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the source of the outbreak, I defined the phylogenetic relationships between various H6N2 isolates, and the previously unpublished gene sequences of an H6N8 virus isolated in 1998 from ostriches in the Leeu Gamka region (A/Ostrich/South Africa/KK98/98).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	CTGGGGTGTGCACCATC CTCC	C Abolnik
H6	H6-661f (forward)	RT-PCR, sequencing	AGCATGAATTTTGCCAA GAG	Lee *et al.* (2001)
	H6-962r (reverse)	RT-PCR, sequencing	GGRCATTCTCCTATCCAC AG	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	TATTGGTCTCAGGGAGC AAAAGCAGGAGT	Hoffmann *et al.* (2001)
	Ba-NA-1413R (reverse)	RT-PCR, sequencing	ATATGGTCTCGTATTAGT AGAAACAAGGAGTTTTT T	Hoffmann *et al.* (2001)
PB2	Ba-PB2-1 (forward)	RT-PCR, sequencing	TATTGGTCTCAGGGAGC GAAAGCAGGTC	Hoffmann *et al.* (2001)
	Ba-PB2-2341R (reverse)	RT-PCR, sequencing	ATATGGTCTCGTATTAGT AGAAACAAGGTCGTTT	Hoffmann *et al.* (2001)
PB1	Bm-PB1-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGC GAAAGCAGGCA	Hoffmann *et al.* (2001)
	Bm-PB1-2341R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGT AGAAACAAGGCATTT	Hoffmann *et al.* (2001)
PA	Bm-PA-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGC GAAAGCAGGTAC	Hoffmann *et al.* (2001)
	Bm-PA-2233R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGT AGAAACAAGGTACTT	Hoffmann *et al.* (2001)
NP	Bm-NP-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGC AAAAGCAGGGTA	Hoffmann *et al.* (2001)
	Bm-NP-1565R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGT AGAAACAAGGGTATTTT T	Hoffmann *et al.* (2001)
M1 M2	Bm-M-1 29 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGC AAAAGCAGGTAG	Hoffmann *et al.* (2001)
	Bm-M-1027R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGT AGAAACAAGGTAGTTTT T	Hoffmann *et al.* (2001)
NS1 NS2	Bm-NS-1 (forward)	RT-PCR, sequencing	TATTCGTCTCAGGGAGC AAAAGCAGGGTG	Hoffmann *et al.* (2001)
	Bm-NS-890R (reverse)	RT-PCR, sequencing	ATATCGTCTCGTATTAGT AGAAACAAGGGTGTTTT	Hoffmann *et al.* (2001)
NA	AINAFSEQ (forward)	sequencing	GGGAGCAAAAGCAGGA GT	C Abolnik
PA	PA1150REV (forward)	RT-PCR, sequencing	GGCACCAGAGAAAGTAG	C Abolnik
PB2	PB21411FOR (forward)	RT-PCR, sequencing	CCTTATAAYGGRCTGTA CTG	C Abolnik
PB1	PB11123FOR (forward)	RT-PCR, sequencing	GTTTATGGTCGTCTYTAG GAACG	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

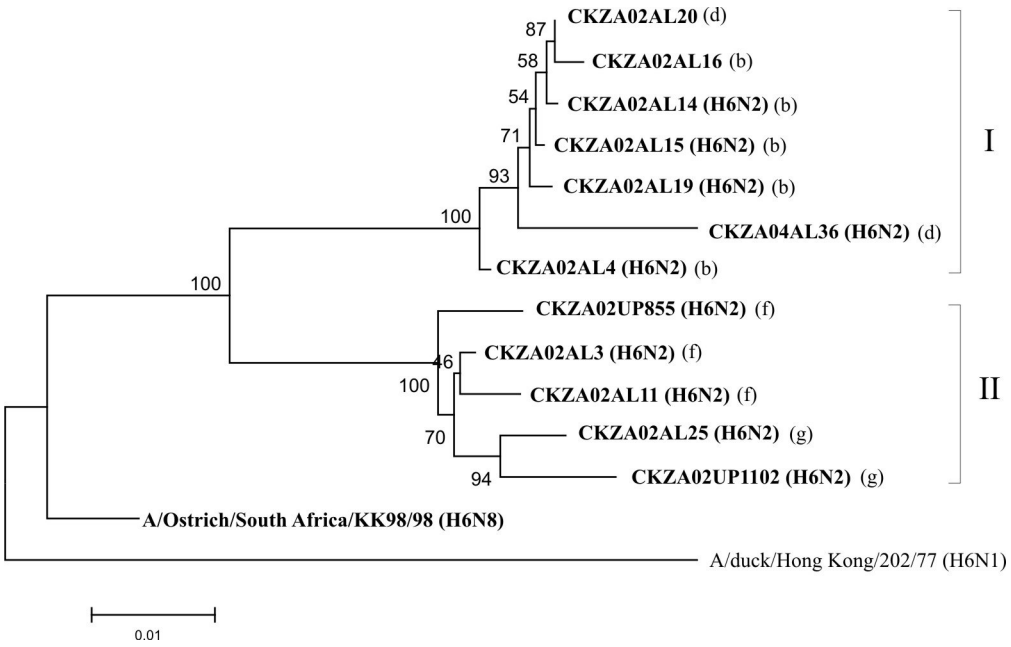


Figure 2.1(a) Dendogram of H6 type hemagglutining gene sequences (1315 nt). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages corresponding to Fig 2.1(c) are indicated (I and II; (a) to (g)).

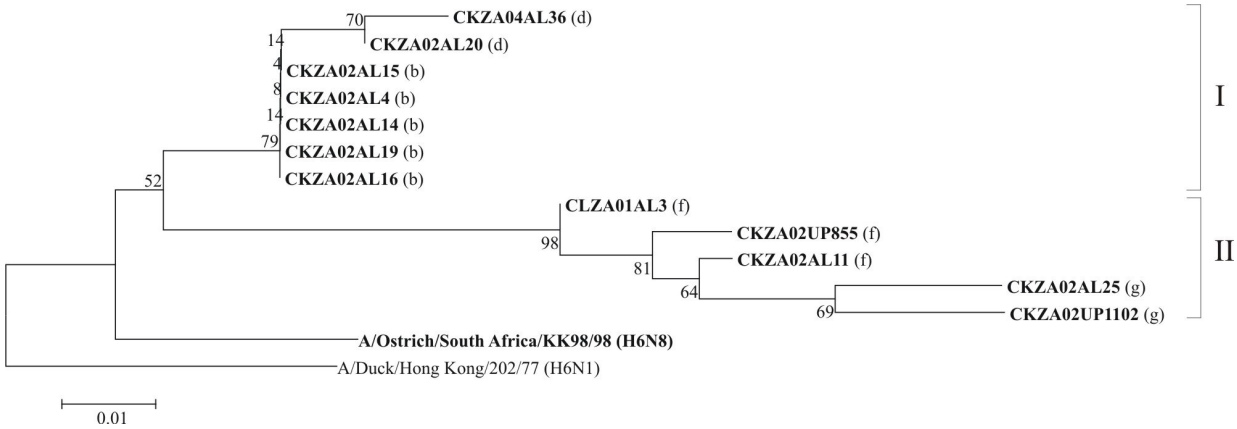


Figure 2.1(b) Dendogram of partial H6 type hemagglutining gene sequences, corresponding to the region between nucleotides 836 and 951 (116 nt) of the viruses presented in Fig 2.1(a). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages corresponding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

