Molecular characterization of highly pathogenic avian influenza clade 2.3.4.4b H5N8 viruses in terns and other coastal birds in South Africa in 2018

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DECLARATION

I, Belinda Margaret Peyrot (student number 18392483) hereby declare that this dissertation, entitled "Molecular characterization of highly pathogenic avian influenza clade 2.3.4.4b H5N8 viruses in terns and other coastal birds in South Africa in 2018", is submitted in accordance with the requirements for the MSc (Veterinary Research) at the University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

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B M Peyrot

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Date

In memory of

Zithuleleni Pasiya

1989 - 2019

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SUMMARY

Title: Molecular characterization of highly pathogenic avian influenza clade 2.3.4.4b H5N8 viruses in terns and other coastal birds in South Africa in 2018

By Belinda Margaret Peyrot

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Degree: MSc (Veterinary Research)

The Gs/GD (goose/Guangdong) highly pathogenic (HP) H5 influenza virus detected in China in 1996 has since evolved into genetically distinct clades and subclades. In June 2017, clade 2.3.4.4b H5N8 highly pathogenic avian influenza (HPAI) caused the first notifiable avian influenza outbreaks in gallinaceous poultry in South Africa. It spread rapidly and caused outbreaks in all provinces of the country. Farmed ostriches, commercial and backyard poultry, zoological collections and wild birds were infected. The first coastal birds that became infected were reported in December 2017, when major die-offs of terns and other coastal bird species started to occur in the southern regions of the Western and Eastern Cape provinces. Outbreaks in the area continued until June 2018. This was the first report of major mortalities from HPAI in coastal birds in South Africa since an outbreak of HPAI H5N3 was reported in common terns (*Sterna hirundo*) in 1961 (Rowan, 1962).

The study objective was to isolate H5N8 HPAI viruses from coastal birds and to carry out molecular characterization by full genome sequencing. Viruses isolated from terns, cormorants, penguins, gulls and an oystercatcher from December 2017 to May 2018, were sequenced using Ion Torrent sequencing. Phylogenetic analysis of the eight gene segments of each isolate was used to compare them with H5N8 viruses responsible for outbreaks in terrestrial avian species in South Africa in 2017, and with selected viruses that circulated in 2017 in other parts of Africa. Radial trees of the gene segments of the coastal bird isolates were also prepared for better visualization of clustering of the viruses to assess reassortment. Two clusters within each of segments 4, 5, 7 and 8 could be distinguished. Three clusters were evident in segment 6. There was relatively greater variation in the polymerase gene segments (Segments 1, 2 and 3) but no distinct clusters within any of them were apparent. No biological significance to the clusters was found. Multiple sequence alignments were investigated to identify genetic markers. No strong adaptive host pressures were evident.

Results indicated that there may have been a single introduction of the virus into the coastal bird population, possibly from the terrestrial wild bird population. The terrestrial strains were basal to the coastal bird strains, but still not closely related. The coastal bird viruses have a common ancestor with the Namibian outbreaks that occurred at the beginning of 2019 and they may have been the source of the Namibian outbreaks. Alternatively, an introduction of the virus into the Namibian birds could have been independent of the South African incursions.

ABBREVIATIONS AND ACRONYMS

AF	Amnio-allantoic fluid
AI	Avian influenza
AIV	Avian influenza virus
APHA	Animal and Plant Health Agency (United Kingdom)
BFAP	Bureau for Food and Agricultural Policy
bp	base pair(s)
CDC	Centre for Disease Control and Prevention (USA)
cds	Coding sequence
Ct	Cycle threshold
D	Aspartic acid
DAFF	Department of Agriculture, Forestry and Fisheries
DOA	Department of Agriculture
DRC	Democratic Republic of the Congo
E	Glutamic acid
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay
EMPRES	Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases
EpiFlu	GISAID EpiFlu™ Database
FAO	Food and Agriculture Organization of the United Nations
FTP	File Transfer Protocol
GISAID	Global initiative on sharing all influenza data
Gs/GD	A/Goose/Guangdong/96
H	Haemagglutinin antigen or subtype
HA	Haemagglutinin
HA_0	Haemagglutinin precursor protein

HA_1 and HA_2	The two sub-units of HA_0 after cleavage
HP	Highly pathogenic/ high pathogenicity
HPAI	Highly pathogenic avian influenza
ICTV	International Committee on Taxonomy of Viruses
IPC	Internal positive control
IUCN	International Union for Conservation of Nature
IVPI	Intravenous Pathogenicity Index
LBM	Live bird market
LP	Low pathogenicity
LPAI	Low pathogenic avian influenza
LPNAI	Low pathogenic notifiable avian influenza
M1	Matrix protein 1
M2	Matrix protein 2
mRNA	Messenger RNA
MSA	Multiple sequence alignment
N	Neuraminidase antigen or subtype
NA	Neuraminidase
NAI	Notifiable avian influenza
NCBI	National Center for Biotechnology Information
NEP	Nuclear export protein (= NS2)
NGS	Next Generation Sequencing
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2 (= NEP)
OFFLU	OIE and FAO Network of Expertise on Animal Influenza
OIE	Office International des Epizooties; World Organisation for Animal Health

PA	Acidic polymerase protein
PB1	Basic polymerase protein 1
PB1-F2	Basic polymerase protein 1 frame 2
PB1-N40	Basic polymerase protein 1 - N40 protein
PB2	Basic polymerase protein 2
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription PCR
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse Transcription PCR
SA	South Africa
SAMUKA	Sango Bay-Musambwa Island-Kagera Wetland System, Uganda
SAN	Specific antigen negative
SANAS	South African National Accreditation System
UP	University of Pretoria
WAHIS	World Animal Health Information System
WCPVL	Western Cape Provincial Veterinary Laboratory
WHO	World Health Organization

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CHAPTER 1

1.1. Introduction

References to avian influenza often generate uneasiness and sometimes even anxiety in the medical and veterinary health community. Being a zoonosis, in some cases this apprehension is well-founded considering the major impact that the disease has had on human and animal health globally over the past few decades. Indeed even the H1N1 influenza virus responsible for the 1918 "Spanish flu" pandemic, which contributed to the deaths of 50 million people, appears to have contained segments of avian origin (Lycett *et al.*, 2019). The World Health Organization (WHO) estimates that annual human influenza epidemics cause between 3 and 5 million cases of severe illness globally (WHO, 2019), and 291 243 to 645 832 seasonal human influenza-associated respiratory deaths are estimated to occur annually (Iuliano *et al*., 2018).

Wild bird species especially anseriforms (ducks, geese and swans) and charadriiforms (gulls and waders) are the natural hosts and reservoirs for all low pathogenicity avian influenza (LPAI) viruses (Lee *et al*., 2017). These LPAI viruses are globally distributed in wild aquatic birds. Highly pathogenic avian influenza (HPAI) viruses do not normally occur in wild bird host reservoirs (Lee *et al*., 2017). However, during HPAI surveillance of apparently healthy wild birds along the Pacific flyway in the USA in late 2014 and early 2015, clade 2.3.4.4 H5Nx viruses were detected (Bevin *et al.,* 2016). In April and May 1961 an HPAI virus, A/Tern/South Africa/1961, caused mass mortalities of common terns (*Sterna hirundo*) along the Southern African coastline from Lambert's Bay to Port Elizabeth (Becker, 1966; Rowan, 1962). Globally this was the first isolation of an HPAI virus from free-living birds and it was an H5N3 virus strain (Swayne, 2008). It was also the only HPAI virus isolated from free-living wild birds until Asian H5N1 strains were detected in captive and free-living wild birds during the Eurasian outbreak in 2002/2003 (Swayne, 2008).

In 2017, outbreaks of clade 2.3.4.4b H5N8 HPAI in domestic and commercial poultry in South Africa resulted in unprecedented economic losses, and the public was reminded of our vulnerability to unpredictable and sometimes devastating animal disease outbreak events. As a result of this outbreak, according to the Bureau for Food and Agricultural Policy (BFAP) an estimated total of 5.4 million birds were culled in South Africa, and the total economic loss including biological losses, direct costs and possible income forgone could be up to R1.87 billion (BFAP, 2018). 2.76 million layers out of the national total of 4.7 million layers that were affected and/or culled were in the Western Cape province (L. Roberts, pers. com).

Fifty-seven years after the 1961 epizootic of H5N3 HPAI in terns in South Africa, an incursion of the H5N8 clade 2.3.4.4b HPAI virus occurred in the wild coastal bird populations along the same coastline. This virus also became a threat to endangered bird species when it entered the wild coastal bird populations of the Western Cape early in 2018. At least three of the species that were affected are listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as endangered, namely African penguins (*Spheniscus demersus*), Cape cormorants (*Phalacrocorax capensis*) and Cape gannets (*Morus capensis*) (BirdLife International, 2018a). African penguins are endemic to Southern Africa with breeding colonies only in Namibia and South Africa. Their very rapid population decline is likely resulting from the impact of commercial fishing and shifts in prey population (IUCN, 2018). Cape gannets are native to Angola, Mozambique, Namibia and South Africa, and are undergoing significant population decline with each generation (BirdLife International, 2018a). Key colonies of Cape cormorants in South Africa and Namibia have also undergone rapid population declines probably as a result of dwindling epipelagic fish stocks. These birds are also highly vulnerable to oiling and outbreaks of avian cholera (BirdLife International, 2018b).

The outbreaks of H5N8 HPAI in wild coastal birds of the Western Cape is the topic of this dissertation.

1.2. Problem statement

Vigilant surveillance is critical to the fight against endemic and emerging AIVs (Suttie *et al*., 2019). Molecular characterization can assist in identifying and tracking molecular markers. These in turn can be used for risk assessment of emerging AIV strains (Suttie *et al.*, 2019).

The Western Cape Department of Agriculture: Veterinary Services Sub-Program Animal Health is responsible for active and passive surveillance of controlled and notifiable animal diseases in the Western Cape province of South Africa. Its Epidemiology Section closely monitored the clade 2.3.4.4b H5N8 HPAI epidemic from when the virus was first detected in avian species in the province in August 2017. The Sub Program Veterinary Laboratory Services was responsible for the bulk of the laboratory testing for AIV.

In coastal bird species, a relatively large die-off of swift terns (*Thalasseus bergii*), Arctic terns (*Sterna paradisaea*), a kelp gull (*Larus dominicanus*) a Hartlaub's gull (*Chroicocephalus hartlaubii*) and a Sandwich tern (*Thalasseus sandvicensis*) was reported at the Bot River estuary about 100km east of Cape Town in December 2017. Simultaneously, coastal bird rehabilitation centres started to see

increasing numbers of sick swift terns. This study was initiated when H5N8 HPAI infection in these coastal birds was confirmed.

At the beginning of January 2018 various role players were approached to increase capacity for coastal bird surveillance. These institutions and individuals (non-profit organizations, private veterinarians, wildlife conservation bodies and a conservation trust) were encouraged to submit diseased or dead birds to the Western Cape Provincial Veterinary Laboratory for AIV testing. This surveillance strategy provided excellent opportunities to include areas that would not normally be monitored by field workers of the Department due to limited access and resources. The assistance provided by these role players was indispensable for the accumulation of valuable data for this study. The spread of this virus to threatened and endangered wild African birds like African penguins, Cape gannets and African oystercatchers was particularly concerning and warranted further investigation. Mortalities of coastal birds continued until May 2018 (Khomenko *et al*., 2018).

1.3. Aims and objectives

1.3.1. To isolate clade 2.3.4.4b H5N8 HPAI viruses from various species of South African coastal birds in the Charadriiformes, Ciconiiformes and Sphenisciformes orders, including terns, gulls, penguins and cormorants from December 2017 to May 2018 in the Western Cape.

1.3.2. To sequence the complete genomes of these viruses, and to:

1.3.2.1. Determine the phylogenic relationships and genomic reassortment patterns in order to trace the source/s of the outbreaks in coastal birds

1.3.2.2. Identify important molecular markers of virulence and host specificity in the encoded protein sequences

1.4. Literature review

1.4.1. Influenza virus genera and Group A influenza

Influenza viruses are classified in the Orthomyxoviridae family and have single-stranded, segmented, negative-sense RNA genomes (Shaw and Palese, 2013). The family comprises seven genera (ICTV, 2019). Influenza A (*Alphainfluenzavirus*) is the most widespread genus, infecting many species including humans, and is the only genus that naturally infects birds (OIE, 2015). Influenza B and C (*Betainfluenzavirus* and *Gammainfluenzavirus* respectively) are mainly human pathogens, but can also affect other mammals. Internal proteins distinguish influenza A from B and C (Webster *et al.*, 1992). The

non-coding regions of the Influenza B virus genome are also longer than those of Influenza A, and Influenza C only has seven segments in its genome (Shaw and Palese, 2013).
Influenza D viruses (*Deltainfluenzavirus*) were first detected in pigs, but the main reservoir species is cattle and it is associated with bovine respiratory disease (Flynn *et al*., 2018). *Isavirus* infects Atlantic salmon. *Thogotovirus* (*Dhori thogotovirus* and *Thogoto thogotovirus*) are arboviruses (i.e. arthropod-borne) and both can cause human infections and disease (Kosoy *et al*., 2015). Viruses in the *Quaranjavirus* genus (e.g. Wellfleet Bay, Johnston Atoll and Quaranfil quaranjaviruses) are also arboviruses and have been isolated from birds and humans (Ballard *et al.*, 2017) (Presti *et al.*, 2009).

Influenza A viruses are classified into numerous subtypes based on combinations of their major surface antigens haemagglutinin (H) and neuraminidase (N) producing an HxNx configuration (OIE, 2015). Sixteen HA subtypes (1 to 16) and 9 NA subtypes (1 to 9) occur in wild and domestic avian species, and the majority of all possible combinations of these surface antigens have been detected (Lycett *et al.*, 2019). The Centre for Disease Control and Prevention (CDC, USA) recognizes 18 different haemagglutinin subtypes and 11 neuraminidase subtypes (CDC, 2017). Two of each of these H and N subtypes, H17N10 and H18N11, have only been detected in bats in Guatemala and Peru, respectively (Tong *et al*., 2013).

As with all RNA viruses, an intrinsic biological feature of influenza viruses is their poor proofreading activity during replication, which results in high genetic variability (Croville *et al*., 2012). Viral RNA polymerase activity is error prone, and the HA segment is subject to a very high mutation rate (Swayne, 2008). Influenza A viruses also readily undergo genetic reassortment, exchanging genetic information when multiple AIVs co-infect a susceptible host cell (Shaw and Palese, 2013). The viruses are therefore constantly evolving. Each new subtype and the strains within subtypes can behave differently and present different risks (OIE, 2018).

1.4.2. Genome structure of Influenza A virus particles

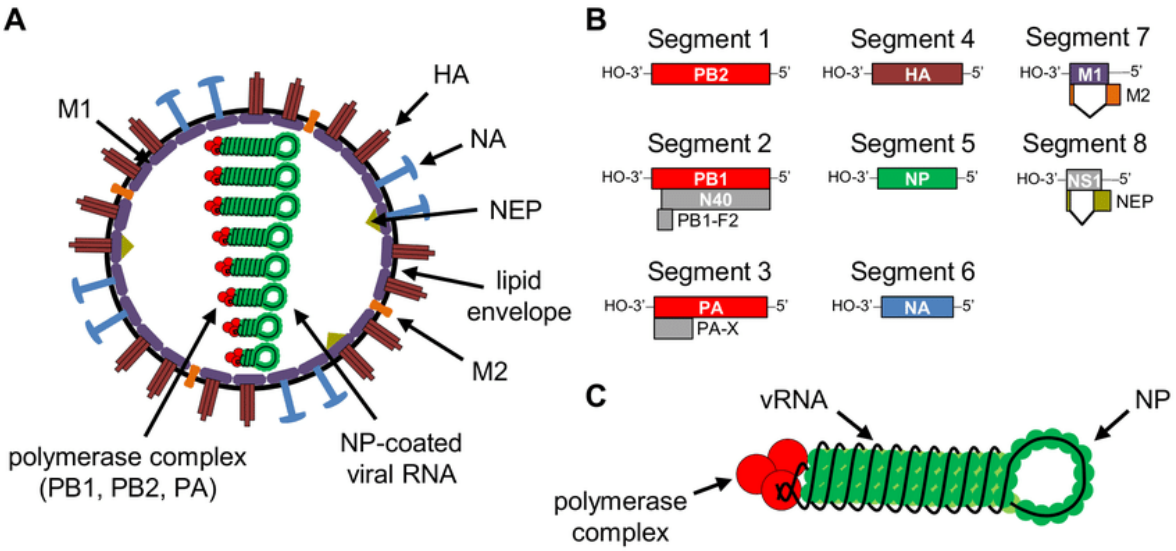

Figure 1 (A) Schematic diagram of a spherical Influenza A virus particle. (B) The eight Influenza A virus genome segments, indicating the proteins they each encode for. V-shapes indicate the introns of the spliced m-RNAs. (C) Diagram of an influenza viral ribonucleoprotein. *Figure: F Heldt* (Heldt *et al.*, 2013).

Influenza A viruses possess eight gene fragments that code for up to 12 proteins (Tauber *et al*., 2012) (Fig. 1.) The surface proteins are the haemagglutinin (HA), neuraminidase (NA) and membrane ion channel (M2) proteins. The internal proteins consist of the nucleoprotein (NP), matrix protein (M1), polymerase basic proteins (PB1 and PB2) and the polymerase acidic protein (PA) (Swayne, 2008b). These eight gene segments have a total length of ≈13.6 kb (Vandegrift *et al.*, 2010).

Table 1 Proteins encoded by influenza A virus

Segment number	**Gene**	**Proteins**	**Functions**
1	Polymerase basic 2 (PB2)	PB2	Component of RNA polymerase complex (RNP), cap recognition
2	Polymerase basic 1 (PB1)	PB1	Component of RNP, elongation
		PB1-F2	Pro-apoptotic activity, interferon antagonist
		PB1 N40	RNA-directed RNA polymerase catalytic activity
3	Polymerase (PA)	PA	Component of RNP, endonuclease activity, protease
4	Haemagglutinin (HA)	HA	Surface glycoprotein and major antigen, host cell receptor sialic acid binding, fusion activity, assembly and budding
5	Nucleoprotein (NP)	NP	RNA binding, RNA synthesis, RNP nuclear export
6	Neuraminidase (NA)	NA	Surface glycoprotein, neuraminidase activity
7	Matrix (M)	M1	Matrix protein, RNP nuclear export, assembly and

			budding
		M2	Membrane protein, ion channel activity, assembly and budding
8	Non-structural (NS)	NS1	Multifunctional, interferon antagonist activity
		NEP/NS2	RNP nuclear export, RNA synthesis regulation

Sources: https://www.uniprot.org/uniprot/R4IS35); Swayne, 2008b

RNA Segment 1 encodes the polymerase PB2 protein, which migrates to the host cell nucleus. Segment 2 encodes the polymerase PB1, which is also contained in the cell nucleus and is responsible for elongation of the viral RNAs. Polymerase PA is encoded by Segment 3. It is also found in the cell nucleus, and forms part of the RNA-polymerase complex (Webster *et al.*, 1992).

Segment 4 encodes the haemagglutinin gene segment HA. This is the major surface antigen of the virus particle, and target of the host immune response. Together with the neuraminidase protein it is used for the subtyping of influenza viruses (e.g. HxNx). It is responsible for fusion of the virion envelope with the host cell membrane. The HA precursor protein is cleaved at the proteolytic cleavage site HA_0 into two subunits, HA_1 and HA_2, by proteases produced by the host cell, and this cleavage is necessary for infectivity because virus-to-cell fusion is mediated by the free exposed amino acid terminus of the HA_2 sub-unit (Webster *et al.*, 1992).

RNA Segment 5 encodes the nucleoprotein NP. It is the second most abundant protein in the virus particle, and encapsidates the viral RNA. It forms part of the RNA-nucleoprotein complex, which travels to the cell nucleus to make new viral m-RNA and template (Swayne, 2008b). The efficient functioning of the RNA-nucleoprotein complex is crucial to developing high titres in the host (Swayne, 2008b). Its phosphorylation pattern is host cell dependent, and may play a role in the virus' host range restriction. Neuraminidase (NA) is encoded by Segment 6. It is the second major surface structural antigen of the virus particle and is an essential membrane glycoprotein. It facilitates the spread of viruses by the release of progeny virions from the host cell. Neuraminidase is also highly susceptible to mutations (Webster *et al.*, 1992). Segment 7 is bicistronic and encodes both internal structural matrix proteins M1 and M2. M1 is the most abundant protein of virus particles and forms a shell around the virion nucleocapsids. The M2 protein functions as an important trigger for viral uncoating (Swayne, 2008b).

Segment 8 encodes the non-structural proteins NS1 and NS2. They are abundant in infected host cells, but are not incorporated into progeny virus particles (Webster *et al.*, 1992).

1.4.3. Inter- and intra-subtype gene reassortment

As a result of segmentation of the influenza virus genome, genetic reassortment is common. The incorporation of RNA segments into virions during replication is partly random. When host cells are infected by two different viruses concurrently, progeny viruses may contain novel genetic combinations

due to genetic reassortment (Webster *et al.*, 1992). Inter-subtype reassortment and the resultant genetic and antigenic variability allow rapid adaption of these viruses (Maljkovic Berry *et al*., 2016). In avian species in South Africa phylogenetic evidence has suggested that new AIV subtypes arose from the reassortment of known subtypes with genes from various wild bird species, especially aquatic birds (Abolnik *et al*., 2010). Farmed ostriches have also been shown to be "mixing vessels" of new subtypes (Abolnik, 2007b).

Substantial segment incompatibility may however limit the success of such reassortment processes. Intra-subtype (i.e. between different lineages of the same sub-type) reassortment patterns of influenza might not be restricted to the same extent due to greater genetic relatedness and segment functional compatibility (Maljkovic Berry *et al*., 2016). As demonstrated by H3N2 in humans, the significance of intra-subtype influenza reassortment affects all aspects of this virus, including its evolution, spread, disease severity and vaccine efficacy (Maljkovic Berry *et al*., 2016). Molecular characterization was used to determine that the first outbreaks of H5N8 HPAI in South Africa in 2017 were not directly related to the virus isolated in Zimbabwe just prior to that (Abolnik *et al.*, 2019). Instead, up to five primary introductions could have occurred, evidently via wild birds (Abolnik *et al.*, 2019). Reassortment patterns demonstrated that some of the genes of the H5N8 viruses isolated from terrestrial birds in South Africa in 2017 shared recent common ancestors with LPAI viruses circulating in the wild bird reservoir as well as West African strains (Abolnik *et al.*, 2019). Molecular analysis of reassortment patterns is therefore an important tool in influenza virus epidemiology and surveillance.

1.5. Phylogenetic analysis and reconstruction

Phylogenetic trees are molecular analyses that are used to depict patterns of evolutionary pathways and relationships between viruses (or other entities e.g. species), with tree branching patterns and branch lengths indicating relative relatedness (De Bruyn, 2014). Nucleotide or amino acid sequence data from each virus are the starting point and are essential when comparing viruses on a molecular level. Only corresponding homologous nucleotides or amino acids can be compared, so these sequences must be aligned in preparation of a matrix known as a multiple sequence alignment (MSA) where each row contains one of the sequences and each column contains a homologous set of nucleotides or amino acids.

Distance-based methods may be used to infer phylogenetic trees from MSAs by measuring the overall genetic distance between all pairs of sequences in the alignment. This produces a matrix of pairwise

genetic distances, from which phylogenetic trees may be constructed using a clustering algorithm. Character-based methods utilize the individual columns of aligned sequences.

Distance-based methods are ideally suited to the initial investigation of evolutionary relationships between sequences because of their computational speed. Character-based methods are slower, but more accurate, because they utilize all information available at each homologous site instead of compressing it into pairs of evolutionary distances and only using that (De Bruyn, 2014) .

1.5.1. Molecular determinants of the AI virus phenotype

Molecular analysis provides genetic information about specific amino acid positions on the genes. The amino acid sequences at certain positions, termed molecular markers, have been reported to be relevant to factors that affect virulence, pathogenicity, transmissibility, replication in hosts, and other aspects of the maintenance of avian influenza viruses in the environment and their continuous reassortment (Suttie *et al.*, 2019). Other molecular markers include residues suggesting mammal-adaption (Salaheldin *et al.*, 2018), and deletions in the stalk of the neuraminidase which occur during circulation of the virus in poultry (Croville *et al*., 2012). Such deletions may contribute to viral adaption to gallinaceous species and a change in tropism from the intestine to the respiratory tract (Croville *et al*., 2012).

The National Centre for Immunization and Respiratory Disease: Influenza Division of the USA Department of Health & Human Services' Centres for Disease Control and Prevention (CDC, USA) has published an inventory of amino acid mutations and/or motifs of H5N1 HPAI that determine important phenotypic characteristics. These include the adaptation of these viruses for humans and other host species (CDC, 2012). The list was developed by the WHO Collaborating Centre for Influenza Reference and Research and was compiled from published and available literature up to 2012 (CDC, 2012). As an example, the zoonotic potential of three H5 HPAI viruses in France during the 2015/2016 winter was evaluated in this way (Briand *et al*., 2017), and the pandemic potential of the strains that were studied was found to be insignificant, because the viruses lacked some important genetic characteristics for efficient human respiratory transmission (Briand *et al*., 2017).

1.5.2. Characterisation of avian influenza by pathogenicity

Avian influenza viruses are characterised as either highly pathogenic avian influenza (HPAI) or low pathogenicity avian influenza (LPAI) viruses. HPAI is deemed such if it fulfils at least one of the following criteria:

- *in vivo* pathotyping – intravenous pathogenicity index in four-to-six-week old chickens is >1.2, or a >75% mortality rate
- multiple basic amino acids at the haemagglutinin gene cleavage site

LPAI in wild birds can evolve into HPAI (usually H5 or H7 subtypes) after introduction into poultry, which can then represent a significant risk for human and animal health (Swayne, 2008; Verhagen *et al.*, 2015a). Biosecurity, including limiting the wild bird/domestic animal interface, is key to decreasing direct or indirect environmental contact, as there is no realistic way of reducing the occurrence of AIV in wild birds (Swayne, 2008). LPAI infections are often asymptomatic, and horizontal transmission occurs when different species of domestic poultry share the same ecosystem (Swayne, 2008).

Sequencing of the HA genes of H5 and H7 subtypes is used to determine the amino acid sequence at the HA_0 cleavage site. The presence of multiple basic amino acids at this cleavage site is one of the primary predictors of pathogenicity in Influenza A viruses (Croville *et al*., 2012). The difference between low pathogenicity and high pathogenicity viruses can be as simple as one amino acid change.

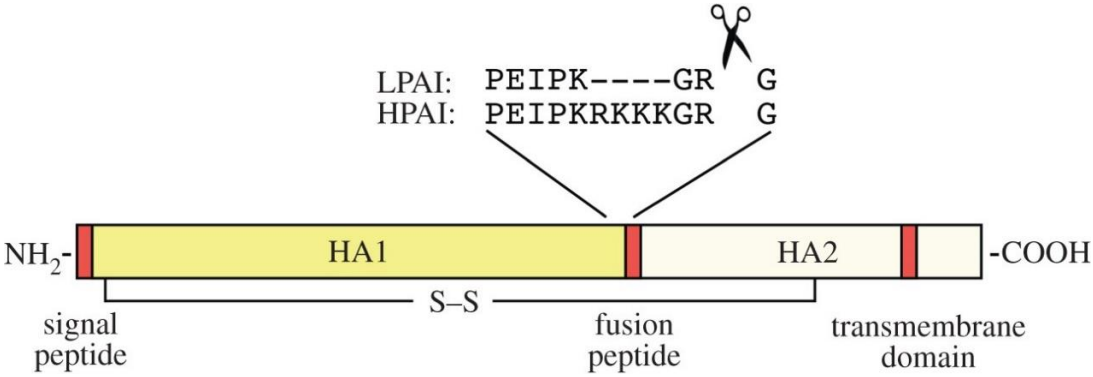

Figure 2 Schematic representation of a post-translational linear haemagglutinin glycoprotein depicting the HA_1 and HA_2 domains and indicating the disulphide bridge. The red boxes indicate regions that are involved in membrane interactions. Examples of cleavage sequences of LPAI and HPAI H5 viruses are presented. *Figure:* Lycett *et al., 2019*

Mono-basic cleavage sites only have one basic amino acid in the -1 position of the cleavage site (Fig. 3). These AIVs can only replicate in epithelial cells of the respiratory and intestinal tract lining, because their HA_0 is only cleaved by trypsin-like proteases that are secreted in these tissues (Abolnik *et al.*, 2016). They are LPAIVs. Because HPAIVs have multiple basic amino acids at their cleavage sites, they have the potential to replicate systemically in the host (OFFLU, 2019). The HA_0 of HPAIVs can be cleaved by

ubiquitous furin-like proteases found in many types of tissues, facilitating replication of the viruses in the respiratory and intestinal tract lining as well as other organs (Abolnik *et al*., 2016)**.**

Cleavage site sequences can be determined and compared to previously published sequences of HPAI viruses of known virulence, a list of which is maintained and periodically updated (OFFLU, 2019).
Strains that have cleavage sites similar to that of previously identified HPAIs are considered to be highly pathogenic regardless of chicken pathogenicity indicators (OIE, 2015).

1.5.3. Notifiable and controlled animal diseases

According to the World Organization for Animal Health (OIE), notifiable animal diseases are
those listed by the governmental veterinary authority of a country that, as soon as detected or suspected, should be brought to the attention of this authority, in accordance with national regulations (OIE, 2019b). The OIE's World Animal Health Information System (WAHIS) is an international platform for reporting notifiable diseases, and provides national veterinary services with information necessary to implement effective surveillance and disease control (OIE, 2018).

In the OIE Terrestrial Animal Health Code, notifiable avian influenza is defined as infection in poultry with an HPAI virus or with H5 and H7 subtypes of low pathogenicity (H5/H7 LPAI), or any influenza A viruses that are highly pathogenic in birds other than poultry, including wild birds. Some H9 viruses, although not notifiable, have still caused production losses in countries in Asia and the Middle East between September 2017 and February 2018 (OFFLU, 2018).

Official on-going surveillance for notifiable avian influenza (NAI) in South Africa is detailed in a Protocol for Compulsory Surveillance in South Africa to Prove Continued NAI Freedom (Horner and Pienaar, 2009). Commercial chicken establishments as well as commercial ostrich operations are tested at least twice a year. Testing comprises serological screening by ELISA for avian influenza antibodies, followed by molecular antigen detection (PCR) at serologically positive holdings.
Surveillance information, including passive surveillance of wild birds, is crucial to help define high-risk areas and populations. Detections of avian influenza, even HPAI, in wild birds alone does not however result in a country losing its HPAI-free status, and in such cases trade restrictions on poultry and poultry products are not justified (OIE, 2018).

Controlled diseases are those that require government control, because they affect individual animal owners, other farmers, the agricultural sector as a whole or consumers of animal products (Department of Agriculture, 2019). The following criteria identify controlled animal diseases:

• Zoonosis: the disease is transmissible to and able to cause disease in humans

• Highly transmissible: the disease may spread rapidly, independent of the actual movement of diseased animals and irrespective of farm boundaries

• Collective control: the disease is best managed by collective control strategies rather than by individual animal owners

• Threat to industry: the disease poses a potential serious threat to the performance of the South African agricultural industry

• Trade sensitive: the disease poses a potentially serious threat to South Africa's international trading status

In South Africa, a disease is classified as a controlled disease if it fulfils at least three of these criteria (Department of Agriculture, 2019).

1.6. Avian influenza virus infections in poultry

Because the main reservoir for AIV is wild aquatic birds, the complete eradication of AI is clearly not possible (Swayne, 2008). A major risk factor for domestic waterfowl and other poultry rearing is outdoor access to infected, free-living wild birds. Inter-species transmission is common in birds, especially when different species share the same ecosystem (Swayne, 2008). Poultry can be infected with AIV from free-living birds when directly exposed to the infected host (Swayne, 2008) . If the virus is poorly adapted, there is inefficient horizontal transmission**.** H5 and H7 subtypes of LPAIV poultry strains can mutate to form HPAI strains in poultry species which, if transmitted back to wild bird species, may enhance global spread (Suttie *et al*., 2019). Another significant source of the virus being introduced onto farms is the use of virus-contaminated, untreated surface water as drinking water for flocks of birds (Swayne, 2008). Some duck species can persistently shed viruses in faeces for 2-4 weeks (Webster *et al.*, 1992). Records indicate that LPAI from free-living waterfowl may remain infective in water sources for up to 207 days at 17ºC (Swayne, 2008). Virus infectivity is dependent on the strain, and the pH, salinity and temperature of the water (Webster *et al.*, 1992).

Other risks include exposure to contaminated fomites, human movements (contaminated hands and clothing) and contaminated airborne dust (Swayne, 2008). Influenza viruses seemingly do not persist in individual animals for extended periods (Webster *et al.*, 1992).

Overall, the most common source of introduction of avian influenza worldwide into poultry occurs at live bird markets (LBM). Virus-naïve birds are constantly being added, and since the viruses present are already adapted to poultry, the new birds are infected in an ongoing cycle (Swayne, 2008). LPAIs from wild birds are more adapted to domestic waterfowl than chickens and at LBMs co-mingling of different bird species, including domestic waterfowl, poses a serious risk.

Transmission of AIV is horizontal. Virus is shed from the conjunctiva, respiratory secretions and faeces into the environment, which is the source of most exposure (Swayne, 2008). Three factors determine the efficiency of transmission: 1) significant viral shedding (source, titre and duration), 2) environment (e.g. stability of the virus in water; organic matter that can protect the virus particles from inactivation), and 3) a low infective dose (Swayne, 2008).

Avian influenza typically manifests as one of two clinical diseases in poultry:

- Mucosal infection with LPAI, usually resulting in a drop in egg production and decreased food and water intake, or
- Systemic infection with HPAI, that may also cause decreased egg production, respiratory symptoms, rales, sinusitis, cyanosis of combs and wattles, head and face oedema, diarrhoea and nervous disorders (Webster *et al.*, 1992).

Disease or mortalities after experimental inoculation of chickens with AIV do not predict pathogenicity in other birds, animals or people (Perdue, 2008).

1.7. Avian influenza outbreaks in South Africa up until 2016

In April and May 1961, mass mortalities of common terns (*Sterna hirundo*) occurred along the Southern African coastline from Lambert's Bay in the west to Port Elizabeth in the east (Rowan, 1962). The outbreaks appeared to have started virtually simultaneously at various locations along this coastline, apparently affecting only common terns, and ceased abruptly. Ring recoveries from some of the birds indicated that they were between one and four years old and had been ringed in the Baltic and North Sea regions (Rowan, 1962). A virus was isolated that initially could not be identified. Field observations indicated that this infection likely spread within bird colonies at their breeding sites, usually shallow waterbodies, by the faecal-oral route (Rowan, 1962). The virus was called A/Tern/South Africa/1961 and later characterised as subtype H5N3 HPAI virus (Swayne, 2008).

