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Reference Sets in Viral Genomics:

Robert J. Gifford

What is a Reference Set?

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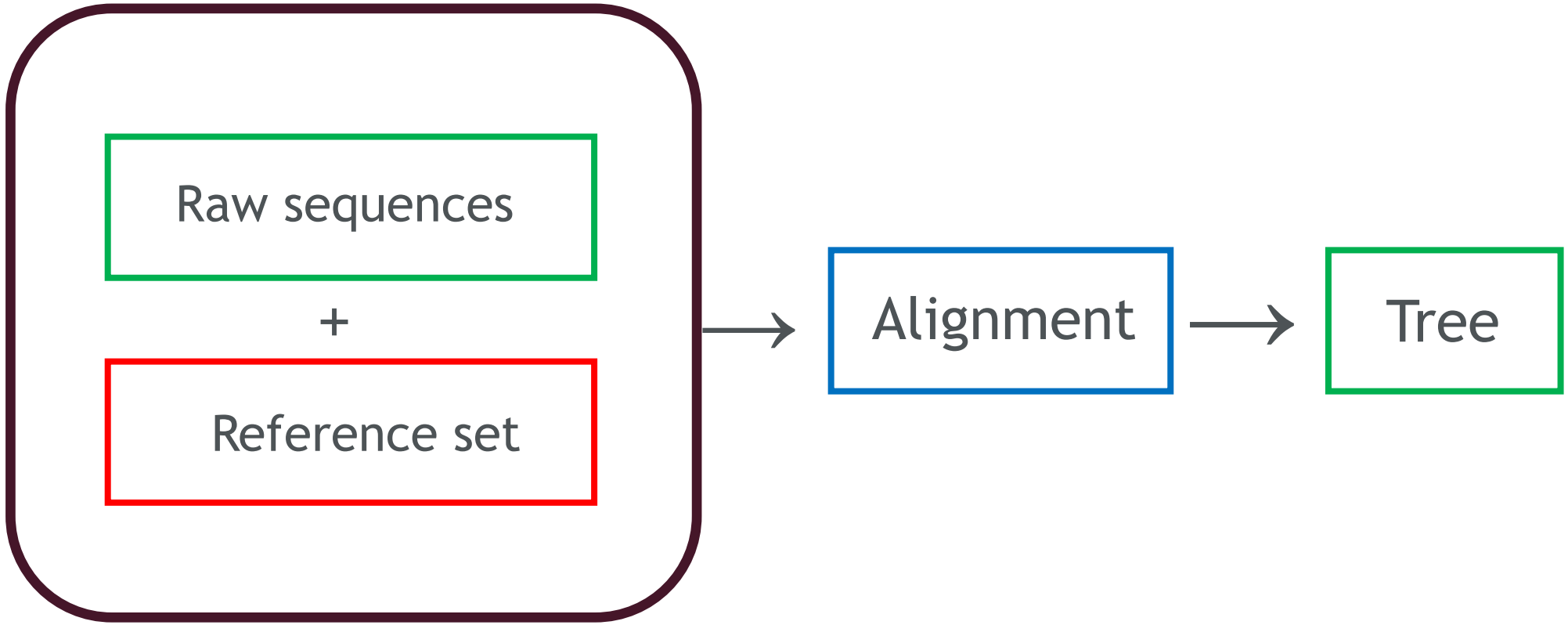
- A *curated collection* of sequences chosen to anchor analysis.

- Provides **context** for:
  - Alignments
  - Phylogenetic trees
  - Genotyping and annotation
- Acts as a **baseline** against which new or unknown sequences are compared.

What is a Reference Set?



Why Do Reference Sets Matter?

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- Shape the **accuracy of alignments**
  - bad references → misaligned regions.
- Influence **phylogenetic trees**
  - different reference sets can shift topology.
- Reduce **bias** by balancing representation.
- Provide **interpretability**
  - results only make sense relative to good context.

