Canadian Bioinformatics Workshops

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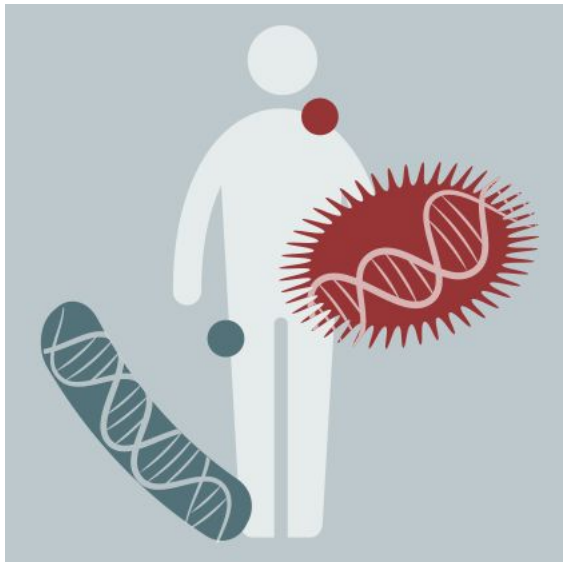
Module 4:Metagenomic Sequencing

Morgan Langille

CBW-IMPACTT Microbiome Analysis

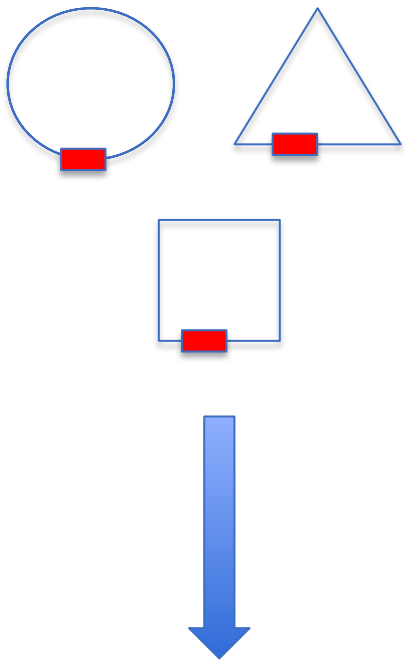
July 5-7, 2023



Learning Objectives

- Contrast metagenomic from amplicon sequencing
- Describe general approaches for determining taxonomic composition from metagenomic data
- Understand microbial functional annotation and tools for annotating metagenomic data with functional labels

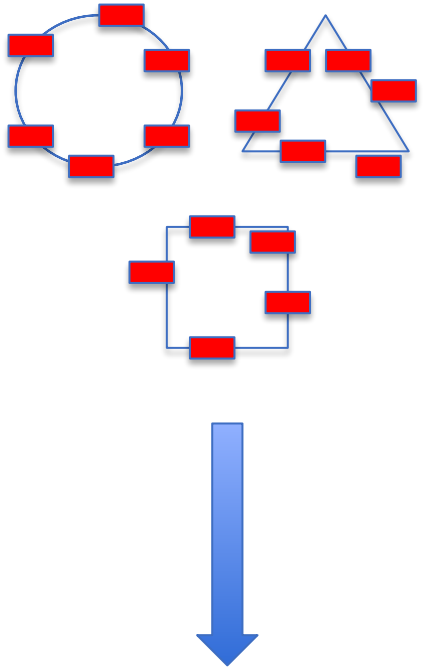
16S rRNA gene sequencing



Who is there?

- 16S: targeted sequencing of the 16S rRNA gene which acts as a marker for identification
  - Well established
  - Relatively inexpensive (~50,000 reads/sample)
  - Only amplifies what you want (no host contamination)

