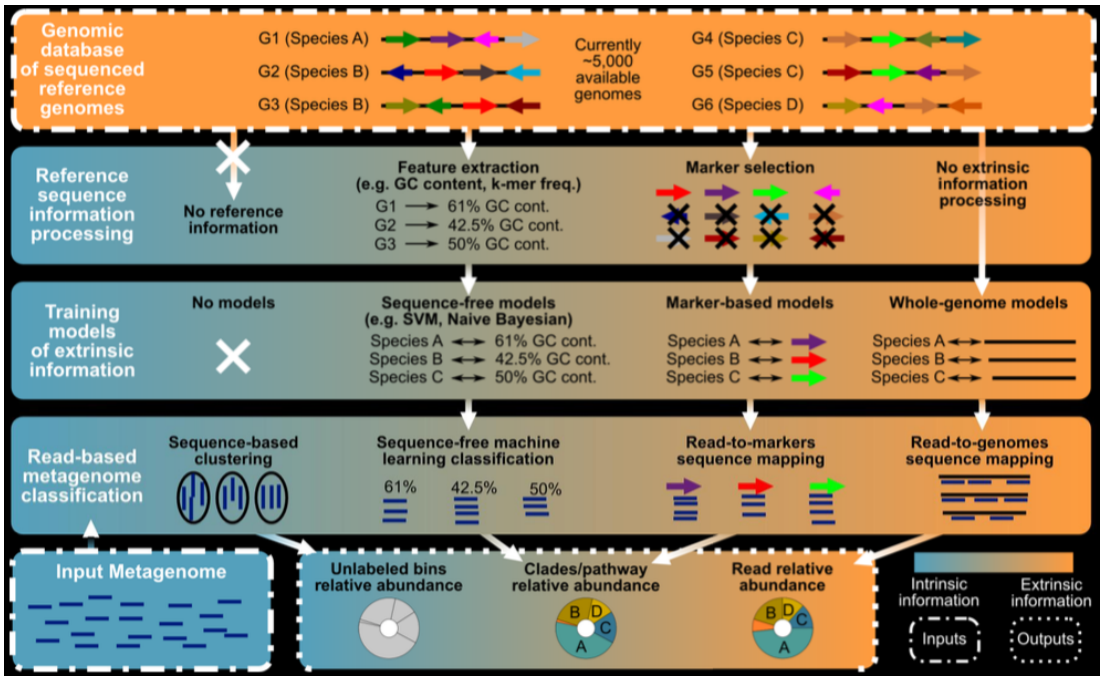


Figure 8.5: An overview of taxonomic profiling approaches

8.4.3 The curatedMetagenomicData resource

Since raw metagenomic sequencing data can be quite difficult to deal with computationally, this database stores features obtained from raw metagenomic datasets uniformly processed (MetaPhlAn or HUMAnN2) and integrated with associated metadata obtained from NCBI, papers and authors. This database is accessible and can be exploited to perform various types of analysis. (reference)

8.4.4 The link between the gut microbiome and colorectal cancer

Colibactin is a genotoxic metabolite produced by *E. coli*: it causes damages to the DNA, possibly causing cancer onset. A fraction (?) of human **colorectal cancer** (CRC) cases are caused by colibactin.

A study published by Segata's group collected stool samples from people having a colonoscopy in Milan (8.7a) and Turin (8.7b). The samples were then categorized after the diagnosis provided by the colonoscopy. The aim was the identification of **biomarkers** associated with the CRC phenotype. Different results were obtained in the two cities.

Comparing this study with similar ones performed around the world (France, China, Austria, USA, Germany and Japan) some biomarkers appeared to be reproducible (8.8a). Moreover, an accuracy around 80% was observed when a machine learning approach (random forest) was applied on all the datasets combined and then the model was applied on a brand new one(8.8b). On the other hand, when each group tried the same approach on their data separately, completely different results were found for each dataset, showing

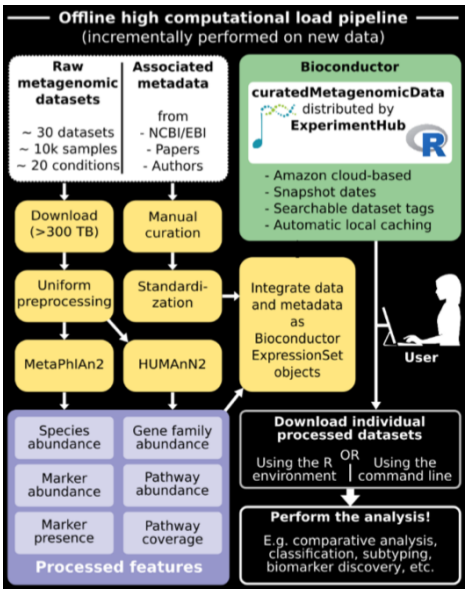
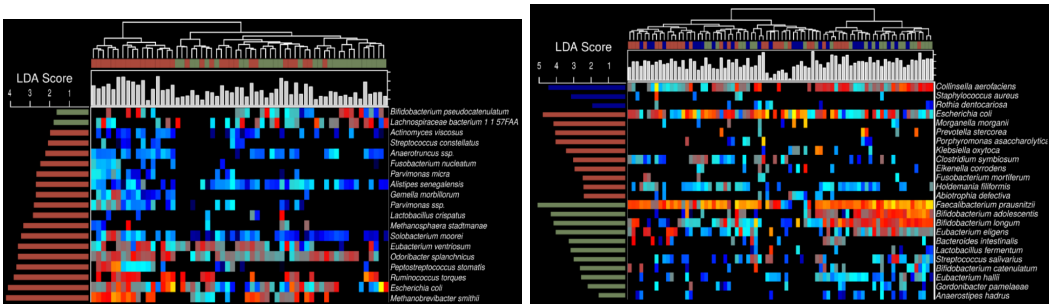


Figure 8.6: CuratedMetagenomicData pipeline



(a) Patients from Milan **(b)** Patients from Turin

Figure 8.7: Taxonomic profiling of gut microbiomes

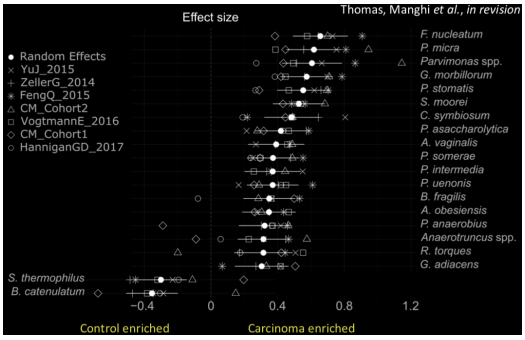
that such a technique could be valid for some cases and completely useful for others.

8.4.4.1 Hypothesis-driven analysis

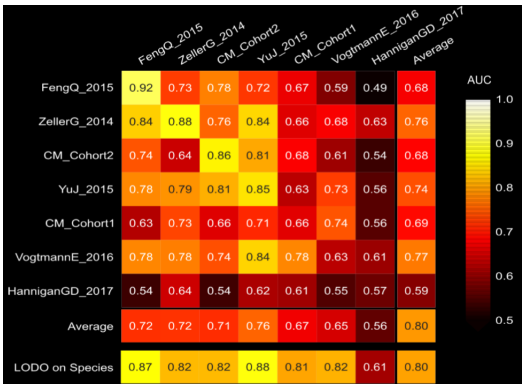
The **cutC** gene appears to be associated with the CRC phenotype (8.9). The problem is that it is present in several microbial species and it is unknown whether the function and the efficiency are maintained.

8.5 PanPhlAn: strain-level profiling

Another tool brought to you by Segata's crew (reference).



