Nanopore Sequencing Technologies

Outbreak tracking of respiratory viruses

Guillaume Croville – ENVT
11/04/2025

Intended Learning Objectives

Specific objectives of this session:

1. Learn about Nanopore sequencing applied to Influenza viruses
2. Learn about Nanopore sequencing for metagenomics

Outline

This session consists of the following elements

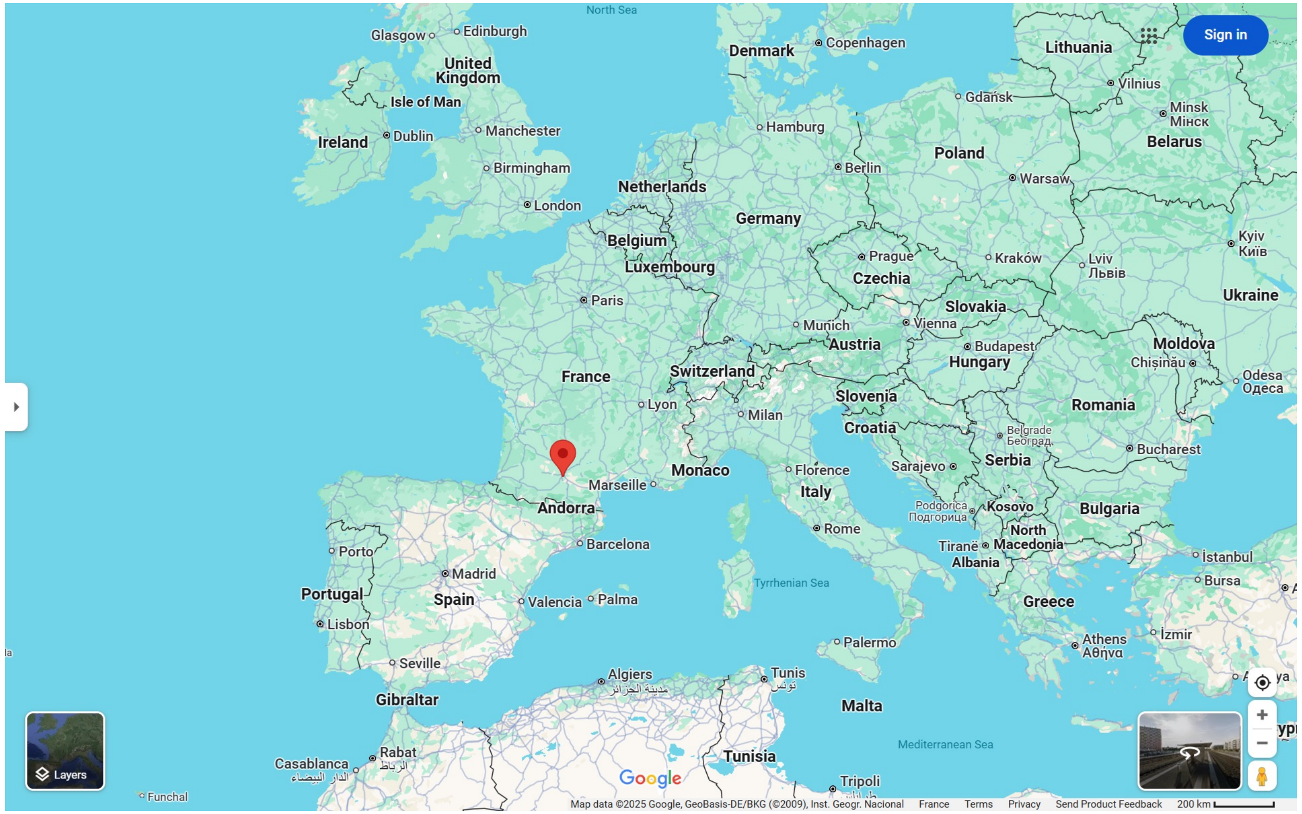
1. Presentation of an Influenza viruses sequencing study
2. Presentation of shotgun metagenomics studies

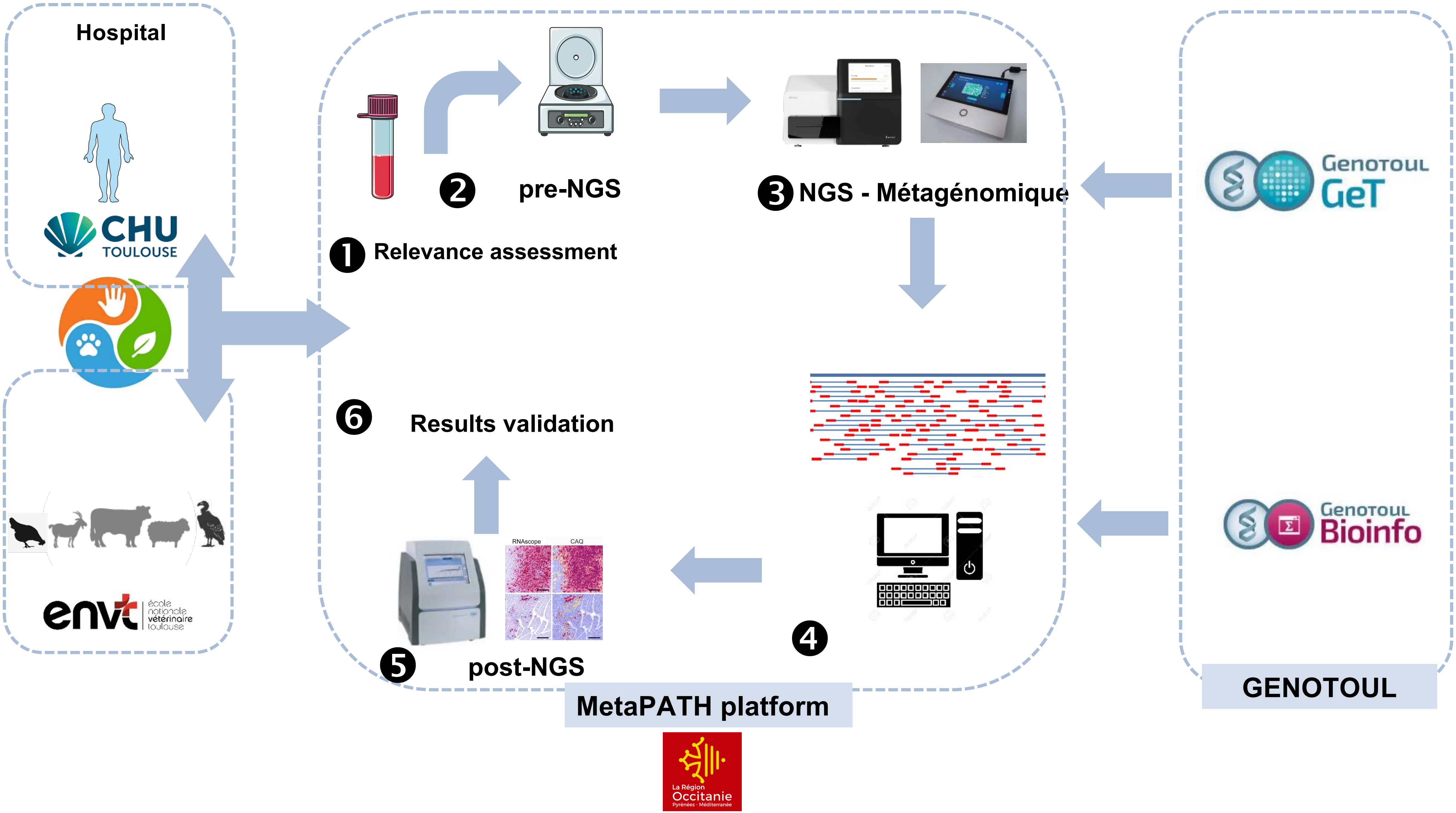
envt national veterinary school toulouse

INRAE

MINISTÈRE
DE L'AGRICULTURE
DE LA SOUVERAINETÉ
ALIMENTAIRE ET DE LA FORÊT
Liberté
Égalité
Fraternité

ecdc EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL





Hospital

❶ **Relevance assessment**

❷ **pre-NGS**

❸ **NGS - Métagénomique**

❹

❺ **post-NGS**

❻ **Results validation**

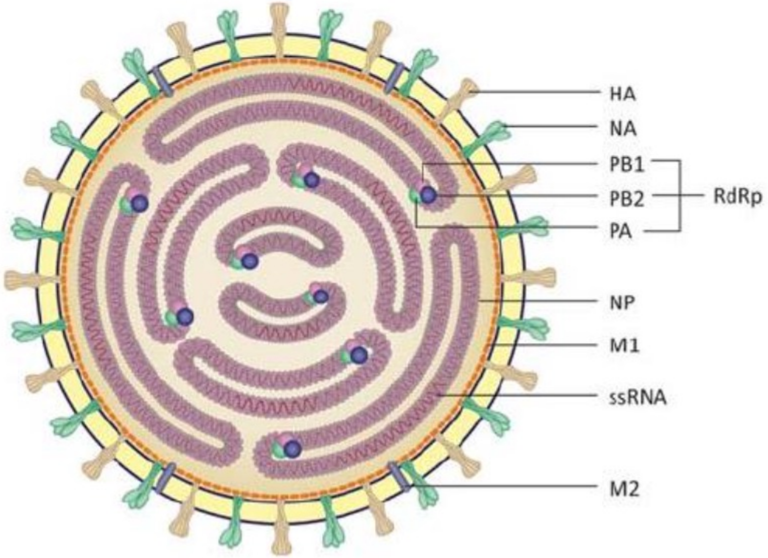
MetaPATH platform

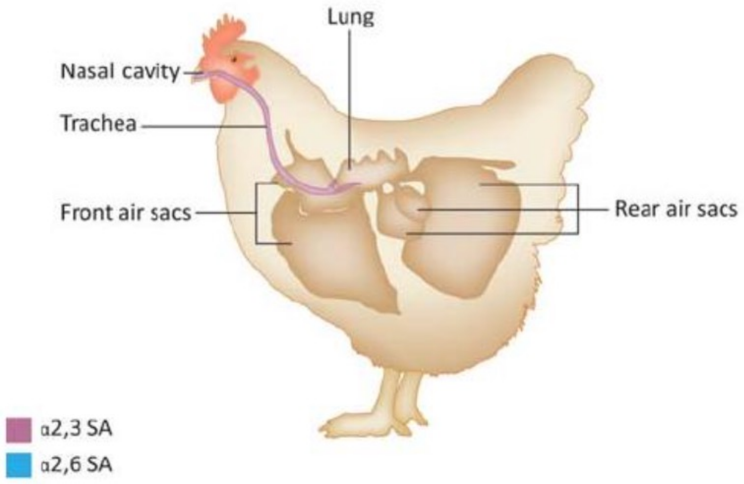
GENOTOUL

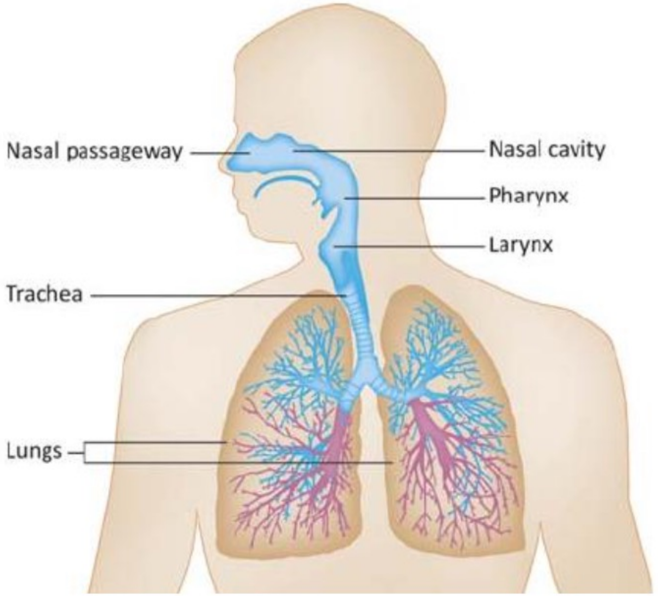
Outbreak tracking of respiratory virus in the field (example of the Avian flu)

Influenza virus genome is:

Avian influenza virus



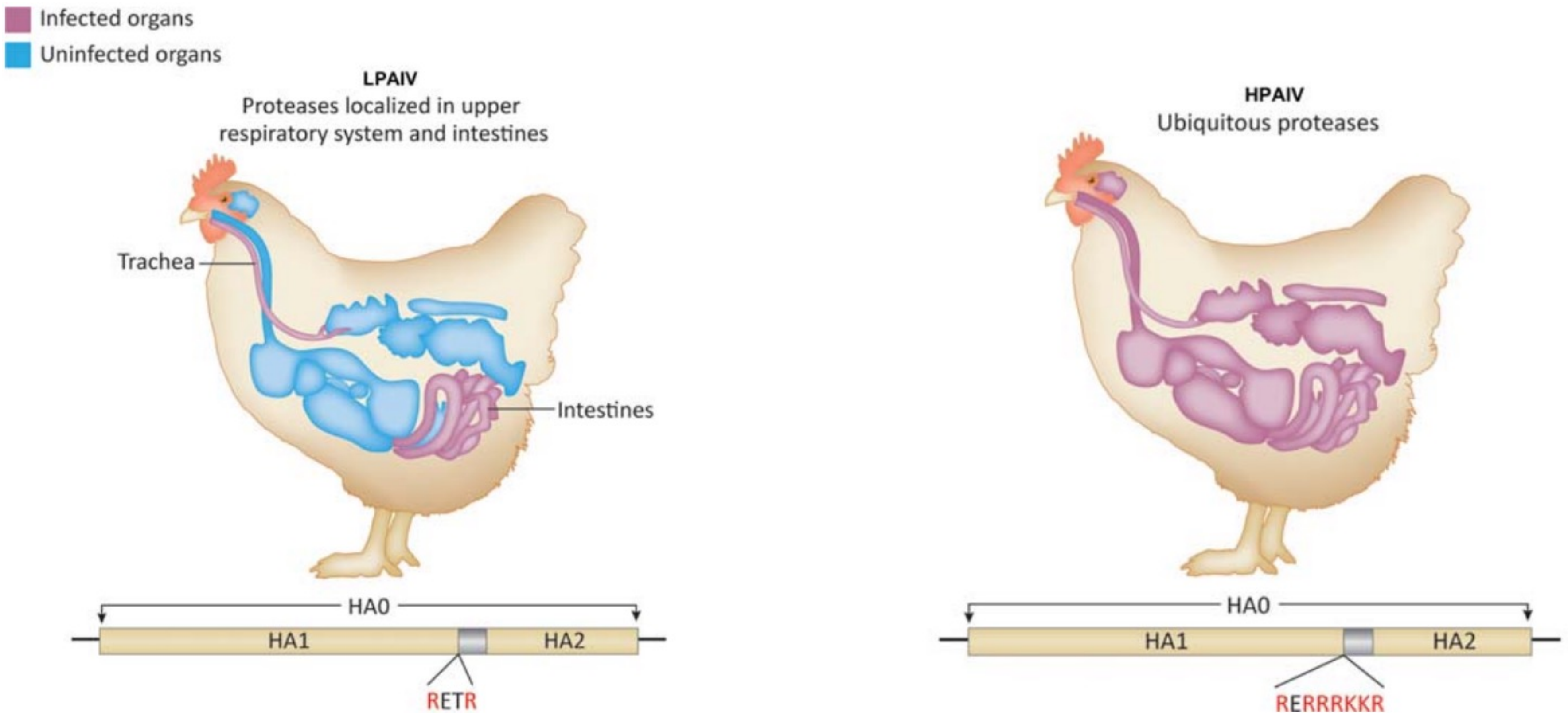




Susan Payne, in Viruses, 2017

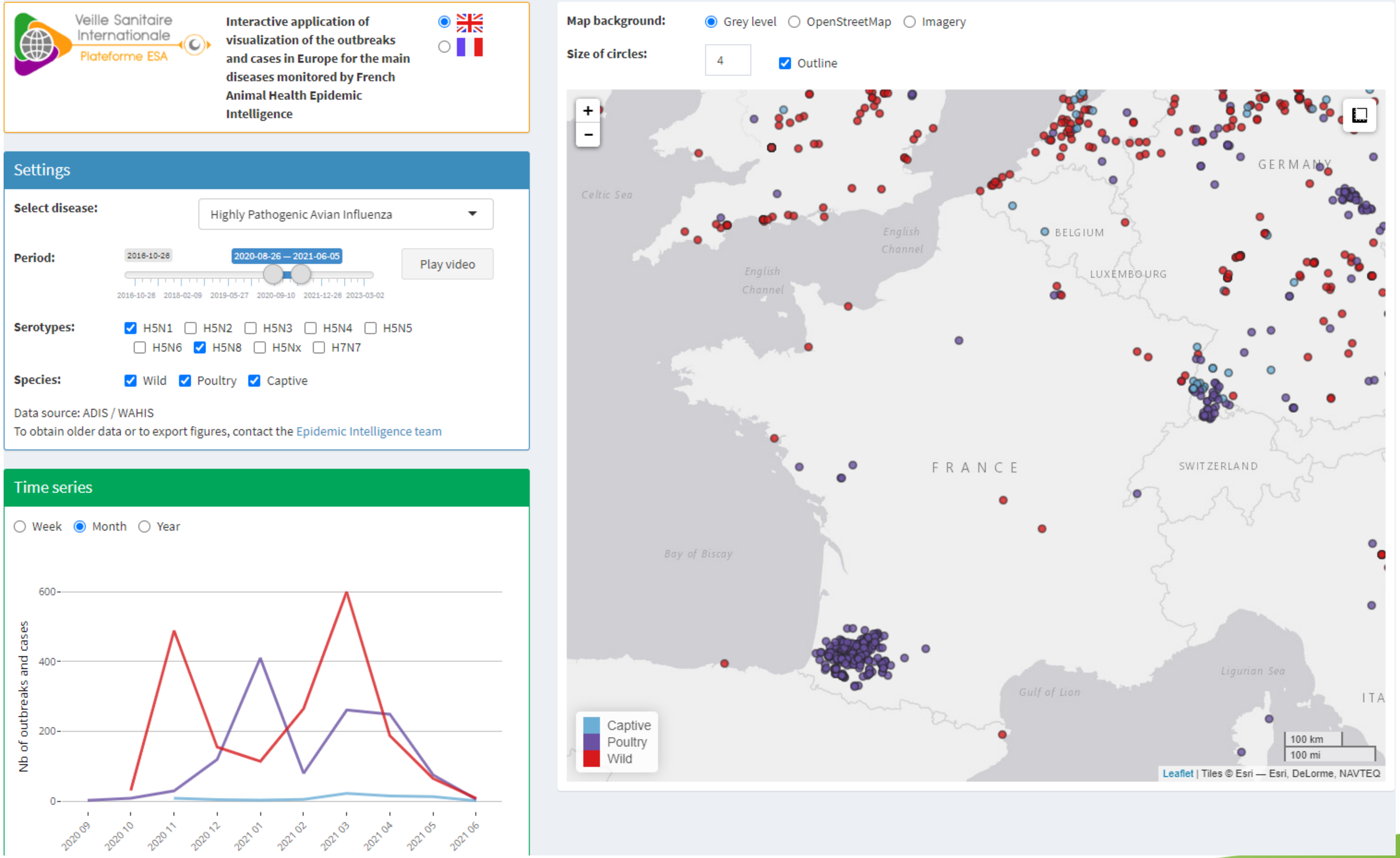
Avian influenza virus

Tissue tropism of LPAIV and HPAIV



Susan Payne, in Viruses, 2017

Avian influenza in France, 2020-2021



Veille Sanitaire Internationale
Plateforme ESA

Interactive application of visualization of the outbreaks and cases in Europe for the main diseases monitored by French Animal Health Epidemic Intelligence

Settings

Select disease: Highly Pathogenic Avian Influenza

Period: 2020-08-26 — 2021-06-05

Serotypes: ☑ H5N1 ☐ H5N2 ☐ H5N3 ☐ H5N4 ☐ H5N5 ☐ H5N6 ☑ H5N8 ☐ H5Nx ☐ H7N7

Species: ☑ Wild ☑ Poultry ☑ Captive

Data source: ADIS / WAHIS
To obtain older data or to export figures, contact the Epidemic Intelligence team

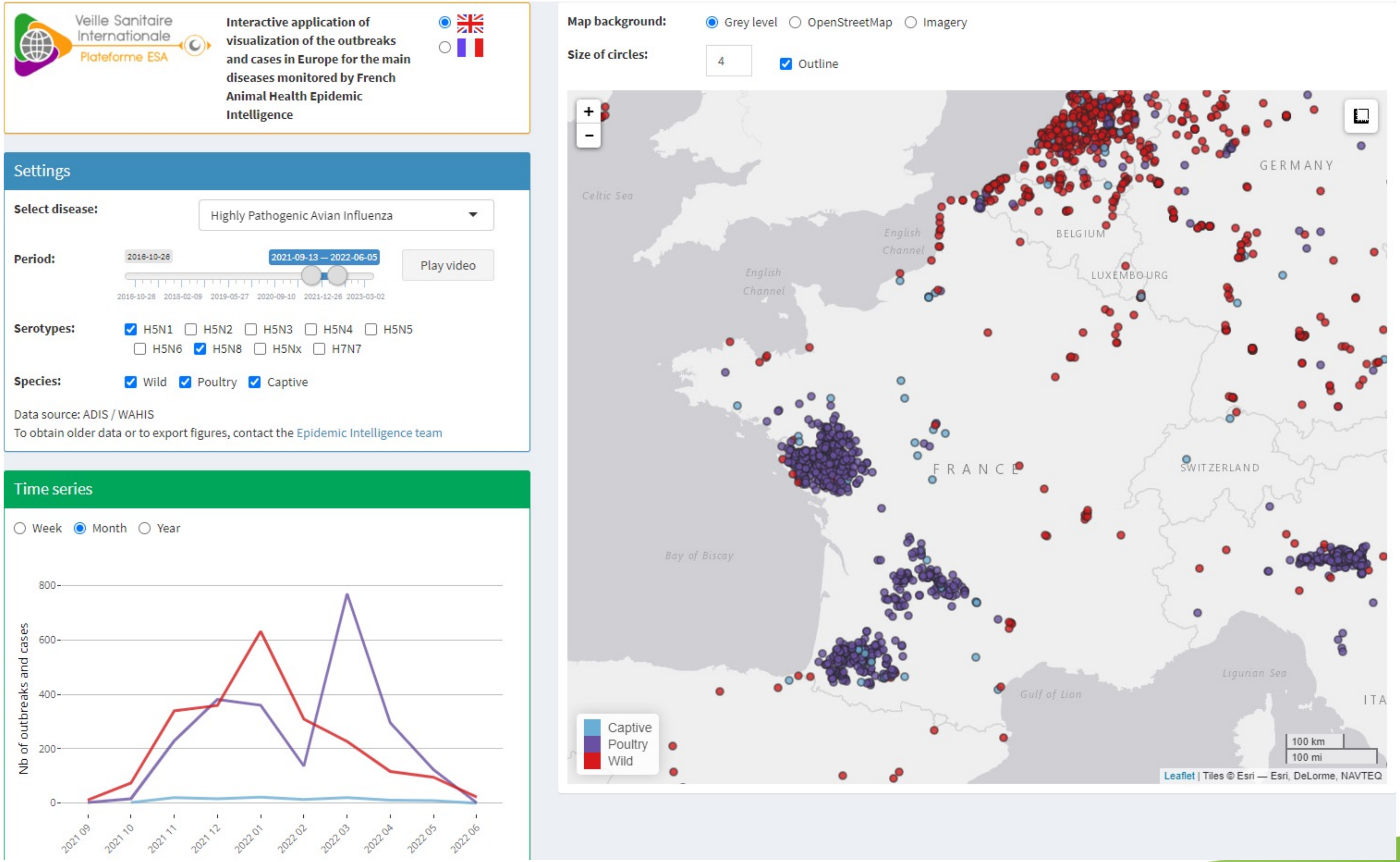
Time series

○ Week ● Month ○ Year

Map background: ● Grey level ○ OpenStreetMap ○ Imagery

Size of circles: 4 ☑ Outline

Avian influenza in France, 2021-2022



Veille Sanitaire Internationale
Plateforme ESA

Interactive application of visualization of the outbreaks and cases in Europe for the main diseases monitored by French Animal Health Epidemic Intelligence

Settings

Select disease: Highly Pathogenic Avian Influenza

Period: 2021-09-13 — 2022-06-05

Serotypes: ☑ H5N1 ☐ H5N2 ☐ H5N3 ☐ H5N4 ☐ H5N5 ☐ H5N6 ☑ H5N8 ☐ H5Nx ☐ H7N7

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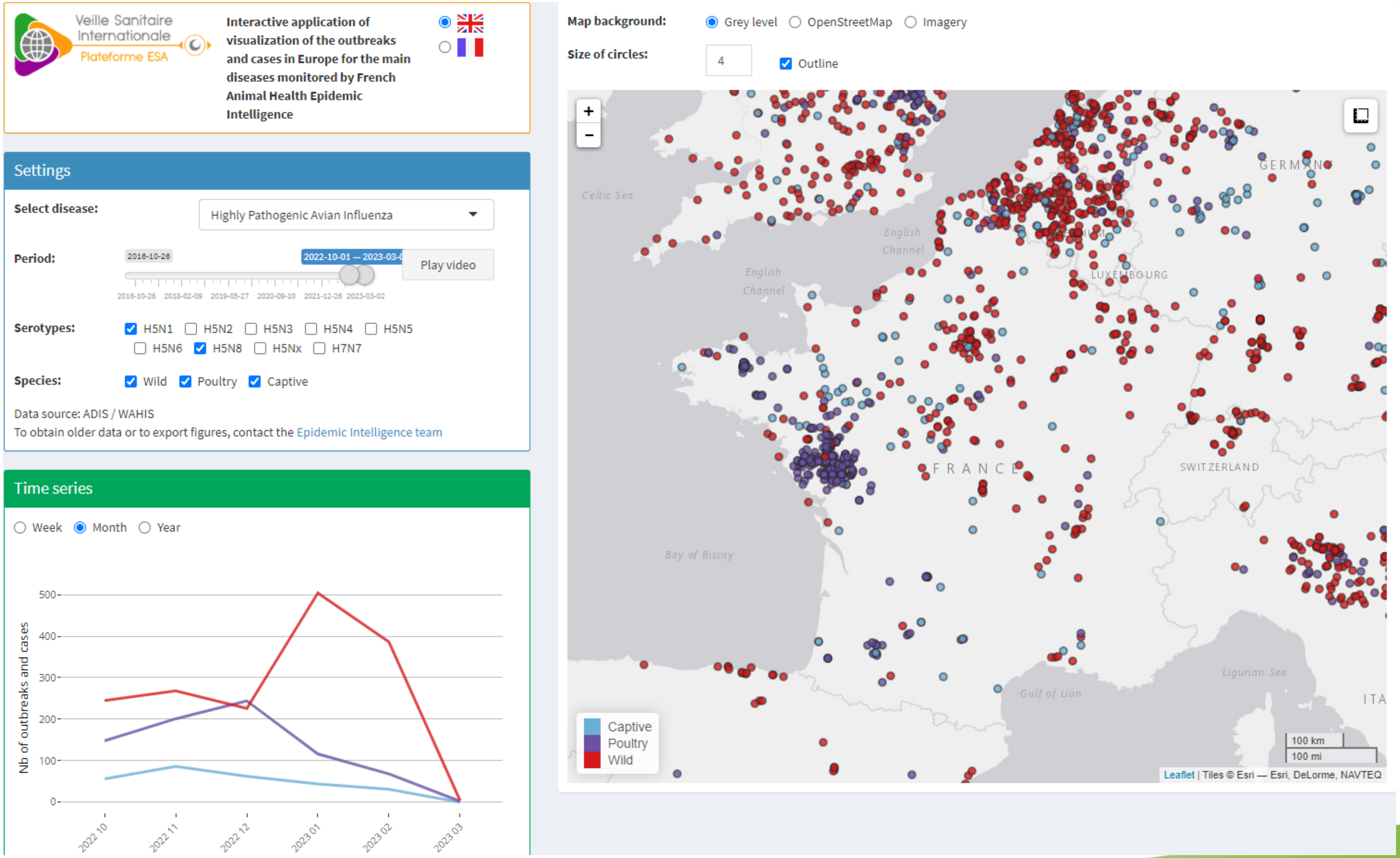
Avian influenza in France, 2022-2023



Veille Sanitaire Internationale
Plateforme ESA

Interactive application of visualization of the outbreaks and cases in Europe for the main diseases monitored by French Animal Health Epidemic Intelligence

Settings

Select disease: Highly Pathogenic Avian Influenza

Period: 2016-10-26 | 2022-10-01 — 2023-03-0

Play video

Serotypes: ☑ H5N1 ☐ H5N2 ☐ H5N3 ☐ H5N4 ☐ H5N5 ☐ H5N6 ☑ H5N8 ☐ H5Nx ☐ H7N7

Species: ☑ Wild ☑ Poultry ☑ Captive

Data source: ADIS / WAHIS

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Time series

○ Week ● Month ○ Year

Map background: ● Grey level ○ OpenStreetMap ○ Imagery

Size of circles: 4 ☑ Outline

Avian influenza in France, 2020-2023

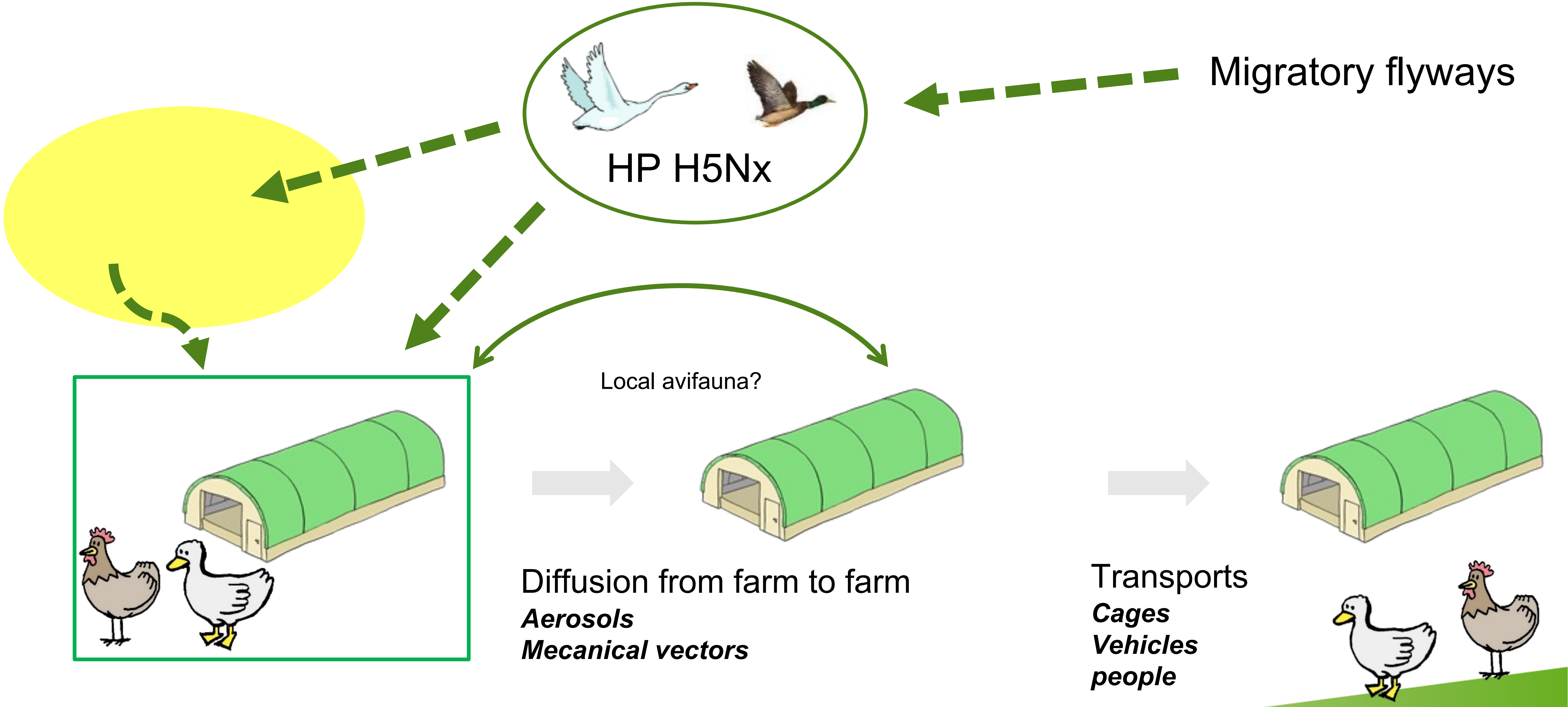
	2020-2021 (H5N8)	**2021-2022** (H5N1)	**2022-2023** (H5N1)
First outbreak	16 November 2020	26 November 2021	1st August 2022
Total outbreaks	514	1476	314

Questions to address during an influenza epizootic:

- Pathotyping (HP vs LP)
- Typing (H*x*N*y*)
- New introduction vs circulating strain
- …

➡ Nanopore sequencing?