Metagenomics

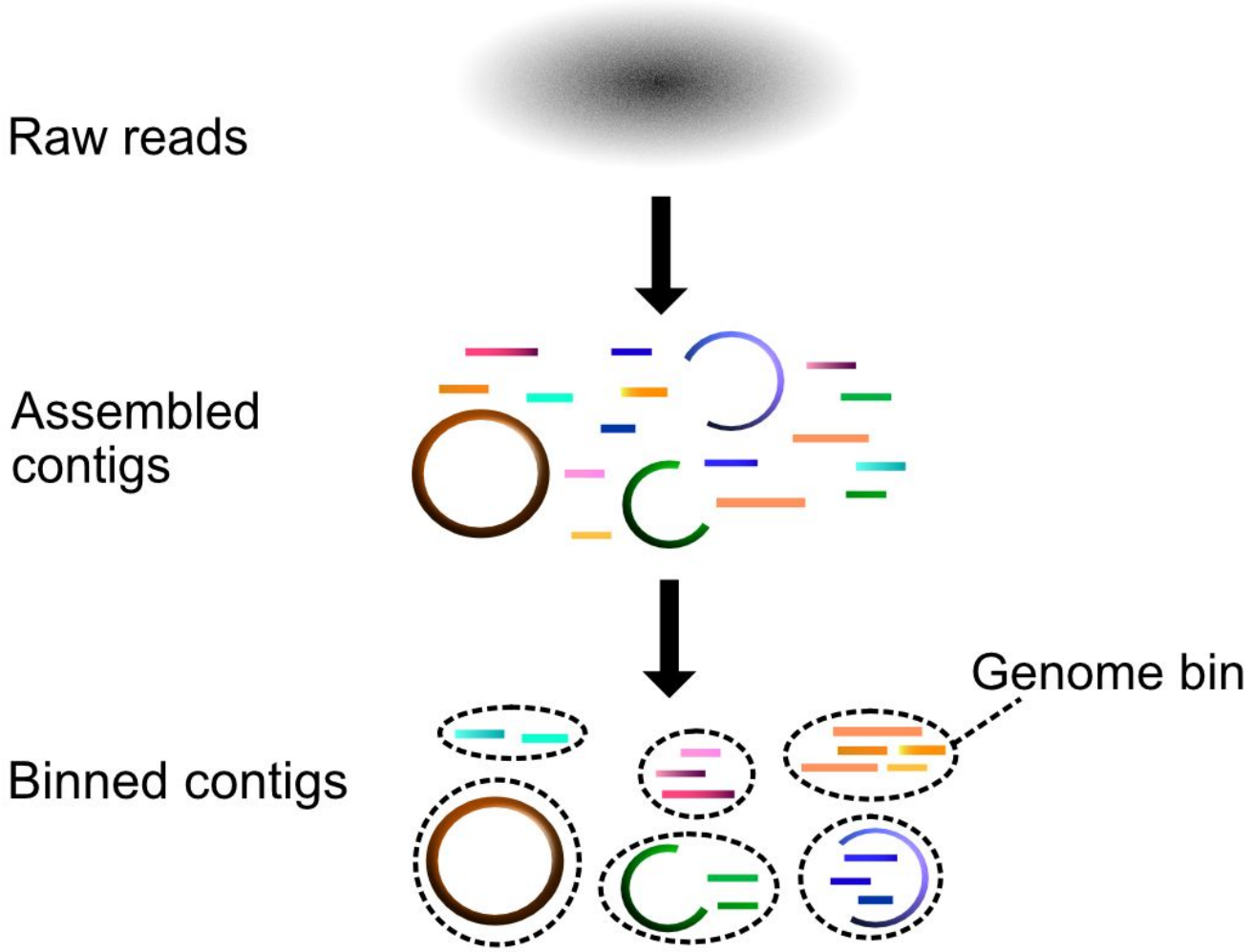


Who is there?
&
What are they doing?

- Metagenomics: sequencing all the DNA in a sample
  - No primer bias
  - Can identify all microbes (bacteria, eukaryotes, viruses)
  - Better taxonomic resolution
  - More expensive (>5-10 million reads/sample)
  - Provides functional information
  - Possibly reconstruct genomes