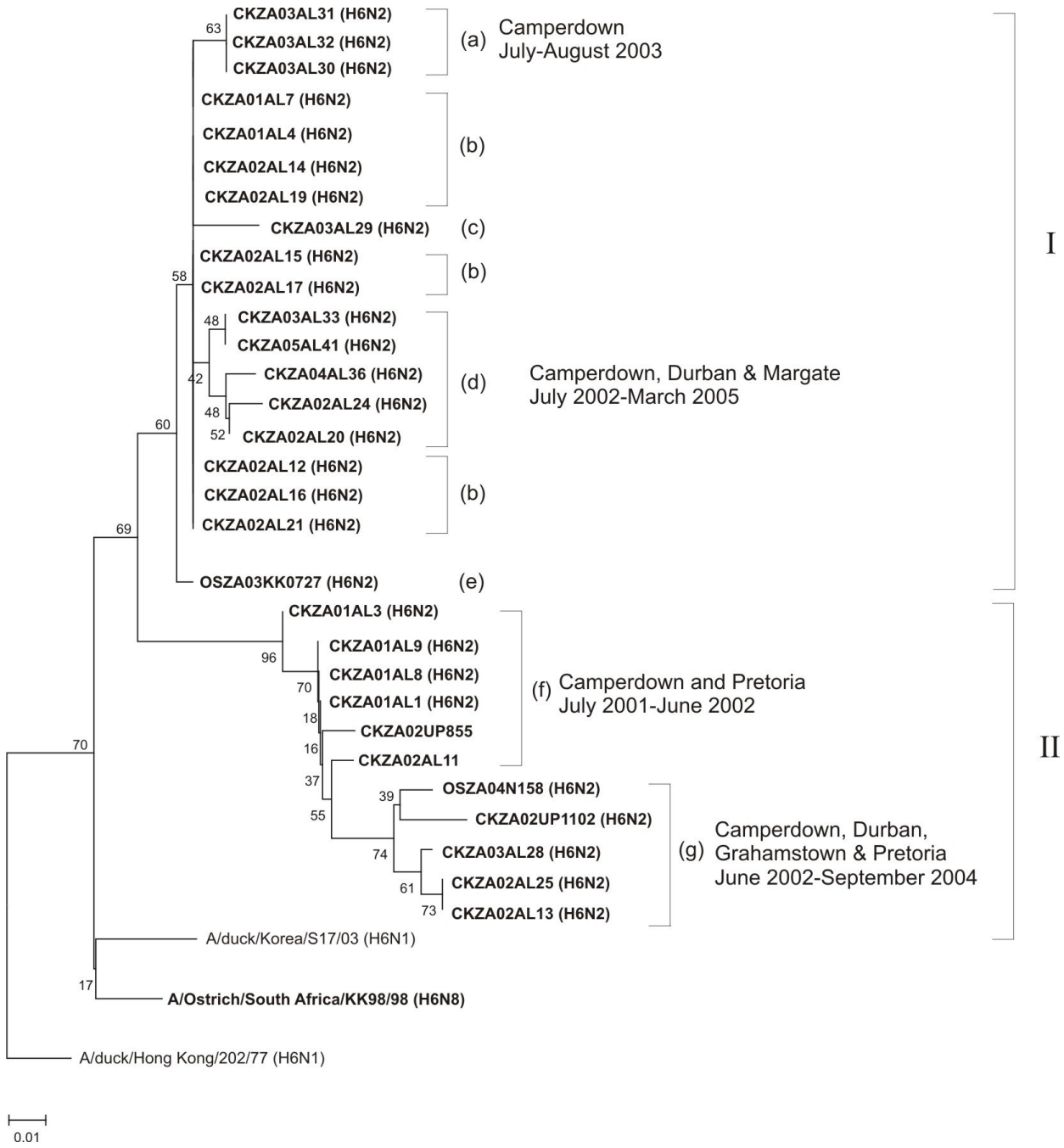


Figure 2.1(c) Dendogram of partial H6 type hemagglutining gene sequences, corresponding to the region between nucleotides 836 and 951 (116 nt). The tree is rooted with A/duck/Hong Kong/202/77. South African isolates are indicated in boldface, and sub-lineages are indicated (I and II; (a) to (g)).

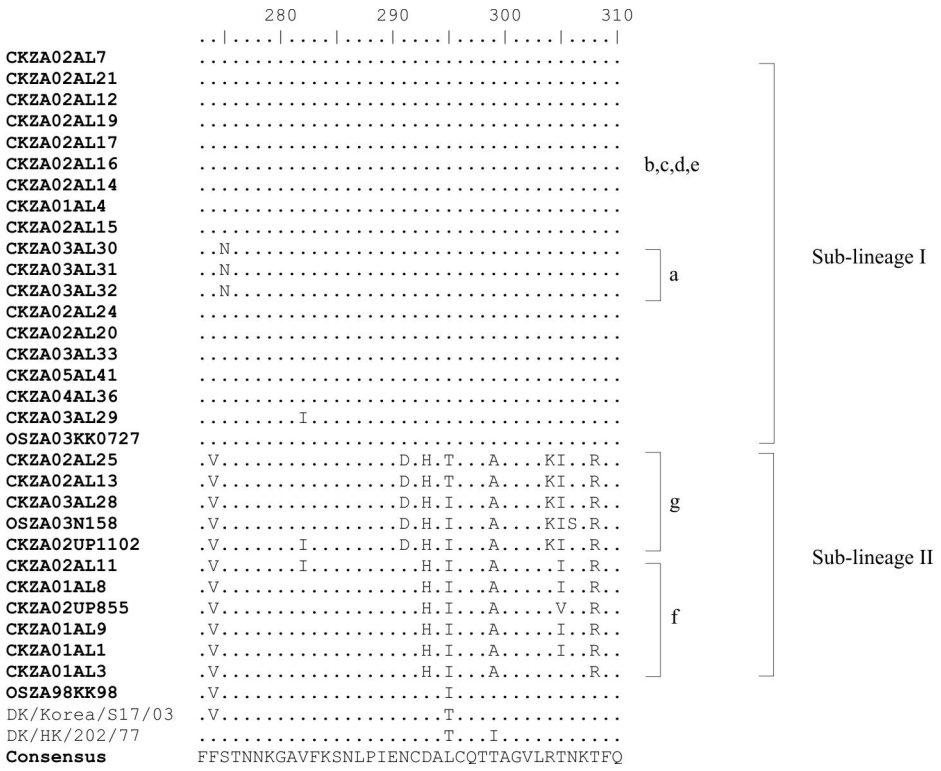


Figure 2.2 Multiple amino acid alignment of partial H6-type hemagglutinin genes (residues 273 to 310).

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:

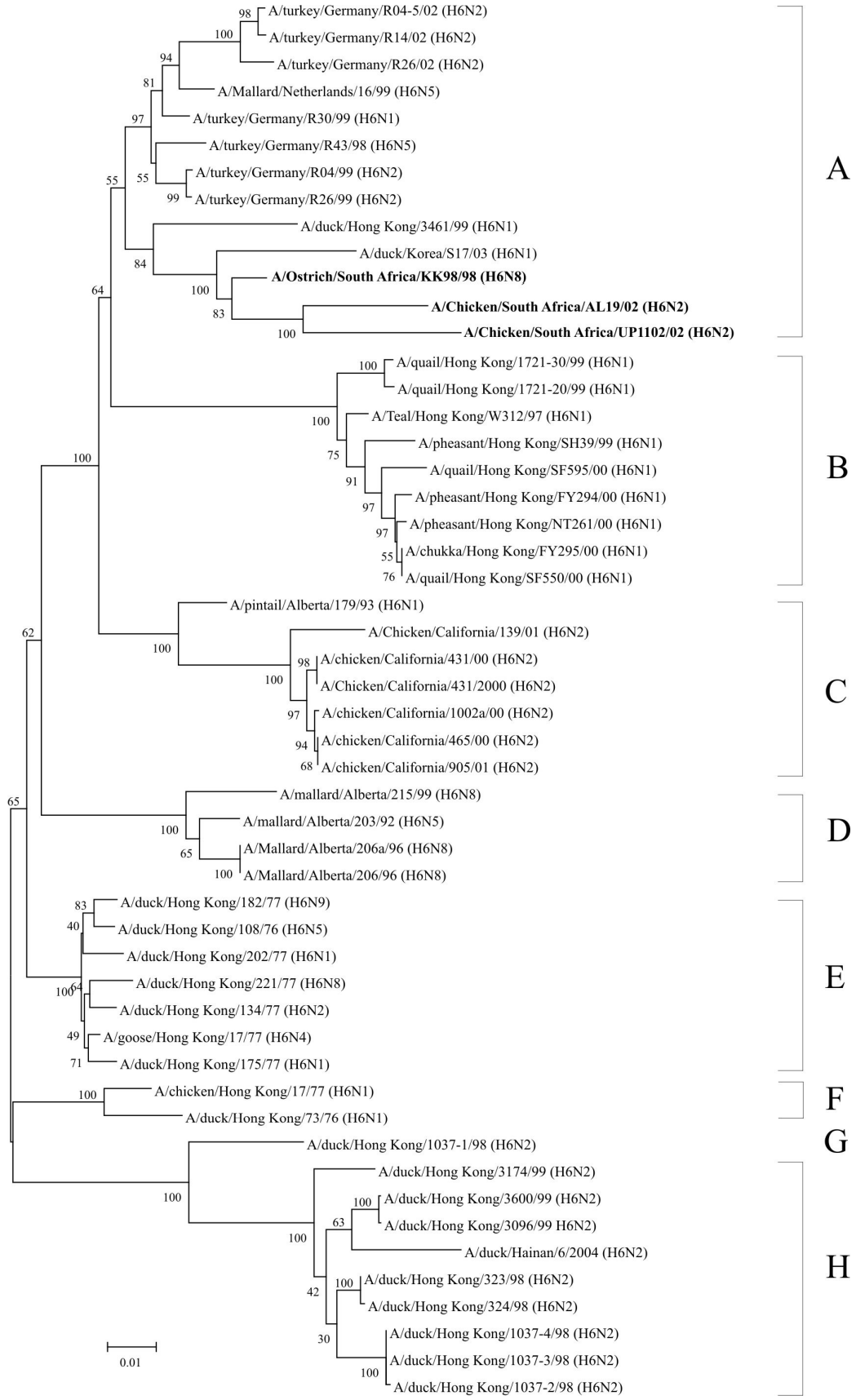


Figure 2.3 Phylogenetic tree inferred from a 1367-nt multiple sequence alignment of the hemagglutinin (H6) genes of South African (in boldface) and other viruses. Sub-lineages A to H are indicated.

