Shotgun metagenomics

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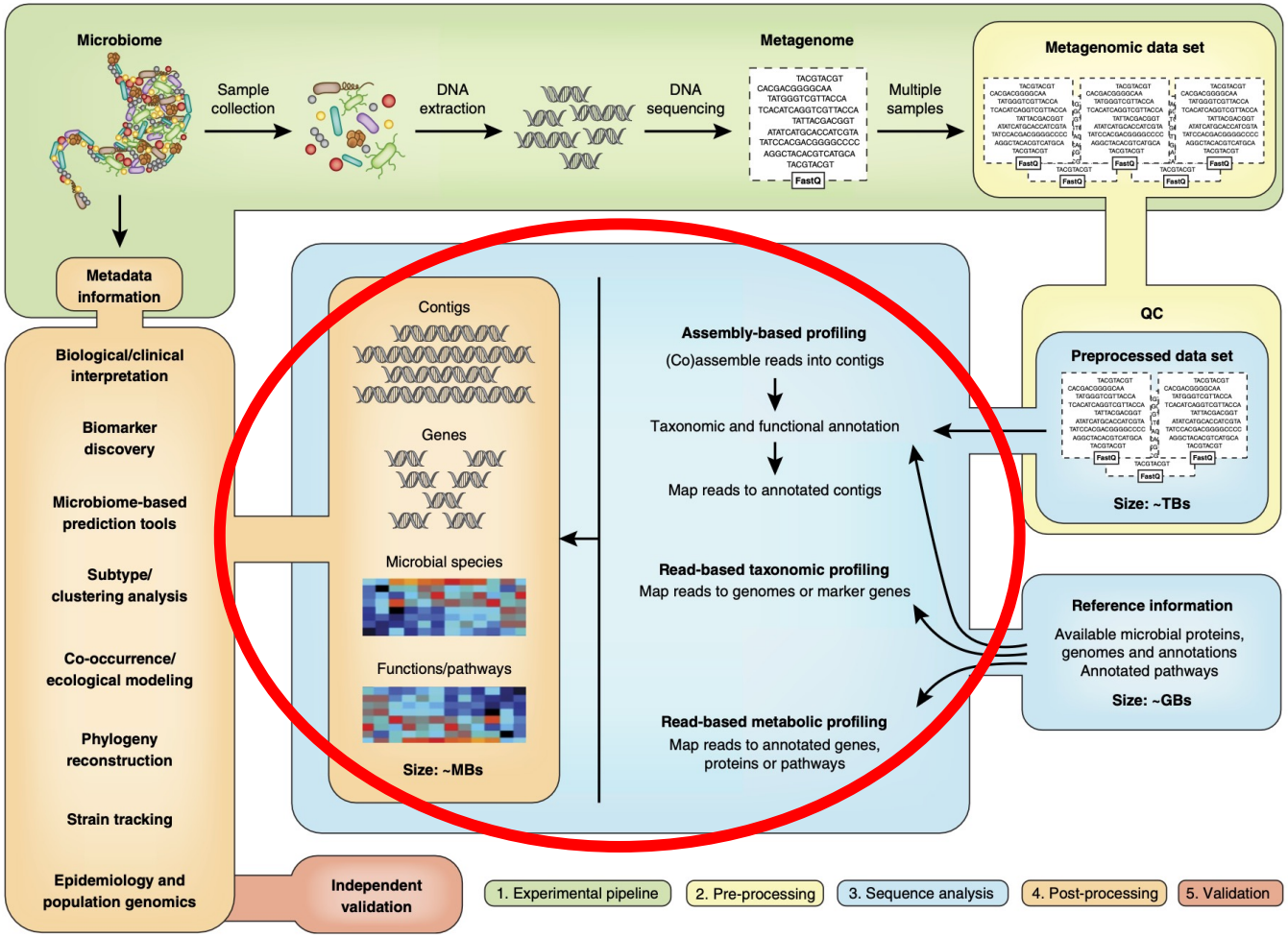
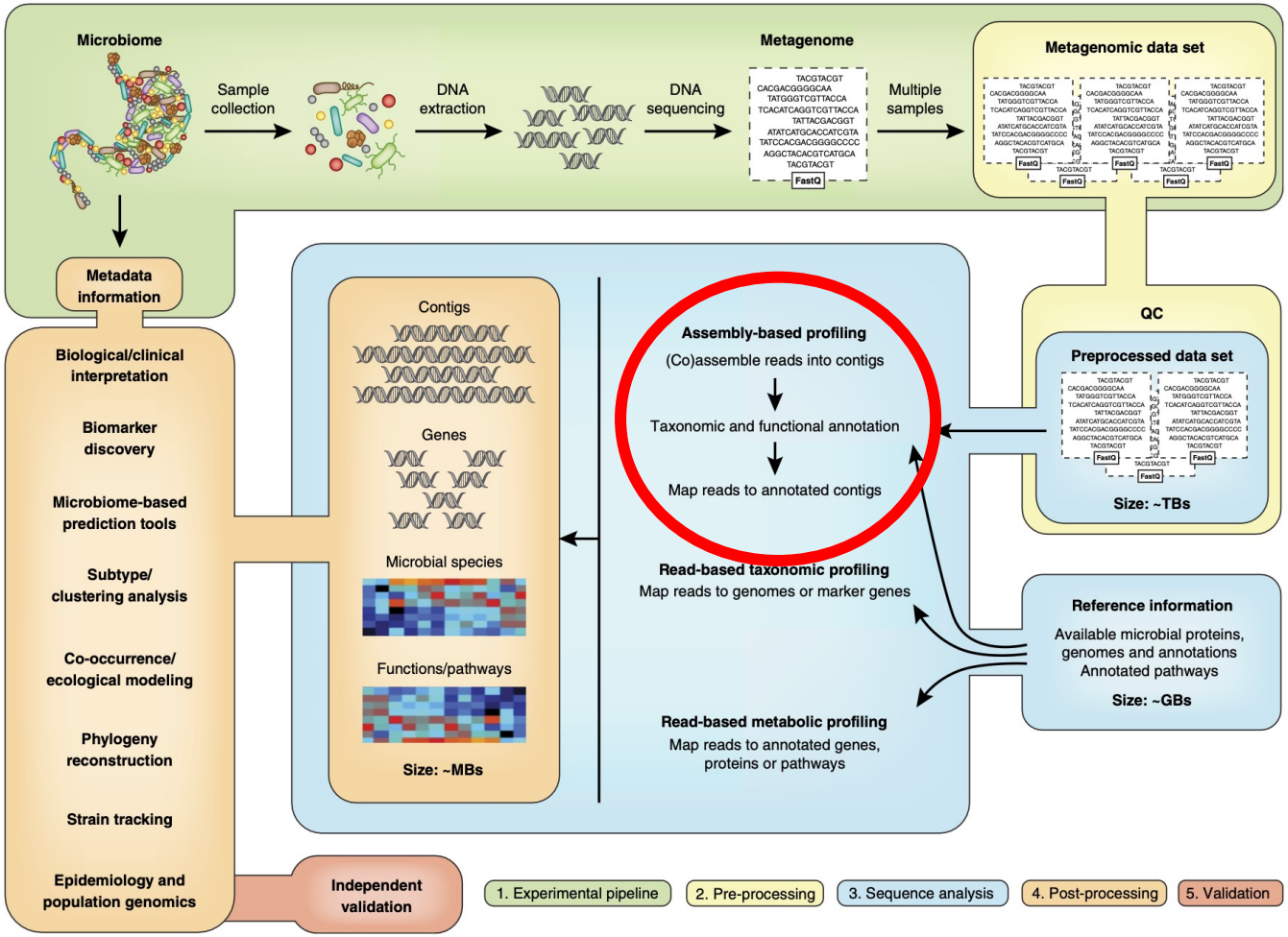