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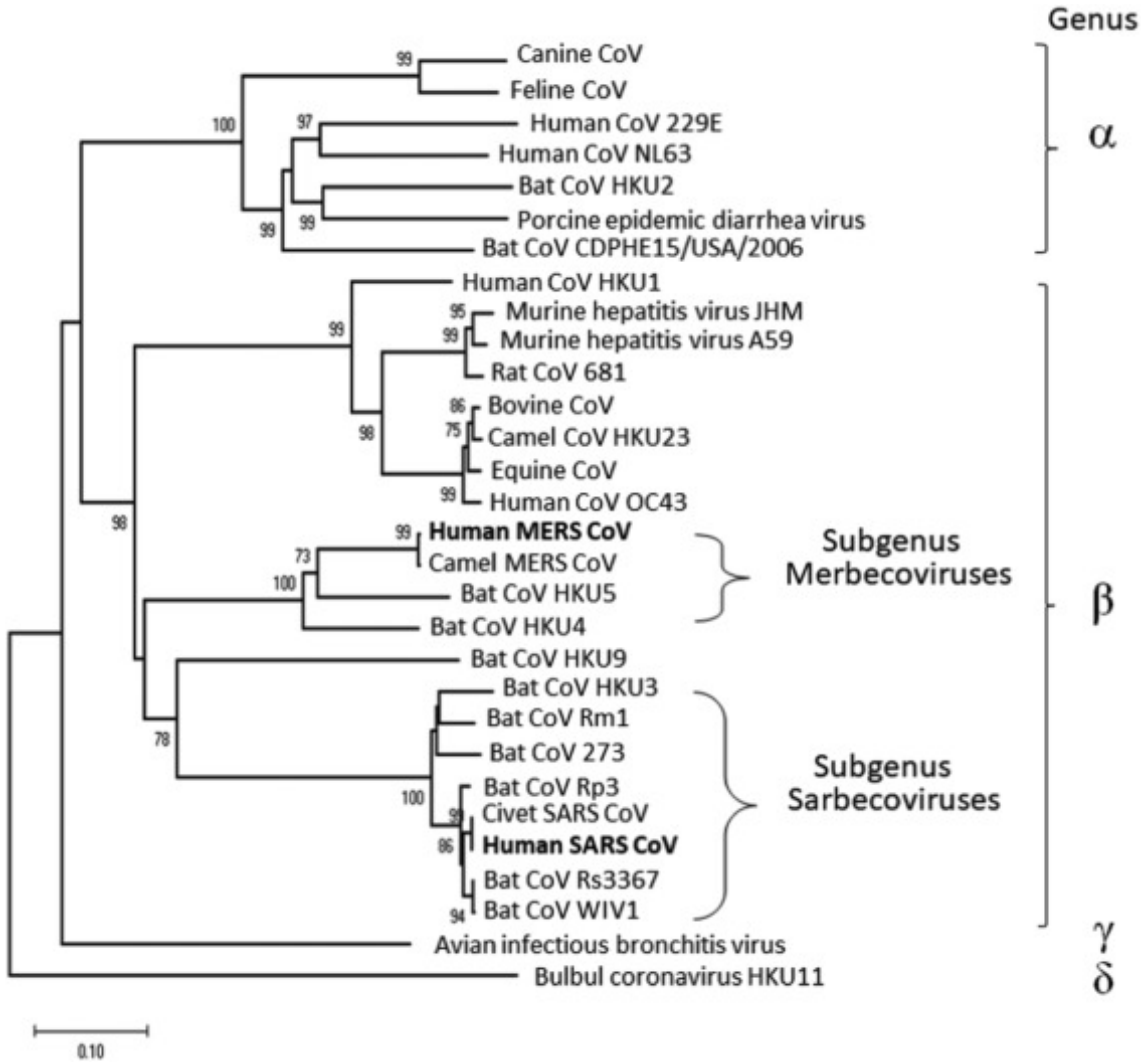

