Molecular epidemiology of Newcastle disease and avian influenza in South Africa

by

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for the degree PhD

Highly pathogenic avian influenza (HPAI) and velogenic Newcastle disease (ND) are devastating diseases of poultry that are notifiable to the World Animal Health Organization (Office International des Epizooties). RT-PCR, DNA sequencing, molecular characterisation and phylogenetic analyses were conducted on South African ND and AI virus strains isolated since the 1990s, to investigate the epidemiology of both diseases in the country. The first recorded outbreak of AI in chickens that started in 2002 was caused by two genetically distinct low pathogenicity avian influenza virus (LPAI) H6N2 genotypes that arose from a common ancestor. The ancestral virus appears to have been produced by reassortment between two ostrich viruses, A/Ostrich/South Africa/KK98/98 (H6N8), and A/Ostrich/South Africa/9508103/95 (H9N2). This highlighted the potential role that ostriches may play as mixing vessels for strains that may spill over into chickens when biosecurity breaks down. LPAI H3N8, H4N8 and H5N1 viruses isolated in 2004 from wild ducks in Gauteng were determined to be Eurasian in origin, but the LPAI H5N1 virus was not closely-related to the highly pathogenic avian influenza (HPAI) Asian (or Genotype Z) H5N1 strain. Pelagic shorebirds are implicated in the introduction of these viruses into South African wetlands that infect sympatric wild ducks and geese that in turn move extensively throughout the country. Interactions between wild ducks and ostriches are well-documented, and the transmission of AIV between these species was confirmed by the genesis of the HPAI

H5N2 ostrich outbreak strain of 2004 from the common ancestor of an LPAI H5N2 virus, isolated from an Egyptian goose (*Alopochen aegypticus*) in the same year.

Since the 1990s, outbreaks of velogenic Newcastle disease virus (NDV) in South Africa were caused by three distinct genotypes. The historic genotype VIII (lineage 3), enzootic since the 1970s, was replaced by genotype VIIb (lineage 5b) in the early 1990s during a panzootic, and VIIb was replaced by genotype VIId (lineage 5d) in 1999. Lineage 5d reemerged in 2003, causing an outbreak in 2004 in commercial and backyard flocks. Phylogenetic evidence indicated that pigeon paramyxmoviruses (ND), similar to the aforementioned genotypes, were introduced into South Africa from Eurasian sources on several occasions. The consecutive replacement of Eurasian NDV genotypes, the lack of a unique South African lineage and the lack of conclusive evidence of a true cyclic reservoir challenges the mindset that NDV is enzootic in South Africa. This is the first time that molecular techniques were used to elucidate the status of these two important diseases in South Africa. It is evident that the region is at risk to the introduction of HPAI strains carried by migratory birds, but that with improved control, the enzootic status of NDV could be reversed, with important economic implications for the poultry industry. Improved biosecurity is therefore key to the prevention of future outbreaks of AI and ND in South Africa.

PAPERS AND CONFERENCE PROCEEDINGS

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Posters

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LIST OF ABBREVIATIONS

°C	:	degrees Celcius
%	:	percentage
A	:	adenine
AGID	:	agar gel immunodiffusion test
AI	:	avian influenza
AIV	:	avian influenza virus
APMV-1	:	avian paramyxovirus type 1
bp	:	base pair
C	:	cytosine
cRNA	:	complimentary RNA
DIVA	:	differentiating infected from vaccinated animals
e.g.	:	for example
ELISA	:	enzyme-linked immunosorbent assay
EM	:	electron microscope
ER	:	endoplasmic reticulum
EU	:	European Union
F_0	:	fusion protein precursor
G	:	guanidine
GIS	:	geographic Information System
GPMV	:	goose paramyxovirus
H_0	:	hemagglutinin protein precursor
HA	:	hemagglutinin protein
HA	:	hemagglutination
HAU	:	hemagglutiniating units
HI	:	hemagglutinin inhibition
HN	:	hemagglutinin-neuraminidase protein
HPAI	:	high pathogenic avian influenza
HPNAI	:	high pathogenic notifiable avian influenza
ICPI	:	intracerebral pathogenicity index
IVPI	:	intravenous pathogenicity index
kDA	:	kilodaltons
KZN	:	KwaZulu-Natal province
L	:	large polymerase protein
LPAI	:	low pathogenic avian influenza
LPNAI	:	low pathogenic notifiable avian influenza

M	:	matrix protein
M1	:	matrix protein-1
M2	:	matrix protein-2
MAb	:	monoclonal antibody
MDT	:	mean death time
min	:	minutes
M-MLV	:	mouse Moloney murine leukemia virus
mRNA	:	messenger RNA
NA	:	neuraminidase protein
NASBA	:	nucleic acid sequence-based assay
NDA	:	National Department of Agriculture
NeuAc	:	N-acetylneuraminic acid
NeuGc	:	N-glycolylneuraminic acid
NI	:	neuraminidase inhibition
ND	:	Newcastle disease
NDV	:	Newcastle disease virus
NP	:	nucleoprotein
NS	:	non-structural
nt	:	nucleotide
NWP	:	North-West province
OIE	:	Office International des Epizooties (World animal health organization)
ORF	:	open reading frame
OVI	:	Onderstepoort Veterinary Institute
P	:	phosphoprotein
PA	:	Polymerase A
PB1	:	Polymerase B1
PB2	:	Polymerase B2
PCR	:	polymerase chain reaction
PPMV-1	:	pigeon paramyxovirus type 1
RBS	:	receptor binding site
RNA	:	ribonucleic acid
RNP	:	ribonucleoprotein
RT-PCR	:	reverse transcription polymerase chain reaction
rRT-PCR	:	real-time reverse transcription polymerase chain reaction
SA	:	sialic acid
SAN	:	specific antibody negative

SAPA	:	South African Poultry Association
sec	:	seconds
SPF	:	specific pathogen free
T	:	thymidine
TNFα	:	tumor necrosis factor alpha
UAE	:	United Arab Emirates
UTR	:	un-translated region
VLA	:	Veterinary Laboratory Agency
vRNA	:	viral ribonucleic acid
RNAP	:	viral RNA-dependent RNA polymerase
vRNP	:	viral ribonucleoprotein
WHO	:	World Health Organization

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CHAPTER ONE

LITERATURE REVIEW

en·zo·ot·ic ("en-z&-'wä-tik)
Function: *adjective*
Etymology: *en-* + epi*zootic*
of animal diseases **:** peculiar to or constantly present in a locality
- **enzootic** *noun*
Merriam-Webster Online Dictionary http://www.m-w.com

1.1 AVIAN INFLUENZA

1.1.1 Introduction

The severe form of avian influenza (AI), historically known as "fowl plague", was first described by the Italian scientist Perroncito in the late 19th century (Perroncito, 1878). AI can cause devastating losses in poultry, with flock mortalities of up to 100%. Coupled with the added economic impact of trade restrictions and embargoes placed on infected areas, all H5 and H7 AI strains and the severe forms of Newcastle disease are notifiable to the World Animal Health Organization (Office International des Epizooties). Highly pathogenic avian influenza (HPAI) is one of the most feared zoonotic diseases today, because of the purported potential of the Asian HPAI H5N1 strain to be involved in the next, supposedly imminent, human influenza pandemic (Webster *et al*., 2006).

1.1.2 Aetiology

Influenza viruses, the aetiological agents of AI, are segmented, negative strand RNA viruses that belong to the family *Orthomyxoviridae*. There are three genera of influenza viruses: type A, type B and type C. Phylogenetically, influenza A and B viruses are more closely related to each other than to influenza C virus (Gammelin *et al*., 1990; Krossoy *et al*., 1999). Type A influenza viruses (but not types B and C) are further divided into subtypes based on the antigenic relationships in the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). At present 16 HA subtypes (H1-H16) and nine neuraminidase subtypes (N1-N9) have been identified. Each virus has one HA and one NA antigen, apparently in any combination (Röhm *et al*., 1996).

1.1.3 Morphology and genome organization of influenza A viruses

Influenza A virions are enveloped and highly pleiomorphic, varying from roughly spherical shapes 80-120 nm in diameter ($300x10^6$ Da) to filamentous particles. The two surface glycoproteins, rod-shaped HA and mushroom-shaped NA, are dispersed as spikes in the host-derived lipid bilayer of the envelope (Fig. 1.1) (Levy *et al*.,

1994). The viral envelope encloses the nucleocapsid containing the genome. The influenza A virus genome is distributed over eight different segments (Fig. 1.2) and encodes at least ten proteins: polymerase A (PA), polymerase B2 (PB2), polymerase B1 (PB1), the two viral surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), matrix proteins M1 and M2, non-structural (NS) proteins NS1 and NS2 and nucleocapsidprotein (NP). The minimal replication unit, the ribonucleoprotein complex, is composed of the viral RNA, NP and three polymerase proteins PB2, PB1 and PA (Neumann *et al*., 2003). Influenza A virus nucleoproteins are antigenically related (and are used to serologically distinguish between influenza types A, B and C), as are their matrix proteins (Zhou *et al*., 1998).

The organization of the eight single-stranded RNA segments within the virion is still poorly understood. Biochemical and biological studies support the idea that each RNA segment exists as a distinct ribonucleoprotein (RNP) complex (Compans *et al*, 1972; Palese & Ritchey, 1977), whereas electron microscopic studies have shown that the internal component released from a disrupted influenza virion is a single large helix (Almeida & Brand, 1975). Genomic RNAs of influenza virus are held in a circular conformation in virions and in infected cells in a terminal panhandle structure form that might play an important role in replication (Hsu *et al*., 1987). Large helical structures have also been observed (Murti *et al*., 1980), and it is possible that multiple RNPs arise from the degradation of helices (Wright & Webster, 2001).

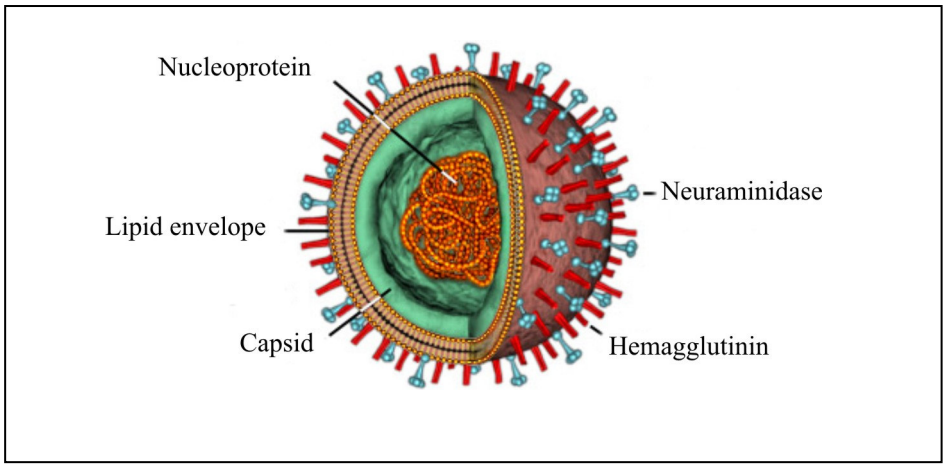


Figure 1.1 Structure of the influenza A virus

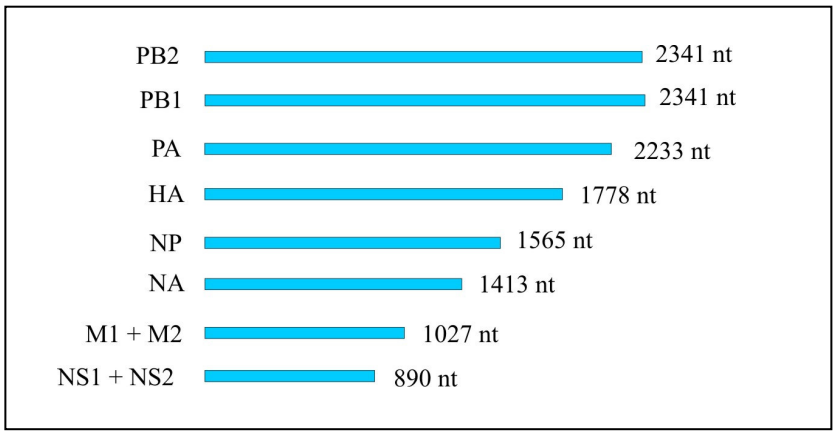


Figure 1.2 Relative sizes of AIV RNA segments

1.1.4 Host range

Type A influenza viruses include the avian, swine and equine strains, as well as human influenza A viruses, which cause pandemics. Natural infections of type A influenza in seals, whales, mink and cats have also been reported. Type B influenza viruses cause influenza epidemics (but not pandemics) in humans, but natural infections of seals are also known to occur. Type C influenza viruses cause mild disease in humans, but have never been associated with large scale epidemics, and have been isolated from dogs and swine (Aiello & Mays, 1998).

Type A are the only influenza viruses known to infect birds, and have been isolated from a large number of avian species covering 12 of the 50 orders (Stallknecht, 1998). The largest numbers of influenza A viruses have been isolated from wild waterbirds including ducks, geese, terns, shearwaters, gulls, as well as a wide range of domestic avian species such as turkeys, chickens, quail, pheasants, geese, ducks, and less frequently, from passerine birds such as starlings and budgerigars (Wright & Webster, 2001). The disease signs associated with influenza A infections vary considerably with the strain of virus and the species of the bird. In ducks, the majority of avian strains of AIV replicate in the lungs and in the cells lining the intestinal tract and infected birds usually show no signs of disease (Webster *et al*., 1978). The viruses gain access by passage through the digestive tract, despite the low pH of the gizzard, and are shed in high concentration in the faeces. The asymptomatic infections in waterfowl, together with the fact that all HA and NA subtypes of influenza A viruses have been isolated from wild waterfowl in most combinations of subtypes, and that mammalian influenza viruses are directly or indirectly derived from this reservoir,

strongly suggest that waterfowl, shorebirds and gulls are the natural hosts and biological reservoirs of AIV (Webster *et al*., 1992). In contrast, influenza viruses that have become established in mammals show a restricted combination of HA and NA types, limited to H1, H2, H3, N1 and N2 types. Certain avian influenza viruses have been transmitted directly to and have caused epidemics in other mammals including H3N8 in horses, H7N7 in seals, and H1N1 in pigs (Baigent & McCauley, 2003). Phylogenetic analyses of amino acid changes led to the surprising discovery that avian influenza viruses, unlike mammalian strains, display low evolutionary rates (Gorman *et al.,* 1990). In fact, influenza viruses in wild aquatic birds appear to be in evolutionary stasis. Nucleotide changes have continued to occur at a similar rate in avian and mammalian influenza viruses, but these changes no longer result in amino acid changes in the avian viruses, whereas all eight mammalian influenza gene segments continue to accumulate changes in amino acids. The high level of genetic conservation suggests that avian influenza viruses in their natural reservoirs are approaching an adaptive optimum, wherein nucleotide changes no longer provide any selective advantage (Wright & Webster, 2001).

1.1.5 Disease

Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses cause HPAI (fowl plague) in which flock mortality may be as high as 100%. Common signs of HPAI are slight to severe drop in egg production, increased mortality, respiratory difficulties, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin and diarrhoea. However, none of these signs can be considered pathognomonic, as similar symptoms are often seen with acute fowl cholera, velogenic Newcastle disease and other respiratory diseases. HPAI is associated only with H5 and H7 AIV strains (Alexander, 2000a).

Low pathogenic avian influenza (LPAI) viruses (all subtypes, including H5 and H7) cause a much milder disease consisting primarily of mild respiratory symptoms, depression and egg production problems in laying birds. The host species, age of the bird, immune status (particularly if the host is concomittently infected with other pathogens), and environmental conditions also contribute to the severity of the disease (Capua *et al*., 2000a). The OIE terrestrial code (2004) differentiates between

notifiable avian influenza (NAI) and strains that are non-notifiable to the OIE. Notifiable avian influenza is defined as an infection of poultry caused by influenza A virus of the H5 or H7 subtype or by any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2 (or as an alternative at least 75% mortality). The notifiable strains can be further divided into highly pathogenic notifiable avian influenza (HPNAI) and low pathogenicity notifiable avian influenza (LPNAI). Therefore, all H5 and H7 strains, whether highly pathogenic or of low pathogenicity, are notifiable to the OIE. The OIE definition also allows a molecular basis for confirming HPNAI, this is discussed in 1.1.8 (p12).

1.1.6 Diagnosis

1.1.6.1 Virus isolation and identification

The classical method of AIV diagnosis is virus isolation in embryonated fowl eggs. Tracheal or cloacal swabs, faeces from live birds or homogenized organs of dead birds are used. The sample or pooled samples are treated with antibiotics and the clarified supernatants are then inoculated into the allantoic sac of nine to eleven-day-old embryonated specific pathogen free (SPF) eggs, or specific antibody negative (SAN) eggs. At least five eggs are inoculated per sample, and incubated for four to seven days at 35-37ºC. Allantoic fluid is harvested from eggs containing dead or dying embryos, and then tested for hemagglutinating (HA) activity. Detection of HA activity (HA test) indicates a high probability of the presence of influenza A virus or of an avian paramyxovirus. The presence of influenza A virus can be confirmed in various other serological tests, including the agar gel immunodiffusion (AGID) that demonstrates the presence of the NP or M antigens, HI tests, and various commercially-available ELISAs. Alternatively, the presence of influenza virus, and subtyping, can be confirmed with the use of reverse-transcription polymerase chain reaction (RT-PCR) or real time reverse-transcription PCR (rRT-PCR). rRT-PCR is able to detect the presence of AIV nucleic acids even if the viruses are no longer viable, and is therefore considered to be a more sensitive method than virus isolation (Swayne 2003; OIE Terrestrial Manual 2004; Cattoli *et al*., 2006).

1.1.6.2 Assessment of pathogenicity

The IVPI test is used as a method of clinically assessing virulence of AIVs. Cultivated virus is injected intravenously into each of ten six-week-old SPF chickens, and the birds are examined at 24-hour intervals for ten days. At each observation, each bird is scored (0) if normal, (1) if sick, (2) if severely sick, and (3) if dead (dead individuals are scored as (3) at each of the remaining daily observations after death). The IVPI is the mean score per bird per observation over the ten-day period. An index of 3.0 means that all birds died within 24 hours, and an index of 0.00 means that no birds showed any clinical sign during the ten-day observation period (OIE Terrestrial Manual, 2004). The OIE and European Union (EU) have adopted the following definition to confirm disease for the purposes of disease control: 'HPAI is defined as an infection of poultry caused by an influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens >1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin' (OIE Terrestrial Manual, 2004). The amino acid sequence at the hemagglutinin cleavage site as a determinant of virulence is discussed in 1.1.8.

1.1.6.3 Nomenclature

Strains of influenza viruses are described by their type, host, place of first isolation, strain number (if any), year of isolation, and antigenic subtype (Fenner *et al*., 1987; Acha & Szyfres 2003) e.g. A/Ostrich/South Africa/2004 (H5N2). For human strains, the host is omitted.

1.1.7 The infection cycle

1.1.7.1 The surface glycoproteins

The main functions of the glycoproteins are to facilitate the entry into and release of viruses from the host cell. HA is the major viral antigen against which neutralizing antibodies are formed. Each HA spike is composed a trimer of rod-shaped protein molecules which, depending on the environment, may be split by a specific peptide bond cleavage into two chains (HA-1 and HA-2) of roughly 20 and 25kDa, held

together by disulphide bonds. The proteolytic cleavage exposes HA-1 at the tip of the spike that carries most of the oligosaccharides of the virion. The oligosaccharides, which compose about 20% of the protein, consist of fructose, galactose, branch-structured mannoses and most importantly, several glucosamines, one of which is attached by N-glucosidic linkages to an asparagine on the protein surface. The sialic (N-acetylneuraminic) acid (SA), usually present at the end of such oligosaccharides is absent in influenza A, owing to the presence of neuraminidase activity on the virion. Erythrocytes of many species also bear polysaccharides on their cell surfaces, to which influenza A viruses bind, thereby causing hemagglutination. The hemagglutination reaction is characteristic of influenza A and is shared only by the paramyxoviruses and a few strains of reovirus, EDS 76 adenovirus, infectious bronchitis virus (if treated with neuraminidase) and bacteria. This characteristic forms the principle on which the hemagglutination (HA) and hemagglutination inhibition (HI) diagnostic tests are based. The HA test will indicate the presence of the virus in a sample, usually alantoic fluid, whereas the HI test is used to detect the presence of specific neutralizing antibodies in serum. In practice, the test serum is incubated with a known influenza A subtype antigen. If any neutralizing antibodies are present, they will bind the HA and NA surface glycoproteins, and prevent the natural hemagglutination reaction. The test involves a serial dilution of the serum against a known concentration of antigen, expressed in HA units (HAU). Agglutination is visually judged against positive and negative controls. The HI titer is defined as the highest dilution of serum causing complete inhibition of 4 HAU of antigen, and may be regarded as positive if there is inhibition at a serum dilution of 1:16 (OIE Terrestrial Manual, 2004).

There are fewer neuraminidase (NA) spikes on the influenza virion surface than hemagglutiniating spikes (about 200 compared with 750). Each NA spike (~200 kDa) consists of a dimer of two identical disulphide-linked 55 kDa glycoproteins. A single oligosaccharide is located on the stalk region, but the terminal knob is the enzymatically active region: the neuraminidase breaks the bond holding sialic acid to the end of many polysaccharide receptors on cell surfaces (although not those for mature influenza virions, because these lack sialic acid). The main function is therefore to remove terminal sialic acid from nascent viral glycoproteins, which facilitates the budding and release of the virions from infected cells, prevention of virus clumping, and the spread of the virus, particularly by helping to penetrate the mucous layer of the host respiratory system. The neuraminidase function therefore

also gradually reverses hemagglutination of erythrocytes. Hemagglutination even occurs at 0°C, but at 37°C the neuraminidase activity is activated and the NA protein cleaves the receptors that hold the virus to the erythrocyte surface. The neuraminidase inhibition (NI) test has also been used to identify the AI neuraminidase type of isolates and to characterize the antibody in infected birds (OIE, 2004).

1.1.7.2 Receptor specificity and attachment

The initial infection involves the attachment of the HA spike to the cellular receptor. The receptor binding site (RBS), which lies near the membrane distal tip of each HA subunit (Weiss *et al*., 1988) binds sialic acid (SA) attached to galactose. Influenza viruses recognise two species of SA (N-acetylneuraminic acid, NeuAc, and N-glycolylneuraminic acid, NeuGlc) which are attached to galactose in SAα-2,3Gal or SAα-2,6Gal linkages (Gambaryan *et al*., 1995). Ability of the virus to replicate in different host species is influenced by both SA species and linkage type in the host, and by amino acids at positions 226 and 228 in the RBS of the virus HA. The host animal might exert selective pressures on receptor specificity of the virus, since the abundance of receptor types on cells at the sites of virus replication varies. Human tracheal epithelium has predominantly NeuAcα-2,6Gal (expressed mainly on noncilliated cells) but recent evidence indicates that ciliated cells, a substantial cellular subset of the respiratory epithelium, express α-2,3 linked sialic acid receptors in sufficient density to allow entry and replication of avian viruses. Ciliated cells therefore most likely serve as primary target cells in those rare cases where avian viruses cause human disease, with sometimes even fatal outcome (Baigent & McCauley, 2003). Equine tissues possess both NeuAc and NeuGc, the major SA-Gal moiety in horse trachea being NeuGcα-2,3Gal; duck intestine contains mainly NeuAcα2,3Gal (also NeuGcα-2,3Gal). This may explain why horses are susceptible to direct transmission of avian viruses (albeit only a single genotype A/Equine/Jilin/1/89, H3N8, has been reported) though apparently not to human viruses. Swine tissues possess both NeuAc and NeuGc, the trachea having both SAα-2,3Gal and SAα-2,6Gal (Ito *et al*., 1998). This confers susceptibility to avian and human viruses, both experimentally and in the field (Baigent & McCauley, 2003), and until recently, it was thought that pigs were required as an intermediate host for transmission of AIV to humans (Gammelin *et al*., 1989; Yasuda *et al*., 1991).

1.1.7.3 Endocytosis and escape into the host cell

In addition to the glycoproteins, a small protein, M2, is also anchored in the viral envelope, and serves as a trans-membrane proton channel (Lamb and Choppin, 1983; Sugrue and Hay, 1991). After the HA spike has docked onto the SA receptor, the cell wall invaginates (mediated by the protein clathrin on the inner wall surface), internalising the virus to a host cell endosome. M2 ion activity is essential for the uncoating process. Once a virion particle has been endocytosed, the low –pH-activated ion channel activity of the M2 protein permits the flow of ions from the endosome to the virion interior, to disrupt protein-protein interactions and free the RNPs from the M1 protein (Lamb & Krug, 2001). Low pH within the endosome also triggers irreversible conformational changes in HA2, exposing the "fusion peptide" and pulling the lipid cell and viral membranes together to fuse them. The virion contents are poured into the host cell, and the vRNPs (viral ribonucleoproteins) are then transported to the nucleus where RNA replication, transcription and assembly of progeny vRNPs occurs (Lamb & Krug, 2001).

1.1.7.4 Transcription, translation and particle assembly

The three types of virus-specific RNAs (mRNAs, template RNAs and vRNAs) are all synthesized in the nucleus. The intact RNPs enter the cell nucleus through a nuclear pore. A unique feature of influenza virus mRNA synthesis is the dependence on host cell nuclear function. This dependence involves the requirement of the virion-associated polymerase on a primer to initiate viral mRNA synthesis. These primers are capped (m^7GpppX^m-containing) RNA fragments, derived by cleavage of host cell RNA polymerase II transcripts. A cap-dependent endonuclease that is intrinsic to the influenza virus polymerase cleaves capped RNAs 10 to 13 nucleotides from their 5' ends (Plotch *et al*., 1981) and the presence of a 5'-methylated cap structure, and not hydrogen bonding between the 12-nt sequence is the actual primer for transcription. The process has been called “cap snatching” (Lamb & Krug, 2001). After priming, mRNAs are transcribed, terminating at the stretch of uridine residues at the 5' end of the gene, which are the templates for the incorporation of polyadenylate (polyA) residues into the new mRNAs. In the nucleus, the viral mRNAs undergo at least some of the same processing steps as cellular RNA precursors. Internal adenosine residues of influenza virus mRNAs are methylated, and two of the viral mRNAs are spliced.

The viral mRNAs travel out through the nuclear pores, and are translated in the cytoplasm (Lamb & Krug, 2001). The virion transcriptase also synthesises viral cRNAs, the template for vRNA synthesis. The second step in replication involves the copying of the template cRNAs into vRNAs, and for this to occur, an alternative type of transcription is utilized that bypasses the need for the capped priming mechanism. Instead, synthesis proceeds due to the presence of 5' triphosphorylated ends of vRNAs (Young & Content, 1971). These new negative-sense viral genomic RNAs become associated with the newly-synthesized nucleoproteins and some M1 proteins that have migrated into the nucleus. The newly formed nucleocapsids and their associated M1 proteins exit the nucleus via nuclear pores (Lamb & Krug, 2001).

The influenza virus integral membrane proteins HA, NA and M2 are synthesized on membrane-bound ribosomes and are translocated across the membrane of the endoplasmic reticulum (ER). Once correctly folded and assembled, proteins are transported out of the Golgi apparatus. These oligosaccharides may be further processed to the complex form, and in HAs containing a furin cleavage site (see 1.1.8), cleavage activation occurs here too. HA (HPAI cleaved, LPAI uncleaved, see 1.1.8), NA and M2 migrate via the Golgi apparatus and become anchored in the regions of the plasma membrane. Although HA appears to be diffusely distributed over the surface, M2 and NA appear to cluster in patches, although the significance of this arrangement is unknown (Lamb & Krug, 2001). Once the integral membrane proteins are in place, matrix protein and new viral nucleocapsids align underneath, and interactions between the cytoplasmic tails of the viral integral membrane proteins likely provide the necessary molecular information for the formation of the budding particle. The progeny virions bud out at the apical surfaces and are initially aggregated at the host cell membrane because they are still sialylated, but the enzymatic activity of NA cleaves these sialic acids, releasing infectious progeny (Lamb & Krug, 2001).

1.1.8 HA, the primary molecular determinant of virulence

Although virulence of avian influenza viruses is multigenic in nature, the HA glycoprotein is recognised as playing a major role (Rott *et al*., 1979; Scholtissek *et al*., 1977). While HPAI viruses appear to exclusively involve H5 and H7 hemagglutinin subtypes, not all H5 and H7 viruses are highly pathogenic. HPAI H5 and H7 viruses

are thought to emerge from low pathogenic precursors only after the latter have been introduced into domestic poultry. This hypothesis is supported by studies which demonstrated that HPAI viruses do not form separate phylogenetic lineages in waterfowl (Röhm *et al*., 1995).

The precursor of each HA monomer is a single polypeptide chain that is cleaved by host proteases into two disulphide-linked subunits, HA-1 and HA-2, exposing a fusion peptide at the newly formed amino terminal end of HA2 (Rott, 1992). In an acidic environment, this peptide undergoes an irreversible conformational change, enabling it to fuse the viral envelope and host cell membranes, a crucial step in influenza virus infectivity (Rott, 1992). Both the peptide sequence at the cleavage site (H_0), and the availability of corresponding host cell proteases directly correlate with virulence in poultry (Rott, 1992).

Low-pathogenicity notifiable strains isolated from feral birds typically have only two basic amino acids at positions –1 & –3 (H7) and -1 & -4 (H5) from the cleavage site (Wood *et al*, 1993). This sequence is recognised and cleaved by extracellular trypsin-like proteases that are secreted only by cells of the respiratory tract and the avian intestinal tracts. Therefore these viruses, although fully infectious, cause relatively mild symptoms because they only replicate in limited cell types and are restricted in their ability to spread in a host and cause anatomically localised infections. In contrast, HPAI viruses have a connecting peptide sequence of several basic amino acids (Arg or Lys) that is recognised and cleaved by a set of intra-cellular subtisilin-like calcium-dependent proteases, most likely to be furin (Stieneke-Grober *et al*., 1992). Intracellular furin-like proteases have a much broader tissue distribution than the trypsin-like proteases, and therefore HPAI viruses produce lethal systemic infections.

The number of basic amino acids at H_0 of HPAI viruses vary from four to six residues, but cleavability is also influenced by the presence of an adjacent oligosaccharide side chain on the HA molecule (see 1.1.9.4). The proposed sequence requirement for HA cleavage by endogenous proteases, when carbohydrate is nearby, is X-X-R-X-R/K-R (where X=nonbasic residues); otherwise it is R/K-X-R/K-R (Ohuchi *et al*., 1989; Vey *et al*., 1992; Horimoto & Kawaoka, 1994). The emergence of HPAI from LPAI has been proposed to occur by a number of mechanisms, viz. (i) the insertion of basic amino acids at H_0, possibly the result of duplication of purine triplets due to a transcription fault of the polymerase complex (Horimoto *et al*., 1995;

Garcia *et al*., 1996); (ii) the progressive accumulation of basic amino acids at the cleavage site by a stepwise process involving amino acid substitutions (Horimoto *et al*., 1995; Spackman *et al*., 2003); and (iii) non-homologous recombination resulting in the insertion of a foreign nucleotide sequence adjacent to the H_0 (Suarez *et al*., 2004). Only four cases of non-homologous recombination at H_0 have been reported in the literature, however all four cases involved the H7 subtype. In two laboratory cases, passage in chicken embryo cells resulted in the insertion of a 54 nt region derived from the 28s rRNA gene into the H_0 cleavage site of an H7N3 isolate (Khatchikian *et al*., 1989), and a 60 nt fragment from the NA gene inserted into the H_0 cleavage site of an H7N7 virus (Orlich *et al.,* 1994). Increased pathogencicity for chickens was observed in both cases. Two field cases involved the 30 nt insertion of a nucleoptotein gene fragment into H_0 of an H7N3 virus in Chile in 2002 (Suarez *et al*., 2004), and the insertion of a 21 nt segment derived from the matrix protein gene into H_0 of an H7N3 virus in Canada in 2004 (Pasick *et al*., 2005)

1.1.9 Other virulence and host range determinants

The molecular determinants and related mechanisms that make certain AIVs highly pathogenic in poultry and mammals are poorly understood. Both viral factors and host factors may determine virulence. Numerous studies have shown that AIV virulence is a polygenic trait, which may require a critical constellation of genes. Thus, only reassortants with a set of genes that function efficiently together in a particular species of host will emerge as viable viruses, since functional interactions between viral proteins, and host proteins, are essential for replication (Scholtissek, 1987).

1.1.9.1 Neuraminidase

The receptor-binding properties of HA should be functionally compatible with the cleavage specificity of NA. The NA stalk, which holds the active site above the virion envelope, varies in sequence and length (Blok & Air, 1982). Thus a short-stalked NA is inefficient in disaggregating progeny virus because the active site cannot access its substrate efficiently. A shortened NA stalk reduces ability of virus to elute from erythrocytes (Els *et al*., 1985; Baigent *et al*., 1999), can reduce virus growth in MDCK cells (Luo *et al*., 1993) and eggs (Castrucci & Kawaoka, 1993) and can

decrease virulence in mice (Castrucci & Kawaoka, 1993). However, naturally occurring avian viruses having short NA stalks are fully virulent in poultry, showing that a long stalk is not essential for virulence in chickens (Baigent & McCauley, 2003). Incompatibility between HA and NA can restrict the virulence of reassortant viruses (Kobasa *et al*., 1999; Saito & Kawano, 1997; Baum & Paulson, 1991; Matrosovich *et al*., 2001).

1.1.9.2 Host basal body temperatures and pH

In avian species, the primary site of AIV replication is the intestinal tract (termed enterotropism), but in humans, human influenza A viruses replicate in the respiratory tract but not the gut, despite the presence of sialic acid in this system. Human viruses are also unable to replicate in the intestine of experimental ducks, despite the fact that their NA has retained a level of α-2,3 cleavage activity (Kobasa *et al*., 2001). The HK/H5N1 virus, considered to be transmitted directly from chickens to humans without prior adaptation in an intermediate mammalian host was able to replicate in the human intestine, causing severe gastrointestinal symptoms suggesting that a biological difference between human-adapted and avian-adapted viruses determines their ability to replicate in the gut. Studies using avian-human reassortant viruses provide evidence that HA and NA are critical for the enterotropism of avian viruses but are not essential for replication in other avian tissues (Hinshaw *et al*., 1983). This is partly due to the fact that the avian virus NAs function better at higher temperature and lower pH than their mammalian-virus counterparts do (Fiszon *et al*., 1989). The human strains replicate well at 37°C, but poorly at 40°C, while avian strains are still able to replicate efficiently at 40°C consistent with the higher body temperature of birds compared with mammals. More significantly for enterotropism, the NA activity of avian H1N1 influenza viruses is more resistant to acid pH than the NA of human- or swine-adapted H1N1 viruses (probably due to amino acid differences around the active site). Thus, avian NAs are capable of functioning in the low pH values in the upper digestive tract (Baigent & McCauley, 2003).

1.1.9.3 Glycosylation and sialylation

HA molecules are glycosylated at four to eleven sites in the head and stem. Glycosylation and sialylation close to the RBS of HA regulate release of avian viruses from cells (Baigent *et al*., 1999; Ohuchi *et al*., 1997), and thereby contributes to virulence and tissue tropism. These glycans can reduce affinity of HA for its receptor, possibly through simple steric hindrance, while SAs attached to the glycans reduce receptor availability by obscuring or completely filling the RBS. SA removal by a compatible NA is essential for HA to show its full receptor binding capacity. In tissue culture, a readily released glycosylated HA and a long-stalked NA favour virus growth (Wagner *et al*., 2000; Baigent & McCauley, 2001). The combination of HA glycosylation and NA stalk length influences host range and host adaptation. Human H1 influenza viruses show hyperglycosylation compared with those from aquatic birds, or pigs (Inkster *et al*., 1993), during the stable adaptation of an avian virus to human hosts. It has been suggested that hyperglycosylation of HA, combined with a compensating deletion in the NA stalk, modifies the progenitor aquatic bird virus preceding the development of virulence during virus evolution in chickens (Matrosovich *et al*., 1999). Hyperglycosylation of the HA head was associated with increased virulence in chickens of an H7N7 virus in Australia (Perdue *et al*., 1995) and an H7N1 virus in Italy (Banks *et al*., 2001). However, this additional glycosylation does not correlate conclusively with virulence of H7 viruses in chickens (Banks *et al*., 2000). To summarise, virulence requires both effective release of virus from infected target cells, and efficient binding of virus to new target cells. This is achieved by compensating changes in receptor-affinity of HA (regulated by amino acid changes in the RBS, and/or glycosylation/sialylation near the RBS) and effective enzyme activity of NA (regulated by amino acid changes to the active site, and by NA stalk length) to ensure an optimal balance in the activities of these two molecules.

1.1.9.4 Polypeptides of the replication complex

Nucleotide sequence analyses indicate that the genes for the virus internal proteins (PB2, PB1, PA, NP, NS and M proteins) cluster into host-species-specific lineages. The possibility that these lineages correlate with virulence and host range has been analysed *in vitro* and *in vivo*. In both animal models and tissue culture, the PB2 polypeptide confers a strong influence on host range. Residue 627 of PB2 is an

important although not the sole determinant of virulence and host specificity. Viruses with a Lys at this position are able to grow more efficiently at lower temperatures than viruses with a Glu residue (Shinya *et al*., 2006). The other polypeptides in the viral replication complex, PB1, PA and NP, also influence AIV host range, and compatibility between these four polypeptides is vital (Baigent & MaCauley, 2003).

Unique amino acid substitutions are associated with host specificity in both PB1 (Kawaoka *et al*., 1989) and PA (Okazaki *et al*., 1989). The combination of human PA with avian PB1 and PB2 or avian PB1 with human PB2 and PA severely restricts replication of reassortants in mammalian cells and squirrel monkeys (Snyder *et al*., 1987). However, the NP gene may have as broader host range since an avian virus with a temperature-sensitive mutation in NP could be rescued by avian and some swine viruses, but not by human strains (Scholtissek *et al*., 1985). Analysis of replication efficiency of reconstituted RNPs showed that functional compatibility between polypeptides of the replication complex is important for replication in mammalian cells (Naffakh *et al*., 2000). Replication was more efficient when PB2 and NP were both derived from the same avian or human virus, or when PB1 was derived from an avian virus whatever the combination of the other genes. This indicates that avian PB1 could have a higher activity, providing a selective advantage to viruses. In this context, it is interesting that the human pandemic viruses of 1957 and 1968 were reassortants having avian PB1 and HA (and NA for the 1957 virus) genes (Kawaoka *et al*., 1989; Scholtissek *et al*., 1987).

1.1.9.5 Matrix proteins

AIV genome segment 7 encodes two polypeptides: matrix protein 1 (M1) and matrix protein 2 (M2), the proton channel. The M protein was generally associated with the restriction of replication of both an H2N2 avian virus in squirrel monkeys (Buckler-White *et al*., 1986), and an H3N8 avian virus in pigs (Kida *et al*., 1994) but it's unknown whether M1 or M2 contributed to the host range restriction (Buckler-White *et al*., 1986). Functional compatibility between the pH at which HA undergoes conformational change, and the pH at which the M2 ion channel opens, ensures that intracellularly cleaved HAs of HPAI viruses can pass through the Golgi apparatus without undergoing a premature irreversible conformational change during virus budding- a requirement for viability of these viruses. Compared with M proteins of an

early human virus (A/PR8/34, H1N1), M proteins of later human viruses have gradually lost the ability to cooperate with avian virus HAs, being unable to form replication-efficient reassortants *in vitro*. This significant finding suggests that currently circulating human viruses may be unable to successfully recombine with avian viruses to produce the predicted H5 pandemic strain that would be transmissible in humans (Scholtissek *et al*., 2002).

1.1.9.6 Non-structural proteins

The non-structural (NS) genes of influenza viruses can be divided into two subtypes, or alleles, A and B (Suarez & Perdue, 1998). All known mammalian virus NS genes are of the A allele type, with one exception: an avian H3N8 virus that became established in horses in China (A/equine/Jilin/89) (Kawaoka *et al*., 1998). This is supported by evidence that reassortant viruses that have an avian virus NS gene in an otherwise human virus genetic background only replicated in squirrel monkeys if the NS gene was an A allele type (Treanor *et al*., 1989). NS1 shows more variation between alleles than NS2 (Suarez & Perdue, 1998; Kawaoka *et al*., 1998) and it has been speculated that since NS1 is implicated in downregulating the anti-viral response of the host, NS1 of allele A may achieve this more effectively in mammals than the B allele. Allele B genes, which include many avian viruses, have the potential to be pathogenic in mammals. Glutamic acid at position 92 in NS1 is associated with high virulence in pigs upon reassortment of the NS gene of H5N1 with A/Puerto Rico/8/34 (Seo *et al*., 2002).

1.1.10 Epidemiology

1.1.10 The prevalence of AIV in feral waterfowl

Feral waterfowl (ducks, geese and shorebirds) are the natural hosts and reservoirs of all influenza A subtypes (Webster *et al*., 1992). Waterfowl in the northern hemisphere migrate south in autumn (around September to October), back north in spring (around April to May), and breed in summer (around November to January) in the nesting places in northern territories such as Alaska, Canada or Siberia (Ito *et al*.,1995). Early phylogenetic analysis of viruses isolated from wild aquatic birds has revealed a

geographical separation of avian influenza viruses into the Eurasian and North American lineages (Gorman *et al* 1990; Kawaoka *et al*., 1998). Influenza viruses, that replicate preferentially in the cells lining the intestinal tract of waterfowl, excrete considerable quantities of AIVs (up to $10^{8.7}$ mean egg infectious doses per gram of faeces) into the environment (Webster *et al*., 1978; Hinshaw *et al*. 1979). High titres of AIVs have been isolated from unconcentrated water samples of different lakes in the breeding areas of ducks in northern high latitudes in summer, and furthermore, the viruses remained viable in the lake water after the ducks left for migration to the south (Hinshaw *et al*. 1979; Ito *et al*., 1995; Okazaki *et al*., 2000). Survival of influenza viruses in water is dependent on the virus strain and the salinity, pH, and temperature of the water; at 17°C some strains remain infectious for up to 207 days, and at 4°C they remain infectious for a longer period (Stallknecht *et al*., 1990a and 1990b). It has also been shown that influenza viruses are preserved in frozen lake water during winter when ducks are absent, and that ducks coming back from the south are infected with these viruses in spring (Hinshaw *et al*. 1980; Ito *et al*., 1995; Okazaki *et al*., 2000).

AIV isolation rates and subtypes in waterfowl vary considerably over time, region, between species and between age groups (Kawaoka *et al*., 1988; Sharp *et al*., 1993 Deibel *et al*., 1985). In *Anseriformes*, the greatest prevalence of AIVs has been observed during late autumn and winter (Sinnecker 1982; Halvorson *et al*., 1985). Continuous surveillance of influenza in migratory birds in Canada and USA showed that the frequency of isolation from migratory ducks coming from northern territories in autumn was high (more than 20% of juveniles), whereas those coming from the south in spring was extremely low (less than 0.25%) (Webster *et al*., 1992). In contrast, higher numbers of viruses were isolated from *Charadriiformes* during the spring (Hanson, 2003), where sampling along the Atlantic coast and the Gulf of Mexico revealed 78% of all isolates originated from Ruddy Turnstones (*Arenaria interpres*) in Delaware Bay (Hanson, 2003). Surveillance activities of wild birds across the world have dramatically increased since the emergence of Asian HPAI H5N1. After the Hong Kong influenza outbreak in 1997, monitoring of migratory ducks and shorebirds started in Taiwan. Since 1998, more than two hundred AI viruses were isolated and at least twenty-five LPAI subtypes viz., H1N1, H1N3, H2N2, H3N6, H3N8, H4N2, H4N6, H4N7, H4N8, H5N2, H5N6, H6N1, H6N2, H6N5, H7N1, H7N3, H7N7, H8N4, H10N3, H10N4, H10N6, H10N7, H11N9 and H14N7 were characterised. This suggested that the winter migratory ducks carried

many AI viruses, representing a large antigenic variation (Cheng *et al*., 2006). In France, free-range mule ducks were surveyed for the presence of AIVs, and LPAI H5N1, H5N2, H6N2, H6N8 and H11N9 were isolated. These viruses were all Eurasian lineage types and separate introductions from the wild bird reservoir were suspected. The N1 gene of the H5N1 isolate was phylogenetically very close to an Italian H7N1 virus (Cherbonnel *et al*., 2006). In Italy 3000 samples from wild bird cloacal swabs were tested for AIVs between 2003 and 2005. Approximately 5% of the samples were positive for AIVs, and in approximately 30% of cases it was possible to isolate viruses. H1, H4, H5, H7, H10 and N1, N2, N3, N4, N6 and N7 subtypes were identified (Cattoli *et al*., 2006). In Norway in 2005, 650 hunted wild species including greylag goose, mallard, wigeon, teal, goosander, tufted duck, common scoter and goldeneye were sampled during AIV surveillance. None of the 200 goose samples were positive for AIVs, but a total of 22.4%, 12.8%, 37.9%, 0%, 0% and 16.6% of the other species, respectively, were positive for AIVs. LPAI H5N2, H1N1, H3N2, H3N8, H6N2, H6N8, H8N4 and H9N2 subtypes were identified. The H5N2 viruses were found to be closely-related to viruses isolated in Sweden and the Netherlands in recent years (Jonassen & Handeland, 2006). In the Netherlands and Sweden, cloacal swabs from ducks, geese, gulls and shorebirds were screened for AIVs. Within the duck, geese and gull species, the prevalence of influenza A virus ranged between 0-60% depending on species, location and season. In shorebirds isolations were rare. All hemagglutinin subtypes except H14 and H15 and all nine neuraminidase subtypes were detected, and sequence and phylogenetic analysis revealed a close relationship between the viruses isolated in the study and all recent HPAI outbreaks in Northern Europe (except Asian HPAI H5N1) (Munster *et al*., 2006). In mainland China in 2004 and 2005, 493 serum samples from 15 migratory species were tested for anti-AIV antibodies. Low-level antibodies against influenza subtypes H2, H9 and H10 were detected in the relict gull, little egret, black-crowned night heron, bar-tailed godwit, whimbrel, and common greenshank, but no viruses were isolated from cloacal and orophyrangeal swabs of live bar-tailed geese (Chen *et al*., 2006). In Russia, 53 strains of influenza viruses were isolated from 1750 cloacal swabs (3% prevalence). Subtypes H3, H4, H5, H9 and H13 were isolated from ducks, terns, gulls, snipes, tree sparrows and muskrats (Lvov *et al*., 2004).

1.1.11 Control of AI

1.1.11.1 Vaccination

Maintaining poultry free from HPAI is essential for the continuation in trade in poultry and poultry products between nations, and erradication by stamping out is still the preferred method for dealing with HPAI. This control strategy is based on the application of sanitary restrictions and culling of all animals that are infected, suspected of being infected, or suspected of being contaminated. Inevitably, this results in mass depopulation, and the financial consequences of such a program are severe. The global poultry industry has undergone substantial changes in the past 20 years, mainly resulting in shorter production cycles and in greater animal densities per territorial unit. Consequently, infectious diseases are significantly more difficult to control, and furthermore, the slaughter and destruction of large numbers of animals is increasingly becoming ethically unacceptible to the general public. Therefore, the OIE has become more flexible in recent years regarding the implementation of control strategies involving vaccination for HPAI outbreaks (Alexander, 1996; Capua & Marangon, 2003; Swayne, 2003). Vaccination may be used solely as a strategy to minimize losses and reduce the incidence of disease, or vaccination may be combined with other strategies for the goal of total eradication.

Experimental and field data show that if birds are sufficiently well immunised against the HA subtype corresponding to that of the challenge virus they will be protected from the worst effects of HPAI and the clinical disease and mortalities associated with LPAI. Vaccination also helps to significantly slow the spread of the infection, and reduces the amount of viruses shed into the environment (Capua & Marangon, 2003; Harder *et al*., 2006; Bublot *et al*., 2006).

The existence of a large number of virus subtypes together with the variation of different strains within a subtype poses a challenge when selecting strains to produce influenza vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly prior concentration. Reverse genetics has enabled the recovery of infectious influenza viruses from plasmid DNAs transfected into tissue culture. This technology permits the construction of high yield 6:2 seed viruses by mixing the 6 plasmid DNAs from a good-growing laboratory strain with the HA and NA DNAs obtained by cloning relevant genes from currently circulating viruses. Furthermore, removal of the basic cleavage peptide of HPAI

strains results in a virus that is attenuated for embryonated eggs, thus allowing high yields to be attained, and viruses that are safe to work with (Palese, 2006).

Whole virus AI vaccines are almost always inactivated because of the reassortment risk associated with live vaccines. Vaccines are prepared from infective allantoic fluid inactivated by betapropiolactone or formalin and emulsified with mineral oil. The inactivated vaccines produced have either been autogenous, i.e. prepared from isolates specifically involved in an epizootic (autogenous vaccines are homologous vaccines), or have been heterologous. Heterologous vaccines use the same HA type as the field virus but contain a heterologous NA. This type of vaccine has the advantage over the homologous vaccine of being distinguished from the field infection, because antibodies produced against the NA can be used as a marker, and this approach is commonly known as the DIVA (Differentiating Infected from Vaccinated Animals) strategy (Capua *et al*, 2002). The internal proteins NS1 and M2 have also been used as markers in a DIVA strategy, as both are abundantly expressed during viral replication in infected cells, illiciting specific antibodies that can be detected, whereas this is not the case with an inactivated, non-replicating vaccine (Lambrecht *et al*., 2006; Dundon *et al*., 2006). Recently vaccines have been developed employing new technologies such as baculovirus derived H5 and H7 haemagglutinins (Crawford *et al*., 1999) fowlpox virus recombinants expressing H7 haemagglutinin (Boyle *et al*., 2000), infectious laryngotracheitis virus (ILTV) expressing H5 or H7 antigens (Luschow *et al*., 2001), recombinant Newcastle disease virus expressing H5 antigens (Römer-Oberdoerfer *et al*., 2006) and DNA vaccines expressing HA antigens (Kodihalli *et al*., 2000). Under field conditions, protection afforded by inactivated vaccines could be undermined by improper vaccination technique, improper storage and handling of vaccines and infections that suppress the immune system of the bird (Swayne, 2003).

1.1.11.2 Chemotherapy

Antiviral drug research shows promise as a broad-spectrum means to control influenza, as an alternative to vaccination. Four different chemotherapeutic substances now exist (Lamb & Krug, 2001; Gubareva *et al*., 2000; Hayden 2001; Roberts, 2001; Flemming, 2001). Amantadine and rimantadine (Lamb & Krug, 2001) function by blocking the M2 ion channel protein of the virus, but viruses become resistant to

amantidine through a single amino acid substitution at positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 protein. Both amantadine and rimantadine may be of substantive benefit in treating influenza A, however side effects on the central nervous system, the liver and kidneys have been reported, and drug-resistant strains have emerged. More useful drugs are zanamivir and osteltamivir which block the action of NA to prevent release of newly formed virus from the infected host cell and its spread within the host (Monto, 2003). However, viruses have been known to become resistant to oseltamivir through a single amino acid substitution at position 119, 152, 274, or 292 in the NA active centre (Gubareva *et al*., 2002). Less is known about the efficacy and safety of the NA inhibitors than the amantadines (Baigent & McCauley, 2003).

1.1.12 The zoonotic potential of avian influenza viruses

1.1.12.1 Historic human influenza epidemics and avian influenza

In humans, influenza is a highly contagious, acute illness for which there are recognisable accounts of epidemics dating back to ancient times, with the earliest documented outbreak of what was probably influenza A occurring in 1173 (Francis, 1953). Seasonal influenza epidemics in man are associated with amino acid changes in the antigenic sites of HA and NA of currently circulating influenza A or B viruses (antigenic drift). The elderly, very young and other immune-compromised groups are particularly susceptible during epidemics, with significant increases in mortalities. Major pandemics are far more serious, but are associated with type A only. They result from introduction of HA and/or NA genes from an animal-derived influenza virus, by reassortment into the genetic background of a currently circulating human virus (antigenic shift). In the 20th century, antigenic shift in human influenza A viruses occurred on four occasions, 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) and 1977 (H1N1), resulting in pandemics. For the worst influenza pandemic, the "Spanish Influenza" of 1918, it has been estimated that between 20 and 40 million people died (Crosby, 1976). Since the entire coding region for the 1918 Spanish influenza strain became available, sequence and phylogenetic analysis suggest that the 1918 Spanish influenza pandemic strain arose from an avian-like influenza virus (Taubenberger & Morens, 2006). Furthermore, reverse genetics was used to regenerate an influenza

virus containing all eight gene segments of the pandemic virus, in order to study the properties associated with its extraordinary virulence (Tumpey *et al*., 2005).

Both human and avian viruses are known to infect pigs readily, therefore it was suggested that pigs acted as "mixing vessels" in which reassortment between human and avian influenza viruses could take place. The reassorted virus therefore contained the necessary genome segments(s) from the virus of human origin to allow replication and spread in the human population, but different haemagglutinin surface glycoproteins, so that the human population could be regarded as immunologically naïve (Scholtissek, *et al*., 1985). This theory was also thought to account for the apparent emergence of pandemics in the 20th century in the Far East where agricultural practices result in high concentrations of people, pigs and waterfowl living in close contact (Shortridge & Stuart-Harris, 1982). Direct transmissions of AIV from animals to humans have occurred, without fatal disease. In 1996 an H7N7 virus was isolated in England from the eye of a woman with conjunctivitis who kept ducks. This virus was shown to be genetically closest in all eight genes to viruses of avian origin (Banks *et al*., 1998). During February of 2003, an outbreak of HPAI H7N7 in chickens in Holland was associated with human conjunctivitis, and caused the death of a veterinarian (Fouchier *et al*., 2004). During the second half of the 1990s, widespread outbreaks in poultry due to the H9N2 subtype were reported in Germany, Italy, Ireland, South Africa, USA, Korea, China, the Middle East, Iran and Pakistan (Banks *et al*., 2000b). In March 1999, two independent isolations of influenza virus subtype H9N2 were made from girls aged one and four who recovered from flu-like illnesses in Hong Kong. Subsequently, five isolations of H9N2 virus from humans on mainland China in August 1998 were reported (Peiris *et al*., 1999a; 1999b).

1.1.12.2 The emergence and spread of Asian HPAI H5N1 (genotype Z)

In May 1997 in Hong Kong, an H5N1 subtype was isolated from a young child who died and by December the same strain had infected 18 people, six of whom died (Shortridge *et al*., 2000). The viruses isolated from the human cases appeared to be identical to viruses first isolated from chickens in Hong Kong in March 1997 following an outbreak of HPAI, and they possessed the typical multi-basic H_0 cleavage sites (Suarez *et al*., 1998). The precursor of this virus was first detected in Guangdong, China, in 1996, when it caused a moderate number of deaths in geese

(Tang *et al*., 1998). This goose virus acquired internal gene segments from influenza viruses later found in quail (A/Quail/HK/G1/97(H9N2)) and the neuraminidase gene segment from a duck virus (A/Teal/HK/W312/97(H6N1)). The newly-assorted virus, A/Goose/Guangdong/96(H5N1), rapidly became widespread in live poultry markets in Hong Kong (de Jongh *et al*., 1997; Sims *et al*., 2003). The HPAI H5N1strain that infected the humans was eradicated from Hong Kong by stamping out, but the goose precursor viruses continued to circulate in geese in southeastern China (Cauthen *et al*., 2000; Webster *et al*., 2002). These strains reassorted with other avian H5N1 viruses that were highly pathogenic in chickens, but not ducks, and again emerged in Hong Kong in 2001 and 2002, and were eradicated on both occasions (Guan *et al*., 2002). Then, in late 2002 a single genotype was responsible for killing most wild, domestic, and exotic waterfowl in two of Hong Kong's nature parks (Guan *et al*., 2004; Sturm-Ramirez *et al*., 2004). This high pathogenicity in ducks was a rare property of AIV, and had not been observed in strains isolated during previous years. An antigenically and molecularly similar virus caused the two confirmed human cases in early 2003 in a Hong Kong family, and this virus was the precursor of the so-called Z genotype that became dominant (Guan *et al*., 2004; Peiris *et al*., 2004). HPAI H5N1 spread in an unprecedented fashion across South East Asia, affecting Vietnam, Thailand, Indonesia, Cambodia, Laos, Korea, Japan, Thailand and later Malaysia, although phylogenetic analysis showed that the viruses that caused the poultry outbreaks in Japan and Korea were of a different lineage, the V genotype (Mase *et al*., 2005; Li *et al*., 2004). After mass die-offs of H5N1-infected bar-headed geese (a migratory species) at Qinghai Lake in western China, an important staging area for migratory waterfowl, the scientific community was greatly concerned that HPAI H5N1 could be spread from southeastern Asia to other regions through migration movements (Webster *et al*., 2006). Worst fears were realised when Russia confirmed H5N1 in poultry in western Siberia. The outbreak spread to affect six administrative regions in Siberia, and dead migratory birds were reported in the vicinity of outbreaks. By August 2005 the infection had spread to neighboring Khazakstan's poultry flocks. Later that month, Mongolia confirmed the deaths of migratory birds at two lakes, caused by HPAI H5N1. By October 2005, H5N1 had caused outbreaks in Turkish turkey flocks, before breaking out in poultry in Romania. By April 2006, the infection had spread to poultry flocks, in order of appearance, in the Ukraine, Cyprus, Iraq, Nigeria, Egypt, India, France, Niger, Bosnia, Azerbaijan, Albania, Cameroon, Myanmar, Afghanistan, Israel, Pakistan, Jordan, Burkina Faso, Germany, Sudan and the Ivory Coast. H5N1 has also been found in dead wild waterfowl in Austria,

Bulgaria, Czech Republic, Denmark, Greece, Hungary, Iran, Italy, Kuwait, Poland, Serbia and Montenegro, Croatia, Slovakia, Slovenia, Sweden, Switzerland and the United Kingdom (www.oie.int.org). The most commonly affected wild birds in Europe have been swans, representing 62.8% of the total, followed by ducks (16.3%), geese (4.5 percent), birds of prey (3.9%) and others (13%) (ProMED, 31 May 2006). Research on viruses isolated from dead birds at Qinghai Lake demonstrated transmission of the virus among migratory geese and confirmed that the virus was indeed carried out of south east Asia by migratory birds (Chen *et al*., 2005). The spread of H5N1 from Eastern Europe to the rest of Europe has been attributed to unusual migration patterns of swans, after a particularly cold spell froze over wintering grounds around the Black Sea region (Brown *et al*., 2006). In contrast, the spread of Asian HPAI H5N1 into and within Africa is thought to be via the importation or illegal movement of infected poultry or poultry products from infected countries in the Middle East/Eastern Europe (FO Fasina, personal communication), rather than via migratory bird movements.

Since the emergence of HPAI H5N1 in 2003 and up to the end of November 2006, 258 human cases had been reported, resulting in 154 human deaths. The human fatalities have occurred in Azerbaijan, Cambodia, China, Djubouti, Egypt, Indonesia, Iraq, Thailand, Turkey and Vietnam (WHO). Hundreds of millions of poultry have been culled since the start of the outbreak in 2003. Most human cases have thought to have arisen via direct contact with the excretions of infected or dead poultry (eating of raw meat, drinking of duck blood, keeping chickens in the house etc). However, increasing reports of clusters of human infections (for example, health workers who treat infected patients, or family clusters) suggest that the extent of person-to-person transmission of HPAI H5N1 has been underestimated (WHO). The evolution of HPAI H5N1 viruses in South East Asia since 1997 has been associated with increasing virulence and an expanding host range. These viruses acquired the unprecedented capability to directly infect humans, to cause neurotropic disease and high mortalities in waterfowl, to be transmitted among felid species, including domestic cats (Kuiken *et al*., 2004), and to cause neurotropic disease and death in ferrets and mice (Govorkova *et al*., 2005). These incremental changes intensified concerns over the pandemic potential of this H5N1 virus. For the human population as a whole, the main danger appears to be if people infected with an avian virus are infected simultaneously with a human influenza virus. In such circumstances reassortment could occur with the potential emergence of a virus fully capable of spread in the

human population, but with an HA for which the human population was immunologically naive. Presumably this represents a very rare coincidence, but one which could result in a true influenza pandemic (Webster *et al*., 2006).

1.1.13 History of avian influenza in South Africa

The first report of avian influenza in South Africa was also the first international report of avian influenza occurring in wild birds. 1300 common terns died along the Western Cape coast between Port Elizabeth and Lamberts Bay in late April 1961. The orthomyxovirus that was isolated from the dead birds was initially named "Tern virus", and later determined to be an HPAI H5N3 strain (Becker, 1966). For thirty years thereafter no isolations of AIV were made in South Africa, but in the early 1980s ostrich farmers in the Oudtshoorn area first observed a syndrome, which they described as "green urine" with respiratory signs (Allwright *et al*., 1993). The green urine syndrome appeared to be a seasonal occurrence, peaking in the autumn and winter months, but was also associated with periods of drought. Then, in 1991, an LPAI H7N1 virus was isolated during an outbreak in young ostriches with symptoms of green urine in Oudtshoorn. The LPAI H7N1 virus had a low pathogenicity for chickens, and the increased mortalities in ostriches were ascribed to factors such as concomittent infections with other pathogens, high population densities, inadequate ventilation and bad hygiene resulting from poor management practises (Allwright *et al*., 1993). Since then, LPAI has been periodically isolated from the Western Cape ostriches. In 1994 an LPAI H5N9 virus was recovered, in 1995 an H9N2 virus, in 1998 an H6N8 virus, and in 2001 an H10N1 virus (Olivier, 2006). Furthermore, an H10N9 virus was isolated from wild birds in the Oudtshoorn region in 1998 (Pfitzer *et al*., 2000). Some literature cites the isolation of an LPAI H5N2 virus from ostriches in Zimbabwe in 1995 and 1996 (Pfitzer *et al*., 2000; Olivier 2006), however this was later determined to be a laboratory contamination (R Horner, personal communication). AI viruses isolated from 2001 onwards are the focus of this study.

1.2 NEWCASTLE DISEASE

1.2.1 Introduction

The earliest description of a poultry disease with symptoms that closely resembled Newcastle disease (ND) originated from an island of Java in 1926 (Kranevald, 1926), but ND may have been present in Korea since as early as 1924 (Konno *et al*., 1929; Alexander, 1997). Doyle (1927) was the first to identify a filterable aetiological agent as the cause of disease in a poultry flock near the town Newcastle on Tyne (UK), and named it accordingly. In the USA, a similar disease called pneumoencephalitis with notable respiratory and neurological symptoms was later discovered to be Newcastle disease. ND has also been known as fowl pest, pseudofowl pest and Ranikhet disease (Lancaster & Alexander, 1975) and like notifiable AIV strains, is notifiable to the OIE.

1.2.2 Aetiology

The etiological agent of Newcastle disease, Newcastle disease virus (NDV), is classified in the *Avulavirus* genus within the family *Paramyxoviridae* in the order *Mononegavirales* (Mayo *et al*., 2002a; 2002b). NDV is also referred to as avian paramyxovirus-1 (APMV-1), one of nine identified serotypes of paramyxoviruses known to infect birds. There is some cross-reaction, albeit low, between the APMV serotypes, especially between APMV-1 and APMV-3 (Box *et al*., 1988). Strains of NDV have been distinguished on the basis of symptoms produced in infected chickens. The following five groups or pathotypes were defined:

Viscerotropic velogenic virus	high mortality with hemorrhagic lesions in the intestines
Neurotropic velogenic virus	high mortality following respiratory and nervous signs, gut lesions usually absent
Mesogenic	moderate (50%) to low mortality with respiratory signs and occasional nervous signs
Lentogenic	no mortality with only mild respiratory signs
Asymptomatic enteric	no mortality with subclinical infection of the intestine, where replication primarily occurs

1.2.3 Disease

The incubation period for NDV infection is four to six days. The symptoms seen in infected birds vary widely and are dependent on factors such as the pathogenicity of the virus, host species, age of the host, infection with other organisms, environmental stress, and immune status. Symptoms may include respiratory and/or nervous signs such as gasping and coughing, drooping wings, dragging legs, twisting of the head and neck (torticollis), circling, depression, inappetence, and complete paralysis. Egg production partially or totally ceases. Eggs are often misshapen, rough-shelled, thin-shelled and contain watery albumen. Birds develop greenish watery diarrhoea, and there is some swelling of the tissues around the eyes and in the neck. Virulent panzootic NDVs usually cause haemorrhagic lesions of the intestinal tract, although there are no pathognomonic gross lesions. Lesions are sometimes seen in other tissues and organs, depending on the tropism of the particular strain. Differential diagnoses include fowl cholera, avian influenza, laryngotracheitis, fowl pox (diphtheritic form), psittacosis (chlamydiosis in psittacine birds), mycoplasmosis, infectious bronchitis, Pacheco's parrot disease (psittacine birds) and also management errors such as deprivation of water, air or feed.

Virulence differences between NDV strains are determined by chicken and chicken embryo inoculation. Four pathogenicity tests are utilized for this differentiation (Alexander, 1997; Pearson *et al*., 1975):

- Intracerebral pathogenicity index (ICPI) test in 1-day-old chicks from specific-pathogen-free (SPF) parents: birds are inoculated intracerebrally and then examined every 24h for eight days. At each observation, the birds are scored: (0) if normal, (1) if sick and (2) if dead. The ICPI is the mean score per bird per observation over the eight-day period. According to the OIE, an isolate with an ICPI equal to or greater than 0.7, or having a dibasic cleavage motif at the F_0 protein cleavage site (see 1.2.7) will be classified as a virulent virus, and its presence is notifiable to the OIE (OIE Manual, 2004).
- Intravenous pathogencity index (IVPI) in 6-week-old SPF chickens.
- Intracloacal inoculation test in 6-to-8-week-old chickens, and
- Mean death time (MDT) in 9-to-10-day-old embryonating eggs. Viruses are characterised as low (lentogens), moderate (mesogens) or high virulence (velogens) based on clinical signs and mortality in chickens along with time to

embryo death postinoculation. Strains are considered to be velogenic if they take <60 hours; mesogenic taking between 60-90 hours; and lentogenic if > 90 hours to kill.

1.2.4 Host Range

ND infections have been established in at least 241 species of birds representing 27 of the 50 bird orders. Domestic fowl, turkeys, pheasants, pigeons, quail and guinea fowl are highly susceptible to infection. Ostriches are less susceptible (Verwoerd, 1995b). Ducks and geese are susceptible but severe disease is rare. Psittacines (parrots) are highly susceptible and can excrete virus for long periods (Kaleta and Baldauf 1988), and many species of wild birds are also susceptible. Newcastle disease viruses have also been reported to infect animals other than birds, ranging from reptiles to humans (Lancaster, 1966).

1.2.5 Morphology and genome structure

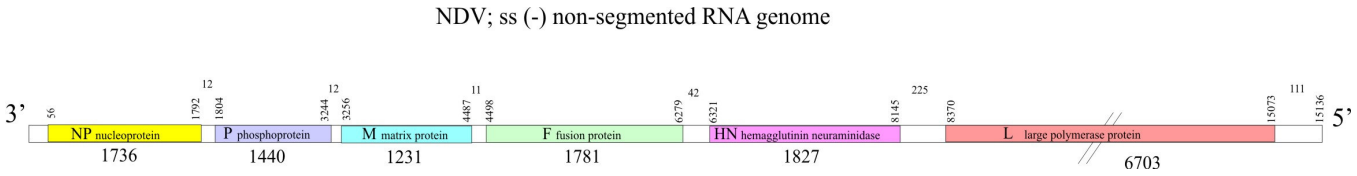


Figure 1.3. Genomic organization of Newcastle disease virus, with relative gene sizes indicated below.

Paramyxovirus virions are pleiomorphic, enveloped particles. Under the electron microscope (EM), NDV appears very similar in size and appearance to avian influenza virus. The virions vary considerably in size; most are 150-350 nm in diameter and spherical in shape, but larger particles (500-600 nm) as well as long filamentous forms are occasionally observed. The glycoproteins are inserted into the lipid envelope (which is derived from the host cell where the virion is assembled), appearing as spikes that protrude from the envelope surface (Bang, 1948; Cunha *et al*., 1947; Elford *et al*., 1947; Horne & Waterson, 1960; Horne *et al*., 1960; Hosaka *et al*., 1966).

NDV has a non-segmented, negative-sense, single stranded RNA genome of 15,186, 15,192 or 15,198 nucleotides (Phillips *et al*., 1998; Huang *et al*., 2004; Czeglédi *et al*., 2006). The genome encodes six proteins, viz. the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN) and the large polymerase protein (L), in the order 3'-NP-P-M-F-HN-L-5' (Fig. 1.3). Apart from these six proteins, NDV extends its genome capacity by the use of two overlapping ORFs in the P gene, V and W. V and W are transcribed as alternative mRNAs that are generated by RNA editing (Steward *et al*., 1993). In NDV, insertion of one nontemplate G residue gives rise to a V-encoding mRNA, while insertion of two nontemplate G residues generates a W-encoding mRNA. Analysis of mRNAs produced from the P gene showed that 68% are P-encoding mRNA, 29% are V-encoding mRNA, and 2% are W-encoding mRNA (Mebatsion *et al*., 2001).

The 3' termini of all non-segmented negative strand RNA virus genomes and antigenomes (including NDV) contain cis-acting sequences (promotors) that allow the viral RNA-dependent RNA polymerase to initiate RNA synthesis (Kolakofsky *et al*., 2005). In paramyxoviruses, the 3'-terminal 12 nucleotides are identical. The 3' end of the genome itself is not an essential part of this replication promotor, but a second promoter element lies downstream of the 3' terminal element, and spacing between the two elements is critical for promoter function: genome analogues of members of the paramyxovirus genus replicate efficiently only if the total genome length is a multiple of six, a requirement which is called the 'rule of six' (Calain & Roux, 1993; reviewed by Kolakofsky *et al*., 1998). At each turn, the helix contains 13 nucleocapsid subunits, each associated with precisely six nucleotides. The terminal and internal promoter elements are thereby aligned on the same face of the helix, thus both promoter elements can simultaneously interact with RNA-dependent RNA polymerase (vRNAP) to initiate RNA synthesis at the 3' end of the nucleocapsid (Lamb & Kolakofsky, 2001). All NDV genomes analysed to date are subject to the rule of six (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Phillips *et al*., 1998).

The ND virion can be viewed as consisting of two structural units: the nucleocapsid (also known as the ribonucleoprotein), which completely covers the RNA genome, and the envelope with its surface projections. The nucleocapsidprotein (NP) is the structural subunit of the nucleocapsid and serves several functions in virus replication, including encapsidation of the genome RNA into an RNase–resistant nucleocapsid,

association with the P-L polymerase during transcription and replication, and interaction with the M protein during particle assembly. When nucleocapsids of the *Paramyxovirinae* are viewed under an EM, successive turns of the helical assembly packed closely together are observed, creating the herringbone pattern that is characteristic of this family (Kolakofsky *et al*., 2005). From the number of turns in the helix and the number of NP subunits per turn, it is calculated that a single nucleocapsid contains 2,200-2,600 subunit molecules (Finch, 1970). Two other proteins, the phosphoprotein (P) and the large (L) protein, are associated with the nucleocapsids, but are recognisable only by immune electron microscopy (Portner & Murti, 1986).

The phosphoprotein (P) plays the central role in viral replication and transcription and has multiple functions. It has been shown that the P protein stabilizes the L protein in the P:L complex which acts as the viral RNA-dependent RNA polymerase (Smallwood *et al*., 1994). A P tetramer (Tabouriech *et al*., 2000) mediates the interaction between the L protein and the N:RNA template (Curran, 1998). The P protein also acts as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by the NP protein (Errington & Emmerson, 1997). The V open reading frame (ORF) is found in the middle of the P gene, it is cysteine-rich and is translated from an mRNA with a single G insertion. The C-terminal contains seven conserved cystein residues and this domain binds two atoms of Zn^{2-}. The V domains of some paramyxoviruses specifically interact with the large subunit of the cellular damage-specific DNA-binding protein (Lin *et al*., 1998), but the consequence of this interaction and the function of V protein cysteine-rich domain remain to be elucidated (Lamb & Kolakofsky, 2001).

The large (L) protein, with a molecular weight of approximately 250 kDa, is the least abundant of the structural proteins (about 50 copies per virion). L is believed to be responsible for all of the catalytic activities of the viral polymerase associated with transcription, including initiation and elongation of transcripts, as well as co-transcriptional modification of RNAs such as capping, methylation, and polyadenylation. Polyadenylation is thought to result from polymerase stuttering on a short stretch of uridylate (U) residues, but the capping step requires both guanylyl and methyl transferase activities, both of which are thought to be provided by the L protein. The function of the RNA polymerase is dictated by the start, stop and restart

template's *cis* signals at the borders of each transcription unit (Bannerjee 1987; Smallwood *et al*., 1999).

1.2.6 The infection cycle

1.2.6.1 Adsorption and penetration

Virion- and infected cell surfaces are characterised by two types of glycoproteins, which mediate early interactions with the target cell: the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins (Scheid & Choppin, 1974). HN proteins are type II integral membrane proteins. Type II proteins possess a sequence that serves both as a signal sequence and as a membrane anchor and are positioned with the amino terminus in the cytoplasm and the carboxyl terminus in the ectodomain. HNs span the membrane once and protrudes from the surfaces of virions and infected cells as tetrameric spikes. The orientation of HN in the membrane is analogous to that of the influenza virus NA. The ectodomain of the HN spike consists of a stalk that supports a terminal globular head in which receptor recognition, NA activity, and all of the known antigenic residues reside (Mirza *et al*., 1993; Thompson *et al*., 1988) (this is in contrast to avian influenza virus in which the receptor-recognition and NA functions reside on independent spike structures). For some strains of NDV, HN is synthesized as a biologically inactive precursor (H_0), and ~90 residues are removed from the C-terminus to activate the molecule (Nagai & Klenk, 1977; Nagai *et al*., 1976a). The HN glycoprotein mediates attachment to sialic-acid containing receptor(s) on the target cell surfaces. NDV recognises both N-acetyl- and N-glycolylneuraminic acid residues (Suzuki *et al*., 1985). The virus, however, elutes rapidly by the action of the neuraminidase even at low temperatures (4°C), and thus the pattern of hemagglutination, which is caused by the virus binding to the receptor of red blood cells, is unstable. Thermostability of the HN glycoprotein is sometimes used as a pathogenicity marker in epidemiological studies (Hanson & Spalatin, 1978). The thermostability of HN is assessed at 56ºC, and hemagglutinating activity is measured at time intervals: the longer the HN protein retains HA activity, the more thermostable it is. Generally, lentogenic field isolates are thermolabile, whereas velogenic viruses have thermostable hemagglutinins (Werner *et al*., 1999). There are several exceptions, however, as thermostable lentogenic field strains have been isolated, and thermostable progeny of vaccine strains have been selected for in the

laboratory (Kim & Spradbrow, 1978; Rosenberger *et al*., 1975; King, 2001). The molecular determinant(s) of HN thermostability have not yet been elucidated.

The complete mechanism of NDV-induced membrane fusion remains unknown. As with many other paramyxoviruses, NDV requires type-specific HN-F interactions that must be present in the same bilayer to induce fusion (Hu *et al*., 1992; Lamb & Kolakofsky; 2001). Since the F proteins of most paramyxoviruses cannot mediate membrane fusion alone and they require the coexpression of the homologues HN protein (Lamb & Kolakofsky, 2001). The fusion-complementing domain in the HN protein has been shown to reside in the HN stalk region, and residues 89, 90 and 94 are likely candidates (Melanson & Iorio, 2004). It has been proposed that the interaction of HN with the cellular receptor induces conformational changes in the HN protein that activates the F protein (Lamb & Kolakofsky; 2001), although the nature of such changes is obscure. The second step of infection is virus penetration, mediated by the fusion of the viral envelope with the lipid bilayer of target cells and resulting in the delivery of the nucleocapsid into cells. The F protein is involved in this process.

The F proteins are type I integral membrane proteins, i.e. the amino-terminal signal sequences are positioned so that the carboxyl terminus is located in the cytoplasm and the amino terminus is in the ectodomain (Chen *et al*., 2001; Russell *et al*., 1994). However, recent evidence suggests that a second topological form may be present and that both exist as a mixed population on the virion surface (McGinnes *et al*., 2003). The NDV F protein is synthesised as a precursor, F_0, the activation of which requires proteolytic cleavage into the disulphide-linked polypeptides F_1 and F_2. This cleavage exposes the hydrophobic amino terminus of F_1, called the fusion peptide, which is inserted into the target cell membrane, thereby disordering the lipid bilayer and preparing it for merger of the membranes (Lamb & Kolakofsky, 2001). Unlike the orthomyxoviruses (AIV), paramyxovirus fusion occurs at neutral pH. Thus, there is no requirement for a low pH-mediated conformational change of their glycoproteins to induce fusion, and paramyxoviruses are not internalised into endosomes. Instead, vRNP complexes are released directly into the cytoplasm, and the cytoplasm is the site for viral transcription and genome replication, as well as viral protein synthesis and processing.

A hallmark cytopathic effect of cell infection by paramyxoviruses occurs later in infection: when proteolytically-cleaved F proteins are expressed at the plasma membrane of infected cells, neighboring cells fuse to form syncytia (giant cell formation), a cytopathic effect that can lead to tissue necrosis *in vivo* and might also be a mechanism of virus spread (Choppin & Scheid, 1980).

1.2.6.2 Transcription and replication

All events of transcription, replication and assembly of paramyxoviruses occur in the cytoplasm. Paramyxovirus ribonucleocapsids, the active template for transcription and replication of the viral genome, function without disassembling (Lamb & Kolakofsky, 2001). Intracellular replication begins with the viral RNA-dependent RNA polymerase (RNAP) (minimally a homotetramer of P and a single L protein) transcribing the N-encapsidated genome RNA (N:RNA) into 5' capped and 3' polyadenylated mRNAs. The viral RNAP begins all RNA synthesis at the 3' end of the genome, and it transcribes the genes into mRNAs in a sequential (and polar) manner by terminating and reinitiating at each of the gene junctions. The junctions consist of a gene-end sequence, at which polyadenylation occurs by the reiterative copying of four to seven U's (followed by release of the mRNA), a short non-transcribed intergenic region, and a gene-start sequence that specifies capping as well as mRNA initiation. The RNAP occasionally fails to reinitiate the downstream RNA at each junction, leading to the loss of transcription of further-downstream sequences. Therefore, paramyxovirus genomes exhibit transcriptional polarity, i.e. transcripts of genes at the 3' end of the virus genome are more abundant than those of genes at the 5' end of the genome, permitting the regulation of the relative amounts of structural (3' genes) and non-structural (5' genes) proteins produced (Lamb & Kolakofsky, 2001).

Later in infection, when viral proteins accumulate, RNA synthesis switches from transcription to replication. This switch is mediated by the binding of NP to the nascent leader RNA. Newly-synthesized NP molecules are recruited by the polymerase complex and are sequentially added to the growing RNA chain. As a result of binding of NP, the polymerase complex ignores the transcription start and stop signals, resulting in the synthesis of full-length antigenomic RNA. The antigenomic RNA, in turn, serves as template for the synthesis of full-length genomic

RNA. Newly synthesized RNP complexes are assembled and transported with viral structural proteins to the plasma membrane (Lamb and Kolakofsky, 2001).

1.2.6.3 Virus assembly and release

Assembly of the virus occurs at the cell surface. The viral intergral membrane proteins are synthesized in the ER and undergo a step-wise conformational maturation before transport through the secretory pathway. Folding and conformational maturation of glycoproteins are not spontaneous events that occur in the cell but instead are assisted by numerous folding enzymes and molecular chaperones. Only correctly folded and assembled proteins are generally transported out of the ER. In the Golgi apparatus, the carbohydrate chains may be modified from the high-mannose to the complex form, and for those F_0 proteins with multiple basic cleavage sites, cleavage occurs in the trans-Golgi network. Finally, the glycoproteins are transported to the plasma membrane (Lamb & Kolakofsky, 2001).

The M protein is the most abundant protein in the virion, and is peripherally associated with the plasma membrane that already contains the glycoproteins (Nagai *et al*., 1976; 1989), although it does not appear to be an intergral membrane protein. The M protein interacts with the plasma membrane, the cytoplasmic domain of a viral glycoprotein(s), and the nucleocapsid (Peeples, 1991). Thus, its role appears to be the central organizer of virus assembly and budding, althought the exact mechanism by which the virus particle is assembled at the plasma membrane is unknown. The protein-protein interactions involved in assembling a virion presumably must be specific, as cellular membrane proteins are largely excluded from the virions. The virus is released from the cell by budding, and the neuraminidase activity of the HN protein hydrolyzes the surface sialic acid residues, preventing virus self-aggregation and enhancing virus spread (Lamb & Kolakofsky 2001).

