Day 4: MPXV phylogenetics & interpretation

MPXV phylogenetics tutorial: *Áine O'Toole*
Quick analysis using NextClade, QC
Background data compilation with Pathoplexus
Running squirrel with EPI2ME for QC and APOBEC3 reconstruction

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Learning Objectives

- Understanding of MPXV genomic epidemiology
- Understanding of host enzyme APOBEC3, what it means for MPXV
- How to run NextClade on new data & interpret results
- How to collect a background dataset to include in analysis
- How to install squirrel in EPI2ME
- How to run squirrel in QC mode
- How to run APOBEC3 reconstruction with squirrel
- How to analyse and interpret an outbreak of MPXV

Primer on MPXV genomic epidemiology

MPXV diversity & genomics

MPXV is a double stranded DNA virus of the family Poxviridae that causes mpox. It has a large, complex genome (~200 kb) and a large gene repertoire. There are two major clades of MPXV, Clades I and II, and recently each has been subdivided into Clades Ia and Ib, and IIa and IIb.

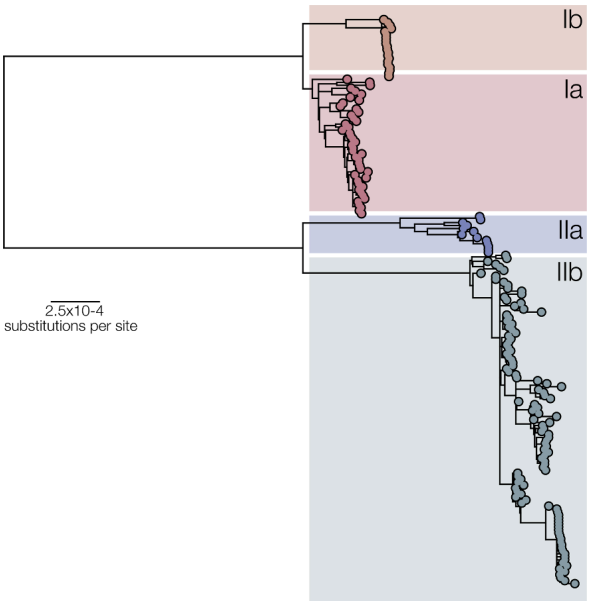


Figure 1. *The known genetic diversity of MPXV is divided into two distinct clades (I and II), and further subdivided into Clades Ia and Ib, and Clades IIa and IIb (Wawina-Bokalanga et al 2025). Historically, cases of Clade I were reported primarily in Central Africa and cases of Clade II were majoritively reported in West Africa. In recent years, cases of Clade II have been reported across the globe.*

The genomes themselves have many repetitive and low-complexity regions that can be challenging to both sequence and analyse. The analytic challenges can be overcome with automated pipelines such as NextClade and squirrel, and alignments and phylogenies can be produced easily without need for the command line. The major challenge that remains for robust genomic epidemiology of MPXV is in knowing how to correctly interpret these results.

MPXV has existed for most of its evolutionary history in a rodent reservoir, and recent spillover events have led to persistence in the human population. In humans, MPXV evolves at a faster rate due to the action of the human enzyme APOBEC3.

APOBEC3 family of cytosine deaminases

APOBEC3 is a family of host enzymes that can bind single stranded DNA and deaminate cytosine bases to uracil. Due to DNA synthesis, this change gets propagated back onto the DNA as a T and what we observe as a result is a C->T mutation. This can then be observed either on the positive strand (as a C->T mutation), or on the negative strand (as a G->A mutation). The function of APOBEC3 enzymes is varied, but has been well studied for its

antiviral function against HIV. APOBEC3F, which is the human enzyme thought to be acting in MPXV infections, has a preference for binding and deaminating TC dinucleotides, so the observed mutations in the MPXV population are TC->TT and GA->AA dinucleotide mutations.

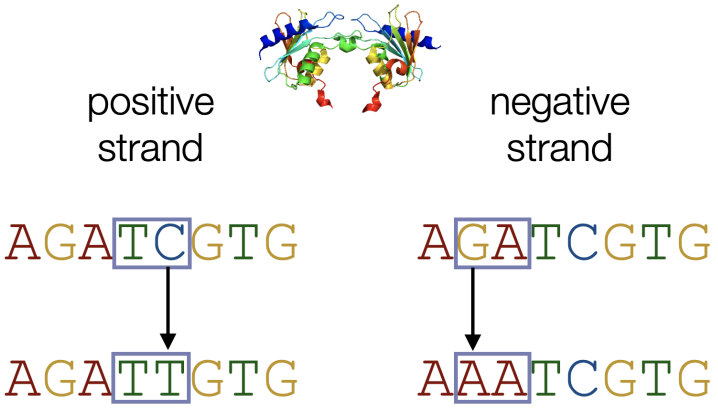


Figure 2. *APOBEC3 is a family of host cytidine deaminases with a track-record of anti-viral function. Humans and other primates have 7 different APOBEC3 genes, whereas rodents only have a single APOBEC3 (Conticello 2008). Human APOBEC3F is thought to be responsible for the enrichment of TC->TT and GA->AA mutations that are characteristic of sustained transmission of MPXV in the human population (Suspène et al 2023). This signature is not observed when the virus circulates in the rodent population (O'Toole et al 2023).*

These mutations are primarily antiviral in function, and are not indicative of MPXV adaptation to humans. To support this, the mutations observed are the residual least deleterious mutations that remain after natural selection has removed those with high fitness-costs to the virus. This means that there is not a constant molecular clock across the MPXV phylogeny, and branch lengths in the rodent population and in the human population need to be interpreted differently. Mutations that are not consistent with APOBEC3 editing will occur at a much lower rate, expecting one couple of years in the MPXV population on average. Genomes with many unique non-APOBEC3 mutations are likely a result of contamination, sequencing issues or bioinformatic error.

Virus evolution during an outbreak

In a virus outbreak, new cases arise as the virus is transmitted from person to person. Often, the number of sampled cases is less than the true number of cases that have occurred in the outbreak (Figure 3A). As the virus transmits, mutations can occur that can be the result of errors during virus replication or as a result of human host defense (e.g. APOBEC3 editing). Not every transmission event gives rise to a mutation and how often a mutation is observed is influenced by a variety of factors, for example the nature of the virus (e.g. RNA, DNA) and the time between observed cases (serial interval) (*Drummond et al 2003)*. Over time mutations accumulate in the virus population, however very deleterious will be selected out through natural selection and likely not observed. The virus genome sequences of sampled cases can allow us to reconstruct the relationships between cases (Figure 3B).

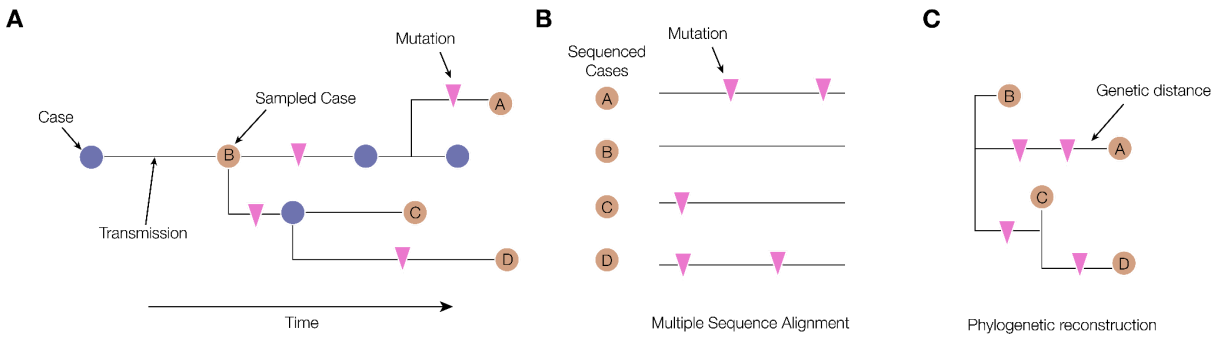


Figure 3. *We can use the mutations that occur as the virus is transmitted from host to host to reconstruct the evolutionary history of the virus in the form of a phylogenetic tree. Not every transmission event will give rise to an observed mutation in the population.*

What is a FASTA file?

FASTA is a standard format used to represent genetic sequence data. For MPXV, we represent our consensus genome sequences in FASTA format. A FASTA file can consist of one or more sequence records, represented by a header line and then the sequence itself. In the below example there are two sequence records.

```
>case001|MPXV|2024-12-03
TTACAGATCATTTATATTCCAAAAATATTAACTATATACGTTTATTATATGATGTTAACGTGTAAATTA
TAAACATTATTTTATGATGCAATTGTCTGACAACCTAGATTGGCATAAGGATATTGATAAGCTCTA
CGAGAATATATTGTTGGACGTTATCGTTTACGAAATAGTTGAGACATCAGAAAGAGGTTTAATAT
TTTTGTGAGACCATCGAAGAGAGAAAGAGAATAAAAATATTTTTTTGTAAAACTTTTTTA
AGACAANNNNNNNNNNNNNNNNNNNNTAGTGATCATATCGTATCACATATTGAAACAG
>case002|MPXV|2024-12-06
TACAGATCATTTATATTCCAAAAATATTAACTATATACGTTTATTATATGATGTTAACGTGTAAATTAT
AAACATTATTTTATGATGCAATTGTCTGACAACCTAGATTGGCATAAGGATATTGATAAGCTCTAC
GAGAATATATTGTTGGACGTTATCGTTTACGAAATAGTTGAGACATCAGAAAGAGGTTTACCATT
TTCAGATGAATAGAGTTATCGATTCAGACACATGCTTTGAGTTTTGTTGAATCGATGAGTGAAGT
ATCATCGGTTGCACCTTCAGATGCCGATCCGTCGACATACTTGAATCCATCCTTG
```

In a FASTA file, the header line always begins with a “>” character, followed by the sequence ID. The ID should not contain any whitespace (like space characters “ ”, or tab characters). It is good practice to include relevant metadata in the header, such as the date of sampling. The sequence follows on the next line and it can be represented in a single long line, or for readability is often represented across a number of lines. In this example the MPXV DNA sequence is shown as ACGT nucleotide characters and any unknown sites are represented as the `N` ambiguity character.

How to read a tree

For a primer on “how to read a phylogenetic tree”, see the detailed guide on the ARTIC website at: https://artic.network/how-to-read-a-tree.html

Background datasets: *The need for context*

Without appropriate context, you are interpreting any newly generated sequence data with only part of the information. Sometimes no other relevant sequences are available and when interpreting the data we need to be aware of that. However, public databases can be very valuable resources for your analysis and for the interpretation of results. In this tutorial, you will learn how to access some background genome sequence data to include with your analysis.

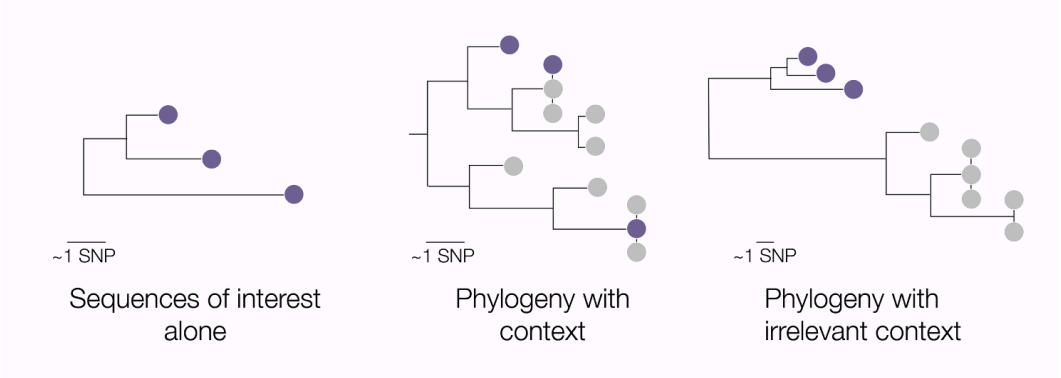


Figure 4. *If all that is used to construct the phylogeny is your sequences of interest alone, it appears that all sequences are linked. However, when relevant genomes are included for additional context this may not be the case. Choosing appropriate sequences for context can be tricky, and you want to include as many as possible without hindering your ability to analyse the data efficiently. Choosing irrelevant contextual data will not help with interpretation.*

What can MPXV genomes tell us?

Some of the first questions that are necessary to answer are:

1. What clades/ lineages are present in the data
2. Whether the observed genomes are part of a known human outbreak or whether they represent a novel spillover event
3. If part of an outbreak, which outbreak are they a part of
4. Are the cases linked
5. What are the most related genomes in public data, possible source attribution

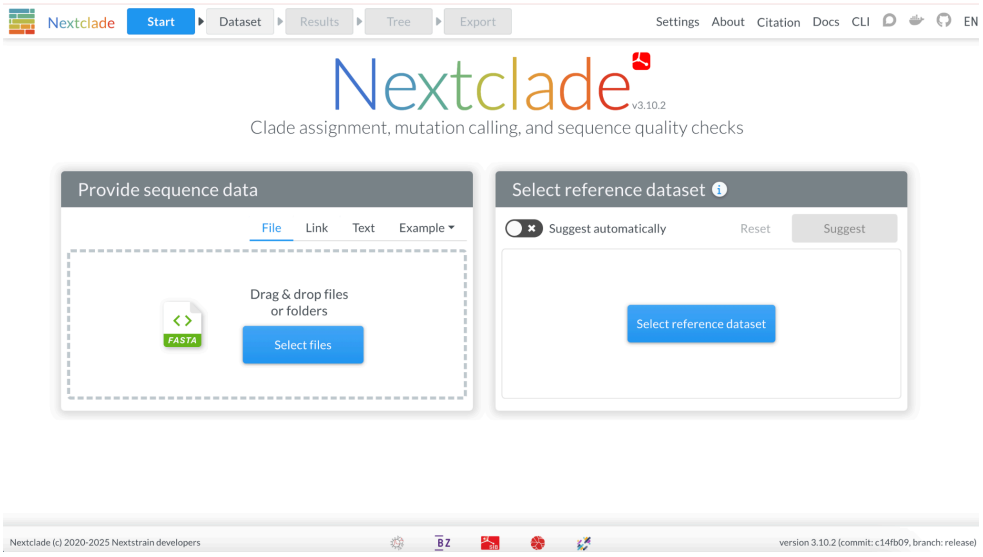
Tutorial: Analysis of MPXV genomes

As part of this tutorial, you will become familiar with running nextclade and squirrel (through the EPI2ME interface). You can run the example outbreak dataset through the tutorial and also the newly generated sequence data to test your ability to apply these new skills to new data.