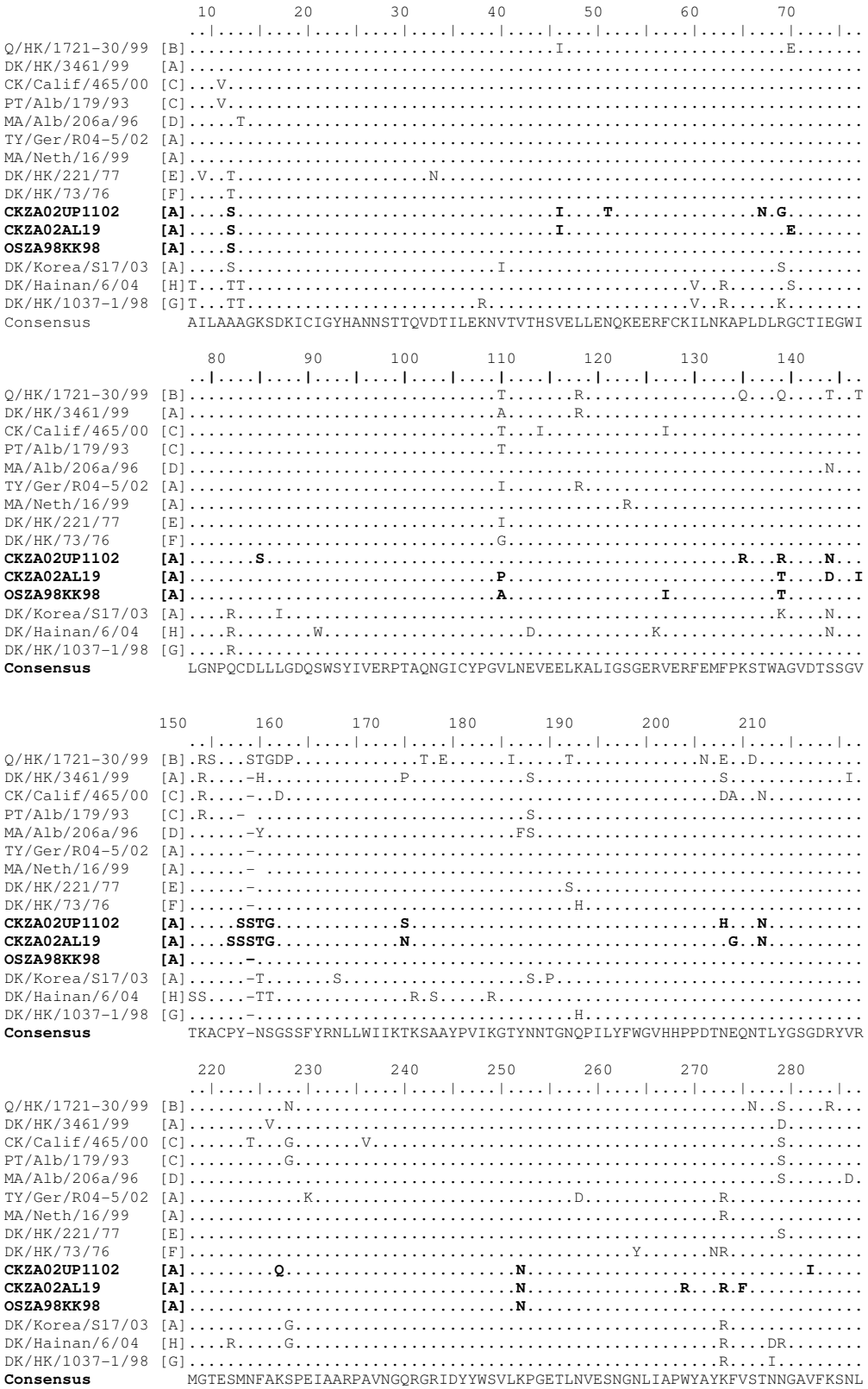
Fig. 2.3 indicates that A/Chicken/South Africa/AL19/2002 (H6N2) and A/Chicken/South Africa/UP1102/02 (H6N2) share a recent common ancestor for their H6-type hemagglutinin genes, and that this hypothetical gene appears to be derived from the H6N8 virus isolated in 1998, A/Ostrich/South Africa/KK98/98. 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

```
                    290       300       310       320       330       340       350
                    ..|....|....|....|....|....|....|....|....|....|....|....|....|..
Q/HK/1721-30/99 [B].....................................................................
DK/HK/3461/99   [A]..............................K......................................
CK/Calif/465/00 [C]..............................K.......G..............................
PT/Alb/179/93   [C].....................................G..............................
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].....N...............................................................
CKZA02UP1102    [A]...D.H.I...A....KI..R.................K.....................................
CKZA02AL19      [A].......L...T.........................K.............P.....................
OSZA98KK98      [A].......I...T.........................................P.....................
DK/Korea/S17/03 [A]...........T.........................................P.....................
DK/Hainan/6/04  [H].....................................................I...................
DK/HK/1037-1/98 [G].F...N...........I....................................................
Consensus          PIENCDATCQTIAGVLRTNKTFQNVSPLWIGECPKYVKSESLRLATGLRNVPQIETRGLFGAIAGFIEGG

                    360       370       380       390       400       410       420
                    ..|....|....|....|....|....|....|....|....|....|....|....|....|..
Q/HK/1721-30/99 [B].....................................VN.........N...................
DK/HK/3461/99   [A].....................................................................
CK/Calif/465/00 [C]...L.................................................................M...
PT/Alb/179/93   [C].....................................................................S.....
MA/Alb/206a/96  [D].....................................R...............................
TY/Ger/R04-5/02 [A].....................................................................
MA/Neth/16/99   [A].....................................................................
DK/HK/221/77    [E].....................................R.....................................S....
DK/HK/73/76     [F].....................K....R...........................................
CKZA02UP1102    [A].....................................A.V.....................G.....
CKZA02AL19      [A]..-..................................A.....................................
OSZA98KK98      [A].....................................................................
DK/Korea/S17/03 [A].....................................R...............................
DK/Hainan/6/04  [H].....................................................................
DK/HK/1037-1/98 [G].....................................................................
Consensus          WTGMIDGWYGYHHENSQGSGYAADRESTQKAIDGITNKVNSIIDKMNTQFEAVDHEFSNLERRIDNLNKR

                    430       440       450       460
                    ..|....|....|....|....|....|....|
Q/HK/1721-30/99 [B].Q.......................M......
DK/HK/3461/99   [A]........................M......
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]..............I.........M......
DK/HK/1037-1/98 [G]........................M......
Consensus          MEDGFLDVWTYNAELLVLLENERTLDLHDANVK
```