1.2.7 F_0, the primary molecular determinant of virulence

In order for the NDV particle to be infectious, the F_0 precursor protein must be cleaved into disulphide-linked F_2 and F_1 polypeptides, so that the fusion peptide on F_1 is exposed. This post translation cleavage is mediated by host cell proteases, and the

cellular site of this cleavage depends upon the sequence at the cleavage site, as for *Orthomyxoviridae*. Some F_0 proteins have a furin recognition site (R-X-K/R-R) and are consequently cleaved in the trans-Golgi membranes by intracellular furin-like enzymes. In this case, the majority of F proteins delivered to the plasma membrane are potentially active F proteins. Thus, cleavage of F_0 requires a double pair of basic amino acids (e.g. Arg and Lys) at residues 112 and 113, and 115 and 116, plus a phenylalanine at residue 117 (Collins *et al*., 1993). Virulent viruses can therefore replicate in a wide range of tissues and organs, resulting in a fatal systemic infection (Rott, 1979). In contrast, F proteins of avirulent viruses have single basic residues at the cleavage site and are delivered to the plasma membrane in an inactive, uncleaved form. In order to direct fusion, these F proteins must be cleaved by an extracellular host cell enzyme (trypsin-like, which recognize a single Arg residue), usually found exclusively in the respiratory and intestinal tracts, thereby restricting replication to these tissues. As with AIV, avirulent NDVs require the addition of exogenous trypsin to facilitiate replication in cell culture (Nagai *et al*., 1976a).

1.2.8 Other virulence determinants

The F_0 protein cleavage site is the primary determinant of virulence, however, comparison of the amino acid sequence of the F_0 protein cleavage site with the intracerebral pathogenicity indices (ICPI) for several NDV viruses has shown that significant differences in virulence can exist between strains with the same velogenic consensus sequence. For instance, virulent field strain Herts/33 with the cleavage site ^{112}RRQRRF^{117} has an ICPI value of 1.88 whereas strains Beaudette C/45 and Komarov with the same sequence showed ICPI values between 1.4 and 1.5 (Collins *et al*., 1993). In addition, NDV isolates recovered from many different bird species with the same velogenic F protein cleavage site were found to exhibit different virulence levels in chickens. These NDV isolates were either mesogenic strains that produced mild pathogenesis or velogenic strains causing severe pathogenesis and death (de Leeuw *et al.*, 2005). Moreover, using reverse genetics Peeters and colleagues (1999) established an infectious clone of the non-virulent La Sota strain (NDFL), and demonstrated that modification of the cleavage site ^{112}GRQGRL^{117} to ^{112}RRQRRF^{117} (NDFLtag) increased the ICPI from 0.00 to 1.28. Although NDFLtag contains exactly the same F_0 protein cleavage site as Herts/33, the ICPI did not reach the level of 1.88, indicating that other factors contribute to virulence. The most likely candidate would

be the HN protein, since it is also involved in viral entry (de Leeuw *et al*., 2005). The V protein is another candidate since it is an interferon antagonist (Park *et al*., 2003; Huang *et al*., 2003) and therefore likely to be involved in pathogenesis and host-range restriction (Mebatsion *et al*., 2001; Park *et al*., 2003).

1.2.9 Zoonotic potential of NDV and the use in cancer treatment for humans

Human infections with NDV have usually resulted from direct contact with the virus, infected birds or carcasses of diseased birds. Human-to-human spread has never been reported, although it is theoretically possible. The most frequently reported clinical signs are conjunctivitis and flu-like symptoms, but infections have never been lethal. Most cases have been reported by laboratory workers, veterinarians, workers in broiler processing plants, and vaccination crews, especially when live vaccines are administered as aerosols or fine dust (Alexander, 2000b).

NDV is one of at least a dozen virus types that are being investigated as alternative therapies for people in whom conventional cancer therapy has failed to stabilize or shrink existing tumors. The observation that tumor regressions occurred during natural virus infections or immunizations were the basis of preliminary studies (Csatary *et al*., 1993; Kirn *et al*., 2001). A particular case history involved a poultry farmer whose metastatic gastric carcinoma underwent regression, coinciding with an outbreak of ND in his chickens. This led to the application of an attenuated NDV vaccine for treatment of a few terminal cancer patients, with favorable results (Csatary *et al*., 1993). Various NDV strains have been used in cancer treatment, because antitumor responses have differed among the isolates used. The mechanism for NDV oncolytic activity is unknown, but NDV has been shown to be a potent inducer of tumor necrosis factor α (TNFα) production by human peripheral blood mononuclear cells, and enhances sensitivity of the tumor cells to the cytolytic effect of TNFα (Lorence *et al*., 1988). Furthermore, NDV may provide anticancer therapy through pleiotrophic modification of the patient's own immune response against the tumor, rather than direct oncolytic activity (Schirrmacher *et al*., 1999).

1.2.10 Genotypes and global epidemiology

Binding pattern analysis of a panel of monoclonal antibodies (mAbs) has been used to separate NDV isolates into specific antigenic groupings (Alexander *et al*., 1997). This system has proved useful for rapidly sorting viruses into broad groups, and the mAb groups described by Alexander and colleagues (1997) have been used as a descriptive basis (Aldous *et al*., 2003). A shortcoming of mAb binding tests is that they fail to differentiate viruses that are antigenically similar, although not genetically identical (Alexander *et al*., 1999; Aldous *et al*., 2003). Restriction enzyme analysis defined six lineages (I to VI) of NDV (Ballagi-Pordany *et al*., 1996) and nucleotide sequencing confirmed these six groups, adding two more lineages, VII and VIII. Several sub-lineages have been described within these eight lineages (Lomniczi *et al*., 1998; Seal *et al*., 1998; Herczeg *et al*., 1999; Yang *et al*., 1999; Herczeg *et al*., 2001; Ke *et al*., 2001; Yu *et al*., 2001). Although considerable genetic diversity has been detected in NDV, viruses sharing temporal, geographic, antigenic or epidemiological parameters tend to fall into specific lineages or clades and this has proven valuable in assessing both the global epidemiology and local spread of ND (Alexander, 2000b, Aldous *et al*., 2003). Thus, three main panzootics of ND have been defined (Alexander, 1997; Lomniczi *et al*., 1998; Sakaguchi *et al*., 1989; Toyoda *et al*., 1989; Ballagi-Pordany *et al*., 1996; Lomniczi *et al*., 1998). Recently, the genetic groupings of ND viruses were revised, and six lineages (1 to 6) were described (Aldous *et al*., 2003):

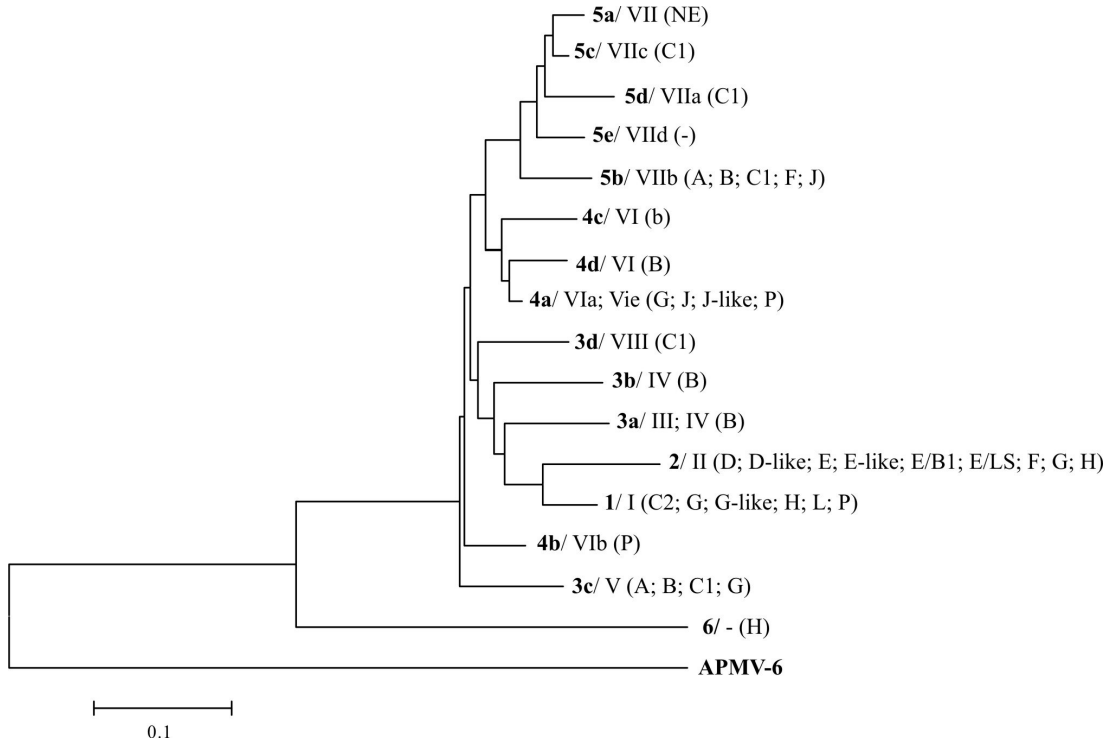


Figure 1.4. Classification of NDV lineages (adapted from Aldous *et al*., 2003). The original genogroups (Lomniczi *et al*., 1998; Alexander *et al*., 1999; Herczeg *et al*., 1999; Yang *et al*., 1999; Herczeg *et al*., 2001, Ke *et al*., 2001; Yu *et al*., 2001, Liang *et al*., 2002) are indicated in Roman numerals, and the corresponding monoclonal antibody-binding groups (Alexander *et al*., 1997) in brackets thereafter. The Neighbour-joining tree is based on a 374 nt region of the F protein and is rooted with avian paramyxovirus 6 (APMV-6)

1.2.10.1 Lineage 1

Lineage 1 was isolated worldwide from 1967 to 2000, the main pathotypes are assymptomatic enteric, but some velogenic viruses are included. The main hosts are ducks and chickens. In recent years, Queensland/V4 and Ulster2C/67 derivatives have been used as live vaccines in many countries. These viruses, with few exceptions, usually cause little or no disease in chickens, replicate primarily in the gut, and are often isolated from feral waterfowl throughout the world (Aldous *et al*., 2003). Two isolates in this group are virulent, apparently derived from an avirulent precursor strain (Gould *et al*., 2001).

1.2.10.2 Lineage 2

Lineage 2 (II) was isolated worldwide from chickens and ostriches from 1945 to 2000, and consists of mixed pathotypes. This lineage contains viruses that had origins in North America, including the virulent Texas GB/48 and the avirulent B1/47 and La Sota/46 strains. B1/47, LaSota and to a lesser extent strain F are widely used as live vaccines throughout the world. Komarov and Roakin, both mesogenic strains, are also used as vaccines in some parts of the world where ND is endemic (Aldous *et al*., 2003).

1.2.10.3 Lineage 3

Sublineage 3a (III; IV) viruses were isolated from 1932 to 1999 in Asia, Australasia and Europe. They are velogenic viruses, isolated mainly from chickens. These include the 'ancient' isolates from Australia (1932) and Japan (1951, Miyadera/51), the highly virulent UK isolate from a field pheasant (1962), and the mesogenic vaccine strain Mukteswar used widely in south east Asia. Sublineage 3b (IV) was isolated from 1933 to 1989 in Asia, Africa and Europe and these viruses are velogenic in quail and chicken, their main hosts. This sublineage includes the Herts/33 virus, one of several reference strains. Sublineage 3c (V) velogenic viruses were isolated from 1970 to 1994 from Africa, America and Europe. Their hosts are chickens, pheasants, turkeys and cormorants. Sublineage 3c is largely composed of isolates derived from or considered to be from the 'second' NDV panzootic in the 1970s, which was influenced by trade in exotic birds (Alexander, 2001). Sublineage 3d (VIII) is composed of velogenic viruses isolated from 1965 to 1994 in South Africa, Asia and Europe from chickens and turkeys (Aldous *et al*., 2003).

1.2.10.4 Lineage 4

Sublineage 4a (VIa; VIe) viruses are velogenic, isolated from 1968 to 1996 in Africa, Europe and the Middle East from falcons and chickens. Sublineage 4b's (VIb) velogenic viruses were isolated from 1984 to 2000 from European and Middle Eastern pigeons, and this sublineage is comprised solely of viruses associated with the ongoing panzootic in pigeons. Sublineage 4c contains velogenic viruses isolated from 1989 to 1999 in Europe and the Middle East from chickens and falcons, whereas

sublineage 4d comprises velogenic viruses isolated from 1989 to 1999 in Europe from chickens, pigeons and ostriches (Aldous *et al*., 2003).

1.2.10.5 Lineage 5

Lineage 5 consists exclusively of velogenic viruses. Sublineage 5a (VII) viruses were isolated in Europe from 1988 to 1996 from chickens and pheasants. Lineage 5b (VIIb) viruses were isolated from 1982 to 2000 from Africa, Asia and Europe from chickens, pheasants, falcons and turkeys. Lineage 5c (VIIc) viruses were isolated from 1984 to 1999 from Asia and Europe. Sublineage 5d (VIId) viruses have been isolated since 1997 from Asia and the Middle East from chickens and ostriches, and sublineage 5e (VIIe) viruses were isolated in Taiwan from 1994 to 1997 from chickens and finches (Aldous *et al*., 2003).

1.2.10.6 Lineage 6

Lineage 6 viruses were isolated worldwide from 1977 to 1998 from chickens and ducks. The majority are lentogenic, but the group does include some velogenic strains. Although their mAb binding pattern is unusual, they react readily with polyclonal sera to NDV strains and share neutralising epitopes on the fusion protein with vaccinal NDVs (Collins *et al*., 1993; Alexander *et al*., 1997). All except one isolate are avirulent. On average, lineage 6 viruses have 50% sequence divergence from other isolates, and with the high degree of genetic heterogeneity, it has been suggested that this group of viruses should be regarded as an APMV subtype distinct from APMV-1 (ie not NDVs). However, the presence of a virulent virus in lineage 6 complicates control and statutory regulations, therefore the *status quo* remains (Aldous *et al*., 2003).

1.2.11 Vaccination and control

There is no treatment for Newcastle disease, therefore a stamping out policy (based on slaughter of infected and potentially infected birds, quarantine procedures, cleaning and disinfection) or vaccination, or a combination may be used to control ND, depending on circumstances. In emergency situations in previously ND-free countries, eradication by stamping out is usually the preferred option. New Zealand,

Papua New Guinea, Fiji and a number of Pacific island countries have non-pathogenic ND strains, but are free of pathogenic strains. Most other countries have experienced outbreaks of virulent strains, and many countries, including those of Africa, Asia, Central and South America are regarded as NDV enzootic (Copland 1987; Spradbrow 1988; Rweyemamu *et al*., 1991). In countries where virulent ND is considered to be enzootic, vaccination is routinely practised. Types of vaccines and vaccination schedules used vary depending on the potential threat, virulence of the field challenge virus, type of production, and production schedules. Both live attenuated and inactivated whole-virus vaccines have been developed, as well as recombinant vaccines. Historically, the first attenuated NDV strain, Hertfordshire (H), was obtained by serial passage in eggs of a virulent field virus isolated in England in 1933. Later, this virulent strain was referred to as Herts'33 and became a standard challenge and reference virus (OIE, 1996).

1.2.11.1 Live attenuated vaccines: Lentogenic strains

The live vaccines used to control NDV around the world are, with very few exceptions, of the lentogenic type and include the strains La Sota/46 (also known as La Sota), B1/47 (also known as B1 or Hitchner/B1), F (Asplin), V4, Ulster and VG/GA. B1/47 and La Sota/46, isolated and developed in the 1940s in the USA (Hitchner & Johnson, 1948; Goldhaft, 1979), are the most commonly used lentogenic live ND vaccines today. Both replicate in the respiratory and alimentary tracts with induction of local as well as humoral immunity. Generally, La Sota vaccines are more virulent and induce a stronger immune response than B1 vaccines (Glisson & Kleven, 1993), but both can be administered in drinking water or in spray, sometimes via the nares or eye. Healthy chicks are vaccinated as early as day 1-4 of life, however if vaccination is delayed until the second or third week, interference with an active immune response by maternal antibody can be avoided.

In 1966, a mild enteric lentogenic strain of NDV was isolated from a healthy chicken in Queensland (Simmons, 1967) and soon after it was discovered that the strain was widely distributed throughout Australia (Anonymous, Australian Veterinary Journal, 1966). The strain (Queensland/66), designated “V4”, was relatively thermostable, and it was demonstrated that it would respond rapidly to selection for enhanced

thermostability. V4 was soon being used as a vaccine. The heat-resistant variant (NDV4-HR) is now the basis of thermostable vaccines for commercial chickens. From a panel of 45 contemporary apathogenic isolates of Australian viruses, a second vaccinal strain, I-2, was selected (Spradbrow *et al*. 1995). The NDV4-HR and I-2 ND vaccines proved to perform well under the adverse conditions in developing countries of Africa and Asia (Alders and Spradbrow, 1998). The NDV4-HR vaccine is a commercial vaccine and can be purchased when foreign exchange is available. For countries where foreign exchange is not readily available, the Australian Centre of International Agricultural Research (ACIAR) has provided the I-2 ND vaccine master seed free of charge to enable a ND vaccine suitable for use in village chickens to be produced locally (Alders and Spradbrow 2001a).

The VG/GA strain was isolated in the early 1990s from commercial turkeys in Georgia in the USA by Pedro Villegas (Beard *et al*., 1993). It also replicated in the respiratory and gastrointestinal tracts of chickens, producing an immune response similar to that induced by B1, but without any recognisable respiratory symptoms (Glisson & Kleven, 1993). It is marketed under the name *AVINEW* in South Africa.

1.2.11.2 Live attenuated vaccines: Mesogenic strains

Mesogenic strains are avirulent for chickens older than eight weeks of age but retain virulence for 3 day-old chickens (ICPI 1.0-1.5). A distinct mesogenic vaccine virus, Mukteswar, was independently developed in India by egg passages. Other mesogenic NDV strains include Roakin, isolated from a field case in the USA, and the Komarov strain. The Komarov strain (also sometimes called the K strain) was obtained by the intracerebral passages through ducklings of a virulent virus isolated in Palestine in 1945 (Komarov & Goldsmit, 1946). The so-called Onderstepoort (OP) strain of Newcastle disease, sold as a live mesogenic vaccine in South Africa, was developed in the late 1970s at Onderstepoort by passaging the imported Komarov strain on cell cultures of monkey kidneys (Anonymous, 1974, Prof L Coetzee, personal communication). Mesogenic vaccines are now very seldom used. Instead, the tendency of the poultry industry is to use vaccines of low reactivity that still provide a high level of protection (Villegas, 1998).

1.2.11.3 Inactivated vaccines

Mycoplasma and some other bacteria, if present, may act synergistically with some live vaccines to aggravate the vaccine reaction after spray administration. When other infections are present in the flock, and where required by law, inactivated (killed) vaccines are used. Furthermore, inactivated vaccines with oil adjuvants give the longest protection. A combination of live and inactivated ND vaccine administered simultaneously is shown to provide the best protection against virulent NDV and has been successfully used in control programmes in areas of intense poultry production (OIE, 1996).

1.2.11.4 Limitations of vaccination

Whether inactivated vaccines, lentogenic mass vaccines, or intramuscularly-injected mesogenic strains are used, repeated vaccination is required to protect chickens throughout life because vaccine-induced immunity is short-lived (8–10 weeks). The frequency of re-vaccination largely depends on the risk of exposure and virulence of the field virus. Proper administration of a high-titered vaccine is essential for induction of a good immune response, as failure to follow instructions (e.g. use of sprays in open or windy houses, or use of chemically treated water to dilute the virus) may result in incomplete or no protection after vaccination. In practice, vaccination of poultry against NDV does not always prevent periodic outbreaks of the disease (Khalafalla *et al*., 1992; Oncel *et al*., 1997; Yang *et al*., 1997, 1999; Roy *et al*., 2000). The factors promoting disease occurrence in vaccinated birds are firstly factors decreasing host resistance to the infection; and secondly changes of the virus allowing it to overcome the 'immunological barrier' (antigenic drift). Panshin and colleagues (2002) investigated the factors affecting antigenic drift in NDV and reminded us that although the AMPV-1 represents a serologically homogeneous group, antigenic variations have been detected by monoclonal antibody studies (Alexander, 1990). So far there is no evidence that antigenic differences between virulent and vaccine viruses have been sufficient to result in failure of the vaccines to provide protection against challenge (Roy *et al*., 2000; Alexander & Parsons, 1986). However, in other studies, it has been demonstrated that protection activity of a mixture of monoclonal antibodies (MAbs) directed against different epitopes of NDV is higher than the protection caused by a single MAb (Umino *et al*., 1990). As far as the immune

response in natural conditions is polyclonal, it is reasonable to expect that just after vaccination, there is an induction of antibodies of a wide spectrum, i.e. directed against various epitopes including those common to both the vaccine and challenging virus. At the period of the maximal antibody induction, the level of those antibodies is sufficient for the protective effect. Afterwards, and especially under immunosuppressive influence, the level of these antibodies may drop below the protective level, enabling the virulent virus to overcome the vaccine barrier, with initiation of the disease (Panshin *et al*., 2002). The most important drawback of vaccination however is that it has a "masking" effect. Vaccinated birds challenged with virulent NDV may become infected and excrete virus, although in relatively small amounts, while remaining apparently healthy, thus enabling the low-level the circulation of a virulent virus in the population (Guittet *et al*., 1993; Parede & Young, 1990; Utterback & Schwarts, 1973; Alexander, 2000b).

1.2.12 Role of wild birds in the perpetuation and spread of Newcastle disease

Newcastle disease virus isolates have frequently been obtained from migratory feral waterfowl and other aquatic birds (Alexander *et al*., 1979; Bannerjee *et al*., 1994; Kellerher *et al*., 1985; Spalatin & Hanson, 1975; Stallknecht *et al*., 1991). These strains are usually of the 'asymptomatic enteric' pathotype but differ from lentogenic strains prevalent in chickens by being thermostable (Spalatin and Hanson, 1974, Kida *et al*., 1980). For example, in a survey of mallard ducks (*Anas platyrhyncos*) trapped in New Zealand in 1997, ten APMV-1 viruses (NDV) were isolated from 321 cloacal and tracheal swabs. All isolates had low pathogenicity indices (0.00-0.16). Phylogenetic analysis showed that nine of the isolates were most similar to a reference strain, D26/76 originally isolated in Japan and also to the Queensland/66 virus isolated in Australia. The tenth isolate was very similar to a virus obtained from a shelduck in France. In these isolates, F_0 cleavage sites were of the sequence ^{112}GKQGRL^{117}, ^{112}ERQGRL^{117} or ^{112}EKQGRL^{117} (Stanislawek *et al*., 2002). In a similar study, five lentogenic NDVs were isolated from white-fronted geese in 2000 over wintering in the San-in district of western Japan (Shenqing *et al*. 2002a), and in recent survey of wild birds in Australia, two non-pathogenic APMV-1 isolates were obtained from cloacal and tracheal swabs from ducks. One of the viruses was V4-like, and the other belonged to monoclonal antibody group H (lineage 6) (Peroulis & O'Riley, 2004). In a similar study, viruses were isolated from faecal samples of

waterfowls in Alaska and Siberia collected from 1991 to 1996. Eight of the avirulent viruses isolated in Siberia and Alaska were closely related to the D26/76 and Queensland/66 viruses. In a South African survey conducted in the Oudshoorn region in 1998, antibodies to NDV were detected in 34 out of 46 serum samples from 14 mostly-aquatic species although no NDVs could be isolated (Pfitzer *et al*., 2000).

Müller *et al*. (1999) not only showed that species of wild geese were serologically positive for NDV, but also that the main resting and wintering areas of migratory waterfowl are likely to be important sites for the inter- and intraspecies transmission of avian diseases. Waterfowl in the northern hemisphere migrate south in autumn, back north in spring, and breed in summer in the nesting places in northern territories such as Alaska, Canada or Siberia. NDVs are preserved in the freezing water during winter when ducks are absent, and ducks returning from the south in spring are reinfected (Takakuwa *et al*., 1998).

The majority of NDVs isolated from wild birds, and especially waterfowl are lentogenic, causing no clinical diseases, but recent genetic comparisons between NDVs in domestic poultry and feral waterfowl suggested that velogenic viruses arose from avirulent strains originating from wild birds. The two most well-known cases are the following: in Ireland in 1990, two outbreaks of ND occurred in poultry. Not only were the viruses isolated highly virulent and apparently identical, but they were also very closely related antigenically and genetically to viruses of low virulence normally isolated from feral waterfowl (Alexander *et al*., 1992; Collins *et al*., 1993). Moreover, the genetic distinctiveness of this group of viruses (lineage 6) compared to all other NDVs suggested that the velogenic viruses that arose from the avirulent ones originated from wild birds (Collins *et al*., 1993). At the molecular level, the virulent viruses had four nucleotide differences at the site encoding the F_0 cleavage site compared to the related viruses of low virulence, which would explain the higher virulence for chickens (Collins *et al*., 1993). Genetic analysis of the viruses isolated during the second case, the 1998-2000 outbreaks in Australia, supported this hypothesis. In the 1960s it was recognised that Australia had its own endemic avirulent strains of NDV, characterised by the Queensland/66 or V4 strain (Simmons, 1967). Molecular characterization has shown that these viruses have avirulent F_0 cleavage site sequences of ^{112}GKQGRL^{117}, and HN extensions of 45 amino acids (Sato *et al*., 1987; Toyoda *et al*., 1989). Certain strains of this particular type are involved with a respiratory form of the disease (Hooper *et al*., 1999). From December

1998 to 1999, outbreaks of virulent ND occurred at Dean Park, Mangrove Mountain, and Tamworth in New South Wales, Australia. Prior to the outbreak, in September 1998, samples were collected from chickens showing respiratory distress on properties close to Peat's Ridge and Mangrove Mountain. Two viruses with the F_0 cleavage site ^{112}RRQGRL^{117} were isolated. It was noted that just two nucleotide substitutions ($G^{4888}\rightarrow A$ and $C^{4894}\rightarrow T$) in either of these sequences would generate a virulent motif (^{112}RRQRRF^{117}) at this site. The Peat's Ridge virus therefore appeared to be the progenitor of the virulent strains, additionally the virulent viruses had only 1.4% sequence diversion from the avirulent progenitor strain (Gould *et al*., 2001). There is mounting evidence that the presence of an unstable virus genome, represented by the Peat's Ridge strain, occasionally generates virulent variant strains in Australia (Kirkland, 2000).

In vivo studies have been performed to investigate the mechanism of mutation of avirulent strains into virulent ones as it was not clear whether the acquisition of virulence takes place in feral bird populations, with subsequent introduction of the virulent mutant into poultry, or whether an avirulent virus is transmitted directly to chickens and then becomes virulent. To investigate this, Shengqing *et al* (2002b) passaged an avirulent wild goose isolate in chickens. After nine consecutive passages by air-sac inoculation, followed by five passages in chick brain, the virus became highly virulent in chickens, producing a 100% mortality rate, and demonstrating typical velogenic properties in pathogenicity tests (MDT >120h $\rightarrow$ 56h; ICPI 0.00 $\rightarrow$ 1.88; IVPI 0h$\rightarrow$2.67h). Sequence analysis at the F_0 cleavage site showed that the original isolate containing the typical avirulent type sequence ^{112}ERQERL^{117}, progressed incrementally to a typical virulent type, ^{112}KRQKRF^{117}, during the repeated passages in chickens. These results clearly demonstrated that avirulent viruses, maintained in wild waterfowl in nature and bearing the consensus avirulent type sequence, have the potential to become velogenic after transmission to and circulation in chicken populations. These results also suggested that chickens provide a mechanism for the selection of virulent viruses from an avirulent background (Shengqing *et al*., 2002b).

Virulent NDVs have also been recovered from wild birds. The most significant outbreaks of virulent ND in feral birds were those reported in double-crested cormorants (*Phalacrocorax auritus*) in North America during the 1990s. Virulent

viruses isolated from dead cormorants appeared to be closely related to the strain that later caused an outbreak in domestic turkeys (Kuiken, 1998). Isolations of virulent viruses from wild aquatic birds that did not develop clinical symptoms have also been reported (Majiyagbe & Nawathe, 1981; Takakuwa *et al*., 1998). In one study, 47 ND viruses were isolated from faecal samples of waterfowls in Alaska and Siberia collected from 1991 to 1996. 29 of these viruses were virulent according to their MDTs, and five of the eleven viruses sequenced, contained the dibasic pair of amino acids at the F_0 cleavage site (^{112}RRQKRF^{117} or ^{112}RRQRRF^{117}), which indicated virulence (Takakuwa *et al*., 1998). In a recent survey of migratory birds in Brazil, five virulent ND viruses were isolated from shorebirds, with ICPIs ranging from 1.68 to 1.72 (Cavalléro, 2004). Therefore, both virulent and potentially virulent strains of NDV are maintained in migratory waterfowl populations in nature (Takakuwa *et al* 1998).

1.2.13 Transmission and spread

Wild birds, especially migratory waterfowl, are therefore often regarded as a reservoir of NDV infection, and are considered a potential risk for the introduction of NDV into NDV-free countries (Kouwenhoven, 1993; Müller *et al*., 1999). This concern is supported by reports of ND outbreaks having occurred in 1991 in Finland, United Kingdom, The Netherlands, Czech Republic and also Austria, where wild birds were presumed to be the primary source of infection (OIE, 1996; 1997). Although the role of wild birds in the epidemiology of NDV is not sufficiently understood, it seems a realistic assumption that infected birds have the potential of spreading the disease very rapidly over long distances (Qureshi, 1988). Like AIV, NDV is spread primarily through direct contact between healthy birds and the bodily discharges of infected birds. The disease is transmitted through infected birds' droppings and secretions from the nose, mouth, and eyes. NDV is highly contagious and spreads rapidly among birds kept in confinement, such as commercially raised chickens. During the active respiratory stage, it can be transmitted through the air, however, the virus is not thought to travel any great distance by this method. All birds in a flock usually become infected within three to four days. Once introduced into poultry, the virus spreads farm-to-farm by the movement of inapparently infected poultry species; on contaminated objects such as boots, clothing, sacks, egg trays, and crates; in manure; or by flies, free-flying birds or mice. NDV can survive for several weeks in a warm

and humid environment, and can survive indefinitely in frozen material. However, the virus is destroyed rapidly by dehydration and by the ultraviolet rays in sunlight (Bram *et al*., 1974; OIE Terrestrial Manual, 2004)

1.2.14 History of Newcastle disease in South Africa

ND was officially diagnosed for the first time in South Africa in 1945, after a severe disease with respiratory, nervous and intestinal symptoms, and high mortalities swept through poultry in the Natal (KwaZulu Natal) province. The diagnosis was made in England, by serum neutralization tests performed at Weybridge (Kaschula *et al.*, 1945). Kashscula believed that ND was already present in Durban in September 1944, and everything seemed to point to the possible introduction of the disease through the Port of Durban. Because the symptoms and autopsy findings in Natal so closely resembled those described by Hudson in Mombassa, Kenya in 1935, it was "almost certain" that the disease had been brought by ship from some harbour on the East Coast of Africa. Hudson considered that the infection had spread south to Lindi, and there were persistent rumours suggesting that the entire African East Coast had been affected (Kaschula *et al.*, 1945). In retrospect, this outbreak appears to have been part of a panzootic that occurred during the Second World War that swept through Italy, Palestine, and the whole of central Europe. It is interesting to note that the rapid spread and devastation caused by ND during the Second World War was partly ascribed to the scarcity of wire, that lead to the absence of efficient barriers between adjacent areas or across water routes (Kluge, 1964).

The 1944/45 outbreak in South Africa was confined almost entirely to the sugar-cane belt of Natal and the free-ranging flocks of the Indian population suffered the heaviest losses. The disease was eventually stamped out, but 100 000 fowl are estimated to have perished. Kaschula (1945) suspected that the Mombassa outbreak in 1935 was not the first African case of ND. His suspicion is corroborated by the following letter that I came across, extracted from an 1892 South African agricultural journal:

Query No. 221- Fowl-sickness (in Fort England, Grahamstown)
Can any of your readers give me some information as to the causes, prevention, and treatment of a disease that from time to time attacks my fowls, and seems to resist all known treatment.The disease apparently begins with a cough, and gasping breathing, the fowl trembles all over, and when it sits down one leg is stretched in front the other behind the body. There seems to be a gradual paralysis of both legs. The patient has all along an excellent appetite, which is maintained to the very end of the disease-invariably death. Some of the cases make gurgling noise when breathing, and they generally live in this dying state for months. Aloes, rusty water, lime, and paraffin-remedies recommended by "fowl" authorities have all been tried-as well as homoeopathic doses of aconite, without benefit. I have two English imported canaries ill, and the symptoms are very similar. As the canaries are valuable birds I am anxious to know of a cure (Greenlees, 1892).

Apart from the prolonged "dying state" described, the symptoms and particularly the neurological ones are consistent with Newcastle disease. The spread from chickens to canaries suggests that the disease was caused by a highly infectious avian pathogen with a wide host range, like APMV-1. Thereafter and up to 1944 there were regular reports of disease outbreaks in poultry in various parts of South Africa. In some cases the diseases were confirmed to be bacterial in origin, but in other cases the etiological agent was never defined (Martinaglia, 1926). For example, another letter written in 1903 described the following symptoms in a flock of mixed-breed, free-ranging fowl at Hamans Kraal (Hammanskraal) near Pretoria:

The fowl becomes unable to walk, and appears to have paralysis of the legs, the wings droop, the foeces [sic] are loose, weakness sets in and the bird dies. We give paraffin externally and caster oil internally. This treatment is no good. What is the right treatment, please? (Rosenbloom, 1903)

The symptoms described here are typical of ND and it seems likely that NDV was present in South Africa long before 1945. Therefore, the account given by Mr Greenlees of Grahamstown in 1892 could be the earliest known description of ND anywhere in the world.

Confirmed ND reappeared in the Windermere Township of the Cape Peninsula in July, 1949, and in the Port Elizabeth area of the Eastern Cape in September. By January 1950, the disease had spread to Natal (KZN) and was also confirmed in Johannesburg. Another outbreak was diagnosed in Durban during January, 1951, and

a smaller outbreak in 1953. With the application of strict control measures, the disease was successfully eliminated until early October, 1954, when an outbreak occurred in Johannesburg. By the end of December 1954, the disease had been stamped out. It is estimated that more than 300 000 fowls died in the outbreaks of the early 1950's, but by then vaccines had been developed, and more than a million birds were vaccinated with an attenuated live virus (De Kock, 1954). ND re-emerged in the northern outskirts of the Pretoria district and the Pretoria Municipal area in August 1961. An intensive control campaign was successfully concluded at the end of November 1961, and no further outbreaks occurred. During November 1961, the disease was diagnosed in Evaton near Vereeniging and again in the same area in June 1962 (Kluge, 1964). All viruses isolated in the outbreaks from the 1940's to the 1960's were of the velogenic type, and caused high mortalities in both small lots of household fowl, and the larger poultry farms which became infected. In July 1960, the mild lentogenic type of Newcastle disease was diagnosed in South Africa for the first time. The strain of virus was so mild that symptoms of infection were apparent only to the careful observer (Kluge, 1964). The distribution of the 1944, 1950 and 1961 outbreaks were localised and mainly confined to small holding and backyard poultry farms, except in the Western Cape areas where the infection spread to 13 larger poultry farms (Kluge 1964). At that time, is was believed that the disease was not enzootic in South Africa and the localised nature of the outbreaks indicated that the infections were probably introduced from some unknown external sources (Kluge, 1964). These outbreaks caused serious losses while they lasted, but the control measures instituted by the State (quarantine and other zoo-sanitary measures, in conjunction with immunization of all birds in the surrounding and proclaimed areas) seemed to effectively control the disease, and eliminated the infection in each instance. The live mesogenic Komarov strain was used as a vaccine at the time, and no immunization against ND was practised or allowed at any time except during outbreaks of the disease and then always done under State supervision. Therefore, ND was not considered to be a disease of any importance for South Africa, especially since there was no evidence, serological or otherwise, to suggest the presence of even lentogenic strains of ND in South Africa (Coetzee & Abrams, 1965).

South Africa experienced one of its most severe ND epidemics from 1970 to 1972. The rapid expansion of the poultry industry since 1960 had created large concentrations of susceptible poultry, and poultry farms accommodating hundreds of thousands of chickens were established in close proximity to one another and near the

urban markets. The method of erradication previously employed to control ND had become impractical because of the rapid dissemination of the infection in various regions of the country (L Coetzee, 1980). The industry suffered great economic losses, and by 1971 ND was believed to be enzootic in the country. By 1974, outbreaks of ND had subsided and occurred only in unvaccinated birds on small poultry farms and among unvaccinated poultry kept in back-yards. Outbreaks were reported fairly frequently in consignments of imported birds at the quarantine station at the Jan Smuts (Johannesburg International) airport. Evidently, these consignments were infected while they were handled by dealers prior to shipment because all birds developed ND virtually without exception from 3-7 days after their arrival in South Africa. Vaccination against ND became a common practise at the airport quarantine station (Anonymous, SAPV-Pluimveebulletin Februarie 1974). Subsequent to the severe outbreak of the 1970's, only small sporadic outbreaks occurred until June 1993. The outbreaks since the early 1990s are the topics of Chapters Four, Five and Six.

1.2.15 The threat of Newcastle disease to sustainable livelihoods

Poultry production in South Africa may be seen as a gradient between two extremes, with highly integrated commercial enterprises with world-class facilities on one hand, and unimproved rural chickens kept by subsistence farmers on the other. During outbreaks, spillover into commercial operations result in severe economic losses, such as the 1993/94 outbreak, that caused an estimated loss of a million broilers a week during its peak (Coetzee, 1994). Because ND is notifiable to the OIE, outbreaks usually result in trade restrictions on poultry and poultry products, with the consequent threat of loss of valuable export markets. Although it is a serious threat to the broiler and layer industries, NDV is not considered an important production disease for ostrich farming, due to its very slow mode of spread (only faecal/oral and drinking water, not respiratory) in ostrich rearing systems (Verwoerd, 1995a).

There has been increasing acknowledgement of the potential of small-scale poultry production to stimulate the socio-economic growth of resource-poor households, and ND is the single largest threat to poultry production in South Africa. The greatest impact, however, is probably on the traditional small-scale production. This is well-illustrated by Kaschula's account of the 1944/1945 outbreak in Natal. As a result of the diminished supplies of mutton during the war, the Indian community (the

workforce at the sugar mills of KZN) were compelled to rely on poultry as a protein source, and as most could not afford to buy birds, they took to raising them on a greater scale than before. In certain districts, the Indian homes were built very close to one another, and the six or twelve fowls belonging to each family roamed through two or three backyards, generally roosting in the highest tree in the neighbourhood. Under these circumstances, there was nothing to check the outbreak. Moreover, in contrast to what happened in the 'native reserves', Indians bought and sold fowls freely and transported them considerable distances. At the sugar mills the Indians usually lived in barracks, and often thousands of birds of all ages were kept there. No barriers existed to prevent the disease killing all the fowls owned by any one group of families once ND was introduced. Kaschula relates how it was a common experience to visit a barracks, to find only the ducks (who were resistant) walking around after a ND outbreak. In contrast, the flocks owned by Europeans were well housed and fed, and losses were limited to those who had purchased table poultry from Indian and African sources. In one such case, a farmer was left with 22 survivors out of a flock of 850 white leghorn chickens. Another lost over 600 leghorns (100% of his flock), but bantams running with these fowls somehow escaped, as did a dozen turkeys on the same farm. The Africans did not suffer as heavy losses as the Indians, only a few very minor outbreaks were reported, apparently because Africans seldom bought fowls to take to their homes and limited movement of fowl between kraals occurred. Kaschula writes that when their birds were moved, they were carried in baskets and crates to trading stores and railway stations for transport to Durban, where an excellent market was established. Very often large numbers were taken at the weekend for sale to the Indians at the sugar mills. From the locations, the poultry moved predominantly in a one-way stream to Durban and the sugar mills, with practically no traffic in the reverse direction.

Nowadays, with the exception of urban areas in northern and southern Africa, most poultry production in Africa is still undertaken through the extensive system at village or family level. Almost every village household keeps domestic fowl (on average between 5 and 20 birds) and it is estimated that village fowl make up more than 80% of the total domestic fowl population in Africa estimated at 1068 million in 1995 (Guèye, 1998). Poultry provides a good source of protein and ready cash for villagers. Moreover, poultry helps to sustain the village economy and contributes to the prevention of urban migration. The benefits of family poultry production go directly to the rural poor, in most cases to the women being most active as caretakers.

Constraints on backyard poultry production include poor nutrition, particularly during the rearing phase, and losses due to predators because of insufficient housing, but ND is frequently responsible for devastating losses in village poultry. For example, Spradbrow (1992) estimated that 90% of the village chickens in Nepal die each year from ND. The extremely high mortalities reported for ND is a major factor that discourages the resource-poor from investing much of their time and scarce resources in expanding flock size. The survivors have high antibody levels and are resistant for a while, but as the levels of antibodies and the level of protection fall, the population becomes susceptible again, and the cycle is repeated. The disease is spread by contact between birds, which is exacerbated by the practise of taking birds to the market from a flock where disease is incubating. However, many aspects of the epidemiology of the disease in the village situation are not fully understood (Awan *et al*., 1994). Although vaccines do exist, even thermostable vaccines which are suited to use in remote rural areas, vaccination is not an accepted method of control in rural chickens. The reasons for this are cost (vaccination in South Africa is not subsidized by the government), large dosage formulations, difficulties associated with catching essentially wild chickens, lack of trained extension staff to administer vaccines and general misconceptions or superstitions about vaccination. Another fear is that vaccinated village flocks, or the small number of survivors of epidemics, could mask the circulation of virulent or potentially virulent viruses therein. Therefore, it has been speculated that village chickens could serve as reservoirs of virulent NDVs, from which spillover into the commercial sector poses a serious threat (Verwoerd, 1995b).

1.3 OBJECTIVES OF THE INVESTIGATION

Highly pathogenic avian influenza (HPAI) and velogenic Newcastle disease are the two most feared diseases of poultry in the world. South Africa's valuable poultry industry, consisting of broiler, layer and ostrich-producing sectors suffer heavy losses due to mortalities, drops in egg production, loss of export markets, and trade restrictions during periodic outbreaks of avian influenza and velogenic Newcastle disease. Newcastle disease is also cited as the biggest threat to sustainable backyard poultry production in developing countries, including South Africa. Whereas Newcastle disease outbreaks occur on average every four years in South Africa and vaccination is widely practised to control the disease, avian influenza is a relatively recent threat to the local poultry industry. LPAI viruses have been periodically isolated from ostriches since the 1990s, but the first outbreak in chickens was recorded in the early 2000's and HPAI broke out for the first time in forty three years in South Africa in ostriches of the Eastern Cape Province in 2004. Global awareness surrounding avian influenza has intensified since Asian HPAI H5N1 arose and spread westwards, and consequently a new emphasis has been placed on surveillance of wild migratory waterfowl, the primary hosts of both diseases. Prior to the work described in this thesis, very little sequence data existed for South African strains of AI and Newcastle disease viruses, and therefore little was known about the true status of these two diseases in South Africa, or what the chances were of introduction of Asian HPAI H5N1 by migratory birds. For decades it has been assumed that Newcastle disease is enzootic in South Africa, periodically spilling over into the commercial sector from an unknown reservoir, which some have suggested to be village chickens. The Newcastle disease project at OVI was initiated on the premise that a novel field strain of Newcastle disease existed in South Africa, with the potential to mutate to the velogenic form, as was reported in Australia in the late 1990s. For avian influenza, the origins of the LPAI strain in ostriches were unknown, although the farmers and veterinarians suspected that wild birds were somehow involved in the periodic outbreaks of disease.

The objectives of this study were to:

- determine the origins of the first outbreak of AIV in chickens (LPAI H6N2) in South Africa in the early 1990s (Chapter 2)
- characterise the 2004 HPAI H5N2 Eastern Cape ostrich outbreak strain and compare it to LPAI H5N1, H5N2, H3N8 and H4N8 viruses isolated from wild

ducks in 2004, determine their origins, the relationship of the H5N2 and H5N1 viruses to the Asian HPAI H5N1 strain, and to characterise any virulence determinants (Chapter 3)

- clarify the status of NDV in South Africa during the 1990s, with incorporation of new sequence data (Chapter 4)
- determine the origins of Pigeon paramyxovirus (PPMV-1) infections in South Africa (Chapter 5) and to
- determine the source of the current outbreak of NDV in South Africa, caused by a "Goose Paramyxovirus" strain and to describe the epidemiology of the disease in South Africa (Chapter 6).

This was the first time that molecular and bioinformatical techniques were applied in South Africa to analyse AIV and NDV strains. Although a strong tradition of serological diagnosis existed in South Africa for both diseases, previously viruses had to be sent to the international reference laboratory at VLA Weybridge (UK) for further characterization.

CHAPTER TWO[1]

PHYLOGENETIC ANALYSIS OF LPAI H6N2 VIRUSES ISOLATED FROM CHICKEN OUTBREAKS (2001-2005)

ABSTRACT

The first recorded outbreak of avian influenza (AI) in South African chickens (low pathogenicity H6N2) occurred at Camperdown, KwaZulu/Natal Province (KZN) in June 2002. To determine the source of the outbreak, I defined the phylogenetic relationships between various H6N2 isolates, and the previously unpublished gene sequences of an H6N8 virus isolated in 1998 from ostriches in the Leeu Gamka region (A/Ostrich/South Africa/KK98/98). I demonstrated that two distinct genetic H6N2 lineages (sub-lineages I and II) circulated in the Camperdown area, which later spread to other regions. Sub-lineages I and II shared a recent common H6N2 ancestor, which arose from a reassortment event between two South African ostrich isolates A/Ostrich/South Africa/9508103/95 and (H9N2) A/Ostrich/South Africa/KK98/98 (H6N8). Furthermore, the H6N2 sub-lineage I viruses had several molecular genetic markers including a 22-amino acid stalk deletion in the neuraminidase (N) protein gene, a predicted increased N-glycosylation, and a D^{144} mutation of the HA protein gene, all of which are associated with the adaptation of AI viruses to chickens. The H6N2 NS1 and PB1 genes shared recent common ancestors with those of contemporary Asian HPAI H5N1 viruses. These results suggest that ostriches are potential mixing vessels for AIV outbreak strains and support other reports that H6 viruses are capable of forming stable lineages in chickens.

[1]Part of the results presented in this chapter are published in *Virus Genes 34 (1), 37-45 (2007)* and *Avian Diseases* (in press).

2.1 INTRODUCTION

Most outbreaks of influenza in domestic poultry are thought to have originated by the transfer of viruses from feral birds (Alexander 2000, Halvorson *et al.*, 1983). The H6 subtype was first isolated from a turkey in 1965, and other H6 viruses were subsequently isolated from shorebirds and wild ducks (Downie & Laver, 1973; Downie *et al.*, 1973). Chickens are generally not considered to be natural hosts for AI viruses and H6 strains have never been associated with mass mortalities in poultry. However, it has been suggested that H6 viruses may be capable of developing stable lineages in chickens (Liu *et al*., 2003), as several H6N1 and H6N2 outbreaks have been recorded in recent years in south-eastern Asia and California (Suarez 2000; Chin *et al.*, 2002).

In July of 1998, anti-H6 antibodies were detected in serum collected from a wild Egyptian goose (*Alopochen aegypticus*) in the Oudtshoorn district, but no H6 viruses were isolated from pooled organs (Pfitzer *et al.*, 2000). Later that winter, an H6N8 virus was isolated from four-month-old slaughter ostrich chicks at Leeu Gamka in the Oudtshoorn region that displayed green urine symptoms with increased mortalities. Then, during 2001, an infection with typical AIV symptoms circulated in the flocks of a commercial operation situated in Worchester in the Western Cape Province of South Africa, but before the aetiological agent could be investigated, sick chickens were sold to cull-buyers in the KwaZulu/Natal (KZN) province (Carine Pienaar, personal communication). There were no isolations of velogenic Newcastle disease in the country in that year (unpublished laboratory data). In June 2002, layers at commercial farms in the Camperdown region of the KZN province developed symptoms of respiratory distress and up to 40% declines in egg production. An orthomyxovirus was isolated and determined to be a low pathogenic avian influenza (LPAI) H6N2 strain at the VLA Weybridge laboratory, United Kingdom. Testing of archival material from the Camperdown region traced the infection back to June 2001, but the exact source of the H6N2 outbreak strain was never determined. The South African H6N2 outbreak, that affected only commercial chickens, continued throughout 2003 and sporadically in 2004 and 2005, and an inactivated homologous H6N2 vaccine was applied to limit the spread of the disease. The purposes of this study were to determine the epidemiological origins of the H6N2 chicken outbreak viruses isolated up to 2005, and to investigate whether these viruses were related to the previously un-sequenced H6N8 virus isolated in 1998 from ostriches.

2.2 MATERIALS AND METHODS

2.2.1 Viruses

H6N2 viruses (Table 2.1) were grown in 9- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual), at Allerton Provincial Veterinary Laboratory, the Poultry Reference Laboratory at the University of Pretoria (UP), the Virology Division at Onderstepoort Veterinary Institute (OVI), or the Klein Karoo (KK) Corporation laboratory in Oudtshoorn. The VLA Weybridge laboratory kindly provided a stock of infective alantoic fluid containing the H6N8 virus isolated in 1998 at OVI, and this virus was named A/Ostrich/South Africa/KK98/98 (H6N8). A/Ostrich/South Africa/9508103/95 (H9N2) was isolated from the Oudtshoorn ostriches in 1995. The virus was submitted to the international reference laboratory at VLA Weybridge, and subsequently, the hemagglutinin gene was sequenced by that group for use in a phylogenetic analysis of other H9 genes from the H9N2 pandemic (Banks *et al.*, 2000b). Later, the N2 glycoprotein and internal genes of A/Ostrich/South Africa/9508103/95 were sequenced and published by a different group (JW Li, KZ Yu, I Brown, KF Shortridge, JSM Peiris and Y Guan, 2002- Genbank record).

Table 2.1. South African LPAI H6N2 and H6N8 viruses isolated from 1998 to 2005

Name[1]	Abbreviation[2]	Collection date	Region
A/Ostrich/South Africa/KK98/98 (H6N8)	OSZA98KK98	Jul/Aug? 1998	Leeu Gamka (WC)
A/Chicken/South Africa/AL1/01 (H6N2)	CKZA02AL1	11/06/2001	Camperdown (KZN)
A/Chicken/South Africa/AL3/01 (H6N2)	CKZA02AL3	19/07/2001	Camperdown (KZN)
A/Chicken/South Africa/AL4/01 (H6N2)	CKZA02AL4	27/08/2001	Camperdown (KZN)
A/Chicken/South Africa/AL7/01 (H6N2)	CKZA02AL7	26/09/2001	Camperdown (KZN)
A/Chicken/South Africa/AL8/01 (H6N2)	CKZA02AL8	09/11/2001	Camperdown (KZN)
A/Chicken/South Africa/AL9/01 (H6N2)	CKZA02AL9	19/11/2001	Camperdown (KZN)
A/Chicken/South Africa/AL10/02 (H6N2)	CKZA02AL10	10/06/2002	Camperdown (KZN)
A/Chicken/South Africa/AL11/02 (H6N2)	CKZA02AL11	12/06/2002	Camperdown (KZN)
A/Chicken/South Africa/AL12/02 (H6N2)	CKZA02AL12	19/06/2002	Camperdown (KZN)
A/Chicken/South Africa/AL13/02 (H6N2)	CKZA02AL13	24/06/2002	Camperdown (KZN)
A/Chicken/South Africa/AL14/02 (H6N2)	CKZA02AL14	28/06/2002	Empangeni (KZN)
A/Chicken/South Africa/AL15/02 (H6N2)	CKZA02AL15	04/07/2002	Camperdown (KZN)
A/Chicken/South Africa/AL16/02 (H6N2)	CKZA02AL16	12/07/2002	Camperdown (KZN)
A/Chicken/South Africa/AL17/02 (H6N2)	CKZA02AL17	16/07/2002	Camperdown (KZN)
A/Chicken/South Africa/AL19/02 (H6N2)	CKZA02AL19	18/07/2002	Botha's Hill (KZN)
A/Chicken/South Africa/AL20/02 (H6N2)	CKZA02AL20	23/07/2002	Camperdown (KZN)
A/Chicken/South Africa/AL21/02 (H6N2)	CKZA02AL21	24/07/2002	Botha's Hill (KZN)
A/Chicken/South Africa/AL24/02 (H6N2)	CKZA02AL24	13/08/2002	Hillcrest (KZN)
A/Chicken/South Africa/AL25/02 (H6N2)	CKZA02AL25	29/10/2002	Verulam (KZN)
A/Chicken/South Africa/AL28/03 (H6N2)	CKZA03AL28	18/03/2003	Chatsworth (KZN)
A/Chicken/South Africa/AL29/03 (H6N2)	CKZA03AL29	24/03/2003	Chatsworth (KZN)
A/Chicken/South Africa/AL30/03 (H6N2)	CKZA03AL30	30/06/2003	Camperdown (KZN)
A/Chicken/South Africa/AL31/03 (H6N2)	CKZA03AL31	01/07/2003	Camperdown (KZN)
A/Chicken/South Africa/AL32/03 (H6N2)	CKZA03AL32	11/08/2003	Hammarsdale (KZN)
A/Chicken/South Africa/AL33/03 (H6N2)	CKZA03AL33	29/09/2003	Margate (KZN)
A/Chicken/South Africa/AL36/04 (H6N2)	CKZA04AL36	01/06/2004	Margate (KZN)
A/Chicken/South Africa/AL39/04 (H6N2)	CKZA04AL39	06/08/2004	Camperdown (KZN)
A/Ostrich/South Africa/N158/03 (H6N2)	OSZA03N158	31/03/2003	Johannesburg (GP)
A/Chicken/South Africa/UP855/02 (H6N2)	CKZA02UP855	09/07/2002	Erasmia (GP)
A/Chicken/South Africa/UP1102/02 (H6N2)	CKZA02UP1102	09/09/2002	Swavelpoort (GP)
A/Ostrich/South Africa/KK0727/03 (H6N2)	OSZA03KK0727	07/09/2003	Oudtshoorn (WC)
A/Chicken/South Africa/AL41/05 (H6N2)	CKZA05AL41	24/03/2005	Margate (KZN)

[1]Name: **A/Ostrich/South Africa/N158/04 (H6N2)**

Influenza A/ Host/ Country/ Sample number/ Year of isolation (Subtype)

[2]Abbreviation: **OSZA03N158**

Host (OS= ostrich; CK= chicken/ Country (ZA=South Africa)/ Sample number

2.2.2 RNA extraction

Viral RNA was extracted from allantoic fluid using TRIzol® reagent (Gibco, Invitrogen), or with the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's instructions.

2.2.3 First strand cDNA synthesis

Reverse transcription was performed with M-MLV reverse transcriptase (Promega) at 42ºC for 90 minutes on 5µl of extracted viral RNA. 3pMol of either the vGEN oligonucleotide, that anneals to the 5' terminal sequence of each of the eight influenza A segments, or the gene-specific oligonucleotides listed in Table 2.3 were used. In some cases, one-step RT-PCR was performed by adding a 20-minute 42ºC incubation step to the thermocycling profile prior to PCR (Table 2.2).

2.2.4 PCR

Full-length genes were amplified for the HA, NA, M, NS, and NP proteins for A/Ostrich/South Africa/KK98/98 (H6N8), A/Chicken/South Africa/AL19/02 (H6N8) and A/Chicken/South Africa/UP1102/02 (H6N8). For the PB2, PB1 and PA genes, approximately 1000nt of the 3' ends were amplified with primers PB11123FOR, PB21411FOR and PA1150REV (Table 2.3). Primer H550 was designed to primer-walk the gap spanning the HA forward and reverse sequences. A partial HA gene region (301 nt) was amplified for the remainder of the H6N2 isolates, and full-length NA genes were amplified for selected H6N2 viruses. An Eppendorf Mastercycler® 5333 or GeneAmp 2400 PCR System (Perkin Elmer) were used. Oligonucleotide primers are listed in Table 2.3.

Table 2.2 Thermal cycling conditions used to amplify AIV genes

Target	Initial denaturation	Denaturation	Annealing	Elongation	Cycles	Final elongation
HA (touchdown)	95°C (5 min)	95°C (30 s)	51°C (30s)	72°C (2 min)	3	72°C (2 min); 4°C (∞)
		95°C (30 s)	48°C (30s)	72°C (2 min)	3	
		95°C (30 s)	45°C (30s)	72°C (2 min)	3	
		95°C (30 s)	42°C (30s)	72°C (2 min)	3	
		95°C (30 s)	41°C (30s)	72°C (2 min)	30	
NA (touchdown)	95°C (5 min)	95°C (30 s)	60°C (30s)	72°C (2 min)	3	72°C (2 min); 4°C (∞)
		95°C (30 s)	58°C (30s)	72°C (2 min)	3	
		95°C (30 s)	56°C (30s)	72°C (2 min)	30	
NP/M/NS PCR (touchdown)	95°C (5 min)	95°C (30 s)	60°C (30s)	72°C (1:30 min)	3	72°C (2 min); 4°C (∞)
		95°C (30 s)	58°C (30s)	72°C (1:30 min)	3	
		95°C (30 s)	56°C (30s)	72°C (1:30 min)	3	
		95°C (30 s)	54°C (30s)	72°C (1:30 min)	30	
PB2/PB1/PA genes (touchdown)	95°C (5 min)	95°C (30 s)	65°C (30s)	72°C (3 min)	3	72°C (2 min); 4°C (∞)
		95°C (30 s)	63°C (30s)	72°C (3 min)	3	
		95°C (30 s)	51°C (30s)	72°C (3 min)	3	
		95°C (30 s)	59°C (30s)	72°C (3 min)	30	
Partial PA, PB2 and PB1	95°C (5 min)	95°C (30 s)	53°C (30s)	72°C (3 min)	30	72°C (2 min); 4°C (∞)

Table 2.3 Oligonucleotide primers used to amplify and sequence AIV isolates

Target gene	Primer name	Application	Sequence (5'→3')	Reference
H6	H550 (forward)	sequencing	CTGGGGTGTGCACCATCCTCC	C Abolnik
H6	H6-661f (forward)	RT-PCR, sequencing	AGCATGAATTTTGCCAAGAG	Lee *et al.* (2001)
	H6-962r (reverse)	RT-PCR, sequencing	GGRCATTCTCCTATCCACAG	Lee *et al.* (2001)
All	vGEN	First strand synthesis	AGCAAAAGCAGG	I Brown
HA	AIHAF (forward)	RT-PCR, sequencing	AGCAAAAGCAGGGGW	D Suarez
	AIHAR (reverse)	RT-PCR, sequencing	AGTAGAAACAAGGGTG	D Suarez
NA	Ba-NA-1 (forward)	RT-PCR	TATTGGTCTCAGGGAGCAAAAGCAGGAGT	Hoffmann *et al.* (2001)
	Ba-NA-1413R (reverse)	RT-PCR, sequencing	ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	Hoffmann *et al.* (2001)
PB2	Ba-PB2-1 (forward)	RT-PCR, sequencing	TATTGGTCTCAGGGAGCGAAAGCAGGTC	Hoffmann *et al.* (2001)
	Ba-PB2-2341R (reverse)	RT-PCR, sequencing	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	Hoffmann *et al.* (2001)
PB1	Bm-PB1-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGCGAAAGCAGGCA	Hoffmann *et al.* (2001)
	Bm-PB1-2341R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGTAGAAACAAGGCATTT	Hoffmann *et al.* (2001)
PA	Bm-PA-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGCGAAAGCAGGTAC	Hoffmann *et al.* (2001)
	Bm-PA-2233R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGTAGAAACAAGGTACTT	Hoffmann *et al.* (2001)
NP	Bm-NP-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGCAAAAGCAGGGTA	Hoffmann *et al.* (2001)
	Bm-NP-1565R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT	Hoffmann *et al.* (2001)
M1 M2	Bm-M-1 29 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGCAAAAGCAGGTAG	Hoffmann *et al.* (2001)
	Bm-M-1027R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	Hoffmann *et al.* (2001)
NS1 NS2	Bm-NS-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	Hoffmann *et al.* (2001)
	Bm-NS-890R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	Hoffmann *et al.* (2001)
NA	AINAFSEQ (forward)	sequencing	GGGAGCAAAAGCAGGAGT	C Abolnik
PA	PA1150REV (forward)	RT-PCR, sequencing	GGCACCAGAGAAAGTAG	C Abolnik
PB2	PB21411FOR (forward)	RT-PCR, sequencing	CCTTATAAYGGRCTGTACTG	C Abolnik
PB1	PB11123FOR (forward)	RT-PCR, sequencing	GTTTATGGTCGTCTYTAGGAACG	C Abolnik

2.2.5 DNA sequencing and phylogenetic analysis

RT-PCR amplicons of the correct sizes were excised from 1% agarose gels and the DNA extracted with the QIAquick® Gel Extraction Kit (Qiagen). Template DNA for sequencing was quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, USA). BigDye® Terminator V3.1 chemistry (Perkin Elmer/Applied Biosystems) and gene-specific primers from Table 2.3 were used for cycle sequencing, according to the manufacturer's instructions. Reactions were electrophoresed on either a 3100 Genetic Analyzer or a 3130 Genetic Analyzer (Applied Biosystems). Sequences were visualised with Chromas Lite 1.0 software (http://www.technelysium.com.au) and edited with BioEdit V.7.5.0.2 (Hall, 1999). Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) were used to identify 50 closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Blast was also used to calculate pairwise nucleotide sequence identities. For the South African H6N2 HA genes, the region analysed corresponds to nucleotides 838 to 954 (116nt) of the complete 1744 nucleotide protein-encoding region for H6 genes (Figs. 2.1(b) and (c)) Some of the H6 viruses did not grow to high titres in chicken eggs, thus amplification of full gene sequences were not possible. The phylogenetic topology for the 116 nt region is similar to that found in the full-length sequences for some of the South African H6N2 H6 genes (Fig 2.1(a) vs. Fig 2.1(b)) and was thus deemed to be a suitable size for phylogenetic comparison. Phylogenies were reconstructed with MEGA 3.1 software (Kumar *et al.*, 2004) using the Neighbour-Joining tree inference method. Sequence statistics directed that the Kimura 2-parameter model of sequence evolution should be used. 1000 bootstrap replicates were performed to assign confidence levels to branches. Neighbour-joining is a clustering method to group pairwise distances. It is the favoured distance calculation method because equal rates of evolution are not assumed. This is important when working with influenza virus sequences, as the viruses have different evolutionary rates in different hosts, and in most sequence alignments, viruses isolated from different hosts were compared. Potential N-glycosylated sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Gene sequences for A/Ostrich/South Africa/KK98/98 (H6N8), A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) were deposited in Genbank under the accession numbers DQ408506-DQ408529.

2.3 RESULTS

2.3.1 H6 Hemagglutinin genes

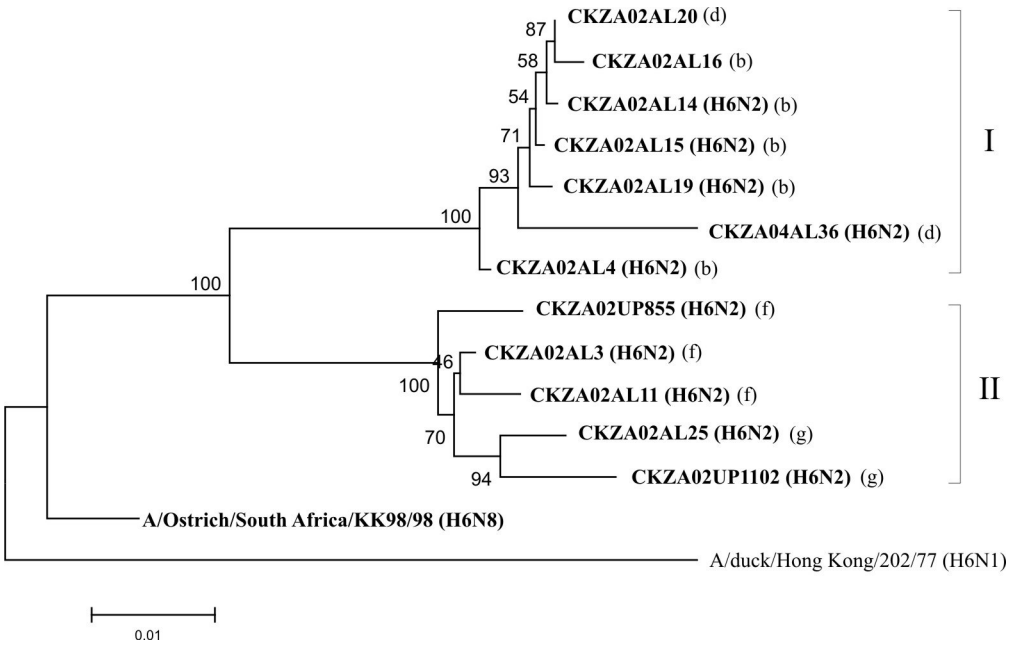


Figure 2.1(a) Dendogram of H6 type hemagglutining gene sequences (1315 nt). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages corresponding to Fig 2.1(c) are indicated (I and II; (a) to (g)).

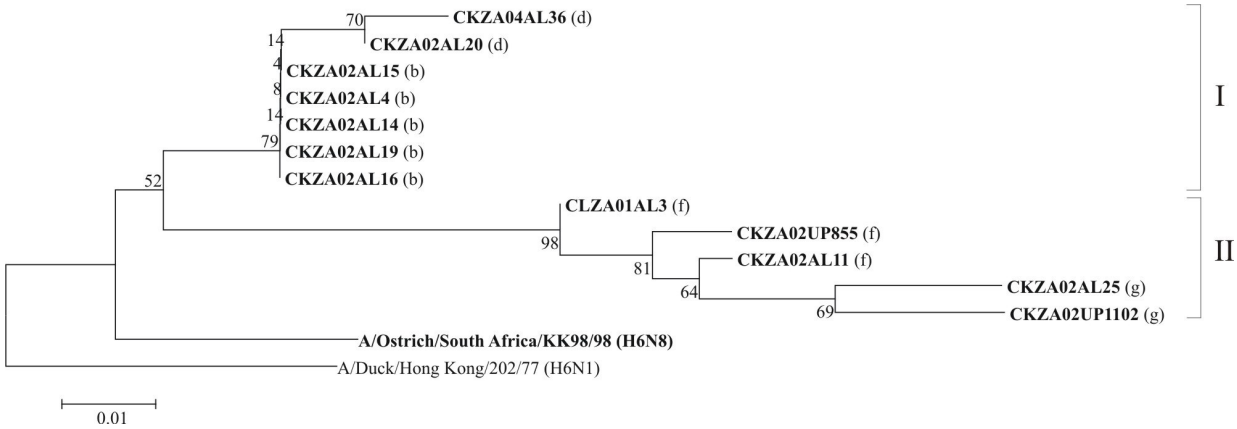


Figure 2.1(b) Dendogram of partial H6 type hemagglutining gene sequences, corresponding to the region between nucleotides 836 and 951 (116 nt) of the viruses presented in Fig 2.1(a). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages corresponding to Fig 2.1(c) are indicated (I and II; (a) to (g)).

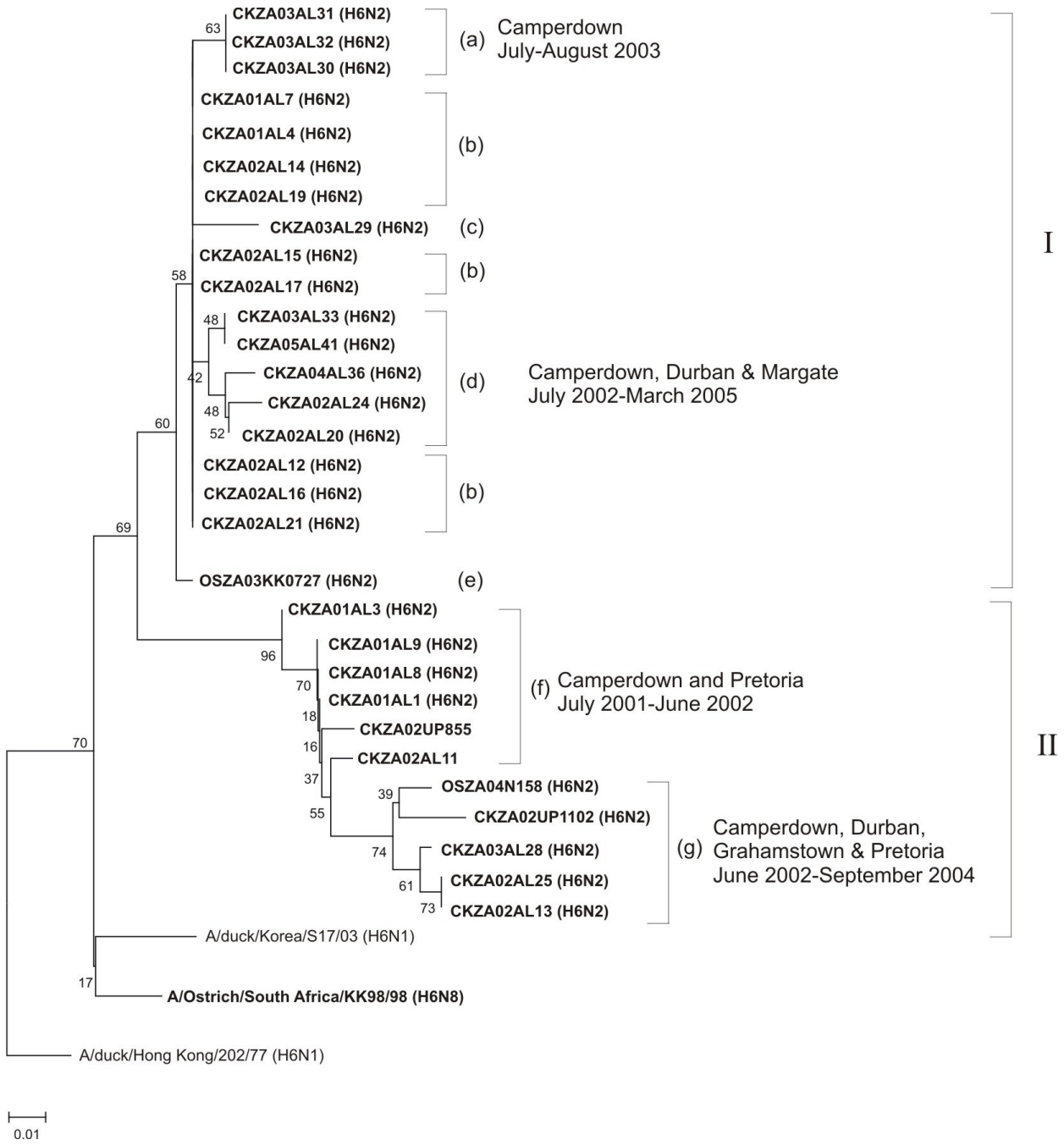


Figure 2.1(c) Dendogram of partial H6 type hemagglutining gene sequences, corresponding to the region between nucleotides 836 and 951 (116 nt). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages are indicated (I and II; (a) to (g)).

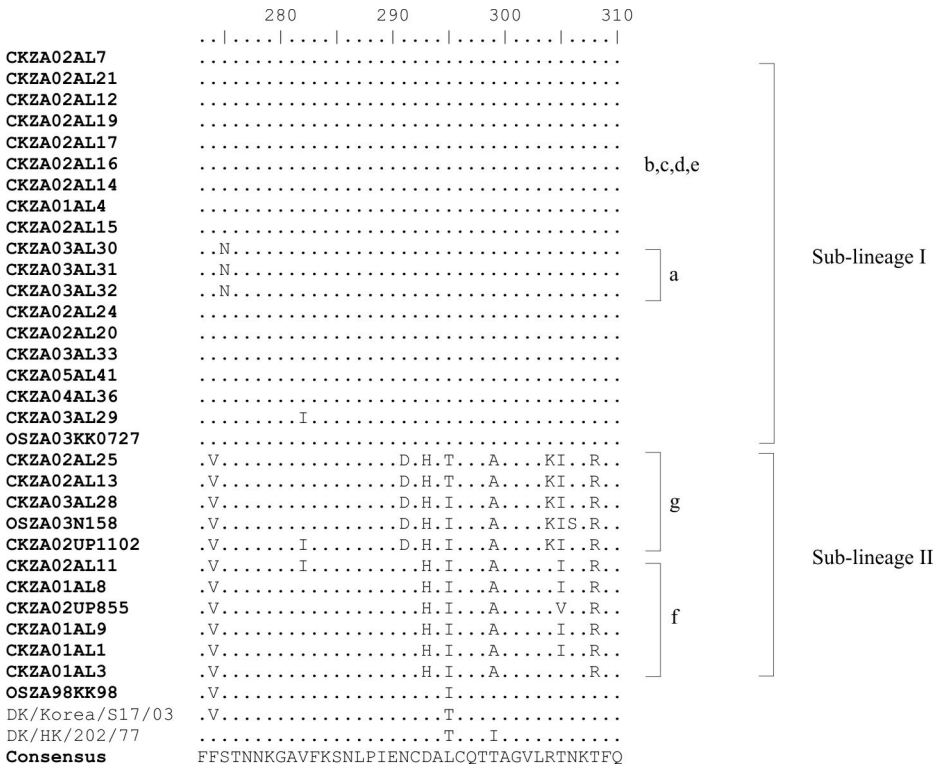


Figure 2.2 Multiple amino acid alignment of partial H6-type hemagglutinin genes (residues 273 to 310).

Two distinct sub-lineages (I and II) of H6 AIVs (Figs 2.1) circulated during the South African outbreak in chickens. Sub-lineage I viruses presented here were isolated only at commercial operations in the KZN Province. The isolates of I(a), CKZA03AL30 and –31 were isolated within a week of each other from the same Camperdown farm, and CKZA03AL32 almost five weeks later from a farm near the neighbouring town of Hammarsdale. This phylogenetic grouping is supported by a shared N^{275} residue in the partial amino acid alignment (Fig. 2.2). The isolates of I(b) were obtained from 2001 to 2002, mostly from the original sites in Camperdown, but also from Botha's Hill near Durban (CKZA02AL14 and CKZA02AL19). CKZA02AL14 was isolated from a farm in Empangeni situated about 170 km up the North Coast, but the farmer had visited a poultry farm in the Camperdown area a week previously. CKZA03AL29 (c) is separated from the rest of the sub-lineage I, evident at the amino acid level as a $V^{282}\rightarrow$I substitution event. This $V^{282}\rightarrow$I is shared by two sub-lineage II viruses, CKZA02UP1102 and CKZA02AL11, although there is no obvious epidemiological connection between these three isolates. The isolates of I(d) originated in Camperdown (CKZA02AL16; July 2002), then appeared in Durban a month later (CKZA02AL24), before being detected in Margate about 150 km away on the South Coast at the end of September 2003 (CKZA03AL33). There it persisted throughout

2004 (CKZA04AL36) and into March 2005 (CKZA05AL41). The ostrich H6N2 virus OSZA03KK0727 (I(e)) was isolated in the Oudtshoorn region in September 2004 and is basal to the chicken viruses within sub-lineage I, but clearly falls within sub-lineage I strain at the amino acid level (Fig.2.2). The long branch lengths could indicate an adaptation to the ostrich host.

Sub-lineage II viruses circulated over the same time period as sub-lineage I but contains isolates from both the KZN Province and the Gauteng Province. Sub-lineage II(f) contains most of the earlier isolates from the initial Camperdown outbreak (including the index case, CKZA01AL1). Sub-lineage II(g) is distinguished from II(f) and sub-lineage I viruses at the amino acid level by two substitutions in the partial H6 sequence, $E^{291}\rightarrow D$ and $R^{304}\rightarrow K$. OSZA03N158 and CKZA02UP1102 were sampled from Booysens, Johannesburg and Swavelpoort, near Pretoria, respectively.

To conduct a full comparison of full-length and internal genes with the A/Ostrich/South Africa/KK98/98 (H6N8) virus, a representative from sub-lineage I, A/Chicken/South Africa/AL19/02 (H6N2) and one from sub-lineage II A/Chicken/South Africa/UP1102/02 (H6N2) were selected. The hemagglutinin genes were compared first:

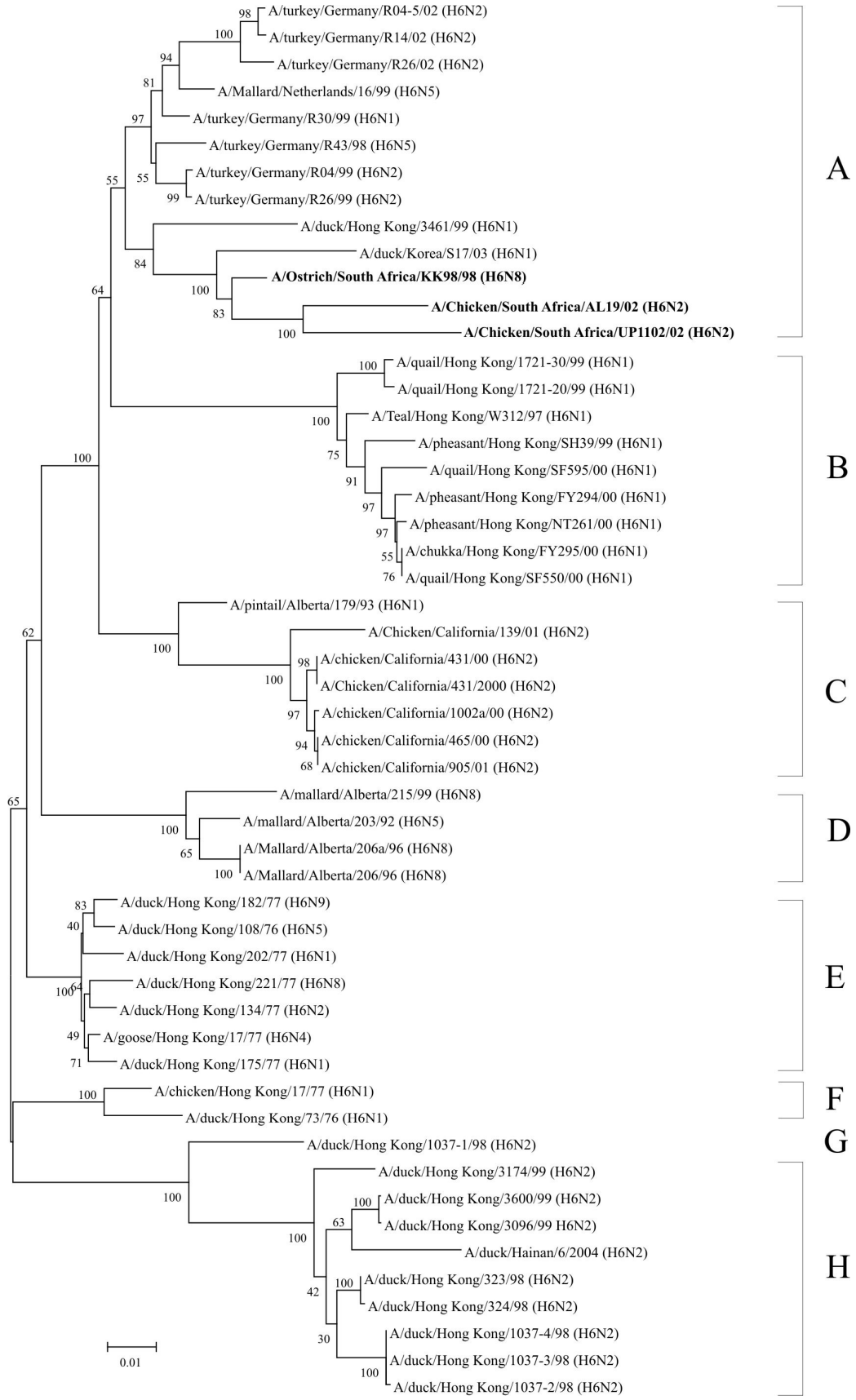


Figure 2.3 Phylogenetic tree inferred from a 1367-nt multiple sequence alignment of the hemagglutinin (H6) genes of South African (in boldface) and other viruses. Sub-lineages A to H are indicated.

