Master Thesis

Development of Galaxy Workflows for Sequence Data Analysis of Notifiable Viral Livestock Diseases

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Declaration

I hereby declare that I am the sole author and composer of my thesis and that no other sources or learning aids other than those listed have been used. Furthermore, I declare that I have acknowledged the work of others by providing detailed references of said work.
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Abstract

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Acronyms

AIV Avian Influenza Virus

AWS Amazon Web Services, Inc.

BAM Binary Alignment Map

BED Browser Extensible Data

BLAST Basic Local Alignment Search Tool

BWA-MEM Burrow-Wheeler Aligner for short-read alignment

CaPV Capripoxvirus

CDC Centres for Disease Control and Prevention

cDNA Coding Deoxyribonucleic Acid

DNA Deoxyribonucleic Acid

COVID-19 Coronavirus Disease 19

drVM detect and reconstruct known Viral genomes from Metagenome

FPV False Positive Variant

HA Hemagglutinin

HPAI Highly Pathogenic Avian Influenza

HTS High-Throughput Sequencing

IAEA International Atomic Energy Agency

ICTV International Committee on Taxonomy of Viruses

ILRI International Livestock Research Institute

INSaFLU "INSide the FLU"

IRIDA Integrated Rapid Infectious Disease Analysis

ITR Inverted Terminal Repeat

iVar intrahost Variant analysis of replicates

IWC Intergalactic Workflow Commission

KSP All k Shortest Path

LPAI Low Pathogenic Avian Influenza

LSD Lumpy Skin Disease

LSDV Lumpy Skin Disease Virus

MAFFT Multiple Alignment using Fast Fourier Transform

MERS Middle East Respiratory Syndrome

MSA Multiple Sequence Alignment

NA Neuraminidase

NGS Next-Generation Sequencing

NP Nucleoprotein

OIE Office International des Epizooties

ONT Oxford Nanopore Technologies

ORF Open Reading Frame

PAIVS Prediction of Avian Influenza Virus Subtype

PCR Polymerase Chain Reaction

PDF Portable Document Format

RNA Ribonucleic Acid

SAM Sequence Alignment Map

SARS Severe Acute Respiratory Syndrome

SARS-CoV-2 Severe Acute Respiratory Syndrome Coronavirus 2

SMRT Single Molecule Real-Time Sequencing

SNP Single-Nucleotide Polymorphism

VETLAB Veterinary Diagnostic Laboratory

WGS Whole-Genome Sequencing

WHO World Health Organization

WOAH World Organization for Animal Health

ZODIAC Zoonotic Disease Integrated Action

1 Introduction

Sharing environments means sharing diseases – this simple relationship expresses how pathogens found in animal populations can spread to humans and have severe impacts. The impact can be as severe as the whole world experienced during the pandemic of Coronavirus Disease 19 (COVID-19) that originated in Wuhan, China in 2019. This highly contagious disease was caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), an infectious virus of presumed zoonotic origin [1]. With more than 757.26 million reported cases and more than 6.85 million confirmed deaths as of February 24, 2023 **TODO: update numbers** `https://covid19.who.int/table`, this pandemic is a public health emergency that has caused estimated costs of 16 trillion U.S. dollars. Apart from this, it invoked an outstanding interest in virology research [2].

Since then, professionals from many different fields, i.e. public health specialists, researchers, biomedical staff, bioinformaticians and veterinarians have put even more effort than before into the monitoring of potentially dangerous viral diseases. International managing institutions with a globally distributed netwok work on safe and healthy environments for animal and human populations. The World Organization for Animal Health (WOAH), founded as Office International des Epizooties (OIE), implements standards in animal health and the handling of zoonoses and other diseases. As an intergovernmental organisation following the multidisciplinary One Health principle, it supports its members in the prevention of animal diseases of concern. National veterinary authorities must notify the WOAH in case they detect

cases of diseases that are listed by the WOAH. The most important definitions, the significance, impacts and surveillance measures of animal diseases are examined below.

1.1 Viral Livestock Diseases

Infectious diseases caused by viruses that affect domesticated animals, like for example cattle, pigs, goats, sheep, and poultry are referred to as viral livestock diseases. The most frequent and known diseases include Foot-and-Mouth Disease, African Swine Fever, Avian Influenza and Newcastle Disease. They can spread quickly among animals, and in some cases are transmitted to humans, making them zoonotic diseases. There are over 200 known types of zoonoses, some of them like rabies being 100% preventable through vaccination and medication [3]. A report from the International Livestock Research Institute (ILRI) states that zoonoses account for approximately 2.5 billion illness cases in humans and 2.7 million deaths annually [4]. The Centres for Disease Control and Prevention (CDC) and its U.S. government partners listed the top eight zoonotic diseases of national concern in a report, filing zoonotic influenza and emerging coronaviruses such as Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) [5]. This joint report is used to tackle the listed diseases with a broader focus [6]. At the same time, not all livestock diseases of viral origin are zoonotic: Around 60% of all known human infectious diseases and approximately 75% of all newly emerging infections are zoonotic [7].

The term livestock is a vague term that generally refers to any breed or animal population that is kept by humans for commercial or useful purpose. According to the 20th Livestock Cencus of the Department of Animal Husbandry and Dairying, given out by the Indian government, India holds the world's largest amount of livestock with 535.78 million animals as of 2019 [8]. Globally, the ice-free surface

that is dedicated to the purpose of livestock whether it is for farmlands or feed production, is up to 26% of the area [9]. Not only food production and economy, but also global trade, the agricultural sector and employment rates highly depend on livestock resources. These numbers illustrate the impressive interconnectedness of the humans with the livestock sector. The consequences of a collapse of this important industry would therefore be significant and far-reaching. As the livestock industry is directly affected by the occurrence of zoonoses in both developed and developing countries, affected parties have a strong interest in avoiding any constraints that might be caused by disease outbreaks.

Historic Outbreaks of Zoonotic Diseases

Historically, zoonoses have shaped serious infectious events. Pathogens that cause zoonotic diseases are viruses (37.7%), and according to surveillance data also bacteria (41.4%), parasites (18.3%), fungi (2.0%) or prions (0.8%) [10]. Prior to the COVID-19 pandemic modern zoonotic diseases like Ebola virus disease and salmonellosis had high infection rates. Influenza viruses cause epidemics each year, and circulate in all parts of the world. Influenza appears in zoonotic and human-only spreads, but the different types of virus can recombine occasionally and cause events such as the 1918 Spanish flu [11, 12]. Especially for poultry, Highly Pathogenic Avian Influenza (HPAI) of the H5 subtype is an ongoing threat [13]. Since its first detection in China, 1996 it has been reported in many avian populations, both domestic and wild. Even though it has adapted to birds as the specific host, the virus can further adapt and in rare cases be transmitted between humans [14]. Avian influenza has caused more recent seasonal outbreaks, such as the 2014-15 outbreak in the United States resulting in almost 50 million birds that died as a consequence of an infection or of depopulation [15]. This is roughly a third of the national stock of laying hens. In 2020, there were several outbreaks reported in Europe, almost all with HPAI viruses from the H5 subtype [16]. It mainly affected farmed ducks due to the high density of animals in the facilities

and the separation from wild birds due to domestication [16]. The latest outbreak of HPAI is spreading worldwide. Started in early 2022 and until today, February 23, 2023 **TODO: update numbers** https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2023.7786 it has led to more than 58 million culled or died birds. Different H5 subtypes have been reported in 37 countries and so far, six human infections were reported in this outbreak [17]. This number is not nearly as high as for the animals affected, but considering that from 2003 to 2022, there were a total of 868 confirmed cases of H5N1 in humans with a mortality rate of 52%, each human infection is a risk [17].

Risk Factors and Impact of Disease Outbreaks

Reasons for recurring huge outbreaks of viral diseases in animal confinements come from the advantageous circumstances for virus transmission as it is warm and humid. In general, animal husbandry practices have evolved in the sense that domestic animal species are raised in relatively small and usually confined spaces at a high density. This domestication has given plenty of opportunities to develop more pathogens of viral and bacterial origin over time. The spread of international trading of farm animals has amplified the number of infected animals and the number of infectious diseases. As transmission routes can differ depending on the disease, the other factor is how easy the infectious agent spreads (transmissibility). Vector-borne diseases are transmitted by living organisms that transfer pathogenic microorganisms to other, uninfected animals or humans. Vectors can be mosquitoes, fleas or ticks. Among others, the World Health Organization (WHO) identifies major globally present vector-borne diseases as malaria, dengue, yellow fever and Zika virus disease [18]. Another transmission mode is direct contact airborne transmission. Environmental factors such as a high temperature, humidity and precipitation can facilitate a virus to spread and keep it alive [19]. Inadequate food and water supplies, overpopulation and mass migration of animals pose additional risks for transmission of animal

diseases.

Outbreaks of livestock diseases do not only affect animal and human health, but also cause high economic losses. Restrictions and containment measures, as well as the culling of animals in the case of confirmed cases of listed diseases, lead to a loss of income for farmers – since livestock and their products, such as milk, eggs or meat, are used for further production, other businesses that rely on these products are also affected by disease outbreaks. Even if infected animals do not die or have to be culled, the medium and long-term consequences of infection can affect the health of the animals. Consequences can be poor growth or poor production and feed conversion. Another impact of depopulating infected animal populations is the loss of biodiversity [20, 21]. Wildlife populations of endangered species experiencing disease outbreak can be decimated, leading to ecological imbalances and interference with natural food chains [22, 23, 24].

As shown, the spread of viral disesaes among animal populations can have enormous impacts on dependent industries, individuals and populations.

Notifiable Animal Diseases

For reasons of biosecurity and surveillance purposes, the WOAH has agreed on a list of notifiable animal diseases that must be reported to in agricultural authorities. This list includes a total of 117 diseases, partly endemic or highly transmissable, such as Foot-and-mouth-disease, lumpy skin disease, peste des petits ruminants, classical swine fever, highly pathogenic avian influenza and Newcastle disease. The list does not cover all known zoonoses and animal diseases since not all of them pose an actual risk for outbreaks.

Reports of illness cases of animals filed by national veterinary authorities are used to detect unusual incidents, including mortality or sickness of animals and have adverse effects on socio-economic or public health. The notifiable animal diseases include more than 50 wildlife diseases which can impact livestock health [25]. As

the surveillance of viral animal diseases is still of highest priority in order to avoid expensive and dangerous outbreaks, this topic is discussed in more detail in the following introductory chapter.

1.2 Prevention, Surveillance and Control

Given the potential danger of disease outbreaks to animal, human and public health, the question is how to detect, monitor, control and prevent outbreaks in farm animal populations.