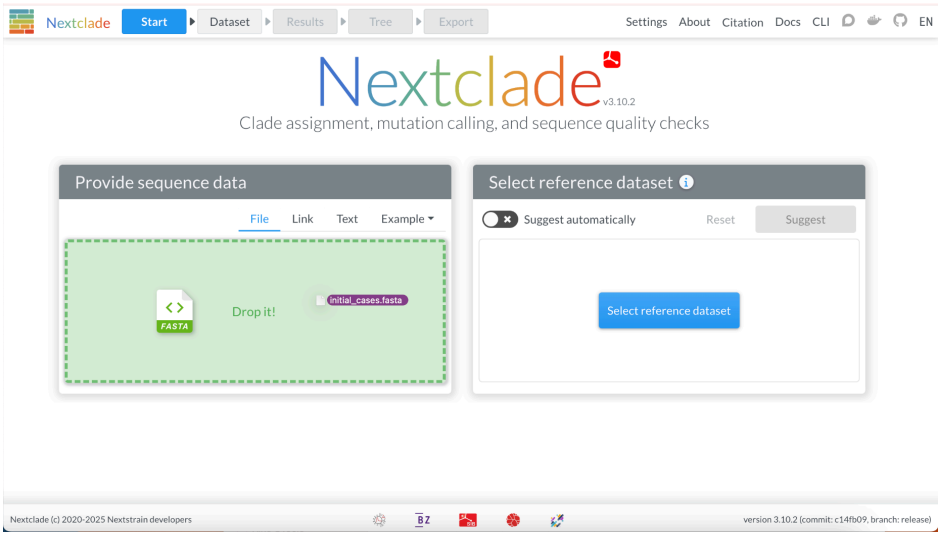
Download the example data here: https://aineotoole.co.uk/initial_cases.fasta
You will have the new consensus genomes from the field bioinformatics tutorial

Running NextClade

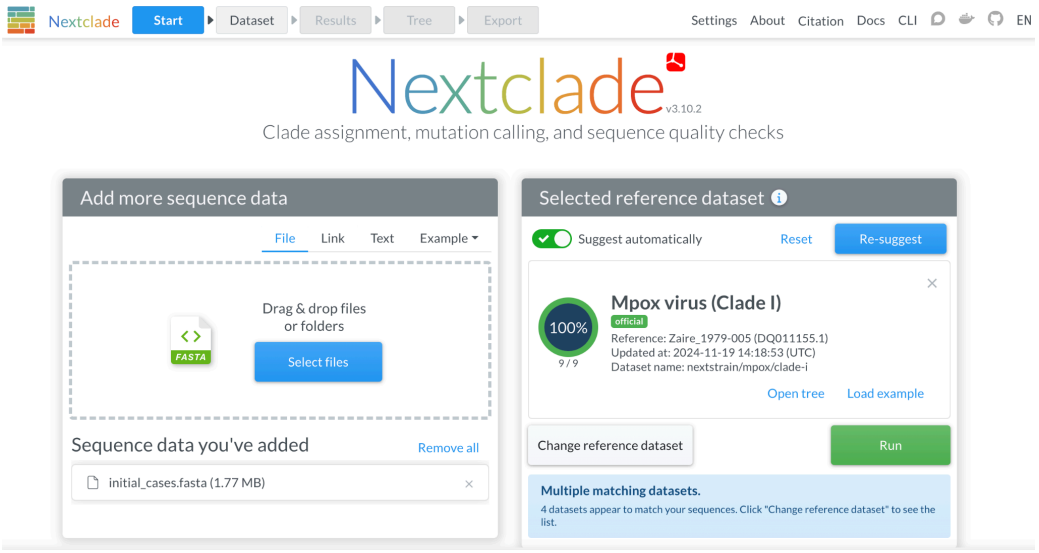
Citing NextClade: Aksamentov, I., Roemer, C., Hodcroft, E. B., & Neher, R. A., (2021). Nextclade: clade assignment, mutation calling and quality control for viral genomes. Journal of Open Source Software, 6(67), 3773, https://doi.org/10.21105/joss.03773



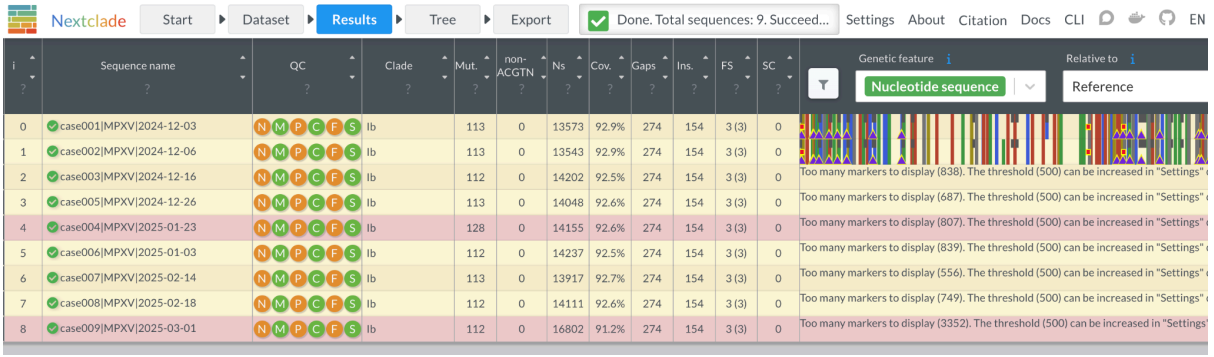
1. Navigate to the NextClade website at https://clades.nextstrain.org/.



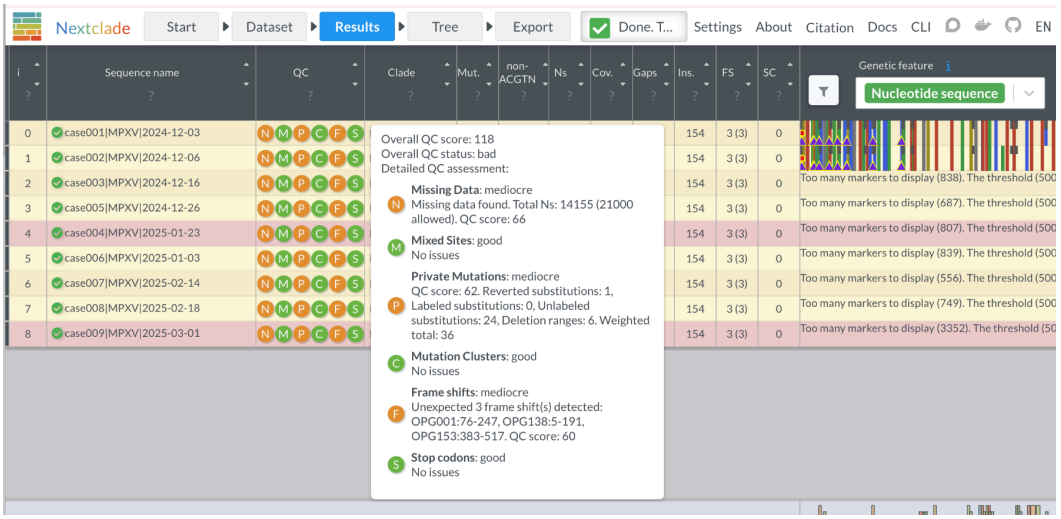
2. Drag and drop the initial_cases.fasta file you've just downloaded into the sequence data box, or select the file in the file browser.



3. In the reference dataset section, toggle the “suggest automatically” switch ON. Select “Suggest” to allow the appropriate reference to be selected. NextClade believes this file contains MPXV Clade I data. Select “Run” in the bottom right to run the pipeline.



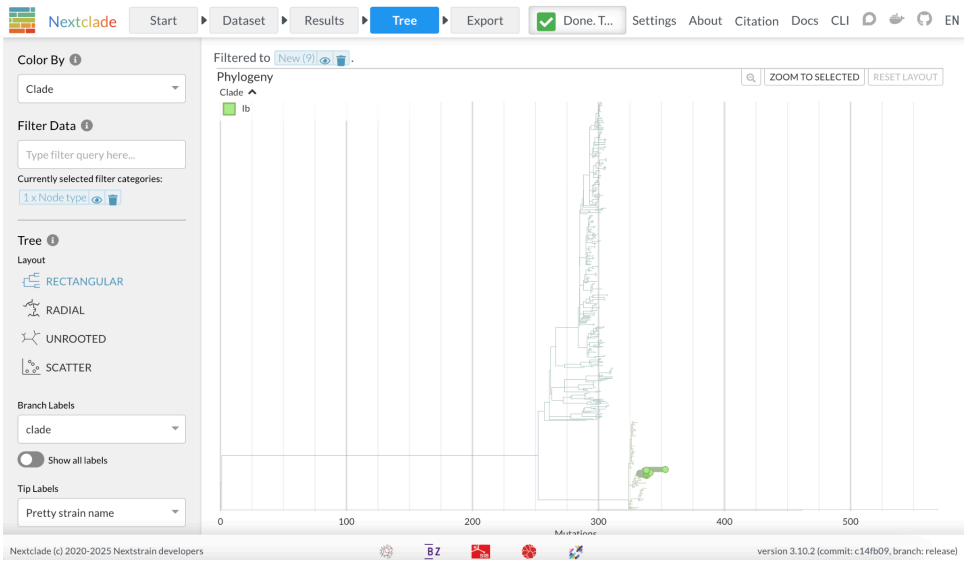
4. When the analysis is complete you should see a results page similar to the above. NextClade has a variety of checks that flag problematic features in genomes, such as low coverage (lots of Ns) and unique mutations in a given sequence.



5. Hover over case004 to see why NextClade has flagged it. You can see it has lots of Ns, lots of Private mutations (mutations only seen in that sequence) and some unexpected frame shifts. A frameshift is caused by an insertion or deletion mutation that causes the *frame* of a coding sequence to change or *shift*. This is flagged here because frameshift mutations are rare as they disrupt gene function. Similarly, hover over case009 and see that it has more Ns than the others.

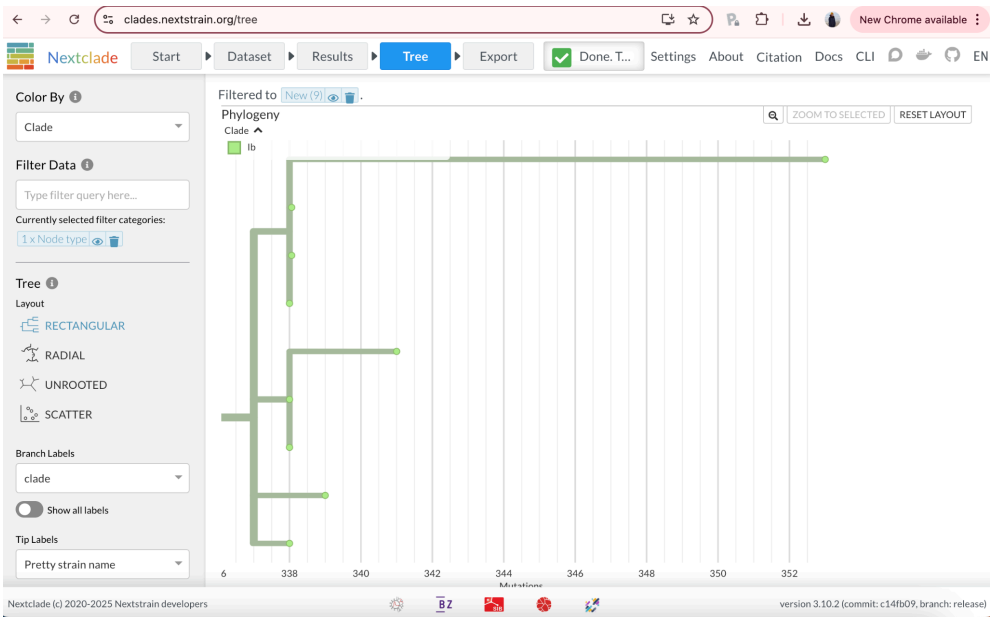
Question:
All sequences contain the flagged frameshifts, what does this say about your data?

Question:
Are there any sequences in this dataset you think may have issues that could impact your interpretation of the results?