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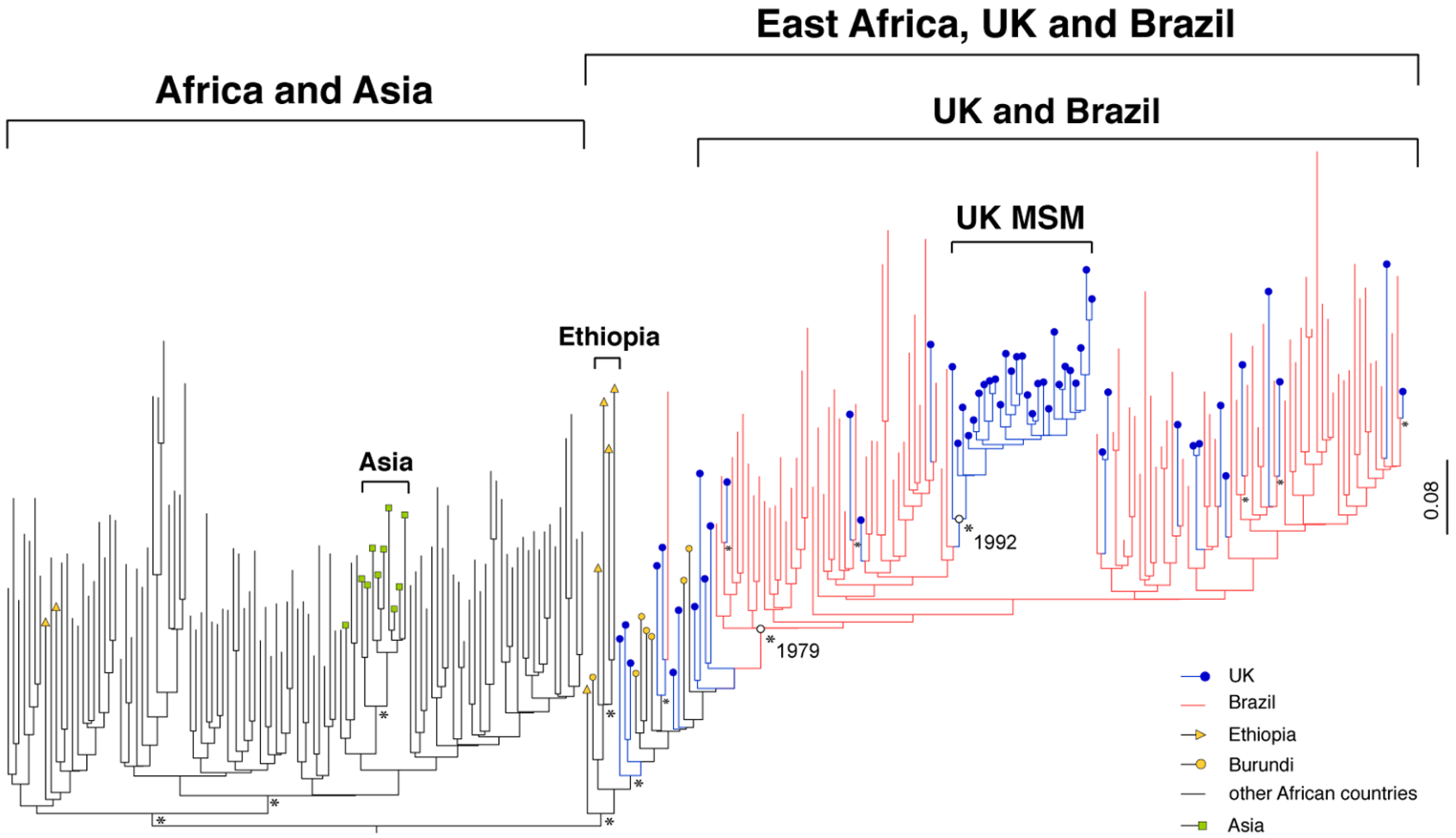

Sources of Reference Sequences

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GenBank

- **Strengths:**
  - Largest open-access virus database.
  - Long-term archive with >1M IAV sequences, >500k IBV.
- **Challenges:**
  - Genome segments stored separately → harder to reconstruct full genomes.
  - Inconsistent isolate metadata (host, location, date, clinical info).
  - Variable accuracy/annotation quality.
  - Limited taxonomy (often only subtype level, lineage rarely consistent).
  - Very large datasets → difficult to manage at scale.