Studies confirmed that avian influenza viruses from Eurasia are intermittently introduced into South Africa by wild birds (Abolnik, 2016). Avian influenza viruses have persisted in Southern African waterfowl for periods of up to six years, and all introductions of AIV in farmed ostriches in South Africa between 1991 and 2013 could be linked to viruses circulating locally in wild birds (Abolnik, 2016). Both in 2004 and 2006 in South Africa, LPAI H5N2 viruses from wild waterfowl were the likely precursors of HPAI H5N2 strains isolated from ostriches (Abolnik, 2007). Phylogenetic evidence also demonstrated that wild birds played a key role in the dissemination of H7N1 (LPAI) in ostriches in 2012 (Abolnik, 2016). Species such as African sacred ibis could have carried this strain between farms, and species such as feral pigeons may have been more localized vectors of fomites (Abolnik *et.al*, 2016). These authors also demonstrated that the 2013 H7N7 (LPAI) seems to have caused an outbreak in ostriches through a single introduction of the virus by wild birds, followed by its spread to other ostriches and locations by wild bird species (Abolnik *et al*., 2016).

Between 1991 and 2001, five LPAIV strains were detected in farmed ostriches in South Africa: H7N1 in 1991, H5N9 in 1994, H9N2 in 1995, H6N8 in 1998 and H10N1 in 2001 (Abolnik, 2007b). In June 2002, the first outbreak of AI (LP H6N2) in South African chickens was reported. It occurred in the KwaZulu-Natal province and comprised two distinct lineages (Abolnik, 2007b). Sub-lineage I only affected commercial chicken holdings, but sub-lineage II affected chickens and ostriches in that province as well as in Gauteng province. Sub-lineage 1 was detected up until March 2005 (Abolnik, 2007b). Evidently, it had most likely originated from ostriches (Abolnik *et al*., 2016). H6N2 is still endemic in South African chicken populations (Abolnik *et al*., 2016).

The first reported outbreak of HPAI in South Africa since the tern outbreak in 1961 was caused by an H5N2 AIV strain in 2004 (Abolnik, 2007a). It caused ostrich mortalities in the Eastern Cape province but did not spill over into poultry. Culling of 26 000 ostriches was carried out in an attempt to control the spread of the disease, and South Africa lost its disease-free status with the World Organization for Animal Health, resulting in trade restrictions, both of which led to serious economic repercussions (Abolnik, 2007a). Wild waterbirds were the probable source of the outbreak, as an LPAI H5N2 was isolated from an Egyptian goose in the neighbouring Western Cape province (Abolnik, 2007a). South Africa was declared disease-free in September 2005, but between June 2006 and August 2006 both HPAI and LPAI H5N2 outbreaks occurred in ostriches in the Western Cape province. Molecular investigations comparing these strains to the 2004 HP strain determined that the H5 genes of the 2006 strains were not directly derived from the 2004 strains (Abolnik, 2007).

Since 2004, AIV surveillance in wild and domestic avian species in South Africa led to the detection of numerous other subtypes of the virus including H1N8, H3N8, H4N2, H4N8, LP H5N1, H6N8, H9N2 and H10N7. An H5N8 strain was also detected in healthy swift terns in 2007 in the Mossel Bay area, but the HA_0 sequence was undetermined (Abolnik *et al*., 2010; Abolnik *et al*., 2016).

During 2011, the third outbreak of HPAI H5N2 occurred in ostriches in South Africa, and the viruses involved had no genetic links to the 2004 and 2006 strains (Abolnik *et al*., 2012). Phylogenetic analyses indicated a lack of reassortment and implied that the source was probably a single point introduction. The progenitor strain likely originated from wild migratory waterfowl from Eurasia and Southern Africa (Abolnik *et al*., 2012). The primary mode of transmission of viruses appeared to be the movement of infected ostriches. This outbreak of HPAI had a devastating effect on the local ostrich industry (Abolnik *et al*., 2012).

Between January 2012 and December 2014, numerous occurrences of AIV in ostriches were reported. The strains involved included H5N2 LPAI, H7N1 LPAI and H7N7 LPAI (Abolnik *et al*., 2016).

1.8. Influenza virus nomenclature

Influenza viruses isolated from non-human hosts are named according to standardised nomenclature (OIE, 2015) including the following descriptive elements:

2.1Antigenic type (e.g. Influenza type A, B or C)	**A**/...
2.2Host of origin (e.g. chicken)	A/**chicken**/...
2.3Geographic origin of the virus (e.g. city, country)	A/chicken/**South Africa**/.....
2.4Unique identification number	A/chicken/South Africa/**17010333**/....
2.5 Year of isolation	A/chicken/South Africa/17010333/**2017.....**
2.6HA and NA subtypes (for Influenza A viruses)	A/chicken/South Africa/17010333/2017**(H5N8)**

Isolates of human origin are named in the same way but exclude the "host of origin" component.

1.9. Clades and the origin of clade 2.3.4.4 Group B

In 1996 an H5N1 HPAI virus, referred to as the goose/Guangdong (Gs/GD) virus was isolated in China from a domestic goose. Its descendants have caused deaths in poultry, wild birds and humans and have spread to at least 80 countries in Asia, Europe, Africa and North America (Lee *et al*., 2017).

The HA gene segment has diversified by sustained global circulation and evolved into ten genetically distinct virus clades, and multiple second, third and fourth order subclades (Lee *et al*., 2017; Smith and Donis, 2015; Abolnik, 2019). The viruses in one of these subclades, the H5 clade 2.3.4.4b HPAI viruses,

have undergone genetic reassortment with local LPAI viruses circulating in wild bird populations, as well as with other clades of H5N1 viruses (Lee *et al*., 2017). Clade 2.3.4.4b HPAI H5Nx viruses have been found reassorted with at least six different NA segments (The Global Consortium for H5N8 and Related Influenza Viruses, 2016). Most of these reassortants were generated in domestic anseriforms (The Global Consortium for H5N8 and Related Influenza Viruses, 2016).

1.10. Migratory birds and their role in dissemination of Avian Influenza

Migratory birds can carry the viruses over great distances along their flyways (Verhagen *et al.*, 2015a). Egypt, for example, is one of the important stop-over sites for wild birds migrating across Europe, Asia and Africa along the East Africa/East Asia and Mediterranean/Black Sea flyways (Selim *et al.*, 2017). Birds following these two different migration routes, but co-mingling at such stop-over sites, are connected in time and space, facilitating the transmission of these pathogens to new areas (Olsen *et al*, 2006). Cumming *et al*. (2008) also noted the possibility that migratory or nomadic wild birds could disseminate HPAI as well over long distances (Cumming *et al*., 2008). This is particularly likely when these migrants carry pathogens that do not significantly affect their health status and therefore their ability to migrate (Olsen *et al.*, 2006). Direct contact with wild birds or indirect contact with materials contaminated with wild-bird faeces were shown to be the most likely source of introduction of H5N8 HPAI into poultry holdings, and not personnel contacts, contaminated water or trade of live animals, animal products or feed (The Global Consortium for H5N8 and Related Influenza Viruses, 2016). The clade 2.3.4.4b H5N8 virus initially spread along two main long-distance migration routes by infected migratory wild birds in 2014: one from East Asia north to the Arctic coast of Eurasia, then west to Europe, and the other from the Korean peninsula northward, then east across the Behring Strait and south along the coast of the North American continent. There was no indication of spread between Europe and North America (The Global Consortium for H5N8 and Related Influenza Viruses, 2016).

1.11. Intercontinental waves of highly pathogenic Avian Influenza virus and the clades involved

Four main intercontinental waves of (HP) AIV of the Gs/GD lineage have been identified of which three (the first, third and fourth waves) also spread to Africa (Sims *et al*., 2017).

The first wave started in 2005 and was caused by Clade 2.2 H5N1 and derivatives (Sims *et al*., 2017). It spread in domestic birds in Asia, the Middle East, Europe and North and West Africa and also caused human infections and deaths (Sims *et al*., 2017). This strain became endemic in Egypt after its introduction there and has since diversified into further sub-clades (Mohamed *et al*., 2019).

The second wave, clade 2.3.2.1c H5N1 viruses, affected poultry and wild birds in Asia and Eastern Europe from 2009 to 2010 (Sims *et al*., 2017). It was initially detected in birds in China and it was a relatively mild wave, which did not persist outside Asia. It reached Korea in the 2013-2014 winter, but it was not the dominant strain. A sub-lineage of H5N8, the 2.3.4.4 Group A or "Buan-like" viruses, became established there (Sims *et al*., 2017). This was the start of the third intercontinental wave, called wave 3a. These Group A viruses circulated in Europe and Asia in 2014 (Sims *et al*., 2017). For the first time a Eurasian HPAI virus spread to North America (The Global Consortium for H5N8 and Related Influenza Viruses, 2016) and reassorted with local wild bird strains of influenza viruses (Sims *et al*., 2017). Concurrently, wave 3b involving H5N1 HPAI clade 2.3.2.1c viruses that were different from the 2009 strain were detected in Russia, the Middle East, West Africa, Eastern Europe and then India. This strain became endemic in Nigeria (Sims *et al*., 2017).

In June 2016, a clade 2.3.4.4 H5N8 HPAI virus detected in the southern Russian Federation marked the start of the fourth wave. Viruses comprising this wave form part of a sub-lineage sometimes referred to as the 2.3.4.4 Group B or "Gochang-like" viruses. They were first detected in China in 2010 (Sims *et al.*, 2017). They are distinct from the Group A or "Buan-like" strains that circulated in Europe in 2014 (Sims *et al*., 2017). The movement of these Group B viruses has been associated with wild bird infections and long distance migrations (Sims *et al*., 2017). This intercontinental wave of outbreaks of Gs/GD lineage H5 HPAI has been more severe in terms of the number of countries affected than the previous intercontinental waves (Sims *et al*., 2017). The 2016-2017 H5N8 viruses reassorted with other LP viruses in wild birds at that time, and some novel strains that included N5 and N6 genes instead of the N8 gene, resulted. The common feature of these H5N8, H5N5 and H5N6 strains is the HA gene, which can be linked back to Gs/GD (Sims *et al*., 2017). A wide range of bird species may be infected and/or transmit clade 2.3.4.4b viruses. Infected birds generally exhibit clinical disease, mortality and pathological features typical of HPAI virus infection, although with reduced virulence when compared to the ancestor Gs/GD H5N1 virus (Lee *et al*., 2017; Sims *et al*., 2017). Of major concern is the tendency of this HA gene to form novel sub-types capable of rapid and global spread (The Global Consortium for H5N8 and Related Influenza Viruses, 2016; Lee *et al*., 2017).

1.12. The global spread of H5N8 Clade 2.3.4.4b HPAI viruses

New subtypes of Asian lineage Gs/GD lineage H5 HPAI viruses belonging to clade 2.3.4.4, like H5N2, H5N5, H5N6 and H5N8 have evolved into subclade 2.3.4.4 Groups A to D. They all originated in China from late 2012 (Lee *et* al., 2017). In 2014, the first outbreaks were reported outside China. A wide range of bird species can be infected and/or transmit clade 2.3.4.4 viruses. The viruses have reassorted with prevailing LPAI viruses and other clades of H5N1 and spread globally in migratory aquatic birds (Lee *et al*., 2017).

Group A consists of H5N8 viruses detected in Asia, Canada, USA and Europe in 2014-2015. Group B strains re-emerged in Qinghai, China in May 2016 (Antigua *et al*., 2019) after their initial detection in 2010 (Sims *et al*., 2017). They reassorted in China with five Eurasian LPAI viruses' segments (PB2, PB1, PA, NP and M) and were detected one month later in Siberia (Lee *et al.*, 2017). There were strong indications that wild waterbirds introduced the virus to the southern Russian Federation due to the absence of domestic poultry in that vicinity (Sims *et al.*, 2016).

On three previous occasions (2005/06, 2009/10 and 2014/15), detections of Gs/GD lineage H5 viruses in south-central parts of Siberia in the Russian Federation occurred prior to detections in avian species further south and west (Sims *et al.*, 2016). This lake is situated in the Ubsu-Nur endorheic basin on the border between Southern Russian Federation and Mongolia. It is a vast wetland and an important stop-over, breeding and moulting site for migratory waterfowl on the Central Asian Flyway (Sims *et al.*, 2016). Several species of wild waterbirds were infected, including black-headed gulls (*Larus ridibundus*), grey herons (*Phalocrocorax carbo*), a great-crested grebe (*Podiceps cristatus*), and a common tern (*Sterna hirundo*) (Sims *et al.*, 2016).

The FAO issued an EMPRES (Emergency Prevention System for Transboundary Animal and Plant Pests and Diseases) article in September 2016 detailing the Russian incursion, and warning that countries to the West and South of Lake Ubsu-Nur should be on high alert for possible introductions of this virus (FAO, 2016). This indeed transpired, and from May 2016 to June 2017 it had spread to 48 countries across Europe and Asia (including the Russian Federation and areas around the Mediterranean) and to at least nine African countries (Sims *et al.*, 2017). Occasional incursions into poultry holdings and captive bird collections also occurred, in some cases possibly because of gaps in farm biosecurity (Globig *et al*., 2018). The FAO re-iterated that initial signs of incursion may actually be the first mortalities in domestic poultry, as the virus is not always fatal for wild birds (FAO, 2016).

Whereas the first outbreaks were often large-scale die-offs, later only single cases were reported (Sims *et al.*, 2017). At least 53 species of wild birds were affected (with mostly the H5N8 subtype), including waterbirds and scavengers (Globig *et al*., 2018). Recommendations were given for intensified passive surveillance, increased public awareness of the importance of biosecurity and prompt reporting of dead wild birds (FAO, 2016).

In Europe, from the June 2016 detection in Siberia up to August 2017, 2067 outbreaks of H5N8 were reported to FAO, including 1112 in poultry and 955 in wild birds (Napp *et al.*, 2018). It resulted in the largest HPAI epidemic in Europe (Napp *et al.*, 2018). In many affected countries, it was first detected in wild birds by passive surveillance (Sims *et al.*, 2017).). The wide host range, and the fact that waterfowl and migratory birds may act as asymptomatic carriers of this lineage, may make their spread unrecognizable, which could explain the success of the recent global spread of these viruses (Lee *et al*., 2017). Although secondary spread between poultry farms in some European countries appears likely, many outbreaks have been caused by several primary incursions into territories (Sims *et al.*, 2017).

Group C viruses are H5N6 strains that were first detected in China and Laos in 2013-2014 and H5N1 viruses from China and Vietnam in 2014 (Lee *et al*., 2017). Group D are H5N6 viruses that were identified in China and Vietnam in 2013/2014 (Lee *et al*., 2017).

1.13. Incursions of clade 2.3.4.4b H5N8 HP avian influenza viruses in Africa

The fourth intercontinental wave of HPAI is the first to spread as far south as Equatorial and Southern Africa, other than one case in Southern Sudan in 2006 (Sims *et al.*, 2017). Ultimately this has been the most severe panzootic in terms of the number of countries affected, extending even to Eastern and Southern Africa (Sims *et al.*, 2017). It has also been more severe than previous waves in terms of the number of wild birds, farms and zoological collections affected (Sims *et al.*, 2017). It has occurred in a range of intensive and extensive farm production systems and avian species (Sims *et al.*, 2017)

In the Nile Delta from February to May 2017, high mortality rates in poultry flocks were reported and H5N8 was isolated from four flocks (Salaheldin *et al.*, 2018). The full genomes of 3 chicken and one duck virus were sequenced and compared to previous H5N8 strains from Egypt and others from Eurasia (Salaheldin *et al.*, 2018). Results suggested that four different introductions of H5N8 virus from Europe, Russia, and Asia into poultry in Egypt occurred (Salaheldin *et al*, 2018). The first incursion of H5N8 was detected in common coots (*Fulica atra*) during active surveillance of migratory birds at a live bird and

fish market in November 2016 (Selim *et al.*, 2017). Molecular investigation of the HA and NA gene segments of these isolates confirmed that wild birds had indeed introduced this virus from Europe into Egypt (Selim *et al.*, 2017)**.** Clade 2.3.4.4b H5N8 viruses have since been detected in Tunisia, Nigeria, Niger, Cameroon, the Democratic Republic of the Congo, Uganda, Zimbabwe, and South Africa (Sims *et al.*, 2017).

The spread of this current panzootic has been ascribed to the seasonal migration of wild aquatic birds (Khomenko *et al*., 2018). However, in contrast to those in more northern, temperate latitudes, migration, and movement patterns of wild waterfowl in Southern Africa are unpredictable and can vary according to rainfall patterns. This, together with the availability of safe annual aggregation sites, results for the most part in a lack of predictable anatid flyways (Cumming *et al*., 2008). The lack of topographical barriers that channel bird movements in Asia and the Americas also makes movements less predictable in Southern Africa (Cumming *et al*., 2008). Eurasian migrating birds can stop over at West African wetlands before crossing the equator and travelling to Southern Africa (Abolnik *et al.*, 2016). Although some of these bird species do not actually travel that entire distance, stop-over sites facilitate the intermingling of intra-Africa migrant species. Consequently, these intra-African movements of African duck species can also facilitate their close contact with Palaearctic-breeding species at wetlands in Sub-Saharan Africa (Cumming *et al*., 2008). Such potential mixing areas include regions near the Senegal and Niger Rivers, floodplains of the Niger River, and Lake Chad (Olsen *et al.*, 2006). The mixing of these viruses and co-infections with different strains creates opportunities for reassortment of AIVs due to the segmented nature of the genome (Abolnik *et al.*, 2016). The 2016 H5N8 HPAI viruses from the Tyva Republic were reassortant strains. Three genes (HA, NA, and NS) were consistent with those of previously characterized H5N8 viruses in the same clade, and the other five genes were acquired from LPAI viruses circulating in wild birds (Sims *et al.*, 2017).

Only four of the 12 species of Palaearctic-breeding anatids reach Southern Africa, and only as vagrants, so direct transmission of AIV from those species to southern African ducks would require interspecies transmission between the two groups at East African wetlands and movement of infected local birds to Southern Africa (Cumming *et al*., 2008). Coastal migrants here, such as terns, rarely make contact with humans or poultry. There is the possibility that granivorous passerines could link waterbirds to poultry by their patterns of foraging near or with poultry, and then roosting, breeding, or drinking at wetlands (Cumming *et al*., 2008). These are important opportunities for LPAI virus transmission between different species as well as between wild and captive birds (Olsen *et al.*, 2006).

Evidence suggests the H5N8 HPAI virus was introduced into Zimbabwe and South Africa by wild birds (Sims *et al.*, 2017). Wild birds were also responsible for its subsequent dispersal (Sims *et al.*, 2017; Abolnik, 2019) The role of migrating wild birds in moving and transmitting Gs/GD lineage viruses has once again been irrefutably established during evaluation of the fourth intercontinental wave of HPAIV (Sims *et al.*, 2017). No northward long-distance transmission of Gs/GD lineage H5 viruses from Egypt or West Africa by migratory birds, even endemic virus strains, has however been recorded (Sims *et al.*, 2017).

1.13.1. Sub-Saharan Africa: Uganda

Major mortalities, estimated at about 60% of a population of about 2000 white-winged terns (*Chlidonias leucopterus*) were reported in Uganda along the shores of Lake Victoria in January 2017 (FAO, 2017). A spillover to domestic birds in Kachanka Village was also detected, and more wild birds died in the Kalangala district, although the cause of death of these was unconfirmed. This area is situated on a major bird migration flyway, the East Asian – East African migratory route (FAO, 2017). The five wetlands of Lake Victoria (Lutemba Bay, Mabamba Bay, Lake Nabogabo, Nabajjuzi and the SAMUKA wetland system) are important stopping points and seasonal shelters for breeding and over-wintering Palaearctic and Afro-tropical migrants, and the presence of HPAI is a significant factor that can contribute to the further spread of these viruses (FAO, 2017). There are fluctuations in the number of waterbird species that occur there, which follow seasonal patterns. Highest counts occur from December to March, and Palaearctic wintering gulls, terns, waders, and white winged terns constitute 70% of the population (FAO, 2017).

Figure 3 White-winged tern (*Chlidonias leucopterus*) [Photo: www.birds.kz]

Breeding of white-winged terns overlap with H5N8 HPAI outbreak areas in countries north of Africa in 2016. This species could have contributed to the incursion of H5N8 HPAI into Uganda, although the role of other wild bird species, e.g. Anatidae, must also be considered (FAO, 2017).

Concerns were raised that the arrival of this H5N8 HPAIV virus in sub-Saharan Africa could potentially result in its further dispersal within these countries (Khomenko *et al*., 2018).

1.13.2. Clade 2.3.4.4b HPAI H5N8 outbreaks in Namibia

Early in January 2019, mass mortalities of African penguins were observed at a breeding colony on Halifax Island on the Southern Coast of Namibia (Umberto *et al.*, 2020) (OIE, 2019). By late February, more than 350 penguin carcasses had been found, making this the most severe mortality event of penguins in Namibia on record (Umberto *et al.*, 2020). Viral RNA was extracted directly from cloacal swabs and a liver sample, and Influenza virus A matrix genes, neuraminidase (N8) genes and haemagglutinin (H5) genes were detected by RT-qPCR (Umberto *et al.*, 2020). Amplicons were sequenced, analysed and then visualised in phylogenetic trees (Umberto *et al.*, 2020). The viruses were confirmed to be HPAI H5N8 Clade 2.3.4.4b viruses (Umberto *et al.*, 2020), and the outbreak was reported to the OIE on 7 February 2019 (OIE, 2019). No other resident wild-bird species on Halifax Island, including swift terns, crowned cormorants, Hartlaub's gulls and African oystercatchers displayed signs of this disease (Umberto *et al.*, 2020).

1.13.3. Introduction of Asian lineage HPAI H5 into poultry and wild birds to South Africa in 2017

In May 2017, the first ever detection of Asian lineage H5 HP Gs/GD clade 2.3.4.4b avian influenza virus was reported in the Southern Rift Valley in Zimbabwe. The first reported outbreak of clade 2.3.4.4b H5N8 HPAI in South Africa occurred on 19 June 2017. This was the first notifiable AI outbreak in gallinaceous poultry ever reported in the country, and the species that would ultimately be affected would include wild birds, commercial poultry and ostriches, backyard poultry and zoological collections (Abolnik *et al*., 2019; Khomenko *et al*., 2018). Phylogenetic analyses confirmed that the first two index cases in Villiers and Standerton, which are 35km apart, were independent introductions of two distinct variants. There were no epidemiological links between the two events. Additionally, neither of these two strains was directly related to the strain that caused the outbreak in Zimbabwe (Abolnik, 2019) or to the viruses isolated in Uganda (Fusaro *et al*., 2019). They are more closely related to viruses isolated in Cameroon and Niger, suggesting independent introduction events (Fusaro *et al*., 2019). Reassortment with Southern African LPAI strains and viruses from West Africa was also demonstrated (Abolnik et al., 2019).

Through on-going routine surveillance, the first outbreak of H5N8 HPAI in the Western Cape province was detected on 2 August 2017 on an ostrich farm (Khomenko *et al*., 2018). Apart from the devastation that it caused in the poultry industry (approximately 70% of commercial layers were lost and 66% of the ostrich farms were quarantined (Roberts, 2018b)), it was also detected in various wild bird species.

These included pigeons (*Columba livia domestica*), spur-winged geese (*Plectropterus gambensis*), helmeted guineafowl (*Numida meleagris*), doves (Columbidae), blue cranes (*Anthropoides paradiseus*), house sparrows (*Passer domesticus*), Peregrine falcons (*Falco peregrinus*), pied crows (*Corvus albus*), sacred ibis (*Threskiornis aethiopicus*), black-headed herons (*Ardea melanocephala*) and Egyptian geese (*Alopochen aegyptiaca*) (Roberts, 2018a). The first coastal bird confirmed to be infected with H5N8 HPAI in South Africa was a swift tern (*Thalasseus bergii*) in the Western Cape, which was sampled on 20 December 2017, and was reported to the OIE on 8 January 2018 (OIE, 2018b).

By the end of 2017, 64 poultry outbreak reports had been submitted (OIE, 2017). Poultry outbreaks abated somewhat in 2018 to bring the total number of reports to 93 (OIE, 2018b) with the virus continuing to circulate in the Western Cape, North-West and Gauteng provinces (Abolnik, 2019). The 2017 South African outbreaks were caused by four reassorted genotypes in the central and Northern regions of South Africa, but by a sole fifth genotype in the Western and Eastern Cape provinces (Abolnik, 2019). When the virus reappeared in poultry between February and April 2018 after a few months' absence, phylogenetic analysis indicated that a previously undetected sixth genotype was involved, which was most likely only introduced into the region in 2018 (Abolnik, 2019).

The last outbreak in coastal birds was reported in May 2018 (OIE, 2019). As of 16 August 2019, the total number of reported outbreaks of this disease, including all avian species, was 104 (OIE, 2019).

1.14. Wild bird ecology

1.14.1. Terns

Terns are classified in the family Laridae, along with other genera which include gulls, skimmers, and noddies, and are found worldwide (Ryan, 2017). Terns breed in various types of habitats including islands, beaches and wetlands (Ryan, 2017). Most terns are migratory, but some are nomadic, their movements prompted by the availability of seasonal food resources (Ryan, 2017). Those that migrate over long distances often stop over at suitable resting sites to feed (Ryan, 2017).

Swift terns (*Thalasseus bergii*) are endemic to Southern Africa and breed in colonies along the coast from Namibia to the Eastern Cape province of South Africa (Ryan, 2017). They migrate locally and are common residents, and they sometimes share breeding locations with gulls (Ryan, 2017). Other sub-species breed similarly in regions including Namibia, Mozambique, East Africa, northern parts of the Indian Ocean and Australia (Allport, 2018).

Common terns (*Sterna hirundo*) are abundant visitors from their North-Western Palaearctic and Baltic breeding grounds, migrating to Southern Africa between August and April (Allport, 2018). Some also breed in areas in Senegal, Nigeria, and Cameroon, and migrate south across the equator to wetlands and the coasts of Southern Africa (Ryan, 2017).

Most Sandwich terns (*Thalasseus sandvicensis*) migrate to estuaries and bays in Southern Africa from breeding grounds in Western Europe, but some may remain here all year round (Ryan, 2017).

Figure 4 Bird species sampled in this study. (a) Crowned cormorant (*Microcarbo coronatus)* [Photo: © 2019 Buckham Birding], (b) Hartlaub's gull (*Chroicocephalus hartlaubii)* [Photo: Heyn de Kock, 2014], (c) African penguin (*Spheniscus demersus*) [Photo: www.aquarium.co.za], (d) Cape cormorant (*Phalacrocorax capensis*) [© 2019 SA Country Life | Caxton Magazines Digital], (e) Sandwich tern (*Thalasseus sandvicensis*) [Photo: Tringa photography www.tringa.org] (f) Swift tern (*Thalasseus bergii*) [Photo: ADU UCT (www.adu.uct.ac.za)], (g) Common tern (*Sterna hirundo*) [Photo: www.hbw.com], (h) Jackal buzzard (*Buteo rufofuscus*) [Photo: Greg Morgan, Cape Bird Club], (i) African oystercatcher (*Haematopus moquini*) [Photo BirdLife Port Natal]

1.14.2. Penguins, gulls, cormorants and oystercatchers

African penguins occur and breed endemically along the Southern African coastline from central Namibia to Algoa Bay (Ryan, 2017). Some birds from the Western Cape disperse up the west coast, and juveniles from Western Cape colonies may migrate to Namibia (Ryan, 2017).

Hartlaub's gulls are endemic to the Benguela upwelling region and are common residents (Ryan, 2017). They breed on offshore islands, large buildings at the coast and up to 30km inland at coastal wetlands (Ryan, 2017).

Cape cormorants occur along the coast from Southern Angola to Algoa Bay. They breed colonially and breeding adults that are feeding chicks may travel up to 60km from their colonies in search of schools of pelagic fish (Ryan, 2017). Crowned cormorants breed along the coast mainly from Central Namibia to Cape Agulhas and are largely non-migrants, but juveniles may disperse up to 500km. They seldom travel more than 10km offshore in search of food (Ryan, 2017).

African oystercatchers are monogamous and establish territories for breeding sites which they defend for life (Underhill, 2014). They occur almost exclusively along the coasts of Namibia and South Africa, but their breeding range only extends from southern Namibia to southern KwaZulu-Natal (Underhill, 2014). Adult birds do not migrate as such due to mesic conditions at their breeding sites, but immature and pre-breeding birds disperse from their natal hatching sites (Rao *et al.*, 2014). This dispersal reduces competition for food and space between juveniles and their parents at breeding sites. A study conducted in 2004-2005 established that 65% of the birds studied dispersed an average distance of 1595km from their natal departure sites in three regions between Lambert's Bay and Dassen Island, north to roosting sites along the entire west coast of South Africa and Namibia (Rao *et al.*, 2014). These dispersal sites are used over multiple years and may also be used as stop-over sites (Rao *et al.*, 2014). Varying numbers of mature birds (2-4yrs) were observed returning to establish their breeding territories in the vicinity of their natal sites (Rao *et al.*, 2014).

1.15. Zoonotic avian influenza viruses

Influenza A viruses are widespread and can infect many avian and mammalian species including humans. Influenza A is also the only genus of influenza viruses that is known to infect birds (OIE, 2018). It is estimated that the "Spanish flu" H1N1 influenza virus pandemic of 1918 could have contributed to the deaths of about 50 million people (Lycett *et al.*, 2019). The next two human pandemics, Asian flu in 1957 and Hong Kong flu in 1968, H2N2 and H3N2 strains respectively, were caused by reassortant viruses that contained glycoprotein genes of avian origin (Lycett *et al.*, 2019). In 2009 the fourth human

pandemic, “swine flu”, arose which was caused by an H1N1 strain. This strain also contained segments of avian origin within its precursor swine reassortant viruses (Lycett *et al.*, 2019).

The highly pathogenic avian influenza (HPAI) viruses that emerged from Guangdong province in China in 1996, Gs/GD, caused millions of deaths among avian species (Fusaro *et al*., 2019). They contain gene segments that most likely originated in birds (Vandegrift *et al.*, 2010), and also caused more than 454 human deaths, demonstrating the links between avian and mammalian influenza infections (Fusaro *et al*., 2019). Since then, with ongoing global circulation, these viruses have evolved rapidly, forming many distinct genetic clades and sub-clades. The gene segment encoding the haemagglutinin gene of the Gs/GD virus is also an ancestor of the HPAI H5N8 viruses (The Global Consortium for H5N8 and Related Influenza Viruses, 2016).

The risk of zoonotic transmission of avian influenza viruses is considered to be very low, however the potential danger of these viruses warrants adequate precautionary measures, including the use of personal protective equipment, for people that are likely to be exposed to the virus (ECDC, 2016). The wild bird/domestic animal interface provides ideal conditions for the continuous introduction and spread of AIV, but infected domestic birds and animals are the more likely source of human infections than wild animals (Perdue, 2008)(Swayne, 2008). Although both H5N1 and H5N8 subtypes of HPAI have been identified as having pandemic potential (ECDC, 2016; FAO, 2018) subtype H7N9 is viewed as the strain most likely to cause a significant pandemic in people (OFFLU, 2018).

In 2017, a survey was conducted in South Africa to investigate the transmission of H5N8 clade 2.3.4.4b AIV from infected birds to humans (Valley-Omar *et al.*, 2020). The people selected for inclusion in the study were in close contact with infected birds or carcases and regarded as being at a potentially higher risk of being infected by the virus, due to occupational exposure. Of the 74 people that were tested, none was positive for the virus, which demonstrates a low risk of human infections (Valley-Omar *et al.*, 2020). Although H5N8 viruses that were isolated possessed known molecular markers of adaption to mammals, this alone may not be enough to enable adaption to humans (Valley-Omar *et al.*, 2020). At the time of writing, no human infections with clade 2.3.4.4b H5N8 HPAI viruses of the fourth intercontinental wave have been reported, and molecular analysis has shown no specific increased affinity of these viruses for humans (ECDC, 2016; Sims *et al.*, 2017; FAO, 2018).

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample collection

Wild birds suspected to be infected with HPAI (H5N8) were collected in the Western Cape province. Staff of conservation authorities, rehabilitation centres, private veterinarians and the Western Cape Department of Agriculture were requested to notify the researcher of any suitable carcases. The locations of wild coastal birds reported to the OIE are indicated with green dots in Figure 5 below.

A sample submission form (Addendum A) was provided for completion by the person submitting samples, to give brief guidelines on sample collection and transport and to ensure that adequate case histories were provided with each case. Information requested included bird species, condition and/or clinical signs, geographic location, and collection date and circumstances. Sample collection equipment (sterile sample jars, icepacks and cooler boxes) was also distributed if requested.

Submissions representing a variety of coastal bird species affected and a variety of geographical regions were encouraged where possible.

Moribund birds, whole bird carcases or tissue samples were submitted to the Provincial Veterinary Laboratory (PVL) in Stellenbosch, either directly or via State Veterinary Animal Health Technicians.

The laboratory is accredited by the South African National Accreditation System (SANAS) (laboratory number V0029) according to ISO17025. Collection of samples for virological analyses was carried out at the PVL by State veterinarians. Photographs of the birds (front and back with wings extended and tail spread open, and with a size indication) were taken where possible to aid and confirm identification of the species. Brain, oropharyngeal and cloacal swab samples were collected during *postmortem* examination using nylon flocked swabs with plastic shafts (80mm Copan FLOQSwabs™, Copan Diagnostics Inc., Murrieta, CA, USA). Pooled samples of organs (usually liver, spleen, lung, trachea and/or brain) for virus isolation were also collected for later virus isolation, if required.

