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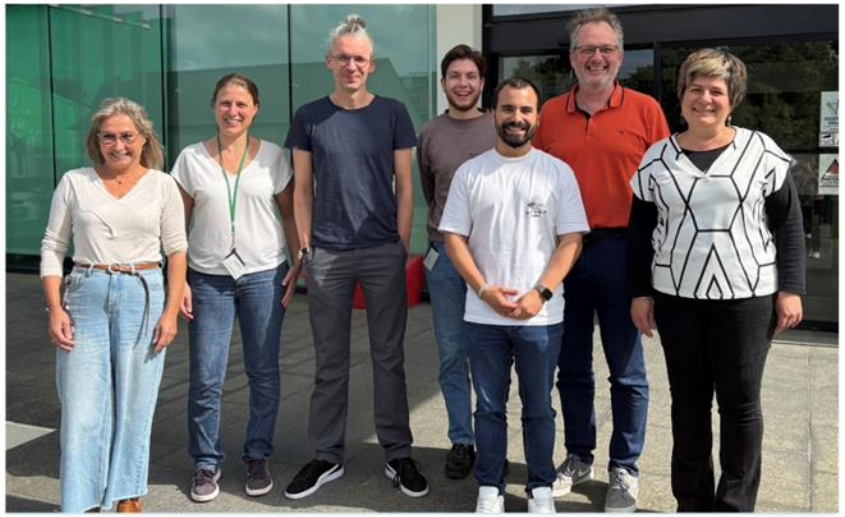
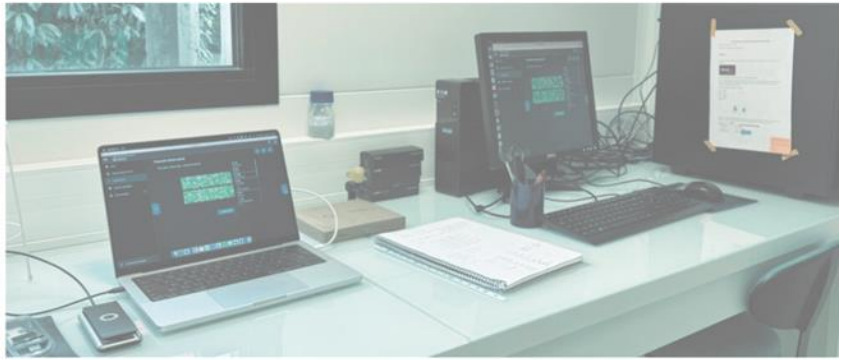
EPPO Jens-Georg Unger Plant Health Fellowship (Call 2024)

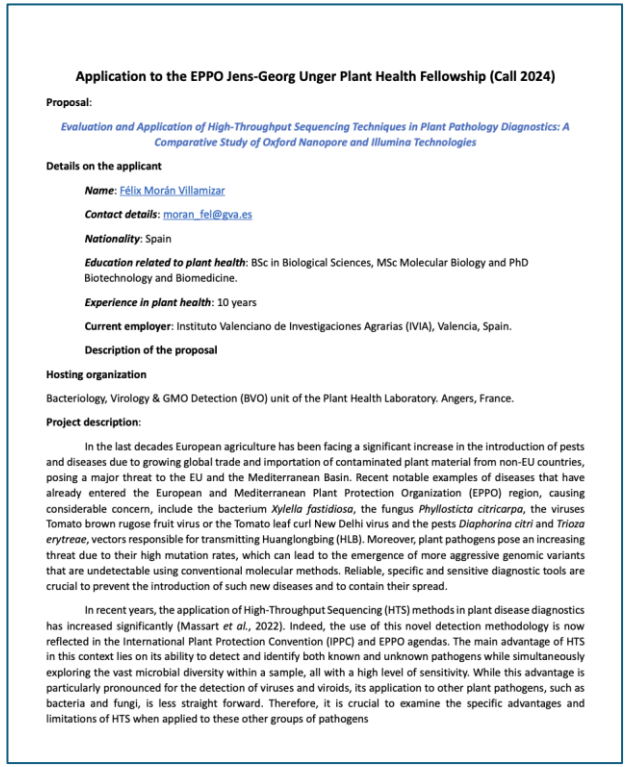
February – May 2025





Evaluation and Application of High-Throughput Sequencing Techniques in Plant Pathology Diagnostics: A Comparative Study of Oxford Nanopore and Illumina Technologies

The proposal



Application to the EPPO Jens-Georg Unger Plant Health Fellowship (Call 2024)

Proposal:

Evaluation and Application of High-Throughput Sequencing Techniques in Plant Pathology Diagnostics: A Comparative Study of Oxford Nanopore and Illumina Technologies

Details on the applicant

Name: Félix Morán Villamizar

Contact details: moran_fel@gva.es

Nationality: Spain

Education related to plant health: BSc in Biological Sciences, MSc Molecular Biology and PhD Biotechnology and Biomedicine.

Experience in plant health: 10 years

Current employer: Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain.

Description of the proposal

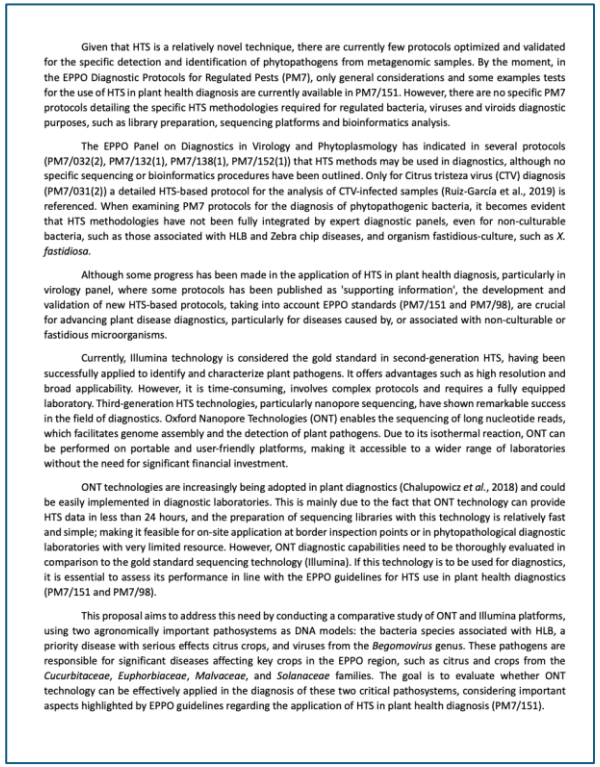
Hosting organization

Bacteriology, Virology & GMO Detection (BVO) unit of the Plant Health Laboratory. Angers, France.