Sources of Reference Sequences

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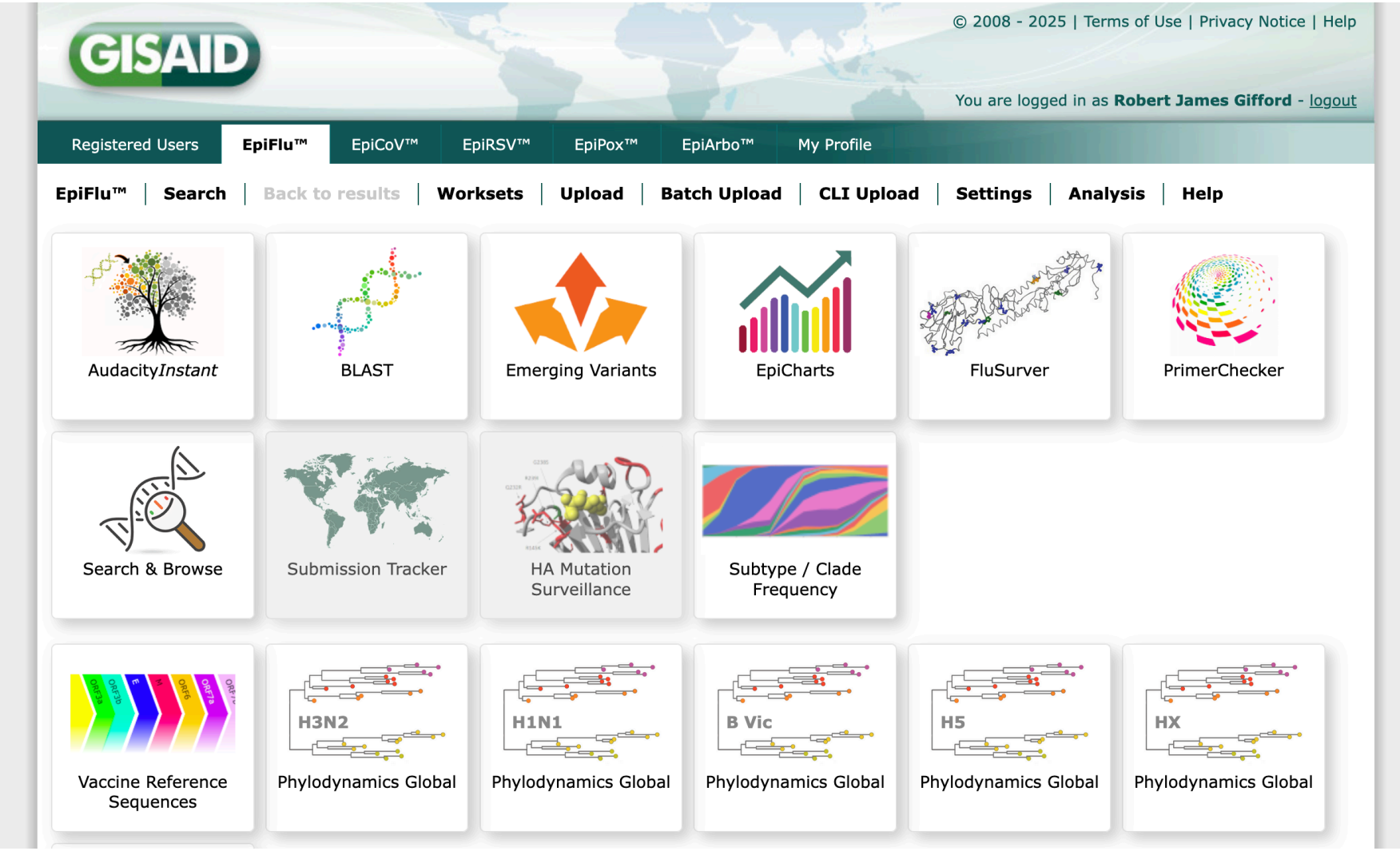
GISAID

- **Strengths:**
  - Rich, curated metadata (host, date, location standardized).
  - Near-real-time coverage of influenza & SARS-CoV-2.
  - Lineage/phylogeny assignments often included.
  - Collaborative ethos → credit to data generators.
- **Challenges:**
  - Controlled access → must register, agree to terms.
  - Restricted redistribution of sequences.
  - Coverage focused on major pathogens (influenza, SARS-CoV-2) — not comprehensive.
  - Metadata occasionally inconsistent across submitters.