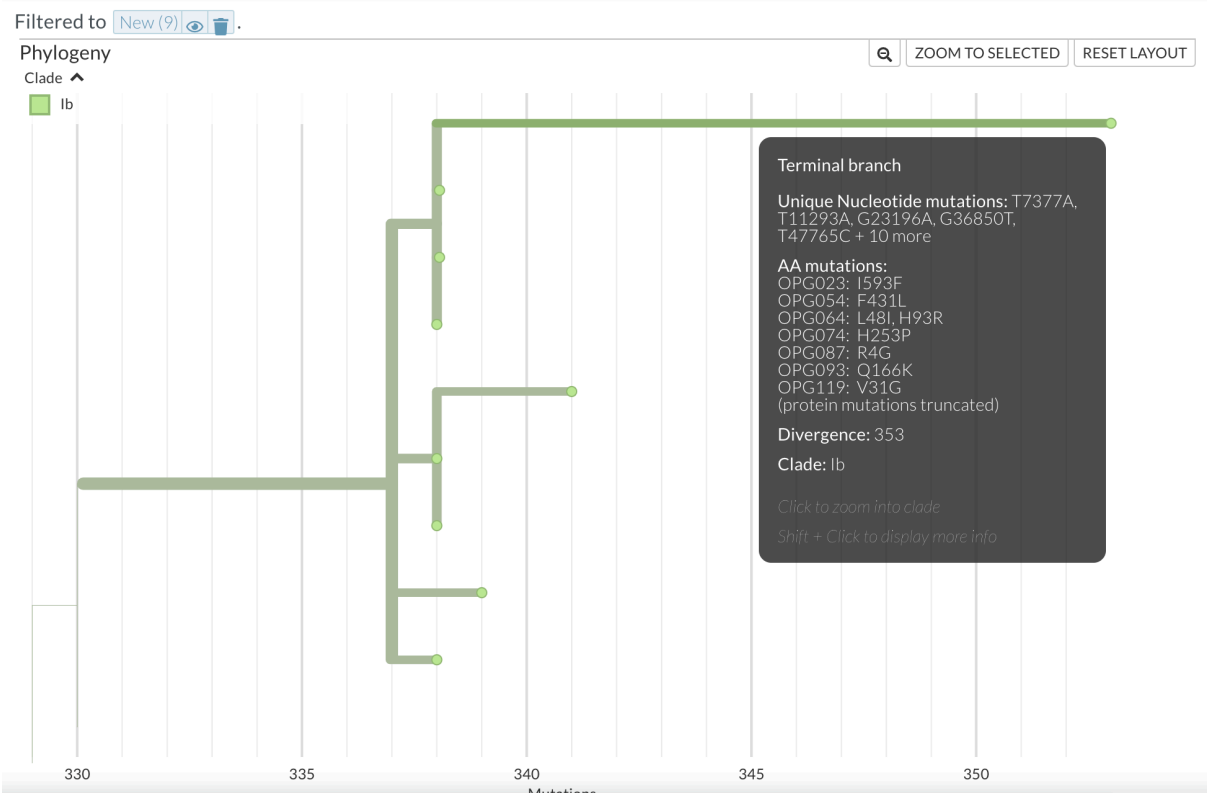
6. Navigate to the `Tree` panel by clicking the `Tree` button on the top of the window. You can see where the data lies in the diversity of Clade I.

   Question: What subclade is the data? Does the data cluster together?



7. Click on your sequences in the tree to zoom in to the data.

   Question:
   What can you say about the sequences?
   How much diversity is there?
   Do you think the cases are linked?

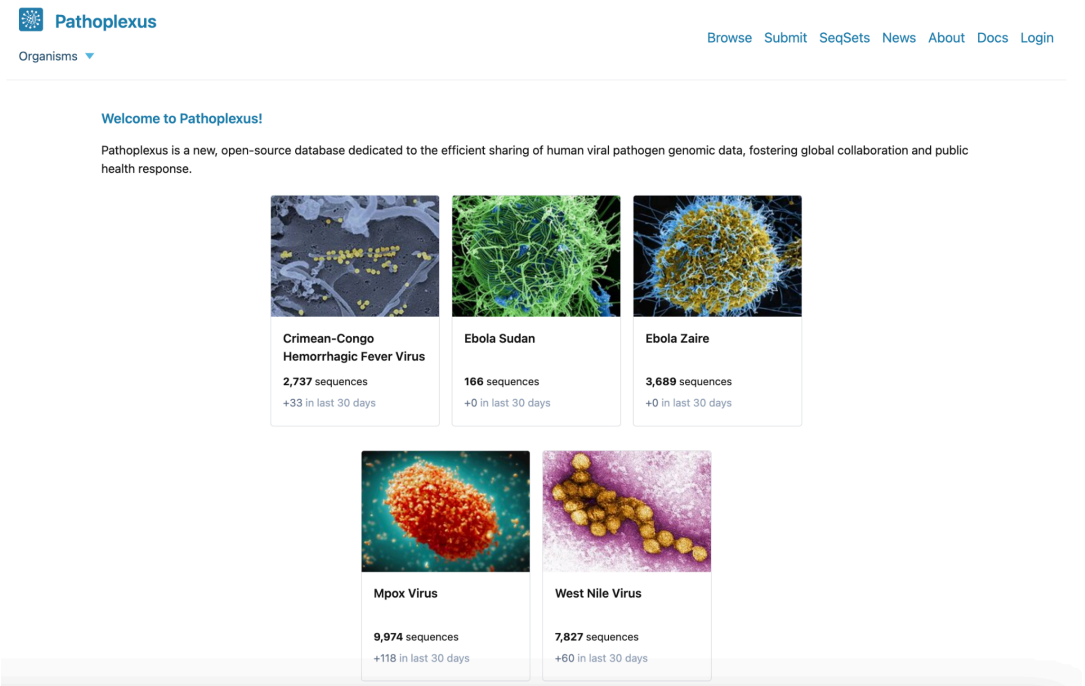


8. Hover over the branch leading to case004 to look at the SNPs present in that sequence only.

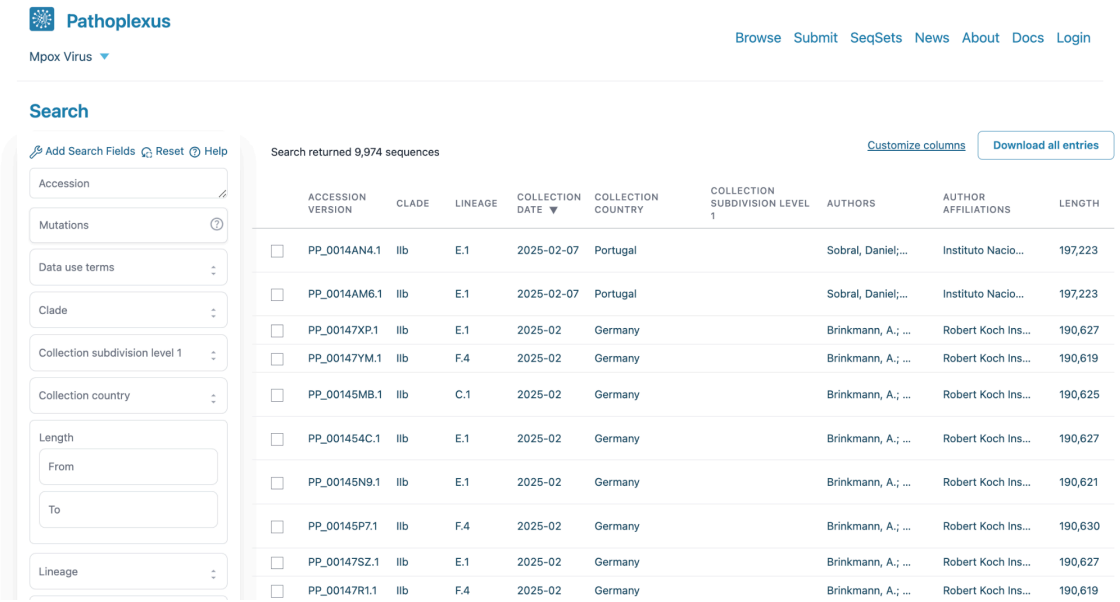
   Question:
   How many unique mutations are there?
   Based on what you know about the rate of MPXV evolution, do you think these mutations are real biological variation?
   Why/ why not?
   What other information might help us interpret this data?

Compiling a background dataset

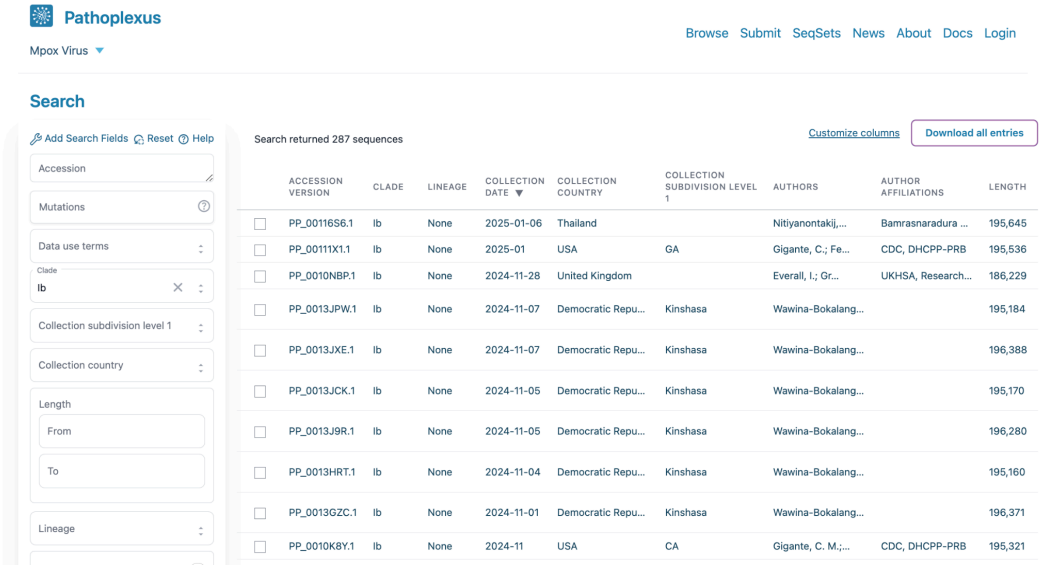
Next, we will gather a background dataset. There are a number of public repositories for data that we will hear more about later in the workshop. Today, we will use Pathoplexus to search for and download some relevant background data.



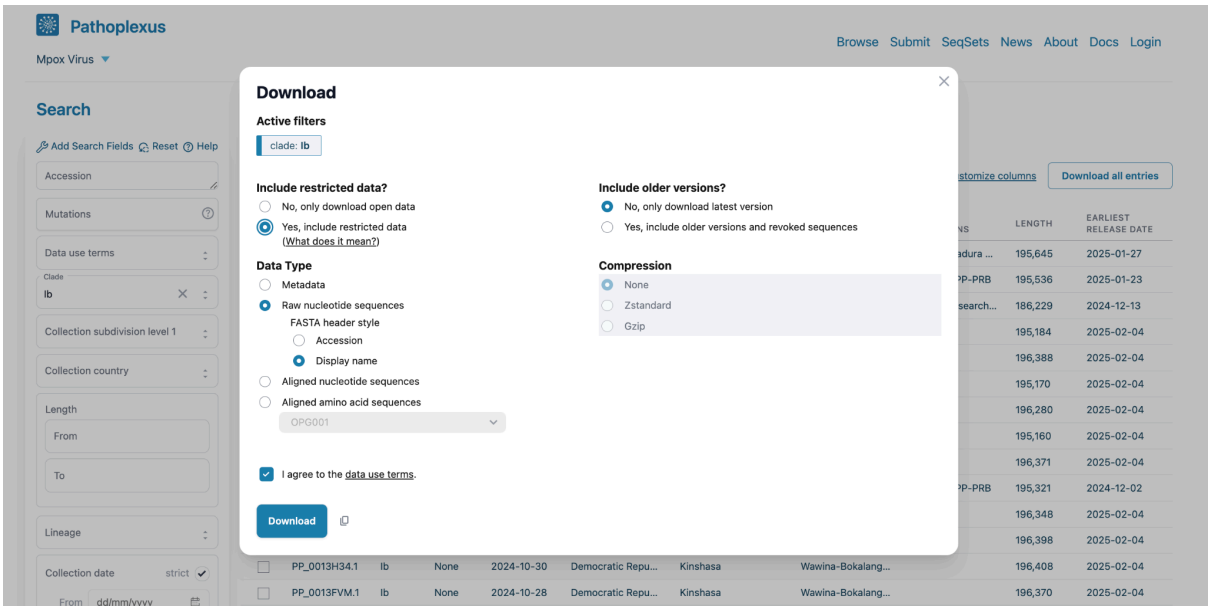
1. Navigate to pathoplexus.org. Scroll down and have a read through the homepage to learn more about Pathoplexus.



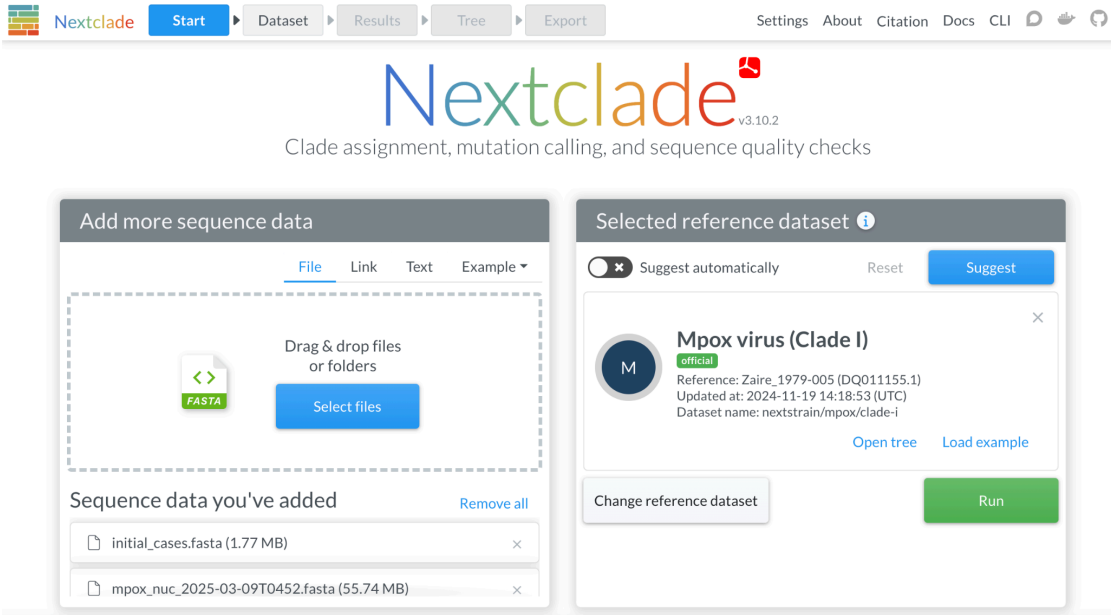
2. Click in to Mpox Virus to see the data available for MPXV.



3. From the quick NextClade analysis, we know our data is Clade Ib. You can play around with the different filters on the left hand panel to specify only Clade Ib, and filter by length, date, country etc. When you're ready to download a dataset, click "Download all entries" in the top right corner.