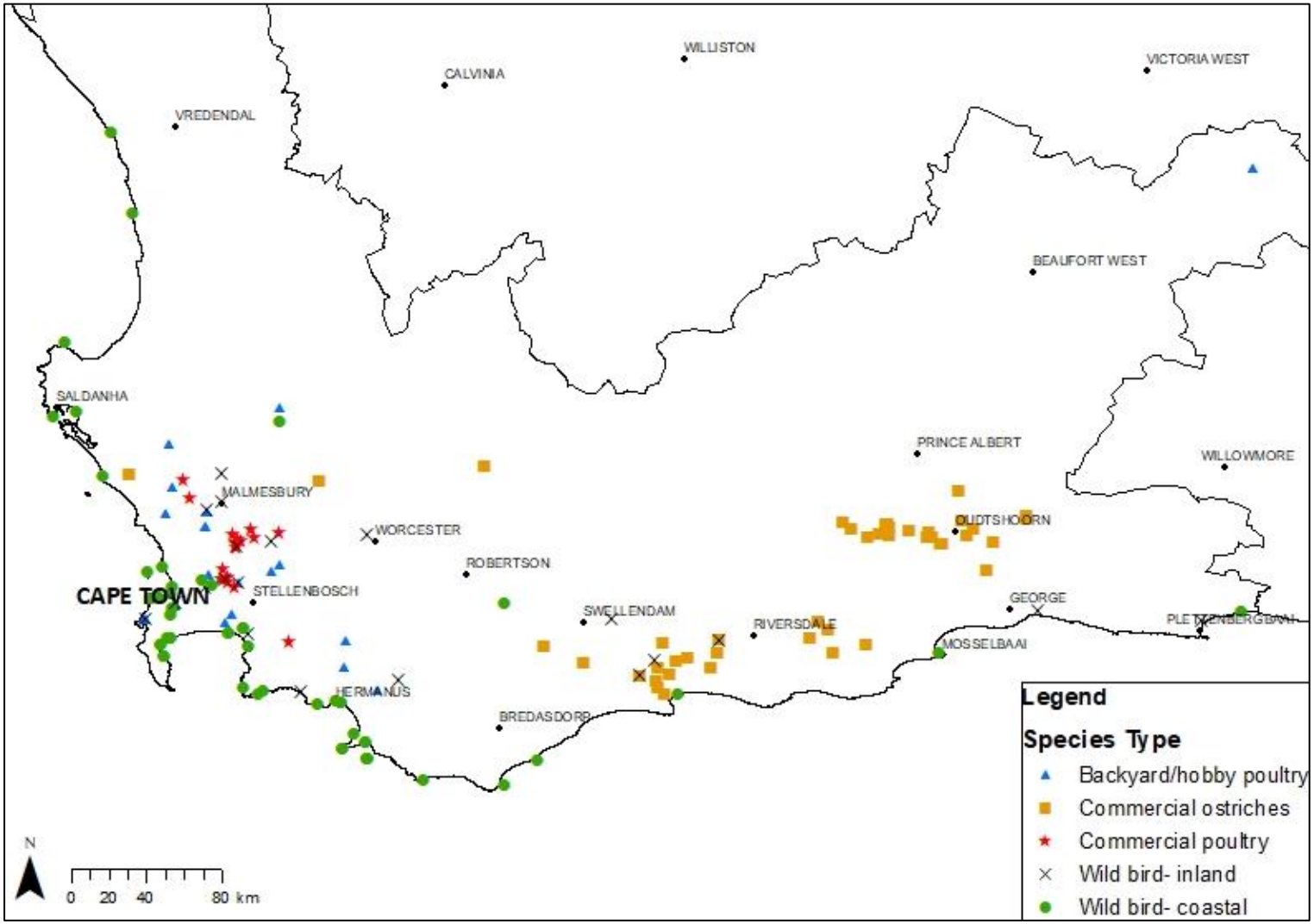

Figure 5 Distribution of HPAI (H5N8) cases (domestic and wild birds) in the Western Cape reported to the OIE from August 2017 to June 2018 [*Map: L Roberts*]

2.2 Screening of samples

Oropharyngeal and cloacal swabs were pooled (per bird) and tested separately from the brain swabs. Swabs were vortexed in ±0.5 ml sterile PBS/Glycerol (50% v/v) per swab. The samples were then screened for the detection of H5N8 RNA using Real-time RT-PCR.

Viral RNA was extracted manually from 150 µl of each swab fluid sample according to the WCPVL standard operating procedures as described by Abolnik *et al*., 2019. Extractions were carried out using the Machery-Nagel Nucleospin® RNA Virus kit, cat.no. 740956.250 (Macherey-Nagel GmbH & Co. KG, Neumann Neander Str. 6-8, D-52355 Düren, Germany) or QIAcube HT® automated extraction system with the cador® Pathogen 96 QIAcube® HT kit (Qiagen), according to the manufacturers' instructions.

Real-time RT-PCR was performed on the RNA extracts using the VetMAX™Gold AIV detection kit (Thermo Fisher: Life Technologies, Cat. no. 4485261) according to the manufacturer's protocol. The assay detects the matrix protein 1, matrix protein 2 and nucleoprotein genes of influenza A viruses.

AIV H5 subtype detection was carried out using the primers and probes described by Slomka *et al*. (2007) as recommended by the Animal and Plant Health Agency (APHA) (2014). The N8 primers and probes described by Hoffmann et al., 2016, and modified as recommended by APHA (2016) were used for N8 subtype detection.

Table 2 Sequences of primers and probe for N8 subtype AIV detection.

Primers/probe	**Sequence**
N8 2016F	5'- YCC CTG YTT TTG GGT CGA AAT GAT -3'
N8 2016R	5'- GCT CCA TCG TGC CAT GAC CA -3'
N8 2016Pro	5'- /56-FAM/TCT AGT AGC /ZEN/TCC ATT GTA ATG TGT GGA GT/3IABkFQ/ -3' *

* ZEN™ *is an internal quencher developed by IDT (Integrated DNA Technologies, Coralville, Iowa, USA)*

2.3 Virus isolation and culture

H5N8 PCR-positive swab samples identified the positive birds, and virus isolations were performed on the corresponding organ pool samples collected from those birds.

Virus isolation was carried out in specific antibody negative (SAN) embryonated white Leghorn hens' eggs according to the method described by the OIE (OIE, 2018). The tissue samples were finely macerated and 10-20 % (v/v) suspensions were prepared in a buffered lactose peptone solution (3.02 g Na_2HPO_4, 0.44 g KH_2PO_4, 10 g bacteriological peptone (Oxoid Ltd, Basingstoke, England), and 50 g lactose per litre). Sample suspensions were centrifuged (1200 rcf, 10 min) and antibiotics were added to

1ml of supernatant (Baytril® [Bayer] 0.75%, Gentamycin sulphate 0.05 %, Amphotericin-B 0.025 %) to suppress bacterial and fungal growth. These suspensions were inoculated into 9 to 11-day-old embryonated eggs. Eggs were incubated for 4 to 7 days and candled daily. Pooled amnio-allantoic fluid (AF) was harvested from all dead eggs and from any live eggs remaining at the end of the incubation period.

Haemagglutination (HA) tests were performed to detect haemagglutinating virus particles. Two-fold serial dilutions of amnio-allantoic fluid in PBS were prepared in 96-well microagglutination plates with V-shaped wells, to a total volume of 25 µl. Chicken red blood cells were collected from donor SAN Leghorn roosters, washed with PBS and suspended to a final concentration of 1% in PBS. 25 µl of this suspension was added to each well of the microtitre plates, and the plates were incubated at room temperature for 30 minutes. A sharp button of red blood cells seen at the bottom of a well indicated agglutination. HA positive samples showed a diffuse film of red blood cells, no button or a very a small button of red blood cells at the bottom of the well.

Harvested AF was also screened to be free of bacteria by microbiological assay using Columbia blood agar (Oxoid Ltd, Basingstoke, England). HA neutralization with specific virus positive antisera was carried out to exclude avian paramyxoviruses. AF was stored at 2-8ºC (up to 7 days) or -80ºC (prolonged storage) according to standard laboratory test methods.

Each amnio-allantoic fluid virus culture was submitted to an independent laboratory (ARC-Onderstepoort Veterinary Research laboratory, Biotechnology Division) for confirmation by real-time RT-PCR. This laboratory is the official national reference laboratory approved by the South African National Department of Agriculture, Forestry and Fisheries (DAFF) for molecular testing. Vetmax reagents, and primers and probes described by Naguib *et al*. 2017 (HP H5 AIV) and by Hoffmann *et al*. 2016 (N8 AIV) were used.

Confirmed H5N8 HPAI virus cultures were selected for further molecular analysis for this study, representing a variety of bird species affected and geographical regions where the birds were found (Fig. 6).

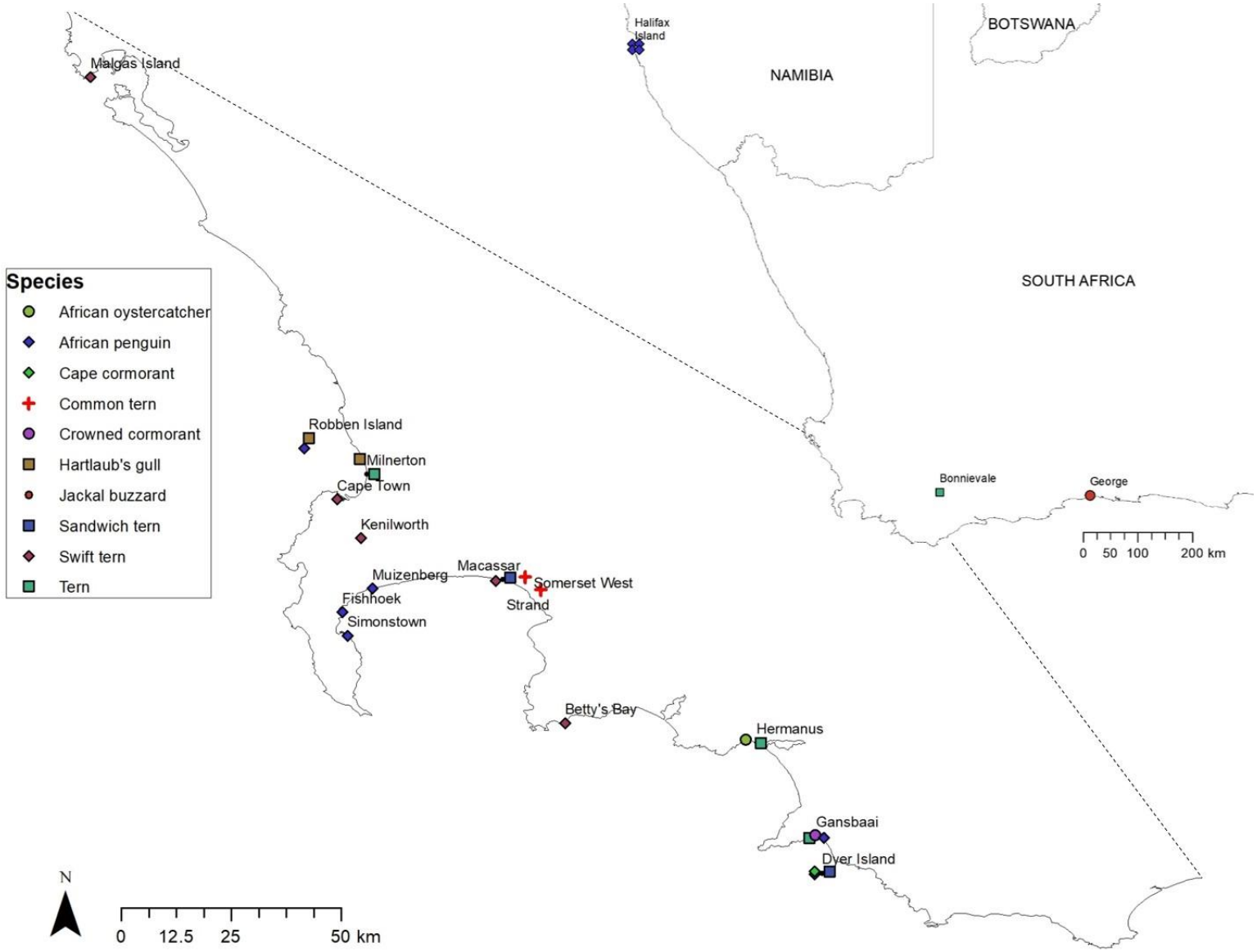

Figure 6 Distribution map of coastal bird species from which viruses were included in this study [*Map: L Roberts*]

2.4 Ion Torrent Next Generation Sequencing

Extracted RNA from the virus cultures was submitted to the Central Analytical Facility of the University of Stellenbosch (Central DNA Sequencing Facility, Rm B102, Block B, JC Smuts Building, Stellenbosch) for Ion Torrent sequencing of complete genomes on the Ion Proton™ system, as described by Abolnik *et al.*, 2019. The RNA samples were evaluated for integrity on a BioAnalyzer2100.

Resulting Ion Torrent reads were downloaded by file transfer protocol (FTP) from the Stellenbosch University server using the confidential login and password provided.

2.5 Genome assembly

Ion Torrent reads were imported into the CLC Genomics Workbench 5.2.1 (QIAGEN CLC bio, Aarhus, Denmark; http://www.clcbio.com) and saved into folders created for each isolate. Gene segments were assembled to reference segments of strain A/Chicken/Czech Republic/206-17_2/2017(H5N8), sourced from GenBank (https://www.ncbi.nlm.nih.gov/nucore) as follows:

KY621531 – segment 1 (polymerase PB2 gene)
KY621532 – segment 2 (polymerase PB1 and PB1-F2 protein genes)
KY621533 – segment 3 (polymerase PA and PA-X protein genes)
KY621534 – segment 4 (haemagglutinin HA gene)
KY621535 – segment 5 (nucleoprotein NP gene)
KY621536 – segment 6 (neuraminidase NA gene)
KY621537 – segment 7 (matrix protein M2 and matrix protein M1 genes)
KY621538 – segment 8 (nuclear export protein NEP and non-structural protein NS1 genes)

Consensus sequences were exported and saved. The complete genome sequences were annotated using the INCUBI Influenza Virus Sequence Annotation Tool (https://www.ncbi.nlm.nih.gov/genomes/FLU/annotation/). This online tool can predict protein sequences encoded by influenza nucleotide sequences, with features used for the GenBank public database sequence submissions. Sequences were deposited in GenBank through its Submissions Portal (https://submit.ncbi.nlm.nih.gov/subs/genbank/). Sequence files in FASTA format (Pearson and Lipman, 1988) were used to create plain text files. All the required isolate information for the sequences to be uploaded was included in an Excel spreadsheet, and then saved as a tab-delimited text file for uploading. This Source Modifier file comprised the following descriptive fields for each

sequence: sequence identifier, organism, isolate, collection date, country, host, serotype and isolation source. Accession numbers are listed in Table 4.

2.6 Reference sequences (Appendix 2 to 9)

In the preparation of phylogenetic trees, sequences of H5N8 HPAI virus segments isolated from terrestrial birds in South Africa in 2017 were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucore) and GISAID Epiflu (https://www.gisaid.org/) public databases. They were selected to represent the different variants described by Abolnik *et al*. (2019). Sequences of the H5N8 HPAI virus detected in Zimbabwe in 2017 were also retrieved from the GISAID EpiFlu database and included in the trees, as were the HA and NA segment sequences of four H5N8 HPAI viruses isolated from African penguins in Namibia in 2019 (Molini *et al*., 2019).

The complete genome sequence of another African penguin H5N8 HPAI virus was provided by Prof. C. Abolnik (Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria) for inclusion in this study. The bird was sampled on 1 March 2018 at Gansbaai. The virus was not isolated, but Deltamune Pty (Ltd) provided RNA extracted from RT-PCR positive swabs to the University of Pretoria and the genome was amplified according to the method described by Zhou *et al*. (2009). The genome was also sequenced by Ion Torrent NGS.

Sequences of H5N8 HPAI viruses isolated in 2017 representing viruses from Egypt, Cameroon, Democratic Republic of Congo and Uganda were also retrieved from GenBank and GISAID Epiflu public databases for inclusion in the phylogenetic trees. One Asian H5N8 HPAI virus, A/Whooper swan/Sanmenxia/01/2016(H5N8), was also included. It was selected randomly to represent the H5N8 clade 2.3.4.4b strains that were isolated in China in 2016.

2.7 Bioinformatic analyses

2.7.1 Multiple sequence alignments

Nucleotide multiple sequence alignments (MSAs) were constructed from consensus sequences for each gene segment, for each virus strain, using BioEdit Sequence Alignment Editor version 7.2.6. (Hall, 1999). They were translated to amino acids to evaluate molecular markers, and the HA_0 sequence of the HA genes.

The inventory of molecular markers compiled by Suttie *et al*. (2019), was consulted to compare the amino acid sequences of these strains with currently known mutations that have been

experimentally verified *in vivo* (Suttie *et al.*, 2019). The cleavage site motifs of the haemagglutinin gene sequences were also compared with those listed in OFFLU's 20 March 2019 version of previously reported highly pathogenic Influenza A cleavage sites (OFFLU 2019).

2.7.2 Phylogenetic reconstruction

Phylogenetic trees for each gene segment were constructed using the Maximum Likelihood method of inferring evolutionary history, with MEGA X software (Tamura et al., 2011; Kumar et al., 2018) based on the Tamura-Nei nucleotide substitution model (Tamura and Nei, 1993). Tree topologies with the highest log likelihood values were selected. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenies were tested with 1000 bootstrap replicates. Trees were drawn to scale with branch lengths representing evolutionary history and indicating the number of nucleotide substitutions per site.

2.7.3 Assessment of reassortment

Radial phylogenetic trees for each genome segment were prepared with the sequences using MEGA X to assess possible clustering within each segment visually (Tamura et al., 2011; Kumar et al., 2018). Clusters were assigned and clustering was tabulated to investigate possible variants or patterns of reassortment between strains. Nucleotide pairwise distance matrices were prepared for each segment, also using MEGA X.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Avian host species and distribution of the samples

Dead birds from between Malgas Island in Saldanha Bay to Wilderness, east of George were received between the beginning of January and the middle of May 2018 (Fig. 6). One bird was collected near Bonnievale, about 80km inland. Most birds had become moribund or died on beaches or at breeding colonies. In some cases, tissue samples were submitted when these could be collected in a veterinary practice where there was a low risk of infecting domestic bird species. Carcases and tissue samples were transported well sealed and on ice. In a few cases, moribund birds were received which died shortly after arrival at the laboratory.

The 24 viruses selected for this study were isolated from six swift terns, five African penguins, two common terns, two Sandwich terns, two Hartlaub's gulls, a Cape cormorant, a crowned cormorant, an African oystercatcher and three unspecified terns. A virus from one scavenger, a jackal buzzard, was also included (Table 3). They were selected to represent isolates from a variety of bird species affected and the geographical regions where the birds were found. The detection of H5N8 HPAI in a jackal buzzard during the course of this study suggests that raptors could be a good passive surveillance source if found dead, due to their scavenging habits on various potentially infected dead birds. Clinical signs of avian influenza in the coastal birds affected by this virus included indications of corneal oedema and weakness, circling and/or the inability to fly, progressing to neurological signs like head tremors, poor balance, torticollis (in the penguins), seizures and death (Roberts, 2018).

Postmortem findings of birds received at the laboratory included poor body condition, green diarrhoea, enlarged livers and severe lung congestion (Figs. 7(b) and (c)). Four abandoned swift tern eggs were also collected at a breeding site where large numbers of bird deaths due to AI were recorded. The contents of these eggs all tested negative by rt-RT PCR so no virus isolation was attempted.

Figure 7(a) Dorsal view of a swift tern (*Thalasseus bergii*) received for *postmortem* examination (*Photo: Meiring van der Merwe*)

Figure 7(b) Vent soiling/ green diarrhoea was evident on numerous carcasses of birds received for testing (*Photo: T. Anthony*)

Figure 7(b) Findings during *postmortem* evaluation of a tern showing cardiac haemorrhages with distended blood vessels, hepatomegaly and poor body condition (*Photo: T. Anthony*)

Table 3 Clade 2.3.4.4b H5N8 HPAI viruses isolated and sequenced for this project

Sampling date	Laboratory identification number	Species	Location	Sample type	# passages (HA titre Log_2)	Embryo mortalities
2017-12-30	18010027	Swift tern	Stony Point	Organ pool	1 (9)	5/5, day 3
2017-12-31	18010028	Swift tern	Kenilworth	Organ pool	3 (4)	5/5, day 2
2018-01-04	18010043	Tern	Hermanus	Swabs: trachea, cloaca, brain	1 (5 and 6)	5/5, day 2 & 3
2018-01-08	18010107	Tern	Gansbaai	Brain	1 (6)	5/5, day 2
2018-01-11	18010106	Jackal buzzard	Wilderness	Swabs: trachea, cloaca	2 (5)	5/5, day 2
2018-01-18	18010259	Common tern	Somerset West	Organ pool	1 (6)	5/5, day 2
2018-01-31	18010422	African penguin	Fishhoek	Organ pool	1 (4)	5/5, day 2
2018-01-31	18010423	African penguin	Muizenberg	Organ pool	1 (5)	5/5, day 3
2018-01-27	18010369	Sandwich tern	Macassar	Organ pool	1 (6)	5/5, day 2
2018-01-27	18010370	Swift tern	Macassar	Organ pool	1 (6)	5/5, day 2
2018-01-29	18010371	Common tern	Strand	Organ pool	1 (5)	5/5, day 2
2018-01-31	18010417	Tern	Milnerton	Organ pool	1 (6)	5/5, day 2
2018-02-15	18020302	Sandwich tern	Dyer Island	Organ pool	1 (6)	5/5, day 2
2018-02-15	18020303	Cape cormorant	Dyer Island	Organ pool	2 (7)	5/5, day 4
2018-02-15	18020304	African penguin	Dyer Island	Organ pool	1 (*)	5/5, day 2
2018-02-22	18020408	African penguin	Simonstown	Organ pool	1 (6 & 8)	4/5, day 2 & 5
2018-03-09	18030213	Crowned cormorant	Gansbaai	Organ pool	1 (3)	5/5, day 3
2018-03-09	18030214	African oystercatcher	Hermanus	Organ pool	1 (3)	5/5, day 2
2018-02-15	18020273	Swift tern	Bonnievale	Organ pool	1 (3)	3/5, day 2

Sampling date	Laboratory identification number	Species	Location	Sample type	# passages (HA titre Log_2)	Embryo mortalities
2018-03-27	18030478	Swift tern	Cape Town	Organ pool	1 (5)	4/5, day 2
2018-04-17	18040275	Swift tern	Malgas Island	Swab: brain	1 (4)	4/5, day 2
2018-04-12	18040224	Hartlaub's gull	Robben Island	Swab: brain	2 (5)	5/5, day 2
2018-04-20	18040367	Hartlaub's gull	Milnerton	Organ pool	2 (5)	4/5, day 2
2018-05-12	18050256	African penguin	Robben Island	Organ pool	2 (4)	4/5, day 2

* Haemagglutination test not done

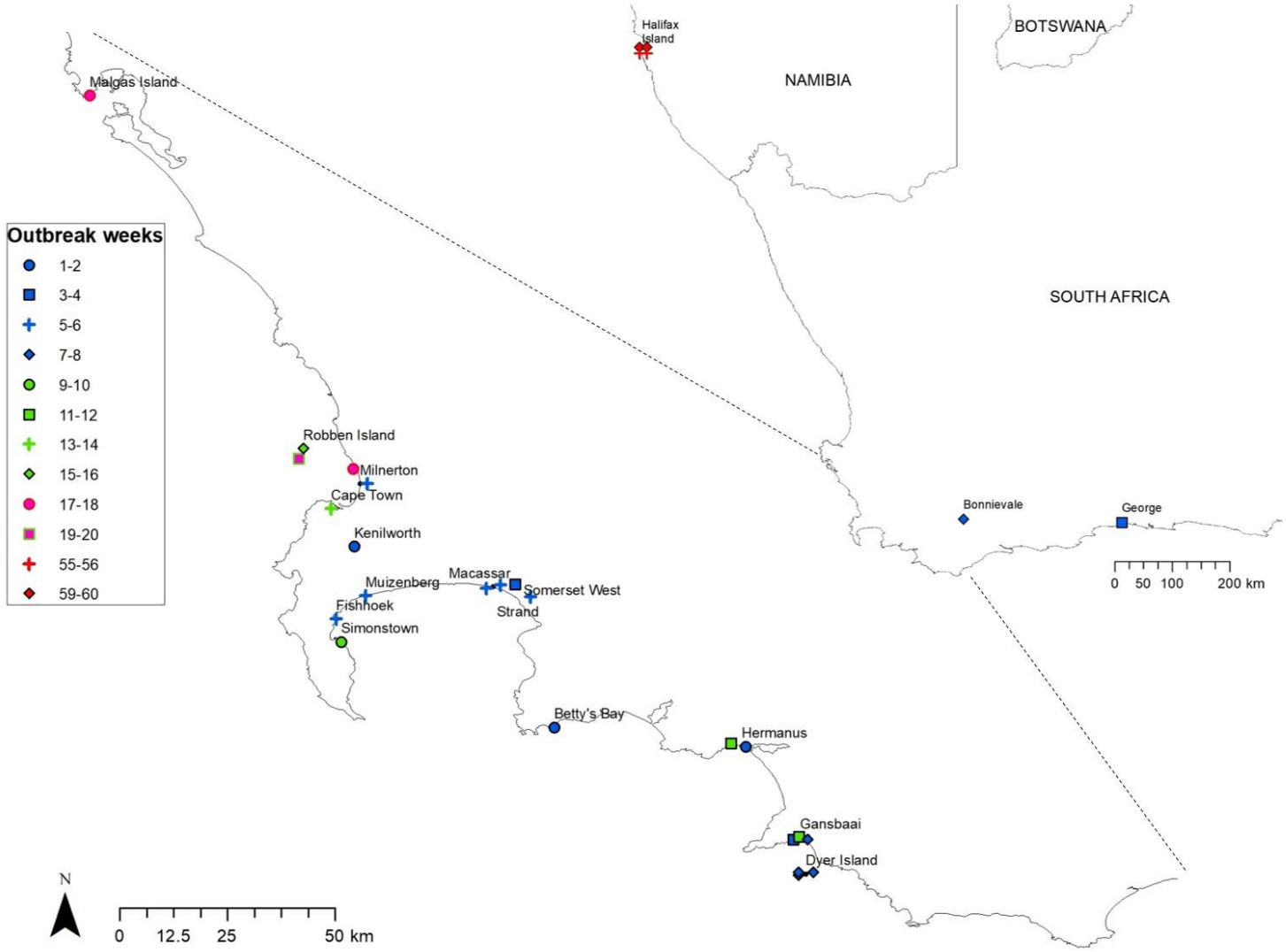

Figure 8 Temporospatial distribution of clade 2.3.4.4b H5N8 HPAI viruses selected for this study. The viruses from Halifax Island, Namibia that were included in the analyses are indicated as red crosses and red diamonds. Locations are indicated in fortnightly increments, according to the progression of the outbreak. [*Map: L Roberts*]

3.2 Virus isolation

3.2.1 Embryo observations

Inoculations of real-time RT-PCR-positive samples caused embryo mortalities, embryo abnormalities and/or positive haemagglutination reactions in the embryonated chicken eggs after one (n=18), two (n=5) or three (n=1) passages (Table 3). The embryos of most of these inoculated eggs died on day two post inoculation. The embryos displayed pathology/abnormalities typical of avian influenza infection, including dwarfism and congestion. Haemagglutination tests carried out on two-fold dilutions of amnio-allantoic fluids yielded titres of between $Log2^{3}$ and $Log2^{9}$.

3.2.2 Percentage positive and negative isolations.

A total of 46 coastal birds were collected and tested for H5N8 AIV testing. PCR screening was carried out on 39 of them (85%).

Virus isolation was attempted on 31 of the birds that tested positive on real-time RT-PCR for avian influenza matrix and nucleoprotein genes (79%). The Ct values of the positive birds ranged between 10.17 and 35.13, although isolation attempts were only successful when the Ct values were below 25.97. Viruses were isolated from 22 of the 31 RT-PCR birds (71%).

Isolation attempts on three PCR-negative birds yielded no viruses. Virus isolation was also attempted on five samples that were not first screened by real-time RT-PCR. All five were positive.

In total, virus isolation was attempted on 39 samples and 27 viruses (69%) were isolated. Three samples (6%) were negative both on real-time RT-PCR and virus isolation. Virus isolation was not attempted on 7 PCR-positive samples, due to capacity constraints.

3.3 Ion Torrent Sequencing.

Quality control performed on the total RNA extracted from each of the viruses indicated RNA Integrity Numbers of between 1 and 2.5. The fragment distribution graphs were acceptable, and the RNA was therefore deemed suitable for the RNASeq protocol (A Vorster, personal communication). Mean Ion Torrent read lengths ranged between 121 and 160 bp (average 149 bp).

Accession numbers of sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/nucore) are listed in Table 4

Table 4 Clade 2.3.4.4b H5N8 HPAI viruses isolated and sequenced for this study, with corresponding GenBank accession numbers

Sampling date	Isolate identification	Accession number
30 December 2017	A/Swift tern/South Africa/18010027/2018(H5N8)	MK734297, MN595247 – MN595253
31 December 2017	A/Swift tern/South Africa/18010028/2018(H5N8)	MK734298, MN595254 – MN595260
04 January 2018	A/tern/South Africa/18010043/2018(H5N8)	MN124541 - MN124548
08 January 2018	A/tern/South Africa/18010107/2018(H5N8)	MN124557 - MN124564
11 January 2018	A/Jackal buzzard/South Africa/18010106/2018(H5N8)	MN124549 - MN124556
18 January 2018	A/Common tern/South Africa/18010259/2018(H5N8)	MN124565 - MN124572
27 January 2018	A/Sandwich tern/South Africa/18010369/2018(H5N8)	MK734294, MN595226 – MN595232
27 January 2018	A/Swift tern/South Africa/18010370/2018(H5N8)	MK734299, MN595261 – MN595267
29 January 2018	A/Common tern/South Africa/18010371/2018(H5N8)	MK734296, MN595240 – MN595246
31 January 2018	A/African penguin/South Africa/18010422/2018(H5N8)	MN124573 - MN124580
31 January 2018	A/African penguin/South Africa/18010423/2018(H5N8)	MN124581 - MN124588
31 January 2018	A/tern/South Africa/18010417/2018(H5N8)	MK734300, MN595268 – MN595274
15 February 2018	A/Swift tern/South Africa/18020273/2018(H5N8)	MN124589 - MN124596
15 February 2018	A/Sandwich tern/South Africa/18020302/2018(H5N8)	MN124597 - MN124604
15 February 2018	A/Cape cormorant/South Africa/18020303/2018(H5N8)	MN124605 - MN124612
15 February 2018	A/African penguin/South Africa/18020304/2018(H5N8)	MN124613 - MN124620
22 February 2018	A/African penguin/South Africa/18020408/2018(H5N8)	MN124621 - MN124628
09 March 2018	A/Crowned cormorant/South Africa/18030213/2018(H5N8)	MN124629 - MN124636
09 March 2018	A/African oystercatcher/South Africa/18030214/2018	MN124637 - MN124644
27 March 2018	A/Swift tern/South Africa/18030478/2018(H5N8)	MN124645 - MN124652
12 April 2018	A/Hartlaubs gull/South Africa/18040224/2018(H5N8)	MN124653 - MN124660
17 April 2018	A/Swift tern/South Africa/18040275/2018(H5N8)	MN124661 - MN124668
20 April 2018	A/Hartlaubs gull/South Africa/18040367/2018(H5N8)	MN124669 - MN124676
12 May 2018	A/African penguin/South Africa/18050256/2018(H5N8)	MN124677 - MN124684

3.4 Phylogenetic analyses

Phylogenetic analyses were performed of each of the eight genomic sequences, incorporating South African, Namibian, and other international reference sequences identified as the closest relatives by BLAST analysis.

Table 5 Final lengths of aligned sequences used for phylogenetic reconstruction

Segment	Gene(s)	Nucleotides (bp)
1	Polymerase (PB2) gene, partial cds	2242
2	Polymerase (PB1) gene, partial cds and putative PB1-F2 gene, complete cds	2264
3	Polymerase acidic (PA) and PA-X protein genes, complete cds	2151
4	Haemagglutinin (HA) gene, partial cds	965
5	Nucleoprotein (NP) gene, complete cds	1453
6	Neuraminidase (NA) gene, partial cds	1128
7	Matrix protein 1 (M1) gene, complete cds, and matrix protein 2 (M2) gene, partial cds	759
8	Non-structural protein 1 (NS1) and nuclear export protein (NEP) genes, partial cds	789

3.4.1 Segment 1: Polymerase PB2 gene

A total of 54 sequences were included in the tree of Segment 1 (PB2) (Fig. 3.5(a). The tree was rooted with A/Whooper swan/Sanmenxia/01/2016(H5N8). All the coastal bird isolates clustered into a unique monophyletic clade supported by a strong bootstrap value of 83 This clade shared a most recent common ancestor with A/chicken/South Africa/17080561-P1/2017(H5N8), its closest relative, which was isolated in the Western Cape province in August 2017. All of these also shared a recent common ancestor (bootstrap value 88) with Variant 4 viruses isolated from terrestrial birds in the Western and Eastern Cape provinces, as described by Abolnik *et al*. (2019).

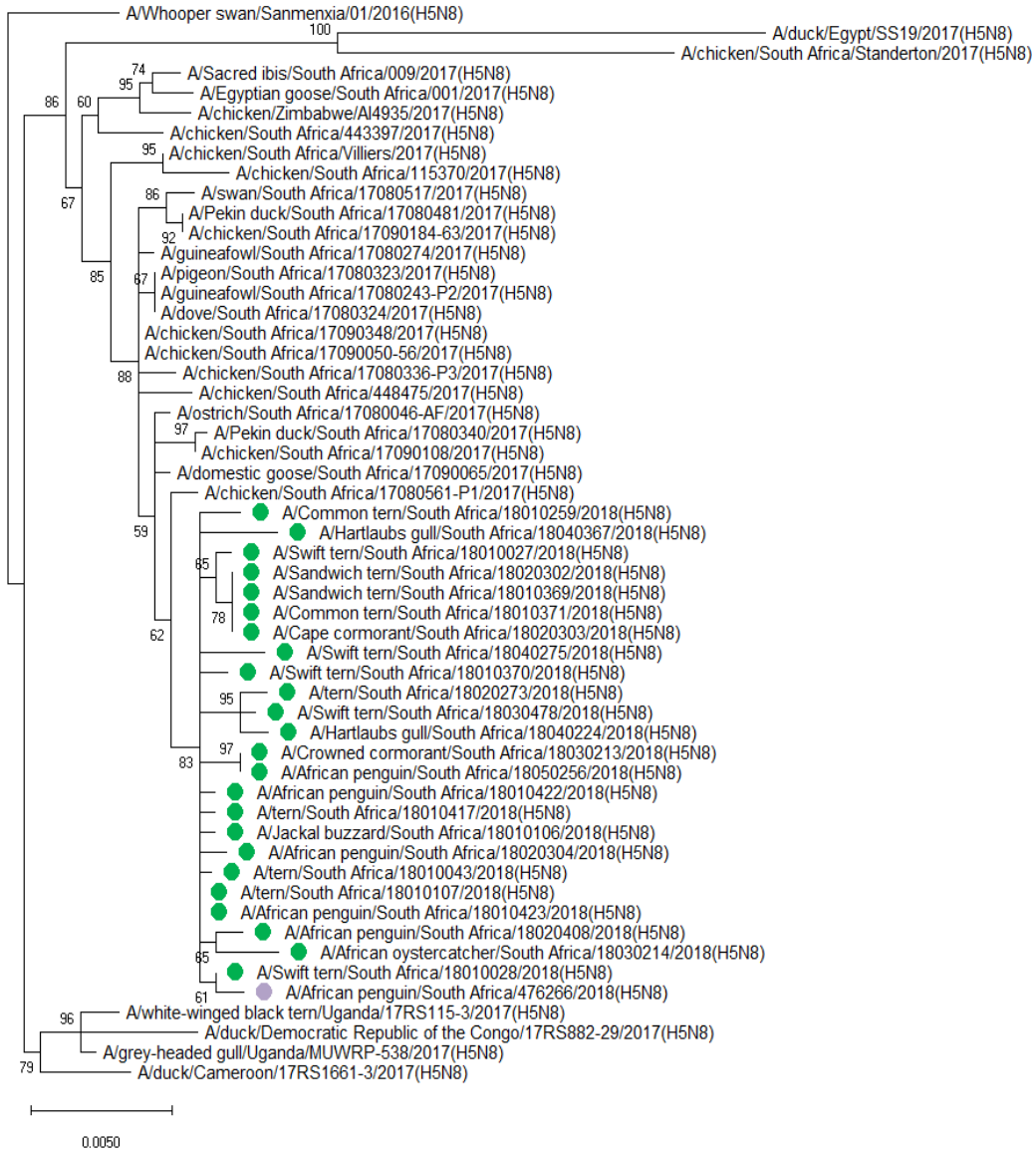

Figure 9(a) Maximum likelihood phylogenetic tree of Polymerase basic 2 (PB2) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP.