(a) *F. nucleatum* found to be enriched in datasets with different origins



(b) Random forest approach on all the datasets shows an accuracy of 0.80 (AUC of 1 = always right prediction, 0.5 = no prediction ability)

Figure 8.8

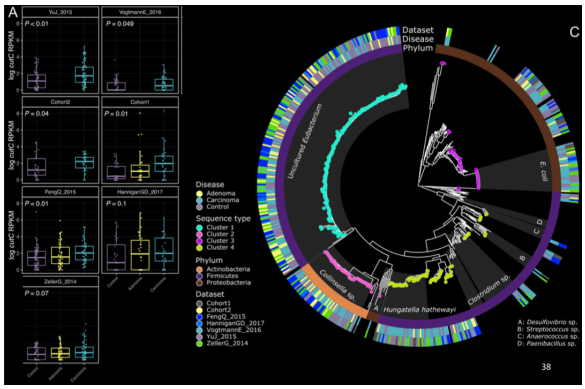


Figure 8.9

PanPhlAn is a tool for strain-level metagenomic profiling that allows to identificate gene composition of individual strains in metagenomic samples.

Challenges:

- Identify the microbial strains present in the metagenome. One could also check whether a specific strain is present but the chances of finding one specific strain in a random sample are low.
- Discover new strains and species.
- Characterize the metagenome genomically.
- Track across samples to find the same strain and eventually prove that transmission of bacterial strains occurred among them.

The *E. coli* pan genome contains about 20.000 gene-families. The goal is to find what strains are present in the metagenome and their abundances.

First all genes are grouped in **functional gene-families** (8.10). Then genes from *E. coli* found in the metagenome are mapped on *E. coli* reference genomes with BowTie2. The coverage is computed for each gene and then they are grouped into gene-families (8.11). Then gene-families are ranked based on their coverage. Multi-copy gene families have really high coverage, while the plot shows a plateau of single-copy gene-families. These families correspond to the strains present in the metagenome and their abundance can be deducted thanks to the base coverage of single-copy genes.

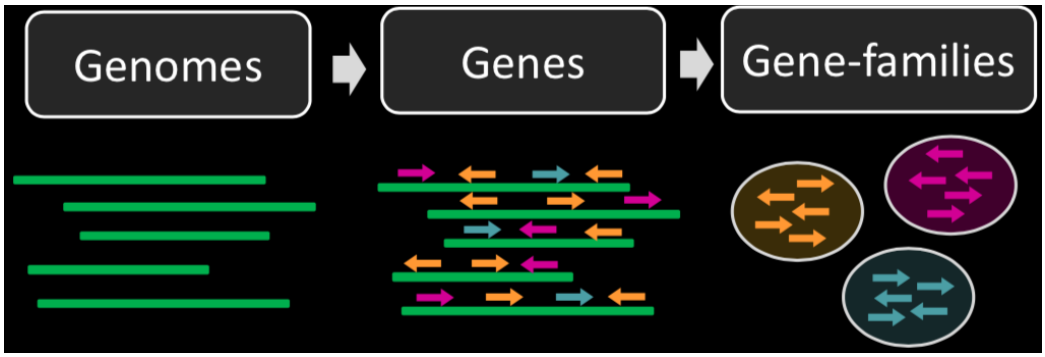


Figure 8.10: Genes are grouped in functional gene-families

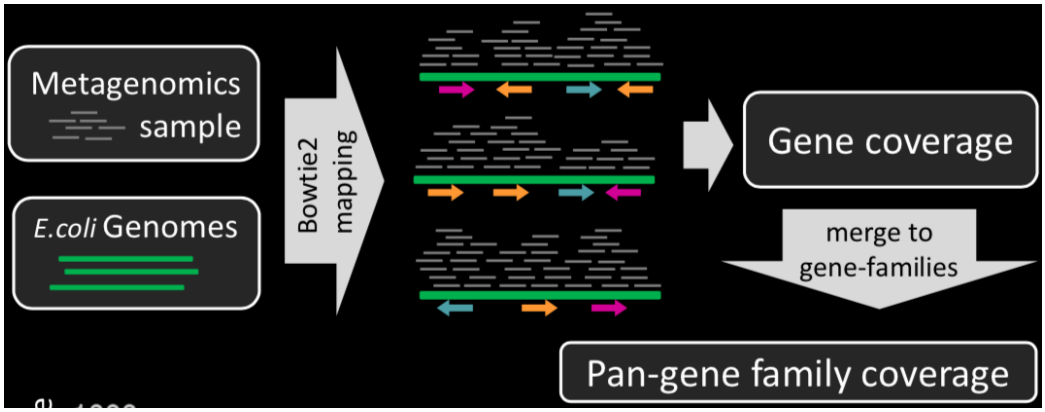


Figure 8.11: Mapping and subsequent coverage of gene-families

8.5.1 Investigating population genomics thanks to PanPhlAn

8.5.1.1 *E. coli* population genomics with PanPhlAn

Scholz, M., Ward, D., Pasolli, E. et al. Strain-level microbial epidemiology and population genomics from shotgun metagenomics. Nat Methods 13, 435–438 (2016).

Figure 8.13a shows the *E. coli* profiling of 1478 shotgun metagenomes carried out with PanPhlAn. Each column is either an *E. coli* strain obtained via shotgun metagenomics or a reference strain and the columns correspond to the gene-families that can be absent

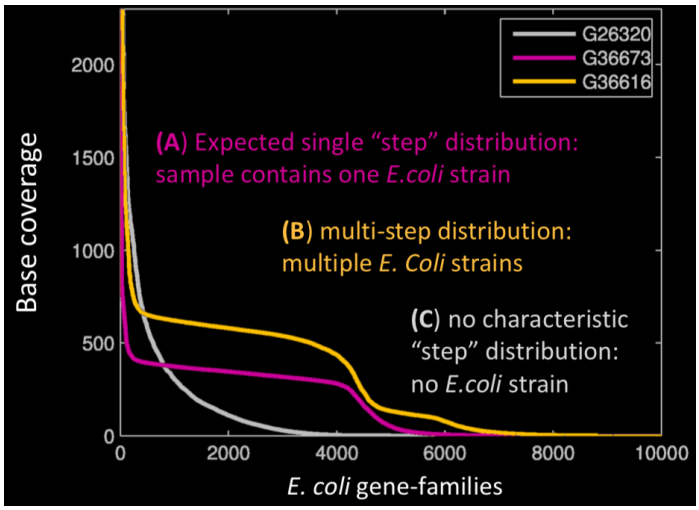
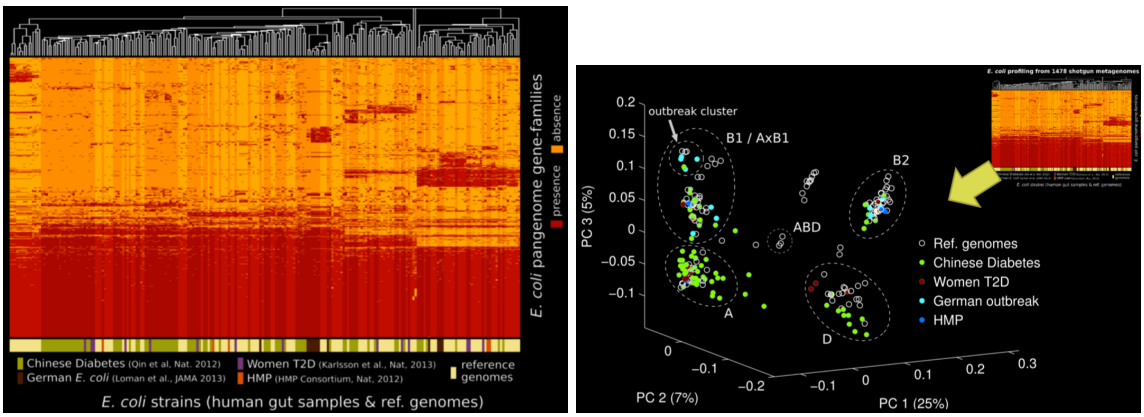


Figure 8.12: *E. coli* gene-family distribution: curve (A) shows the typical gene-families distribution: multi-copy genes with extremely high coverage, a plateau of single-copy genes and a tail of non-present gene-families. Curves like (C) should be discarded from the analysis because they indicate that no *E. coli* strain is detected in that sample.

or present in each strain. The strains are then clustered (8.13b) based on which gene-families are present in order to study the population genomics: in this case we can see that the strains isolated from the German *E. coli* outbreak cluster together, while other strains are present in several different areas of the world.



