Detection/characterisation of undescribed genetically modified bacteria by statistical analysis of high throughput sequencing data

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Supervision :

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ANSES Ploufragan

15/03/2023

1. Introduction
2. Objectives
3. Data preparation
4. Calculation of the distances
5. Design of the prediction model
6. Results
7. Conclusion
8. Perspectives

1. **Introduction**
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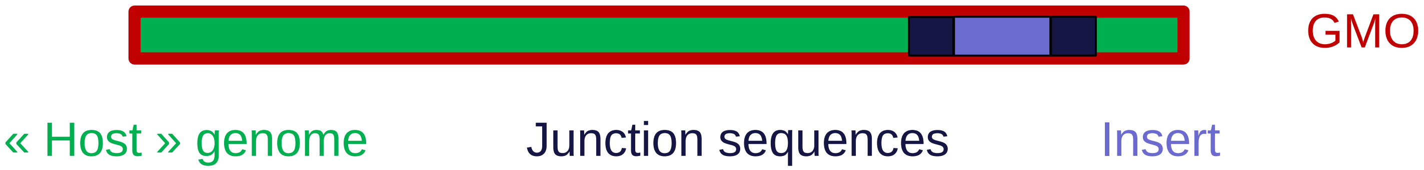
A – Genetically Modified Organism : GMO

- Living being whose genetic material has been modified in a non-natural way

- Simplified description of the structure of a GMO



GMO

« Host » genome

Junction sequences

Insert

- Insert : often CDS(s) (coding sequence)

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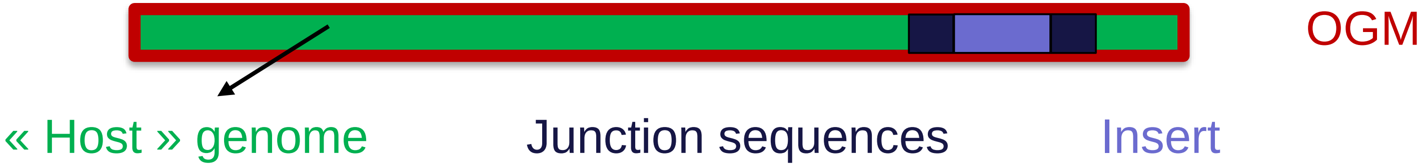

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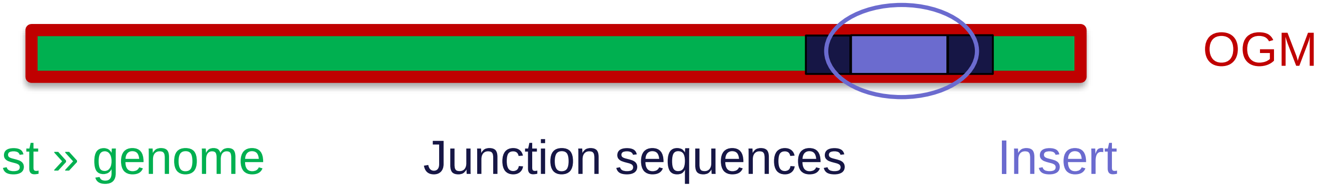

- Insert : often CDS(s) (coding sequence)

B – Existing detection methods

- For known GMO
  - Methods based on protein detection or DNA detection
  - Use of qPCR : greater sensitivity and acurate quantification
  - GMOseek [Morisset et al. 2014]

Credit: Vit Kovalcik/Shutterstock.com

B – Existing detection methods

- For partially known GMO
  - New sequencing techniques coupled with
    - Molecular methods [Fraiture *et al.*, 2017, 2018]
    - Bioinformatics/Statistics [Willems *et al.*, 2016]
  - DNA walking [Fraiture *et al.*, 2015, 2018]
- For unknown GMO
  - No method available so far

C – State of the art

- Current detection limits

	Detection of known and partially known GMO		**Detection of unknown GMO**	
	Prokaryotes	Eukaryotes	Prokaryotes	Eukaryotes
Intergenic sequences	✔	✔	✘	✘
Truncated gene	✔	✔	~	~
Fused gene	✔	✔	~	~
Insertion/deletion in a gene	✔	✔	✘	✘
% of point mutations ≥ 9%	✔	✔	✘	✘
% of point mutations < 9%	✔	✔	✘	✘

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A – General information

- No method available for the detection of unknown GMO

- Creation of a method to address this issue
  - **DUGMO** : Detection of Unknown Genetically Modified Organism [Hurel *et al.*, BMC Bioinformatics 2020] https://github.com/ANSES-Ploufragan/DUGMO

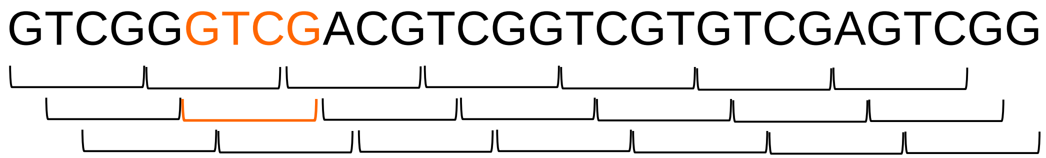
- Basic idea of the method
  - Identify the vocabulary differences between the host genome and the insert

B – General principle of DUGMO

- Particularity : use the CDS of the host genome
- Specific genomic vocabulary
  - Species-specific
  - Composed of words

Nucleotidic sequence

Species 1



GTCGGGTCGACGTCGGTCGTGTCGAGTCGG

4-letter words

B – General principle of DUGMO

- Particularity : use the CDS of the host genome
- Specific genomic vocabulary
  - Species-specific
  - Composed of words

Nucleotidic sequence

Species 1	GTCGGGTCGACGTCGGTCGTGTCGAGTCGG
	Over-represented 4 letter-words
Species 2	AGGCTCAGCTGAGCTTCAGTCGTGTACTAGC

C – Steps in DUGMO

1. Data preparation

    - Cleaning, assembly, annotation pipeline
    - Sorting of the CDSs in the sample
    - Filtering of the databank of known GMOs

2. Characterisation of the « host » genome CDSs based on their over-represented words

3. Calculation of the distances to support the comparaison to the host genome CDSs

4. Design of a prediction model

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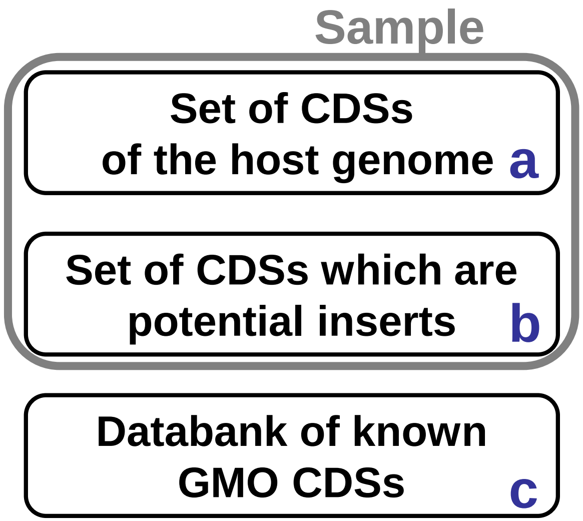

Sample

Set of CDSs of the host genome a

Set of CDSs which are potential inserts b

Databank of known GMO CDSs c

- Characterizes what we are not looking for
- Other CDSs, absent from the pangenome
- Non-species sequence variability

- Computation of distances to characterize :
  - the vocabulary of **a**
  - the vocabulary of **c**
  - the vocabulary of each sequence in the set **b**

- Design of a prediction model

C – Steps in DUGMO

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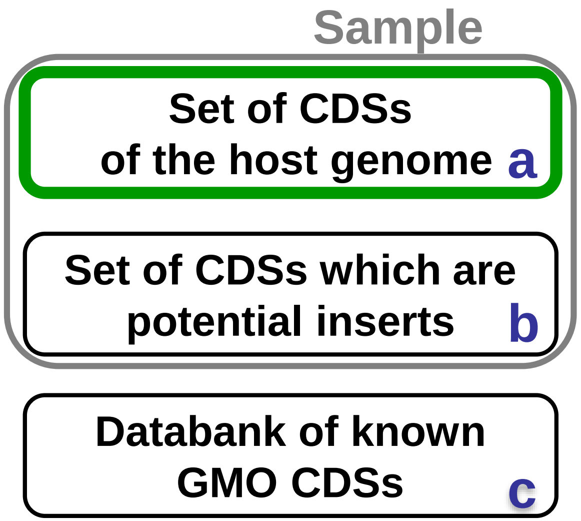

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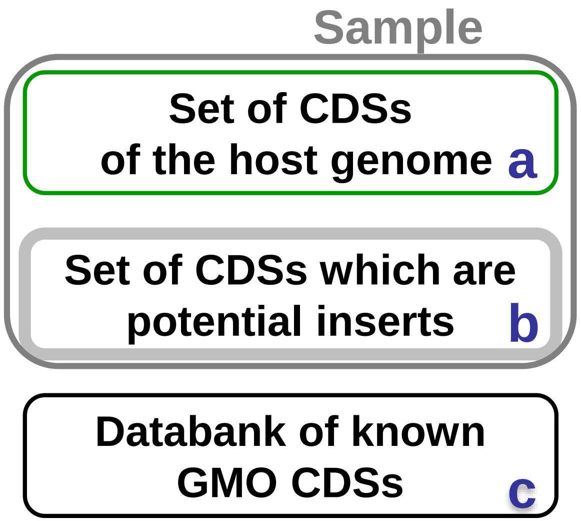

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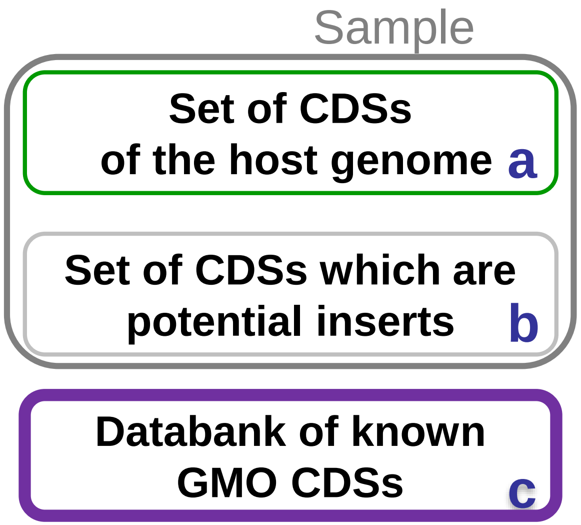

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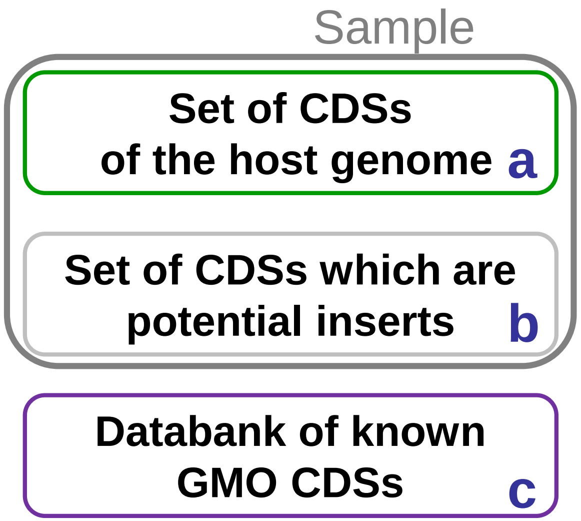

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C – Steps in DUGMO

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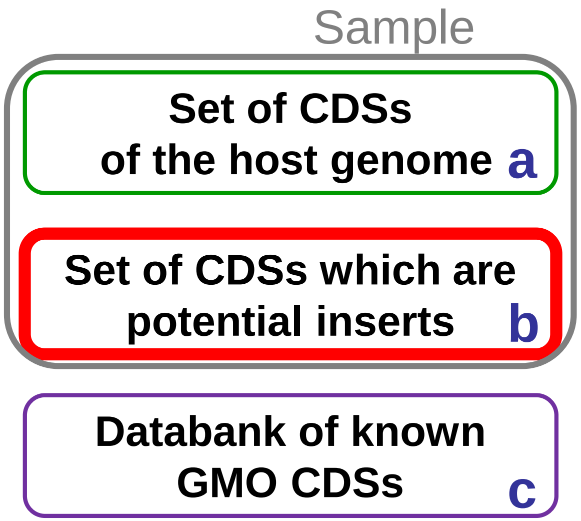