c

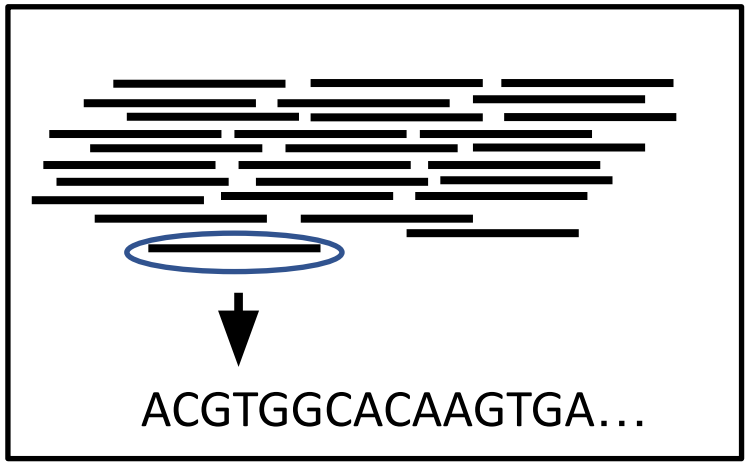
Metagenome-based genome assembly



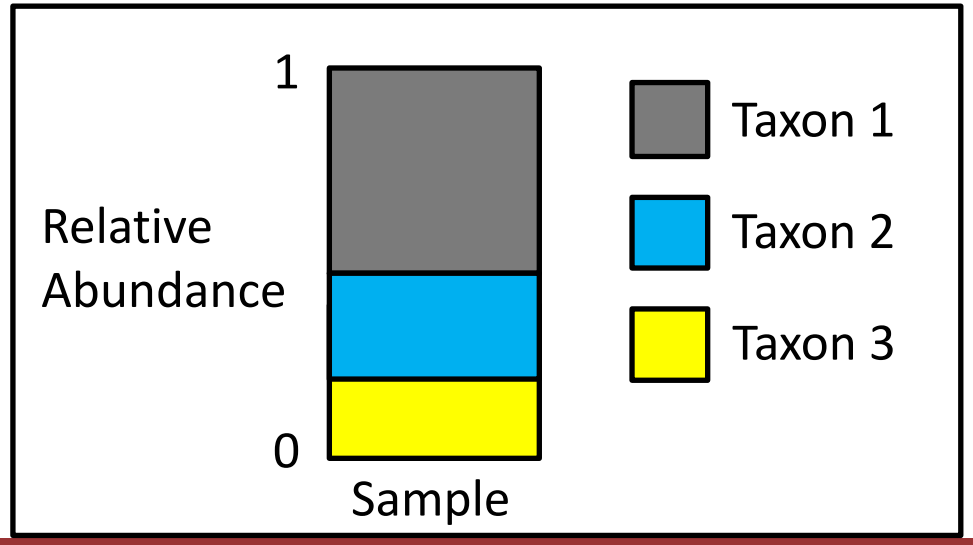
Douglas & Langille 2021

Taxonomic Profiling

With this raw data:



How do we get this output?



Challenges identifying taxa from metagenomics data

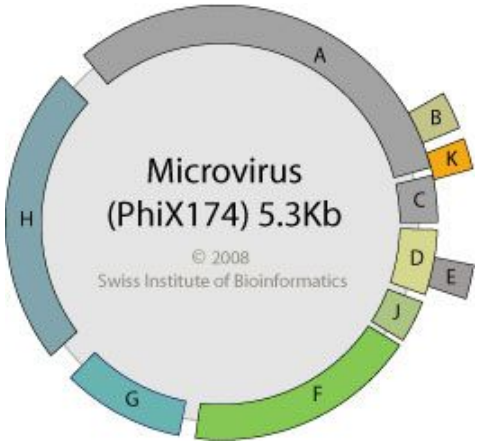
- Reads are randomly assorted
- Reads are *usually* short (~100-150bp)
- Spotty genome coverage due to sequencing depth
- Lateral gene transfer
- Computational time (Large # reads vs huge databases)

Initial bioinformatic processing steps

- Many initial steps are similar to 16S studies
- De-multiplexing and lane merging
- Quality filtering
- Stitching paired end reads --> not usually
- **Removal of unwanted host-associated reads**

Identifying “contaminant” reads

- Contaminant reads are usually associated with the sampled host (e.g. human, mouse, plant, etc.)
- Typically removed by mapping reads to host reference genome (e.g. bwa, Bowtie2)
- Should filter for Phi X which is used as a sequencing control and is not always removed



Reference Based Approaches

- "All reads" approach
  - Attempts to assign taxonomic classification to as many reads as possible
  - Similarity search is computationally demanding
  - May be hard to assign accurate taxonomy to a short read (e.g., repetitive sequence, LGT, no homologs, etc.)

- Marker approaches
  - Uses one or more genome markers to determine the taxonomic composition
  - Only uses a minor subset of the data and thus hard to link to functions downstream
  - Very dependent on choice of markers

Marker Based

- Single Gene
  - Identify and extract reads hitting a single marker gene (e.g. 16S, cpn60, or other “universal” genes)
  - Use existing bioinformatics pipeline (e.g. QIIME, etc.)
- Multiple Gene
  - Several universal genes
    - mOTUs2 (Milanese et al, 2019)
      - Uses 10 universal single copy genes
  - Clade specific markers
    - MetaPhlAn4 (Blanco-Míguez et al., 2023)

MetaPhlAn4

- Combines ~1 million bacterial and archaeal genomes
  - ~25% from isolate or single cell sequencing
  - ~75% from metagenomically assembled genomes (MAGs)

- Groups genomes into species level genome bins (SGBs, at 5% genomic identity)
  - ~22,000 are known SGBs. ~5,000 unknown SGBs (>5 MAGs)

- 5.1 million unique and core marker genes
  - 10 to 200 markers per genome

- MGS reads are profiled against all markers using Bowtie, filtered, and normalized to produce taxonomic profiles.

- Limited to identifying bacterial and archaea previously identified

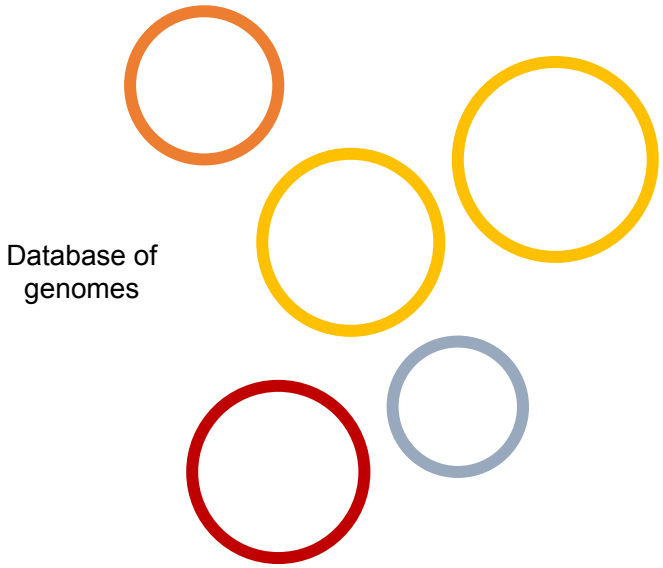
All Reads Approaches

- Kraken/Bracken
- Centrifuge
- Kaiju
- And others!

