UNIVERSITÀ DI TRENTO
Dipartimento di Biologia Cellulare, Computazionale e Integrata
CIBIO

Master Degree in
Quantitative and Computational Biology

# Project group
# Computational Microbes Genomics

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# Contents

# 1 Introduction

## 1.1 Metagenome Sequencing, Assembly, and Binning

Metagenome sequencing enables the construction of metagenomes-assembled genomes (MAGs). A MAG can be seen as a microbial genome obtained by a preliminary passage of genome assembly of high quality contigs. This kind of analysis enables us to identify novel species thanks to a passage of annotation and taxonomic classification [1].

A typical metagenome project involves a specific pipeline, a step of sample processing and sequencing, a step of assembly and finally a step of binning followed by genome-annotation. This whole process is than completed with a statistical analysis [2].

Metagenomics is possible thanks to the study of DNA genomes, the sequencing is possible using a variety of novel sequencing technologies and platforms like Roche 454 sequencing, Illumina sequencing, and ion torrent Personal Genome Machine (PGM) [3].

Thanks to the proccess of assembly it is possible to reconstruct genomes. This method is based on a process of aligment and merging of overlapping sequences, creating large contiguous regions (contigs) [4].

After the process of assembly is completed, contigs are grouped by their organism of origin into bins, using a process known as binning [5]. The selection of high quality bins enables the identification of MAGs, these are characterized by a high completness and low levels of contamination and are used to operate taxonomic annotation and gene prediction [6]. These can be grouped together in the same species genome bin (SGB) if they exceed a certain threshold of nucleotide identity, with a treshold of the 5% for genomic identity. It is possible to assign a taxonomic label based on the presence (or not) of characterized genomess [7]. If a genome with associated taxonomy is not availabe, we talk about known SGB (kSGB), while in the opposite case, we talk about unknown clades (uSGB) [8].

With the term pangenome, we indicate the union of the *core genome*, containing genes present in all strains, and the *dispensable genome*, also called *accessory genome*, containing genes present in two or more strains and genes unique to single strains [9].

DA SPIEGARE IL PANGENOME ANAYSIS E LE ALTRE ANALISI CHE FACCIAMO??

## 1.2 uSGB 15132

We were provided with a set of 30 high-quality prebinned metagenomes grouped in the same uSGB labelled SGB15132.

The bins have a completeness higher that 97.3, as shown in the Figure 1.1 and the maximum redundacy registered is equal to 2.25.

DA AGGIUNGERE LE INFO SULLE PROTEINE GUARDANDO I BIN? MA QUI O NELLA PARTE DI ANNOTATION??

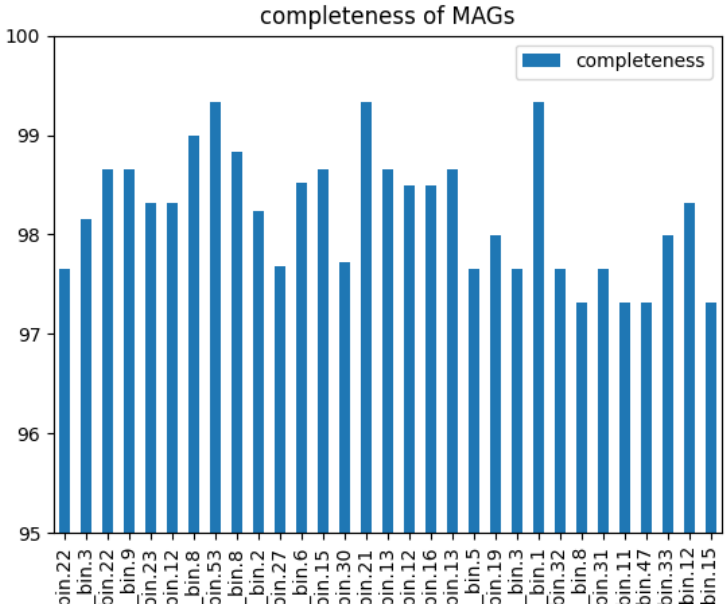


Figure 1.1: Completeness distribution of the given MAGs.