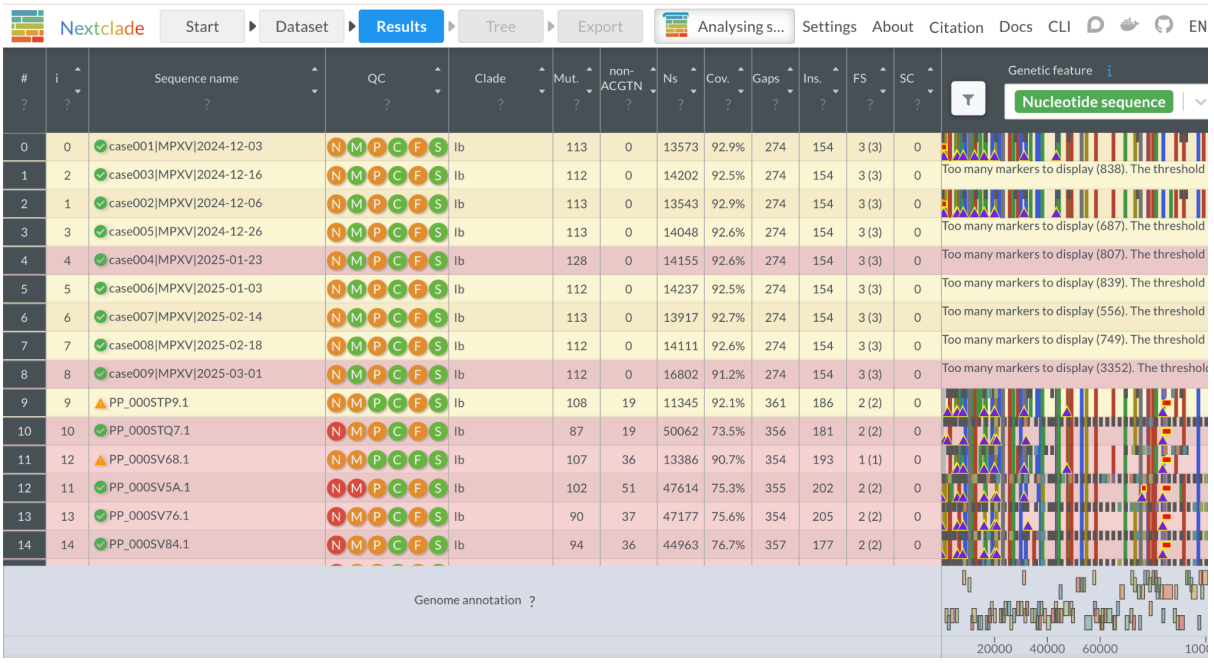


4. A dialogue box will pop up. You can decide whether you want to include restricted data or not. We will include it because our use of the data will just be for background purposes, which is allowed under the restricted data terms of use. To read more about the terms of use, see https://pathoplexus.org/about/terms-of-use/restricted-data. Select "raw nucleotide sequences" to download. The data can be downloaded without compressing, or with either Zstandard or Gzip compressions. We will download the sequences with no compression.

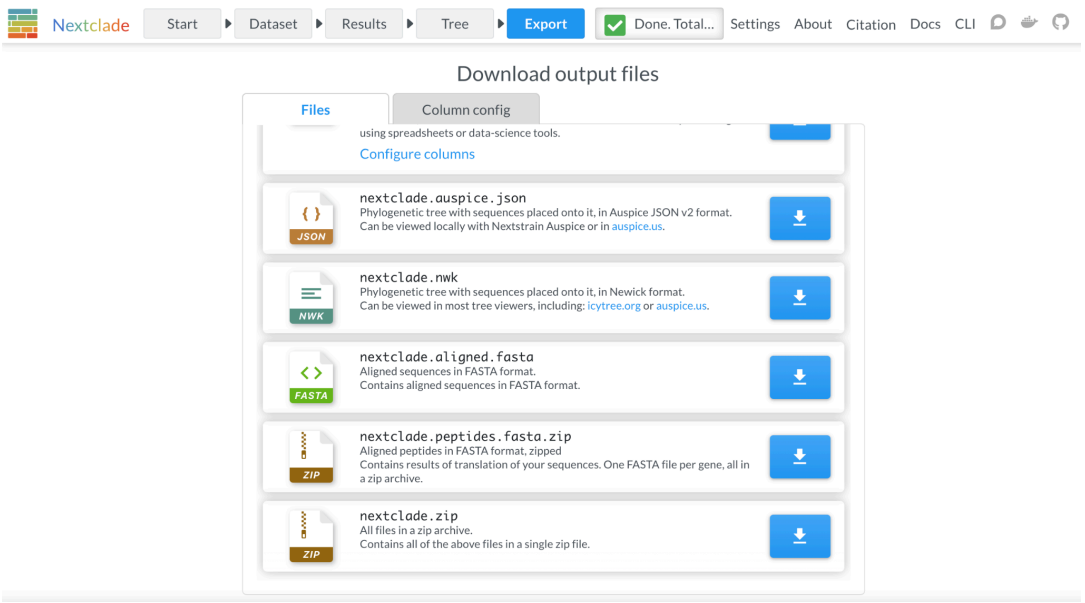


5. Return to the NextClade tab and navigate to the “Start” window by clicking the button in the top panel. Drag and drop the newly downloaded file with background sequences into the “Add more sequence data” box. You should see your additional file appear below in the “Sequence data you’ve added” panel.

6. Ensure the reference dataset is set to Mpox virus (Clade I) and click “Run”.

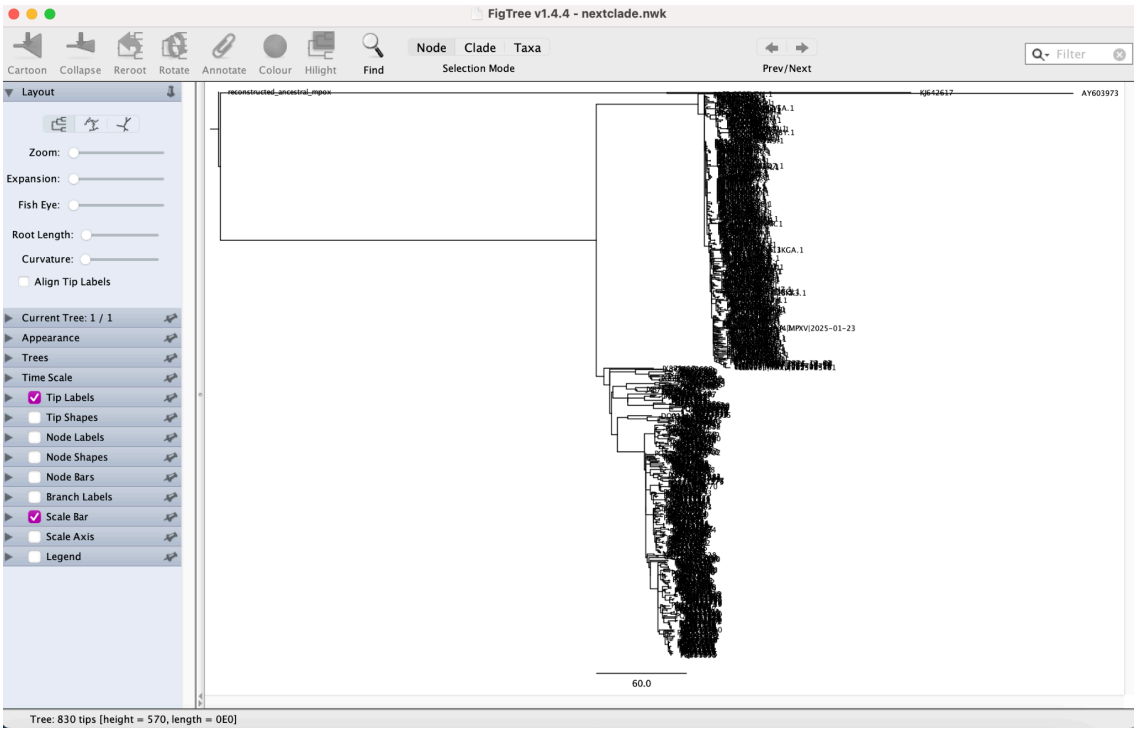


7. The “Results” window will show the case data alongside the background data. The analysis may take a little time as there are quite a few sequences to process. When the analysis has finished running, select “Tree” from the top panel to look at the tree that NextClade has produced.

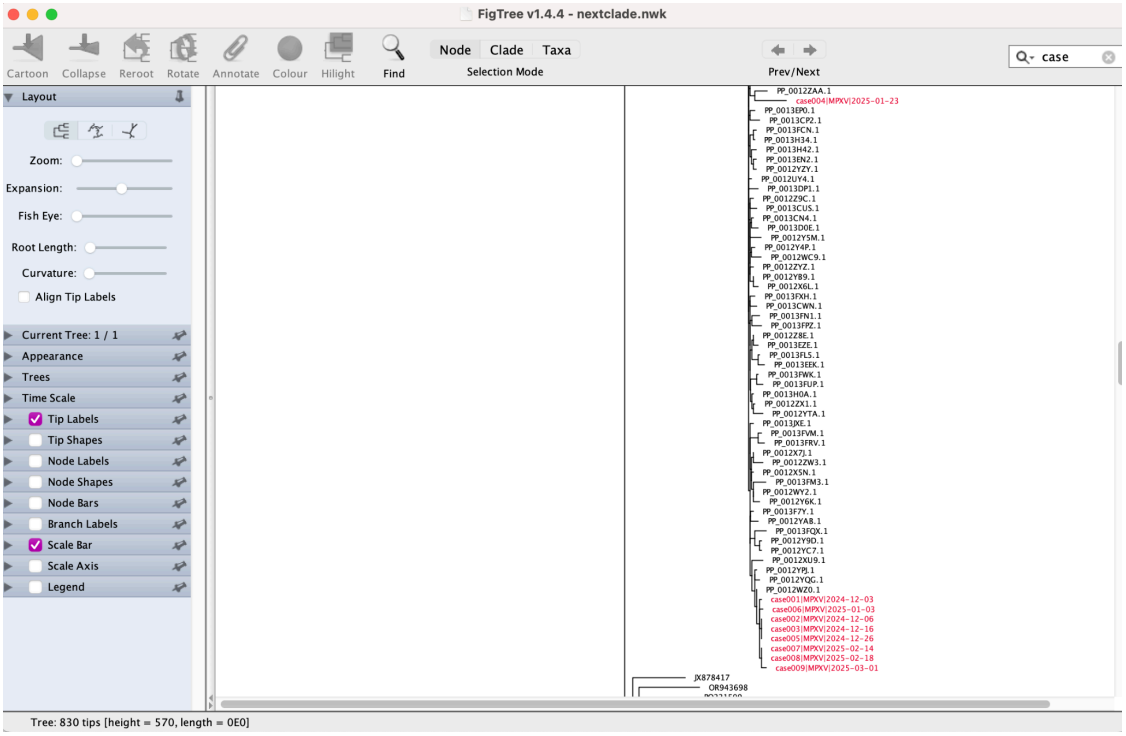


8. Navigate to the “Export” window by clicking “Export” in the top panel. Scroll down until you see the `nextclade.nwk` download. This is the tree file.

   Click the download button ( ) to download the file to view in FigTree.



9. Open the newick (nwk) tree file in FigTree. Use the search bar in the top right to search for “case”. The sequences of interest will be highlighted. Select “Colour” to change the colour of the labels to make the sequences of interest clearer to see. You can expand the tree by sliding the “Expansion” dial in the left hand “Layout panel”.



10. Scroll to see your sequences of interest.

    Question:

    Do they cluster together?

    What does this phylogeny tell us about the cases?

    What do we not know?

Exercise: Repeat on newly generated data

Repeat these steps with the newly generated data to investigate the Clades and quality of the sequences you have generated during the workshop.

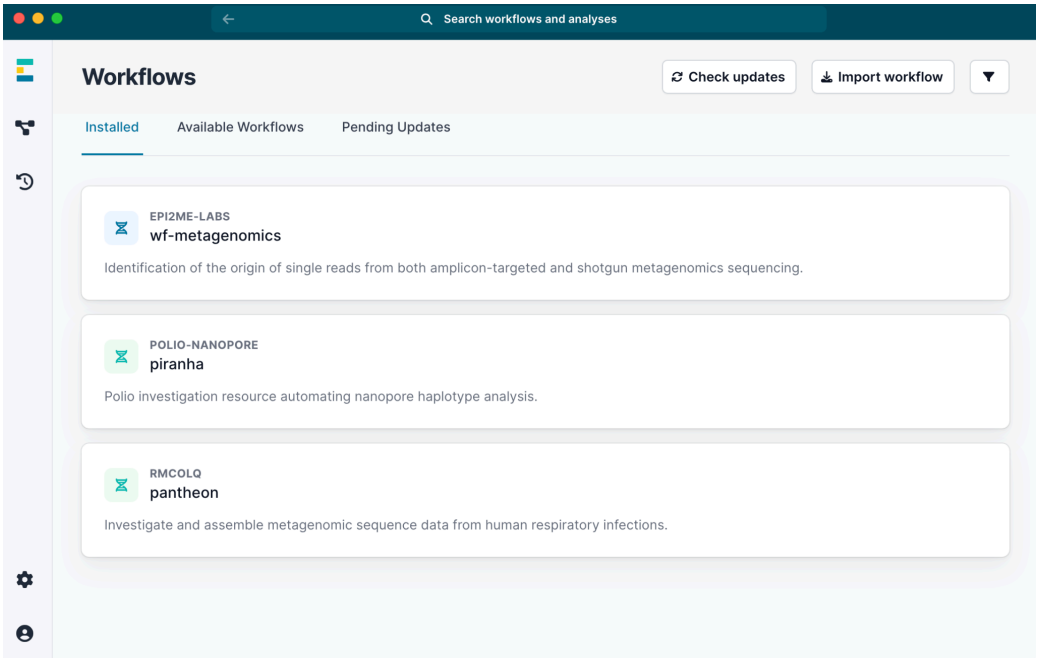
Introduction to squirrel

If the cases you're dealing with are part of known, sustained human outbreaks. It may not be necessary to take your analysis any further beyond what NextClade already provides. However, if it is unclear whether the data you're looking at is part of a known human outbreak, or if you are interested in APOBEC3 reconstruction for the data, squirrel can run this analysis.