(a) **(b)**

Figure 8.13: *E.coli* population genomics with PanPhlAn

8.5.1.2 PanPhlAn on *Eubacterium rectale*

Thanks to PanPhlAn, it was possible to identify many subtypes of *E.rectale* even though only one reference genome was available at the time (8.14).

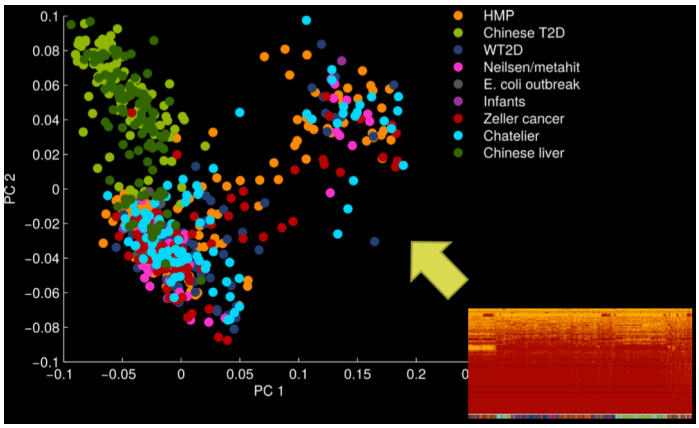


Figure 8.14: PanPhlAn on *Eubacterium rectale*

8.5.1.3 The infant gut microbiome in disease

Ward et al. Cell Reports 2016

Necrotizing Enterocolitis (NEC) is a devastating disease that affects mostly the intestines of premature infants. This study took samples from a cohort of 173 infants, 151 of them were preterm and 30 of them had NEC. They obtained 460 shotgun metagenomic samples and 284 shotgun metatranscriptomic samples, all of which were coupled with clinical data of the patients. The heatmap shows MetaPhlAn profiling of different bacterial species for all the samples: about 20% of the patients have a high *E. coli* predominance (8.15a: yellow circle). PanPhlAn was employed to investigate the *E. coli* strains found in these patients: of the 4 identified clades, only 2 are associated with NEC (8.15b).

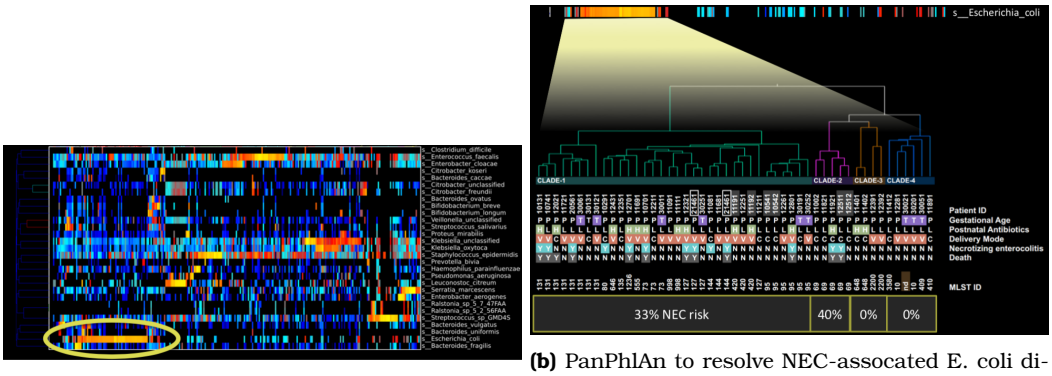
8.5.2 StrainPhlAn: a complementary approach

As usual, tool by Segata and friends(reference)

The idea is to base the classification on the **genetic variance** of core genomes: look for unique combinations of **SNPs** in genes that are always present and analyze their variance to find some SNPs with a variance different from the others that could characterize a new strain.

StrainPhlAn exploits MetaPlAn to compute species-level abundances thanks to species-specific markers and then aligns the marker genes present in the samples to find the SNPs. The SNPs are then analyzed to build a phylogenetic tree.

This approach can be applied to many different species. or *E. rectale* it seems to have a higher resolution compared to the previous approach.



(a) MetaPhlAn2 profiling of the cohort of infants

(b) PanPhlAn to resolve NEC-assocated E. coli diversity

Figure 8.15: The infant gut microbiome in disease

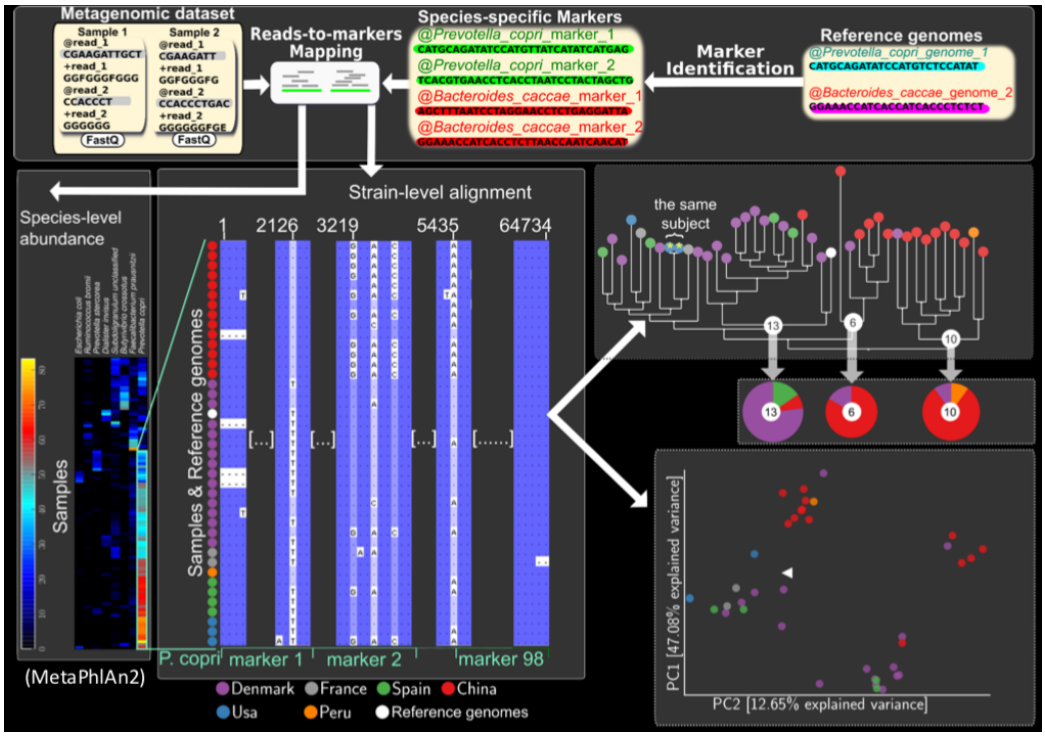


Figure 8.16: StrainPhlAn pipeline

8.5.3 StrainPhlAn applications

8.5.3.1 The stability of strains in the human gut

This study analyzes samples of the human gut microbiome obtained from different continents. The barplots (8.17) show the distances measured between results of SNPs analysis in different regions around the world. There are almost no pairs with zero or low distance