Sources of Reference Sequences



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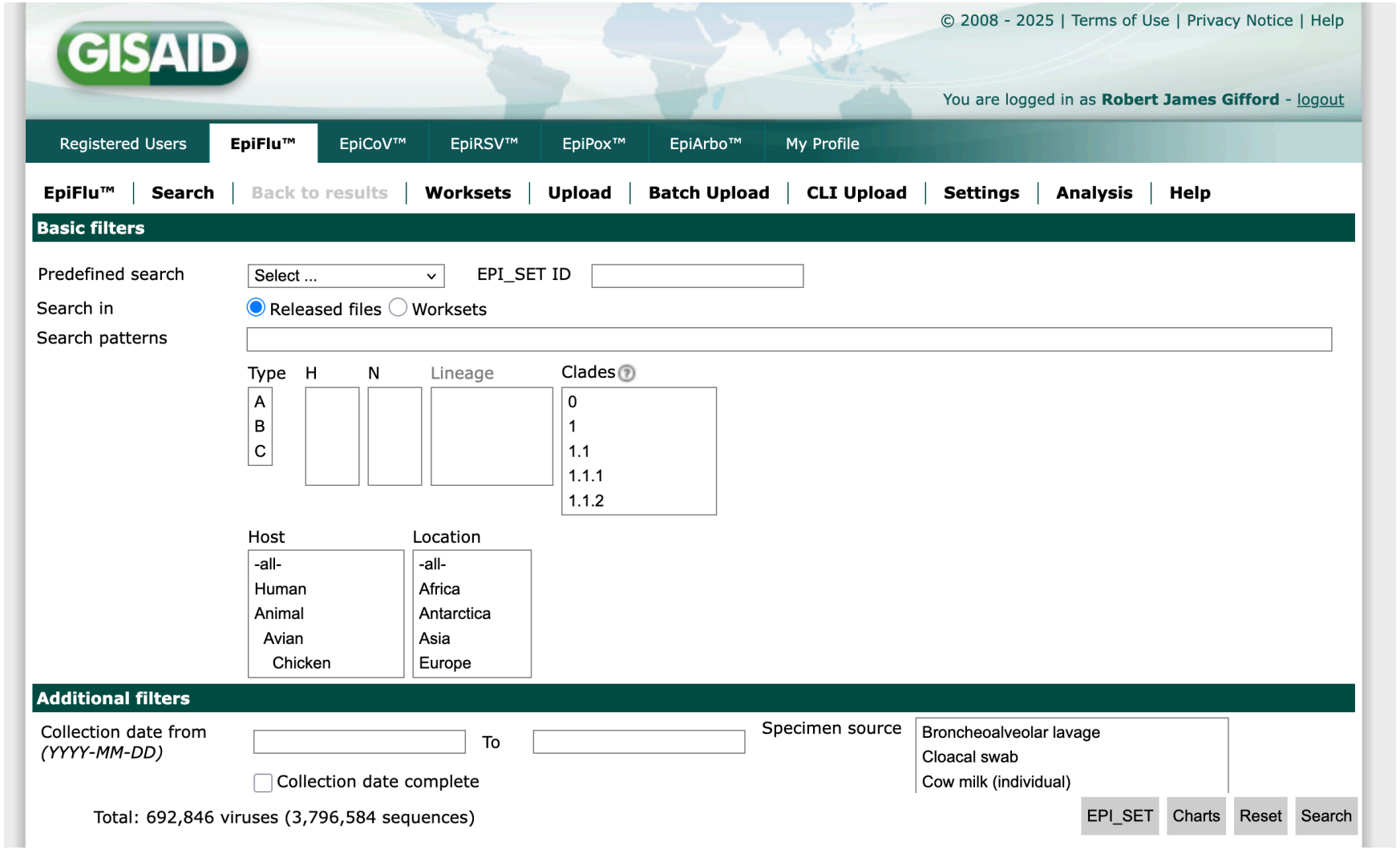

Sources of Reference Sequences

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NextStrain

- **Strengths**
  - Provides curated reference datasets for specific pathogens.
- **Challenges**
  - Reference sets tailored to specific builds → not comprehensive.
  - Coverage focused on major pathogens (e.g. Flu, SARS-CoV-2, Ebola).
  - Less suited for deep historical or lineage-wide reference curation.
  - Not a general-purpose sequence archive.

Sources of Reference Sequences

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PathoPlexus

- **Strengths**
  - Open-source, non-profit platform for *all human viral pathogens*.
  - Integrates with Nexstrain, INSDC (NCBI/ENA/DDBJ).
  - Community-driven governance, transparent policies.
  - Offers SeqSets and APIs for filtering/search.
  - Flexible data-sharing: immediate open or embargoed.
- **Challenges**
  - Still emerging → limited pathogen coverage so far.
  - Focused on human pathogens, not broader host range.
  - Curation depth varies by virus.
  - Dependent on community participation for sustainability.