The spread of HPAIVs from wild birds to farms



Migratory flyways

HP H5Nx

Local avifauna?

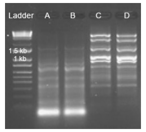
Diffusion from farm to farm
Aerosols
Mecanical vectors

Transports
Cages
Vehicles
people

Workflow

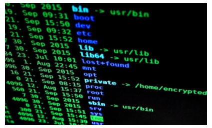
Day 1

- Sampling
- (On-field screening)
- RNA extraction
- Influenza real-time PCR
- Influenza mPCR



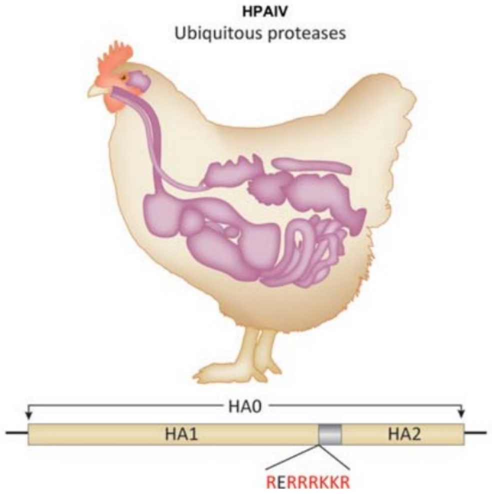
Day 2

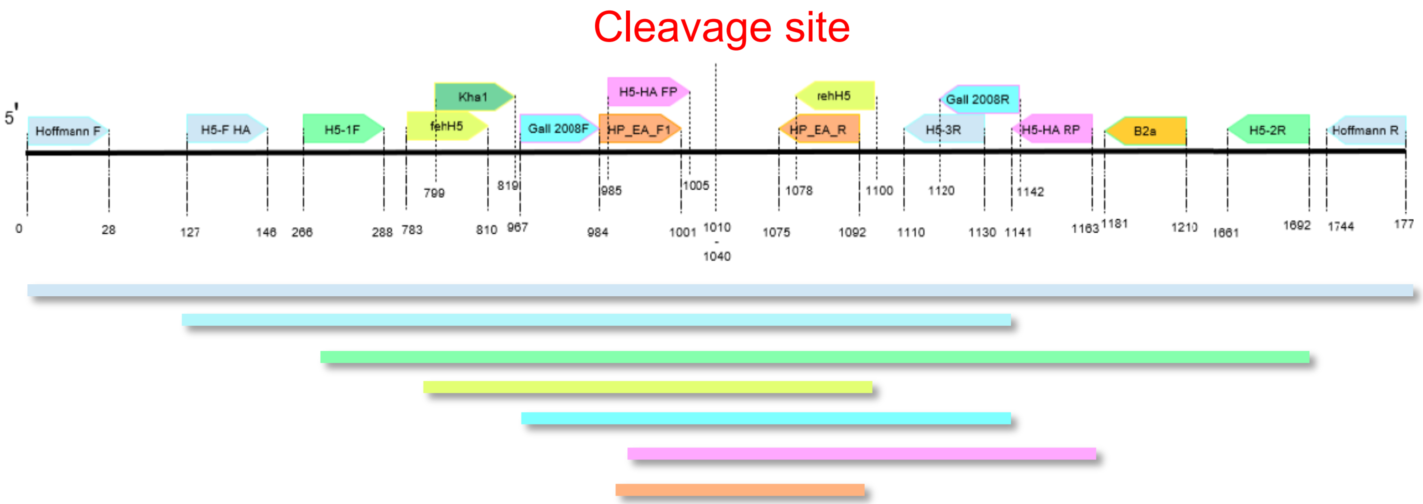
- Library prep
- Sequencing
- Bioinformatics

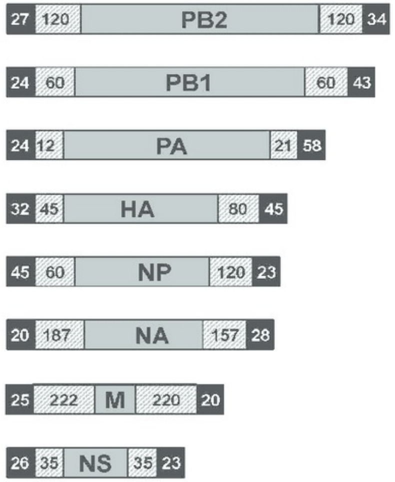


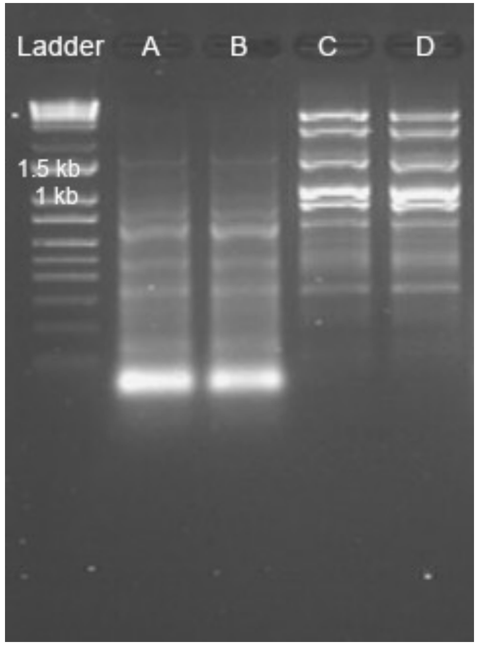
```
Sep 2015 bin -> usr/bin
19. Sep 09:31 boot
21. Sep 15:50 dev
19. Sep 09:32 etc
21. Sep 15:52 home
30. Sep 2015 lib -> usr/lib
30. Sep 2015 lib64 -> usr/lib
34 23. Jul 10:01 lost+found
96 1. Aug 22:45 mnt
996 30. Sep 2015 opt
16 21. Sep 15:52 private -> /home/encrypted
0 21. Sep 08:15 proc
4096 12. Aug 15:37 root
560 21. Sep 15:50 run
7 30. Sep 2015 sbin -> usr/bin
4096 30. Sep 2015 srv
0 21. Sep 15:51 sys
300 21. Sep 15:45 tmp
4096 12. Aug 15:39 usr
```

Influenza PCR

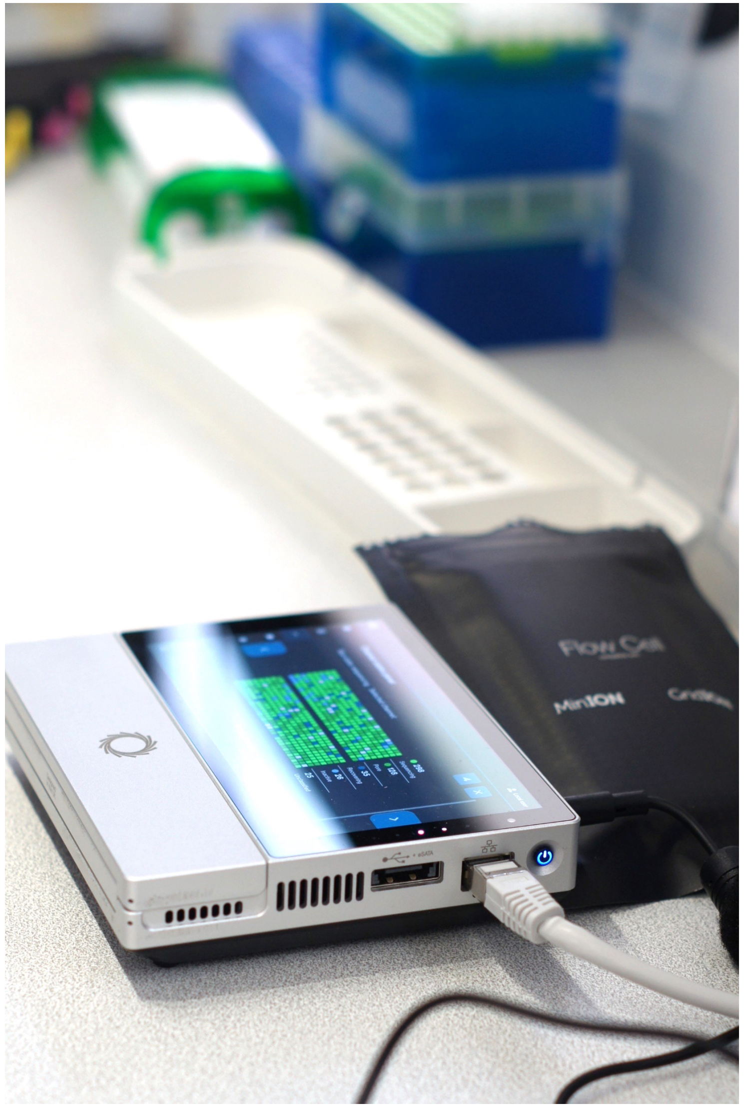


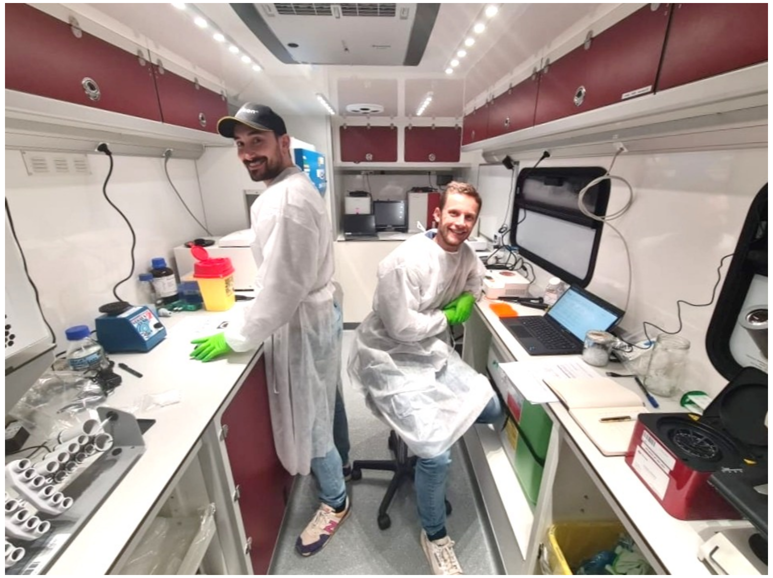

Cleavage site





A lab in a truck



Oxford Nanopore sequencing (3 samples)

Kit type: SQK-NBD112-24

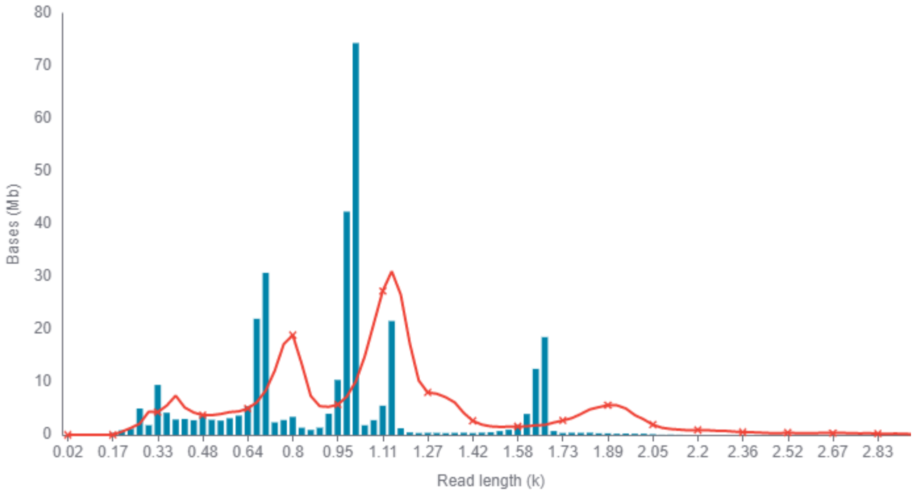
Flow cell type: FLO-MIN106

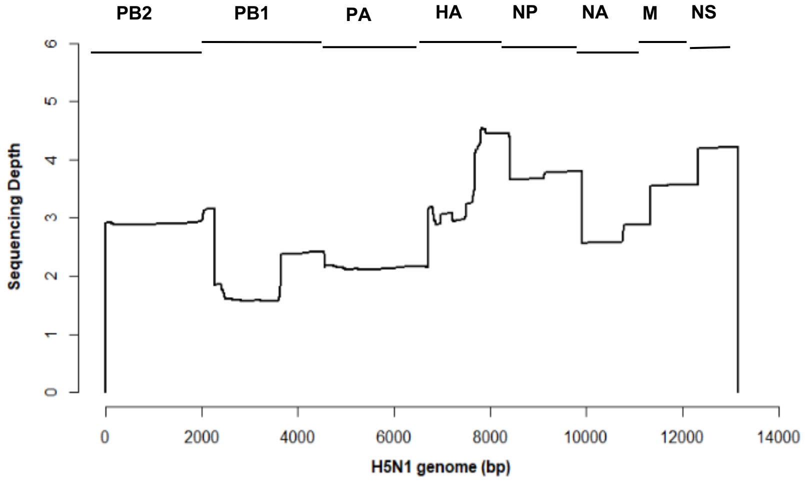
Run duration: 1 hour 45 minutes

Reads generated: 477.13 k

Bases called: 331,72 Mb

N50: 1,02 kb





Bioinformatics

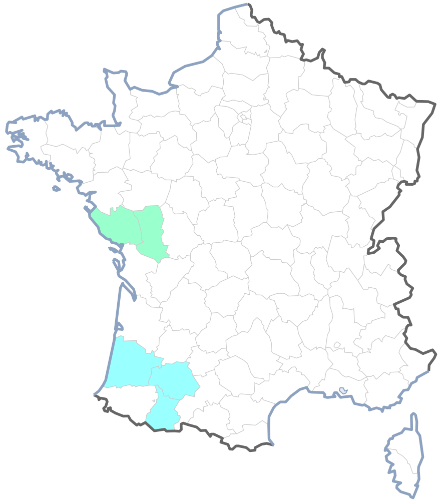
```
minimap2 -ax map-ont reference.fa mysample.fastq > mysample.sam

samtools sort mysample.sam > mysample.sort.bam

samtools index mysample.sort.bam

samtools flagstat mysample.sort.bam > mysample_flagstat

samtools depth mysample.sort.bam > mysample_samdepth

samtools mpileup -A -Q 0 mysample.sort.bam | ivar consensus -p prefix -q 10 -t 0 -m 1
```

Minimap2: Li, H. 2018
Samtools: Li, 2011
iVar: Grubaugh *et al.* 2019

Avian influenza outbreaks tracking 2020-2022

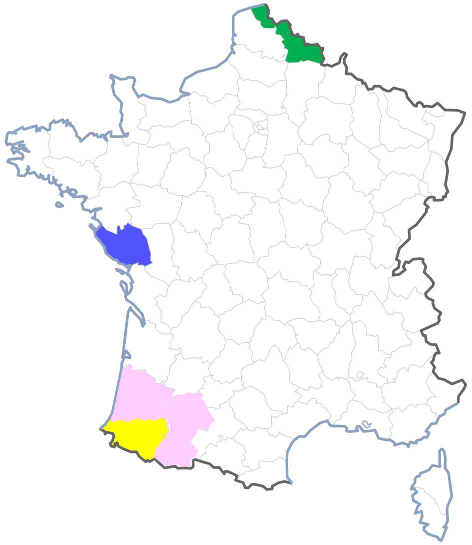
2020-2021 (H5N8)

- 11 samples : tracheal swabs, feathers, dust
- CT value : 14 to 28,5
- Flongle FC
- 28 to 474Mb per sample



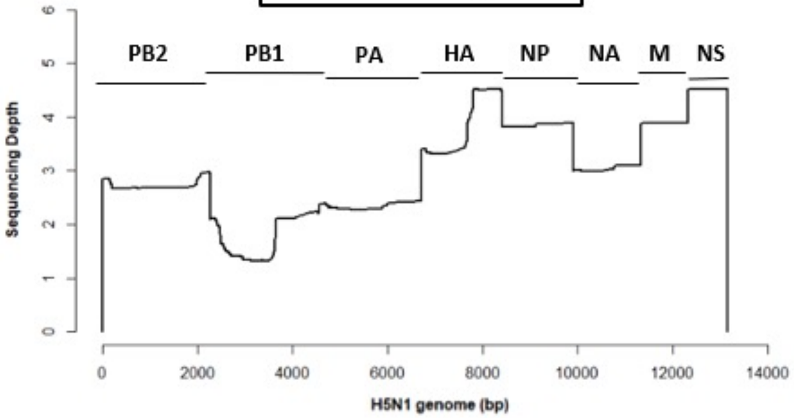
2021-2022 (H5N1)