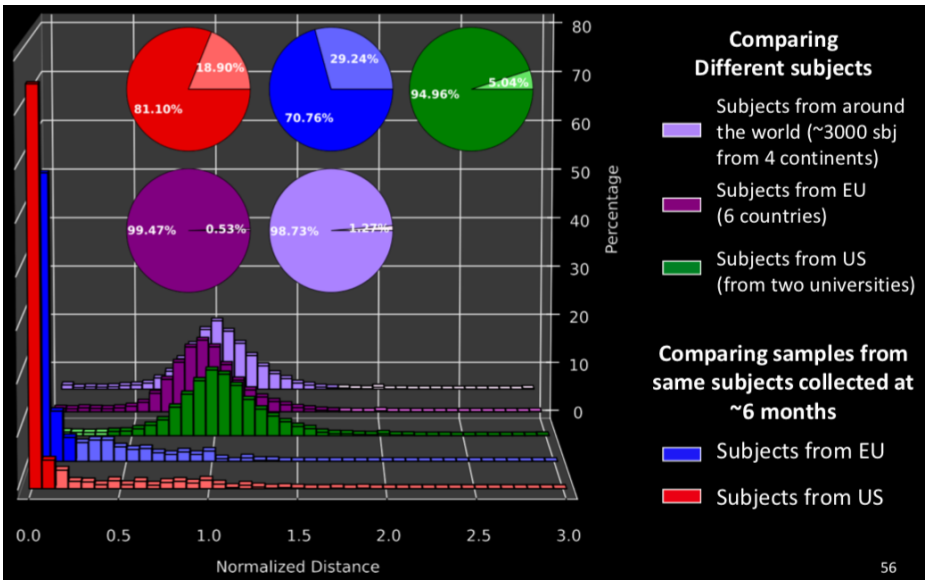


Figure 8.17: The stability of strains in the human gut

and the results do not change if only subjects from the EU or US are considered. On the other hand, the red and blue barplots (8.17) show that comparing the SNPs of samples coming from the same individual but collected 6 months apart they show great similarity. This indicates that there is some stability in the human gut microbiome: usually the strains present in one's gut microbiome tend to remain almost the same. Nevertheless, changes of diet or other habits can bring variations in their abundances.

8.5.3.2 Identification of subspecies

Some bacteria are strongly represented in the human population's microbiomes and their presence is detected in all continents. Everything changes when looking at **subspecies**: some of them are highly region-specific. Un Figure 8.18, each color indicates a different country: it is clear that when looking at subspecies of these common microbes, they are mostly associated with only one country/continent.

8.5.4 Uncharacterized species

A great amount of information about the human is still unknown:

- **Functional unknowns**: genes for which we still do not know the function because they do not match any functional database.
- **Unknown species/strains**: genes not matching any of the known reference genomes.
- **Undetected unknowns**: things we do not know and were not even observed yet.

Westernized metagenomes are more characterized than non-westernized ones:

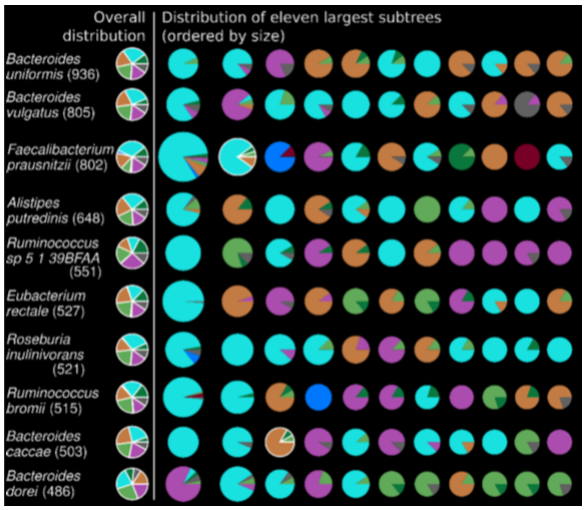


Figure 8.18: Association of sub-species structures with geography

Pasolli, Edoardo et al. "Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes from Metagenomes Spanning Age, Geography, and Lifestyle." Cell vol. 176,3 (2019

This study performed a large-scale metagenomic assembly on data from uncharacterized regions of the world. They were able to reconstruct 70.000 high quality genomes and 85.000 medium-quality ones (with 50% completeness)

Workflow (8.19):

- Assembly of the reads into contigs
- Binning of contigs into putative genomes (MAGs = Metagenome-Assembled Genomes)
- Quality control

The MAGs are then clustered (also thanks to reference genomes) in **Species-level Genome Bins** (SGBs) by measuring the distance scores between couples of putative genomes. SGBs are divided into known **kSGBs** (if they contain at least one reference genome), unknown **uSGBs** (they are not identified but can be used to detect novel marker genes) and non-human SGBs.

In this study they also characterized a new bacterium: the **Cibiobacter** (this is how they named it but sadly they had to change). Interesting fact: Madagascar-associated strains of Cibiobacter uniquely possess the trp operon for tryptophan metabolism.

8.5.5 Applications of strain-level metagenomic profiling

8.5.5.1 *E.rectale* refined population genomics

Karcher, N., Pasolli, E., Asnicar, F. et al. Analysis of 1321 Eubacterium rectale genomes

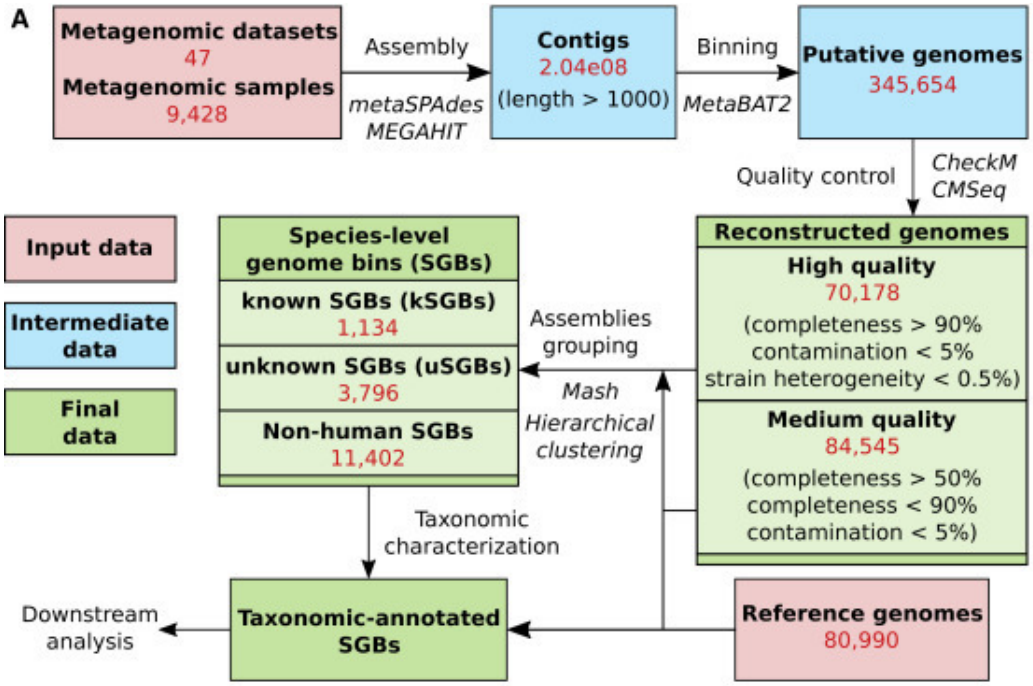


Figure 8.19: Workflow of large-scale metagenomic assembly

from metagenomes uncovers complex phylogeographic population structure and subspecies functional adaptations. Genome Biol 21, 138 (2020)

Thanks to the advancements brought by strain-level metagenomic profiling, a new subtype of *E. rectale* was discovered. Subtype 3 lacks the operon coding for motility and other genes that become useless for the bacteria if it is not motile. On the other hand, they present more copies of the **CAZy** genes: these are involved in metabolism and are required by non-motile bacteria in order to be more efficient in the exploitation of carbon sources since they cannot move to reach them.