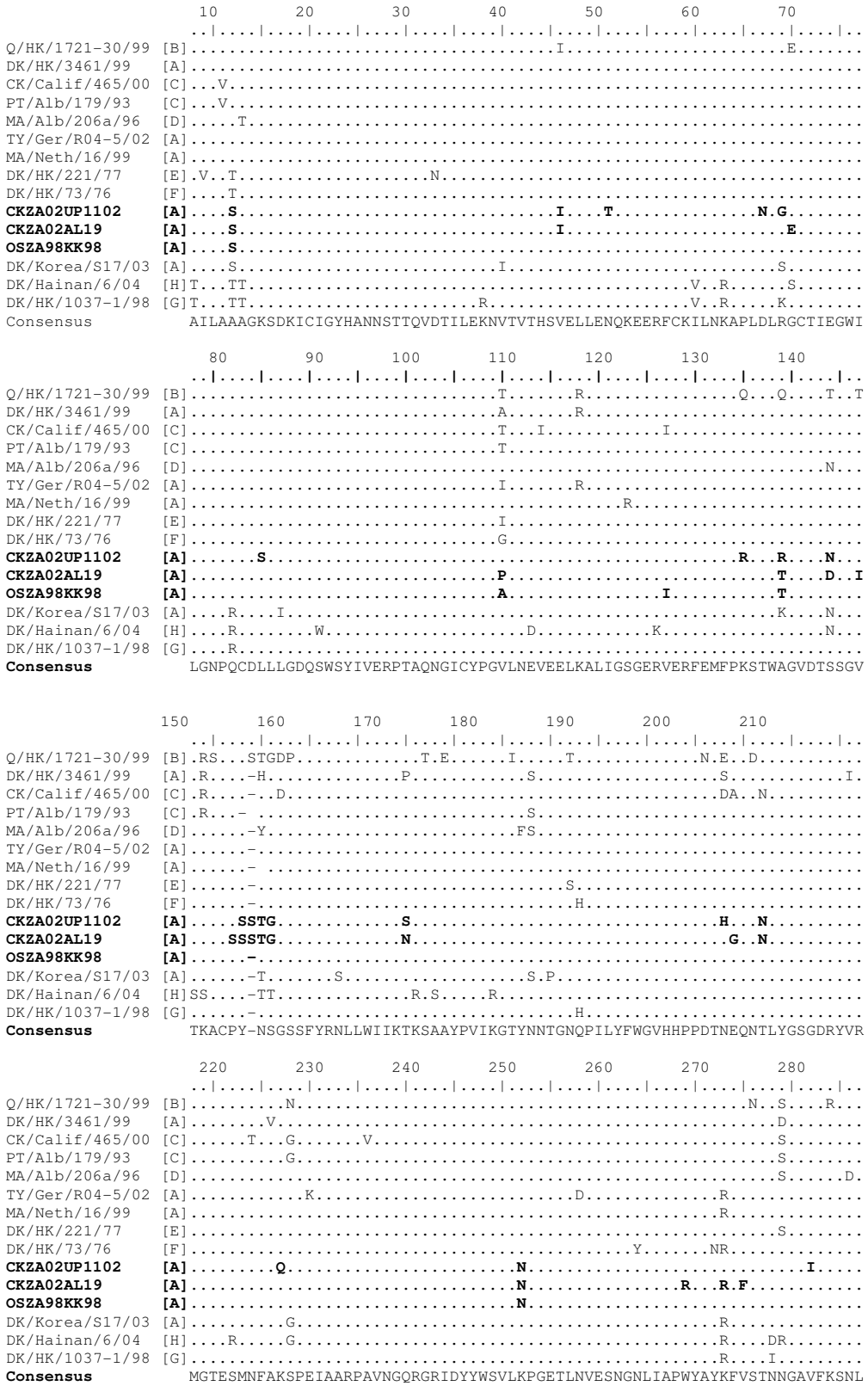
Fig. 2.3 indicates that A/Chicken/South Africa/AL19/2002 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) share a recent common ancestor for their H6-type hemagglutinin genes, and that this hypothetical gene appears to be derived from the H6N8 virus isolated in 1998, A/Ostrich/South Africa/KK98/98. At the amino acid level (Fig. 2.4), the shared residues I^{46}, N^{207}, K^{327} and A^{398} and the $^{153}SSTG^{157}$ motif (including a unique S^{154} insertion) support a common ancestor for the A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) H6 genes. The phylogenetic relationship between the H6N2 chicken viruses with A/Ostrich/South Africa/KK98/98 (H6N8) is supported by an N^{252} substitution. Other H6 genes in sub-lineage A originated in Korea, Hong Kong, Germany and the Netherlands.

A T^{299} residue was common to the A/Ostrich/South Africa/KK98/98 (H6N8), Korean and A/Chicken/South Africa/AL19/02 (H6N2) viruses, but not A/Chicken/South Africa/UP1102/02 (H6N2). It is difficult to assess whether A/Chicken/South Africa/AL19/02 (H6N2) or A/Chicken/South Africa/UP1102/02 (H6N2) is more closely-related to the ostrich H6N8 virus. A/Chicken/South Africa/UP1102/02 had 18 unique amino acid substitutions (T^{51}, N^{67}, G^{69}, S^{85}, R^{135}, R^{139}, S^{170}, H^{203}, Q^{227}, I^{282}, D^{291}, H^{293}, A^{299}, K^{304}, R^{308}, V^{400}, and G^{422}), but shared the K^{273} and I^{295} substitutions with A/Ostrich/South Africa/KK98/98 (H6N8), whereas A/Chicken/South Africa/AL19/02 (H6N2) had an R^{273} and a unique L^{295} residue, respectively. A/Chicken/South Africa/AL19/02 had only eight unique residues in comparison (P^{110}, D^{144}, I^{147}, S^{152}, N^{170}, G^{204}, R^{269} and F^{276}), but contained a unique codon deletion at position 360. This virus shared a unique T^{139} residue with A/Ostrich/South Africa/KK98/98 (H6N8), and a unique T^{299} residue with both the ostrich H6N8 and A/Duck/Korea/S17/03 (H6N1) H6 genes. This relationship between A/Chicken/South Africa/AL19/02(H6N2), A/Ostrich/South Africa/KK98/98 (H6N8) and A/Duck/Korea/S17/03 (H6N1) also extends to the hemagglutinin peptide cleavage site as these three viruses shared the unique sequence ^{339}PQIEPRGLR^{347}, whereas that of A/Chicken/South Africa/UP1102/02 was ^{339}PQIETRGLF^{347}.

HA molecules are glycosylated at four to eleven sites in the head and stem. Glycosylation and sialylation close to the RBS of HA regulate release of avian viruses from cells (Baigent *et al*., 1999; Ohuchi *et al*., 1997), thereby contributing to virulence and tissue tropism. The three South African H6 peptide sequences were examined for potential N-glycosylation sites. Four such sites were predicted in

A/Ostrich/South Africa/KK98/98 (positions 27, 39, 182 & 305), whereas A/Chicken/South Africa/UP1102/02 had only three (positions 27, 39 and 311) and A/Chicken/South Africa/AL19/02, five (positions 27, 39, 170, 183 and 306).

Further sequence analysis of the H6 HA gene revealed the insertion of an aspartic acid residue between positions 144 and 145 (H3 numbering) of the H6 HA in contemporary terrestrial isolates, and it was proposed that the corresponding change in the gene sequence could be used as a genetic marker to distinguish terrestrial isolates from aquatic ones. The aspartic acid corresponds to a proposed antigenic site of the HA surface (Wiley *et al.*, 1981; Wilson *et al.*, 1981). Fig. 2.4 indicates that A/Ostrich/South Africa/KK98/98 (H6N8) has a S^{144} residue at this position, shared by ducks, turkeys, chickens and quails (and in this case not a clear indicator of terrestrial or aquatic host specificity). A/Chicken/South Africa/UP1102/02 (H6N2) has an N^{144} residue, shared by other duck isolates, but A/Chicken/South Africa/AL19/02 (H6N2) has an aspartic acid residue, D^{144}, like the contemporary terrestrial south-eastern China H6 viruses. It is probable that D^{144} is an artefact of viral adaptation to the chicken host and could therefore be a reliable terrestrial marker, as suggested by Chin *et al*. (2002).


10 20 30 40 50 60 70
Q/HK/1721-30/99 [B]
DK/HK/3461/99 [A]
CK/Calif/465/00 [C]
PT/Alb/179/93 [C]
MA/Alb/206a/96 [D]
TY/Ger/R04-5/02 [A]
MA/Neth/16/99 [A]
DK/HK/221/77 [E]
DK/HK/73/76 [F]
CKZA02UP1102 [A]
CKZA02AL19 [A]
OSZA98KK98 [A]
DK/Korea/S17/03 [A]
DK/Hainan/6/04 [H]
DK/HK/1037-1/98 [G]
Consensus AILAAAGKSDKICIGYHANNSTTQVDTILEKNVTVTHSVELLENQKEERFCKILNKAPLDLRGCTIEGWI
80 90 100 110 120 130 140
Q/HK/1721-30/99 [B]
DK/HK/3461/99 [A]
CK/Calif/465/00 [C]
PT/Alb/179/93 [C]
MA/Alb/206a/96 [D]
TY/Ger/R04-5/02 [A]
MA/Neth/16/99 [A]
DK/HK/221/77 [E]
DK/HK/73/76 [F]
CKZA02UP1102 [A]
CKZA02AL19 [A]
OSZA98KK98 [A]
DK/Korea/S17/03 [A]
DK/Hainan/6/04 [H]
DK/HK/1037-1/98 [G]
Consensus LGNPQCDLLLGDQSWSYIVERPTAQNGICYPGVLNEVEELKALIGSGERVERFEMFPKSTWAGVDTSSGV
150 160 170 180 190 200 210
Q/HK/1721-30/99 [B]
DK/HK/3461/99 [A]
CK/Calif/465/00 [C]
PT/Alb/179/93 [C]
MA/Alb/206a/96 [D]
TY/Ger/R04-5/02 [A]
MA/Neth/16/99 [A]
DK/HK/221/77 [E]
DK/HK/73/76 [F]
CKZA02UP1102 [A]
CKZA02AL19 [A]
OSZA98KK98 [A]
DK/Korea/S17/03 [A]
DK/Hainan/6/04 [H]
DK/HK/1037-1/98 [G]
Consensus TKACPY-NSGSSFYRNLLWIIKTKSAAYPVIKGTYNNTGNQPILYFWGVHHPPDTNEQNTLYGSGDRYVR
220 230 240 250 260 270 280
Q/HK/1721-30/99 [B]
DK/HK/3461/99 [A]
CK/Calif/465/00 [C]
PT/Alb/179/93 [C]
MA/Alb/206a/96 [D]
TY/Ger/R04-5/02 [A]
MA/Neth/16/99 [A]
DK/HK/221/77 [E]
DK/HK/73/76 [F]
CKZA02UP1102 [A]
CKZA02AL19 [A]
OSZA98KK98 [A]
DK/Korea/S17/03 [A]
DK/Hainan/6/04 [H]
DK/HK/1037-1/98 [G]
Consensus MGTESMNFAKSPEIAARPAVNGQRGRIDYYWSVLKPGETLNVESNGNLIAPWYAYKFVSTNNGAVFKSNL

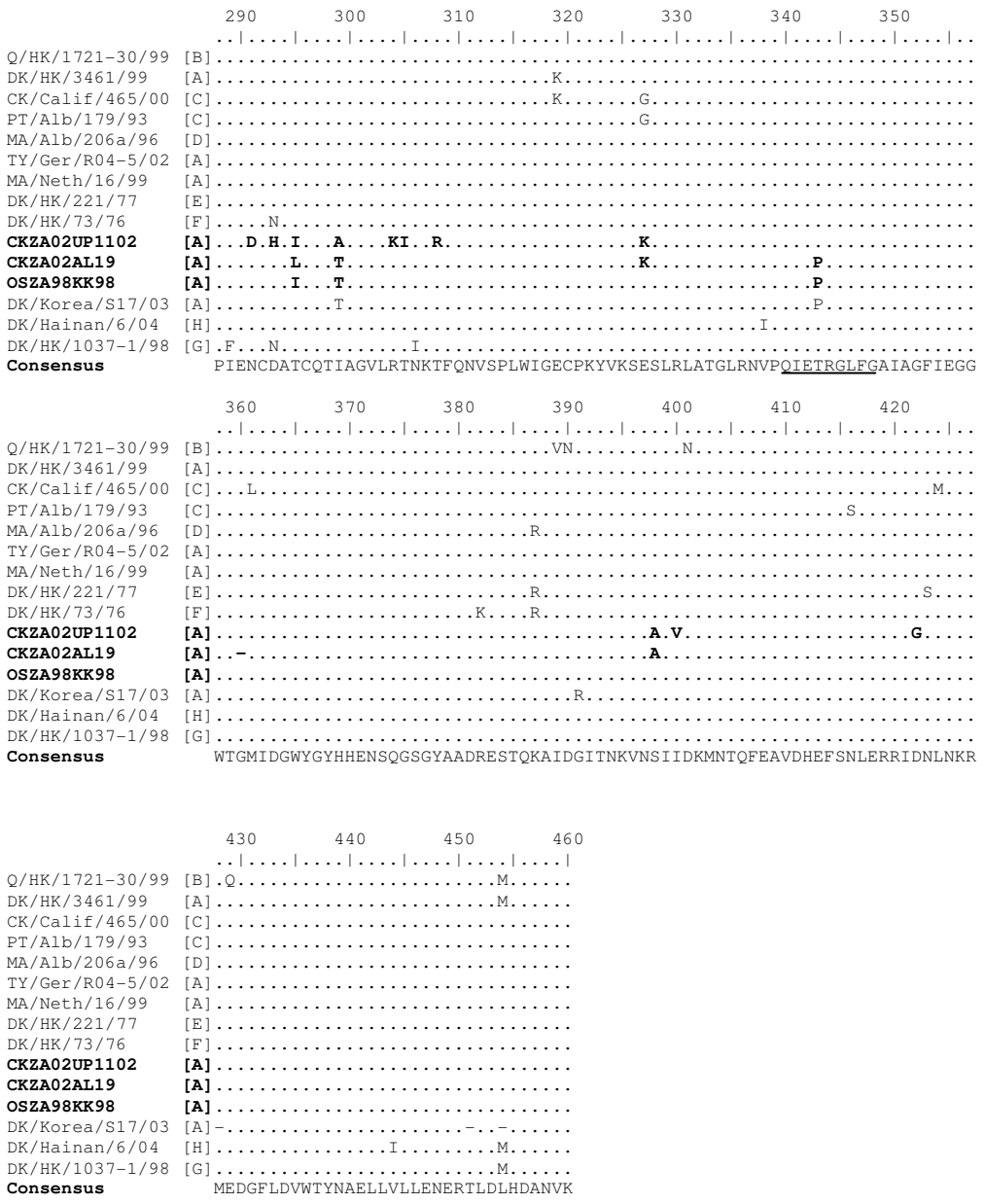


Figure 2.4 Amino acid alignment of full-length H6 genes. The hemagglutinin peptide cleavage site (H_0) at position 339 to 357 is underlined. South African viruses are indicated in boldface. Sub-lineages are indicated in square brackets.

2.3.2 Neuraminidase genes

2.3.2.1 N8 Neuraminidase genes

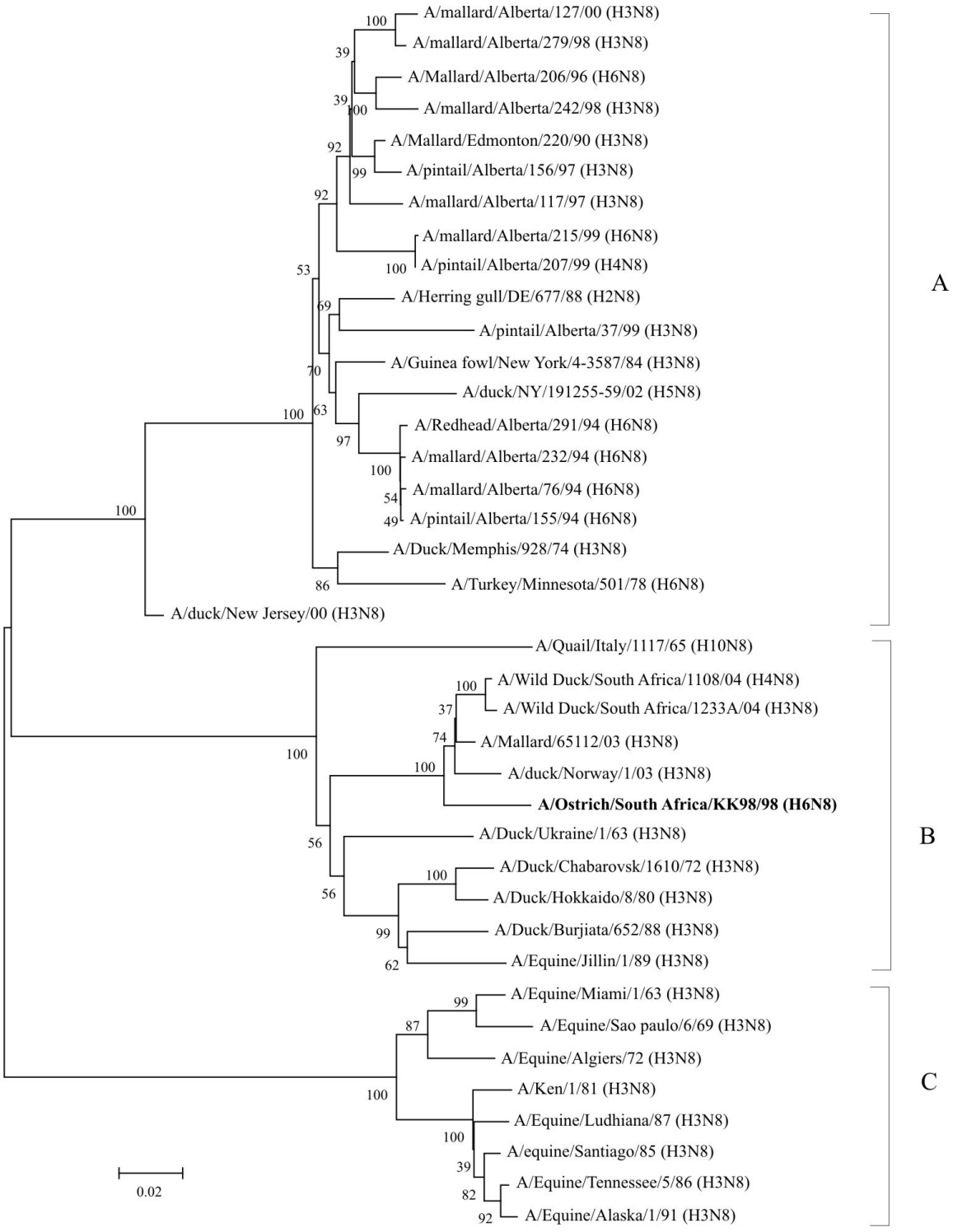


Figure 2.5 Phylogenetic tree inferred from a 1314-nt multiple sequence alignment of the neuraminidase (N8) genes of A/Ostrich/South Africa/KK98/98 (H6N8) (in boldface) and other viruses. Sub-lineages A to C are indicated.

Seroarcheological data indicate that an H2N8 virus caused the human influenza epidemic of 1889-1890 and that an H3N8 virus may have been responsible for the human epidemic of 1900 (Mulder and Masurel, 1958). The type N8 neuraminidase genes have evolved into at least three sub-lineages (Saito *et al.*, 1993), A to C, clearly distinguished in Figure 2.5. Sub-lineage A represents the North American lineage, comprising isolates from Canada and the USA. Most of these are either H6N8 or H3N8 viruses, although H5N8 and H2N8 viruses have been isolated. Sub-lineage B represents the Eurasian lineage, made up of strains from Italy, South Africa, Norway, Ukraine, Russia, China and Japan. The strains from Ukraine, Russia, Japan and China are all of the H3N8 subtype. The third lineage, C, contains the equine H3N8 viruses, isolated in many countries from 1963 to 1991, including the prototype of the N8 equine influenza virus, "equine 2 virus (H3N8)" that was isolated in Miami in 1963 (Waddell, 1963). A/Equine/Jilin/1/89 (sub-lineage B) was the causative virus of the 1989 influenza pandemic among horses in Northeast China (Webster and Guo, 1991). Although classified as an H3N8 subtype, it differed both antigenically and at the nucleotide/peptide sequence level from other equine 2 viruses, and is considered to be of avian virus origin (Guo *et al.*, 1992)

It is evident that the splitting within the N8 gene must have occurred some time before 1963 (the date of the earliest isolate), supported by relatively long branch lengths and bootstrap values of 100. Two other South African N8 viruses, A/Wild duck/South Africa/1108/04 (H4N8) and A/Wild duck/South Africa/1233A/04 (H3N8) (Chapter 3) are also included in Fig. 2.5. The topology indicates they are probably not direct descendants of the ostrich N8 gene, although these viruses had an earlier common ancestor. There were no deletions in the stalk region of the N8 genes, and therefore an amino acid alignment is not presented.

2.3.2.2 N2 Neuraminidase genes

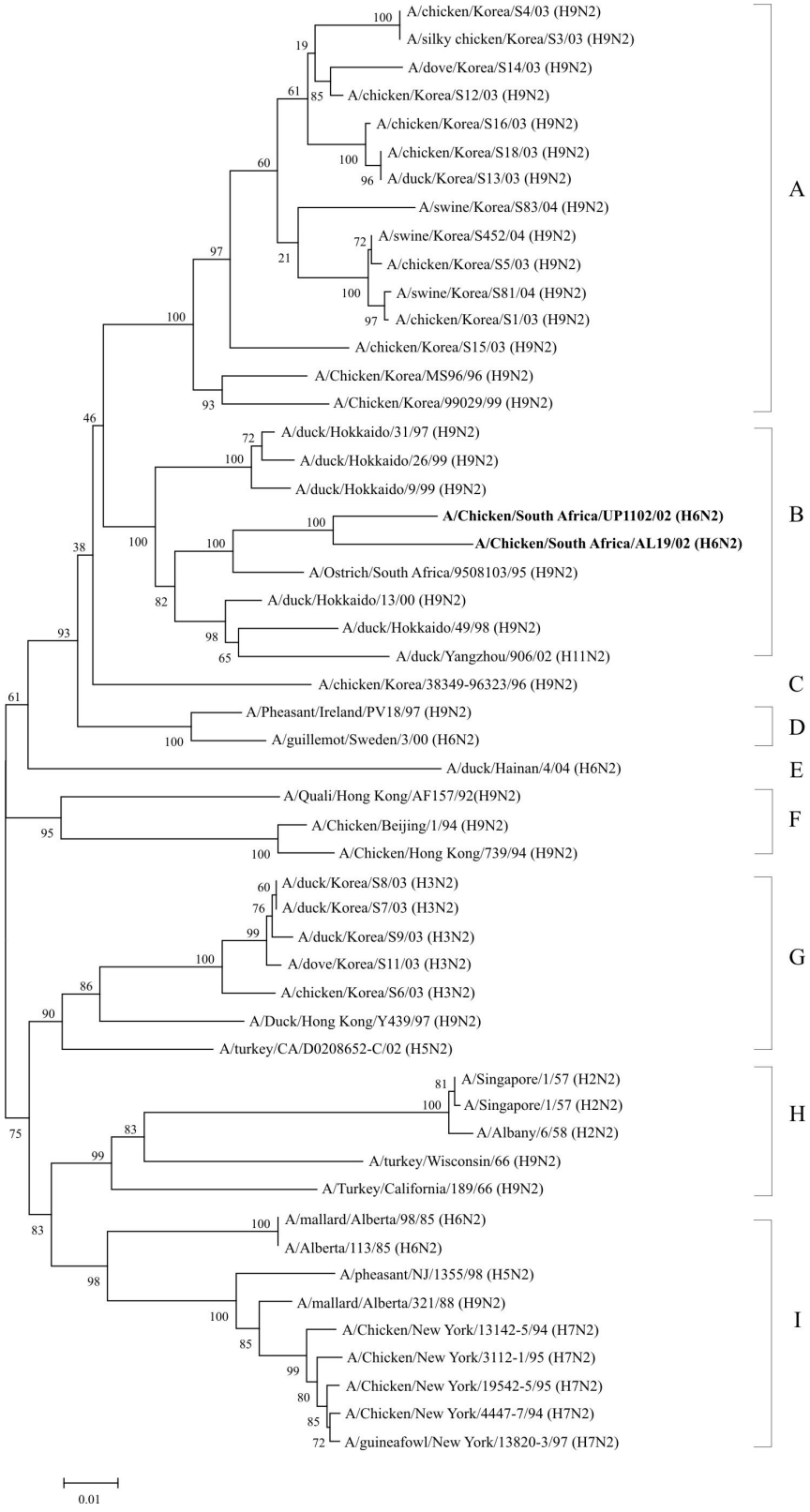


Figure 2.6 Phylogenetic trees inferred from a 1104-nt multiple sequence alignment of the neuraminidase (N2) genes of South African H6N2 (in boldface) and other viruses. Deletions or insertions were excised to simplify the phylogenetic analysis. Sub-lineages A to I are indicated.

Fig. 2.6 indicates that the closest relative to A/Chicken/South Africa/AL19/02 and A/Chicken/South Africa/UP1102/02 type N2 neuraminidase genes is the N2 gene from A/Ostrich/South Africa/9508103/95 (H9N2) isolated in 1995 from ostriches in the Oudtshoorn region, supported by a booststrap value of 100. Analysis of the amino acid sequences of the South African virus N2 genes and those of selected viruses from the sub-lineages A to I reveal several features that support the phylogenetic relationships. Firstly, A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) N2 genes originated from a common source, indicated by the shared unique characters L^{88}, E^{127} and N^{208} (shared only by A/Pheasant/Ireland/PV18/97) (Figure 2.7). The $K^{338}\rightarrow R$ substitution present in the two South African chicken virus N2 genes is unique within sub-lineage B, but present in sub-lineages E to I. Secondly, the relationship of the two South African chicken virus N2 genes to that of A/Ostrich/South Africa/9508103/95 (H9N2) (and other sub-lineage B genes) is supported by shared residues G^{41} and L^{82}. The A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) N2 genes possess several unique features: A/Chicken/South Africa/AL19/02 (H6N2) had ten unique amino acid substitutions, viz. M^{31}, T^{44}, T^{81}, A^{116}, Q^{143}, T^{153}, L^{240}, V^{262}, G^{271} and T^{275}, but the most noteworthy unique feature is the 22-amino acid deletion (52-78) in the stalk region of the NA gene. The predicted sequence of the N2 NA protein of the six Californian H6N2 chicken viruses from 2000 to 2001 also contained a deletion in the stalk region, but it was only 18 amino acids in length (represented by CK/California/139/01 in Fig. 2.6). No large deletions were found in the stalk regions of the aquatic birds sequenced in that study, indicating that the region may represent an adaptation for growth in chickens (Webby *et al.*, 2002; Kinde *et al.*, 2003). The deletion is absent in all other N2 genes within sub-lineage B, including A/Chicken/South Africa/UP1102/02 (H6N2). The latter had only eight unique amino acid substitutions, E^{39}, T^{42}, S^{77}, E^{86}, S^{161}, L^{210}, M^{249} and F^{332} within its N2 gene.

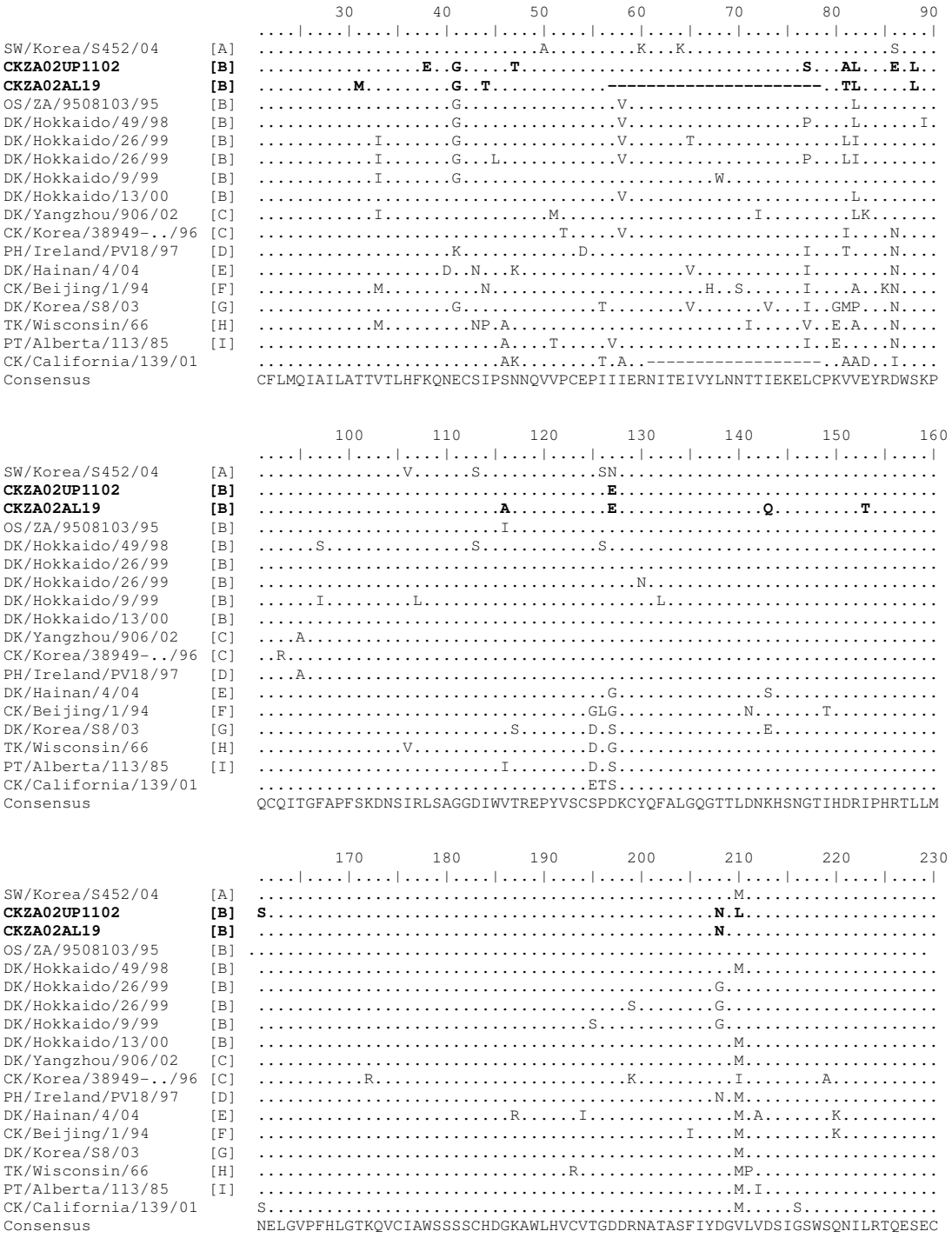


Figure 2.7 Multiple alignment of full-length N2 peptide sequences. Lineages are indicated in square brackets, and deleted amino acids are represented by (-).

Fig. 2.7 continued

```
                                240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
SW/Korea/S452/04          [A]   ..................................Q.....................................
CKZA02UP1102              [B]   ................M.....................................I.....
CKZA02AL19                [B]   .........L.....................V.........G...T.....................
OS/ZA/9508103/95          [B]   .......A.........................V.....................K.....
DK/Hokkaido/49/98         [B]   ................V.......VE.....................................R....
DK/Hokkaido/26/99         [B]   ..................................................................
DK/Hokkaido/26/99         [B]   ..................................................................
DK/Hokkaido/9/99          [B]   ..................................................................
DK/Hokkaido/13/00         [B]   ..................................................................
DK/Yangzhou/906/02        [C]   I.......................V..........................................
CK/Korea/38949-../96      [C]   ..............G.....................................................
PH/Ireland/PV18/97        [D]   ........................R....I...Q..................................
DK/Hainan/4/04            [E]   .......A..........V.......AR.................................I.....
CK/Beijing/1/94           [F]   ........................R.................V..........E.............
DK/Korea/S8/03            [G]   ........................R..........................................
TK/Wisconsin/66           [H]   .G....GP.....EG...N....V..NR....I.....................................
PT/Alberta/113/85         [I]   ...S.....................R.................V..........N.............
CK/California/139/01            ................K.............A...................Q.SN.............
Consensus                       VCINGTCTVVMTDGSASGRADTRILFIKEGKIVHISPLSGSAQHIEECSCYPRYPDVRCVCRDNWKGSNR
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```
                                310       320       330       340       350       360       370
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
SW/Korea/S452/04          [A]   .....K..............................................................
CKZA02UP1102              [B]   ............................F.....R...................S............
CKZA02AL19                [B]   ....................................R...............................
OS/ZA/9508103/95          [B]   ......V.............................................................
DK/Hokkaido/49/98         [B]   .........R...........................................................
DK/Hokkaido/26/99         [B]   .....S.....................................................S.....
DK/Hokkaido/26/99         [B]   .....S.....................................................S.....
DK/Hokkaido/9/99          [B]   .....S.....................................................S.....
DK/Hokkaido/13/00         [B]   ............N.............................................................
DK/Yangzhou/906/02        [C]   .....................................................................
CK/Korea/38949-../96      [C]   ........................E.....................................
PH/Ireland/PV18/97        [D]   .....................................................................
DK/Hainan/4/04            [E]   .VV.............................S...R.................D...........R..
CK/Beijing/1/94           [F]   .VLY............................S...R.......A.........N...I......K...
DK/Korea/S8/03            [G]   .V.............................S...R.................NE............
TK/Wisconsin/66           [H]   .V...K......N.G............S.........R.................N.D.....
PT/Alberta/113/85         [I]   .V.............................S...R.................N.............
CK/California/139/01            ..............................S...R.................D.............
Consensus                       PIIDINMADYSIDSSYVCSGLVGDTPRNDDSSSNSNCKDPNNERGNPGVKGWAFDYGNDVWMGRTISKDS
```

```
                                380       390       400
                                ....|....|....|....|....|....|....|....
SW/Korea/S452/04          [A]   .......K..D.....................................
CKZA02UP1102              [B]   .....................................
CKZA02AL19                [B]   .....................................
OS/ZA/9508103/95          [B]   .....................................
DK/Hokkaido/49/98         [B]   ........................I............
DK/Hokkaido/26/99         [B]   ...F.....................................
DK/Hokkaido/26/99         [B]   ................................C....
DK/Hokkaido/9/99          [B]   ................................C....
DK/Hokkaido/13/00         [B]   ................................C....
DK/Yangzhou/906/02        [C]   ..............M.....................
CK/Korea/38949-../96      [C]   .......K......M.....................
PH/Ireland/PV18/97        [D]   ..............A.....................
DK/Hainan/4/04            [E]   .......K.....I.....................V
CK/Beijing/1/94           [F]   .........V...........I..P....SD.....
DK/Korea/S8/03            [G]   .......K.....................................
TK/Wisconsin/66           [H]   ...............P.............S.........
PT/Alberta/113/85         [I]   ......K.....AI......T.................
CK/California/139/01            .......K..D..A.......T.................
Consensus                       RSGYETFRVIGGWTTANSKSQVNRQVIVDNNNWSGYSGI
```

To determine the presence or absence of the NA-stalk deletion in the rest of the South African H6N2 isolates, and to investigate whether there were any variations in the size of the deletion, the amplified NA genes of selected viruses were sequenced from the 5' end. The results are presented in Fig. 2.8.

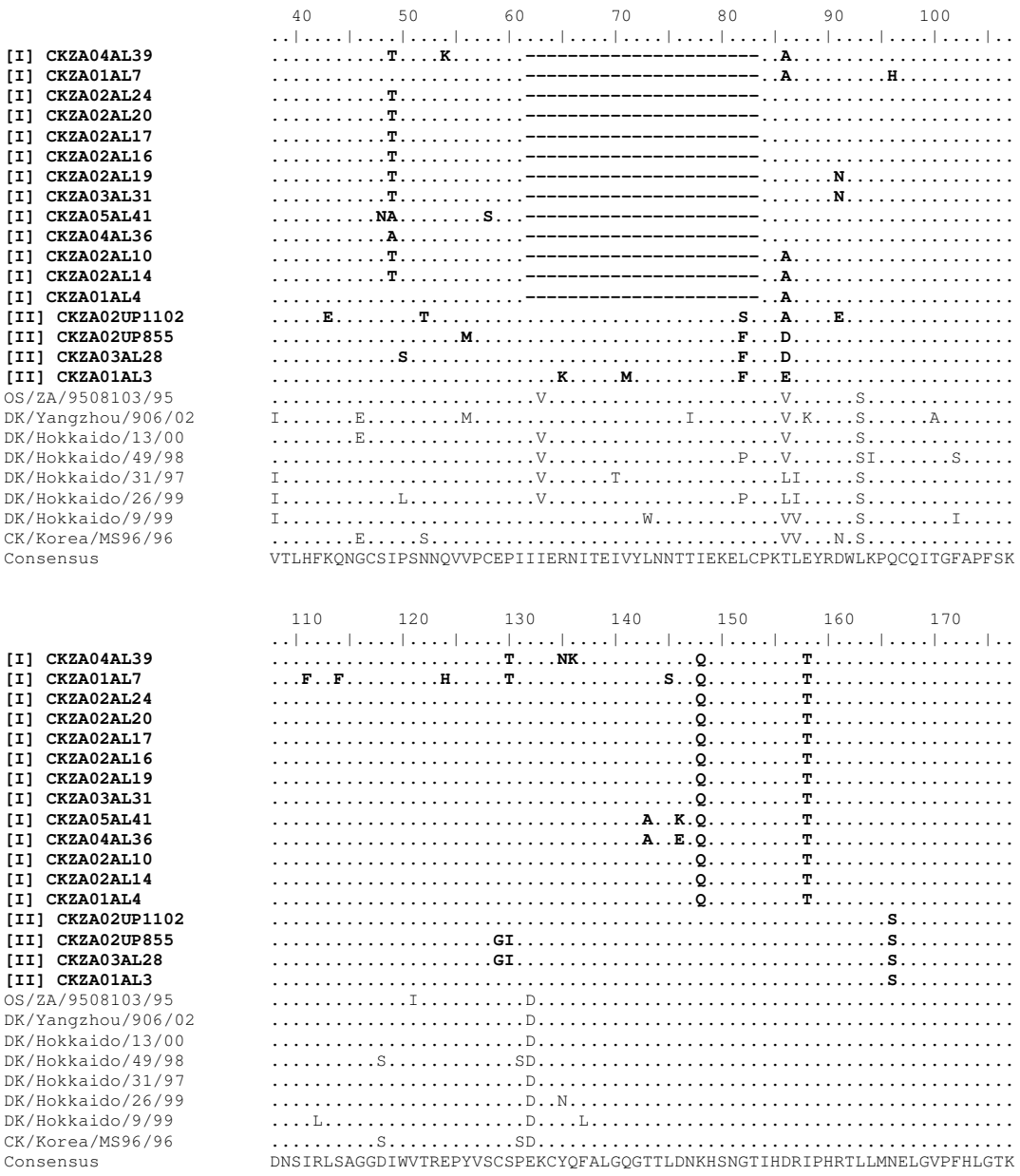


Figure 2.8 Multiple amino acid alignment of the N-stalk region of selected South African H6N2 (in boldface) and reference virus N2 genes. Sub-lineages I and II are indicated in brackets. Deleted amino acids are represented by (-).

Analysis of some of the South African N2 genes (Fig. 2.8) shows that all those containing the NA stalk deletion (CKZA04AL39- and 36; CKZA01AL7- and –4; CKZA02AL24-, -20, -17, -16, -19, -10 and 14; CKZA03AL31 and CKZA05AL41) belong to H6 hemagglutinin sub-lineage I (Fig. 2.1), whereas the remainder that lack the deletion (CKZA02UP1102, CKZA02UP855, CKZA03AL28 and CKZA01AL3), were sub-lineage II HA type (Fig 2.1). There were no additional insertions or deletions, which suggests a clonal expansion of each of the ancestral viruses, and that the progeny were relatively stable thereafter. Next, the internal gene sequences were analysed.

2.3.3 Matrix protein (M) genes

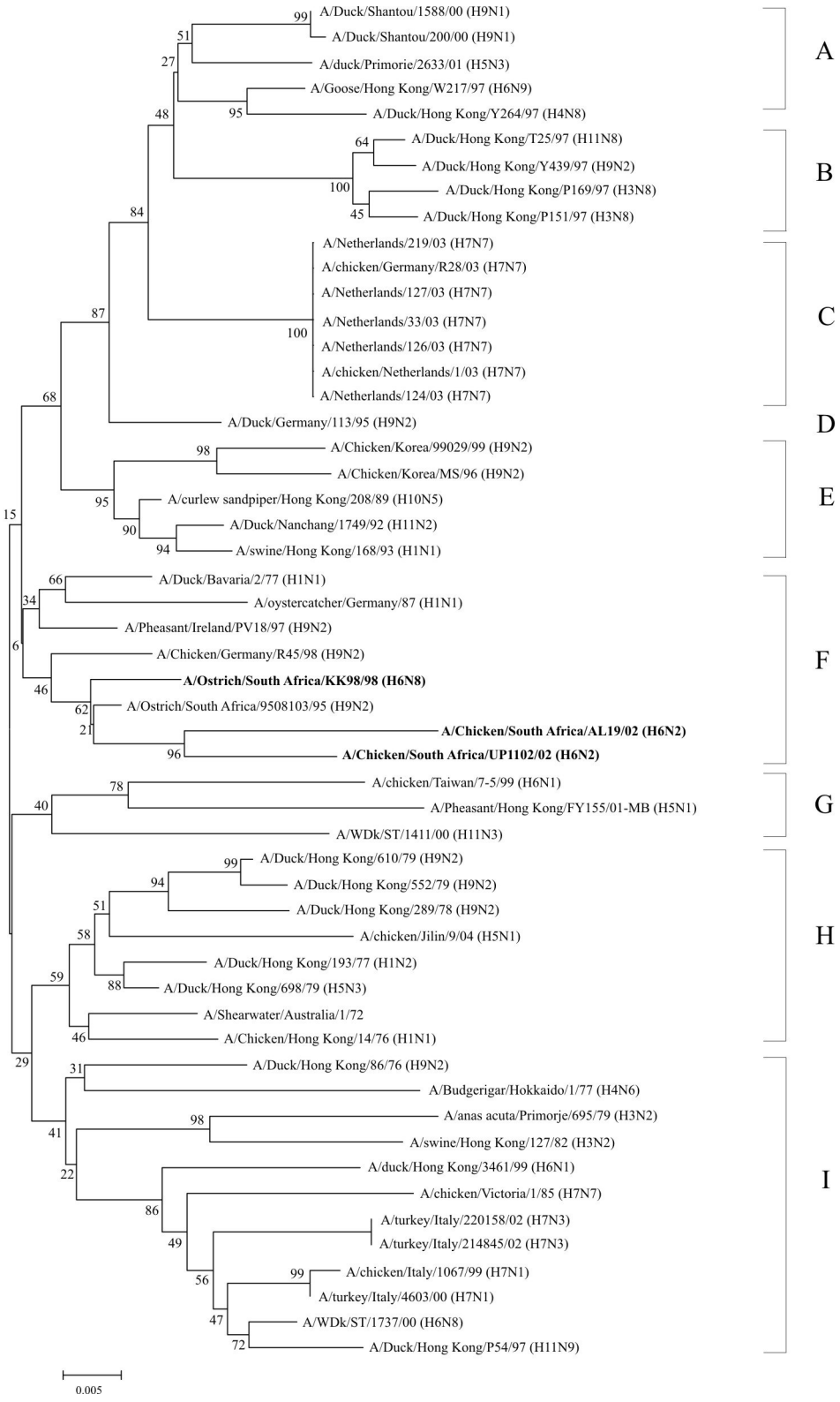


Figure 2.9 Phylogenetic tree inferred from a 797-nt multiple sequence alignment of the matrix (M) protein genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

Fig. 2.9 illustrates that A/Chicken/South Africa/AL19 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) shared recent common ancestors for their matrix protein genes. The nodes at which A/Ostrich/South Africa/9508103/95 (H9N2) and A/Ostrich/South Africa/KK98/98 (H6N8) virus M genes diverged are so close that it is impossible to conclusively identify which of these two viruses is the source of the M gene to the H6N2 viruses. Two other H9N2 viruses from the late 1990s (A/Pheasant/Ireland/PV18/97 and A/Chicken/Germany/R45/98) are located within sub-lineage F, suggesting that this particular lineage of M gene was common among H9N2 viruses at the time. Therefore the A/Ostrich/South Africa/9508103/95 (H9N2) virus is most likely to be the original source of the M gene, for both the H6N8 virus and either directly or indirectly (via H6N8) of the progenitor to the H6N2 chicken viruses.

2.3.4 Nonstructural protein (NS1) genes

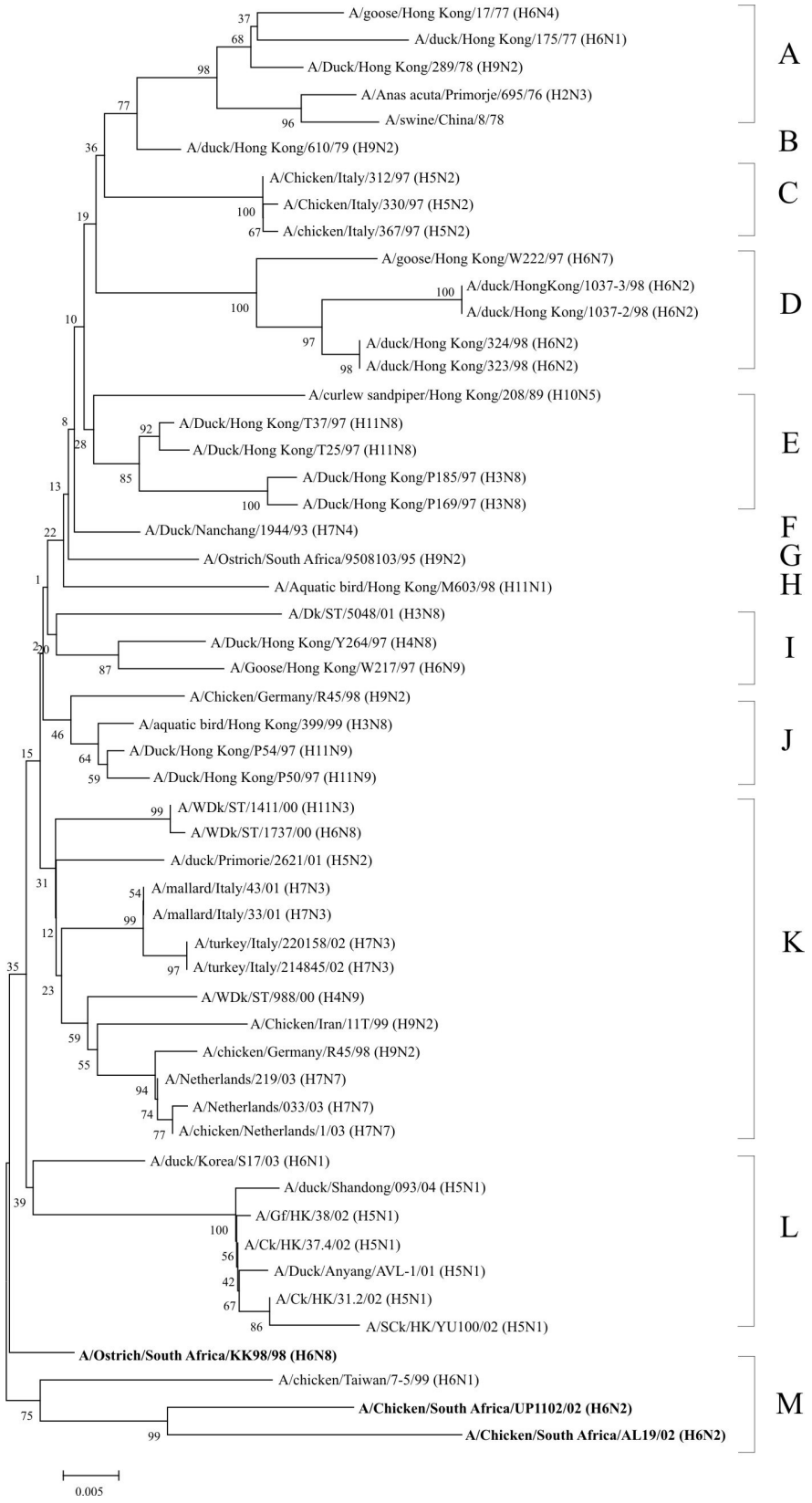


Figure 2.10(a) Phylogenetic tree inferred from a 763-nt multiple sequence alignment of the nonstructural (NS1) protein genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to M are indicated.

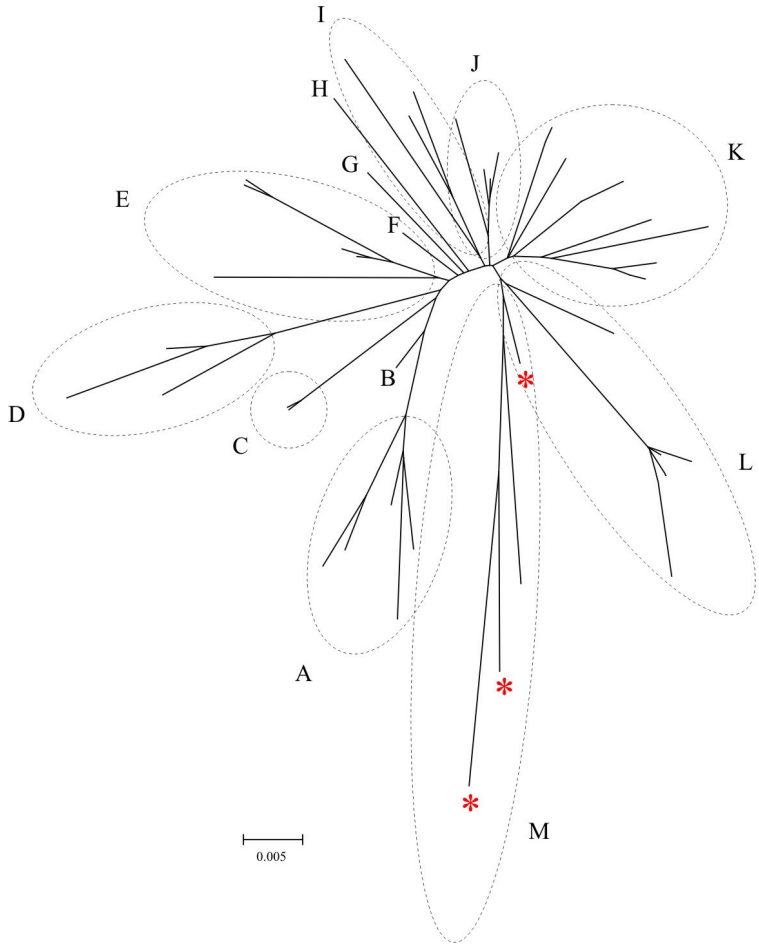


Figure 2.10(b) Radial version of Fig.2.10(a). South African viruses are indicated by "*".

All viruses in Figs. 2.10(a) and (b) belong to NS1 allele A. A/Ostrich/South Africa/KK98/98 (H9N2) and the two H6N2 representatives, A/Chicken/South Africa/UP1102/02 and A/Chicken/South Africa/AL19/02 are grouped in sub-lineage M, but sub-lineage M is separated from sub-lineage L by a low confidence bootstrap value node, therefore the grouping may not be significant Sub-lineage L contains NS1 genes of Korean and Chinese HPAI H5N1 viruses isolated from 2001 to 2004. The closest genetic relative to the South African chicken H6N2 virus NS1 genes is the NS1 of A/chicken/Taiwan/7-5/99 (H6N1). The branch lengths for the South African H6N2 viruses are particularly long, indicating that many point mutations occurred since the time that they split from their root. Generally, the chicken H6N2 viruses are a full percent closer to the H6N8 ostrich virus in nucleotide sequence identities than to the Taiwanese H6N1 virus (Table 2.4b). Three other genes from A/chicken/Taiwan/7-5/99 (H6N1) appear in the present study; its M gene falls within sub-lineage G in Fig. 2.9, the PA gene falls within sub-lineage D of Fig. 2.12, and the PB1 gene is the sole representative of sub-lineage G in Fig. 2.13. None of these genes have a closer

phylogenetic relationship to the South African viruses, and therefore, despite the topology in Fig. 2.10, it is more likely that the H6N8 virus, and not A/chicken/Taiwan/7-5/99 (H6N1), was the source of the NS1 gene. Interestingly the A/Ostrich/South Africa/9508103/95 (H9N2) NS1 gene was located in the unrelated sub-lineage G.

2.3.5 Nucleocapsidprotein (NP) genes

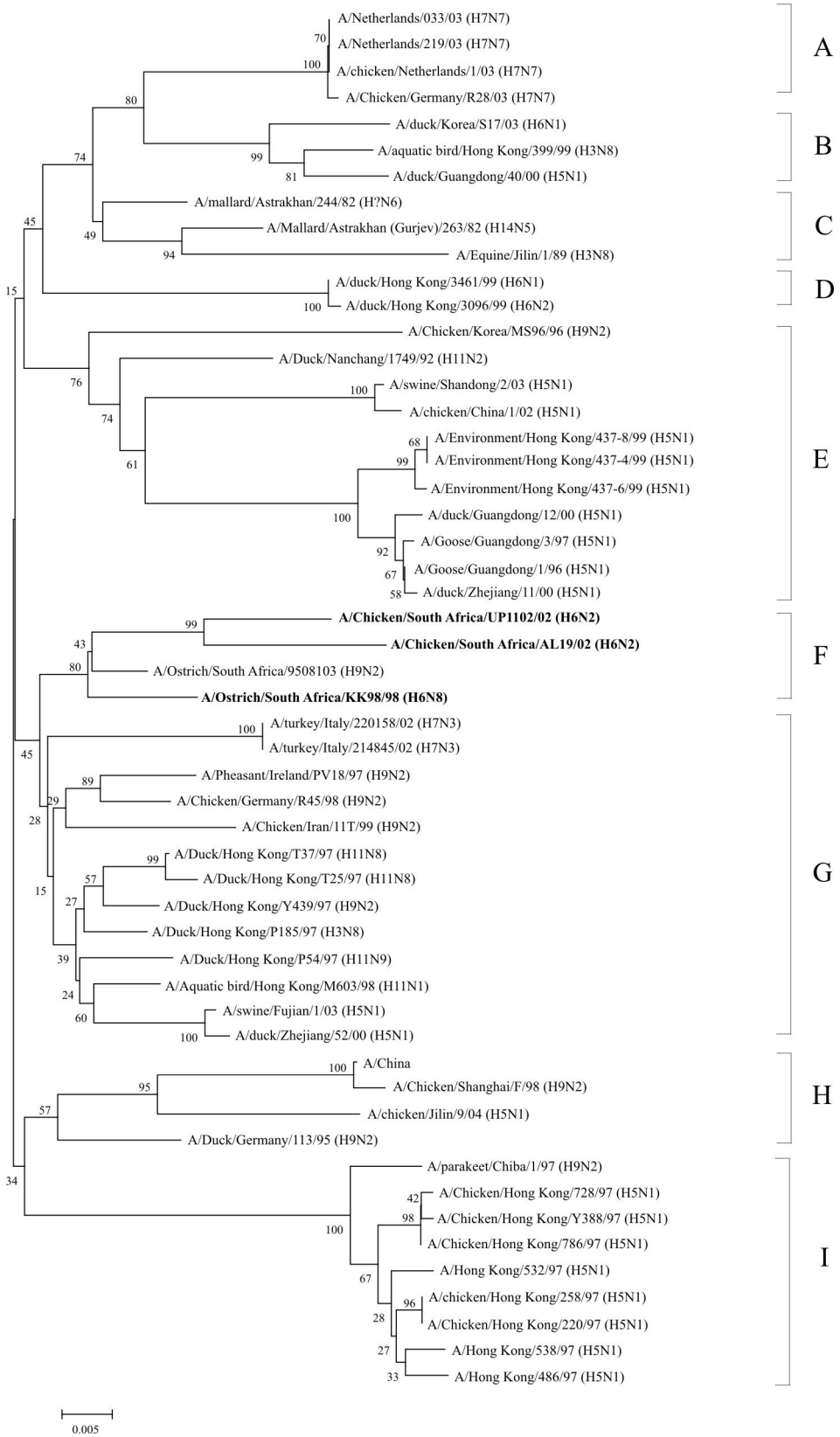


Figure 2.11 Phylogenetic tree inferred from an 863-nt multiple sequence alignment of the nucleocapsidprotein (NP) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated alongside

Sub-lineage F is comprised solely of the NP genes of South African isolates, and is separated by a low bootstrap value of 45 from sub-lineage G, containing isolates with a wide diversity in geographical origins and subtypes (Fig. 2.11). A high bootstrap support value of 80% groups A/Ostrich/South Africa/9508103/95 (H9N2) with the cluster containing the NP genes of A/Ostrich/South Africa/KK98/98 (H6N8) A/Chicken/South Africa/UP1102/02 (H6N2) and A/Chicken/South Africa/AL19/02 (H6N2). It appears that the H9N2 ostrich virus was the source of the NP genes for the other South African viruses. Furthermore, the proximity of three other H9N2 viruses in lineage G, A/Pheasant/Ireland/PV18/97, A/Chicken/Germany/R45/98 and A/chicken/Iran/11T/99 confirms that the H9N2 virus was the original source of the NP genes.

2.3.6 Polymerase A (PA) genes

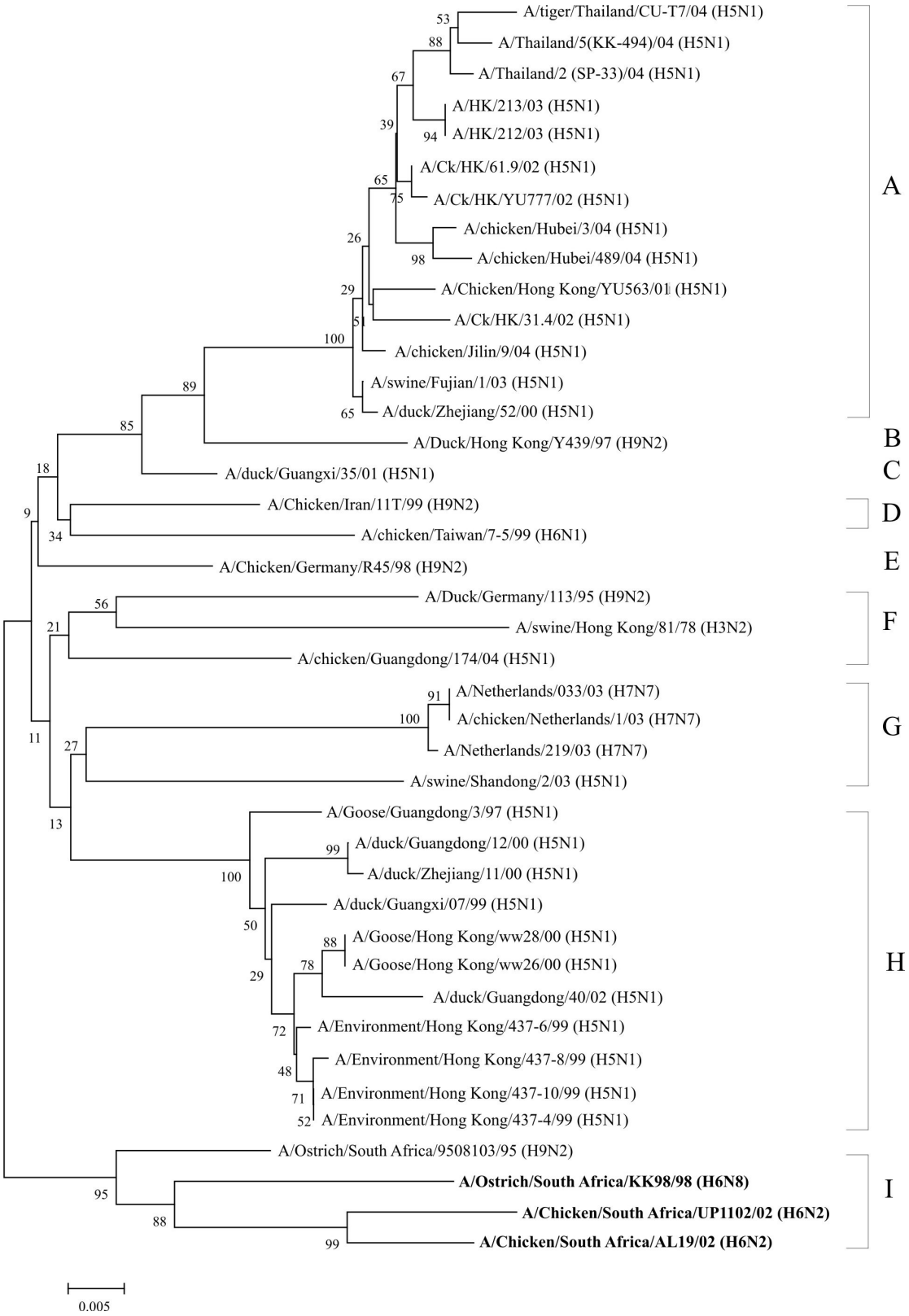


Figure 2.12 Phylogenetic tree inferred from a 735-nucleotide multiple sequence alignment of the polymerase A (PA) genes of South African H6N2, H6N8 (in boldface; *) and other viruses. Sub-lineages A to I are indicated.

Fig 2.12 illustrates that the PA genes of the South African viruses fall within a single sub-lineage (I), and that the topology within this sub-lineage is supported by high bootstrap values of between 95 and 98%. It suggests that the PA gene was passed from A/Ostrich/South Africa/9508103/95 (H9N2) to A/Ostrich/South Africa/KK98/98 (H6N8) and then to the common ancestor of the South African H6N2 viruses.

2.3.7 Polymerase B1 (PB1) genes

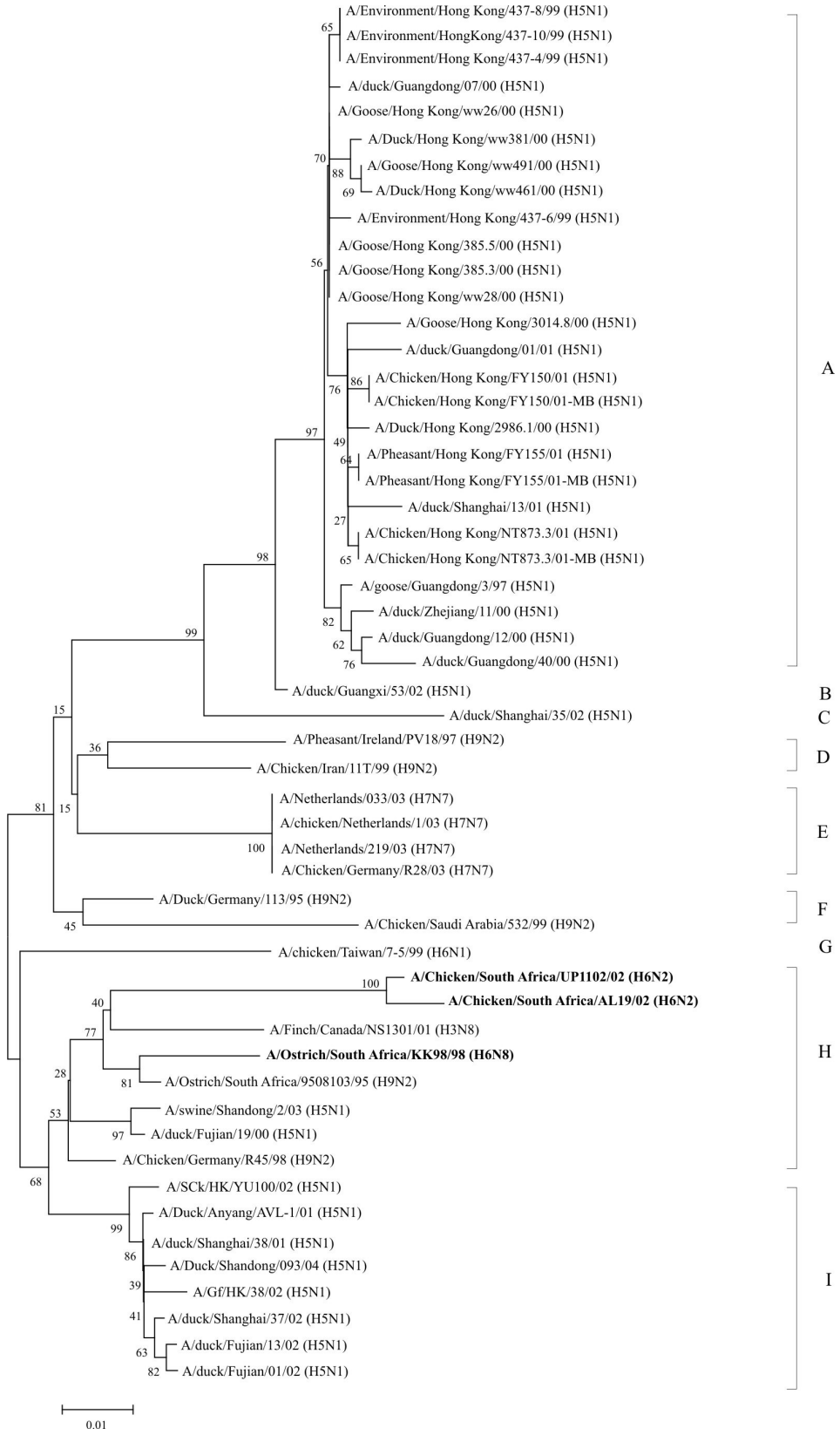


Figure 2.13(a) Phylogenetic tree inferred from a 668-nt multiple sequence alignment of the polymerase B1 (PB1) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

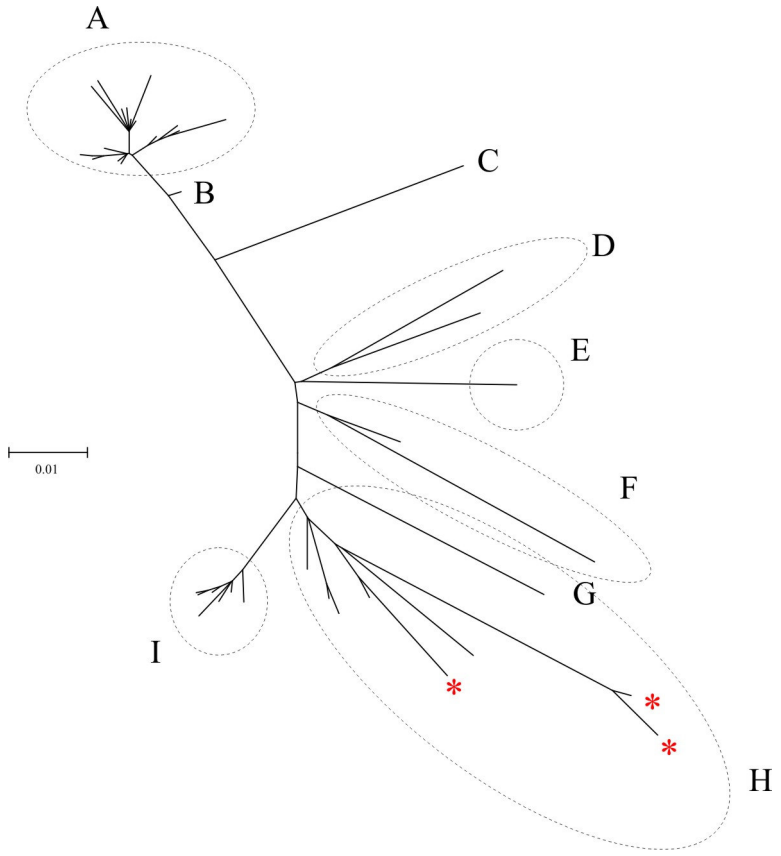


Figure 2.13(b) Radial version of Fig. 2.13(a). South African viruses are indicated by "*".

The South African ostrich (H9N2 and H6N8) and chicken (H6N2) virus PB1 genes cluster within sub-lineage H, along with PB1 genes of viruses isolated in the Far East, Germany and Canada (Fig. 2.13). Chicken/South Africa/UP1102/02 (H6N2) and A/Chicken/South Africa/AL19/02 (H6N2) PB1 genes shared a recent common ancestor, with 98% nucleotide sequence identities (Table 2.4a). Furthermore, the PB1 genes of A/Ostrich/South Africa/KK98/98 (H6N8) and A/Ostrich/South Africa/9508103/95 (H9N2) also share common ancestry (98% sequence identities), supported by a high bootstrap value of 81%. The phylogenetic relationships between the South African ostrich H9N2 and H6N2 chicken virus PB1 genes and A/Finch/Canada/NS1301/01 (H3N8) are poorly-supported with a low bootstrap value (40%). A/Finch/Canada/NS1301/01 (H3N8) was isolated in Canada from sick quarantined birds imported from the Netherlands (Pasick *et al.*, 2003), and should therefore be considered a Eurasian lineage virus (John Pasick, personal communication). No other genes similar to those of the A/Finch/Canada/NS1301/01 (H3N8) virus have been detected in South Africa. The nodes from which the common ancestor of the chicken H6N2, the finch H3N8, and the ostrich H9N2 and H6N8

viruses PB1 genes arise are very closely-situated, making it difficult to determine the exact origin of the H6N2 PB1 gene common ancestor. The lower nucleotide sequence homologies between the South African and ostrich and chicken virus PB1 genes (Table 2.4a) could be due to an increased mutation rate after the species barrier was crossed. It should be noted that sub-lineage H, containing the South African PB1 genes, shares a common ancestor with sub-lineage I, containing Asian HPAI H5N1virus PB1 genes.

2.3.8 Polymerase B2 (PB2) genes

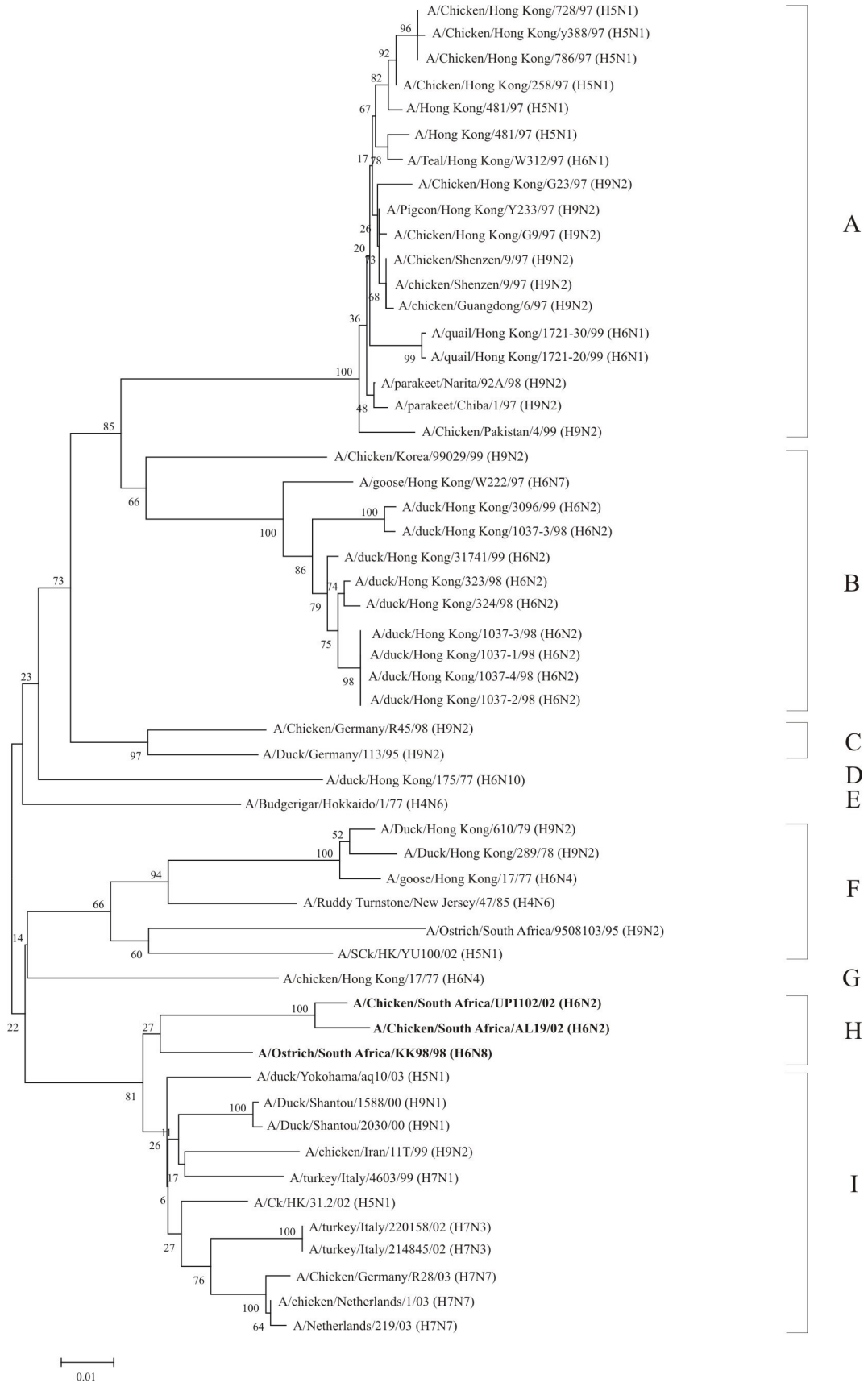


Figure 2.14 Phylogenetic tree inferred from a 738-nt multiple sequence alignment of the Polymerase B2 (PB2) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

The South African H6N2 and H6N8 PB2 genes cluster within sub-lineage H (Fig. 2.14), and sub-lineage H PB2 genes share a common origin with those of sub-lineage I, containing contemporary isolates from China, Japan and Iran (H5 and H9 types) and Italy, Germany and Holland (H7 types). A/Ostrich/South Africa/9508103/95 (H9N2) is located in sub-lineage F, where its PB2 gene shared only 85 to 87% sequence identities with the South African H6N2 and H6N8 viruses (Table 2.4a). It appears that the PB2 gene from A/Ostrich/South Africa/KK98 (H6N8) is the most recent common ancestor and probable donor of the PB2 gene to the common ancestor of A/Chicken/South Africa/UP1102/02 (H6N2) and A/Chicken/South Africa/AL19/02 (H6N2).

In summary of the phylogenetic analyses, Figure 2.15 illustrates the sources of the genes of the H6N8 and H6N2 viruses. The PB1, PA, NP, M and NS genes were passed from the H9N2 ostrich virus that circulated since 1995 to the H6N8 ostrich virus of 1998, and in turn these genes were passed on to the H6N2 chicken viruses first isolated in 2001. The N2 gene was passed directly from the H9N2 virus to the common ancestor of the H6N2 viruses. The H6N2 viruses derived their PB2 and HA genes directly from a very close relative of the H6N8 ostrich virus

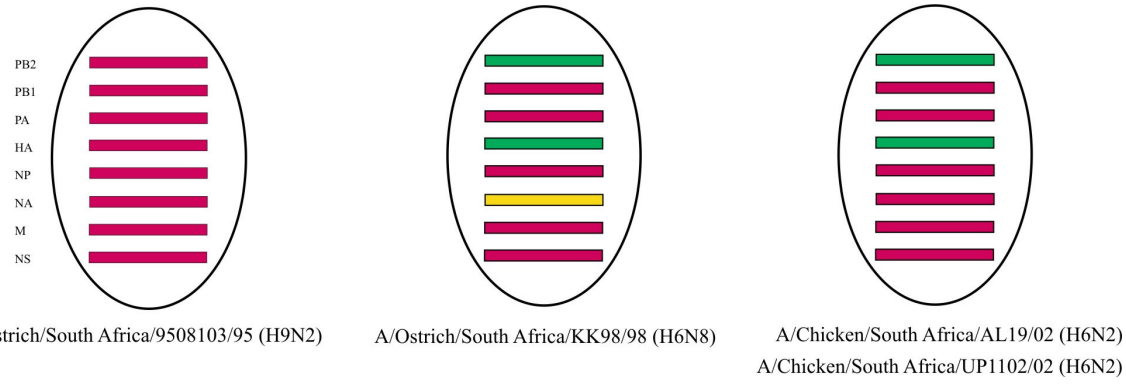


Figure 2.15 Schematic representation of the origins of genes of South African H6N2 viruses

Table 2.4(a) Percent nucleotide identities between genes

	A	B	C	D	E	F
Gene	**CKZA02AL19 vs CKZA02UP1102**	**CKZA02AL19 vs OSZA98KK98**	**CKZA02UP1102 vs OSZA98KK98**	**CKZA02UP1102 vs OSZA95(H9N2)[1]**	**CKZA02AL19 vs OSZA95(H9N2)[1]**	**OSZA98KK98 vs OSZA95(H9N2)[1]**
HA	94%	95%	94%	-	-	-
NA	95%	-	-	95%	96%	-
M	96%	96%	97%	97%	96%	98%
NP	96%	96%	96%	97%	96%	98%
NS	94%	94%	95%	94%	93%	97%
PA	97%	94%	94%	95%	95%	95%
PB1	98%	93%	94%	95%	94%	98%
PB2	98%	94%	95%	86%	85%	87%
Ave *excl	**96.0**	**94.57**	**95.0**	**95.5**	**95.0**	**97.2**

Table 2.4(b)

Gene	**CKZA02AL19 vs CKTW99(H6N1)[2]**	**CKZA02UP1102 vs CKTW99(H6N1)[2]**	**OSZA98KK98 vs CKTW99(H6N1)[2]**	**OSZA95(H9N2)[1] vs CKTW99(H6N1)[2]**
NS	93%	94%	97%	95%

Table 2.4(c)

Gene	CKZA02AL19 vs FNCA01(H3N8)[3]	CKZA02UP1102 Vs FNCA01(H3N8)[3]	OSZA98KK98 Vs FNCA01(H3N8)[3]	OSZA95(H9N2)[1] Vs FNCA01(H3N8)[3]
PB1	93%	94%	95%	96%

[1]A/Ostrich/South Africa/9508103/95 (H9N2)

[2]A/chicken/Taiwan/7-5/99 (H6N1) (Fig. 2.8(a))

[3]A/Finch/Canada/NS1301/01 (H3N8) (Fig. 2.11(a))

Column A represents the sequence identities between the genes of the two chicken H6N2 viruses, A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2). Columns B and C represent the comparisons of the nucleotide sequences of these two viruses, respectively, to A/Ostrich/South Africa/KK98/98 (H6N8). Columns D and E represent the comparison of A/Chicken/South Africa/UP1102/02 (H6N2) and A/Chicken/South Africa/AL19/02 (H6N2) to A/Ostrich/South Africa/9508103/95 (H9N2). The last column, F, represents the comparison of the two South African ostrich viruses, A/Ostrich/South Africa/9508103/95 (H9N2) and A/Ostrich/South Africa/KK98/98 (H6N8).

2.4 DISCUSSION

In this chapter, I demonstrated that the H6N2 viruses, although divided between two distinct genotypes (sub-lineage I and sub-lineage II), probably shared a recent common ancestor and that this ancestral virus arose from a reassortment event between close relatives of the 1995 H9N2 virus, A/Ostrich/South Africa/9508103, and the H6N8 virus, (A/Ostrich/South Africa/KK98/98) isolated in 1998. Both H6N2 sub-lineages I and II were isolated at Camperdown early on, sometimes even from the same farm. Although both sub-lineage I and II became widespread in the KZN Province, only sub-lineage II viruses were detected in the northern Gauteng province in this study. The outbreak was restricted to commercial chickens, and the probable mode of transmission was via the movement of eggs between commercial operations (S Bisschop, personal communication) or by vendors of spent hens (a.k.a. cull buyers) whilst moving between farms and their depots. A case where a breeder company in the North-West Province brought H6N2-infected male breeders to replace existing males was also reported (S Bisschop, personal communication).

H6N2 sublineages I and II circulated during roughly the same period, but sub-lineage I viruses contained multiple genetic markers associated with the adaptation of avian influenza viruses to chickens. Firstly, a 22-amino acid deletion in the stalk region of the NA gene was observed. NA stalk deletions have been shown to reduce the enzymatic activity of the protein (Luo *et al*., 1993) and, presumably, adversely affect the spread of the virus to uninfected cells. Secondly, changes in the HA gene that compensate for a shortened NA stalk have been described, including increased glycosylation near the receptor binding site thereby decreasing receptor binding affinity (Matrosovich *et al*., 1999; Wagner *et al*., 2000). Sub-lineage I displayed this hyperglycosylation, with five predicted N-glycosylations sites in the HA gene compared to only three in sub-lineage II, which did not contain the NA-stalk deletion. Thirdly, the presence of D^{144}, a proposed genetic marker that distinguishes terrestrial bird isolates from aquatic ones (Chin *et al.,* 2002), was observed in the sub-lineage I HA gene. These adaptations combined with the example of a particular strain (Fig 1, sub-lineage I(d)) persisting in a small geographical region (KZN South Coast) isolated for three years, supports the findings of other investigators (Suarez, 2000; Webby *et al*., 2003; Woolcock *et al*., 2003) that H6 viruses are capable of forming stable lineages in chickens.