- 19 samples : tracheal swabs, cloacal swabs feathers, dust
- CT value : 13 to 27
- Flongle FC
- 49 to 205Mb per sample

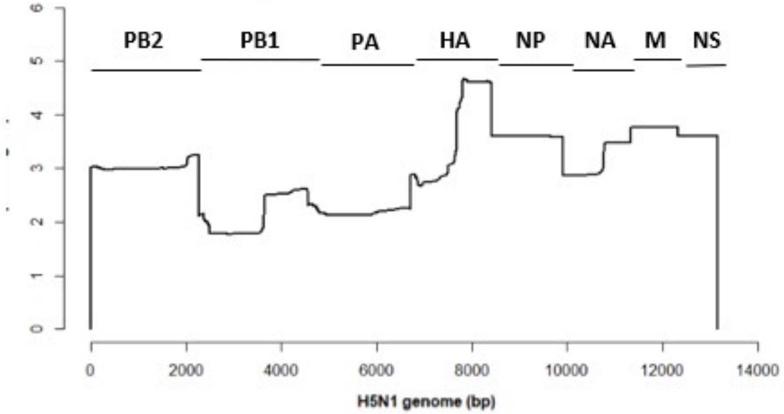


Outbreaks tracking 2020-2022

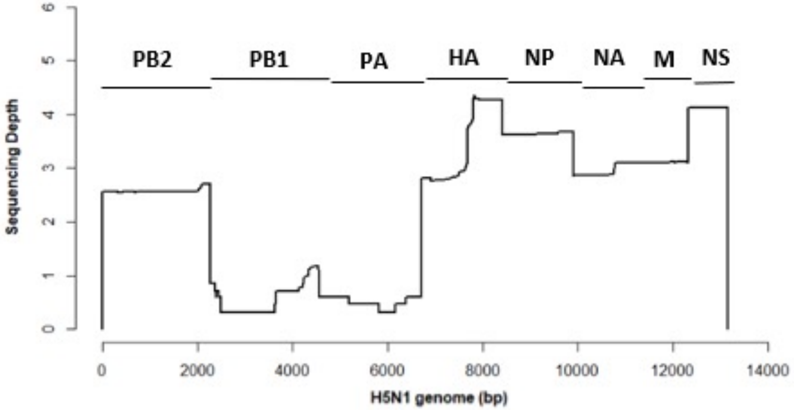
LAB21-328_Z1



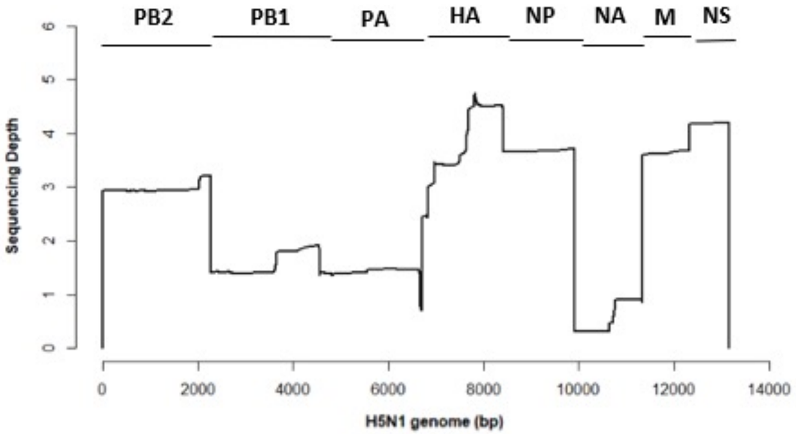
LAB21-328_Z7



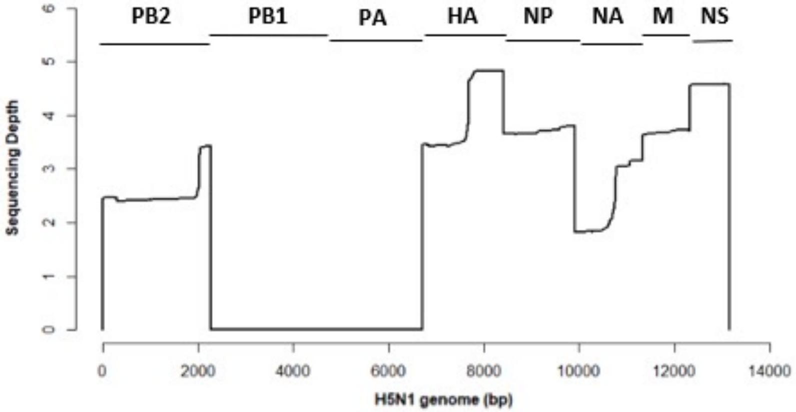
LAB21-329_Z7



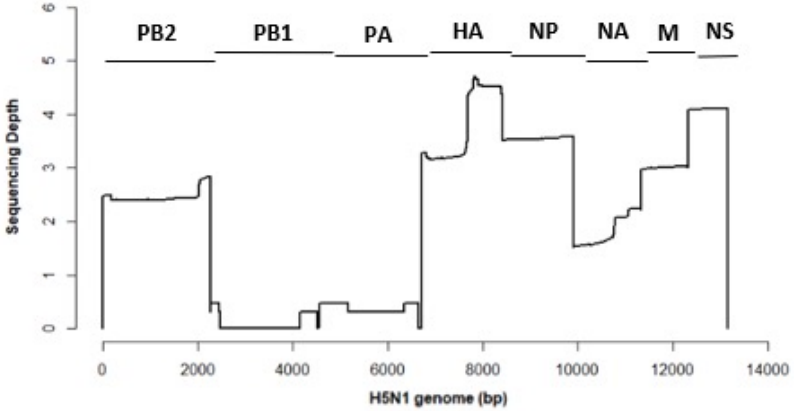
LAB21-343_P11



LAB21-347_P5

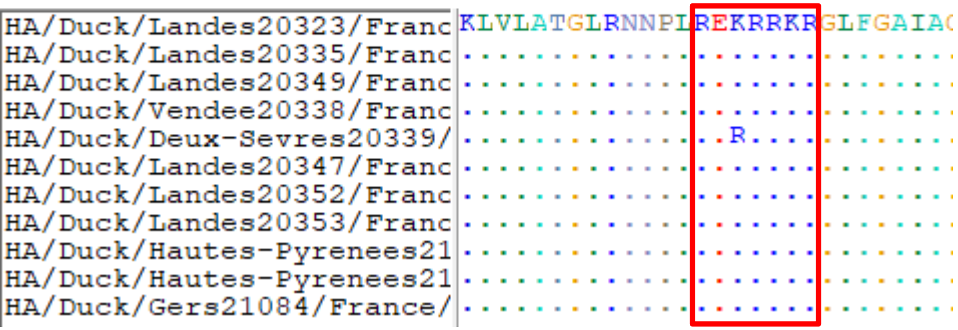


LAB21-347_P8



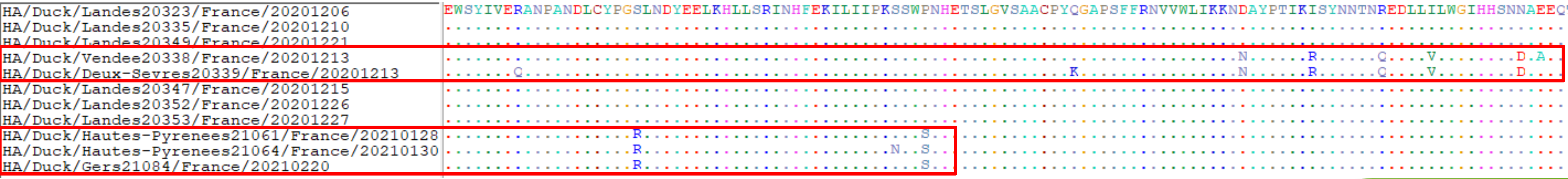
Outbreaks tracking 2020-2022

Pathotyping



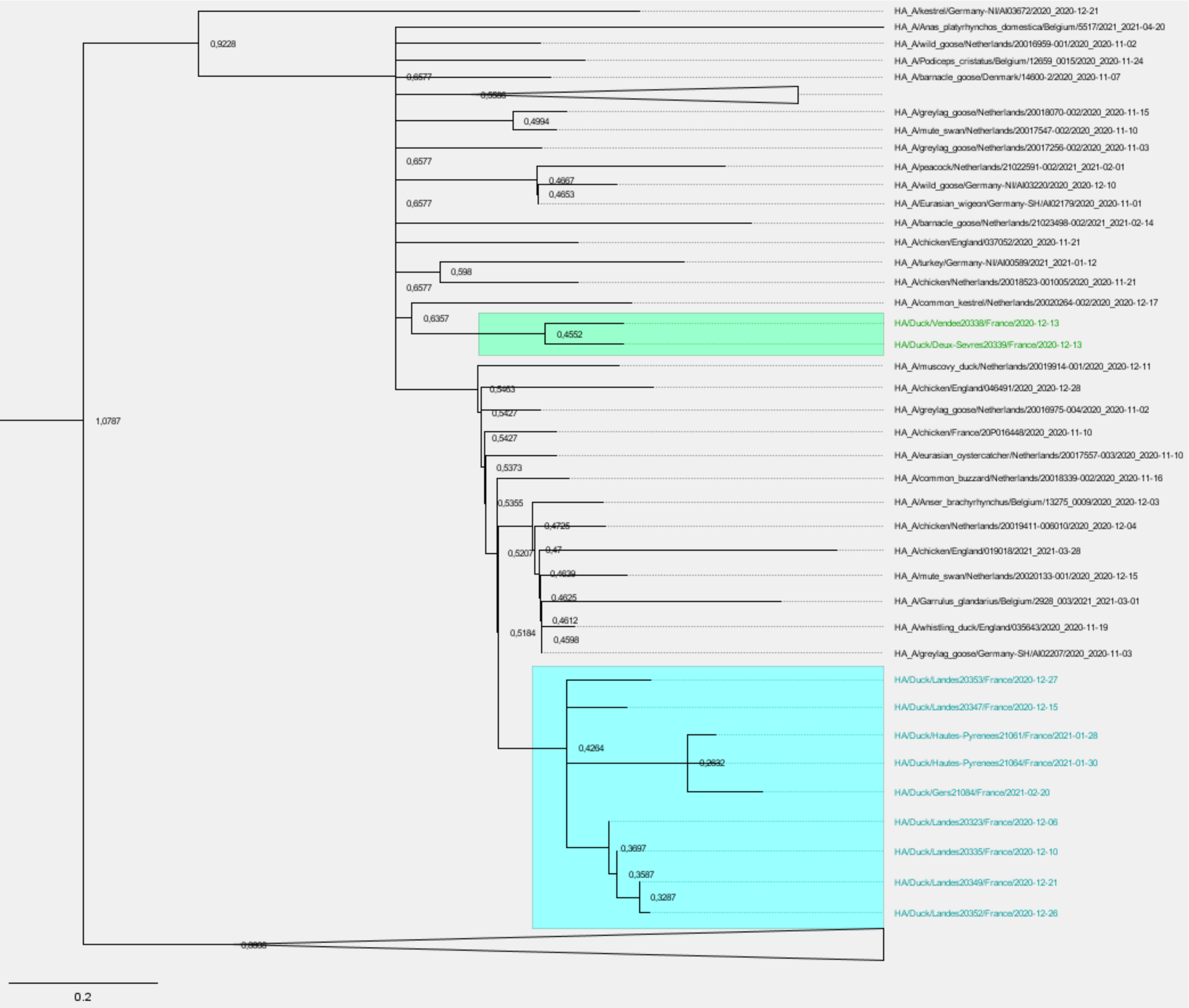
```
HA/Duck/Landes20323/Franc KLVLATGLRNNPLREKRRKRGLFGAIA
HA/Duck/Landes20335/Franc ...........................
HA/Duck/Landes20349/Franc ...........................
HA/Duck/Vendee20338/Franc ...........................
HA/Duck/Deux-Sevres20339/ ...............R...........
HA/Duck/Landes20347/Franc ...........................
HA/Duck/Landes20352/Franc ...........................
HA/Duck/Landes20353/Franc ...........................
HA/Duck/Hautes-Pyrenees21 ...........................
HA/Duck/Hautes-Pyrenees21 ...........................
HA/Duck/Gers21084/France/ ...........................
```

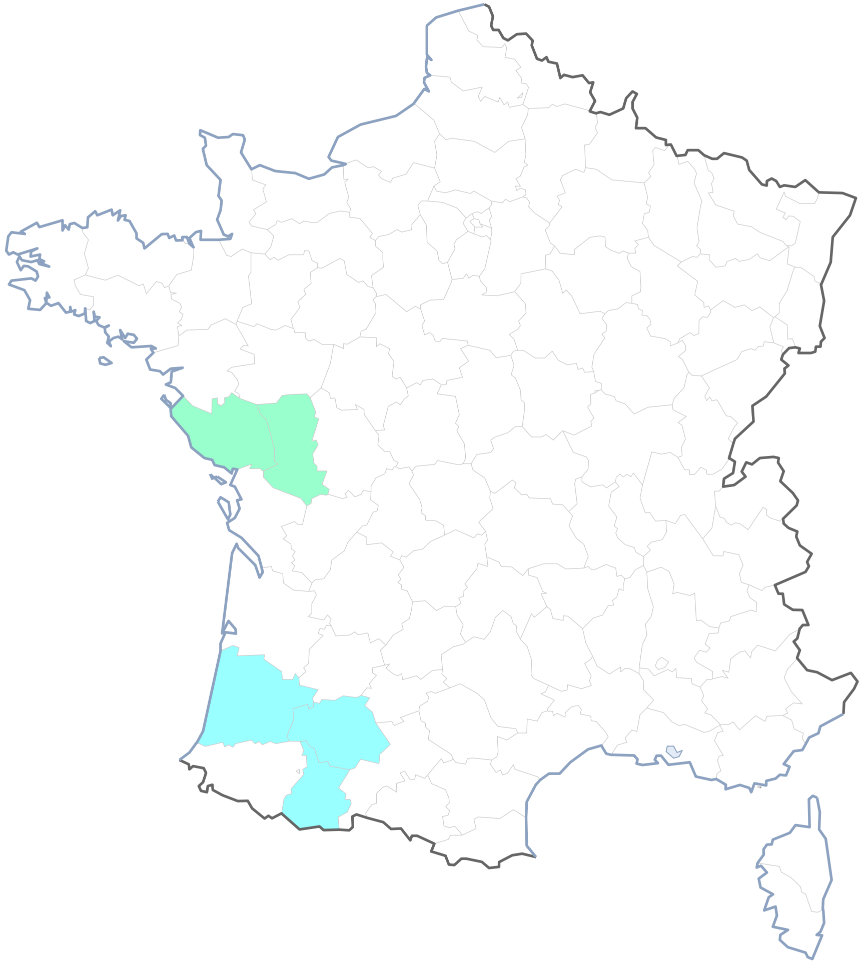
Identification of cluster specific mutations



```
HA/Duck/Landes20323/France/20201206         EWSYIVERANPANDLCYPGSLNDYEELKHLLSRINHFEKILIIPKSSWPNHETSLGVSAACPYQGAPSFFRNVVWLIKKNDAYPTIKISYNNTNREDLLILWGIHHSNNAEEQ
HA/Duck/Landes20335/France/20201210         .................................................................................................................
HA/Duck/Landes20349/France/20201221         .................................................................................................................
HA/Duck/Vendee20338/France/20201213         ................................................................................N......R......Q....V........D.A..
HA/Duck/Deux-Sevres20339/France/20201213    .......Q.......................................................K................N......R......Q....V........D....
HA/Duck/Landes20347/France/20201215         .................................................................................................................
HA/Duck/Landes20352/France/20201226         .................................................................................................................
HA/Duck/Landes20353/France/20201227         .................................................................................................................
HA/Duck/Hautes-Pyrenees21061/France/20210128 ...................R............................S................................................................
HA/Duck/Hautes-Pyrenees21064/France/20210130 ...................R.........................N..S................................................................
HA/Duck/Gers21084/France/20210220           ...................R............................S................................................................
```