# 2 Methods

## 2.1 Softwares and parameters used

- What software you used for each purpose, what parameters

### 2.1.1 Genome annotation (Prokka)

Prokka is a fast and accurate command line software tool used to annotate prokaryoyic genomes. It produces standards-compliant output files that can be used for further analysis or viewing in genome browsers.

Prokka expect one single input file in a FASTA format, containing an assembled genome. The process of annotation is possible thanks to the comparison of the gene codes with a large database of known sequences, identifying the best match as the most significative one and therefore associating the labelling and the relevant features to the gene codes. Prokka use this method in an hieratical manner, using initially small and reliable databases moving only at the end of the process to protein family databases. Prokka produces several outputs file, listed in the Figure 2.1 [10].

We need to specify several parameters, specifically, the input files, our MAGs, the output directory `--outdir` and the parameter `--kingdom Bacteria` that is needed to specify the annotation mode, to make the prokka more fast.

```
prokka --kingdom Bacteria --outdir SGB15132_prokka_output .f*
```

| Suffix | Description of file contents |
|---|---|
| .fna | FASTA file of original input contigs (nucleotide) |
| .faa | FASTA file of translated coding genes (protein) |
| .ffn | FASTA file of all genomic features (nucleotide) |
| .fsa | Contig sequences for submission (nucleotide) |
| .tbl | Feature table for submission |
| .sqn | Sequin editable file for submission |
| .gbk | Genbank file containing sequences and annotations |
| .gff | GFF v3 file containing sequences and annotations |
| .log | Log file of Prokka processing output |
| .txt | Annotation summary statistics |

Figure 2.1: Prokka outputs files [10].

### 2.1.2 Pangenome analysis and Phylogenetic analysis (Roary, Roary + FastTree)

Roary is a tool that enables the construction of large-scale prokaryote pangenomes, identifying the core and accessory genes.

The input file to Roary is a GFF file containing sequences features.

Roary collects the coding regions from the annotated input genome. It operates a clustering process creating a network and defining a phylogenetic tree. A matrix is therefore obtained and the pangenome (core genes and accessory genes) is defined. The process of clustering is based on the minimum percentage of identity, setted to 95% by default [11].

The main output of Roary is a tree obtained using the presence and absence of the accessory genes. It is a tree used to have an initial insight of the data, grouping in a quick way the genomes based on their accessory genes [11]. It can be visualized using iTOL, an online tool for phylogenetic tree display [12].

Roary returns three graphs, the newick tree associated to the pangenome table, a pie chart of the breakdown of genes and the number of isolate they are present in, a graph with the frequency of genes versus the number of genomes. [13]

There are some main parameters that need to be specified to roary, specifically, the input `.gff` files; the output directory `-f roary_out` ; the -i parameter, specifing the percentage identity of blastp, here used at 95%, `-i 95` ; the -cd parameter, percentage of isolates a gene must be in to be considered part of the core genome, here setted at 90%, `-cd 90` .

```
roary .gff -i 95 -cd 90
```

With Roary it is also possible to perform a core gene alignment to generate a more reliable tree. A core-genome alignment is more scalable with respect to the whole-genome alignment. It is an alignment useful to identify the core genes conserved in all aligned genomes and that can be very useful to infer the phylogeny [14].