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

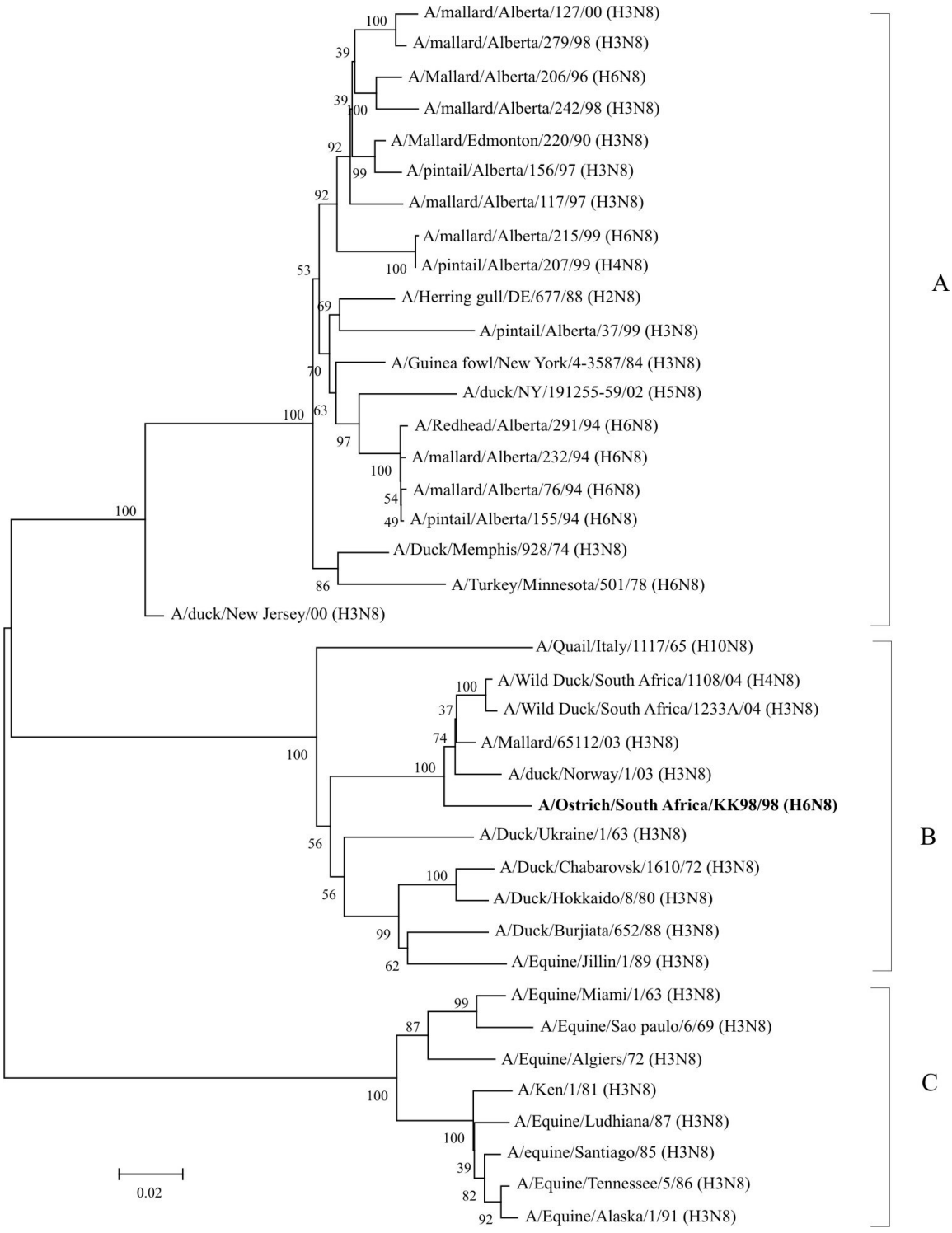


Figure 2.5 Phylogenetic tree inferred from a 1314-nt multiple sequence alignment of the neuraminidase (N8) genes of A/Ostrich/South Africa/KK98/98 (H6N8) (in boldface) and other viruses. Sub-lineages A to C are indicated.

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

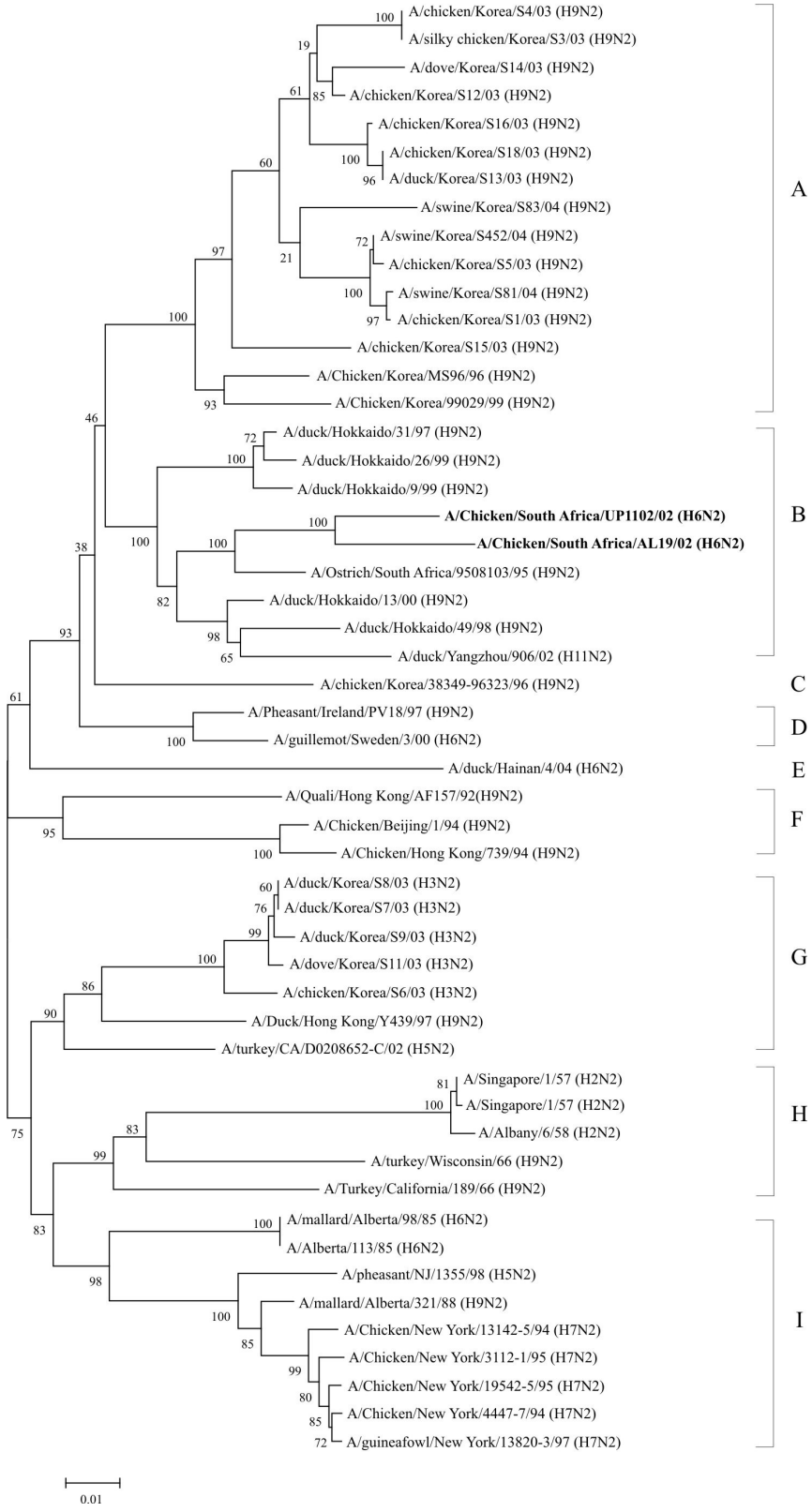


Figure 2.6 Phylogenetic trees inferred from a 1104-nt multiple sequence alignment of the neuraminidase (N2) genes of South African H6N2 (in boldface) and other viruses. Deletions or insertions were excised to simplify the phylogenetic analysis. Sub-lineages A to I are indicated.

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.

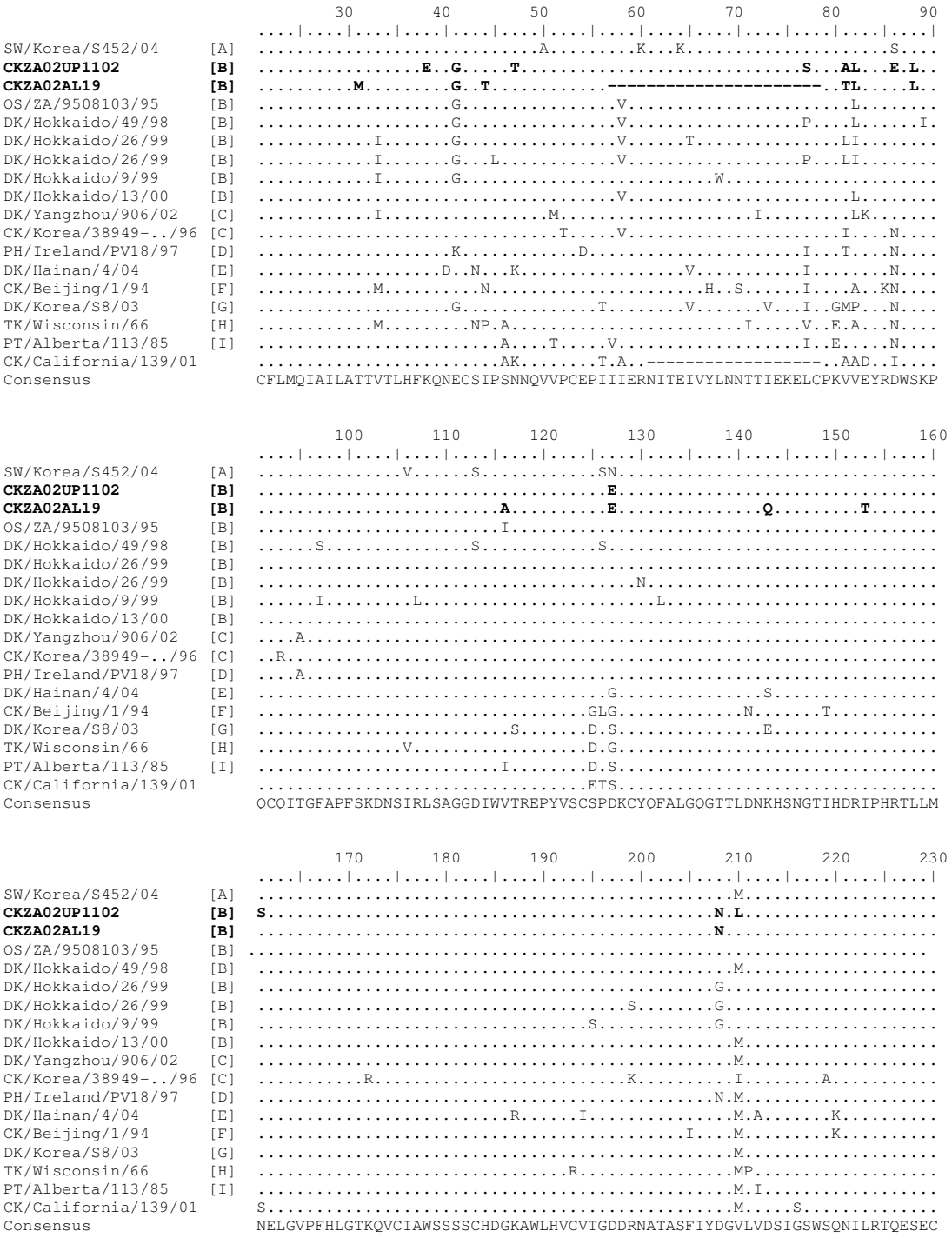


Figure 2.7 Multiple alignment of full-length N2 peptide sequences. Lineages are indicated in square brackets, and deleted amino acids are represented by (-).