Sources of Reference Sequences

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GLUE

- **Strengths**
  - Openly available isolate-organised table (iav_nuccore_isolates.tsv).
  - Metadata cleaned, standardised, and cross-checked using GLUE.
  - Links isolates to all 8 genome segments → easy accession retrieval.
  - Simple to filter/subsample with Unix tools.
  - Fits directly into Nextclade/Augur/Auspice workflows.
- **Challenges**
  - Not leveraging GLUE's full interactive framework (command layer, modules).
  - Updates depend on periodic refresh, not real-time feeds.
  - Coverage only for influenza (not all viruses).
  - Some metadata fields less detailed than GISAID (e.g. passage history).

Virus Reference Sequences

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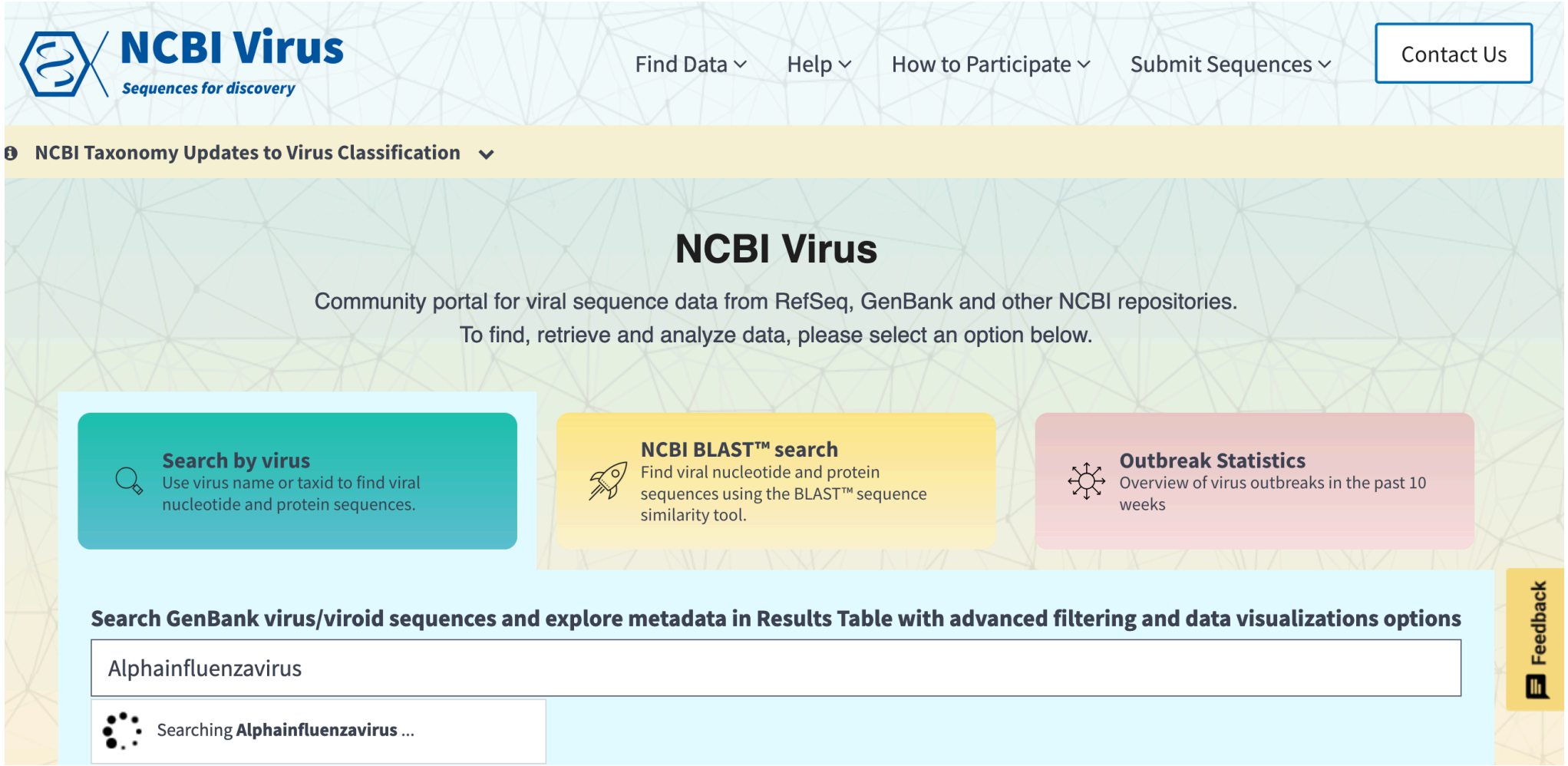