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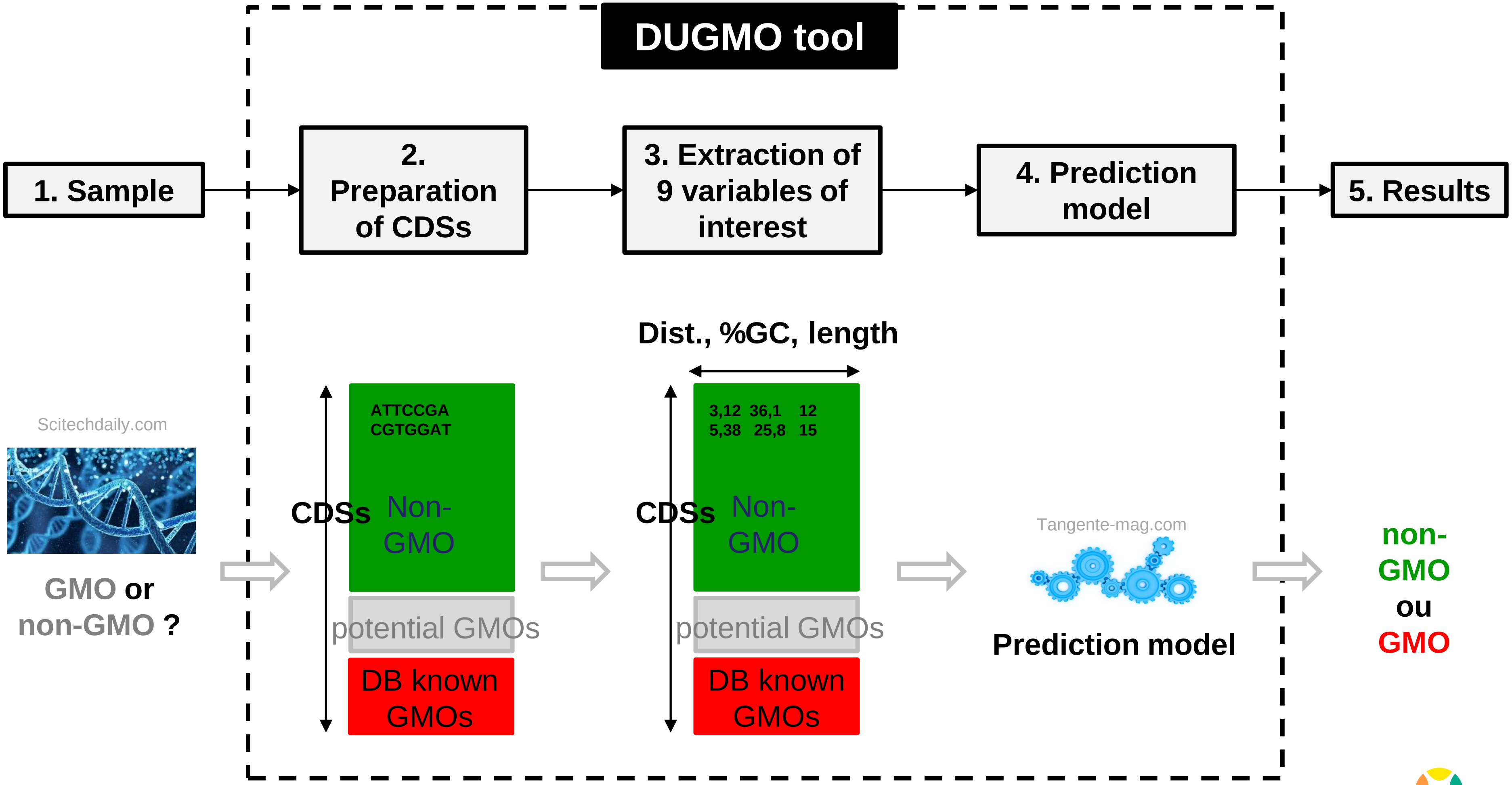
- Design of a prediction model **=> GMO ?**

C – Steps in DUGMO



DUGMO tool

1. Sample

2. Preparation of CDSs

3. Extraction of 9 variables of interest

4. Prediction model

5. Results

Scitechdaily.com

GMO or non-GMO ?

CDSs

ATTCCGA
CGTGGAT

Non-GMO

potential GMOs

DB known GMOs

Dist., %GC, length

CDSs

3,12 36,1 12
5,38 25,8 15

Non-GMO

potential GMOs

DB known GMOs

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Prediction model

non-GMO
ou
GMO

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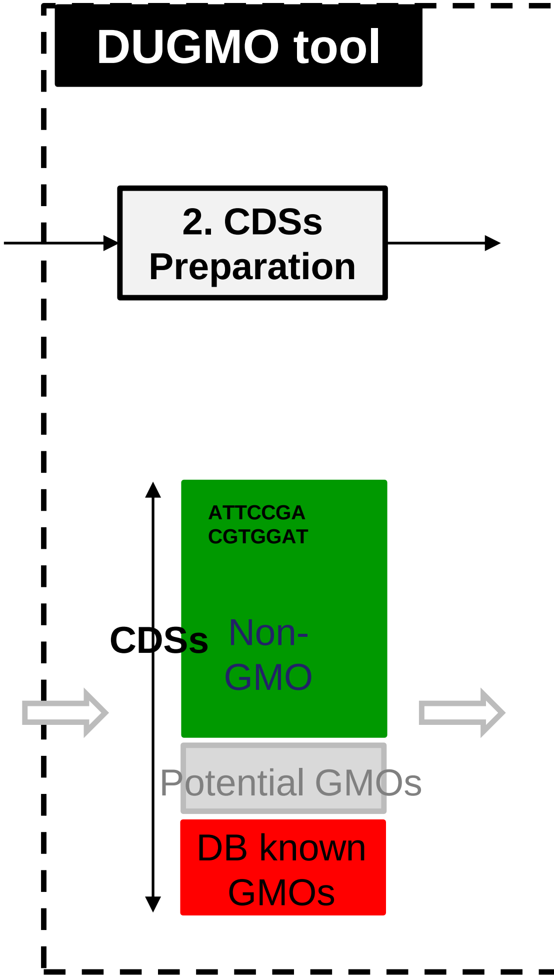
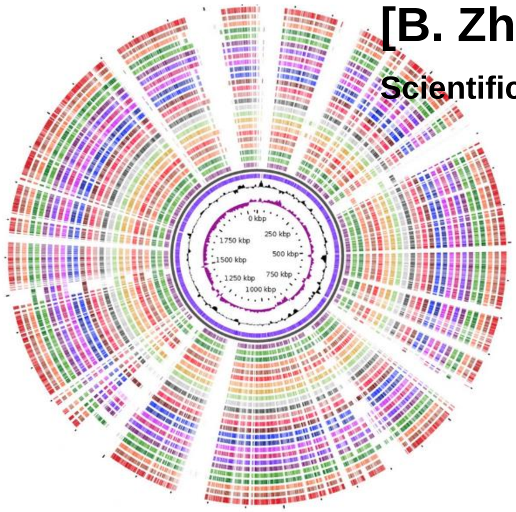
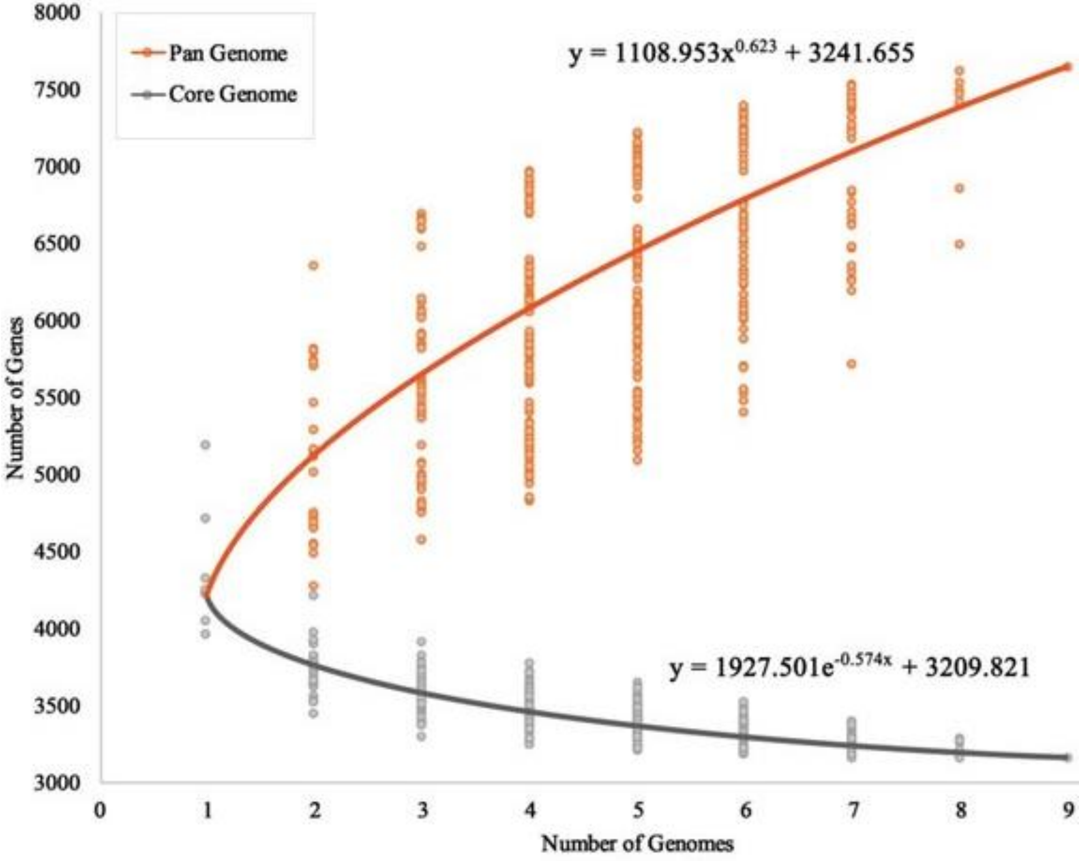
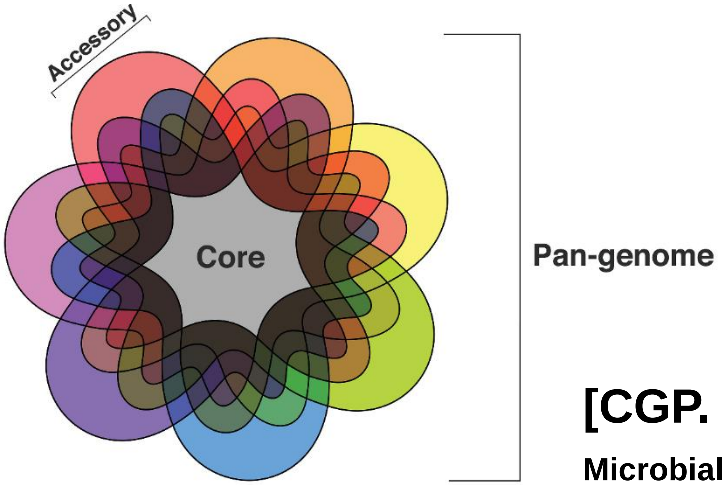


A – Pangenome: wild-type and representative of species



[B. Zheng et al.
ScientificReports 2017]





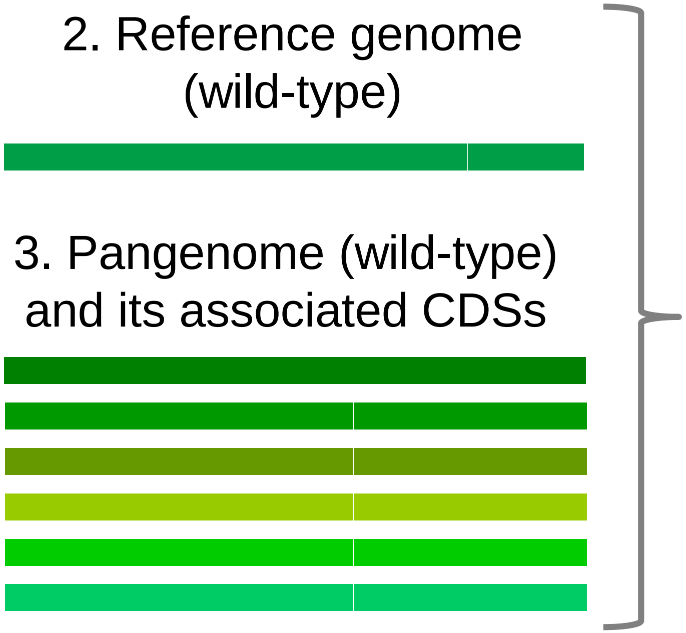
[CGP. McCarthy et al.
Microbial genomics 2019]