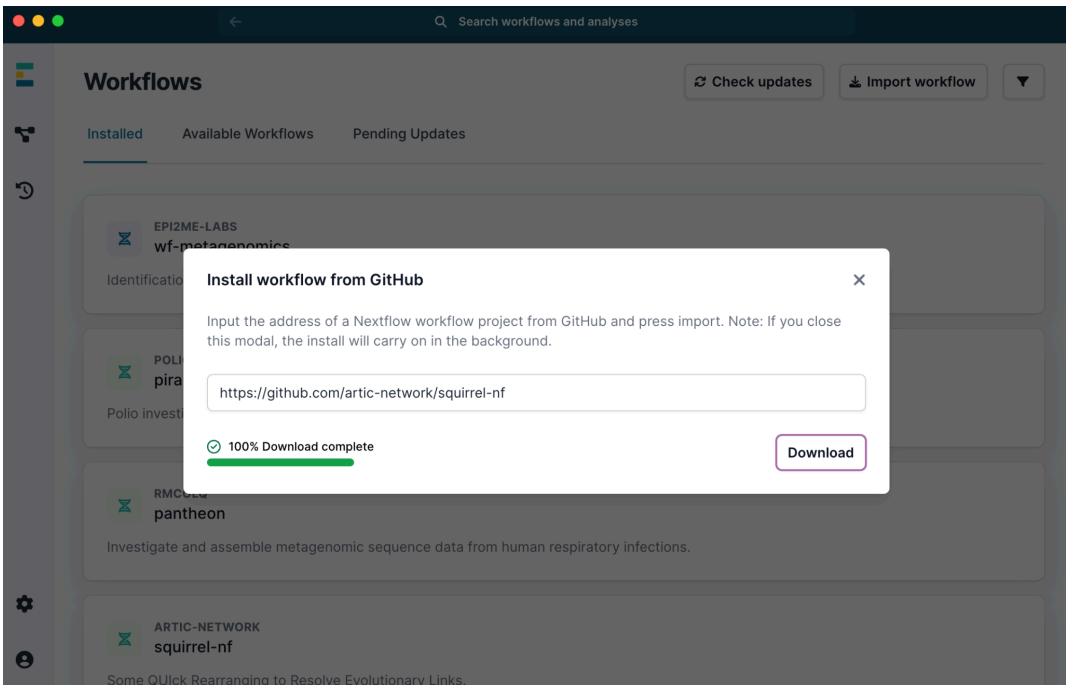
In QC mode, squirrel can flag potential issues in the MPXV sequences that have been provided for alignment (e.g. SNPS near tracts of N, clusters of unique SNPs, reversions to reference alleles and convergent mutations) and outputs these in a mask file for investigation. We suggest you use this information to examine the alignment and pay close attention to the regions flagged. Squirrel can then accept this file with suggested masks and apply it to the sequences before doing phylogenetics.

Enrichment of APOBEC3-mutations in the MPXV population are a signature of sustained human-to-human transmission. Identifying APOBEC3-like mutations in MPXV genomes from samples in a new outbreak can be a piece of evidence to support sustained human transmission of mpox. Squirrel can run an APOBEC3-reconstruction and map these mutations onto the phylogeny.

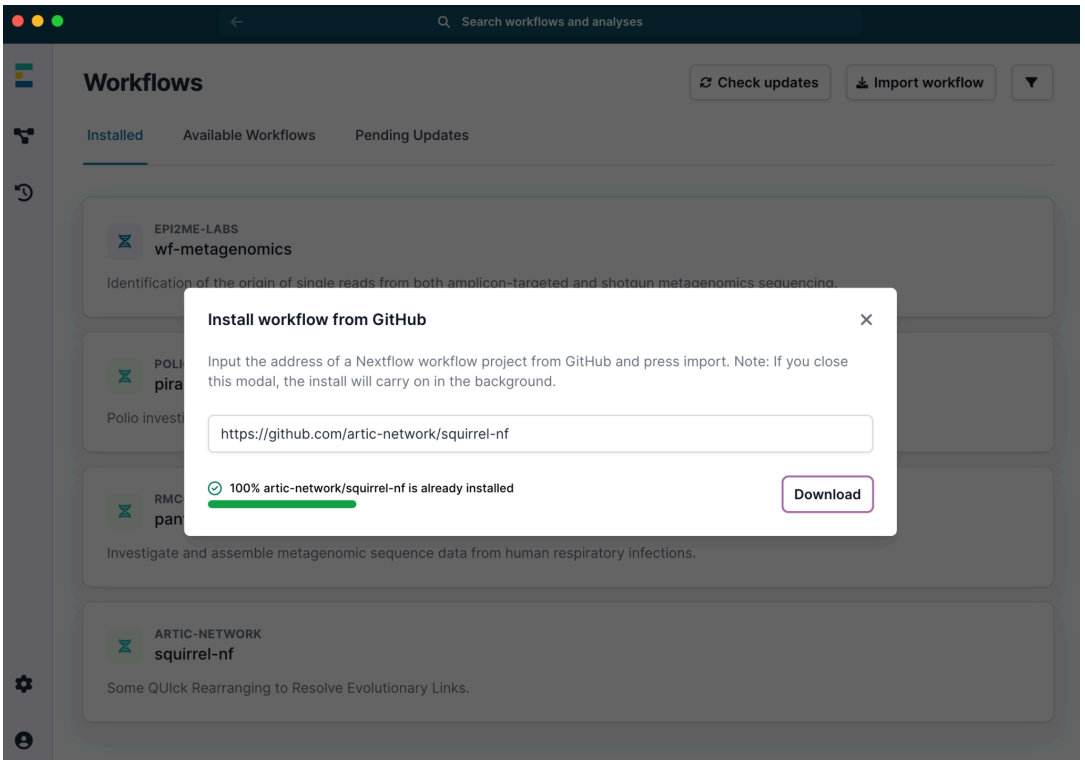
Installing squirrel using EPI2ME



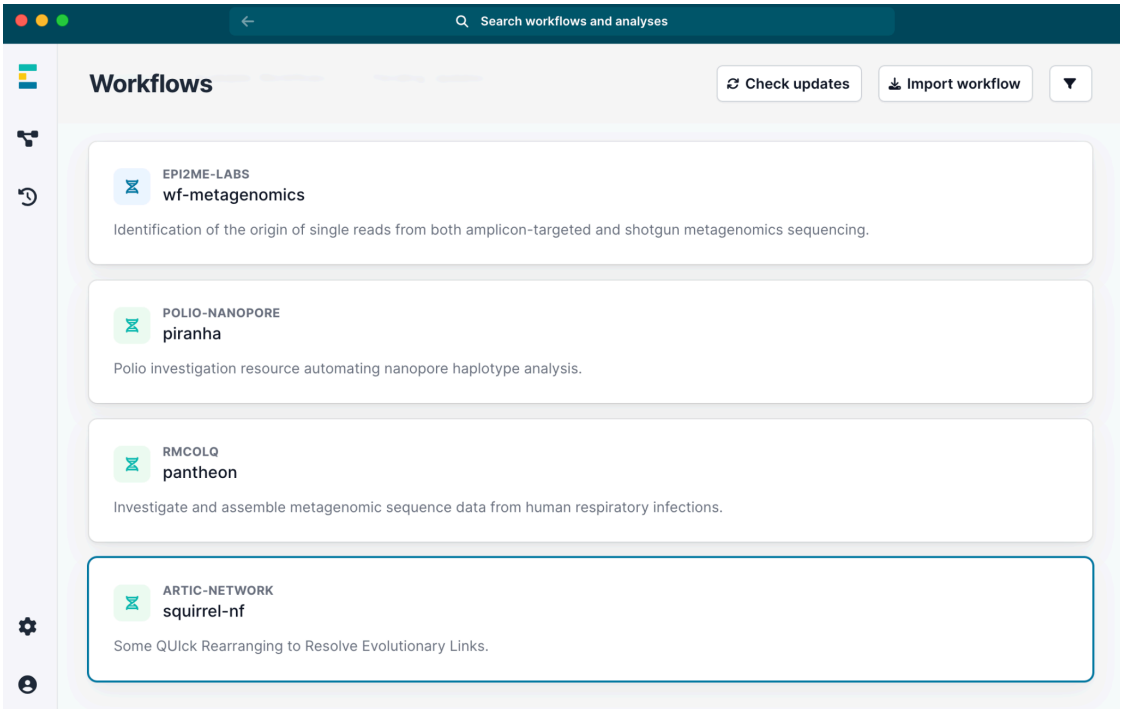
1. Open EPI2ME window and view workflows. If you haven't previously installed the squirrel workflow, it will not appear in the list of installed workflows. Click on the "Import workflow" button in the top right corner.



2. This will open a dialogue window as shown. Copy and paste the address to the squirrel workflow into the url box and click “Download”.
   The address is:
   https://github.com/artic-network/squirrel-nf



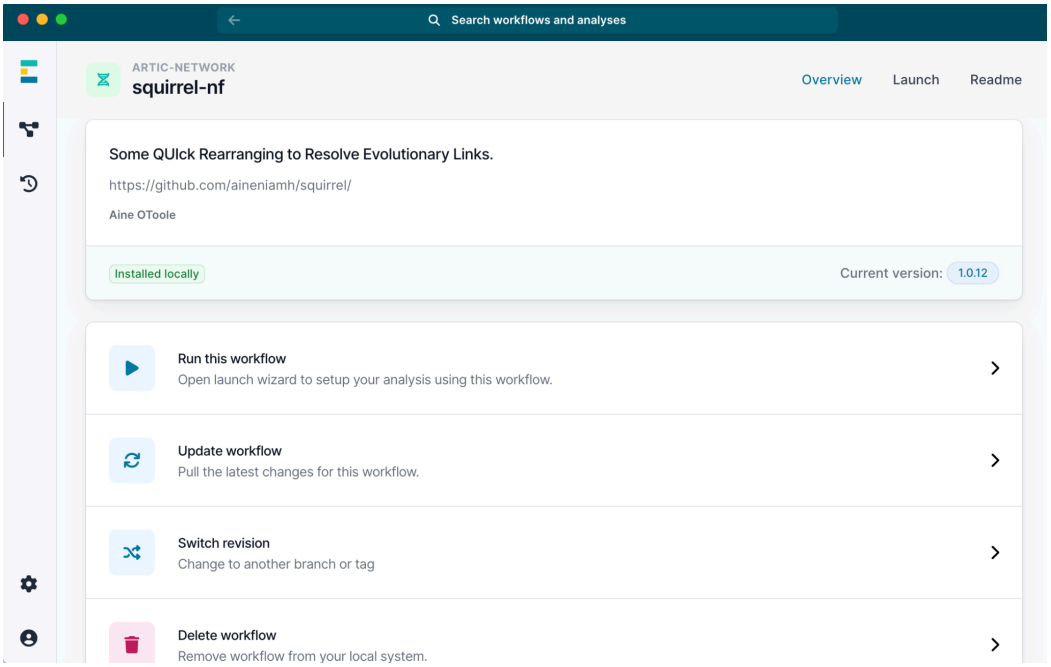
3. When the download has completed, click the top right X to close the dialogue box.



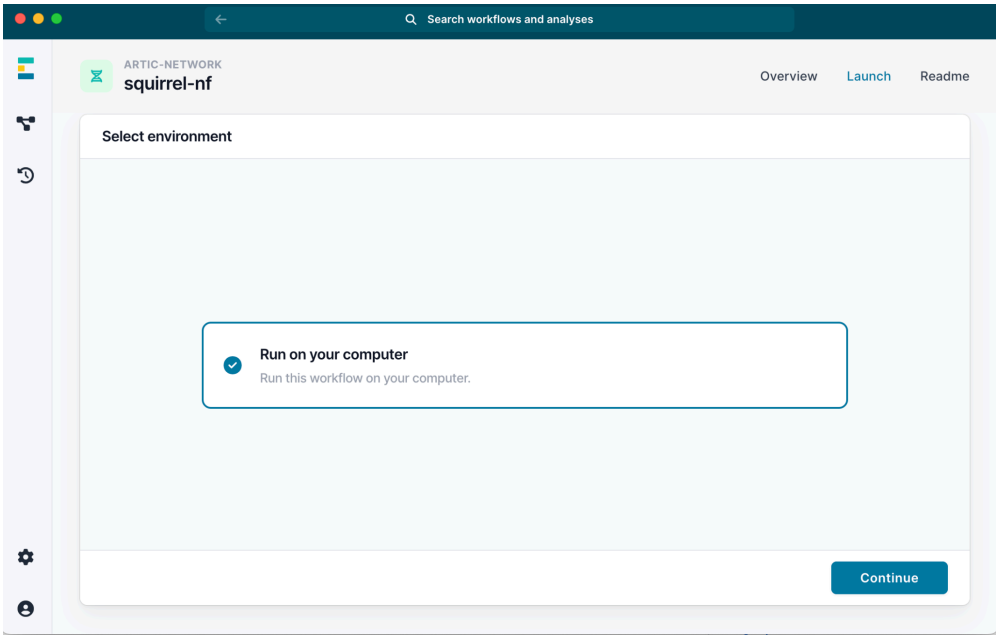
4. You will return to the installed workflows page, where you can now see squirrel available as an installed workflow under the label `squirrel-nf`. You are now ready to run squirrel.