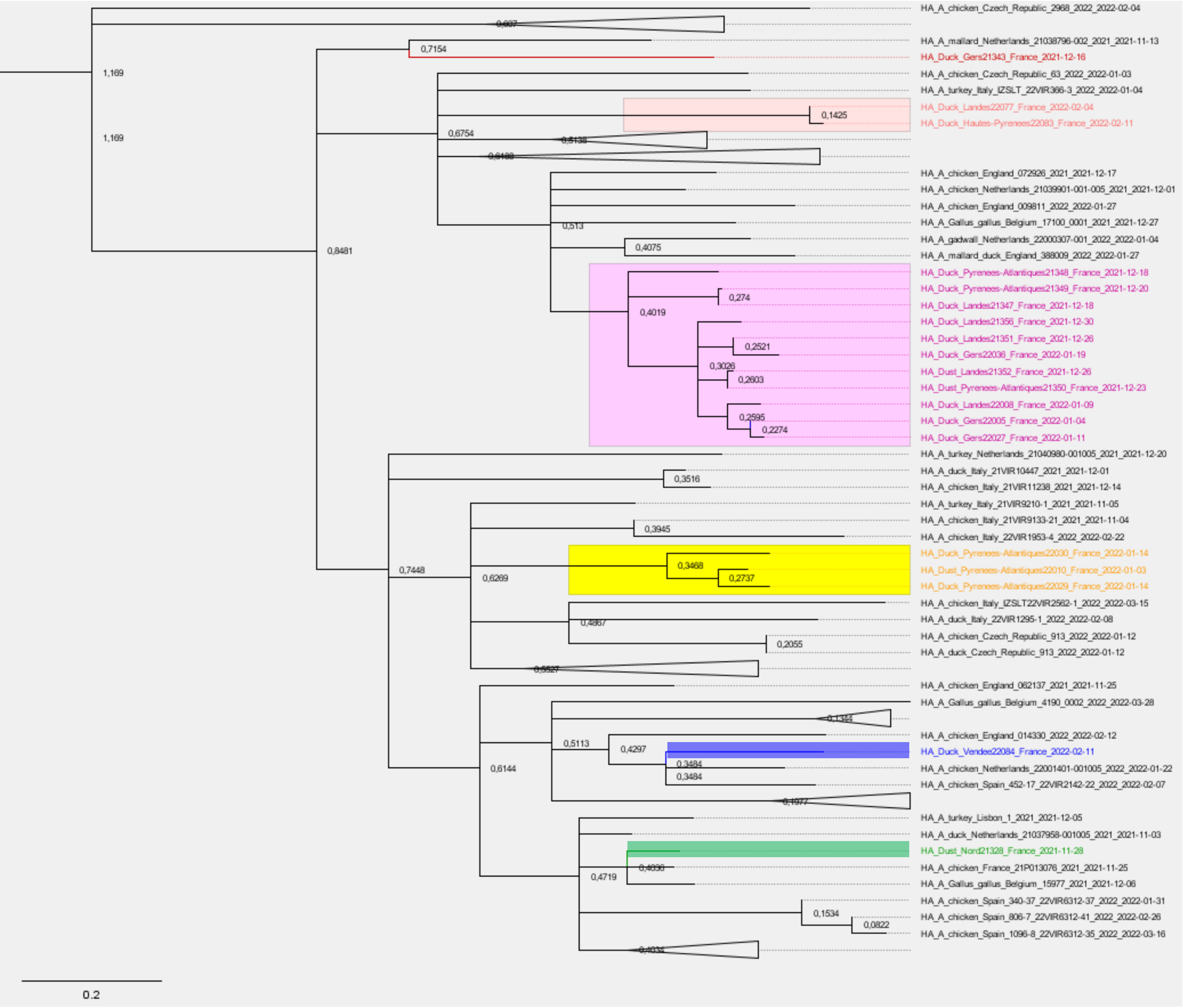
Outbreaks tracking 2020-2021

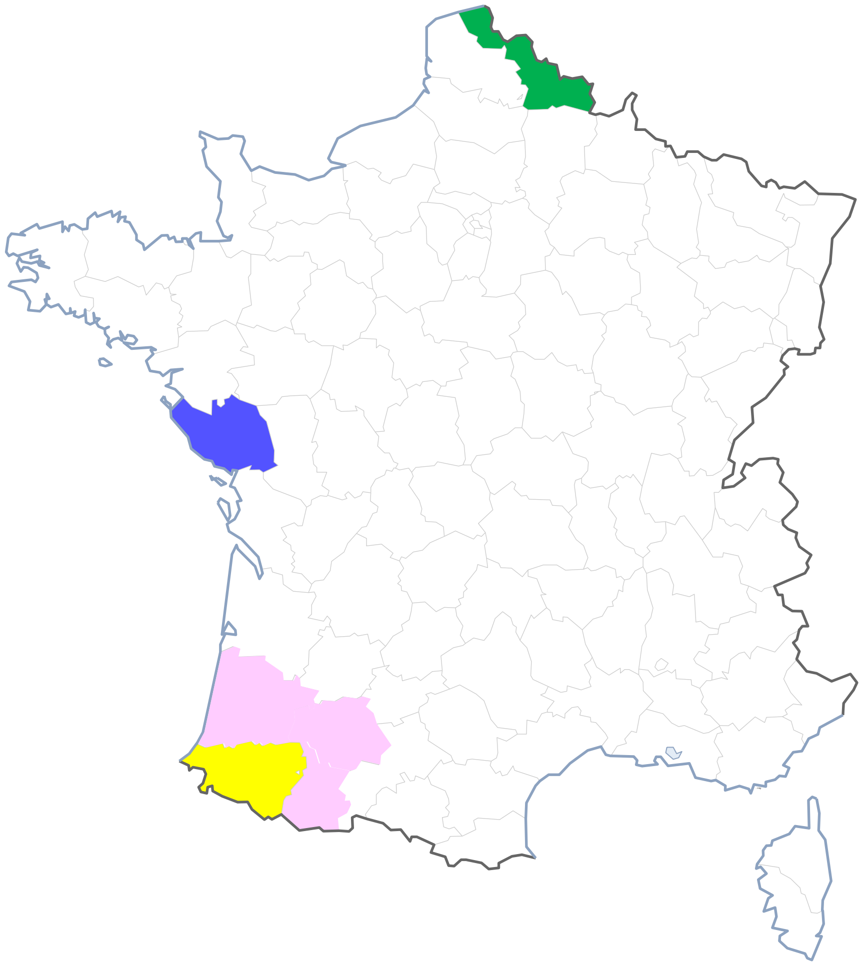




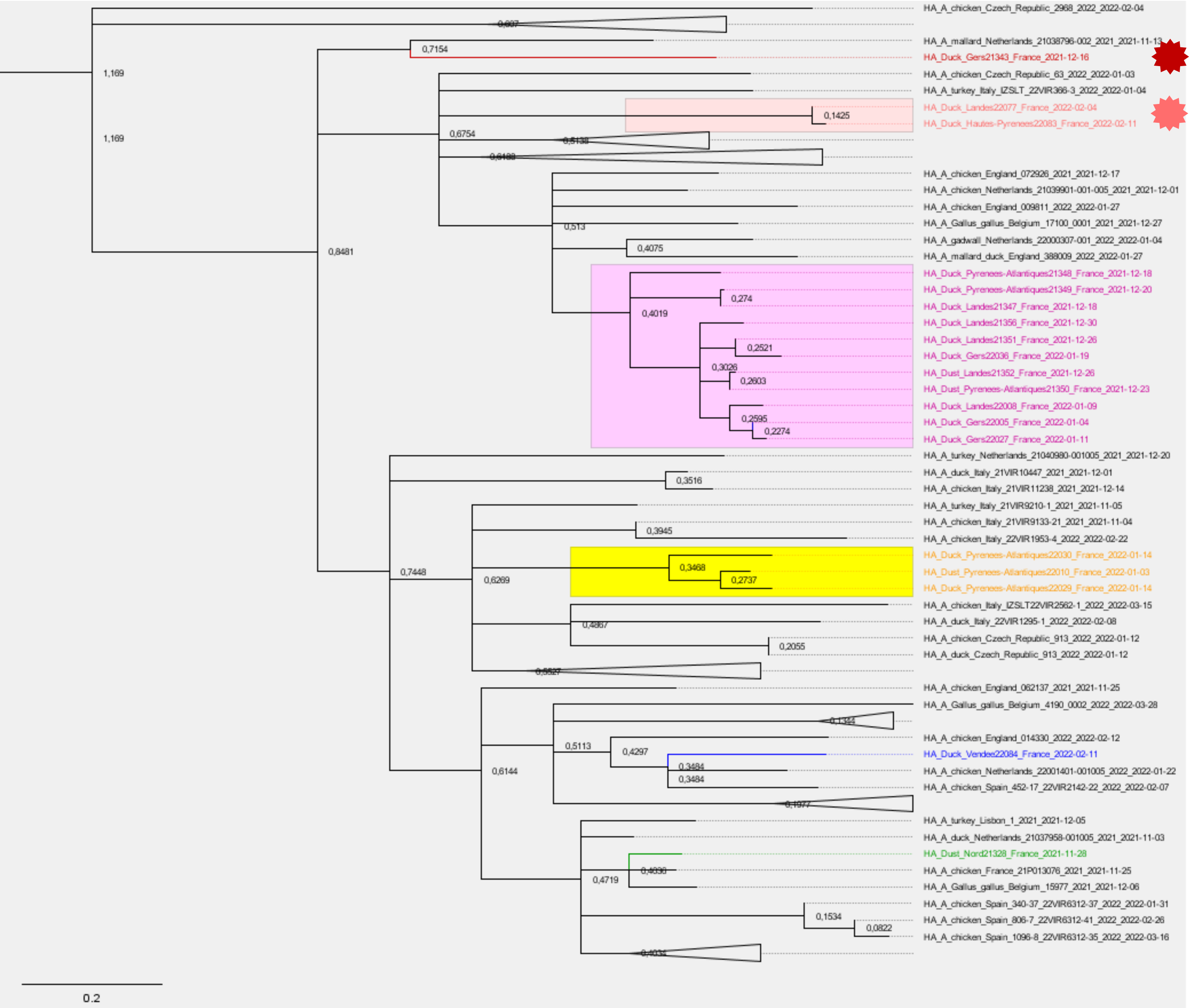
→ Two different outbreaks clusters

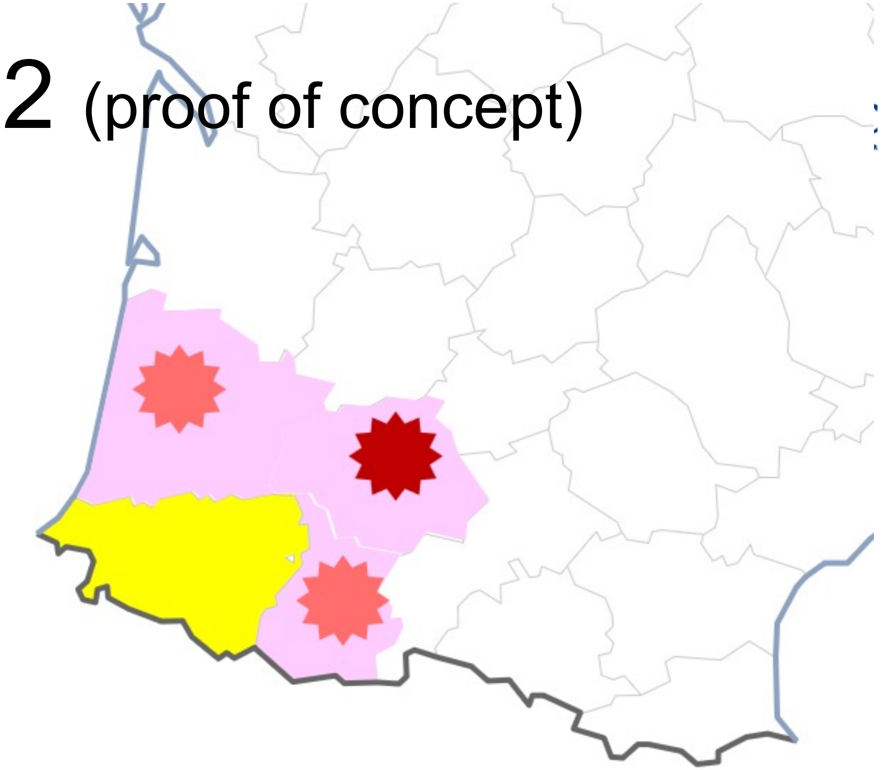
Outbreaks tracking 2021-2022





Outbreaks tracking 2021-2022 (proof of concept)





→ Introductions (✸) not linked with circulating strains

An amplicon-based nanopore sequencing workflow for rapid tracking of avian influenza outbreaks, France, 2020-2022.

Croville G, Walch M, Sécula A, Lèbre L, Silva S, Filaire F, Guérin JL.

Front Cell Infect Microbiol. 2024 Jan 22;14:1257586. doi: 10.3389/fcimb.2024.1257586. eCollection 2024.

PMID: 38318163 **Free PMC article.**

Conclusion

- Combination of mPCR with ONT:
  - Typing, pathotyping, phylogeny in <48h
  - Whole genome sequencing / good seq. depth
- MK1C 😍
- (Expired) MinION Flowcell 😍

Nanopore sequencing for metagenomic studies

Metagenomics is:

Metagenomics

Metagenomics aims to study the **microbiome**.

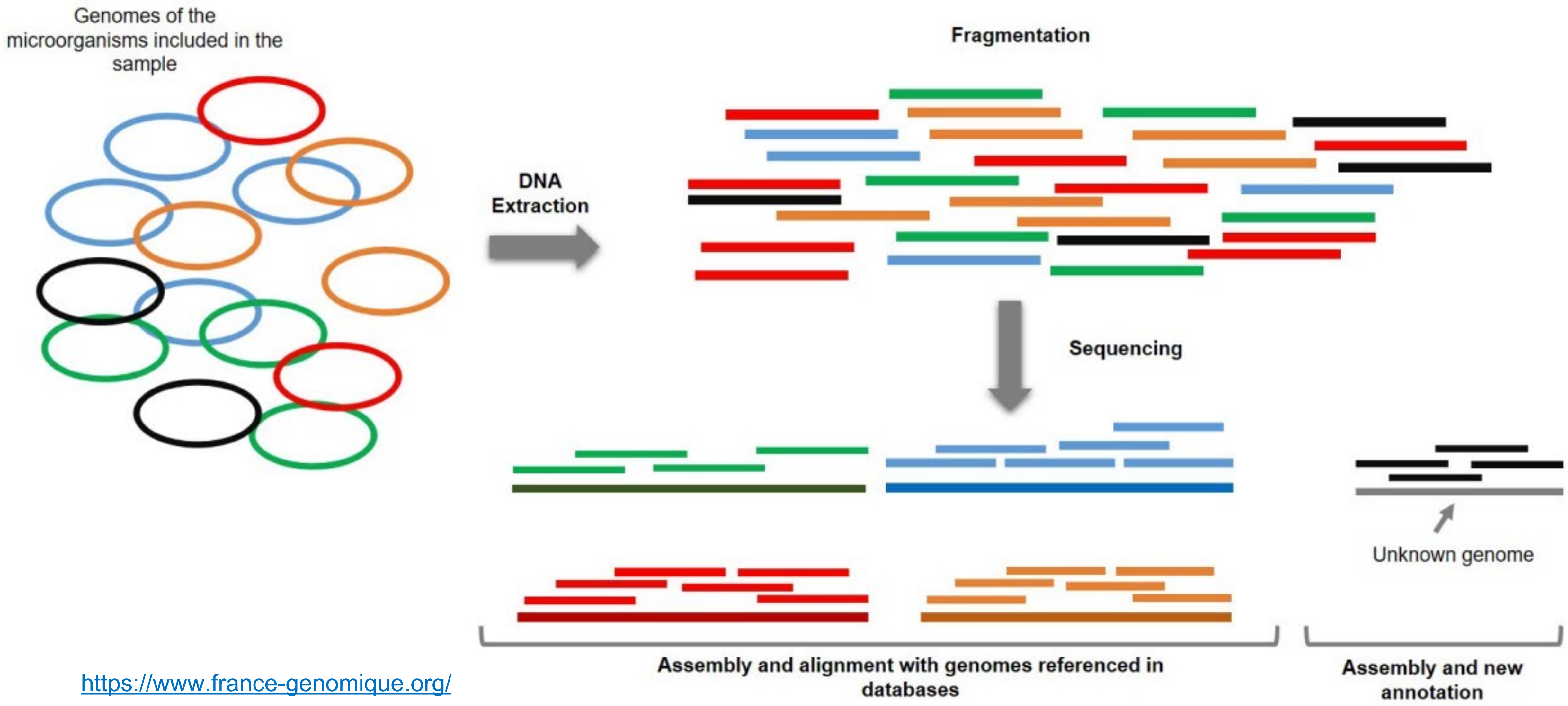
Microbiome: the set of micro-organisms (bacteria, viruses, fungi, yeasts, plankton…) living in a specific environment (intestine, ocean, soil, air, etc.).

