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.

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[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

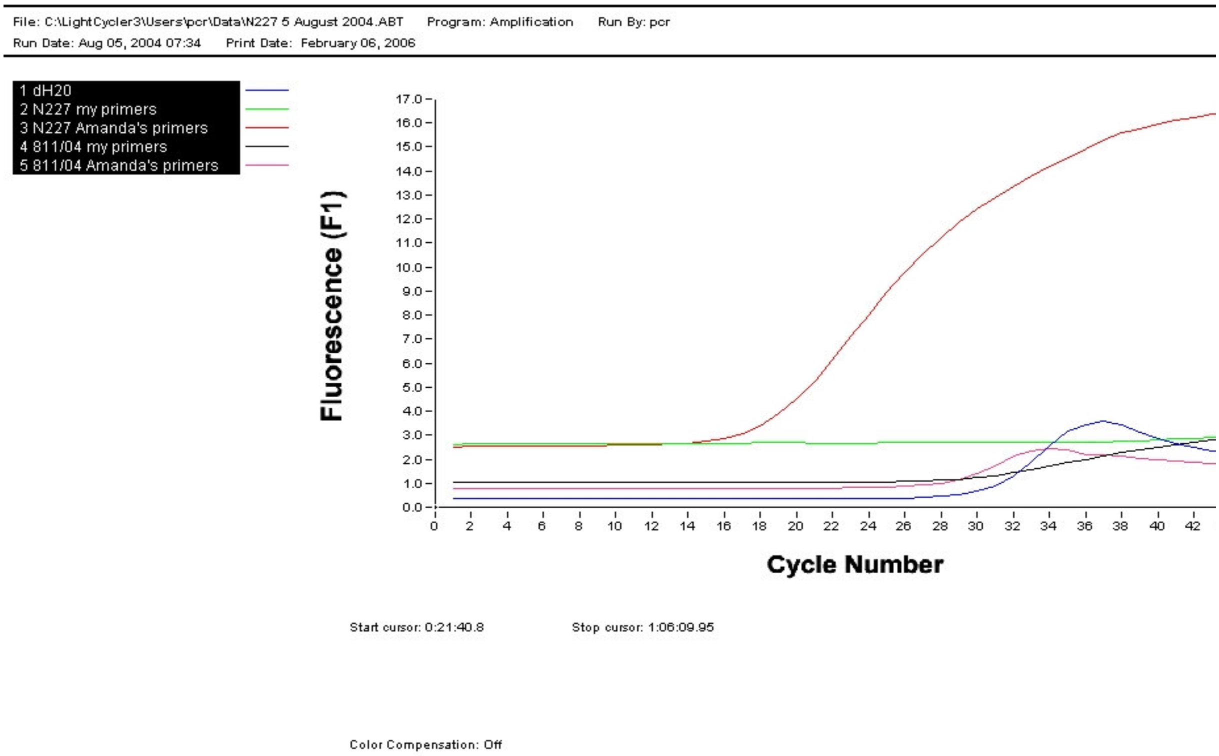
DNA sequencing and phylogenetic analyses were performed as described in Chapter Two, p60. For each gene, the highest similarity scores were retrieved from Genbank by BLAST searches. Dendograms of the midpoint-rooted Neighbour-joining trees are presented for each gene.