The main parameters sto be specified are the `-e` parameter,needed to perform a core gene alignment; the `-n` parameter, to use mafft as the tool for the multiple sequence alignmet, making the process faster and finally the parameter `-p` , needed to specify the number of threads, increasing therefore the speed [11].

```
roary .gff -i 95 -cd 90 -e -n -p 8 .
```

The core gene aligment can be used to construct a phylogenetic tree. This is possible using FastTree, a tool for constructing large phylogenies, estimating their reliability. FastTree exploit Neighbor-Joining and nearest neighbor interchanges to create a phylogentic tree. [15] Specifically, we used FastTreeMP, that allow the parallelization of the steps needed in computing a tree [16]. The tree is obtained using the following code,

```
FastTreeMP -gtr -nt -out core_gene.tre core_gene_alignment.aln ,
```

in which the parameter `-gtr` , the generalized time-reversible model (to be used with nucleotide alignments only), the parameter `-nt` is used to specify that the alignment is performed on nucleotides.

### 2.1.3 Taxonomic assignment (PhyloPhlAn 3.0)

PhyloPhlAn 3.0 is an accurate and rapid tool to perform microbial genome characterization and phylogenetic analysis both of newly assembled microbial genomes and metagenomes. PhyloPhlAn 3.0 can integrate public genome resources/information to the genomes in input and is also accurate at the strain and species level and allow the assign to each bin obtained via metagenomic assembly its closest species-level genome bins [17]. There are some main parameters needed to be specified, mainly, the input folder with the `-i` parameter; the output folder, with the parameter `-o` ; the `--nproc` parameter, used to specify the CPUs that can be used; the `-n` parameter that allow us to decide how many SGBs (sorted by increasing average genomic distance) will be reported for each input bin in the output file; the `--database_update` parameter to update the databases file, the `-d` parameter to specify the name of the output database adn finally, the `--verbose` parameter to print to the bash. The final command is the following:

```
phylophlan_metagenomic -i phylo -o phylo_out --nproc 4 -n 1 --database_update -d CMG2324 --verbose
```

The phylophlan_metagenomic script has three different types of outputs: (1) list of the top -n/–how_many SGBs sorted by their average Mash distance, (2) closest SGB, GGB, FGB, and reference genomes, and (3) "all vs. all" matrix of all pairwise Mash distances. Output 1 Each line reports the bin name and the list of the closest SGBs (sorted by their increasing average Mash distance) in a tab-separated fashion. The information of each SGB are separated by :. Similar to Output 1., with the difference that the information reported are for the closest SGB, then the closest GGB, followed by the closest FGB, and finally the closest reference genomes, according to their respective Mash distances. Output 3 In this case, phylophlan_metagenomic produces a square matrix of all pairwise distances of the only input bins against themselves.

### 2.1.4 Association with host data

# 3 Results and discussion

## 3.1 Genome annotation

After the gene annotation process, possible using prokka, we were able to identify that the number of the CDS (protein coding sequence) is slightly variable, spanning from a minimun value of 2651 to a max of 3935. For each MAG, more or less a half of the CDS are known proteins, while the other half is represented by RNA or hypotetical proteins.

DA FARE IL GRAFICO CON LIVELLO CDS, LIVELLO UNKNOWN PROTEINS E ??? COME HA FATTO VITTORIA

## 3.2 Pangenome analysis

Each SGB strain was found to contain an average of 1317 genes that are present in every strain (core genome), plus 8407 genes that are absent in more than one strains (strain $< 30$) (accessory genome). This can be seen in the Figure 3.1, showing the frequency plot of the genes per genome, this plot gives a general overview of the frequency of genes within a whole genome set, typically these plots have an shape and most genes can be detected in a single genome or in all genomes [18]. Here it is shown that the number of genes present in all the genomes correspond to the number of core genes. The accessory genes are also divided into genes present in only one strain (cloud genome) or genes present in two or more strains but not all strains (shell genome) [9]. The figure 3.2 show the subparts constituting the pangenome, associated with the total number of genes.