To avoid the impact that a disease outbreak can have, the best method is to avoid the disease in the first place. This leads to the principle of prevention, which sees its main task as reducing the overall risk of a virus spreading. Corresponding measures are vaccinations and hygiene standards. For viral material that reassorts over time as the number of infections increases, the potential for a virus to exploit host cell genes that favour viral growth and survival may be high [26]. Other disease prevention practises include disinfection and good animal husbandry. Practitioners in the field or in veterinary clinics are obliged to follow this principle of prevention. In-depth strategies to prevent viral diseases depend heavily on the characteristics of the virus, taking into account transmission modes, environmental stability, zoonotic risk and pathogenesis. Exclusion of infected livestock and vaccination of potentially infected flocks is increasingly practised worldwide [26]. The spatial spread of disease can be contained through quarantine, separation from wildlife populations, testing and regular inspections of imported animals.

Surveillance of viral diseases involves the collection of basic information about the disease, including incidence, prevalence and transmission patterns; the systematic and regular collection and analysis of these data is crucial to obtain a detailed overview of the spread. This need for data has led the WOAH to publish the above-mentioned list of notifiable diseases. Based on the data collected, authorities can inform their decisions on the allocation of resources for disease control and other containment

activities [26, 27].

Common methods for animal diseases surveillance include notifiable diseases reporting, laboratory-based surveillance and population-based surveillance. General awareness among veterinary diagnosticians and practitioners is another key to an effective surveillance system. Most countries have their own national veterinary authorities, coordinated by the WOAH to enable a coordinated exchange of information [27]. Since efforts in tackling viral disease outbreaks or mass vaccination are very expensive, official budgets from the governments are needed. This makes it a political responsibility to prevent and control animal diseases.

One important component of modern and accurate surveillance systems of viral diseases is the access to relevant data. Technologies to produce Deoxyribonucleic Acid (DNA) sequencing data have developed to be very cost and time efficient which makes the study of infectious diseases better and faster. At the same time, the amount of DNA sequencing data produced with Next-Generation Sequencing (NGS), also known as High-Throughput Sequencing (HTS) platforms prove this change. NGS platforms include IonTorrent, Illumina and Oxford Nanopore Technologies (ONT). Advances in the biotechnological application and evaluation of these data are revolutionalising the field on the molecular level [28]. Sequencing technologies take a key role in describing viral diversity in humans and animals, in detecting pathogens and co-infections, in epidemiologic research about the evolution of viral material and in metagenomic characterisation of new microbial material. This is done by constructing the complete genetic information of a virus, the genome, where the nucleic acids store this information in single or double strands in a linear or circular sequence. With NGS methods, the genome sequence can be precisely determined. More detailed methods that are used for viral animal disease surveillance with NGS-based technologies are described in Chapter 2.

1.3 Motivation and Objectives of the Thesis

Bioinformatics and data analysis are crucial for understanding and monitoring viral diseases. However, there is a lack of knowledge and resources in many parts of the world. This is particularly true for poorer countries with small laboratories and national health organisations that are not well equipped with modern sequencers and surveillance systems. Additionally, transporting clinical samples across international borders is difficult and expensive. Nonetheless, efforts are made to establish global networks such as the Zoonotic Disease Integrated Action (ZODIAC). It is an initiative by the International Atomic Energy Agency (IAEA), launched in 2021, with five major objectives: (1) Strenghthening member states' detection, diagnostic and monitoring capabilities, (2) Developing and making novel technologies available for the detection and monitoring of zoonotic diseases, (3) Making real-time decision-making support tools available for timely interventions, (4) Understanding the impact of zoonotic diseases on human health and (5) Providing access to an agency coordinated response for zoonotic diseases [29]. In collaboration with technical experts from different fields and from all over the world, and to support the Veterinary Diagnostic Laboratory (VETLAB) Network, the ZODIAC project has the resources to provide standardised, easy-access, public and integrated pipelines for virus surveillance on a long-term. This will enable laboratories and veterinarians to monitor and analyse their samples more effectively, leading to early detection and prevention of viral livestock diseases.

Due to the outstanding research efforts brought about by the COVID-19 pandemic, analysis pipelines for SARS-CoV-2 samples were developed on the Galaxy platform. Galaxy and the implementation of pipelines are discussed in more detail in Chapter 3. Using the knowledge and application of SARS-CoV-2and transferring it to other viruses will lead to a more comprehensive understanding of viral diseases and better prevention strategies.

This work is part of the ZODIAC project and supports pillar (2) in the develop-

ment of integrated pipelines that enable laboratories, veterinarians and other health professionals to analyse their data from samples obtained with HTS technologies. The zoonoses studied are avian influenza A for subtype identification and a poxvirus pipeline for determining poxvirus genomes sequenced as half-genomes in a tiled-amplicon approach. The poxvirus pipeline has been tested with samples of lumpy skin disease virus. These two viruses have been chosen for the availability of test samples that were used for validation of the pipelines and for their relevance concerning animal and public health risk.

In summary, the lack of bioinformatics knowledge and resources in poorer countries poses a major challenge to effective, globally integrated viral animal diseases surveillance systems. However, established global networks such as ZODIAC together with VETLAB can provide the necessary resources to enable effective surveillance and analysis of viral animal diseases. This in turn will lead to early detection, insights into transmission routes and changes of the virus, prevention of disease outbreaks and ultimately protect public health and reduce the impact of viral diseases on livestock.

2 State-of-the-Art

In the demand for an effective, high-quality approach to the analysis of isolates from infected animals, molecular studies help to investigate characteristics of the sample. Genome analysis has become an integral part of animal disease surveillance, especially since the advent of high-throughput sequencing technologies in the last 15 years. Next-generation techniques and applications are described below, the state of the art in poxvirus and avian influenza virus detection and analysis, and lastly the drawbacks of the methods discussed.

2.1 High-throughput Technologies in Genomics and Virology

When comparing DNA sequencing technologies, there are differences in speed, throughput and volume of sequences. The term ”next-generation” in NGS used to describe newer technologies in the field implies a next step in the evolution of sequencing technologies. As sequencing machine technologies evolve rapidly, there are gradations such as ”second-generation” and ”third-generation”. Following the original 1977 Sanger sequencing method using radioactivity and gels, second-generation sequencers are advancements of Sanger sequencing that uses sequencing by synthesis [30]. In second-generation methods, reactions run in parallel and drastically reduce overall costs compared to Sanger sequencing. They produce short sequence reads length and are able to detect reads without using electrophoresis. Reads are equal to single fragments of DNA or Ribonucleic Acid (RNA). Third-generation sequencing

technologies typically generate longer primary reads of DNA (and RNA) molecules while maintaining the massive parallelism of the technology and taking advantage of this benefit [31]. The nowadays most commonly used next-generation technologies for DNA sequencing and their applications are described below.

2.1.1 NGS Platforms and Applications

By far the biggest player in the field of DNA sequencing is the Illumina platform, first developed by Solexa and Lync Therapeutics [32]. Illumina sequencing is based on bridge amplification, which creates clusters of copies of each DNA fragment. This technique involves repeated synthesis reactions with proprietary modified nucleotides containing a different fluorescent label for each of the four bases A, T, C and G. The reactions are performed over 300 or more rounds, and fluorescent detection allows for faster detection through direct imaging. An Illumina sequencer outputs data in the form of sequence reads, which are short DNA fragments ranging from 50 to 600 base pairs in length depending on the specific instrument and protocol used [32, 31, 30]. The output data from an Illumina sequencer typically is in the form of raw sequence files in FASTQ format, which contain the base calls and corresponding quality scores for each read. These reads can be used for downstream analyses such as viral genome assembly and variant calling.

ONT is a third-generation paradigm shifting sequencing technology. It measures changes in ionic current across membranes as single-stranded DNA nucleotides pass through a nanopore [33]. Nanopore-based DNA sequencing technologies are purchasable as a portable, small MinION (by ONT) device, allowing experts to use it for applications where space requirements or portability are important [34, 33]. The cyclic mode of sequencing used in second-generation approaches is replaced by sequencing in real-time with read lengths of up to 10,000 basepairs [33]. Despite its advantages, the main caveat of ONT is its relatively high error rate compared to

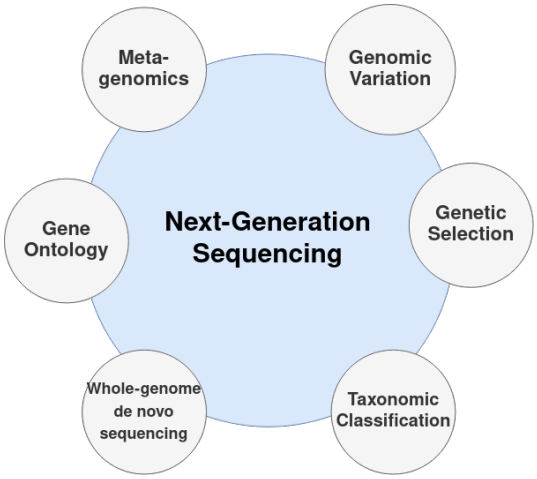

Figure 1: Overview of next-generation sequencing technology applications in virology.

other HTS methods [35]. This makes ONT less suitable for single-nucleotide variant analysis that is required in some diagnostic applications [36, 37].

Other frequently used second-generation platforms are Roche/454 sequencing, Ion Torrent (Thermo Fisher) technology and SOLiD (Sequencing by Oligonucleotide Ligation and Detection). Third-generation platforms include Single Molecule Real-Time Sequencing (SMRT) by PacBio and nanopore sequencing [38].

As NGS platforms are widely used in biomedical and clinical contexts, some of the most important applications in diagnostic virology are depicted in Figure 1. In virology, metagenomics can be used to identify viruses in complex clinical samples [39]. It allows for the detection of known and novel viruses without prior knowledge of the infectious agent. Metagenomics involves the sequencing of all genetic material in a sample, including viral genomes, to identify the presence of viruses. Once a virus is identified, genomic variation refers to differences in the DNA sequence of a virus between different strains or isolates. These variants can be used for tracking the spread of an outbreak, identification of sources of an infection, or determination of the level of virus virulence [40]. Variant detection is only possible with NGS data, as they provide insight to the genome on a nearly every-base level and allow to reliably

interpret and identify the many different possible variants [41].

Genetic selection describes to the process by which certain viral strains become more prevalent in a population over time due to selective pressures. In diagnostic virology, genetic selection is used to track the evolution of a virus in the course of time and determine which strains are most likely to cause outbreaks or epidemics. This is of special interest in the backtracing of infected animals to know where the virus came from. Using gene ontology, functions and interactions of genes are described. This is crucial to identify the genes responsible for specific viral functions and to understand how these functions contribute to viral pathogenesis.

Based on their genetic and structural characteristics, viruses are classified to existing systems, called taxonomic classification. This clustering analysis can be used for the type identification of a virus causing infections and determination of its potential for transmission and pathogenicity [42].

Whole-genome de novo assembly is the reconstruction of an entire viral genome without prior knowledge of its genetic sequence, being a costly and time-intensive method with potentially high error rates. Similar to metagenomics, this technique can be used to identify novel viruses, to study mutations in viral genomes and to track the evolution of a virus over time [31].