3. Metagenome assembly and annotation



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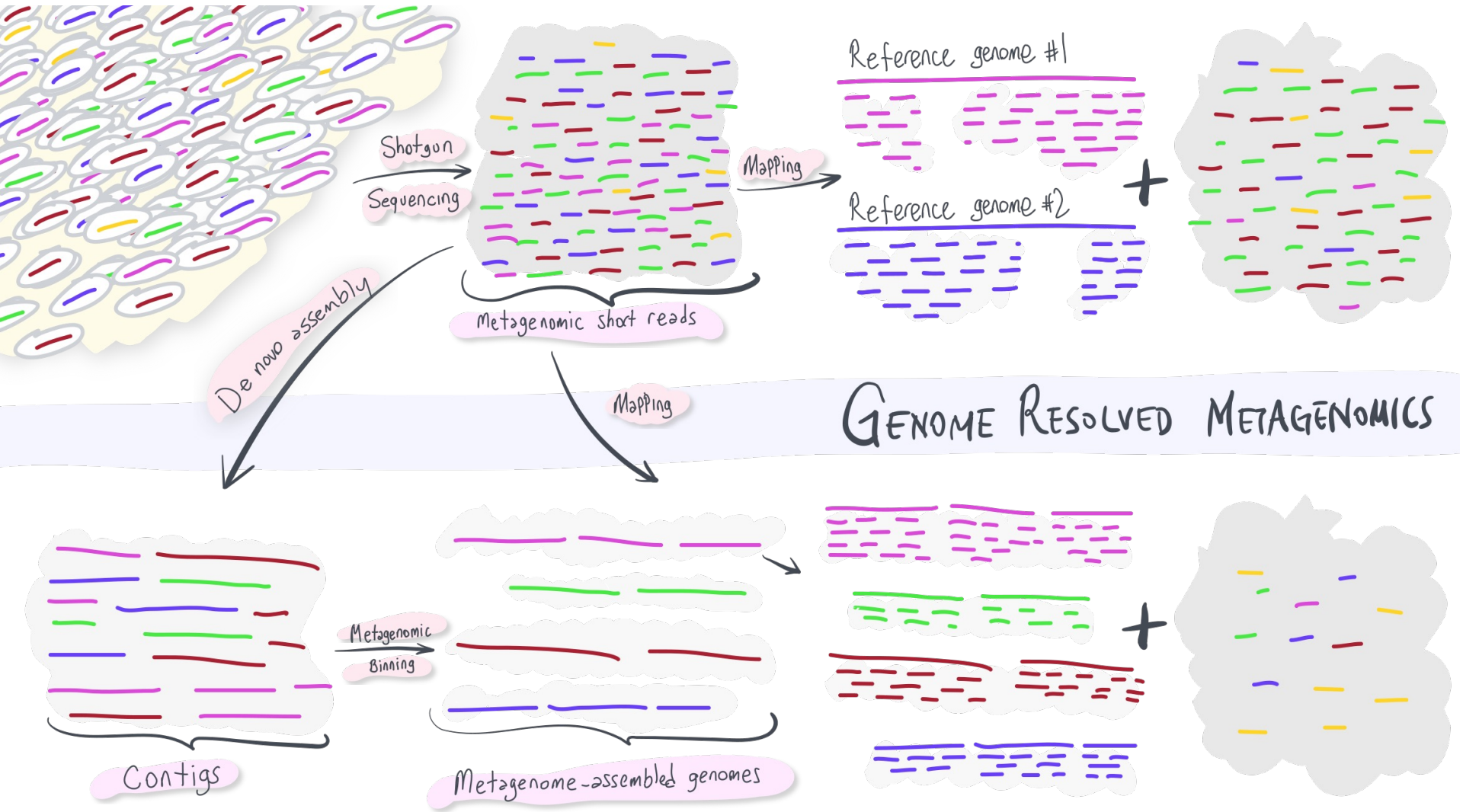
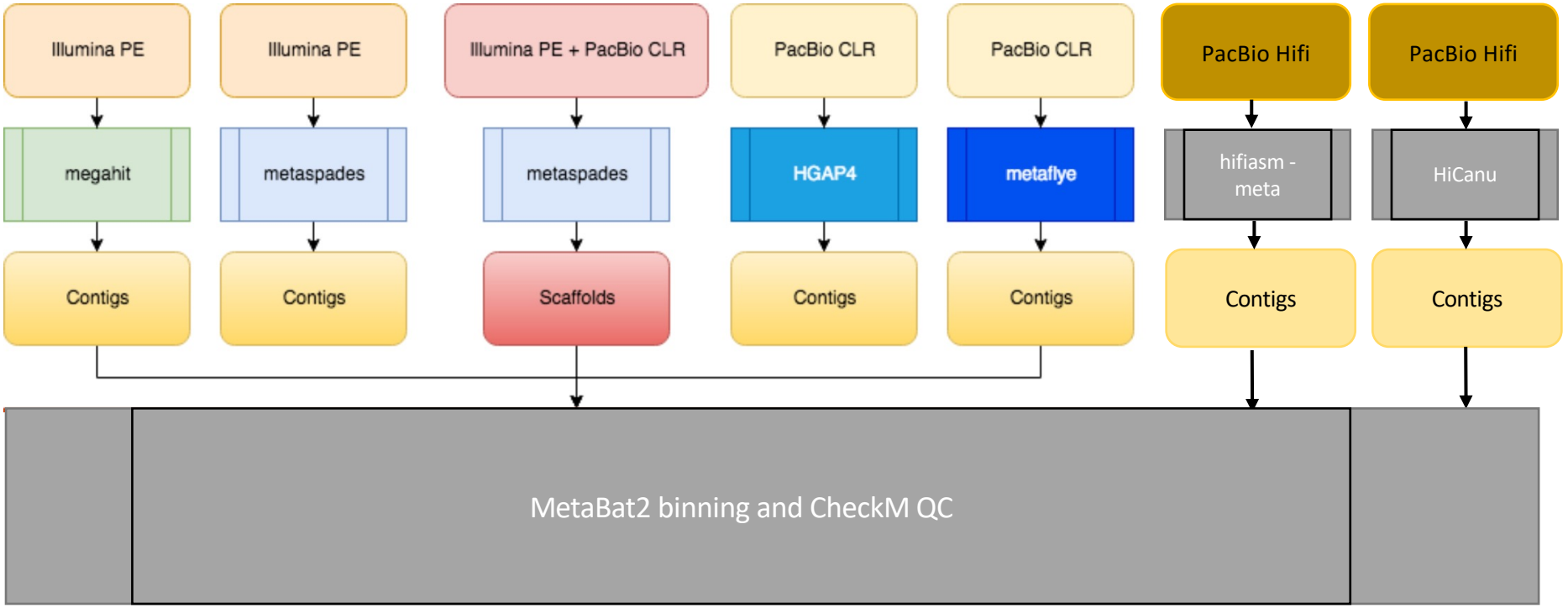
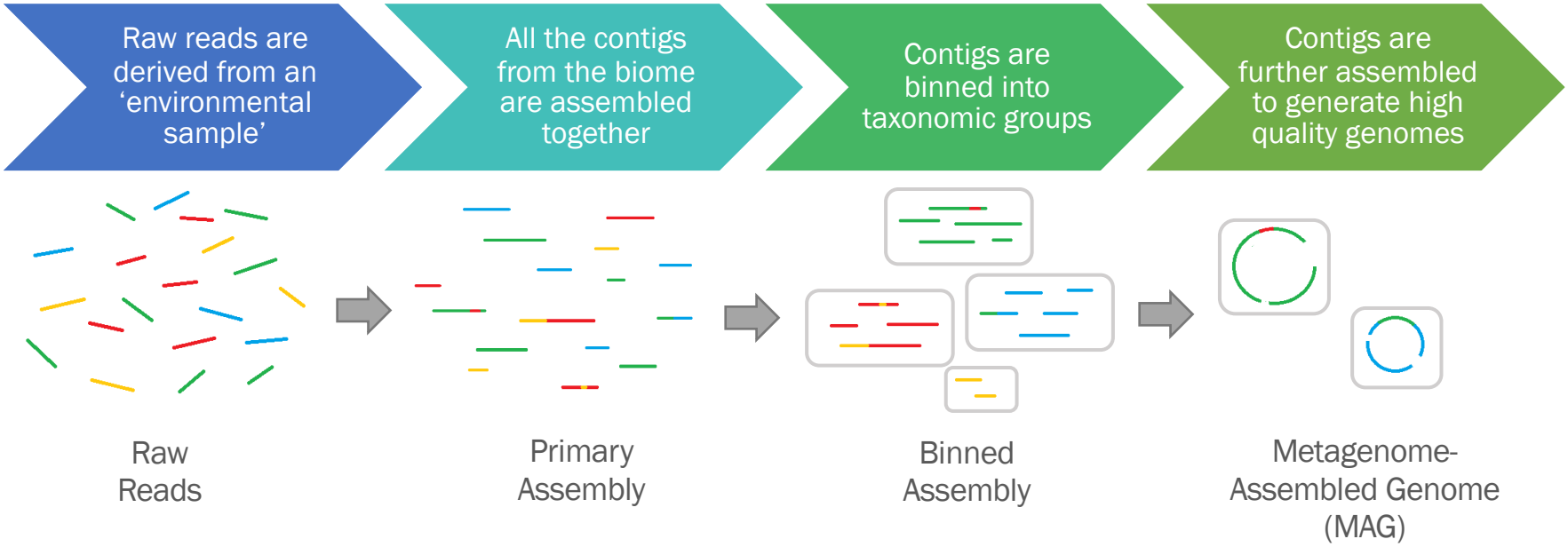


https://merenlab.org/momics/#genome-resolved-metagenomics

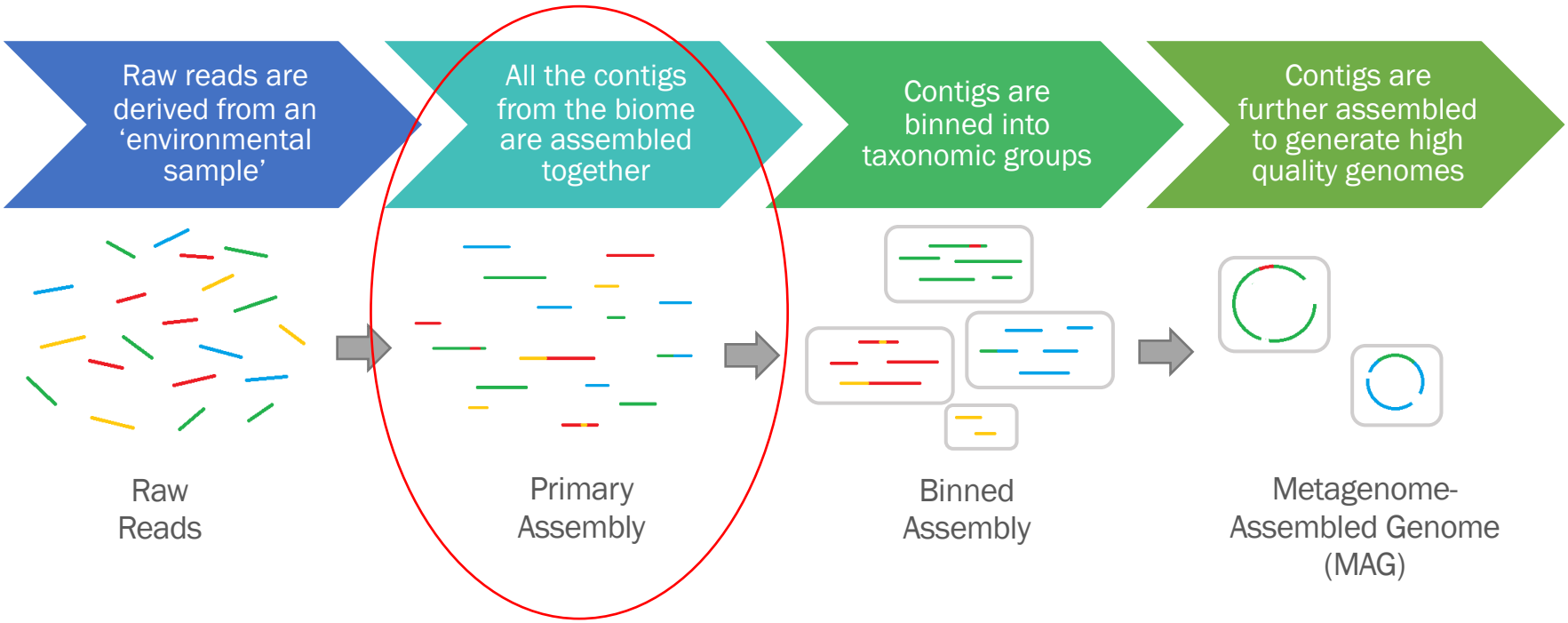
Metagenome assembly and binning workflow



Structuring Metagenomic Studies: Types Of Metagenomic Assembly



Structuring Metagenomic Studies: Types Of Metagenomic Assembly

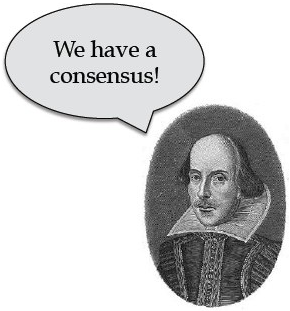


ETH zürich University of Zurich[UZH] functional genomics center zurich f·g·c·z

The problem

- ***Reads***

```
ds, Romans, count
ns, countrymen, le
Friends, Rom
send me your ears;
crymen, lend me
```



- ***Overlaps***

```
Friends, Rom
     ds, Romans, count
          ns, countrymen, le
               crymen, lend me
                    send me your ears;
```

- ***Majority consensus***

```
Friends, Romans, countrymen, lend me your ears;
```

Dr. Torsten Seemann

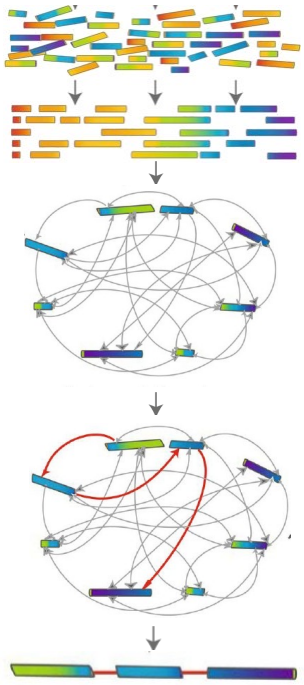
Overlap-Layout-Consensus

assembly algorithms

De Brujin graph

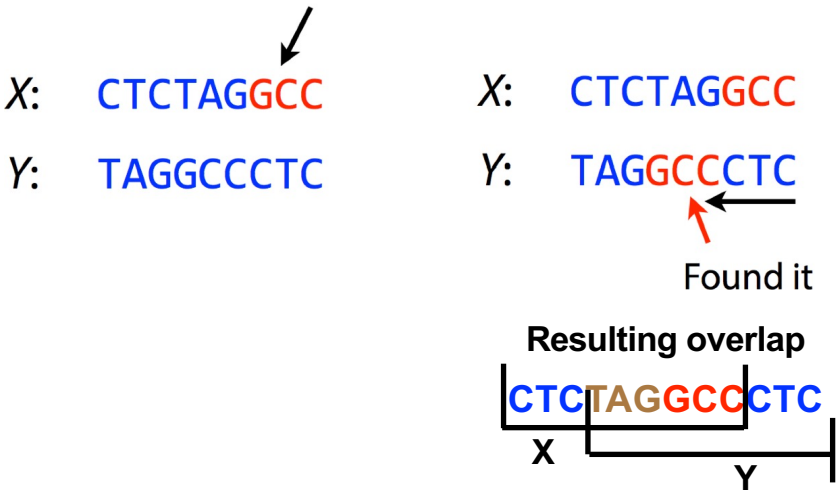
Overlap-layout-consensus (OLC)

- Overlap
  - Find all overlaps between reads
  - Build overlap graph: nodes=reads, edges=overlaps
- Layout
  - Simplify graph: errors/SNPs, repeats
  - Define assembly path
- Consensus
  - Align reads along assembly path
  - Consensus bases are called using weighted voting

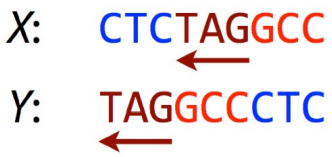


Overlap

Look for this in *Y*, going right-to-left



Extend to left; in this case, we confirm that a length-6 prefix of *Y* matches a suffix of *X*



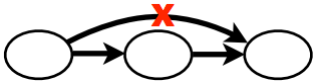
- We need to do this for every pair of reads
- Typically is the slowest part of the process

Overlapping is typically the slowest part of assembly

Consider a second-generation sequencing dataset with hundreds of millions or billions of reads!