A data set containing the parents of a hypothetical re-assorted virus, and two progeny genotypes that continue independently along their own evolutionary paths provides a rare opportunity to explore evolutionary rates and mechanisms. Sub-lineage I and -II H6N2 viruses recently diverged from their common ancestor, as few point mutations accumulated between them. By comparison of the gene nucleotide sequences, the PB1 and PB2 genes were found to be the most conserved between the H6N2 sub-lineages I and II. The PB1 and PB2 proteins are components of the ribonucleoprotein complex (RNP). Other phylogenetic studies have indicated that the evolution of these two genes appear to be less dependent on host factors than the other genes, and that avian PB2 lineages display less mutation than their human-derived counterparts. The conservative evolutionary rate of PB1 and PB2 explains their relative lack of host specificity (avian PB2 genes have reassorted into human strains) and predicts that there may not be significant host adaptation barriers to prevent these genes from becoming integrated into virus gene pools of alternate hosts. Unlike the NP gene (and possibly the PA gene), the PB2 and PB1 genes are not likely to be involved in the maintenance of host-specific virus gene pools (Gorman *et al*., 1990). Conversely, the HA and NS1 genes displayed the highest heterogeneity between H6N2 sub-lineages I and II. This phenomenon was also observed in isolates from a Mexican H5N2 outbreak that circulated unabated for 16 months starting in 2000 (García *et al*., 1997). The estimated evolutionary rates obtained for human influenza H1 and H3 genes ranged from 0.61 to 7.0 x 10^{-3} nucleotide substitutions per year (Gorman *et al*., 1992), but it was established that the Mexican chicken-origin H5N2 viruses acquired 28.1 x 10^{-3} nucleotide and 8.8 x 10^{-3} amino acid changes per year. These results suggested that nucleotide substitution rates in the HA gene of the H5 subtype AIVs increase significantly once the virus is introduced into commercial poultry.

The substitution rate for the Mexican isolates NS1 sequences was determined to be 19.5 x 10^{-3} per site per year (Gorman *et al*., 1992; García *et al*., 1997). The NS gene encodes two proteins: NS1 and NS2. The NS1, a non-structural protein, is localised in the nucleus independently of other viral proteins. Here it presumably interacts with host nuclear factors. NS2 is a structural protein found in the virions, that interacts with the M1 protein, and displays greater conservation than NS1 (Richardson & Akkika, 1991, Ludwig *et al*., 1991, Yasuda *et al*., 1993). The evolutionary rates of the NS1 protein have been determined to differ between species, and these differing rates of evolution are possibly due to specific interaction of NS1 with the host nuclear proteins (Kawaoka *et al*., 1998). A value of 97% sequence identities between ostrich isolates compared to 94% for chicken isolates suggests that the rate at which point

mutations in the NS1 gene are accumulated in ostriches is even slower than that of chickens.

Several studies have identified the NS1 gene as a potential molecular clock (Buonagurio *et al*., 1986; Nakajima *et al*., 1990) but it follows that the clock theory should only be applied if sequences of the same species are being compared. If the experimentally-determined mutation rate of 19.5 x 10^{-3} nucleotide substitutions per site per year for the Mexican H5N2 NS1 genes is extrapolated to a 6% (94% sequence identities) divergence between the two 2002 progeny H6N2 chicken virus NS1 genes, a theoretical value of 36.9 months is obtained for the point from which they split from their common ancestor. A/Chicken/South Africa/AL19/02 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) were isolated in July and September 2002, respectively, placing the evolutionary split at 37 months earlier, in the winter of June 1999. This predicted time span is most consistent with the isolation of H6N8 from ostriches a year earlier in 1998.

The most likely vectors for the introduction of AIV into the Western Cape ostrich population are the wild waterfowl with which ostriches are in contact with through the attraction of wild birds to water and feed troughs, or who graze on ostrich pastures each winter. In fact, H6 antibodies were detected in the serum of an Egyptian goose in the Oudtshoorn region during the winter of 1998 (Pfitzer *et al*., 2000), around the same time that the H6N8 virus was isolated from the ostriches. The isolation of the H9N2 virus from ostriches in 1995 coincided with a global pattern of H9N2 outbreaks during the 1990s. Phylogenetic analyses of H9 subtype outbreak viruses from across the world indicated that they originated from separate introductions from feral birds, that H9 viruses are heterogeneous, and that the H9 pool is maintained in *Charadriiformes* (Banks *et al.*, 2000). The latter finding is interesting, and the proposed role of the shorebirds in the introduction of AI viruses into South Africa is discussed in the closing chapter. There is mounting evidence that ostriches exhibit atypical (for poultry) and often sub-clinical responses to infections with AI viruses, even with highly pathogenic strains (Manvell *et al.,* 2003; Clavijo *et al*., 2003). Therefore, it is possible that mature ostriches may act as mixing vessels for strains of avian influenza viruses without showing clinical disease (Clavijo *et al.,* 2003). It is not clear when or how the disease initially spread from ostriches to chickens as ostrich and commercial poultry production are separated by geographical and climatic boundaries. Wild waterfowl are one possibility, as the immune response in waterfowl

is not long lasting, and waterfowl can be re-infected with the same strain (Kida *et al*., 1980), H6 in this case.

The results of this chapter suggest that ostriches may act as mixing vessels for AIV subtypes, although wild waterfowl are also good candidates. These viruses potentially pose a threat to the poultry industry if there is a breakdown in the implementation of biosecurity measures. In the case of H6N2 in South Africa, the infection seems to have spread by the movement of infected chickens and not by wild waterfowl, since the outbreak was limited to commercial chickens. Furthermore, the presence of two internal genes, viz. NS1 and PB1, sharing recent common ancestors with those of current Asian HPAI H5N1 strains in the South African AIV gene pool is a cause for concern, particularly since notifiable strains have been isolated from South African ostriches in the past and the reassortment of HPAI strains is a possibility. The potential for long-term maintenance of avian influenza viruses in ostriches and local waterfowl is an area that requires more research.

CHAPTER THREE[1]

PHYLOGENETIC ANALYSIS OF AVIAN INFLUENZA VIRUSES ISOLATED FROM OSTRICHES AND WILD WATERFOWL IN SOUTH AFRICA IN 2004

ABSTRACT

South Africa recorded its first case of high pathogenicity avian influenza (HPAI) since 1961, when an H5N2 strain caused mortalities in ostriches of the Eastern Cape province in 2004. In the same year, LPAI H5N2, H5N1, H3N8 and H4N8 viruses were isolated from wild ducks in the Gauteng and Western Cape provinces. Phylogenetic and genetic characterization indicated that the LPAI H5N2 virus, detected in an Egyptian goose in the Western Cape province, was the probable precursor to the ostrich outbreak strain. The ostrich H5N2 virus had acquired the characteristic multi-basic amino acid insertion at H_0, typical of HPAI strains, but lacked an N-stalk deletion. A second potential virulence determinant, a deletion in the NS1 gene was also detected, however, the IVPI value obtained was characteristic of an LPAI virus. The LPAI H5N1 virus lacked molecular determinants associated with virulence for poultry and mammals, and none of the South African H5 viruses were closely-related to Asian HPAI H5N1 strains. Reassortment within the South African gene pool was evident, indicating the mixing of wild duck populations and exchange of viruses from geographically-separated regions of the country. Additionally, a North American lineage H5 gene was detected by RT-PCR. All other genes shared recent common ancestors with Eurasian viruses, suggesting that AIVs are periodically introduced into South Africa via migratory birds.

[1]Part of the results presented in this chapter were published in *Developments in Biologicals* 124:189-199 (2006)

3.1 INTRODUCTION

Waterfowl play an important role in the maintenance, re-assortment and transmission of AIVs. All sixteen HA and nine NA-types have been isolated from ducks, geese and shorebirds, suggesting that they are the hosts of origin (Webster *et al*., 1992; Alfonso *et al*., 1995; Hanson *et al*., 2003). Although data on the prevalence on AIV in South African wild waterfowl is limited, South Africa was the location of the first ever report of the isolation of influenza A viruses from wild birds, an event that coincided with the first documented case of HPAI in South Africa. In April of 1961, over 1300 migrant Common terns (*Sterna hirundo*) died along the Cape coast between Port Elizabeth and Lamberts bay. A virus was isolated and was determined to be an HPAI H5N3 strain (Becker, 1966).

The second case of HPAI in South Africa involved an outbreak of an H5N2 strain in ostriches in 2004. After an unreported initial three month period characterized by low-grade AIV infection in ostriches, in mid-July 2004 mortalities suddenly and inexplicably increased to 44.5% in ostrich flocks on two farms around the towns of Bedford and Somerset-East in the Middleton area of the Eastern Cape Province. The birds displayed symptoms of respiratory distress, swelling, exudates from the eyes, fluorescent green diarrhoea, depression and emaciation before collapse and death (Adriaan Olivier, personal communication). Serological tests indicated the presence of an H5-type AI virus, and a quarantine zone was enforced by the National Directorate of Animal Health, but by then the disease had already spread to other ostrich farms within the 30 km quarantine radius. Organ samples were collected from ostriches on ten farms for virus isolation, but only one AI virus was isolated. Real-time RT-PCR (rRT-PCR), sequencing and molecular characterisation of the HA and NA surface glycoproteins indicated that the viral isolate was an HPAI H5N2 strain (results of this chapter). By the 6th of August 2004, the OIE had been notified of the outbreak and the molecular characterization results were confirmed by staff at the Weybridge laboratory (UK), who also performed pathogenicity tests in chickens. In late September 2004, forward and backward tracing and extensive serological testing led to the discovery of a new pocket of virus on an ostrich farm near Fort Brown, north of Grahamstown, 160 km away from the original outbreaks. Apparently this farm had exchanged birds with farms in the area of the original outbreak, prior to enforcement of the quarantine measures. Sero-positives in the Camdeboo and Ikwezi municipalities were later detected by forward and backward tracing (Department of

Agriculture). During the Eastern Cape outbreak, a total of seventeen out of 186 (9.1%) of ostrich farms tested positive for the H5 serotype by HI tests, and tracheal or cloacal swabs from six out of 136 (4.4%) farms tested positive for H5 virus by RT-PCR. A further five farms were found to be positive for the presence of H5 virus by nucleic acid sequence-based assay (NASBA), but no viruses could be isolated (M Romito, personal communication). Fortunately, no chickens or pigs were found to be serologically positive for H5 AIV during the Eastern Cape ostrich outbreak, and only a single worker on one of the infected ostrich farms sero-converted, but did not develop any clinical symptoms (G Gerdes, personal communication).

In an attempt to control the disease and prevent the spread to other regions, government authorities decided to cull all the poultry and ostriches in the affected area. A total of 25945 ostriches were culled with compensation being paid to the farmers on 37 infected and in-contact farms. Following the AI outbreak in the Eastern Cape, the National Directorate of Animal Health ordered all provinces to conduct serological surveillance for AI. Thus, 42 ostrich farms in the Western Cape were found to be serologically positive for H5 AIV, but attempts at virus isolation or virus detection by RT-PCR and NASBA were unsuccessful. Neither farmers, state- nor private veterinarians in the Western Cape had reported mortalities or any symptoms. All suspicious farms were put under quarantine and movement restrictions were imposed. Fortunately no sero-conversion was observed on the 42 farms, and no new H5-seropositive farms were detected. The control measures seemed to have been successful and South Africa officially declared itself free of HPAI on 13 September 2005.

Wild waterfowl have been the prime suspects in the introduction of avian influenza into Western Cape ostriches since the early 1990s (D Verwoerd, personal communication). About 20 species of the order *Anseriiformes* (ducks and geese) occur naturally in South Africa, and most of them are indigenous to the region. In addition, about 90 species of the order *Charadriiformes* (gulls, terns and waders) have been recorded in southern Africa, and at least two dozen of these are Palaearctic migrants that breed on the Siberian tundra and over winter in South Africa annually (Appendix 4; Underhill *et al*., 1999). Data on the prevalence of AIV infections in southern African waterfowl are limited, and prior to the surveillance of waterfowl described in this chapter, the only other survey was conducted in Oudtshoorn (Western Cape province) in the winter of 1998. In that study, specimens from 262 wild birds

including the Egyptian goose (*Alopchen aegypticus*), yellowbilled duck (*Anas undulata*), shelduck (*Tadoma cana*), African black duck (*Anas sparsa*), redbilled teal (*Anas erythrorhyncha*), hadeda ibis (*Bostrychia hagadash*), sacred ibis (*Threskiomis aethiopicus*), cattle egret (*Ibis bubulcus*), redknobbed coot (*Fulica cristata*), cormorant (*Phalacrocorax spp*.), darter (*Anhinga melanogaster*) and hammerhead stork (*Scopus umbretta*) were collected. Eight H10N9 viruses were isolated from organ pools, although the birds were serologically negative for H10N9 antibodies. One bird was serologically positive for H6 virus. These results confirmed that wild birds in the Oudtshoorn area had been infected with H10N9 and H6 avian influenza viruses (Pfitzer *et al*., 2000). The role of wild birds in the introduction of H5N2 to the Cape ostriches was speculatory, until a low pathogenicity H5N2 virus was detected in an Egyptian goose (*Alopochen aegypticus*) from one of the Western Cape ostrich farms in June 2004, just one month prior to the disease outbreak in the Eastern Cape. During the same period, H3N8, H4N8 and H5N1 viruses were isolated from indigenous wild ducks at the Blesbokspruit wetland in the Gauteng province. The objectives of this study were to characterise the 2004 HPAI H5N2 Eastern Cape ostrich outbreak virus and to phylogenetically compare it to LPAI H5N2, H5N1, H3N8 and H4N8 viruses isolated from wild ducks in the same year. Furthermore, the virulence markers of the H5N2 and H5N1 viruses and their phylogenetic relationships to the Asian HPAI H5N1 outbreak strain were investigated to determine their threat to human health.

3.2 MATERIALS AND METHODS

3.2.1 Viruses

3.2.1.1 Ostrich virus

Organ samples from 1500 ostriches with symptoms of AI that died on ten farms in the Eastern Cape were sent to both the Stellenbosch Provincial Veterinary Laboratory (SPVL) and Onderstepoort Veterinary Institute (OVI). Both SPVL and OVI isolated an H5N2 virus from the same sample. Thus, two viruses were isolated, but the cases were identical. After characterisation (this chapter) the virus was named A/Ostrich/South Africa/N227/04 (H5N2).

3.2.1.2 Wild duck viruses

From April to July 2004, 53 specimens of local waterfowl including yellow billed ducks (*Anas undulata*), red billed teals (*Anas erythrorhyncha*), red knobbed coots (*Fulica cristata*), Cape teals (*Anas capensis*) and Cape shovellers (*Anas smithi*i) were hunted by a trained falcon at the Blesbokspruit wetlands (26º 17' S; 28º 30' E) in the Gauteng province (D. Verwoerd). Homogenates of organ pools (Table 3.1) were injected into 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs (standard procedures, OIE manual). Three hemagglutinating agents were isolated and confirmed to be orthomyxoviruses under electron microscopy (EM), at the Department of Veterinary Tropical Diseases, University of Pretoria (Onderstepoort). Organs from an Egyptian goose were submitted by Dr Adriaan Olivier (Klein Karoo Laboratory) to OVI for testing. The bird had been culled by a farmer in the Oudtshoorn region on the 10th of June 2004. Initially, RNA extraction was performed directly on tissue homogenates and an AI virus was detected by RT-PCR. Later attempts at virus isolation were unsuccessful, possibly because repeated freeze-thawing of the tissues had destroyed any viable viruses. After characterization, this virus was named A/Egyptian Goose/AI23/04 (H5N2).

The viruses isolated in South Africa in 2004 are listed in Table 3.1. Serotypes were established by nucleotide sequencing (results of this chapter).

Table 3.1 Viruses isolated/detected in South Africa during winter of 2004

Isolate	Serotype	Location	Host	Accession numbers
A/Ostrich/South Africa/N227/04	HPAI H5N2	Sommerset East region, Eastern Cape province	Ostrich	Pending[1]
A/Duck/South Africa/1108/04	LPAI H3N8	Blesbokspruit, Gauteng province	Cape teal/ Cape shoveller	EF041487-EF041494
A/Duck/South Africa/1108/04	LPAI H5N?	Blesbokspruit, Gauteng province	Cape teal/ Cape shoveller	EF041503
A/Duck/South Africa/1233A/04	LPAI H4N8	Blesbokspruit, Gauteng province	Red-billed teal	EF041495-EF041502
A/Duck/South Africa/811/04	LPAI H5N1	Blesbokspruit, Gauteng province	Yellow-billed duck	EF041479-EF041486
A/Egyptian Goose/AI23/04	LPAI H5N2	Oudtshoorn, Western Cape province	Egyptian goose	Pending[1]

[1]The genetic sequences for A/Ostrich/South Africa/N227/04 and A/Egyptian Goose/AI23/04 will be published in collaboration with VLA Weybridge as part of a collaborative phylogenetic study of H5N2 viruses isolated in Europe and South Africa.

3.2.2 RNA extraction

Viral RNA was extracted from allantoic fluid using TRIzol® reagent (Gibco, Invitrogen), or the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's instructions. The Egyptian goose virus RNA was extracted by Josephine Mitchell, PCR Diagnostic Laboratory from tissue homogenates using a MagnaPure system (Roche).

3.2.3 Real-time RT-PCR

Real-time RT-PCR (rRT-PCR) was initially performed on the ostrich HPAI H5N2 virus because the fastest possible determination of the pathotype was required in the outbreak situation. A LightCycler system (Roche) was used. rRT-PCR was performed with an RNA Master SYBR Green I V3 kit (Roche Molecular Biochemicals) and H5-specific oligonucleotides were used to amplify the region containing the critical H_0 pathotype determinant. Each reaction was carried out with 5 µl of extracted RNA and 15 µl reaction mixtures consisting of 0.7 µl of each primer at a concentration of 5 pMol each (H5HA1 and H5HA2, Starick *et al*., 2000), 1.3 µl MnOAc, 7.5 µl RNA Master SYBR Green I mix (containing Tth DNA polymerase, reaction buffer, dNTP

[with dUTP instead of dTTP] and SYBR Green I), and 4.8 µl of dH_20. The thermal cycling protocol is detailed in Table 3.2. Amplicons were subjected to melting curve analysis. The melting temperatures of the peaks were analysed using the best-fit analysis software provided by Roche Molecular Biochemicals. rRT-PCR products were visualised on 1% agarose, and the 534 bp amplicon was excised for DNA extraction and cycle sequencing.

Table 3.2 LightCycler Experimental protocol to amplify the HPAI H5N2 HA gene

PROGRAM 1- REVERSE TRANSCRIPTION			
Cycle Program Data	*Value*		
Cycles	1		
Analysis Mode	None		
Temperature Targets	**Segment 1**		
Target Temperature (°C)	61		
Incubation time (h:min:s)	00:20:00		
Temperature Transition Rate (°C/s)	20.0		
Secondary Target Temperature (°C)	0		
Step Size (°C)	0.0		
Step Delay (Cycles)	0		
Acquisition Mode	None		
PROGRAM 2- DENATURATION			
Cycles	1		
Analysis Mode	None		
Temperature Targets	*Segment 1*		
Target Temperature (°C)	95		
Incubation time (h:min:s)	00:00:30		
Temperature Transition Rate (°C/s)	20.0		
Secondary Target Temperature (°C)	0		
Step Size (°C)	0.0		
Step Delay (Cycles)	0		
Acquisition Mode	None		
PROGRAM 3- AMPLIFICATION			
Cycles	45		
Analysis Mode	Quantification		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target Temperature (°C)	95	55	72
Incubation time (h:min:s)	00:00:01	00:00:10	00:00:20
Temperature Transition Rate (°C/s)	20.0	20.0	2.0
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	None	Single
PROGRAM 4- MELTING CURVE ANALYSIS			
Cycles	1		
Analysis Mode	Melting Curve		
Temperature Targets	Segment 1	Segment 2	Segment 3
Target Temperature (°C)	95	64	95
Incubation time (h:min:s)	00:00:30	00:00:01	00:00:00
Temperature Transition Rate (°C/s)	20.0	20.0	0.2
Secondary Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	None	Cont.
PROGRAM 5- COOLING			
Cycles	1		
Analysis Mode	None		

Temperature Targets	Segment 1
Target Temperature (°C)	40
Incubation time (h:min:s)	00:00:30
Temperature Transition Rate (°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

3.2.4 First strand cDNA synthesis and PCR

Reverse transcription was performed as described in Chapter Two, p60. For the Egyptian goose virus, partial HA and full-length NA, M and NS gene sequences were obtained. Subsequent attempts to re-extract RNA, synthesise cDNA and PCR-amplify the remaining genes were unsuccessful and limited by RNA quantities.

3.2.5 DNA sequencing and phylogenetic analysis

DNA sequencing and phylogenetic analyses were performed as described in Chapter Two, p60. For each gene, the highest similarity scores were retrieved from Genbank by BLAST searches. Dendograms of the midpoint-rooted Neighbour-joining trees are presented for each gene.

3.3 RESULTS

3.3.1 Hemagglutinin genes

3.3.1.1 Hemagglutinin (H5) genes

3.3.1.1.1 Realtime RT-PCR detection of the HPAI H5N2 ostrich H5 gene

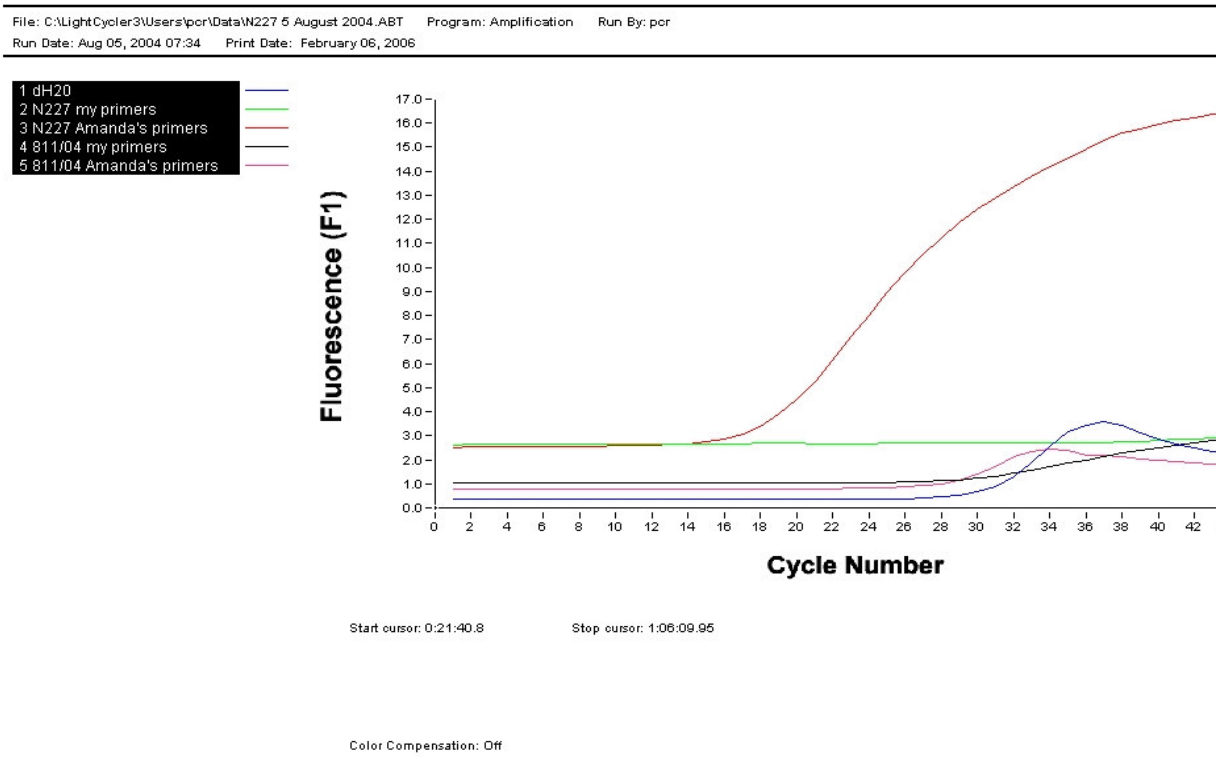


Figure 3.1(a) rRT-PCR amplification curve for A/Ostrich/South Africa/N227/04(H5N2) partial HA sequence (red). dH20 (blue) represents the negative water control. Black and pink indicate a failed positive control. Two oligonucleotide sets were tested: "My primers" represents the H5-specific oligonucleotides of Lee *et al* (2001) whereas "Amanda's primers" are the H5HA1/HA2 oligonucleotide pair (Starick *et al*, 2002).

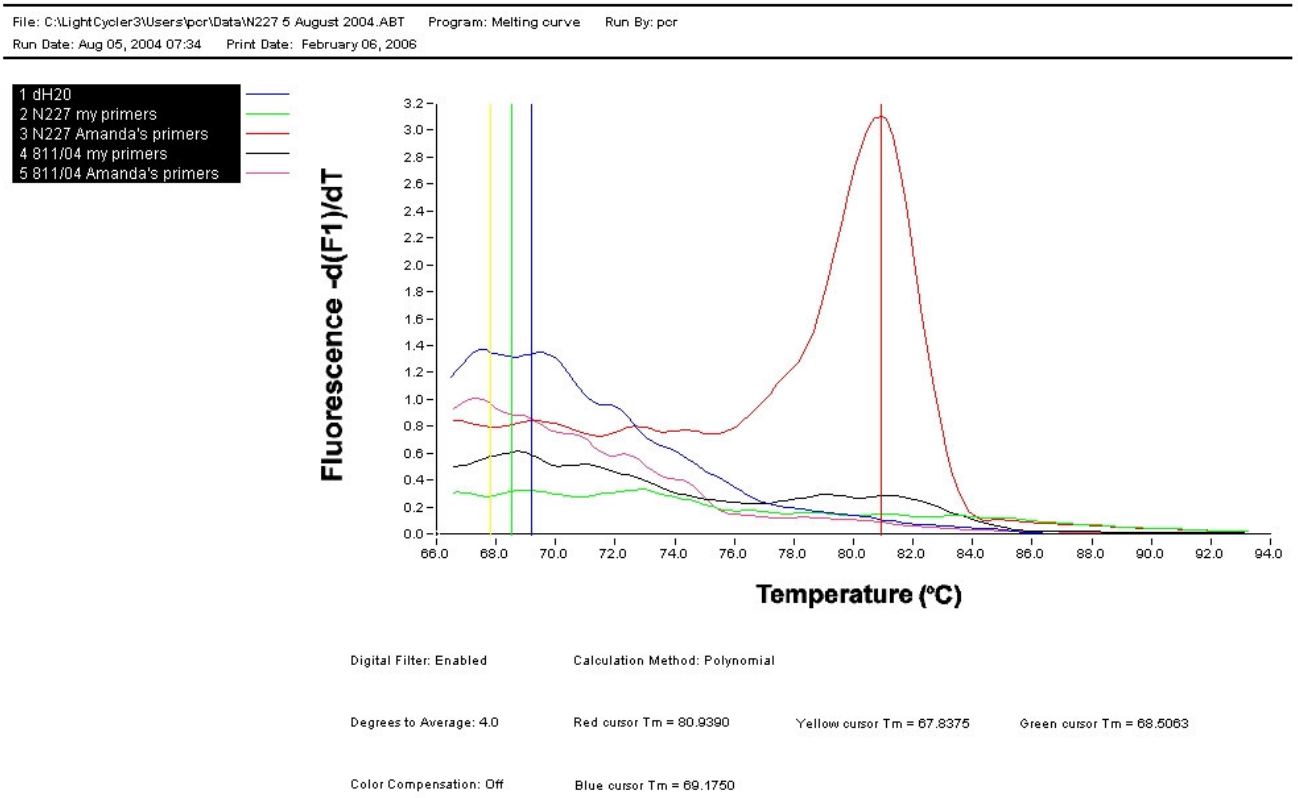


Figure 3.1(b) Melting curve for A/Ostrich/South Africa/N227/04(H5N2) partial HA sequence

Although the positive H5 control (811/04) failed to amplify (possibly due to degraded RNA), the A/Ostrich/South Africa/N227/04 (H5N2) partial H5 amplicon was detectable after 16 PCR cycles (Fig. 3.1(a)). The amplification product had a melting temperature of 80.9ºC (red, Fig. 3.1(b)). After agarose gel electrophoretic separation of the amplification products, the A/Ostrich/South Africa/N227/04 H5 amplicon was excised and subjected to nucleotide sequencing. The predicted amino acid sequence at H_0 was determined to be PQREKRRKKRGLF (Fig 3.3), the multiple basic amino acids insertion is underlined. The virus was clearly HPAI according to the OIE definition (OIE Terrestrial Manual, 2004), and the relevant authorities were immediately notified.

3.3.1.1.2 Phylogenetic comparison of H5 genes

The full-length HA gene of A/Ostrich/South Africa/N227/04 (H5N2) was amplified and sequenced, for comparison with the sequence from A/Wild duck/South Africa/811/04 (H5N1), the partial A/Egyptian goose/South Africa/AI23/04 (H5N2) HA gene, and related H5 genes. The full-length sequences were analysed first:

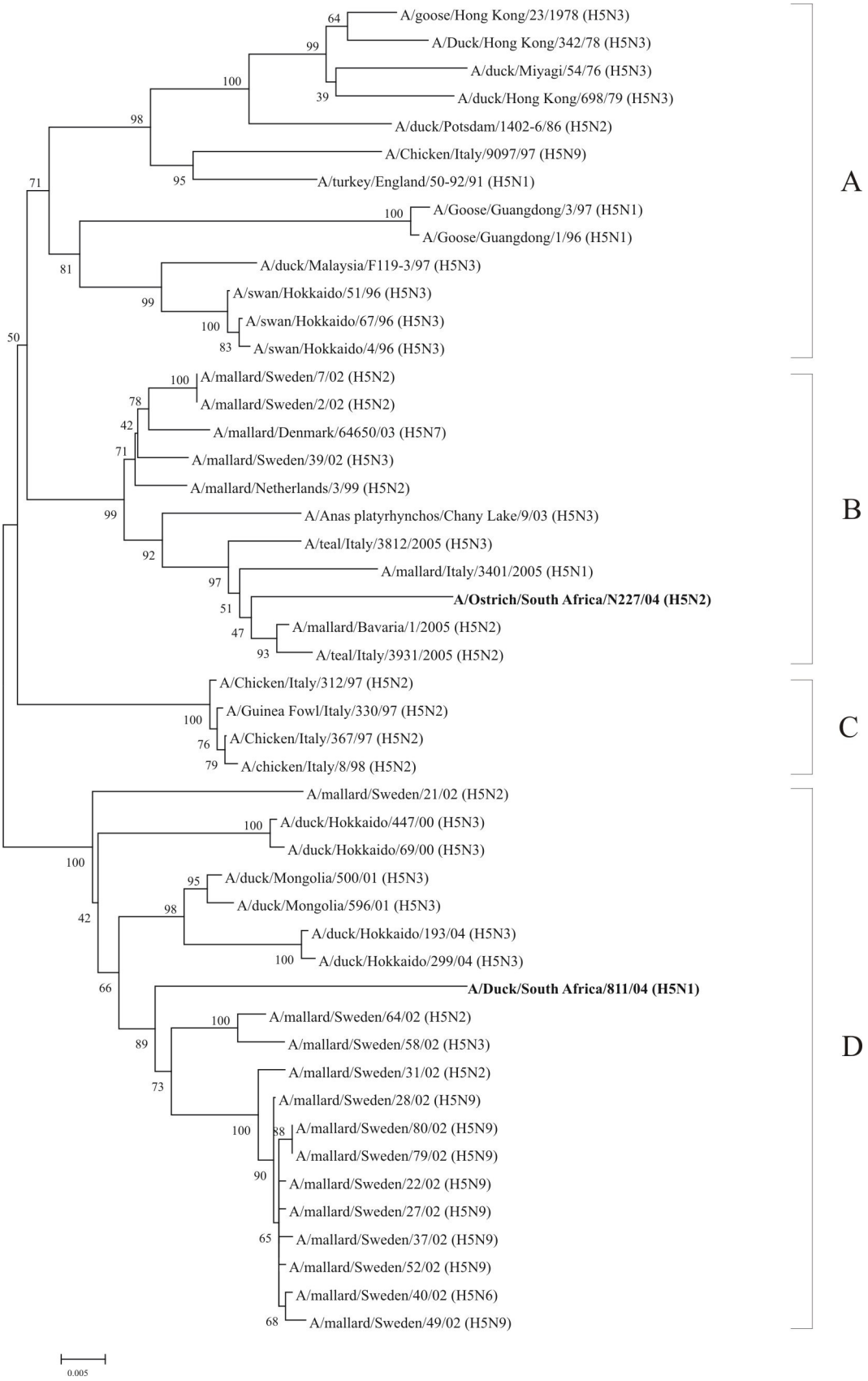


Figure 3.2 Phylogenetic tree inferred from a 1338-nt multiple sequence alignment of the HA (H5) genes of the outbreak strain A/Ostrich/South Africa/N227/05 (H5N2) and A/Wild duck/South Africa/811/04 (H5N1) (in boldface) and related sequences. Sub-lineages A to D are indicated

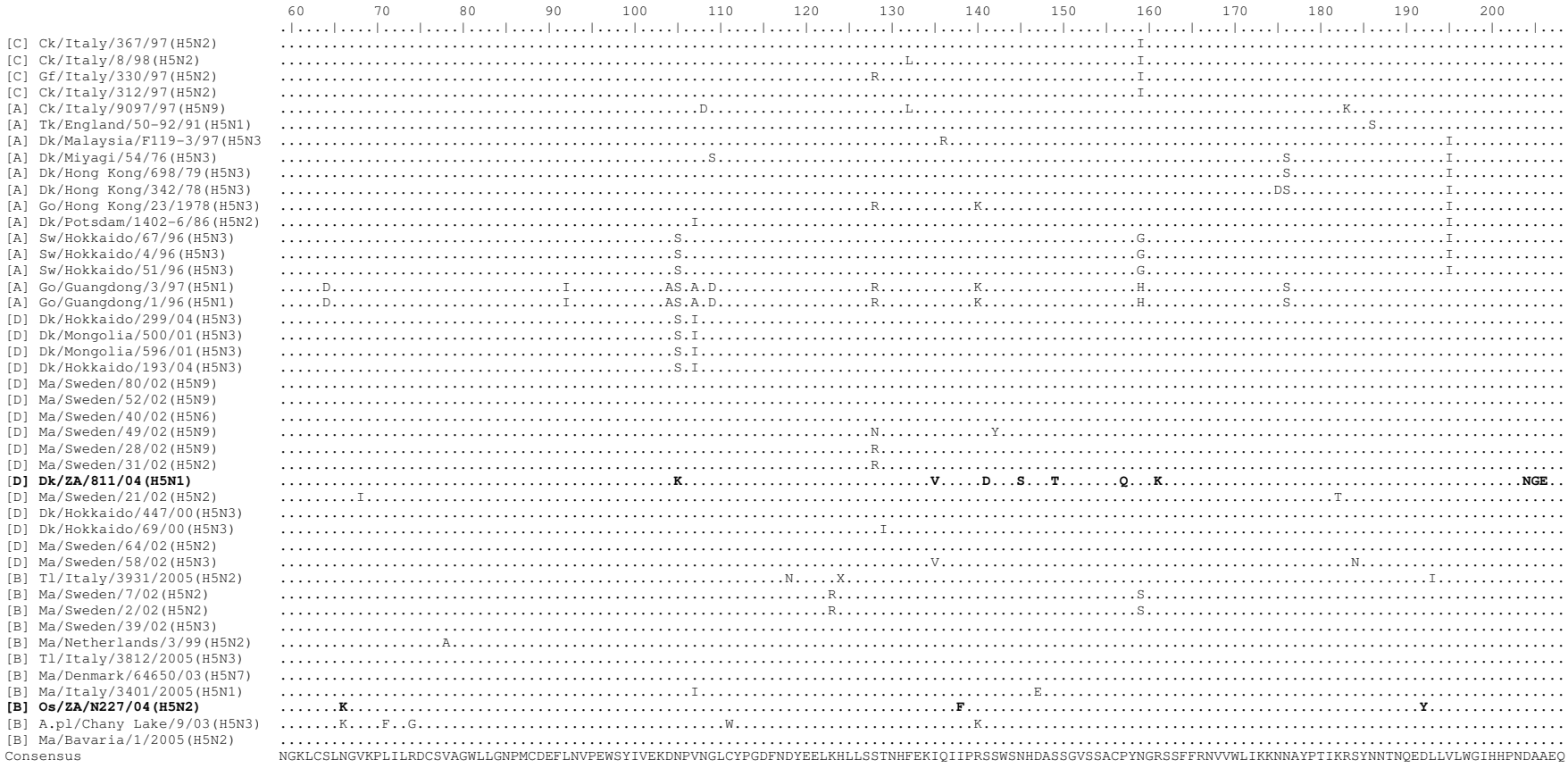


Figure 3.3 Multiple H5 HA amino acid alignment. The South African viruses are indicated in boldface, and the H_0 cleavage site is underlined. Sub-lineages are indicated in square brackets.

Fig 3.3 continued

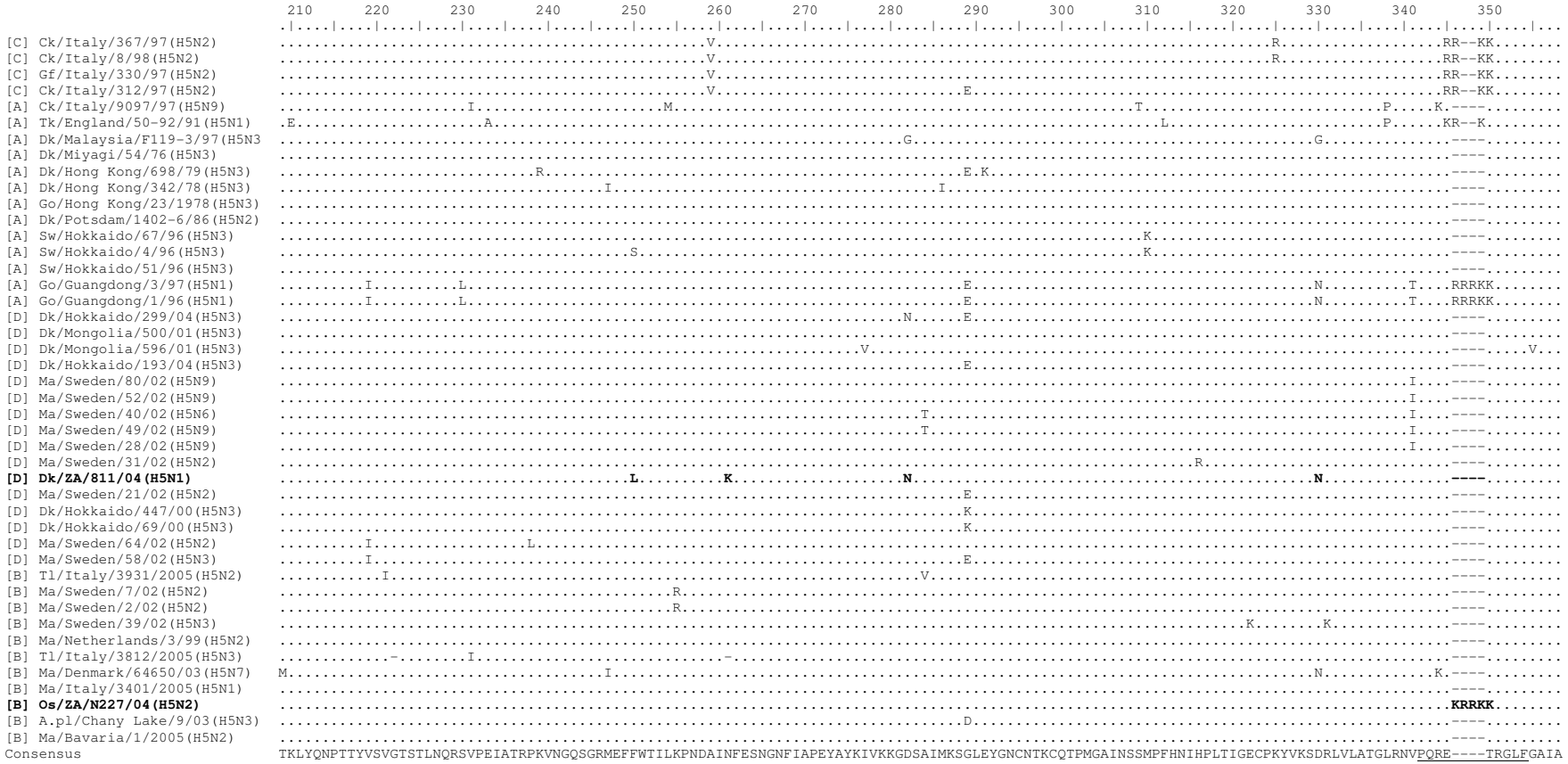
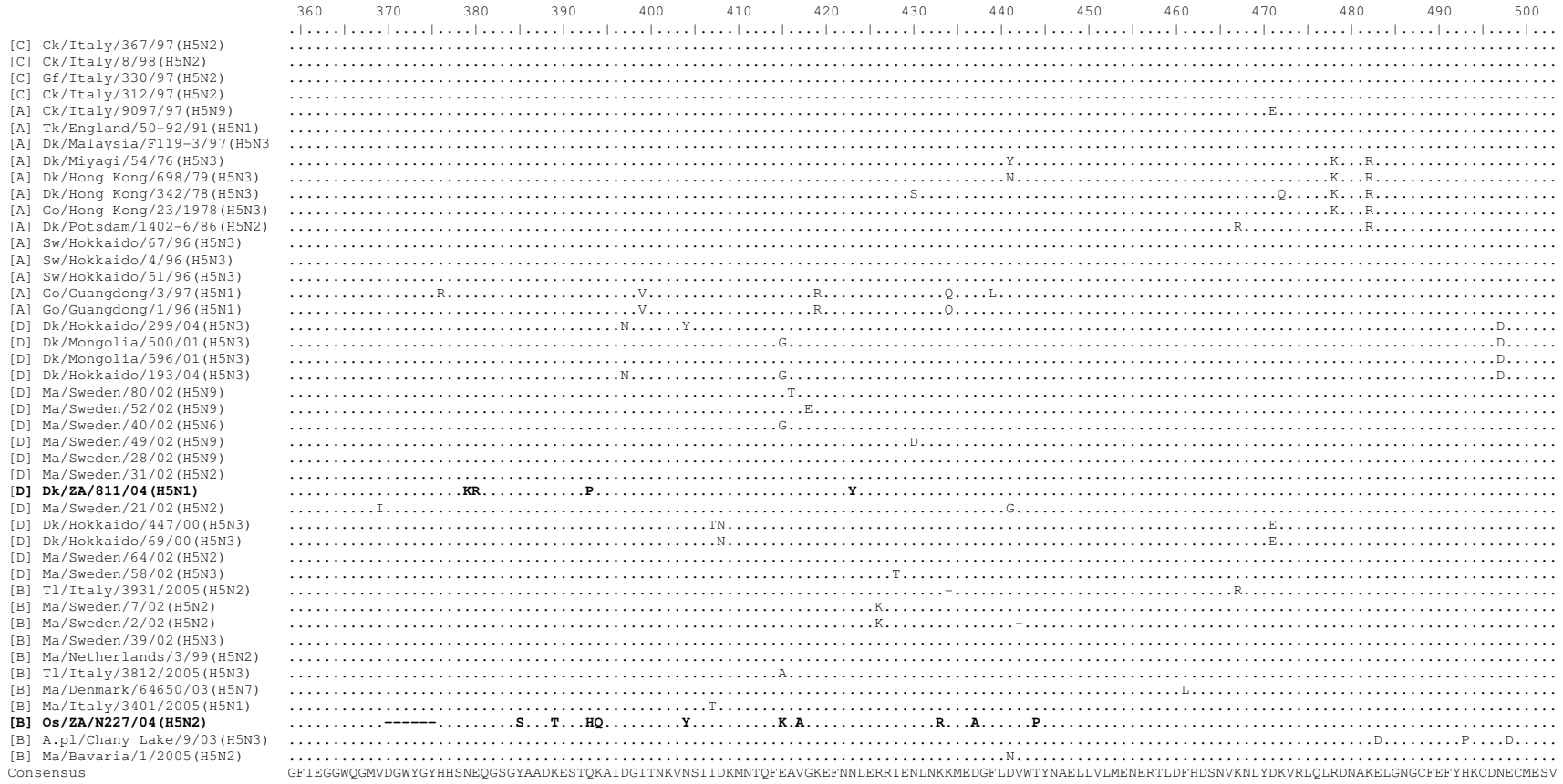


Fig 3.3 continued



The A/Ostrich/South Africa/N227/04 (H5N2) H5 gene is located in sub-lineage B (Fig. 3.2), where it shares recent common ancestors with the hemagglutinin genes of LPAI H5N1, H5N2, H5N3 and H5N7 viruses isolated in Russia and Europe since 1999. The nucleotide sequence identity between the A/Ostrich/South Africa/N227/04 (H5N2) H5 gene and other genes in sub-lineage B varies from 95 to 97%. The highest sequence identity, which is not reflected in the topology of Fig. 3.2, is with the A/Anas platyrhynchos/Chany Lake/9/03 (H5N3) H5 gene. At the amino acid level (Fig 3.3), this genetic relationship is supported by a shared unique K^{66} residue. The ostrich H5 gene was the only one within sub-lineage B that contained a multiple basic amino acid insertion (Fig. 3.3). A/Ostrich/South Africa/N227/04 (H5N2) H5 contained many unique amino acid substitutions at F^{135}, Y^{191}, S^{385}, T^{389}, H^{393}, Q^{394}, Y^{404}, K^{415}, A^{417}, R^{433}, A^{437}, P^{444}, D^{478}, P^{492} and D^{498}, and these markers could reflect an ostrich-specific adaptation, although this needs to be investigated. Three potential N-glycosylation sites were predicted in HPAI A/Ostrich/South Africa/N227/04 (H5N2) H5 gene sequence, at positions 186, 214 and 307. Therefore, hyperglycosylation was not observed within the HPAI A/Ostrich/South Africa/N227/04 (H5N2) H5 gene.

The Duck/South Africa/811/04 virus, in sub-lineage D, shares recent common ancestors with homologous H5 genes of H5N9, H5N2 and H5N3 viruses isolated from wild ducks in Asia and Northern Europe in 2003. The Swedish H5 viruses were isolated from cloacal swabs of trapped mallards collected during autumn (southwards) migration in 2003 (Bragstad *et al*., 2005). All of these viruses are low pathogenic, containing the amino acid sequence PQRETRGLF at the H_0 cleavage site (Fig 3.3). Duck/South Africa/811/04 is the only H5 virus within sub-lineage D that contains an N1-type neuraminidase gene. Six potential N-glycosylation sites were predicted to occur in the Duck/South Africa/811/04 H5 gene, at positions 31, 43, 186, 214, 307 and 413. No additional glycosylation site at position 158 (by H3 numbering) was detected, which is in accordance with findings on other nonpathogenic H5 viruses isolated from aquatic birds (Matrosovich *et al*., 1999). The Duck/South Africa/811/04 H5 gene contained the following thirteen unique amino acid substitutions: I^{37} and A^{57} (not shown in Fig. 3.3), S^{145},T^{149}, Q^{157}, N^{204}, G^{205}, E^{206}, L^{251}, K^{262}, K^{379}, R^{380}, P^{393}, Y^{423}.

Therefore, the hemagglutinin genes of A/Duck/South Africa/811/04(H5N1) and A/Ostrich/South Africa/N227/04(H5N2) are not closely-related to each other (with

only 89% nucleotide sequence identities), and neither are they closely-related to the Asian HPAI H5N1 outbreak strains, represented in Fig 3.2 by A/Goose/Guangdong/3/97 (H5N1) and A/Goose/Guangdong/1/96 (H5N1) in sub-lineage A. Next, the H5 partial Egyptian goose HA sequence was included in the phylogenetic analysis:

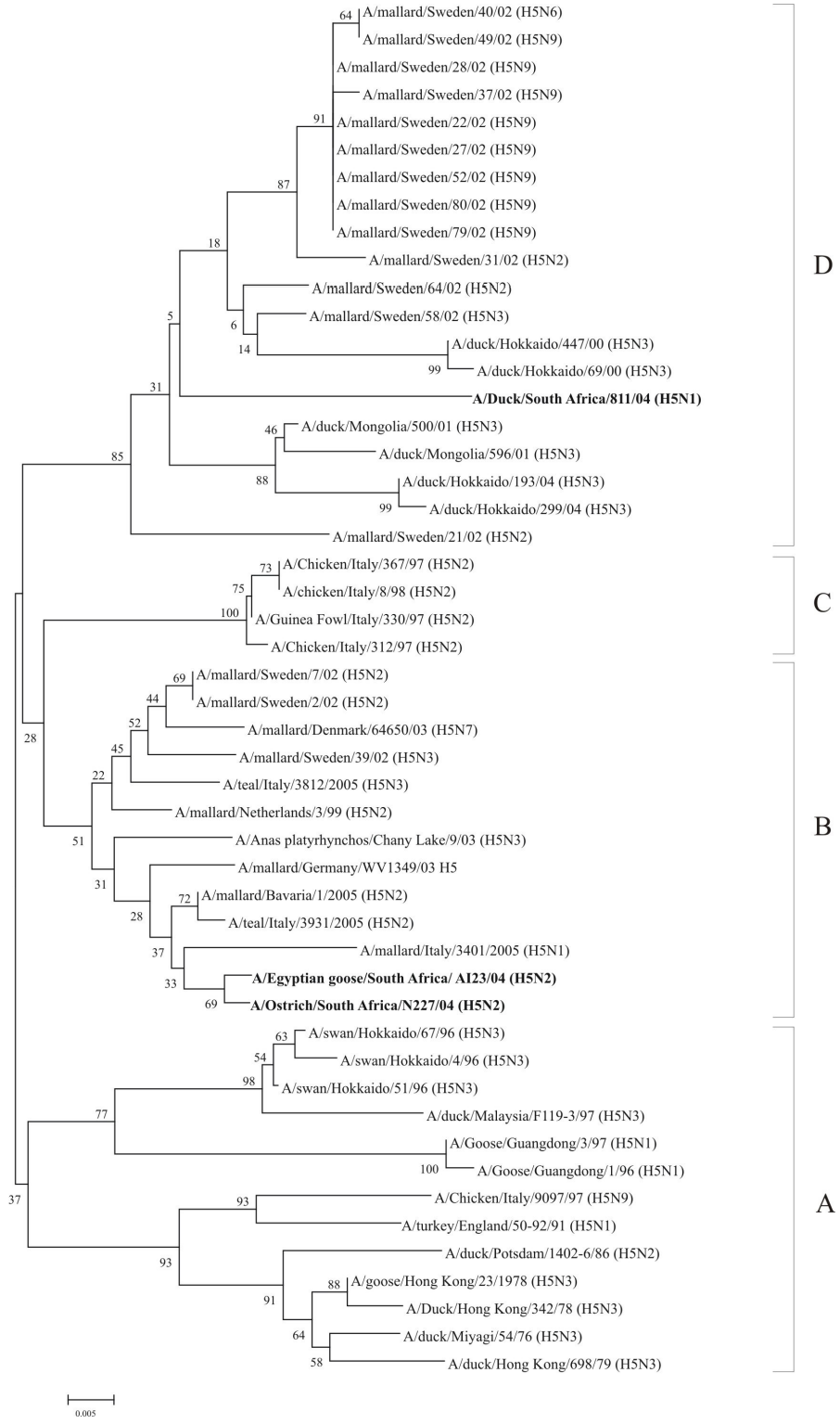


Figure 3.4 Phylogenetic tree inferred from a 373-nt multiple sequence alignment of the H5 hemagglutinin genes including that of A/Egyptian Goose/AI23/04 (H5N2) (in boldface). Sub-lineages A to D are indicated, corresponding to Fig 3.2.

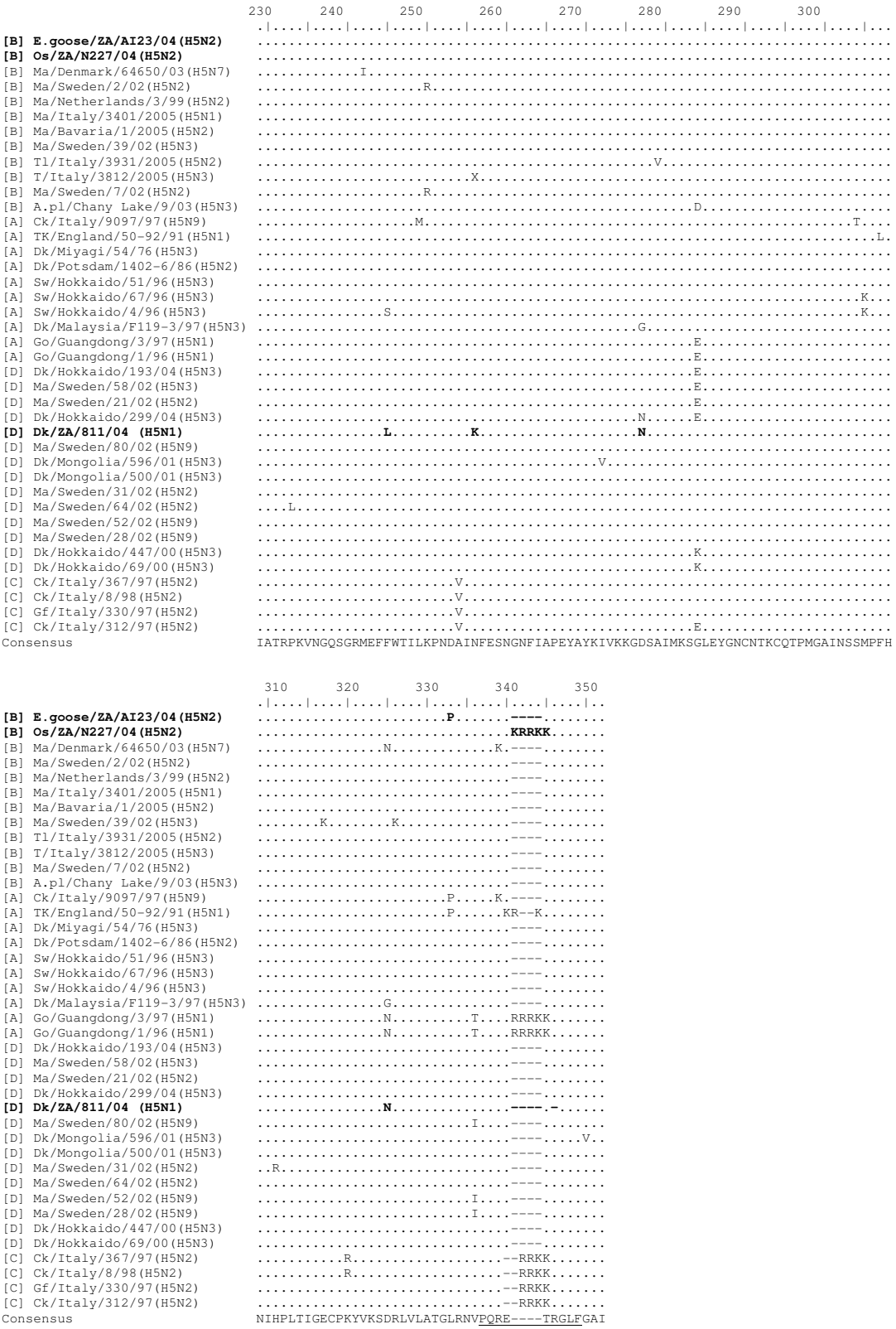


Figure 3.5 Multiple amino acid sequence alignment of partial H5 gene sequences including the H_0 cleavage site (underlined).

Although a shorter region (373 nt compared to 1338 nt) was analysed in Fig 3.4 the topology remains similar to Fig. 3.2. The Egyptian goose virus H5 gene is the closest relative to A/Ostrich/South Africa/N227/04 (H5N2) H5 gene, sharing 99% sequence identity (H_0 cleavage site excluded). The A/Egyptian Goose/AI23/04 (H5N2) H5 sequence however lacked the multiple basic amino acid insertion. Only one unique amino acid substitution was detected in the partial sequence for the Egyptian goose virus viz., $L^{333} \rightarrow P$, although this mutation was also present in the H5 genes of A/chicken/Italy/9097/97 (H5N9) and A/Turkey/England/50-92/91 (H5N1) (sub-lineage A).

3.3.1.1.3 RT-PCR Detection of an H5 virus co-infection of A/Duck/South Africa/1108/04 (H3N8) and phylogenetic analysis of the partial sequence

During routine RT-PCR testing for H5, a faint H5-specific amplicon was obtained with A/Duck/South Africa/1108/04 (H3N8) RNA. After the nucleotide sequence was determined, phylogenetic analysis was performed to determine the closest genetic relative (Fig. 3.6). The virus was determined to be an LPAI virus with an H_0 cleavage site sequence of PQRETRGLF (Fig. 3.7), but unusually, phylogenetic data indicated that the partial sequence had 99.6 % nucleotide sequence identity (across 308 nt) to A/mallard duck/ALB/57/1976 (H5N2), and was thus a North American lineage strain. Furthermore, the closest relative was a historic virus, isolated in 1976.

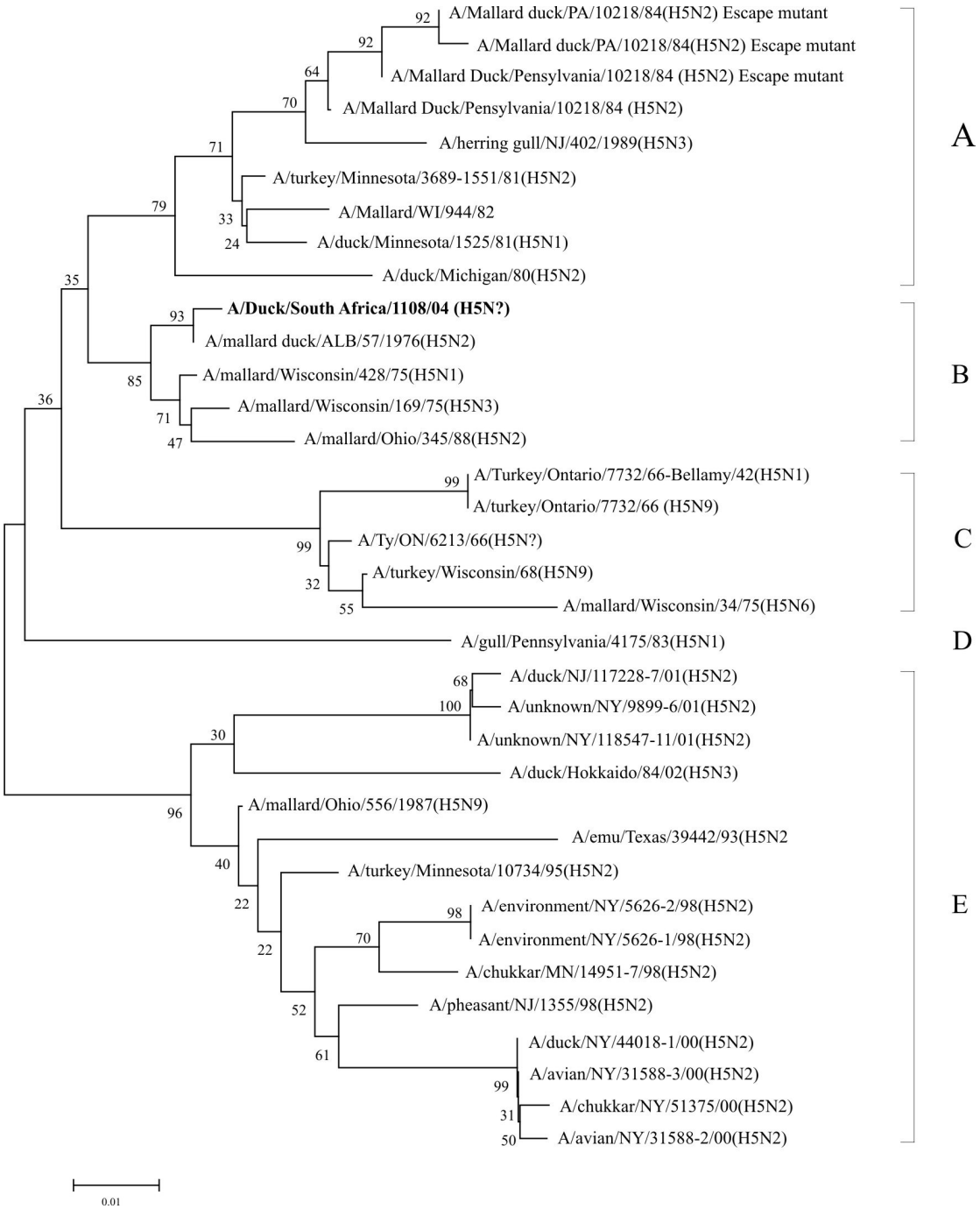


Figure 3.6 Phylogenetic trees inferred from a 308-nt multiple sequence alignment of a partial H5-type virus detected by RT-PCR in A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to E are indicated.

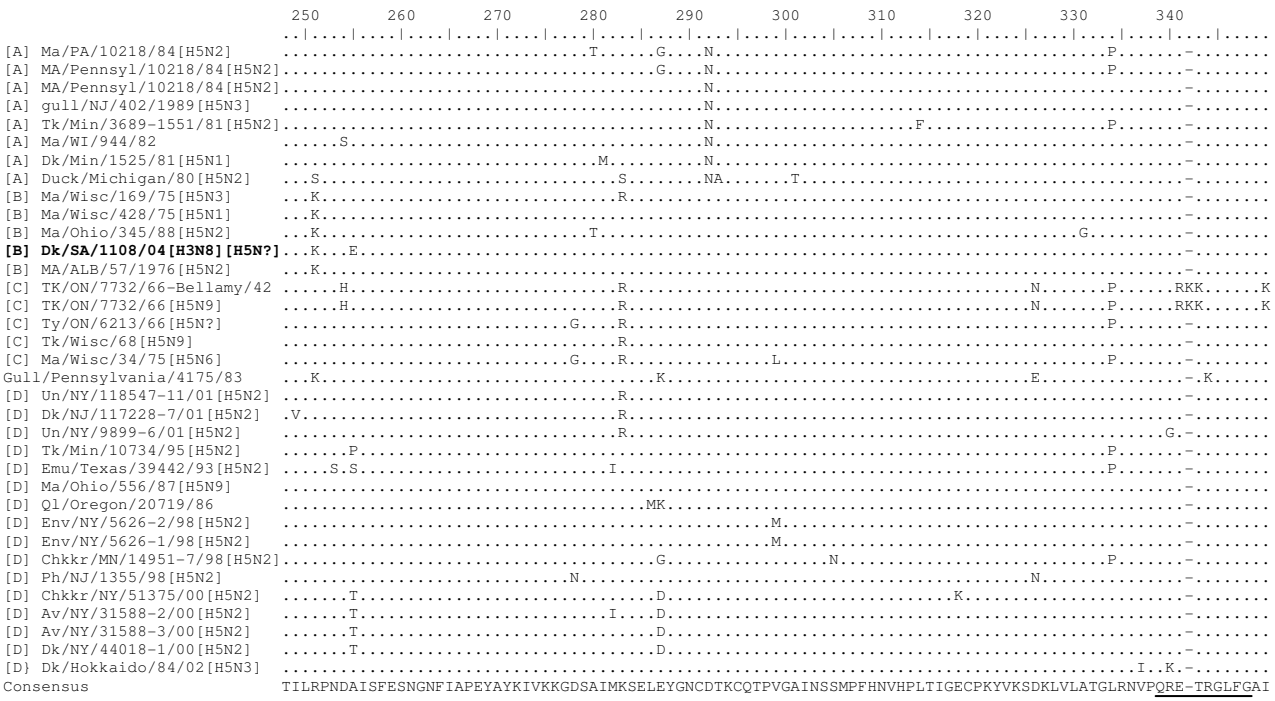


Figure 3.7 Partial peptide sequence alignment of North American lineage H5 genes and the South African sequence (boldface) including the H_0 cleavage site (underlined).

3.3.1.2 Hemagglutinin (H3) genes

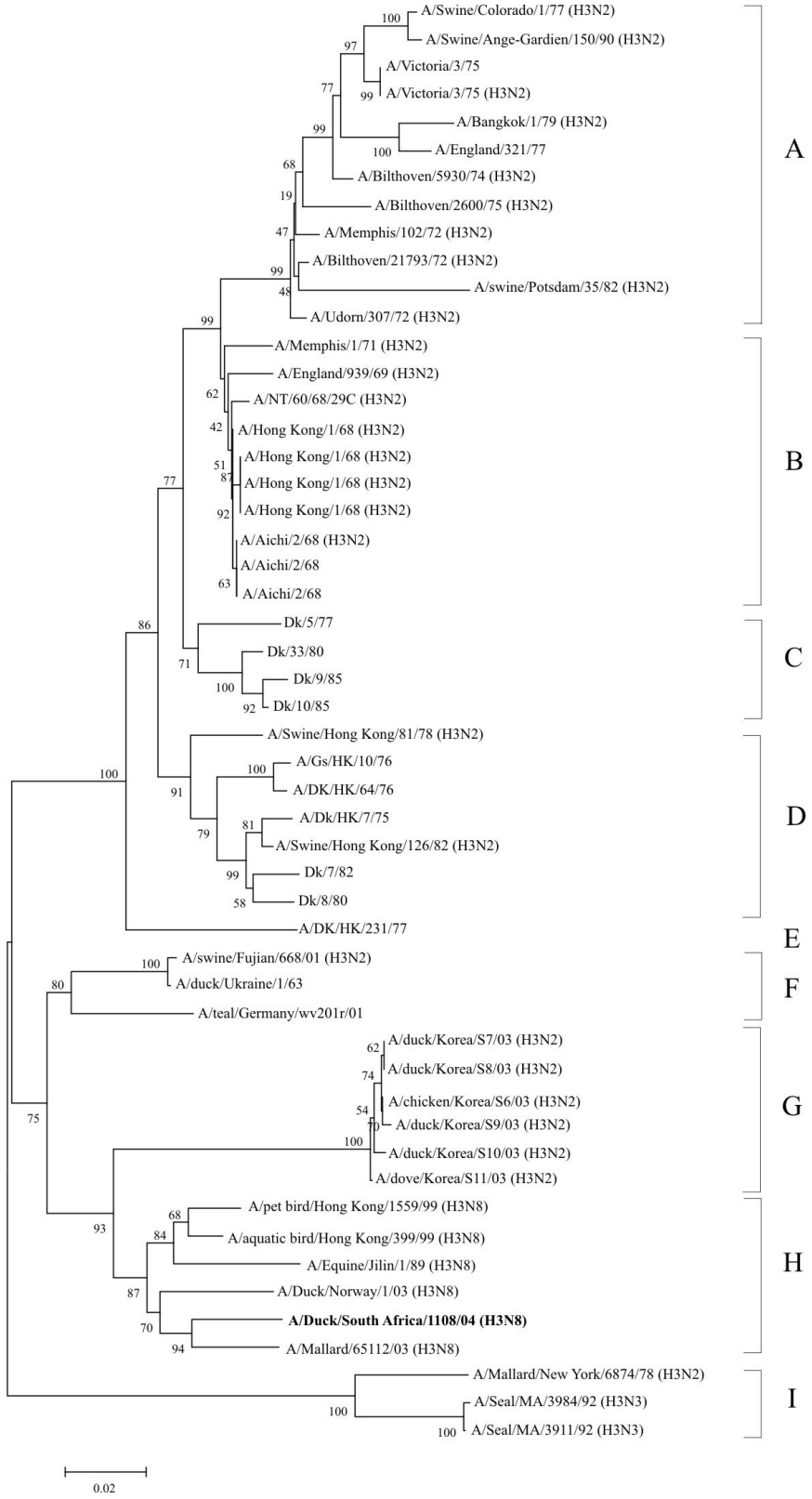


Figure 3.8 Phylogenetic tree inferred from a 1104-nt multiple sequence alignment of the H3 genes of A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to I are indicated.

H3 genes are separated into several lineages (Fig. 3.8): American mallard and seal (sub-lineage I), historic American (sub-lineages A and B), and historic Eurasian (sub-lineages C, D and E), and contemporary Eurasian viruses (sub-lineages F, G ang H). The Duck/South Africa/1108/04 (H3N8) H3 gene falls within sub-lineage H, with H3 viruses from China (Hong Kong, Jilin) and duck isolates collected in 2003 from Northern Europe (Norway and Denmark). The Danish virus, Mallard/65112/03 (H3N8), shared 95% nucleotide sequence identity with the Duck/South Africa/1108/04 H3 gene. Sub-lineage G viruses, Korean H3N2 viruses from 2003, shared a common ancestor with sub-lineage H. The H_0 cleavage site sequence was PEKQTRGLF, which is typical of H3 sequences.

3.3.1.3 Hemagglutinin (H4) genes

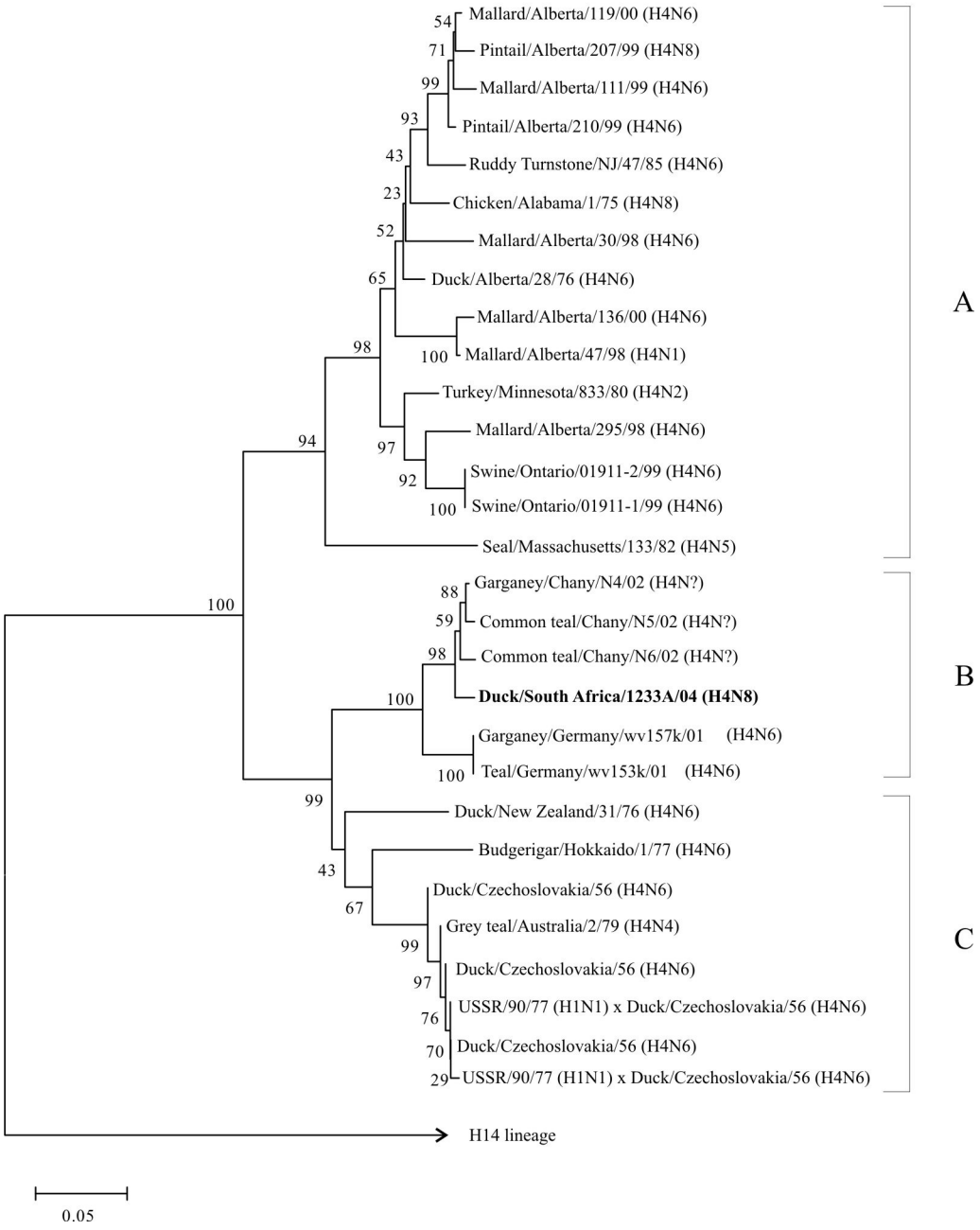


Figure 3.9 Phylogenetic trees inferred from a 459-nt multiple sequence alignment of the H4 genes of A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A, B and C are indicated.

The phylogenetic distinction between the Eurasian (Fig. 3.9, sub-lineages B and C) and North American lineage viruses (sub-lineage A) is clearly demonstrated within the H4 subtype. Sub-lineage C contains historic H4 genes isolated within a wide geographical range (New Zealand, Australia, Japan and Czechoslovakia) between 1956 and 1979, whereas sub-lineage B contains genes from contemporary isolates from lake Chany in Russia, Germany, and the South African H4 gene of Duck/South Africa/1233A/04 (H4N8). The South African virus H4 gene shares very high sequence identity (98%) with the isolates from common teals (*Anas crecca*) and garganey (*Anas querquedula*) tested for AIV in the Chany lake region, indicating that the South African H4 gene was very recently derived from this gene pool. The H_0 peptide cleavage sequence was PEKASRGLF, which is typical of H4 sequences.

3.3.2 The Neuraminidase genes

3.3.2.1 Neuraminidase (N2) genes

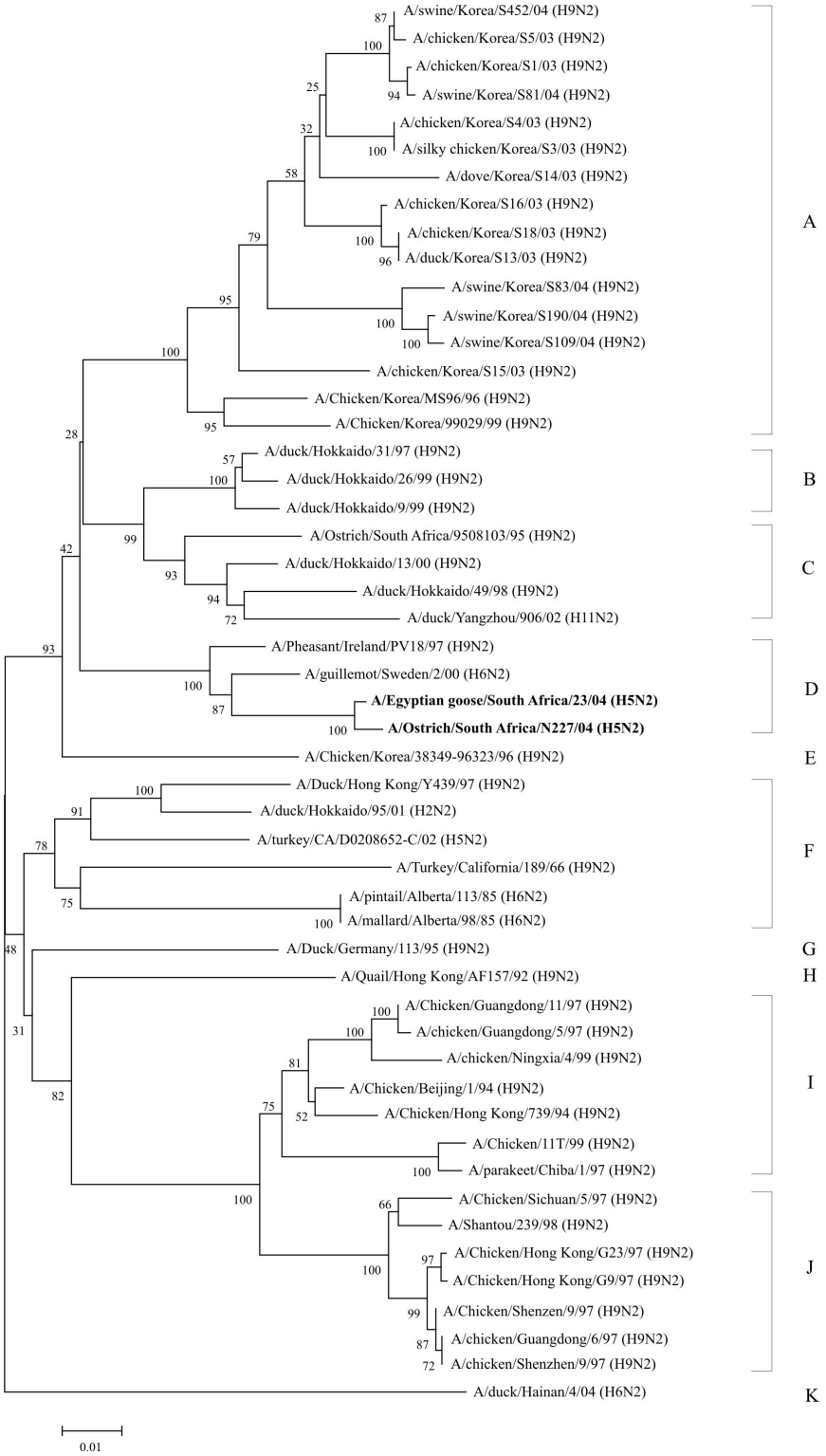


Figure 3.10 Phylogenetic tree inferred from a 1073-nt multiple sequence alignment of the NA (N2) genes of the outbreak strain A/Ostrich/South Africa/N227/05 (H5N2), A/Egyptian

Goose/AI23/04 (H5N2) (both in boldface) and related viruses. Sub-lineages A to K are indicated

The full-length NA sequence was obtained for LPAI A/Egyptian Goose/AI23/04 (H5N2) for comparison with HPAI A/Ostrich/South Africa/N227/04 (H5N2). Pairwise comparisons revealed a 99% sequence identity between the two sequences at the nucleotide level, and from the deduced amino acid sequences that a unique T^{102} substitution was shared (Fig. 3.11). Phylogenetically, the closest relatives in the Genbank database are the N2 genes of a Swedish isolate, A/guillemot/Sweden/2/00 (H6N2) and A/Pheasant/Ireland/PV18/97 (H9N2) (Fig. 3.10, sub-lineage D). 96% nucleotide sequence similarity was observed between the N2 genes of A/guillemot/Sweden/2/00 (H6N2) and A/Pheasant/Ireland/PV18/97 (H9N2) and the South African viruses. Only 92% sequence identity was observed between the N2 gene of A/mallard/Bavaria/2005 (H5N2), (a close genetic relative in the case of the H5 gene) and the South African H5N2 viruses' N2 genes. Sub-lineage D N2 genes are defined by several shared amino acid characters, viz. D^{55}, A^{70}, N^{183}, R^{233}, I^{238} and T^{393}. The phylogenetic and amino acid analyses suggests that there is no close genetic relationship between the N2 genes of A/Ostrich/South Africa/9508103/95 (H9N2) (sub-lineage C) and the H5N2 viruses of the current study. Instead, the N2 gene of the common ancestor of A/Egyptian Goose/AI23/04 (H5N2) and A/Ostrich/South Africa/N227/04 (H5N2) appears to be a more recent introduction to the South African gene pool. NA-stalk deletions were absent from the A/Egyptian Goose/AI23/04 (H5N2) and A/Ostrich/South Africa/N227/04 (H5N2) N2 genes.