3.4.2 Segment 2: Polymerase (PB1) gene and putative PB1-F2 gene

This phylogenetic tree included 52 sequences and was rooted with A/Whooper swan/Sanmenxia/01/2016(H5N8) (Fig. 9(b)). Multiple branches appeared in the tree showing considerable variation in this segment. All of the coastal bird isolates clustered together onto one branch, and shared a less recent common ancestor with H5N8 viruses isolated in the Western and Eastern Cape provinces in August and September 2017 from six different terrestrial bird species, with a high bootstrap value of 98.

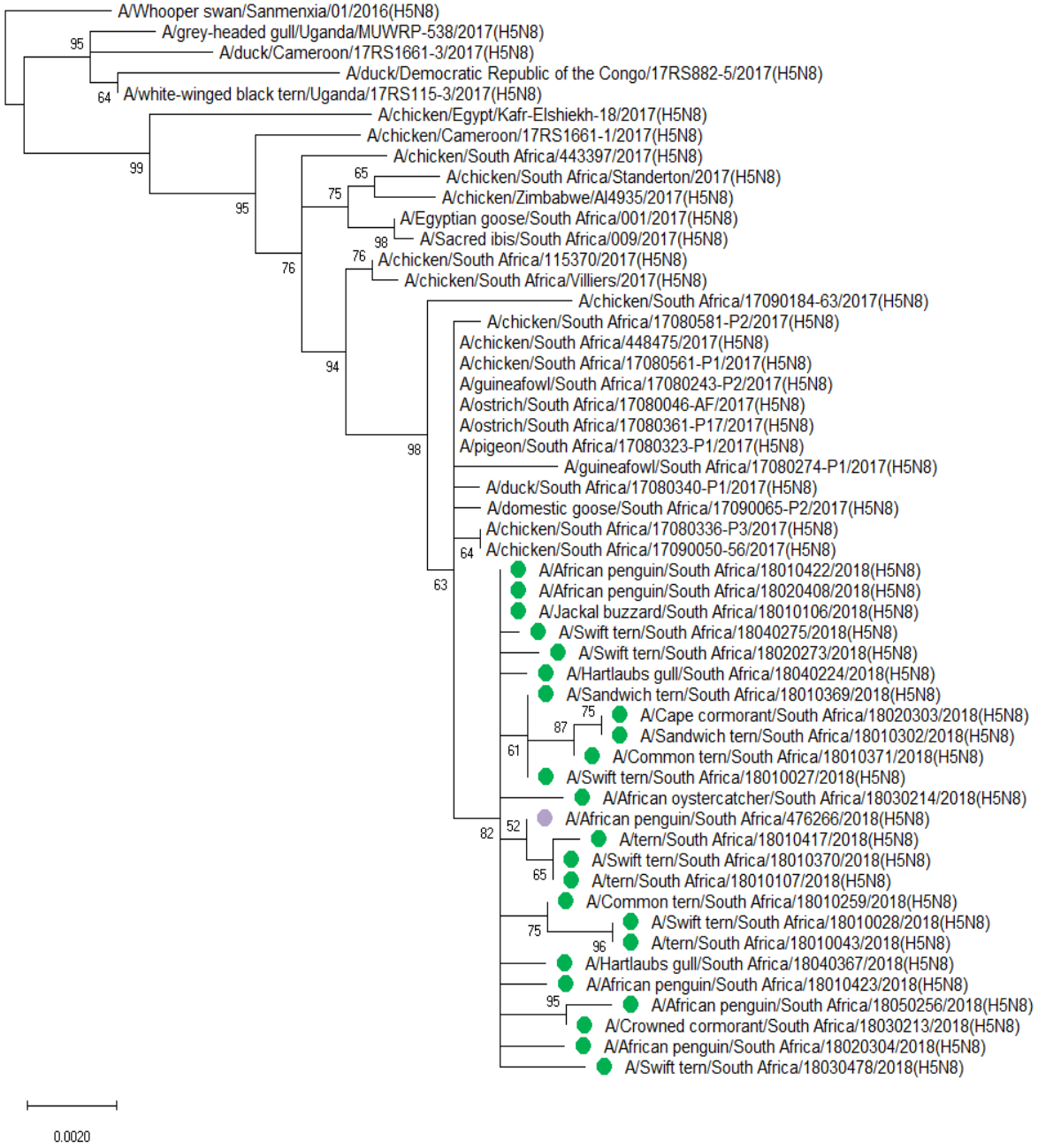

Figure 9(b) Maximum likelihood phylogenetic tree of Polymerase basic 2 (PB2) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP.

3.4.3 Segment 3: Polymerase acidic (PA) and PA-X protein genes

The PA segment analysis included 58 nucleotide sequences. It was rooted on A/chicken/South Africa/Standerton(H5N8), which was one of the first H5N8 HPAI viruses detected in South Africa in 2017 (Fig. 9(c)). The coastal bird isolates formed a unique monophyletic clade with a bootstrap value of 88.

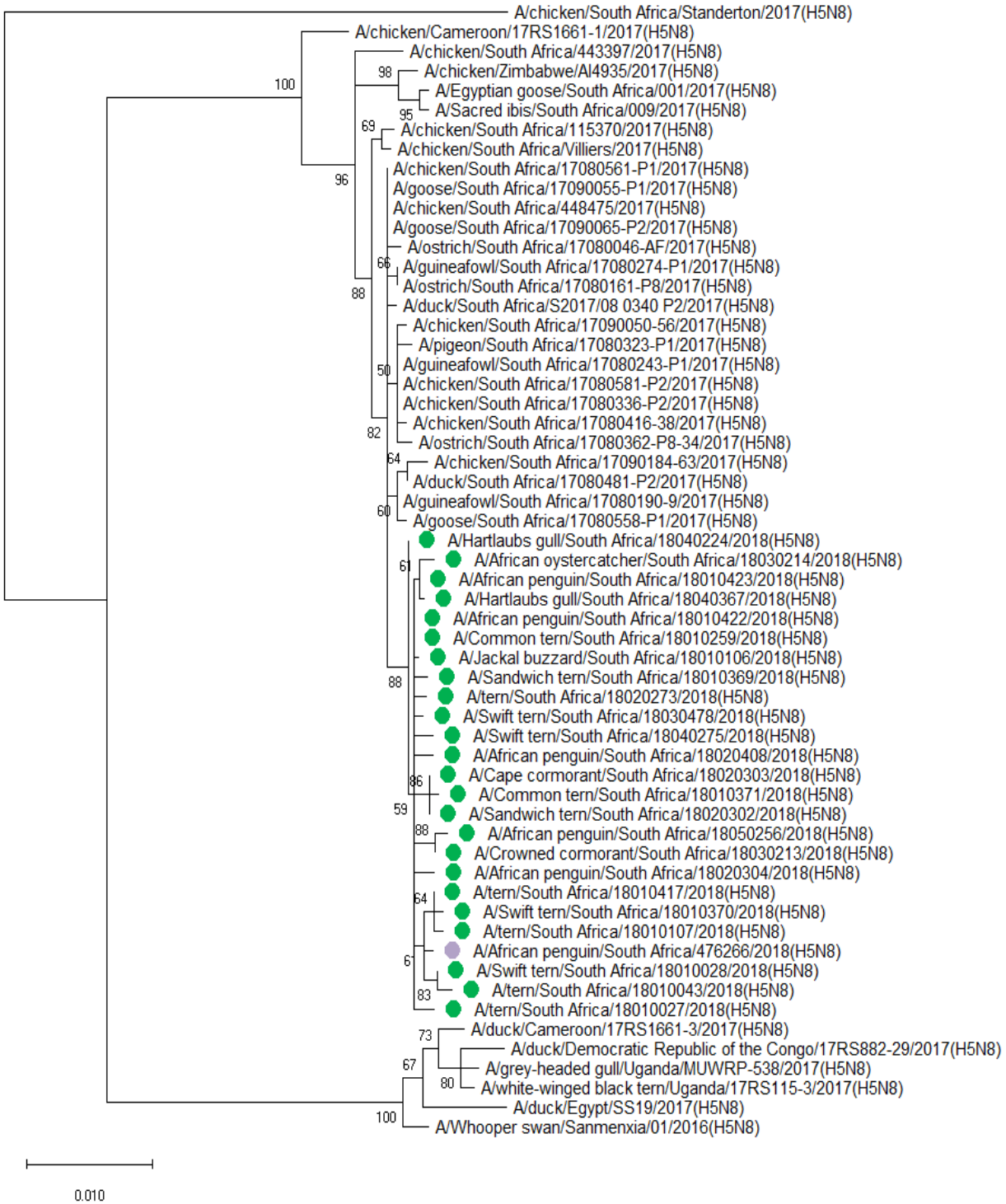

Figure 9(c) Maximum likelihood phylogenetic tree of Polymerase acidic (PA) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP.

3.4.4 Segment 4: Haemagglutinin (HA) gene

The haemagglutinin segment tree included 65 sequences (Fig. 9(d)). The tree was rooted with A/Whooper swan/Sanmenxia/01/2016(H5N8). All of the coastal bird isolates formed a unique monophyletic clade within the Southern African sub-cluster. They share a recent common ancestor with H5N8 viruses isolated in the Western Cape in August and September 2017, with a virus isolated from Mpumalanga province (A/chicken/South Africa/Villiers/2017) in June 2017, and one each from the Eastern Cape (A/chicken/South Africa/448475/2017) and Free State (A/chicken/South Africa/115370/2017) provinces isolated in September 2017. The four Namibian strains isolated in 2019 (indicated with red), form their own sub-clade and show the most divergence. One of the gull isolates, A/Hartlaubs gull/South Africa/18040367/2018, shared a most recent common ancestor with the Namibian isolates, with a bootstrap value of 66.

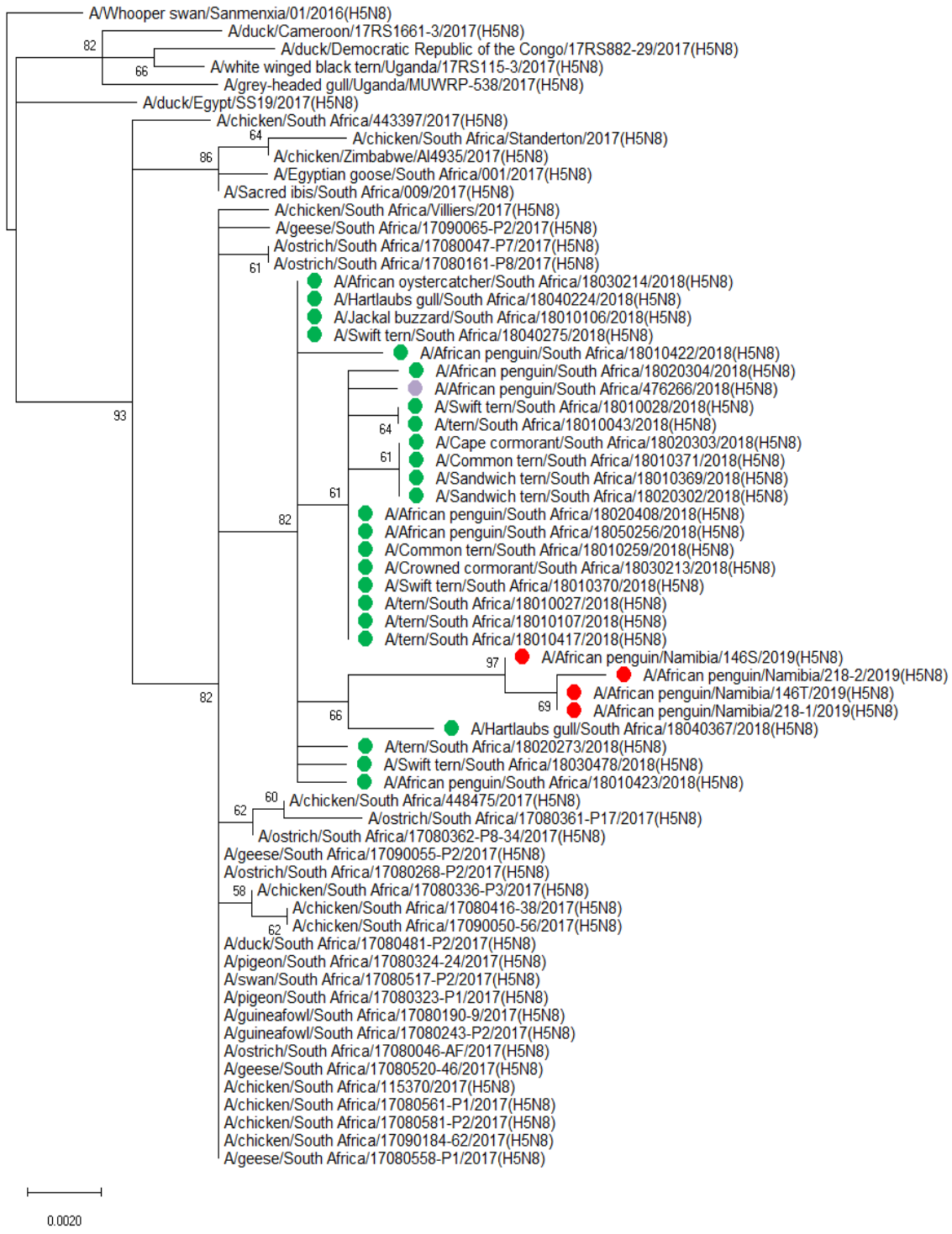

Figure 9(d) Maximum likelihood phylogenetic tree of Haemagglutinin (HA) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP. Red indicates viruses detected in penguins in Namibia in 2019.

3.4.5 Segment 5: Nucleoprotein (NP) gene

Fifty-seven sequences were included in the Nucleoprotein gene tree (Fig. 9(e)). The coastal bird isolates formed a unique phylogenetic clade. The bootstrap value is low (42) but this clade still falls within the larger Southern African H5N8 cluster of terrestrial bird isolates with a bootstrap value of 99.

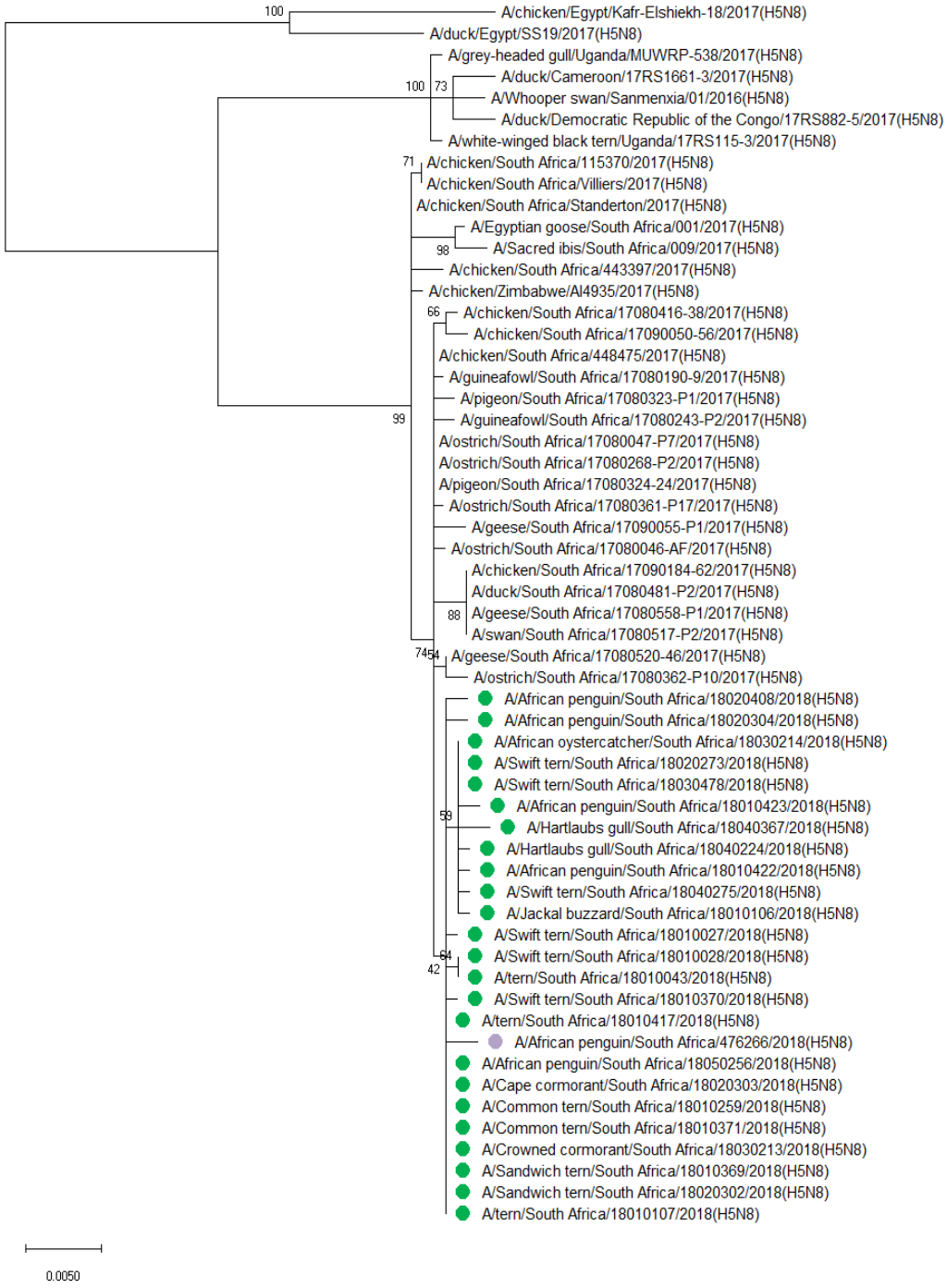

Figure 9(e) Maximum likelihood phylogenetic tree of Nucleoprotein (NP) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP

3.4.6 Segment 6: Neuraminidase (NA) gene

The phylogenetic tree of this segment contained 68 sequences (Fig. 9(f)). The tree was rooted with A/Whooper swan/Sanmenxia/01/2016(H5N8). The coastal bird isolates, including the 2019 Namibian isolates, formed a unique monophyletic clade with a low bootstrap value of 47. They also clustered together with viruses isolated from seven different terrestrial bird species in the Western Cape and Eastern Cape provinces in August and September 2017. The four viruses detected in penguins in Namibia in 2019 shared a common ancestor but were still nested within a broader cluster of South African coastal bird virus isolates. Two viruses isolated from a Cape cormorant (A/Cape cormorant/South Africa/18020303/2018(H5N8)) and a Sandwich tern (A/Sandwich tern/South Africa/18020302/2018(H5N8)), at the same location and at the same time, shared a most recent common ancestor.

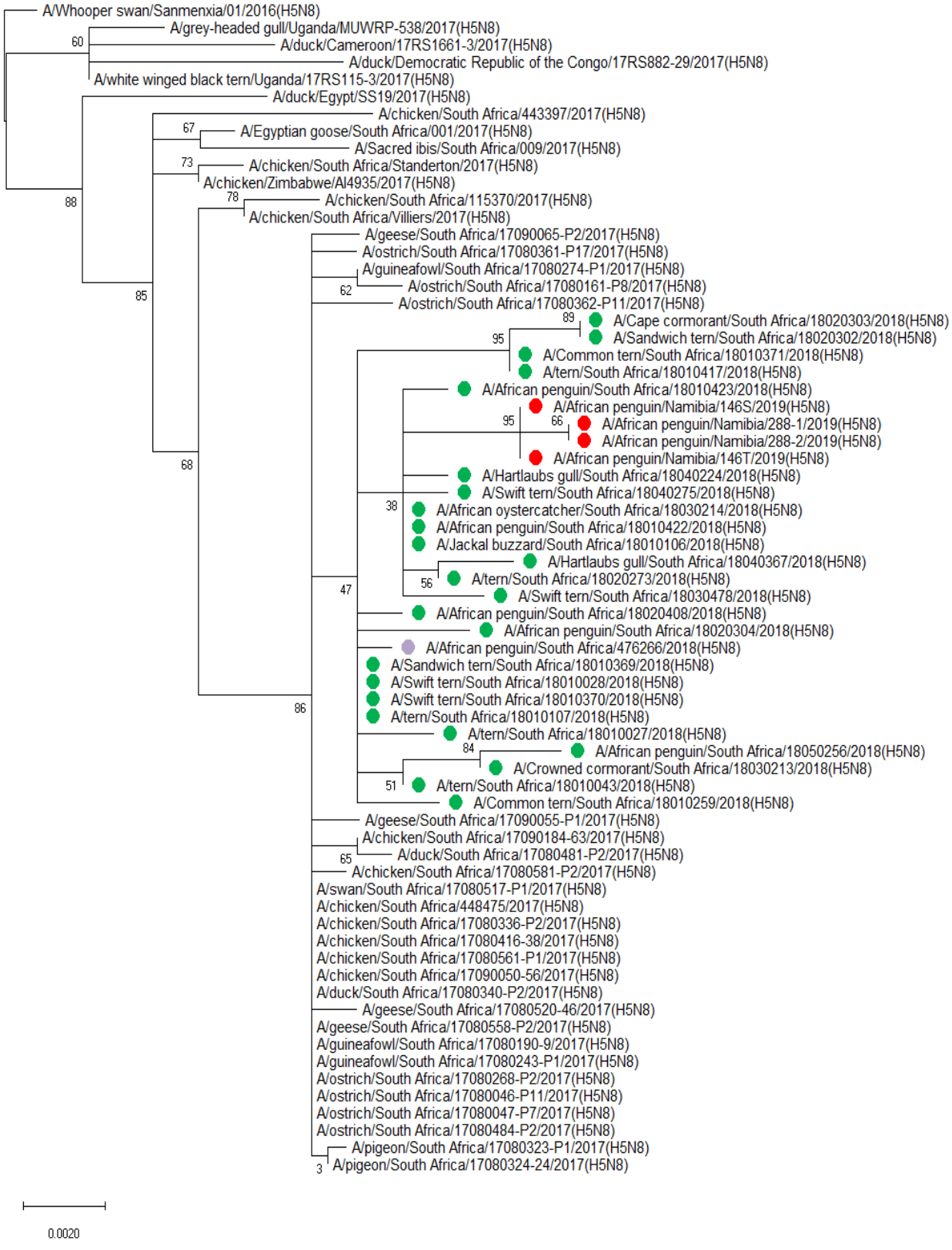

Figure 9(f) Maximum likelihood phylogenetic tree of Neuraminidase (NA) gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP. Red indicates viruses detected in penguins in Namibia in 2019.

3.4.7 Segment 7: Matrix protein 1 (M1) gene and matrix protein 2 (M2) gene

The M1 and M2 segment analysis included 55 nucleotide sequences (Fig. 9(g)). It was rooted on A/chicken/Egypt/Kafr-Elshiekh-18/2017(H5N8). The coastal birds formed a unique monophyletic clade, with a bootstrap value of 63.

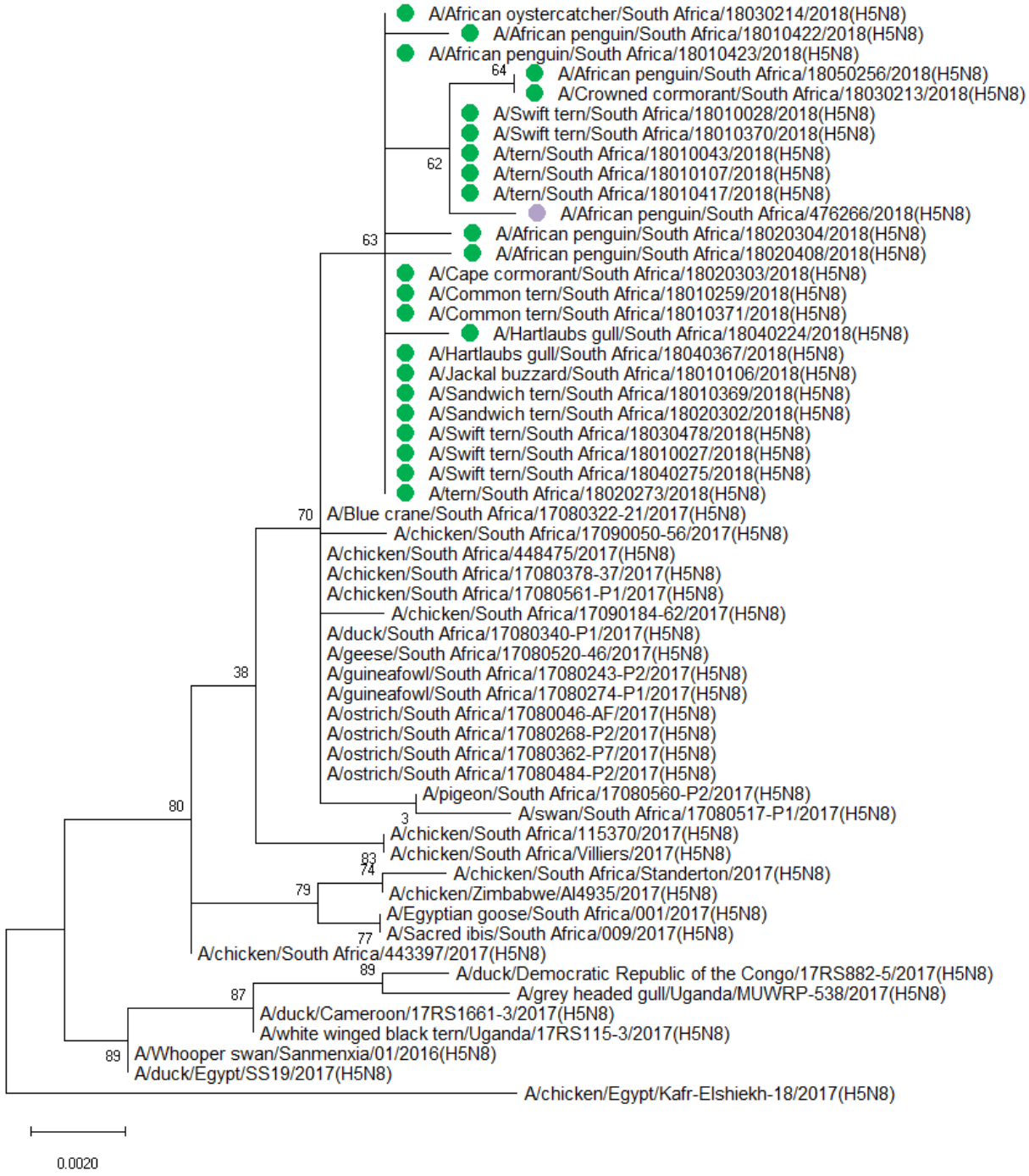

Figure 9(g) Maximum likelihood phylogenetic tree of Matrix 1 and 2 (M1 and M2) genes nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP.

3.4.8 Segment 8: Non-structural protein 1 (NS1) and nuclear export protein (NEP) genes

The tree of the NS protein genes included 53 nucleotide sequences (Fig. 9(h)). It was rooted on A/Whooper swan/Sanmenxia/01/2016(H5N8). The coastal bird isolates did not share one unique common ancestor. They are dispersed within a cluster that includes H5N8 viruses from the Western Cape and Eastern Cape provinces that were isolated from August to September 2017, from avian species including chickens, ostriches, a pigeon and a blue crane.

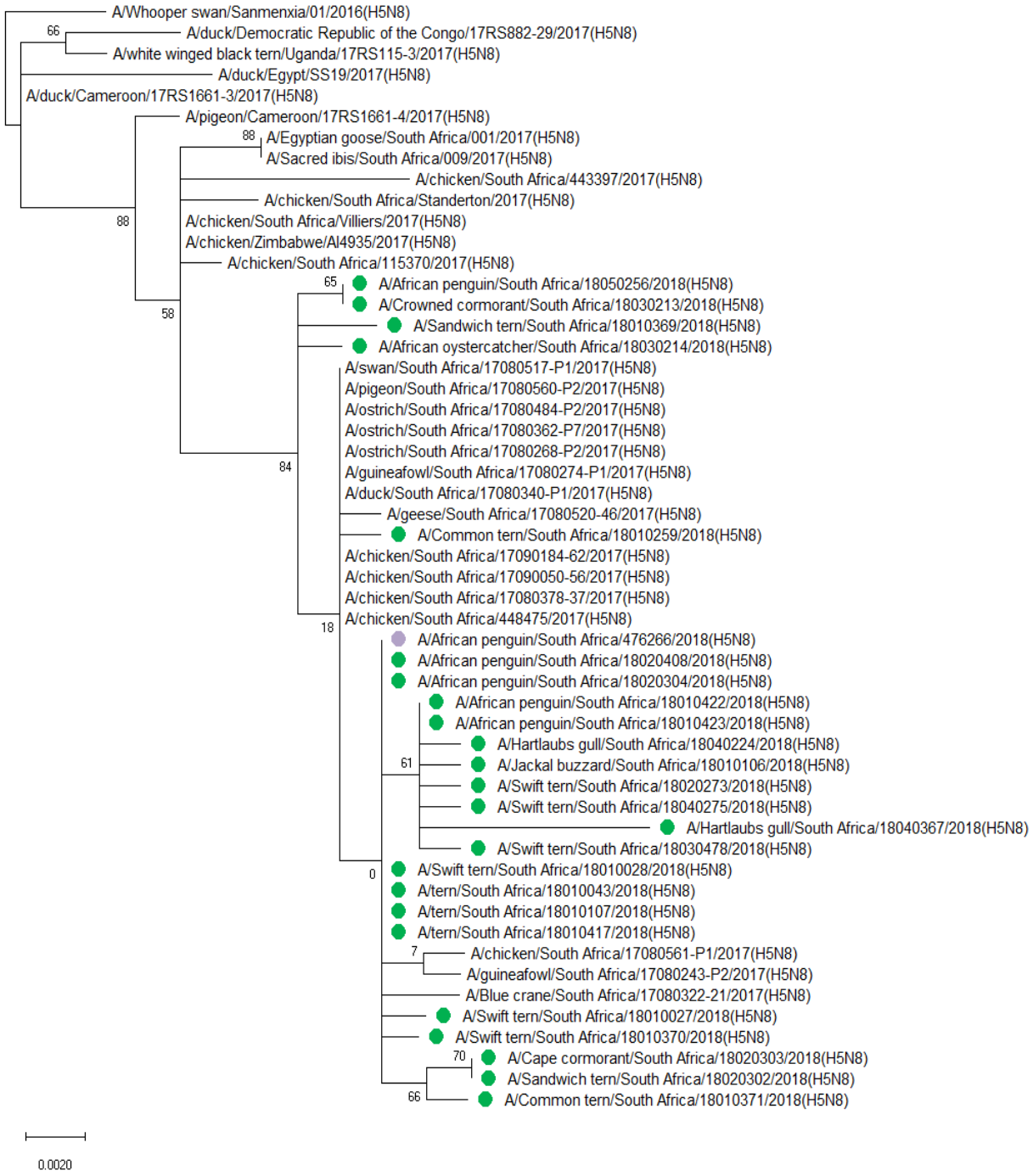

Figure 9(h) Maximum likelihood phylogenetic tree of NS1 and NEP gene nucleotide sequences. Branch lengths indicate number of substitutions per site. Green indicates viruses isolated for this study. Lavender indicates the penguin virus sequence provided by UP.

3.5 Investigation of inter-segmental reassortment

Radial phylogenetic trees of all gene segments were prepared for better visualization of clustering of the viruses to assess reassortment (Figs. 10(a) to (h)). Only the coastal bird isolates were included in these trees. Two clusters within each of segments 4, 5, 7 and 8 could be distinguished, randomly designated as Clusters I and II. Three clusters were evident in segment 6. There is no correlation between the cluster numbers of the different segments.

There was relatively greater variation in the polymerase gene segments (Segments 1, 2 and 3) but no distinct clusters within any of them were apparent (Figs. 10(a) to (c)).

Figure 10(a) Radial maximum likelihood tree of segment 1 (not to scale). No obvious clustering of isolates was evident.