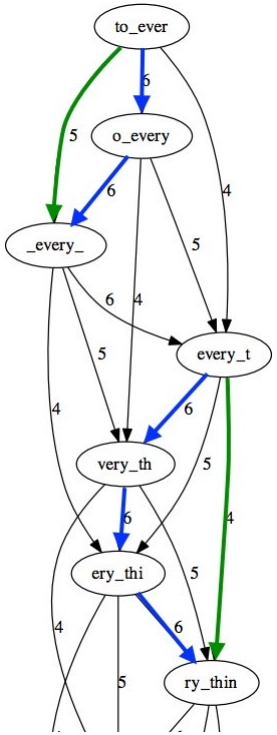
Layout – pop bubbles

- Bubbles are formed with transitively inferable edges
  - Edges can be inferred from others



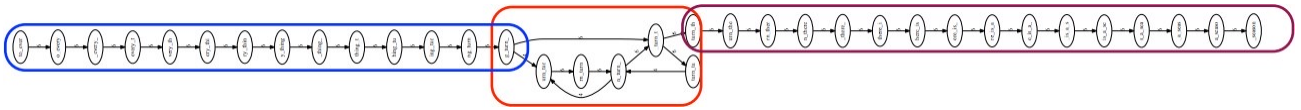
  - The green ones can be inferred from the blue ones

- Popping bubbles collapses small differences (i.e. minor heterozygosity)



Layout – traverse only unambiguous paths

- Repeats, paralogous genes etc form ambiguous paths
  - **Branches**
- Report only paths corresponding to non-branching stretches
  - Reliable stretches of unique DNA sequences
- Leave ambiguous stretches unresolved



Contig 1
to_every_thing_turn_

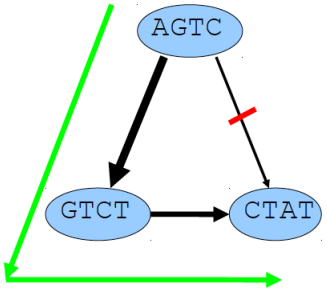
Contig 2
turn_there_is_a_season

Unresolvable repeat

Example – layout and consensus

- Find a path which visits each node once
  - Hamiltonian path/cycle is NP-hard (this is bad)
  - solution will be a set of paths which terminate at decision points
- Form a consensus sequences from paths
  - use all the overlap alignments
  - each of these collapsed paths is a contig

- Optimal path shown in green
- Un-traversed weak overlap in red
- Consensus is read by outputting the overlapped nodes along the path
- `aGTCTCTat`



Consensus

At each position, ask: what nucleotide (and/or gap) is here?

Complications: (a) sequencing error, (b) ploidy

Say the true genotype is AG, but we have a high sequencing error rate and only about 6 reads covering the position.

De Bruijn graph

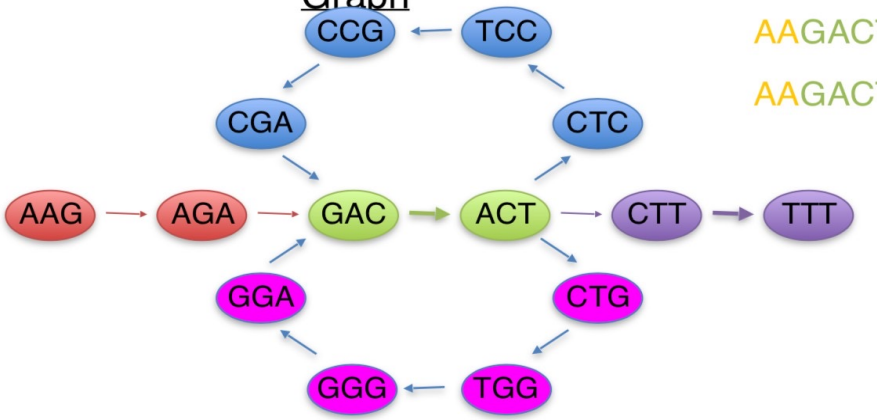
- Reads are split into K-mers (sequences of length K)
- K-mers represent the edges of the graph
- The nodes are the suffix and the prefix of length K-1 shared by edges

De Brujin graph (DBG)

Reads

AAGA
ACTT
ACTC
ACTG
AGAG
CCGA
CGAC
CTCC
CTGG
CTTT
…

de Bruijn Graph



Potential Genomes

AAGACTCCGACTGGGACTTT

AAGACTGGGACTCCGACTTT

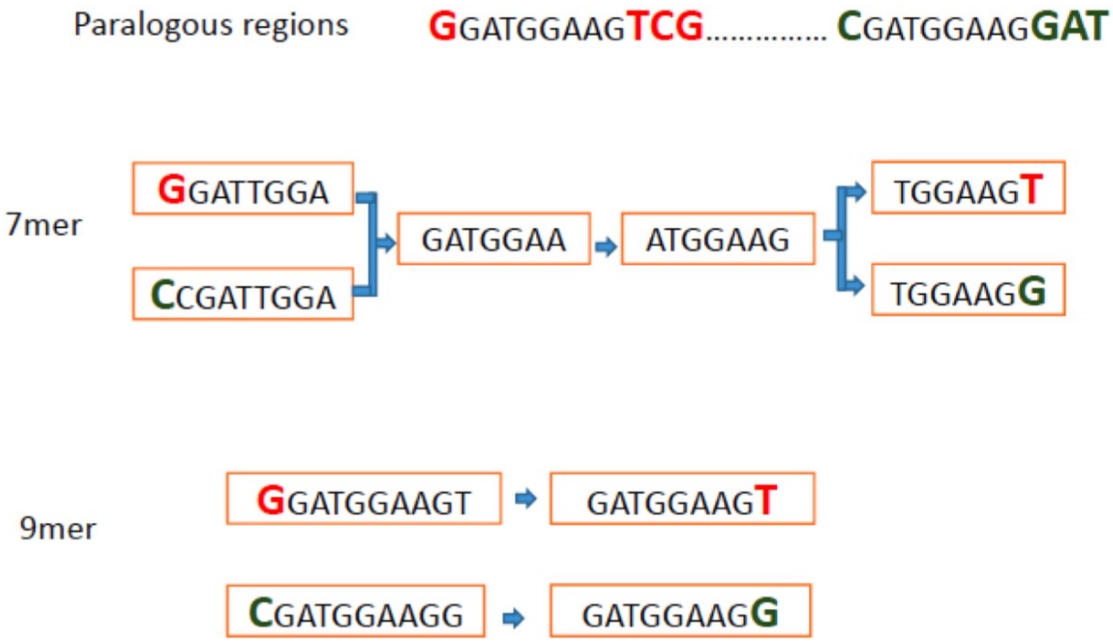
…

The assembly is an Eulerian path (it contains all the edges)

A directed, connected graph is Eulerian if and only if it has at most 2 semi-balanced nodes and all other nodes are balanced

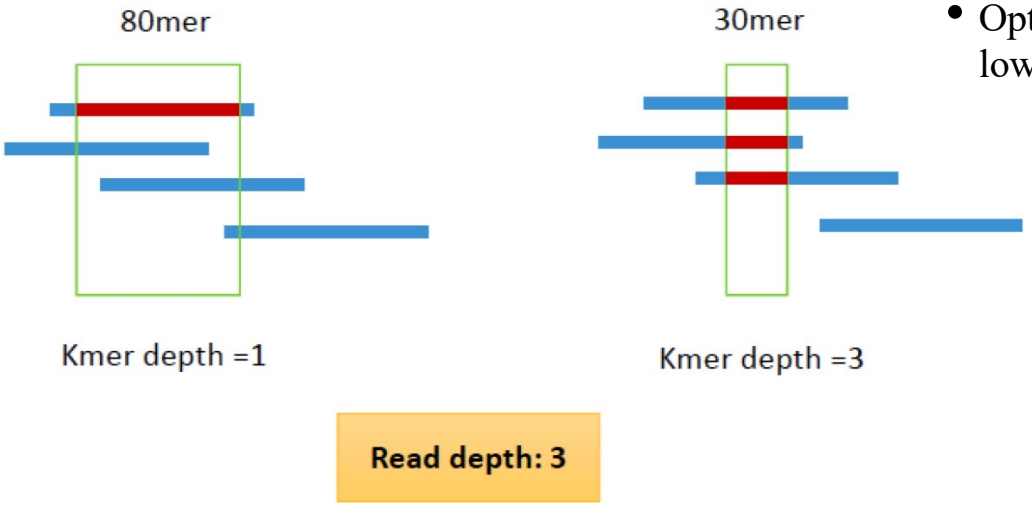
Impact of kmer length

- Short kmers would collapse paralogous regions, and result in more branches



Impact of kmer length

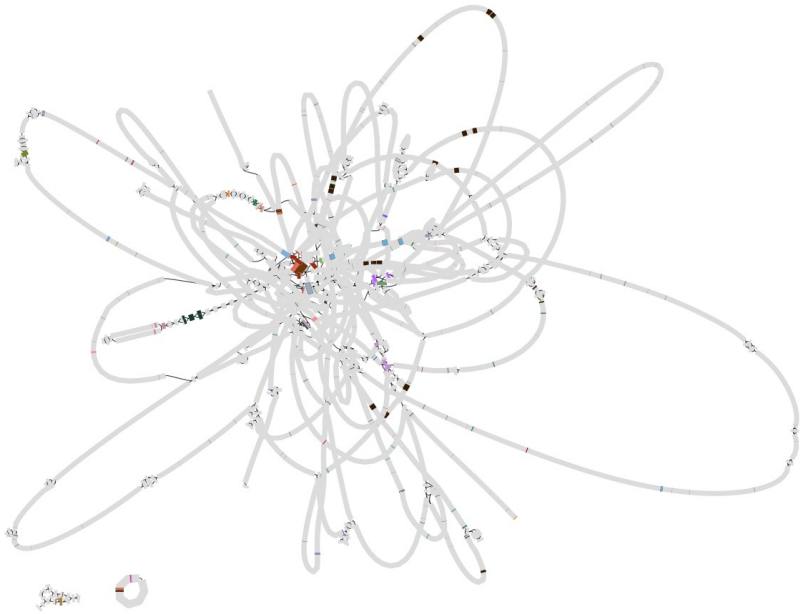
- Longer kmers – lower kmer depth

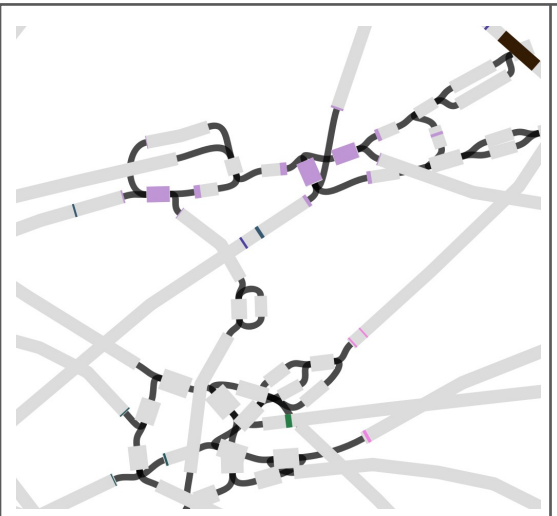


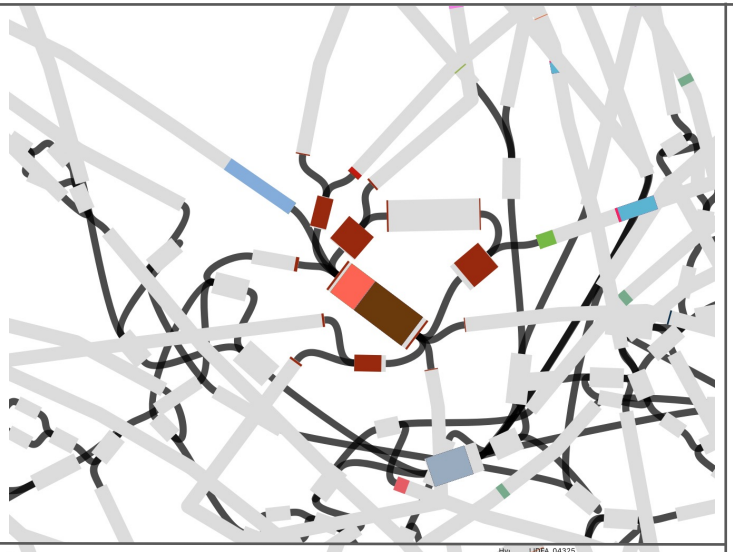
- A selection of k-mers is always used to get the optimal assembly.
- Megahit and Metaspades are DBG assemblers.
- Optimal k-mer length has to be chosen for low- and high- abundance species