2.1.2 Detecting Viral Pathogens and Viral Genome Construction

For NGS methods to be a viable tool in diagnosis and analysis of viral animal diseases, the methods must be efficient and reliable. Almost all downstream analyses depend on the data obtained by sequencing, hence it is imperative to choose the most appropriate method for each application. Metagenomic-based approaches use whole-genome sequencing to characterise viral diversity in animal, human and environmental samples.

The reconstruction from HTS-generated reads to assemble the full-length genome can be made using different approaches, depending on the preparation of the NGS data.

During sequencing, Polymerase Chain Reaction (PCR) amplification is a widely used technique to amplify specific regions of nucleic acids by producign many copies of the targetted sequence. PCR can be used to sequence for example specific genes in a viral metagenomic sample. Another approach, developed by the ARTIC network is based on amplicons, i.e. fragments of the genetic sequence that cover the whole genome and are then sequenced on an Illumina platform. Amplicons are generated by tiled primers that start a PCR to generate the amplicons. For each amplicon, two primers for each end of the region of interest are needed, hence the expression tiled or tiling primers. The distance between the primers determines the size of the produced amplicon **TODO: sources**.

The detection of rare and novel infectious pathogens and the study of mutations in the genome are crucial for developing a deeper understanding of livestock viromes and potential zoonotic agents. In addition, it has been shown that NGS data provide a genome resolution high enough with low error rates to detect non-culturable organisms as well as co-infections that have not been detected using traditional microbiological approaches [43]. Metagenome sequencing often relies on a low number of pathogenic reads to detect and to make diagnostic calls. As sequencing depth directly influences genome coverage that can be obtained, the optimal amount of data to cover the complete genome is necessary. It has been shown that for a full virus genome to be represented, NGS data generated from ribo-depleted total RNA with a minimum length of one million high-quality reads works best [44]. Nevertheless, validation pipelines and confirmatory tests are needed for NGS approaches to pathogen detection and genome construction [45].

2.1.3 Data Analysis Issues

Since the surveillance of viral animal diseases with NGS is advancing rapidly, it is important that regions and health organisations that experience high damage of viral outbreaks but do not have their own facilities and know-how have access to

the needed tools and knowledge. Costs for NGS sequencers are still high and the access to appropriate laboratories is not given everywhere. Networks like VETLAB and standardisation of techniques, for example freely available and published by the WOAH, can enable professionals worldwide independent of their equipment on site. In the scope of the ZODIAC project, this aspect is addressed by providing protocols for each step from taking samples of potentially infected animals to the detailed analysis and derived actions [29].

NGS methods themselves have downsides that need to be considered when applying these techniques. Generally, chimerical sequences are formed during sequencing, which may be interpreted as false positives for novel organisms. Chimeric products are artifacts originating from joining sequences and are represented by point mutations, insertions and deletions. Chimera formation also occurs during PCR amplification [46].
During bioinformatics analysis steps using algorithms with computationally expensive steps, the choice of the algorithm as well as its configuration settings have huge impact on the final results obtained. This includes algorithms in steps such as filtering for quality, clustering and sequence classification [47]. The cleaning step or filtering phase eliminates low-quality reads from the dataset, whereas the error correction process distinguishes true variants from those caused by experimental noise. This is based on the concept that errors occur randomly with low frequency, while true mutations tend to be clustered, and their frequency can be measured [48]. Longer reads avoid this problem because contigs must not be assembled in the first place, avoiding clustering and filtering errors. This is why the shift in third-generation and later sequencing platforms is towards longer reads again. Due to the relatively high error rates of HTS technologies, that base on the sequencing process itself, PCR amplification of the viral material, and reverse transcription of viral RNA to Coding Deoxyribonucleic Acid (cDNA), it is crucial to include quality checks and filtering steps when using the HTS data [49].
Each application of software with NGS data requires expertise in resolving limitations

and drawbacks of specific methods. This in turn requires skills and experience in the field and the careful interpretation of results. Still, NGS provides a large pool of methods which eases this task, although available algorithms for genome assembly and amplicon analysis have drawbacks and limitations [50].

2.2 Tools for Genomic Analysis with NGS Data

TODO

A variety of suites and software packages is available to process NGS-generated data. Depending on the user's reasearch interest and procedures, tools are used independently or subsequently. Pursuing the goal to construct the full-length genome from short NGS-sequenced raw reads in FASTQ format, the text-based format to represent nucleotide sequences with quality scores for each base serves as the primary input for any analysis steps. For the central steps of the bioinformatics pipelines described in Section 2.3.2, Section 2.4.2, Section 2.6 and importantly the newly designed pipelines in Section 3.3.1 and Section 3.3.2 **TODO: add FMDV?**, tools and software suites are presented in the following.

Preprocessing – fastp, Trimmomatic

fastp is 2-5 times faster than Trimmomatic or Cutadapt
Trimmomatic requires separate file with adapters

Classification of Reads – VAPOR, BLASTn

[51] [52]

Reference-based Alignment – BWA-MEM

minimap2 for ONT/PacBio/Illumina
for >100bp length Illumina reads, minimap2 is 3x faster as BWA-MEM/Bowtie2 (same accuracy)
Bowtie2 for higher coverage contigs?
[53]

De novo Assembler – SPAdes

[54]

Consensus Calling – iVar consensus

[55]

Phylogenetic Tree Construction – IQ-Tree

[56]

2.3 Poxvirus Analysis

Among the family of poxviruses, there are some diseases that circulate in livestock and pose a risk so that they are on the list of notifiable animal diseases. Among others, monkeypox, sheeppox and goat pox are the diseases of concern. In the following, characteristics of poxviruses and current approaches to analyse NGS data of poxviruses are described.

2.3.1 Poxviruses

Throughout human history, poxviruses have played a significant role with variola being the most notorious as it is the causative agent of smallpox. Smallpox has been described in Chinese texts dating back to the 4th Century AD, and evidence of pox-like scars found on Egyptian mummies suggests the disease may have existed as far back as the 2nd millennium BC [57]. The discovery of a vaccine for smallpox made it the first disease to be eradicated by human efforts, and variola was the first human virus to be successfully eliminated [58]. Modern vaccinology owes its origins to Edward Jenner's discovery in the late 18th century that zoonotic infections with the "cowpox virus" provided immunity to smallpox [57]. Furthermore, vaccinia virus, which is now used for smallpox vaccination, was the first animal virus to be observed using electron microscopy and the first to be utilized as a vector for transporting foreign genes into animals. This is why poxviruses are among the best-known viruses. The family of poxviruses, *Poxviridae*, is a family of double-stranded DNA viruses. Its natural hosts are vertebrates and arthropods and there are currently 83 species within 22 genera in this family. The family is divided into two subfamilies, *Entemopoxvirinae* (insect-infecting viruses) and *Chordopoxvirinae* (vertebrate-infecting viruses).
Historically, poxviruses were classified based on disease symptoms and the animal species that was infected. Humans, cows, sheep, goats, horses and pigs have been studied to determine not only clinical symptoms but with the aim to classify poxviruses. This genus classisification has been confirmed by recent comparative genome analysis [59]. Symptoms of disease caused by a poxvirus infection are skin lesions that can differ in size. Depending on the type of poxvirus, the papules can vary from small and pearly papules in infections of Lumpy Skin Disease Virus (LSDV) to larger crusts and spread generalised pustules in infections with the variola virus. Other general symptoms include fever, headache and rash.

Table 1 shows ten representatives of the 18 Chordopoxvirus genera according to the newest International Committee on Taxonomy of Viruses (ICTV) Taxonomy Release

Genus	Virus Species	Natural Hosts
Avipoxvirus	Canarypox virus	Songbirds
	Fowlpox virus	Chickens, turkeys
Capripoxvirus	Sheep pox virus	Sheep
	Lumpy skin disease virus	Cattle
Centapoxvirus	Yokapox virus[1]	Humans, mosquitoes
Cervidpoxvirus	Deerpox virus	Deer
Crocodylidpoxvirus	Crocodilepox virus	Crocodiles
Leporipoxvirus	Myxoma virus	Rabbits, hares
Molluscipoxvirus	Molluscum contagiosum virus[1]	Humans, primates, birds, dogs
Orthopoxvirus	Variola virus (Smallpox)	Humans (eradicated)
	Mpox virus[1]	Humans, primates
	Cowpox virus[1]	Humans, cats, cows, elephants
	Vaccinia virus[1]	Humans, cattle, buffalos, rabbits
	Camelpox virus	Camels
Parapoxvirus	Pseudocowpox virus[1]	Humans, cattle
	Orf virus[1]	Humans, sheep, goats, etc.
Suipoxvirus	Swinepox virus	Pigs
Yatapoxvirus	Yaba monkey tumour virus[1]	Humans, rhesus monkeys

[1] Zoonotic disease

Table 1: Representative viruses from ten Chordopoxvirus genera.

from 2021, while at least five genera contain zoonotic poxviruses [60]. Orthopoxviruses have the biggest impact on human and animal health, and are remarkable for their broad host spectrum ranging from humans to wild and domestic animals [58]. The Chordopoxvirus subfamily is characterised by its large, linear double-stranded genome. Size varies between 134 to 365 kilobases [61, 62]. Chordopoxvirus genomes contain 130 to 328 Open Reading Frames (ORFs), and typically, two identical Inverted Terminal Repeats (ITRs) are located at both ends of poxvirus genomes.
Vaccination is available for smallpox, and the vaccine is even considered protective

against symptoms of all orthopoxvirus infections. It is recommended for laboratory staff that works with mpox, cowpox, vaccinia and variola [63]. For animals, there is a smallpox-based vaccine that is used to protect elephants against cowpox [64]. Sheep and goats are broadly vaccinated with an orf vaccine, which is, similar to smallpox vaccine, a live virus. The effective vaccination against existing poxvirus diseases and further microbiological studies, as well as similarities between poxviruses, motivate the expansion of existing data analysis pipelines that work for a specific poxvirus so that they can also work with other poxviruses.

Lumpy Skin Disease Virus

Lumpy Skin Disease is caused by the lumpy skin disease virus belonging to the *Capripoxvirus* (CaPV) genus within the family of poxviruses, subfamily *Chordopoxvirinae* [65]. The Lumpy Skin Disease (LSD) virus genome is a double-stranded linear DNA molecule of circa 151 kilobasepairs in length. It contains between 147 and 156 open reading frames. Similar to other poxviruses, the LSDV genome consists of a central coding region which is bounded by two identical ITR regions with a length of circa 2,400 basepairs at both ends of the genome. This is a key characteristic to consider during reconstruction of the genome. With a sequence identity of over 96% with the other CaPV genus members sheep pox and goatpox, the LSDV genome is highly similar to the other CaPV genomes [66].

LSDV is not known to be transmissiable to humans and therefore not a zoonosis. Natural hosts of LSDV are cattle and Asian water buffalos. Although CaPV is considered to be host specific, sheep pox and goatpox strains can naturally cross-infect in both host species. There have been no cases of natural infection of sheep or goats with LSDV reported [67]. The three CaPV viruses are the most serious poxvirus diseases of livestock in terms of economic losses in the case of an outbreak.