Project description:

In the last decades European agriculture has been facing a significant increase in the introduction of pests and diseases due to growing global trade and importation of contaminated plant material from non-EU countries, posing a major threat to the EU and the Mediterranean Basin. Recent notable examples of diseases that have already entered the European and Mediterranean Plant Protection Organization (EPPO) region, causing considerable concern, include the bacterium *Xylella fastidiosa*, the fungus *Phyllosticta citricarpa*, the viruses Tomato brown rugose fruit virus or the Tomato leaf curl New Delhi virus and the pests *Diaphorina citri* and *Trioza erytreae*, vectors responsible for transmitting Huanglongbing (HLB). Moreover, plant pathogens pose an increasing threat due to their high mutation rates, which can lead to the emergence of more aggressive genomic variants that are undetectable using conventional molecular methods. Reliable, specific and sensitive diagnostic tools are crucial to prevent the introduction of such new diseases and to contain their spread.

In recent years, the application of High-Throughput Sequencing (HTS) methods in plant disease diagnostics has increased significantly (Massart *et al.*, 2022). Indeed, the use of this novel detection methodology is now reflected in the International Plant Protection Convention (IPPC) and EPPO agendas. The main advantage of HTS in this context lies on its ability to detect and identify both known and unknown pathogens while simultaneously exploring the vast microbial diversity within a sample, all with a high level of sensitivity. While this advantage is particularly pronounced for the detection of viruses and viroids, its application to other plant pathogens, such as bacteria and fungi, is less straight forward. Therefore, it is crucial to examine the specific advantages and limitations of HTS when applied to these other groups of pathogens



Given that HTS is a relatively novel technique, there are currently few protocols optimized and validated for the specific detection and identification of phytopathogens from metagenomic samples. By the moment, in the EPPO Diagnostic Protocols for Regulated Pests (PM7), only general considerations and some examples tests for the use of HTS in plant health diagnosis are currently available in PM7/151. However, there are no specific PM7 protocols detailing the specific HTS methodologies required for regulated bacteria, viruses and viroids diagnostic purposes, such as library preparation, sequencing platforms and bioinformatics analysis.

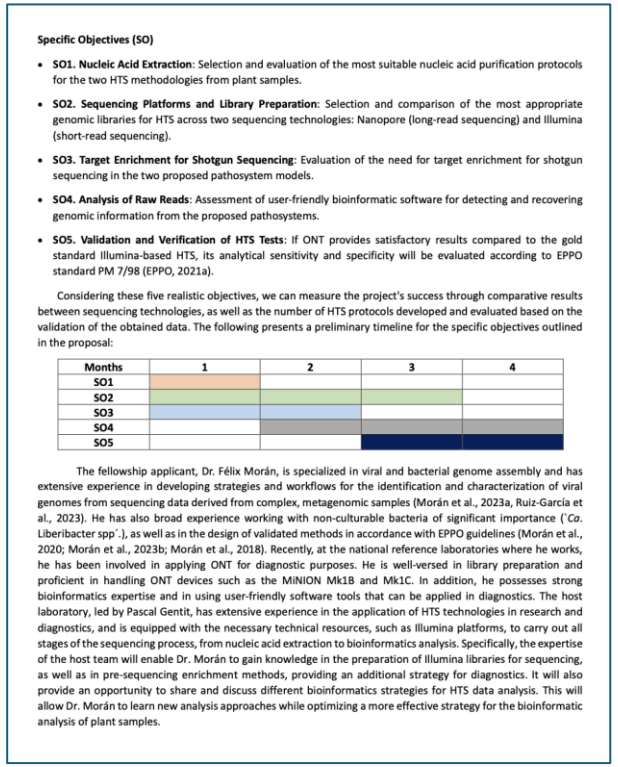
The EPPO Panel on Diagnostics in Virology and Phytoplasmology has indicated in several protocols (PM7/032(2), PM7/132(1), PM7/138(1), PM7/152(1)) that HTS methods may be used in diagnostics, although no specific sequencing or bioinformatics procedures have been outlined. Only for Citrus tristeza virus (CTV) diagnosis (PM7/031(2)) a detailed HTS-based protocol for the analysis of CTV-infected samples (Ruiz-García et al., 2019) is referenced. When examining PM7 protocols for the diagnosis of phytopathogenic bacteria, it becomes evident that HTS methodologies have not been fully integrated by expert diagnostic panels, even for non-culturable bacteria, such as those associated with HLB and Zebra chip diseases, and organism fastidious-culture, such as *X. fastidiosa*.

Although some progress has been made in the application of HTS in plant health diagnosis, particularly in virology panel, where some protocols has been published as 'supporting information', the development and validation of new HTS-based protocols, taking into account EPPO standards (PM7/151 and PM7/98), are crucial for advancing plant disease diagnostics, particularly for diseases caused by, or associated with non-culturable or fastidious microorganisms.

Currently, Illumina technology is considered the gold standard in second-generation HTS, having been successfully applied to identify and characterize plant pathogens. It offers advantages such as high resolution and broad applicability. However, it is time-consuming, involves complex protocols and requires a fully equipped laboratory. Third-generation HTS technologies, particularly nanopore sequencing, have shown remarkable success in the field of diagnostics. Oxford Nanopore Technologies (ONT) enables the sequencing of long nucleotide reads, which facilitates genome assembly and the detection of plant pathogens. Due to its isothermal reaction, ONT can be performed on portable and user-friendly platforms, making it accessible to a wider range of laboratories without the need for significant financial investment.

ONT technologies are increasingly being adopted in plant diagnostics (Chalupowicz *et al.*, 2018) and could be easily implemented in diagnostic laboratories. This is mainly due to the fact that ONT technology can provide HTS data in less than 24 hours, and the preparation of sequencing libraries with this technology is relatively fast and simple; making it feasible for on-site application at border inspection points or in phytopathological diagnostic laboratories with very limited resource. However, ONT diagnostic capabilities need to be thoroughly evaluated in comparison to the gold standard sequencing technology (Illumina). If this technology is to be used for diagnostics, it is essential to assess its performance in line with the EPPO guidelines for HTS use in plant health diagnostics (PM7/151 and PM7/98).

This proposal aims to address this need by conducting a comparative study of ONT and Illumina platforms, using two agronomically important pathosystems as DNA models: the bacteria species associated with HLB, a priority disease with serious effects citrus crops, and viruses from the *Begomovirus* genus. These pathogens are responsible for significant diseases affecting key crops in the EPPO region, such as citrus and crops from the *Cucurbitaceae*, *Euphorbiaceae*, *Malvaceae*, and *Solanaceae* families. The goal is to evaluate whether ONT technology can be effectively applied in the diagnosis of these two critical pathosystems, considering important aspects highlighted by EPPO guidelines regarding the application of HTS in plant health diagnosis (PM7/151).