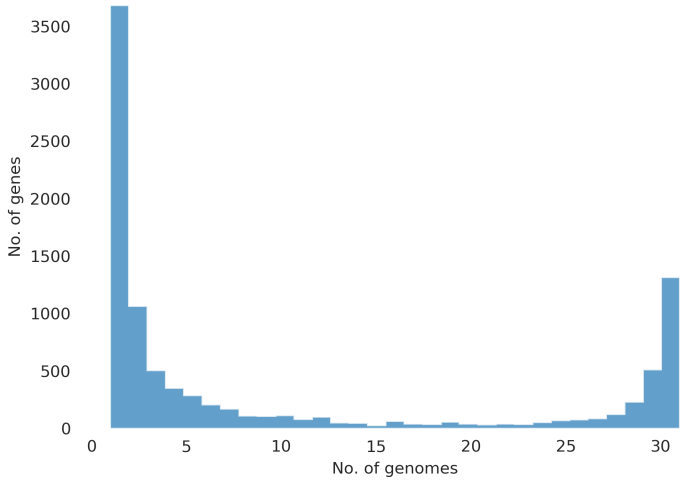


Figure 3.1: Pangenome Frequency

Looking at the Figures 3.3 and 3.4 we can obtain/derive an idea on the pangenome, specifically if it is open or closed. It is important to remember that these results were obtained using only 30 metagenomes, making the process of discussion more complex. Even though, looking at the Conserved vs Total genes plot in Figure 3.3, we can argue that the total genes initial slope is very high but at the end, when almost all the genomes were added, the slope start to decrease. The conserved gene line is almost stationary, with litte changes when the genomes are all added. Only looking at this graph it is difficult to define if the pangenome is open or closed, therefore, the observation of the Unique vs New genes plot can be useful. Looking at the Figure 3.4, we can see that, adding new genomes doesn't give new information, the number of new genes is almost at zero when we add the last genome. This help us to state with a good security that the pangenome is closed.

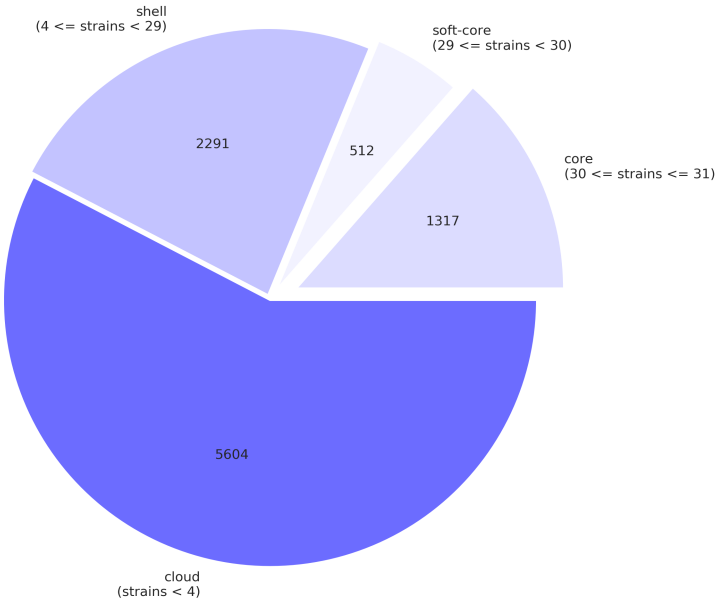


Figure 3.2: Core and accessory genome

## 3.3 Phylogenetic analysis and association with host data

comparison of phylogenetic trees based on accessory gene presence/absence or on core gene alignment. Do you detect clusters of strains? How do they associate with the metadata?