Cattle infected with the LSDV typically show symptoms like fever, reduced feed and water uptake and characteristic skin nodules. The number of lesions varies from

a few to many, covering the whole body [68]. From these symptoms alone, it is impossible to differentiate the diagnosis between sheep pox, goatpox and lumpy skin disease. Even with classical methods like cell culture and electron microscopy the highly similar viruses cannot be distinguished. Nowadays, PCR and sequencing are the techniques used to provide the sensitive detection of CaPV [69].

LSDV has spread from the African continent and since 2019 reached major cattle producer countries in Asia, mainly India, Republic of China and Bangladesh. Other bigger outbreaks in south-west Europe were reported in 2014 to 2018, although these countries opted for a strict vaccination program and successfully eliminated LSDV from the region [70]. In African and Asian countries, veterinarians struggle to fight endemic LSDV outbreaks because of a lacking financial support by governments, justified by low mortality and morbidity rates.

One strain of LSDV that has been extensively studied is the Neethling strain, first isolated in Kenya in 1958. It constitutes the strain used for the live attenuated vaccine that is widely used, if accessible, for cattle against LSDV outbreaks. Some countries use sheep pox vaccines to protect cattle against LSD, even though it does not bring complete immunity. Nevertheless they are used in regions where all CaPV are prevalent [71].

"More recently a novel strain of LSDV was discovered in Russia (`https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0207480`). The LSDV Saratov (2017) strain appears to have been formed by recombination between field and vaccine strains, something Gershon and his colleagues had predicted many years ago given the close similarities between Capripoxviruses." (`https://www.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-70-2-485`, `https://onlinelibrary.wiley.com/doi/pdf/10.1111/tbed.13322?casa_token=BkNNRCE42tMAAAAA:aYmyJUcWrmTz7nSFFF3OsTChokhrdtazx0USNUGP3PCkNURQSfHJzA3bb1eGcUK76sN`

2.3.2 Pipelines for Genomic Analysis with Poxvirus NGS Data

The need for rapid identification of a virus sample to distinguish between species of poxviruses requires sensitive analysis of NGS data. Challenges in alignment against a reference are the identical ITRs at both ends of Capripoxviruses, which is omitted from many pipelines and not part of the analysis, as well as the high identity of 96-97% between the three Capripoxviruses. In order to reach a sufficiently high coverage in all parts of the genome, the reference and the reads can be split into two parts to map against the identical ITR regions. With a tiling approach, there is no ambiguity in where to map a read from the ITRs to. However, the reads have to be sequenced in two pools, which is not a standard protocol. These challenges make it difficult to differentiate between LSDV, goatpox and sheeppox [66].

A Whole-Genome Sequencing (WGS) approach to distinguish capripoxviruses is described by Mathijs et al. [72]. They develop a sequencing protocol in two pools to separate the ITR regions. After pre-processing, the pools of reads are de novo assembled with SPAdes and the resulting contigs of each pool are merged into a single contig. To find the correct merging location, an overlap of one amplicon in the middle is assembled in both pools. The test results with various samples show that this approach reconstructs nearly complete CaPV genomes. The presented tiling amplicon approach is not usable as an automated pipeline, but can be implemented using the tool specifications in the article. Other viral genomes have been examined in a similar tiling amplicon approach with Illumina, ONT or PacBio sequenced data [73, 74, 55, 75].

A pipeline of Zhao et al. was designed to study the whole genome of monkeypox samples [76]. After the de novo assembly step, a neural network method is used for smart gap filling between the assembled contigs. The method shows that gap filling of a genome is an *all k shortest path* (KSP) problem and can be used in an automated pipeline from HTS reads to the whole genome sequence. They conclude that it is a promising method to find the ”correct” sequence but it did not find

the correct sequence assembly for five cases in a sample sequence of monkeypox. Therefore, this method can be used as a guiding first-shot feature, but should not be used for sensitive analyses. Also, the neural-KSP method requires knowledge in how to finetune the pipeline parameters.
Other methods to detect the species of capripoxvirus of a given sample is nucleic acid extraction and real-time PCR [77]. This approach is based on the presence of specific genes to distinguish between capripoxviruses, but since it does not work with NGS data, it does not allow for more analyses and is not comparable to the previous methods.

2.4 Avian Influenza Virus Analysis

NGS-based sequencing data from AIV samples need profound processing to gain insights into the subtype and variants within the sequence. In the following, the causative agent for avian influenza, avian influenza virus, is described in detail and state-of-the-art methods in the form of automated pipelines for the analysis of such data are presented.

2.4.1 Avian Influenza Virus

Informally known as bird flu, avian influenza is a viral infectious disease that affects wild birds and poultry. The AIV has occasionally crossed the species barrier and infects mammals, including humans. This makes it a high-priority zoonotic viral disease that has been designated as notifiable by WHO and WOAH [25]. Avian influenza occurs in two variants that determine severity: Low Pathogenic Avian Influenza (LPAI) and HPAI, with only HPAI cases requiring notification. The virus spreads indirectly via contaminated material, e.g. feed, water supplies, feces or feathers. It is transmitted directly from bird to bird via the air, mainly through

the transregional movement of wild birds and through long distance bird migration. Humans become infected through close contact with infected livestock or wild birds, and most reported human avian influenza infections are from farm workers and others who are exposed in markets, production or clinical contexts [14].
Symptoms of severe illness are characterised by influenza-like signs such as fever, nasal discharge, coughing and conjunctivitis. This applies to infections in both human and mammals, while infected birds show signs such as swollen heads, loss of appetite, breathing difficulties and a decrease in egg production.

AIV contains a negative-sense, single-stranded segmented RNA genome, and due to the segmented nature of the virus, co-infection of different influenza strains can lead to reassortment events. Avian influenza viruses are members of the *Orthomyxoviridae* family and the four species of influenza viruses A, B, C and D are distinguished on the basis of the presence of the Nucleoprotein (NP) and matrix (M1) proteins [14]. AIV subtypes are determined by the Hemagglutinin (HA) and Neuraminidase (NA) segments, which include all known influenza A virus subtypes H1-H16 in combination with N1-N11, resulting in subtype designations such as H5N1 or H7N9 [14, 78]. To be infectious, a virus particle must contain one of eleven proteins in each of the eight unique segments PB2 (poymerase), PB1/PB1-F2 (polymerase), PA/PA-X (polymerase), HA, NP, NA, M1/M2 and NS1/NEP (distinct non-structural proteins). Mutations in the HA and NA genes occur relatively frequently due to the prone-error RNA polymerase in the viral genome which lacks the proof-reading exonuclease activity. LPAI subtypes H5 and H7 usually infect poultry, although the natural hosts of avian influenza A are wild waterfowl. These subtypes can transform into HPAI during circulation in poultry stocks by recombination with other gene segments or the host genome [79]. Both LPAI and HPAI infections have been reported in domestic poultry, i.e. ducks and chickens, turkeys, caged birds, aquatic birds and wild birds. As the different influenza species can infect different animal hosts, all of them can infect pigs and humans.
Influenza A strains are the most virulent virus species, and have caused all major

historic flu outbreaks through reassortment. Subtypes H5, H7 and H9 are responsible for the largest outbreaks of AIV that also spread to humans [80]. The first confirmed report of human infection with an animal avian influenza virus dates to 1958, and since then 16 subtypes have been detected in humans [81]. Zoonotic spillover events have becomeincreasingly common since the early 20th century and have led to major endemics such as a huge H5 outbreak in the U.S. in 2014-2015. It resulted in more than 25 million bird deaths [82]. Another current outbreak, resulting in more than 58 million dead birds and costs of around 661 million U.S. dollars began in 2022 and is spreading across the U.S. [83]. Vaccination against HPAI in poultry are used worldwide to ward off avian influenza. They also serve as a preventive measure in the event of an outbreak to reduce the risk of introducing the virus into poultry populations [84, 85].

2.4.2 Pipelines for Genomic Analysis with Avian Influenza Virus NGS Data

Surveillance systems in the field of genotyping emerging viral strains include classical phylogenetic methods far classifying viral strains, assessing tree topologies, distinguishing between novel and emerging strains, and discovering novel disease-causing variants [41]. These analyses are essential given the high genetic variability of the genome, and since it consists of eight segments, specific bioinformatics workflows are required for the analysis.

The challenge in identifying subtypes and detecting variants lies in the diversity of HA and NA genes, the main targets of the host immune response. The HA and NA genes have evolved into several subfamilies and require a dynamic reference selection approach for sequencing analysis. There are a growing number of web platforms, suites and pipelines that enable the analysis of influenza-specific samples with NGS data and resources for further analysis, e.g. Influenza Research Database/Fludb [86], EpiFLU/GISAID [87], Nextflu [88], NCBI Influenza Virus Resource [89], FluNet [90]

and OpenFluDB [91]. Many existing suites for automated analysis of influenza samples are based on SARS-CoV-2 research and have been adapted for the similarly large influenza genome. "INSide the FLU" (INSaFLU) and Prediction of Avian Influenza Virus Subtype (PAIVS) are two pipelines specifically designed for the analysis of NGS-generated (avian) influenza samples and are discussed in more detail below.

INSaFLU

One prominent pipeline for viral metagenomic detection and routine genomic surveillance, INSaFLU, provides a web-based protocol for data generated by Illumina, Ion Torrent or ONT sequencers [92]. It is the first influenza-focused suite to process NGS data to automatically generate output data and answer key questions in influenza genomic surveillance. These include type and subtype identification, reference-based mapping, consensus sequence generation, and phylogenetic tree construction. The INSaFLU pipeline consists of steps that cover some of the objectives in parallel: (1) Reads quality analysis and improvement, (2a) classification, (2b) mutation detection and consensus generation, (3a) intra-host minor variant detection, (3b) alignment/phylogeny and (3c) coverage analysis. Using the output data of step (3b), a downstream integrative phylogenetic and geotemporal analysis with Nextstrain can be started. A reference sequence for the mapping step must be provided as input data from the beginning. Currently, INSaFLU is accepting NGS data from influenza, SARS-CoV-2 and monkeypox samples [92]. The INSaFLU pipeline is installed locally via the command-line on any server instance, which requires technical knowledge to set up, but can also be used via the website. The pipeline steps cannot be customised via the web interface, instead general configurations can be set at the beginning. The pipeline is constantly being developed to integrate new features and modules.

PAIVS

PAIVS (Prediction of Avian Influenza Virus Subtype) is a pipeline specifically designed for avian influenza virus samples. It consists of five steps: (1) pre-processing, (2a) reference-based alignment or (2b) de novo assembly, (3) subtyping, (4) variant calling and identification of the closest sequences by (5) Basic Local Alignment Search Tool (BLAST) for nucleotides [93]. PAIVS uses a similar approach to INSaFLU, but leaves it up to the user to decide whether to include a de novo assembly step. The results are presented in a downloadable format for the user and include a graphical summary. The pipeline is written in Python and is freely available on `http://ircgp.com/paivs`, being a web-based platform only available in Korean [93]. This is a very limiting factor for the usability of PAIVS.