Running squirrel for sequence QC

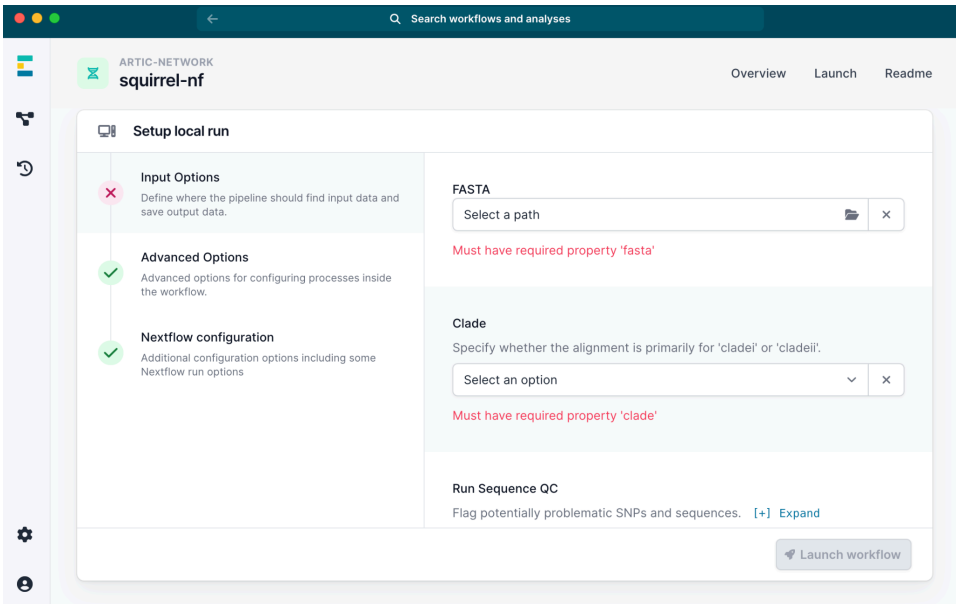
If squirrel is run in QC mode, it will flag sites that it believes may need to be masked from the alignment and produce a csv mask file summarising the sites. This file can then be provided to squirrel to redo the alignment with additional masking.



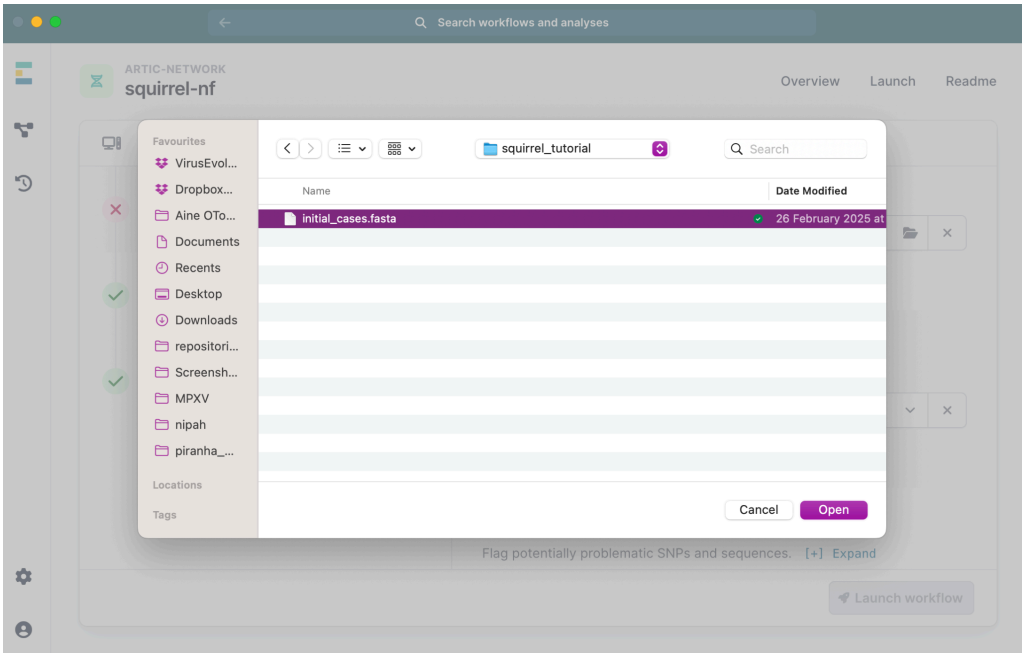
1. Click the squirrel workflow. You will be directed to this page, which has options to run, update and delete the workflow, as well as changing revision versions. We will run the workflow by clicking “Run this workflow”.



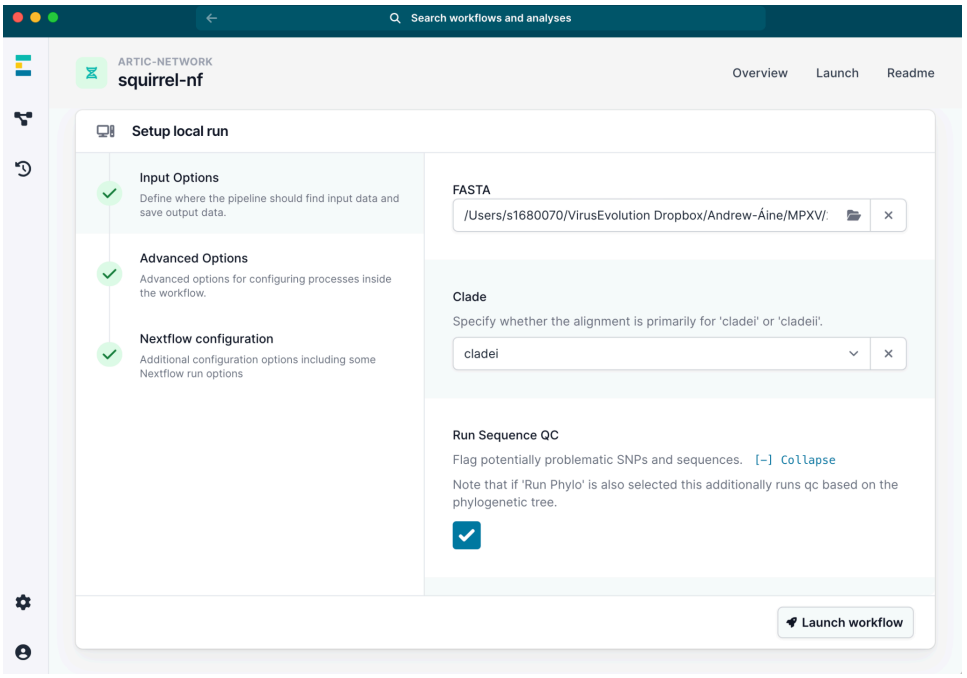
2. Select “Run on your computer” by clicking inside the box, and then click “Continue” on the bottom right corner.



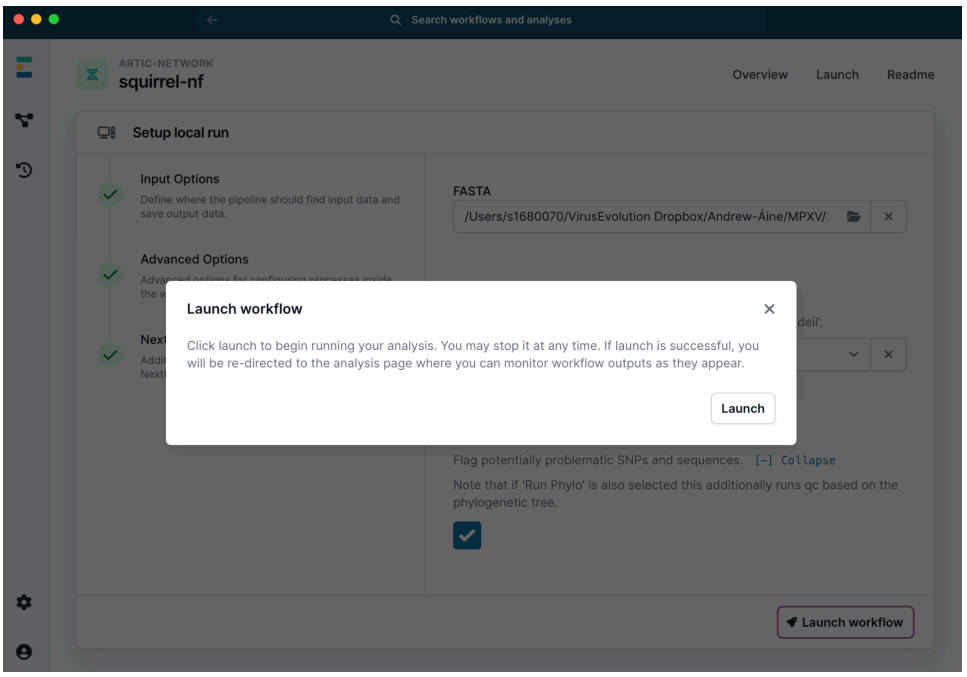
3. This will bring up the setup screen as shown above. Along the left hand panel, there are “input options”, “advanced options” and “nextflow configuration”. The “advanced options” and “nextflow configuration” are already completed (green ticks) so we don’t need to change those settings. We do need to change some input options. This first pass we are going to run in `Sequence QC` mode. Select a FASTA file to input into squirrel by selecting the folder icon.



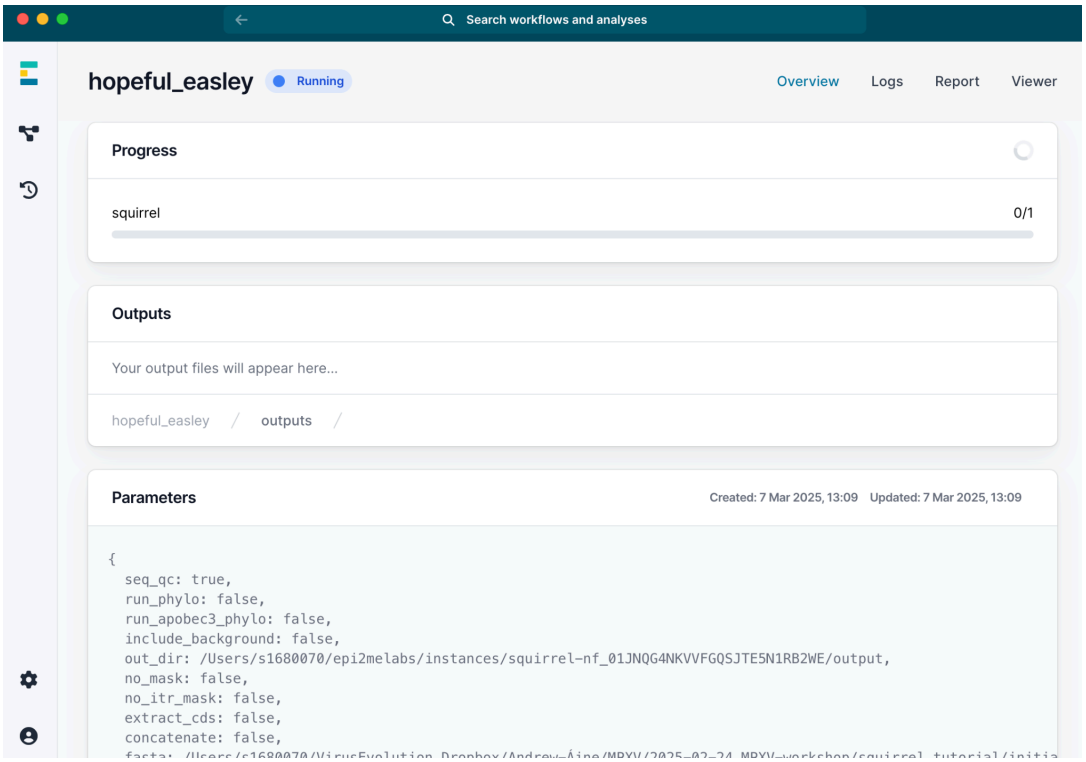
4. Navigate in the file browser to the file you wish to run through squirrel. In this case we will run “initial_cases.fasta”, which has some simulated Clade Ib genome sequences.



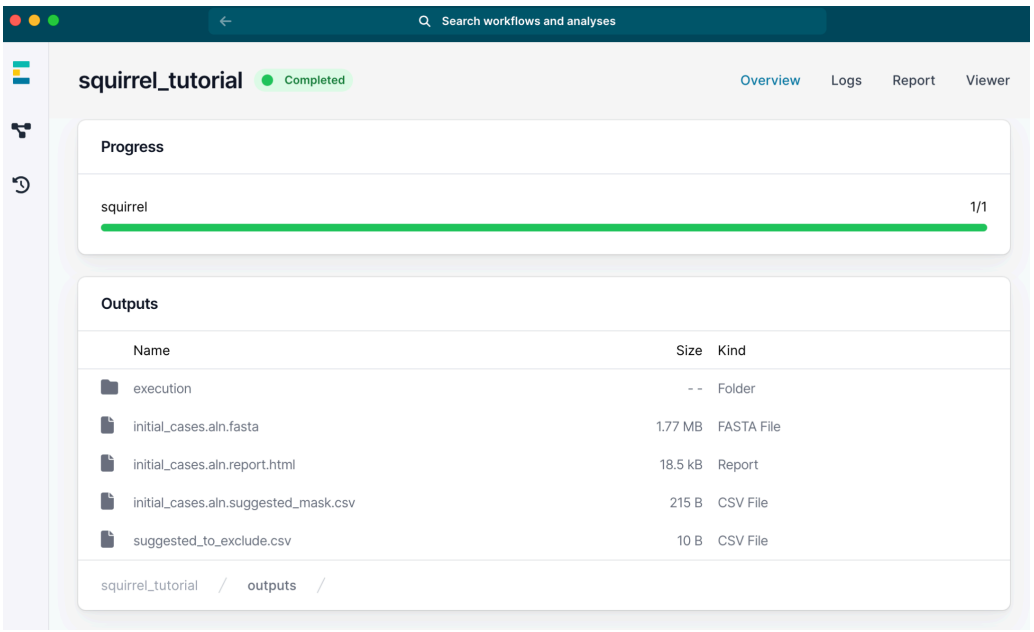
5. Select cladei for the clade and tick the box under “Run Sequence QC”, and then select “Launch workflow”.