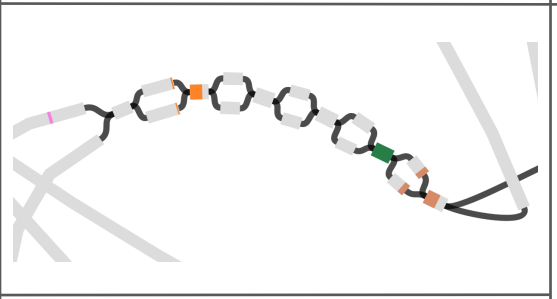
Visualising the assembly graph

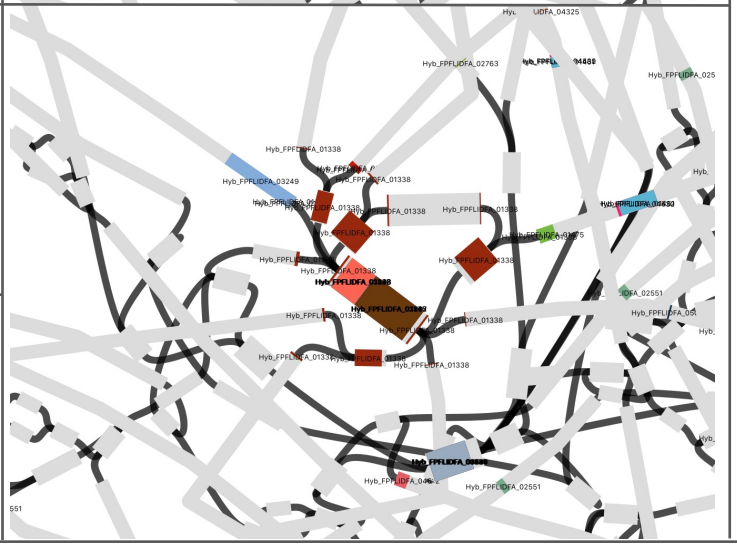
- Tools such as metaSPAdes convert the de Bruijn graph to an assembly graph
- Bandage allows the visualisation and querying of this graph

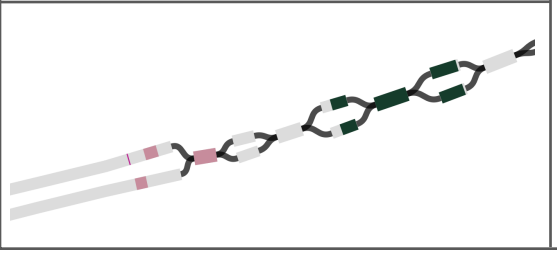










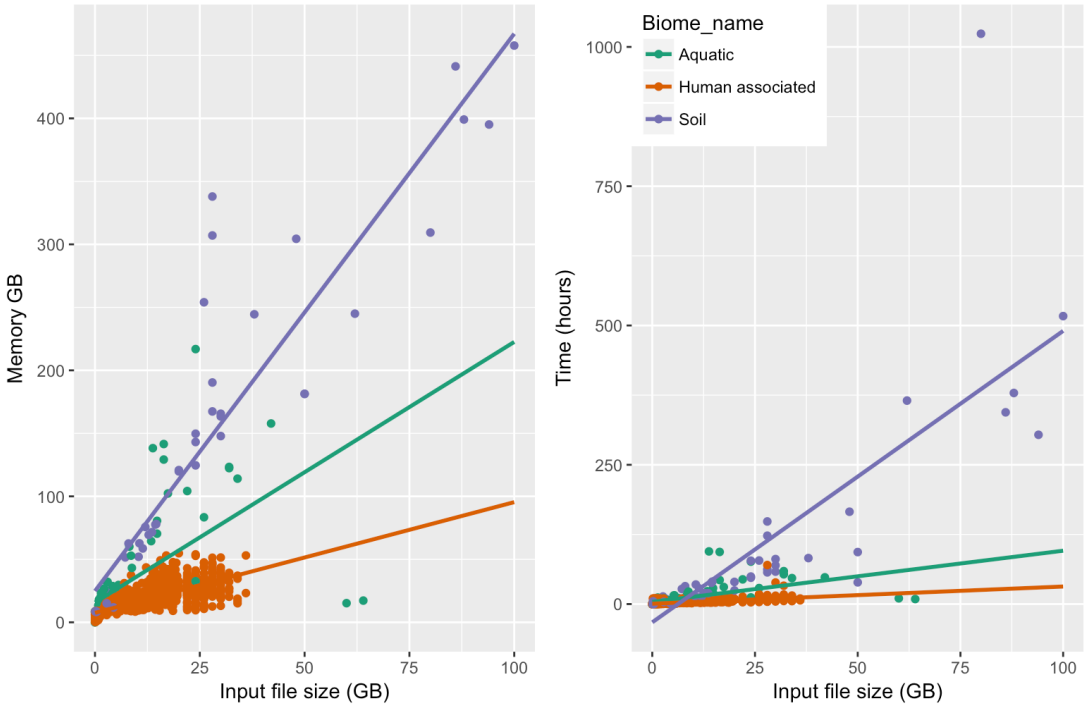


Width of edges proportional to read coverage

Some examples of multiple possible contiguity in which lost proteins are involved.

Conserved domains have multiple matches.

Computational resources are limiting for assemblies

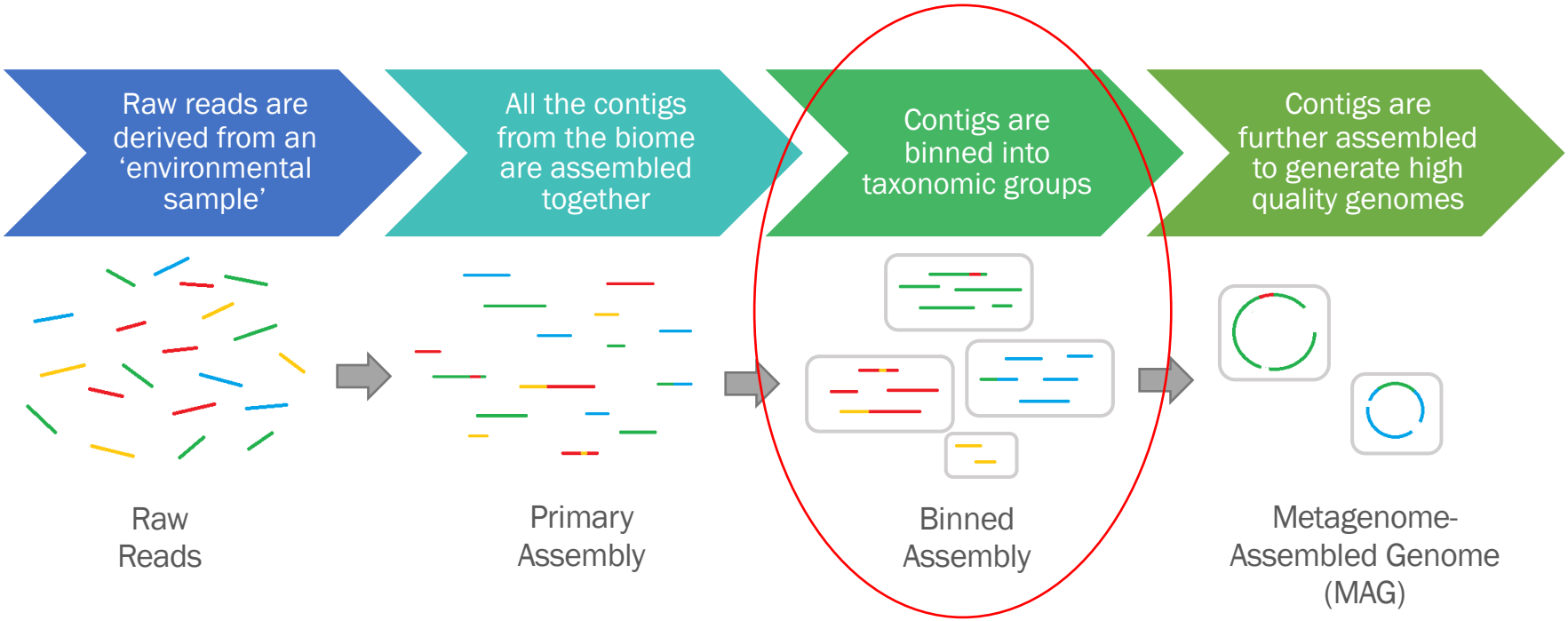


Complications of assembling shotgun metagenomics data

- Different abundance
  - Highly abundant genomes are over-sequenced
    - higher coverage ≠ repeats
  - Low abundant genomes – low coverage kmers are real
- Different relatedness
  - Unique but highly conserved regions in a single genome might be repetitive in a metagenome
- Simple coverage statistics can no longer be used to detect the repeats
- Distinguishing true biological differences from sequencing errors becomes nearly impossible

Ghurye JS. 2016. Yale J Biol Med.

Structuring Metagenomic Studies: Types Of Metagenomic Assembly



Binning: two ways

- Taxonomy-independent binning – unsupervised approach
- Taxonomy-dependent binning – supervised approach

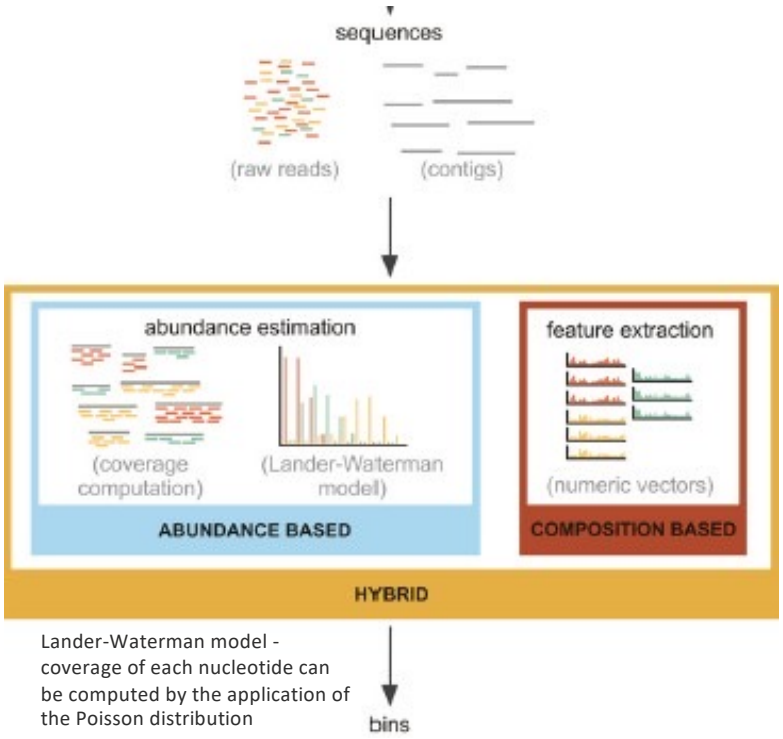
Taxonomy-independent binning

Composition-based

- Assumes nucleotide composition as closest proxy for species similarity - based on an assumption that the genome composition is unique for each taxon
- Can also be reference agnostic, no assembly
- Only reads information (e.g., GC %, K- mers)
- Fast (no need to align)

Abundance-based

- Assumes sequence abundances as closest proxy for species similarity
- *De novo* assembly for initial contigs
- Abundance of contigs via read mapping
- Binning of contigs
- Concerned with k-mer abudance rather than conent