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

                                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.

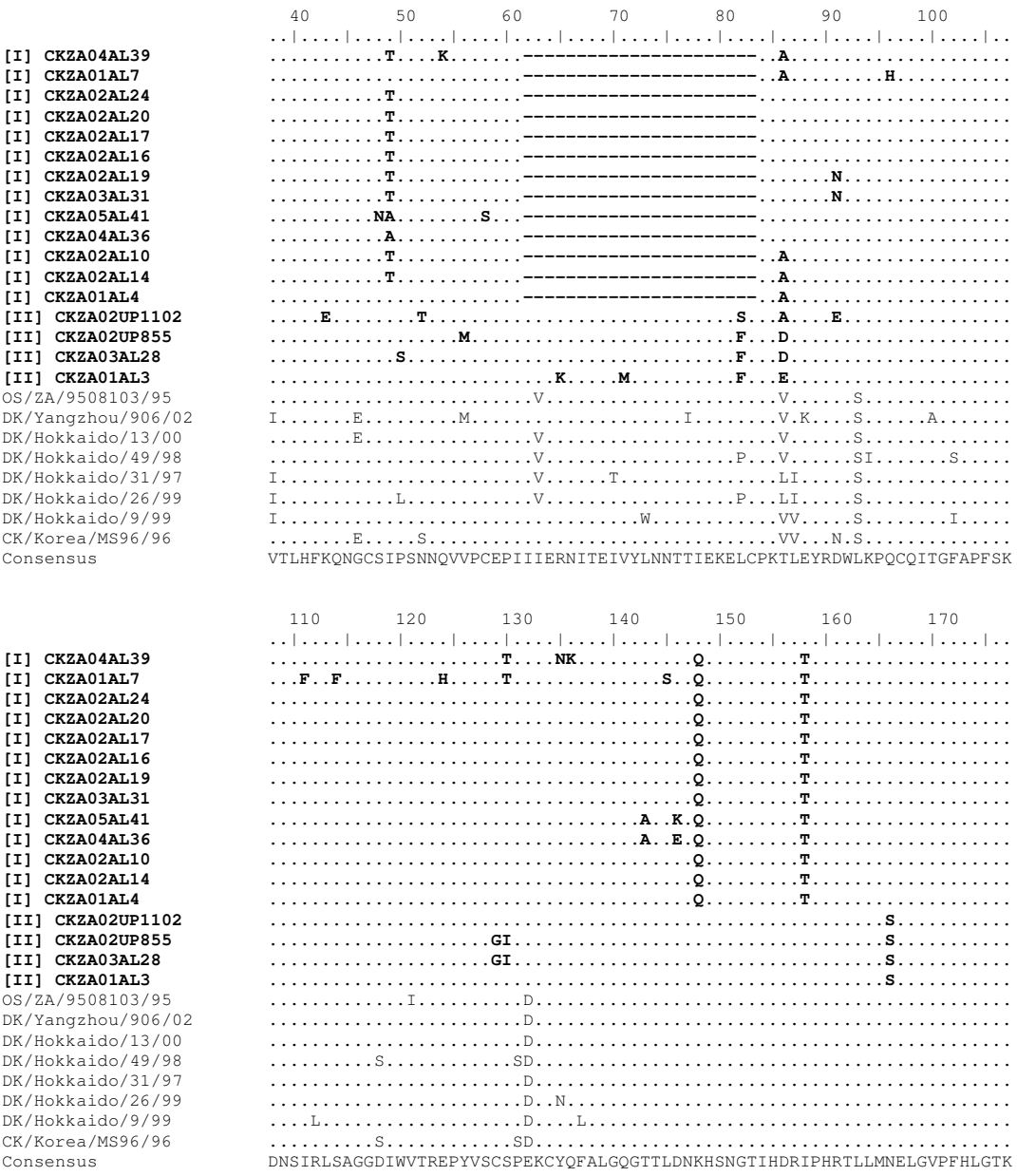


Figure 2.8 Multiple amino acid alignment of the N-stalk region of selected South African H6N2 (in boldface) and reference virus N2 genes. Sub-lineages I and II are indicated in brackets. Deleted amino acids are represented by (-).

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

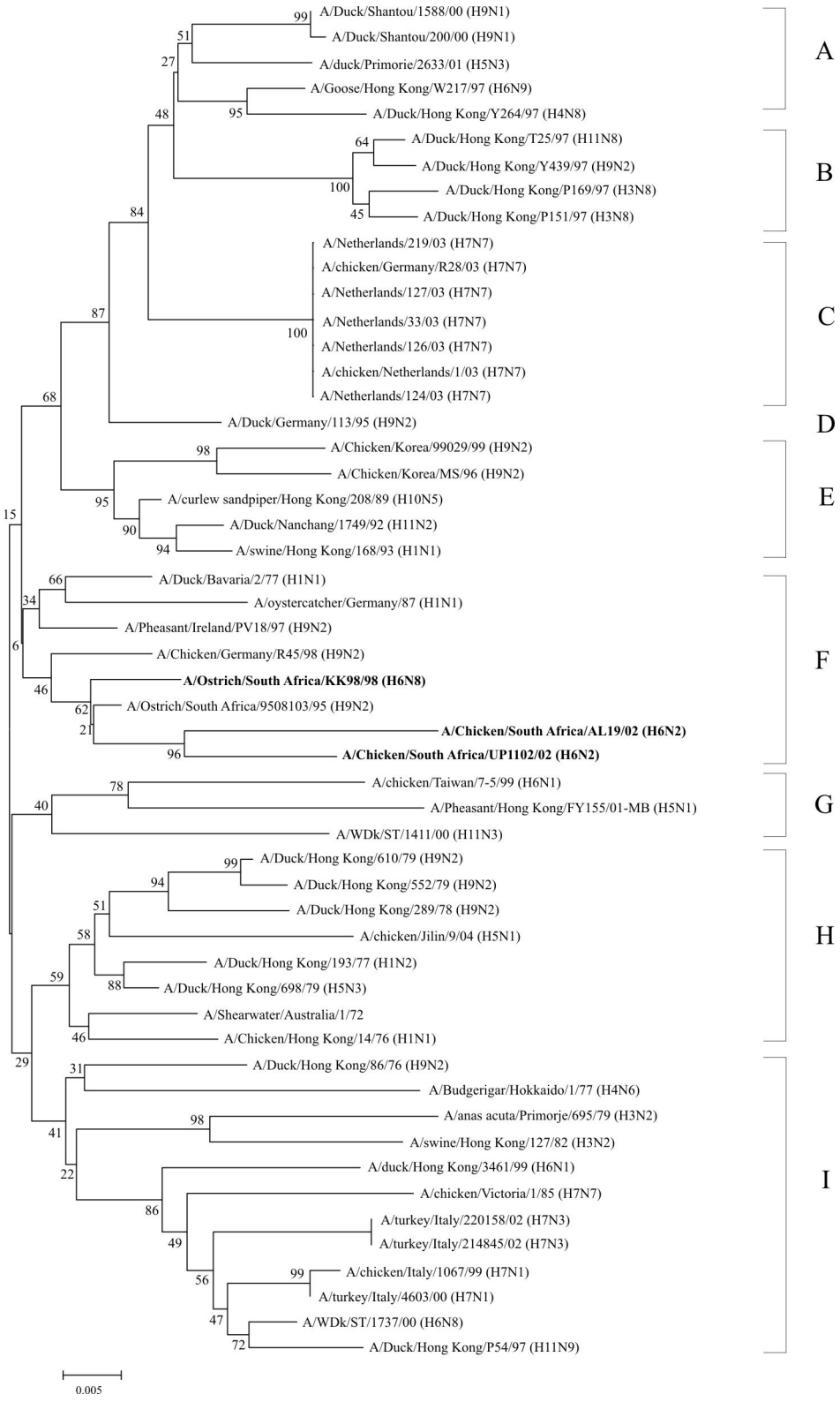


Figure 2.9 Phylogenetic tree inferred from a 797-nt multiple sequence alignment of the matrix (M) protein genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