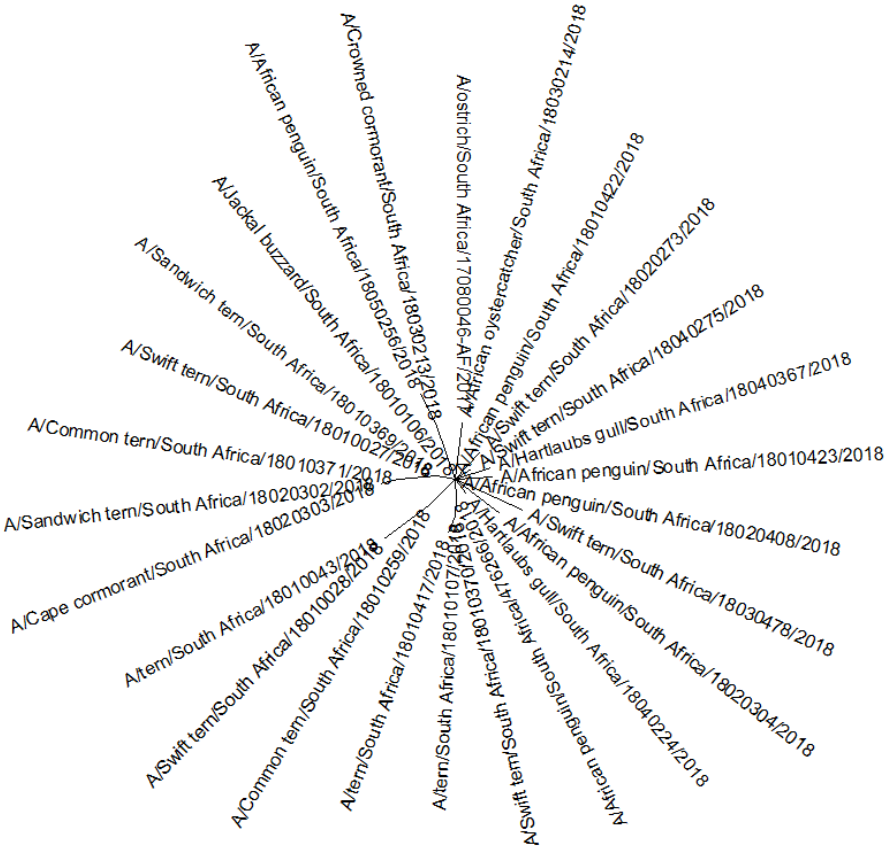

Figure 10(b) Radial maximum likelihood tree of segment 2 (not to scale). No obvious clustering of isolates was evident

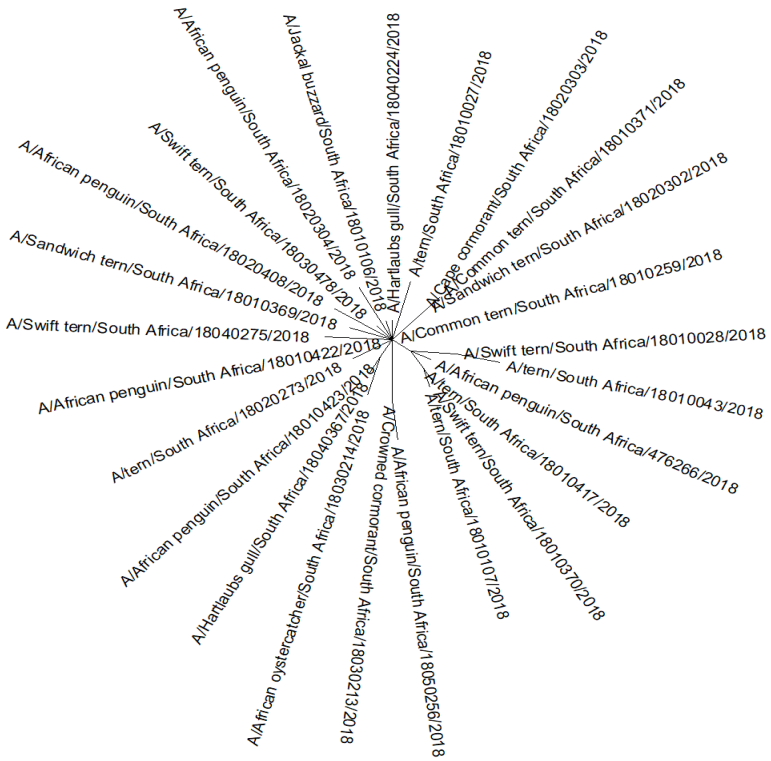

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Figure 10(c) Radial maximum likelihood tree of segment 3 (not to scale). No obvious clustering of isolates was evident.

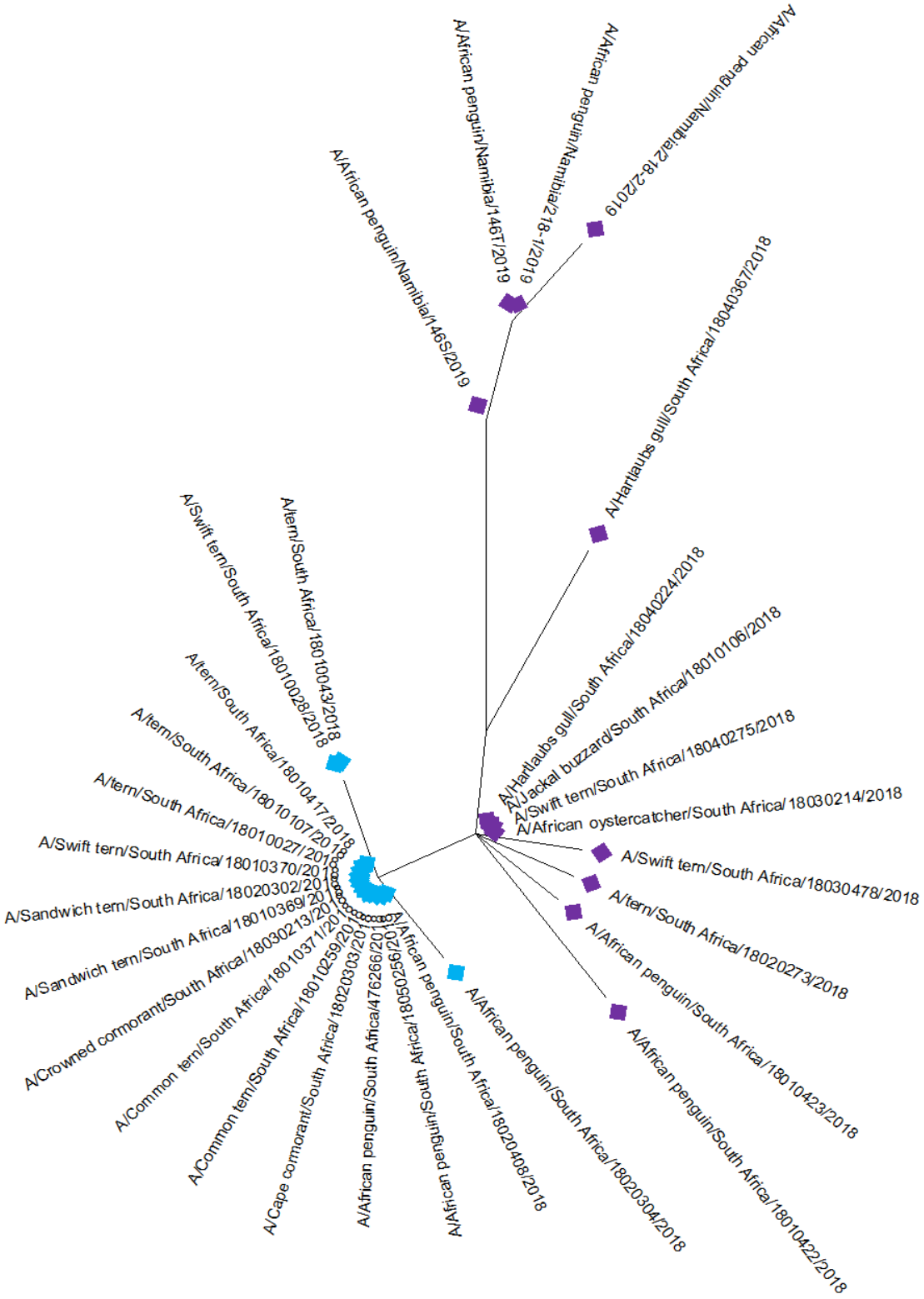


Figure 10(d) Radial maximum likelihood tree of segment 4 (not to scale). Two clusters were assigned. Purple indicates Cluster 1. Blue indicates Cluster 2.

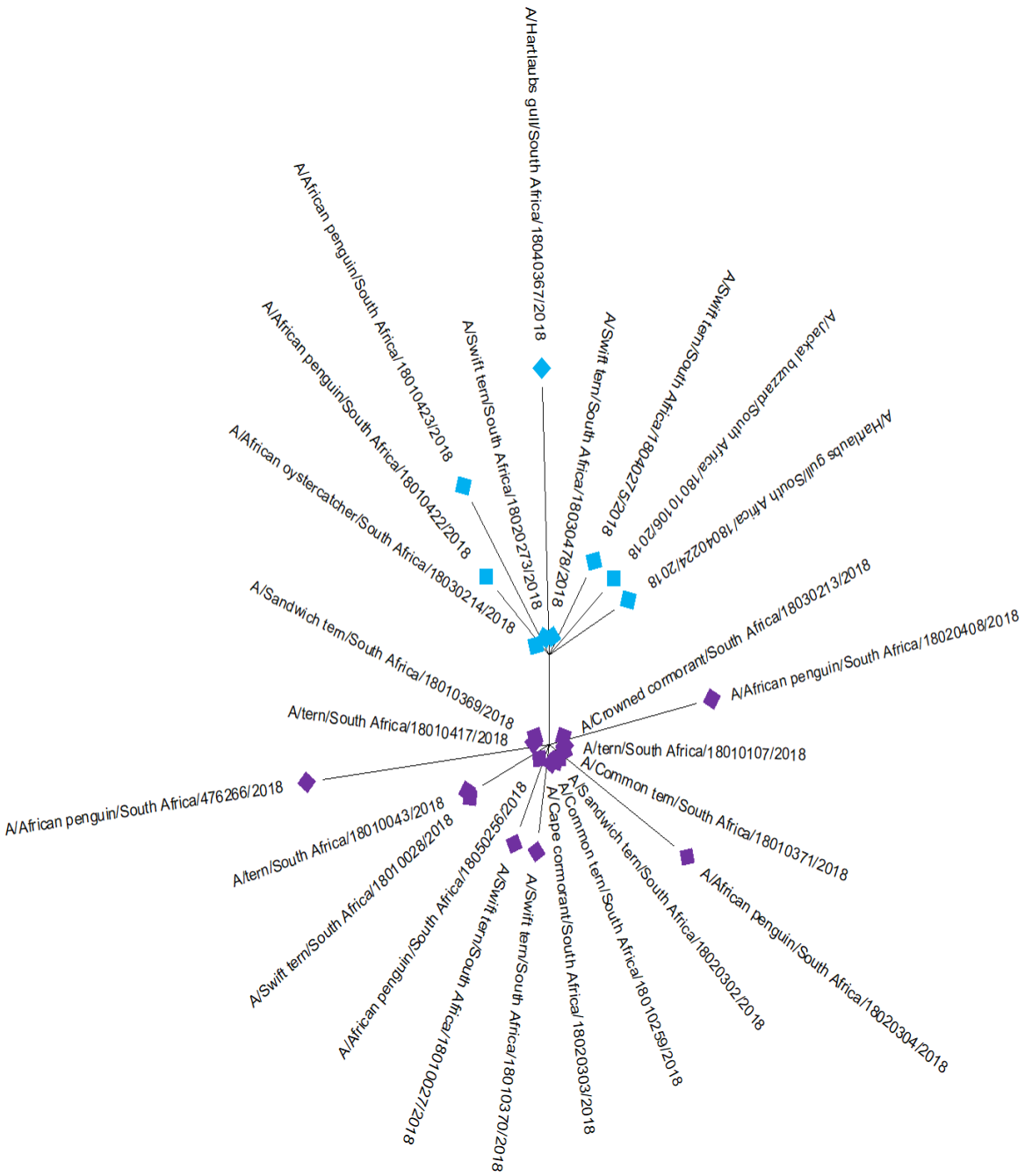


Figure 10(e) Radial maximum likelihood tree of segment 5 (not to scale). Two clusters were assigned. Purple indicates Cluster 1. Blue indicates Cluster 2.

Figure 10(f) Radial maximum likelihood tree of segment 6 (not to scale). Three clusters were assigned. Purple indicates Cluster 1. Blue indicates Cluster 2. Pink indicates Cluster 3.

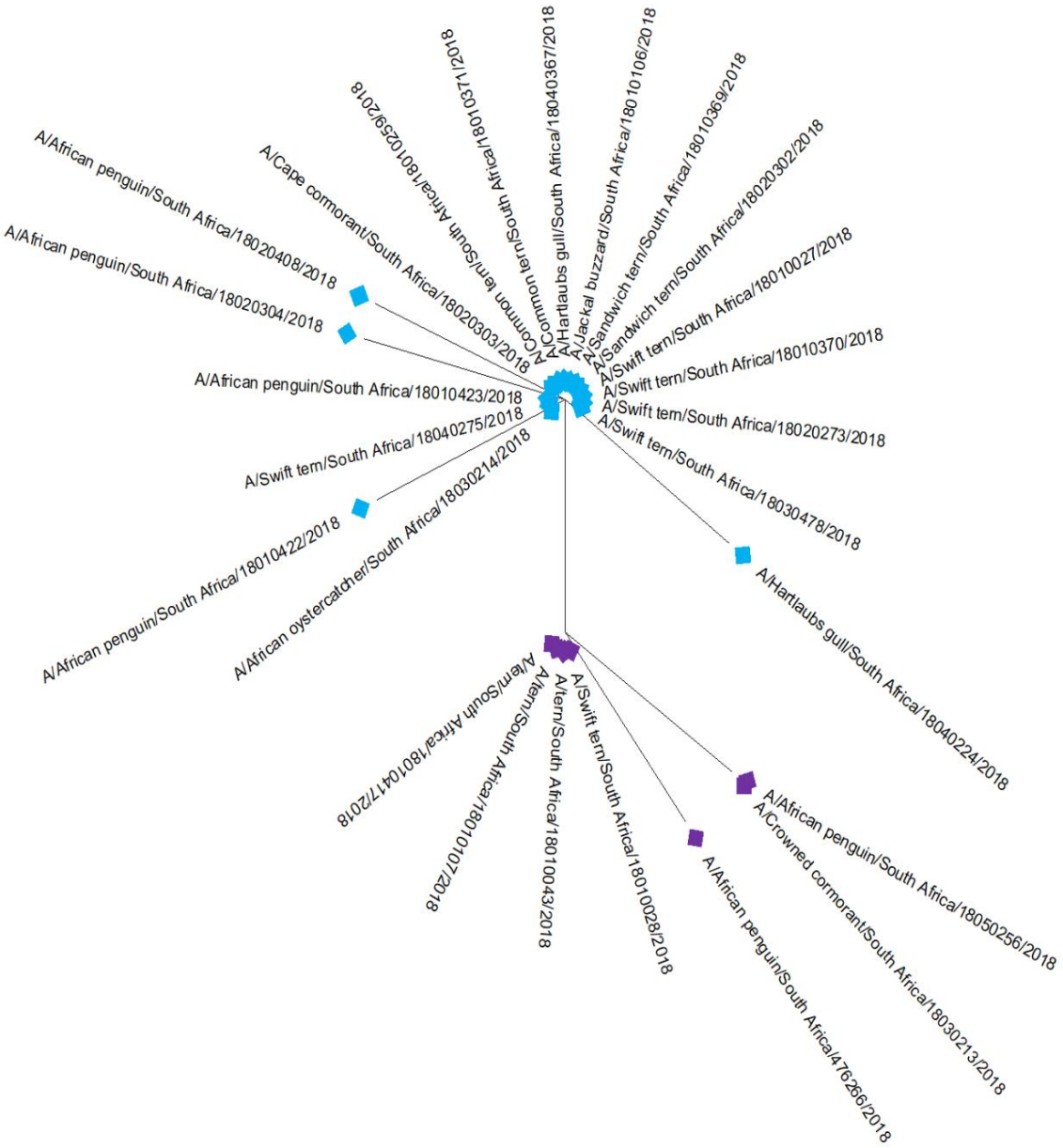


Figure 10(g) Radial maximum likelihood tree of segment 7 (not to scale). Two clusters were assigned. Purple indicates Cluster 1. Blue indicates Cluster 2.

Figure 10(h) Radial maximum likelihood tree of segment 8 (not to scale). Two clusters were assigned. Purple indicates Cluster 1. Blue indicates Cluster 2.

Clustering pairwise distances

The Distance Analysis approach was used to analyze variation within aligned sequences. Pairwise distance analysis uses a distance algorithm to compare two aligned sequences at a time, and the number of changes (base substitutions and insertion/deletions) are presented as a percentage of the overall sequence length. A matrix of all possible pairs of sequences is then constructed, reflecting pairwise distances.

Each segment of each coastal bird virus was evaluated. The most variation, 0.004%, was found in the NA segment (Segment 6). The highest similarity was found within the NP (Segment 5) and M (Segment 7) genes. The variation in these was 0.001% (Appendix 10 to 17).

Reassortment patterns

Genotype groups were assigned according to how the gene segments of each virus clustered together (Table 6). Some amino acid motifs observed in the sequences of certain gene segments were consistently present in all the virus strains within one genotype, mostly to the exclusion of the others.

Table 6 Reassortment patterns of clade 2.3.4.4b H5N8 HPAIV sequences analysed and genotypes assigned, arranged chronologically by sampling date. Segments are shaded according to clusters assigned (Fig's. 10(a) to (h)). There is no colour correlation between segments. Locations are shaded according to genotypes assigned.

Sample ID	Date	Seg1	Seg2	Seg3	Seg4	Seg5	Seg6	Seg7	Seg8	Genotype	Location
A/Swift tern/South Africa/18010027/2018	2017/12/30	1	1	1	2	1	3	2	2	A	Betty's Bay
A/Swift tern/South Africa/18010028/2018	2017/12/31	1	1	1	2	1	3	1	2	B	Kenilworth
A/tern/South Africa/18010043/2018	2018/01/04	1	1	1	2	1	3	1	2	B	Hermanus
A/tern/South Africa/18010107/2018	2018/01/08	1	1	1	2	1	3	1	2	B	Gansbaai
A/Jackal buzzard/South Africa/18010106/2018	2018/01/11	1	1	1	1	2	2	2	1	C	George
A/Common tern/South Africa/18010259/2018	2018/01/18	1	1	1	2	1	3	2	2	A	Somerset West
A/Sandwich tern/South Africa/18010369/2018	2018/01/27	1	1	1	2	1	3	2	2	A	Macassar
A/Swift tern/South Africa/18010370/2018	2018/01/27	1	1	1	2	1	3	2	2	A	Macassar
A/Common tern/South Africa/18010371/2018	2018/01/29	1	1	1	2	1	1	2	2	D	Strand
A/African penguin/South Africa/18010422/2018	2018/01/31	1	1	1	1	2	2	2	1	C	Fishhoek
A/African penguin/South Africa/18010423/2018	2018/01/31	1	1	1	1	2	2	2	1	C	Muizenberg
A/tern/South Africa/18010417/2018	2018/01/31	1	1	1	2	1	1	1	2	E	Milnerton
A/African penguin/South Africa/476266/2018	2018/02/10	1	1	1	2	1	3	1	2	B	Gansbaai
A/African penguin/South Africa/18020304/2018	2018/02/15	1	1	1	2	1	3	2	2	A	Dyer
A/Cape cormorant/South Africa/18020303/2018	2018/02/15	1	1	1	2	1	1	2	2	D	Dyer
A/Sandwich tern/South Africa/18020302/2018	2018/02/15	1	1	1	2	1	1	2	2	D	Dyer
A/tern/South Africa/18020273/2018	2018/02/15	1	1	1	1	2	2	2	1	C	Bonnievale
A/African penguin/South Africa/18020408/2018	2018/02/22	1	1	1	2	1	3	2	2	A	Simonstown
A/African oystercatcher/South Africa/18030214/2018	2018/03/09	1	1	1	1	2	2	2	2	E	Hermanus
A/Crowned cormorant/South Africa/18030213/2018	2018/03/09	1	1	1	2	1	3	1	2	B	Gansbaai
A/Swift tern/South Africa/18030478/2018	2018/03/27	1	1	1	1	2	2	2	1	C	Cape Town - city
A/Hartlaubs gull/South Africa/18040224/2018	2018/04/12	1	1	1	1	2	2	2	1	C	Robben Island
A/Swift tern/South Africa/18040275/2018	2018/04/17	1	1	1	1	2	2	2	1	C	Malgas
A/Hartlaubs gull/South Africa/18040367/2018	2018/04/20	1	1	1	1	2	2	2	1	C	Milnerton
A/African penguin/South Africa/18050256/2018	2018/05/12	1	1	1	2	1	3	1	2	B	Robben Island
A/African penguin/Namibia/146S/2019	2019/01/17				1		2			C or E-like	Namibia
A/African penguin/Namibia/146T/2019	2019/01/17				1		2			C or E-like	Namibia
A/African penguin/Namibia/218-1/2019	2019/02/05				1		2			C or E-like	Namibia
A/African penguin/Namibia/218-2/2019	2019/02/05				1		2			C or E-like	Namibia

Spatial distribution of genotypes

Genotype C showed the widest distribution range from George to Malgas Island and included Bonnievale. The distribution of Genotype A viruses was restricted to locations in Walker Bay and False Bay. All of the Gansbaai isolates belong to the same genotype (Genotype B).

Duration of genotype detections

The longest period of detection of any genotype was Genotype B, occurring from 31 December 2017 to 12 May 2018. Detections of Genotype C viruses would extend for a significantly longer period than Genotype B if the 2019 Namibian isolates grouped with it as well. Genotype C virus was isolated from a very large swift tern breeding colony in Cape Town where large numbers of birds were dying. This was the first time in coastal birds in 2018 that such high numbers of birds in one place succumbed to the virus (L. Roberts, pers. com). A Genotype C virus was also responsible for the next big swift tern die-off further up the West Coast on Malgas Island. Genotype D viruses were only isolated on 29 January and 15 February 2018.

Species diversity

Three birds of different species, namely an African penguin, a Cape cormorant and a Sandwich tern were all sampled on 15 February 2018 and at the same location yet represented two different genotypes (Genotypes A and D). Generally no trends of any one genotype occurring in a specific bird species was observed, although the sample sizes of each species varied considerably and were too small to substantiate any trends.

Namibian outbreak

Comparisons with the genotypes of the Western Cape isolates suggest that the 2019 Namibian viruses could be linked to Genotypes C or E because the their HA and NA segments grouped together with the Western Cape Genotype C and Genotype E viruses. This however can only be postulated based on analyses of the HA and NA genome segments because complete genomes of the Namibian isolates were not available at the time of writing.

Another possibility is that, due to the length of time between the last Western Cape virus isolation and the first virus detection in Namibia, the Namibian outbreak may have been as a result of an independent introduction. In that case, they may represent a completely separate genotype of clade 2.3.4.4b H5N8 HPAI viruses. This inference can made based on the presence of lysine at position 276 of their haemagglutinin genes (Segment 4), while all the other isolates had glutamic acid in that position (Fig. 11(f)). This would make them unique among all the coastal bird isolates.

3.6 Identification of molecular markers

The alignments of all the nucleotide sequences used for the phylogenetic analyses were translated to amino acids to identify molecular markers.

Multiple amino acid sequence alignments of each gene were prepared to detect substitutions, comparing the sequences with each other to identify consistencies and patterns within the coastal bird isolates.

3.6.1 Segment 1 - polymerase (PB2) gene

A mutation known to increase virulence in chickens, K627E, was not observed (Suttie et al., 2019). The D701N mutation observed in this gene has been shown to increase viral replication in mammalian cells, and to increase virulence in mice and contact transmission in guinea pigs (Suttie et al., 2019). This substitution can partially function similar to E627K (Abolnik et al., 2016). The motif has not been found in waterfowl but was detected in an H5N2 HPAI strain isolated from ostriches in 2004 (Abolnik et al., 2016).

3.6.2 Segment 2: Polymerase (PB1) and putative PB1-F2 gene

The D3V mutation seen in the PB1 genes of all the viruses has been experimentally demonstrated to increase polymerase activity and viral replication in mammalian and avian cell lines (Suttie et al., 2019). The D622G substitution observed in all strains has been shown to increase polymerase activity in mice. Viruses 18020303, 18010371, 18010369, 18020302 and 18010027 all have an S654G substitution reinforcing the observation that they share a common ancestor.

Two substitutions in the PB1-F2 gene, N28S and L34S, confirm that viruses 18010028 and 18010043 share a recent common ancestor as observed in the phylogenetic tree. No molecular markers affecting biological characteristics were present in the PB1-F2 protein section.

3.6.3 Segment 3: Polymerase acidic (PA) gene

All the PA genes have S37A, P190S, N383D and N409S substitutions. These motifs have mostly been shown to affect polymerase activity in mammalian cell lines, but N383D also increases polymerase activity in avian cell lines (Suttie *et al*., 2019). Twelve of the genes have glycine at position #657, which occurs on the second domain (C-PA) region of the PA gene.

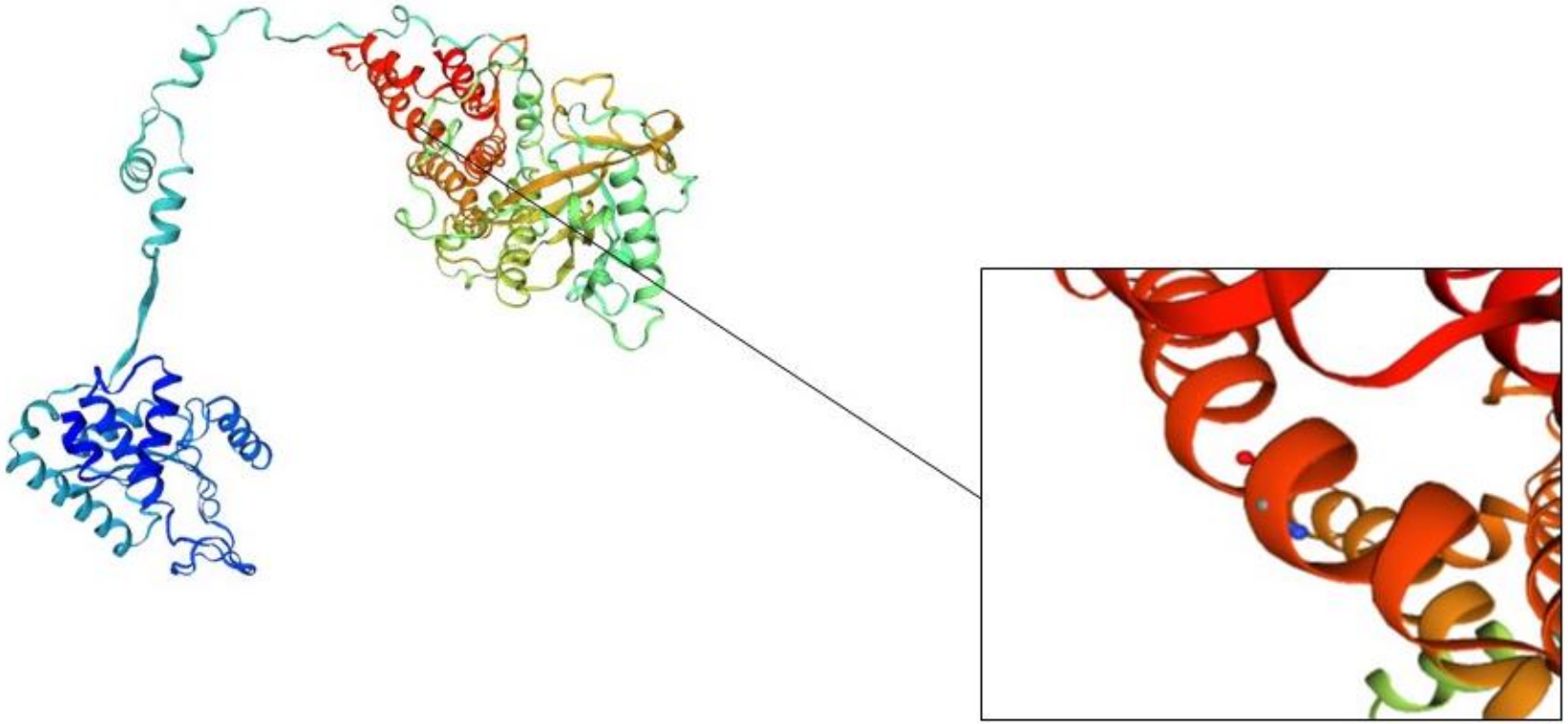


Figure 11(d) Diagram of the polymerase acidic gene of Influenza A (H5N8) virus showing the N-PA and C-PA domain regions connected by a linkage region. Position #657 occurs on the C-PA domain (insert). *Source: https://swissmodel.expasy.org/*

Segment 3 (cont.): Polymerase acidic (PA-X) gene

No known molecular markers were evident in the PA-X genes (Suttie et al., 2019). Three viruses, 18020303, 18010371 and 08020302 share a common ancestor, and have an R199G substitution.

3.6.4 Segment 4: Haemagglutinin (HA) gene

The cleavage site (HA0) motif of all the HA gene segments was PLREKRRKR*GLF (shaded in green in fig. 11(f)), with the multiple basic amino acids lysine and arginine confirming that these viruses are indeed HPAI strains. The cleavage site motif is listed in OFFLU's 20 March 2019 version of reported Influenza A haemagglutinin cleavage sites (OFFLU 2019).

Three substitutions in the HA protein (D94N, S154N and Q222L) present in all the strains are indicative of increased virus receptor binding avidity by α2-6 sialic acid linkages present in the respiratory tract of humans (Suttie et al., 2019). This is considered a key factor for the development of human AIV pandemics (Suttie et al., 2019). An E276K substitution was observed in all four of the Namibian isolates. Three of the Namibian viruses were isolated from tissue samples (Umberto et al., 2020) and have an M306I substitution. The fourth virus was isolated from a swab taken from one of the same birds from which tissue samples were collected, and has methionine in position 306 consistent with all the other isolates. There appear to be minimal selection or adaptive changes in the HA gene despite the wide host range of the viruses.

3.6.5 Segment 5: Nucleoprotein (NP) gene

Glycine is present in position 375 of the nucleoprotein gene of all Genotype C isolates. One molecular marker, A184K, present in all the strains has been experimentally verified to increase virulence in chickens (Suttie *et al*., 2019).

3.6.6 Segment 6: Neuraminidase (NA) gene

No experimentally verified molecular markers associated with increased virulence were detected in the neuraminidase gene. Aspartic acid in position 396 of Cluster 1 NA genes supports the grouping of that cluster.

3.6.7 Segment 7: Matrix protein 1 (M1) gene

In the M1 proteins, all strains had N30D and T215A substitutions. These indicate increased virulence for mice (Suttie et al., 2019). Additionally an I43M substitution also present has been experimentally verified to result in increased virulence in mice, chickens and ducks (Suttie et al., 2019).

Segment 7: Matrix protein 2 (M2) gene

Serine occurs at position 13 of the matrix-2 (Segment 7-M2) of all the Genotype B viruses. No known virulence markers were present in the M2 proteins.

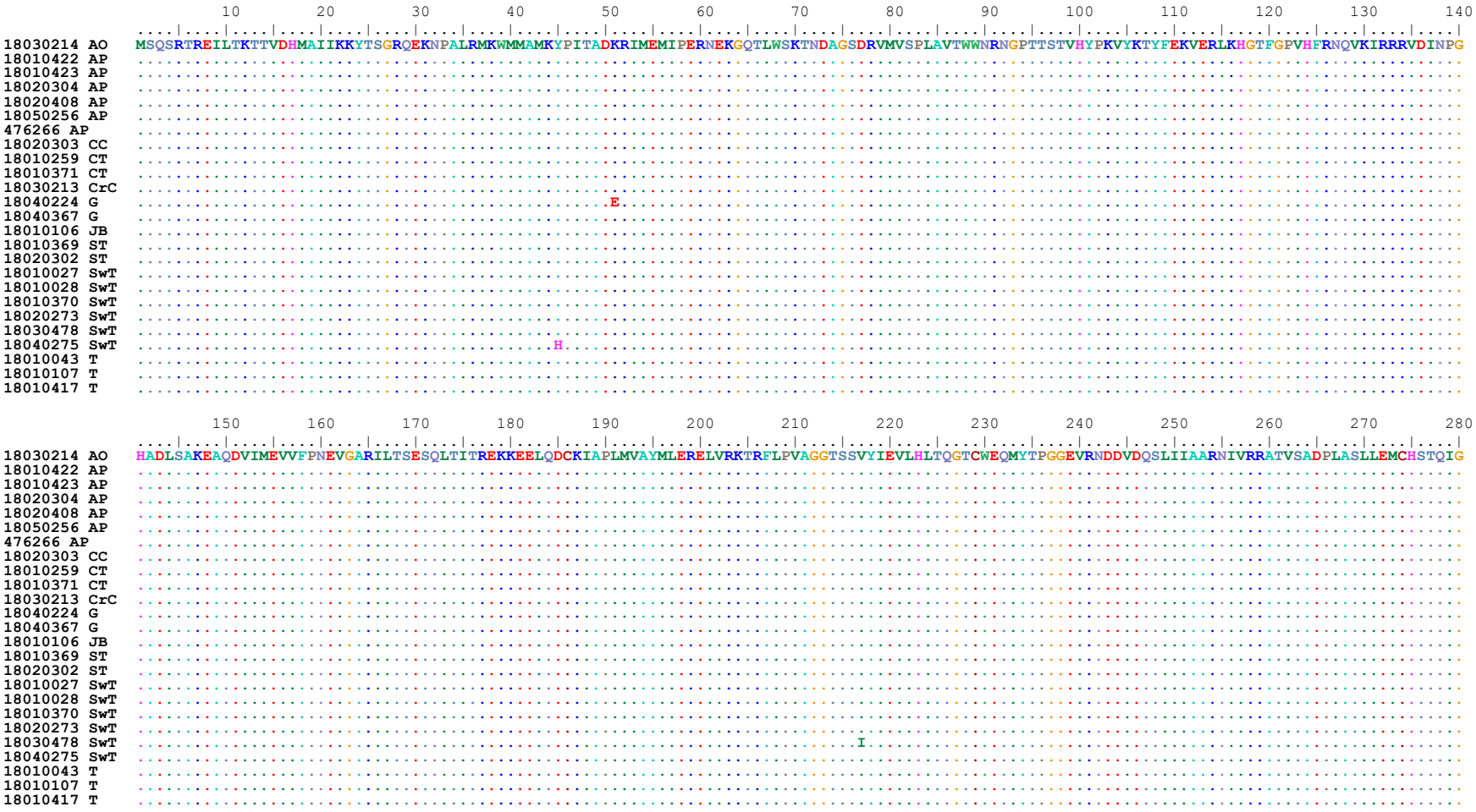
3.6.8 Segment 8: Non-structural protein 1 (NS1) gene

Seven experimentally verified molecular markers were present in the NS1 proteins of all the strains. One of them, V149A, can cause increased virulence and decreased interferon response in chickens (Suttie *et al*., 2019). The other six, P42S, K55E, K66E, L103F, I106M and C138F, have been associated with viral response in mice and mammalian cell lines (Suttie *et al*., 2019)

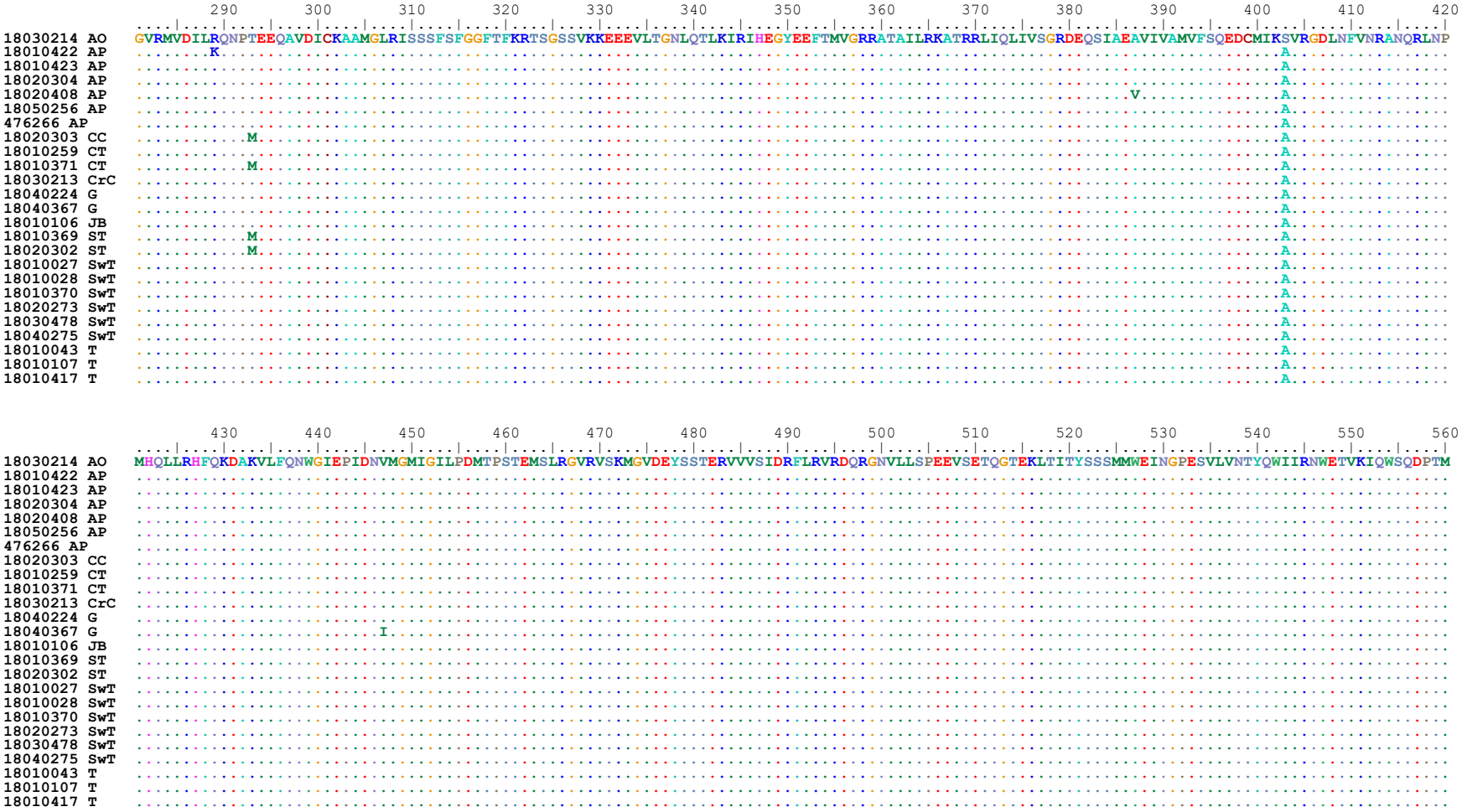
Segment 8: Nuclear export protein (NEP) gene

Histidine occurs in position 92 of the nuclear export protein genes of all Genotype C isolates. No virulence markers were observed in the NEP proteins.