8.5.5.2 *Prevotella copri* is strongly lifestyle-associated

Tett, Adrian et al. "The Prevotella copri Complex Comprises Four Distinct Clades Underrepresented in Westernized Populations." Cell host & microbe vol. 26,5 (2019)

P. copri is a frequent bacterium in the gut microbiome and it tends to be an on/off one: if it is present, it is often dominant. 4 *P. copri* clades with the ability of degrading complex fibers were found. They are called clades just because it is not yet confirmed that they can be considered as new strains. The interesting part is that westernized populations are associated with a lower prevalence of these clades and with a lower probability of presenting all the 4 clades together (8.21). This is probably caused by our diet: westernized populations tend to eat less complex fibers than non-westernized ones. This was confirmed by the analysis of the microbiome found in Otzi (3.300 BC) and some ancient Mexican

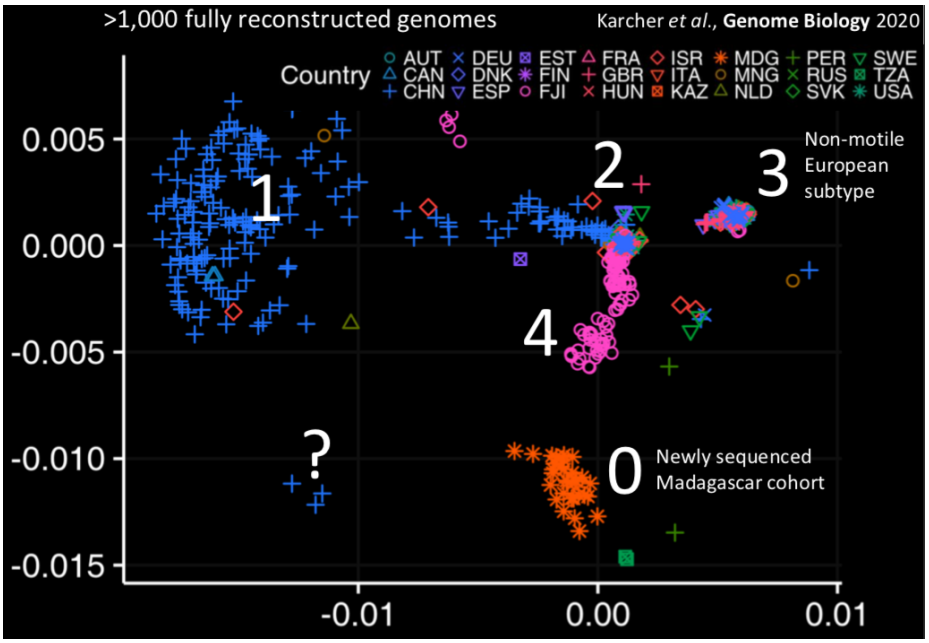


Figure 8.20: *E.rectale* refined population genomics

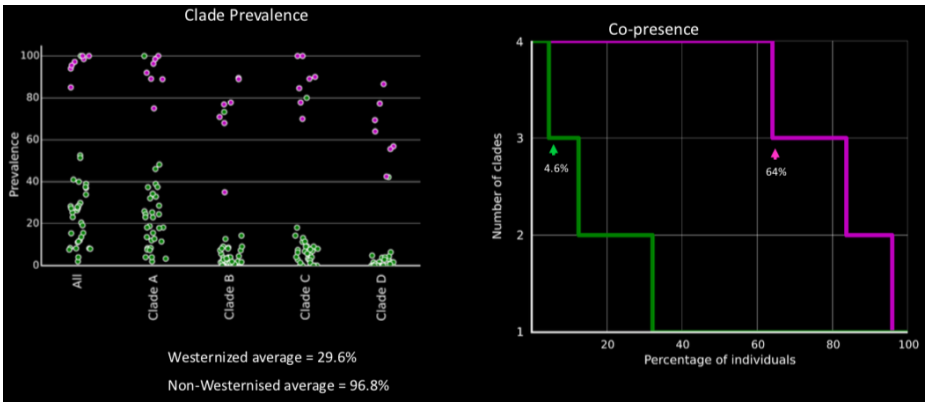


Figure 8.21: *Prevotella copri* is strongly (Western) life-style associated

coprolites (nice term to say cacca mummificata) (600-1300 AD). The *P. copri* clades were found in these samples, indicating that we are possibly losing *P. copri* in the westernized populations.

8.5.5.3 Identification of *Akkermansia* candidate subspecies

Karcher, N., Nigro, E., Punčochář, M. et al. Genomic diversity and ecology of human-associated Akkermansia *species in the gut microbiome revealed by extensive metagenomic assembly. Genome Biol 22, 209 (2021)*

Only two subspecies of *Akkermansia* have been described and characterized so far: *A. muciniphila* and *A. glycaniphila*. In this paper, they used PhyloPhlAn3 (Segata's tool for phylogenetic analysis) to identify 4 MAGs that are candidate *Akkermansia* species. Moreover, they observed that these candidate *Akkermansia* species are mutually exclusive for what concerns hosts: they were rarely found coexisting in the same sample. They also appear to have different associations in respect to *A. muciniphila*: one is associated with decreased host body mass index (BMI) but the others are not (8.22).

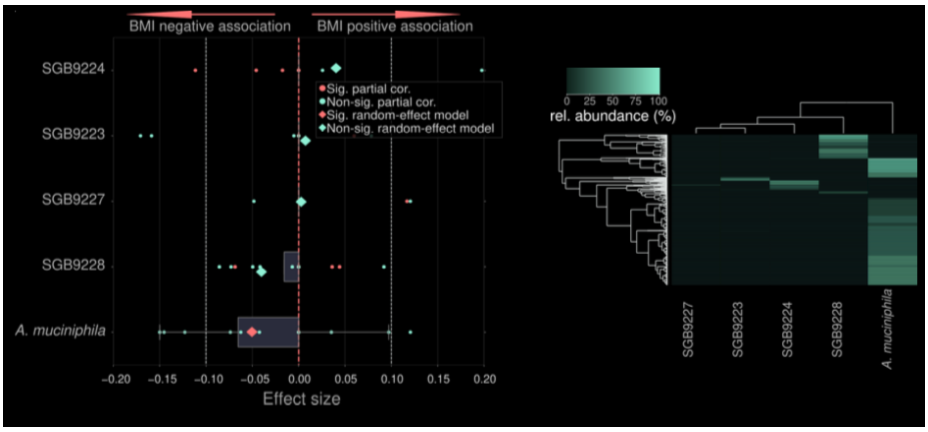


Figure 8.22: Distinct associations and co-exclusion for the *Akkermansia* (candidate) species

They also identified putative bacteriophages with spacer hits from *Akkermansia* candidate species and found that viral detectability correlates strongly with the relative abundance of the *Akkermansia* candidate species, suggesting an intimate ecological interplay.

Analyzing *A. muciniphila* subspecies, they determined that these are host-specific: some are found only in mice and some only in humans.

8.5.5.4 An example of eukaryotic microorganism: *Blastocystis*

Beghini, Francesco et al. "Large-scale comparative metagenomics of Blastocystis*, a common member of the human gut microbiome." The ISME journal vol. 11,12 (2017)*

They developed a pipeline to detect *Blastocystis* subtypes and applied it on 12 large datasets composed of 1689 subjects of different geographic origin, disease status and lifestyle.

They confirmed that *Blastocystis* is a component of the healthy gut microbiome and found a higher prevalence in non-westernized individuals. Moreover, they were able to construct and functionally profile 43 new *Blastocystis* genomes.

A strong association of specific microbial communities with *Blastocystis* was confirmed by the high predictability of the microorganism colonization based on the species-level composition of the microbiome.

8.5.5.5 Bacteriophages profiling

Just know that it is possible.