- Most of these methods use a k-mer based searching solution along with other heuristics to speed up large similarity searches

- Many use a lowest common ancestor approach for taxon classification after similarity search

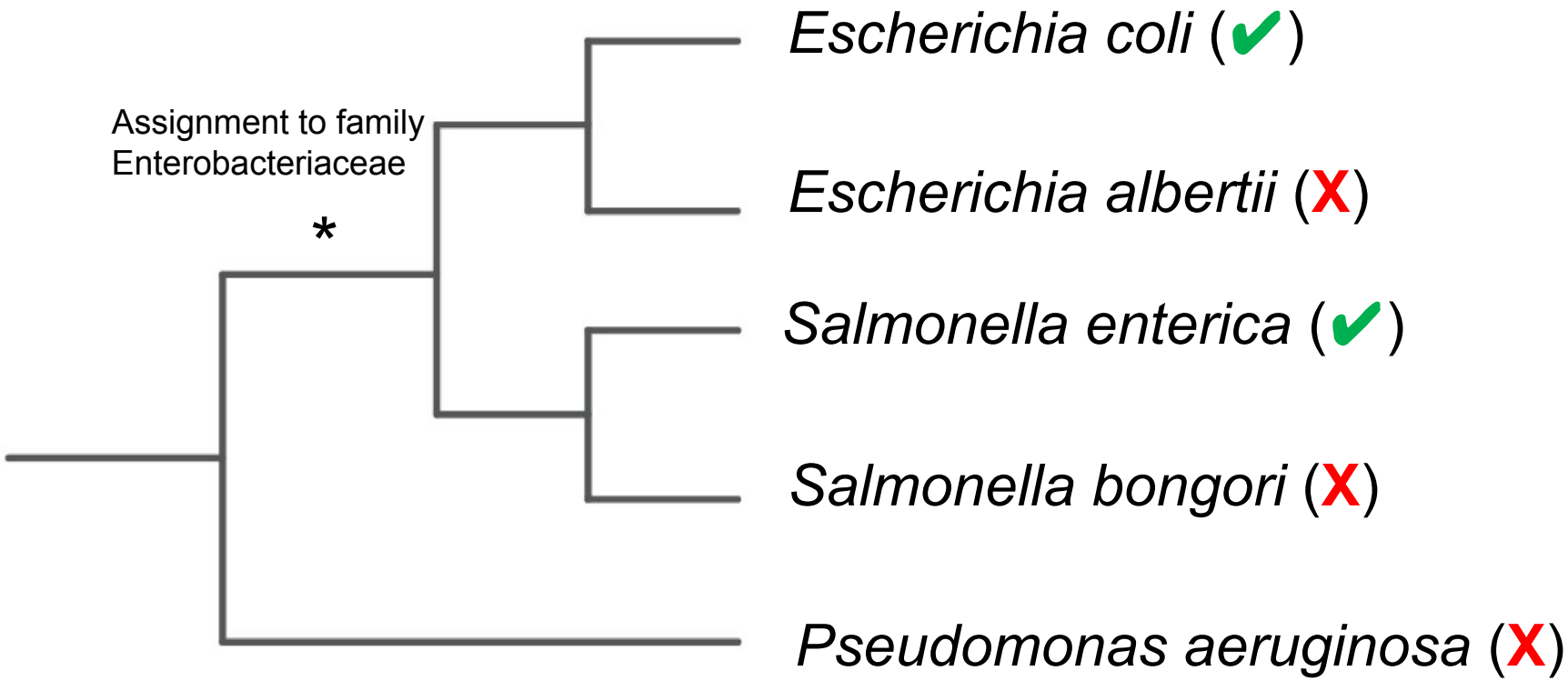
k-mer-based approaches



Sub-sequences of length *k* (*k*-mers)

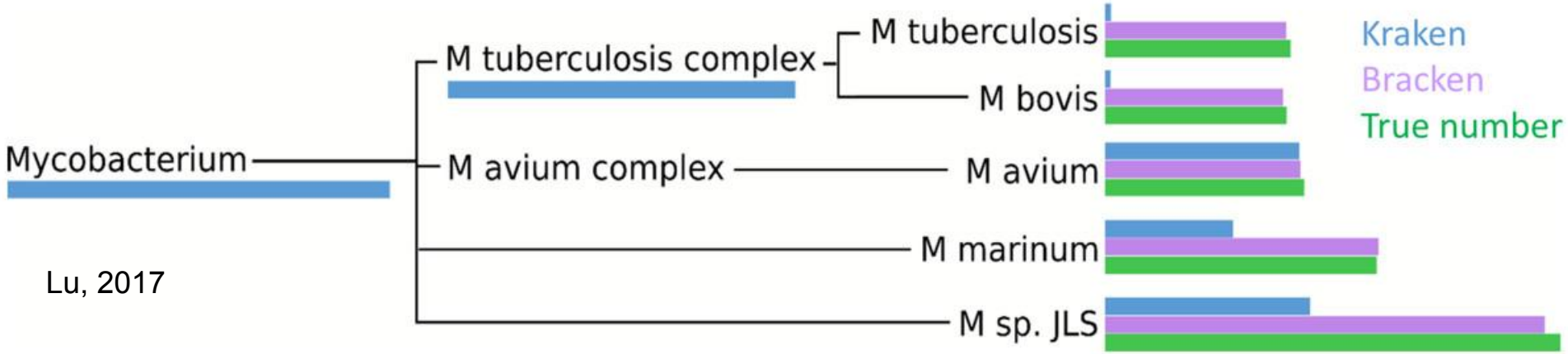
```
ATCGATCGATCGATCGATCGATCGATCGATC
ATCGA
 TCGAT
  CGATC
   GATCG
    ATCGA
     TCGAT
      CGATC
       GATCG
        ATCGA
         TCGAT
          CGATC
           GATCG
            ATCGA
             TCGAT
```