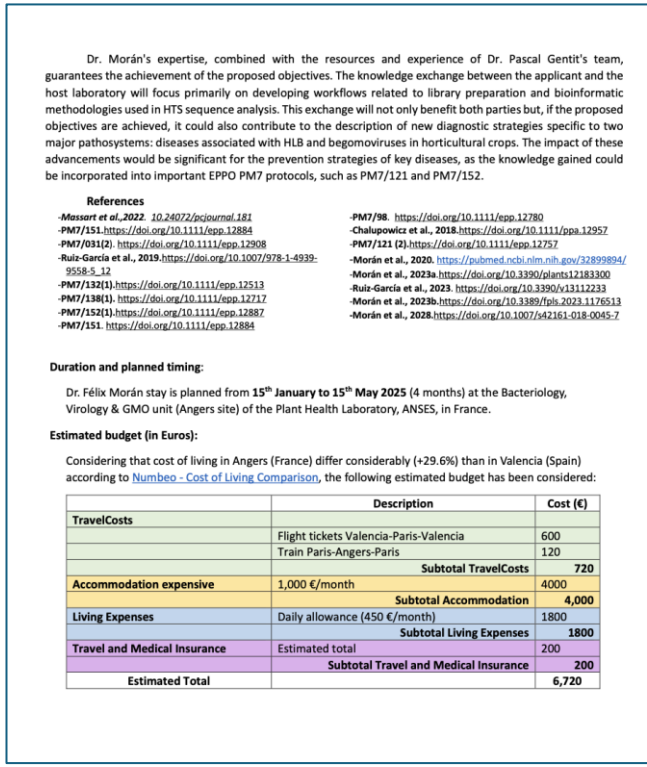
Specific Objectives (SO)

- **SO1. Nucleic Acid Extraction**: Selection and evaluation of the most suitable nucleic acid purification protocols for the two HTS methodologies from plant samples.
- **SO2. Sequencing Platforms and Library Preparation**: Selection and comparison of the most appropriate genomic libraries for HTS across two sequencing technologies: Nanopore (long-read sequencing) and Illumina (short-read sequencing).
- **SO3. Target Enrichment for Shotgun Sequencing**: Evaluation of the need for target enrichment for shotgun sequencing in the two proposed pathosystem models.
- **SO4. Analysis of Raw Reads**: Assessment of user-friendly bioinformatic software for detecting and recovering genomic information from the proposed pathosystems.
- **SO5. Validation and Verification of HTS Tests**: If ONT provides satisfactory results compared to the gold standard Illumina-based HTS, its analytical sensitivity and specificity will be evaluated according to EPPO standard PM 7/98 (EPPO, 2021a).

Considering these five realistic objectives, we can measure the project's success through comparative results between sequencing technologies, as well as the number of HTS protocols developed and evaluated based on the validation of the obtained data. The following presents a preliminary timeline for the specific objectives outlined in the proposal:

Months	1	2	3	4
SO1				
SO2				
SO3				
SO4				
SO5				

The fellowship applicant, Dr. Félix Morán, is specialized in viral and bacterial genome assembly and has extensive experience in developing strategies and workflows for the identification and characterization of viral genomes from sequencing data derived from complex, metagenomic samples (Morán et al., 2023a, Ruiz-García et al., 2023). He has also broad experience working with non-culturable bacteria of significant importance (`*Ca.* Liberibacter spp´.), as well as in the design of validated methods in accordance with EPPO guidelines (Morán et al., 2020; Morán et al., 2023b; Morán et al., 2018). Recently, at the national reference laboratories where he works, he has been involved in applying ONT for diagnostic purposes. He is well-versed in library preparation and proficient in handling ONT devices such as the MiNION Mk1B and Mk1C. In addition, he possesses strong bioinformatics expertise and in using user-friendly software tools that can be applied in diagnostics. The host laboratory, led by Pascal Gentit, has extensive experience in the application of HTS technologies in research and diagnostics, and is equipped with the necessary technical resources, such as Illumina platforms, to carry out all stages of the sequencing process, from nucleic acid extraction to bioinformatics analysis. Specifically, the expertise of the host team will enable Dr. Morán to gain knowledge in the preparation of Illumina libraries for sequencing, as well as in pre-sequencing enrichment methods, providing an additional strategy for diagnostics. It will also provide an opportunity to share and discuss different bioinformatics strategies for HTS data analysis. This will allow Dr. Morán to learn new analysis approaches while optimizing a more effective strategy for the bioinformatic analysis of plant samples.



Dr. Morán's expertise, combined with the resources and experience of Dr. Pascal Gentit's team, guarantees the achievement of the proposed objectives. The knowledge exchange between the applicant and the host laboratory will focus primarily on developing workflows related to library preparation and bioinformatic methodologies used in HTS sequence analysis. This exchange will not only benefit both parties but, if the proposed objectives are achieved, it could also contribute to the description of new diagnostic strategies specific to two major pathosystems: diseases associated with HLB and begomoviruses in horticultural crops. The impact of these advancements would be significant for the prevention strategies of key diseases, as the knowledge gained could be incorporated into important EPPO PM7 protocols, such as PM7/121 and PM7/152.

References

-***Massart et al.,2022***. *10.24072/pcjournal.181*
-**PM7/151**.https://doi.org/10.1111/epp.12884
-**PM7/031(2)**. https://doi.org/10.1111/epp.12908
-**Ruiz-García et al., 2019**.https://doi.org/10.1007/978-1-4939-9558-5_12
-**PM7/132(1)**.https://doi.org/10.1111/epp.12513
-**PM7/138(1)**. https://doi.org/10.1111/epp.12717
-**PM7/152(1)**.https://doi.org/10.1111/epp.12887
-**PM7/151**. https://doi.org/10.1111/epp.12884
-**PM7/98**. https://doi.org/10.1111/epp.12780
-**Chalupowicz et al., 2018**.https://doi.org/10.1111/ppa.12957
-**PM7/121 (2)**.https://doi.org/10.1111/epp.12757
-**Morán et al., 2020**. https://pubmed.ncbi.nlm.nih.gov/32899894/
-**Morán et al., 2023a**.https://doi.org/10.3390/plants12183300
-**Ruiz-García et al., 2023**. https://doi.org/10.3390/v13112233
-**Morán et al., 2023b**.https://doi.org/10.3389/fpls.2023.1176513
-**Morán et al., 2028**.https://doi.org/10.1007/s42161-018-0045-7

Duration and planned timing:

Dr. Félix Morán stay is planned from **15th January to 15th May 2025** (4 months) at the Bacteriology, Virology & GMO unit (Angers site) of the Plant Health Laboratory, ANSES, in France.