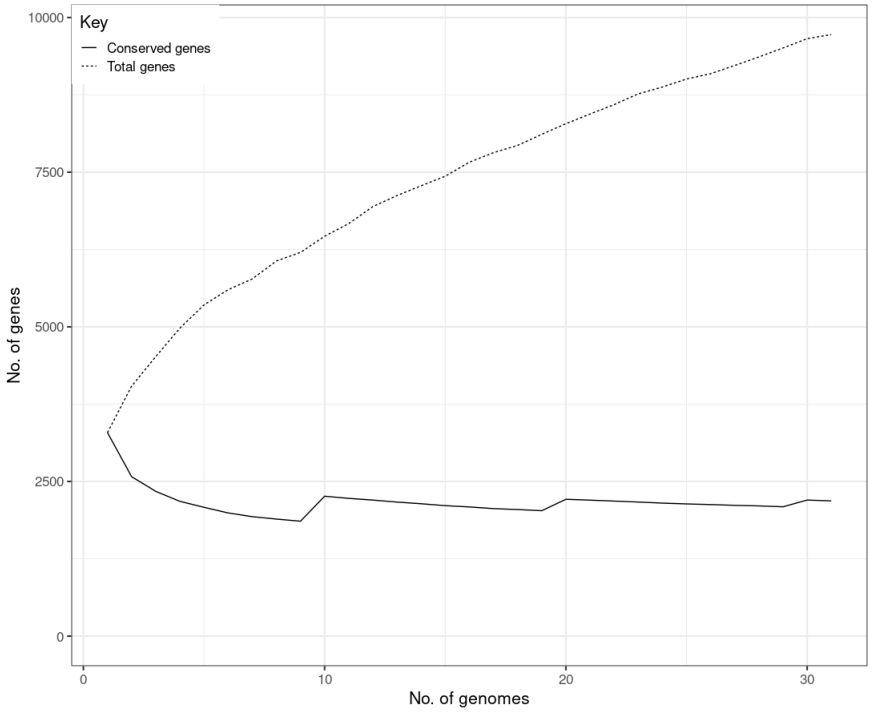


Figure 3.3: Conserved vs Total genes

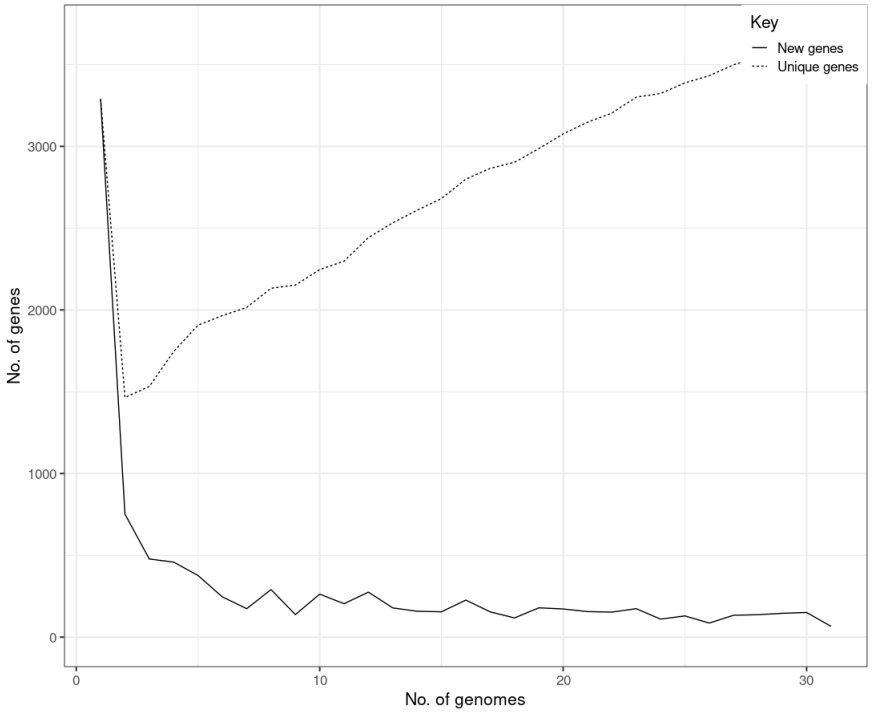


Figure 3.4: Unique vs New genes

# 4 Conclusion

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