Segment 1 - polymerase (PB2) gene



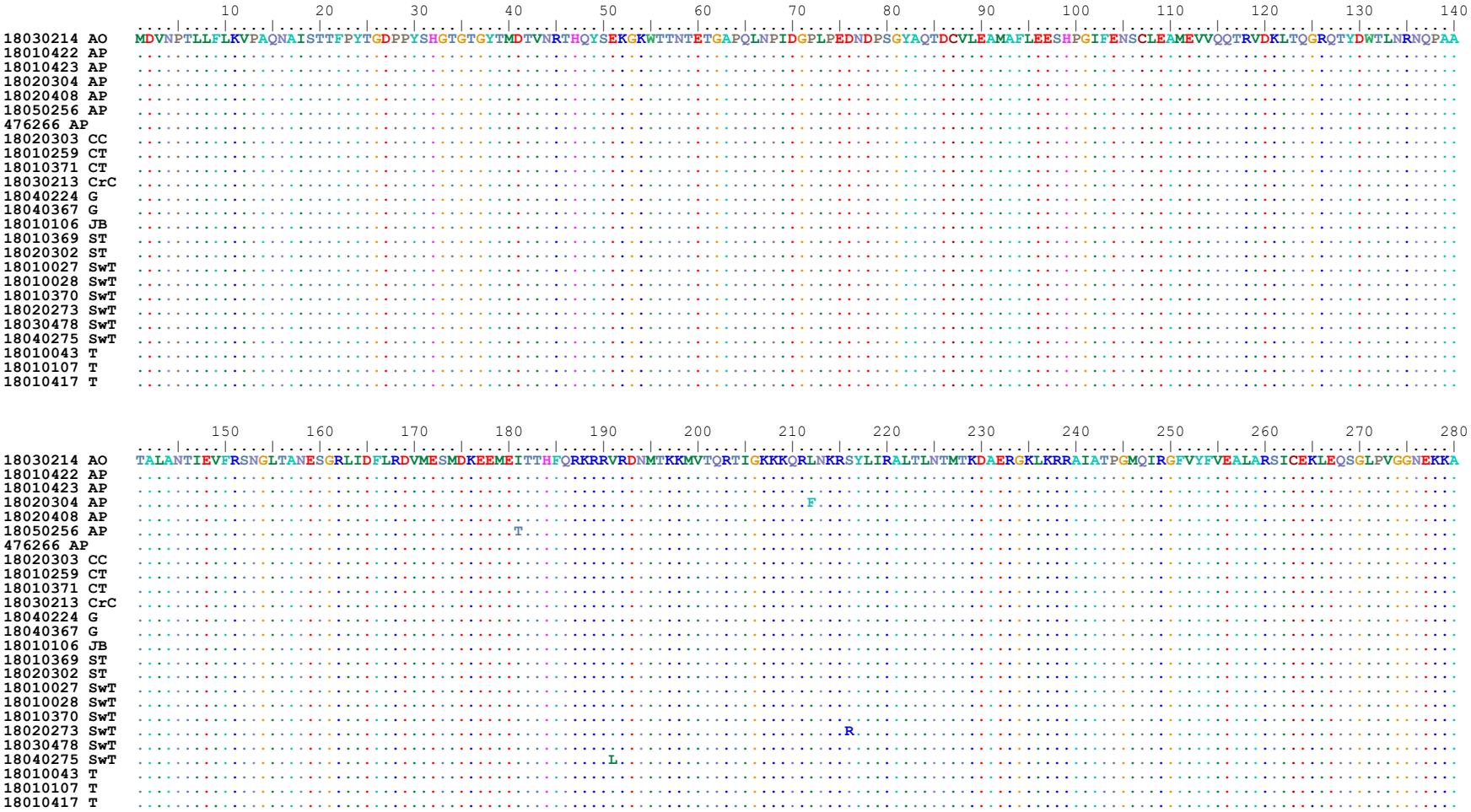
Segment 1 - polymerase (PB2) gene (cont.)



Segment 1 - polymerase (PB2) gene (cont.)

Figure 11(a) Multiple amino acid sequence alignment for segment 1, encoding the PB2 gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 2: Polymerase (PB1) and putative PB1-F2 gene



Segment 2 - polymerase (PB1) and putative PB1-F2 gene (cont.)

Segment 2 - polymerase (PB1) and putative PB1-F2 gene (cont.)

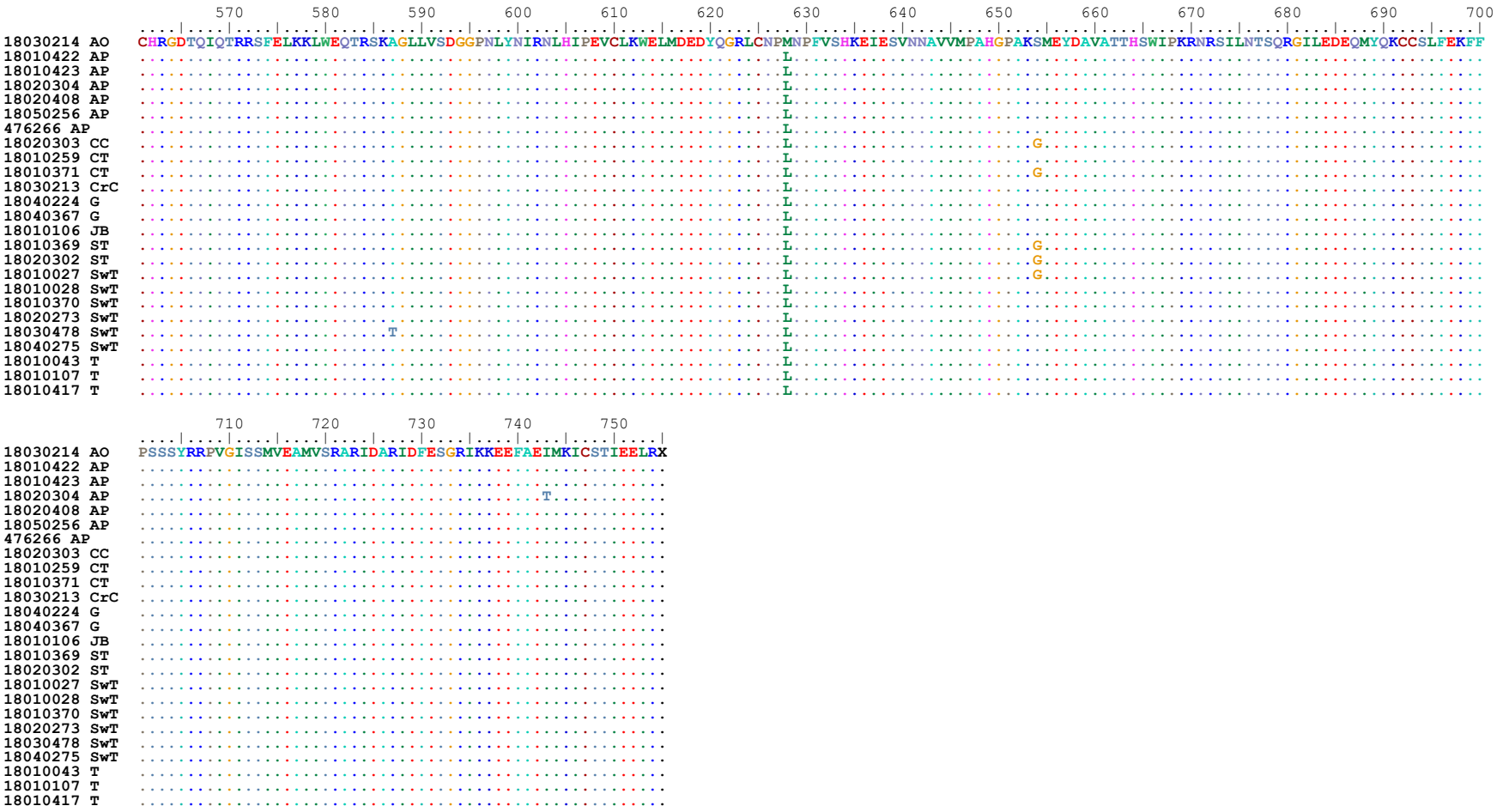
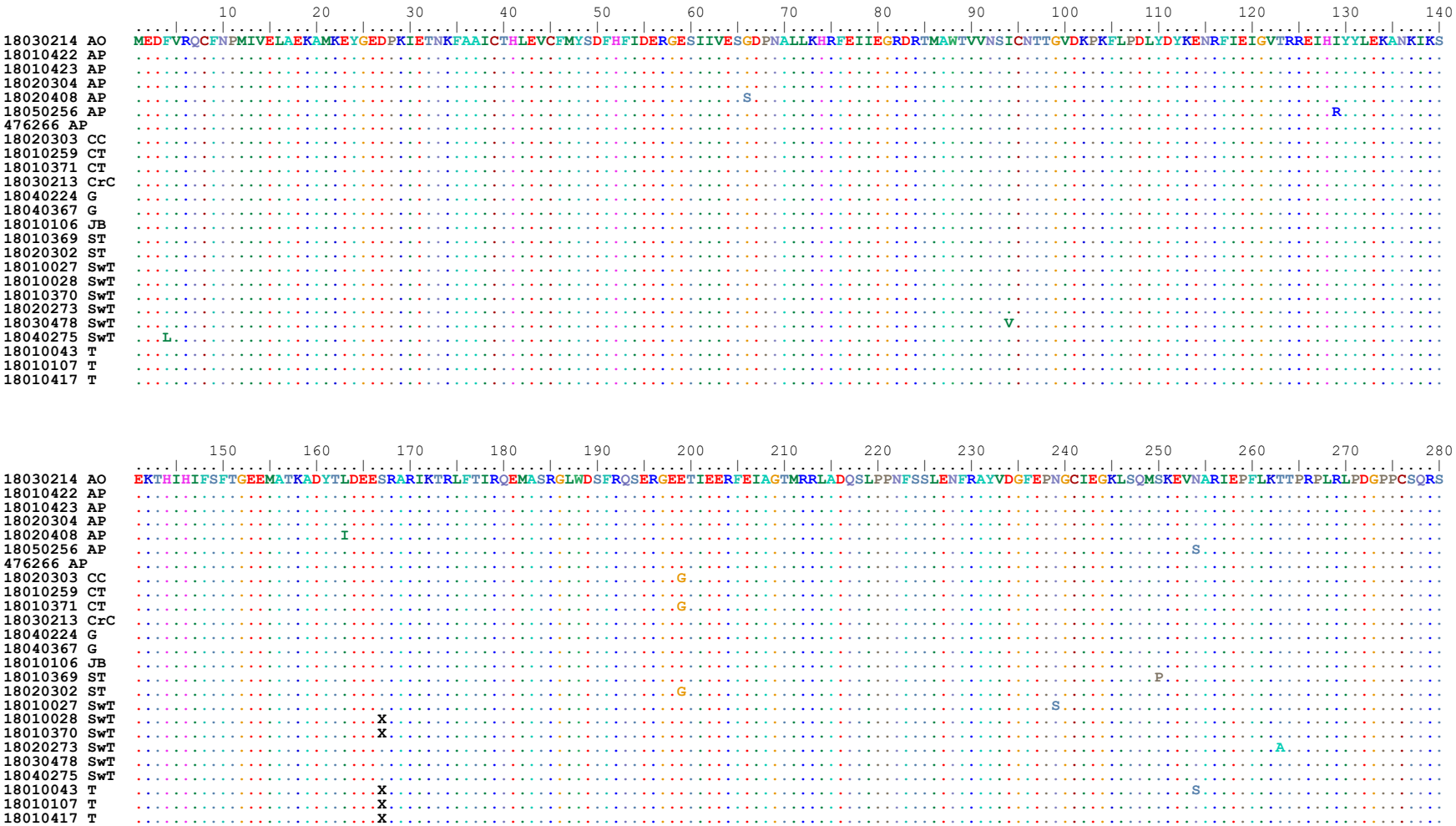


Figure 11(b) Multiple amino acid sequence alignment for segment 2, encoding the PB1 gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 3: Polymerase acidic (PA) gene



Segment 3- polymerase acidic (PA) gene (cont.)

Segment 3 - polymerase acidic (PA) gene (cont.)

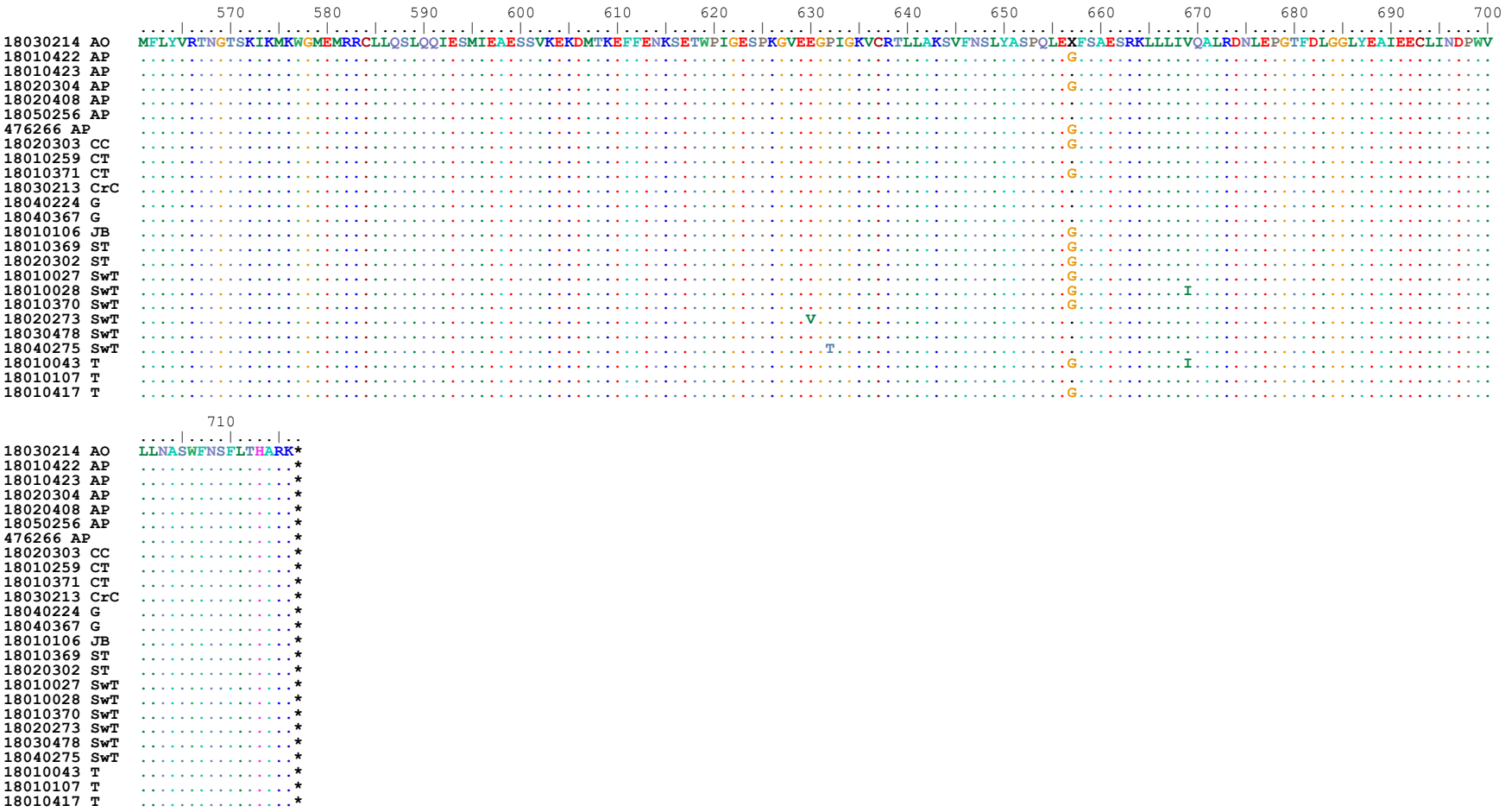


Figure 11(c) Multiple amino acid sequence alignment for segment 3, encoding the PA gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 3: Polymerase acidic (PA-X) gene

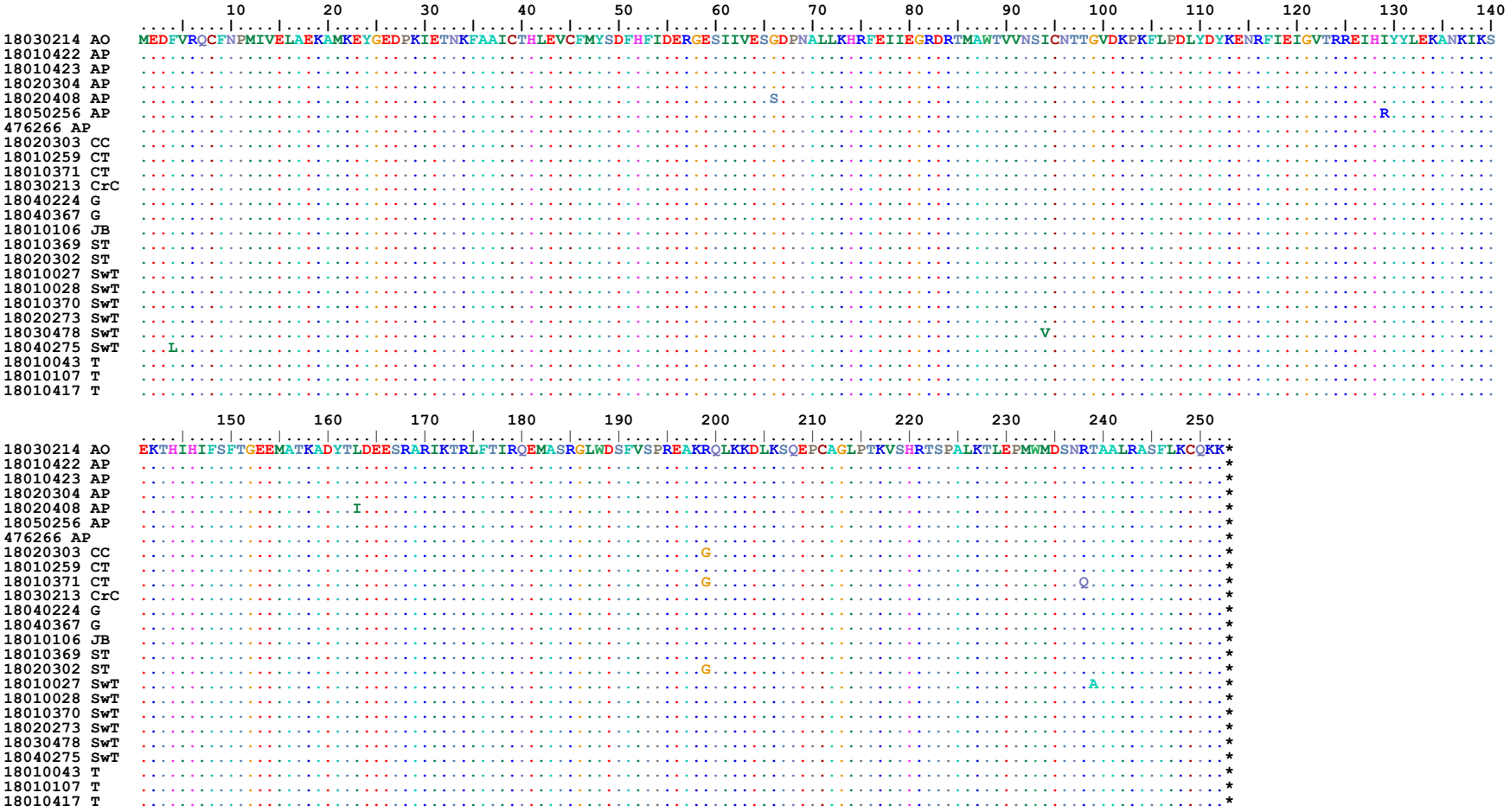
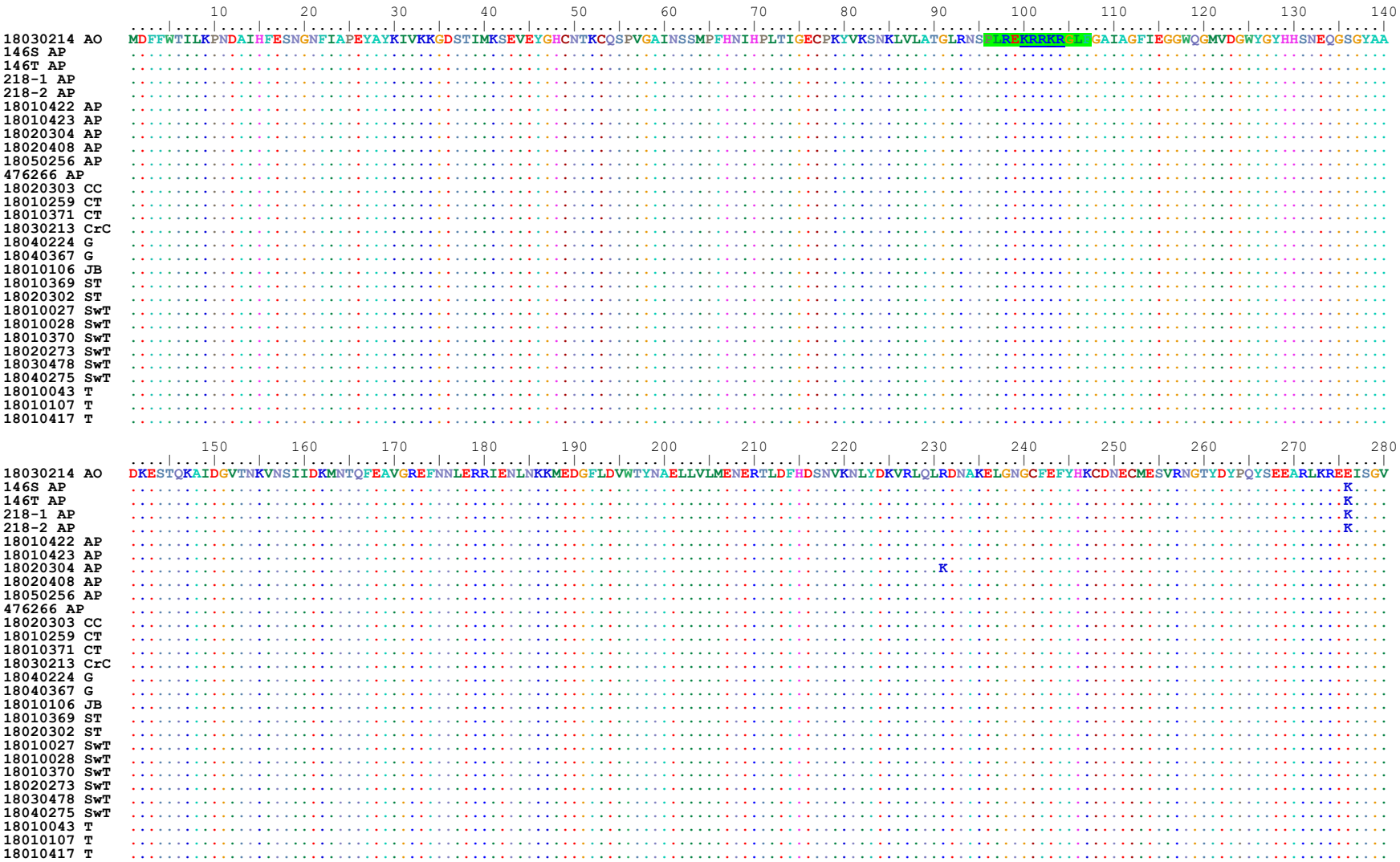


Figure 11(e) Multiple amino acid sequence alignment for segment 3, encoding the PA-X gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 4: Haemagglutinin (HA) gene



Segment 4 - haemagglutinin (HA) gene (cont.)

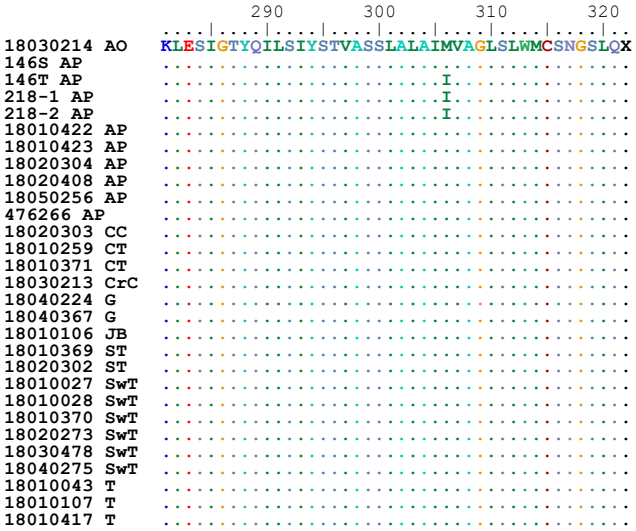
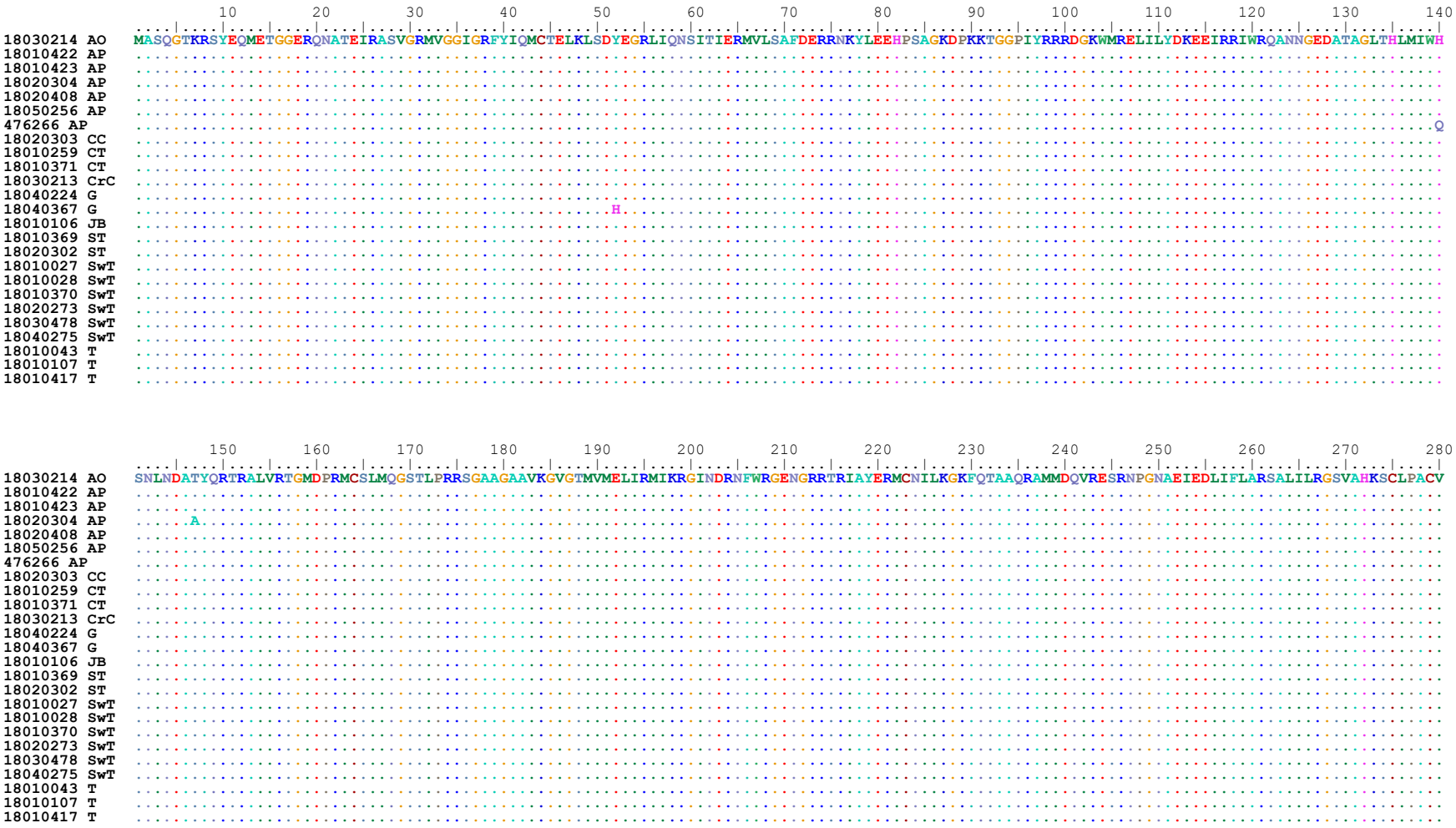


Figure 11(f) Multiple amino acid sequence alignment for segment 4, encoding the HA gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 5: Nucleoprotein (NP) gene



Segment 5 - nucleoprotein (NP) gene (cont.)