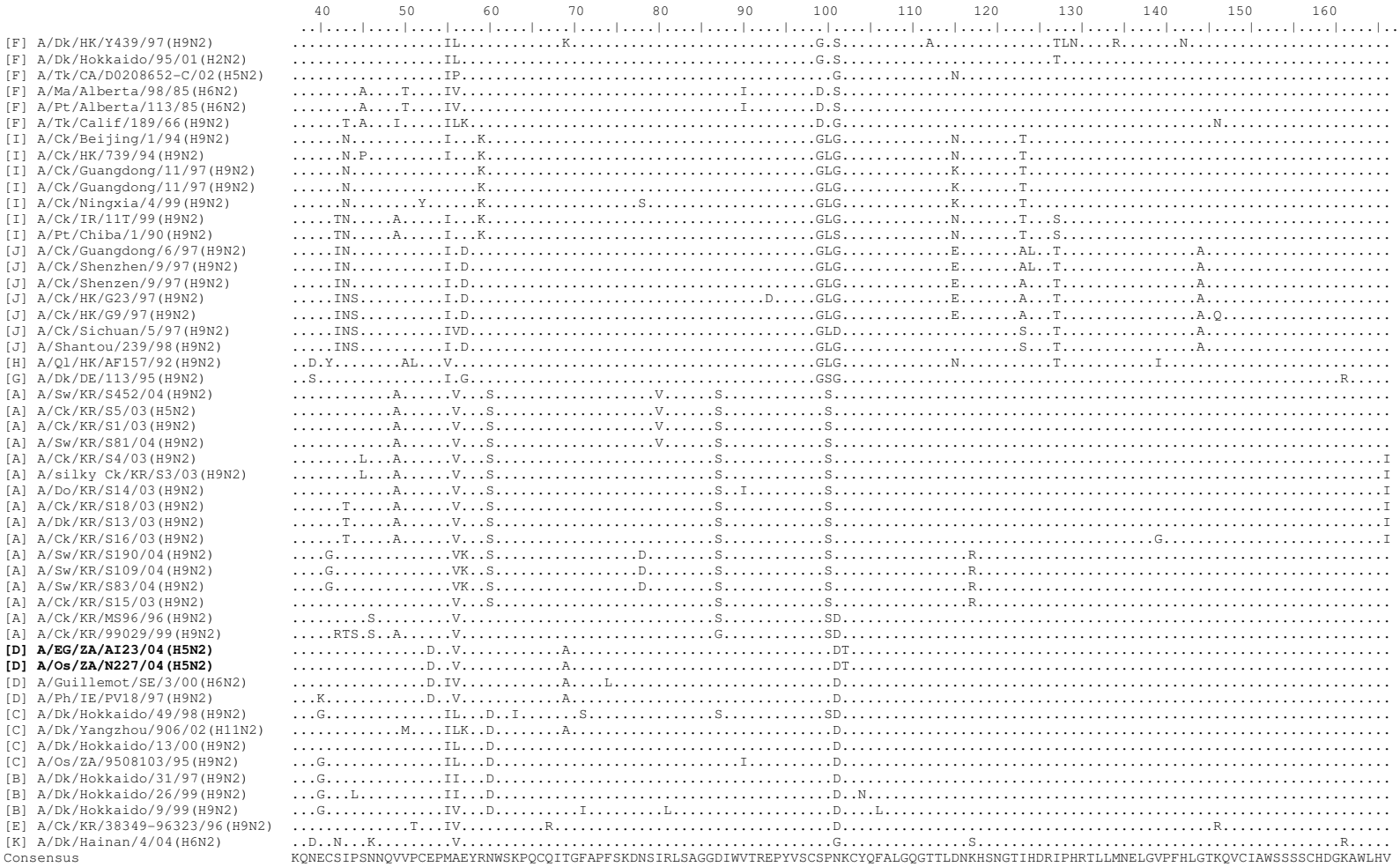


Figure 3.11. Multiple amino acid alignment of N2 genes. South African viruses sequenced in this study are indicated in boldface

Figure 3.11 continued

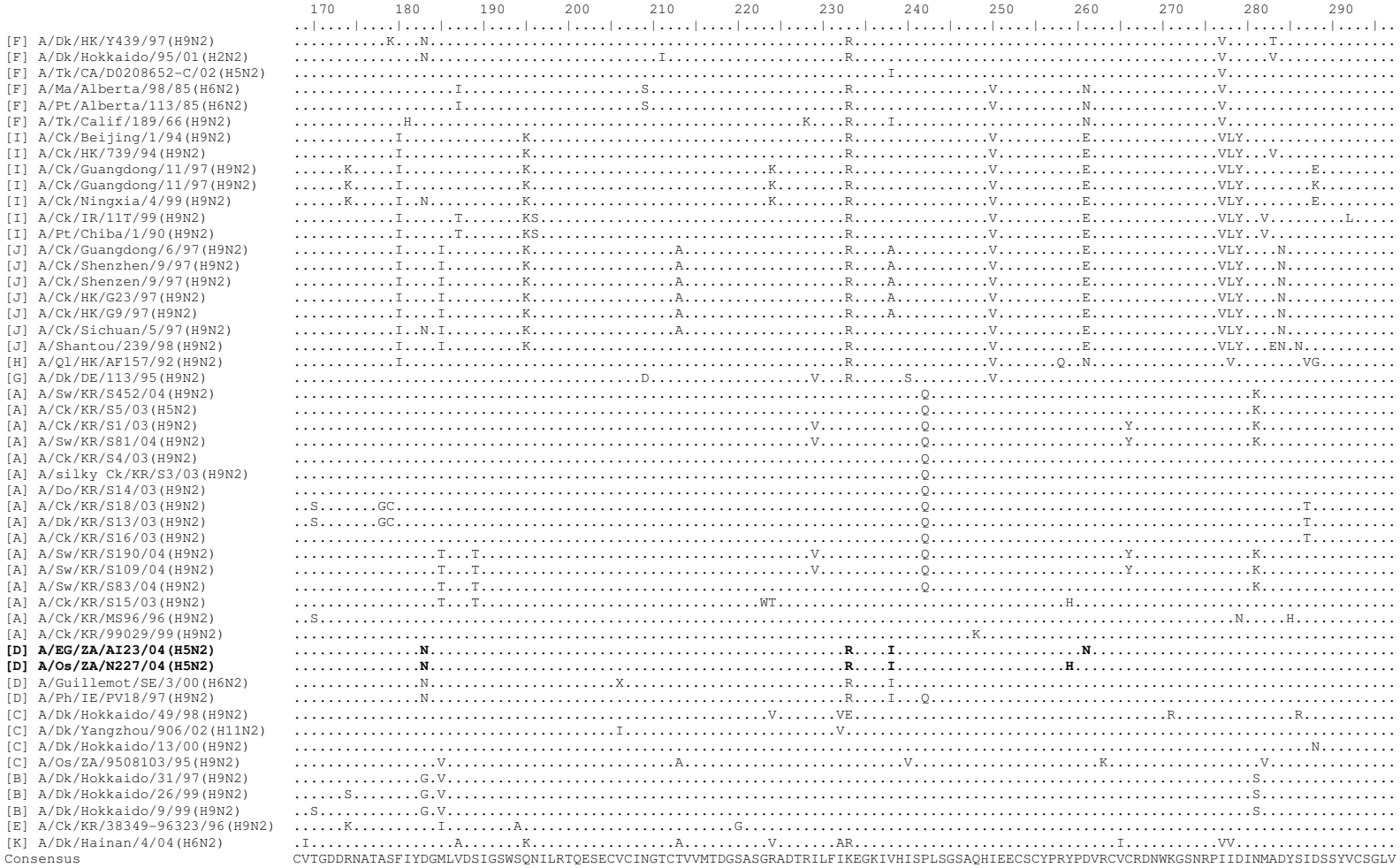
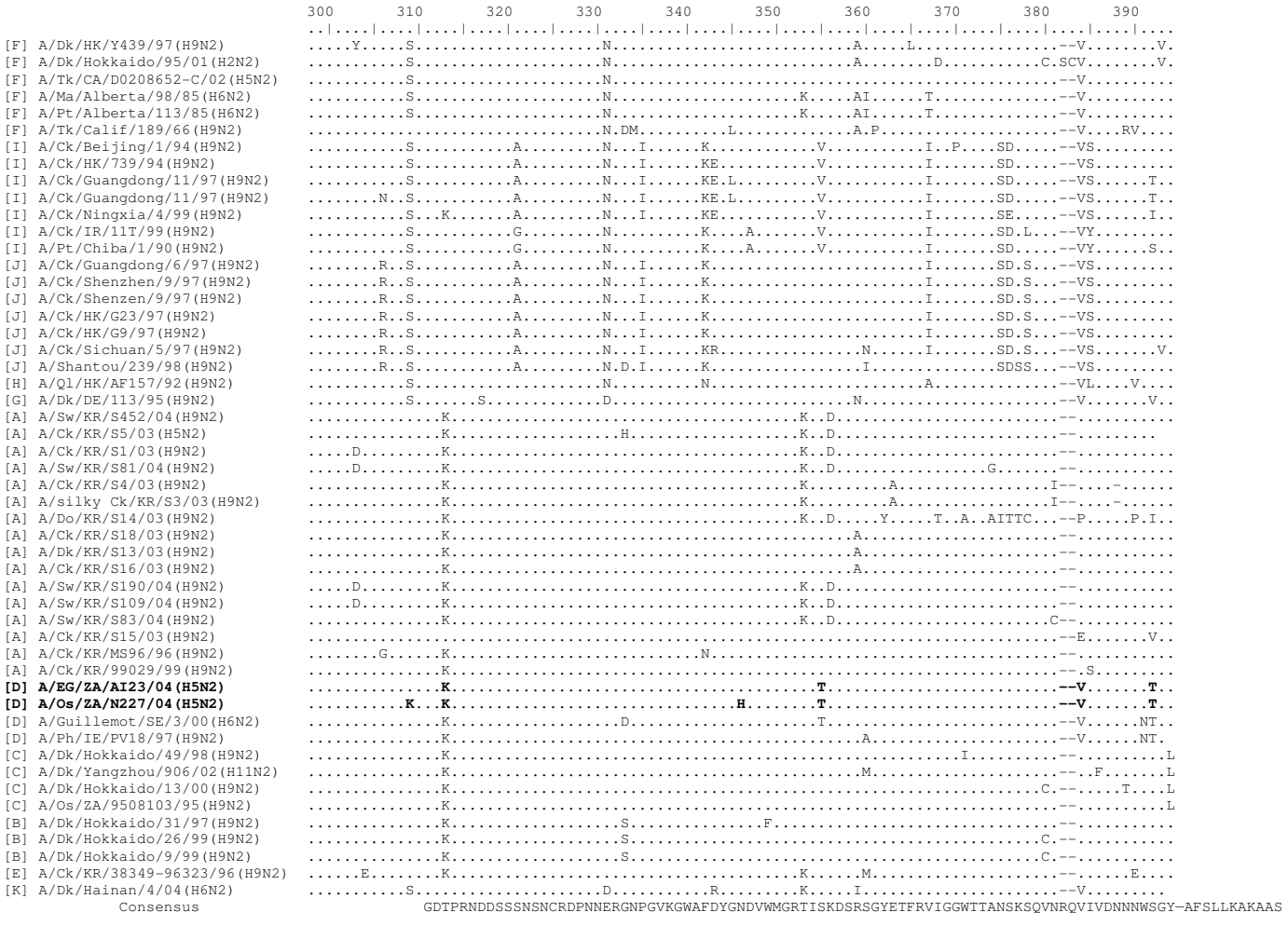


Figure 3.11 continued



3.3.2.2 Neuraminidase (N8) genes

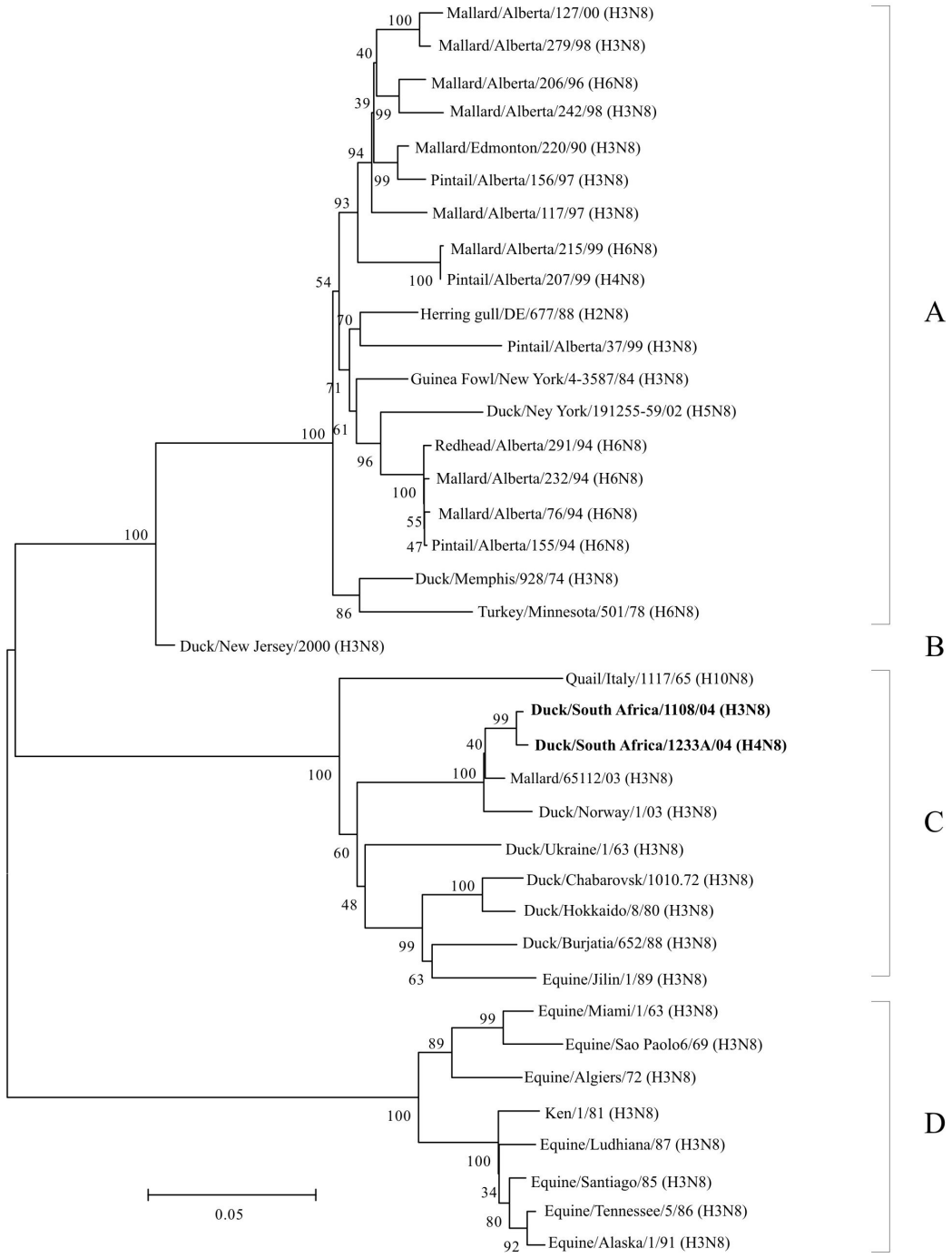


Figure 3.12 Phylogenetic tree inferred from a 1305-nt multiple sequence alignment of the N8 genes of A/Duck/South Africa/1233/04 (H4N8) and A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to D are indicated.

N8-type genes are split between the Equine, North American and Eurasian lineages (Saito *et al*., 1993). The N8 genes of A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233A/04 (H4N8) viruses fall within the Eurasian lineage (Fig. 3.12, sub-lineage C). These two N8 genes shared 99% sequence identity at the nucleotide level, which possibly indicates a common source. 98% and 97% nucleotide sequence identities were shared with the Danish virus A/Mallard/65112/03 (H3N8), and A/Duck/Norway/1/03 (H3N8) N8 genes, respectively. The close genetic relationships suggest that the South African N8 genes were recently derived from a northern European gene pool, and that an H3N8 virus was probably the original source of both South African N8 genes.

3.3.2.3 Neuraminidase (N1) genes

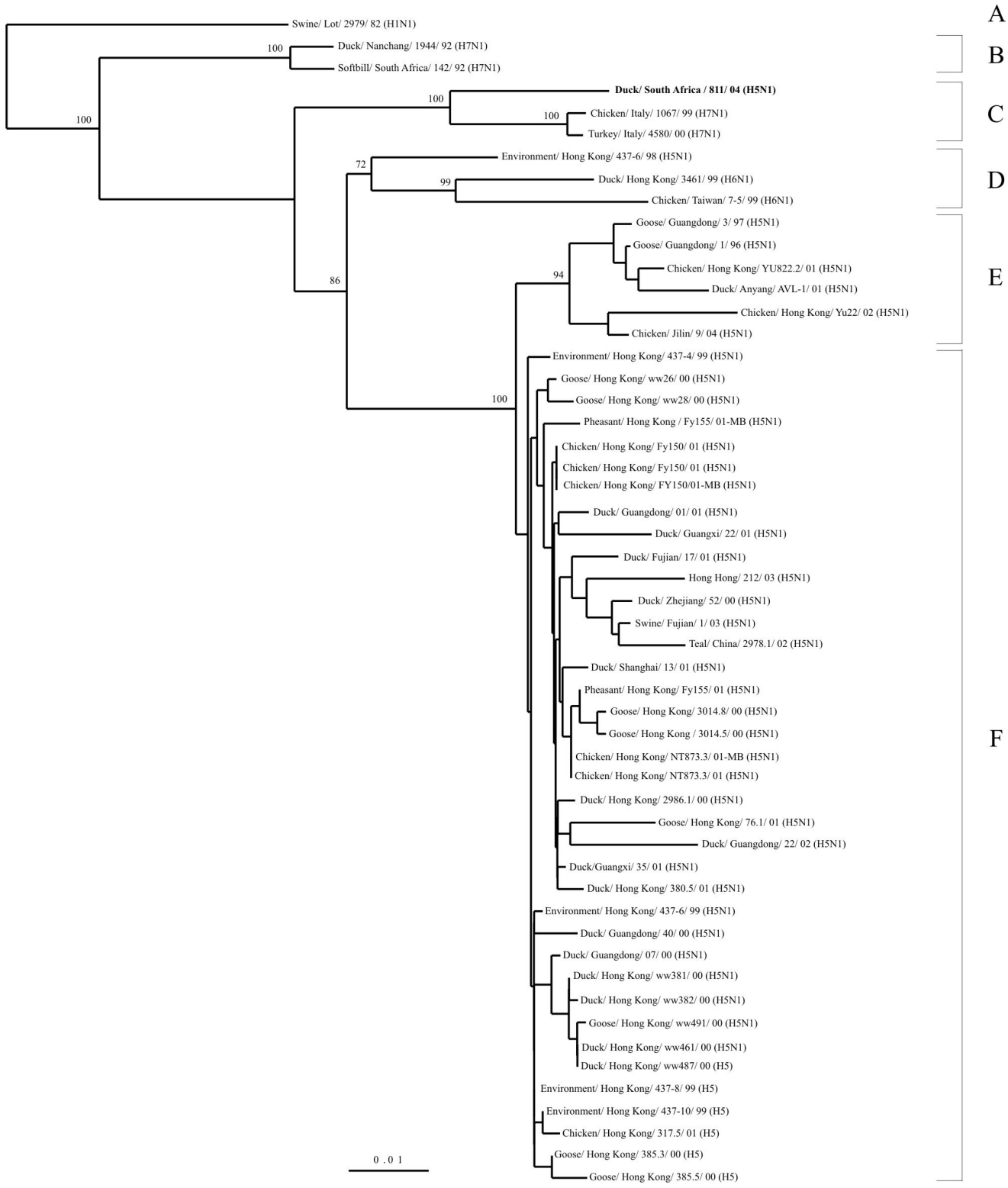


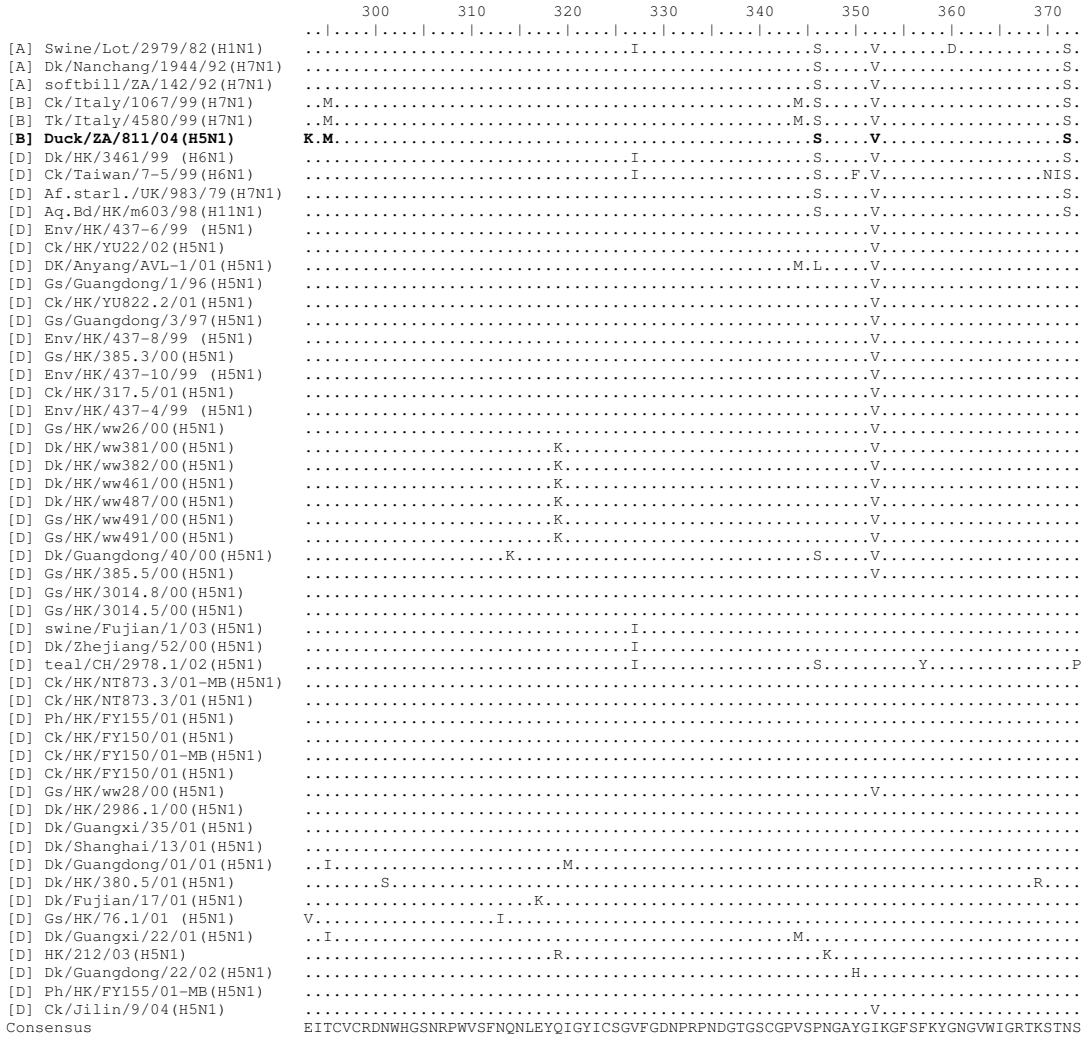
Figure 3.13 Phylogenetic trees inferred from a 1025-nucleotide multiple sequence alignment of the N1 genes of A/Duck/South Africa/811/04 (H5N1) (in boldface) and related sequences. Sub-lineages A to F are indicated.

The N1 gene of A/Duck/South Africa/811/04 (H5N1) shares a common ancestor and 96% nucleotide sequence identity with each of the N1 genes of two Italian viruses, A/Chicken/Italy/1067/99 (H7N1) and A/Turkey/Italy/4580/99 (H7N1) (Fig. 3.13, sub-lineage C). Another South African N1 gene, that of A/softbill/SouthAfrica/142/92 (H7N1) is located in sub-lineage B and unlikely to be the progenitor of A/Duck/South Africa/811/04 (H5N1). The Italian viruses were isolated from outbreaks in poultry during 1999 (Mannelli *et al*., 2005). Sub-lineage C N1 genes are clearly phylogenetically separated from those of the Asian HPAI H5N1 outbreak strains, located in sub-lineages E and F. A/Duck/South Africa/811/04 (H5N1) lacked a deletion in the stalk region of the neuraminidase gene, however, the Italian H7N1 N1 genes contained deletions of varying lengths (45 and 78 amino acids) (Fig. 3.14). Unique shared amino acid characters (T^{87} and M^{295}) distinguish the sub-lineage C viruses from other sub-lineages, but A/Duck/South Africa/811/04 (H5N1) additionally contained the unique amino acid substitutions of Y^{50}, V^{208}, S^{230} and K^{260}.

Figure 3.14 Multiple amino acid sequence alignment of neuraminidase (N1 genes). Sub-lineages are indicated in square brackets.

Fig. 3.14 continued

Fig. 3.14 continued



3.3.3 Matrix protein (M) genes

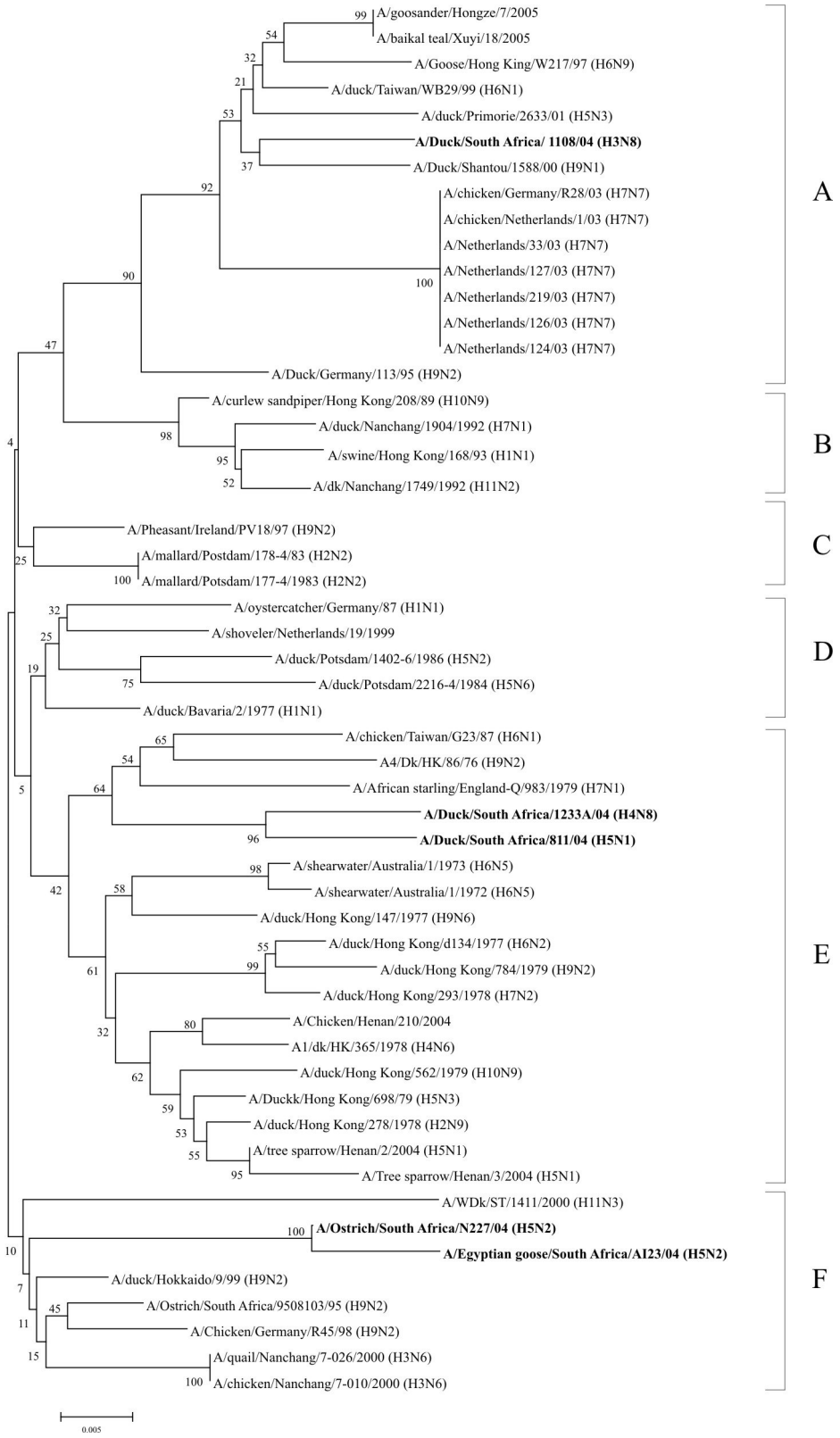


Figure 3.15 Phylogenetic tree inferred from a 674-nt multiple sequence alignment of the M genes of South African AI viruses isolated in 2004 (in boldface) and related sequences. Sub-lineages A to F are indicated.

The M genes of A/Ostrich/South Africa/N227/04 (H5N2) and A/Egyptian Goose/South Africa/AI23/04 (H5N2) (Fig. 3.15, sub-lineage F) share 99% nucleotide sequence identities and thus indicate a very recent common ancestor. Other phylogenetic relationships within sub-lineage F are weakly-supported and nucleotide sequence identity varies from 98% to 95%. Two of the Blesbokspruit isolates, A/Duck/South Africa/811/04 (H5N1) and A/Duck/South Africa/1233A/04 (H4N8) cluster together in sub-lineage E with M genes of viruses isolated in the Far East, Europe and Australia, whereas the Duck/South Africa/1108/04 (H3N8) virus M gene is completely separated (sub-lineage A) where it shares ancestors with M genes of viruses from Northern Europe and the Far East. One explanation for the long branch lengths displayed by the M protein genes in Fig 3.15 could be genetic drift, caused by host immunological pressure, since the M1 gene is expressed in large quantities during infection cycle and is immunogenic (Lambrecht *et al*., 2006).

3.3.4 Nonstructural protein (NS1) genes

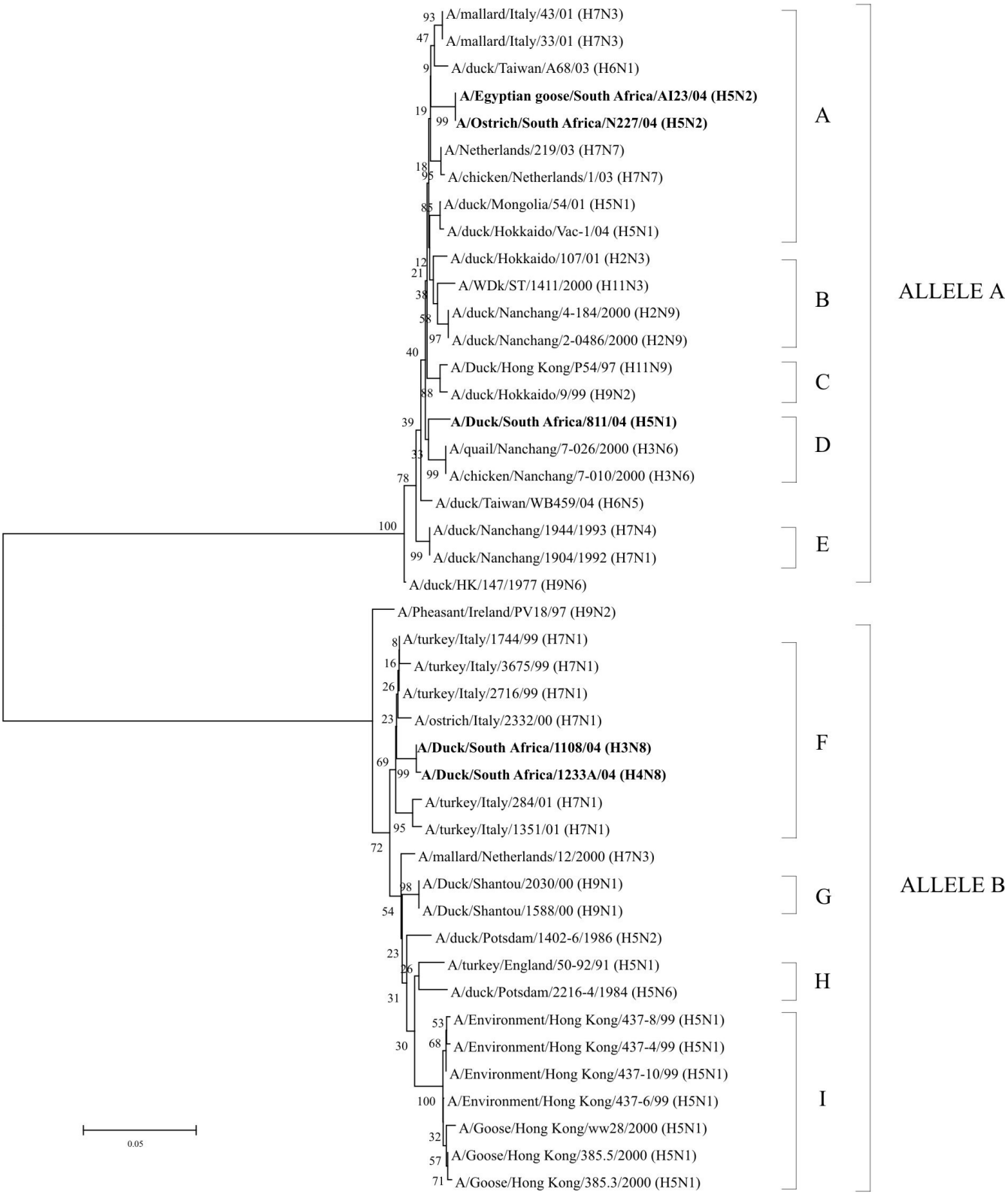


Figure 3.16 Phylogenetic tree inferred from a 675-nt multiple sequence alignment of the NS1 genes of South African AI viruses isolated in 2004 (in boldface) and related sequences. Alleles A and B and sub-lineages A to I are indicated.

Both NS1 allele A and allele B are represented by SA AI viruses isolated in 2004 (Fig. 3.16). Phylogenetically, the NS1 genes of A/Ostrich/South Africa/N227/04(H5N2) and A/Egyptian Goose/AI23/04 (H5N2) NS1 genes are almost identical (sub-lineage A). The only difference between the South African H5N2 NS1 genes is a three amino acid deletion in A/Ostrich/South Africa/N227/04 (H5N2) NS1 (positions 77 to 80, Fig. 3.17). Positions 80 to 85 in Fig. 3.17 correspond to positions 88 to 92 in Thai HPAI H5N1 NS1 genes of 2004 (underlined in Fig. 3.17). Multiple amino acid sequence alignments of contemporary Asian HPAI H5N1 NS gene sequences revealed that HPAI H5N1 NS1 genes in the 1997 group had full-length NS1 genes, whereas those collected during 2000, 2001 and more recently, 2004 Thai HPAI H5N1 NS1 genes, contained a five amino acid (AIASS/V) deletion (Viseshakul *et al.,* 2004). The three amino acid deletion in A/Ostrich/South Africa/N227/04 (H5N2) NS1 is adjacent to the five amino acid deletion in contemporary Asian HPAI H5N1 NS1 genes. Although no specific function has been ascribed to this region yet, the C-terminus of the NS protein has been shown to play an important role in inhibiting interferon expression (Geiss *et al*., 2002).

A/Duck/South Africa/811/04 (H5N1) NS1 gene falls within sub-lineage D of the allele A, and thus appears to be derived from a different source to the NS1 genes of the South African H5N2 viruses. In contrast, A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233A/04 (H4N8) NS1 genes fall within allele B (Fig. 3.16). They shared 99% nucleotide sequence identity with each other, and recent common ancestors with Italian outbreak strains of H7N1 viruses from 1999-2000.

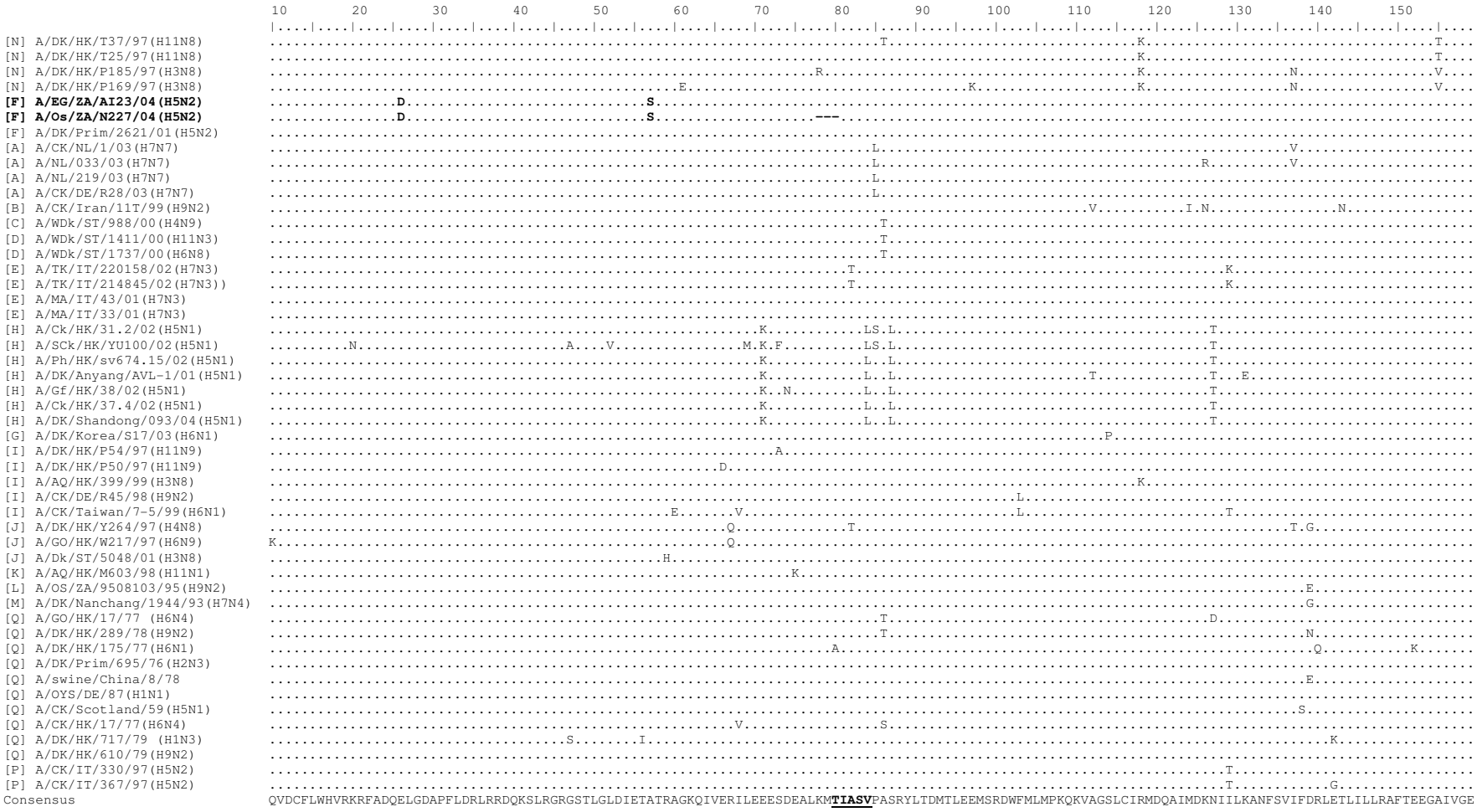
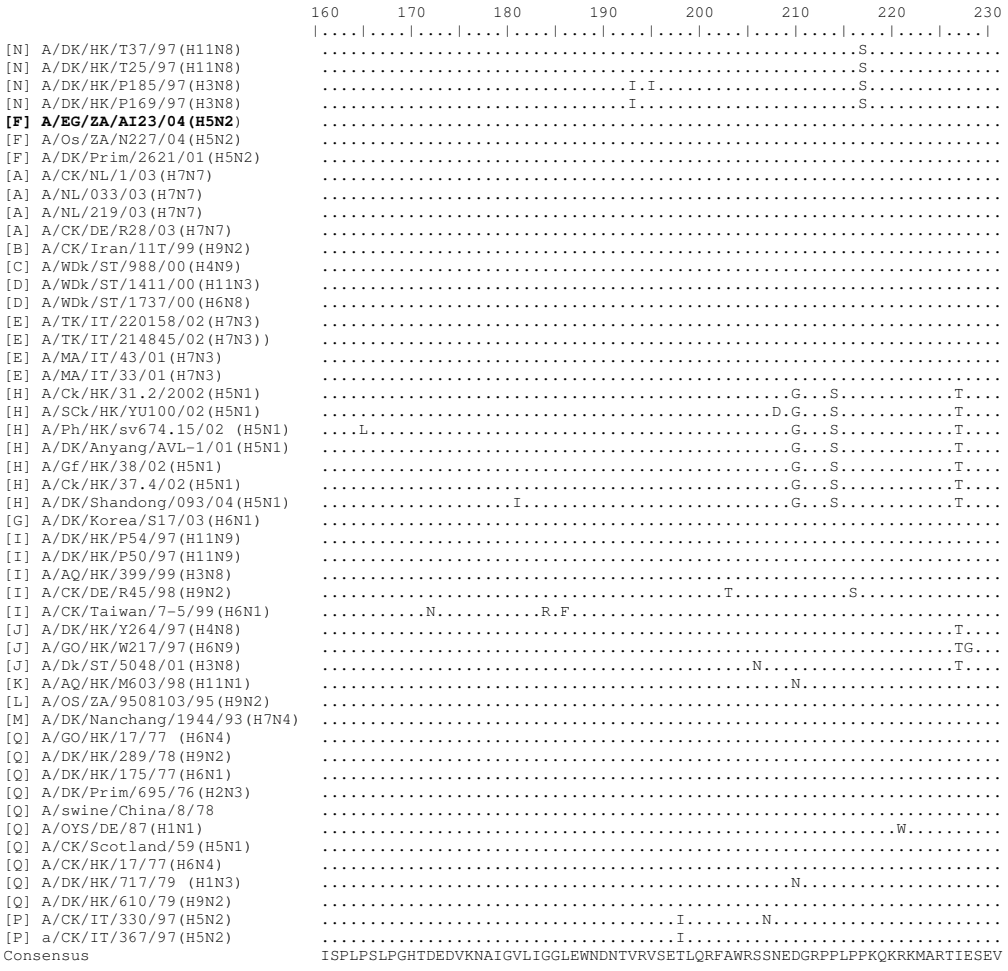


Figure 3.17 Multiple amino acid alignment of the NS1 protein genes of the South African H5N2 viruses and related genes. The position of the 2004 Thai HPAI H5N1 viruses' NS1 deletion is underlined.

Fig. 3.17 continued



3.3.4 Nucleocapsidprotein (NP) genes

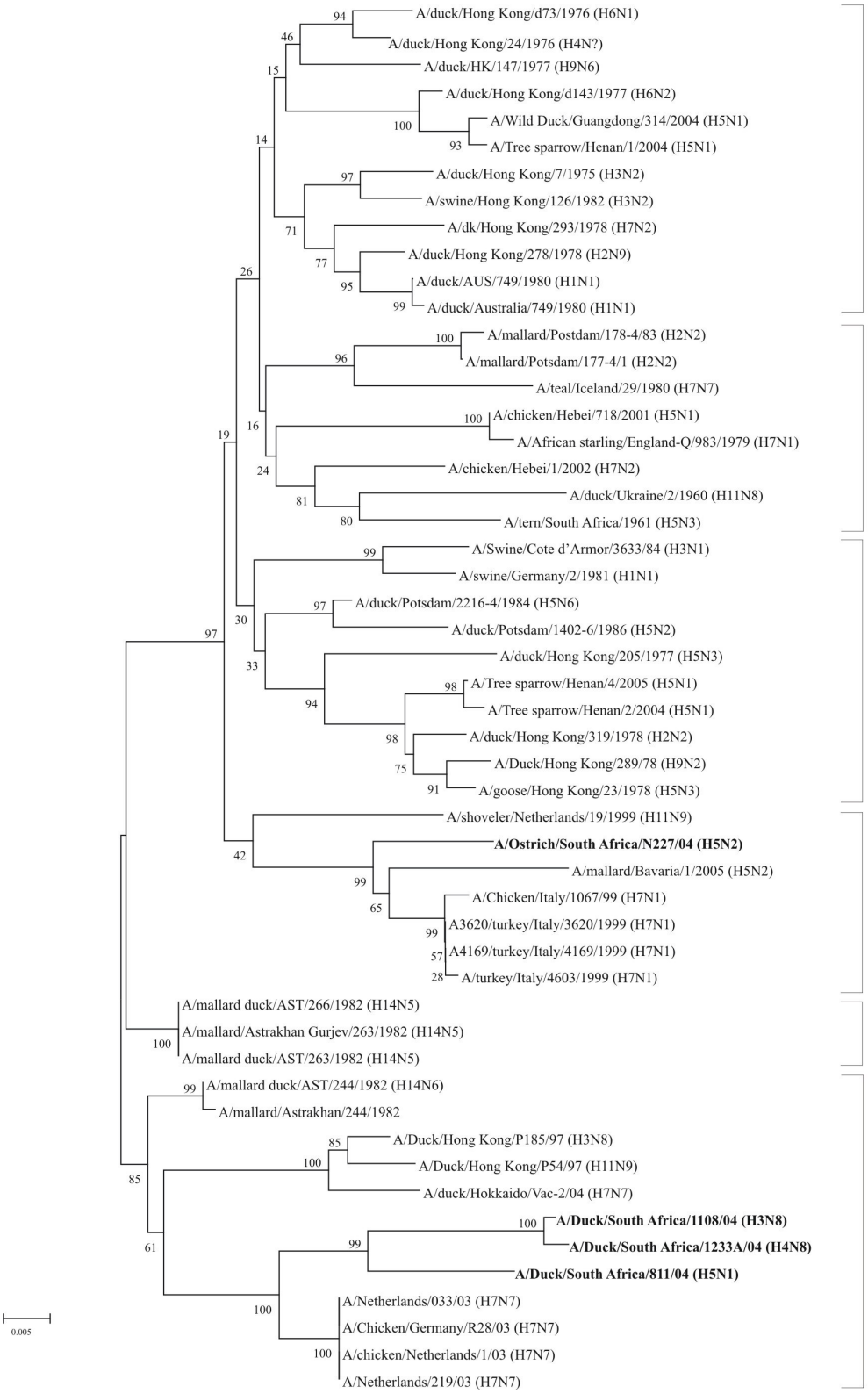


Figure 3.18 Phylogenetic tree inferred from a 776-nt multiple sequence alignment of the NP genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to F are indicated.

The nucleocapsidprotein gene of A/Ostrich/South Africa/N227/04 (H5N2) NP is located within within sub-lineage D (Fig. 3.18) along with those of Italian H7N1 and Dutch H11N9 viruses isolated in 1999. The South African ostrich and Italian H7N1 NP genes share 98% nucleotide sequence identity. These Italian H7N1 isolates were isolated in poultry outbreaks in northern Italy during the winter of 1999-2000. Initially, the H7N1 was LPAI, but later mutated to the HPAI form (Mannelli *et al*., 2005). In comparison, the NP gene of A/Mallard/Bavaria/1/2005 (H5N2) shared only 97% nucleotide sequence identity with A/Ostrich/South Africa/N27/04 (H5N2).

The NP genes of A/Duck/South Africa/1108/04 (H3N8), A/Duck/South Africa/1233A/04 (H4N8) and A/Duck/South Africa/811/04 (H5N1) fall within sub-lineage F and share a recent common ancestor, although A/Duck/South Africa/1108/04 (H3N8) and Duck/South Africa/1233A/04 (H4N8) NP possibly originated from a common source, as they share 99% nucleotide sequence identity, whereas the similarity between either of these and Duck/South Africa/811/04 (H5N1) is only 96%. The phylogenetic relationship of the South African virus genes with those of H7N7 viruses from Germany and the Netherlands suggests epidemiologic origins in a northern European gene pool for the South African LPAI H5N1, H3N8 and H4N8 virus NP genes.

3.3.6 Polymerase A (PA) genes

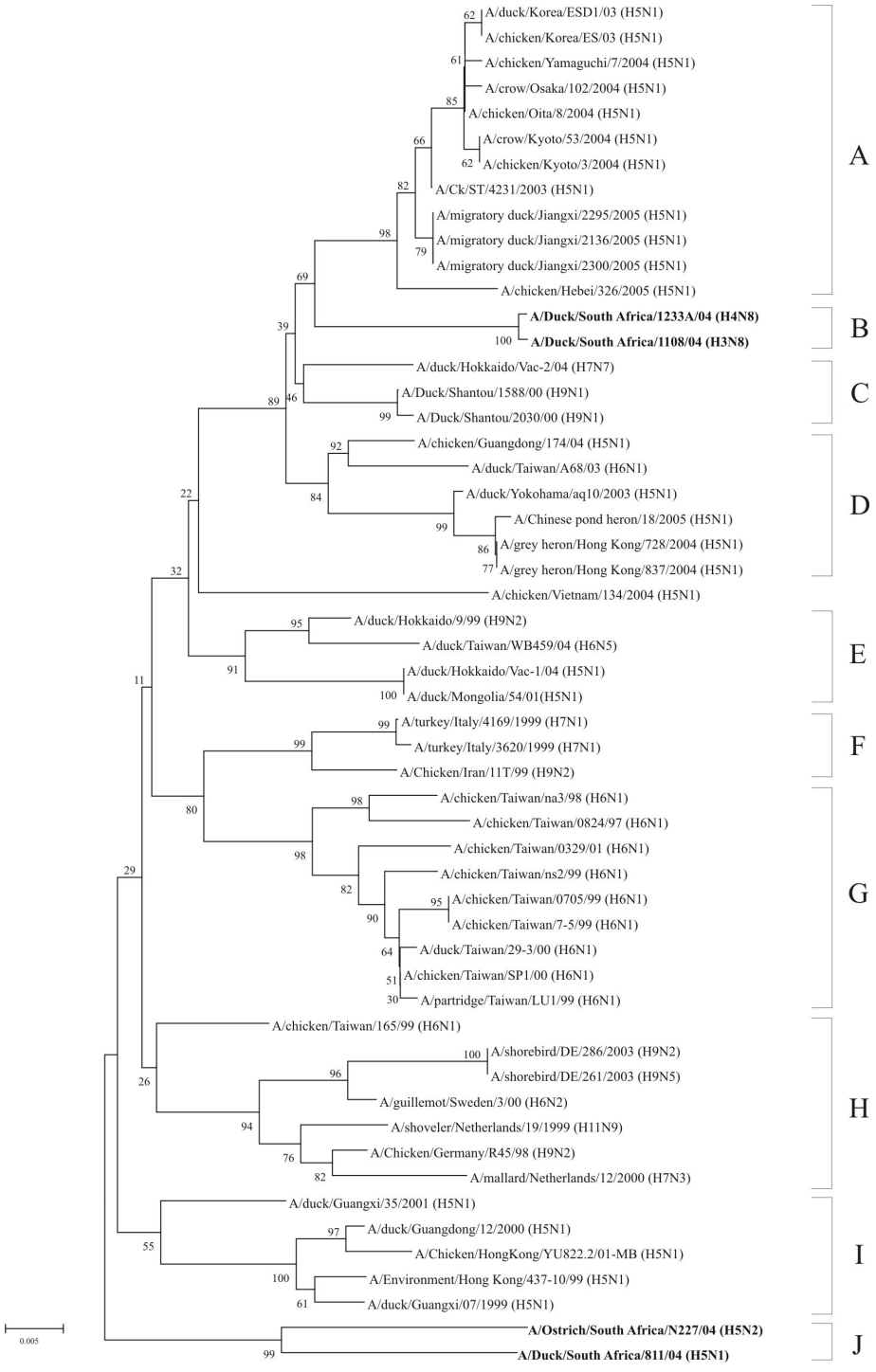


Figure 3.19 Phylogenetic tree inferred from a 695-nt multiple sequence alignment of the PA genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to J are indicated.

Fig. 3.19 indicates that the A/Duck/South Africa/811/04 (H5N1) and A/Ostrich/South Africa/N227/04 (H5N2) PA genes share a common ancestor (sub-lineage J), although the long branch lengths suggest that the evolutionary relationship might be more distant. Alternatively, it may reflect host adaptation. As with the NP genes, A/Duck/South Africa/1108/04 (H3N8) and Duck/South Africa/1233A/04 (H4N8) PA genes (sub-lineage B) share 99% nucleotide sequence identity and a very recent common ancestor. The sub-lineage B PA genes share 97% nucleotide sequence identity with those of sub-lineage A, which is comprised of recent HPAI H5N1 viruses from China and Japan (Mase *et al*., 2005).

3.3.7 Polymerase B1 (PB1) genes

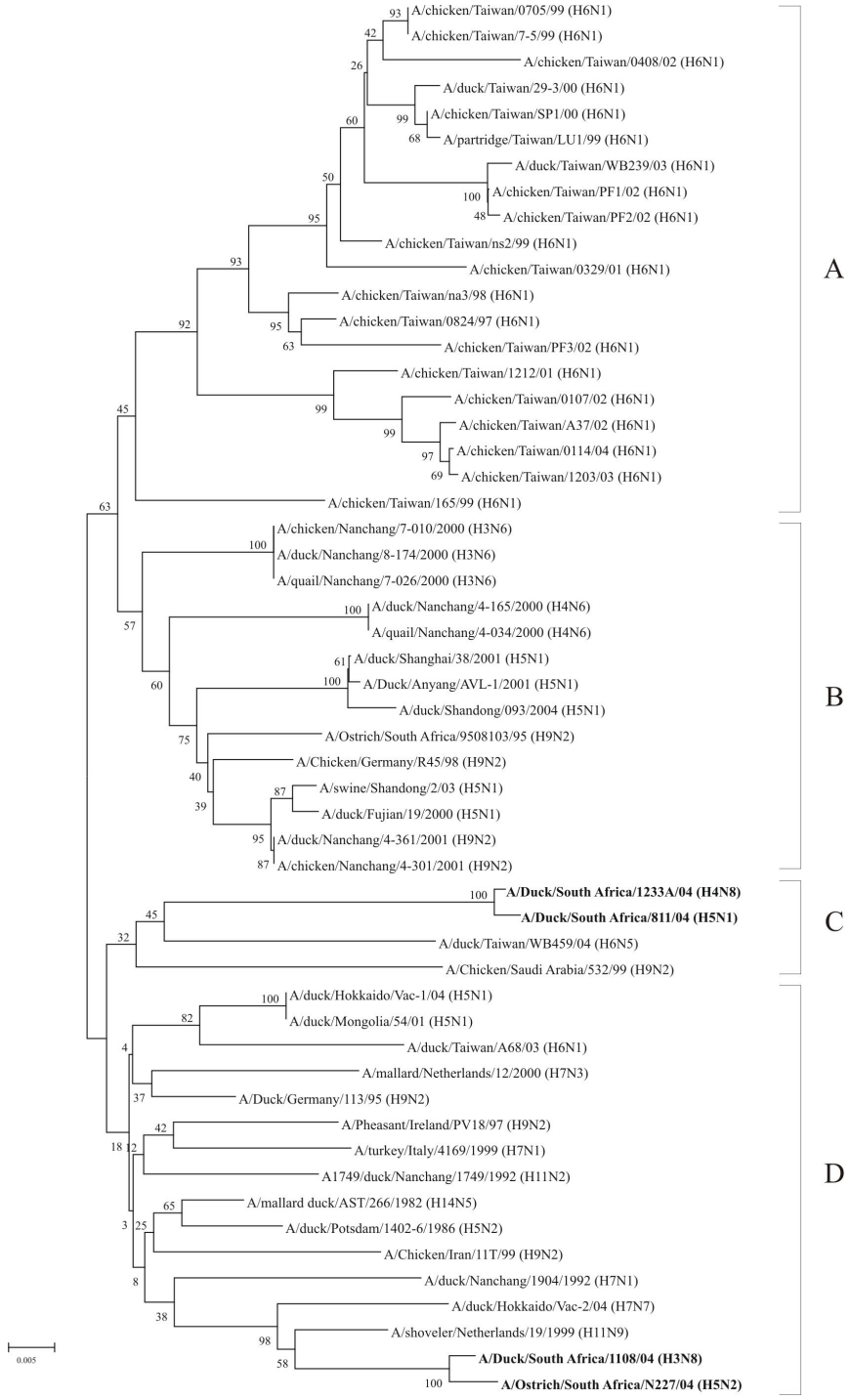


Figure 3.20 Phylogenetic tree inferred from a 753-nt multiple sequence alignment of the PB1 genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to D are indicated.

Phylogenetic analysis (Fig. 3.20) indicated that the HPAI H5N2 ostrich virus PB1 gene shared a recent common ancestor with the homologous gene of A/Duck/South Africa/1108/04 (H3N8) (sub-lineage D). 99% nucleotide sequence identities were shared between the PB1 genes of these two viruses. Sub-lineage D PB1 genes have been identified in AIVs of a wide variety of subtypes from Asia and Europe since the 1980s.

The PB1 genes of A/Duck/South Africa/811/04 (H5N1) and A/Duck/South Africa/1233A/04 (H4N8) also share a very recent common ancestor (99% sequence identity, sub-lineage C). These 2004 viruses fall within sub-lineages distinct from that of the 1995 ostrich strain A/Ostrich/South Africa/958103/95 (H9N2).

3.3.8 Polymerase B2 (PB2) genes

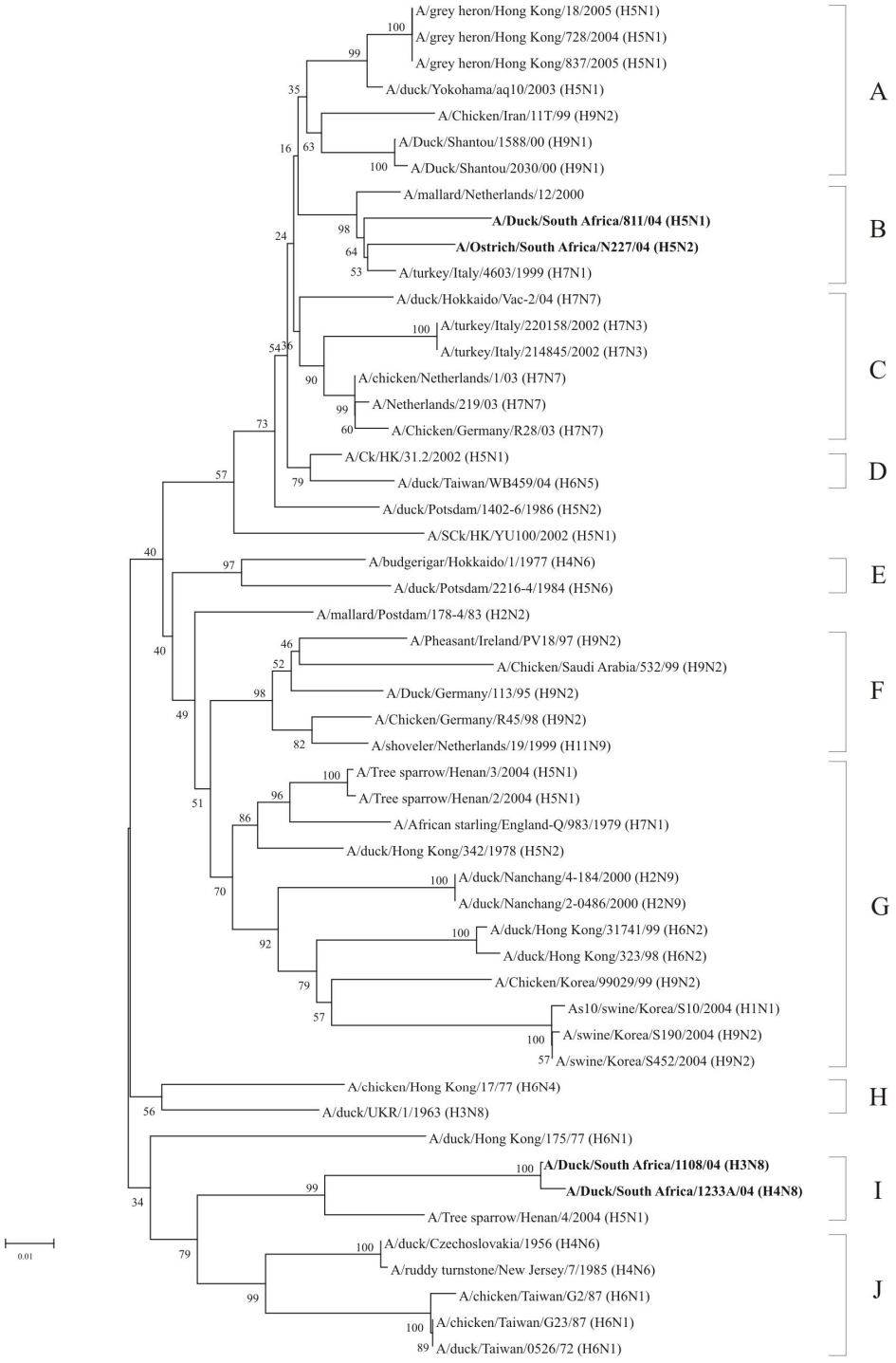


Figure 3.21 Phylogenetic trees inferred from a 726-nt multiple sequence alignment of the PB2 genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to J are indicated.

The A/Ostrich/South Africa/N227/04 (H5N2) PB2 gene falls within sub-lineage B (Fig. 3.21), and as in the case of the PA gene, shares a recent common ancestor with the PB2 gene of A/Duck/SA/811/04 (H5N1). PB2 gene sequences from viruses isolated in Italy and the Netherlands are also included in sub-lineage B. In addition to the insertion of basic amino acids at H_0, the amino acid at position 627 of PB2 has also been associated with the pathogenicity of Asian H5N1 viruses for mammals (Hatta *et al*., 2001; Xu *et al*., 1999). In Fig 3.22, the A/Ostrich/South Africa/N227/04 (H5N2) PB2 partial gene is aligned with the full-length PB2 A/Thailand/1(KAN-1)/04 (H5N1) PB2 gene (that lacked the virulence determinant), to identify position 627. In both viruses position 627 is occupied by an (E) residue confirming that A/Ostrich/South Africa/N227/04(H5N2) PB2 lacks the molecular virulence determinant. The PB2 genes of A/Duck/South Africa/1108/04 (H3N8) and A/ Duck/South Africa/1233A/04 (H4N8) shared 99% sequence identity. The most recent common ancestral sequence in Genbank is the PB2 gene from A/Tree sparrow/Henan/4/2004, an HPAI H5N1 strain.

```
                                10        20        30        40        50        60        70        80        90       100
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  ----------------------------------------------------------------------------------------------------
A/Thailand/1(KAN-1)/04(H5N1)  MERIKELRDLMSQSRTREILTKTTVDHMAIIKKYTSGRQEKNPALRMKWMMAMKYPITADKRIIEMIPERNEQGQTLWSKTNDAGSDRVMVSPLAVTWWN

                               110       120       130       140       150       160       170       180       190       200
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  ----------------------------------------------------------------------------------------------------
A/Thailand/1(KAN-1)/04(H5N1)  RNGPATSAVHYPKVYKTYFEKVERLKHGTFGPVHFRNQVKIRRRVDINPGHADLSAKEAQDVIMEVVFPNEVGARILTSESQLTITKEKKEELQDCKIAP

                               210       220       230       240       250       260       270       280       290       300
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  ----------------------------------------------------------------------------------------------------
A/Thailand/1(KAN-1)/04(H5N1)  LMVAYMLERELVRKTRFLPVAGGTSSVYIEVLHLTQGTCWEQMYTPGGEVRNDDVDQSLIIAARNIVRRATVSADPLASLLEMCHSTQIGGIRMVDILRQ

                               310       320       330       340       350       360       370       380       390       400
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  ----------------------------------------------------------------------------------------------------
A/Thailand/1(KAN-1)/04(H5N1)  NPTEEQAVDICKAAMGLRISSSFSFGGFTFKRTSGSSVKKEEEVLTGNLQTLKIRVHEGYEEFTMVGRRATAILRKATRRLIQLIVSGRDEQSIAEAIIV

                               410       420       430       440       450       460       470       480       490       500
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  ------------------------------------------------------DNVMGMIGILPDMTPSTEMSLRGVRVSKMGVDEYSCTERVVVSIDR
A/Thailand/1(KAN-1)/04(H5N1)  AMVFSQEDCMIKAVRGDLNFVNRANQRLNPMHQLLRHFQKDAKVLFQNWGIEPIDNVMGMIGILPDMTPSTEMSLRGVRVSKMGVDEYSSTERVVVSIDR

                               510       520       530       540       550       560       570       580       590       600
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  FLRVRDQRGNVLLSPEEVSETQGTEKLTITYSSFMMWEINGPESVLVNTYQWIIRNWETVKIQWSQDPTMLYNKMEFEPFQSLVPKAARGQYSGFVRTLF
A/Thailand/1(KAN-1)/04(H5N1)  FLRVRDQRGNVLLSPEEVSETQGTEKLTITYSSSMMWEINGPESVLVNTYQWIIRNWETVKIQWSQDPTMLYNKMEFEPFQSLVPKAARGQYSGFVRTLF

                               610       620       630       640       650       660       670       680       690       700
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A/Ostrich/ZA/N227/04(H5N2)  QQMRDVLGTFDTVQIIKLLPFAAAPPEQSRMQFSSLTVNVRGSGMRILVRGNSPVFNYNKATKRLTVLGKDAGALTEDPDEGTAGVESAVLRGFLILGKE
A/Thailand/1(KAN-1)/04(H5N1)  QQMRDVLGTFDTVQIIKLLPFAAAPPEQSRMQFSSLTVNVRGSGMRILVRGNSPVFNYNKATKRLTVLGKDAGALTEDPDEGTAGVESAVLRGFLILGKE

                               710       720       730       740       750
                        ....|....|....|....|....|....|....|....|....|....
A/Ostrich/ZA/N227/04(H5N2)  NKRYGPALSINELSNLAKGEKANVLIGQGDVVLVMKRKRDSS
A/Thailand/1(KAN-1)/04(H5N1)  NKRYGPALSINELSNLAKGEKANVLIGQGDVVLVMKRKPDTSILTDSQTATKRIRMAIN
```

Figure 3.22 Pairwise amino acid alignment of a full-length PB2 gene with A/Ostrich/South Africa/N227/04 (H5N2) PB2. The amino acid at position 627 (boxed) is a glutamate (E) residue.

3.4 DISCUSSION

In 2004, South Africa recorded its first outbreak of HPAI in forty three years when an H5N2 strain caused mortalities in ostrich flocks of the Eastern Cape Province. In the event of an H5 or H7 avian influenza outbreak, the immediate need is to determine the pathotype (by determining the amino acid sequence at the H_0 cleavage site) and this was the key role that I played in the 2004 HPAI H5N2 outbreak. Furthermore, I identified the NA-type as N2 by RT-PCR and sequencing, as NA typing by neuraminidase–inhibition test (NI) was not available in SA at the time. During active surveillance conducted prior to the outbreak, four other AIVs were isolated or detected in wild duck organ samples in two different provinces, Gauteng and the Western Cape. The serotypes were established as LPAI H5N1, LPAI H5N2, H3N8 and H4N8 by sequence analysis. I was particularly interested in determining whether the LPAI H5N2 virus, detected in an Egyptian goose from the Western Cape province, was genetically related to the Eastern Cape HPAI H5N2 outbreak strain. Apart from phylogenetic analysis to determine the sources of all the viruses isolated in 2004, molecular characterization was done to assess whether the H5N2 and H5N1 strains were related to the Asian HPAI H5N1 strains and posed a threat to human health, and whether they contained any known virulence determinants.

Phylogenetic analysis of the H5 genes confirmed that the LPAI H5N2 Egyptian goose virus was the closest relative to the HPAI H5N2 Eastern Cape ostrich outbreak strain. HPAI viruses arise from avirulent H5 and H7 lineages in poultry by duplication of basic amino acid sequences at the cleavage site of HA, and it appears that these mutations occur only after the viruses have moved from their natural host to poultry (Banks *et al*., 2000a, Rohm *et al*., 1995; Perdue *et al*., 1997). Such events have been recorded in the USA (1983-1984), Mexico (1994-1995), Italy (1999-2000) and Chile (Swayne & Suarez, 2000). In full agreement with these reports, the H_0 cleavage site of the Egyptian goose virus was PQRETRGLF, and the ostrich virus sequence was PQREKRRKRGLF, containing the multiple basic amino acid insertion that is typical of highly pathogenic strains. Support for the theory that the HPAI H5N2 ostrich virus arose from a virus like the LPAI H5N2 Egyptian goose virus was provided by the phylogenetic results of the N2,

M and NS genes, where the LPAI and HPAI viruses were shown to be very closely related. A potential virulence determinant was identified in a three amino acid deletion in the NS1 gene of the ostrich virus, that was absent from the LPAI Egyptian goose virus NS1 gene. This deletion occurred at a position adjacent to a five amino acid deletion observed in Thai HPAI H5N1 viruses isolated since 2001 (Viseshakul *et al.,* 2004). The ostrich virus lacked any virulence determinants associated with the internal genes, had no deletion in the N2-stalk region and lacked additional N-glycosylation sites in the HA gene. Despite the molecular virulence determinants at H_0 and possibly NS1, the ostrich virus did not appear to be highly pathogenic to chickens: an IVPI test was carried out at VLA Weybridge (UK) on the ostrich virus and a value of 0.63 was obtained. However, after a further passage in embryonated eggs, the IVPI had increased to 1.19 in chickens, and when the virus was isolated from cloacal swabs from birds used in the initial IVPI, the IVPI value obtained with the reisolated virus was 2.73. Interestingly, birds that survived the first two IVPI tests exhibited marked cyanosis of wattles, combs and legs and became depressed, but by the end of the ten-day test period the birds had returned to an apparently normal clinical state. No additional changes at the H_0 cleavage sites were observed (Manvell *et al*., 2005). Therefore, the HPAI H5N2 virus had the potential to become highly pathogenic for chickens. Fortunately it did not spill over into or circulate amongst chickens, as indicated by negative serological data from the outbreak area and the rest of the country (Truuske Gerdes, personal communication). Phylogenetic analysis confirmed that A/Ostrich/South Africa/N227/04 (H5N2) was not closely-related to contemporary Asian H5N1 strains, and this particular H5 lineage has never been associated with human deaths.

The H5N1 virus isolated from a yellow-billed duck, A/Duck/South Africa/811/04, was not genetically closely-related to the Asian HPAI H5N1 viruses, and analysis of the peptide sequences at H_0 (PQRETRGLF) confirmed that the Duck/South Africa/811/04 (H5N1) is a low pathogenic strain. Additional N-glycosylation sites were not detected in the HA protein sequence, the N1 gene lacked a stalk deletion, and virulence markers were absent from the PB2 and NS1 genes.

Influenza A viruses possessing the H3 hemagglutinin are one of the most frequently isolated subtypes from feral birds (Kida *et al*., 1987), and are also one of the most genetically stable subtypes, able to remain viable for prolonged periods in the environment (Webster *et al*., 2006). Since 1998, H3 viruses and H3N8 in particular have been isolated from feral ducks in Northern Europe (Denmark, the Netherlands, Sweden and Norway), Russia and China, but not in southern European countries such as France or Italy (Cheng *et al*., 2006; Jonassen & Handeland, 2006; Lvov *et al*., 2004; Munster *et al*., 2006; Cherbonnel *et al*., 2006; Cattoli *et al*., 2006). I demonstrated that the A/Duck/South Africa/1108/04 (H3N8) H3 gene was derived from a recent common ancestor of a Danish H3N8 virus isolated from a mallard in 2003.

The HA gene from A/Duck/South Africa/1233/04 (H4N8) was very closely-related to Russian H4 virus genes isolated from wild ducks and garganey at lake Chany in 2002. Twenty three H4 viruses (H4N2, H4N6, H4N8 and H4N9) have been isolated from South Eastern Siberia and the North Pacific from 2000 to 2002, and the high degree of H4 sequence homology between isolates from the birds and muskrats in south-eastern Siberia suggest that the water is the common source of the infection (Lvov *et al*., 2004). H4N8 was also recently isolated from wild ducks in China, the Netherlands, Sweden and H4 (not N8) in Italy (Cheng *et al*., 2006; Munster *et al*., 2006; Cattoli *et al*., 2006). The H_0 cleavage sites of the H3N8 and H4N8 viruses were PEKQTRGLF and PEKASRGLF, which is typical for H3 and H4 viruses, respectively. The N8 genes of both viruses probably originated from a common source.

Phylogenetic analysis of the internal protein genes of the South African HPAI H5N2, LPAI H5N2, H5N1, H3N8 and H4N8 viruses isolated in 2004 revealed that some reassortment has occurred within the South African gene pool, as summarised in Fig. 3.23:

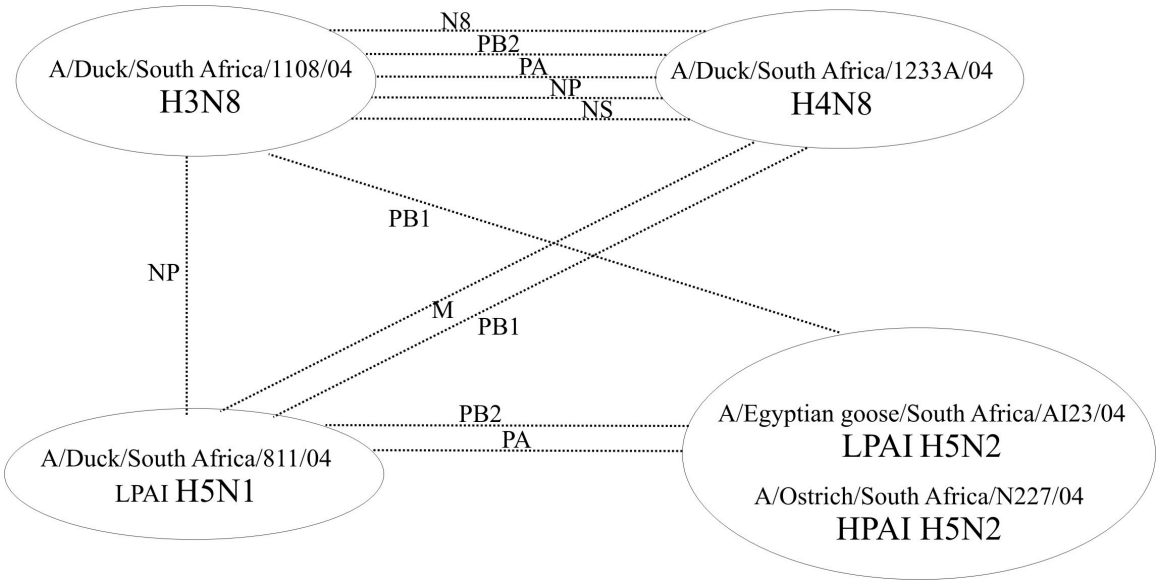


Figure 3.23. Reassortment between South African AIVs isolated in 2004.

The HPAI H5N2 virus and its LPAI progenitor obtained the polymerase genes (PB2, PB1 and PA) from the same gene pool as the LPAI H5N1, H3N8 and H4N8 viruses that were collected at Blesbokspruit in the Gauteng province. This demonstrates that the wild waterfowl populations from geographically separated regions in southern Africa do mix and exchange viruses.

Phylogenetic analysis of glycoprotein and internal genes demonstrated that the South African LPAI viruses were recently derived from the Eurasian gene pool, particularly the northern European countries of Germany, Norway, Denmark, Sweden and the Netherlands. Russia features prominently, followed by Italy, Mongolia, Japan and China. High sequence identities were observed between genes of viruses isolated in South Africa and in these countries, however, the movements of South African duck and goose species are restricted to sub-Saharan Africa, as indicated by ringing and capture data (Underhill *et al*., 1999). In some cases, the movements of feral waterfowl have been modified by the construction of permanent water bodies and the provision of agricultural grain as a predictable food source during the non-breeding season. Prior to these becoming available, migration to subtropical regions would have been necessary (Underhill *et al*., 1999). Some species, like the Southern Pochard and Comb duck have been recorded to

move between SA and countries north of the equator (Underhill *et al*., 1999). Viruses from West African ducks can theoretically be passed to southern African ducks that move (albeit rarely) that far North. Although very little sequence data exists for AIVs from other African countries, different species follow the Black-Sea Mediterranean flyway to West Africa each year (Appendix 3). A likely source for the introduction of Eurasian viruses into South Africa is via migratory waders, and this will be discussed in greater detail in the closing chapter.

The detection of a North American lineage virus in a Eurasian zone is unusual but not unrecorded: H2N3 and H2N2 viruses isolated from migratory ducks in Japan contained PB2 and PA genes, respectively, that belonged to American lineage viruses, whereas the other genes were Eurasian. The PA gene of the Japanese H2N2 virus Dk/Hokkaido/95/01 was closely related to Mal/NY/6750/78 (H2N2), thus at least one other case where two genetically closely-related viruses appear more than twenty years apart has been described in the literature (Liu *et al*., 2004). Swedish guillemots sampled on the island of Bonden in the northern Baltic Sea were found to contain H6N2 viruses that contained PA, NP and NA gene segments from the Eurasian lineage, but PB2, PB1, HA, M and NS gene segments from the American lineages (Wallensten *et al*., 2006). Conversely, North American viruses containing genes of Eurasian lineage have also been reported (Shafer *et al*., 1993; Makarova *et al*., 1999). The American and Eurasian lineages may have emerged because of non-overlapping migration routes, as most wild birds follow north-south migrations routes that are separate for the two hemispheres (Makarova *et al*., 1999). Birds that do cross the Atlantic do so at times when North-South migrations are over, for example, the terns and gulls that cross the Atlantic from Canada arrive in Europe after the European birds have migrated south to Africa. On rare occasions, unusual weather conditions may bring the two populations into contact, allowing the inter-regional transmission of viruses. Turnstones are one of the pelagic migrant species that may play a role in this transmission of AIVs between the Eurasian and American continents (Curry-Lindahl, 1975), and they are one of the migrant species that visit South Africa annually (Underhill *et al*., 1999). Influenza viruses remain viable in frozen lake water after migration and get preserved over winter (Ito *et al*., 1995), allowing a non-seasonal

migrant to become infected with a virus that was deposited some months before. The frozen lake theory could also be the mechanism whereby viruses are able to lie apparently dormant for several decades, which could explain how a North American H5 virus that was last detected in 1976 appeared in South African ducks in 2004.

Once pelagic migrants arrive in South Africa, their habitats are not limited to shorelines and coastal estuaries, instead, they are frequent inhabitants of freshwater inland dams, pans, wetlands and water treatment works, and here they have contact with endemic duck and goose species. Viruses shed by waders into the water bodies would be ingested by ducks and geese and would be amplified in these reservoir hosts, which move extensively throughout the country. The majority of ostrich farms in South Africa are located in the Karoo area of the Western Cape province, a semi-arid region with sparse natural grazing and an annual rainfall of only 228 mm. Ostrich farms are often concentrated around rivers and riverine areas, and are used by the ostrich farmers in cultivating irrigated lucerne pastures for ostriches and sheep. Wild indigenous and migratory birds are found in abundance in this area and accumulate in vast numbers on ostrich farms, where they graze alongside ostriches and concentrate in great numbers around the watering troughs and feeders. In this way, ostrich water and feed become contaminated with faecal material (Sinclair *et al*., 2005).

There are some indications that the H5N2 ostrich infection originated in the Western Cape province, as there was serological evidence of H5 infections on 42 Western Cape ostrich farms, without any reports of clinical disease. The LPAI virus must have circulated in ostriches for some time before the HPAI sequence PQREKRRKKRGLF was generated, although it is unknown how many passages or what time frame is required for these mutations to occur. It could be a step-wise acquisition of basic amino acids into H_0, and at least some of the steps could have occurred in the Western Cape ostriches. Interspecies transmission of avian influenza not only flows from waterfowl to terrestrial birds, but also in the opposite direction, as evidenced by viruses analysed from birds in southern China and possibly Hong Kong (Li *et al*., 2003). Therefore, translocated infected ostriches could have been the source of the infection to the Eastern Cape

Province, or alternatively, wild birds possibly introduced the virus into Eastern Cape ostriches.

The link between wild birds and AIV outbreaks in ostriches was conclusively shown by the results presented in this chapter, and this highlights the importance of bio-security in commercial ostrich farming, and the potential of LPAI viruses from wild waterfowl to mutate to HPAI in terrestrial poultry. An AIV prevalence rate of 6% in South African wild ducks in 2004 (three out of 53 organ samples) is in accordance with similar international studies (Kawaoka *et al*., 1988; Sharp *et al*., 1993 Deibel *et al*., 1985; Hinshaw *et al*., 1985, Cattoli *et al*., 2006, Munster *et al*., 2006) but as reported, this tends to vary somewhat, since no viruses were detected in active surveillance of wild ducks in South Africa since 2004 (unpublished laboratory data). This is also the first report of AIV isolation from Cape Teal (*Anas capensis*), Cape Shoveller (*Anas smithii*), Yellow-billed ducks (*Anas undulata*) and Red-billed ducks (*Anas erythrorhynchos*) in South Africa, and the first report of AIV isolation from wild ducks in the Gauteng province. The reassortment observed between internal genes of viruses from the Western Cape and Gauteng provinces highlights the extensive movements and mixing of wild waterfowl populations in the region. However, wild ducks, particularly those species considered to be agricultural pests like the yellow-billed duck and Egyptian geese make good sentinels for viruses introduced by migratory birds, and can be targeted for active surveillance on annual basis. Their role as sentinels is becoming become increasingly important as Asian HPAI H5N1 continues to spread across the world.

CHAPTER FOUR[1]

PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN VELOGENIC NEWCASTLE DISEASE OUTBREAK STRAINS OF THE 1990s (GENOTYPES VIII AND VIIb)

ABSTRACT

A previously-published report found that two separate genotypes of exotic NDV circulated in South Africa during the 1990s. In this study, new and existing data was collated to better define the epidemiology of Newcastle disease in South Africa in the 1990s. The results confirmed that genotype VIII/lineage 3d became enzootic in South Africa since it was introduced from south East Asia in the 1960s and 1970s, but that South Africa was not the source for subsequent outbreaks in China, Japan and Italy. Genotype VIII viruses appear to have attenuated, as the most recent isolates were not as virulent as those of the early 1990s. A second genotype, VIIb (lineage 5b), entered South Africa in the early 1990s and swept across the country as part of a panzootic. Although it was previously suggested that the infection was introduced into southern Europe from South Africa, phylogenetic data indicated that instead, strains identical to early South African viruses had been present in Spain and Portugal at least one to two years prior to the outbreaks in South Africa, and that introduction possibly occurred on multiple occasions from Europe. The chronological and geographical separation of South African genotype VIIb strains during the 1990s was consistent with reports from other countries, and the epidemiological pattern suggested that Genotype VIIb was sustained in South Africa throughout the 1990s via the translocation of infected poultry.