GenBank Influenza Virus Entry

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```
REFERENCE   3  (bases 1 to 890)
  CONSRTM   The NIAID Influenza Genome Sequencing Consortium
  TITLE     Direct Submission
  JOURNAL   Submitted (01-AUG-2005) on behalf of TIGR/Wadsworth-NYSDOH/NCBI,
            National Center for Biotechnology Information, NIH, Bethesda, MD
            20894, USA
COMMENT     REVIEWED REFSEQ: This record has been curated by NCBI staff. The
            reference sequence was derived from CY002068.
            COMPLETENESS: full length.
FEATURES             Location/Qualifiers
     source          1..890
                     /organism="Influenza A virus (A/New York/392/2004(H3N2))"
                     /mol_type="viral cRNA"
                     /strain="A/New York/392/2004"
                     /serotype="H3N2"
                     /isolation_source="gender:M; age:16y"
                     /host="Homo sapiens"
                     /db_xref="taxon:335341"
                     /segment="8"
                     /lab_host="RhMK 1 passage(s)"
                     /geo_loc_name="USA: Tompkins County, NY"
                     /collection_date="21-Dec-2004"
     gene            27..864
                     /gene="NEP"
                     /locus_tag="FLUAVH3N2_s8p1"
                     /gene_synonym="NS2"
                     /db_xref="GeneID:3655157"
     CDS             join(27..56,529..864)
                     /gene="NEP"
```

GenBank Influenza Virus Entry

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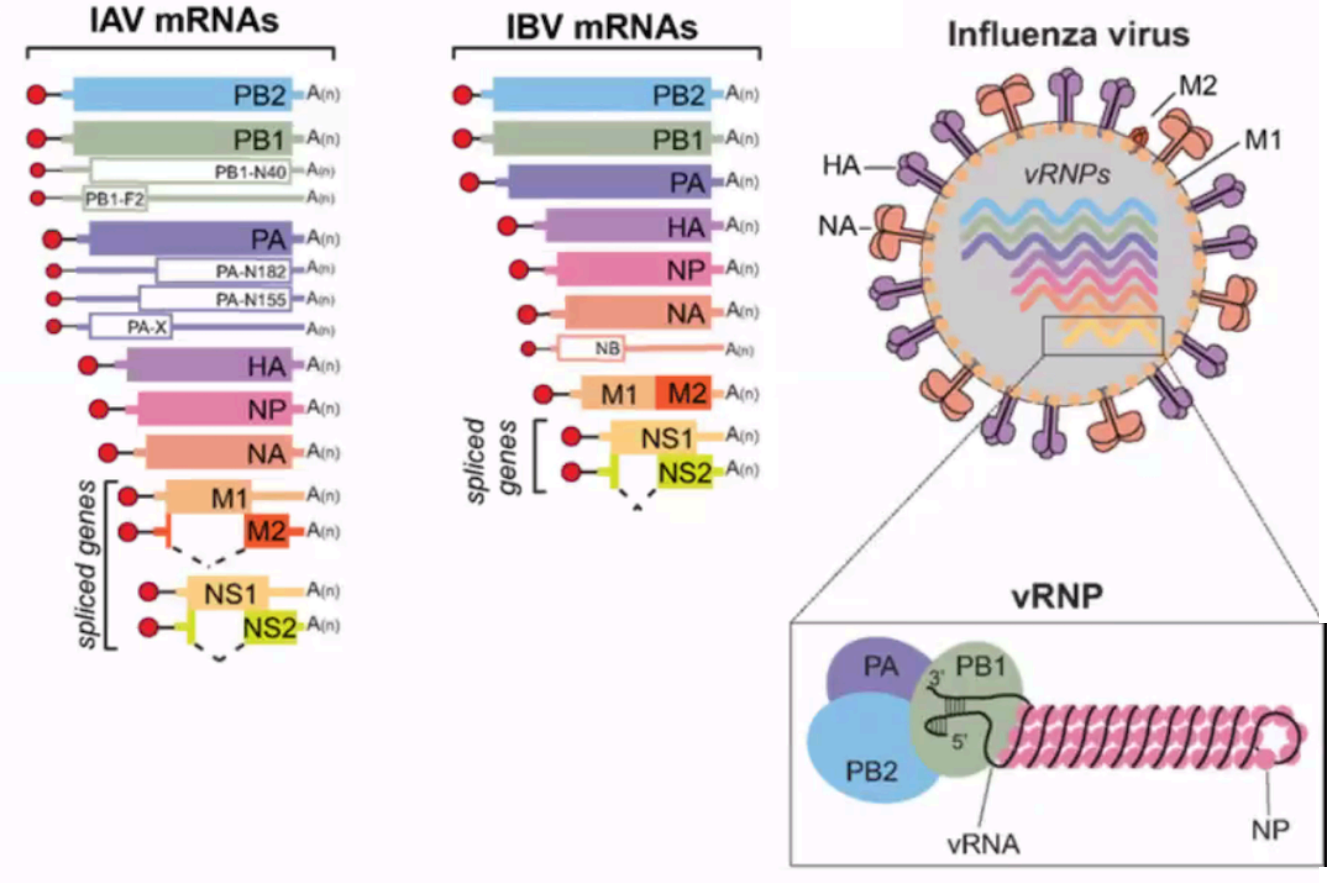
- Each genome segment = separate record.
- Isolate details partly in strain name, partly in extra fields.
- Metadata (host, location, date) often inconsistent or missing.
- For influenza, formatting is *more standardised* than for many viruses — but still patchy.

Influenzavirus Genomes



Selecting Reference Sets (Web)

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- **GenBank:**
  - Searchable, but limited filters.
  - Hard to select by segment or isolate fields.
- **GISAID:**
  - Advanced filters (host, date, clade, geography).
  - Better for curated sets — but export restrictions.
- **Nextstrain:**
  - Provides curated, ready-to-use reference sets.
  - Useful as examples/templates, not always comprehensive.