Estimated budget (in Euros):

Considering that cost of living in Angers (France) differ considerably (+29.6%) than in Valencia (Spain) according to Numbeo - Cost of Living Comparison, the following estimated budget has been considered:

	Description	Cost (€)
TravelCosts		
	Flight tickets Valencia-Paris-Valencia	600
	Train Paris-Angers-Paris	120
	Subtotal TravelCosts	**720**
Accommodation expensive	1,000 €/month	4000
	Subtotal Accommodation	**4,000**
Living Expenses	Daily allowance (450 €/month)	1800
	Subtotal Living Expenses	**1800**
Travel and Medical Insurance	Estimated total	200
	Subtotal Travel and Medical Insurance	**200**
Estimated Total		**6,720**

Tips:

-The proposal should be clear and focused on addressing a relevant gap or problem in plant health.

-It is highly recommended that the proposal is discussed and evaluated in advance with the hosting organization. This helps optimize the duration of the stay and ensures that the objectives are realistic and achievable.

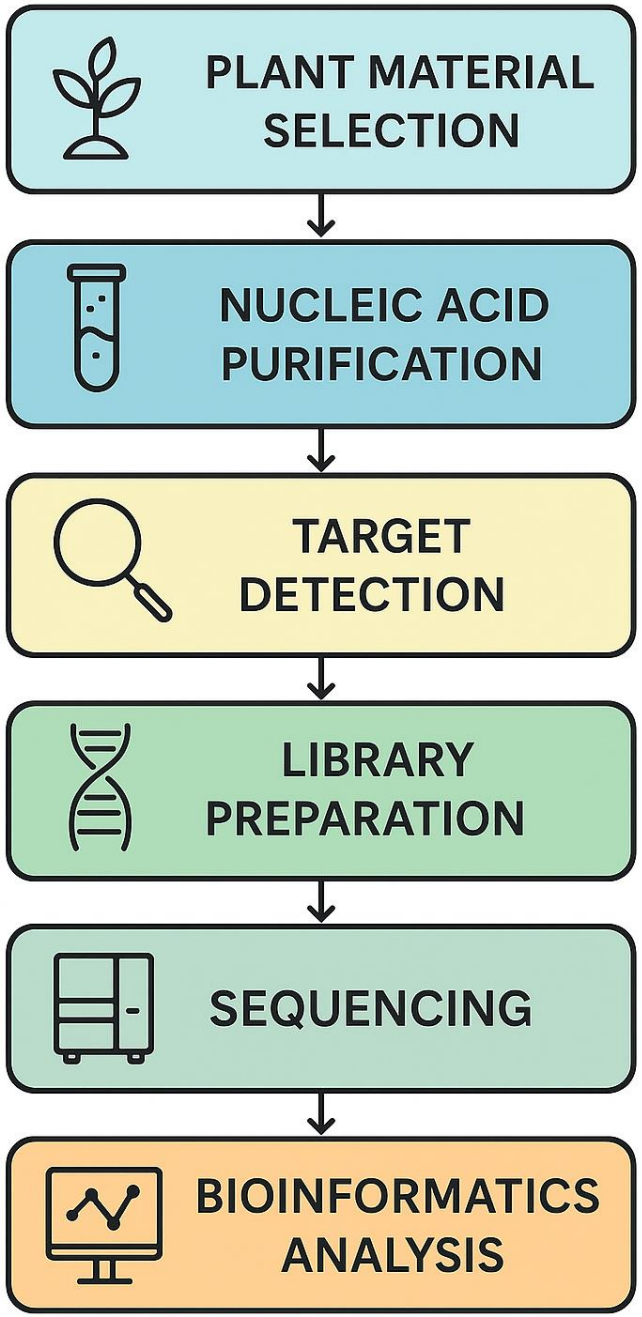
-Include a timeline figure to clearly outline the planned activities and milestones throughout the fellowship.

Proposal summary

- **Main idea**: to address a diagnostic gap in the application of HTS within EPPO PM7 protocols.

- This study presents a comparative evaluation of Oxford Nanopore Technologies (ONT) and Illumina platforms using two agronomically relevant DNA-based pathosystems: Huanglongbing (`*Ca*. Liberibacter spp´) and Begomoviruses.

- The main objective was to assess whether ONT technology can be effectively implemented for the diagnosis of these pathosystems, taking into account key requirements outlined in EPPO guidelines for HTS-based diagnostics (PM7/151).



`*Ca*. L asiaticus´

HLB

+Begomo

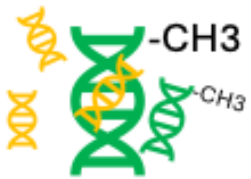
SPLCV; ToLCNDV

-Nextera (Amplicones)
-Ilumina **DNA prep**

illumina

RawData
Ilumina

Total-DNA

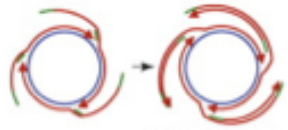


NEBNext Microbiome

Processed DNA

Enriched DNA

RCA



Long-PCR

Oxford NANOPORE Technologies

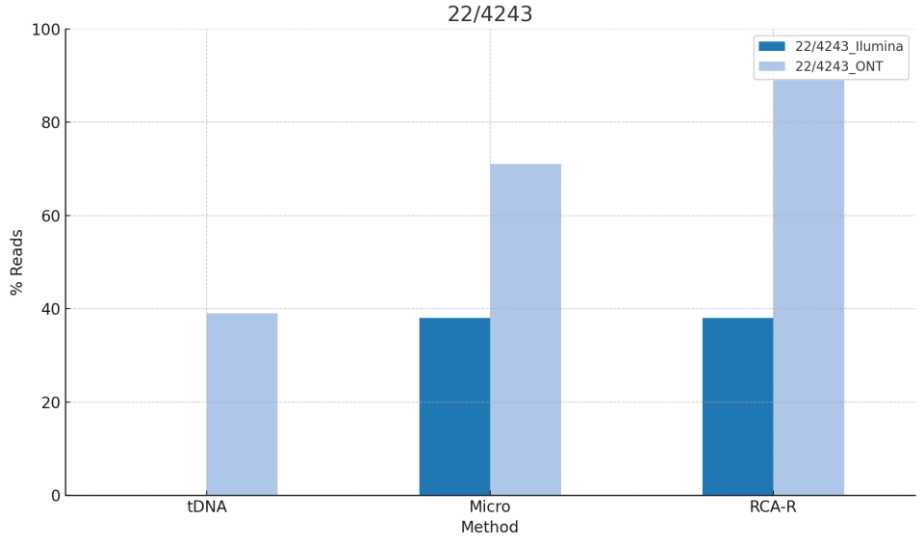
Rapid DNA libraries
-Rapid DNA
-Barcoding 16S