[1]Part of the results presented in the chapter were published in Archives of Virology 149(3):603-619 (2004)

4.1 INTRODUCTION

Exotic Newcastle disease has been considered to be enzootic in South Africa since 1971 (L Coetzee, personal communication), and therefore trade in South African poultry and unprocessed poultry products are banned by many countries. After severe outbreaks in the 1970's, only sporadic outbreaks occurred in South Africa over the next decade. Then, in June 1993, a neuro/respirotropic NDV was isolated from an outbreak at a large commercial poultry producer near Hartebeespoort, Gauteng province. Within six months ND had spread throughout southern Africa, causing devastating losses in all types of poultry. An estimated million chickens per week died at the peak of the outbreak. The major outbreak of 1993/94 was eventually brought under control by improved vaccination procedures and bio-security measures (Huchzermeyer & Gerdes 1993; Verwoerd, 1995a; 1995b), but sporadic outbreaks continued to occur for the remainder of the decade (unpublished laboratory data).

The presence of genotypes VIIb (lineage 5b) and VIII (lineage 3d) in southern Africa and their involvement in the NDV outbreaks of the 1990s was first described by Herczeg and colleagues (1999). Genotype VIII which has predominantly been isolated in southern Africa (Herczeg *et al*, 1999), occurred in western China between 1979 and 1985 (Liang *et al*., 2002), Japan in 1991 (Mase *et al*., 2002), Taiwan in 1995 (Tsai *et al*., 2004) and Italy in 1994 (Aldous *et al*., 2003). Phylogenetic evidence has indicated that genotype VIII viruses possibly originated in Southeast Asia (Mase *et al*., 2002; Tsai *et al.,* 2004). Similarly, genotype VII appeared in Taiwan and Indonesia in the 1980's (Yu *et al.* 2001; Lomniczi *et al*., 1998). It was responsible for the fourth pandemic and was the progenitor to the VIIa, VIIb, VIIc and VIId genotypes. Genotype VIIa has been reported in the Far East and Europe, VIIb in the Far East, Middle East, Europe, India and southern Africa, VIIc in the Far East and Europe and VIId in the Far East and South Africa (Lomniczi *et al.*, 1998; Jørgensen *et al*., 1998; Alexander *et al*., 1999; Yang *et al.,* 1999; Herczeg *et al*., 2001; Liang *et al*., 2002; Wehman *et al*., 2003a; 2003b; Abolnik *et al*., 2004a; Bogoyavlenskiy *et al*., 2005).

In a previous study (Herczeg *et al*., 1999), six South African genotype VIII, seven genotype VIIb, and six genotype VIIb Mozambican viruses were analysed phylogenetically. Results suggested that genotype VIII viruses were maintained in South Africa by enzootic infections since the 1960s, whereas genotype VIIb was introduced more recently. However, data were insufficient to make accurate inferences about the sources of the two genotypes, nor could it describe the epidemiology of NDV in the region. In 2003, a larger NDV sequence data set was published by the international reference laboratory, VLA Weybridge in the UK. That study contained over 300 strains of exotic NDV submitted by countries across the world, and the authors concurred with Herczeg and colleagues that genotype VIIb had spread from southern Africa to southern Europe in the early 1990s (Aldous *et al*., 2003).

For NDV to be enzootic in South Africa, a source or reservoir must exist from which periodic outbreaks arise. Village chickens have been suspected of being these reservoirs, but wild birds have also been implicated (Verwoerd, 1995b). From 1990 to 2002, 155 ND viruses were collected by national veterinary laboratories across South Africa. These viruses were pathotyped at OVI by nucleotide sequencing of the region at the fusion protein (F_0) cleavage site and phylogenetic analyses. Sixty five of the viruses were identified as being virulent genotype VIIb and VIII viruses. The remaining viruses were typed as vaccine strains or their close derivatives (Abolnik *et al*., 2004a). In this chapter, I collated all available sequence data for genotype VIIb and VIII with sequence data for sixty five additional South African virulent strains isolated during the outbreaks of the 1990s. The objectives were to better define the epidemiology of NDV in South Africa during the 1990s and, if possible, to determine the source of the outbreaks.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

Velogenic ND viruses (Table 4.1) were grown in 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual, 2000), at Allerton Provincial Veterinary Laboratory, the Poultry Reference Laboratory (University of Pretoria), and Stellenbosch Provincial Veterinary Laboratory. ICPI and MDT tests were performed at Allerton Provincial Veterinary Laboratory according to standard procedures (OIE manual, 2000).

4.2.2 RNA extraction

Viral RNA was extracted from infective allantoic fluid using the QIAamp® Viral RNA Mini Kit (QIAGEN), TriReagent™ (SIGMA) or TRIzol® reagent (Gibco, Invitrogen), according to the manufacturer's instructions.

4.2.3 RT-PCR

Random hexamers were used to generate first strand cDNA according to the method described by Sambrook *et al.* (1989). The following oligonucleotide primers were used to amplify a 1180 base pair fragment spanning the regions between nucleotides 581 of the fusion protein and nucleotides 610 of the matrix protein, which includes the F_0 cleavage site. Reaction mixtures were cycled 35 times at 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min.

M610	5'- CTGTACAATCTTGCGCTCAATGTC -3'	(forward primer)
NDVF581	5'- CTGCCACTGCTAGTTGTGATAATCC -3'	(reverse primer)

4.2.4 DNA sequencing and phylogenetic analysis

DNA was sequenced using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions, and was analysed with an ABI377™ automated sequencer. Sequences of as little as 250 base pairs give meaningful phylogenetic analyses for NDV, comparable with much longer sequences (Seal *et al*., 1995; Lomniczi *et al*., 1998). A commonly used region in NDV phylogenetic studies is a 374 nt fragment at the 3' end of the fusion protein, which includes the region encoding the nuclear localization signal sequence and the precursor fusion protein cleavage site (F_0). Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 374 nucleotide (nt) region were used to identify 50 closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Preparation of Fig. 4.4 (variable sites only of the nucleotide sequence alignment) was done with MEGA v3.1. The results are presented as rooted neighbour joining trees with 1000 bootstrap trials to assign confidence values to topology. Virulent viruses were rooted with the lentogenic La Sota/46 virus sequence.

Table 4.1 Velogenic South African ND viruses characterised in this study

NDV Strain	Collection date	District	Genotype	Accession number
ZA11/B/91	22/03/1991	Pietermaritzburg	VIII	AF532140
ZA13/L/91	08/08/1991	Richmond	VIII	AF532142
ZA16/GF/91	12/09/1991	Richmond	VIII	AF532143
ZA19/B/91	10/12/1991	Lion's River/ Merrivale	VIII	AF532752
ZA52/BB/94	03/03/1994	Camperdown area	VIIb	AF532749
ZA53/BB/94	04/03/1994	Camperdown	VIIb	
ZA54/BB/94	07/03/1994	Camperdown	VIIb	
ZA60/B/94	21/03/1994	Camperdown area	VIIb	AF532750
ZA71/B/94	21/04/1994	Camperdown area	VIIb	AF532751
ZA76/L/94	18/05/1994	Lion's River/Merrivale	VIIb	
ZA87/X/94	20/06/1994	Pretoria	VIIb	
ZA108/L/94	15/07/1994	Estcourt	VIIb	AF532139
ZA110/X/94	19/07/1994	Pretoria	VIIb	AF532141
ZA133/B/94	01/09/1994	Camperdown	VIIb	
ZA170/B/95	09/03/1995	Lower Tugela/ Umhlali	VIIb	AF532144
ZA172/B/95	18/04/1995	Lower Tugela/ Umhlali	VIIb	
ZA903/UP/96	21/03/1996	Pretoria	VIIb	
ZA983/UP/96	10/11/1996	Mpumahlanga province	VIIb	
ZA100/L/94	06/07/1997	Durban	VIIb	
ZA256/X/97	22/07/1997	Irene, Pretoria	VIIb	AF532146
ZA825/UP/97	31/11/1997	Mokopane	VIIb	AY210499
ZA839/UP/97	17/10/1997	Kuruman	VIIb	AY210494
ZA842/UP/97	17/10/1997	Pretoria	VIIb	AY210504
ZA837/UP/97	18/10/1997	Wakkerstroom	VIIb	
ZA928/UP/97	05/12/1997	Gabarone, Botswana	VIIb	AY210495
ZA982/UP/97	06/12/1997	Kuruman	VIIb	
ZA96/UP/98	06/02/1998	Tzaneen	VIIb	
ZA148/UP/98	07/03/1998	Gabarone, Botswana	VIIb	AY210507
ZA7351/Rvsdl/98	??/07/1998	Riversdale	VIIb	AY210502
ZA7381/98	??/07/1998	Western Cape	VIIb	AY210512
ZA700/UP/98	12/07/1998	Vorna Valley	VIIb	
ZA699/UP/98	12/07/1998	Johannesburg	VIIb	
ZA704/UP/98	12/07/1998	Rustenburg	VIIb	AY210508
ZA751/UP/98	25/07/1998	Pretoria	VIIb	
ZA753/UP/98	25/07/1998	Ermelo	VIIb	
ZA762/UP/98	25/07/1998	Johannesburg	VIIb	
ZA756/UP/98	25/07/1998	Vereeniging	VIIb	
ZA734/UP/98	25/07/1998	Rustenburg	VIIb	

ZA984/UP/98	27/07/1998	Gabarone, Botswana	VIIb	AF109876
ZA775/UP/98	01/08/1998	Mokopane	VIIb	
ZA798/UP/98	15/08/1998	Cullinan	VIIb	
ZA9357/Moslb/98	??/09/1998	Mosselbaai	VIIb	
ZA933/UP/98	13/09/1998	Pretoria	VIIb	
ZA949/UP/98	13/09/1998	Phalaborwa	VIIb	
ZA959/UP/98	13/09/1998	Mokopane	VIIb	
ZA955/UP/98	13/09/1998	Bronkhorstpruit	VIIb	
ZA917/UP/98	13/09/1998	Witbank	VIIb	
ZA983/UP/98	20/09/1998	Rustenburg	VIIb	AY210509
ZA1003/UP/98	20/09/1998	Makapanstad	VIIb	
ZA296/L/98	20/09/1998	Durban	VIIb	AF532148
ZA922/UP/98	21/09/1998	Johannesburg	VIIb	
ZA444/B/98	??/10/1998	Hammarsdale	VIIb	
ZA3291/Klpmts/99	??/03/1999	Klapmuts	VIIb	AY210503
ZA309/B/99	12/03/1999	Paulpietersburg	VIIb	AF532151
ZA309/B/99	12/03/1999	Paulpietersburg	VIIb	
ZA1320/UP/99	31/07/1999	Pretoria	VIIb	AY210510
ZA1251/UP/99	01/08/1999	Halfway House	VIIb	AY210501
ZA8057/99	??/08/1999	Western cape	VIIb	
ZA922/UP/98	21/09/1999	Johannesburg	VIIb	
ZA335/B/99	26/10/1999	Camperdown	VIIb	AF532740
ZA598/UP/00	02/06/2000	Pretoria	VIII	AY210496
ZA606/UP/00	02/06/2000	Pretoria	VIII	AY210497

4.3 RESULTS

Phylogenetic analysis of the 65 strains isolated in South Africa from 1990 to 2000 confirmed previously published results that two separate genotypes were responsible for outbreaks during this period: genotype VIII (lineage 3d) and genotype VIIb (lineage 5b). Genotype VIII was analysed first:

4.3.1 Genotype VIII (lineage 3d)

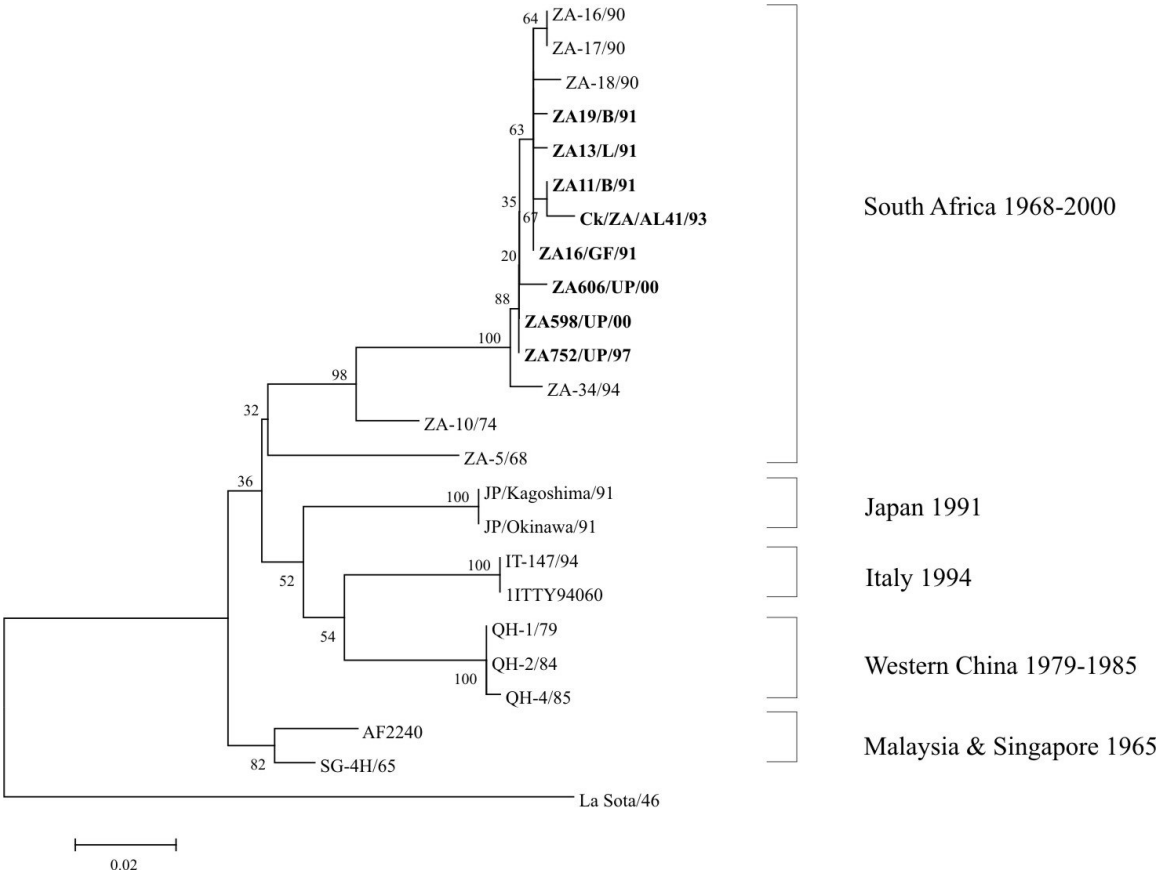


Figure 4.1 Dendogram of a 374 nt 3'-end region of the fusion proteins of South African genotype VIII (lineage 3d) viruses. Viruses whose fusion protein sequences were determined in this chapter are indicated in boldface.

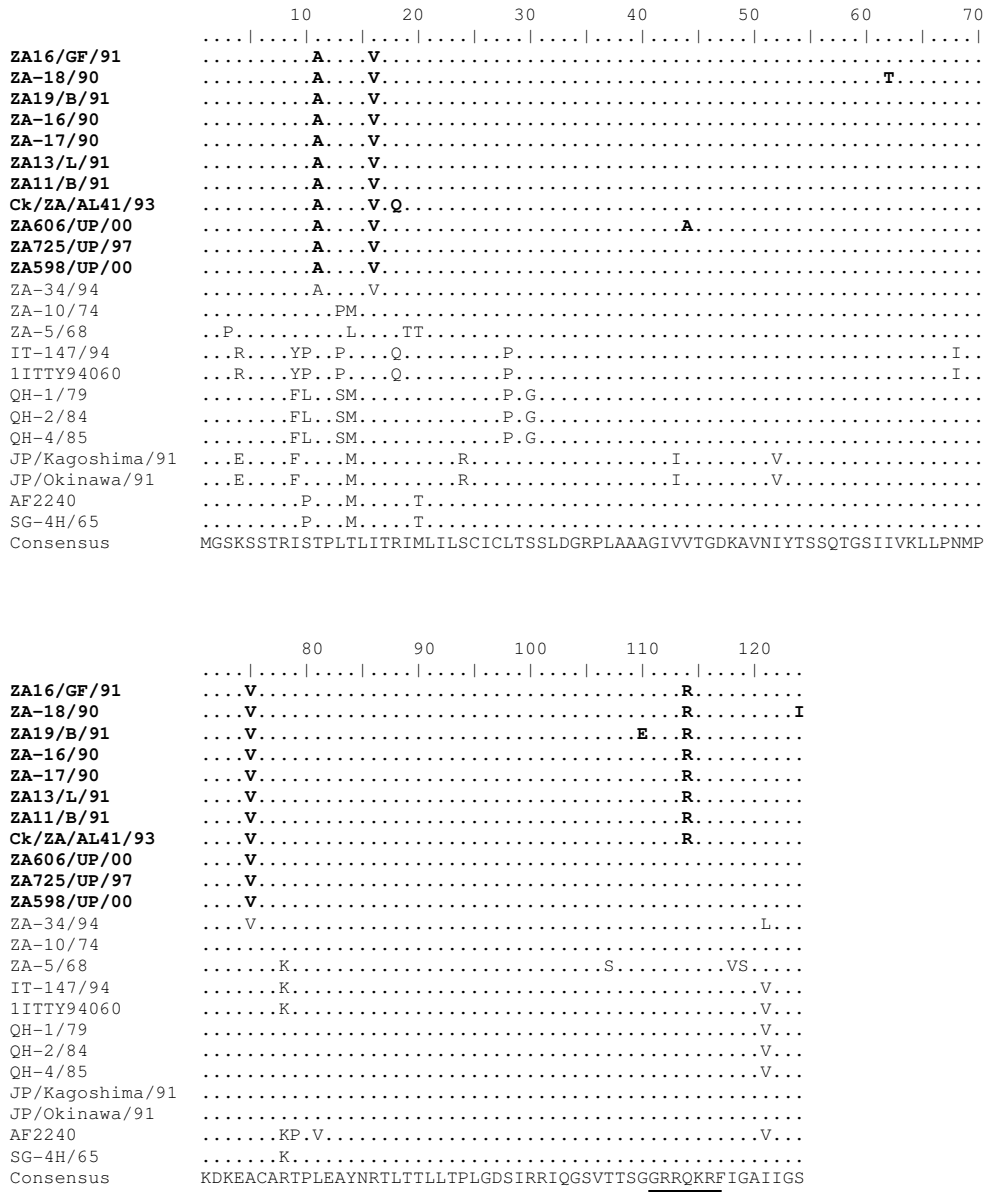


Figure 4.2 Multiple amino acid alignment of partial fusion (F) protein genes of genotype VIII viruses, including the F_0 cleavage site (underlined). Viruses whose nucleic acid sequences were determined in this study are indicated in boldface.

Genotype VIII/lineage 3d is composed of only 23 isolates from Singapore, Malaysia, Italy, Japan, China and South Africa ranging in years of isolation from 1965 to 2000. Of these, fourteen viruses (60.8%) originated from South Africa, and eight of the fourteen were contributed by the present study. Fig. 4.1 indicates that the South African viruses are distinct from viruses isolated from other regions, but that all contemporary genotype VIII isolates (including South Africa) are likely to be derived from the South East Asian strains isolated in the 1960s. The earliest South African viruses from 1968 (ZA-5/68) and 1974 (ZA-10/74) are basal to the contemporary South African strains, although the 1968 genotype is only supported by a 32% bootstrap value. In contrast, the 1974 virus is supported by a bootstrap value of 98%, supporting suggestions that this genotype was enzootic in South Africa since the 1970s (Herczeg *et al*., 1999; Aldous *et al*., 2003).

The South African genotype VIII viruses sequenced in this study (ZA11/B/91, ZA19/B/91, ZA13/L/91, ZA16/GF/91) were phylogenetically interspersed with four viruses analysed in a previous study (ZA-16/90, ZA-17/90, ZA-18/90 and ZA-34/94) (Herczeg *et al*., 1999). An IVPI value of 2.89 was obtained for ZA/11/B/91, confirming the highly virulent pathotype of genotype VIII viruses. The SA genotype VIII viruses isolated from 1990 to 1993 contained a unique R^{114} for Q substitution (Fig. 4.2). Herczeg and colleagues (1999) suggested that the R^{114} contributed to the high pathogenicity of genotype VIII. After 1994, Genotype VIII viruses went undetected until 1997 (ZA752/UP/97), and were then isolated on two more occasions in 2000 (ZA598/UP/00, ZA606/UP/00). Unlike the viruses of the early-to-mid 1990s (KZN province), these later isolates were obtained from the Pretoria region (Gauteng province). South African genotype VIII viruses isolated from 1994 onwards contained the conventional Q^{114} in the F_0 cleavage site (Fig. 4.2), and did not appear to be as virulent as those isolated in the early 1990s. For example, ZA598/UP/00, which was isolated east of Pretoria, only caused a 2% drop in egg production with no clinical symptoms apart from nasal excretions (unpublished laboratory report data). The phylogenetic results clearly indicate that South Africa was not the source of genotype VIII viruses causing outbreaks in Japan, China or Italy.

4.3.2 Genotype VIIb (Lineage 5b)

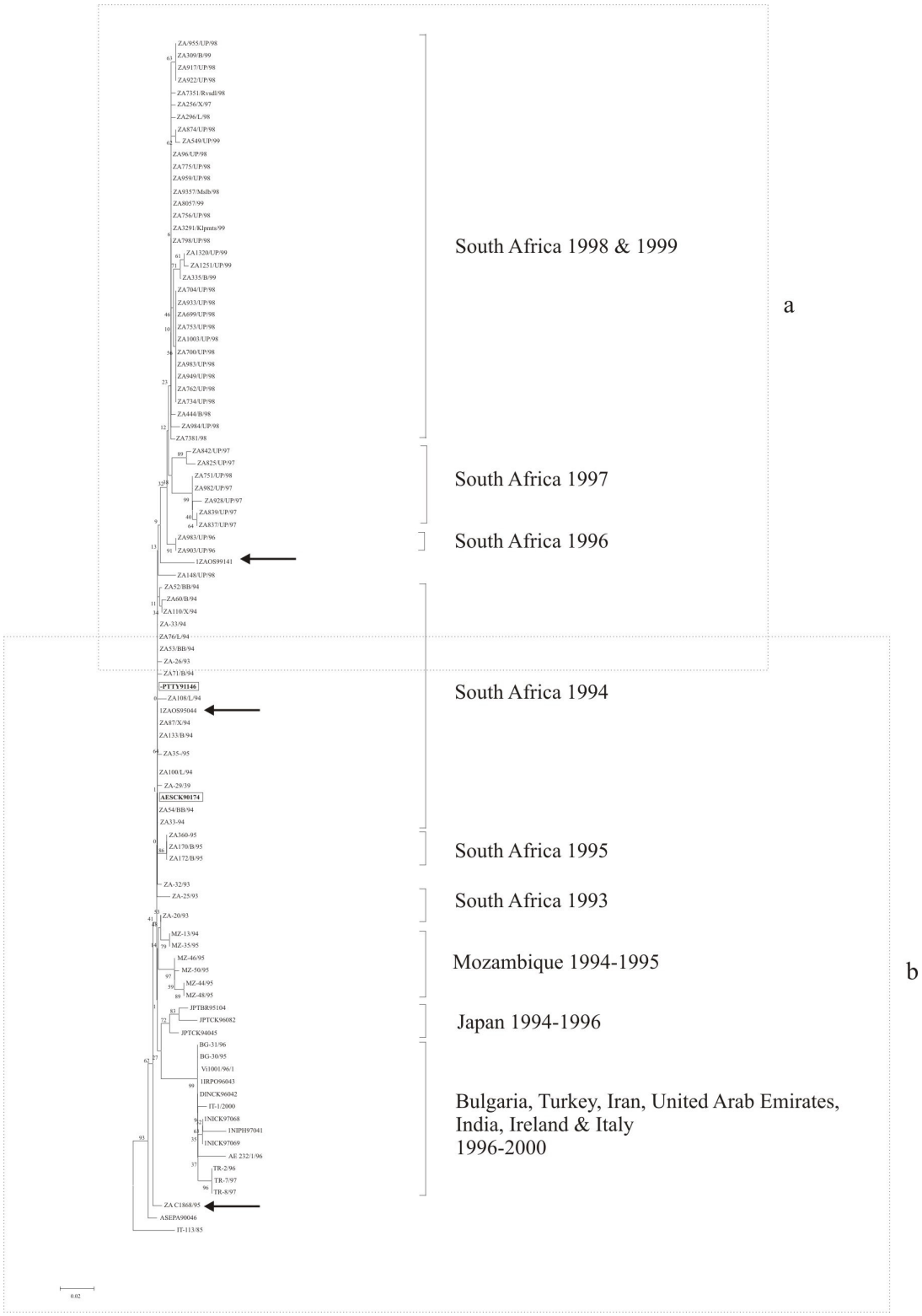


Figure 4.3(a) Phylogenetic tree of genotype VIIb isolates. South African viruses whose nucleic acid sequences were determined in this study are indicated in boldface. Ostrich isolates are indicated by arrows. Portuguese and Spanish viruses are boxed. Enlargements of views (a) and (b) are presented in Figs. 4.3(b) and 4.3(c) respectively.

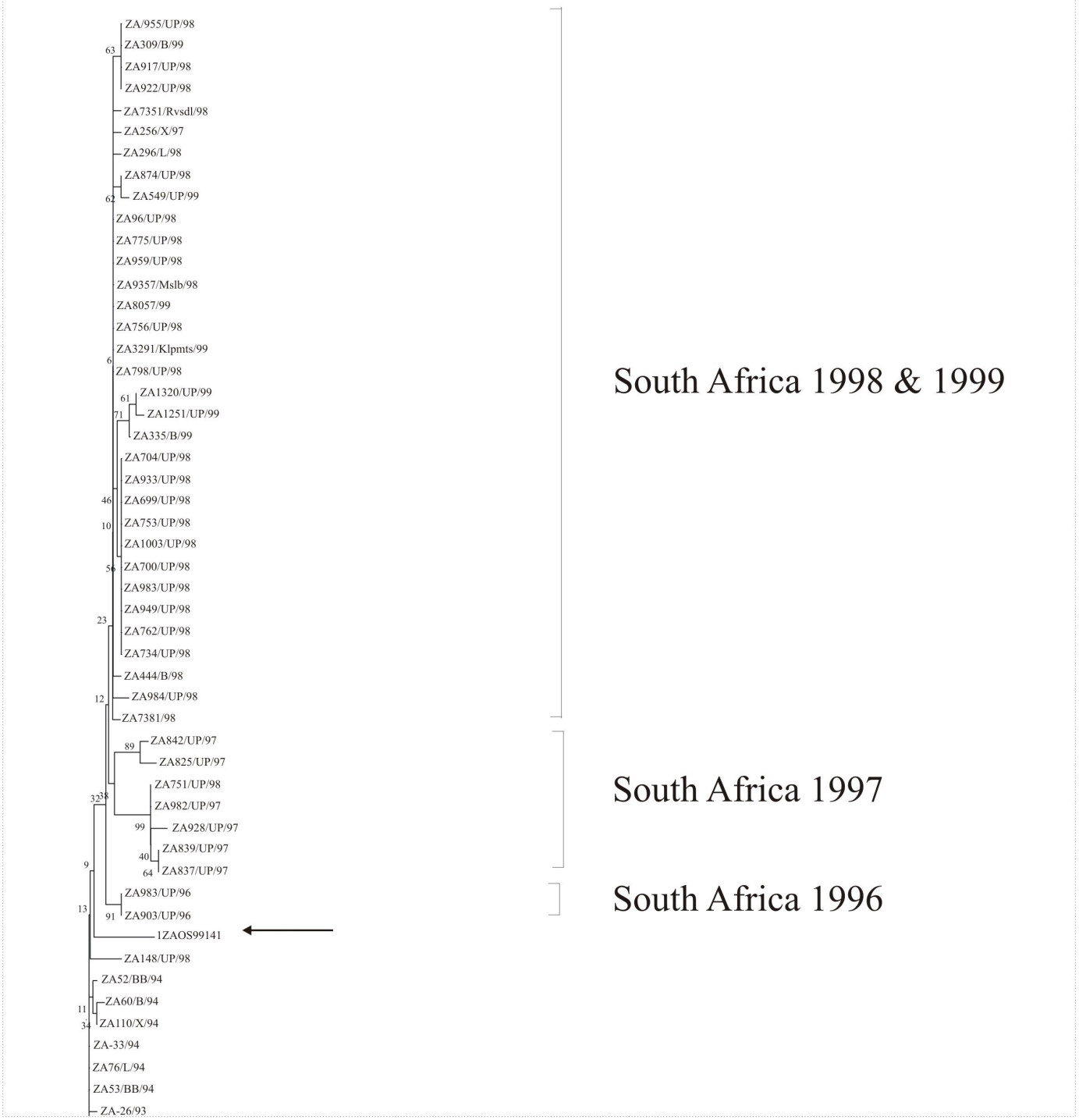


Figure 4.3(b). Enlargement of view (a)

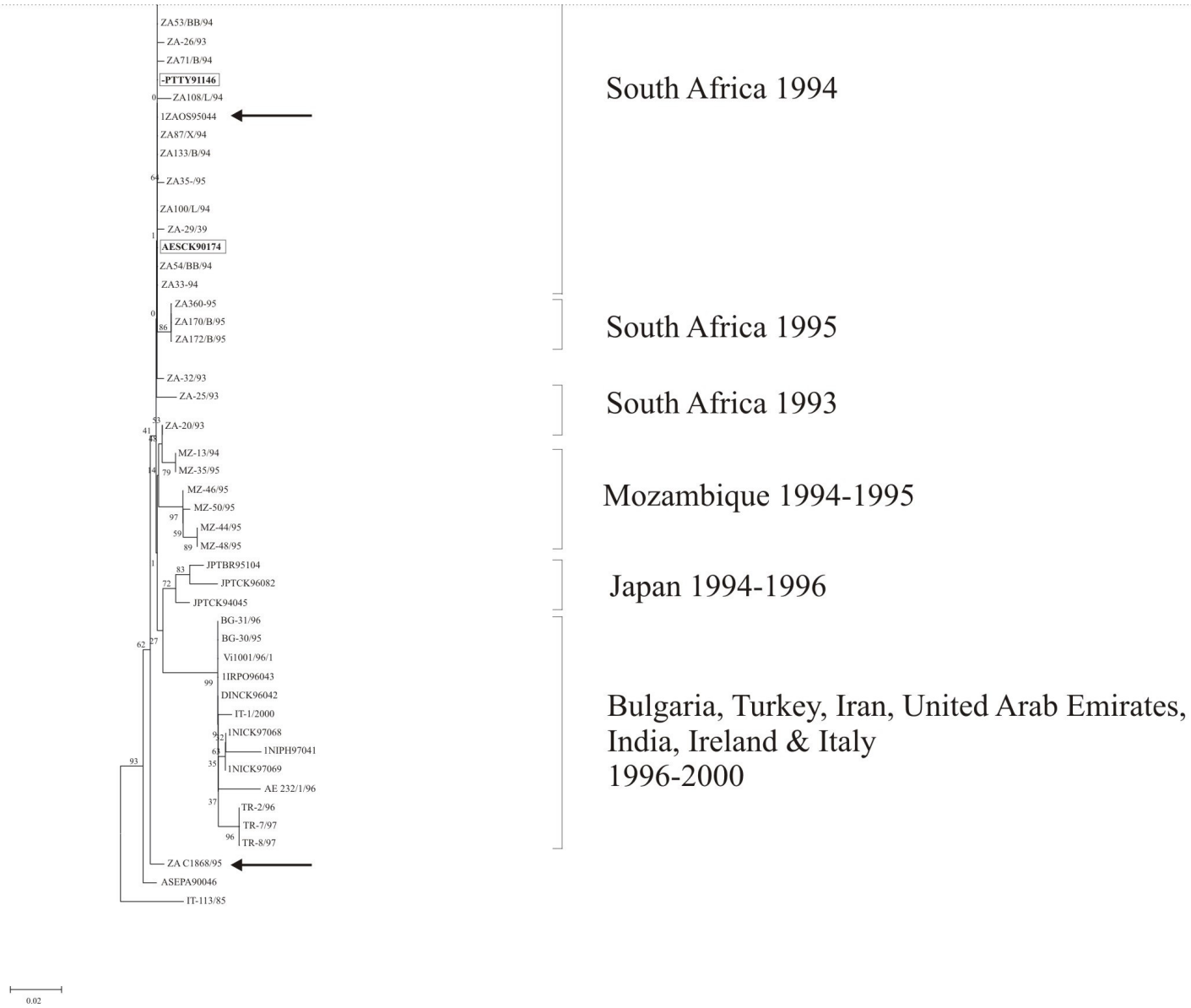


Figure 4.3(c). Enlargement of view (b)

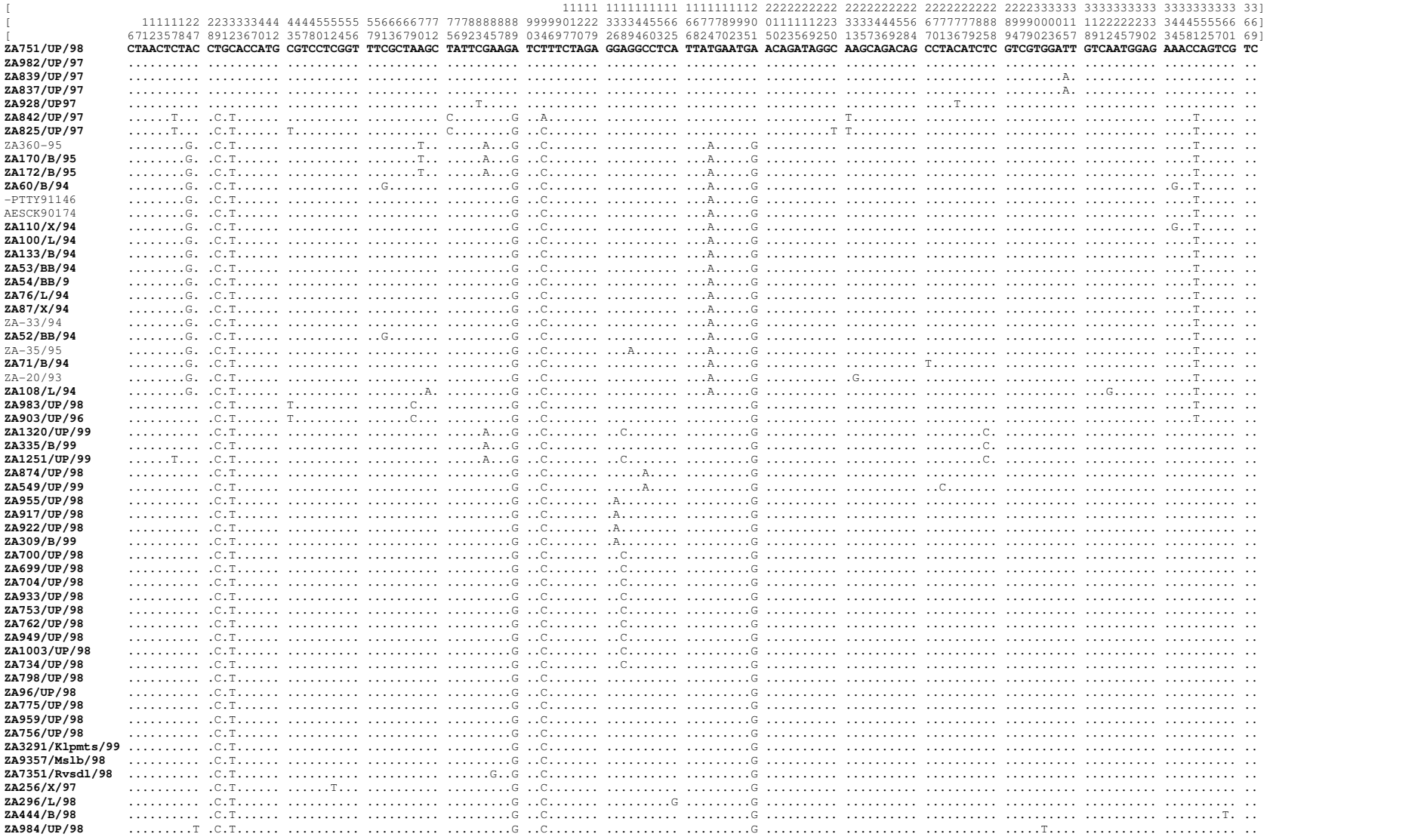
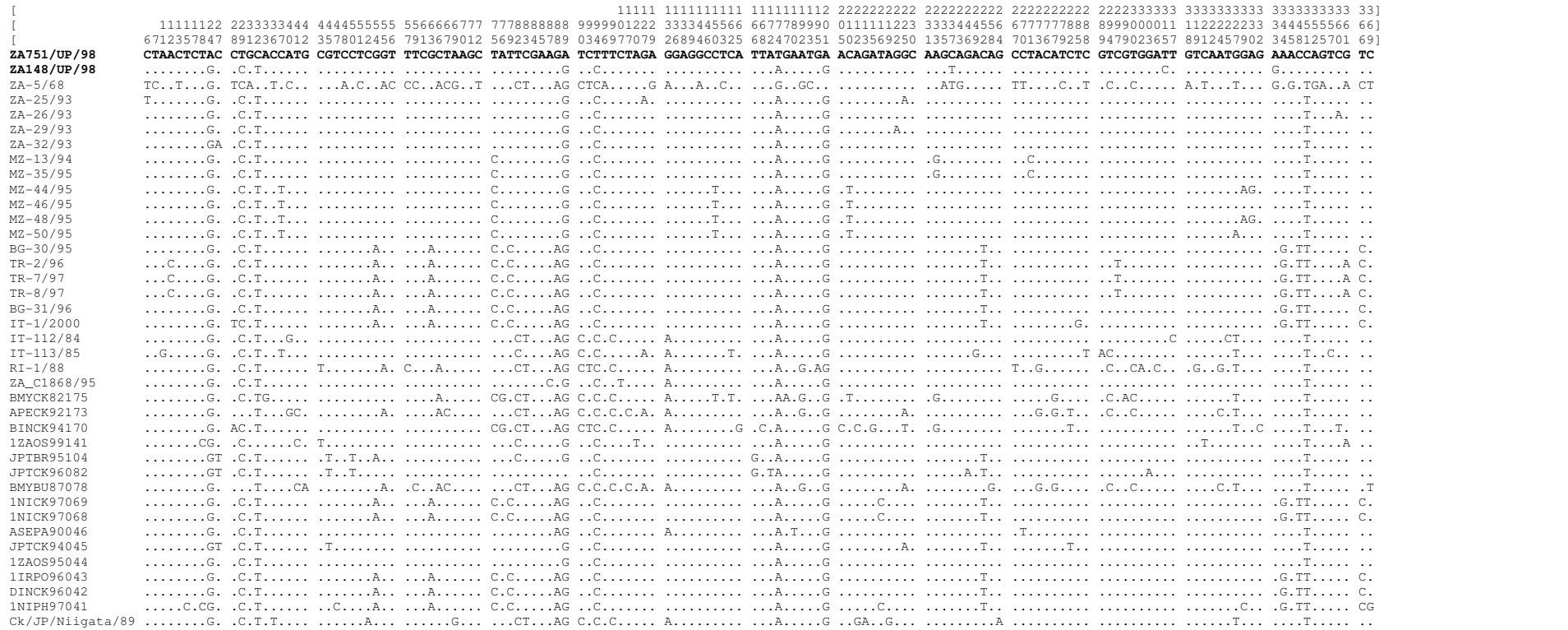


Figure 4.4 Muliple nucleotide sequence alignment of partial fusion (F) protein genes of genotype VIIb viruses. Only variable sites are presented, and sequences contributed by the present study are indicated in boldface

Fig. 4.4 continued



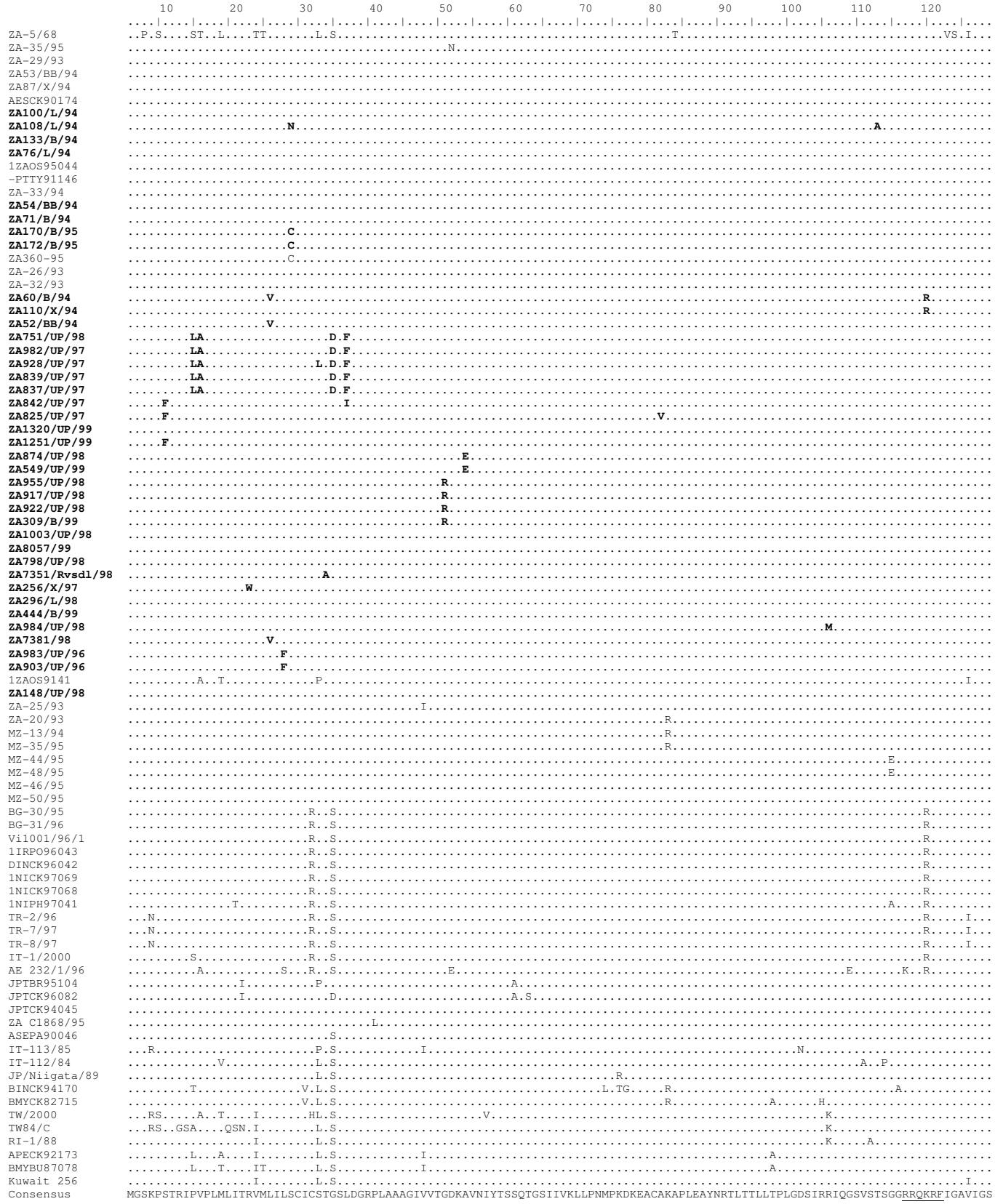


Figure 4.5 Multiple amino acid alignment of partial fusion (F) protein genes of genotype VIIb viruses, including the F_0 cleavage site (underlined). Viruses whose nucleic acid sequences were determined in this chapter are indicated in boldface.

Figs. 4.3(a) to (c) illustrate the phylogenetic relationships between South African genotype VIIb viruses and the closest relatives in the Genbank database. The early southern African genotype VIIb isolates (1993 to 1995) sequenced in this study are interspersed with previously-published isolates ZA-33/94, ZA-26/93, ZA-35/95, ZA-29/93, ZA-38/94, ZA-25/93 and ZA-20/93 from the same period, and are phylogenetically separated from later isolates. The chronological separation is supported at the nucleotide level by a unique A^{174} substitution (Fig. 4.4) that is also present in a South African ostrich isolate, 1ZAOS91141 isolated in 1991 (Aldous *et al*., 2003). Within this early group, two smaller sub-lineages are evident. The first sub-lineage is composed of isolates ZA60/B/94, ZA52/BB/94 and ZA110/X/94. The first two viruses were isolated in Camperdown in March 2004, whereas ZA110/X/94 was isolated in Pretoria four months later (Table 4.1). Phylogenetically, ZA60/B/94 appears to be more closely-related to ZA110/X/94 and these two virus share a non-synonymous $A^{344}\rightarrow G$ substitution (Fig. 4.4) that resulted in a $K^{115}\rightarrow R$ mutation within the F_0 cleavage site (Fig. 4.5). The $K^{115}\rightarrow R$ mutation was also found within isolates from Bulgaria, Turkey, Iran, the United Arab Emirates, India, Ireland and Italy between 1996 and 2000 (Fig 4.3; Fig. 4.5). ZA60/B/94 and ZA52/BB/94 shared a non-synonymous G^{61} substitution (Fig. 4.4) that resulted in a $L^{21}\rightarrow V$ mutation (Fig. 4.5). Despite having a virulent F_0 cleavage site motif, ZA60/B/94 had a mean death time value between 60 and 90 hours, which classified it as a mesogenic and not a velogenic virus. The attenuation of undefined virulence determinants elsewhere in the genome of ZA60/B/94 is expected to account for the decreased pathogenicity.

The second sub-lineage within the early genotype VIIb viruses is composed of three isolates, viz. ZA170/B/95, ZA172/B/95 and ZA360-95, which are identical at the nucleotide sequence level. Nucleotide substitutions $A^{70}\rightarrow T$ and $G^{84}\rightarrow A$ were observed (Fig. 4.4). The former resulted in a unique $S^{24}\rightarrow C$ substitution (Fig. 4.5). ZA360-95 was isolated from an ostrich (Alexander *et al.,* 1999) and ZA170/B/95 and ZA172/B/95 from broilers in the Lower Tugela/Umhlali area of KZN. Despite a lack of nucleotide sequence data from provinces other than KZN, the spread of the early major outbreak is well documented (Huchzermeyer & Gerdes, 1993; Verwoerd *et al* 1995a; 1995b; 1997; 1999). From the phylogenetic evidence presented here, it seems likely that ZA-20/93 or a close genetic relative gave rise to the Mozambican outbreak strains. The two most interesting inclusions into the early South African clade were two viruses from Spain

and Portugal (AECK90174 and -PTTY91148), that were isolated from poultry in 1990 and 1991, respectively (Aldous *et al*., 2003). These two viruses were identical at the nucleotide sequence level to ZA-29/93, ZA53/BB/94, ZA87/X/94, ZA100/L/94, ZA133/B/94, ZA76/L/94, 1ZAOS95044, ZA-33/94 and ZA54/BB/94, the earliest southern African isolates apart from 1ZAOS9141.

Chronological phylogenetic separation was also evident for viruses isolated in 1996, 1997, and 1998 to 1999 (Fig. 4.3). The 1996 isolates ZA983/UP/96 and ZA903/UP/96 shared a unique non-synonymous $A^{69}\rightarrow C$ substitution (Fig. 4.4) that produced a unique F^{24} residue (Fig. 4.5). The 1997 isolates ZA752/UP/97, ZA825/UP/97, ZA839/UP/97, ZA842/UP/97, ZA837/UP/97, ZA928/UP/97, ZA982/UP/97 and a single 1998 isolate (ZA751/UP/98) shared numerous synonymous and non-synonymous nucleotide substitutions (Fig. 4.4), and were distinguished at the amino acid level by unique L^{10}, A^{11}, D^{30} and F^{32} residues (ZA842/UP/97 had an I^{32} mutation) (Fig.4.5).

Within the 1997 sub-lineage, other nucleotide and amino acid residues that indicated recent common ancestors for some of the strains were evident (Fig. 4.4, Fig. 4.5). For example, ZA842/UP/97 and ZA825/UP/97 shared an $S^{6}\rightarrow F$ substitution. ZA842/UP/97 was isolated in October 1997 in Pretoria and ZA825/UP/97 in November in Mokopane (Limpopo province).

ZA751/UP/98, ZA982/UP/97, ZA928/UP/97, ZA839/UP/97 and ZA837/UP/97 contained T^{29}, C^{32}, A^{89}, T^{94} and A^{201} substitutions (Fig. 4.4) that indicated a shared ancestral virus. This variant was first detected in Kuruman (Northern Cape province) in mid-October 1997, and almost simultaneously in Wakkerstroom (Mpumahlanga province). Two months later it appeared in Gaborone, Botswana, and was re-isolated in Kuruman, indicating that it persisted there for at least two months. Finally, the 1997 variant was detected in July 1998 in Pretoria.

In the 1998/1999 sub-lineage, ZA955/UP/98, ZA309/B/99, ZA917/UP/98 and ZA922/UP/98 contain a unique $G^{136}\rightarrow A$ non-synonymous substitution (Fig. 4.4) that resulted in a unique $G^{46}\rightarrow R$ mutation (Fig. 4.5). This variant circulated in a relatively small area of approximately 115 square kilometres between Witbank, Bronkhorstspruit

and Johannesburg (all in the Gauteng province) in September 1998 before moving to Paulpietersburg in northern KZN in March 1999.

A genetic variant that arose in 1998 was characterized by a synonymous $A^{138}\rightarrow C$ substitution (Fig. 4.4). These viruses, ZA700/UP/98, ZA699/UP/98, ZA704/UP/98, ZA933/UP/98, ZA983/UP/98, ZA753/UP/98, ZA762/UP/98, ZA949/UP/98, ZA1003/UP/98 and ZA734/UP/98 were identical at the nucleotide sequence level (Fig. 4.4) and are represented by ZA1003/UP/98 in Fig. 4.5. The first isolations occurred near Johannesburg, Vorna Valley (Midrand, Gauteng), and Rustenberg (North West province) almost simultaneously, and then appeared two weeks later in Ermelo (Mpumahlanga) in July 1998. It was also re-isolated in Johannesburg and Rustenberg at the same time that it was detected in Ermelo. Two months later the variant had spread to Pretoria, then to Phalaborwa (Limpopo province), before being isolated in Makapanstad near Pretoria.

ZA1320/UP/99, ZA1251/UP/99 and ZA335/B/99 shared synonymous $G^{84}\rightarrow A$ and unique $T^{285}\rightarrow C$ substitutions (Fig. 4.4). Furthermore, the two earlier isolates ZA1320/UP/99 and ZA1251/UP/99 shared the synonymous $A^{138}\rightarrow C$ substitution with several 1998 isolates, ZA704/UP/98 and homologues (Fig. 4.4), suggesting that the 1999 variants arose from them. The 1999 variant was first detected in Pretoria at the end of July 1999 before spreading to Halfway House (Gauteng) in August, and to Camperdown in October.

Viruses excluding the distinguishing features discussed above, except for the $T^{351}\rightarrow C$ synonymous substitution (Fig. 4.4) that separates the early from the late VIIb viruses, were grouped together. This sub-lineage consisted of the isolates ZA798/UP/98, ZA96/UP/98, ZA775/UP/98, ZA959/UP/98, ZA756/UP/98, ZA3291/Klpmts/99, ZA9357/Moslb/98 and ZA8057/99. These viruses were isolated from Tzaneen (Limpopo) in February 1998, then Mokopane in June, Vereeniging (Gauteng) in July, Cullinan (Gauteng) in August, back in Mokopane in September and then in Mosselbay (Eastern Cape) in September and Klapmuts (Western Cape) near Paarl in March 1999. This indicated a southward spread, with the persistence in Mokopane for at least three months. ZA8057/99 was also isolated in August 1999 in the Western Cape Province, although the exact location is unknown. ZA7351/Rvsdl/98 and ZA7381/98 which are

separate variants, were detected in July 1998 which suggests at least two separate NDV introduction events into the Western Cape region.

Other viruses isolated in 1997 and 1998 were closely related to those discussed above, but contained generally synonymous point mutations at the nucleotide sequence level. ZA444/B/98 only caused about 5-10% mortality. This particular outbreak was not widespread as only a few farms in the district were affected and NDV was only detected on routine virus isolation attempts. It lasted only a few weeks, which was attributed to a heavy ND vaccination program in place on those farms at that time. ZA984/UP/98 was the second isolate of this study that originated from Gaborone, Botswana. The unique $R^{101} \rightarrow M$ substitution (Fig 4.5), and other synonymous substitutions at the nucleotide sequence level (Fig. 4.4) could perhaps be attributed to geographical separation after a probable earlier introduction from South Africa.

4.4 DISCUSSION

In a previous study (Herczeg *et al*., 1999), it was established that two separate genotypes of NDV circulated in South Africa during the early 1990s. The first, genotype VIII or lineage 3d, had been isolated from South Africa in 1968, 1974 and in the early 1990s up to 1994. It is believed that this genotype was enzootic in South Africa since the 1960s. The results of the current chapter, whereby eight additional viruses were added to genotype VIII, one of the smallest exotic NDV lineages in the world, confirmed that genotype VIII was maintained in an enzootic infection in South Africa since at least the 1970s. Most isolations of genotype VIII were made in the KwaZulu/Natal Province, one of the largest and most intensive poultry-producing areas in South Africa (Appendix 1), but three more viruses were detected in the latter part of the decade. After 1994, genotype VIII did not appear to be as virulent as it initially was (R Horner, personal observation). Whether this was due to improved and widespread vaccination in the 1990s, or attenuation, such as the mutation of the $R^{114} \rightarrow Q$ in the F_0 cleavage site is unknown. Vaccinated chickens are still able to shed viruses but remain clinically healthy, and this could account for the failure to detect genotype VIII between 1994 and 1997. June 2000 was the last time that genotype VIII was isolated in South Africa, or anywhere else in the world according to published data.

The incurrence of genotype VIIb (lineage 5b) into South Africa in the early 1990s is concurrent with the rise of this pandemic strain in many parts of East Asia and Europe in the 1990s. The data presented by Herczeg and colleagues in 1999 was limited. It did however demonstrate that South Africa was the likely source of the pandemic VIIb strain to Mozambique in 1993. They furthermore concluded that genotype VIIb had been introduced to South Africa first, and then to southern Europe, based on a divergence of only 2.4-4.5 % between the South African cluster and one containing strains from the United Arab Emirates, Turkey and Bulgaria. Aldous and colleagues (2003), who published the most comprehensive study on NDV phylogenetics yet, found that another strain, FI 1001/96, had only 2.7% sequence divergence from ZA 33/94, and therefore agreed with Herczeg and colleagues that VIIb had spread from southern Africa to Europe. However, in that extensive study, two pivotal sequences were published: **-**PTTY91146 isolated in Portugal from a turkey in 1991, and AESCK90174 isolated from a chicken in Spain in 1990. Neither was noted in the phylogenetic tree

amongst the South African isolates (Aldous *et al*., 2003) despite the fact that these two European viruses shared 100% nucleotide sequence identities with the early South African epidemic strains, therefore I suggest that the VIIb strain responsible for the outbreaks in South Africa originated in Southern Europe, and not the opposite. Genotype VIIb may even have been introduced from abroad into South Africa on multiple occasions: strain 1ZAOS9141 (Aldous *et al*., 2003) was isolated from a South African ostrich in 1991, long before the index case at Hartebeespoort in June 1993. That strain shared only 97% nucleotide sequence identities with the early South African genotype VIIb viruses compared to the Spanish (1990) and Portuguese (1991) viruses. It is quite possible that day-old chicks, eggs or unprocessed poultry products were imported into South Africa from Europe before the infection was reported there. The chronological and geographical separation of South African genotype VIIb strains during the 1990s is consistent with reports from other countries (Aldous *et al*., 2003). The epidemiological pattern suggests that Genotype VIIb (and probably VIII) was spread throughout South Africa via the movement of infected poultry, rather than by wild birds. Most locations of isolation lie along major transportation routes and fall within or are close to the main poultry-producing areas in South Africa (Appendix 1). Spent hen vendors and their roles in the dissemination of ND will be discussed in greater detail in the final chapter. Furthermore, no evidence was found that indigenous village chickens acted as sub-clinical long-term reservoirs for infection of the formal industry. These birds are usually unvaccinated and are highly-susceptible to virulent NDV strains. In contrast, commercial chickens are vaccinated, and therefore likely to maintain and even shed virulent NDV strains while remaining clinically healthy. Vaccination combined with the transportation of infected birds and equipment across the country could thus have played the critical role in sustaining the initial 1993/94 outbreak throughout the remainder of the decade.

South African poultry infected with exotic NDV strains are clearly a threat to neighbouring trade partners when bio-security measures fail, as indicated by the spread of genotype VIIb from South Africa to Mozambique and Botswana in the 1990s. The initial routes of entry into South Africa of genotypes VIII and VIIb were probably via importation (illegal or otherwise) of infected poultry, although introduction by wild birds cannot be ruled out. Fortunately, genotype VIIb disappeared after 1999 (unpublished laboratory data), but in the same year a new genotype, VIId, arose and this is discussed in Chapter Six.

CHAPTER FIVE[1]

PHYLOGENETIC ANALYSIS OF PIGEON PARAMYXOVIRUSES ISOLATED IN SOUTH AFRICA

ABSTRACT

Pigeon paramyxovirus type- 1 (PPMV-1), a variant of Newcastle disease virus that primarily affects doves and pigeons, has been present in South Africa since at least the mid-1980s, and has since become widespread. Phylogenetic evidence indicated that pigeon paramyxovirus-type viruses were introduced into South Africa on at least two occasions, based on the presence of two separate subgroups, 4bi and 4bii, that have been circulating in Europe and Japan since the early 1990s. Sub-group 4bi is a recent introduction into South Africa, but subgroup 4bii may have been introduced earlier. PPMV-1 was probably introduced via the importation of infected racing pigeons, their eggs or other pigeon products from Europe, and smuggling remains a problem. Two cases of chicken infection with PPMV-1 were reported since 2002. Even though the disease symptoms of PPMV-1 infections in chickens were mild, it has been experimentally determined by other groups that passage in chickens causes the virus to gain virulence. The threat to poultry production and biodiversity of indigenous dove and pigeon species in southern Africa highlights the importance of monitoring the spread of this subtype.

[1]Part of the results presented in this chapter were published in the Onderstepoort Journal of Veterinary Research, 70:157-160 (2004)

5.1 INTRODUCTION

Pigeon paramyxovirus type 1 (PPMV-1) are antigenic variants of avian paramyxovirus-1 (APMV-1; Newcastle disease virus) that were identified by monoclonal antibody binding studies (Alexander *et al.* 1984; 1985a; King 1996; Lana *et al.*, 1988). Pigeon paramyxovirosis was first described in Iraq in 1978 (Kaleta *et al.*, 1985). In 1981, PPMV-1 strains were responsible for outbreaks among racing and show pigeons in Europe (Biancifiori & Fioroni, 1983, Alexander *et al.*, 1985; Wilson, 1986, Vindevogel & Duchatel, 1988; Kaleta, 1992a,b; Ujvári *et al*., 2003) and it re-emerged in 1985 causing a panzootic (Aldous *et al*., 2004). Prior to the rise of PPMV-1 in the early 1980s, only sporadic incidences of ND were reported in pigeons, usually in association with ND epizootics in chickens (Stewart, 1971, Hilbrich, 1972; Vindevogel *et al.*, 1972; Pearson & McCann, 1975; Kaleta & Baldauf, 1988). These secondary cases, however, did not have the tendency to spread either among pigeon flocks or from pigeons to chickens (Alexander *et al.*, 1984).

PPMV-1 causes an often fatal disease in pigeons, associated with neurological signs like torticollis and paralysis, and the excretion of large volumes of green, watery diarrhoea. Increased embryo mortality during breeding or deformed feathers during moulting is observed if infected during those periods (Alexander *et al.*, 1984; 1985b; Lemahieu 1985). The incubation period of the disease varies from one to six weeks (Wilson, 1986). Most PPMV-1 strains have reduced (intermediate) virulence for chickens (Alexander & Parsons, 1986; Kissi, 1988; Werner *et al.*, 1999; Meulemans *et al.*, 2002). In some cases ICPI values are typical of mesogenic strains, but in most cases, PPMV-1 isolates have increased their virulence for chickens after chicken passages, and therefore represent a threat to poultry production (Alexander & Parsons, 1986; King 1996; Kommers *et al.*, 2002). PPMV-1 isolates have furthermore been characterized by pathogenicity tests and sequencing of the F protein (Meulemans *et al.*, 1986; Alexander *et al.*, 1993; Collins *et al.*, 1993, 1994, 1996). Most of the PPMV-1 viruses isolated from 1983 to 1987 in Europe and Japan had the F_0 sequence of ^{112}GRQKRF^{117}, and had a mean ICPI of 1.44 (Collins *et al.*, 1994; Mase *et al*., 2002). In later years, the emergence of PPMV-1 isolates possessing the F_0 site motifs ^{112}RRQKRF^{117}, ^{112}RRKKRF^{117} or ^{112}RRRKRF^{117} motifs was demonstrated (Meulemans *et al.*, 2002; Terregino *et al.*, 2003; Mase *et al*., 2002). The ^{112}RRQKRF^{117}

motif was present in the majority of the isolates but the ICPIs of PPMV-1 isolates having this motif was highly variable (0.68 to 1.38), but generally lower (mean, 0.69), than reported for PPMV-1 viruses isolated in the years 1983 and 1984 (Terregino *et al.*, 2003). The wide variation in the pathogenicity of the variant PPMV-1 for chickens is therefore not related to variation in the amino acid motif at the F_0 cleavage site, nor is it due to production of HN_0 which may also influence pathogenicity (Collins *et al.*, 1994). There appears to be some other property of the PPMV-1 virus which influences virulence, although to date studies have not been able to identify it (Collins *et al*, 1994; 1996). Phylogenetic analysis has classified PPMV-1 strains into a discrete lineage, VIb (Lomniczi *et al*., 1998), recently re-classified as lineage 4b. Lineage 4b could be further divided into subgroups 4bi and 4bii (Aldous *et al*., 2004). Furthermore, phylogenetic analyses revealed that four distinct subgroups of lineage VIb, Iraqi, early European, North American and recent European have emerged and circulated in the past decades. Subgroups early European and North American strains were responsible for the main streams of infection in the 1990s (Aldous *et al.*, 2004).

The mixing of pigeons in association with races and the extensive trade in these birds and their products is likely to be the cause of the rapid dissemination of this disease in racing pigeon communities (Aldous *et al.*, 2004). Although the disease in racing and show pigeons has been controlled by vaccination, PPMV-1 has become panzootic and continues to circulate in many countries worldwide (Alexander *et al.*, 1997; Werner *et al.*, 1999; Kommers *et al.*, 2001; Zanetti *et al.*, 2001; Meulemans *et al.*, 2002; Alexander *et al.*, 1985a; Shirai *et al.*, 1986; Gelb *et al.*, 1987; Pearson *et al.*, 1987; Abu-Elzein *et al.*, 1999; Zanetti *et al.*, 2001; Eisa & Omer 1984; Pienaar & Cilliers, 1987). In 1984, PPMV-1 spread to domestic chickens in Great Britain, causing more than 23 outbreaks. Feedstuffs stored at Liverpool docks became infected with pigeon faeces and carcasses of feral pigeons infected with PPMV-1 were considered the source of the virus in most of those outbreaks (Alexander *et al.*, 1985a). Similarly, in 2001, an outbreak of PPMV-1 in commercial layer chickens in Canada was linked to feed contaminated by faeces of PPMV-1-infected wild pigeons (Toro *et al.*, 2005). Besides pigeons, doves and chickens, PPMV-1 viruses have also been isolated from kestrels, falcon, cockatoos, budgerigar, pheasant, swan and a robin (Alexander *et al.*, 1985a; Lister *et al.*, 1986; Johnston & Key, 1992; Kaleta, 1992b; Werner *et al.*, 1999; Monne *et al*., 2006, Aldous *et al*., 2003).

Outbreaks of ND in doves and pigeons have been occasionally reported in various parts of South Africa since the 1980s and PPMV-1 was initially isolated in South Africa during an outbreak in September 1986. Symptoms in the affected pigeons were similar to those observed in the European outbreaks. Six viruses with mean death times that varied from 92 to 118 hours were isolated, and subsequent infectivity trials showed that they were avirulent for 60- and 4-week-old chickens infected by the intra-cloacal and intra-tracheal routes (Pienaar & Cilliers, 1987). Unfortunately, these isolates could not be located for inclusion in the present study. From 2002 to the present, PPMV-1 viruses were isolated from doves, pigeons and chickens in South Africa. The objectives of this chapter were to determine the phylogenetic relationships of seventeen South African PPMV-1 isolates collected since 2002, to determine whether a unique South African lineage exists (on the hypothesis that PPMV-1 has been endemically maintained since the 1980s), and to determine the source of the outbreaks.

5.2 MATERIALS AND METHODS

5.2.1 Viruses

ND viruses were grown in 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual), at Allerton Provincial Veterinary Laboratory, the Poultry Reference Laboratory (University of Pretoria), and Stellenbosch Provincial Veterinary Laboratory. ICPI and MDT tests were performed at Allerton Provincial Veterinary Laboratory. South African isolates of PPMV-1 are listed in Table 5.1, along with their collection dates, and hosts and regions.

5.2.2 RNA Extraction

Viral RNA was extracted from allantoic fluid using TRIzol® reagent (Gibco, Invitrogen), according to the manufacturer's instructions.

5.2.3 RT-PCR

A one-step RT-PCR was performed using the oligonucleotide pair described in Chapter Four (p160), with the addition of MMLV-reverse transcriptase and the following modification to the thermal cycling protocol: incubation at 42ºC for 20 min., followed by 35 cycles of 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min.

5.2.4 DNA sequencing and phylogenetic analysis

DNA was sequenced using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions, and was analysed with an ABI3130™ Genetic Analyser. Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 374 nucleotide (nt) region of the 3' end of the F protein, including the F_0 cleavage site, were used to identify 50 closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). Preparation of Fig. 5.3 (variable sites of the multiple nucleotide sequence alignment) was done with MEGA v3.1.

Phylogenies were also reconstructed with MEGA 3.1 software (Kumar *et al.*, 2004) using the Neighbor-Joining tree inference method with the Kimura 2-parameter substitution model (commonly used in AI phylogenetic studies), and 1000 bootstrap replicates assign confidence levels to branches. Only the variable sites, also prepared with MEGA 3.1 software, are presented in the multiple nucleotide sequence alignment (Fig. 5.3).

Table 5.1 South African Pigeon Paramyxovirus isolates analysed in this study

Isolate	Date of collection	Place	Host	Accession number
ZA469/PPMV1/02	01/12/2002	Mooirivier	28 week old layers	AY445669
PIZA04N230	06/08/2004	Oudtshoorn	Racing pigeons	EF030962
DOZA05N240	24/01/2005	Kimberly	Doves	EF030953
DOZA05N247	09/03/2005	Kuilsrivier	Laughing dove	EF030954
PIZA05N277	16/05/2005	Pretoria	Pigeon	EF030963
DOZA05AM68313	18/08/2005	Brits/Rustenberg	Laughing dove	EF030952
DOZA05N417	24/10/2005	Montagu	Doves	EF030955
DOZA05N539	19/12/2005	Bellville	Doves	EF030956
DOZA06N549	16/01/2006	Cape Town	Doves	EF030957
DOZA06N589	06/03/2006	Darling	Doves	EF030958
DOZA06N591	27/02/2006	Kimberly	Doves	EF030959
CKZA06N606	10/03/2006	Sibasa	4 week old broilers	EF030951
DOZA06UP470	16/03/2006	Polokwane	Dove	EF030961
PIZA06N642	01/04/2006	Cape Town	Pigeon	EF030950
DOZA06N621	03/04/2006	Stellenbosch	Doves	EF030960
PIZA06N635	18/04/2006	Stellenbosch	Pigeon	EF030964

5.3 RESULTS

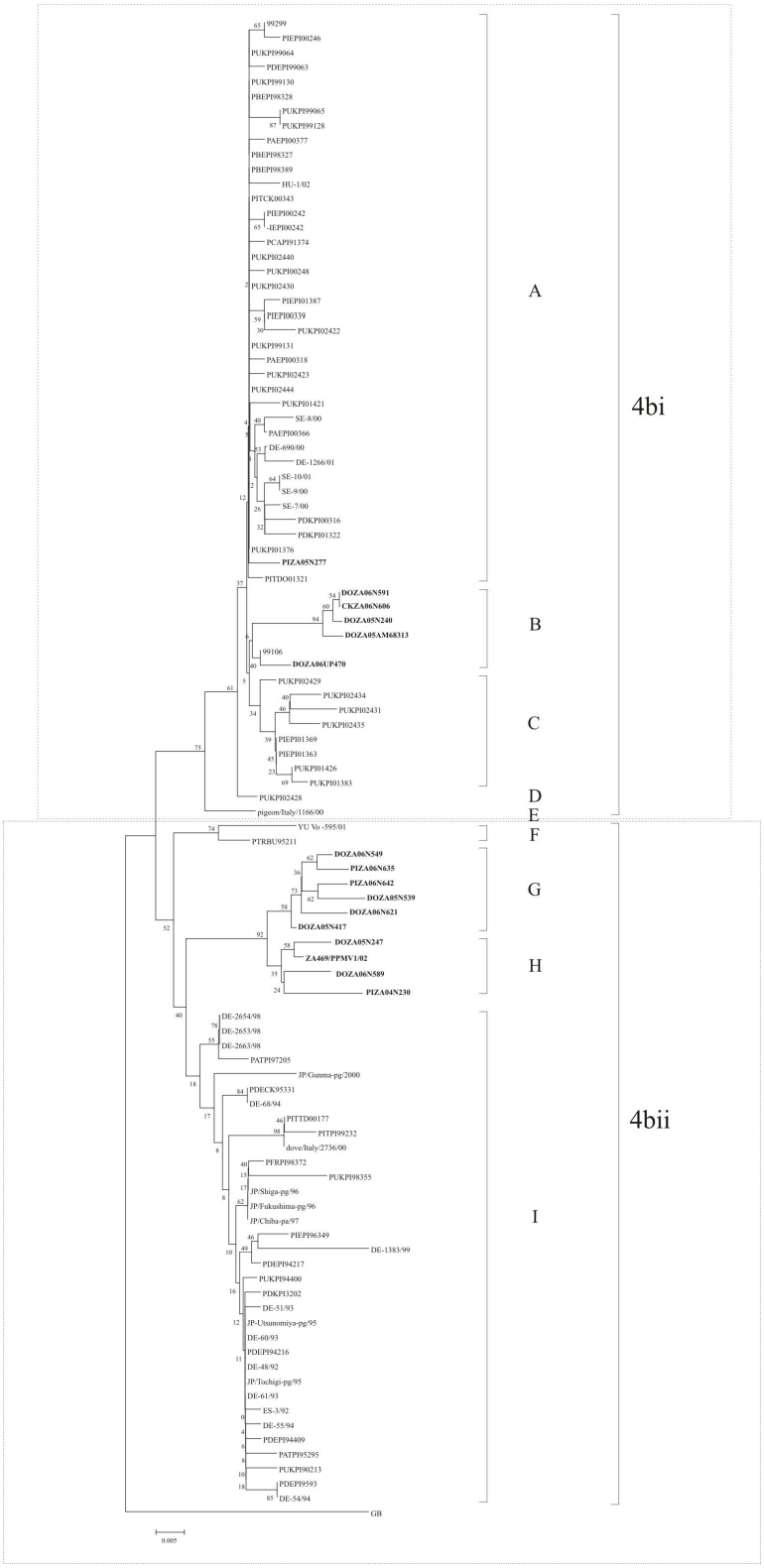


Figure 5.1(a) Dendogram inferred from the 374 bp region of the 3' end of the fusion protein (F) gene of PPMV-1 strains isolated in South Africa (boldface) and strains from Genbank. Enlargements of the regions representing subgroups 4bi and 4bii are presented separately in Figs 5.1(b) and 5.1(c) respectively.

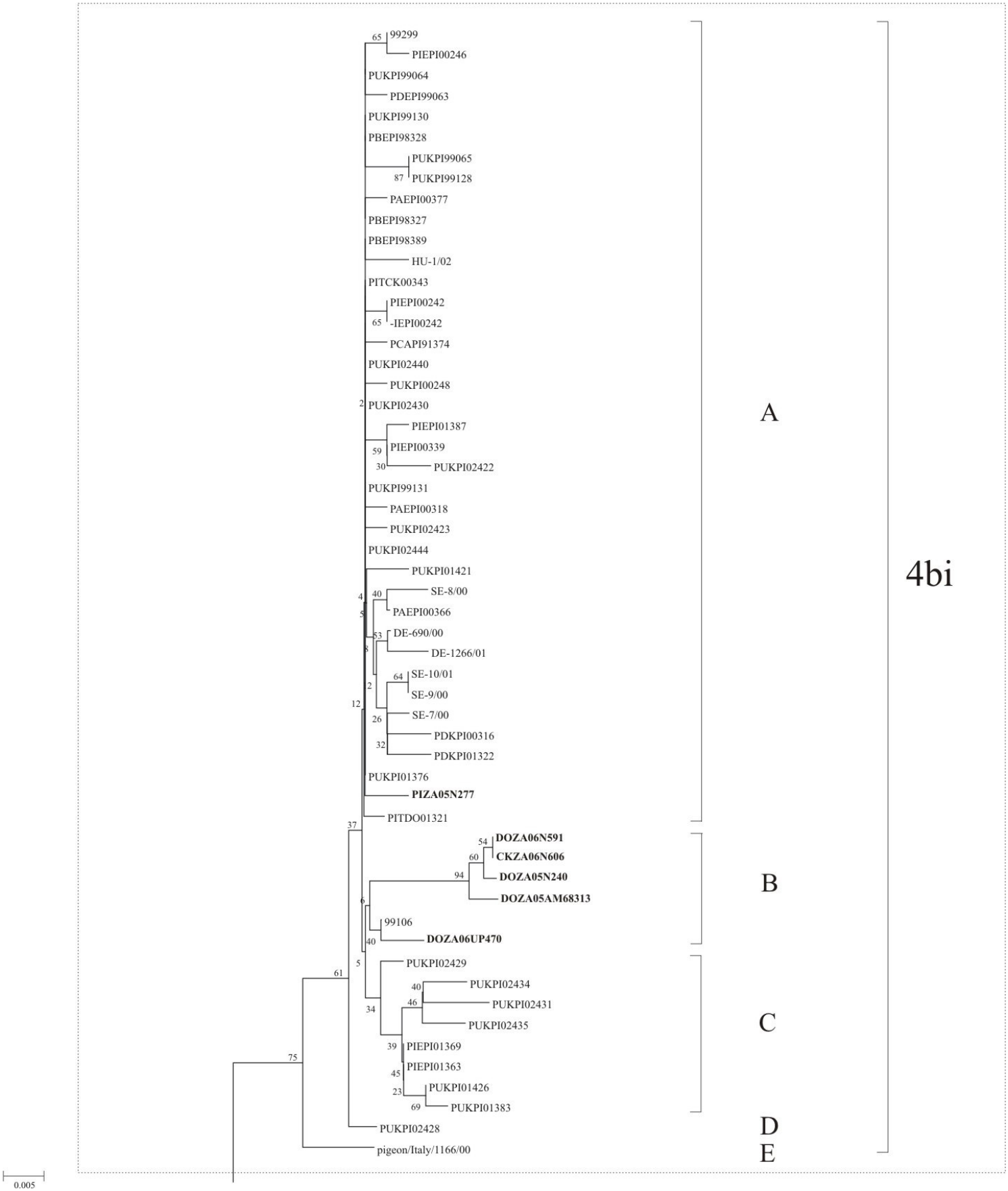


Figure 5.1(b) Enlargement of Fig 5.1(a) depicting Subgroup 4bi phylogeny

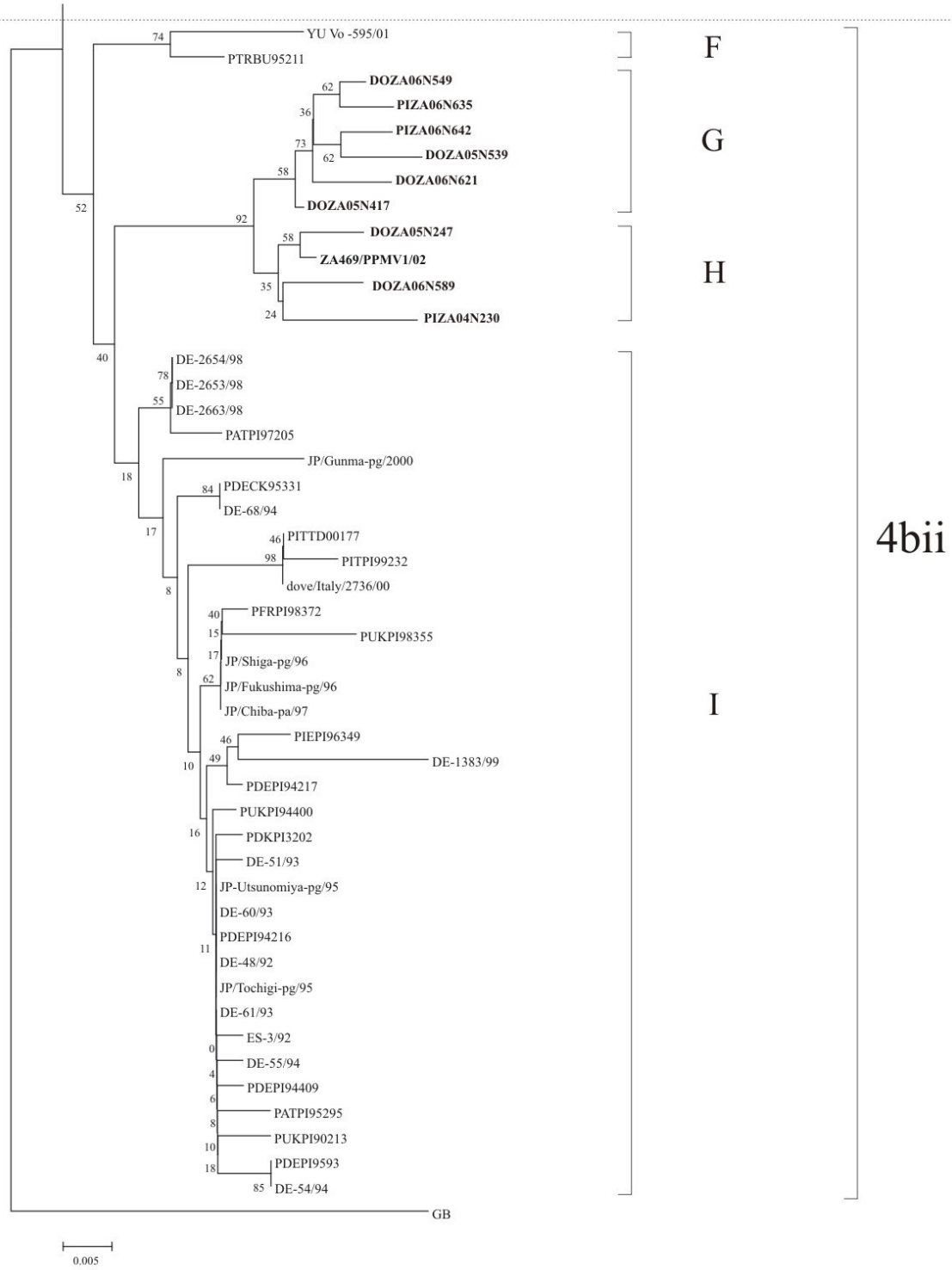


Figure 5.1(c) Enlargement of Fig 5.1(a) depicting Subgroup 4bii phylogeny

Figure 5.2 Multiple amino acid alignment of the translated 374-nt region of the fusion (F) protein of South African PPMV-1 strains (underlined) and other viruses, including the F_0 cleavage site (underlined).