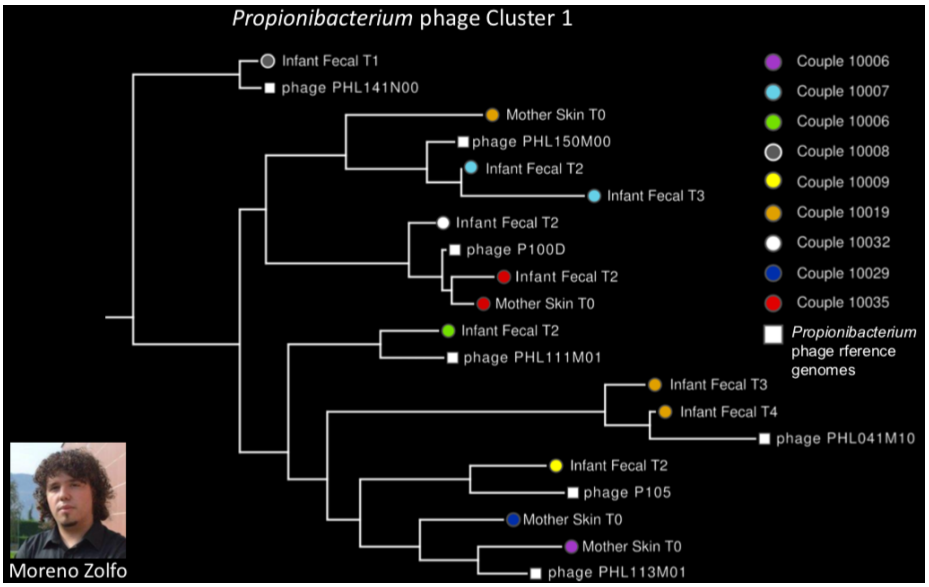


Figure 8.23: *Propionibacterium* phage Cluster 1

8.5.6 HUMAnN2: Functional profiling

The task of mapping nucleotide sequences to proteins (that can be done by blastX on a smaller scale) is important but computationally challenging. Multiple bacteria can be responsible for the same function in the microbiome. Thanks to this redundancy, functions are more conserved than bacterial prevalence: the abundance of a bacteria can vary but its function remains efficient because another microbe supplies it. For functional profiling, the idea is to reduce the reference dataset by mapping the genes only across proteins that we know are present in the bacteria found in the sample. Then the remaining unclassified reads can be mapped to a comprehensive protein database 8.24. HUMAnN2 performs species-level functional profiling of metagenomes and metatranscriptomes.

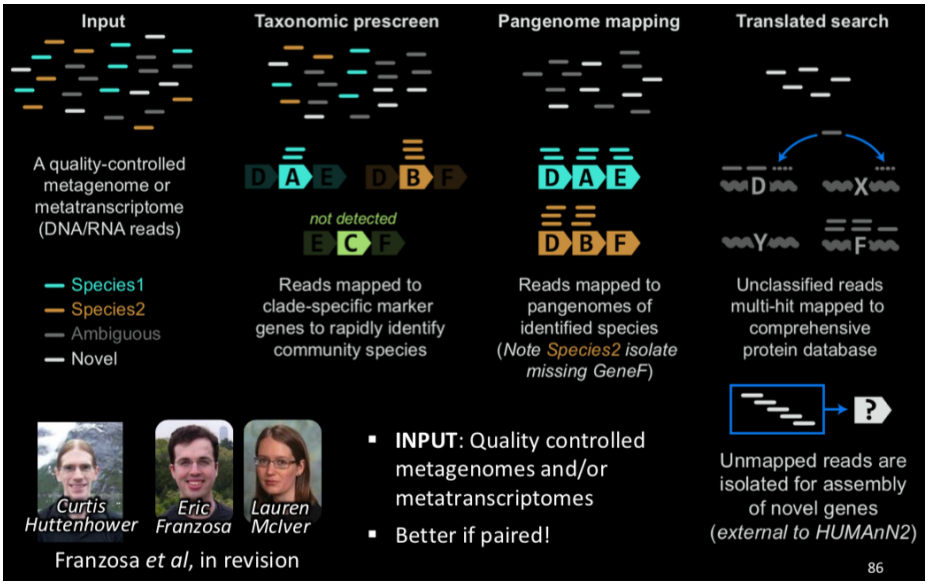


Figure 8.24: HUMAnN2 workflow

Chapter 9

Staphylococcus aureus

Staphylococcus aureus is a gram positive bacterim (it has a peptidoglican layer into the cell wall) and it is a facultative anaerobe bacterium. This is very crucial for *S. aureus* epidemiology because it is able to colonize nostrils where there is oxygen, but it is also able to colonize organs that are inside the body. It is one of the main players in common food poisoning. It is also involved in the menstrual toxic shock syndrome. It plays a key role, also, in other serious disease, like osteomyelitis which in an infection of the bones or sepsis that is a systemic infections. It is a common skin colonizer and for this reason 25% of the people probably have *S. aureus* in their body, but it is also the cause of very bad skin infection. The main reason for *S. aureus* to be tricky to treat because of is immune evasion strategies.

9.1 Immune evasion strategies

S. aureus has two different main strategies that used in order to stop the immune system of the host from getting rid of it.

1. It prevents the engagement of the host immune system, so it is not be recognized by the host immune system. That is done by different proteins that are present on the surface of *S. aureus*.

   - Adhesins bind complement factors to inhibit complement activation cascade.
   - Leukocidins are instead a number of toxins that are selectively killing the adapting immune cells, so killing those immune cells that would be able to kill *S. aureus*.
   - Immunoglobulin binding proteins bind and immobilize IgGs, so they cannot start the cascade of activation of the immune systems.
   - Proteases that cleave the immunoglobulin that are responsible for the activation.

   There are four different kind of classes of proteins that are all try to hide the *S. aureus* to the host immune system.

2. Overactiovation of the non-specific immune system. It is able to trigger a lot of inflammation to cytokine release, that is a non-specific reaction of the body, and also

to facilitate invasion of the so-called non-professional phagocytes, the neutrophiles. There is the production of autholysins, that facilitate invasion of non-professional phagocytes, and super-antigens, that activate T cells and trigger the cytokine release. This could be an advantage to *S. aureus* because when a neutrophiles phagocytes a bacterium or any pathogen can happen that the microbiome is uptaken by the neutrophile, is killed through degranulation and ROS production. The neutrophile then undergoes apoptosis and it is removed by macrophages and so we have a resolution of the infection. If *S. aureus* is present, we have the neutrophile that uptake him. However, *S. aureus* stops the apoptosis of the neutrophile and is able to divide inside it and be screened by the immune system of the host and then, with the leukocidins, it can cause some holes in the neutrophile and also the release of its content outside causing an extra inflammation.

9.2 Antibiotic resistance in *S. aureus*

In the 1940s we have the introduction of penicillin G that is quickly followed by emergence of resistance, the penicillinase. In 1959 was found the methicillin, a semisynthetic penicillinase-resistant β-lactam antibiotics, but in 1960 we have already some cases of resistance to this antibiotic from an hospital in UK (MRSA). In 1960 MRSA emerged in many countries.

9.2.1 Methicillin-resistant *S. aureus* (MRSA)

S. aureus is resistant to *beta*-lactam and is also able to acquire other resistances, event to last resource antibiotics, like vancomycin, linezolid, daptomycin.

Because *S. aureus* is a gram-positive bacterium, the peptidoglycan layer of the cell wall is extremely important for the correct assembly of the cell membrane and the β-lactam antibiotics have the ability to act as substrate analog causing an impaired transpeptidation of the peptidoglycan and the creation of a defective cell wall during cell division.

1. In absence of β-lactam antibiotics, we have the normal cell-wall biosynthesis.
2. In presence of β-lactam antibiotics, we have the binding of the antibiotic to the PBP active site and therefore the peptidoglycan cannot be transpeptidated and the peptidoglycan layer of the cell wall cannot be produced and so we have the cell death during division.
3. In presence of the β-lactam antibiotics, but with mutated PBP (PBP2a, aka MecA), β-lactam is not able to bind the modified PBP. So, the peptidoglycan can be normally transpeptidated and *S. aureus* can produce the cell wall and proliferate.