Lowest Common Ancestor (LCA) Approach



Kraken & Bracken

- Kraken does the (fast) searching and assigning taxonomy to reads
- However, many reads may be placed at a high taxonomic level (e.g. phylum or family) because they are conserved across genomes
- Increasing genomes results in more reads being pushed to higher levels
- Bracken is run after Kraken to improve estimates of species abundance in a sample



Lu, 2017

Big question: Which is best?

- Difficult to assess comparisons between tools
  - Often different (and often changing) databases
  - Choice of testing dataset (often mock/simulated communities)
  - Choice of tool options/cutoffs
  - Depends who you ask ☺️
  - Underlying differences in approaches

MICROBIAL GENOMICS
Volume 9, Issue 3

Research Article | Open Access

From defaults to databases: parameter and database choice dramatically impact the performance of metagenomic taxonomic classification tools

Robyn J. Wright[1], Andrè M. Comeau[2], Morgan G. I. Langille[1,2]
View Affiliations

Published: 03 March 2023 | https://doi.org/10.1099/mgen.0.000949

Comparison Summary

- Metaphlan3
  - Fast & low computational requirements,
  - Simple bioinformatic setup (default db and parameters are good)
  - Good for human microbiome studies
  - Good precision (at the cost of some recall)

- Kraken2
  - Good for human AND environmental microbiome studies
  - Confidence cutoff should be changed from default (~0.5)
  - Use as big a database as your computational resources allow (database size equates to amount of memory required)

Functional Composition

- Taxonomic composition answers “Who is there?”
- Functional composition answers “What are they doing?”
- Metagenomics provides the opportunity to catalog the set of genes from an entire community and compare those differences across samples

What do we mean by function?

- General categories
  - Photosynthesis
  - Nitrogen metabolism
  - Glycolysis

- Specific groups of orthologs
  - Nifh
  - EC: 1.1.1.1 (alchohol dehydrogenase)
  - K00929 (butyrate kinase)

Various Functional Databases

- COG
  - Well known but original classification (not updated since 2003)
- SEED
  - Used by the RAST and MG-RAST systems
- PFAM
  - Focused more on protein domains
- UniRef
  - Has clustering at different levels (e.g. UniRef100, UniRef90, UniRef50)
  - Most comprehensive and is constantly updated
- KEGG
  - Very popular, each entry is well annotated, and often linked into “Modules” or “Pathways”
  - Full access requires a license fee
- MetaCyc
  - Very popular and the primary alternative/replacement for KEGG

Metagenomics Annotation Systems

- Web-based (These all provide functional and taxonomic analysis, plus hosts your data.)
  - MGnify (i.e. EBI Metagenomics Server)
  - MG-RAST
  - IMG/M

- Graphical user interface:
  - MEGAN
    - Provides several visualizations

- Local-based (many more not listed here)
  - Carnelian
    - K-mer based approach, calls genes first with fragGeneScan
  - HUMAnN3
    - Popular and fast
  - Microbiome Helper
    - Custom pipeline (used in lab)

Challenges in Functional Annotation

- Similar challenges to microbial genome annotation
- Partial gene fragments
- Mixed communities
  - Including microbial euks and viruses!
- Large amounts of data! Speed and scalability are very important!
- **Bias from gene lengths**
- **Inferring modules pathways with multiple organisms**

Keys steps of a functional annotation pipeline

- Similarity search approach
  - Mappers like BWA and Bowtie are very fast but limited to DNA space and very similar sequences
  - Protein alignments like DIAMOND and mmSeqs2 are slower but more sensitive in protein space
    - Note that tools like BLAST are usually too slow for any metagenomics annotation
- Database
  - Larger databases are more comprehensive and will annotate larger portions of your samples
  - Smaller databases can be used if focused on well-annotated functions