Metagenomic can be performed using 2 approaches:

– **Targeted metagenomics** or **metabarcoding**.

– **Shotgun metagenomics.**

https://www.france-genomique.org/



Genomes of the microorganisms included in the sample

DNA Extraction

Fragmentation

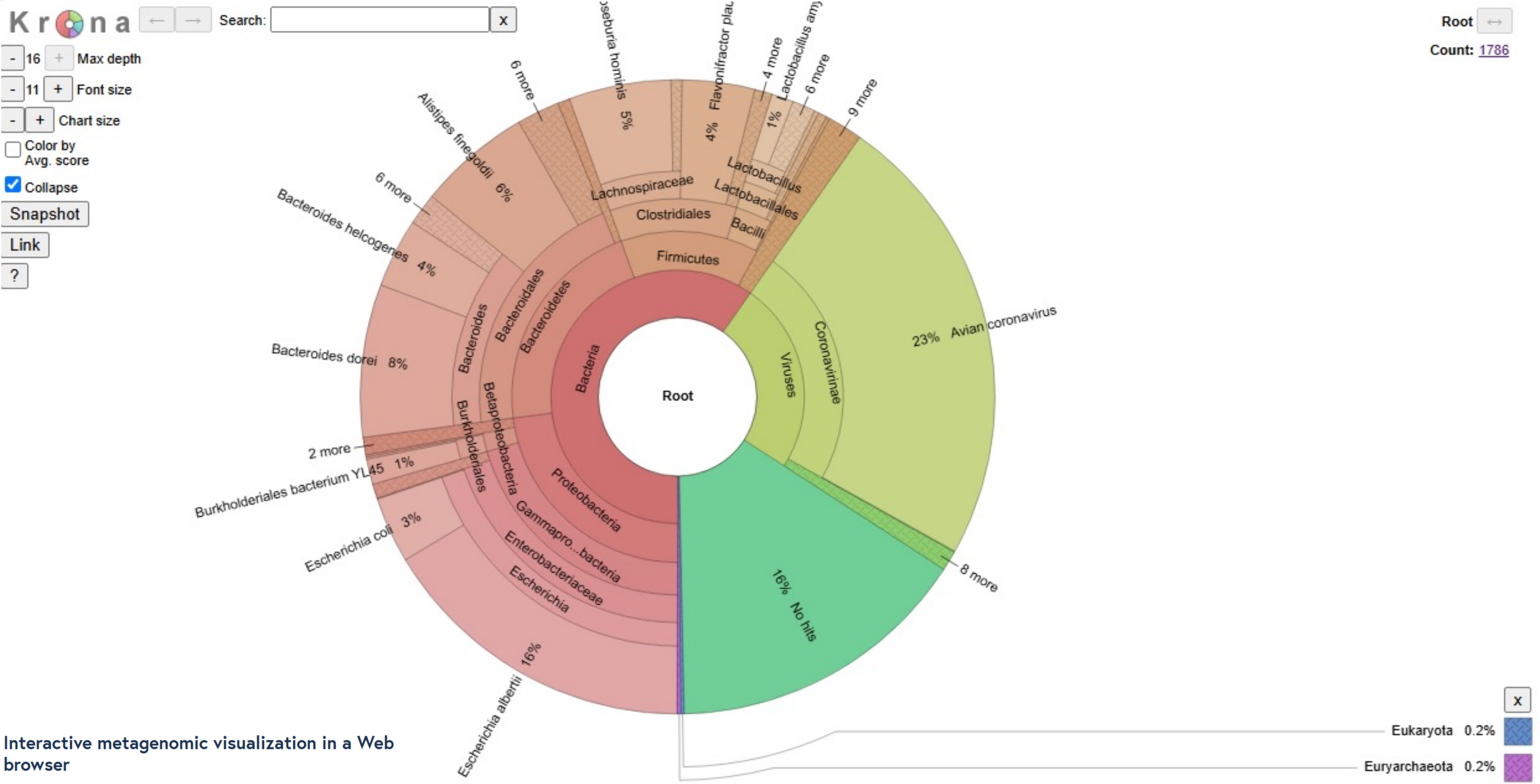
Sequencing

Unknown genome

Assembly and alignment with genomes referenced in databases

Assembly and new annotation

https://www.france-genomique.org/



- 16 + Max depth
- 11 + Font size
- + Chart size

☐ Color by Avg. score

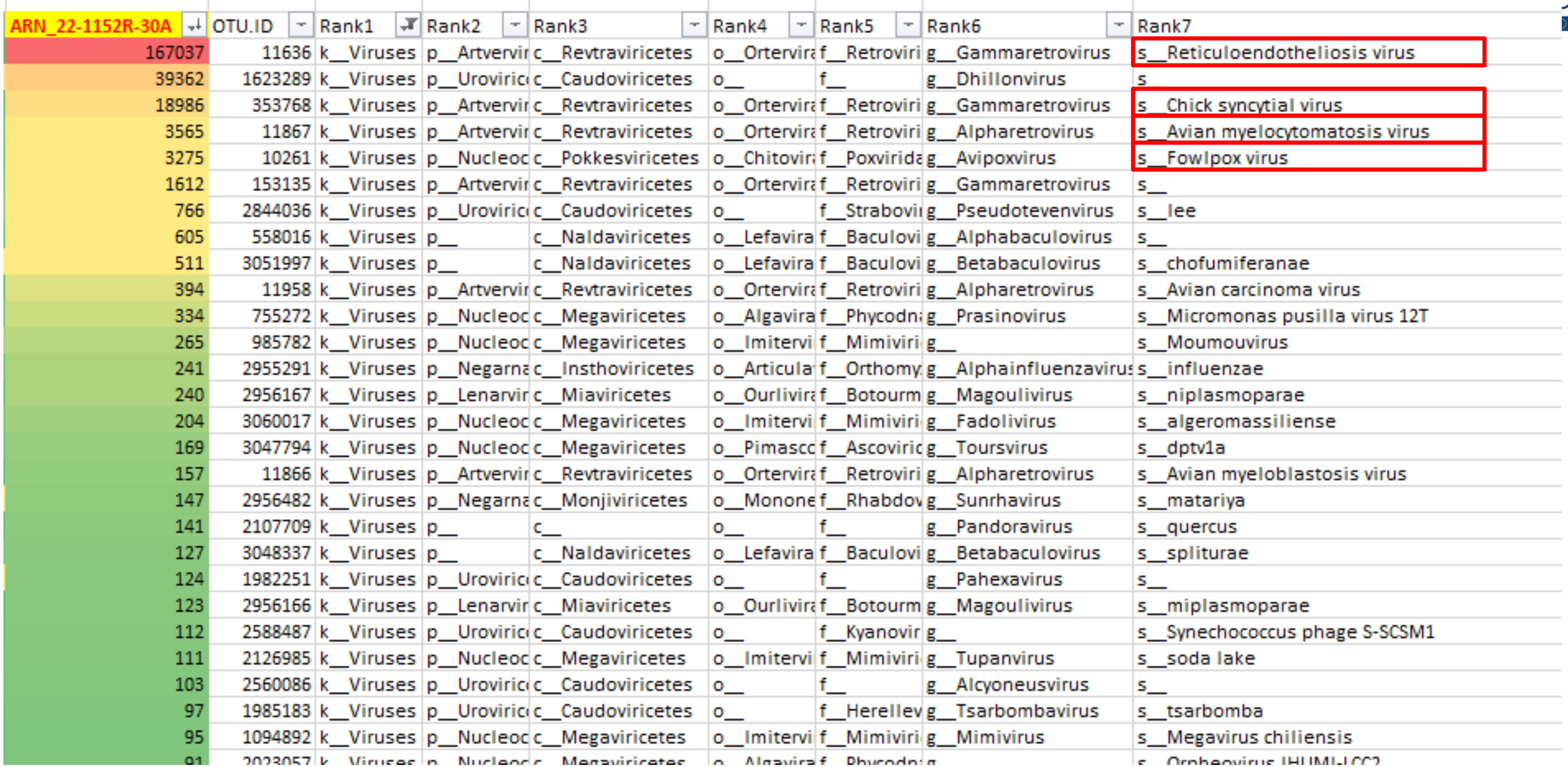
☑ Collapse

Count: 1786

Interactive metagenomic visualization in a Web browser

Brian D Ondov, Nicholas H Bergman & Adam M Phillippy

BMC Bioinformatics **12**, Article number: 385 (2011) | Cite this article



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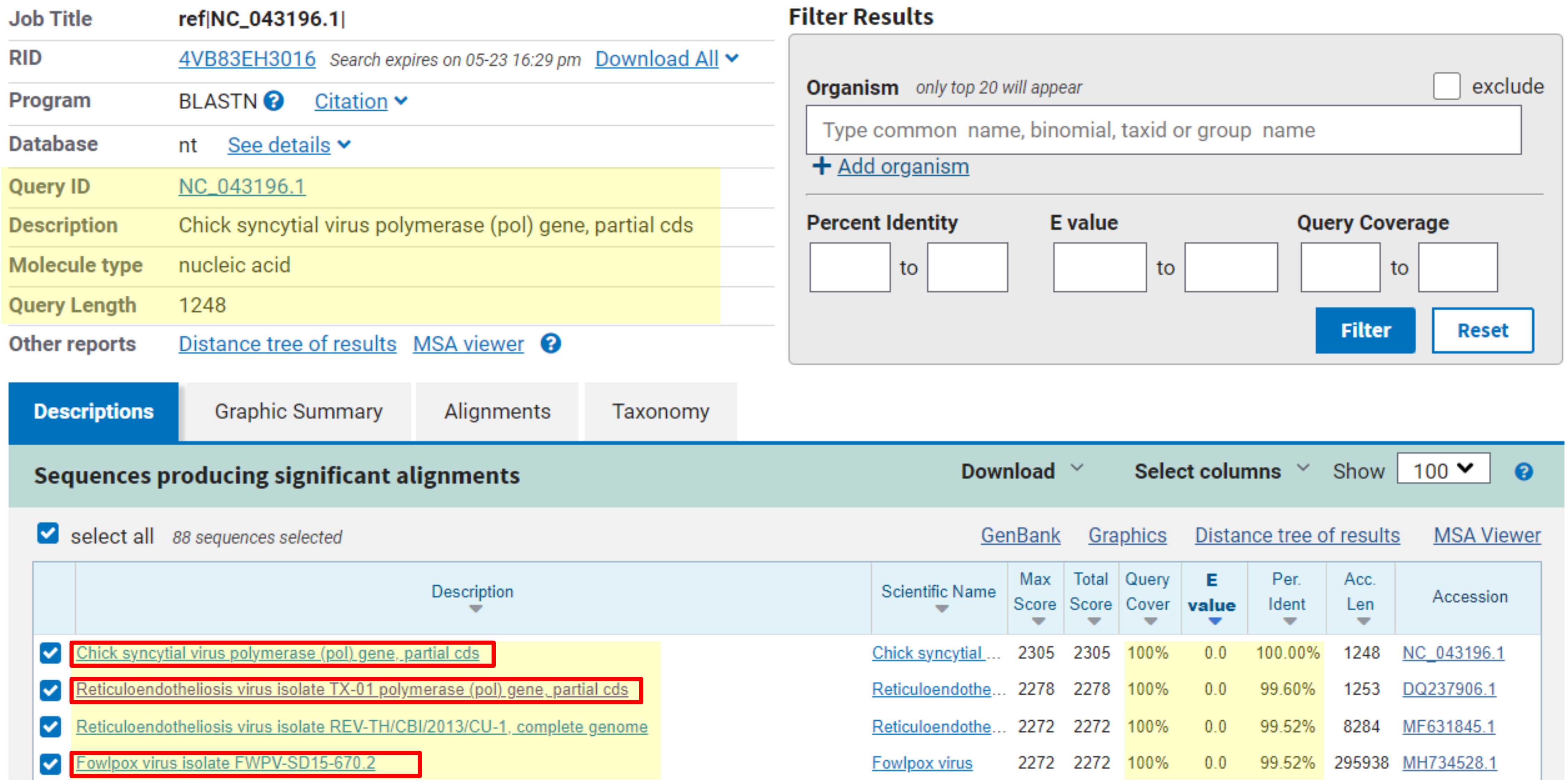
39362	1623289	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__	g__Dhillonvirus	s
18986	353768	k__Viruses	p__Artvervir	c__Revtraviricetes	o__Ortervira	f__Retroviri	g__Gammaretrovirus	s__Chick syncytial virus
3565	11867	k__Viruses	p__Artvervir	c__Revtraviricetes	o__Ortervira	f__Retroviri	g__Alpharetrovirus	s__Avian myelocytomatosis virus
3275	10261	k__Viruses	p__Nucleoc	c__Pokkesviricetes	o__Chitovir	f__Poxvirida	g__Avipoxvirus	s__Fowlpox virus
1612	153135	k__Viruses	p__Artvervir	c__Revtraviricetes	o__Ortervira	f__Retroviri	g__Gammaretrovirus	s__
766	2844036	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__Strabovi	g__Pseudotevenvirus	s__lee
605	558016	k__Viruses	p__	c__Naldaviricetes	o__Lefavira	f__Baculovi	g__Alphabaculovirus	s__
511	3051997	k__Viruses	p__	c__Naldaviricetes	o__Lefavira	f__Baculovi	g__Betabaculovirus	s__chofumiferanae
394	11958	k__Viruses	p__Artvervir	c__Revtraviricetes	o__Ortervira	f__Retroviri	g__Alpharetrovirus	s__Avian carcinoma virus
334	755272	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Algavira	f__Phycodna	g__Prasinovirus	s__Micromonas pusilla virus 12T
265	985782	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Imitervi	f__Mimiviri	g__	s__Moumouvirus
241	2955291	k__Viruses	p__Negarna	c__Insthoviricetes	o__Articula	f__Orthomy	g__Alphainfluenzavirus	s__influenzae
240	2956167	k__Viruses	p__Lenarvir	c__Miaviricetes	o__Ourlivira	f__Botourm	g__Magoulivirus	s__niplasmoparae
204	3060017	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Imitervi	f__Mimiviri	g__Fadolivirus	s__algeromassiliense
169	3047794	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Pimascc	f__Ascoviric	g__Toursvirus	s__dptv1a
157	11866	k__Viruses	p__Artvervir	c__Revtraviricetes	o__Ortervira	f__Retroviri	g__Alpharetrovirus	s__Avian myeloblastosis virus
147	2956482	k__Viruses	p__Negarna	c__Monjiviricetes	o__Monone	f__Rhabdov	g__Sunrhavirus	s__matariya
141	2107709	k__Viruses	p__	c__	o__	f__	g__Pandoravirus	s__quercus
127	3048337	k__Viruses	p__	c__Naldaviricetes	o__Lefavira	f__Baculovi	g__Betabaculovirus	s__spliturae
124	1982251	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__	g__Pahexavirus	s__
123	2956166	k__Viruses	p__Lenarvir	c__Miaviricetes	o__Ourlivira	f__Botourm	g__Magoulivirus	s__miplasmoparae
112	2588487	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__Kyanovir	g__	s__Synechococcus phage S-SCSM1
111	2126985	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Imitervi	f__Mimiviri	g__Tupanvirus	s__soda lake
103	2560086	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__	g__Alcyoneusvirus	s__
97	1985183	k__Viruses	p__Uroviric	c__Caudoviricetes	o__	f__Herellev	g__Tsarbombavirus	s__tsarbomba
95	1094892	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Imitervi	f__Mimiviri	g__Mimivirus	s__Megavirus chiliensis
91	2023057	k__Viruses	p__Nucleoc	c__Megaviricetes	o__Algavira	f__Phycodna	g__	s__Orpheovirus IHUMI-LCC2