[D. Sinha et al.
BMC genomics 2021]

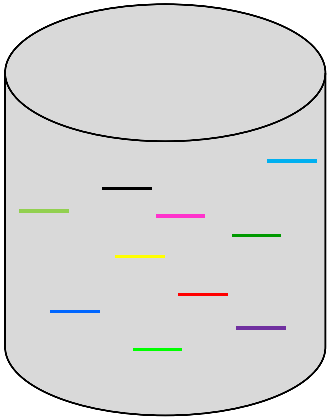
A – Data required as input to the software

illumina.com

1. High throughput sequencing data


2. Reference genome (wild-type)
3. Pangenome (wild-type) and its associated CDSs

Species of the potentially GM bacterium



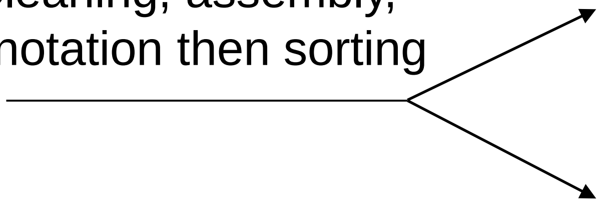
4. Databank of known GMO inserts

B – Objectives

- Data cleaning, assembly, annotation of sequencing data
- Sorting of the sample CDSs
- Filtering of the database of known GMO inserts

illumina.com

High throughput sequencing data



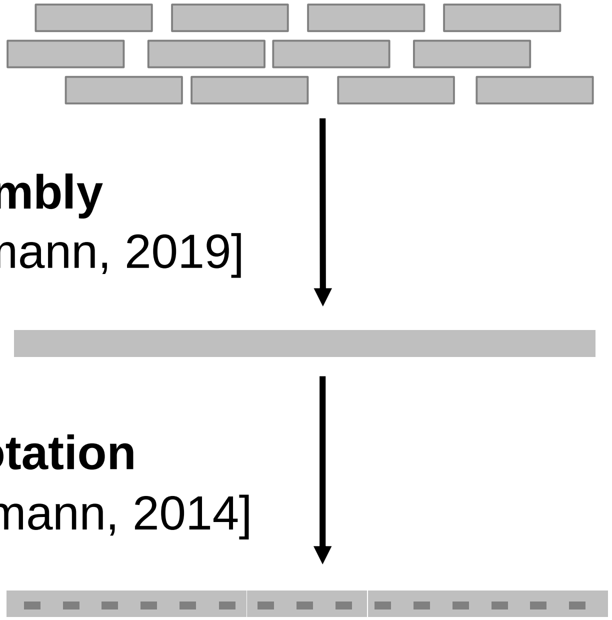
Cleaning, assembly, annotation then sorting

CDSs related to the host genome

CDSs which are potential GMO inserts

B – Cleaning pipeline

- Assembly and annotation of high throughput sequencing data



potentially GM sample

Reads

Assembly
Shovill [Seemann, 2019]

Whole genome
contigs/scaffolds

Annotation
Prokka [Seemann, 2014]

CDSs

C – Sorting of the sample CDSs

- Step 1 : Comparison of the potental GMO CDSs with pangenome's CDSs

Potential GMO CDSs

Pangenome CDSs

C – Sorting of the sample CDSs

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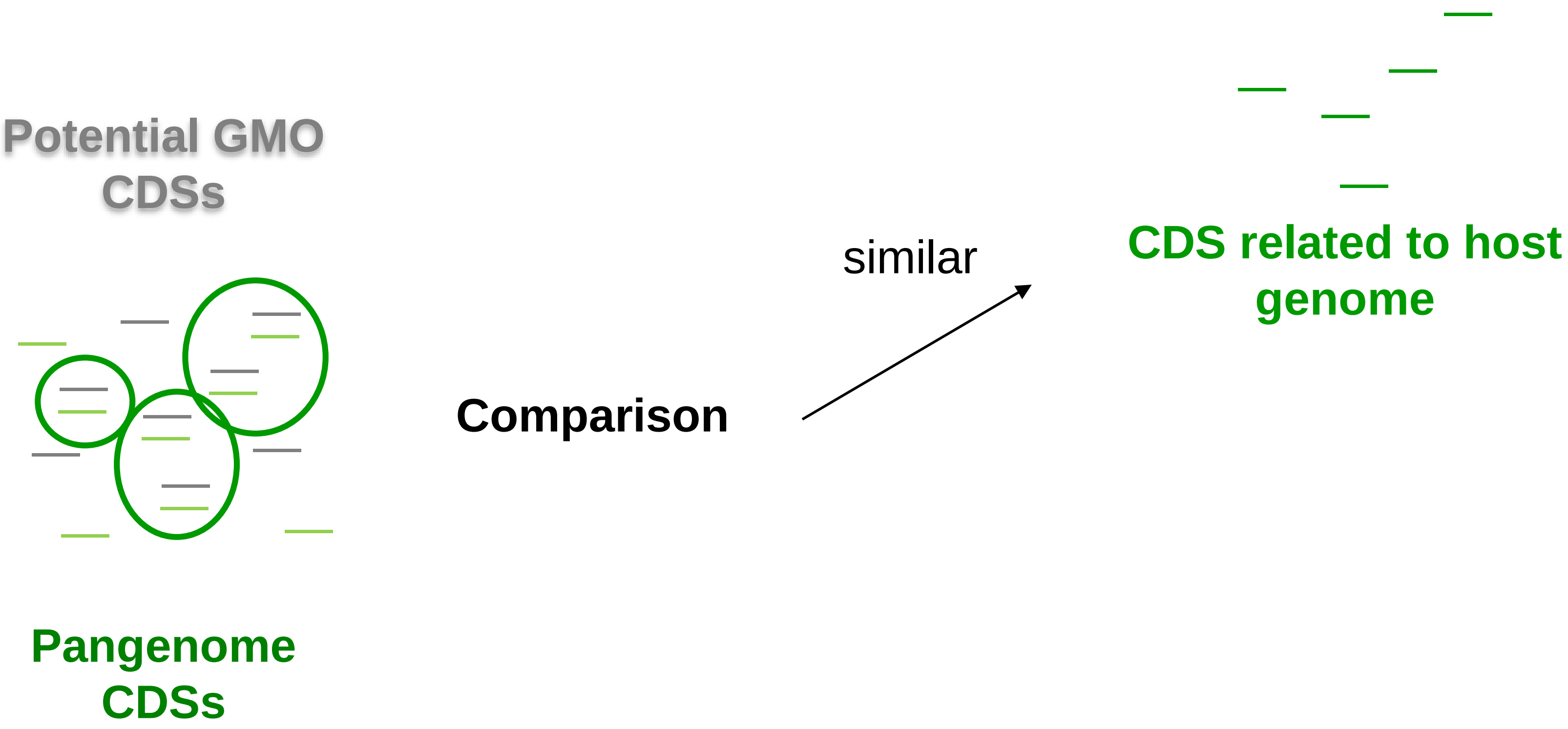

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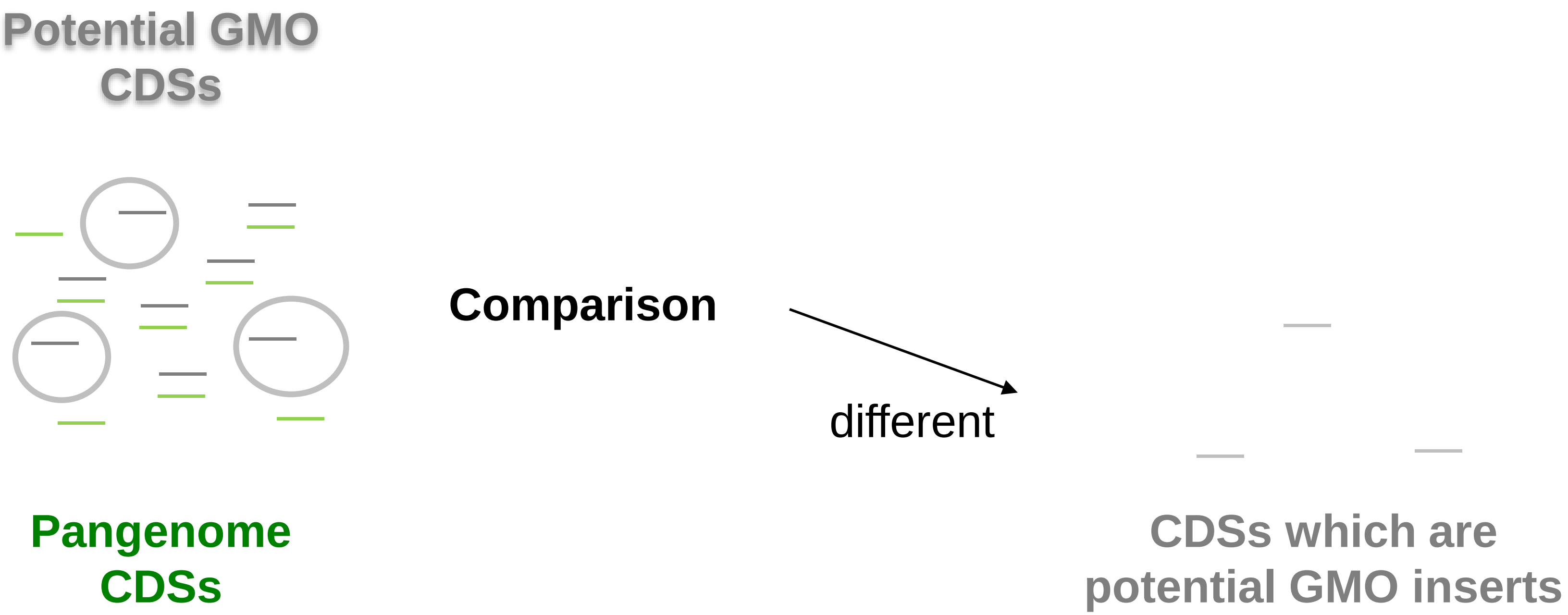