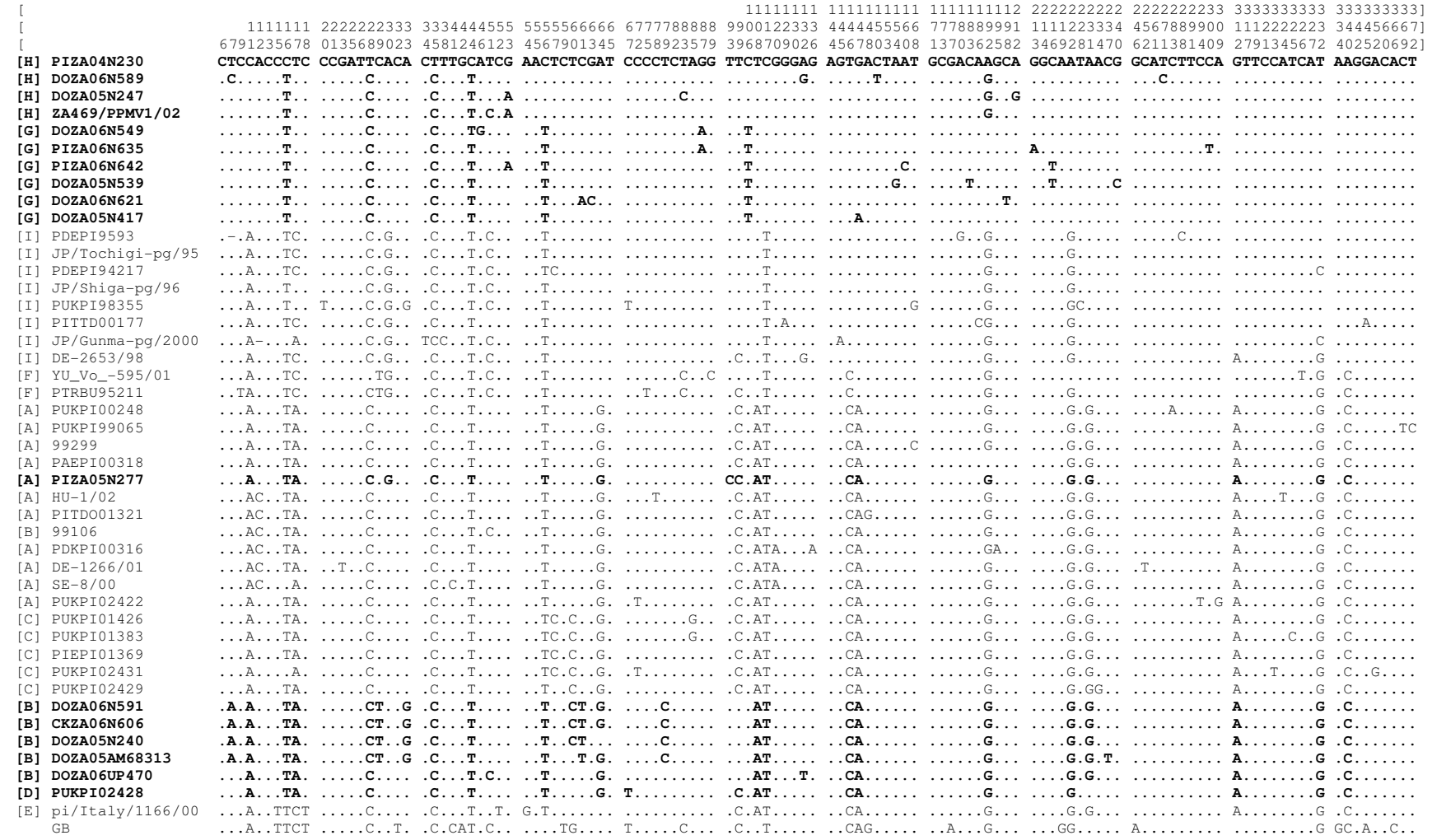


Figure 5.3 Nucleotide sequence alignment of variable sites within the 374 nt region of the 3' end of the fusion (F) protein. The South African isolates (in boldface) are compared with selected representatives of sub-lineages A to I.

The South African PPMV1 isolates do not cluster together as a geographical entity, but instead are split between the two subgroups 4bi and 4bii, wherein sub-lineages A to I were distinguished (Figs. 5.1(a) to (c)). Sub-lineage A (Fig. 5.1(a)) consists of PPMV-1 isolates from England, Ireland, Belgium, the United Arab Emirates, Hungary, Germany and Sweden, dating from 1998 to 2002 (with a single isolate, PCAPI91374, from 1991). One of the South Africa isolates, PIZA05N277, isolated in Pretoria in May 2005 from a pigeon, falls within sub-lineage A and shared 99% sequence identity with the other sub-lineage A viruses but only 98-99% and 94-95% sequence identities with South African sub-lineage B and sub-lineages G+H viruses, respectively. PIZA05N277 contained unique synonymous nucleotide substitutions A^{30}→G and T^{93}→C that distinguished it from the other isolates.

Sub-lineage B (Fig. 5.1(a)) consists of five South African viruses and one French virus. The South African sub-lineage B viruses were isolated from January 2005 to March 2006 initially from Kimberly (Northern Cape), then the Brits/Rustenburg area and finally the Polokwane area (including Sibasa) in the Limpopo Province. Isolates were predominantly obtained from doves, but CKZA06N606 was isolated from four-week old broiler chickens, and this was only the second reported case of PPMV-1 being isolated from fowl in South Africa (Abolnik *et al.,* 2004b). The French virus, 99106, was isolated during outbreaks in 1999 in racing pigeons (Baberzange & Jestin, 2003). The phylogenetic relationship between 99106 and the rest of the sub-lineage B viruses is probably not significant, as the grouping with DOZA06UP470 is based on a single shared C^{51} nucleotide (Fig 5.3). In fact, 99106 contained the unique K^{4}→N substitution (Fig. 5.2) that grouped it with other sub-lineage A viruses of European origin.

The phylogenetic grouping of isolates DOZA06N591, CKZA06N606, DOZA05N240 and DOZA05AM68313 is supported by unique S^{3}→T, P^{10}→L and C^{22}→R substitutions (Fig. 5.2). Although DOZA06UP470 is included into sub-lineage B, it lacked the aforementioned characteristics. However, at the nucleotide sequence level (Fig. 5.3), DOZA06UP470 contained the T^{99} character that is unique to sub-lineage B within sub-group 4bi.

Sub-lineage C (Fig. 5.1(a)) consists of viruses from England and Ireland isolated from 2001 to 2002, whereas sub-lineages D and E consists of European strains that are outliers to the rest of the subgroup 4bi strains.

Subgroup 4bii (Fig. 5.1(b)) contains the sub-lineages F to I. Sub-lineage F is composed of only two strains, isolated in Turkey and Yugoslavia. All South African subgroup 4bii strains share a $K^{4}\rightarrow T$ substitution that distinguishes them from viruses of other regions (Fig. 5.2). Sub-lineages G and H, containing only South African strains, are phylogenetically related and share a common ancestor (supported by a high bootstrap value of 92). Despite low levels of support for sub-lineages G and H (58% and 35% respectively), the viruses within these lineages are distinct from one anther at the amino acid level by virtue of unique $I^{19}\rightarrow T$ (Sub-lineage H) and $P^{36}\rightarrow S$ (sub-lineage G) substitutions (Fig. 5.2). The sub-lineage G strains, DOZA06N549, PIZA06N635 PIZA06N642, DOZA05N539, DOZA06N621 and DOZA05N417 were isolated from pigeons and doves from Cape Town, Stellenbosch, Bellville, and Montagu (all situated in the Western Cape province) over a six-month period from 2005 to 2006.

Sub-lineage H contains the earliest South African isolate of the study, ZA469/PPMV1/02. ZA469/PPMV/02 was isolated from 28-week-old layers with symptoms of moderate mucoid tracheitis from the Mooi Rivier area in the KZN province, in December 2002. The thermostability value obtained for ZA469/PPMV/02 was 60 min, which is typical for velogenic ND viruses, but the MDT value was over 90 hours, which is which is typical for lentogenic viruses (Abolnik *et al.,* 2004b). ZA469/PPMV1/02 shared a $R^{18}\rightarrow Q$ substitution with DOZA05N247, indicating a possible common source and furthermore suggests that the infection spread from KZN to the Western Cape. The other two sub-lineage H viruses, PIZA04N230 and DOZA06N589, were isolated from Oudtshoorn racing pigeons in August 2004, and doves (Darling, near Cape Town) in March 2006.

Finally, sub-lineage I consists of the rest of the subgroup 4bii viruses, isolated since the early 1990s with a wide geographical distribution including Germany, Austria, Italy, Japan, France, the UK, Denmark and Spain.

5.4 DISCUSSION

In this chapter, phylogenetic evidence indicated that pigeon paramyxoviruses were introduced into South Africa on at least two occasions, based on the presence of two separate subgroups, 4bi and 4bii, that have been circulating in Europe and Japan since the early 1990s.

The close phylogenetic association between sub-lineage B (4bi), a single isolate from sub-lineage A (PIZA05N277) and recent isolates from the UK in particular, suggest that sub-group 4bi was recently introduced into South Africa. Subgroup 4bii, represented by sub-lineages G and H, may have been introduced earlier, as the long branch-lengths and branching order suggest that that these viruses have been circulating independently in South Africa for an extended period, although they descended from a common ancestor. Reports of PPMV-1 in South Africa date back to the mid-1980s, and the infection appears to be widespread (Pienaar & Cilliers, 1987; Dirk Verwoerd, personal communication). The epidemiology of PPMV-1 in South Africa is difficult to describe with this limited data set, which is biased towards isolates made in the last two years. The recent increase in sample submissions from dead doves and pigeons to veterinary laboratories is probably due to the heightened public awareness caused by the international HPAI H5N1 outbreaks. An interesting phenomenon is that the South African subgroup 4bi viruses (sub-lineages A and B) are restricted to the northern regions of the country (Limpopo, Gauteng and the north-eastern Northern Cape provinces), whereas the subgroup 4bii viruses have only been found in the southern regions to date (KwaZulu-Natal and Western Cape provinces).

The routes of entry of PPMV-1 into South Africa are speculative, but most likely via the importation of infected racing pigeons, their eggs or other pigeon products from Europe. The breeding of doves and pigeons and particularly pigeon racing is a popular pastime in South Africa, as in many other regions of the world. For example, the Sun City Million Dollar Pigeon Race, since its inception in 1995, has attracted thousands of entrants annually from all over Europe, North America the Far East and Australia, but the three biggest international entrants are Germany, the UK and the USA. The entrants are placed under mandatory 30-day quarantine and are vaccinated on arrival (http://www.scmdpr.com). Although this huge international event is well-controlled, illegal smuggling of valuable pigeon racing stock into South Africa is not unheard of.

In at least one incident of its kind, racing pigeons were smuggled into South Africa by boat. In this scenario, the South African client would have contact with the crew member of a ship from abroad carrying the valuable birds. The South African client would enter the ship to allegedly visit the crew member, carrying on the exact number of pigeons being smuggled into the country, and would allow the Port Authority to inspect his South African birds. The exchange then takes place on the ship, and the client disembarks with the new birds. Once at sea, the exchanged pigeons are released from the deck, and fly back to their master. Alternatively, pigeon eggs have been smuggled into the country by carrying them onto the airplane in clothing pockets (Dirk Conradie, personal communication).

Two cases of chicken infection with PPMV-1 have been reported in South Africa. The first occurred in Mooirivier in December 2002 (Abolnik *et al*, 2004b) and the second in Sibasa, near Polokwane in March 2006. This highlights the importance of the threat of PPMV1 to poultry. Even though disease symptoms of PPMV-1 infections in chickens are mild, it has been experimentally determined that passage in chickens causes the virus to gain pathogenicity (Alexander & Parsons 1986; King 1996; Kommers *et al.* 2002). In addition to the threat that PPMV-1 poses to poultry production in South Africa, it potentially threatens biodiversity too. Besides exotic feral pigeons (*Columba livia)* and abundant resident species such as the Redeyed dove (*Streptopelia semitorquata*), Cape turtle dove (*Streptopelia capicola*), Laughing dove (*Streptopelia senegalensis*), Namaqua dove (*Oena capensis*), and Rock pigeon (*Columba guinea*), southern Africa is home to rarer species such as the Mourning dove (*Streptopelia decipiens*), Bluespotted dove (*Turtur afer*), Emeraldspotted dove (*Turtur chalcospilos*), Cinnamon dove (*Aplopelia larvata*), Tambourine dove (*Turtur tympanistria*), Rameron pigeon (*Columba arquatrix*), Green pigeon (*Treron calva*), and Delegorgue's pigeon (*Columba delegorguei*). The results presented here indicate the presence of four discrete lineages of PPMV-1, of which two have become established in South African chickens and doves, and seem to be becoming enzootic. The threat to poultry production and biodiversity of indigenous dove and pigeon species highlights the importance of monitoring the infection in South Africa.

CHAPTER SIX

THE MOLECULAR EPIDEMIOLOGY OF NDV GENOTYPE 5d/VIId ("GOOSE PARAMYXOVIRUS") IN SOUTH AFRICA FROM 1999-2006

ABSTRACT

Phylogenetic analysis of velogenic Newcastle disease viruses isolated in South Africa showed that outbreaks that affected poultry in the Kwa Zulu-Natal province from 1999 to 2000 were not caused the same genetic type as the pandemic strain of the early and mid-1990's (VIId), but instead by strains of lineage 5d, previously only described in the Far and Middle East and recently Greece, also known as "Goose paramyxovirus". In 2003 lineage 5d re-emerged in a single outbreak in the same area, and almost exactly one year later an epidemic started that swept across the country. 257 Newcastle disease viruses were characterised in this study. Multiple species including chickens (commercial, ornamental and indigenous), peacocks, Hadeda Ibis (*Bostrychia hagedash*) chicks, geese, ostriches, pheasants and doves were found to be susceptible to lineage 5d strains, that had ICPI values ranging from 1.89 to 1.91. This is the first report of isolation of lineage 5d from peacocks, Hadeda Ibis, doves and ostriches. A second sub-genotype, recently introduced from an east-Asian source circulated at the start of the outbreak in 2004, but this strain disappeared after a few months. Epidemiological analysis revealed a close association between outbreaks and the national/regional road system, indicating that translocation of infected poultry was again the major mode of spread. The supply of vaccinated spent birds by the cull buyer industry into rural areas creates a possible reservoir for the maintenance of velogenic NDV strains in South Africa that periodically spill over into commercial flocks, causing outbreaks.

6.1 INTRODUCTION

Generally, APMV-1 strains are infectious to waterfowl such as geese and ducks without causing overt clinical symptoms, and these species therefore usually act only as carriers of the virus (Yin & Liu, 1997; Takakuwa *et al*, 1998; DJ Alexander, 1997a). However, paramyxovirus infection outbreaks have occurred frequently in Chinese geese since 1997 (Liu *et al*., 2003). In 1999, an outbreak with an incidence of 50-70% occurred in Shanghai goose flocks. Clinical signs varied from ruffled feathers and slight depression to severe systemic infection, resulting in high mortality and characterized by very rapid spread (Jinding *et al*., 2005). Although the mortality rates in adult geese were only 10 to 20%, up to 100% mortalities were observed in young geese under two weeks of age. A novel virus isolate, SF02, was determined to be the etiological agent of these outbreaks and was named "Goose paramyxovirus" (GPMV) (Zou *et al*., 2002; Liu *et al*., 2003). GPMV has been confirmed to be avian paramyxovirus-1 (Newcastle disease virus) by genomic and serotype analyses, and all but one of the strains of goose origin fell into genotype VIId (Liu *et al*., 2003). GPMV is also highly pathogenic to chickens, pigeons, partridges and ducks (Zou & Gong, 2003). MDTs of 45.6 – 60 h and ICPIs of 1.80-1.94 have been recorded (Liu *et al*., 2003) but chickens inoculated with either live or inactivated LaSota vaccines were fully protected from disease and death against challenge with VIId (Liu *et al*., 2003). The phylogeny of NDV was recently revised, and genotype VIId was renamed as lineage 5d (Aldous *et al*., 2003).

At the genomic level, GPMV contains an extra 6nt fragment in the un-translated region (UTR) between the NP and P genes, but this insertion also occurs in NDVs in genotypes VI, VII, VIII and IX while it is absent from NDVs in genotypes I, II, III and IV. The insertion was not only limited to contemporary lineages (VI onwards) as it was also detected in viruses from the 1940s and 1960s (Huang *et al*., 2004). The 3' leader of GPMV genome shares high identity with APMV-6 and other APMV-1 viruses, whereas its 5' trailer is more variable (Zou *et al*., 2005). The HN protein is six amino acids shorter compared to avirulent strains, but this trait is also shared by other virulent strains (Huang *et al*., 2004).

From 1999 to 2000, an outbreak of velogenic viscerotropic NDV occurred in a single commercial flock and village chickens in a relatively small geographic area of the

KwaZulu/Natal province. PCR and sequencing identified the causative strain as a genotype VIId/ lineage 5d (Goose Paramyxovirus) strain (Abolnik *et al*., 2004a). For almost three years thereafter there were no reports of NDV outbreaks nor were any virulent viruses isolated by national laboratories. Then, towards the end of September 2003, a virulent ND virus was isolated from village chickens near Pietermaritzburg, close to the locations of the outbreaks in 1999 and 2000, however, the infection did not seem to spread. Almost exactly one year later in October 2004, a paramyxovirus outbreak started in commercial flocks of the Camperdown/ Richmond districts. Although the index case was a commercial farm, workers reported that a disease with similar symptoms had been killing the fowl in their villages for several weeks already, and a sample was obtained from the village for testing. The infection continued to spread throughout KZN commercial and backyard flocks, and to and throughout most provinces in 2005 and 2006. The purpose of this chapter was to genetically characterize 257 virulent ND viruses collected from outbreaks in South Africa from 1999 to 2006, to describe the epidemiology of the disease, and if possible, to determine the origins of the infections.

6.2 MATERIALS AND METHODS

6.2.1 Viruses

Velogenic ND viruses (Table 6.1) were grown in 9-to-11 day old specific pathogen free (SPF) embryonated chicken eggs by standard procedures (OIE manual of standards, 2000), at Allerton Provincial Veterinary Laboratory, the Tropical Diseases Division (University of Pretoria), Stellenbosch Provincial Veterinary Laboratory, and Deltammune (formerly Avimmune) Laboratory. ICPI and MDT tests were performed at Allerton Provincial Veterinary Laboratory. Isolates are listed in Table 6.1 along with their collection dates, hosts and regions.

Table 6.1 Virulent ND viruses isolated in South Africa from 1999-2006 (n=257)

Isolate[1]	Collection date	Location	Host[2]	Accession number[3]
CKZA99AL308	12/03/1999	New Hanover/Wartburg	Chickens	AF532150
CKZA99AL328	27/08/1999	Thornville	Village fowl	
CKZA99AL331	02/09/1999	Stanger	Village fowl	AF532739
CKZA99AL337	02/11/1999	Durban	Village fowl	
CKZA00AL344	04/01/2000	Durban	Village fowl	
CKZA00AL377	20/10/2000	Stanger	Village fowl	
CKZA00AL378	20/12/2000	Port Shepstone	Village fowl	AF532745
CKZA03AL482	26/09/2003	Pietermaritzburg	Village fowl	EF030966
CKZA04AL491	30/09/2004	Pietermaritzburg	Broilers Breeders	
CKZA04AL495	08/10/2004	Hopewell Village	Village fowl	
CKZA04AL496[4]	**19/10/2004**	**Estcourt**	**Village fowl**	
CKZA04AL497	22/10/2004	Camperdown	Village fowl	
CKZA04AL498	28/10/2004	Glencoe	Village fowl	
CKZA04AL499	28/10/2004	Howick	Broilers	
CKZA04AL500	**03/11/2004**	**Umzimkulu**	**Village fowl**	**EF030967**
CZA04AL501	03/11/2004	Lower Umfolozi	Village fowl	
CKZA04AL502	**09/11/2004**	**Pietermaritzburg**	**Village fowl**	**EF030968**
CKZA04AL503	12/11/2004	Camperdown	Layers	
CKZA04AL504	12/11/2004	Hluhluwe	Village fowl	
CKZA04AL508	12/11/2004	Camperdown	Layers	
CKZA04AL506	15/11/2004	Camperdown	Village fowl	
CKZA04AM59503	16/11/2004	Pietermaritzburg	Chickens	
CKZA04AL510	18/11/2004	Durban	Village fowl	
CKZA04AL509	22/11/2004	Camperdown	Chickens	
CKZA05AI514	25/11/2004	Witsieshoek	Village fowl	
CKZA04AL511	26/11/2004	Lower Umfolozi	Village fowl	
CKZA04AL512	29/11/2004	Estcourt	Broilers	
CKZA04AL512	**29/11/2004**	**New Hanover**	**Village fowl**	**EF030969**
CKZA04AL514	30/11/2004	New Hanover	Village fowl	
CKZA05N238	11/01/2005	Rustenberg	Chickens	
CKZA05N239	18/01/2005	Pretoria	Chickens	
CKZA05AI600	21/01/2005	Phutadithaba	Village fowl	

CKZA05AL516	31/01/2005	New Hanover	Broilers	
CKZA05N241	16/02/2005	Frankfort	Village fowl	
CKZA05AL517	22/02/2005	New Hanover	Broilers	
CKZA05AL518	22/02/2005	New Hanover	Broilers	
CKZA05N242	23/02/2005	Zeerust	Chickens	
PHZA05N243	02/03/2005	Middleburg	Pheasant	
CKZA05N2343	02/03/2005	Middleburg	Chickens	
CKZA05UP300	03/03/2005	Hartebeeshoek	Village fowl	
CKZA05N246	04/03/2005	Randfontein	Chickens	
CKZA05N244	07/03/2005	Middleburg	Chickens	
CKZA05AL521	14/03/2005	Port Shepstone	Broilers	
CKZA05AL522	17/03/2005	Camperdown	Broilers Breeder	
CKZA05AL525	22/03/2005	New Hanover	Layers	
CKZA05AL526	29/03/2005	New Hanover	Layers	
CKZA05UP408	05/04/2005	Bapsfontein	Chickens	
CKZA05AL527	06/04/2005	Camperdown	Broilers	
CKZA05N255	07/04/2005	Kroonstad	Chickens	
CKZA05N257	08/04/2005	Kroonstad	Chickens	
CKZA05AL530	13/04/2005	Pietermaritzburg	Village fowl	
CKZA05AL531	14/04/2005	Port Shepstone	Broilers	
CKZA05N259	14/04/2005	Kroonstad	Chickens	
CKZA05UP483	15/04/2005	Halfway House	Layers	
CKZA05N265	25/04/2005	Lichtenburg	Chickens	
CKZA05AL532	03/05/2005	Ixopo	Broilers	
CKZA05N272	09/05/2005	Lehurutshe	Village fowl	
CKZA05N275	12/05/2005	Kroonstad	Chickens	
CKZA05UP675	17/05/2005	Kuruman	Chickens	
CKZA05N278	18/05/2005	Vryburg	Chickens	
CKZA05N279	20/05/2005	Vryburg	Chickens	
CKZA05N280	23/05/2005	Ellisras	Chickens	
CKZA05UP754	01/06/2005	Honeydew	Layers	
CKZA05N285	07/06/2005	Vrede (Kroonstad)	Chickens	
CKZA05N300	07/06/2005	Moumong (Brits)	Village fowl	
CKZA05AL536	08/06/2005	Camperdown	Layers	
CKZA05N284	10/06/2005	De Aar	Chickens	
CKZA05N286	10/06/2005	Stutterheim	Chickens	
CKZA05N290	10/06/2005	De Aar	Chickens	EF030971
CKZA05UP803	12/06/2005	Mokopane	Chickens	
CKZA05N287	15/06/2005	Warden (Kroonstad)	Village fowl	
CKZA05N288	15/06/2005	Kudumane (Kroonstad)	Village fowl	
CKZA05M102	17/06/2005	Sibasa	Chickens	
CKZA05AM66479	20/06/2005	Lanseria	Chickens	
CKZA05AL537	23/06/2005	New Hanover	Broilers	
CKZA05AL538	24/06/2005	New Hanover	Layers	
CKZA05N289	28/06/2005	Clarens (Kroonstad)	Chickens	
CKZA05N302	28/06/2005	Vredefort (Kroonstad)	Chickens	
CKZA05AM66832	28/06/2005	Delmas	Chickens	
CKZA05N292	29/06/2005	Warden (Kroonstad)	Village fowl	
CKZA05N294	01/07/2005	Puthaditjhaba (Kroonstad)	Village fowl	
CKZA05M115	04/07/2005	Vryburg	Village fowl	
CKZA05N293	05/07/2005	Hoopstad (Kroonstad)	Village fowl	
CKZA05UP931	05/07/2005	Honeydew	Layers	
CKZA05N301	07/07/2005	Marapong (Ellisras)	Village fowl	
CKZA05AL540	07/07/2005	Port Shepstone	Village fowl	

CKZA05AL541	13/07/2005	Umzimkhulu	Village fowl	
CKZA05UP976	15/07/2005	Noordheuwel	Broilers	
CKZA05UP1008	20/07/2005	Tiegerpoort	Broilers	
CKZA05N305	20/07/2005	Kimberly	Chickens	
CKZA05N306	20/07/2005	Amatole (East London)	Chickens	
CKZA05N304	21/07/2005	Heilbron (Kroonstad)	Village fowl	
CKZA05UP1063	24/07/2005	Makapaanstad	Village fowl	
CKZA05AL543	25/07/2005	Camperdown	Broilers	
CKZA05AL545	25/07/2005	Camperdown	Broilers	
CKZA05N310	25/07/2005	Kwelera (East London)	Chickens	
CKZA05UP1049	26/07/2005	Kameeldrif East	Layers	
CKZA05UP1050	26/07/2005	Newcastle	Layers	
CKZA05AL547	26/07/2005	Mount Currie	Village fowl	
CKZA05AM67794	27/07/2005	Pietermaritzburg	Chickens	
CKZA05AL548	27/07/2005	Mooirivier	Village fowl	
CKZA05AL549	27/07/2005	Underberg	Village fowl	
CKZA05N309	28/07/2005	Vaalwater (Ellisras)	Chickens	
CKZA05N311	28/07/2005	Hartebeespoortdam	Chickens	
CKZA05UP997	29/07/2005	Rustenburg	Layers & Fowl	
GOZA05N317	29/07/2005	Smithfield (Bloemfontein)	Goose	
CKZA05UP1085	01/08/2005	Magaliesburg	Layer chicks	
CKZA05UP1099	02/08/2005	Siyabushwa	Village fowl	
CKZA05UP1097	02/08/2005	Magaliesburg	Broilers	
CKZA05UP1098	02/08/2005	Makapaanstad	Village fowl	
CKZA05UP1111	03/08/2005	Queenstown	Village fowl	
CKZA05UP1134	05/08/2005	Pretoria	SPF Chickens	
CKZA05AL550	05/08/2005	Ixopo	Layers	
CKZA05UP1133	05/08/2005	Soshanguve	Village fowl	
CKZA05UP1139	05/08/2005	Germiston	Village fowl	
CKZA05N312	11/08/2005	Mmabatho	Village fowl	
CKZA05N327	11/08/2005	Kimberly	Chickens	
CKZA05UP1151	11/08/2005	Rustenburg	Layers	
CKZA05N315	12/08/2005	Kgotsong (Bothaville)	Village fowl	
CKZA05AL551	15/08/2005	Ixopo	Layers	
CKZA05AL560	15/08/2005	Camperdown	Broilers	
CKZA05UP1180	16/08/2005	Lichtenburg	Village fowl	
CKZA05UP1181	16/08/2005	Lichtenburg	Village fowl	
CKZA05UP1178	17/08/2005	Warrenton	Layers	EF030975
CKZA05N328	18/08/2005	Grootrietvlei	Chickens	
CKZA05N340	18/08/2005	Witsieshoek	Village fowl	
CKZA05AL552	19/08/2005	Pietermaritzburg	Village fowl	
CKZA05N349	19/08/2005	Ventersdorp	Village fowl	
CKZA05UP1207	22/08/2005	Pretoria	Village fowl	
CKZA05UP1219	22/08/2005	Pretoria North	Layers	
CKZA05N336	22/08/2005	East London	Village fowl	
CKZA05N337	22/08/2005	Butterworth	Village fowl	
CKZA05N336	22/08/2005	East London	Village fowl	
CKZA05UP1221	23/08/2005	Bronkhorstspruit	Broilers breeders	
CKZA05UP1216	23/08/2005	Delmas	Broilers	
CKZA05N350	23/08/2005	Standerton	Chickens	
CKZA05AL553	24/08/2005	Camperdown	Broilers	EF030970
CKZA05AL554	24/08/2005	Ixopo	Layers	
CKZA05N386	24/08/2005	Vredefort	Chickens	
CKZA05UP1233	25/08/2005	Cullinan	Broilers	

CKZA05UP1236	25/08/2005	Bronkhorstspruit	Chickens	
CKZA05N334	25/08/2005	Ventersdorp	Village fowl	
CKZA05N347	25/08/2005	Ventersdorp	Village fowl	
CKZA05UP1176	26/08/2005	Pretoria	Village fowl	
CKZA05N338	26/08/2005	Amsterdam	Village fowl	
CKZA05UP1235	26/08/2005	Bronkhorstspruit	Broilers	
CKZA05UP1250	26/08/2005	Brits	Broilers	
CKZA05UP1251	29/08/2005	Olifantsfontein	Layers	
CKZA05N339	29/08/2005	Bethlehem	Village fowl	
CKZA05AL558	29/08/2005	New Hanover	Broilers	
OSZA05N333	30/08/2005	Brits	Ostrich	
CKZA05UP1278	31/08/2005	Bryanston	Layers	
CKZA05N360	31/08/2005	Heilbron	Village fowl	
CKZA05N342	01/09/2005	Heilbron	Village fowl	
DOZA05N341	02/09/2005	Bethlehem	Dove	
CKZA05UP1306	05/09/2005	Middelburg	Broilers	
CKZA05UP1307	05/09/2005	Randfontein	Layers	
CKZA05UP1333	05/09/2005	Kuruman	Village fowl	
CKZA05N348	05/09/2005	Lydenburg	Village fowl	
CKZA05UP1402	05/09/2005	Pretoria	Free range chickens	
CKZA05UP1412	05/09/2005	Makhado	Chickens	
CKZA05UP1314	06/09/2005	Muldersdrif	Chickens	
CKZA05UP1279	08/09/2005	Kudumane	Free range Chickens	
CKZA05N355	08/09/2005	Brits	Village fowl	
CKZA05N356	09/09/2005	Lydenburg	Chickens	EF030972
CKZA05UP1353	09/09/2005	Meyerton	Free range Chickens	EF030976
CKZA05UP1355	12/09/2005	Pretoria	Broilers	
CKZA05UP1364	13/09/2005	Bramley	Free range Chickens	
CKZA05AL563	14/09/2005	Howick	Bantams	
CKZA05M202	15/09/2005	Standerton	Chickens	
CKZA05N357	19/09/2005	Bloemfontein	chicks	
CKZA05UP1411	19/09/2005	Kuruman	Chickens	
CKZA05M203	20/09/2005	Makhado	Chickens	
CKZA05N362	21/09/2005	Knysna	Chickens	
CKZA05AL566	21/09/2005	Eston	Broilers	
CKZA05M207	22/09/2005	Walkerville	Chickens	
CKZA05UP1446	22/09/2005	Randfontein	Free range Chickens	
CKZA05M208	23/09/2005	Lerato	Chickens	
CKZA05N387	27/09/2005	Nelspruit	Chickens	
CKZA05N404	27/09/2005	Klerksdorp	Village fowl	
CKZA05M233	28/09/2005	Thaba Nchu	Chickens	
CKZA05N405	29/09/2005	Port Elizabeth	Chickens	
CKZA05AL568	30/09/2005	Mooi River	Village fowl	
CKZA05N406	03/10/2005	Fouriesburg	Chickens	EF030973
CKZA05N403	04/10/2005	Port Alfred	Chickens	
CKZA05N408	04/10/2005	Kroonstad	Chickens	
CKZA05UP1510	05/10/2005	Bronkhorstspruit	Chickens	
CKZA05UP1514	05/10/2005	Germiston	Village fowl	
CKZA05UP1565	05/10/2005	Kuruman	Chickens	
CKZA05UP1649	05/10/2005	Germiston	Village fowl	
CKZA05N407	06/10/2005	Bloemfontein	Chickens	
CKZA05N402	07/10/2005	Pretoria	Chickens	
CKZA05M239	07/10/2005	Hertzogville	Chickens	
CKZA05AL570	10/10/2005	Camperdown	Broilers breeders	

CKZA05AL571	13/10/2005	Durban	Chickens	
CKZA05M243	17/10/2005	Delmas	Chickens	
CKZA05N429	17/10/2005	Queenstown	Village fowl	
CKZA05AL575	19/10/2005	Camperdown	Chickens	
CKZA05AL576	20/10/2005	Ezakheni	Chickens	
CKZA05M272	25/10/2005	Mokopane	Broilers	
CKZA05N421	27/10/2005	Murraysburg	Chickens	
CKZA05AL573	28/10/2005	Durban	Chickens	
CKZA05M275	02/11/2005	Nelspruit	Chickens	
CKZA05N516	03/11/2005	Coligny	Broilers	
CKZA05N442	04/11/2005	Bloemfontein	Chickens	
CKZA05M287	04/11/2005	Naboomspruit	Chickens	
HIZA05N500	04/11/2005	Middleburg	Hadeda ibis chicks	
CKZA05UP796	05/11/2005	Tarlton	Chickens	
CKZA05UP1835	05/11/2005	Rynfield	Chickens	EF030977
CKZA05UP1879	05/11/2005	Naboomspruit	Broilers	
CKZA05M279	07/11/2005	Nelspruit	Chickens	
CKZA05M288	09/11/2005	Randfontein	Chickens	
CKZA05N496	10/11/2005	Potchefstroom	Chickens	
CKZA05N517	14/11/2005	Tzaneen	Chickens	
CKZA05N519	14/11/2005	Lindley	Chickens	EF030974
CKZA05AL578	14/11/2005	Dundee	Chickens	
PCZA05N518	23/11/2005	Pretoria	Peacock	
CKZA04AL580	28/11/2005	Howick	Chickens	
CKZA06N515	01/12/2005	Wolmaranstad	Chickens	
CKZA05N540	19/12/2005	Haarlem	Chickens	
CKZA06AM73356	04/01/2006	Klerksdorp	Chickens	
CKZA06UP30	05/01/2006	Pretoria	bantam	
CKZA06UP35	05/01/2006	Kroonstad	Broilers	
CKZA06UP97	13/01/2006	Bramley	Village fowl	
CKZA06UP113	17/01/2006	Kuruman	Village fowl	
CKZA06N553	19/01/2006	Pretoria	Broilers	
CKZA06N555	20/01/2006	Pretoria	Broilers	
CKZA06N556	20/01/2006	Zonderwater	Broilers	
CKZA06AM74097	24/01/2006	Paarl	Chickens	
CKZA06UP171	25/01/2006	Kuruman	Village fowl	
CKZA06UP182	26/01/2006	Viljoenskroon	Broilers	
CKZA06UP184	27/01/2006	Kuruman	Village fowl	
CKZA06UP187	27/01/2006	Skuinsdrift	Broilers	
CKZA06AL585	30/01/2006	Pietermaritzburg	Chickens	
CKZA06AL586	08/02/2006	Uitenhage	Chickens	
CKZA06N573	17/02/2006	Van Wyk's Rivier (Paarl)	Chickens	
CKZA06N574	17/02/2006	Van Wyk's Rivier (Paarl)	Chickens	
CKZA06AL590	03/03/2006	Camperdown	Chickens	
CKZA06N602	07/03/2006	Gedults Rivier (St Albans)	Chickens	
CKZA06AL591	08/03/2006	Pietermaritzburg	Chickens	
CKZA06N607	14/03/2006	East London	Chickens	
CKZA06N616	15/03/2006	Sommerset East	Village fowl	
CKZA06N609	23/03/2006	Worchester	Chickens	
CKZA06N610	23/03/2006	Malmesbury	Chickens	
CKZA06N608	24/03/2006	Rietgat (Brits)	Chickens	
CKZA06UP529	29/03/2006	Bapsfontein/Tiegerpoort	Broilers	
CKZA06N630	31/03/2006	Mokopane	Chickens	
CKZA06N642a	03/04/2006	Calitzdorp	Chickens	

CKZA06N642b	03/04/2006	Plettenberg Bay	Chickens	
CKZA06N628	13/04/2006	Ladismith	Chickens	EF030978
CKZA06N654	20/04/2006	Grahamstown	Chickens	EF030965
CKZA06SB29	01/05/2006	Robertson	Chickens	
CKZA06SB56	03/05/2006	Vredenburg	Chickens	
CKZA06N641	05/05/2006	Stellenbosch	Chickens	EF030979
CKZA06N651	11/05/2006	Stellenbosch	Chickens	EF030980
CKZA06N652	11/05/2006	Stellenbosch	Chickens	EF030981
CKZA06UP736	05/06/2006	Marble Hall	Layers	

[1]Viruses were named according to host, year of isolation and the original lab number. CK=chicken, OS=ostrich, DO=dove, HI=hadeda ibis, GO=goose, PC=peacock, PH=pheasant; N=OVI Biotechnology, M=OVI Virology, SB=Stellenbosch, UP=University of Pretoria, AL=Allerton, AM=Avimmune (Deltammune) e.g. CKZA06AL591= Chicken/South Africa/2006/Allerton lab number 591.

[2]Where available, the type of chicken is specified

[3]Only one representative of each sub-lineage was selected, except for sub-lineage (o)

[4]Sub-lineage (o) viruses are indicated in boldface

6.2.2 RNA Extraction

Viral RNA was extracted from allantoic fluid using TRIzol® reagent (Gibco, Invitrogen), according to the manufacturer's instructions.

6.2.3 RT-PCR

A one-step RT-PCR was performed using the oligonucleotide pair described in Chapter Four (p160), with the addition of MMLV-reverse transcriptase and the following modification to the thermal cycling protocol: incubation at 42ºC for 20 min, followed by 35 cycles of 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min. Amplification products were analysed by electrophoresis on 1% agarose gel. Bands of the correct size were excised and DNA purified using the QiaQuick Gel Extraction Kit (Qiagen).

6.2.4 DNA sequencing and phylogenetic analysis

DNA was sequenced using the ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions, and was analysed with an ABI3130™ Genetic Analyser. Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) of the 374 nucleotide (nt) region of the 3' end of the F protein, including the F_0 cleavage site, were used to identify closely-related sequences to include in multiple sequence alignments, which were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html). The preparation of Fig. 6.2 (variable sites of the multiple nucleotide sequence alignment) was done with MEGA v3.1 software. The region from 61 to 374 was used for the final phylogenetic analysis and multiple alignments, because of shorter reference sequences in Genbank. The results are presented as a rooted neighbour joining tree with 1000 bootstrap trials to assess nodal support values. The majority (n=149) of the South African lineage 5d nucleotide sequences were identical across the 374-bp region analysed, and are represented by a single virus as indicated in Fig. 6.1(a).

6.2.5 Geographic Information System maps

Maps of the distributions of isolates were constructed using ArcGIS 9 (ESRI) software, and are located in Appendix 2.

6.3 RESULTS

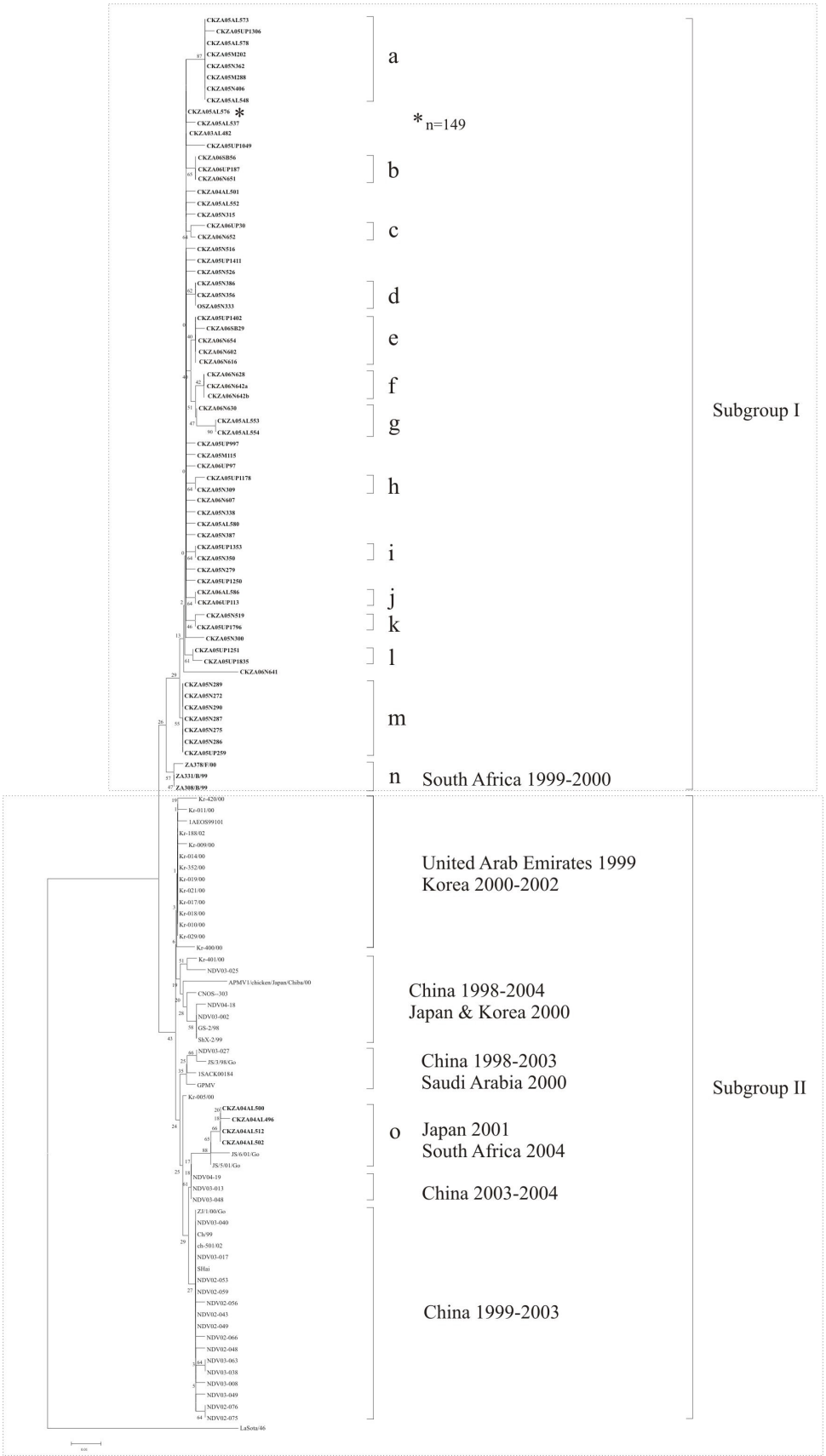


Figure 6.1(a) Phylogenetic tree based on nucleotide sequences of South African lineage 5d viruses (in boldface) and reference strains. The tree is rooted with LaSota/46 virus. Sub-lineages (a) to (o) are indicated. Enlargements of Subgroups I and II are presented in Figs. 6.1(c) and 6.1(d) respectively.

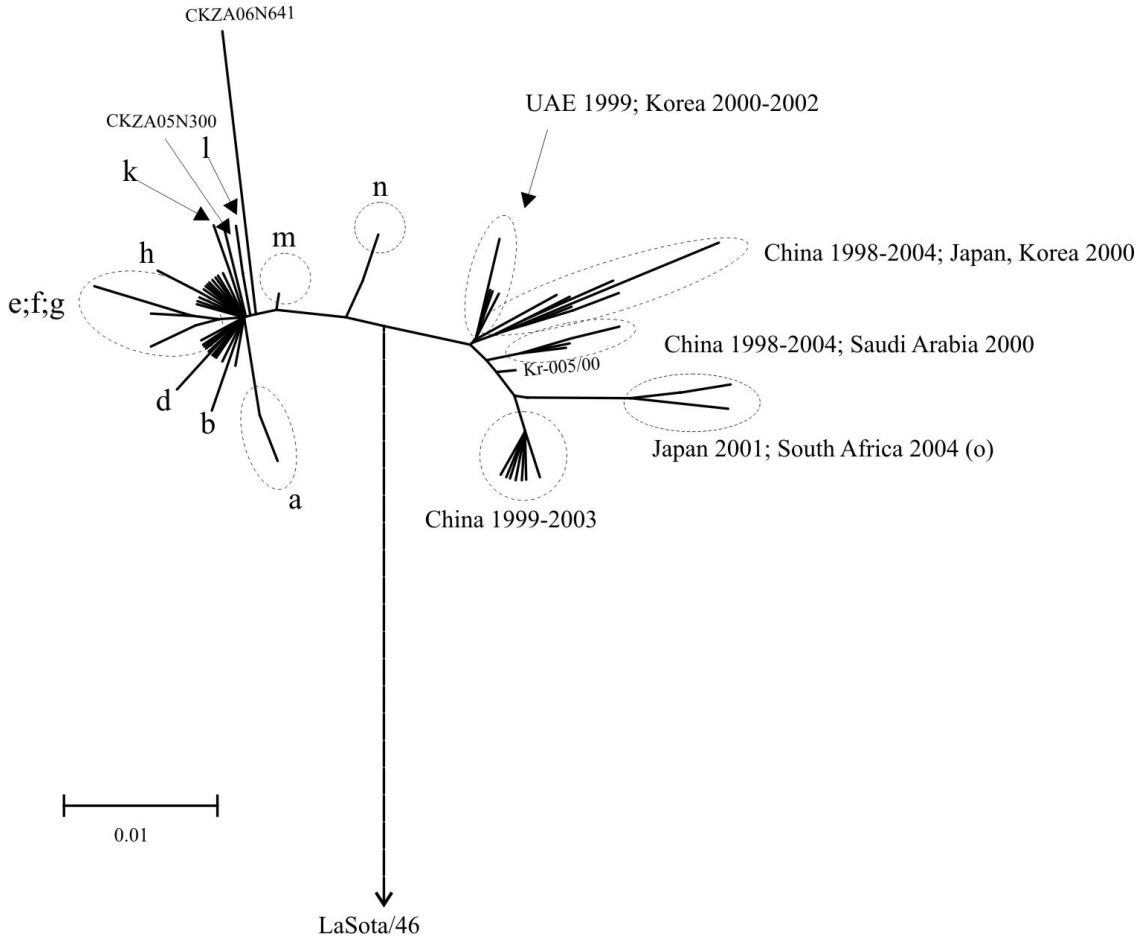


Figure 6.1(b) Radial (unrooted) version of Fig. 6.1(a)

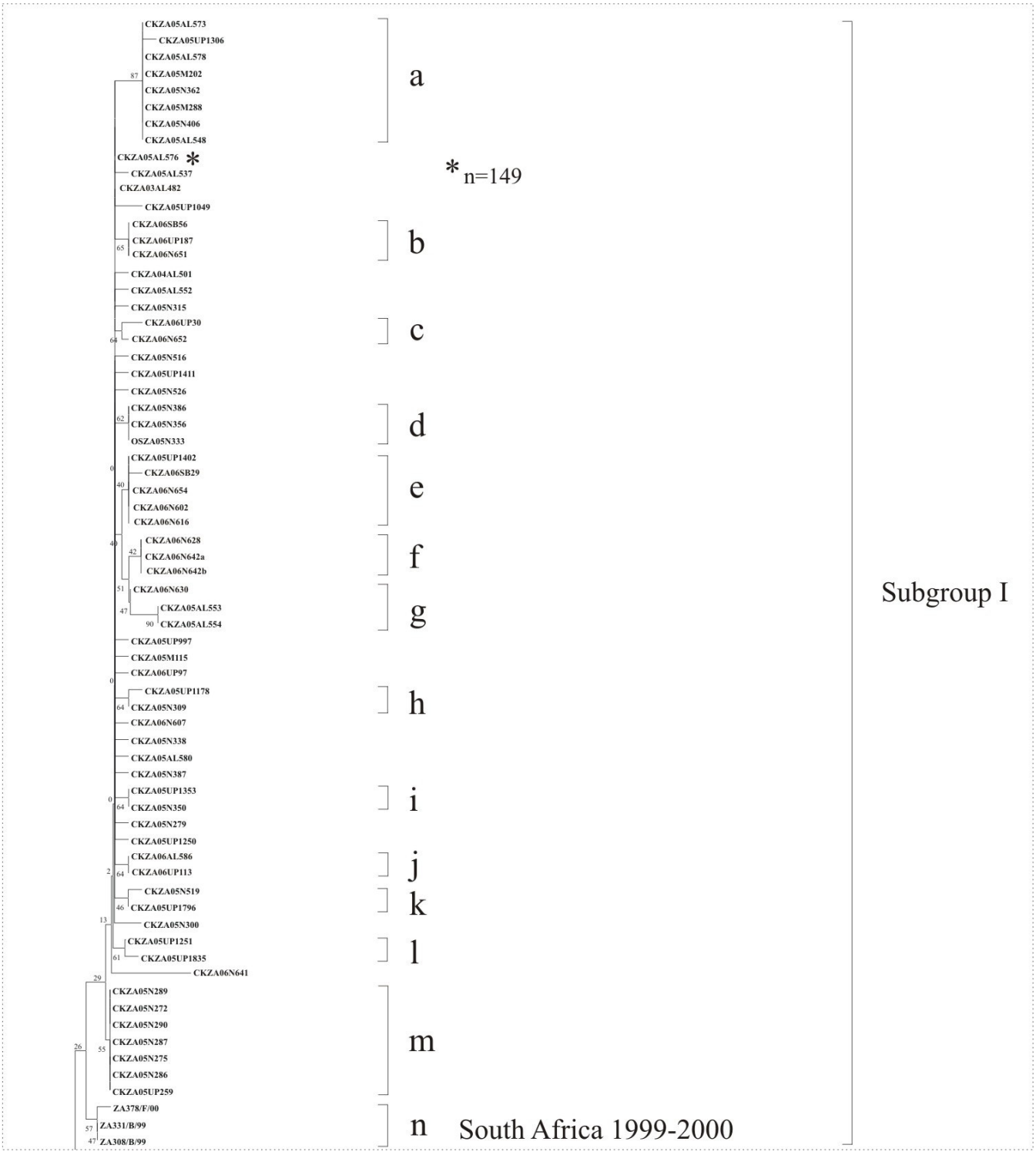


Figure 6.1(c) Enlargement of Fig 6.1(a) depicting Subgroup I phylogeny

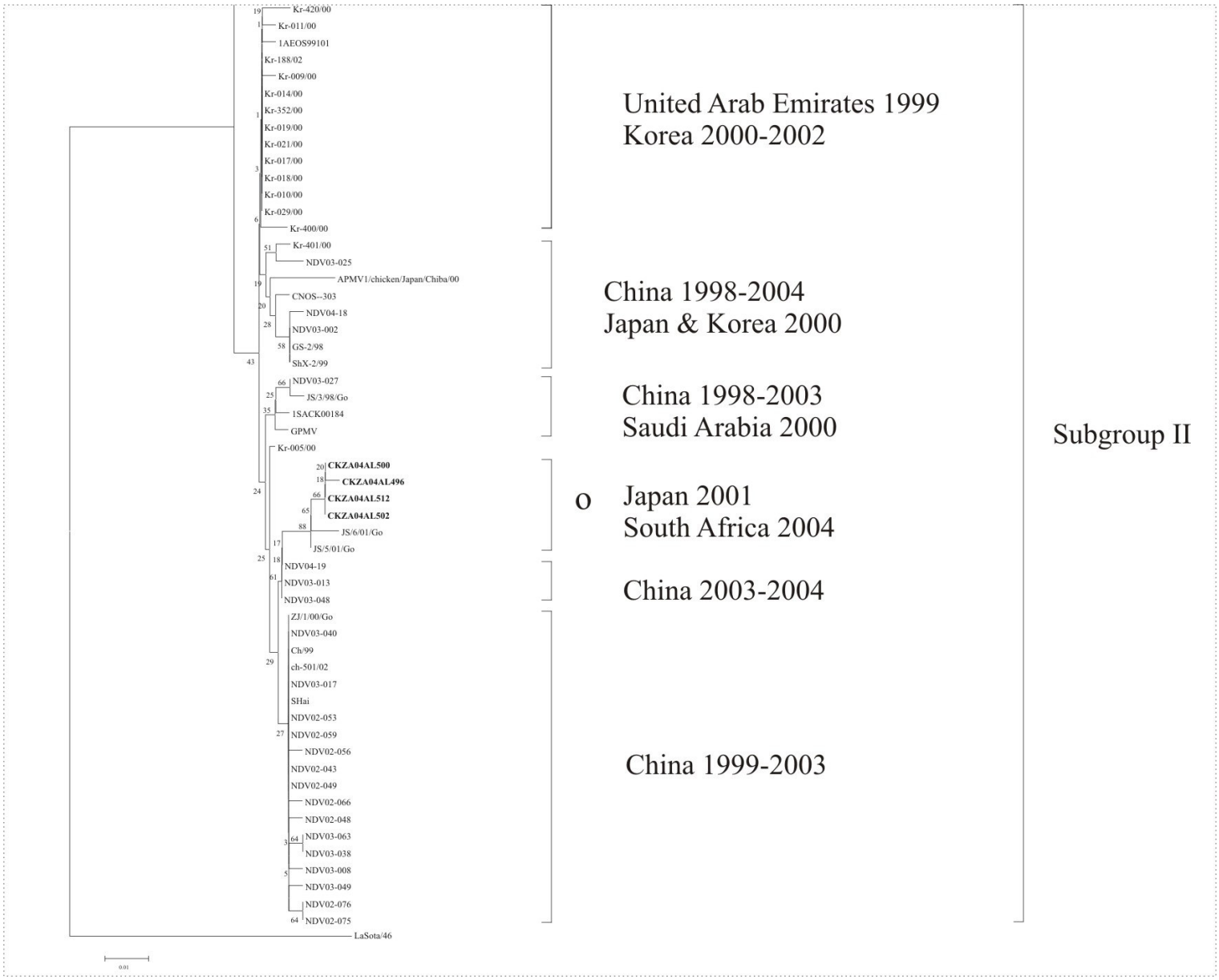


Figure 6.1(d) Enlargement of Fig 6.1(a) depicting Subgroup II phylogeny

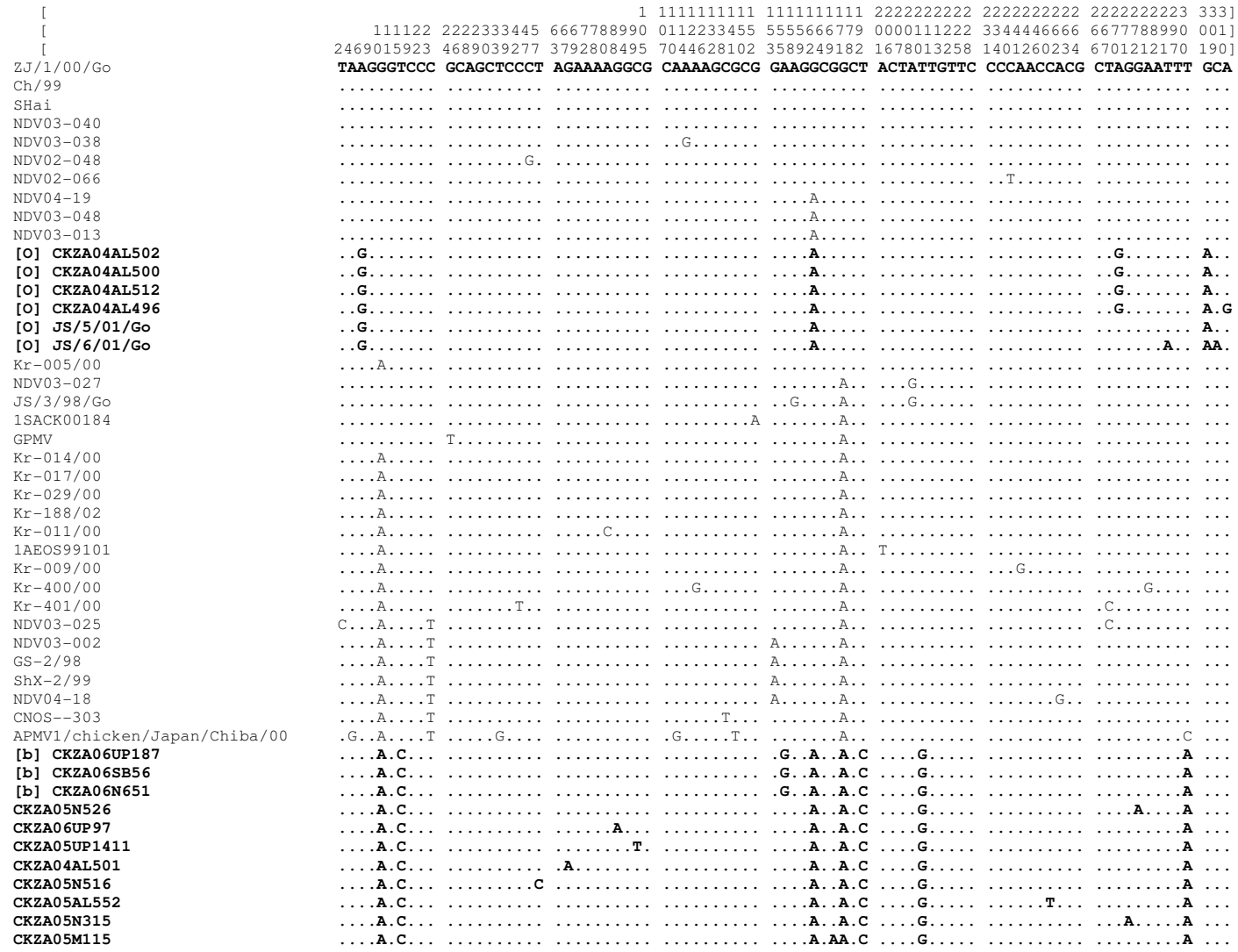
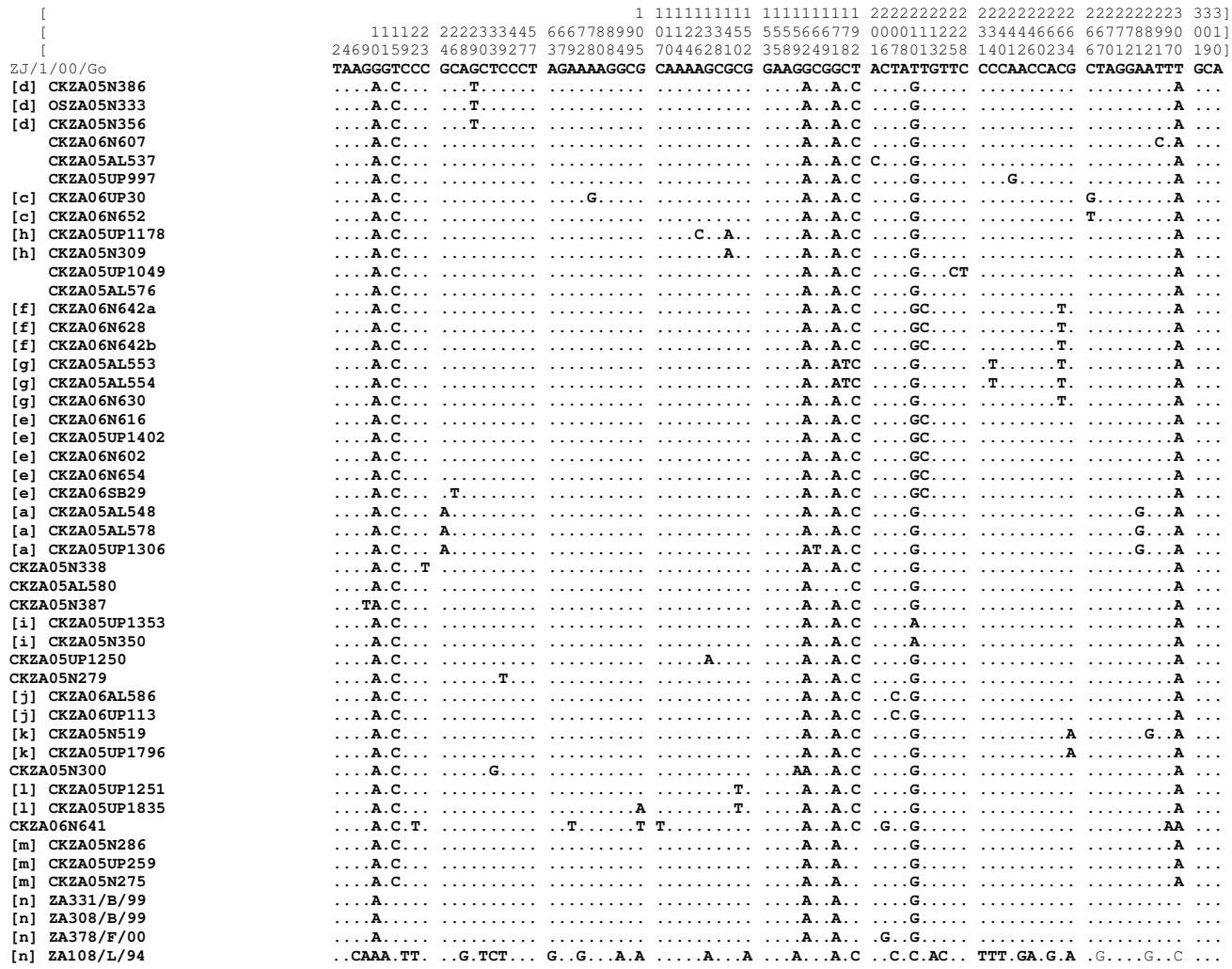


Figure 6.2 Multiple nucleotide sequence alignment of variable sites only within the fusion protein gene (nt 61-374) of selected South African (in boldface) and other lineage 5d strains. Sub-lineages (a) to (o) are indicated in square brackets.

Fig. 6.2 continued



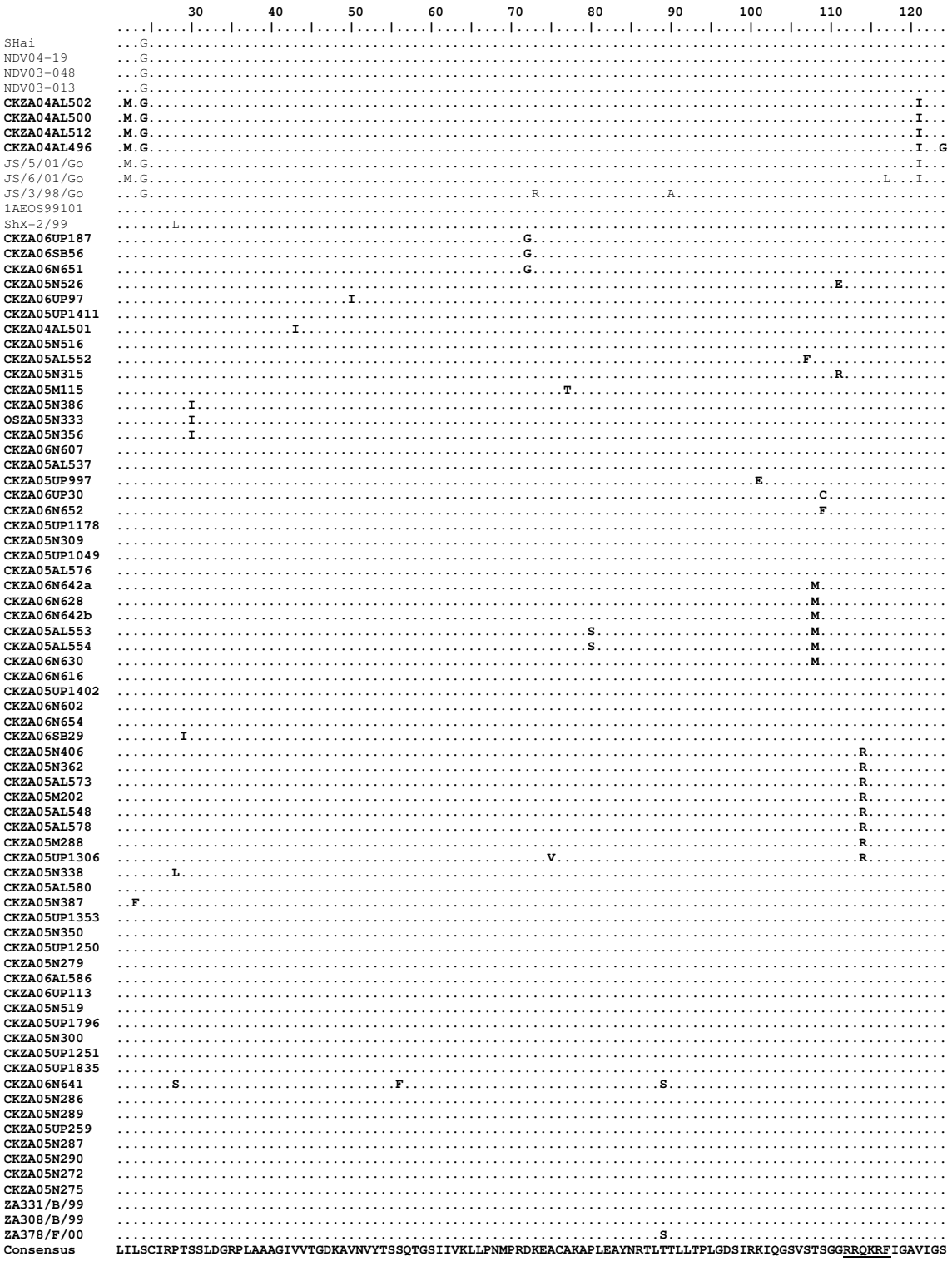


Figure 6.3 Multiple amino acid alignment (residues 21 to 124 of the fusion protein) of South African (in boldface) and other lineage 5d strains. The F_0 cleavage site is underlined.

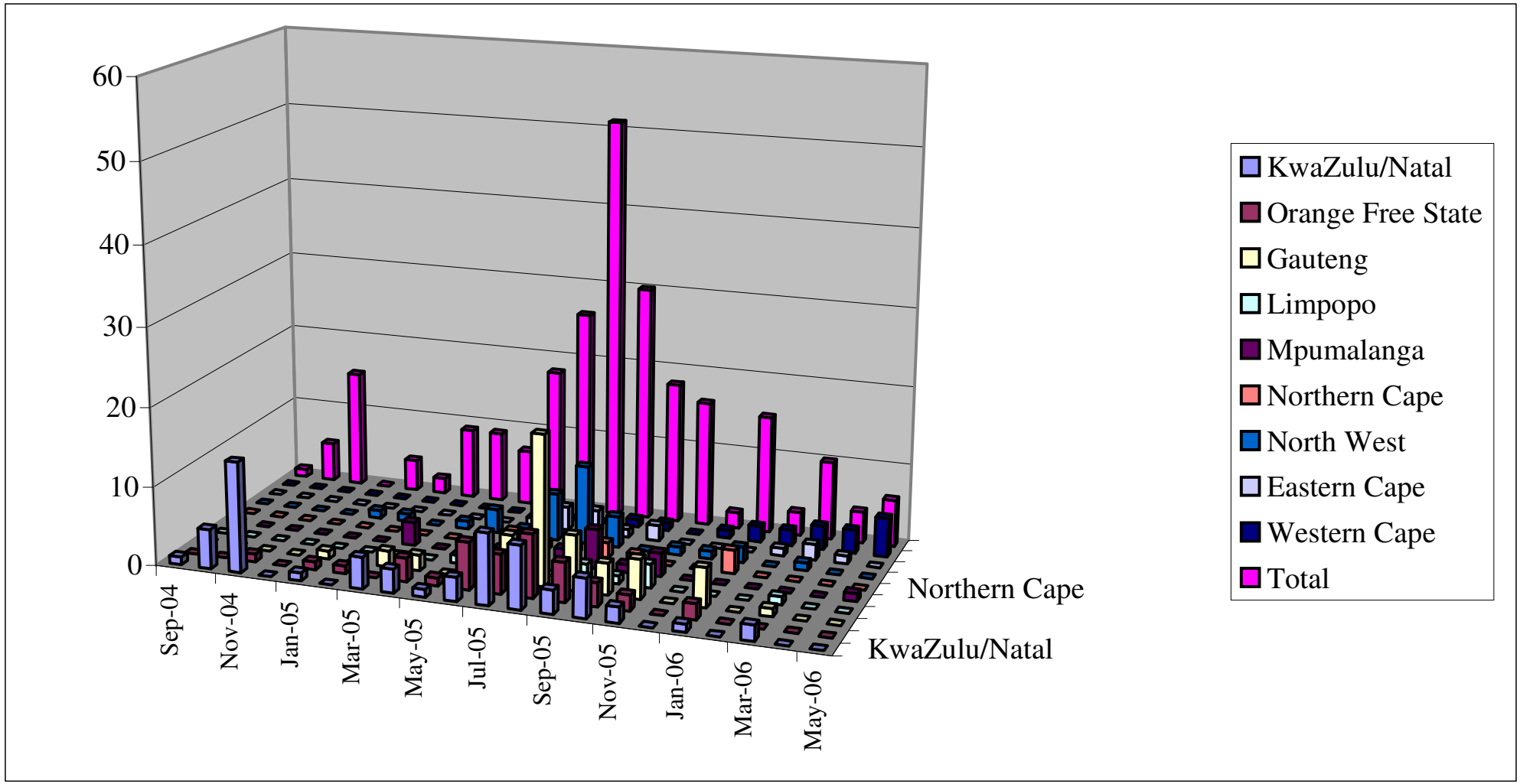


Figure 6.4 Prevalence of NDV GPMV isolates collected from September 2004 to May 2006

6.3.1 South African lineage 5d strains are closely-related to strains from the Far East

Phylogenetic analysis (Figs. 6.1(a) and (b)) indicates that South Africa ND outbreak strains of 1999-2000, 2003 and 2004-2006 are closely-related and may be classified as lineage 5d by their close sequence homology to viruses isolated in the middle and Far East since 1999. Furthermore, it was evident that two separate genotypes of lineage 5d, or "goose paramyxovirus" were responsible for outbreaks in KZN in late 2004. 253/257 (98.4%) of South African lineage 5d strains form a separate group from other lineage 5d strains from the Far and Middle East and will be referred to as subgroup I (sub-lineages (a) to (n)) in this chapter, whereas other international viruses and the second genotype of South African lineage 5d (sub-lineage (o)) will be referred to as subgroup II (Fig 6.1(a))

6.3.2 The ND outbreak in KwaZulu/Natal from 1999-2000

Seven velogenic viruses (CKZA99AL308, CKZA99AL328, CKZA99AL331, CKZA99AL337, CKZA00AL344, CKZA00AL377 and CKZA00AL378) were isolated between 1999 to 2000 in a relatively small area in KZN (Appendix 2i, p275; Appendix 2 xv, p). In Fig 6.1(a) the group is represented by three viruses, ZA378/F/00, ZA331/B/99 and ZA308/B/99 and form sub-lineage (n). The earliest of the viruses, CKZA99L308, was isolated from 25-day old broilers in the New Hanover/Wartburg area in March 1999, but only village chickens were affected thereafter. Sub-lineage (n) is located at the root of the majority of the SA viruses (Fig. 6.1(b)). This suggests that they gave rise to the later South African lineage 5d viruses (subgroup I). At the nucleotide sequence level (Fig. 6.2), they contain a T^{15} and T^{300} residues that are absent from SA viruses isolated after 2003, but are shared with viruses from the Far and Middle East, supporting the theory of a recent origin in the Far East.

6.3.3 The ND outbreak in KwaZulu/Natal in 2003

CKZA03AL482 was isolated from an outbreak in backyard chickens that killed 17 chickens out of a flock of 18 birds. This single outbreak near Pietermaritzburg occurred almost three years after the initial outbreak in the same geographic area (Appendix 2i,

p275, red dot). At the nucleotide sequence level, CKZA03AL482 is indistinguishable from 149 other subgroup I 2004 outbreak strains (Fig. 6.1), indicating that the same strain re-emerged in 2004 to cause the epidemic.

6.3.4 The rise of the epidemic strain in KwaZulu/Natal

Appendix 2i (p275) maps the locations of the 1999/2000 (sub-lineage (n)), 2003, and initial 2004 isolations. The positions of the three outbreaks overlap, and correspond to a major poultry-producing area in South Africa (Appendix 1) that is also populated with rural villages in the Valley-of-1000-Hills region of KwaZulu/Natal. The index case in the 2004 lineage 5d outbreak was CKZA04AL491, isolated from 8- and 12-week old broiler breeders at a site of a large commercial operation in Hopewell, near Pietermaritzburg on 30 September 2004. A house on each of two sites was affected and a total of 1,901 out of 100,000 birds died (Department of Agriculture (DoA)).

About a week later, on 8 October 2004 the second isolate (CKZA04AL495) was made after reports were received of illness and deaths amongst village chickens at Hopewell Village in the Richmond district, approximately 200 meters from the breeder farm. The villagers reported that chickens had been dying in the township for several weeks prior to the outbreak at the commercial farm. CKZA04AL495 had an ICPI of 1.91, which is characteristic of lineage 5d (Lin *et al*., 2003) and long heat stability.

Newcastle disease also occurred on a broiler rearing site, east/south-east of the breeder site. 5,000 out of 360 000 birds died within three days. The commercial operation suspected that a break in the biosecurity caused the jump from the neighbouring township (DoA).

The next isolate, CKZA04AL496 was the first sub-group II (Fig. 6.1(a), sub-lineage (o)) isolate, from a township near Estcourt. This virus had an ICPI of 1.89. The other sub-lineage (o) isolates were CKZA04AL500, CKZA04AL502 and CKZA04AL512, isolated throughout November 2004. These viruses shared very close genetic relationships (98.7-99.7%) with two Chinese isolates, JS/6/01/Go and JS/5/01/Go, but only 95.2 to 97.1% sequence identities with subgroup I. At the amino acid level (Fig. 6.3), the close genetic relationship to viruses from China is illustrated by shared M^{22}, G^{24} and I^{121} residues. This

suggests that sub-lineage (o) is a recent introduction to SA, rather than having evolved from the 1999/2000 viruses like the sub-group I viruses.

A few other notable cases from the early outbreak cases include CKZA04AL506 that was isolated from a flock of free-range bantams kept by a commercial farmer for ornamental purposes. Although the commercial chickens were well-vaccinated and therefore fully-protected, 180 out of 200 of the bantams died, demonstrating the high pathogenicity of lineage 5d viruses for chickens. A second case involved CKZA04AL509, isolated during an outbreak that destroyed most of 3000 to 4000 pullets on a small chicken farm, but the owner admitted that his vaccination had been poor. Thirdly, the location of CKZA05AL516 (commercial chickens) was geographically very close to that of CKZA04AL514 (village chickens) although the outbreaks were two months apart. Labourers reported that chickens had been dying in large numbers over December and January 2004. All indications are that this is the second recorded case of the strain spreading from village to commercial chickens.

6.3.5 The spread of the outbreak throughout South Africa

From the period September to November 2004, the outbreak remained confined to KZN and was defined by the presence of two separate genotypes, sub-group I and sub-group II. Sub-group II (sub-lineage (o), Appendix 2xvi, p277) disappeared, but by January 2005 (Appendix 2ii, p275) sub-group I had spread northwards to the OFS, Gauteng and North-West provinces. The index case in the north appears to be a large commercial producer, who sourced hatching eggs from KZN that likely were contaminated (S. Bisschop, personal communication). ND seemed to be spreading westward towards Botswana in mid-February. During March, April and May 2005 (Appendix 2iii, p276) the outbreak was still continuing in KZN, and a cluster of outbreaks in Kroonstad village chickens persisted. The disease had also spread eastwards into Mphumalanga, northwards into Limpopo and was progressing steadily through the North West Province (NWP) into the Northern Cape. From June to August 2005 (Appendix 2iv, p276) the main cluster occurred in the Gauteng Province but ND continued to disperse widely throughout the infected regions, appearing for the first time in the Eastern Cape in June 2005. In the period September to November 2005 (Appendix 2v, p277) the Western Cape also became infected and ND moved further eastwards in Mphumalanga towards Mozambique. At this time the infection still continued

in KZN, particularly along the N2 highway. Towards the end of 2005 and beginning of 2006 (Appendix 2vi, p277) the number of cases had diminished dramatically in KZN but clusters of outbreaks still occurred in Gauteng, Northern Free State/southern NWP and on the Northern Cape border. From March to June 2006 (Appendix 2vii, p278) the outbreak was dying out in the northern and eastern regions, but was becoming established in the Eastern and Western Cape provinces. The general distribution is depicted in Fig. 6.4, and indicates that the outbreak peaked around September 2005. It is clear from the figure that in 2006 outbreaks were ceasing in all provinces except the Western Cape, where cases were on the increase heading into the winter months.

6.3.6 Mapping of specific genetic variants to gain insight into how ND is spread in South Africa

The natural tendency of RNA viruses to undergo antigenic drift results in the rise of specific sub-populations during the course of an outbreak. These genetic variants give clues to the spread of viruses in a geographic region, since they are unique and easily traceable. Several sub-lineages ((a) to (m); Appendices 2viii to 2xvi, pp278-282) arose during the course of the South African lineage 5d outbreaks that started in 2004. They are discussed in the order that they appear in the phylogenetic tree, Fig. 6.1(a).

Sub-lineage (a) consists of CKZA05N406, CKZA05N362, CKZA05AL573, CKZA05M202, CKZA05AL548, CKZA05AL578, CKZA05M288 and CKZA05UP1306. This group is defined at the nucleotide sequence level by unique $G^{24}\rightarrow A$ and $A^{281}\rightarrow G$ substitutions (Fig. 6.2), the latter resulting in a unique Q→R substitution within the F_0 cleavage site (Fig. 6.3). The (a) sub-lineage (Appendix 2viii, p278) first arose in KZN (Mooiriver, village fowl) in July 2005 and then appeared in September in Middleburg, Standerton and Knysna only days apart. At the beginning of October 2005 it re-appeared in Fouriesburg in the Free State and again in Durban towards the end of the month. Sub-lineage (a) was finally isolated in November 2005 in Randfontein and Dundee. Generally, the distribution is clustered in the poultry-producing corridor between Durban and Johannesburg (Appendix 1, p) with the single incursion into the Western Cape Province.

Sub-lineage (b) consists of three viruses, CKZA06SB56, CKZA06N651 and CKZA06UP187 (Appendix 2ix, p279), that are defined by A^{155}→G mutations (Fig. 6.2) and result in a non-synonymous D^{72}→G substitution (Fig 6.3). The first location was a broiler operation at Skuinsdrift, NW Province at the end of January 2006, and by May 2006 it had reached Vredenburg and Stellenbosch in the Western Cape.

Sub-lineage (c) consists of only two viruses, CKZA06N652 and CKZA06UP30 that may not be phylogenetically related, but are grouped together on account of containing different mutations at exactly the same position in the F protein. The former contains a C^{166}→T and the latter a C^{266}→G non-synonymous substitutions resulting in unique F and C amino acid residues at position 109, respectively (Fig. 6.3).

Sub-lineage (d) consists of three viruses, CKZA05N386 and CKZA05N356 isolated from chickens, and OSZA05N333 isolated from an ostrich. CKZA05N386 and OSZA05N333 were both isolated near the end of August 2005, from Vredevort in the northern OFS and Brits in NW province, respectively, and CKZA05N356 was isolated in September in Lydenburg (Appendix 2x, p279). This small genetically-distinguished group is defined by a unique G^{29}→T non-synonymous mutation (Fig 6.2) that produced a unique S^{30}→I substitution at the amino acid level (Fig. 6.3).

Sub-lineage (e) viruses (CKZA05UP1402, CKZA06SB29, CKZA06N654, CKZA06N602, CKZA06N616) lack distinguishing amino acid substitutions, but at the nucleotide sequence level (Fig. 6.2) they share a T^{211}→C substitution that is also shared by CKZA06N642a, CKZA06N642b and CKZA06N628 (sub-lineage (f)). This particular variant (Appendix 2xi, p280) first arose in Pretoria in September 2005 before spreading to Gedult's rivier and Sommerset East in the Western Cape in March 2006, Grahamstown in the Eastern Cape in April and Robertson, Western Cape, in May. Therefore, sub-lineage (e) appeared to have originated in Gauteng in 2005 before spreading to and becoming prevalent in the Cape provinces in 2006. Sub-lineage (f) viruses may have shared a common ancestor with sub-lineage (e) as discussed earlier, and were restricted within the Western Cape Province in April 2006 (Appendix 2xii, p280), but the lineage (f) viruses also share a synonymous C^{263}→T mutation with CKZA05AL553, CKZA05AL554 and CKZA06N630 of sub-lineage (g). Sub-lineage (g) consists of viruses isolated in KwaZulu/Natal during August 2005 (CKZA05AL553 and CKZA05AL554) and Mokopane at the end of March 2006

(CKZA06N630) (Appendix 2xiii, p281). Therefore, its possible the ancestral virus of sub-lineages (e), (f) and (g) first arose in the latter part of 2005 in KZN before being spread to the Northern regions and finally down to the Western and Eastern Cape provinces where they continued to spread in 2006.