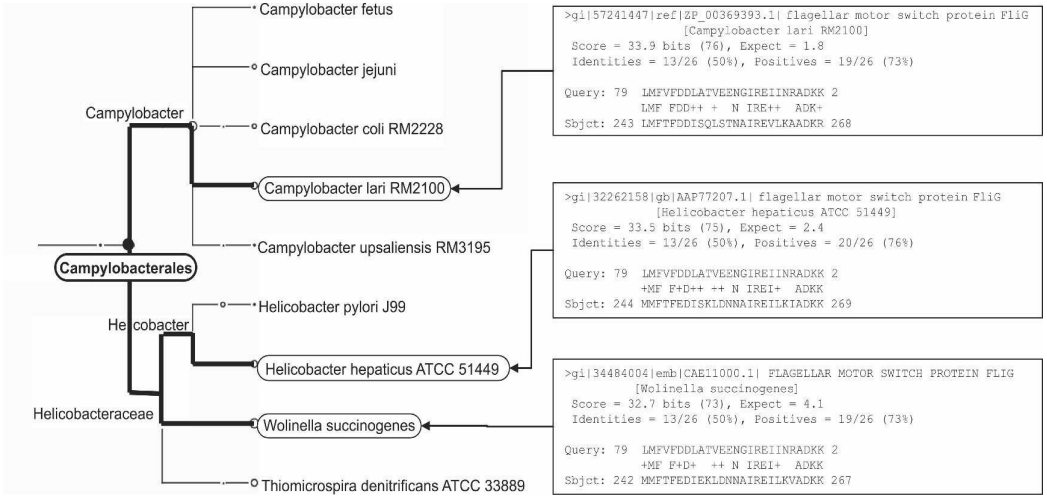
Composition- vs abundance-based approach

Composition	Abundance
Clear visualization of analysed microbiomes	Complex populations with low abundance communities
More reliable with long sequences cannot be used to classify very short fragments, because of the substantial variation of DNA composition patterns within a single genome	Capable of classifying short reads
No need to perform *de novo* assembly	More reliable with multiple samples

Taxonomy-dependent binning (LCA)

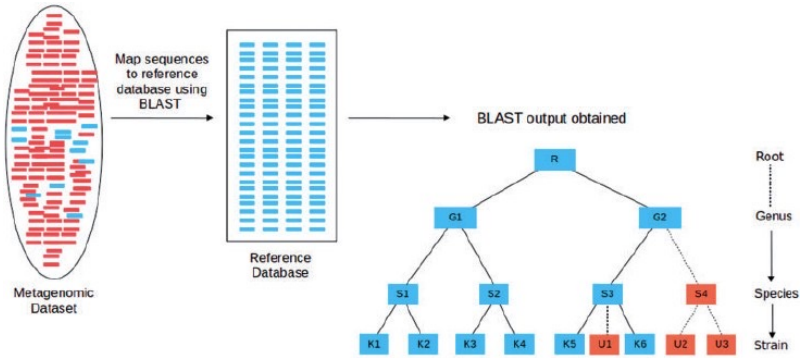
Sequence-based

- Assumes similarity to sequences in a database as closest proxy for species similarity
- Multiple potential hits usually reported (30-50)
- Lowest common ancestors approach (LCA) to resolve ambiguities
- Standard LCA limited in the case of long/multiple ORF contigs (e.g., Eukaryote metagenomes)
- LCA* recently proposed (Hanson et al., Bioinformatics 2016)



Huson et al., Gen. Res., 2007

LCA approach

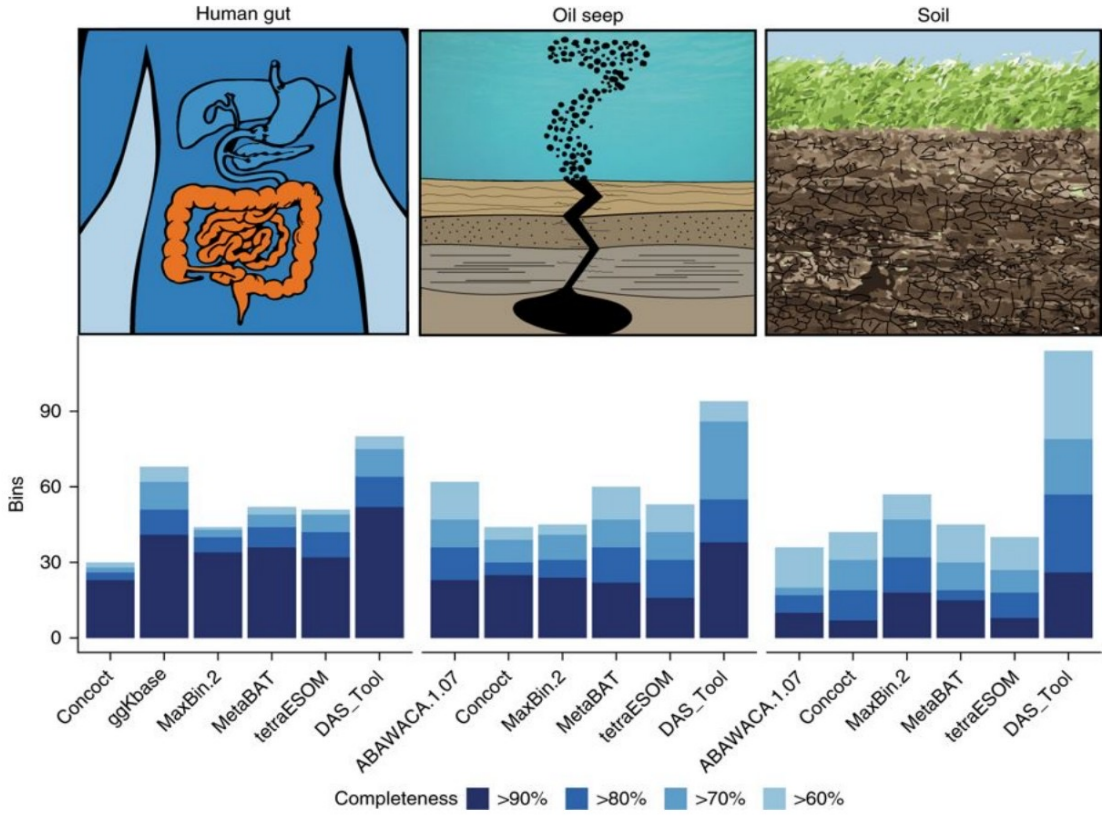


PRO	CONS
Leveraging existing information	Computationally intensive/time consuming (alignment and eventual downstream assembly)
Amenable to shorter reads	Matching bit-scores sometimes inadequate metric
Rare microorganisms identifiable provided sufficient coverage	Problematic with sample from mostly unexplored environments

Reads originate from	Significant BLAST Hits	Assingment Strategies	
		Best BLAST Hit Approach	LCA
K1	K1, K2, K3	K1 (√)	G1 (√)
U1	K5, K6	K5 (X)	S3 (√)
U2 and U3	K5, K6	K5 (X)	S3 (X)

Selection of metagenome binning methods

- Use multiple binning methods when possible



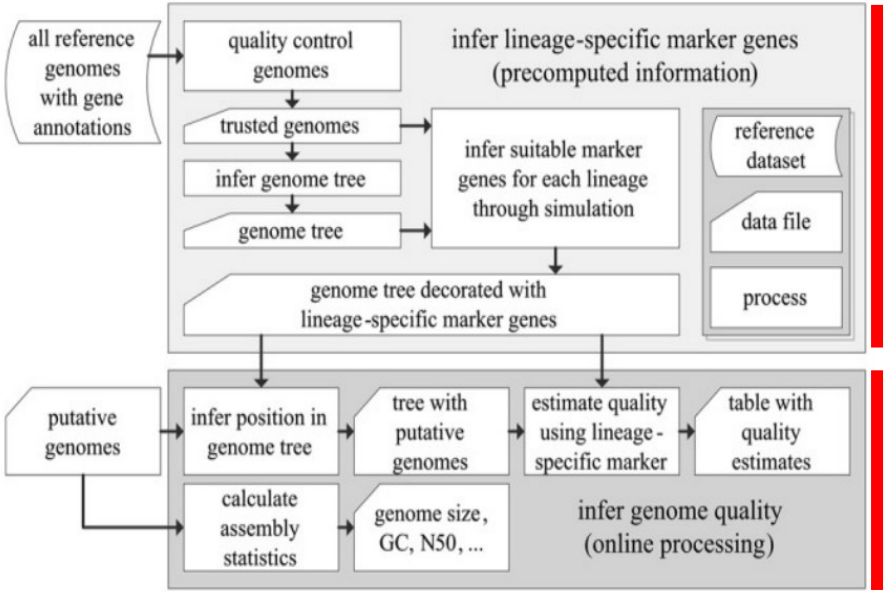
Sieber CMK et al. 2018.Natural Microbiology

Assessing the quality of reconstructed genome bins

- Estimate completeness and cleanness
  - The number and copy number of marker genes
  - Universal and lineage specific marker gene sets

- checkM
  - Utilize the set of marker genes specific to the position of a genome within a reference genome tree
  - Can distinguish contamination introduced by multiple strains from that introduced by more divergent taxa: amino acid identity (AAI) between multicopy genes
    - a gene identified as single copy in ≥97% of genomes is considered to be a marker gene
    - often encode essential functions and are frequently organized into operons
    - often also used collocated marker sets