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

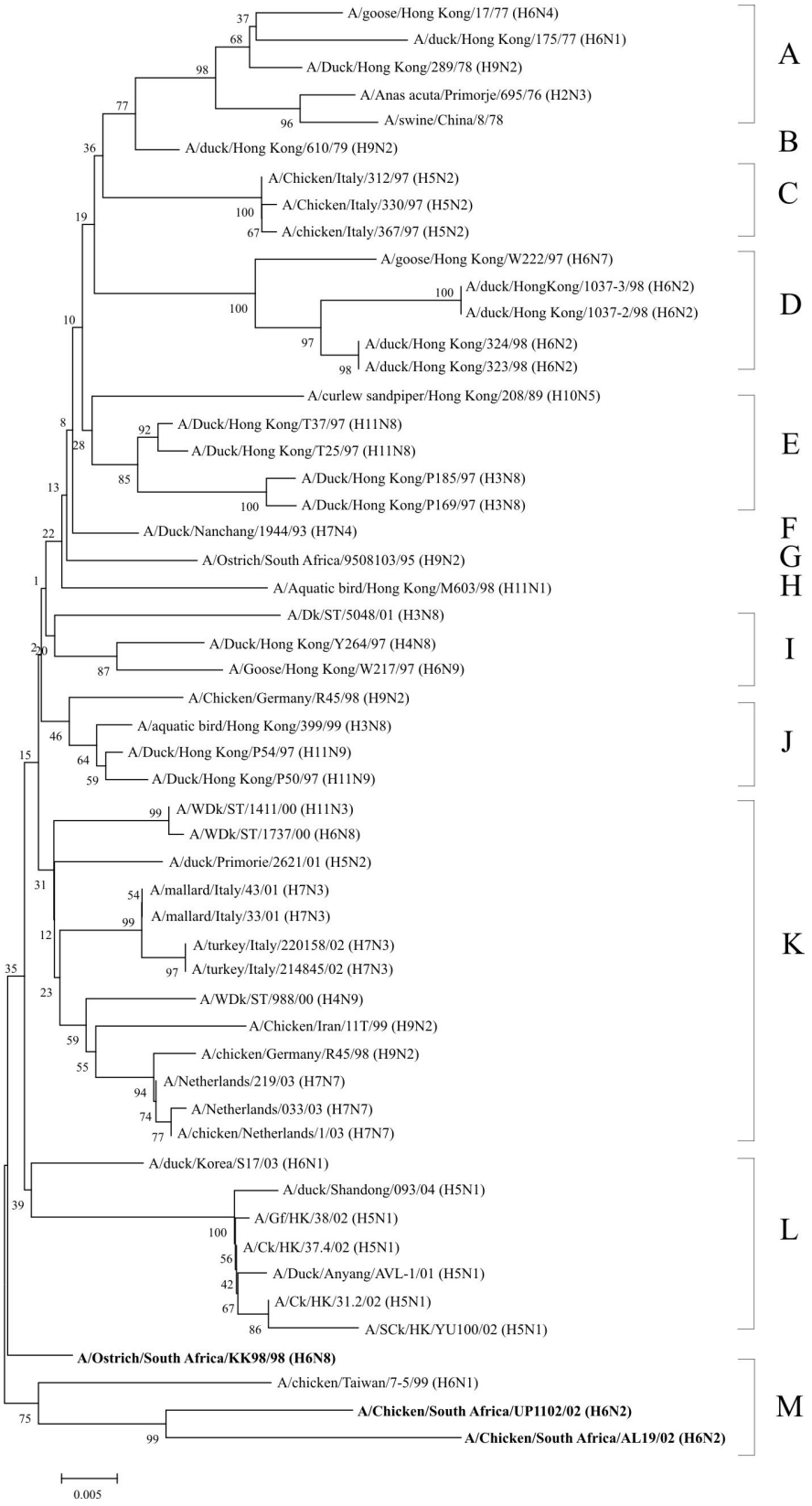


Figure 2.10(a) Phylogenetic tree inferred from a 763-nt multiple sequence alignment of the nonstructural (NS1) protein genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to M are indicated.

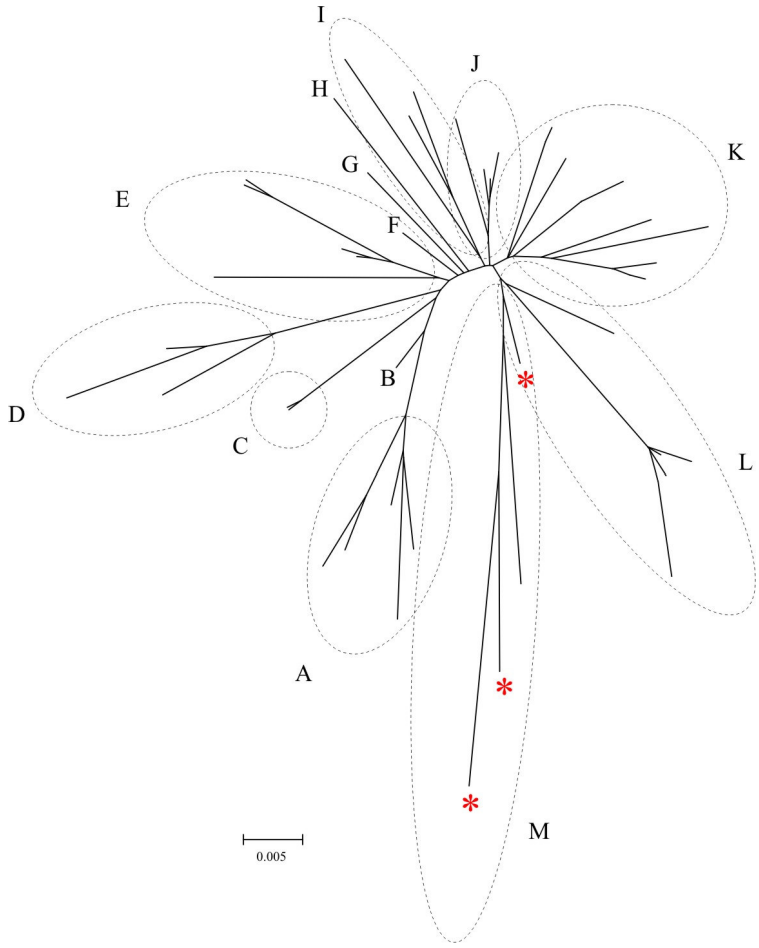


Figure 2.10(b) Radial version of Fig.2.10(a). South African viruses are indicated by "*".