Selecting Reference Sets (CLI)

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- **Entrez Tools (NCBI):**
  - esearch + efetch → programmatic access.
  - Reproducible queries (e.g. by host, segment, date).

- **Nextstrain Augur:**
  - Higher-level filtering & downsampling.
  - Integrates metadata + sequence selection.

Why Use Docker?

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- **Consistency:** same environment across all computers.
- **Portability:** runs on Linux, Mac, Windows.
- **Reproducibility:** ensures identical results for everyone.
- **Convenience:** no dependency hell (prepackaged software).

Quality Control of Reference Sets

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Nextclade

- Remove **low-quality or incomplete** sequences.
- Check for **frame shifts, stop codons, sequencing errors.**
- Confirm **correct genotyping** (e.g. HA subtype for influenza).
- Tools: **Nextclade** (genotyping + QC in one step).

Alignment & Tree Building

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- **Multiple Sequence Alignment (MSA):**
  - Align sequences against reference set.
  - Anchors homologous sites across genomes.
- **Tree Building:**
  - Infer evolutionary relationships from aligned data.
  - Reference set provides stable “scaffold” for new sequences.
- **Tools (Nextstrain, Docker):**
  - Alignment → `augur align`
  - Phylogeny → `augur tree + augur refine`

Key Points

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- Reference sets = **foundation** of viral sequence analysis.
- Good sets are: **representative, high-quality, balanced, fit-for-purpose.**
- Sources differ:
  - **GenBank** = comprehensive but messy.
  - **GISAID** = curated but controlled.
  - **Pathoplexus/NextStrain/GLUE** = pre-curated, niche
- Web portals = **exploration**.
- Command line + Docker = **reproducibility**.
- **QC, alignment, and tree building** depend on having a robust reference set.

Now It’s Your Turn

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We’ll walk through:

- **Downloading sequences** (GenBank + GISAID web).
- **Cleaning and preparing sequences & metadata**
- **Running QC** with Nextclade.
- **Filtering** with Augur.
- **Building and viewing** a tree with Nextstrain.

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OVER TO YOU!!

Photo by Stefan Els