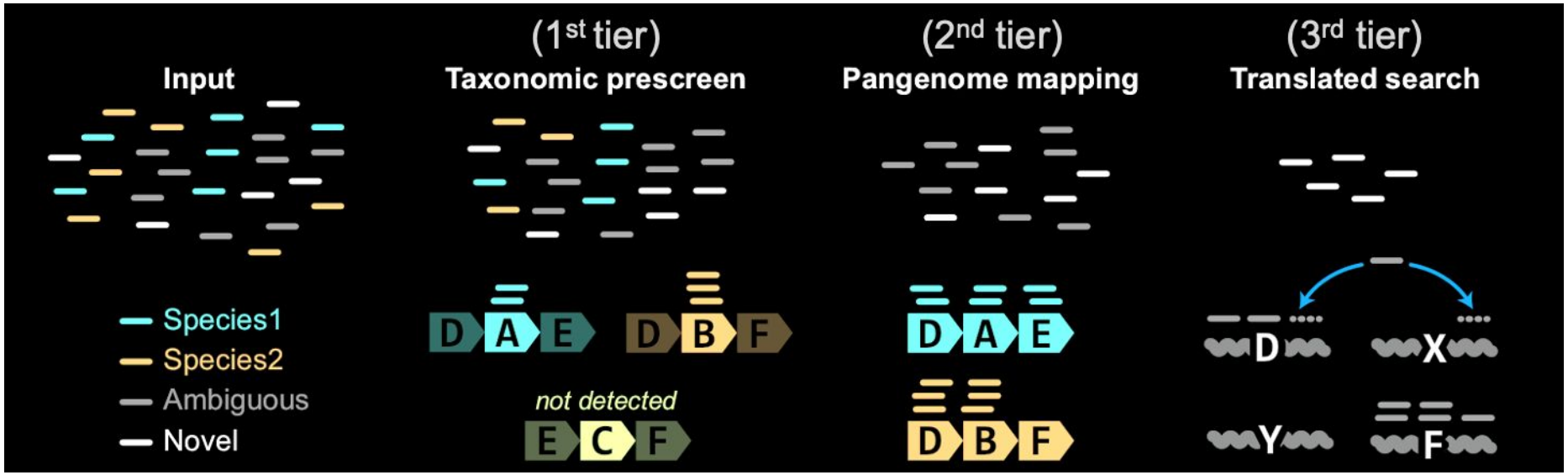
“Normalization”

- Larger genes are more likely to be sequenced than small genes.
- Thus, normalize gene annotations by their length is very common
  - Results are often reported as RPKM (reads per kilobase per million)
- Some methods may also account for similarity % to db, genome size, scaling factor (so not small decimals)

Pathway Inference

- Goal is to reduce spurious pathways
- A KO/EC can map to one or more KEGG/MetaCyc Pathways
  - Just because a gene is found in a pathway doesn't mean that it exists in the community
  - If a pathway has 20 genes and only 2 genes are observed in the community (but at high abundances) what should be the abundance of the pathway?
  - MinPath attempts to estimate the abundance of these pathways and remove spurious noise

Humann



- 1st: Metaphlan3 used to identify species
- 2nd: Reads are mapped with Bowtie to pangenomes of species identified by Metaphlan
- 3rd: Left over reads are translated searched (nucleotide vs protein database) using DIAMOND