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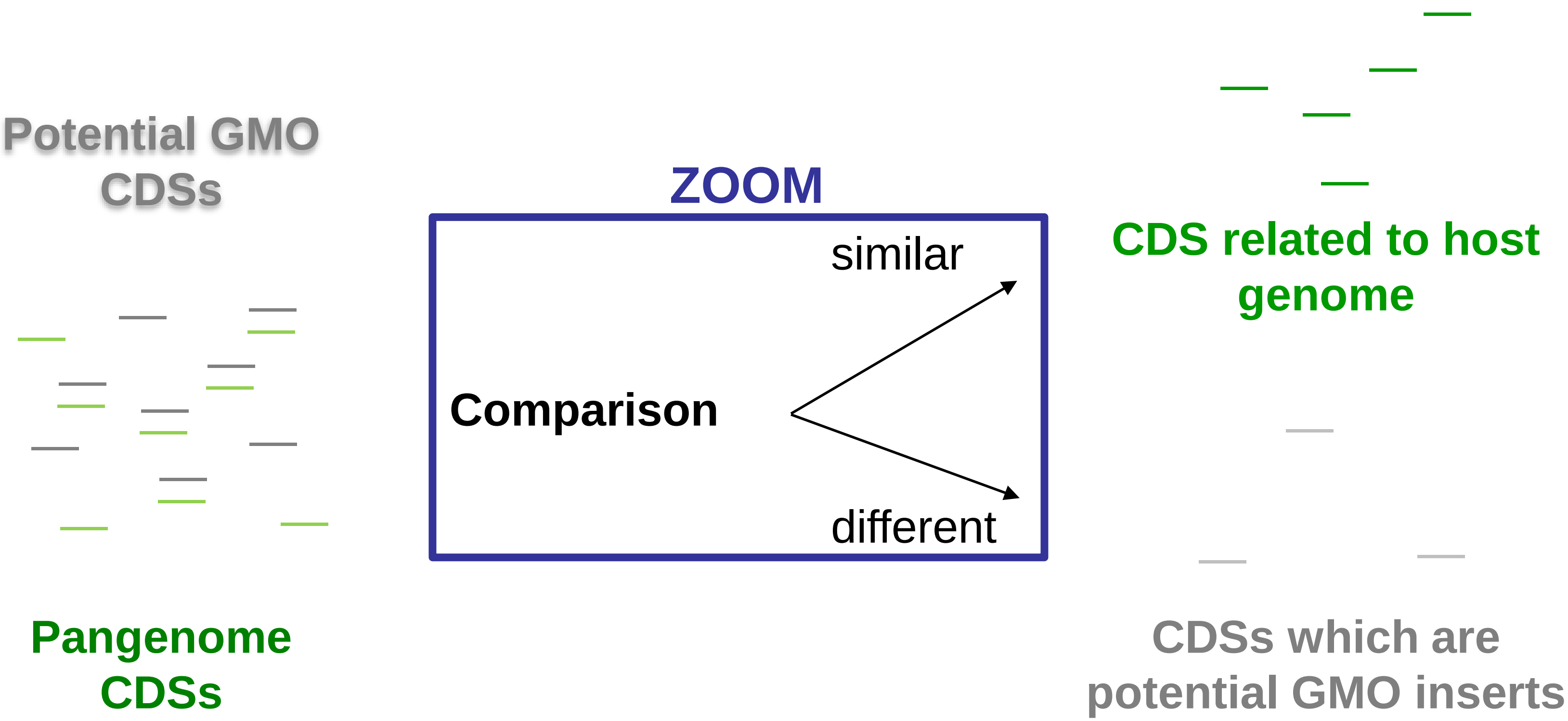


Potential GMO CDSs

Pangenome CDSs

ZOOM

Comparison

similar

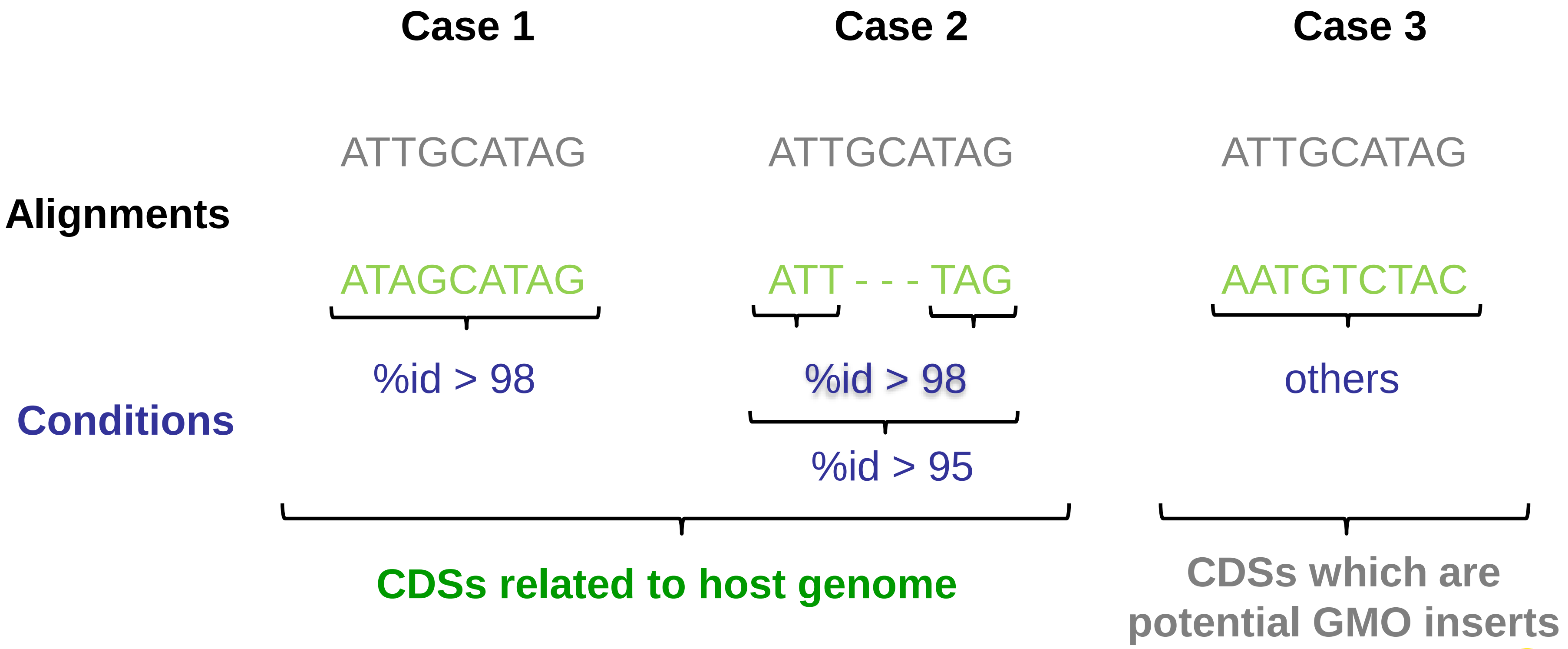
different

CDS related to host genome

CDSs which are potential GMO inserts

C – Sorting of the sample CDSs

- Step 1 : Comparison of the potental GMO CDSs with pangenome’s CDSs



Case 1 | **Case 2** | **Case 3**

Alignments

Case 1: ATTGCATAG / ATAGCATAG

Case 2: ATTGCATAG / ATT - - - TAG

Case 3: ATTGCATAG / AATGTCTAC

Conditions

Case 1: %id > 98

Case 2: %id > 98, %id > 95

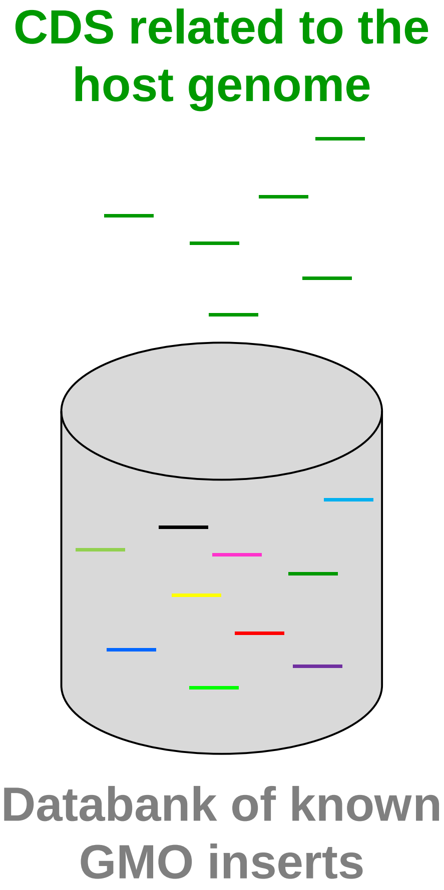
Case 3: others

CDSs related to host genome

CDSs which are potential GMO inserts

D – Filtering the GMO insert databank

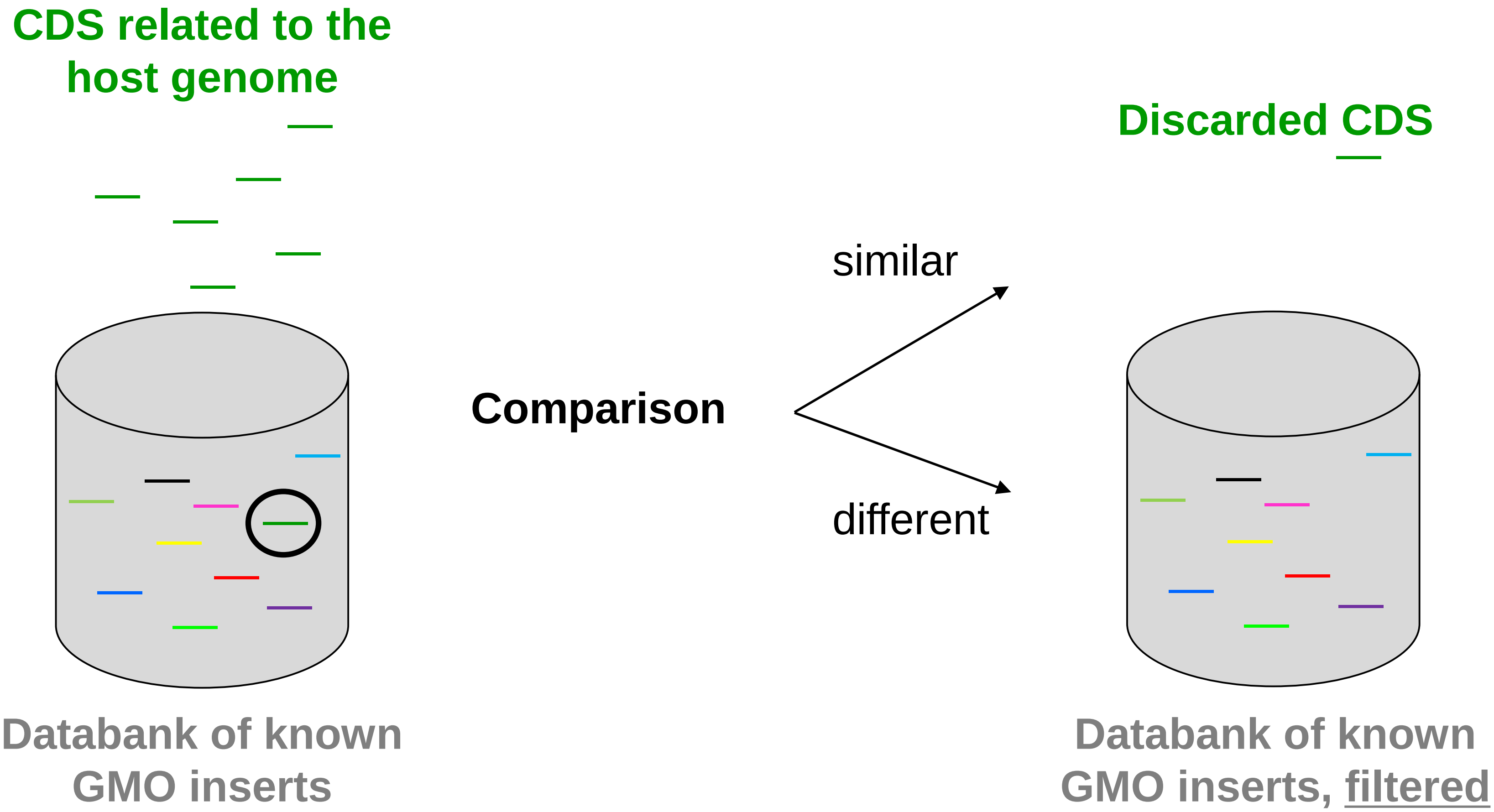
- Using the host genome CDSs



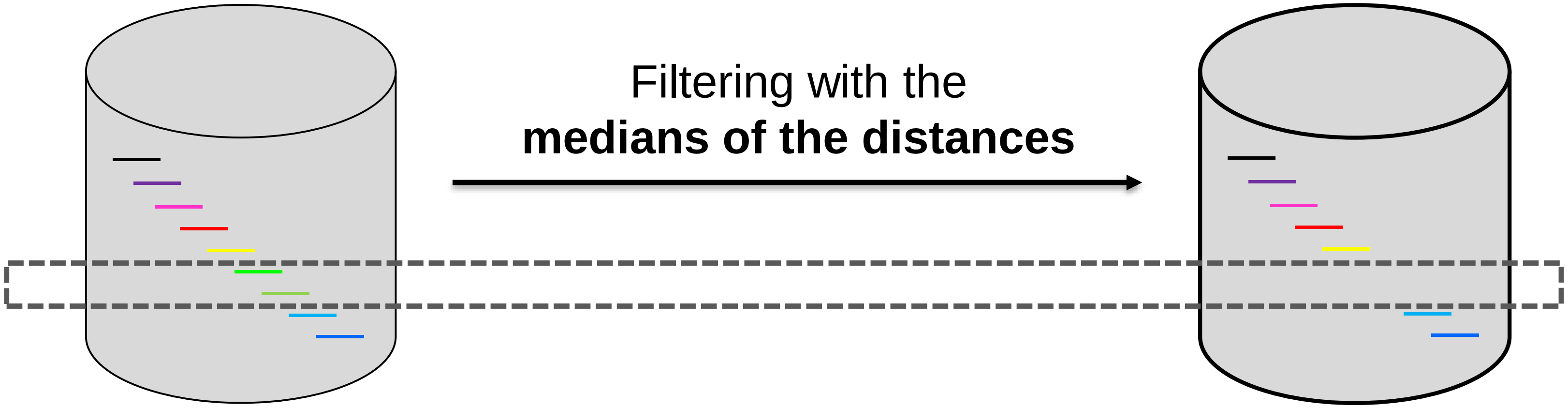
Comparison criteria identical to step 1

D – Filtering the GMO insert databank

- Using the host genome CDSs


CDS related to the host genome
Discarded CDS
similar
Comparison
different
Databank of known GMO inserts
Databank of known GMO inserts, filtered