9.2.2 Methicillin resistance: where is it encoded?

The resistance to methicillin, but more in general to β-lactam, is not encoded on a plasmid. It is encoded on a mobile genetic element that is called staphylococcal chromosome cassette *mec* (SCC*mec*). SCC*mec* are mobile genetic elements that are wide spread across staphylococci genome. They commonly carries genes that might confers some increased

fitness for specific environments. This type of mobile genetic elements can be easily integrated into the genome and also can easily excises from the genome. That means that is really easy for a MSSA to integrate the mobile genetic element in case of a strong selective pressure that might be given by the presence of antibiotic. When the antibiotic is not more present, it is easy for MSSA to excise the mobile genetic element and return to the basic state of methicillin asset of the *S. aureus*. The mobile genetic element is not maintained inside the cell. It is integrated into the cell or it is excised and released outside.

9.2.3 Methicillin-resistant *S. aureus* (MRSA)

S. aureus is not well recognized for the problems that it causes. There are 80 thousands new patients per year in US that have invasive infections (not colonization), so that means that are people that are sick. The mortality rate is 20%. Hospitalize patients, immune compromise patients or patients with conditions like cystic fibrosis are very expose to *S. aureus*. This is why, in 2017, the World Health Organization insert *S. aureus* resistant to meticillin and partially resistant to vancomycin or completely as a high priority bug for the research and the development of new antibiotics. Is the fifth one in the list. An article of three years ago estimated about 5 millions deaths associated with bacterial antibiotic resistances (not *S. aureus* only) in 2019. The World Health Organization has estimated that by 2025 there will be 10 millions deaths per years because of antibiotic resistance.

9.2.3.1 *S.aureus* worldwide

There are lot of studies that focused on MRSA or *S. aureus* infections, but the problem is that there are quite biases toward specifically lineages. With the term lineages, we refer to a specific strain or a group of strains that are known to be particularly hyper-virulent or resistant or affecting a specific population (e.g. cystic fibrosis patients). So, only a part of the pool of infections that *S. aureus* can cause. There is a greater underestimation of the strains that are sensitive to methicillin for no reason because if *S. aureus* can be sensitive to methicillin, so it can be resistant to all the other antibiotics. There is a great variability in MRSA that can change epidemiology:

- 60s-70s. There were lots of hospitals associated infections, so people go to the hospital, get a surgery and get *S. aureus*. Also, nowadays *S. aureus* is a key player in post-surgery infections.
- 80s-90s. The community start talking about community-associated *S. aureus* infections and methicillin resistance of *S. aureus* because the study starting focusing on the dissemination, also in healthy people.
- 2000s. There was a great studies on livestock-associated MRSA. Zoonotic infections are pretty relevant, but treatment with antibiotics cause resistances in that community. Livestock diseases and resistances are serious consequences.

9.3 Whole genome epidemiology, characterization, and phylogenetic reconstruction of *S. aureus* strains in a pediatric hospitals

This work is a full pipeline of what you do if you want to do a survey of the general populations of *S. aureus* in a specific place.

Figure 9.1

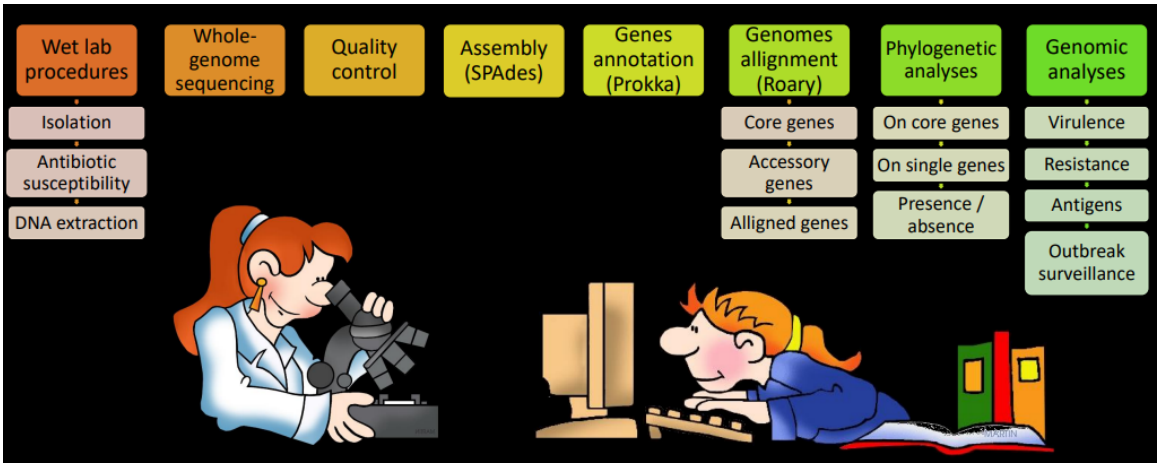


Figure 9.2: Wet and dry lab workflow

9.3.1 Methods

They had 11 operative units that were quite separated.

They started with 234 *S. aureus* isolates:

- antibiotic susceptibility test in vitro
- whole-genome sequencing

After, they selected 184 (N50>50k) so they discarded 50 of the isolates because they want high-quality genome to do a number of analysis. They did not select patients on the base of their infections or the status, because some of these people were not there because of *S. aureus*. People come from different departments such as cystic fibrosis , day hospital, first aid, immunology, infections diseases, intensive care, oncology, pediatrics, respiratory physio-pathology, surgery or week hospital. 135 single patients were selected because them wanted all single patients isolates.

- Average nr of contigs = 51 (12-138)
- Average N50 = 206k (50k-970k)

Also the materials of the samples were different. They had some bronchoaspiration material, sputum, nasal swab, pharyngeal swab, lesion swab and other materials, which included blood and similar.

9.3.2 Typing methods

Usually the typing of *S. aureus* is based on four different typing methods. All these methods have been created for wet-lab work. They are:

1. Multilocus sequence typing (MLST).
2. *S. aureus* proteins A (spa). It is one of the major determinant of virulence on *S. aureus*.
3. Staphylococcal cassette chromosome *mec* (SSC*mec*). Looked at the presence or absence.
4. Proton-Valentine Leukocydin (PVL). Looked at the presence or absence.

9.3.3 MLST

1. Characterizing isolates by sequencing fragments of house-keeping genes.
2. Each isolate is characterized by its allelic profile at the house keeping loci $\rightarrow$ sequence type (ST).
3. Based on multilocus enzyme electrophoresis (MLEE). We should do that for all the genes and because the large amount of PCRs (945) it is not really fast. The allelic profile is much faster since you need only to sequence and then compare.

MLST take fragments of house-keeping genes and look at the sequence of them to assign an allelic profile. That means that if we have 3 strains A, B and C, if we look at the first gene (abcZ) and at a some position we have G it will be allele of type 1, while if we have a C it will be of type 2. We do this kind of analysis with all the genes. Then we put the allelic profiles of all the 7 house-keeping genes that are important for *S. aureus* and we have a sequence of numbers that we can compare to a database of sequence types to know of lineage we are looking at.

9.3.4 Spa-typing

1. Single locus DNA-sequencing of the repeat region of the Staphylococcus protein gene (spa).
2. Can be used to further discriminate STs.
3. Repeats are assigned a numerical code and the spa-type is deduced from the order of repeats.

The Staphylococcus protein A typing looks at the differences in the repeat sequence that is internal in the Staphylococcus protein A gene. This gene has a part of repeats that can be in different positions. The order of the repeats is the determinant of the spa-type. They have done 135 PCRs.