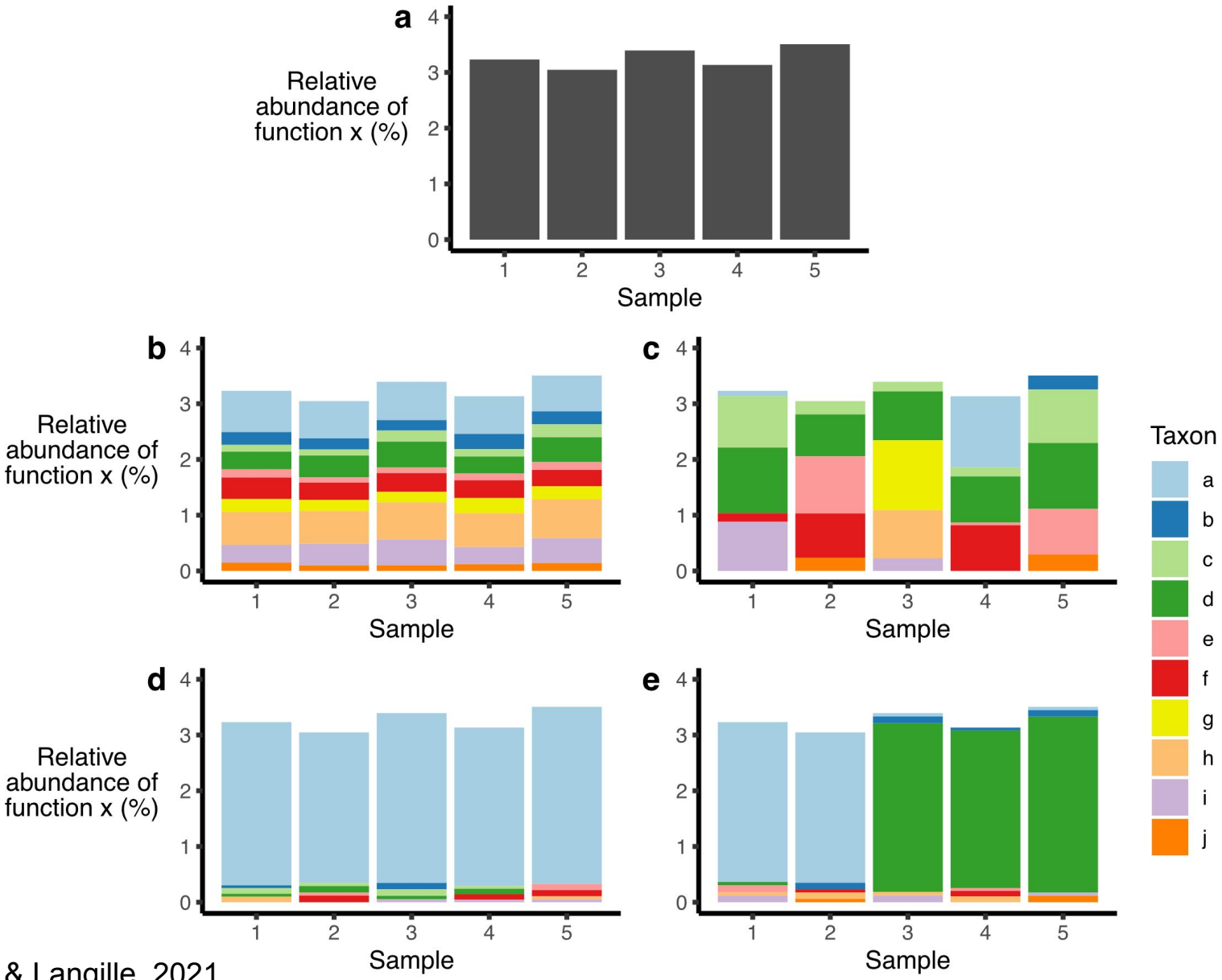
HUMAnN2: stratified output

UniRef gene cluster	Gene name	Total gene abundance (RPK)
UniRef90_R6K3Z5	IMP dehydrogenase	600.95
	Bacteroides_caccae	234.76
	Bacteroides_dorei	107.38
	Bacteroides_ovatus	92.18
	Bacteroides_stercoris	83.95
	Bacteroides_vulgatus	57.27
	unclassified	25.41

Per-species unclassified

MetaCyc pathway	abundance	coverage
PWY-7221: GTP biosynthesis	200.35	1
	120.23	1
	11.12	0