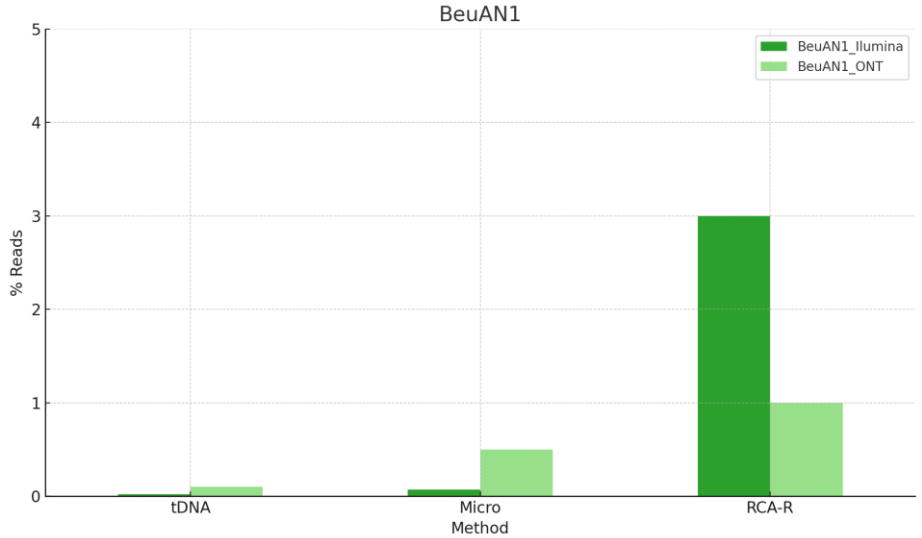
RawData
Nanopore

Data output comparison

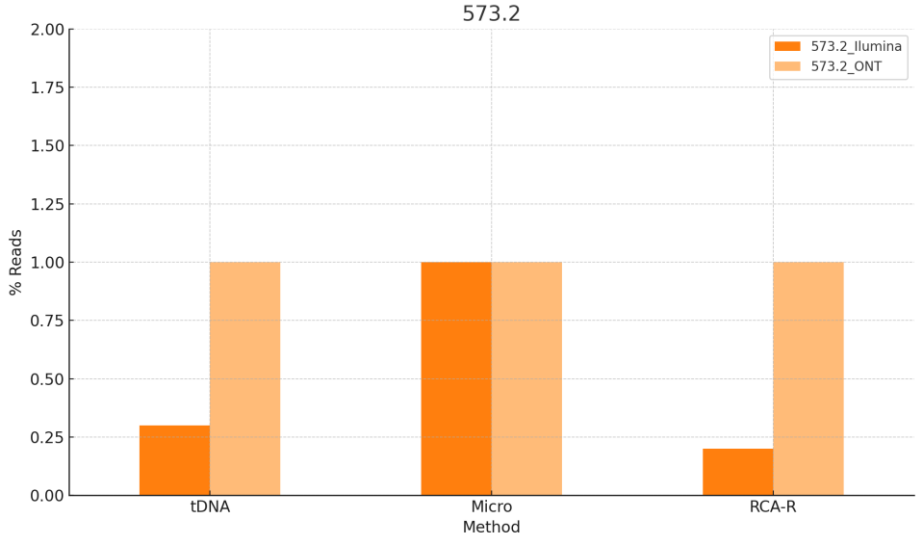
Relative titter of viral/bacteria after Host Remove (Q>20) classified by Kraken2