D – Filtering the GMO insert databank

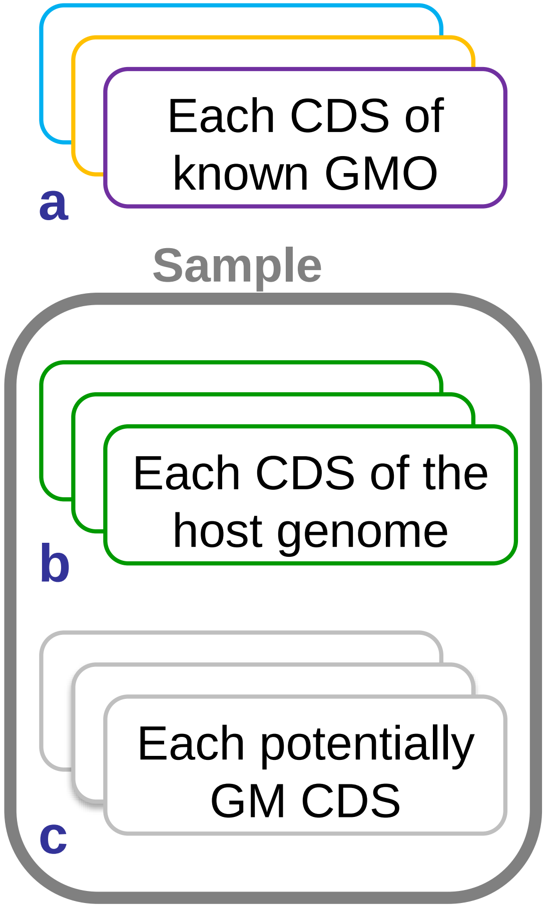


Filtering with the **medians of the distances**

Databank of known GMO inserts

Databank of known GMO inserts, filtered

C – Get the three needed distinct datasets



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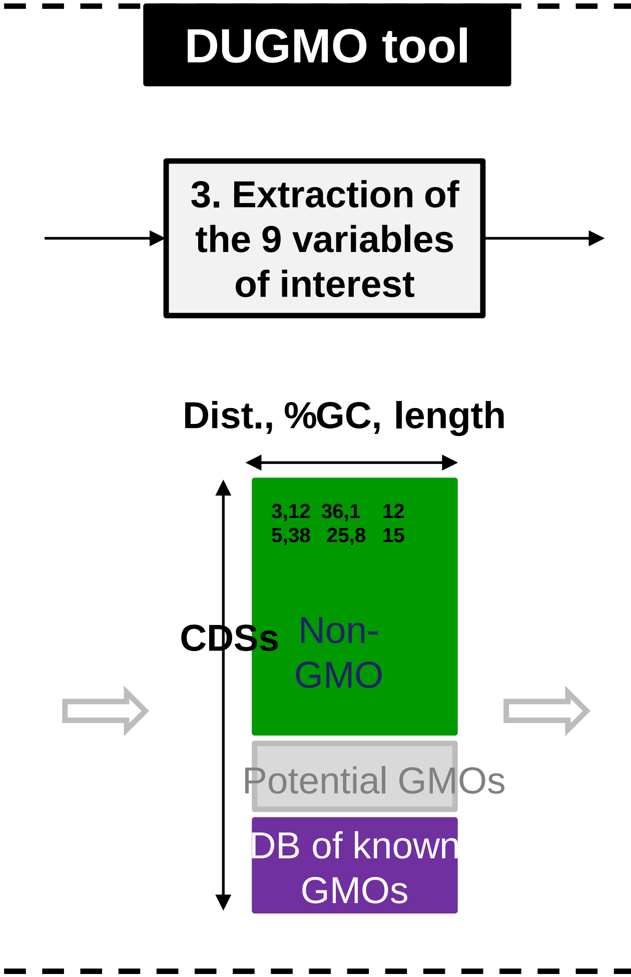

A – Objectives

- Objective : characterization of the host genome CDSs and of the two other CDS sets

- Approach : determine the best combinations of parameters

- Purpose : establish explanatory variables for the development of the prediction model

B – Characterization of the genomic vocabulary

- R’MES [Schbath, S. and Hoebeke, M. 2011]: Searches for the exceptional words in a sequence
  - Determine the number of occurrences of each word and its exceptional character
  - It defines a set of words that are over-represented in the host genome

- Three distance formula and two types of calculations
  - Euclidean distance
  - Kullback-Leibler distance [Trifonov et Rabadan, 2010]
  - Bray-Curtis distance

B – Characterization of the genomic vocabulary

- R’MES : Searches for the exceptional words in a sequence
    - Determine the number of occurrences of each word and its exceptional character
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- Three distance formula and two types of calculations
    - Euclidean distance
    - Kullback-Leibler distance [Trifonov et Rabadan, 2010]
    - Bray-Curtis ~distance (dissimilarity measurement)

B – Characterization of the genomic vocabulary

- Type of distance calculation named « in Frequencies »
  - Concatenation of the third codon positions into a new sequence

	Word size	Running R'mes
AGTACGTCAGGTAGTATCCAGCTAATG	27	impossible
TGATTCGAG	9	fast

  - 10% of over-represented words

- Type of distance calculation named « in Proportions »
  - Whole CDS
  - All words

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AGTACGTCAGGTAGTATCCAGCTAATG

TGATTCGAG

Arginine

CGU
CGC
CGA
CGG

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C – Combinations of parameters

- List of tested parameters :
  - Word sizes
  - Order of the Markov model
  - Percentage of over-/under-represented words
  - Concatenation of the third codon positions

D – Explanatory variables retained in DUGMO for one CDS

- Bray-Curtis distances
  - « In frequencies »
    - F L9M7 : Word size 9 and Markov model order 7
  - « In proportions »
    - P L3M1 : Word size 3 and Markov model order 1
    - P L4M2 : Word size 4 and Markov model order 2
- Average exceptionality scores in the host genome for L4M2 et L9M7
- Count density per nucleotide for 4-letter and 9-letter words

$$\frac{\text{Sum of all word counts}}{\text{CDS length}}$$

- Percentage of GC
- Length

D – Explanatory variables retained in DUGMO for one CDS

- Bray-Curtis distances
  - « In frequencies »
    - F L9M7 : Word size 9 and Markov model order 7
  - « In proportions »
    - P L3M1 : Word size 3 and Markov model order 1
    - P L4M2 : Word size 4 and Markov model order 2
- Average exceptionality scores provided by R’MES in the host genome for L4M2 and L9M7
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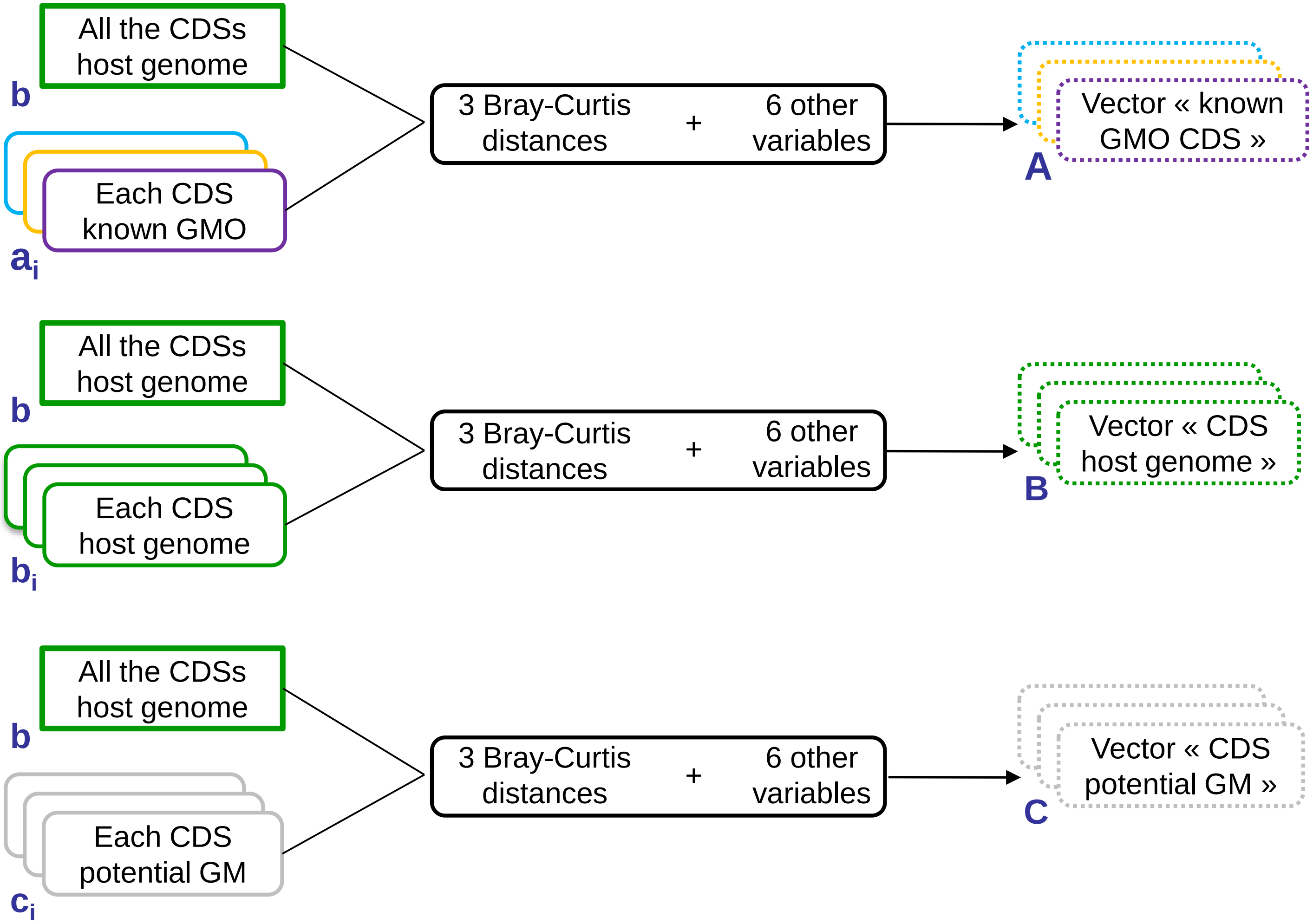
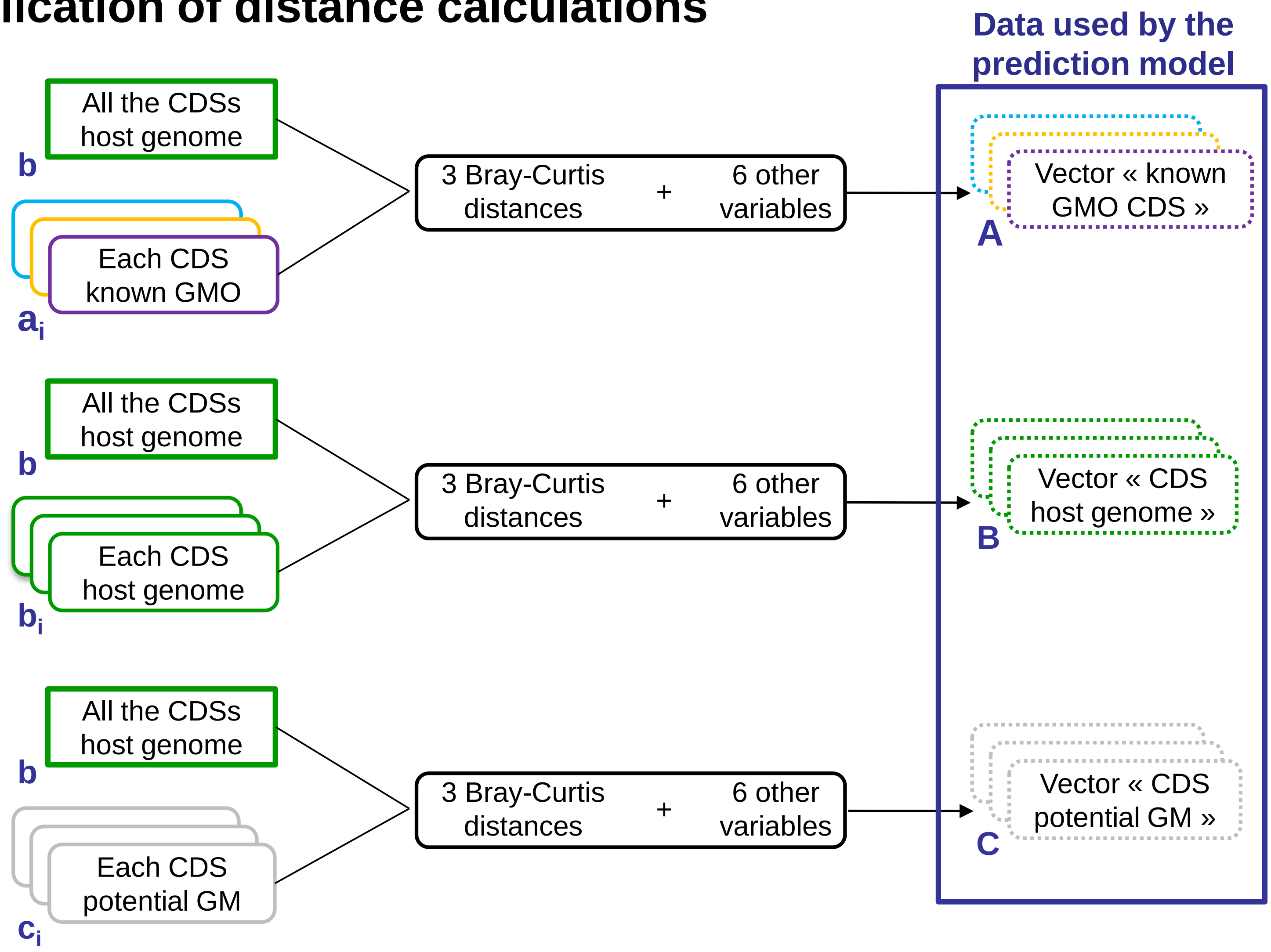
E – Application of distance calculations