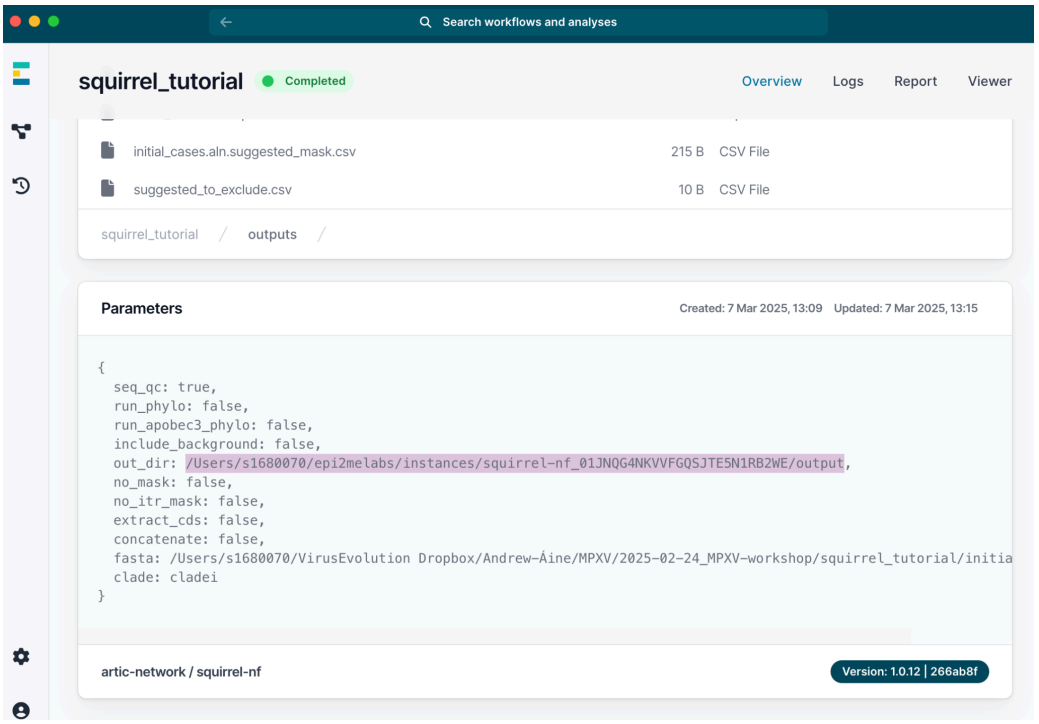
6. A dialogue box will appear. To run the workflow, select “Launch”.



7. EPI2ME will select a random name for the workflow run. In the above example it's "hopeful_easley". The name doesn't matter and only functions to create a unique run ID. The window shows the workflow is "Running" with the blue box.



8. When the workflow has finished running, it will change to green and say "Completed" as shown above.

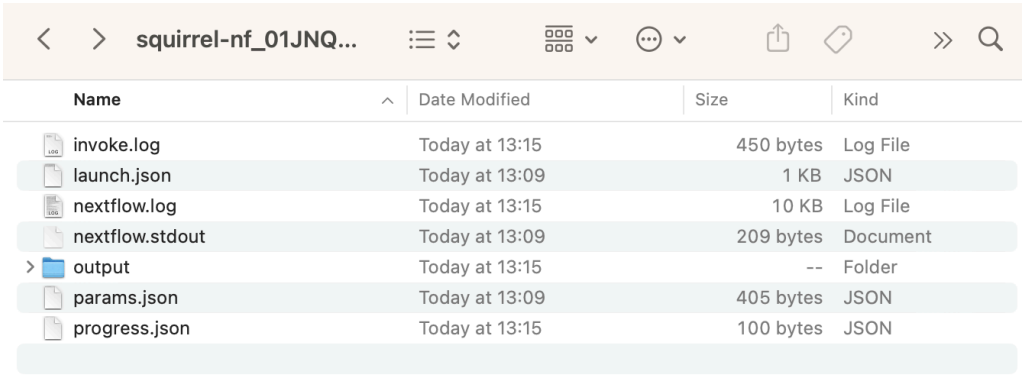


9. Scroll down to the “Parameters” section, which lists the workflow setup parameters. One of the pieces of information here describes the out_dir, which is the location that EPI2ME stores the output files from the workflow run. Navigate to this location in a file browser to find the output files from the squirrel analysis run. You can open the output folder by scrolling down and clicking “Open folder”.

   To find the folder in your file browser manually follow the following instructions. There will be different ways to navigate to this location depending on your operating system.
   For a windows machine, open up file explorer and on the left hand side panel you should be able to navigate to:

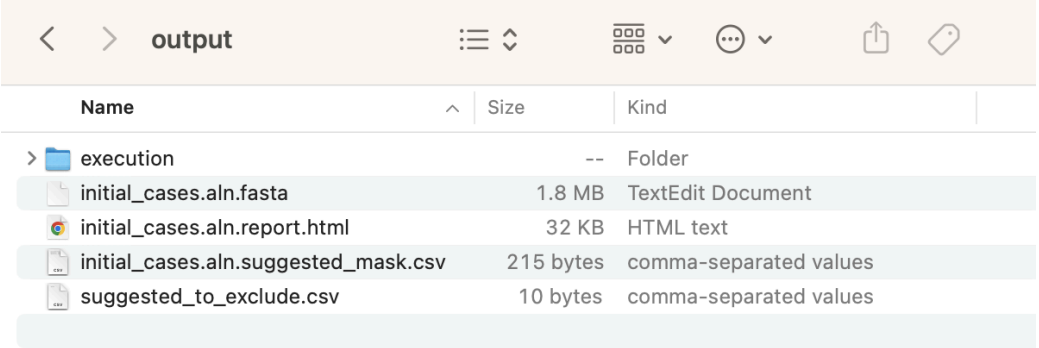
   *This PC > Local Disk(C) > Users > *your_username* > epi2melabs > instances*

   The most recent directory in this location will be the squirrel workflow that has just run. If you’ve previously run other workflows, such as artic_field_bioinformatics, they will also be shown here in this directory.



10. Within the most recent directory, you'll see a folder called "output", click into this to access the output of the EPI2ME squirrel run.

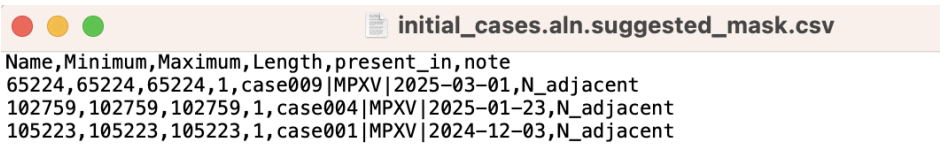
Understanding the squirrel QC output



Contents of the squirrel QC output directory:

- initial_cases.aln.fasta
  The file that ends in `.aln.fasta` is the aligned genome sequence data.
- initial_cases.aln.report.html
  The html file is the file displayed within the EPI2ME report window when the run finishes
- initial_cases.aln.suggested_mask.csv
  This suggested mask file is the file that we want out of our QC run. MPXV sequencing can be challenging, and the suggested mask file will flag any mutations in your genome that seem to be problematic/ a result of assembly or sequencing error.
- suggested_to_exclude.csv
  This file contains a list of any sequences in the provided file that are very incomplete or problematic, so are suggested to be excluded from the alignment for phylogenetics. Whether to exclude the sequences is left to the user's discretion.

Double click the suggested mask file to open it:

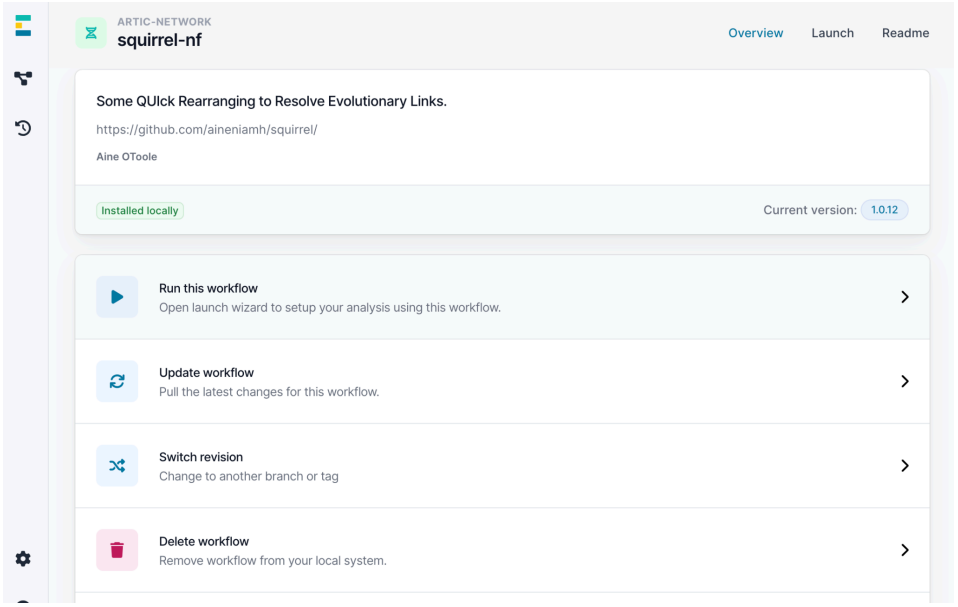


The file describes 3 sites with changes that squirrel believes are due to error because they occur immediately next to an N base (or block of Ns), which suggests they occur in an area of low read coverage.

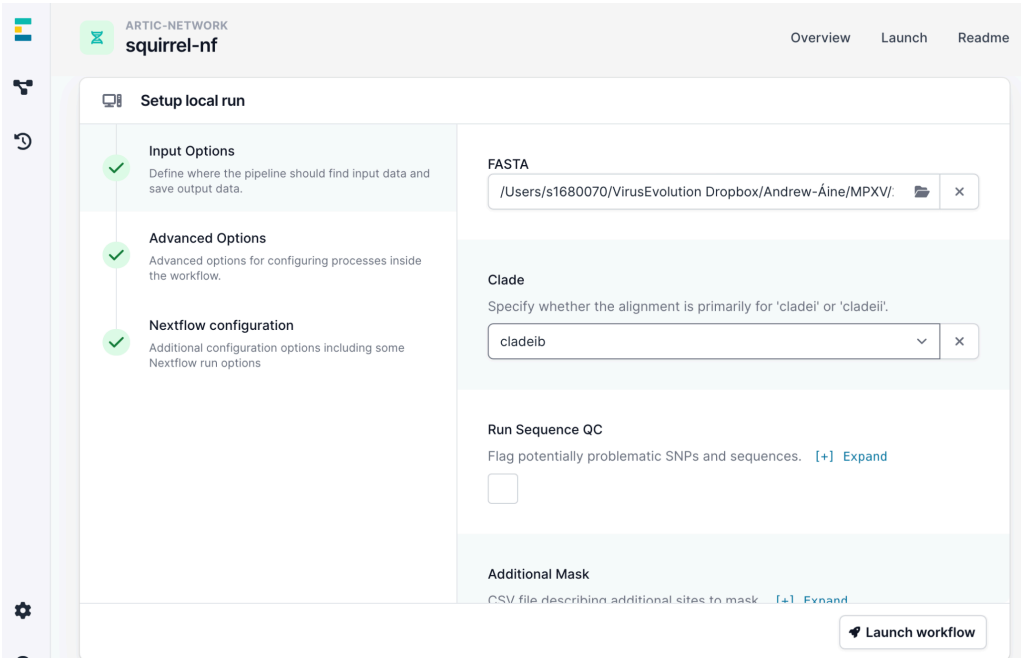
When you next run squirrel, you can supply this file and the mutations that are likely due to error can be masked out.

Running squirrel using EPI2ME for APOBEC3 reconstruction

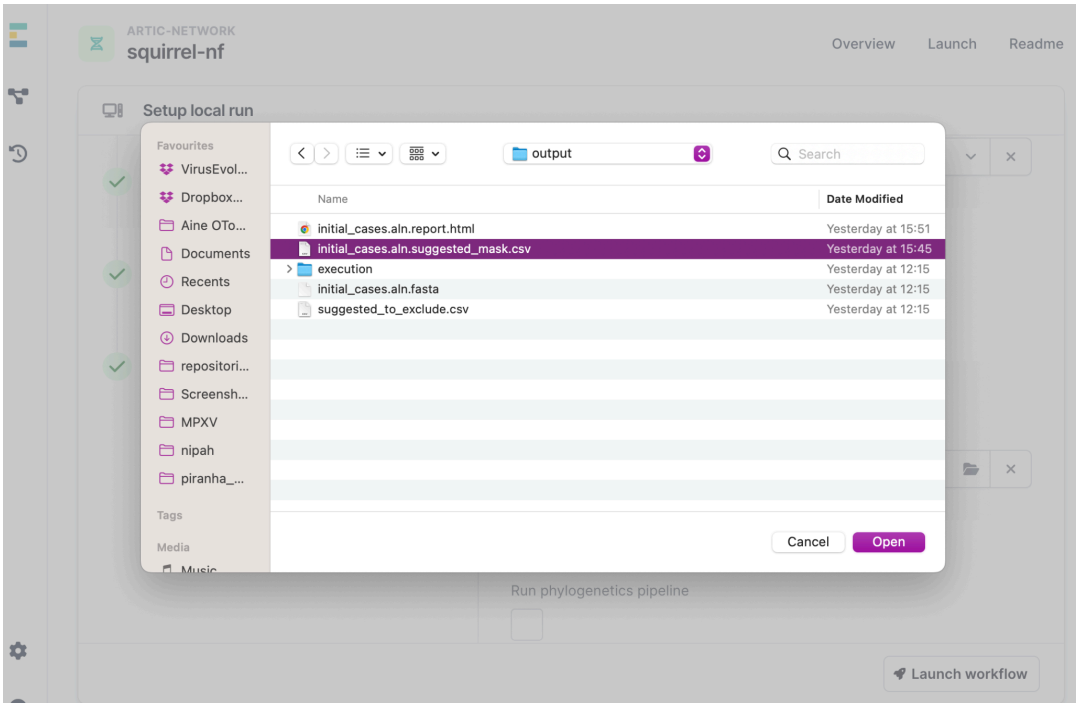
We will now run APOBEC3 reconstruction and phylogenetics through squirrel, using the suggested_mask file produced in the QC analysis.