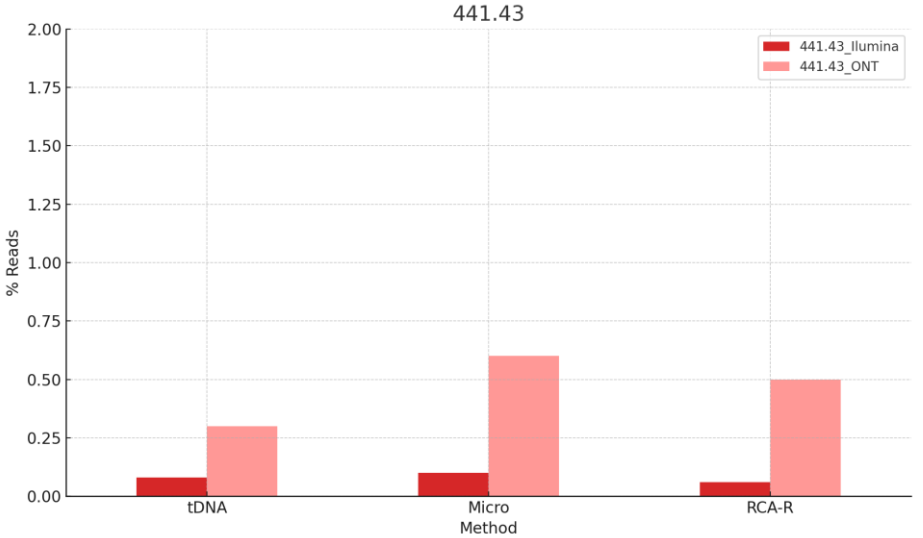
Viruses





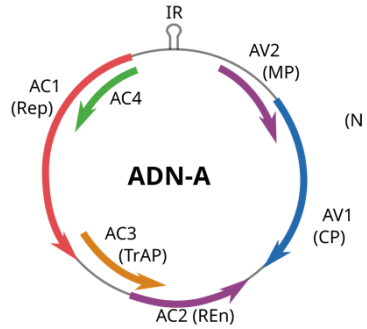
Bacteria



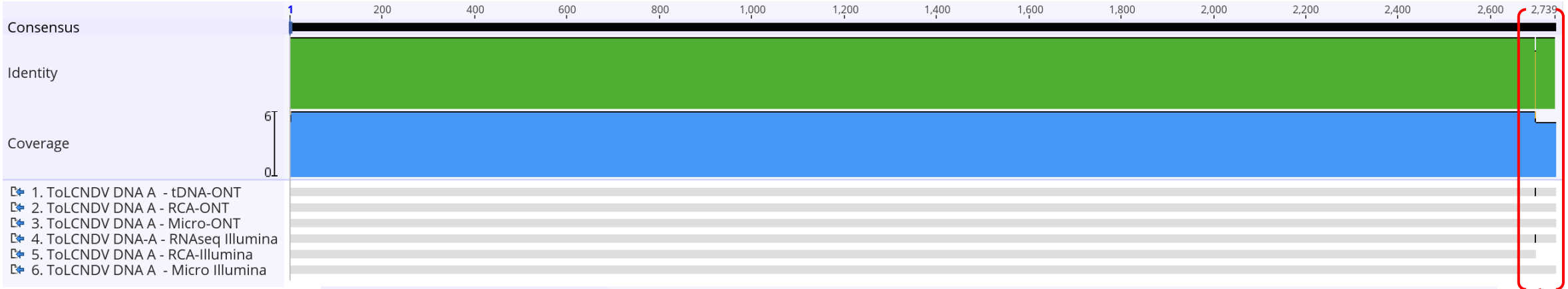


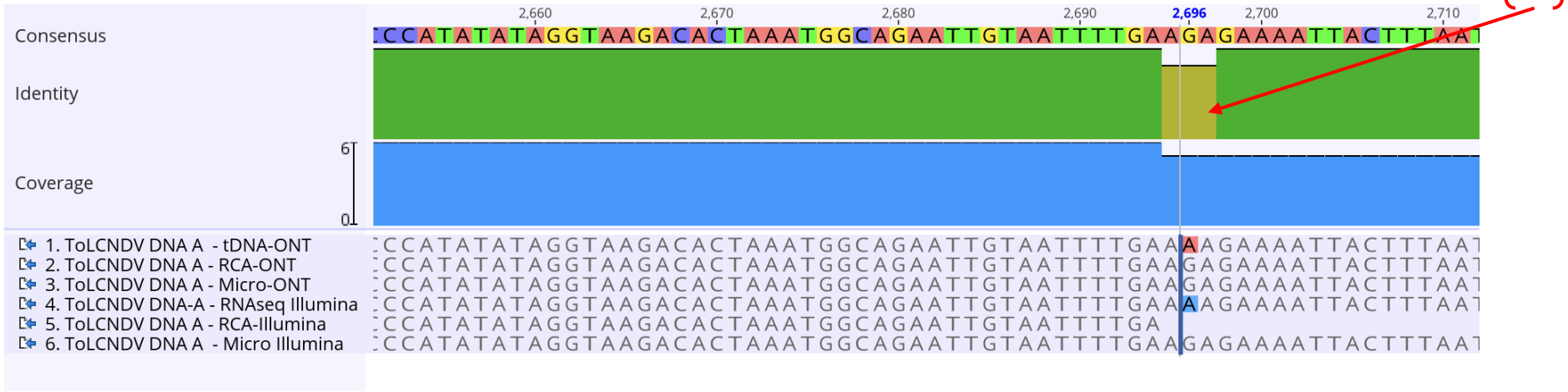
Data output comparison

ToLCNDV



Sample 22/4243





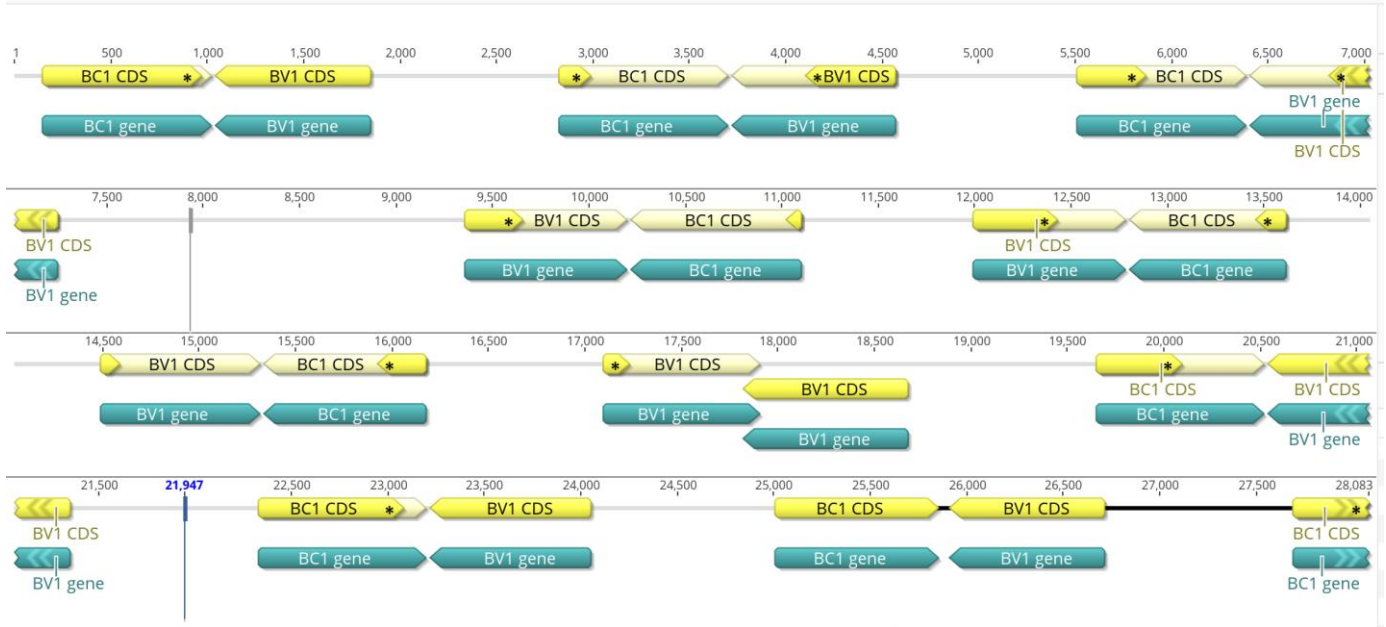
1. tDNA - ONT
2. RCA – ONT**
3. Micro -ONT
4. tRNA - Illumina
5. RCA –Illumina **
6. Micro - Illumina