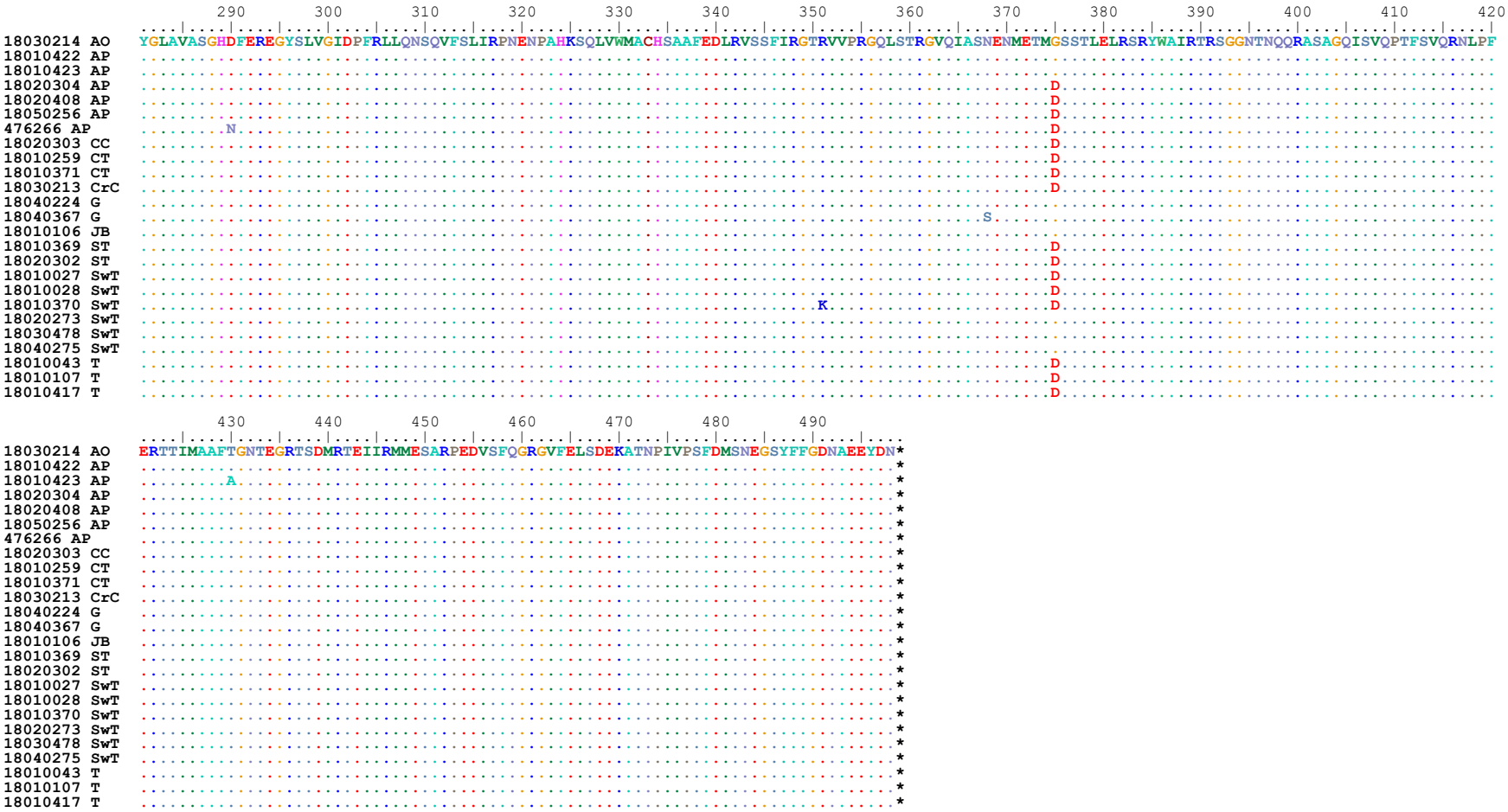
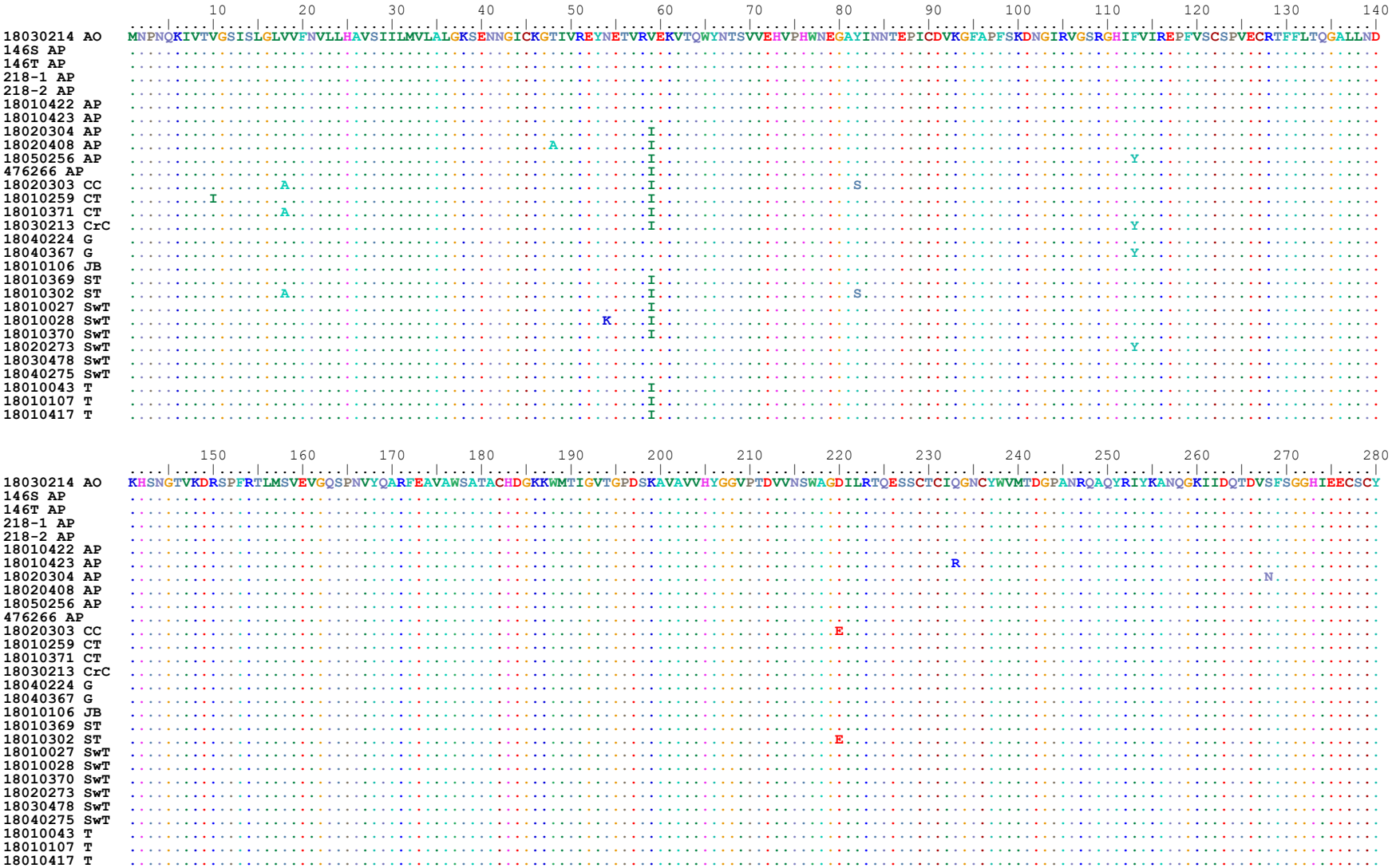


Figure 11(g) Multiple amino acid sequence alignment for segment 5, encoding the NP gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 6: Neuraminidase (NA) gene



Segment 6 - neuraminidase (NA) gene (cont.)

Figure 11(h) Multiple amino acid sequence alignment for segment 6, encoding the NA gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 7: Matrix protein 1 (M1) gene

Figure 11(i) Multiple amino acid sequence alignment of segment 7 encoding the M1 gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 7: Matrix protein 2 (M2) gene

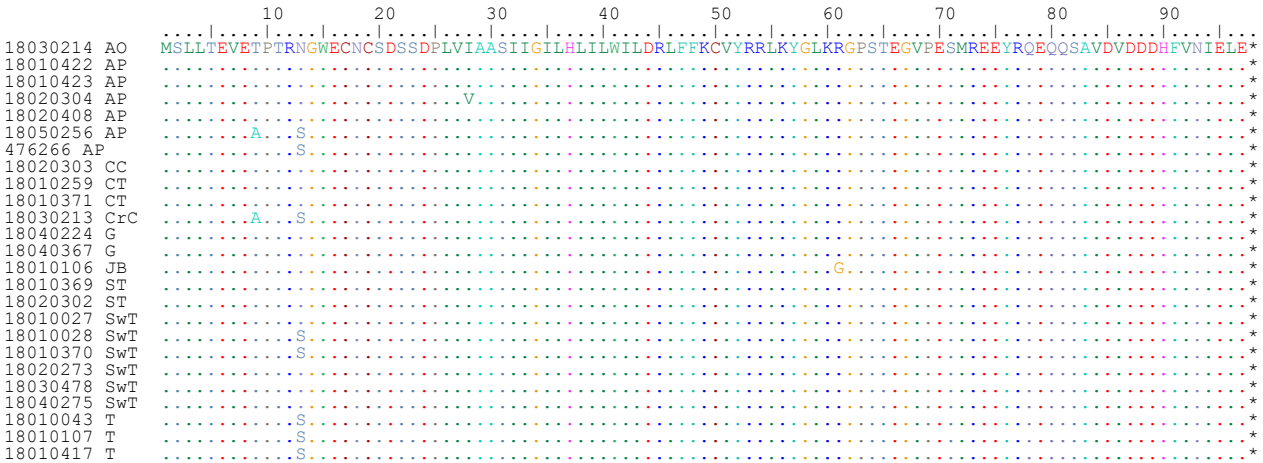


Figure 11(j) Multiple amino acid sequence alignment of segment 7 encoding the M2 gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 8: Non-structural protein 1 (NS1) gene

Figure 11(k) Multiple amino acid sequence alignment for segment 8, encoding the NS1 gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

Segment 8: Nuclear export protein (NEP) gene

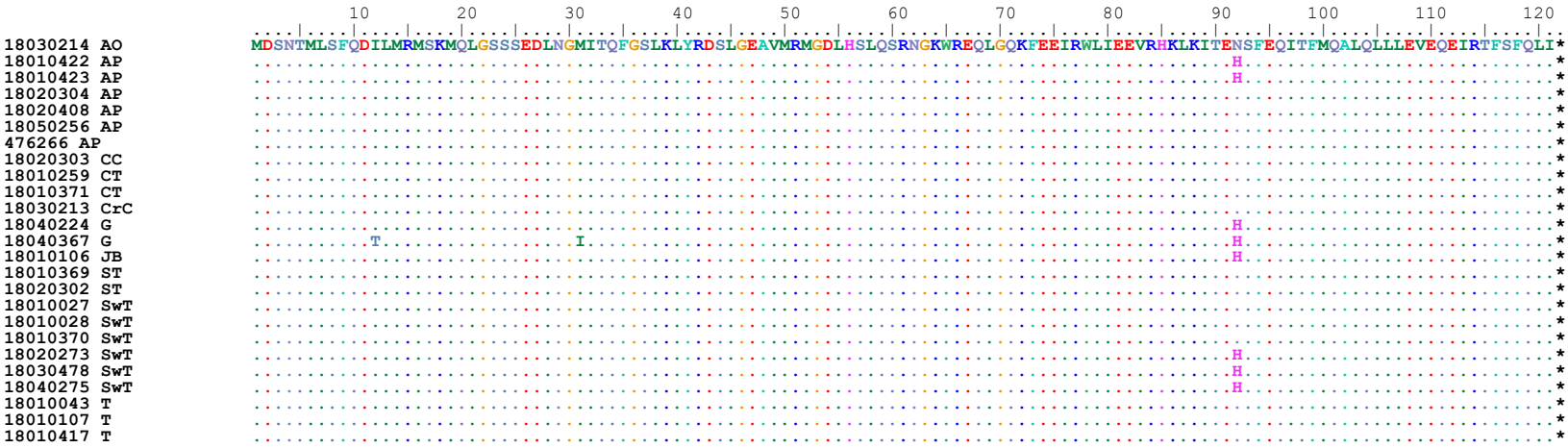


Figure 11(l) Multiple amino acid sequence alignment for segment 8, encoding the NEP gene. Isolates are listed according to sample identification numbers only, with abbreviations indicating the bird species. AO (African oystercatcher), AP (African penguin), CC (Cape cormorant), CT (common tern), CrC (crowned cormorant), G (Hartlaub's gull), JB (jackal buzzard), ST (Sandwich tern), SwT (swift tern), T (tern).

CHAPTER 4

CONCLUSIONS

This study was centred around the first highly pathogenic avian influenza outbreak reported in wild coastal birds in South Africa since 1959. Affected birds were first detected in December 2017, when the poultry industry in the Western Cape province was still reeling from the effects of the outbreak of strains of the same virus during the previous four months.

Phylogenetic analysis of the H5N8 HPAI viruses responsible for coastal bird mortalities up until June 2018 provided information regarding the origins of these H5N8 HPAI viruses within the Western Cape province in various coastal bird species, including threatened and endangered birds. No direct link between the terrestrial outbreaks and the coastal bird outbreaks was apparent in the phylogenetic trees. There was no evidence of multiple, recurrent spillover events from land to coastal species. In some gene segments though, the coastal bird strains' closest relatives were poultry strains rather than migratory birds. Long branch lengths could indicate unsampled ancestor viruses.

The detection of outbreaks of clade 2.3.4.4b H5N8 HPAI in coastal birds coincided with the start of the annual summer holiday season, which generally sees a high influx of people to beaches and coastal areas in the Western Cape. Areas of coastline that were more easily accessible at that time may be over-represented. The likelihood that diseased or dead birds would be found and reported to authorities at that time and in the ensuing summer months by members of the public may have increased. Additionally, by that stage of the epidemic, media exposure could have resulted in greater public awareness, perhaps more so than if this had been a disease of lesser public interest. The outbreaks in coastal birds could have started earlier or could have corresponded with the terrestrial bird outbreaks in the Western Cape over the preceding four months, but were not detected.

Care should be taken to ensure that the number of isolates from any particular bird species, as well as the distribution of bird species in these coastal areas are interpreted in the correct context and without bias. No conclusions can be drawn as to the total numbers of outbreaks, the total range of species involved, or the areas affected.

Factors responsible for these HPAIV H5N8 outbreaks in coastal birds of the Western Cape, and the means of their introduction into this population, are as yet unknown. Sympatric species such as sacred ibis and Egyptian geese may be implicated. Future research could include comparisons of the viruses isolated in the coastal birds with the terrestrial bird strains by full genome analysis to determine if there are any common molecular markers of clinical disease. Similarly, comparisons with LPAI viruses

circulating in the wild bird population could be carried out. The identification of molecular markers that might predict pathogenicity in coastal bird species could shed light on possible reasons why this is the first HPAIV isolated in the Western Cape province that has caused considerable mortalities in these birds since the 1961 outbreak. No species specific molecular markers could be identified because of the small sample size. Future research could also investigate molecular markers that may indicate temporal or geospatial influences.

The Namibian outbreaks of HPAIV H5N8 that occurred in penguins in early 2019 raise questions and present opportunities for further investigation. Regarding the introduction of the viruses into that population, migration patterns of African penguins along the Southern African coastline is well documented. The range of their breeding colonies extends from islands off the Namibian coast eastwards to Algoa Bay (Whittington *et al*., 2005). They may travel considerable distances between colonies, and birds from the Eastern Cape have been known to travel over 1700km to Ichaboe Island on the Namibian coast (Whittington *et al*., 2005). It is possible that the viruses could have been transmitted to the Namibian penguins by penguins from the Western Cape. In 2005 Whittington *et al.* concluded that food resources for African penguins along these coastlines were adequate to support the movement of birds between and within these territories (Whittington *et al*., 2005). Since then, however, fish stocks have been significantly reduced due to overfishing and climate change (Sherley *et al.*, 2017). Juvenile penguins in particular target environmental cues associated with abundant prey but suffer high mortalities due to fish depletion in these ecological traps (Sherley *et al.*, 2017).

The penguin breeding colony where the viruses were detected is shared by many other coastal bird species. Sympatric species include gulls, terns, cormorants and African oystercatchers (*Haematopus moquini*) (J. Kemper, pers. comm.). At the time of writing, there is circumstantial evidence from phylogenetic analysis of the H gene that gulls may be implicated as vectors, introducing the virus into the Namibian populations from the Western Cape. In the phylogenetic tree one of the gull isolates, 18040367, formed a sub-cluster with the four Namibian isolates.

Another possibility is that, due to the length of time between the last Western Cape virus isolation and the first virus detection in Namibia, the Namibian outbreak may have been as a result of an independent introduction. In that case, they may represent a completely separate genotype of clade 2.3.4.4b H5N8 HPAI viruses. With the current data this inference can made based on the presence of lysine at position 276 of their haemagglutinin genes while all the other isolates had glutamic acid in that position (Fig. 11(f)). This would make them unique among all the coastal bird isolates.

The impact of influenza viruses on wild animal host survival, reproduction and behaviour are largely unknown, and these factors have important conservation and disease management implications. In coastal birds, long-distance movements of viruses are possible during pre-clinical periods of infection (Vandegrift *et al*., 2010). Large numbers of coastal birds regularly migrate from Europe to South Africa (Vandegrift *et al*., 2010). Increased active environmental monitoring especially at wild bird breeding and feeding sites may identify locations and periods of heightened risk for the transmission of AIV in coastal birds and migratory species.

Further study of these viruses, including phylogeographic and phylodynamic analyses is warranted to investigate factors that may have led to this HPAI virus outbreak in coastal birds at the end of 2017. Analyses that model the rate of molecular evolution with molecular clock models (e.g. BEAST) can be performed. Full genome analyses could give an indication whether the D94N, S154N and Q222L substitutions in the HA protein may be as a result of selection or simply random. Single point mutations in the HA gene of the Namibian viruses could also be further investigated with stronger insights from full genome analyses.

Contributing factors need to be investigated by extensive environmental and/or epidemiological surveys. Whether the infection burden pressure in the poultry population in the Western Cape province prior to the introduction into coastal birds was unique should be explored. Population density of sympatric species and shared habitat use, especially at the first coastal areas that were affected, should be explored. Although in temperate and boreal regions temperature anomalies can affect bird migration, in Sub-Saharan Africa the main trigger for wild bird movement is rainfall and the ensuing food and water availability (Fusaro, *et al*., 2019). Consideration should be given to effects of the preceding drought and climate change on movement patterns and habitats of these birds to determine to what extent, if at all, these factors played a role in the incursions of H5N8 in wild coastal birds in South Africa. Relay transmission in various birds migrating south through Africa from Europe, and locally within Southern Africa, might not concur with the timing of their arrivals and incursion of the virus. Such information may aid in determining factors such as whether gulls may indeed be implicated as vectors of the virus from the Western Cape to Namibia.

The full genomes of twenty-three of these viruses, isolated from terns, cormorants, penguins, gulls and an oystercatcher from December 2017 to May 2018, were sequenced using Ion Torrent sequencing. Results indicated that there may have been a single introduction of the virus into the coastal bird population, possibly from the terrestrial wild bird population. The terrestrial strains were basal to the coastal bird strains, but still not closely related. The coastal bird viruses have a common ancestor with the Namibian outbreaks that occurred at the beginning of 2019 and they may have been the source of

the Namibian outbreaks. Alternatively, an introduction of the virus into the Namibian birds could have been independent of the South African incursions. Future research to determine why these viruses reached Southern Africa when previously globally circulating strains did not, and to evaluate the possibility that this may be a cyclic threat, would contribute greatly to international HPAI control.

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APPENDICES

APPENDIX 1: Sample submission form completed for each bird/sample submitted to the laboratory

Avian Influenza Virus in Terns in the Western Cape
Western Cape Provincial Veterinary Laboratory

Sample submission process for virus culture

Sample details:

Bird species identification: _____

Received live or dead? ☐ Live ☐ Dead Date received: _____

If live: euthanased or died naturally? ☐ Euthanased ☐ Naturally Date of death: _____

Clinical signs: _____

_____

Other comments/information: _____

_____

GPS co-ordinates of place where bird was found, if available:

Longitude (e.g. -34.123456)	
Latitude (e.g. 19.123456)	

Date of sample collection: _____

Sample type (organs or carcass): _____

Sending veterinarian: _____

Details and suggestions:

Required information	*Suggestions*
Species identification	Photographs: front and back, with wings extended, tail spread open, and size indication (lay a ruler next to the carcass when taking the photo, check ruler is in focus).
GPS co-ordinates of place where it was found, if available	On Google maps: click on the screen on the right spot and the co-ords should pop up in the search box. If not available, an indication of where the bird was found
Sample type	Organ pool: spleen, kidney, liver, lung, trachea, brain. In sterile container, chilled. Please do NOT freeze the sample. Put an ice pack in the sample box. Only about 5 grams of pooled tissue is required.

Dear veterinarian,

Thank you very much for your willingness to participate in this project. You are assisting us to collect this very valuable data for epidemiological analysis of the highly pathogenic AI virus currently circulating in South Africa. Your services will be acknowledged in any published data that may originate from this survey.

Above is a guideline for what we require for each bird sampled. Please bear in mind that we are attempting to culture the *LIVE VIRUS* (it's not a PCR test), so getting the samples as soon as possible and maintaining the cold chain is imperative.

If sampling and collection of the tissue is NOT possible, we would be grateful to receive the entire bird carcass and do the sampling ourselves. Although this is not ideal, due to possible deterioration of the condition of the carcass (autolysis) and resulting bacterial contamination, it would still be acceptable. Please provide sample details (see above) for each bird. For multiple cases keep each sample pool separate and mark clearly (i.e. one pool per bird).

Please contact us for any queries:

(Ms) Belinda Peyrot
Tel: 021 887 0324
E-mail: belindap@elsenburg.com

Dr Tasneem Anthony
Tel: 021 887 0324
E-mail: tasneema@elsenburg.com

Physical address:
Helderfontein
Helshoogte Road
Stellenbosch
7599

Courier:
Skynet (account number C 15976)

If lost, please return this form to: Belinda Peyrot, WCPVL, P/Bag X5020, Stellenbosch, 7600

APPENDIX 2: Accession numbers of reference strains included in Segment 1 (Polymerase basic 2) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595233	
A/chicken/South Africa/115370/2017(H5N8)		MH165801	
A/chicken/South Africa/17080336-P3/2017(H5N8)	S2017/08_0336_P3		EPI1187455
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1188007
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1187894
A/chicken/South Africa/17090108/2017(H5N8)		MH165833	
A/chicken/South Africa/17090184-63/2017(H5N8)	S2017/09_0184_63		EPI1187945
A/chicken/South Africa/17090348/2017(H5N8)		MH165761	
A/chicken/South Africa/443397/2017(H5N8)		MH165633	
A/chicken/South Africa/448475/2017(H5N8)		MH165737	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165529	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165521	
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973222	
A/domestic goose/South Africa/17090065/2017(H5N8)		MH165705	
A/dove/South Africa/17080324/2017(H5N8)		MH165649	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223867
A/duck/Democratic Republic of the Congo/17RS882-29/2017(H5N8)			EPI1223821
A/duck/Egypt/SS19/2017(H5N8)			EPI1018177
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165545	
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)		MG881943	
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1188137
A/guineafowl/South Africa/17080274/2017(H5N8)		MH165625	
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1187949
A/Pekin duck/South Africa/17080340/2017(H5N8)		MH165665	
A/Pekin duck/South Africa/17080481/2017(H5N8)		MH165673	
A/pigeon/South Africa/17080323/2017(H5N8)		MH165641	
A/sacred ibis/South Africa/009/2017(H5N8)		MH165609	
A/swan/South Africa/17080517/2017(H5N8)		MH165681	
A/white-winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569644	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320440	

[1] *not isolated*

APPENDIX 3: Accession numbers of reference strains included in Segment 2 (Polymerase basic 1) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595234	
A/chicken/Cameroon/17RS1661-1/2017(H5N8)			EPI1223866
A/chicken/Egypt/Kafr-Elshiekh-18/2017(H5N8)			EPI1104273
A/chicken/South Africa/115370/2017(H5N8)		MH165802	
A/chicken/South Africa/443397/2017(H5N8)		MH165634	
A/chicken/South Africa/448475/2017(H5N8)		MH165738	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165530	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165522	
A/chicken/South Africa/17080336-P3/2017(H5N8)	S2017/08_0336_P3		EPI1106512
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1106459
A/chicken/South Africa/17080581-P2/2017(H5N8)	S2017/08_0581_P2		EPI1106514
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1187894
A/chicken/South Africa/17090184-63/2017(H5N8)	S2017/09_0184_63		EPI1106504
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973223	
A/domestic goose/South Africa/17090065-P2/2017(H5N8)	S2017/09_0065_P2		EPI1106510
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223865
A/duck/Democratic Republic of the Congo/17RS882-5/2017(H5N8)			EPI1223828
A/duck/South Africa/17080340-P1/2017(H5N8)	S2017/08_0340_P1		EPI1106508
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165546	
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200926
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1106513
A/guineafowl/South Africa/17080274-P1/2017(H5N8)	S2017/08_0274_P1		EPI1106506
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1106502
A/ostrich/South Africa/17080361-P17/2017(H5N8)	S2017/08_0361_P17		EPI1106500
A/pigeon/South Africa/17080323-P1/2017(H5N8)	S2017/08_0323_P1		EPI1106511
A/sacred ibis/South Africa/009/2017(H5N8)		MH165610	
A/white-winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569643	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320441	

[1] *not isolated*

APPENDIX 4: Accession numbers of reference strains included in Segment 3 (Polymerase acidic) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595235	
A/chicken/Cameroon/17RS1661-1/2017(H5N8)			EPI1223866
A/chicken/South Africa/115370/2017(H5N8)		MH165803	
A/chicken/South Africa/443397/2017(H5N8)		MH165635	
A/chicken/South Africa/448475/2017(H5N8)		MH165739	
A/chicken/South Africa/17080336-P2/2017(H5N8)	S2017/08_0336_P2		EPI1106478
A/chicken/South Africa/17080416-38/2017(H5N8)	S2017/08_0416_38		EPI1106482
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1106458
A/chicken/South Africa/17080581-P2/2017(H5N8)	S2017/08_0581_P2		EPI1106485
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1106486
A/chicken/South Africa/17090184-63/2017(H5N8)	S2017/09_0184_63		EPI1106490
A/chicken/South Africa/Standerton/2017(H5N8)		MH165531	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165523	
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973224	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223864
A/duck/Democratic Republic of the Congo/17RS882-29/2017(H5N8)			EPI1223829
A/duck/Egypt/SS19/2017(H5N8)			EPI1018179
A/duck/South Africa/17080340-P2/2017(H5N8)	S2017/08_0340_P2		EPI1106481
A/duck/South Africa/17080481-P2/2017(H5N8)	S2017/08_0481_P2		EPI1106483
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165547	
A/goose/South Africa/17080558-P1/2017(H5N8)	S2017/08_0558_P1		EPI1106484
A/goose/South Africa/17090055-P1/2017(H5N8)	S2017/09_0055_P1		EPI1106487
A/goose/South Africa/17090065-P2/2017(H5N8)	S2017/09_0065_P2		EPI1106466
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200939
A/guineafowl/South Africa/17080190-9/2017(H5N8)	S2017/08_0190_9		EPI1106471
A/guineafowl/South Africa/17080243-P1/2017(H5N8)	S2017/08_0243_P1		EPI1106472
A/guineafowl/South Africa/17080274-P1/2017(H5N8)	S2017/08_0274_P1		EPI1106475
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1106468
A/ostrich/South Africa/17080161-P8/2017(H5N8)	S2017/08_0161_P8		EPI1106470
A/ostrich/South Africa/17080362-P8-34/2017(H5N8)	S2017/08_0362_P8_34		EPI1106456
A/pigeon/South Africa/17080323-P1/2017(H5N8)	S2017/08_0323_P1		EPI1106477
A/sacred ibis/South Africa/009/2017(H5N8)		MH165611	
A/white winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569642	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320442	

[1] *not isolated*

APPENDIX 5: Accession numbers of reference strains included in Segment 4 (Haemagglutinin) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/Namibia/146S/2019(H5N8)		MK571753	
A/African penguin/Namibia/146T/2019(H5N8)		MK571754	
A/African penguin/Namibia/218-1/2019(H5N8)		MK571806	
A/African penguin/Namibia/218-2/2019(H5N8)		MK571807	
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595239	
A/chicken/South Africa/115370/2017(H5N8)		MH165804	
A/chicken/South Africa/443397/2017(H5N8)		MH165636	
A/chicken/South Africa/448475/2017(H5N8)		MH165740	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165532	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165524	
A/chicken/South Africa/17080336-P3/2017(H5N8)	S2017/08_0336_P3		EPI1093378
A/chicken/South Africa/17080416-38/2017(H5N8)	S2017/08_0416_38		EPI1104300
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1104414
A/chicken/South Africa/17080581-P2/2017(H5N8)	S2017/08_0581_P2		EPI106289
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1104305
A/chicken/South Africa/17090184-62/2017(H5N8)	S2017/09_0184_62		EPI1106294
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973225	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223854
A/duck/Democratic Republic of the Congo/17RS882-29/2017(H5N8)			EPI1223833
A/duck/Egypt/SS19/2017(H5N8)			EPI1018180
A/duck/South Africa/17080481-P2/2017(H5N8)	S2017/08_0481_P2		EPI1104310
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165548	
A/geese/South Africa/17080520-46/2017(H5N8)	S2017/08_0520_46		EPI1104366
A/geese/South Africa/17080558-P1/2017(H5N8)	S2017/08_0558_P1		EPI1104401
A/geese/South Africa/17090055-P2/2017(H5N8)	S2017/09_0055_P2		EPI1098532
A/geese/South Africa/17090065-P2/2017(H5N8)	S2017/09_0065_P2		EPI1104203
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200946
A/guineafowl/South Africa/17080190-9/2017(H5N8)	S2017/08_0190_9		EPI1104387
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1104391
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1104381
A/ostrich/South Africa/17080047-P7/2017(H5N8)	S2017/08_0047_P7		EPI1103576
A/ostrich/South Africa/17080161-P8/2017(H5N8)	S2017/08_0161_P8		EPI1104253
A/ostrich/South Africa/17080268-P2/2017(H5N8)	S2017/08_0268_P2		EPI1098487
A/ostrich/South Africa/17080361-P17/2017(H5N8)	S2017/08_0361_P17		EPI1094821
A/ostrich/South Africa/17080362-P8-	S2017/08_0362_P8_34		EPI1106299

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
34/2017(H5N8)			
A/pigeon/South Africa/17080323-P1/2017(H5N8)	S2017/08_0323_P1		EPI1104396
A/pigeon/South Africa/17080324-24/2017(H5N8)	S2017/08_0324_24		EPI1094811
A/sacred ibis/South Africa/009/2017(H5N8)		MH165612	
A/swan/South Africa/17080517-P2/2017(H5N8)	S2017/08_0517_P2		EPI1104315
A/white winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569637	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320443	

[1] *not isolated*

APPENDIX 6: Accession numbers of reference strains included in Segment 5 (Nucleoprotein) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595236	
A/chicken/Egypt/Kafr-Elshiekh-18/2017(H5N8)			EPI1104268
A/chicken/South Africa/115370/2017(H5N8)		MH165805	
A/chicken/South Africa/17080416-38/2017(H5N8)	S2017/08_0416_38		EPI1187891
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1187895
A/chicken/South Africa/17090184-62/2017(H5N8)	S2017/09_0184_62		EPI1187998
A/chicken/South Africa/443397/2017(H5N8)		MH165637	
A/chicken/South Africa/448475/2017(H5N8)		MH165741	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165533	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165525	
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973226	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223859
A/duck/Democratic Republic of the Congo/17RS882-5/2017(H5N8)			EPI1223840
A/duck/Egypt/SS19/2017(H5N8)			EPI1018181
A/duck/South Africa/17080481-P2/2017(H5N8)	S2017/08_0481_P2		EPI1187898
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165549	
A/geese/South Africa/17080520-46/2017(H5N8)	S2017/08_0520_46		EPI1187904
A/geese/South Africa/17080558-P1/2017(H5N8)	S2017/08_0558_P1		EPI1188016
A/geese/South Africa/17090055-P1/2017(H5N8)	S2017/09_0055_P1		EPI1187908
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200942
A/guineafowl/South Africa/17080190-9/2017(H5N8)	S2017/08_0190_9		EPI1188142
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1188138
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1187950
A/ostrich/South Africa/17080047-P7/2017(H5N8)	S2017/08_0047_P7		EPI1187621
A/ostrich/South Africa/17080268-P2/2017(H5N8)	S2017/08_0268_P2		EPI1187540
A/ostrich/South Africa/17080361-P17/2017(H5N8)	S2017/08_0361_P17		EPI1187537
A/ostrich/South Africa/17080362-P10/2017(H5N8)	S2017/08_0362_P10		EPI1187618
A/pigeon/South Africa/17080323-P1/2017(H5N8)	S2017/08_0323_P1		EPI1188134
A/pigeon/South Africa/17080324-24/2017(H5N8)	S2017/08_0324_24		EPI1187530
A/sacred ibis/South Africa/009/2017(H5N8)		MH165613	
A/swan/South Africa/17080517-P2/2017(H5N8)	S2017/08_0517_P2		EPI1187901
A/white-winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569640	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320444	

[1] *not isolated*

APPENDIX 7: Accession numbers of reference strains included in Segment 6 (Neuraminidase) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2017(H5N8)[1]		MN595237	
A/African penguin/Namibia/146S/2019(H5N8)		MK898935	
A/African penguin/Namibia/146T/2019(H5N8)		MK898936	
A/African penguin/Namibia/288-1/2019(H5N8)		MK898937	
A/African penguin/Namibia/288-2/2019(H5N8)		MK898938	
A/chicken/South Africa/115370/2017(H5N8)		MH165806	
A/chicken/South Africa/443397/2017(H5N8)		MH165638	
A/chicken/South Africa/448475/2017(H5N8)		MH165742	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165534	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165526	
A/chicken/South Africa/17080336-P2/2017(H5N8)	S2017/08_0336_P2		EPI1106430
A/chicken/South Africa/17080416-38/2017(H5N8)	S2017/08_0416_38		EPI1104302
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1104416
A/chicken/South Africa/17080581-P2/2017(H5N8)	S2017/08_0581_P2		EPI1106291
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1104307
A/chicken/South Africa/17090184-63/2017(H5N8)	S2017/09_0184_63		EPI1104378
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973227	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223858
A/duck/Democratic Republic of the Congo/17RS882-29/2017(H5N8)			EPI1223841
A/duck/Egypt/SS19/2017(H5N8)			EPI1018182
A/duck/South Africa/17080340-P2/2017(H5N8)	S2017/08_0340_P2		EPI1104224
A/duck/South Africa/17080481-P2/2017(H5N8)	S2017/08_0481_P2		EPI1104312
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165550	
A/geese/South Africa/17080520-46/2017(H5N8)	S2017/08_0520_46		EPI1104368
A/geese/South Africa/17080558-P2/2017(H5N8)	S2017/08_0558_P2		EPI1104233
A/geese/South Africa/17090055-P1/2017(H5N8)	S2017/09_0055_P1		EPI1104373
A/geese/South Africa/17090065-P2/2017(H5N8)	S2017/09_0065_P2		EPI1104205
A/grey-headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200959
A/guineafowl/South Africa/17080190-9/2017(H5N8)	S2017/08_0190_9		EPI1104388
A/guineafowl/South Africa/17080243-P1/2017(H5N8)	S2017/08_0243_P1		EPI1104260
A/guineafowl/South Africa/17080274-P1/2017(H5N8)	S2017/08_0274_P1		EPI1104238
A/ostrich/South Africa/17080046-P11/2017(H5N8)	S2017/08_0046_P11		EPI1106420
A/ostrich/South Africa/17080047-P7/2017(H5N8)	S2017/08_0047_P7		EPI1103578
A/ostrich/South Africa/17080161-P8/2017(H5N8)	S2017/08_0161_P8		EPI1104255
A/ostrich/South Africa/17080268-P2/2017(H5N8)	S2017/08_0268_P2		EPI1098489

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/ostrich/South Africa/17080361-P17/2017(H5N8)	S2017/08_0361_P17		EPI1094823
A/ostrich/South Africa/17080362-P11/2017(H5N8)	S2017/08_0362_P11		EPI1103655
A/ostrich/South Africa/17080484-P2/2017(H5N8)	S2017/08_0484_P2		EPI1106438
A/pigeon/South Africa/17080323-P1/2017(H5N8)	S2017/08_0323_P1		EPI1104398
A/pigeon/South Africa/17080324-24/2017(H5N8)	S2017/08_0324_24		EPI1094814
A/sacred ibis/South Africa/009/2017(H5N8)		MH165614	
A/swan/South Africa/17080517-P1/2017(H5N8)	S2017/08_0517_P1		EPI1106419
A/white winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569639	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320445	