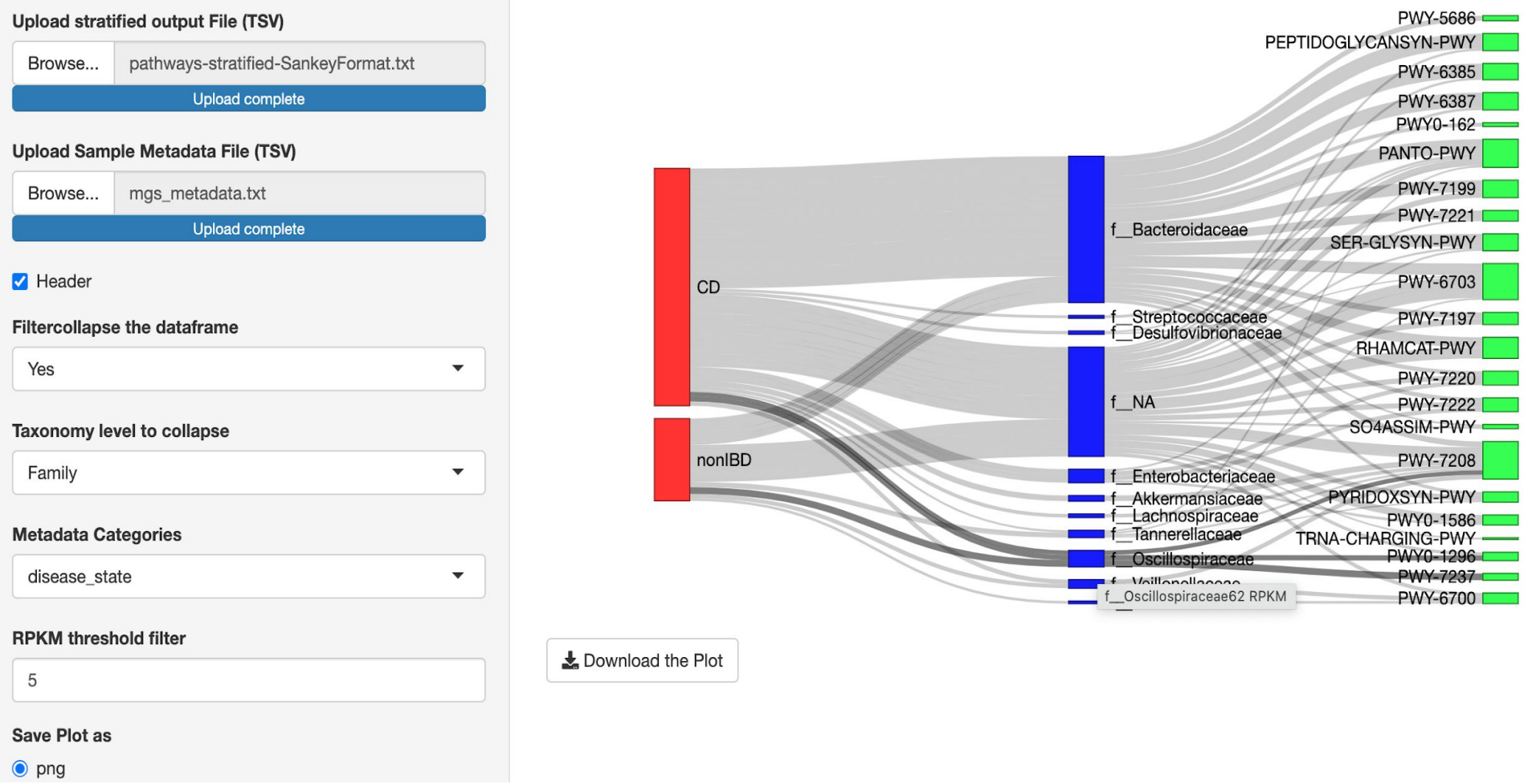
Functional Contributions



Douglas & Langille, 2021

JARRVIS

- Just Another stRatified Rpkm VISualizer



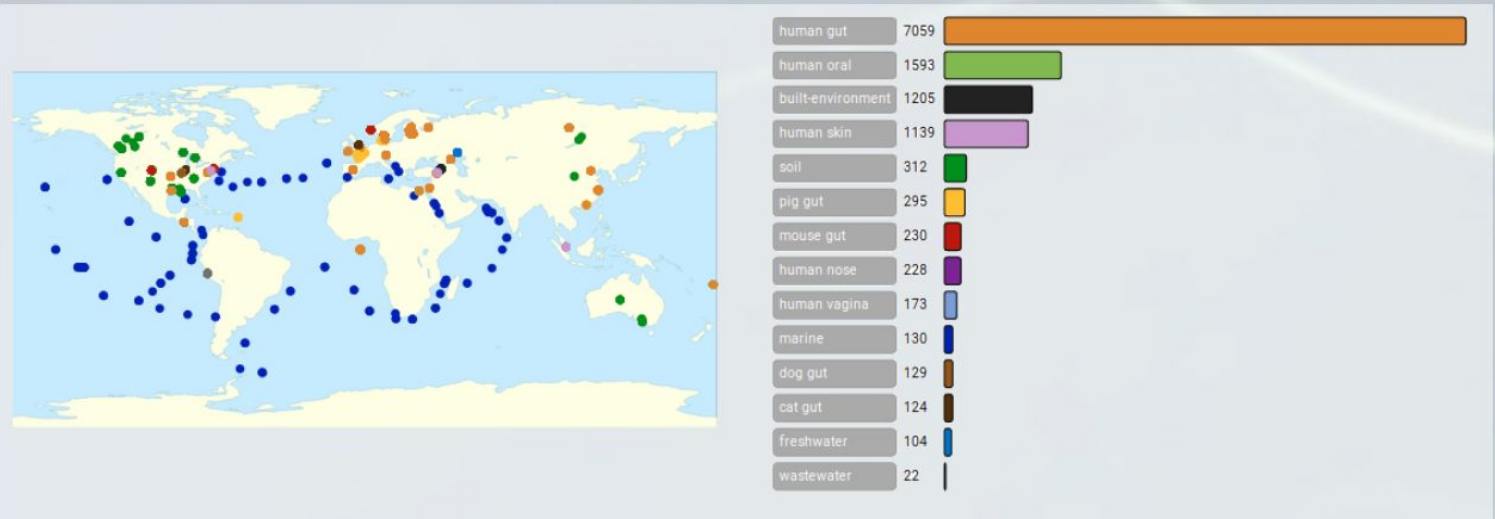
Specialized gene annotation systems/dbs

- Depending on research question, other specialized tools and databases can be used
- Virulence factors
  - VFDB
- Antimicrobial resistance (AMR) genes
  - CARD
- Carbohydrate metabolizing genes
  - CAZy
- Phage and prophage
  - VIBRANT, VirSorter, VirFinder, and MARVEL
- Large microbiome catalogues
  - Based on clustering of previous MGS reads

Global Microbial Gene Catalog v1.0

The **Global Microbial Gene Catalog** is an integrated, consistently-processed, gene catalog of the microbial world, combining metagenomics and high-quality sequenced isolates. A total of 2.3 billion ORFs from 13,174 metagenomes (covering 14 habitats) and the complete ProGenomes2 database were clustered together at 95% nucleotide identity to build a catalog of 302,655,267 unigenes.

Geographical and habitat distribution of samples used to build this catalogue



Community Function Potential

- Important that meta**genomics,** is not meta**transcriptomics**, and not meta**proteomics**
- These annotations suggest the functional **potential** of the community
- The presence of these genes/functions does not mean that they are biologically active (e.g. may not be transcribed)
- DNA may also be from dead cells

We are on a Coffee Break & Networking Session

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