9.3.5 SCC*mec*

During the sequencing of the region containing *mecA* it was find a distinct mobile genetic element named the staphylococcal chromosome cassette *mec* (SCC*mec*).

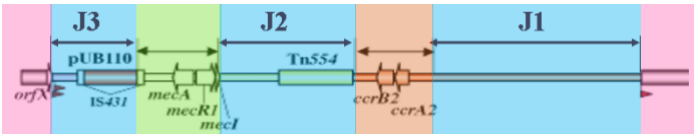


Figure 9.3

1. *mec* gene complex (*mecA* + *mecI* and *mecR*). *mecA* is responsible for the resistance, *mecI* is the inhibitor of *mecA*, *mecR* (1 or 2) is the inhibitor of the inhibitor (*mecI*). *mecA* is always present, the other two might be present or not and that will give a typing of the cassette.
2. Cassette chromosome recombinase (*ccr*) gene complex helps making the typing of the cassette.
3. 3 Joining (J) regions are responsible for other resistances. And can be also used for subtyping regions.
4. Specific inverted and directed repeats containing the insertion site recognized by the *ccr*-encoded recombinase.
5. 11 (from I to XI) types of cassette, different in size and gene content
    - SCC*mec* typing on *mec* and *ccr* gene complexes
    - Suptyping on J regions

There were made 675 PCRs.

9.3.6 PVL

PVL is a regarded as an important indicator of *S. aureus* virulence. The PVL factor is encoded in a prophage that secretes two toxins LukS-PV and LukF-PV. It is a good indicator of how invasive an infections can be.

- assemble in the membrane of host white blood cells, monocytes, and macrophages.
- form a ring with a central pore through which immune-cell contents leak
- the ring also acts as a superantigen and we have the suppression of adaptive immune response.

There were made 135 PCRs. In total were made 1890 PCRs to get the typing of the community.

9.3.7 The cohort

There are 1464 core genes that are present in at least 99% of the strains and there are some trees that are quite specific and they matched very well with the MLST typing. We have lot of information about the sample type, the operative unit, the PVL presence, the SCC*mec* type and also the presence of virulence genes. For virulence genes they made a list of all the very well known genes that are thought to be virulence genes in *S. aureus* and they just reported the presence or absence of them. In this little cohort there were:

- 28 STs (14 CCs)
- 41 *spa*-types
- 4 SCC*mec* types
- 27.4% PVL+

There are 8373 core genes in total that are present in at least 1 isolates.

9.3.8 Typing highlights common clones and newly sequenced ones

9.3.9 Co-presence of local, global, animal-associated and hypervirulent clones

Lineage	Epidemic clone	# of isolates	Notes
ST228-I	South German / Italian	16	Regional
ST22-IV	E-MRSA-15	13	
ST1-IV	USA400 / CA-MRSA-7	11 (5 var)	
ST5-IV	USA800	10	Paediatric
ST8-IV (PVL-)	USA500 / E-MRSA-2/6	4	
ST8-IV (PVL+)	USA300	2	Highly virulent
ST45-IV	Berlin / USA600	2	Highly virulent (bacteremia)
ST152-V	Balkan clone	2	Highly virulent
ST5-II	USA100 / New York / Japan	1	
ST247-I	Iberian / E-MRSA-5 clone	1	
ST5-I	E-MRSA-3	1	

Figure 9.4

1. Highly virulent STs
   - USA300 ST8-SCC*mec*IV PVL+
   - ST239 HA-MRSA → high transmissibility and quickly develops into bacteria
   - ST45-SCC*mec*IV → bacteremia, MSSA isolated from infectious diseases
   - ST121 MSSA → from lesion swabs
   - ST152-SCC*mec*V → severe infections
2. Livestock-associated MRSA (LA-MRSA), like ST398 and ST97. Increasing in non at-risk individuals

3. They found also ST395 lineage that is very peculiar and it is usually not found in humans. It was find in a child that was not at risk. It is particularly interesting because he can exchange DNA with the coagulase negative *S. aureus* (CoNS) because it has modified wall teichoic (WTA).

Livestock-associated MRSA (LA-MRSA) cause mastitis. They found also in children that were not expose.

9.3.10 Genomic signature of chronic versus acute *S. aureus* infections

Correlation of specific departments with virulent STs and PVL+:

- CF + intensive care → PVL + ST121
- first aid + infectious diseases → PVL
- infectious diseases → ST45

Sample types:

- lesion swabs → MSSA, ST121 and PVL. Here they found virulent and not resistant infections, and that make sense because it is an acute infections.
- Lung isolates (bronchoaspiration material + sputum) → ST128, PVL and MSSA.

They observed that chronic infections are usually less virulent, while normally acute infections are more virulent.

9.3.11 Variability in SCC*mec* cassettes

They took cassettes and they did genomically analysis to specifically check the genes that are present. When you focused on a specific part of the genome, you can look in depth which genes are present or absent and also the functions of each genes. They found two cassettes harboring extra genes that were resistant at antibiotics (kanamycin, bleomycin and trimethoprim).

9.3.12 Diversity of virulence factors and antigens

You can look also at specific class of genes, like immune evasion genes. Some immune invasion genes are present in almost all of the isolates. The resistant to vancomycin is never present. But is present the resistant to penicillin. There is only one sample (first aid) positive for Edin (epidermal cell differentiation inhibitor) → translocation into the bloodstream. One USA300 isolate positive for the arginine catabolic mobile element (ACME) and that increased the pathogenicty. Higher prevalence of:

- resistance genes in chronic infections
- virulence genes in acute infections

Toxins primarily responsible for *S. aureus* skin manifestations (Eta and Etb) were strongly associated with ST121 and lesions. Staphylococcal enterotoxins are present in infections department, but are not also present in CF and intensive care departments.

9.3.13 Virulence factors with available vaccines targets

There are no vaccine approved now for *S. aureus*. They took a list of genes that encode for the target of these vaccines and they checked for the prevalence in the community of isolates and also the presence of SNPs or deletions. There are different strategies for the development of vaccine:

1. highly prevalent genes, but with an high degree of variability/indels
2. most virulent or lethal infections
3. non-virulence genes, more prevalent and conserved than virulence ones

The mentioned antigens are part of the formulation of putative vaccines tested in published clinical trial, with only a few of them getting favorable results and no approved vaccine to date.

9.3.14 Phylogenetic of specific STs highlights the aggressive spread of a novel independently acquired ST1 clone

They investigated the hypothesis that some of the prevalent STs could be hospital-associated clones:

1. isolates sharing the same ST, SCC*mec*, and *spa* types, were not monophyletic subtrees when considering external genomes for the same STs. There is and independent acquisition of the clones and there is no evidence of transmission in the hospital.
2. two ST121 MSSA isolates from two patients in the same time window were found to be almost identical.
3. all but two isolates belonged to the same sub-lineage, typed as SCC*mec*IV t127 PVL-.

It was used a Bayesian phylogenetic modelling approach integrating in the analysis all the ST1 reference genomes publicly available and the two ST1 SCC*mec*V:

1. Meyer's clone emerged 6 to 28 years ago as a specific branch of the ST1 tree (26-160 years old)
2. An isolate obtained in a recent study investigating the spread of a ST1 SCC*mec*IV t127 clone in Irish hospitals and carrying a virulence and resistance profile very close to the one of our cohort (differences in gene presence: 2/79 and 0/18 respectively) is phylogenetically rooted inside the Meyer's cluster

ST1 SCC*mec*IV t127 is not specific of the Meyer's hospital, but might represent a newly arising community clone that is now spreading in the nosocomial environment of different countries.

9.3.15 Conclusions

With a whole genome sequencing approach we can:

1. type and phylogenetically reconstruct a large *S. aureus* cohort
    - observe emerging / unexpected clones
    - spot potential outbreaks
2. test for antibiotic resistances and virulence factors
3. discovery of variants in genes of interest or of unknown relevant genes
4. track strain transmission among patients