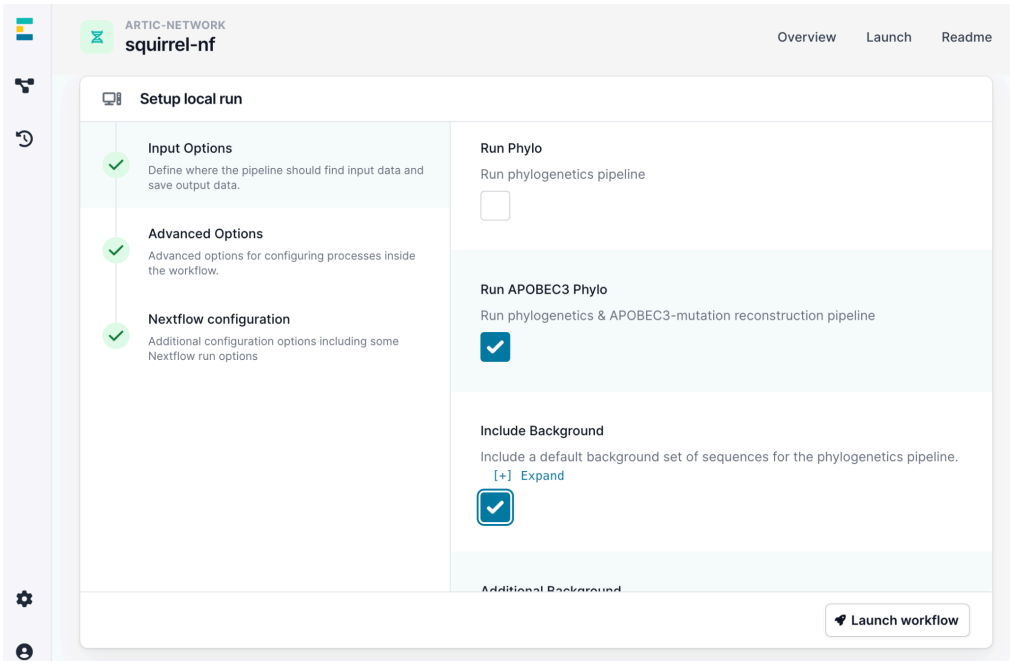
1. Navigate to the squirrel-nf workflow page by clicking the workflow panel button on the left hand side ( ), and then selecting squirrel-nf.



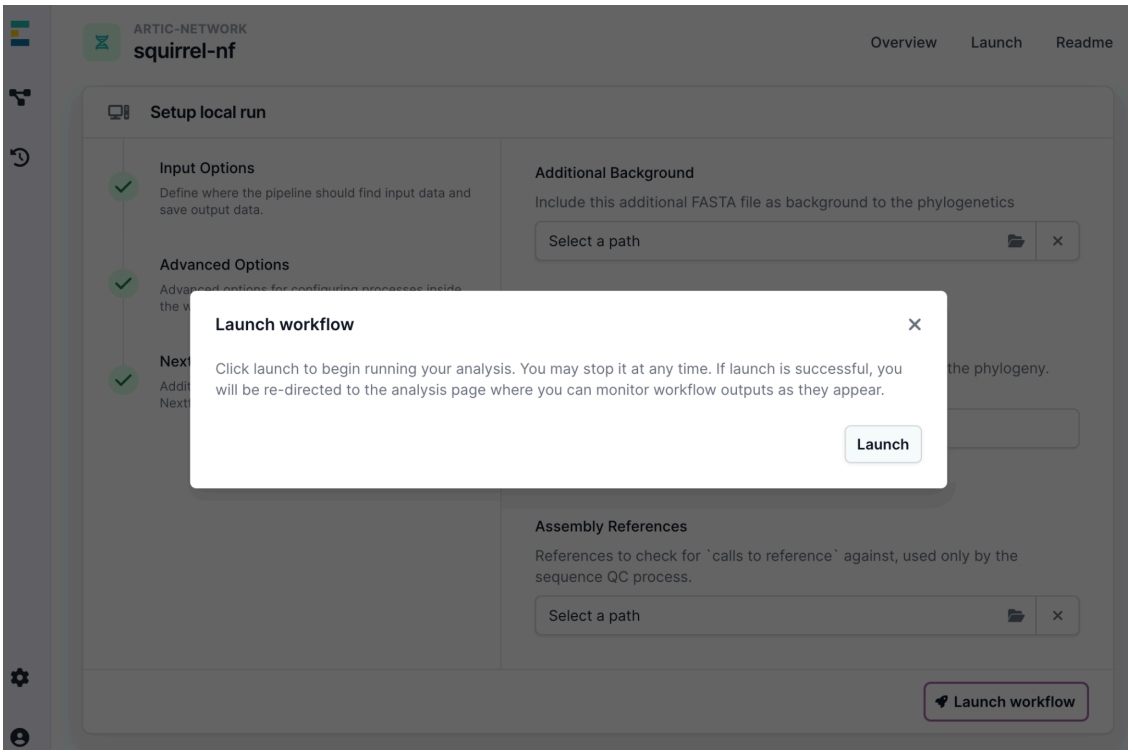
2. Run this workflow and click through as before until you get to the setup screen above. Select the initial_cases.fasta file as before, and you can select cladeib from the Clade dropdown menu. We do not need to run sequence QC this time, so you don't need to tick that box.



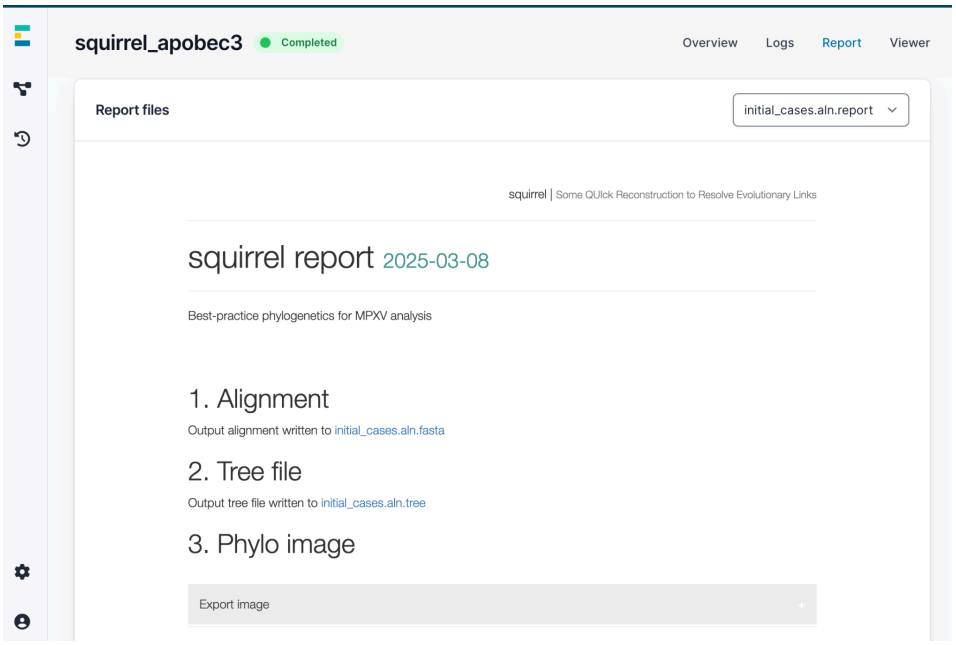
3. Scroll down to the “Additional Mask” file panel and navigate to the epi2melabs workflow output as you did previously. Select the suggested_mask.csv file.



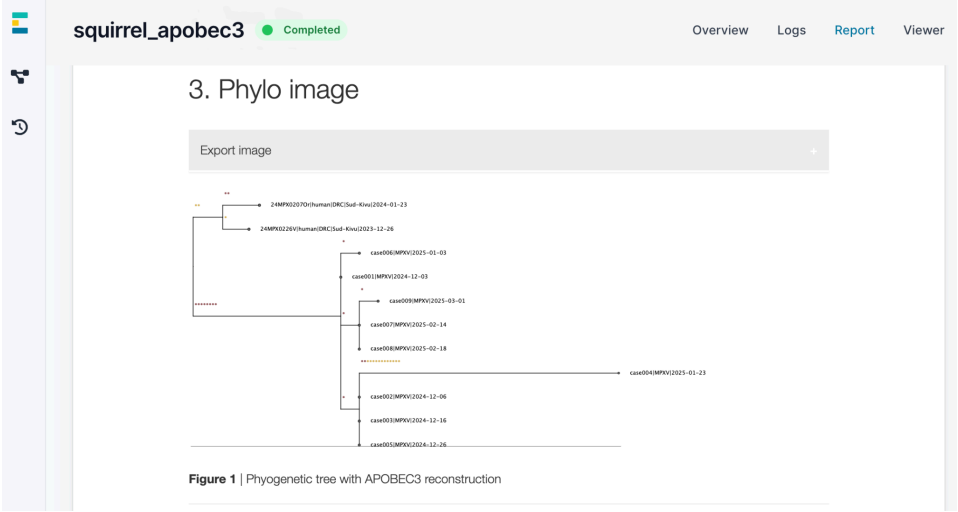
4. Continue to scroll down the page and select “Run APOBEC3 Phylo” and “Include Background”. This will run the full phylogenetic analysis with APOBEC3 reconstruction and include some minimal background Clade Ib sequences in the analysis.



5. Click “Launch workflow” and then “Launch” when the dialogue box appears to run the workflow. You can rename the workflow as before by scrolling down and selecting “Rename analysis”.



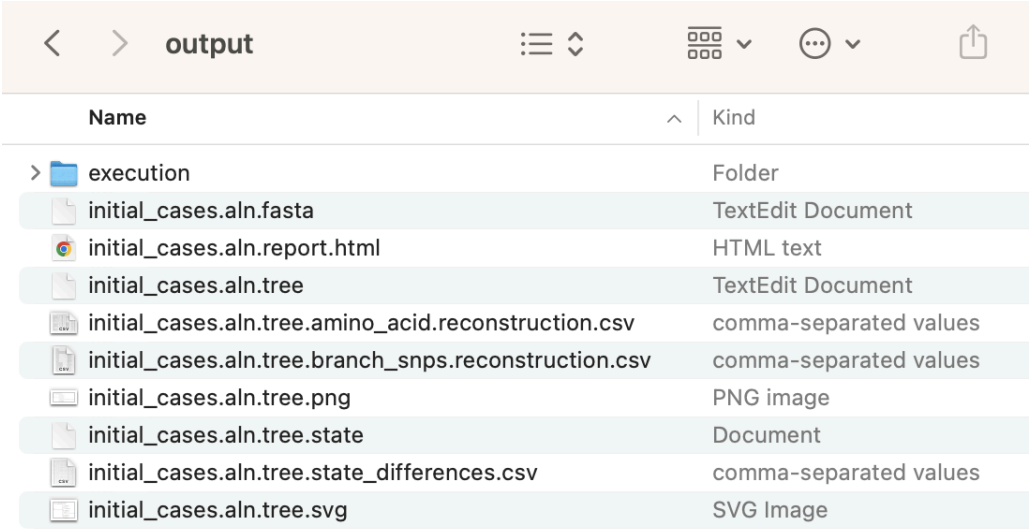
6. When the workflow has finished running (the status says “Completed” in green at the top), you can navigate to the Report viewer, by clicking “Report” in the top right.



7. Scroll down to view the image of the phylogenetic tree with APOBEC3 reconstruction. Mutations are displayed along branches as dots, with red dots representing APOBEC3-type mutations that are characteristic of human-to-human transmission and yellow dots representing non-APOBEC3 type mutations. Locate the samples in the phylogenetic tree and identify the background sequences that have been included too.

   Question:
   What sort of SNPs are seen across the branches of the tree?
   What can you say about case004?
   Are all the sequences in the initial_cases.fasta file linked?
   What might provide more information about whether the cases are related?

8. Return to the Overview window and scroll down until you see the option “Open folder”. Open the folder to explore the output files.



9. The output files from the squirrel and APOBEC3-reconstruction analysis are shown above.

- *`initial_cases.aln.fasta`*

The alignment file, with alignment scaffolded against a clade-specific reference. By default one of the ITR regions and a curated set of problematic regions is masked as Ns.

- *`initial_cases.aln.tree`*

The output maximum likelihood tree file from IQTREE2 with Node labels that correspond to the reconstruction Node labels. This tree can be viewed in various tree viewers, for example FigTree.

- *`initial_cases.aln.tree.state`* and *`initial_cases.aln.tree.state_differences.csv`*

The output ancestral state reconstruction file from IQTREE2 and the compiled list of unambiguously variable sites from squirrel.

- *`initial_cases.aln.tree.branch_snps.reconstruction.csv`*

A report of individual site changes mapped to specific branches and their dinucleotide context.

- *`initial_cases.aln.tree.amino_acid.reconstruction.csv`*

A report of each mutation that occurs across the phylogeny, their location, dinucleotide context, APOBEC3 status, which gene they're present in, codon position, amino acid change and a prediction of how extreme that amino acid change is with Grantham score.

- *`initial_cases.aln.tree.png` and `initial_cases.aln.tree.svg`*

Visualisation of reconstructed phylogeny showing whether mutations are consistent with APOBEC3 editing or not.

- *`initial_cases.aln.report.html`.* Summary report of analysis run.

Exercise:

Give squirrel a try with your newly generated MPXV genome sequence data. Squirrel analysis is clade specific, so we have provided you with a clade I file and clade II file.

References

Conticello, S.G. 2008. https://doi.org/10.1186/gb-2008-9-6-229

Drummond et al 2003
https://www.sciencedirect.com/science/article/abs/pii/S0169534703002167

O'Toole et al 2023 https://www.science.org/doi/10.1126/science.adg8116

Suspène et al 2023 https://pubmed.ncbi.nlm.nih.gov/37224627/

Wawina-Bokalanga et al 2025
https://www.medrxiv.org/content/10.1101/2024.11.15.24317404v1

Useful links

https://clades.nextstrain.org/

https://github.com/aineniamh/squirrel

https://nanoporetech.com/products/analyse/epi2me

https://artic.network/how-to-read-a-tree.html

https://artic.network/mpxv/mpxv-phylogenetics-epi2me-sop.html