CheckM



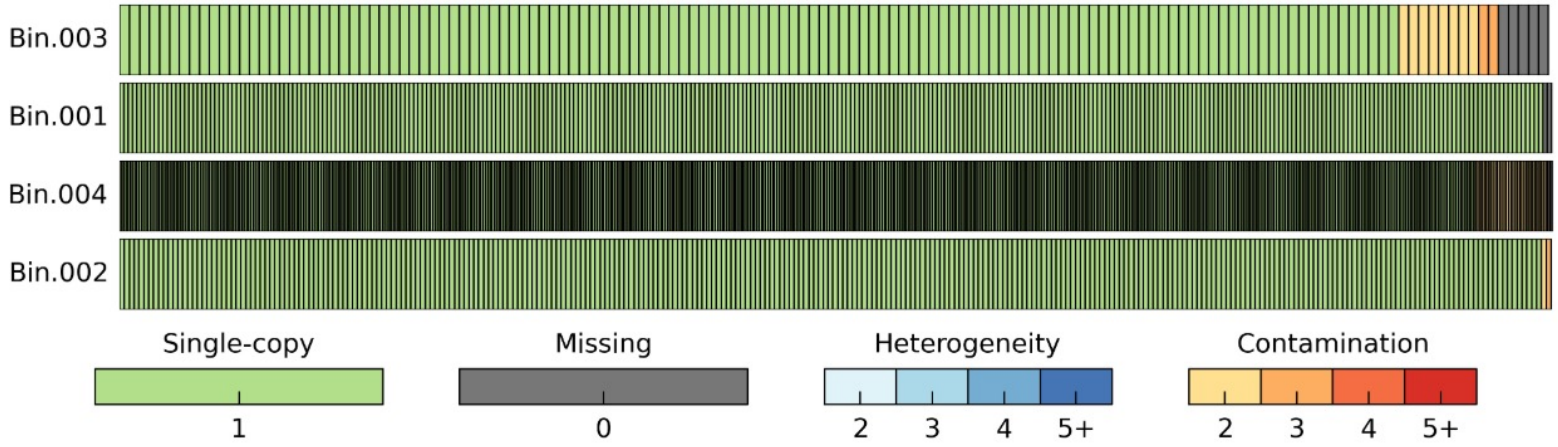
Creating a CheckM database

Assesing your bins against the database

Parks DH et al. 2015. Genome Research

CheckM

CheckM PLOT | CheckM Table



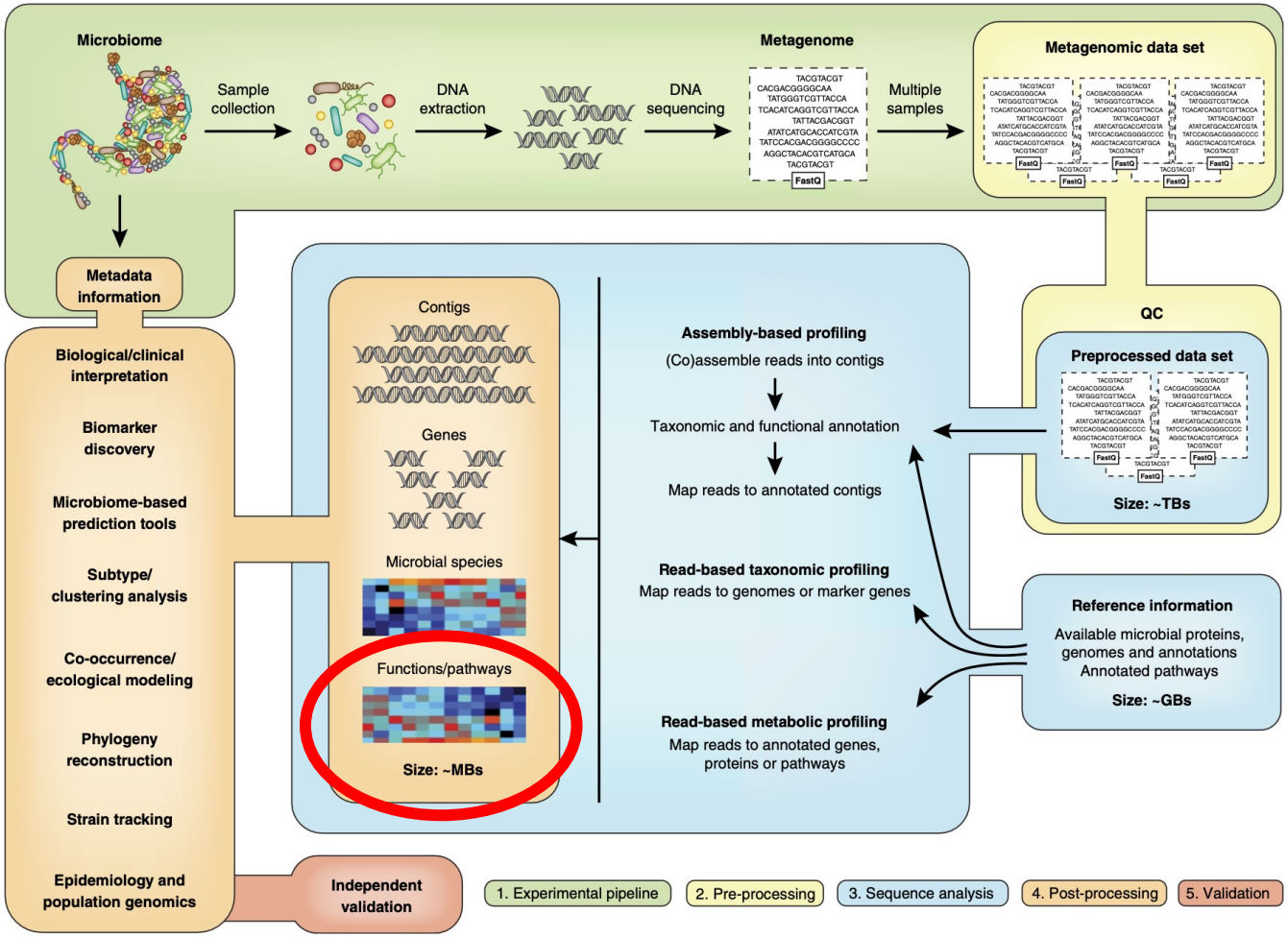
What makes a good assembly?

- N50
  - minimum contig length that contains 50% of the assembled bases
- Long contigs (subjective)
- Does the assembly contain what you expect
  - MetaQUAST, BLAST
- Assembly Likelihood Evaluation framework (ALE) - https://bioinformaticshome.com/tools/wga/descriptions/ALE-Assembly.html
  - tool can be used to detect errors in metagenomes as well by pinpointing single base errors, indels, genome re-arrangement and chimera
  - without the need of a reference genome

Which tools are commonly used for robust taxonomic classification?

MG-RAST (GenBank annotation)
(https://www.mg-rast.org/)

GTDBTk
(https://github.com/Ecogenomics/GTDBTk)
(https://gtdb.ecogenomic.org/)



3. Metagenome assembly and annotation

Protein prediction: Prodigal

What does Prodigal do?

- **Predicts protein-coding genes**: Prodigal provides fast, accurate protein-coding gene predictions in GFF3, Genbank, or Sequin table format.
- **Handles draft genomes and metagenomes**: Prodigal runs smoothly on finished genomes, draft genomes, and metagenomes.
- **Runs quickly**: Prodigal analyzes the *E. coli K-12* genome in 10 seconds on a modern MacBook Pro.
- **Runs unsupervised**: Prodigal is an unsupervised machine learning algorithm. It does not need to be provided with any training data, and instead automatically learns the properties of the genome from the sequence itself, including genetic code, RBS motif usage, start codon usage, and coding statistics.
- **Handles gaps, scaffolds, and partial genes**: The user can specify how Prodigal should deal with gaps and has numerous options for allowing or forbidding genes to run into or span gaps.
- **Identifies translation initiation sites**: Prodigal predicts the correct translation initiation site for most genes, and can output information about every potential start site in the genome, including confidence score, RBS motif, and much more.
- **Outputs detailed summary statistics for each genome**: Prodigal makes available many statistics for each genome, including contig length, gene length, GC content, GC skew, RBS motifs used, and start and stop codon usage.

Protein prediction: PROKKA

Prokka trustworthy databases, moving to medium-sized but domain-specific databases in a hierarchical manner.

1.An optional **user-provided set of annotated proteins**. These are expected to be trustworthy curated datasets and will be used as the primary source of annotation. They are searched using BLAST+ blastp (Camacho *et al.* , 2009).

2.All bacterial **proteins in UniProt** (Apweiler *et al.* , 2004) that have real protein or transcript evidence and are not a fragment. This is ~16 000 proteins, and typically covers >50% of the core genes in most genomes. BLAST+ is used for the search.

3.All proteins from **finished bacterial genomes in RefSeq** for a specified genus. This captures domain-specific naming, and the databases vary in size and quality, depending on the popularity of the genus. BLAST+ is used for this and is optional.

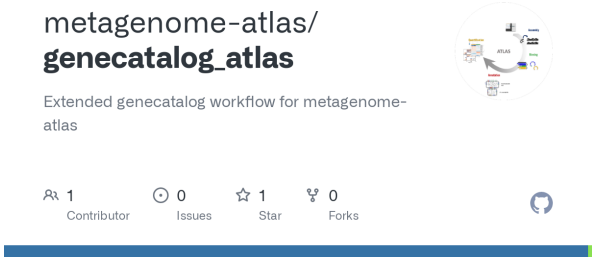
4.A series of hidden Markov model profile databases, including **Pfam** (Punta *et al.* , 2012) and **TIGRFAMs** (Haft *et al.* , 2013). This is performed using hmmscan from the HMMER 3.1 package (Eddy, 2011).

5.If no matches can be found, label as 'hypothetical protein'.

Annotation

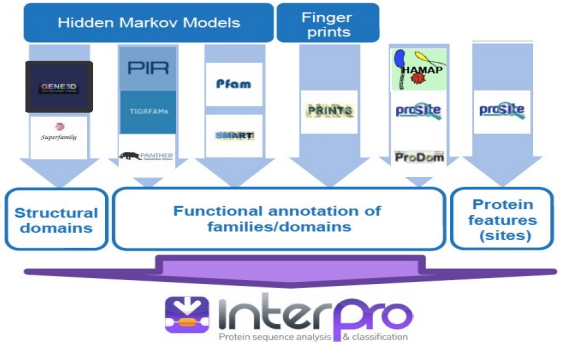
- After binning/reads assembly (preferred)
  - Large contigs (30,000+ bp) expected to have optimal results
  - Genome annotation tools used quite successfully

A lot of pipelines do it automatically as a next step of the workflow:

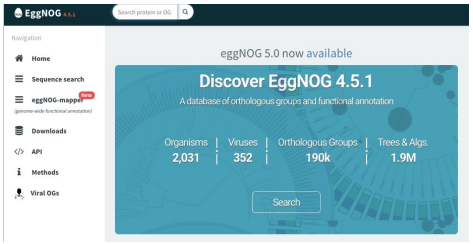


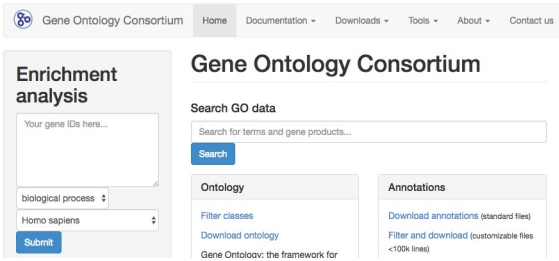
Annotation

Annotation of protein domains (Interpro scan)



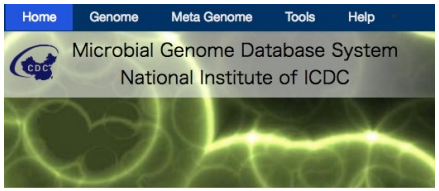
Orthology based gene funtion prediction (EggNOG, GO Consotrium, OMA GO annotation, Pannzer2)