Sub-lineages (h (i), (j), (k) and (l) only consists of two viruses each, and point mutations are synonymous (Fig 6.2)

Sub-lineage (m) consists of six viruses, CKZA05N272, CKZA05N275, CKZA05N290, CKZA05N286, CKZA05N287 and CKZA05N289. At the amino acid sequence level they are indistinguishable from the majority of SA strains (in the region analysed), but at the nucleotide sequence level they contain a $C^{192}\rightarrow T$ substitution that is shared by the 1999/2000 viruses of sub-lineage (n) and international viruses. Sub-lineage (m) (Appendix 2xiv, p281) was first detected in Lehurutshe near the Botswanan-border village fowl in May 2005 and almost simultaneously in Kroonstad. By June it had spread to de Aar, Northern Cape Province, and Stutterheim in the Eastern Cape Province, but appeared to persist in the Kroonstad area as it was re-isolated near Warden and Clarens in June 2005. Sub-lineage (m) appears to have been restricted to village chickens.

6.4 DISCUSSION

In this chapter the largest amount of NDV sequence data from an outbreak in South Africa thus far was collected and analysed. For the first time we were able to monitor the spread of an outbreak in real-time. I demonstrated that the outbreaks that mainly affected village chickens in KZN from 1999 to 2000 were not caused the same genetic type as the pandemic strain of the early and mid-1990's (VIId), but instead by strains of lineage 5d, previously only described in the Far and Middle East and were therefore probably a recent introduction into South Africa. From 2001-2003 a serological survey of village chickens was conducted to determine whether village chickens could play the role of a reservoir for virulent NDV. The serological test does not distinguish between vaccine and field strains, and village chickens are not usually vaccinated. Only a few sero-positives were found in rural Zulu chickens: about 10% of 166 birds in one district had antibodies against NDV and in another district (n=179) 11% HI positives were detected with titres of up to 2^{10} (unpublished laboratory data). In 2003 lineage 5d re-emerged in a single outbreak in the same area as the 1999/2000 outbreaks, but did not seem to spread. Phylogenetic evidence showed that the 1999/2000 strain was most likely the progenitor of the 2003 virus. Almost exactly one year later a virus identical to the 2003 strain re-emerged in KZN, causing an epidemic that continues to date.

Both commercial and village chickens were affected in the 2004-2006 outbreak and epidemiological and molecular evidence suggests that the outbreak first appeared in villages around the commercial operations. Spillovers were attributed to poor bio-security practices, and outbreaks in commercial operations in KZN were usually associated with poor vaccination practices although sometimes mortalities were reported in well-vaccinated flocks (R Horner, personal communication). The mechanism by which lineage 5d was able to overcome the vaccination barrier in these cases is unknown and requires further investigation. Another interesting phenomenon was the apparently recent introduction of a separate genotype of Lineage 5d into KZN. Four isolations were made of a sub-genotype that shared a very recent common ancestor with two Chinese goose viruses isolated in 2001 (JS5/98/Go and JS6/98/Go). This subgroup circulated concurrently with the subgroup I lineage 5d viruses, but appeared to die out and has not been isolated since. From December 2004, lineage 5d spread northwards along the major poultry-producing corridor between KZN and Gauteng/ North West Provinces, affecting both commercial and backyard flocks.

The index case in the Transvaal region appears to have been contaminated hatching eggs sourced from the supplier in KZN. Over the next few months the disease spread westwards to the Northern Cape, southwards to the Cape provinces, and eastwards into Mphumalanga. The epidemic peaked in September 2005 and by 2006 it had begun to subside in most regions, except for the Western and Eastern Capes where cases were still increasing. The Western and Eastern Cape outbreaks were characterized by at least three separate introduction events from northern regions, as indicated by the mapping of specific genetic variants of lineage 5d. Most large commercial poultry producers have spread their operations across the country (for example, day-old chicks or eggs produced in one province are distributed to layer or broiler operations in another province) (personal communication, Kevin Lovell, South African Poultry Association). GIS mapping also revealed a close association between outbreaks and national/regional roads, which proves that infected chickens and their products are moved extensively and over long distances by road in South Africa. Apart from the large producers, the cull-buyer industry is probably one of the main role-players in this dissemination and this will be discussed in the concluding chapter.

Genotypes VIIc, VIId and VIIe have been predominant among NDV infections in China in recent years, accounting for 78% of the total strains isolated since 1996 (Liu *et al*., 2003). Outbreaks caused by Genotype VIId/lineage 5d were also recently reported in Greece from December 2004 to January 2005 and from July to April 2005 (OIE Disease Information, 8 July 2005 Vol 18, #27; www.oie.int). At the time of writing sequence data was not available for comparison. Goose paramyxovirus is unusual because of its pathogenicity for waterfowl. In the South African epidemic, mortalities were recorded in multiple species including chickens (commercial, ornamental and indigenous), peacocks, hadeda ibis (*Bostrychia hagedash*) chicks, geese, ostriches, pheasants and doves. This is the first report of isolation of lineage 5d from peacocks, hadeda ibis, doves and ostriches. Most of the wild species could have become infected by scavenging off infected dumped chicken waste which is illegal but unfortunately still common (David Allen, personal communication). The molecular determinant of the high virulence of GPMV for waterfowl is not known, but is has been suggested that the sequence differences in the intragenic regions of HN and P genes of GPMV might cause the differences of RNA editing efficiency of P gene and the expression of V protein, which increases virulence (Zou *et al*., 2005).

The reservoir for the maintenance of lineage 5d in South Africa from 2000 to 2004 is unknown. Although village chickens are unvaccinated and highly-susceptible to lineage 5d strains, an infection passed from flock to flock in the district of the Valley-of-1000-Hills in KZN, that lies adjacent to the poultry-producing region, could easily have gone unreported due to the remoteness, and evidence of a low-level infection was indicated by the 2001-2003 serological survey. Layer hens are vaccinated and spent birds sold by cull-buyers into the township and rural regions are able to be infected with and shed virulent NDV strains for up to six months without developing clinical symptoms. The stress of being bundled into cages on open trucks and driven across the country could trigger virus shedding (Shahn Bisschop, personal communication), and these birds are therefore ideal candidates for the reservoirs of virulent NDV in South Africa. The possibility of a wild reservoir cannot be ruled out, but since partridges and pheasants have been found to be highly susceptible to infection with lineage 5d (Zhou & Gong, 2003), the francolin species and guinea fowl both common in KZN are not expected to be the asymptomatic reservoirs. It is also unknown how lineage 5d (on two occasions) was introduced into KZN, and various possibilities are discussed in the concluding chapter.

CHAPTER SEVEN

CONCLUDING REMARKS

Highly pathogenic avian influenza and velogenic Newcastle disease are the two most serious diseases of poultry worldwide and cause massive mortalities during outbreaks. While NDV is recognised as the most important disease threatening backyard poultry production for sustainable rural livelihoods in developing countries, HPAI has the status of having zoonotic potential. Asian HPAI H5N1 (genotype Z) has recently been at the receiving end of much media attention, most of it hype surrounding the theoretical possibility that HPAI H5N1 could be involved in the next human pandemic, in which millions of immunologically-naïve humans are predicted to die. South Africa regained its HPAI-free status in October 2005, but this was once again suspended in July 2006 when an outbreak of HPAI H5N2 was reported on an ostrich farm in the Riversdale area of the Western Cape Province.

Similarly, South Africa is not free of NDV and therefore trade restrictions apply. During 2004/2005, the South African poultry meat industry again made the largest contribution to the national gross value of agricultural production with 15.6% of R68 026 million (~$9 700 million). In 2004, almost 600 million broilers were slaughtered for consumption in South Africa (Trends in the Agricultural Sector 2005, NDA). The ostrich farming industry in South Africa is the single largest in the world producing 65% of the world's ostrich meat and export income amounts to R1,2 billion annually with 90% of both leather and meat products being exported (South African Ostrich Business chamber). Although AI and ND outbreaks can potentially have a huge impact on the economy, very little was known about the status of these two diseases in South Africa. It has for many years been assumed that NDV is enzootic here, periodically spilling over from an unknown reservoir. Similarly, the origin of the sporadic AI infections of ostriches was unknown. Therefore, the objectives of my investigation were to analyse genetically and phylogenetically the AIV and NDV viruses that were isolated across South Africa since the early 1990s. During this time, South Africa also experienced its first outbreak of HPAI since the 1960s. This was the first time that molecular techniques were applied in South Africa to analyse AIV and NDV strains.

In Chapters Two and Three, I demonstrated for the first time that South African AIV strains share recent common ancestors with viruses isolated predominantly in Russia (particularly Lake Chany and Primorje), northern Europe (Germany, the Netherlands, Norway, Denmark and Sweden), Italy and China. No South African viruses have yet been isolated in other regions, according to published reports. Although LPAI viruses have been isolated in 12 bird orders, the large majority of isolations were reported in the orders Anseriformes (in particular in the family Anatidae: ducks, swans and geese) and Charadriiformes (shorebirds and relatives). While anatidae certainly seem to play the major role in the ecology and transmission of LPAI viruses to domestic poultry in Europe and Asia (Brown *et al*., 2006), southern African ducks and geese are only short-to-medium distance migrants, and do not come into direct contact with the wild duck and goose populations of Europe and Asia. They are thus unlikely to be directly involved in the initial introduction of LPAI into South Africa. However, about two dozen species of the orders Ciconiiformes (storks) and Charadriiformes (waders and terns) are Palaearctic migrants that over winter annually in Southern Africa (Underhill *et al*., 1999) (Appendix 4). Siberia, and in particular the West Siberian Lowland, is the breeding area of many different migratory species in the Palaearctic, where birds arrive during springtime, following different routes from Europe, Asia, and Africa. The breeding season in these northern most regions of Eurasia is usually very brief, and migratory bird populations start migrating southward with their newly-hatched juveniles to escape the first frosts in early autumn. This results in the build up of pre-migration concentrations of many species of waterfowl in the south of the breeding areas, where maturation and moulting take place before the main southern migration starts. This is a critical stage in the LPAI virus transmission and redistribution process because of the density of waterfowl and the presence of immunologically naïve individuals, which may undergo several subsequent infection episodes, shedding large quantities of the virus in their faeces (Halvorson *et al*. 1985; Stallknecht and Shane 1988). As the autumn migration involves juveniles, it is characterised by short flights and several stopover sites along the way, which enhances the risk of AI virus transmission, when compared to the spring migration (northwards) that involves much longer distance flights. There is thus a strong seasonality, both in the prevalence of LPAI viruses in the wild bird populations, and in the timing of the high risk period on the migration route. Three main routes for Palaearctic migrants from Siberia to Africa have been described viz. the Black Sea/Mediterranean, West Asian/East African, and East Atlantic flyways (Appendix 3). In the northern hemisphere, these flyways

intersect with the Central Asian flyway and the East Asian/Australasian flyway. The possible persistence of AI viruses in cold water (Stallknecht *et al*. 1990) in the Arctic region and the transmission within populations during pre-migration concentrations also contributes to the large-scale redistribution of AI viruses along the different migration paths. Based on the sequence data presented in this study, it seems likely that the East Atlantic flyway is at least one of the major routes used by the specie(s) that carries LPAI into South Africa on southbound migration. This flyway utilizes Lake Chany, one of the largest freshwater lakes in Russia. From there, migrants move through northern Europe, via important stopover sites like the Wadden Sea. The Wadden Sea ranks among the most important wetlands to migratory waterbirds in the world. Its vast area of intertidal mudflats hosts numerous bird species breeding in the tundra from arctic Canada in the west to northern Siberia in the east. This may also be the ecosystem where the North American lineage H5 gene described in Chapter Three was introduced into the gene pool. From there the journey continues down the West African coast, where birds would fly for one week at a time and stop for about two to rest and refuel before continuing (Les Underhill, personal communication). Birds start to arrive in South Africa around August and September, and most have arrived by November. At the end of summer they start preparing to depart on the spring migration northwards, that usually starts around April. Alternatively, some populations may make use of the Black Sea/Mediterranean flyway and the West Asian/East African flyway, moving southwards through Eastern Europe and down the fertile African Rift Valley (Underhill *et al*., 1999). At some point in their lifecycle in the overwintering grounds of southern Africa, pelagic migrants shed viruses into the environment where they are ingested by and infect sympatric species, since LPAI has been isolated on several occasions from wild waterfowl in South Africa.

In Chapters Two and Three, molecular data for the first time provided conclusive evidence that wild ducks are linked with ostrich influenza outbreaks. The interaction between wild ducks and ostriches on the winter lucerne grazing pastures of the Klein Karoo is well-documented but the conversion of an LPAI virus originating from wild ducks to an HPAI virus in ostriches in 2004 was a seemingly rare event. Consequently, biosecurity awareness among Cape Ostrich farmers led to changes in farm management practises to minimise the contact between free-ranging ostriches and wild waterfowl, by fencing off rivers on ostrich farms, preventing contact with wild birds at ostrich feed points and by regular disinfection

of feed and water troughs (Adriaan Olivier, personal communication). Hopefully these biosecurity measures will help to reduce the risks.

Very little is known about the length of time that ostriches are able to maintain LPAI viruses. The results of Chapter Two suggested that ostriches possibly act as mixing vessels for LPAI strains and as a reservoir pose a threat to the chicken-producing industry when biosecurity breaks down. LPAI H6N2 still seems to be circulating in South Africa, based on PCR-detection of the H6 gene in ostriches, doves and NDV-infected chickens in four provinces, but interestingly, preliminary phylogenetic evidence suggests that this H6 gene is identical to the original H6 gene from the 1998 ostrich virus (A/Ostrich/South Africa/KK98/98) instead of that from the 2002 H6N2 chicken viruses (Abolnik *et al*., Avian Diseases, in press). However, the NA type was confirmed to be N2 by NI tests performed at Allerton Provincial Veterinary laboratory. These findings (not presented in this work) strongly suggest that the progenitor H6N2 virus to sub-lineages I and II is still circulating in ostriches and/or poultry.

South Africa therefore seems to be at the end of a "migratory funnel" for pelagic shorebirds, where birds from a wide geographic distribution and vast wetland ecosystems eventually converge at much smaller water bodies in South Africa. Why then are outbreaks caused by LPAI and HPAI viruses such relatively rare events in South Africa? I propose that a combination of the following factors might play a major role:

i. The primary vectors of AI viruses to South Africa (Charadriiformes) are not the major hosts

Anatidae harbour the highest diversity and prevalence of avian influenza viruses (EFSA 2005; Stallknecht and Shane 1988) and historical outbreaks of HPAI in poultry have been linked to LPAI strains circulating in ducks (Campitelli *et al*. 2004; Munster *et al.* 2005). Domestic ducks have been shown to be able to excrete large amount of H5N1 virus while remaining relatively healthy (Hulse-Post *et al.* 2005), and direct contacts especially between wild Anatidae and domestic wildfowl are believed to be more common than with other wild bird groups. None of the Anatidae from Europe and Asia migrate as far South as southern Africa (Underhill *et al*., 1999).

ii. Ostriches, seemingly the link between wild birds and domestic poultry in South Africa, are not ideal hosts

Ostriches are generally not considered to be highly-susceptible to AIV. Clinical disease and mortality usually seen where isolations of LPAI have been made were usually associated with concomitant infection with other pathogens, high population densities, inadequate ventilation and bad hygiene resulting from poor management practises. Green urine syndrome has not been conclusively or exclusively tied to AIV infection (Adriaan Olivier, personal communication). Transmissibility of AIV between ostriches also seems to be poor as they have to be in very close contact, and a higher viral load may be required to infect an ostrich than other smaller poultry species. Furthermore, the IVPI of the HPAI H5N2 virus from 2004 was initially low for chickens, and only increased with serial passage in chickens (Manvell *et al*., 2005) which suggests that virulence determinants in ostriches do not evolve as they do in chickens. The results of Chapter Two also suggest that the evolutionary rates of AI viruses are slower in ostriches than in other poultry.

iii. Ostrich farming and poultry-producing areas are geographically separated

Appendix 1 illustrates the distribution of poultry production in South Africa. Ostriches thrive in drier climates thus the main ostrich-producing areas are concentrated in the arid regions of the Cape Provinces. The Klein Karoo has low (288 mm per year) and unreliable precipitation that falls mainly in the winter months. It is bordered by the Outeniqua, Langeberg and Swartberg mountain ranges and is thus geographically separated from the major chicken-producing industries that are concentrated in KwaZulu/Natal, Gauteng and North-West provinces and to a lesser extent Mpumahlanga, Orange Free State and Western Cape provinces. As a result, there are not many opportunities for ostriches and chickens to mix.

iv. Consumer preference for chicken over duck meat in South Africa

Consumer demand for duck meat in South Africa is not as large as in other countries, and chicken remains the poultry staple. The raising of ducks by subsistence farmers is

uncommon, probably because the scarcity of water in the country. This is in comparison to the large formal and informal scale of domestic duck farming in other regions, such as Europe and East Asia. Recent evidence showed a strong association between free-grazing ducks in rice paddies, and incidence of HPAI H5N1 in Thailand (Gilbert *et al*., 2006). Therefore, an important link in the ecology of HPAI viruses, viz. intensively and widespread farmed domestic ducks is absent in South Africa.

v. Lack of live bird market systems and integrated farming practises

Unlike the developing countries in Southeast Asia and the rest of Africa, live bird markets are limited in South Africa and most poultry is kept as backyard flocks. Integrated farming is not practised at the scale as in Asian countries. The cull-buyer industry, that basically trades spent hens of the backs of open trucks into townships and rural areas could be one of the factors that limit the number of large live bird markets in SA.

vi. Climate

Generally, South Africa has far less surface water than countries in the northern hemisphere, and therefore wild waterfowl congregate at smaller, fewer water bodies distributed across the country. It was recently demonstrated that in chicken manure (with a pH of 8.23 and 17.7% moisture), HPAI H5N1 lost infectivity within 24 hours at 25ºC and within 15 minutes at 40ºC (Chumpolbanchorn *et al.,* 2006). A virus in waterfowl faeces will probably not remain viable for very long on a hot and dry Karoo day, where surface temperatures can reach the upper-thirties in summer, but this could also explain why AIV outbreaks occur mainly in the wintertime in ostriches, as cool wet winter weather allows viruses to survive for longer in the environment, and immuno-suppressed, hungry ostriches are more susceptible to microbial infections. In South Africa (and Australia), the highest UV radiation values in the world are attributed to a thinner ozone layer and less cloud coverage. A combination of all these factors would possibly decrease the viral load in the environment, and further reduce the availability of an infectious load that is required to infect ostriches.

However, despite the supposedly rare introduction of a notifiable strain with pathogenic potential into the ostriches, the 2004 HPAI H5N2 outbreak in ostriches had devastating economic consequences. A total of 7232 ostriches or 32.2% of the population on the five infected farms died between June and mid August 2004, and 26 000 Eastern Cape Ostriches were culled to curb the spread of the infection. This, coupled with ban on the export of ostrich meat from South Africa that lasted thirteen months, caused a financial loss of R600 million (~$86 mil) and 4000 jobs losses within the industry (Anton Kruger, South African Ostrich Business Chamber).

It is accepted that migratory birds played a role in moving HPAI H5N1 from eastern Asia into Europe, and this has emphasised the fact that no country on the migration paths of wild waterfowl can consider themselves to be free of the disease or free from the risk of introduction. But what about Newcastle disease in South Africa? Is it really enzootic? According to the definition on page 1, an enzootic disease should be *peculiar to* or *constantly present* in a locality. Since the 1970s, the government, poultry industry, and international community have believed that ND is enzootic in South Africa, based on the perception that it is more or less constantly present in the region (disappearing on average every four years to some unknown reservoir before sporadically reappearing). However, if ND was *truly* enzootic then surely a unique South African strain or lineage should exist that has persisted here for decades?

In Chapters Four, Five and Six I explored the molecular epidemiology of avian paramyxoviruses isolated in South Africa since the early 1990s. I demonstrated that distinct genetic lineages of virulent ND viruses were periodically introduced into South Africa, completely replacing their predecessors in each case, and that they were always prevalent in other geographic regions first. In two cases during the current outbreak of goose paramyxovirus (lineage 5), and genotype VIII of the 1970s and 1980s, the Far East was the recent and direct source of infection. However, in the case of the panzootic strain of the 1990s (VIIb) and pigeon paramyxoviruses (on at least two recent occasions), the infections appear to have been European in origin (UK, Spain and Portugal). Therefore, introduction of ND into South Africa appears to occur via a variety of mechanisms. These could be:

i. Illegal importation of exotic birds and poultry products

All birds entering South Africa legally are quarantined and tested at state laboratories and must be accompanied by a veterinary health certificate from the country of origin, proving freedom of infection from NDV (and AIV). Unfortunately, anecdotal evidence suggests that exotic birds such as parrots and racing pigeons illegally enter South Africa from time to time. Cock-fighting, particularly with birds brought from East Asia, or belonging to ship crew, is another illegal activity that poses a risk for the introduction of avian diseases. A significant source of infection could be via the approval of importation of frozen chickens from infected countries by corrupt officials. A recent example involved the importation of frozen chickens from China (personal communication, Kevin Lovell, South African Poultry Association)

ii. Contaminated harbour swill

By law, swill off international ships must be destroyed, but cases of illegal swill dumping are still reported in South Africa. Humans, wild birds and animals feeding off or handling contaminated ship galley waste are potential vectors for virulent strains, particularly from the Far East in the case of NDV. However, ND outbreaks in South Africa have either started in Durban or Hartebeesport (Gauteng province), but never from other major harbours such as Richards Bay, Port Elizabeth or Cape Town. Infected swill has definitely played a role in the introduction of infectious diseases into South Africa before. In 2000, Foot and Mouth disease was introduced into KZN with contaminated swill (Brückner *et al*., 2002), and Classical Swine Fever was probably also introduced into the Western Cape in 2005 by illegal swill feeding of pigs (Truuske Gerdes, personal communication).

iii. Potential introduction by wild migratory birds

Paramyxoviruses isolated from wild birds are usually categorised within the highly divergent lineage 1 (Aldous *et al*., 2003). However, some birds have also been able to carry velogenic strains without showing clinical symptoms. Some examples include the isolation of five virulent NDVs with ICPIs ranging from 1.68 to 1.72 from Semipalmated Sandpipers (*Calidris pusilla*) and Ruddy Turnstones (*Arenaria*

interpres) in Brazil from April to May 2004. The latter shorebird specie is a frequent Palaearctic visitor to South Africa (Appendix 3). In another case, a lineage 5b (VIIb) virus, Sterna/Astrakhan/Z275/2001, was isolated from a shorebird in the Volga River Delta (Usachev *et al*., 2006). At the time of writing the sequence was not available for comparison with South African strains. Therefore, like LPAI, virulent NDVs may be brought to South Africa periodically by migratory shorebirds.

KZN seems to be the hot spot for NDV outbreaks. The commercial poultry-producing region in KZN lies adjacent to the "Valley of 1000 Hills", a remote area with poor infrastructure that is well-populated by Zulu villages, most of which house backyard chicken flocks. Detection of ND in such regions relies on reporting. Although indigenous village chickens have been suggested to be the assymptomatic reservoirs of velogenic NDV, there is very little evidence to support this, especially since they are unvaccinated and highly susceptible to velogenic NDV strains. I suspect that there is no true asymptomatic reservoir for NDV in South Africa, but that outbreak strains have either (a) circulated unreported in Zulu chickens and somehow avoided sampling in serological surveys (village chickens are essentially wild and almost impossible to catch, and there are thousands of villages in rural KZN), and/or (b) been present in low levels in vaccinated spent layers bought off cull-buyers or immune survivors of outbreaks (a small percentage of birds do survive outbreaks of velogenic NDV).

Whatever the initial source of infection is, once the disease is introduced into commercial poultry it does not remain localised for long. I was able to demonstrate by mapping of specific genetic variants, that outbreaks were sustained for years (for example throughout the 1990s in the case of genotype VIIb) via road transportation of infected poultry and/or their products. This is facilitated firstly by the large commercial producers themselves, who transport hatching eggs and day-old chicks between their operations, and secondly by the cull-buyers.

The spent-hen industry in South Africa is large and lucrative. Broilers (table chickens) do not play a large role in this industry, because their lot is eventually the abattoir. In contrast, commercial layers have a commercial lifespan of about 70 weeks, and broiler/breeders of about 65 weeks. After their peak productivity has passed commercial farmers sell them to cull buyers (a.k.a spent hen vendors). In Europe and elsewhere, commercial chickens have

almost no resale value after their usefulness has expired, however, in South Africa, a bird that was initially bought for R25 (~$3.50) can be sold for R17 (~$2.40), allowing the farmer to recoup some of his expenses. Therefore, in South Africa a layer hen still has a value at the end of her production life, and this makes the layer market very competitive, as about 16 million layer hens are in production in South Africa at any given time.

Cull buyers pick up the culls in large batches of thousands of birds with trucks, and transport them to central depots. Some large commercial operations even have their own cull depots, and in this way, birds from different farms are brought together. From the depot, fowl (perhaps several hundred or less) are sold to smaller buyers, who then supply birds to informal markets, including townships and rural areas. Where the market is good, large cull buyers will often travel over long distances, for example, between Gauteng and the Western Cape provinces, or between Gauteng and KZN. Therefore, culls that originate in the Cape can very easily, and are commonly, sold in KZN. KZN seems to be a nett buyer of cull birds. This is apparently due to the demand for these birds by Indian and black buyers. Not only are they less expensive, at R17 per bird, but older chickens are coveted because they apparently make better curry. The cull buyer industry is legal, although largely unregulated and uncontrolled. Most large commercial farms conduct exit bleeds to ascertain the disease status in the birds, but this is mostly surveillance for AI and not NDV. Furthermore, these tests are performed by contracted laboratories and veterinary services and not state facilities. The South African Poultry Association (SAPA) recognised the role that cull buyers play in the dissemination of NDV in South Africa some time ago, and attempted to control and regulate the industry, without success. The main problem is that the South African poultry industry allows the cull buyer industry to exist, because it's lucrative (S Bisschop, personal communication).

Molecular epidemiology applied for the first time in South Africa to assess the status of ND has shown that NDV in South Africa should be considered to be *exotic* and not *enzootic*, and this finding may have important implications for the industry. It is clear that if better control is enforced, particularly in the regulation of the cull buyer industry, South Africa may be able to regain its NDV-free status. This should be done at government-level, possibly by restricting the movements of poultry to those that have been tested and issued with a movement permit by the Directorate of Animal Health. Furthermore, better control

can be enforced during periods of outbreak by restricting the movement of poultry between provinces or infected areas. It is ironic that the formal poultry industry, that is most heavily impacted by ND outbreaks and the resultant trade restrictions, is the main contributor to the problem by moving infected birds and their products across the country and by selling off vaccinated spent hens that perpetuate and spread the infection in rural areas.

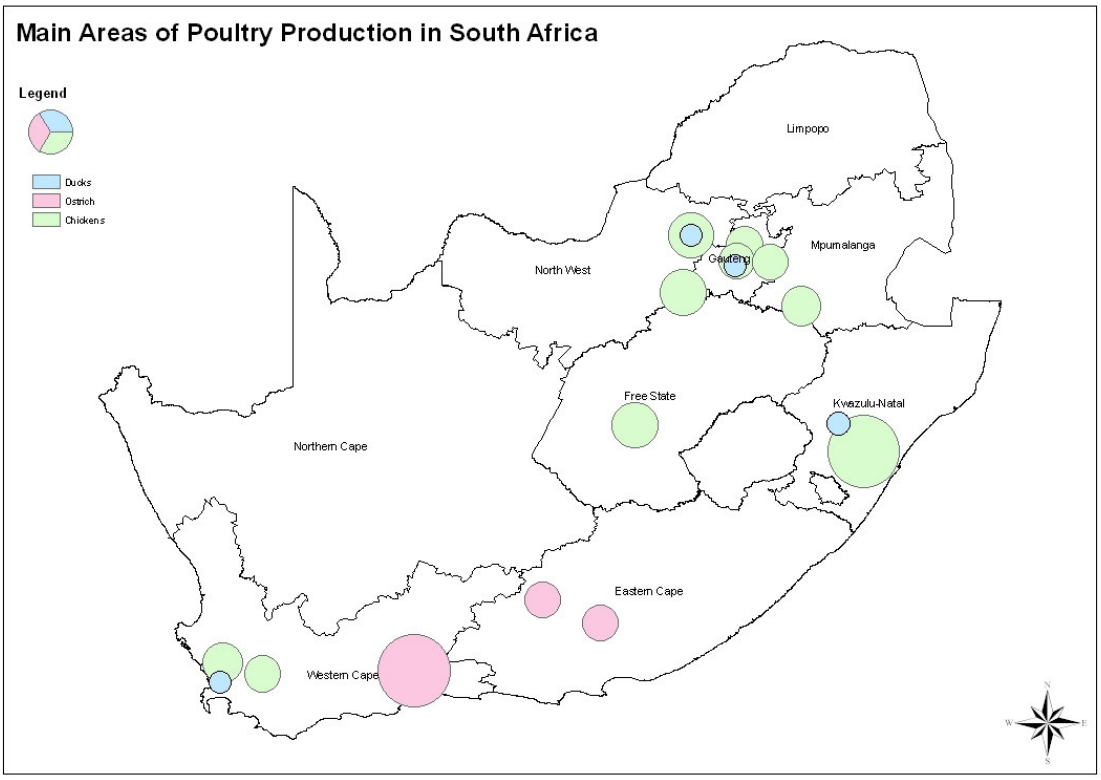
Current, ongoing and future research

The genetic link between South African and Eurasian AI and ND viruses raises many questions. One of our immediate needs is to conclusively prove that pelagic shorebirds are indeed the vectors of these viruses to South Africa, to determine which species or populations are involved, and exactly which routes they follow. As a spin-off of this work a national surveillance project funded by the DoA was initiated at OVI to monitor the presence of AIVs and APMV-1 in wild waterfowl. In this program, faecal samples from migrants are tested as birds arrive in the spring and throughout summer, and in winter, the indigenous wild ducks and geese are sampled as indicators of which viruses were recently introduced and which are still circulating. It is hoped that by monitoring our wild bird species that they will act as an early warning system for the entry of HPAI H5N1 and other notifiable AIVs into South Africa. Data of the prevalence and distributions of AIVs will also be used in the generation of prediction and risk models in collaboration with other institutes. It is also unknown whether the shorebird and wild duck reservoirs shed constantly or intermittently, and in the latter case which factors trigger shedding. Physiological stressors may be involved. For example, migration restlessness (triggered by changes in photoperiodism) was experimentally linked to the shedding in migratory birds of a spirochete that causes Lyme disease (Gylfe *et al*., 2000). This information will be critical for the design of effective future surveillance programs. Since the incursion of HPAI H5N1 into Europe, it has become vital to monitor the Russian and northern European waterfowl populations of the wetlands that South African shorebirds are likely to use as stopover sites during their southbound migrations. The data presented here predicts that once HPAI H5N1 becomes established in those shorebird populations, that it's likely to reach South Africa within a year or two. Of course, the risk of introduction of HPAI H5N1 via illegal poultry or exotic bird importation from one of the African or other states is also a cause for concern.

Characterisation of the 2006 HPAI H5N2 Western Cape ostrich outbreak virus is underway, and preliminary results indicate that the virus is not directly linked to the 2004 Eastern Cape outbreak strain, since the 2006 strain was produced by multiple reassortments with wild duck viruses, and furthermore contains three amino acids less at H_0. A probable LPAI H5N2 precursor was isolated from a Western Cape ostrich farm. Many unanswered questions surround the interaction between AI viruses and the ostrich host, and this is a research area in which South Africa is well-positioned to take the lead. For example, what is the biological link between green urine and AIV infection (if any)? Why are ostriches less susceptible to HPAI strains? And most importantly, what biological factors are involved in the conversion of LPAI to HPAI in ostriches, again highlighted by recent events in the Western Cape. A reverse genetics system recently developed at OVI will assist us in answering this question, and others related to the molecular determinants of virulence of AIV, for example what contribution does the NS1 deletion in the 2004 HPAI H5N2 virus make to the pathogenicity of the virus for ostriches? Reverse genetics technology will furthermore enable us to genetically alter local strains for enhanced growth and reduced pathogenicity for use as inactivated vaccines.

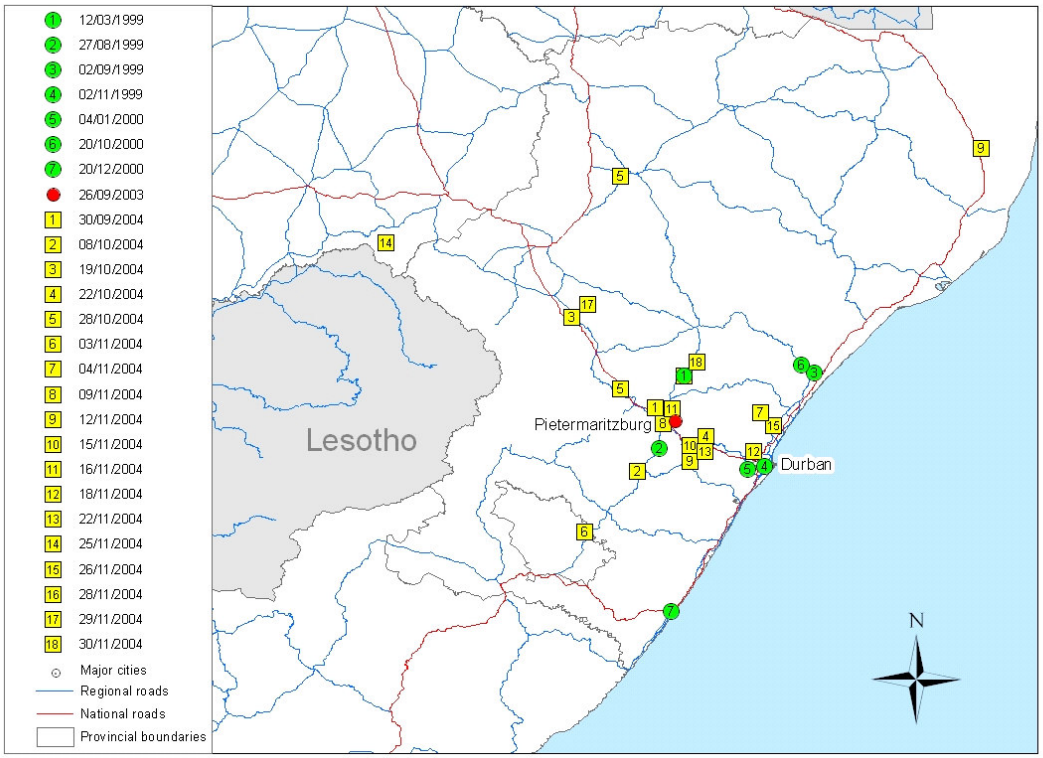
It is likely that HPAI H5N1 will continue to spread and cause periodic outbreaks, similar to H9N2 in the 1990s, and it is evident that when uncontrolled, ND is able to spread rapidly within months throughout the whole of South Africa through the movement of infected poultry. Using NDV as a model for the spread of HPAI H5N1 in chickens is clear that we could face a humanitarian disaster of epic proportions if biosecurity were to break down. The largest densities of commercial poultry producers are clustered around heavily-populated areas in Gauteng and KZN, and both provinces are home to high incidences of HIV-positive immune-compromised individuals. With a mortalility rate of slightly above 50% in relatively healthy people, the effect of HPAI on an immune-compromised population could be disastrous. Only if biosecurity and control of avian diseases in South Africa is drastically improved in a partnership between government and private industry, can future disasters be averted.

APPENDIX 1

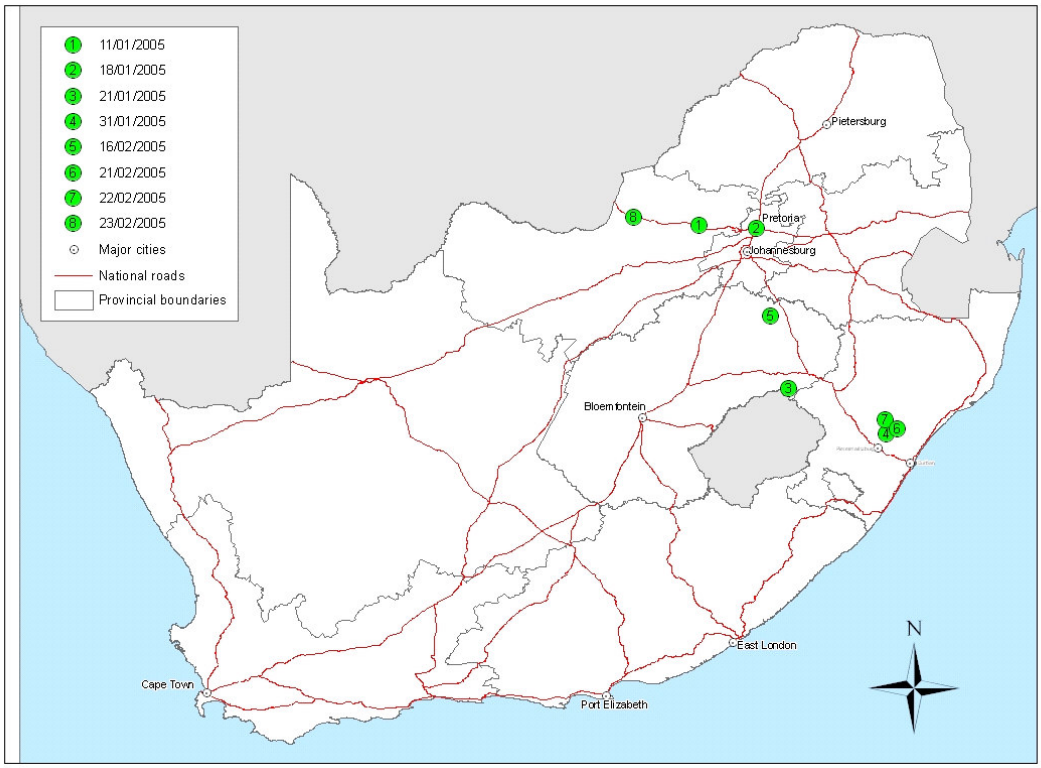


Main poultry producing areas in South Africa (c/o Dr Grietjie de Klerk, National Department of Agriculture)

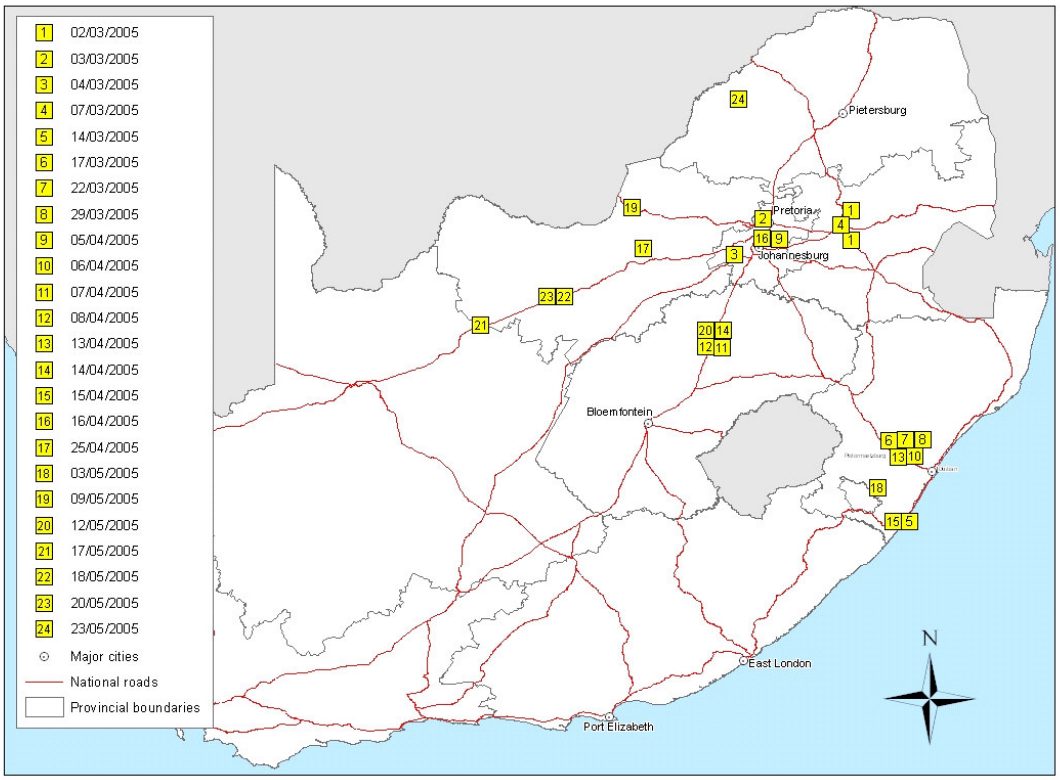
APPENDIX 2



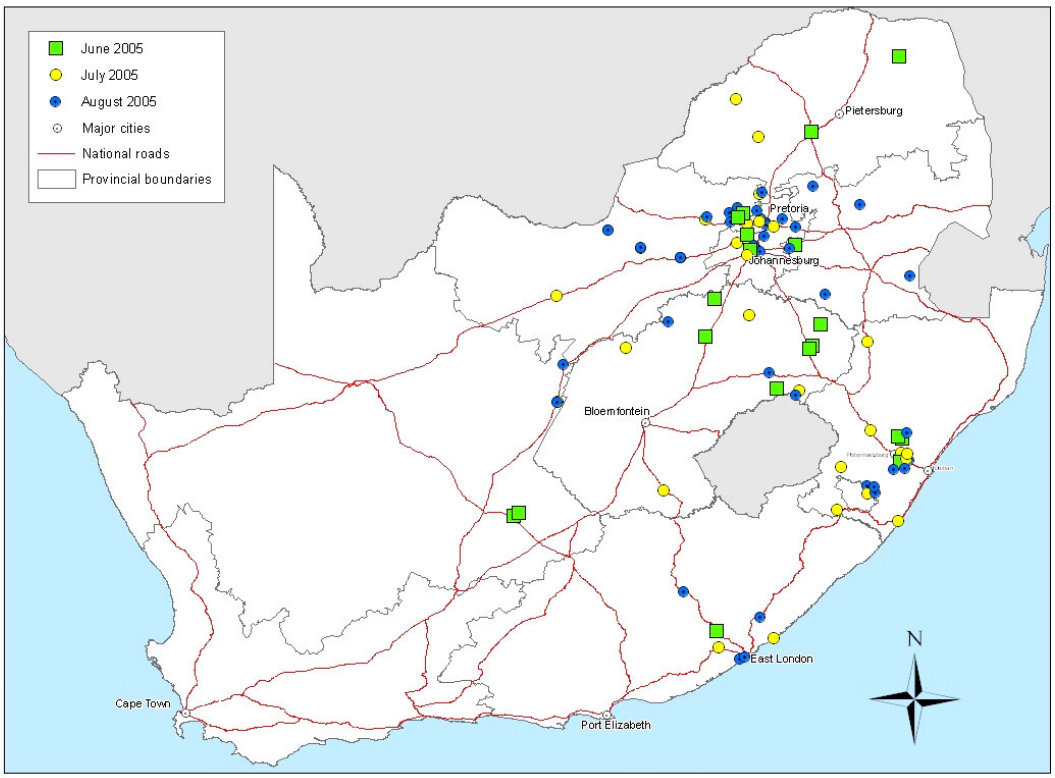
Appendix 2i Distribution of GPMV isolates from 1999-2000 (green circles), the single isolate in 2003 (red dot), and the 2004 cases (yellow squares) from September, October and November 2004



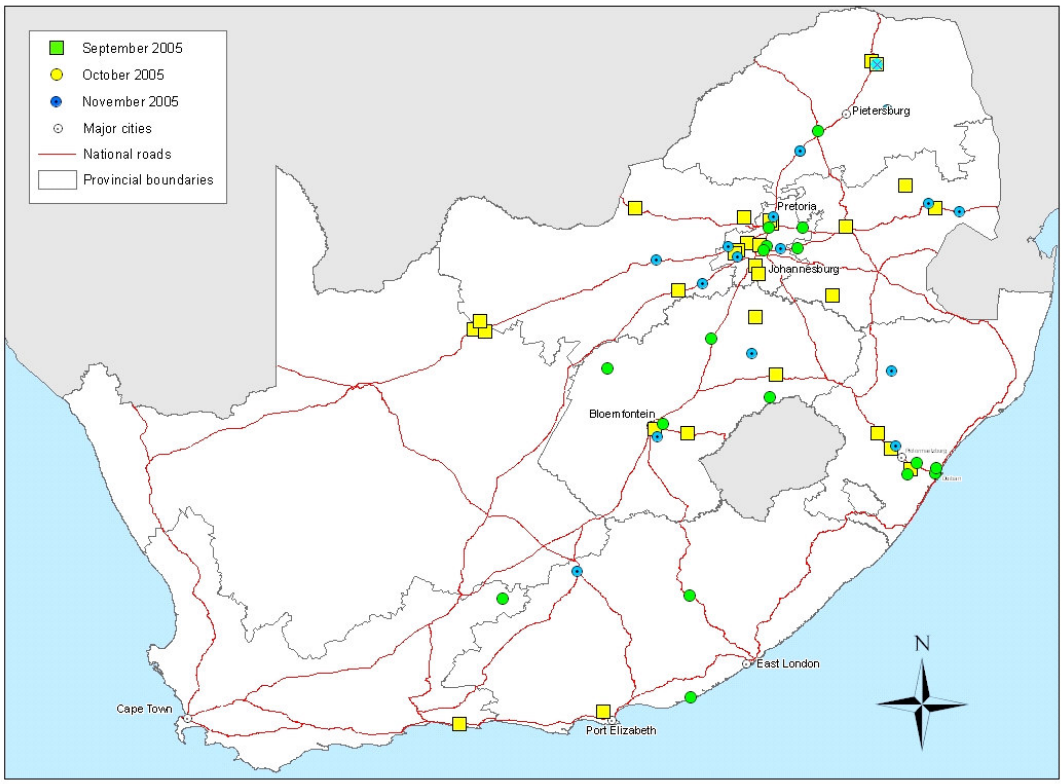
Appendix 2ii Distribution of GPMV isolates from January and February 2005



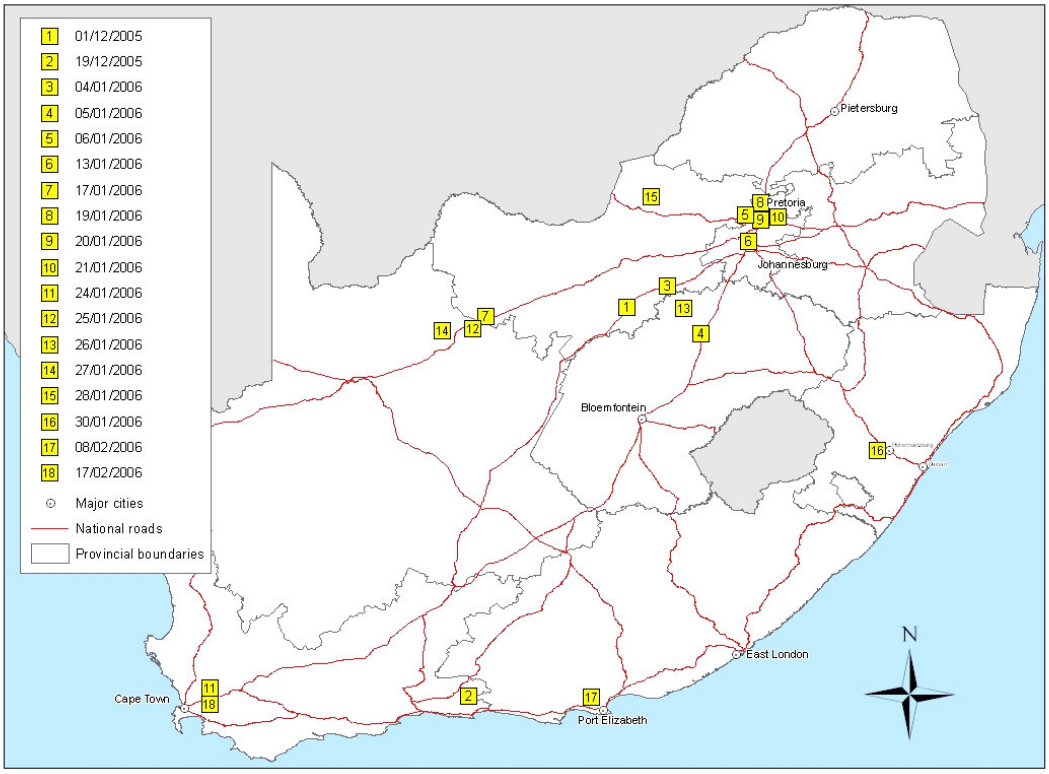
Appendix 2iii Distribution of GPMV isolates from March, April and May 2005



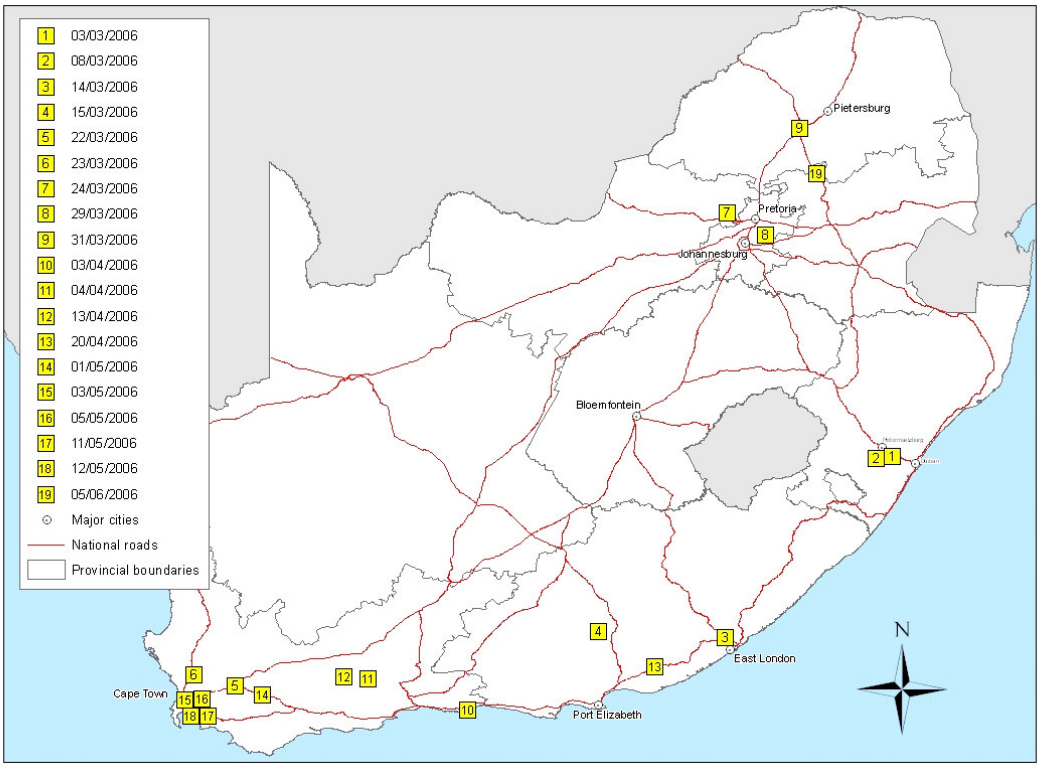
Appendix 2iv Distribution of GPMV isolates from June, July and August 2005



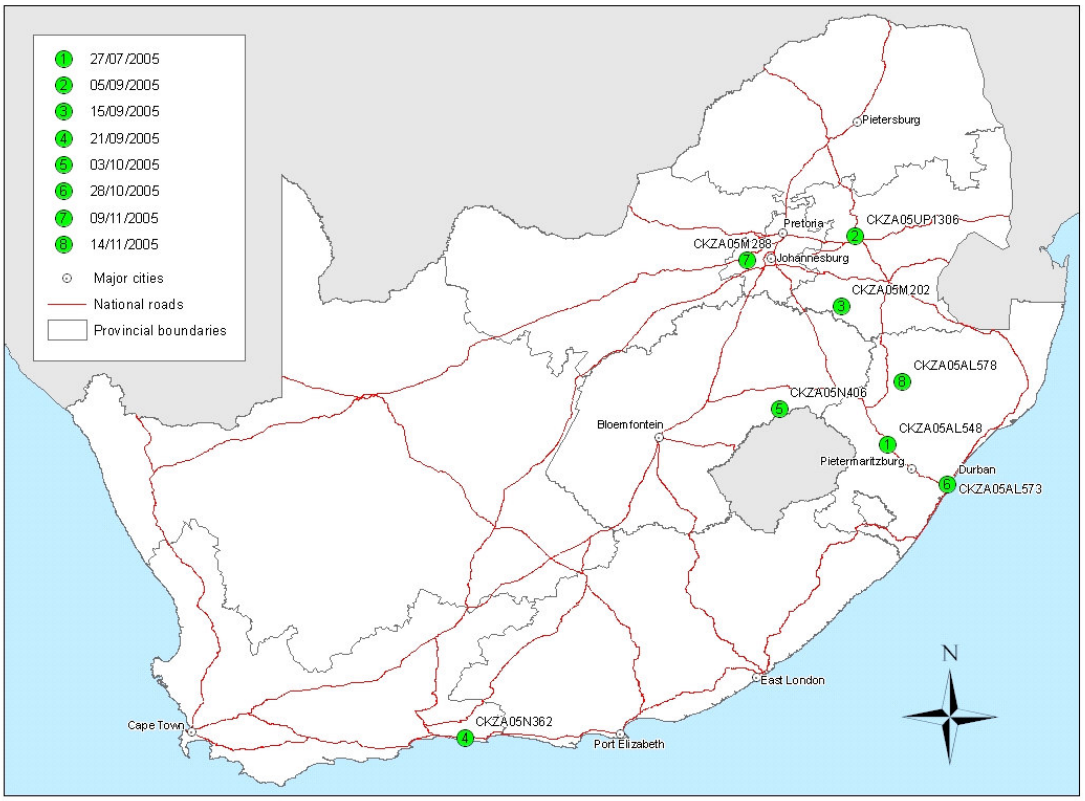
Appendix 2v Distribution of GPMV isolates from September, October and November 2005



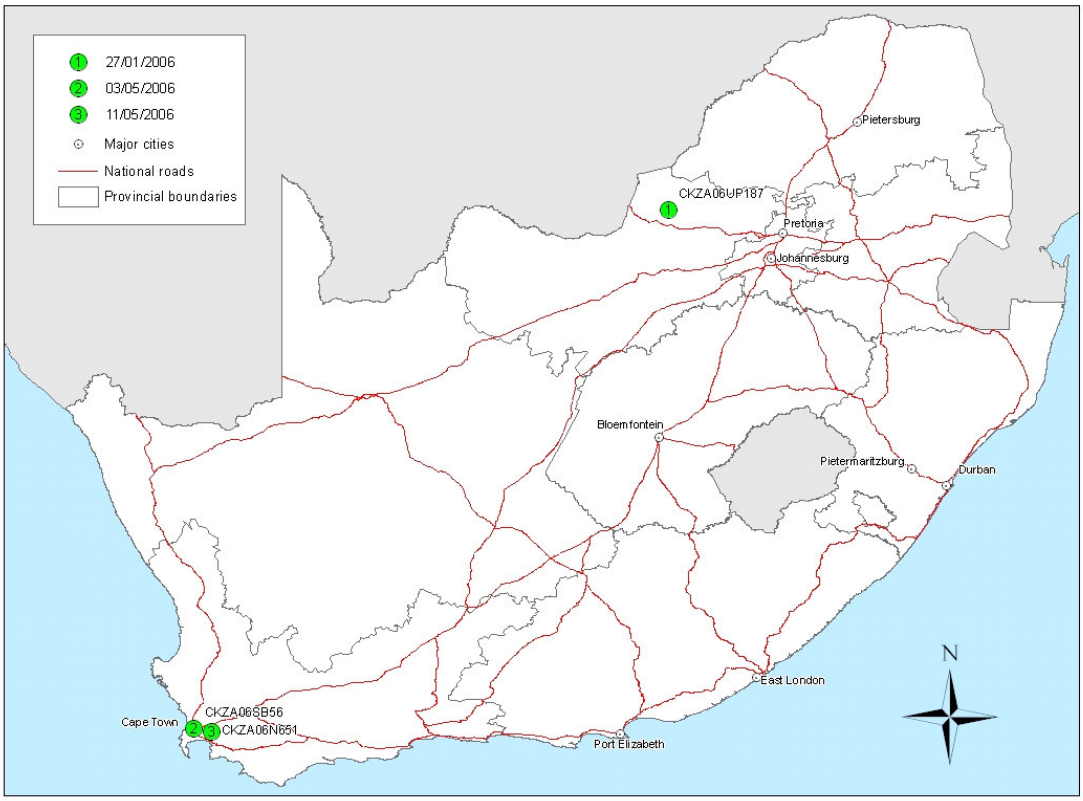
Appendix 2vi Distribution of GPMV isolates from December 2005, January and February 2006.



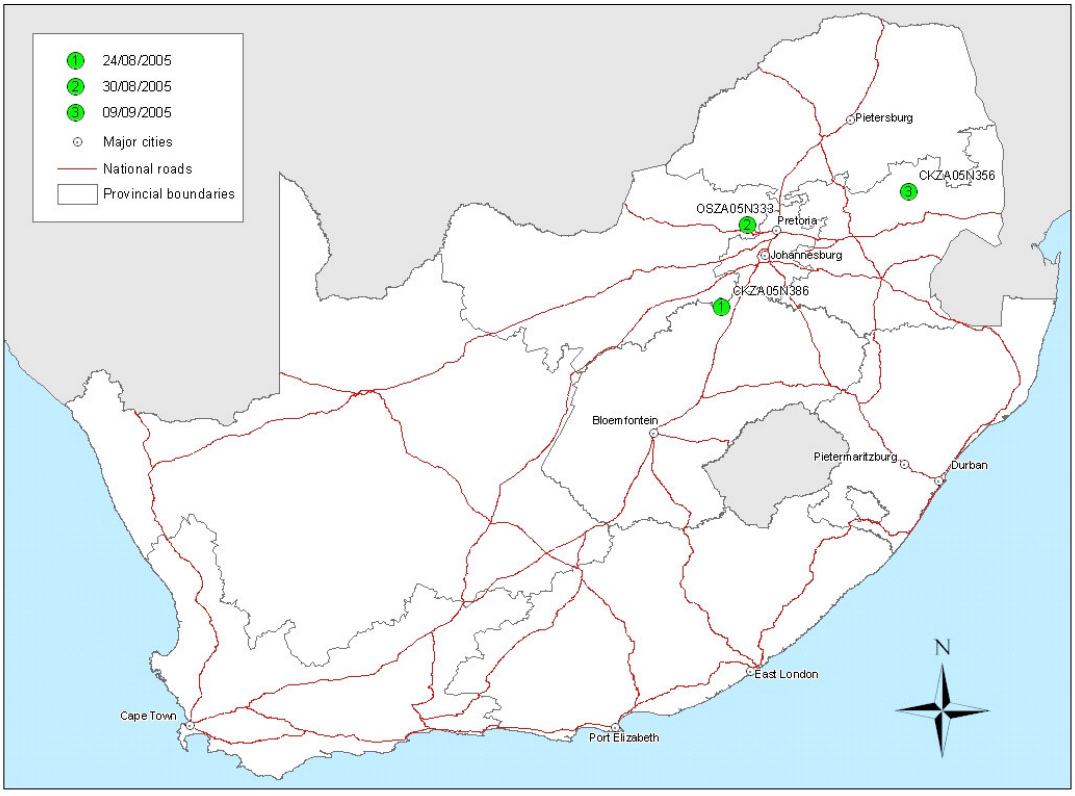
Appendix 2vii Distribution of GPMV isolates from March, April, May and June 2006



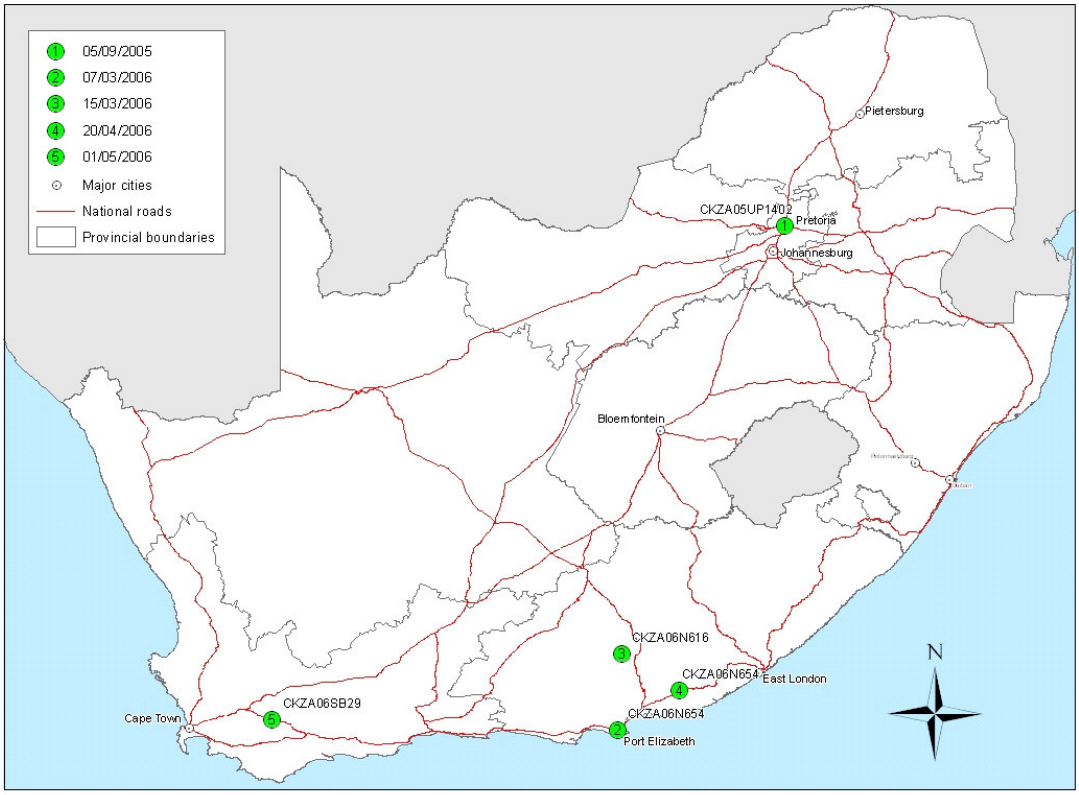
Appendix 2viii Distribution of GPMV sub-lineage (a)



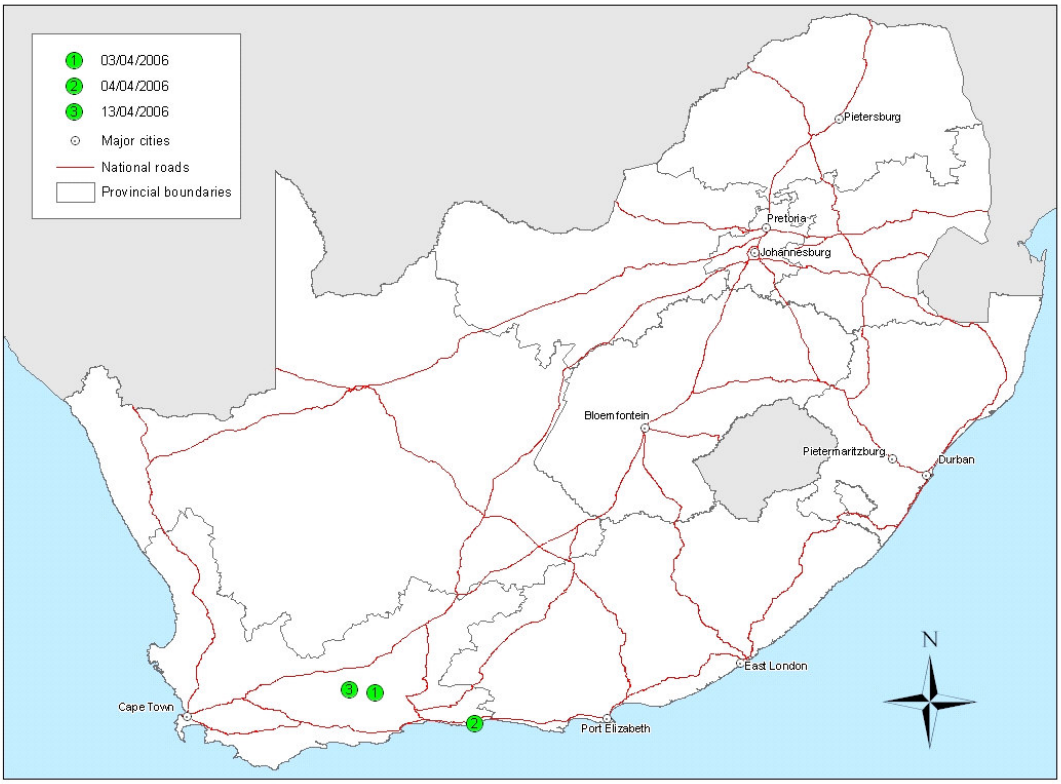
Appendix 2ix Distribution of GPMV sub-lineage (b)



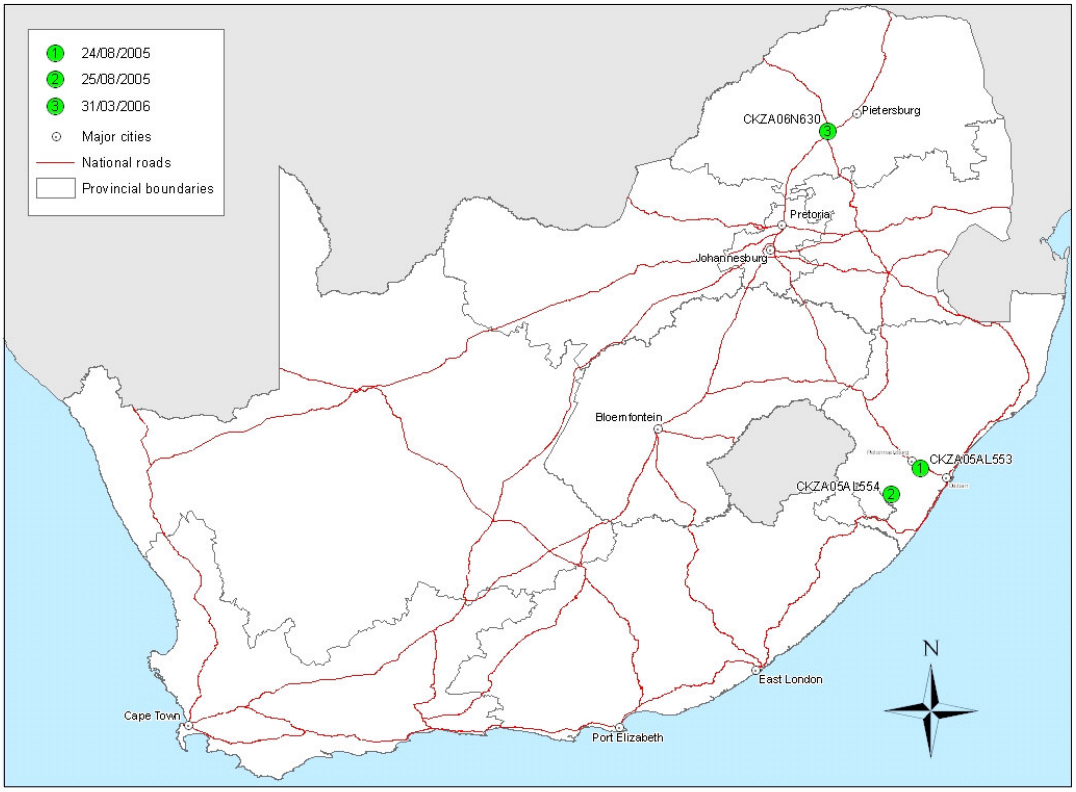
Appendix 2x Distribution of GPMV sub-lineage (d)



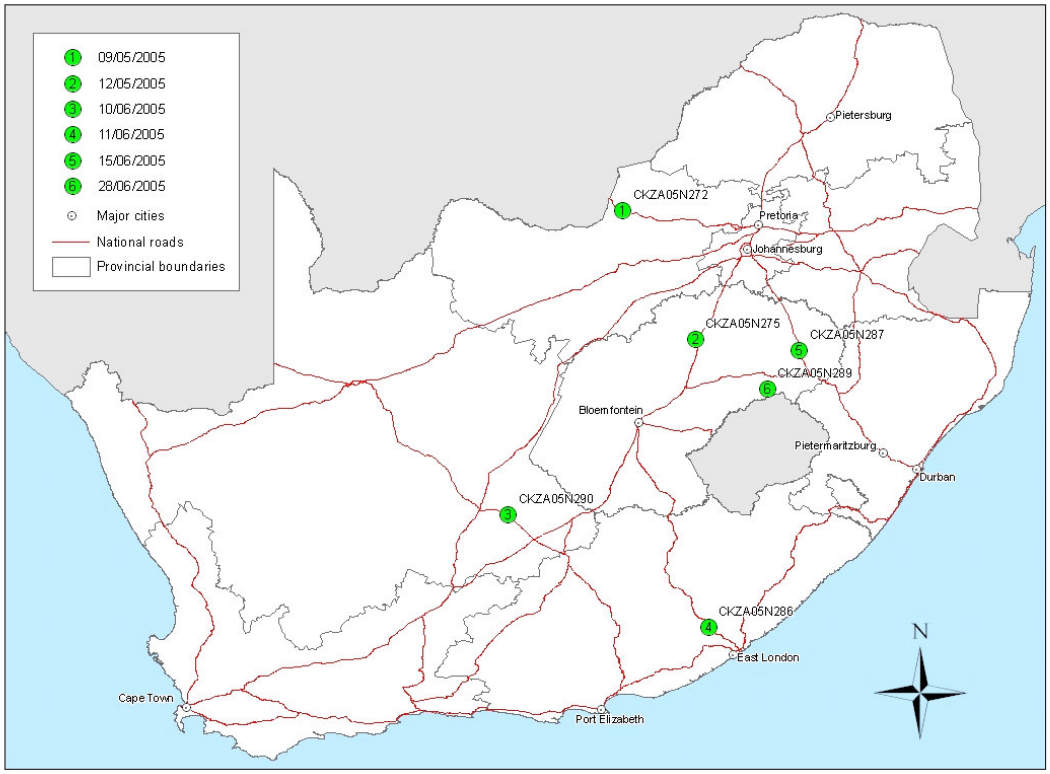
Appendix 2xi Distribution of GPMV sub-lineage (e)



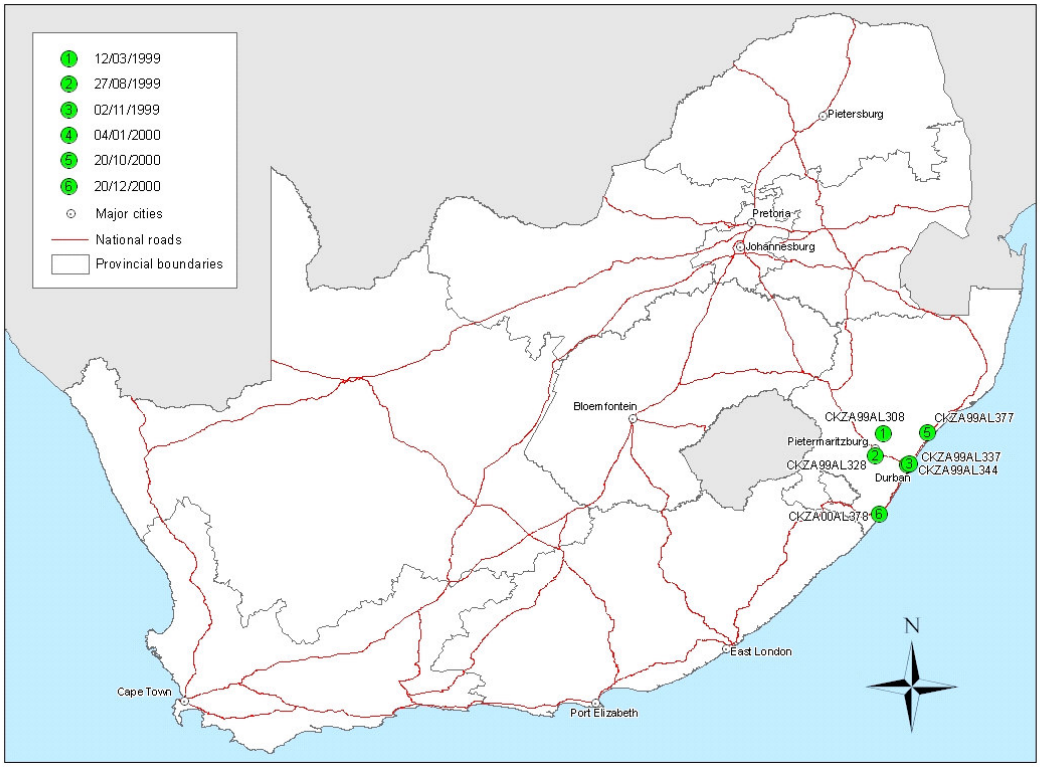
Appendix 2xii Distribution of GPMV sub-lineage (f)



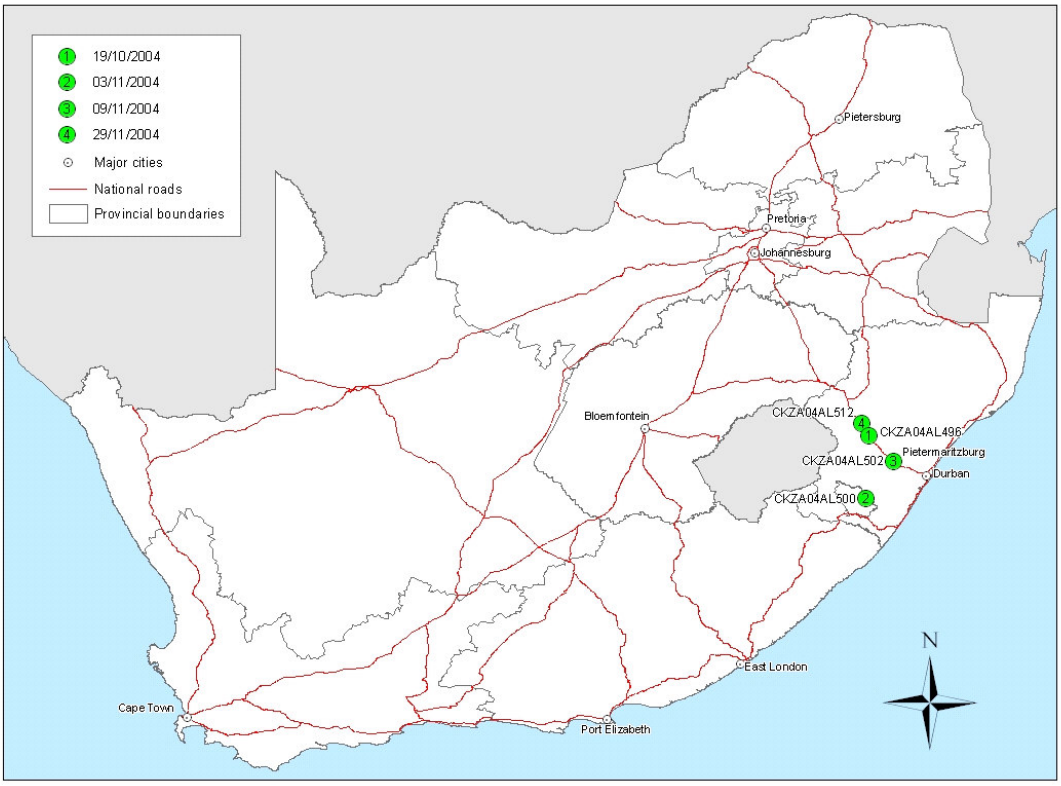
Appendix 2xiii Distribution of GPMV sub-lineage (g)



Appendix 2xiv Distribution of GPMV sub-lineage (m)



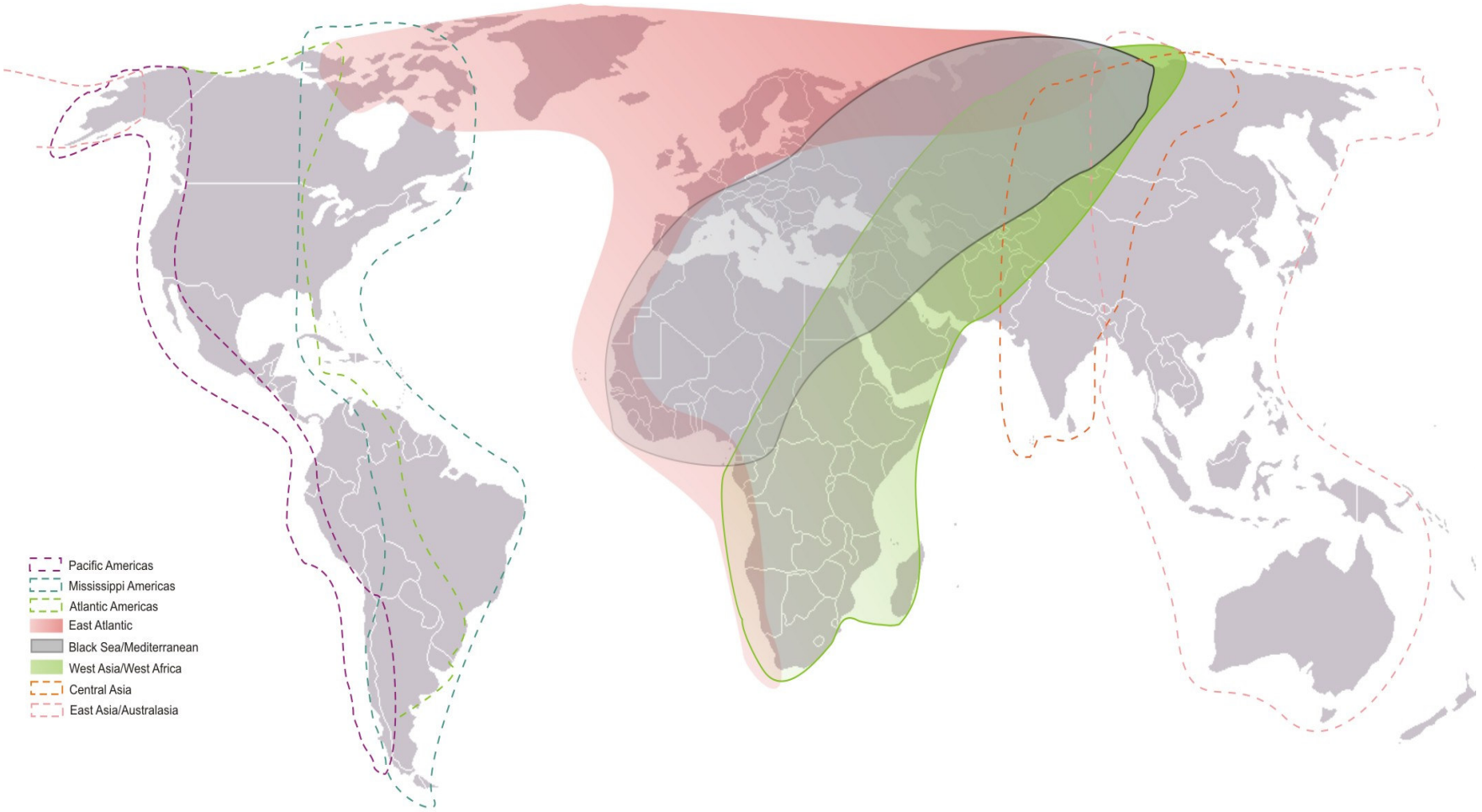
Appendix 2xv Distribution of sub-lineage (n), the 1999/2000 outbreak strains



Appendix 2xvi Distribution of GPMV sub-lineage (o) (sub-group II) strains of 2004

APPENDIX 3

Migratory flyways



Adapted from www.fao.org/.../EMPRES_ Watch_global_flyways.gif

APPENDIX 4

List of Palaearctic migrant waterfowl that over winter in Southern Africa

(Underhill *et al*., 1999)

Order Ciconiiformes

Black-crowned night heron *Nycticorax nycticorax*

European White stork *Ciconia ciconia*

Black stork *Ciconia nigra*

Order Charadriiformes Sub-order Charadrii

Grey plover *Pluvialis squatarola*

Common Ringed Plover *Charadrius hiaticula*

Terek Sandpiper *Xenus cinereus*

Common Sandpiper *Acitis hypoleucos*

Wood Sandpiper *Tringa glareola*

Common Greenshank *Tringa nebularia*

Red Knot *Calidris canutus*

Curlew Sandpiper *Calidris ferruginea*

Little Stint *Calidris minuta*

Sanderling *Calidris alba*

Ruff *Philomachus pugnax*

Bar-tailed Godwit *Limosa lapponica*

Whimbrel *Numenius phaeopus*

White-winged Tern *Chlidonias leucopterus*

Marsh sandpiper *Tringa stagnatilis*

Great Snipe *Gallinago media*

Order Charadriiformes sub-order Lari

Sandwich tern *Thalasseus sandvicensis*

Common tern *Sterna hirundo*

Arctic tern *Sterna paradisaea*

Black tern *Chlidonias niger*

White-winged tern *Chlidonian leucopterus*