All the CDSs host genome
b
Each CDS known GMO
a$_i$
3 Bray-Curtis distances + 6 other variables
Vector « known GMO CDS »
A
All the CDSs host genome
b
Each CDS host genome
b$_i$
3 Bray-Curtis distances + 6 other variables
Vector « CDS host genome »
B
All the CDSs host genome
b
Each CDS potential GM
c$_i$
3 Bray-Curtis distances + 6 other variables
Vector « CDS potential GM »
C

E – Application of distance calculations



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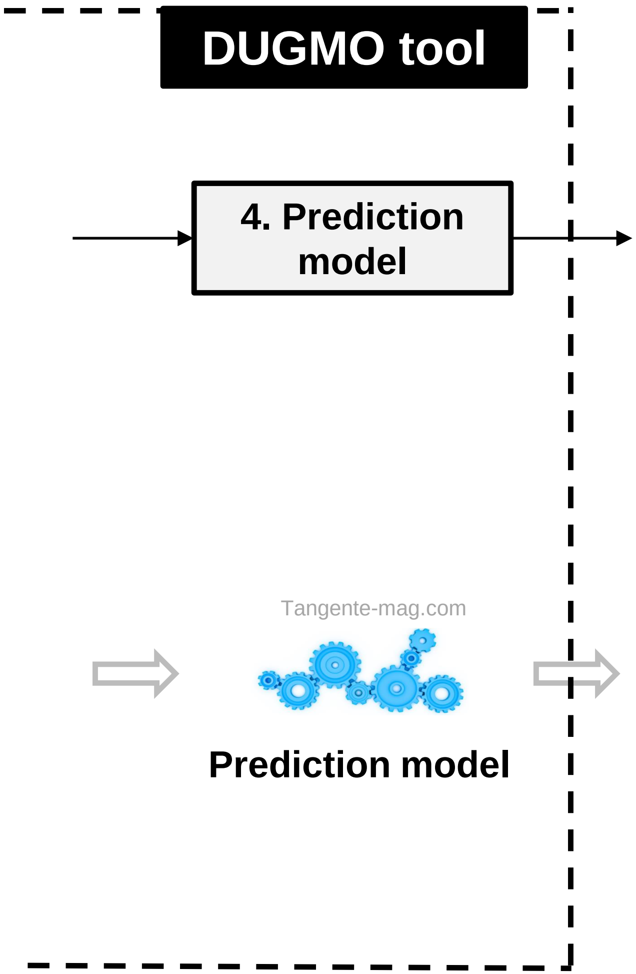


DUGMO tool

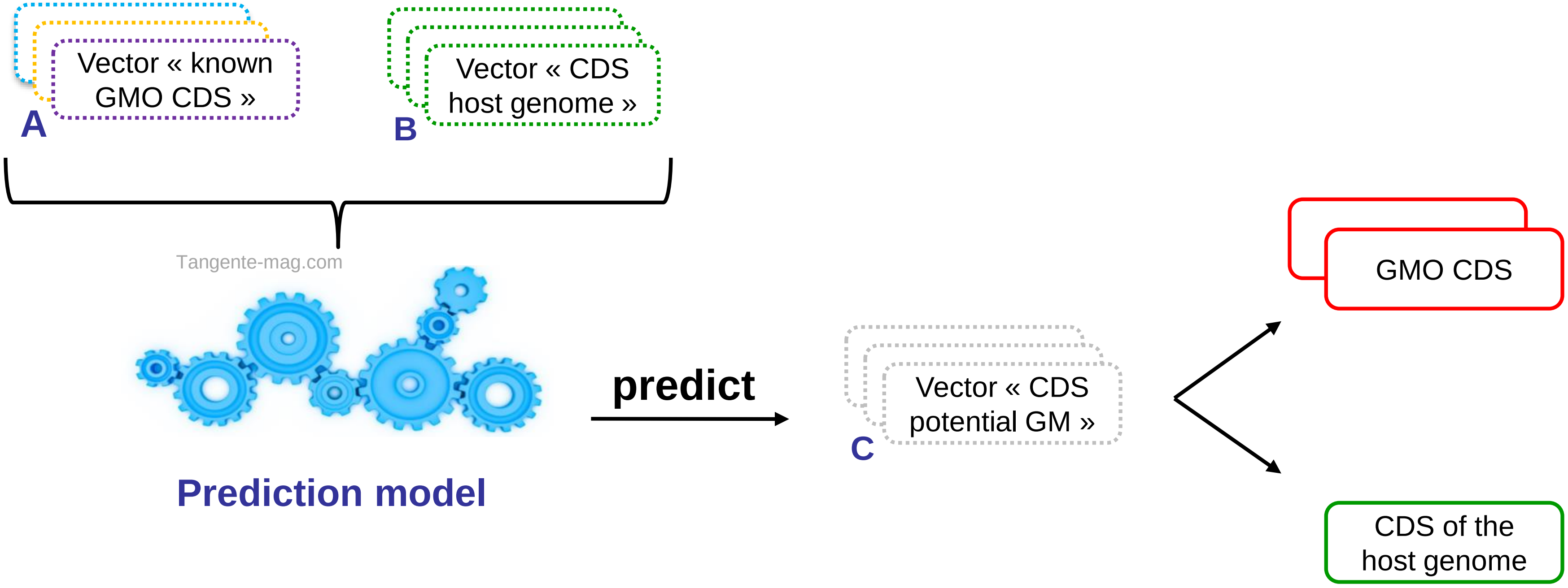
4. Prediction model

Tangente-mag.com

Prediction model

A – Objectives

- Approach : use of Machine Learning methods
- Purpose : predict proven CDSs of GMO inserts



A
Vector « known GMO CDS »

B
Vector « CDS host genome »

Tangente-mag.com

Prediction model

predict

C
Vector « CDS potential GM »

GMO CDS

CDS of the host genome

B – Machine Learning

- 12 tested methods

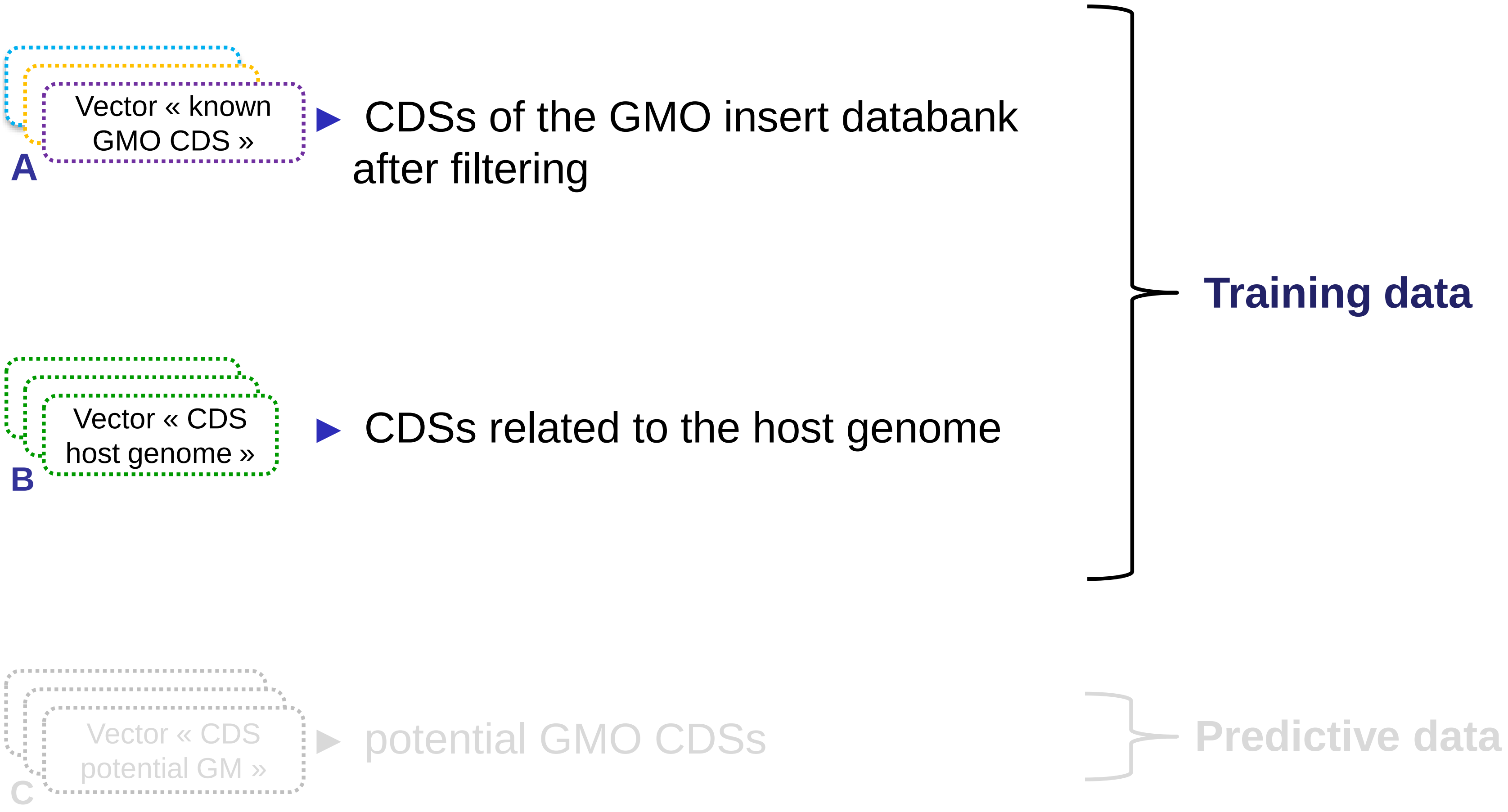
parametric

- Logit : generalized linear model
- StepLDA : linear discriminant analysis
- StepQDA : quadratic discriminante analysis
- Plsda : partial least squares regression

non parametric including decision trees

- NN : neural networks
- SvmRadial : support vector machines
- KNN : K nearest neighbors
- C5.0 : classification algorithm
- Rpart : recursive partitionig trees
- RF : random forests
- Treebag : classification trees with bagging
- Xgboost : extreme gradient boosting