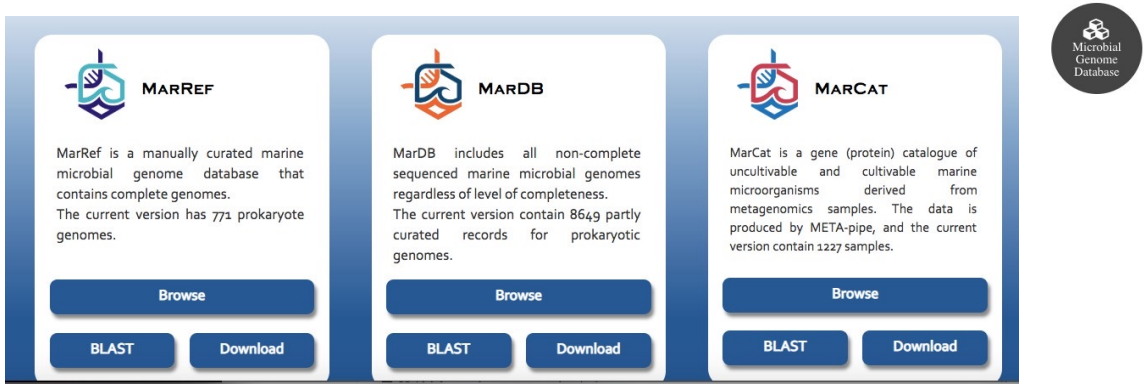


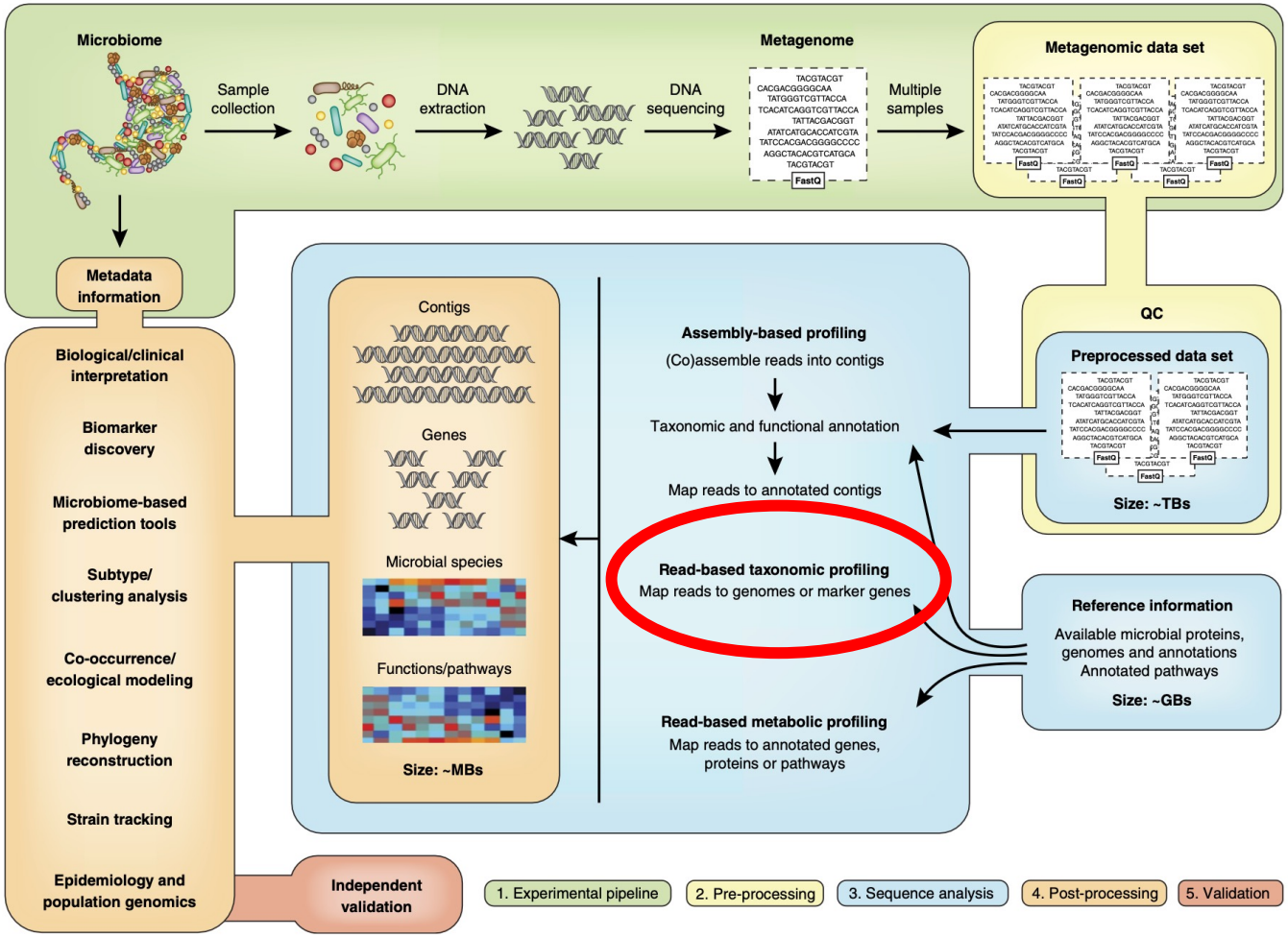


Metabolic pathway prediction (KEGG)

Using ecosystem-specific databases for annotation and taxonomy

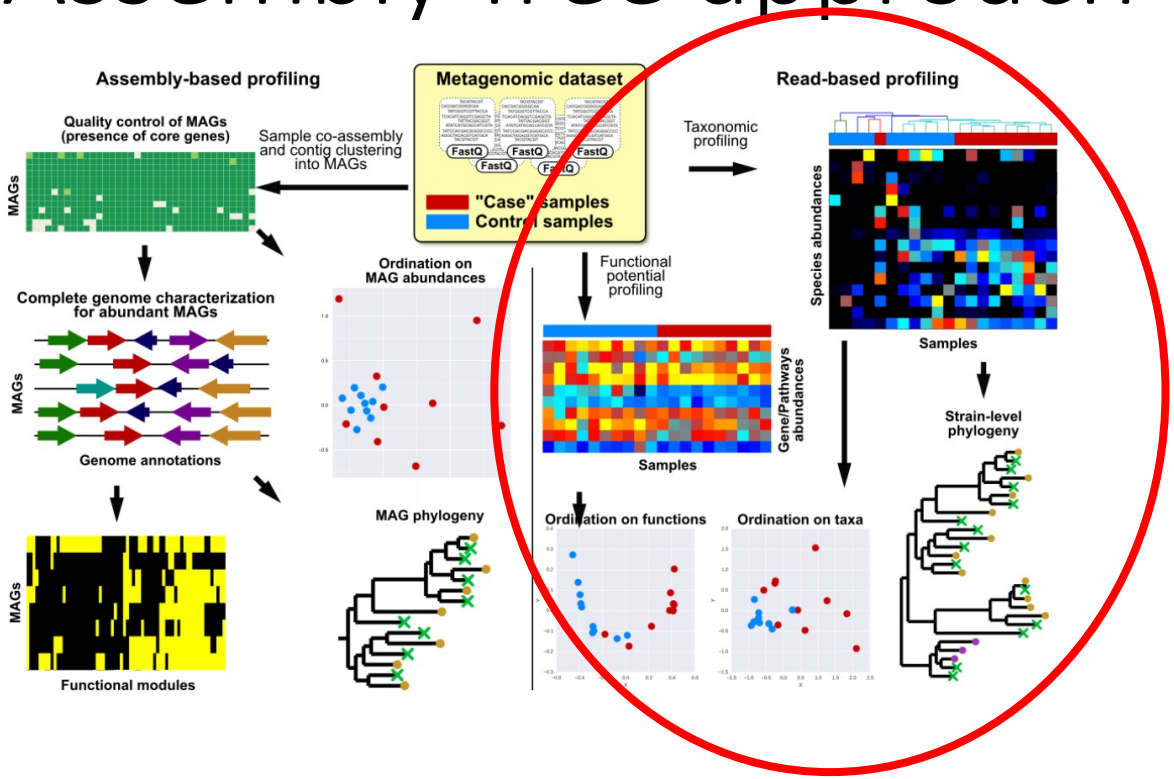






4. Assembly – free taxonomic profiling and annotation

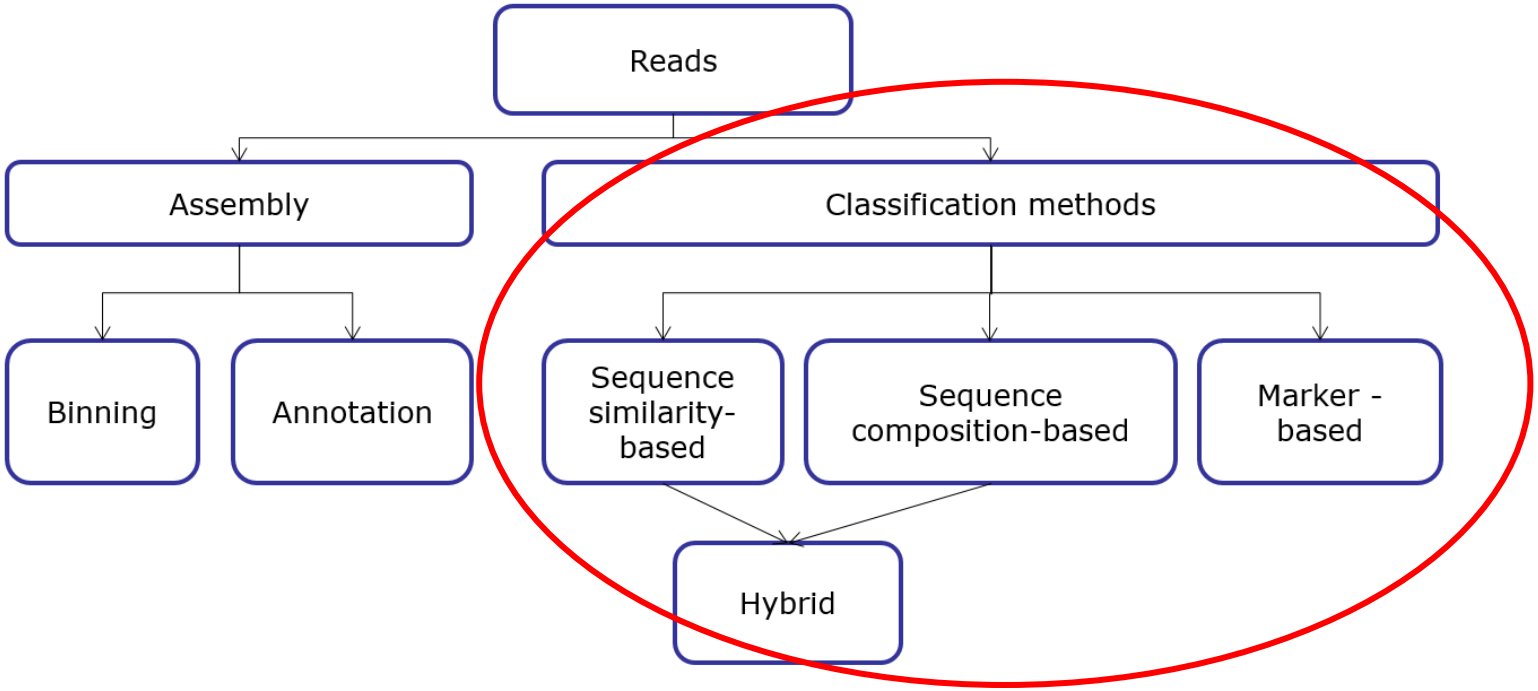
Assembly-free approach



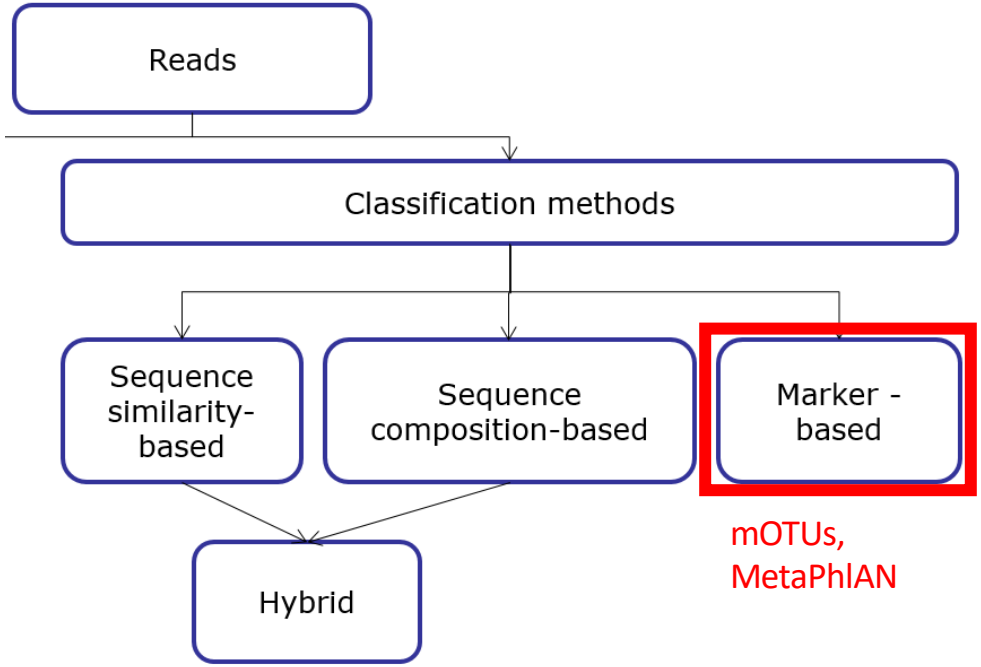
Why?