2.5 Foot-and-Mouth Disease Virus Analysis

2.5.1 Foot-and-Mouth Disease Virus

Cloven hoofed animals, small positive-sense ssRNA virus (8.3kb) Aphthovirus genus, Picomaviridae family, 7 distinct serotypes (with different subtypes each), high heterogenity of the virus in the host populations –> WGS needed for accurate variant calling

2.5.2 Pipelines for Genomic Analysis with Foot-and-Mouth Disease Virus NGS Data

* `https://academic.oup.com/bib/article/21/5/1766/5565040?login=true&casa_token=1DxsiOURgvsAAAAA:m9Fyy4W5xLE-6y2jMvn8EkQHIayhjrgXZJ3sSTNnTgx61D6TLP3MhmZIIRO-k`
alignment with Bowtie2

* ONT serotyping: https://www.frontiersin.org/articles/10.3389/fvets.2021.656256/full and mapping, consensus sequence generation, BLASTn, But: many SNPs found (due to high error rates of 5-10% in MinION sequencer)

* https://assets.researchsquare.com/files/rs-2396402/v1/7ab80566-9e56-40a3-8117-07191dbcf2a9.pdf?c=1672243503 mapping with Bowtie2, samtools, variants calling (Mutect2), SnpEff for annotation, phylogenetic analysis. GATK4 pipeline

2.6 General Pipelines for Genomic Analysis with Viral NGS Data

In the following, pipelines are presented that can be used with unspecified or unknown virus data. They cover some general parts of the previously mentioned pipelines but focus mainly on virus discovery, assembly and consensus sequence generation.
ViReflow is a pipeline for viral consensus sequence generation and provides a mapping-based approach to variant calling and many optional downstream analyses such as de novo assembly and lineage assignment [94]. The pipeline is based on the Reflow suite, and all computations run in an Amazon Web Services, Inc. (AWS) container in a cloud. Reflow emphasises versioning, testing and workflow sharing and does not provide a user-friendly web interface. Instead, it is accessible via a command-line interface. As a result, it may not be as easy to use as Galaxy and its workflows, including workflow development, as this requires programming in Go language. Similar to other pipelines, ViReflow was originally created for the consensus genome construction of SARS-CoV-2 samples and has been extended for use with all viral genomes [94].
Another automated pipeline for viral genome assembly, lineage assignment, mutation and intra-host variant detection is V-Pipe, a computational pipeline assessing genetic diversity and introducing a new alignment method *ngshmmalign* specifically for small and highly diverse viral genomes. It includes local and global haplotype reconstruction

and a module for detection of flow cell cross-contamination [95]. Although V-Pipe is suitable for all viral genomes, it was tested for the identification of the eight influenza segments and successfully identified them from the test sample.

Other freely available pipelines for the analysis of viral genomes from NGS data with several focuses in genomics are VirFind [96] and Integrated Rapid Infectious Disease Analysis (IRIDA) [97]. These pipelines focus on rapid identification of viral materials and do not provide steps for detailed downstream analyses. Automated pipelines for metagenomic NGS data are detect and reconstruct known Viral genomes from Metagenome (drVM) and VirMAP [98, 99]. However, they do not consider the segmented influenza genome and do not provide output data for custom downstream analyses. To our knowledge, there is no freely available pipeline that uses a mapping-based approach that focuses on the viral segments of the AIV genome and uses the closest possible reference for each segment. For the various possible downstream analyses, depending on the specific research question, it is critical for a pipeline to provide data outputs and endpoints that enable user-specific assays. **TODO: concerning Pox?** A Galaxy workflow covering the above points has been developed in this thesis and is described in the following chapter.

3 Materials and Methods

TODO

3.1 Galaxy Platform

Galaxy is a web-based scientific platform that has become a major player in many fields of life sciences and bioinformatics. Founded in 2007 it has provided an emerging amount of resources and tools to empower scientists and researchers to work with biomedical datasets. The platform is free to use and collaborative, making it one of the biggest of its kind. Resources on Galaxy cover genomics, metagenomics, transcriptomics, proteomics, drug discovery and non-biology fields like natural language processing and social sciences.

Galaxy's primary objective is to make analyses more accessible, reproducible, and easier to communicate among researchers. The platform's distinctive and success is attributed to four core elements: a very active community, a public server for analyses, an open-source software ecosystem, and the Galaxy ToolShed. The community adheres to the FAIR practises (Findable, Accessible, Interoperable and Reusable) [100].

The Galaxy community is thriving, with over 124,000 users who also contribute to subcommunities. The public server for analyses provides access to public datasets and workflows. The open-source software ecosystem ensures automated setup and

deployment of all tools and services, making it simple for beginners and professionals to use. The Galaxy ToolShed is a server dedicated to hosting, sharing, and installing tools used on the platform. A Galaxy tool is the abstraction layer that makes external software usable from within Galaxy with a frontend, i.e. lets users use the program with all its parameters and inputs from within Galaxy.
Galaxy workflows are a key feature that allow the user to stack tools in a chain and to configure them so that the workflow user only has to upload his or her data for the input fields. The automation of tools in a chain is used for modular, longer analyses that are executed repeatedly.
Workflows that are available on and accepted by the Intergalactic Workflow Commission (IWC; `https://github.com/galaxyproject/iwc`) are conform with the community's best practise standards and tested on the latest Galaxy release. Dockstore and WorkflowHub automatically publish the IWC workflows and guarantee the availability in a Docker-based environment on Dockstore [101] and on the workflow collaboratory WorkflowHub [102].

Important contributions of Galaxy, as stated by the Galaxy Community (2022), include Vertebrate Genome Project assembly workflows and collaborations on SARS-CoV-2 research. Another toolkit leveraged in Galaxy is Galaxy-ML, a set of tool that provides a suite for analyses based on machine learning. With growing publicity, more topics are covered by and moved to Galaxy. It has contributed to over 5,700 scientific publications and has many tutorials available for researchers to use. Training material and ready-to-use workflows facilitate professionals and beginners in the field to use Galaxy for their research purposes.

The platform is continuously enhanced, and it still attracts around 2,000 new users every month, indicating the quality and significance of the project. The team and infrastructure of Galaxy initially come from the Nekrutenko lab in the Center for Comparative Genomics and Bioinformatics at Penn State, the Taylor lab at Johns Hopkins University, and the Goecks Lab at Oregon Health & Science University. All

of these organisations have contributed significantly to the success of Galaxy. There are 138 public servers available worldwide as of 2023, while the most prominent general-purpose server instances are hosted by teams at University of Freiburg, Germany (for UseGalaxy.eu), Texas Advanced Computing Center (for UseGalaxy.org) and Genomics Virtual Laboratory, formerly at the University of Queensland (for UseGalaxy.org.au). These main public servers are synchronised in their tools and set of reference tools [100].

3.2 Workfow Requirements

TODOwhich problems should the pipeline solve? For a detailed genetic analysis of the AIV genome, several consecutive steps are required after taking a sample from an infected host and sequencing it. NGS data need to be preprocessed in order to remove reads that are too short, have low base quality or include NGS platform-specific adapters that are ligated to the read ends and need to be trimmed.

what is ”ampliconic” sequence analysis? -> amplicon is the region of DNA/RNA of interest, using primers during PCR to bind to these regions (define the boundaries) at both ends. DNA polymerase enzymes extend the primers and synthesise new strands

ARTIC Illumina-sequenced data

SARS-CoV-2 Pipeline as Baseline.
(annotated) variants are of interest

tested workflow, includes minimal steps: * Quality control
* Mapping
* Filtering
* Trimming

* Consensus Sequence Construction

Plus Variant Calling and genome annotation;

Plus phylogenetic ranking "to assign a SARS-CoV-2 genome sequence the most likely lineage based on a chosen nomenclature system" (Pangolin)

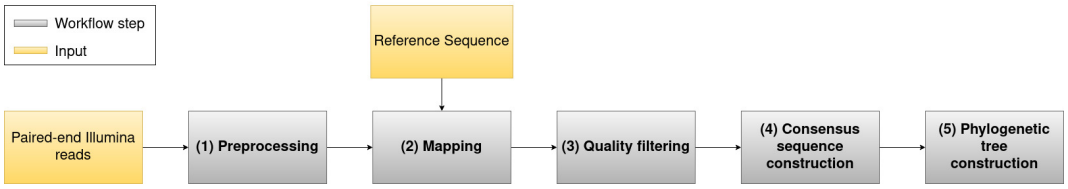

Figure 2: General workflow for the consensus genome construction and analysis of paired-end reads.

Requirements for Poxvirus Workflow

TODO: use ARTIC data As explained in Section 2.3, the genome of most poxviruses is bound by identical sequences located at the termini of the genome. It is shown that the size of such differs for some poxviruses, such as rabbitpox and vaccinia virus, while monkeypox, cowpox and capripoxviruses have shorter ITRs [103]. For a whole-genome reconstruction from HTS-generated reads, alignment algorithms look for the unambiguous location of a read. Since this is impossible for repeated sequences neither for reference-based mapping approaches nor de-novo assembly, a new approach has to be used that splits the sequencing reads into two parts, separating the identical sequences and running alignment algorithms for each of the splits. To build the full-length genome, the alignments need to be glued together. This approach requires the reads to be sequenced in two pools with two libraries. A similar protocol has been described by Mathijs et al. [72].

Other requirements for a reference-based surveillance of the genomics of poxviruses include the availability of the primer scheme that was used for amplicon-based

sequencing. The Browser Extensible Data (BED) file containing the primers, their positions and the pool identifier is essential for the correct linking of the alignments when splitting the pipeline into two parts and merging it back together.

Requirements for AIV Workflow

The main objectives of surveillance of AIV on the genetic level are to get phylogenetic insights and to check for new variants that could occur in the HA and NA proteins as a consequence of reassortment.

A pipeline for an avian influenza virus sample that should build a consensus sequence in order to check for mutations needs a reference sequence that it can compare the sequence to. The alignment step requires a reference sequence to map against. A main caveat of many existing pipelines is the user's choice of reference sequence, since it is an arbitrary choice and there are many different reference sequences to choose from. Therefore, a dynamic approach that is sensitive enough for the segmented structure of the AIV genome is needed. The diversity of HA and NA segments' sequences is significant enough to make it challenging to map sequenced reads to a single, full-length influenza A reference sequence. Although this approach may be effective for the other six segments, the mapping software would frequently be unable to locate sufficient plausible matches for sequenced reads of HA and NA origin to continue with the analysis. By using a splitting approach that finds the best reference sequence from a database, the expensive assembly is avoided and mapping can be conducted.

Compared to analyses with similarly large genomes such as SARS-CoV-2 and due to the segmented structure of the AIV genome, duplicates among the mapped reads of the AIV sample should not be dismissed but kept for maintaining a reasonable high coverage for the further analyses.

multisample, VAPOR, mapping (de novo assembly for control?)