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

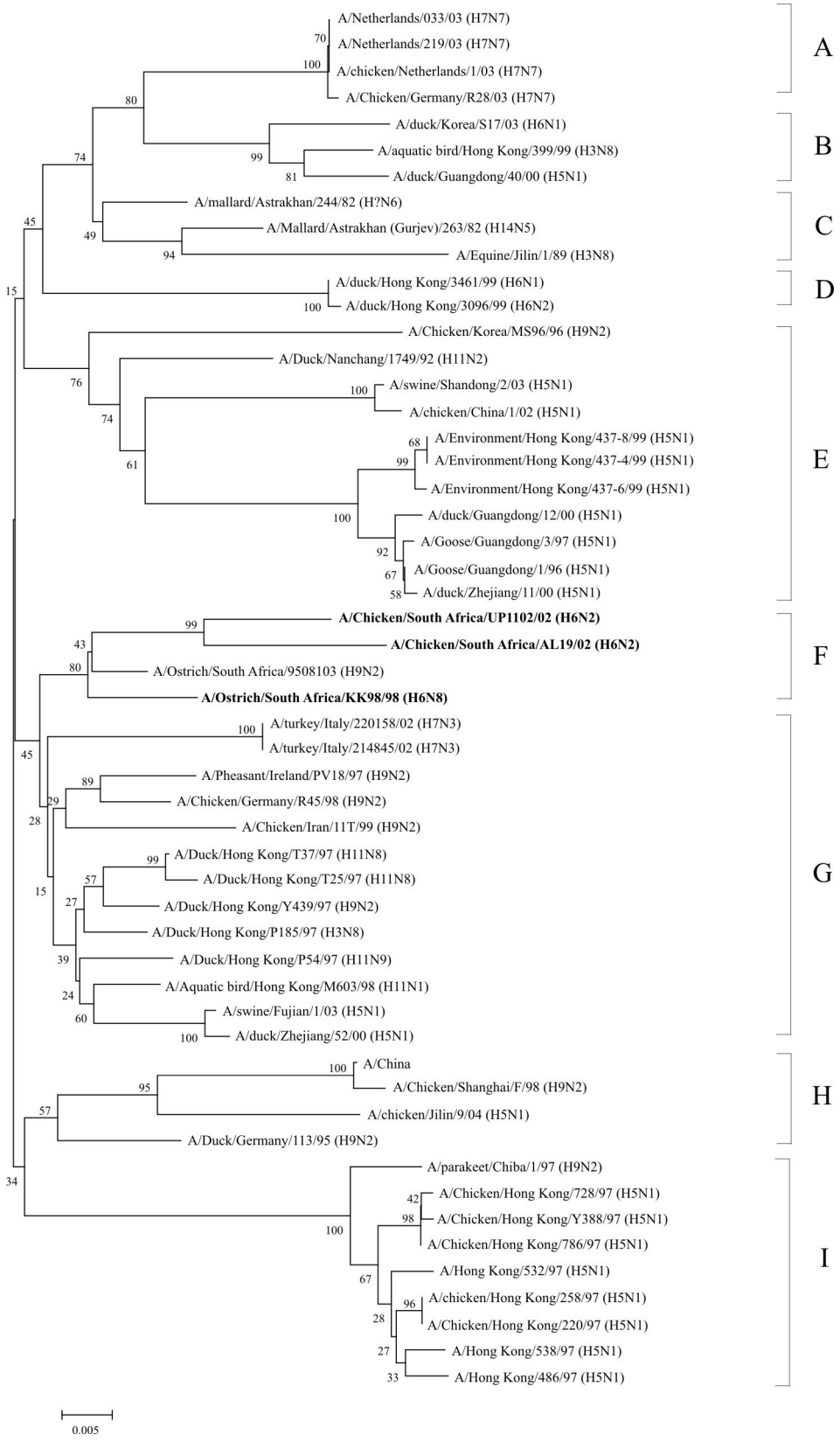


Figure 2.11 Phylogenetic tree inferred from an 863-nt multiple sequence alignment of the nucleocapsidprotein (NP) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated alongside

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

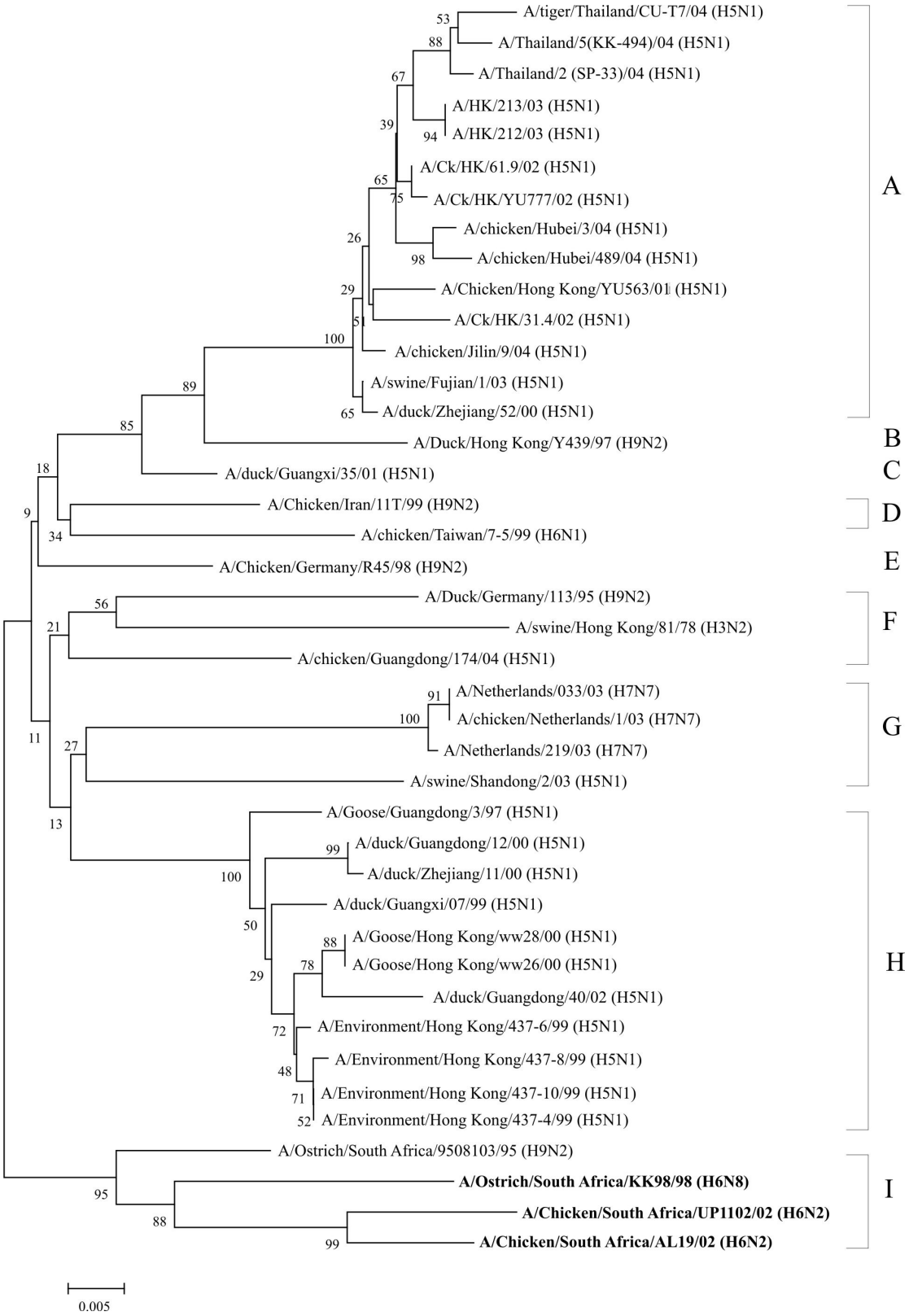


Figure 2.12 Phylogenetic tree inferred from a 735-nucleotide multiple sequence alignment of the polymerase A (PA) genes of South African H6N2, H6N8 (in boldface; *) and other viruses. Sub-lineages A to I are indicated.

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

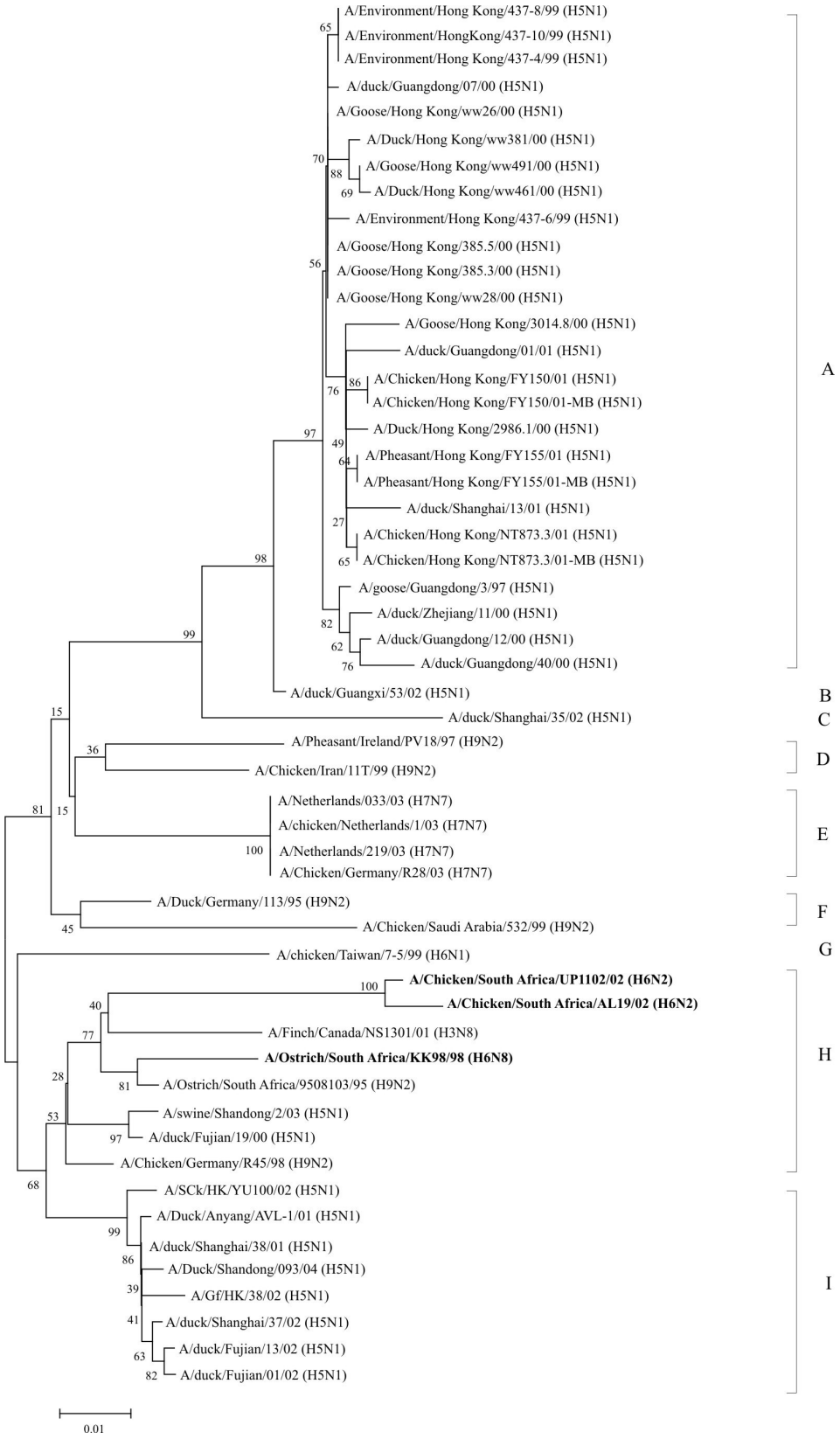


Figure 2.13(a) Phylogenetic tree inferred from a 668-nt multiple sequence alignment of the polymerase B1 (PB1) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