- speed up computation – assembly methods take a long time
- make it possible to profile low-abundance organisms that cannot be assembled de novo – but database dependent
- might have improved sensitivity
- can answer questions about presence/absence

Assembly-free approach



Assembly-free approach



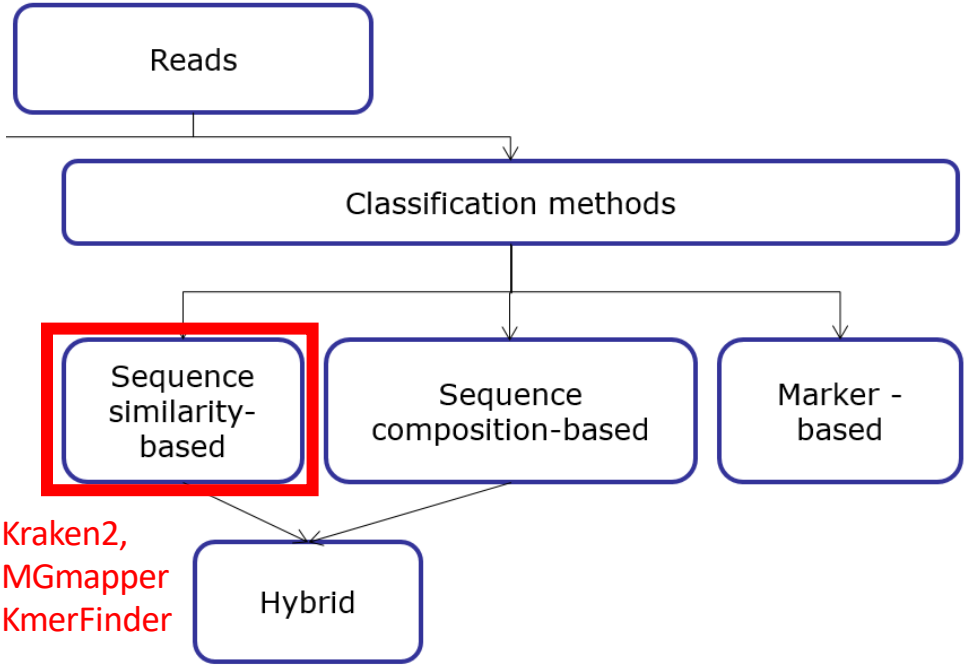
mOTUs, MetaPhlAN

MetaPhlAn 3.0

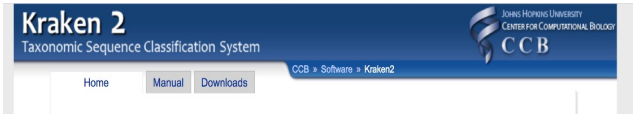
(http://huttenhower.sph.harvard.edu/metaphlan/)

- 2,887 genomes available from the Integrated Microbial Genomes (IMG) system – 2 million potential markers

Assembly-free approach



Kraken2,
MGmapper
KmerFinder

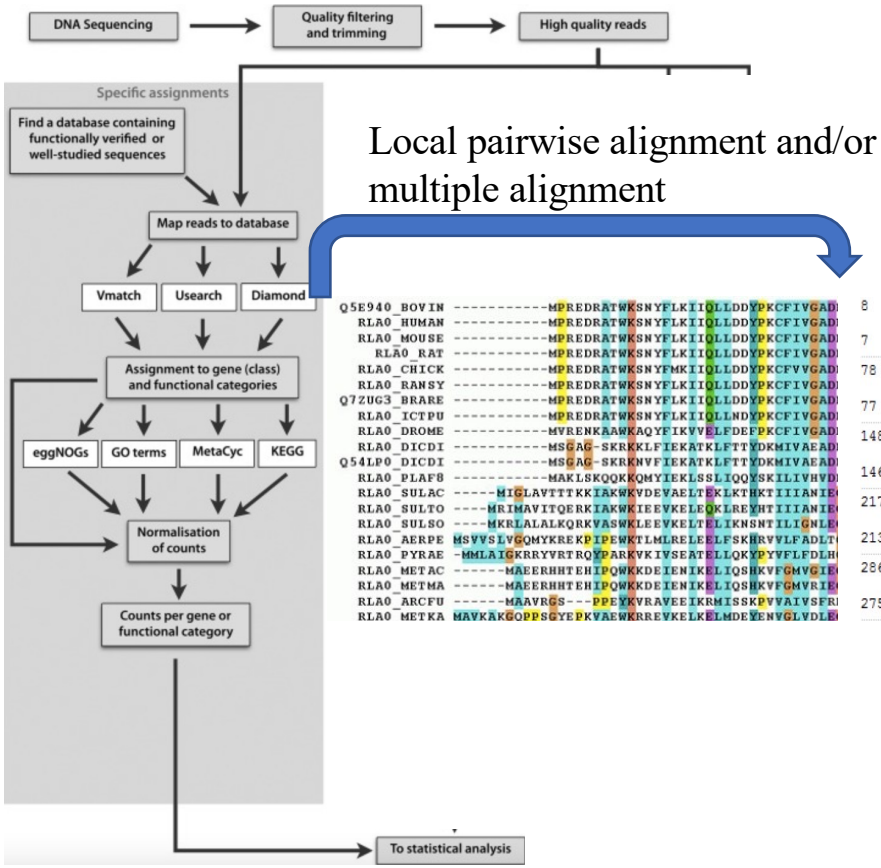


(**https://ccb.jhu.edu/software/kraken2/**)

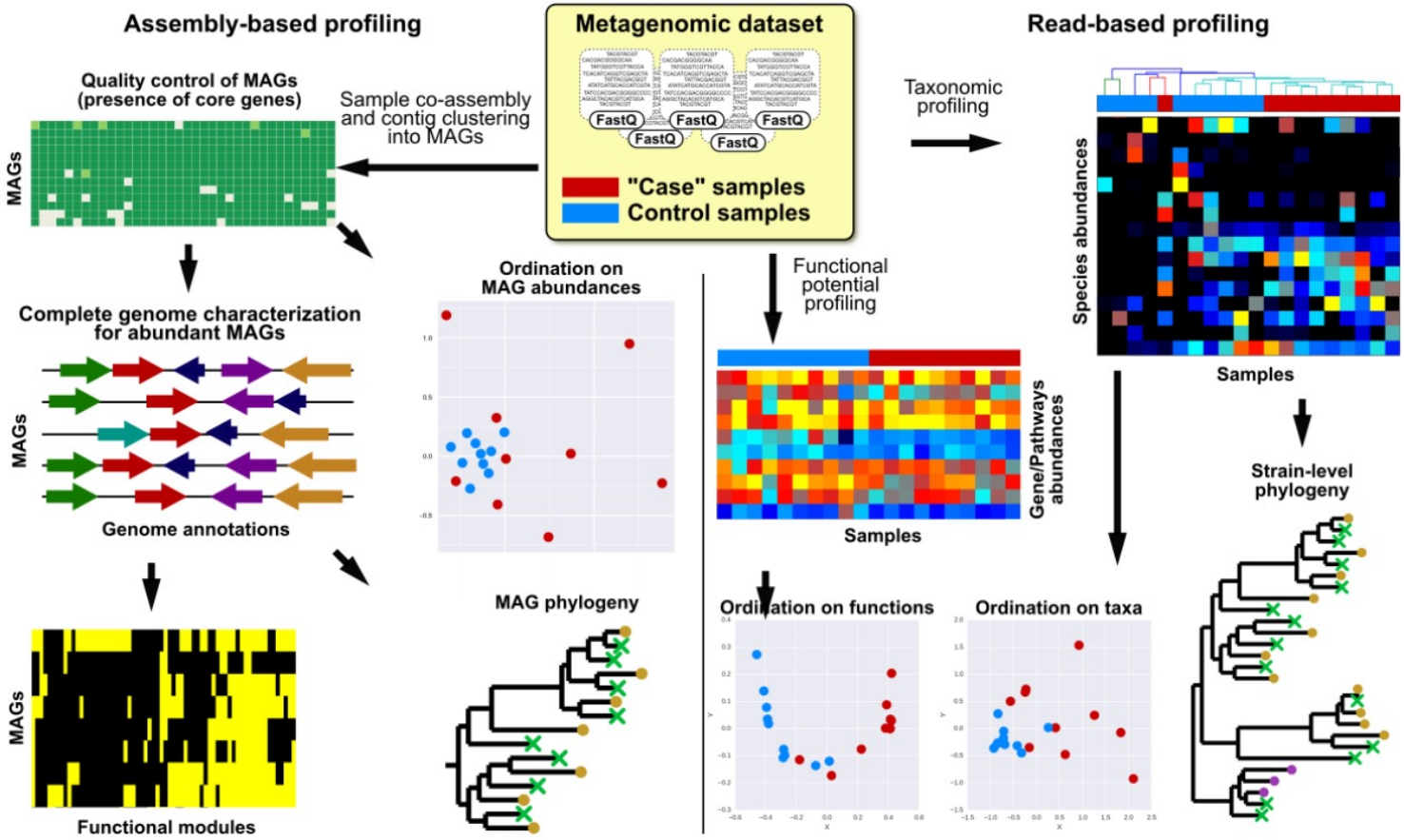
KmerFinder 3.2

(https://cge.cbs.dtu.dk/services/KmerFinder/)

Annotation

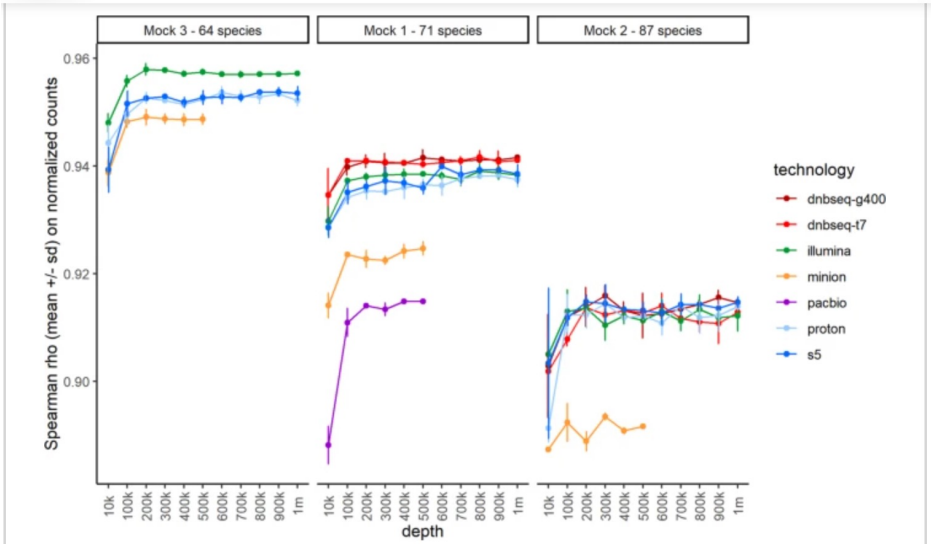

DNA Sequencing
Quality filtering and trimming
High quality reads
Specific assignments
Find a database containing functionally verified or well-studied sequences
Map reads to database
Vmatch
Usearch
Diamond
Assignment to gene (class) and functional categories
eggNOGs
GO terms
MetaCyc
KEGG
Normalisation of counts
Counts per gene or functional category
To statistical analysis
Local pairwise alignment and/or multiple alignment

Conclusions



Illumina/ONT/Pacbio – Benchmarking Nov 2022

Benchmarking second and third-generation sequencing platforms for microbial metagenomics



Sequencer	Ion Proton P1 (spades)	Ion S5 (spades)	Illumina HiSeq 3000 (spades)	DNBSeq G400 (spades)	DNBSeq T7 (spades)	ONT MinION R9 (metaflye)	PacBio Sequel II (metaflye)
Nb Reads (M)	20	20	2×10	2×10	2×10	0.696	0.524
Nb Contigs	45,510	43,879	40,147	44,887	44,603	1,283	**437**
Largest Contig (bp)	384,996	794,907	1,599,668	1,063,396	1,002,925	4,324,150	**7,147,004**
N50 (bp)	7,847	9,089	13,707	8,519	8,184	759,940	**2,013,697**
Genome Fraction(%)	54.767	55.257	61.897	49.397	47.365	44.955	**68.197**
Mismatches per 100kbps	83.29	89.12	47.55	77.22	107.52	339.99	**18.3**
Indels Per 100kbps	77.8	50.03	3.53	3.23	3.67	764.45	11.76
Fully Unaligned Contigs	1,497	1,339	975	735	1,368	231	**6**
Fully Unaligned Length (bp)	900,150	821,545	620,805	426,856	711,992	6,279,694	**134,713**
NB full genome*	5	5	12	7	7	22	**36**

https://www.nature.com/articles/s41597-022-01762-z

Bash commands cheat sheet

pwd
– show the path to the current directory, check where you are
cd <dir>
– go into this directory
cd ..
– exit this directory/go to the previous directory
ls
– check the contents of the directory
ls -alt
– check the contents of the directory but also file sizes/date of creation/last author
cat <file>
– show the contents of the file
head <file>
– show only the 10 first lines of a file
tail <file>
– show only the 10 last lines of a file
grep <pattern> <file>
– find a pattern/phrase/word in a file
cp <file1> <file2>
– copy one file into another file
mv <file1 <file2>
– rename a file

Tutorial

- Time to get some work done! Lets go back to https://github.com/zajacn/metagenomics_course_FGCZ