3.3 Workflow Development

"Reference-based genomic Surveillance" (INSaFLU)

3.3.1 Poxvirus Illumina Amplicon Workflow

The proposed Galaxy workflow for poxvirus samples that were HTS-sequenced with a tiling amplicon approach is available on the WorkflowHub, Dockstore and Galaxy EU **TODO: links**. It aims at constructing the full genome from Illumina-sequenced reads and providing alignment files, the constructed consensus sequence and intermediate results and reports that give insights into reads and mapping quality and mapping coverage. A table with these outputs and the respective datatypes is provided in Supplementary Table 2.
To account for the repeated ITRs at the ends of the poxvirus genome, the workflow is based on a tiled-amplicon approach. During the first steps, the reads of the two pools from each genome half are treated individually. Input data for the workflow are the reads from pool1 and pool2, sourced from the sequencing with two libraries; the used primer scheme in BED file format that contains an indicator for pool1 or pool2 in the *SCORE* column, and a reference sequence that is used for mapping.

As a first step, (1) the provided reference sequence is prepared for the mapping of the two pools. Hence, the primer scheme is needed to compose the exact intervals so that the remaining bases are N-masked. For mapping pool1 against the reference, the second half is N-masked and therefore the interval for the remaining bases is built, while the second half of the reference sequence is N-masked. The masking

Figure 3: Simplified Poxvirus PE reads iVar-based workflow.

starts at the minimal start position of the first primer of pool2. Respectively for the mapping of pool2, the interval of the remaining bases is constructed by taking the maximal end position of the pool1 primers and the full length of the reference sequence so that the masking of the first half can be conducted.

The workflow is designed to process multiple samples in one run, thus the samples of both pools are sorted by the order of how they are listed in pool1. Before mapping, (2) the reads of both pools are preprocessed with `fastp` to trim the ends. The following (3) mapping takes the corresponding masked reference sequence for each genome-

half. A statistics report for each alignment is generated to check for the quality of the mapping. The alignments are (4) filtered for quality and the pool identifiers (*pool1/pool2*) are prepended to the sample names so that using external software to check variants, the pool and sample identification is maintained. The next step (5) joins the two alignments while retaining the identifiers for each sample and pool. For the mapping of the whole-genome, a coverage report is generated so that the ITRs and the part where the mappings are merged can be inspected. (6) Primer-trimming with `iVar trim` removes the loose primer ends and cleans the alignment for the consensus sequence construction. The (7) consensus sequence is called with `iVar consensus` for which the user can use either provided default settings (minimum quality score to count base: 20, minimum allele frequency threshold to call SNV: 0.7, minimum allele frequence to call indel: 0.8) or enter their own values before starting the workflow. The workflow with a complete list of the 47 steps, used tools and their versions as well as outputs and connections between the tools is provided in Supplementary Table 6.

3.3.2 AIV Illumina Reads Workflow

We propose a fully automated pipeline for the analysis of Illumina-sequenced paired-end reads from avian influenza samples. The workflow is integrated in the Galaxy platform and available via **TODO: link**. It is designed to take one input sample at a time and besides a summarising results report, the outputs of the analysis steps can be used for further research based on the user's interest. The workflow is outlined in Figure 4, where the nine main steps of the workflow are visualised. The full workflow of 48 steps with the tools, tool version and paramters can be found in Supplementary Table 6.

One novelty of the workflow is the consideration of the different segments of the influenza virus genome. After uploading paired-end reads and a reference sequence database, which is available online too **TODO: link**, the workflow builds a hybrid ref-

erence from the given database for each of the segments of the genome. The reference sequence database consists of eight FASTA files, one per segment, containing numerous full-length sequences for a given segment. The provided database file consists of 56 sequences for each segment. If a user decides to upload their own references, it is important to follow the sequence identifier pattern so that the extraction of sequence identifiers works: *>segment_name|influenza_strain|subtype|accession_number*. For example, one entry's identifier is *>PB1|A/duck/Manitoba/1953|A/H10N7|KF435047.1* followed by the sequence in the next line.

After (1) preprocessing of the reads with `fastp` to dismiss reads shorter than 30 basepairs and automatic trimming PolyG tails of the Illumina reads, the database of reference sequences is used to (2) find the closest possible reference for each of the segments. The tool `VAPOR` outputs a table with a scoring based on the graph construction, and should not be confused with the identity of the sequence compared to the reference. As `VAPOR` is running once per segment but has independent inputs, this step is executed in parallel. `VAPOR` is a graph-based classifier that maps k-mers to a weighted De Bruijn graph [51]. Its benchmarking shows that it runs significantly faster than BLAST and default configuration leads to reasonable matches similar to Mash, as long as the given sample is not very different from or novel to the provided sequences in the reference database.
Using the highest scoring sequences from the eight `VAPOR` runs, a hybrid reference sequence is built. To control the statistics of the graph and adapt the configuration, a table with the highest `VAPOR` scores of each run is generated.
The hybrid reference sequence is composed of the eight segments and is used for the third step of the pipeline, (3) mapping with `BWA-MEM`. The segment names in the hybrid reference genome are truncated and shortened to just the segment identifier. Mapping of the preprocessed reads against the prepared hybrid reference is run with default parameters of `BWA-MEM`. The Burrow-Wheeler Aligner for short-read alignment (BWA-MEM) algorithm aligns 70-1000 basepairs long reads by seeding alignments with maximal exact matches, and extending the seeds using the affine-gap

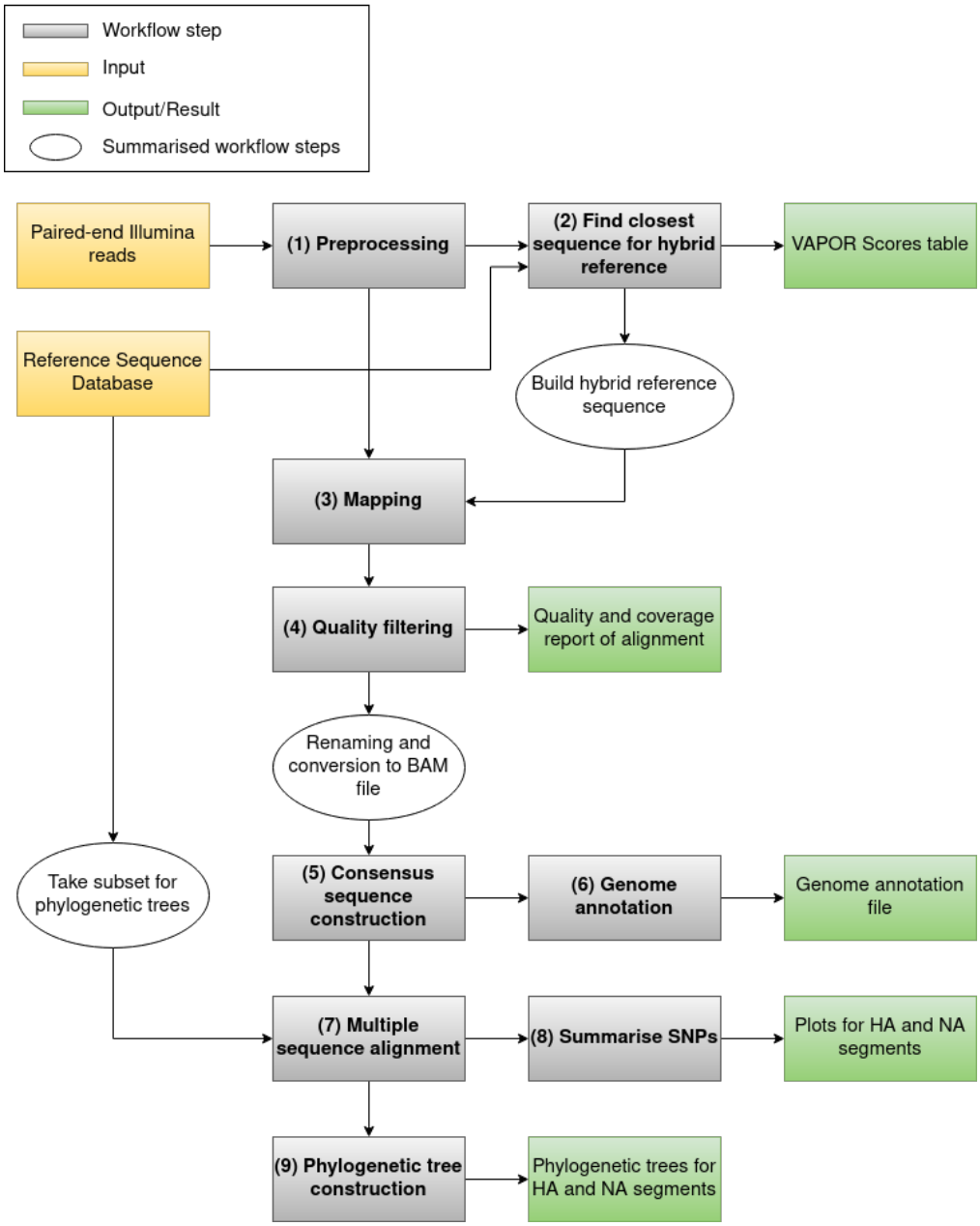

Figure 4: Simplified AIV workflow with the most important steps.

Smith-Waterman algorithm [53]. After mapping, the resulting Binary Alignment Map (BAM) dataset is (4) quality filtered using `Samtools view`. Reads with a minimum quality of 20 and those that are paired and mapped in a proper pair are

kept. The alignment and quality results as well as coverage statistics for each segment are reported using `QualiMap BamQC`.

The subsequent steps before generating the consensus sequence of the sample prepare the BAM file and deconstruct the mapped reads into a collection of eight datasets and relabel the elements, so that (5) `iVar consensus` can perform consensus sequence construction in parallel. Per-segment consensus construction is run with a minimum quality score threshold of 20, minimum frequency threshold of 0.7, minimum depth to call consensus of 10, does not exclude regions with smaller depth than the minimum threshold and uses N instead of - for regions with less than the minimum coverage. These settings accept any base as the consensus base for a genome position with a base calling quality of 20 or higher in order to avoid false bases that come from sequencing errors. If there is no consensus base to be found with the above thresholds, an N is inserted instead.

The next step using the consensus sequence is (6) generating genome annotation files with `Prokka`. As the input sample is a viral genome, the *Kingdom* parameter is set to *Viruses*. With this file, open reading frames can be predicted using web-tools and further downstream analyses can be started.

To place the consensus sequence of the avian influenza segments in a set of samples frm the reference sequences, (7) a multiple sequence alignment for a user-specified number of sequences (i.e. determines the size of the resulting phylogenetic trees) is conducted with `MAFFT` (Multiple Alignment using Fast Fourier Transform) and the consensus sequence is added using `MAFFT add`. The multiple sequence alignment is also used for (8) a visualisation of SNPs, produced with the `snipit` tool.

As a final step, (9) phylogenetic trees for the HA and NA segments are built using `IQ-Tree`. The taxonomy of the sample segments visualised in the phylogenetic trees give insight into spatial and temporal spread of the genome. The consensus sequence from the input sample is assigned to the most likely lineage [56].