BLAST of the CSV polymerase gene



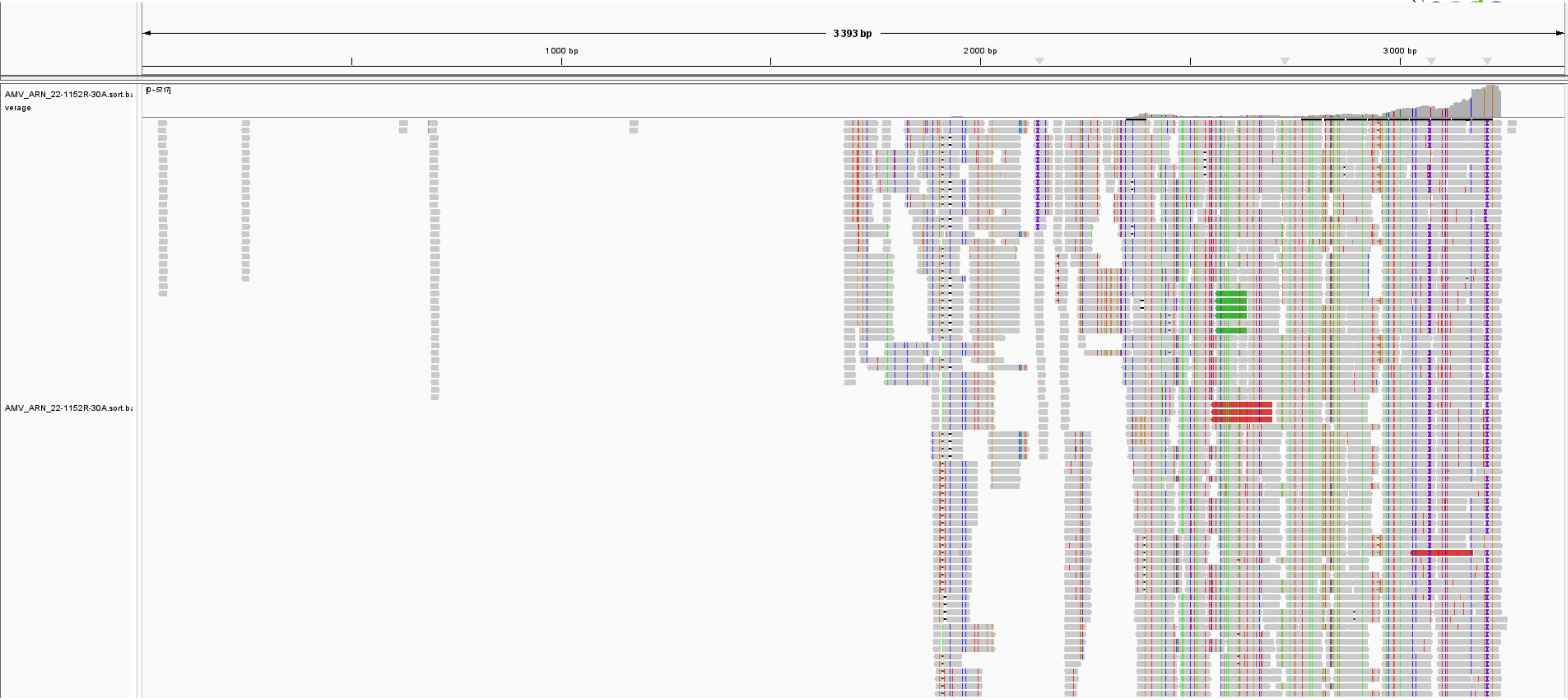
Job Title	ref|NC_043196.1|
RID	4VB83EH3016 *Search expires on 05-23 16:29 pm* Download All
Program	BLASTN Citation
Database	nt See details
Query ID	NC_043196.1
Description	Chick syncytial virus polymerase (pol) gene, partial cds
Molecule type	nucleic acid
Query Length	1248
Other reports	Distance tree of results MSA viewer

Sequences producing significant alignments

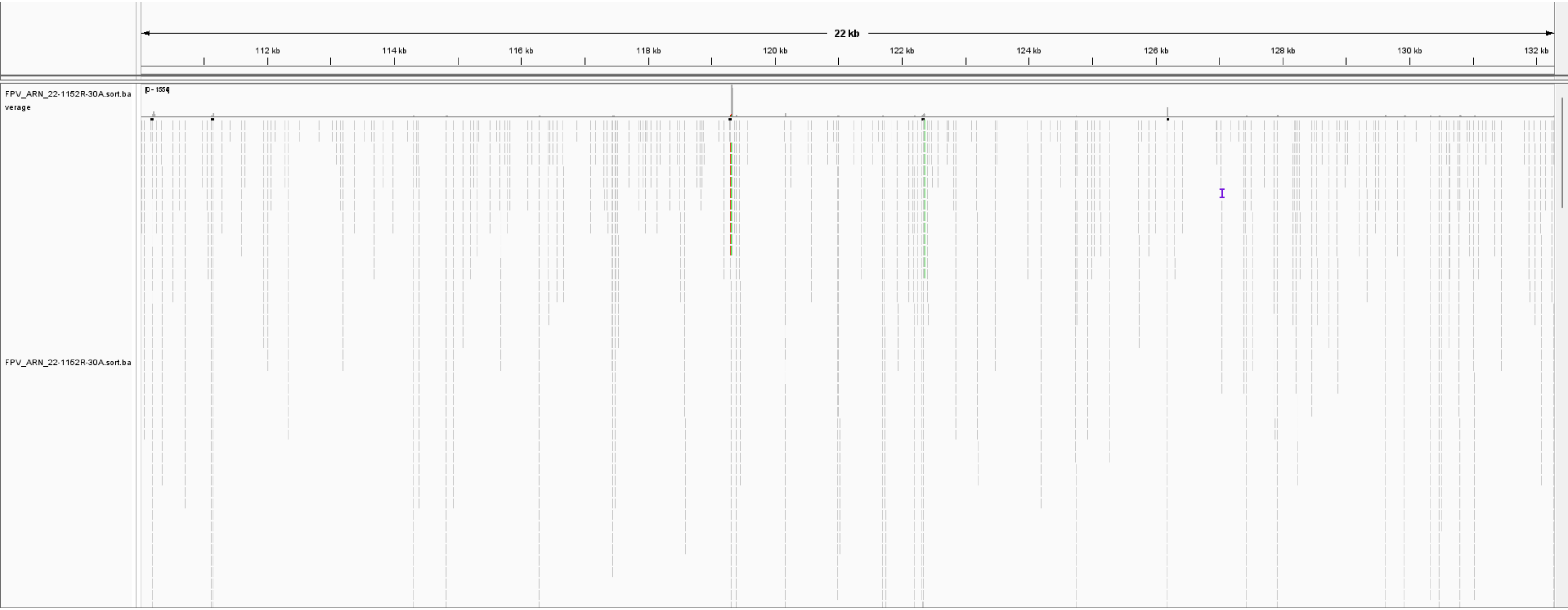
☑ select all *88 sequences selected*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Chick syncytial virus polymerase (pol) gene, partial cds	Chick syncytial ...	2305	2305	100%	0.0	100.00%	1248	NC_043196.1
☑	Reticuloendotheliosis virus isolate TX-01 polymerase (pol) gene, partial cds	Reticuloendothe...	2278	2278	100%	0.0	99.60%	1253	DQ237906.1
☑	Reticuloendotheliosis virus isolate REV-TH/CBI/2013/CU-1, complete genome	Reticuloendothe...	2272	2272	100%	0.0	99.52%	8284	MF631845.1
☑	Fowlpox virus isolate FWPV-SD15-670.2	Fowlpox virus	2272	2272	100%	0.0	99.52%	295938	MH734528.1

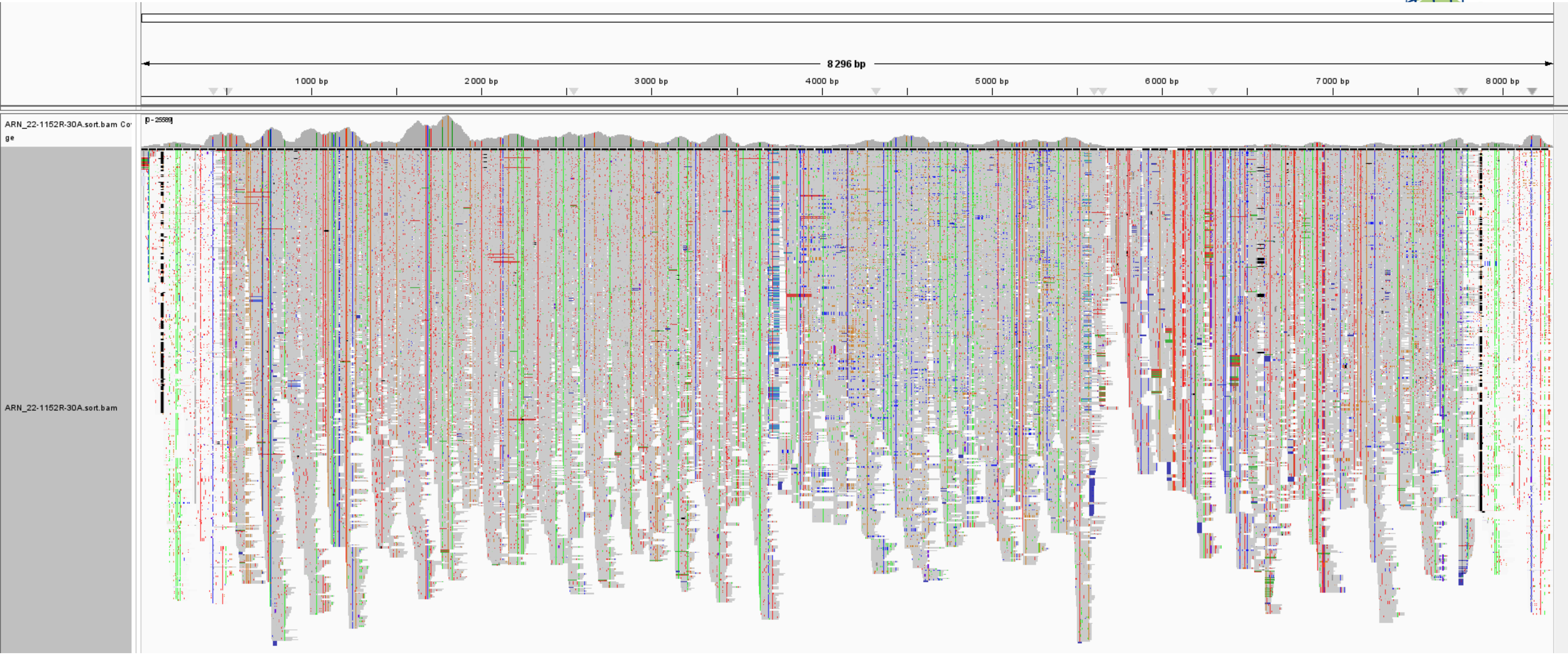
Avian myelocytomatosis virus



Fowlpox virus



Reticuloendotheliosis virus



Integrative genomics viewer

James T Robinson, Helga Thorvaldsdóttir, Wendy Winckler, Mitchell Guttman, Eric S Lander, Gad Getz
& Jill P Mesirov

Nature Biotechnology **29**, 24–26 (2011) | Cite this article

Shotgun metagenomics, Example of Bat CoV sequencing

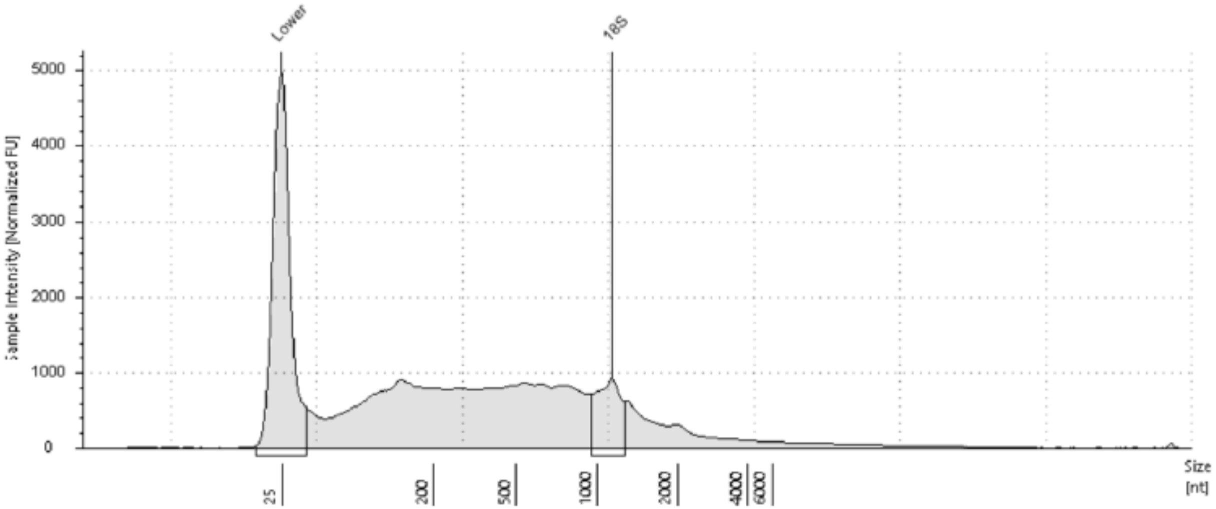
Metagenomics analysis of bat droppings

01606490 © Bernard Castelein / naturepl.com

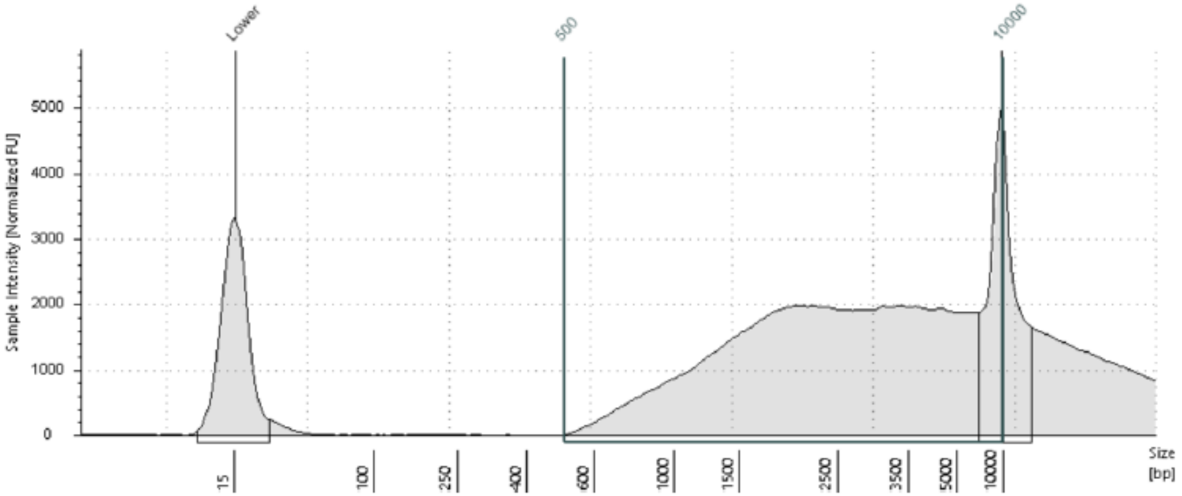
- **Sample type**: bat guano
- **Wetlab**:
  - Nucleic acids extraction (magnetic beads, Mag Fast protocol)
  - Random amplification of RNA (SMART9N protocol - Claro *et al.* 2023)
  - Oxford Nanopore Technologies sequencing
- **In silico**:
  - Taxonomic sequence classification (Kraken2 - Wood *et al.* 2019)
  - Sequence alignment (Minimap2 - Li, H. 2018)

Examples of TapeStation results

Total RNA



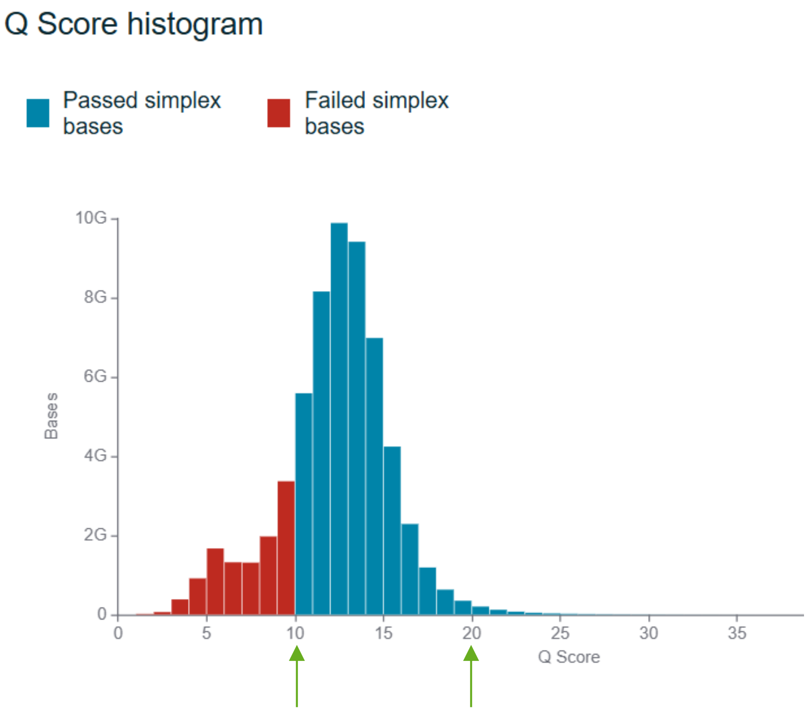
ds cDNA
(SMART9N amplification)



Results

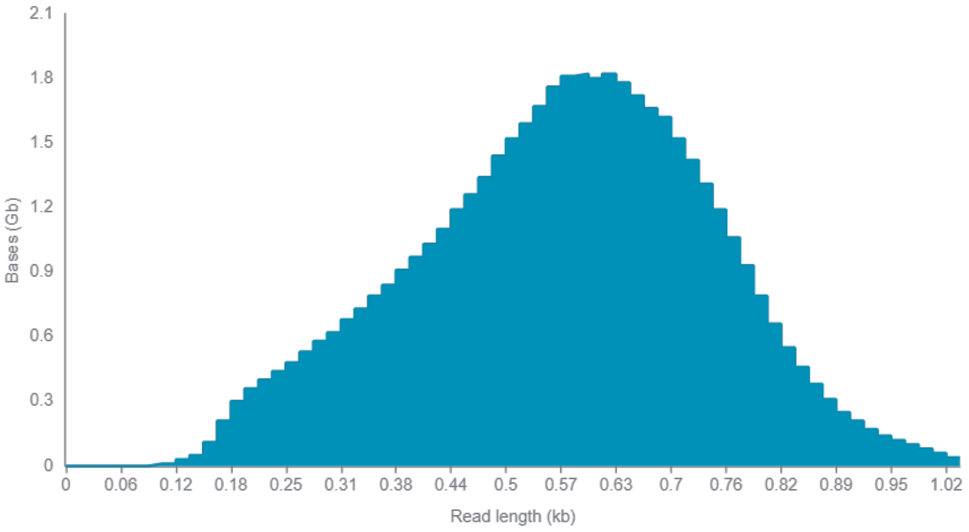
- 21 bat samples sequenced using PromethION flowcells on P2Solo device.