C – Used data



A

Vector « known GMO CDS »

▶ CDSs of the GMO insert databank after filtering

B

Vector « CDS host genome »

▶ CDSs related to the host genome

Training data

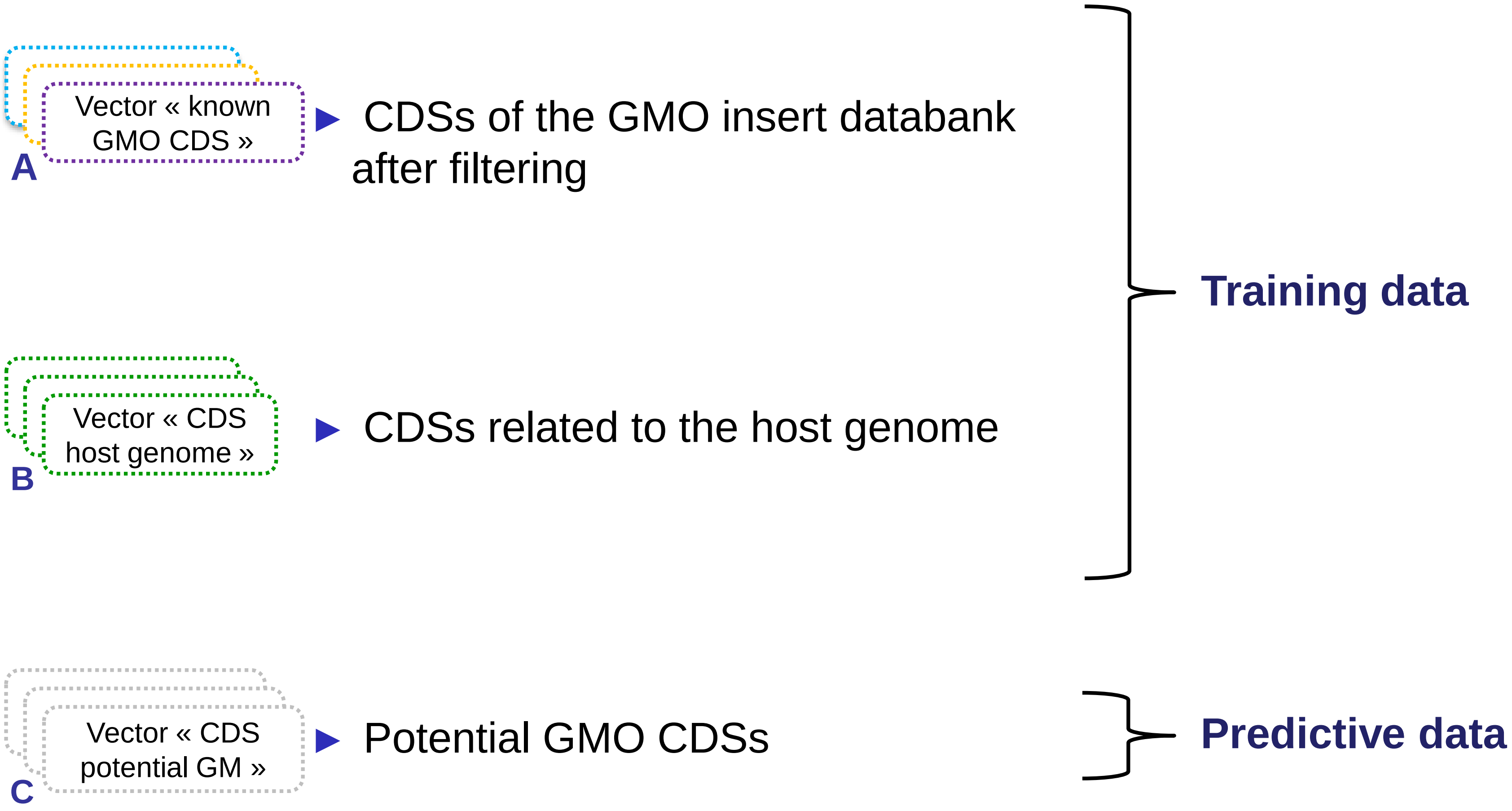
C

Vector « CDS potential GM »

▶ potential GMO CDSs

Predictive data

C – Used data



A – Vector « known GMO CDS » ▶ CDSs of the GMO insert databank after filtering

B – Vector « CDS host genome » ▶ CDSs related to the host genome

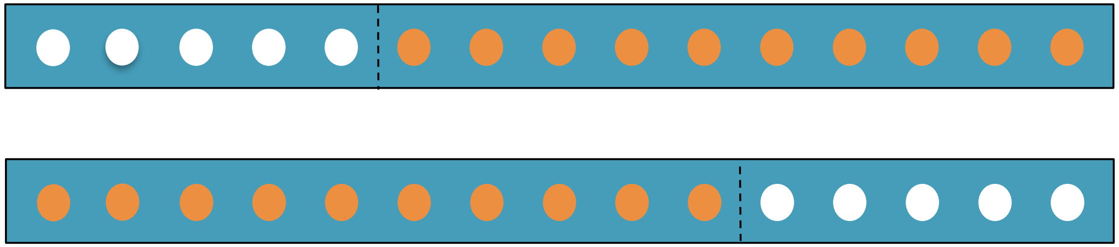
Training data

C – Vector « CDS potential GM » ▶ Potential GMO CDSs

Predictive data

D – Comparison of the tested methods

- Centered, reduced, and stratified data
- Optimisation of the parameters of each method in 10-fold cross-validation
- 2-fold cross-validation comparison of methods



2-fold cross-validation

E – Selection criteria

- Confusion matrix

		Predictions of a model	
		GMO	**Non-GMO**
Real data	**GMO**	True positives	False negatives
	Non-GMO	False positives	True negatives

- **Specificity : ++**
- **False positive rate: --**
- **Sensitivity : ++**
- **False negative rate : +++**

F – Final choice

- Union of the results of two methods
  - **RF** : Random Forests
  - **Logit** : Generalized linear model

	Results for prediction data		
	Logit	**RF**	**Union of RF and Logit**
False negative rate	0.04	0.01	0.01
Specificity	0.94	0.98	0.99
Sensitivity	0.95	0.98	0.99
False positive rate	0.05	0.01	0.09

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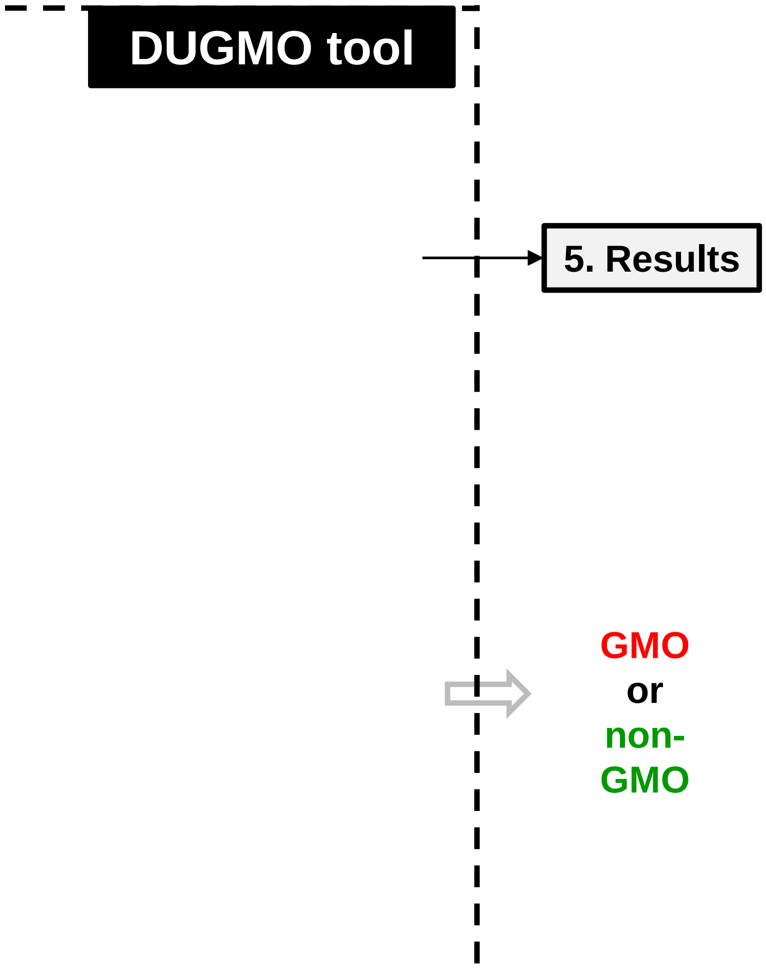
8. Perspectives



DUGMO tool

5. Results

GMO
or
non-GMO

A – Tested data

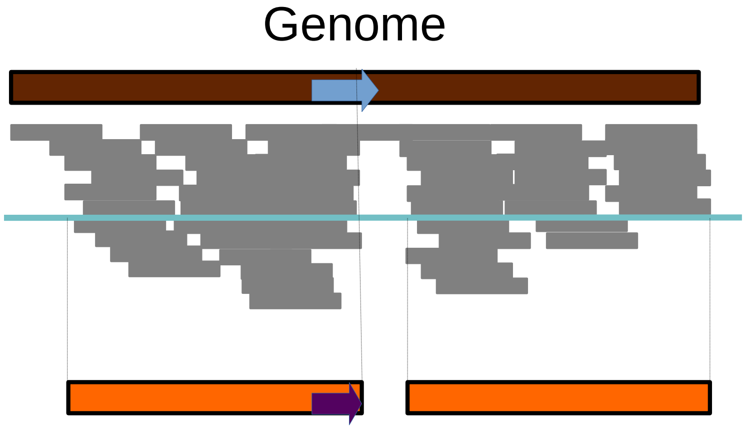
- **1 wild-type** genome and **1 GM** genome of *B. subtilis* bacterium
- **3 GM** genomes of *E. coli* bacterium
- **6 wild-type** genomes of bacteria
  - *Campylobacter jejuni*
  - *Lactococcus lactis*
  - *Listeria monocytogenes*
  - *Mycobacterium tuberculosis*
  - *Staphylococcus aureus*
  - *Salmonella* Typhimurium
- **42 synthetic GM** genomes
  - Combinations : 6 wild-type bacteria + 7 exogenous genes

B – Global results

- With the 2 *B. subtilis* genomes
  - WT: No insert ✓
  - GM: 25 detected inserts + ✓
    12 false negatives (maximum)
- With the 3 GM genomes of *E. coli*
  - **3** known inserts are detected ✓
  - **1 false positive** ✗ → ✓
- With the 48 synthetic WT genomes
  - **47**: No insert ✓
  - **1 false positive** (*M. tuberculosis)* ✗ → ✓
- With the 48 synthetic GM genomes
  - **47**: insert found ✓
  - **1 false negative** (*S. aureus* including a *C. jejuni* gene) ✗

C – GM *Escherichia coli*

- In the genome modified with a gene from *S. pyogenes* : 1 false positive
  - *arlS* gene
  - **Truncated gene because of the assembly**