The presented workflow avoids the computationally expensive de novo assembly, instead uses a mapping approach with a dynamically composed reference sequence

of close sequences for each of the eight influenza segmets. This accounts for a high quality mapping and is evaluated in Chapter 4.2. To control and look up intermediate outputs, quality reports are emitted during the workflow process and after finishing, can be downloaded as a Portable Document Format (PDF) for each workflow run. Due to a variety of possible downstream analyses that can be of the user's interest, the pipeline provides intermediate results of the individual steps so that they can be used with other tools. An overview of these outputs with their datatype is provided in Table 4. Possible downstream analyses are discussed in Chapter 5.

3.3.3 FMDV Illumina Amplicon Workflow

multisample, VAPOR, mapping (de novo assembly for control?)

4 Results and Workflow Evaluation

real-world data provided by Belgian Sciensano laboratory to test the workflow.

4.1 Poxvirus Illumina Workflow

Samples by Sciensano by Elisabeth Mathijs

IWC link, primer scheme. tested with LSDV data, pipeline outputs on 20L70, 20L81

4.1.1 LSDV Datasets 20L70 and 20L81

4.2 AIV Workflow

Samples by Sciensano s4+s8, Tunesian?

point out output for downstream analyses

4.2.1 AIV Datasets U2012100-n21_S8 and U2008751-n5_S4

Quality report, snipit plots, IQ-Tree for HA/NA, consensus reference, VAPOR scores

4.3 FMDV Workflow

Samples by Pirbright Institute by Dr. Graham Freimanis

4.4 Complexity Analysis of the Workflows

complexity of each tool
multiple inputs -> sequences of fewer complexity can be neglected
plots for many/few samples and long/short reads

for optimisation purposes.

how is server capacity allocated for a workflow run?

5 Discussion

5.1 Contribution to the Field

Workflows that solve common problems, provide useful information, are user-friendly, customisable, extendable

single sample vs. multi sample (reality check, what is needed?)
further pox viruses, pipelines can be more or less easily applied/adjusted
limitations
LSDV interesting for all poxviruses and adjustable due to ITRs and tiled-amplicon approach
AIV downstream - everything is possible. Highlight key minor assets that indicate adaption to mammals -> databases needed to check against, detect mutation of isolates?
Generally: annotate on amino acid layer (most information)

Make phylogenetic trees publicly accessible, not one sample per strain but in high resolution and greater details, strains from different countries,

"The high sensitivity of the NGS technology ensures that major kinds of viral pathogens in mixed samples can be detected." One strength of NGS is that it can be used to detect emerging viral diseases with a high genetic variation. Like AIV. Since

it can analyse a full sequence instead of targeting a specifig gene. -> makes sense to use virus-specific primers for PCR or NGS

”Comparison of the whole genome sequences of recent LSDV isolates from the 2015–2016 epidemic in southern Europe revealed only a limited number of point mutations between the isolates” WGS is essential to capture all genetic variation at once

In sequencers, falso positive variants (False Positive Variant (FPV)) must be avoided (happens when too many amplifiation cycles are made)

5.2 Future Directions

further validation and improvement of the developed pipelines, expansion to other viral livestock diseases, integration with existing surveillance systems; expand the VETLAB network to entitle even more professionals to professionally analyse their samples.

AIV workflow offers many possible directions for downstream analysis:

* consensus sequence for each segment -> compare consensus sequence to others can help identify outbreaks and patterns of transmission, get more insights how the virus spreads and its evolution * Prokka annotation file. Predict the protein coding regions of the virus, to understand the function of the viral proteins and how they interact with host cells * SNPs relative to the reference sequence * Multiple Sequence Alignment (MSA) and phylogenetic tree for broad or detailed phylolgenetic analysis and understand evolutionary relationships between the sample and other strains. could also use clusters or subtypes within the sample. make trees available so that new isolates can be immediately arranged * more visualisation of the data

* long-term objective: build public high-resolution databases to enable researchers to detect mutation of an isolate. this is crucial for a global surveillance system to work.

6 Conclusion

Summary of objectives, achievements and discussion

By relying on raw read data rather than assembled genomes and allowing every result to be traced back to its raw data, it goes a step beyond current surveillance efforts.

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Appendix

No.	Output	From Tool	Datatype	Remark
12	Quality report of pool1/pool2 input reads	fastp	HTML	For web-view
29	Alignment of pool1/pool2 against half-masked reference sequence	Map with BWA-MEM	BAM	Available as SAM file in hidden datasets
32	Statistics report of mapping of pool1/ pool2	Samtools stats	HTML	For web-view
31	Quality filtered alignments of pool1/ pool2	Samtools view	BAM	Available as SAM file in hidden datasets
39	Merged alignments	Samtools merge	BAM	Available as SAM file in hidden datasets
40	Quality and coverage reports of merged alignments	QualiMap BamQC	HTML	For web-view
41	Primer-trimmed aligned reads	iVar trim	BAM	Available as SAM file in hidden datasets
43	Consensus sequences of each sample	iVar consensus	FASTA	List of one FASTA file per sample
47	Combined consensus genomes	Concatenate datasets	FASTA	Consensus sequences of all samples in one file

Table 2: Outputs of the Poxvirus workflow that can be used for downstream analyses.

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
2	-	Upload Primer scheme with pool1/pool2 identifiers in SCORE column	-	-	BED
9	2	Select pool1 primers	Select 1.0.4	that: Matching the pattern: [p|P]ool1	BED
10	2	Select pool2 primers	Select 1.0.4	that: Matching the pattern: [p|P]ool2	BED
14	9	Get end position of pool1	Datamash 1.1.0	Operation to perform on each group: Type: maximum On column: 3	BED
15	10	Get start position of pool2	Datamash 1.1.0	Operation to perform on each group: Type: minimum On column: 2	BED
19	14	Parse integer as text	Parse parameter value 0.1.0	-	text_param
20	15	Parse integer as text	Parse parameter value 0.1.0	-	text_param
1	-	Upload reference sequence	-	-	FASTA
8	1	Get the full length	Compute sequence length 1.0.3	-	tabular
13	8	Get the length value from table	Cut 1.0.2	Cut columns: c2	tabular
18	13	Parse integer as text	Parse parameter value 0.1.0	-	text_param

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
23	19, 18	Build interval for masking the second half of the reference	Compose text parameter value 0.1.1	1: components value from 19 2: components Enter text that should be part of the computed value: - 3: components value from 18	text_param
24	20	Build interval for masking the first half of the reference	Compose text parameter value 0.1.1	1: components Enter text that should be part of the computed value: 1- 2: components value from 20	text_param
27	1, 23	Mask reference for mapping of pool1	maskseq 5.0.0	Regions to mask: value from 23	FASTA
28	1, 24	Mask reference for mapping of pool2	maskseq 5.0.0	Regions to mask: value from 24	FASTA
3	-	Upload paired-end reads of pool1 run	-	-	list of fastq/ fastqsanger pairs
4	-	Upload paired-end reads of pool2 run	-	-	list of fastq/ fastqsanger pairs
11	3	Get the sample names from pool1	Extract element identifiers 0.0.2	-	TXT
17	4, 11	Sort samples of pool 2 according to pool1	Sort collection 1.0.0	Sort type: from file	list of fastq/ fastqsanger pairs

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
16	11	Place one sample identifier per file	Split file 0.5.0	Select the file type to split: Text files Base name for new files in collection: split_file	TXT
21	16	Parse identifier as text	Parse parameter value 0.1.0	-	text_param
25	21	Append _pool1 to the sample identifiers	Compose text parameter value 0.1.1	1: components value from 21 2: components Enter the text that should be part of the computed value: _pool1	text_param
26	21	Append _pool2 to the sample identifiers	Compose text parameter value 0.1.1	1: components value from 21 2: components Enter the text that should be part of the computed value: _pool2	text_param
12	3	Preprocessing of pool1 reads	fastp 0.23.2	Single-end or paired reads: Paired collection PolyG tail trimming: Automatic trimming for Illumina NextSeq/ NovaSeq data	fastq/ fastqsanger, HTML, JSON
22	17	Preprocessing of pool2 reads	fastp 0.23.2	Single-end or paired reads: Paired collection PolyG tail trimming: Automatic trimming for Illumina NextSeq/ NovaSeq data	fastq/ fastqsanger, HTML, JSON

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
29	27, 12, 25	Mapping of pool1 against half-masked reference sequence, retaining read group identifier	Map with BWA-MEM 0.7.17.2	Single or Paired-end reads: Paired Collection Set read groups information? Set read groups (SAM/BAM specification) Platform/technology used to produce the reads (PL): ILLUMINA	BAM
30	28, 22, 26	Mapping of pool2 against half-masked reference sequence, retaining read group identifier	Map with BWA-MEM 0.7.17.2	Single or Paired-end reads: Paired Collection Set read groups information? Set read groups (SAM/BAM specification) Platform/technology used to produce the reads (PL): ILLUMINA	BAM
32	29	Generate statistics of mapping	Samtools stats 2.0.4	-	tabular
34	30	Generate statistics of mapping	Samtools stats 2.0.4	-	tabular
35	12, 32	Aggregate quality reports of pool1 reads and mapping statistics	MultiQC 1.11	1: Results Which tool was used to generate logs? fastp 2: Results Which tool was used to generate logs? Samtools Type of Samtools output? stats	HTML
37	22, 34	Aggregate quality reports of pool1 reads and mapping statistics	MultiQC 1.11	1: Results Which tool was used to generate logs? fastp 2: Results Which tool was used to generate logs? Samtools Type of Samtools output? stats	HTML

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
31	29	Quality filter the mapped reads of pool1	Samtools view 1.15.1	What would you like to look at? A filtered/ subsampled selection of reads Filter by quality: 20 Require that these flags are set: Read is paired, Read is mapped in a proper pair	BAM
33	30	Quality filter the mapped reads of pool2	Samtools view 1.15.1	What would you like to look at? A filtered/ subsampled selection of reads Filter by quality: 20 Require that these flags are set: Read is paired, Read is mapped in a proper pair	BAM
36	31, 33	Create paired collection from both pool mappings	Zip collections 1.0.0	Input 1: value from 31 Input 2: value from 33	BAM
38	36	Add rules to distinguish between pool1 and pool2	Apply rules 1.1.0	1. Add column for identifier0. 2. Add column for identifier1. 3. Set columns A and B as List Identifier(s)	BAM
39	38	Merge the alignments of both pools	Samtools merge 1.15.1	Alignments in BAM format: value from 38	BAM
40	39	Generate quality and coverage report of mapping to check for the middle part of the merged pools	QualiMap BamQC 2.2.2d	-	fastq/ fastqsanger, HTML
42	40	Remove failed reads from the dataset	Filter failed datasets 1.0.0	Input Collection: value from 40	fastq/ fastqsanger, HTML
44	42	Place the two datasets from nested collection into a list of reports	Flatten collection 1.0.0	Input Collection: value from 42 Join collection identifiers using: underscore	fastq/ fastqsanger
46	44	Aggregate results from the reports	MultiQC 1.11	1: Results Which tool was used to generate logs? Qualimap	HTML

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
41	39, 2	Trim the aligned reads to remove primers	ivar trim 1.3.1	BED file with primer sequences and positions: value from 39 Filter reads based on amplicon info: Yes, drop reads that extend beyond amplicon boundaries Include reads not ending in any primer binding sites? Yes	BAM
5	-	Enter minimum quality score to call base	-	-	integer
6	-	Enter allele frequency to call SNV	-	-	float
7	-	Enter allele frequence to call indel	-	-	float
43	41	Consensus sequence construction from trimmed and merged alignments	ivar consensus 1.3.2	Minimum quality score threshold to count base: value from 5 Minimum frequency threshold: value from 6 Minimum indel frequency threshold: value from 7 Minimum depth to call consensus: 50 How to represent positions with coverage less than the minimum depth threshold: Represent as N	FASTA
45	43	Relabel consensus sequences per sample	Text transformation 1.1.1	File to process: value from 43 SED Program: /^>/s/Consensus_(.*) _threshold_.*/\1	FASTA
47	45	Get combined consensus genomes in a multifasta file	Concatenate datasets 0.1.1	Datasets to concatenate: value from 45	FASTA

Table 3: The full Poxvirus workflow with tools, parameters and input/output connections.