- 125 millions reads (64 Gigabases) with a minimum Qscore of 10.

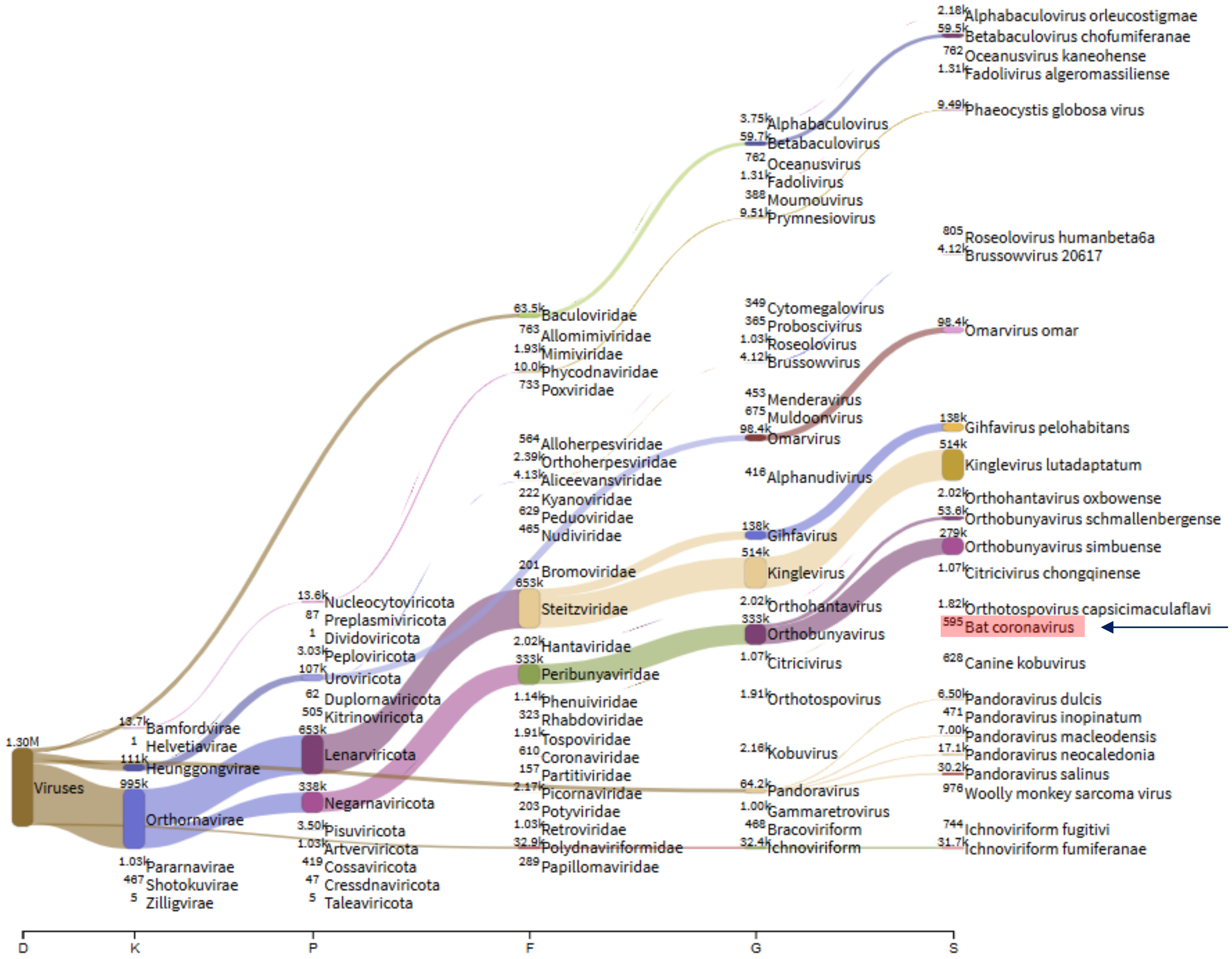


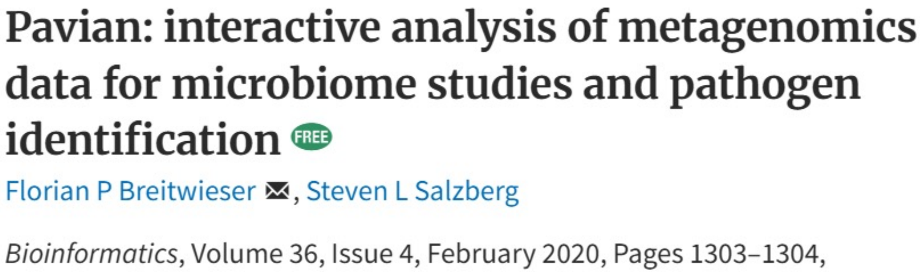
Fidelity: 90% 99%

- Size distribution, up to 1Kb



- Taxonomic sequence classification (Kraken2, Wood et al. 2019)



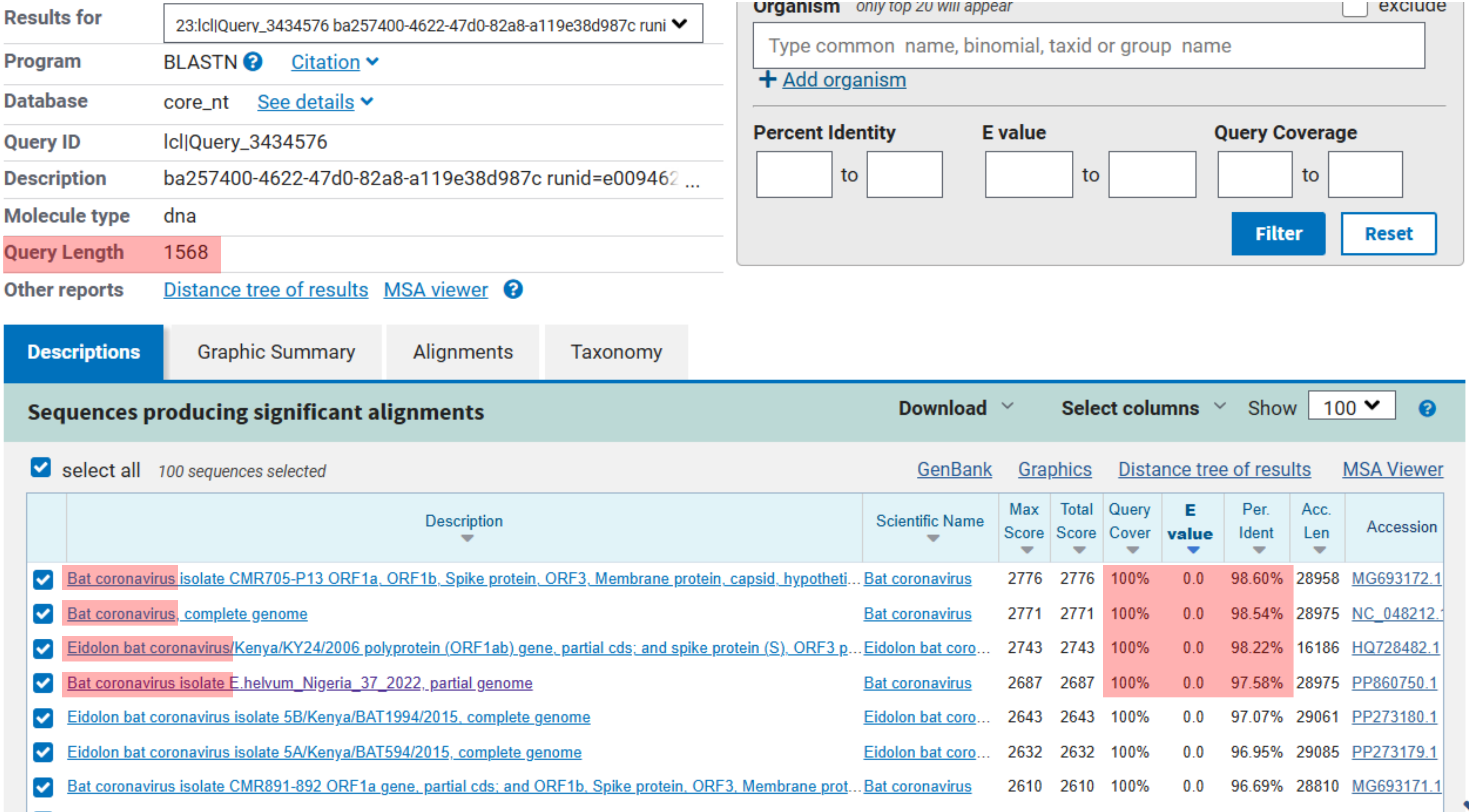


Pavian: interactive analysis of metagenomics data for microbiome studies and pathogen identification FREE

Florian P Breitwieser ✉, Steven L Salzberg

Bioinformatics, Volume 36, Issue 4, February 2020, Pages 1303–1304,

- BLAST analysis to confirm taxonomic affiliation



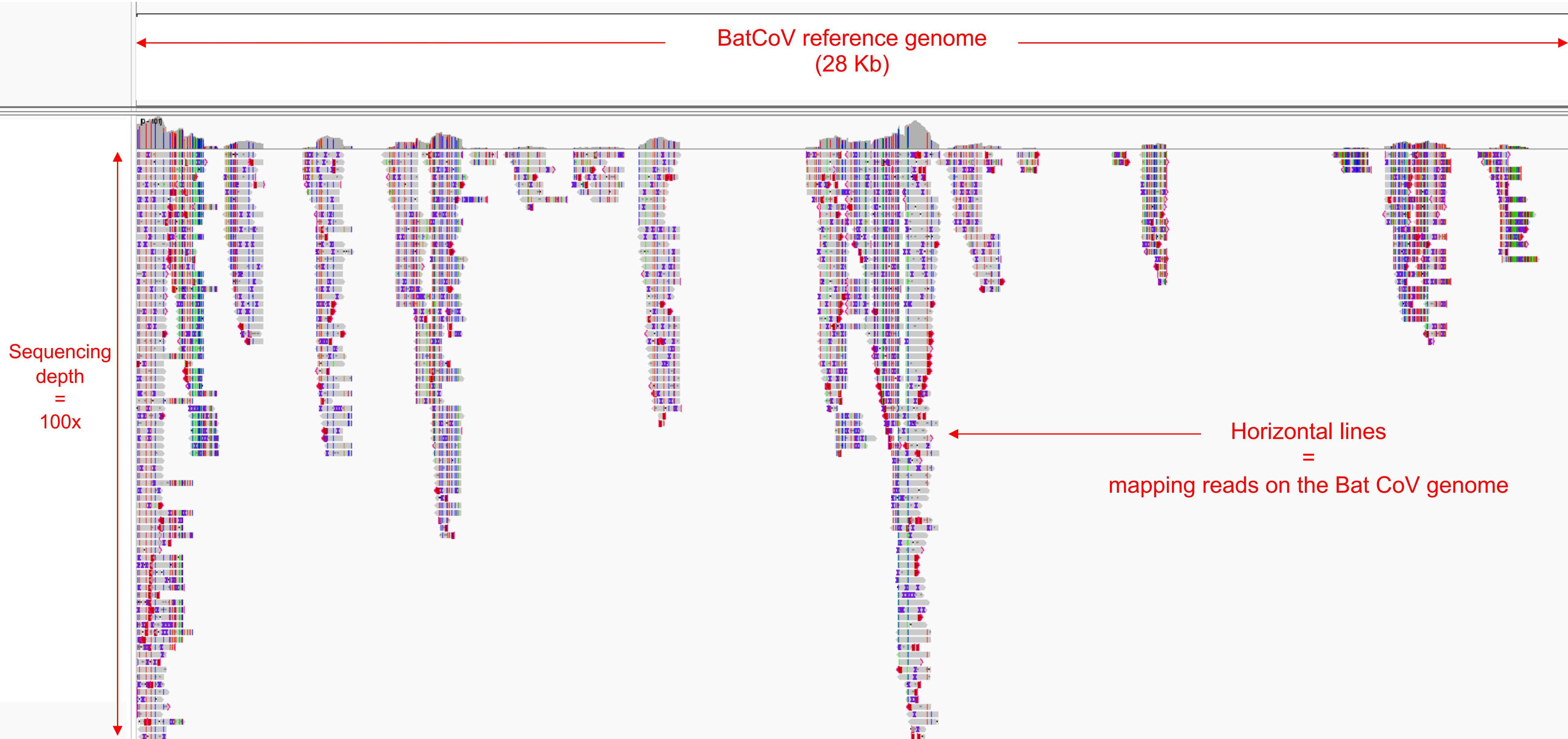
Results for	23:lcl|Query_3434576 ba257400-4622-47d0-82a8-a119e38d987c runi
Program	BLASTN Citation
Database	core_nt See details
Query ID	lcl|Query_3434576
Description	ba257400-4622-47d0-82a8-a119e38d987c runid=e009462 ...
Molecule type	dna
Query Length	1568
Other reports	Distance tree of results MSA viewer

Sequences producing significant alignments

☑ select all *100 sequences selected*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Bat coronavirus isolate CMR705-P13 ORF1a, ORF1b, Spike protein, ORF3, Membrane protein, capsid, hypotheti…	Bat coronavirus	2776	2776	100%	0.0	98.60%	28958	MG693172.1
☑	Bat coronavirus, complete genome	Bat coronavirus	2771	2771	100%	0.0	98.54%	28975	NC_048212.1
☑	Eidolon bat coronavirus/Kenya/KY24/2006 polyprotein (ORF1ab) gene, partial cds; and spike protein (S), ORF3 p…	Eidolon bat coro…	2743	2743	100%	0.0	98.22%	16186	HQ728482.1
☑	Bat coronavirus isolate E.helvum_Nigeria_37_2022, partial genome	Bat coronavirus	2687	2687	100%	0.0	97.58%	28975	PP860750.1
☑	Eidolon bat coronavirus isolate 5B/Kenya/BAT1994/2015, complete genome	Eidolon bat coro…	2643	2643	100%	0.0	97.07%	29061	PP273180.1
☑	Eidolon bat coronavirus isolate 5A/Kenya/BAT594/2015, complete genome	Eidolon bat coro…	2632	2632	100%	0.0	96.95%	29085	PP273179.1
☑	Bat coronavirus isolate CMR891-892 ORF1a gene, partial cds; and ORF1b, Spike protein, ORF3, Membrane prot…	Bat coronavirus	2610	2610	100%	0.0	96.69%	28810	MG693171.1

- Sequence alignment vs reference genome



BatCoV reference genome
(28 Kb)

Sequencing depth
=
100x

Horizontal lines
=
mapping reads on the Bat CoV genome

In summary

Oxford Nanopore Technologies sequencing:

- Can be used on the field
- Allows outbreack tracking
- Allows shotgun metagenomics studies

Acknowledgements

UMR 1225 IHAP

Team VIRAL

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The creation of this training material was commissioned by ECDC to Institut Pasteur with the direct involvement of Guillaume Croville