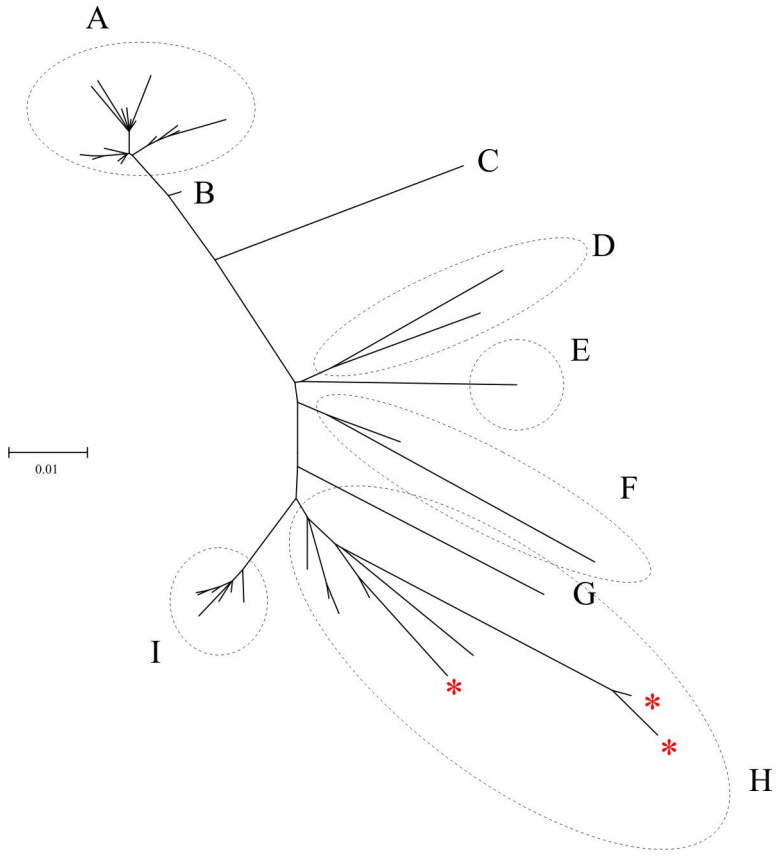


Figure 2.13(b) Radial version of Fig. 2.13(a). South African viruses are indicated by "*".

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

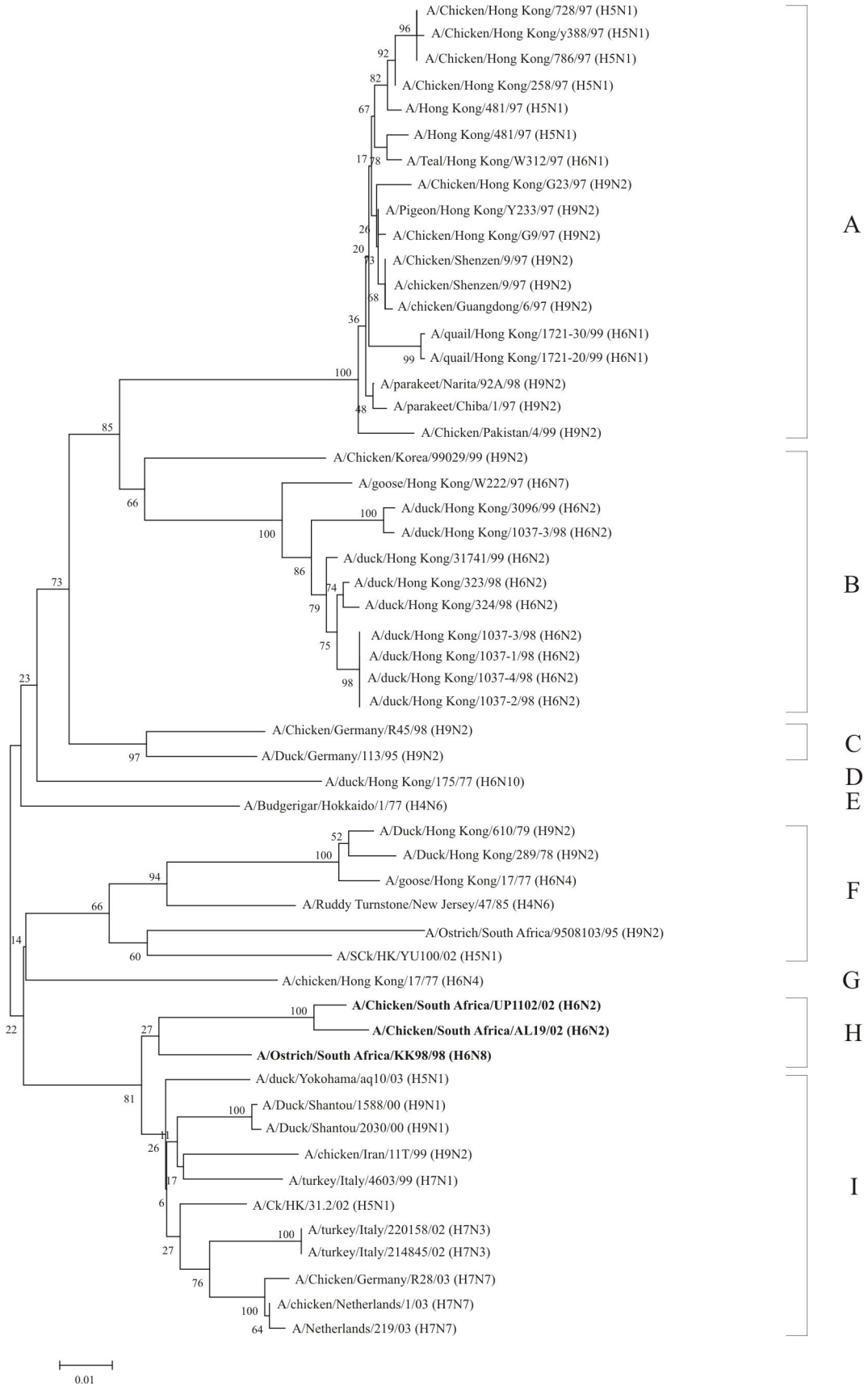


Figure 2.14 Phylogenetic tree inferred from a 738-nt multiple sequence alignment of the Polymerase B2 (PB2) genes of South African H6N2, H6N8 (in boldface) and other viruses. Sub-lineages A to I are indicated.

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

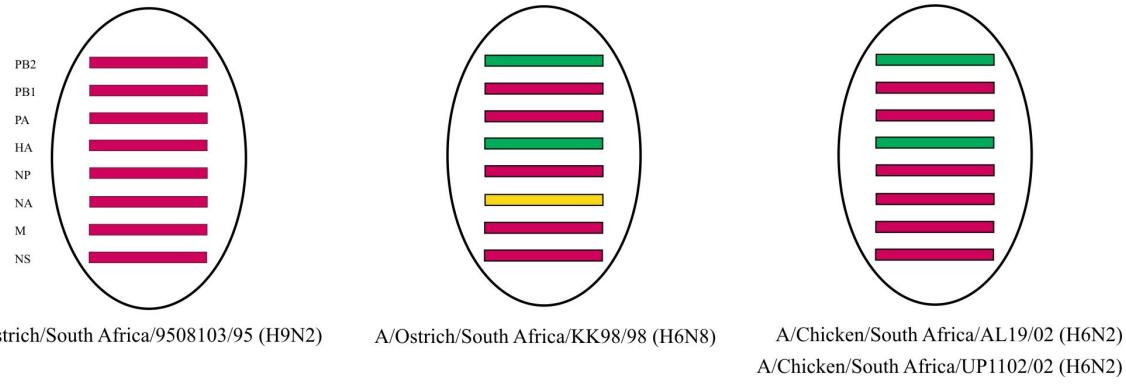


Figure 2.15 Schematic representation of the origins of genes of South African H6N2 viruses

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.