No.	Output	From Tool	Datatype	Remark
6	Quality report of input reads	fastp	HTML	In report
8	Scores of closest references	VAPOR	tabular	One table per segment
24	Scores of sequences chosen for hybrid reference sequence	VAPOR	tabular	Overview of scores
25	Alignment of reads against hybrid reference sequence	Map with BWA-MEM	BAM	Available as SAM file in hidden datasets
27	Quality filtered alignments	Samtools view	BAM	Available as SAM file in hidden datasets
31	Quality and coverage report on alignment	QualiMap BamQC	HTML	In report
36	Consensus sequence	iVar consensus	FASTA	One FASTA file per segment
41	Consensus sequences with segment names as sequence identifiers	Collapse Collection	FASTA	Contains eight sequences
42	Plots to visualise SNPs	snipit	PNG	HA and NA plots in report
44	Genome annotation file	Prokka	FAA	In report
43	Phylogenetic trees	IQ-Tree	iqtree	HA and NA trees in report

Table 4: Outputs of the Avian Influenza Virus workflow that can be used for downstream analyses.

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
1	-	Upload reference sequence database per segment	-	-	list
2	-	Upload paired-end Illumina reads	-	-	pair of fastq/ fastqsanger
6	2	Preprocessing	fastp 0.20.1	Single-end or paired reads: Paired Collection Length required: 30 PolyG tail trimming: Automatic trimming for IlluminaNextSeq/NovaSeq data Cut by quality in front (5'): Yes Cut by quality in tail (3'): Yes Cutting mean quality: 30	pair of fastq/ fastqsanger, HTML JSON
8	1, 6	Find closest reference per segment	VAPOR 1.0.2	Type of sequencing data: Paired-end as collection Desired output: Return scores of best matches Limit number of reported matches to: 0 Kmer Length: 21 Read kmer filtering threshold: 0.1 Coverage threshold for k-mer culling: 5 Minimum k-mer proportion: 0.0 Fraction of best seeds to extend: 1.0	tabular
9	8	Get sequence identifier	Replace 1.1.4	Find pattern: ^.+\t>()$ Replace with: $1 Find-Pattern is a regular expression: Yes Replace all occurences of the pattern: Yes Find and Replace text in: entire line	tabular
12	9	Get the first identifier of each segment	Select first 1.0.2	Select first: 1	tabular
16	1, 12	Extract sequences from the database according to the identifiers	seqtk_subseq 1.3.1	Select source of sequence choices: FASTA/Q ID list	FASTA
20	16	Place all closest reference sequences into one FASTA file	Collapse Collection 5.1.0	-	FASTA
23	20	Shorten the identifiers	Replace 1.1.4	Find pattern: >([^]]+).+$ Replace with: >$1 Find-Pattern is a regular expression: Yes Replace all occurences of the pattern: Yes Find and Replace text in: entire line	FASTA

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
25	6, 23	Mapping against hybrid reference sequence	Map with BWA-MEM 0.7.17.2	Single or Paired-end reads: Paired Collection Select analysis mode: 1.Simple Illumina mode BAM sorting mode: Sort by chromosomal coordinates	BAM
10	8	Pick columns with sequence identifier and score	Cut 1.0.2	Cut columns: c6,c1	tabular
14	10	Get the first identifier of each segment	Select first 1.0.2	Select first: 1	tabular
18	14	Place all closest reference sequences in one table	Collapse Collection 5.1.0	-	tabular
3	-	Generate a text file with names ”segment 1”, ”segment 2” etc.	Create text file 1.1.0	1: selection Characters to insert: segment 1 Specify the number of iterations by: User defined number How many times? 1 (repeat until segment 8)	TXT
22	3, 18	Build table with segment name, sequence identifier and score	Paste 1.0.0	-	tabular
24	22	Reorder the columns	Cut 1.0.2	Cut columns: c3,c1,c2	tabular
27	25	Quality filter the mapped reads	Samtools view 1.9	What would you like to look at? A filtered/ subsampled selection of reads Filter by quality: 20 Require that these flags are set: Read is paired, Read is mapped in a proper pair	BAM
29	27	Generate coverage and quality report of alignment	QualiMap BamQC 2.2.2d	Skip duplicate reads: Unselect all Number of bins to use in across-reference plots: 40	SAM
31	29	Generate HTML report	MultiQC 1.9	Which tool was used to generate logs? Qualimap (BamQC or RNASeq output)	HTML
26	25	Convert header to SAM format	Samtools view 1.9	What would you like to look at? Just the input header (-H) What would you like to have reported? The header in ... Output format: SAM	SAM
28	26	Get header lines starting with @SQ	Select 1.0.4	that: Matching the pattern: ^@SQ.+	SAM
30	28	Rewrite segment names and get subtype information from identifier	Replace 1.1.4	Find pattern: ^\@SQ\tSN:(.*)\tLN:([0-9]+) Replace with: $1^$2 Find-Pattern is a regular expression: Yes Replace all occurences of the pattern: Yes Find and Replace text in: entire line	SAM

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
32	30	Place the alignment of one segment in one file	Split file 0.5.0	Select the file type to split: Text files Specify number of output files or number of records per file? Number of records per file Chunk size: 1 Base name for new files in collection: split_file Method to allocate records to new files: Alternate output files	SAM
33	30	Get columns with sequence identifiers	Cut 1.0.2	Cut columns: c1	tabular
34	27, 32	Filter by mapped intervals	Samtools view 1.9	What would you like to look at? A filtered/ subsampled selection of reads Filter by regions: Regions from BED file	BAM
35	34, 33	Rename sequence identifiers to segment names	Relabel identifiers 1.0.0	How should the new labels be specified? Using lines in a simple text file.	BAM
36	35	Consensus sequence construction	ivar consensus 1.3.1	Minimum quality score threshold to count base: 20 Minimum frequency threshold: 0.7 Minimum depth to call consensus: 10 Exclude regions with smaller depth than the minimum threshold: No Use N instead of - for regions with less than minimum coverage: Yes	FASTA
38	36	Use readable sequence identifiers	Replace 1.1.4	Find pattern: ^>Consensus_(.*)_threshold_.* Replace with: >$1 Find-Pattern is a regular expression: Yes Replace all occurences of the pattern: Yes Find and Replace text in: entire line	FASTA
41	38	Create multifasta file containing the eight consensus sequences	Collapse Collection 5.1.0	-	FASTA
44	41	Create genome annotation file from consensus sequence	Prokka 1.14.6	Kingdom: Viruses	FAA
11	9	Get identifiers of the 10 best scores per segment	Select first 1.0.2	Select first: 10	tabular
15	1, 11	Retrieve sequences of the 10 best scores per segment from reference database	seqtk_subseq 1.3.1	Select source of sequence choices: FASTA/Q ID list	tabular
19	15	Multiple sequence alignment of the 10 best scores per segment	MAFFT 7.508	Data type: Nucleic acids Matrix selection: No matrix	FASTA
4	-	Set size of phylogenetic trees	-	-	integer

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
13	4, 9	Select the first X lines from the VAPOR scores table	Select first 1.0.2	-	tabular
5	1	Dismiss B strain if present in the reference sequence database	Filter FASTA 2.3	Criteria for filtering on the headers: Regular expression on the headers Regular expression pattern the header should match: \>\S*|A	FASTA
7	5	Dismiss H17 and H18 subtypes if present in the reference sequence database	Filter FASTA 2.3	Criteria for filtering on the headers: Regular expression on the headers Regular expression pattern the header should match: H1N|H2|H3|H4|H5|H6|H7|H8|H9|H10| H11|H12|H13|H14|H15|H16	FASTA
17	7, 13	Retrieve the sequences for the phylogenetic trees from the filtered reference sequence database	seqtk_subseq 1.3.1	Select source of sequence choices: FASTA/Q ID list	FASTA
21	17	Multiple sequence alignment of the sequences for phylogentic trees	MAFFT 7.508	Data type: Nucleic acids Matrix selection: No matrix	FASTA
37	36	Rename sequence identifiers from consensus sequences	Replace 1.1.4	Find pattern: ^>.+ Replace with: >sequenced_sample Find-Pattern is a regular expression: Yes Replace all occurences of the pattern: Yes Find and Replace text in: entire line	FASTA
39	37, 19	Add consensus sequences to the alignment	MAFFT add 7.508	What do you want to add to the alignment: A single sequence Preserve the original alignment: Yes Preserve the original order of sequences: Yes	FASTA
42	39	Summarise SNPs relative to the reference sequence of each segment	snipit 1.0.7	The reference sequence ...: should be picked via its ID ID of reference sequence: sequenced_sample Order of samples in the plot: Sort by number of mutations Invert sort order: Yes Flip plot orientation: Yes	Collection of PNG
45	42	Get SNP plot for HA segment	Extract dataset 1.0.1	How should a dataset be selected? Select by index Element index: 3	PNG
46	42	Get SNP plot for NA segment	Extract dataset 1.0.1	How should a dataset be selected? Select by index Element index: 5	PNG

Output No.	Input No.	Description	Tool	Parameters	Output Datatype
40	38, 21	Add relabeled consensus sequences to the alignment	MAFFT add 7.508	What do you want to add to the alignment: A single sequence Preserve the original alignment: Yes	FASTA
43	40	Build phylogenetic trees for each segment from the specific MSAs	IQ-TREE 2.1.2	Specify sequence type: DNA	nhx, mldist, iqtree
47	43	Get phylogenetic tree for HA segment	Extract dataset 1.0.1	How should a dataset be selected? Select by index Element index: 3	iqtree
48	43	Get phylogenetic tree for NA segment	Extract dataset 1.0.1	How should a dataset be selected? Select by index Element index: 5	iqtree

Table 5: The full AIV workflow with tools, parameters and input/output connections.