[1] *not isolated*

APPENDIX 8: Accession numbers of reference strains included in Segment 7 (Matrix proteins) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595238	
A/Blue crane/South Africa/17080322-21/2017(H5N8)	S2017/08_0322_21		EPI1188146
A/chicken/Egypt/Kafr-Elshiekh-18/2017(H5N8)			EPI1104270
A/chicken/South Africa/115370/2017(H5N8)		MH165807	
A/chicken/South Africa/443397/2017(H5N8)		MH165639	
A/chicken/South Africa/448475/2017(H5N8)		MH165743	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165535	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165527	
A/chicken/South Africa/17080378-37/2017(H5N8)	S2017/08_0378_37		EPI1188148
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1188009
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1187896
A/chicken/South Africa/17090184-62/2017(H5N8)	S2017/09_0184_62		EPI1187999
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973228	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223856
A/duck/Democratic Republic of the Congo/17RS882-5/2017(H5N8)			EPI1223848
A/duck/Egypt/SS19/2017(H5N8)			EPI1018183
A/duck/South Africa/17080340-P1/2017(H5N8)	S2017/08_0340_P1		EPI1187860
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165551	
A/geese/South Africa/17080520-46/2017(H5N8)	S2017/08_0520_46		EPI1187905
A/grey headed gull/Uganda/MUWRP-538/2017(H5N8)			EPI1200927
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1188139
A/guineafowl/South Africa/17080274-P1/2017(H5N8)	S2017/08_0274_P1		EPI1187874
A/ostrich/South Africa/17080046-AF/2017(H5N8)	S2017/08_0046_AF		EPI1187951
A/ostrich/South Africa/17080268-P2/2017(H5N8)	S2017/08_0268_P2		EPI1187539
A/ostrich/South Africa/17080362-P7/2017(H5N8)	S2017/08_0362_P7		EPI1187889
A/ostrich/South Africa/17080484-P2/2017(H5N8)	S2017/08_0484_P2		EPI1187957
A/pigeon/South Africa/17080560-P2/2017(H5N8)	S2017/08_0560_P2		EPI1187990
A/sacred ibis/South Africa/009/2017(H5N8)		MH165615	
A/swan/South Africa/17080517-P1/2017(H5N8)	S2017/08_0517_P1		EPI1187954
A/white winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569638	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320446	

[1] *not isolated*

APPENDIX 9: Accession numbers of reference strains included in Segment 8 (Non-structural protein) phylogenetic tree

Virus isolate name	WCPVL isolate #	GenBank accession number	GISAID Epiflu accession number
A/African penguin/South Africa/476266/2018(H5N8)[1]		MN595239	
A/Bluecrane/South Africa/17080322-21/2017(H5N8)	S2017/08_0322_21		EPI1188147
A/chicken/South Africa/115370/2017(H5N8)		MH165808	
A/chicken/South Africa/17080378-37/2017(H5N8)	S2017/08_0378_37		EPI1188150
A/chicken/South Africa/17080561-P1/2017(H5N8)	S2017/08_0561_P1		EPI1188010
A/chicken/South Africa/17090050-56/2017(H5N8)	S2017/09_0050_56		EPI1187897
A/chicken/South Africa/17090184-62/2017(H5N8)	S2017/09_0184_62		EPI1188000
A/chicken/South Africa/443397/2017(H5N8)		MH165640	
A/chicken/South Africa/448475/2017(H5N8)		MH165744	
A/chicken/South Africa/Standerton/2017(H5N8)		MH165536	
A/chicken/South Africa/Villiers/2017(H5N8)		MH165528	
A/chicken/Zimbabwe/AI4935/2017(H5N8)		MF973229	
A/duck/Cameroon/17RS1661-3/2017(H5N8)			EPI1223862
A/duck/Democratic Republic of the Congo/17RS882-29/2017(H5N8)			EPI1223849
A/duck/Egypt/SS19/2017(H5N8)			EPI1018184
A/duck/South Africa/17080340-P1/2017(H5N8)	S2017/08_0340_P1		EPI1187861
A/Egyptian goose/South Africa/001/2017(H5N8)		MH165552	
A/geese/South Africa/17080520-46/2017(H5N8)	S2017/08_0520_46		EPI1187906
A/guineafowl/South Africa/17080243-P2/2017(H5N8)	S2017/08_0243_P2		EPI1188140
A/guineafowl/South Africa/17080274-P1/2017(H5N8)	S2017/08_0274_P1		EPI1187875
A/ostrich/South Africa/17080268-P2/2017(H5N8)	S2017/08_0268_P2		EPI1187538
A/ostrich/South Africa/17080362-P7/2017(H5N8)	S2017/08_0362_P7		EPI1187890
A/ostrich/South Africa/17080484-P2/2017(H5N8)	S2017/08_0484_P2		EPI1187958
A/pigeon/Cameroon/17RS1661-4/2017(H5N8)			EPI1223875
A/pigeon/South Africa/17080560-P2/2017(H5N8)	S2017/08_0560_P2		EPI1187911
A/sacred ibis/South Africa/009/2017(H5N8)		MH165616	
A/swan/South Africa/17080517-P1/2017(H5N8)	S2017/08_0517_P1		EPI1187955
A/white winged black tern/Uganda/17RS115-3/2017(H5N8)		MH569641	
A/Whooper swan/Sanmenxia/01/2016(H5N8)		KY320447	

[1] *not isolated*

APPENDIX 10: Pairwise distance matrix of Segment 1 PB2 gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	18030214																								
B	18010422	0.003																							
C	18010423	0.003	0.000																						
D	18020304	0.004	0.001	0.001																					
E	18020408	0.003	0.002	0.001	0.002																				
F	18050256	0.004	0.002	0.001	0.002	0.003																			
G	476266	0.004	0.002	0.001	0.002	0.003	0.003																		
H	18020303	0.004	0.001	0.001	0.002	0.002	0.002	0.002																	
I	18010259	0.004	0.002	0.001	0.002	0.003	0.003	0.003	0.002																
J	18010371	0.004	0.001	0.001	0.002	0.002	0.002	0.002	0.000	0.002															
K	18030213	0.004	0.002	0.001	0.002	0.003	0.000	0.003	0.002	0.003	0.002														
L	18040224	0.005	0.003	0.002	0.003	0.004	0.004	0.004	0.003	0.004	0.003	0.004													
M	18040367	0.005	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005												
N	18010106	0.003	0.001	0.000	0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.003	0.003											
O	18010369	0.004	0.001	0.001	0.002	0.002	0.002	0.002	0.000	0.002	0.000	0.002	0.003	0.004	0.001										
P	18020302	0.004	0.001	0.001	0.002	0.002	0.002	0.002	0.000	0.002	0.000	0.002	0.003	0.004	0.001	0.000									
Q	18010027	0.004	0.001	0.001	0.002	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.003	0.004	0.001	0.001	0.001								
R	18010028	0.003	0.001	0.000	0.001	0.002	0.002	0.001	0.001	0.002	0.001	0.002	0.003	0.003	0.001	0.001	0.001	0.001							
S	18010370	0.004	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.004	0.001	0.002	0.002	0.002	0.001						
T	18030478	0.004	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.001	0.004	0.002	0.003	0.003	0.003	0.002	0.003					
U	18040275	0.005	0.003	0.002	0.003	0.004	0.004	0.004	0.003	0.004	0.003	0.004	0.004	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.004				
V	18010043	0.003	0.001	0.000	0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.003	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.003			
W	18010107	0.003	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.003	0.000	0.001	0.001	0.001	0.000	0.001	0.002	0.002	0.000		
X	18010417	0.003	0.001	0.000	0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.003	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.003	0.001	0.000	
Y	18020273	0.005	0.003	0.002	0.003	0.004	0.004	0.004	0.003	0.004	0.003	0.004	0.002	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.001	0.004	0.003	0.002	0.003

Average distance = 0.002

APPENDIX 11: Pairwise distance matrix of Segment 2 PB1 gene

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A 18030214																								
B 18010422	0.001																							
C 18010423	0.002	0.001																						
D 18020304	0.003	0.001	0.002																					
E 18020408	0.001	0.000	0.001	0.001																				
F 18050256	0.004	0.002	0.003	0.004	0.002																			
G 476266	0.002	0.000	0.001	0.002	0.000	0.003																		
H 18020303	0.003	0.002	0.003	0.003	0.002	0.004	0.002																	
I 18010259	0.002	0.001	0.002	0.002	0.001	0.003	0.001	0.003																
J 18010371	0.003	0.001	0.002	0.003	0.001	0.004	0.002	0.000	0.002															
K 18030213	0.003	0.001	0.002	0.003	0.001	0.001	0.002	0.003	0.002	0.003														
L 18040224	0.002	0.000	0.001	0.002	0.000	0.003	0.001	0.002	0.001	0.002	0.002													
M 18040367	0.002	0.001	0.002	0.002	0.001	0.003	0.001	0.003	0.002	0.002	0.002	0.001												
N 18010106	0.001	0.000	0.001	0.001	0.000	0.002	0.000	0.002	0.001	0.001	0.001	0.000	0.001											
O 18010369	0.002	0.000	0.001	0.002	0.000	0.003	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.000										
P 18020302	0.003	0.002	0.003	0.003	0.002	0.004	0.002	0.000	0.003	0.000	0.003	0.002	0.003	0.002	0.001									
Q 18010027	0.002	0.000	0.001	0.002	0.000	0.003	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.000	0.000	0.001								
R 18010028	0.004	0.002	0.003	0.004	0.002	0.004	0.002	0.004	0.001	0.004	0.004	0.003	0.003	0.002	0.003	0.004	0.003							
S 18010370	0.002	0.001	0.002	0.002	0.001	0.003	0.000	0.003	0.002	0.002	0.002	0.001	0.002	0.001	0.001	0.003	0.001	0.002						
T 18030478	0.002	0.001	0.002	0.002	0.001	0.003	0.001	0.003	0.002	0.002	0.002	0.001	0.002	0.001	0.001	0.003	0.001	0.003	0.002					
U 18040275	0.003	0.002	0.003	0.003	0.002	0.004	0.002	0.004	0.003	0.003	0.003	0.002	0.003	0.002	0.002	0.004	0.002	0.004	0.003	0.003				
V 18010043	0.002	0.000	0.001	0.002	0.000	0.003	0.001	0.002	0.001	0.002	0.002	0.001	0.001	0.000	0.001	0.002	0.001	0.003	0.001	0.001	0.002			
W 18010107	0.004	0.002	0.003	0.004	0.002	0.004	0.002	0.004	0.001	0.004	0.004	0.003	0.003	0.002	0.003	0.004	0.003	0.000	0.002	0.003	0.004	0.003		
X 18010417	0.002	0.001	0.002	0.002	0.001	0.003	0.000	0.003	0.002	0.002	0.002	0.001	0.002	0.001	0.001	0.003	0.001	0.002	0.000	0.002	0.003	0.001	0.002	
Y 18020273	0.003	0.001	0.002	0.003	0.001	0.004	0.001	0.003	0.002	0.003	0.003	0.002	0.002	0.001	0.002	0.003	0.002	0.003	0.000	0.002	0.003	0.002	0.003	0.000

Average distance = 0.002

APPENDIX 12: Pairwise distance matrix of Segment 3 PA gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	18030214																								
B	18010422	0,001																							
C	18010423	0,001	0,000																						
D	18020304	0,001	0,000	0,000																					
E	18020408	0,004	0,003	0,003	0,003																				
F	18050256	0,004	0,003	0,003	0,003	0,005																			
G	476266	0,003	0,001	0,001	0,001	0,004	0,004																		
H	18020303	0,003	0,001	0,001	0,001	0,004	0,004	0,003																	
I	18010259	0,001	0,000	0,000	0,000	0,003	0,003	0,001	0,001																
J	18010371	0,004	0,003	0,003	0,003	0,005	0,005	0,004	0,001	0,003															
K	18030213	0,003	0,001	0,001	0,001	0,004	0,001	0,003	0,003	0,001	0,004														
L	18040224	0,001	0,000	0,000	0,000	0,003	0,003	0,001	0,001	0,000	0,003	0,001													
M	18040367	0,001	0,000	0,000	0,000	0,003	0,003	0,001	0,001	0,000	0,003	0,001	0,000												
N	18010106	0,001	0,000	0,000	0,000	0,003	0,003	0,001	0,001	0,000	0,003	0,001	0,000	0,000											
O	18010369	0,003	0,001	0,001	0,001	0,004	0,004	0,003	0,003	0,001	0,004	0,003	0,001	0,001	0,001										
P	18020302	0,003	0,001	0,001	0,001	0,004	0,004	0,003	0,000	0,001	0,001	0,003	0,001	0,001	0,001	0,003									
Q	18010027	0,005	0,004	0,004	0,004	0,007	0,007	0,005	0,005	0,004	0,007	0,005	0,004	0,004	0,004	0,005	0,005								
R	18010028	0,003	0,001	0,001	0,001	0,004	0,004	0,000	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005							
S	18010370	0,004	0,003	0,003	0,003	0,005	0,005	0,001	0,004	0,003	0,005	0,004	0,003	0,003	0,003	0,004	0,004	0,007	0,001						
T	18020273	0,001	0,000	0,000	0,000	0,003	0,003	0,001	0,001	0,000	0,003	0,001	0,000	0,000	0,000	0,001	0,001	0,004	0,001	0,003					
U	18030478	0,003	0,001	0,001	0,001	0,004	0,004	0,003	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005	0,003	0,004	0,001				
V	18040275	0,003	0,001	0,001	0,001	0,004	0,004	0,003	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005	0,003	0,004	0,001	0,003			
W	18010043	0,003	0,001	0,001	0,001	0,004	0,004	0,000	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005	0,000	0,001	0,001	0,003	0,003		
X	18010107	0,003	0,001	0,001	0,001	0,004	0,004	0,000	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005	0,000	0,001	0,001	0,003	0,003	0,000	
Y	18010417	0,003	0,001	0,001	0,001	0,004	0,004	0,000	0,003	0,001	0,004	0,003	0,001	0,001	0,001	0,003	0,003	0,005	0,000	0,001	0,001	0,003	0,003	0,000	0,000

Average distance = 0.002

APPENDIX 13: Pairwise distance matrix of Segment 4 HA gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB
A	18030214																												
B	18010422	0,002																											
C	18010423	0,001	0,003																										
D	18020304	0,002	0,004	0,003																									
E	18020408	0,001	0,003	0,002	0,001																								
F	18050256	0,001	0,003	0,002	0,001	0,000																							
G	476266	0,001	0,003	0,002	0,001	0,000	0,000																						
H	Namibia_ 146S	0,004	0,006	0,005	0,006	0,005	0,005	0,005																					
I	Namibia_ 146T	0,005	0,007	0,006	0,007	0,006	0,006	0,006	0,001																				
J	Namibia_ 288-1	0,005	0,007	0,006	0,007	0,006	0,006	0,006	0,001	0,000																			
K	Namibia_ 288-2	0,006	0,008	0,007	0,008	0,007	0,007	0,007	0,002	0,001	0,001																		
L	18020303	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007																	
M	18010259	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000																
N	18010371	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000															
O	18030213	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000														
P	18040224	0,000	0,002	0,001	0,002	0,001	0,001	0,001	0,004	0,005	0,005	0,006	0,001	0,001	0,001	0,001													
Q	18040367	0,003	0,005	0,004	0,005	0,004	0,004	0,004	0,005	0,006	0,006	0,007	0,004	0,004	0,004	0,004	0,003												
R	18010106	0,000	0,002	0,001	0,002	0,001	0,001	0,001	0,004	0,005	0,005	0,006	0,001	0,001	0,001	0,001	0,000	0,003											
S	18010369	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001										
T	18020302	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001	0,000									
U	18010027	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001	0,000	0,000								
V	18010028	0,002	0,004	0,003	0,002	0,001	0,001	0,001	0,006	0,007	0,007	0,008	0,001	0,001	0,001	0,001	0,002	0,005	0,002	0,001	0,001	0,001							
W	18010370	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001	0,000	0,000	0,000	0,001						
X	18020273	0,001	0,003	0,002	0,003	0,002	0,002	0,002	0,005	0,006	0,006	0,007	0,002	0,002	0,002	0,002	0,001	0,004	0,001	0,002	0,002	0,002	0,003	0,002					
Y	18030478	0,001	0,003	0,002	0,003	0,002	0,002	0,002	0,005	0,006	0,006	0,007	0,002	0,002	0,002	0,002	0,001	0,004	0,001	0,002	0,002	0,002	0,003	0,002	0,002				
Z	18040275	0,000	0,002	0,001	0,002	0,001	0,001	0,001	0,004	0,005	0,005	0,006	0,001	0,001	0,001	0,001	0,000	0,003	0,000	0,001	0,001	0,001	0,002	0,001	0,001	0,001			
AA	18010043	0,002	0,004	0,003	0,002	0,001	0,001	0,001	0,006	0,007	0,007	0,008	0,001	0,001	0,001	0,001	0,002	0,005	0,002	0,001	0,001	0,001	0,000	0,001	0,003	0,003	0,002		
AB	18010107	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001	0,000	0,000	0,000	0,001	0,000	0,002	0,002	0,001	0,001	
AC	18010417	0,001	0,003	0,002	0,001	0,000	0,000	0,000	0,005	0,006	0,006	0,007	0,000	0,000	0,000	0,000	0,001	0,004	0,001	0,000	0,000	0,000	0,001	0,000	0,002	0,002	0,001	0,001	0,000

Average distance = 0.003

APPENDIX 14: Pairwise distance matrix of Segment 5 NP gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	18030214																								
B	18010422	0,001																							
C	18010423	0,001	0,002																						
D	18020304	0,002	0,003	0,003																					
E	18020408	0,002	0,003	0,003	0,003																				
F	18050256	0,001	0,001	0,002	0,001	0,001																			
G	476266	0,003	0,003	0,004	0,003	0,003	0,002																		
H	18020303	0,001	0,001	0,002	0,001	0,001	0,000	0,002																	
I	18010259	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000																
J	18010371	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000															
K	18030213	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000	0,000														
L	18040224	0,001	0,001	0,002	0,003	0,003	0,001	0,003	0,001	0,001	0,001	0,001													
M	18040367	0,002	0,003	0,003	0,004	0,004	0,003	0,005	0,003	0,003	0,003	0,003	0,003												
N	18010106	0,001	0,001	0,002	0,003	0,003	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,003											
O	18010369	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000	0,000	0,000	0,001	0,003	0,001										
P	18020302	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000	0,000	0,000	0,001	0,003	0,001	0,000									
Q	18010027	0,001	0,002	0,003	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,002	0,003	0,002	0,001	0,001								
R	18010028	0,001	0,002	0,003	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,002	0,003	0,002	0,001	0,001	0,001							
S	18010370	0,001	0,002	0,003	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,002	0,003	0,002	0,001	0,001	0,001	0,001						
T	18020273	0,000	0,001	0,001	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,002	0,001	0,001	0,001	0,001	0,001	0,001					
U	18030478	0,000	0,001	0,001	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,002	0,001	0,001	0,001	0,001	0,001	0,001	0,000				
V	18040275	0,001	0,001	0,002	0,003	0,003	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,002	0,002	0,002	0,001	0,001			
W	18010043	0,001	0,002	0,003	0,002	0,002	0,001	0,003	0,001	0,001	0,001	0,001	0,002	0,003	0,002	0,001	0,001	0,001	0,000	0,001	0,001	0,001	0,002		
X	18010107	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000	0,000	0,000	0,001	0,003	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,001	
Y	18010417	0,001	0,001	0,002	0,001	0,001	0,000	0,002	0,000	0,000	0,000	0,000	0,001	0,003	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,000

Average distance = 0.001

APPENDIX 15: Pairwise distance matrix of Segment 6 NA gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB
A	18030214																												
B	18010422	0,000																											
C	18010423	0,001	0,001																										
D	18020304	0,004	0,004	0,004																									
E	18020408	0,002	0,002	0,003	0,004																								
F	18050256	0,005	0,005	0,006	0,007	0,005																							
G	476266	0,002	0,002	0,003	0,004	0,002	0,005																						
H	Namibia_ 146S	0,003	0,003	0,004	0,006	0,004	0,008	0,004																					
I	Namibia_ 146T	0,003	0,003	0,004	0,006	0,004	0,008	0,004	0,000																				
J	Namibia_ 288-1	0,004	0,004	0,004	0,007	0,005	0,009	0,005	0,001	0,001																			
K	Namibia_ 288-2	0,004	0,004	0,004	0,007	0,005	0,009	0,005	0,001	0,001	0,000																		
L	18020303	0,005	0,005	0,006	0,007	0,005	0,009	0,005	0,008	0,008	0,009	0,009																	
M	18010259	0,002	0,002	0,003	0,004	0,002	0,005	0,002	0,004	0,004	0,005	0,005	0,005																
N	18010371	0,004	0,004	0,005	0,006	0,004	0,008	0,004	0,007	0,007	0,008	0,008	0,001	0,004															
O	18030213	0,004	0,004	0,004	0,005	0,004	0,002	0,004	0,006	0,006	0,007	0,007	0,007	0,004	0,006														
P	18040224	0,001	0,001	0,002	0,004	0,003	0,006	0,003	0,004	0,004	0,004	0,004	0,006	0,003	0,005	0,004													
Q	18040367	0,003	0,003	0,004	0,006	0,004	0,006	0,004	0,005	0,005	0,006	0,006	0,008	0,004	0,007	0,004	0,004												
R	18010106	0,000	0,000	0,001	0,004	0,002	0,005	0,002	0,003	0,003	0,004	0,004	0,005	0,002	0,004	0,004	0,001	0,003											
S	18010369	0,001	0,001	0,002	0,003	0,001	0,004	0,001	0,004	0,004	0,004	0,004	0,004	0,001	0,004	0,003	0,002	0,004	0,001										
T	18020302	0,005	0,005	0,006	0,007	0,005	0,009	0,005	0,008	0,008	0,009	0,009	0,000	0,005	0,001	0,007	0,006	0,008	0,005	0,004									
U	18010027	0,003	0,003	0,004	0,004	0,003	0,006	0,003	0,005	0,005	0,006	0,006	0,006	0,003	0,005	0,004	0,004	0,005	0,003	0,002	0,006								
V	18010028	0,001	0,001	0,002	0,003	0,001	0,004	0,001	0,004	0,004	0,004	0,004	0,004	0,001	0,004	0,003	0,002	0,004	0,001	0,000	0,004	0,002							
W	18010370	0,001	0,001	0,002	0,003	0,001	0,004	0,001	0,004	0,004	0,004	0,004	0,004	0,001	0,004	0,003	0,002	0,004	0,001	0,000	0,004	0,002	0,000						
X	18020273	0,001	0,001	0,002	0,004	0,003	0,004	0,003	0,004	0,004	0,004	0,004	0,006	0,003	0,005	0,003	0,002	0,002	0,001	0,002	0,006	0,004	0,002	0,002					
Y	18030478	0,002	0,002	0,003	0,005	0,004	0,007	0,004	0,004	0,004	0,005	0,005	0,005	0,004	0,004	0,005	0,003	0,004	0,002	0,003	0,005	0,004	0,003	0,003	0,003				
Z	18040275	0,001	0,001	0,002	0,004	0,003	0,006	0,003	0,004	0,004	0,004	0,004	0,006	0,003	0,005	0,004	0,002	0,004	0,001	0,002	0,006	0,004	0,002	0,002	0,002	0,003			
AA	18010043	0,002	0,002	0,003	0,004	0,002	0,004	0,002	0,004	0,004	0,005	0,005	0,005	0,002	0,004	0,002	0,003	0,004	0,002	0,001	0,005	0,003	0,001	0,001	0,003	0,004	0,003		
AB	18010107	0,001	0,001	0,002	0,003	0,001	0,004	0,001	0,004	0,004	0,004	0,004	0,004	0,001	0,004	0,003	0,002	0,004	0,001	0,000	0,004	0,002	0,000	0,000	0,002	0,003	0,002	0,001	
AC	18010417	0,004	0,004	0,005	0,006	0,004	0,008	0,004	0,007	0,007	0,008	0,008	0,001	0,004	0,000	0,006	0,005	0,007	0,004	0,004	0,001	0,005	0,004	0,004	0,005	0,004	0,005	0,004	0,004

Average distance = 0.004

APPENDIX 16: Pairwise distance matrix of Segment 7 M gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	18030214																								
B	18010422	0,001																							
C	18010423	0,000	0,001																						
D	18020304	0,001	0,003	0,001																					
E	18020408	0,001	0,003	0,001	0,003																				
F	18050256	0,003	0,004	0,003	0,004	0,004																			
G	476266	0,003	0,004	0,003	0,004	0,004	0,003																		
H	18020303	0,000	0,001	0,000	0,001	0,001	0,003	0,003																	
I	18010259	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000																
J	18010371	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000															
K	18030213	0,003	0,004	0,003	0,004	0,004	0,000	0,003	0,003	0,003	0,003														
L	18040224	0,001	0,003	0,001	0,003	0,003	0,004	0,004	0,001	0,001	0,001	0,004													
M	18040367	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001												
N	18010106	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000											
O	18010369	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000										
P	18020302	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000	0,000									
Q	18010027	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000	0,000	0,000								
R	18010028	0,001	0,003	0,001	0,003	0,003	0,001	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,001	0,001							
S	18010370	0,001	0,003	0,001	0,003	0,003	0,001	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,000						
T	18020273	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000	0,000	0,000	0,000	0,001	0,001					
U	18030478	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000	0,000	0,000	0,000	0,001	0,001	0,000				
V	18040275	0,000	0,001	0,000	0,001	0,001	0,003	0,003	0,000	0,000	0,000	0,003	0,001	0,000	0,000	0,000	0,000	0,000	0,001	0,001	0,000	0,000			
W	18010043	0,001	0,003	0,001	0,003	0,003	0,001	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,000	0,000	0,001	0,001	0,001		
X	18010107	0,001	0,003	0,001	0,003	0,003	0,001	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,000	0,000	0,001	0,001	0,001	0,000	
Y	18010417	0,001	0,003	0,001	0,003	0,003	0,001	0,001	0,001	0,001	0,001	0,001	0,003	0,001	0,001	0,001	0,001	0,001	0,000	0,000	0,001	0,001	0,001	0,000	0,000

Average distance = 0.001

APPENDIX 17: Pairwise distance matrix of Segment 8 NS gene

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	18030214																								
B	18010422	0,003																							
C	18010423	0,003	0,000																						
D	18020304	0,001	0,001	0,001																					
E	18020408	0,001	0,001	0,001	0,000																				
F	18050256	0,003	0,003	0,003	0,001	0,001																			
G	476266	0,001	0,001	0,001	0,000	0,000	0,001																		
H	18020303	0,004	0,004	0,004	0,003	0,003	0,004	0,003																	
I	18010259	0,001	0,001	0,001	0,000	0,000	0,001	0,000	0,003																
J	18010371	0,004	0,004	0,004	0,003	0,003	0,004	0,003	0,003	0,003															
K	18030213	0,003	0,003	0,003	0,001	0,001	0,000	0,001	0,004	0,001	0,004														
L	18040224	0,004	0,001	0,001	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004													
M	18040367	0,010	0,008	0,008	0,009	0,009	0,010	0,009	0,012	0,009	0,012	0,010	0,009												
N	18010106	0,004	0,001	0,001	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004	0,003	0,009											
O	18010369	0,004	0,004	0,004	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004	0,005	0,012	0,005										
P	18020302	0,004	0,004	0,004	0,003	0,003	0,004	0,003	0,000	0,003	0,003	0,004	0,005	0,012	0,005	0,005									
Q	18010027	0,003	0,003	0,003	0,001	0,001	0,003	0,001	0,004	0,001	0,004	0,003	0,004	0,010	0,004	0,004	0,004								
R	18010028	0,001	0,001	0,001	0,000	0,000	0,001	0,000	0,003	0,000	0,003	0,001	0,003	0,009	0,003	0,003	0,003	0,001							
S	18010370	0,003	0,003	0,003	0,001	0,001	0,003	0,001	0,004	0,001	0,004	0,003	0,004	0,010	0,004	0,004	0,004	0,003	0,001						
T	18020273	0,004	0,001	0,001	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004	0,003	0,009	0,003	0,005	0,005	0,004	0,003	0,004					
U	18030478	0,004	0,001	0,001	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004	0,003	0,009	0,003	0,005	0,005	0,004	0,003	0,004	0,003				
V	18040275	0,004	0,001	0,001	0,003	0,003	0,004	0,003	0,005	0,003	0,005	0,004	0,003	0,009	0,003	0,005	0,005	0,004	0,003	0,004	0,003	0,003			
W	18010043	0,001	0,001	0,001	0,000	0,000	0,001	0,000	0,003	0,000	0,003	0,001	0,003	0,009	0,003	0,003	0,003	0,001	0,000	0,001	0,003	0,003	0,003		
X	18010107	0,001	0,001	0,001	0,000	0,000	0,001	0,000	0,003	0,000	0,003	0,001	0,003	0,009	0,003	0,003	0,003	0,001	0,000	0,001	0,003	0,003	0,003	0,000	
Y	18010417	0,001	0,001	0,001	0,000	0,000	0,001	0,000	0,003	0,000	0,003	0,001	0,003	0,009	0,003	0,003	0,003	0,001	0,000	0,001	0,003	0,003	0,003	0,000	0,000

Average distance = 0.003

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YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Molecular characterization of Clade 2.3.4.4 H5N8 HPAI viruses causing outbreaks in terms (*Thalasseus* spp. and *Sterna* spp.) in South Africa**
PROJECT NUMBER	**REC027-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**BM Peyrot**

STUDENT NUMBER (where applicable)	
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Prof Celia Abolnik**

APPROVED	Date **5 June 2018**
CHAIRMAN: UP Research Ethics Committee	Signature A.M. Dinan

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UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCES

Plagiarism policy agreement

The University of Pretoria places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

While academic staff teaches you about referencing techniques and how to avoid plagiarism, you too have a responsibility in this regard. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article or a website) without acknowledging the source and pass it off as your own. In effect, you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim), but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it. You are not allowed to use work previously produced by another student. You are also not allowed to let anybody copy your work with the intention of passing it off as his/her work.

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The declaration which follows must accompany all written work submitted while you are a student of the University of Pretoria. No written work will be accepted unless the declaration has been completed and attached.

Full names of candidate: Belinda Margaret Peyrot

Student number: 18392483

Date:

Declaration

I understand what plagiarism is and am aware of the University's policy in this regard.

Signature of candidate: .....

Signature of supervisor: .....

This document should be completed, signed and submitted to student administration two months after registration.

RECORD OF AGREEMENT ON PLACES AND DATES OF MEETINGS, MILESTONES AND DEADLINES

(to be completed at the time when the Agreement is signed)

MAY 2018	SKYPE		
AUG 2018	SKYPE		
NOV 2018	SKYPE		
FEB 2019	SKYPE		
MAY 2019	SKYPE		
AUG 2019	SKYPE		
NOV 2019	SKYPE		
FEB 2020	SKYPE		

Initial – Student

Initial – Supervisor

This document must not be altered, signed and submitted in any other way than electronically

THE SUPERVISOR Prof Ceko Aboink (name)

accepts and undertakes the following roles and responsibilities:

1. Abiding by the relevant rules and regulations of the University.
2. Assisting the student in building knowledge and research skills in the specific area of postgraduate study and relevant to the level of the degree.
3. Ensuring that the proposed research project is feasible, of an appropriate level for the degree under consideration, and that the necessary resources and facilities will be available to enable the student to complete the research timeously.
4. Providing information on the conditions to be met in order to achieve satisfactory progress/ performance and assisting with the construction of a written time schedule which outlines the expected completion dates of various stages of the research work.
5. Being accessible to the student by attending meetings in line with a schedule agreed upon in advance by the supervisor and the student, and being prepared for the meetings.
6. Implementing an arrangement for student supervision in cases where the supervisor is away from the University e.g. sick leave, sabbatical leave, or leaves the employ of the University, and communicating these arrangements to the student timeously.
7. Accepting submission of written work at intervals agreed on by the student and supervisor, providing constructive comment and criticism within a time frame jointly agreed on at the start of the research, and informing the student, in writing, of any inadequacy relating to progress or work, in relation to the expectations previously agreed on by the student and supervisor.
8. Assisting the student with the production of the dissertation, providing guidance on technical aspects of writing including discipline-specific requirements.
9. Assisting with the publication of research articles as appropriate and agreeing the ownership of research results in accordance with the University's policy on intellectual property.
10. Contributing to the student's academic development by introducing her or him to relevant academic and professional networks through conferences, seminars and other events where possible.

THE STUDENT and THE SUPERVISOR:

1. confirm that we have read and understood this Memorandum of Agreement and
2. agree to accept its content for the duration of the period of study in respect of the degree as specified below.

Initial – Student	
Initial – Supervisor	

This document should be completed, signed and submitted to student administration two months after registration

Memorandum of Agreement between Postgraduate Student and Supervisor

THE STUDENT Belinda Margaret Peyrot (name)

accepts and undertakes the following roles and responsibilities:

1. Abiding by the relevant rules and regulations of the University.
2. Working independently under the guidance of the supervisor, and ensuring that she or he stays abreast of the latest developments in the field of study.
3. Agreeing with the supervisor, and abiding by, a time schedule which outlines the expected completion dates of various stages of the research work (See Supervisor section, #4 below).
4. Attending pre-scheduled meetings with the supervisor, and being adequately prepared for these consultation sessions (See Supervisor section, #5 below).
5. Submitting written work at times agreed upon by the student and the supervisor.
6. Taking account of the feedback provided by the supervisor before subsequent submission of written work.
7. Undertaking to submit the dissertation within the prescribed time for the completion of the degree unless exceptional circumstances arise, and to plan accordingly.
8. Accepting responsibility for the overall coherent structure of the final dissertation and, as far as possible, submitting written work that is free of spelling mistakes, grammatical errors and incorrect punctuation.
9. Undertaking to submit draft papers for publication, taking into account advice provided by the supervisor.
10. Informing the supervisor of any absence or circumstances that may affect the research progress and time line.

Initial – Student	
Initial – Supervisor	

This document should be completed, signed and submitted to student administration two months after registration

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FACULTY OF VETERINARY SCIENCE

Memorandum of Agreement (MoA) for Academic Supervision of Postgraduate Students

This document should be read in conjunction with the following University of Pretoria policy documents:

the University of Pretoria General Regulations applicable to postgraduate study
the University Code of Ethics for Research,
the University Plagiarism Policy,
the Policy for the Preservation and Retention of Research Data
the Intellectual Property Policy,
the Guidelines for Postgraduate Supervision and
the Declaration of Originality form.

These documents are all available on the University of Pretoria web site (http://www.up.ac.za) and on request from the Registrar's Division

Clear mediation mechanisms are available to deal with any grievances, personal problems or disagreements that may arise between a postgraduate candidate and the supervisor.

(Refer to the General Regulations and Information of the University of Pretoria pertaining to the Student Communication Channel, Section B.15)

Name of student:	Belinda M Peyrot
Student number:	18392483
Degree:	MSc
Department:	Production Animal Studies
School:	
Faculty:	Veterinary Science

Initial – Student	
Initial – Supervisor	

The document should be completed, signed and submitted to student administration one month after registration