** Contig deeper

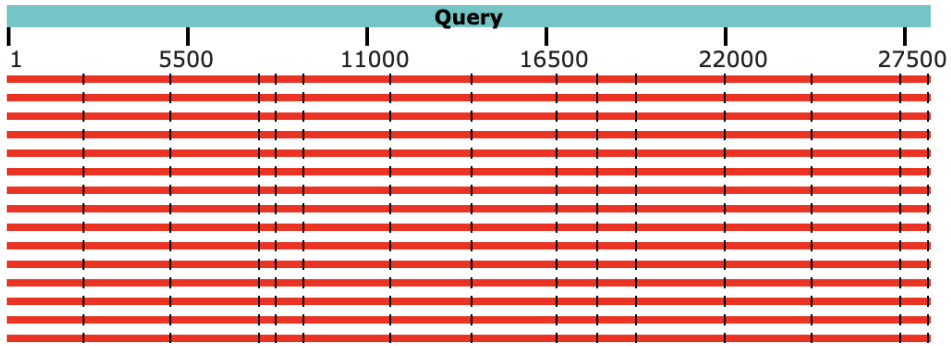
Reads ONT- RCA-R

- Problem for analysis by de novo assembly

RCA-R (ONT)
Example of a single read



Tomato leaf curl New Delhi begomovirus



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V.1.0 by Morán et al. 2025

Detection and identification of DNA viruses from ONT, RCA-R-ONT, and Illumina data

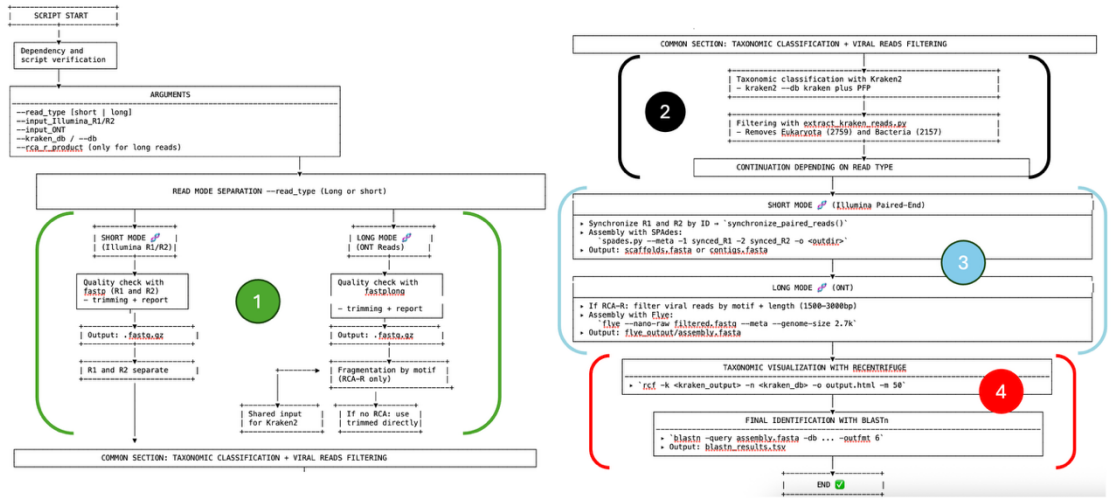
Checking dependencies...
Dependencies verified.

```
usage:

ViroCatch v1.0 - Pipeline for viral DNA detection from HTS data

optional arguments:
  -h, --help              show this help message and exit

Arguments:
  --read_type {long,short}  Tipo de lectura: 'short' (Illumina) o 'long' (ONT)
  --input_ONT               Archivo FASTQ de ONT (lecturas largas)
  --input_Illumina_R1       FASTQ R1 de Illumina
  --input_Illumina_R2       FASTQ R2 de Illumina
  --outdir                  Kraken2 database directory
  --threads THREADS         Número de hilos de procesamiento
  --db                      Base de datos BLASTn (preformateada con makeblastdb)
  --kraken_db               Directorio de base de datos Kraken2- Download from https://benlangmead.github.io/aws-indexes/k2
  --memory_mapping          Activar --memory-mapping en Kraken2
  --quality_threshold       Umbral de calidad minima para trimming
  --motif MOTIF             Motivo para fragmentación RCA-R y filtrado - Por defecto TAATATTA
  --rca_r_product           Activar procesamiento RCA-R para ONT
```



README

ViroCatch

ViroCatch - HTS DNA-Virus Detection Pipeline-Draft

ViroCatch is a multi-stage bioinformatics Python pipeline for detecting and assembling DNA-Viral genomes from Illumina, Oxford Nanopore (ONT), and RCA-R Amplicon sequencing data.

> Versión 1.0 - Developed by Félix Morán

If you use ViroCatch please cite:

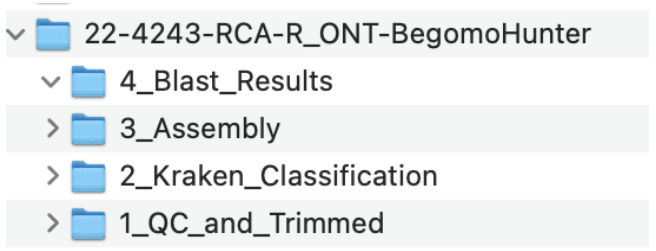
status STABLE | python 3.6+ | license MIT