Legend

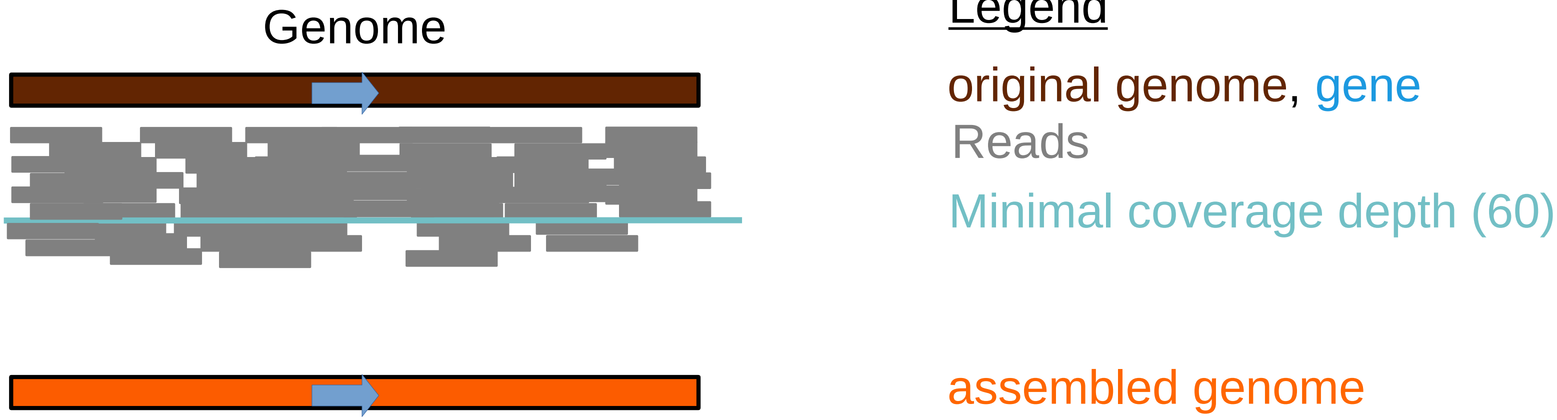
Original genome, gene
Reads
Minimal coverage depth (60)

assembled genome, truncated gene

C – GM *Escherichia coli*

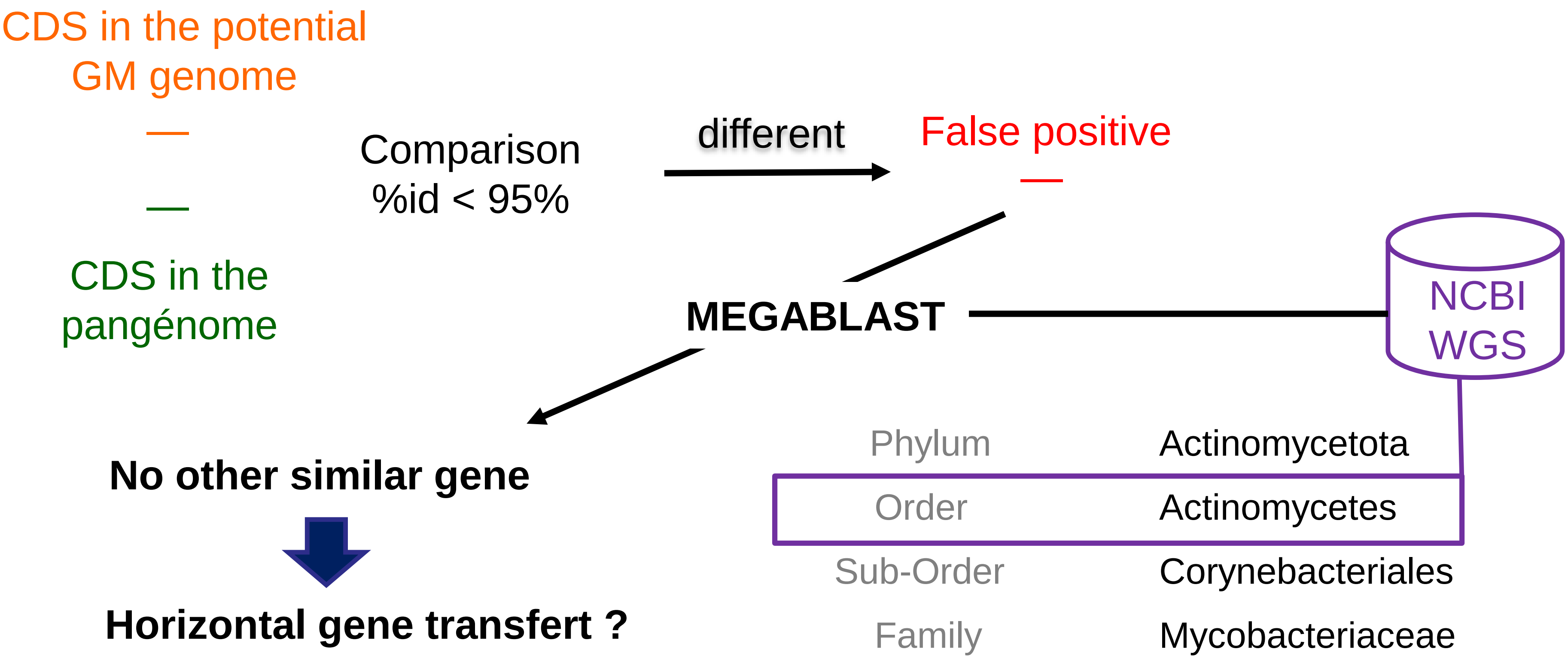
- In the genome modified with a gene from *S. pyogenes* : ~~1 false positive~~

- Solution : **minimal coverage depth of 60** (bacteria)



D – Wild-type *Mycobacterium tuberculosis*

- One false positive detected by DUGMO: **labelled GM** while it **is not**



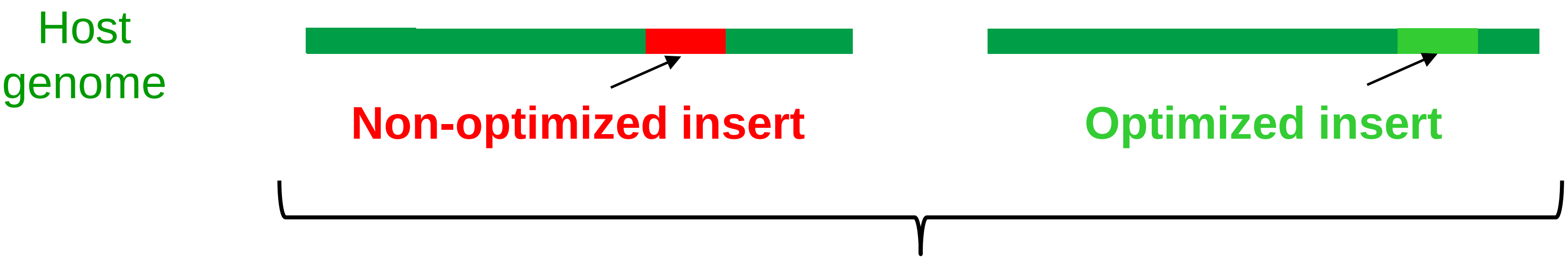
No other similar gene

Horizontal gene transfert ?

Phylum	Actinomycetota
Order	Actinomycetes
Sub-Order	Corynebacteriales
Family	Mycobacteriaceae
Genus	Mycobacterium

E – Synthetic data

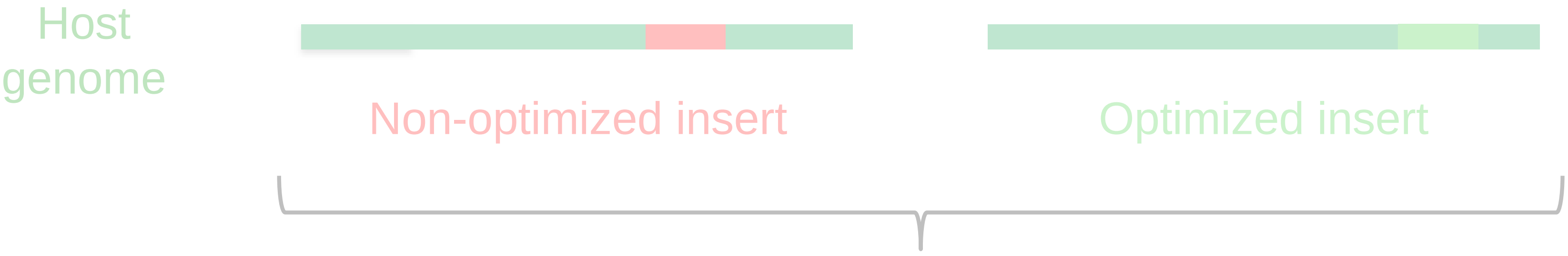
- Objectives
  - Test the sensitivity of the method to **dicodon optimization**



Detection by DUGMO ? **YES**

E – Synthetic data

- Objectives
  - Test the sensitivity of the method to dicodon optimization



Host genome

Non-optimized insert

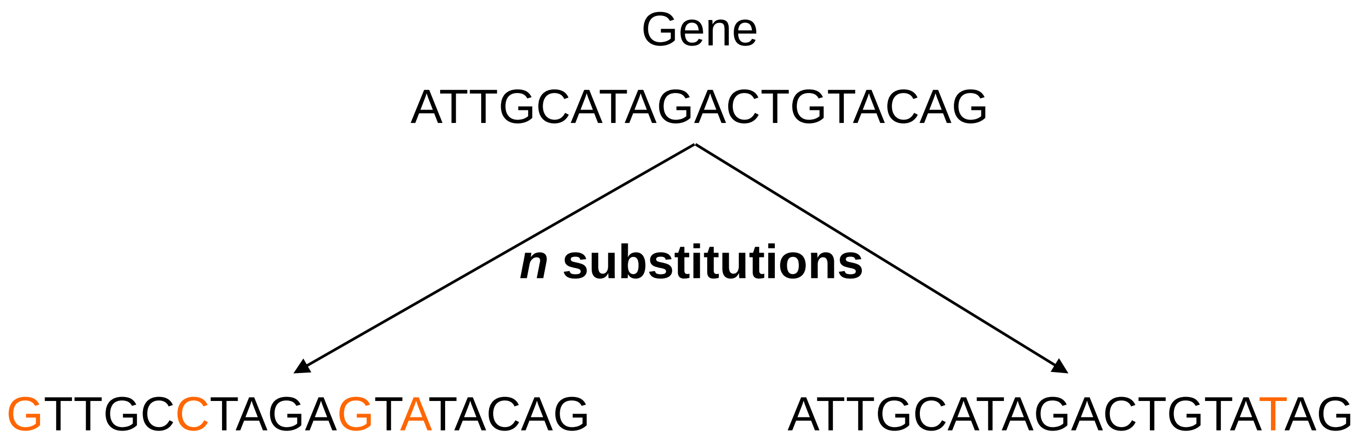
Optimized insert

Detection by DUGMO ? YES

  - Test the **detection threshold** of the method

E – Synthetic data

- Generation of **mutations in a wild-type gene** of *B. subtilis*



Gene

ATTGCATAGACTGTACAG

n **substitutions**

GTTGCCTAGAGTATACAG

ATTGCATAGACTGTATAG

DUGMO results	Detection	No detection

- Results : **substitution rate ≥ 9%** ➡ **detection** of the GM gene

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C – Summary: before

	Detection known or partially known GMO		Detection unkown GMO	
	Prokaryotes	Eukaryotes	Prokaryotes	Eukaryotes
Intergenic sequence	✔	✔	✘	✘
Truncated gene	✔	✔	~	~
Fused gene	✔	✔	~	~
Insertion/deletion in a gene	✔	✔	✘	✘
% of point mutations ≥ 9%	✔	✔	✘	✘
% of point mutations < 9%	✔	✔	✘	✘

C – Summary: after

	Detection known or partially known GMO		Detection unknown GMO	
	Prokaryotes	Eukaryotes	Prokaryotes	Eukaryotes
Intergenic sequences	✔	✔	✘	✘
Truncated gene	✔	✔	✔	⌛
Fused gene	✔	✔	✔	⌛
Insertion/deletion in a gene	✔	✔	✔	⌛
% of point mutations ≥ 9%	✔	✔	✔	⌛
% of point mutations < 9%	✔	✔	✘	✘

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A – General

- ▶ Adapt the method for application to other organisms

tokopedia.com Tetra Import Glowfish

- ▶ Possibility to provide assembly data as input (Illumina, Pacbio)

GloFish®

Ackowledgements

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Région BRETAGNE

- **Funders**
  Anses

Région Bretagne

Thank you for your attention

https://github.com/ANSES-Ploufragan/DUGMO

Answers reflect my personal opinion, they are not the expression of the ANSES opinion

18 months post-doc position in bioinformatics

DUGMO for Eukaryotic genomes

Conditions:
- out of France for 18 months from May 2020
- work in a P2+ lab

Contact: fabrice.touzain@anses.fr

https://github.com/ANSES-Ploufragan/DUGMO