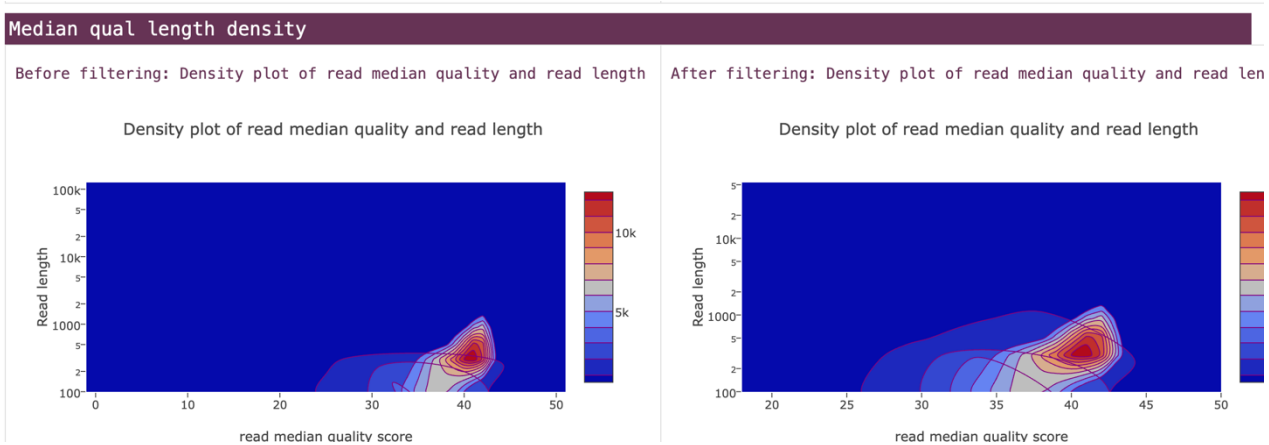
COMMANDS

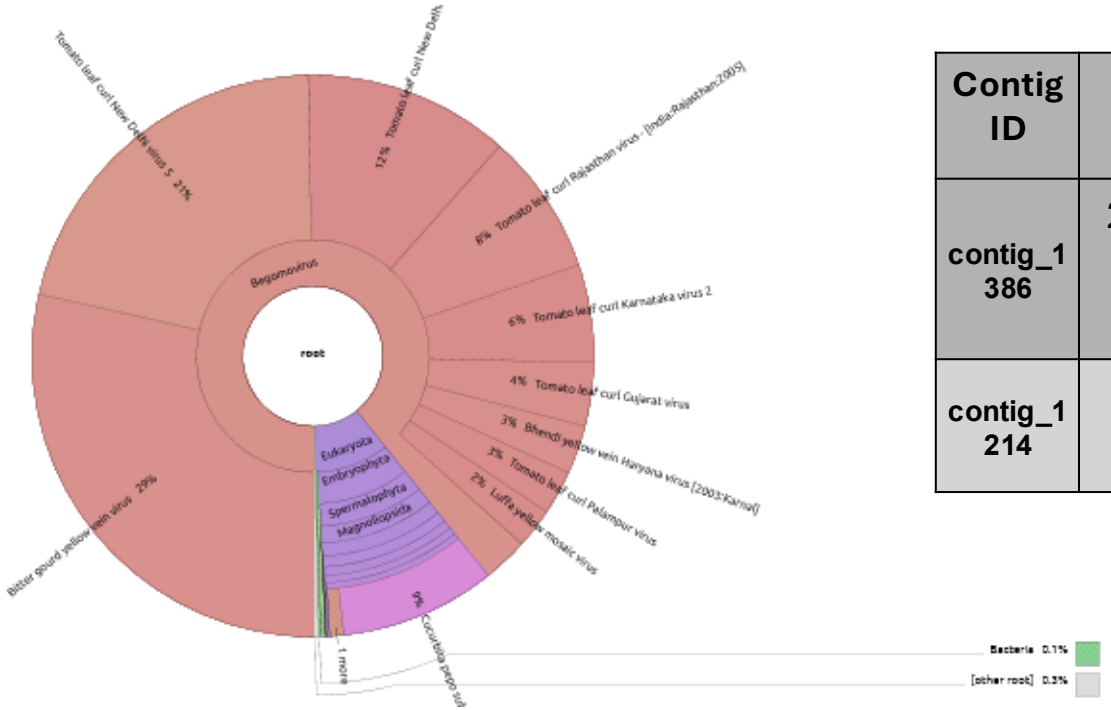
```
python ViroCatch.py
--read_type <long|short>
--input_ONT <ONT.fastq.gz>
--input_Illumina_R1 <R1.fastq.gz>
--input_Illumina_R2 <R2.fastq.gz>
--outdir <output_directory>
--threads \ By default: 8 --db <blast_db_path>
```

Oxford NANOPORE Technologies

illumina®







Contig ID	Size (nt)	BLASTN Sbjct	%identity	Query cover
contig_1386	2766	MW760328.1 Chilli leaf curl virus isolate CHL54, complete genome DNA-A	90%	100%
contig_1214	614	KR052157.1_Papaya_satellite_isolate_Mohali,_complete_sequence	81.967	

Begomovirus and others DNA viruses - ANSES collection

Distinct viral species: 18
Unique hosts (matrices): 8
Distinct countries/geographical origins: 12

Sample Id	Type	Name of virus	Isolate	Host	Origin	RCA-R	Real-time PCR Li et al 2004 (30ng) Average CTs	
						Gel Band	RCA-R 1:10	Total DNA
1	Begomovirus	African cassava mosaic virus (bipartite)	17/22.2 ACMV	Tabac	DSMZ (Ivory coast)	+	19	27
2	Begomovirus	Bean golden mosaic virus (bipartite)	17/23.2 BGMV		DSMZ (Guatemala)	+	10	14
3	Begomovirus	Chili leaf curl virus (monopartite)	17/104.1 ChiLCV	Pepper	India	+	6	15.2
4	Begomovirus	Pepper golden mosaic virus (bipartite)	17/104.4 PepGMV	Pepper	Mexico	+	10	15
5	Begomovirus	Potato yellow mosaic virus (bipartite)	16/257.4 PYMV	Tomate	The Martinique	+	21	27
6	Begomovirus	Sri Lankan cassava mosaic virus (bipartite)	19/182 SLCMV	Manioc lyoph	DSMZ PC-0424	+	12	21
8	Begomovirus	Tomato leaf curl virus Comores (monopartite)	12/49 (EL25) ToLCYTV	Tomate	Mayotte	+	20	-
10	Begomovirus	Tomato yellow leaf curl virus (monopartite)	16/34.2 et .3 TYLCV	Tomate fruit	New Caledonia	+	12	-
11	Begomovirus	Tomato yellow leaf curl virus Sardinia (monopartite)	EL18 09/12/98 TYLCSV	Tomate	Spain	+	14	28
12	Begomovirus	Tomato leaf yellow leaf curl thailand	21/409.2 TYLCTHV	Nicotiana benthamiana		+	8	13
13	Begomovirus	Chayote yellow mosaic virus	21/409.5	Nicotiana benthamiana		+	4	10
14	Begomovirus	Watermelon chlorotic stunt virus (bipartite)	21/409.7	Watermelon		+	-	21
15	Begomovirus	Tomato yellow leaf curl	15/117	Tomato		+	-	-
16	Begomovirus	Tomato mottle virus (bipartite)	14/14 (EL28) ToMoV	Tomate	Florida USA	+	10	-
17	Begomovirus	Abutilon mosaic virus (bipartite)	17/87 AbMV	Abutilon	The Netherlands	+	23	25
18	Begomovirus	TYLCV_IL__CLCuGeB	25V001469	Tomate	CIRAD MONTPELLIER	+	13	14
19	Becurtovirus	Geminivirus beet curly top Iran virus-BCTIV	24v0002359		Evaglobal	+	NO tested	NO tested
20	Nanovirus	Faba bean necrotic stunt virus-FBNSV	24V001217		INRAE Montpellier	+	NO tested	NO tested
21	Curtovirus	Beet curly top virus-BCTV	24V002358		MTA_24_R_V_01 USDA-WA USA	+	NO tested	NO tested

RCA-Enriched Oxford Nanopore HTS Workflow for the Diagnosis of Circular DNA Plant Viruses

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EPPO STANDARD ON DIAGNOSTICS

PM 7/98 (6) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity

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EPPO STANDARD ON DIAGNOSTICS

PM 7/151 (1) Considerations for the use of high throughput sequencing in plant health diagnostics[1]

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