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

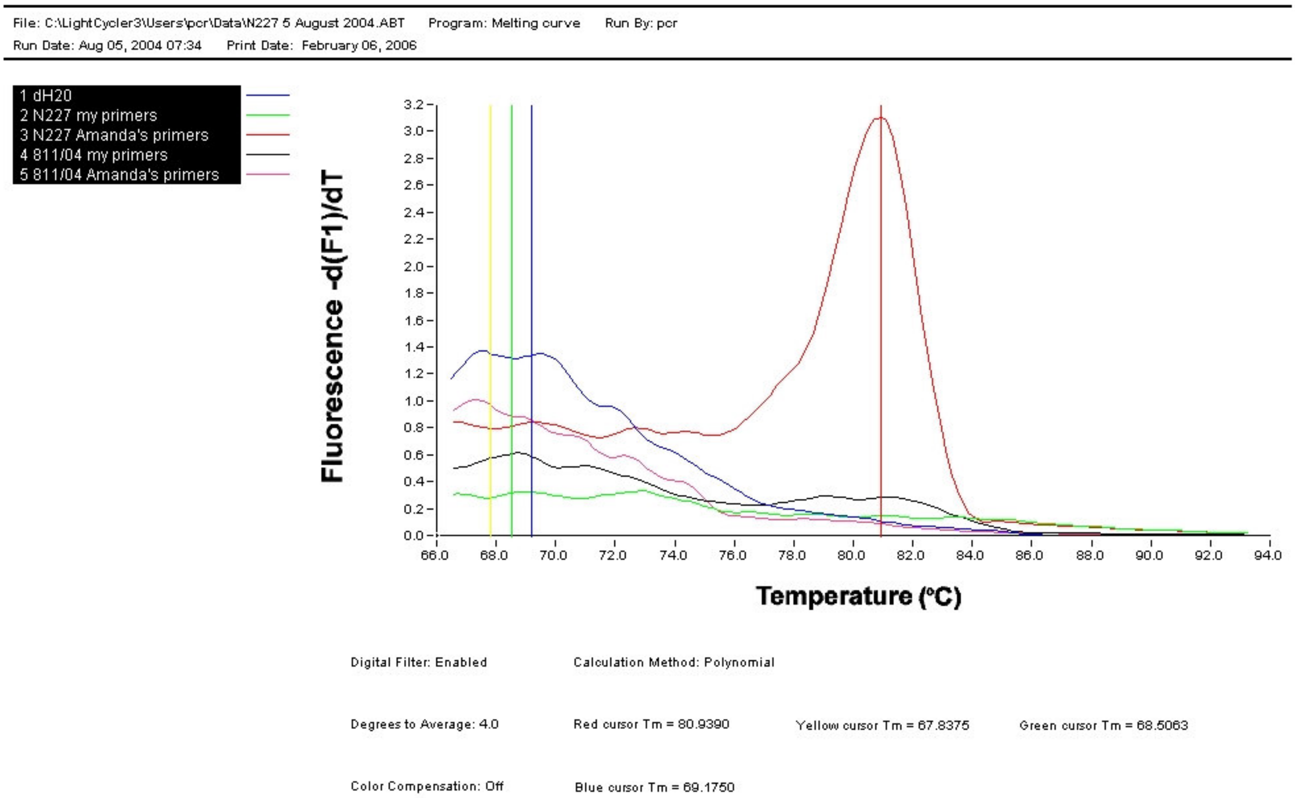


File: C:\LightCycler3\Users\pcr\Data\N227 5 August 2004.ABT Program: Amplification Run By: pcr
Run Date: Aug 05, 2004 07:34 Print Date: February 06, 2006

Start cursor: 0:21:40.8 Stop cursor: 1:06:09.95

Color Compensation: Off

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).



File: C:\LightCycler3\Users\pcr\Data\N227 5 August 2004.ABT Program: Melting curve Run By: pcr
Run Date: Aug 05, 2004 07:34 Print Date: February 06, 2006

Digital Filter: Enabled
Calculation Method: Polynomial
Degrees to Average: 4.0
Red cursor Tm = 80.9390
Yellow cursor Tm = 67.8375
Green cursor Tm = 68.5063
Color Compensation: Off
Blue cursor Tm = 69.1750

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 PQRE$\underline{\text{KRRKK}}$RGLF (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:

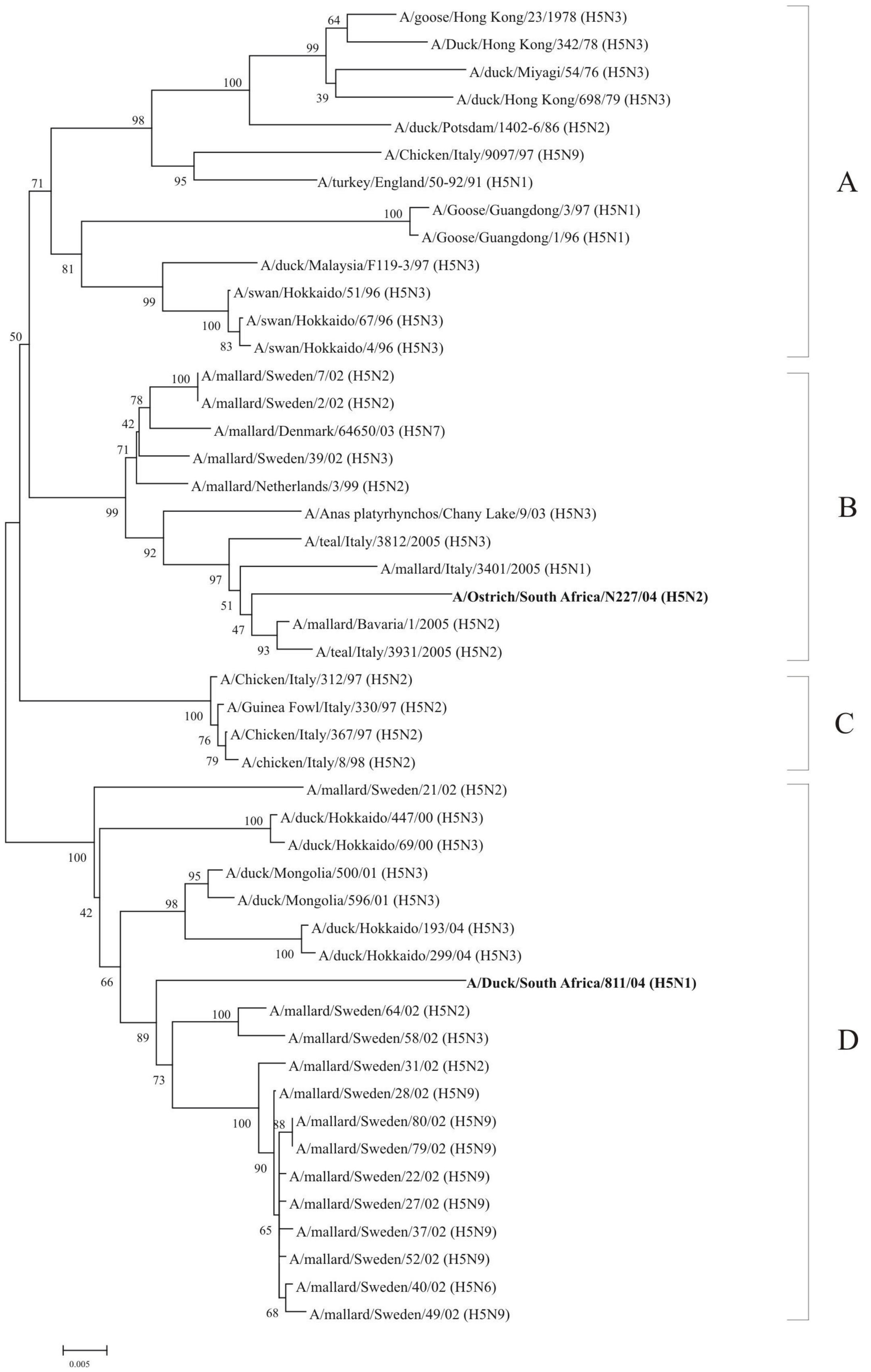


Figure 3.2 Phylogenetic tree inferred from a 1338-nt multiple sequence alignment of the HA (H5) genes of the outbreak strain A/Ostrich/South Africa/N227/05 (H5N2) and A/Wild duck/South Africa/811/04 (H5N1) (in boldface) and related sequences. Sub-lineages A to D are indicated

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                                 60        70        80        90       100       110       120       130       140       150       160       170       180       190       200
                                .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
[C] Ck/Italy/367/97(H5N2)       ....................................................................................................I.................................................
[C] Ck/Italy/8/98(H5N2)         .........................................................................L..........................I.................................................
[C] Gf/Italy/330/97(H5N2)       .....................................................................R..............................I.................................................
[C] Ck/Italy/312/97(H5N2)       ....................................................................................................I.................................................
[A] Ck/Italy/9097/97(H5N9)      .................................................D.......................L..................................................K.........................
[A] Tk/England/50-92/91(H5N1)   ...............................................................................................................................S......................
[A] Dk/Malaysia/F119-3/97(H5N3  .............................................................................R..........................................................I.............
[A] Dk/Miyagi/54/76(H5N3)       ..................................................S..................................................................S..................I.............
[A] Dk/Hong Kong/698/79(H5N3)   .....................................................................................................................S..................I.............
[A] Dk/Hong Kong/342/78(H5N3)   ....................................................................................................................DS..................I.............
[A] Go/Hong Kong/23/1978(H5N3)  .....................................................................R...........K......................................................I.............
[A] Dk/Potsdam/1402-6/86(H5N2)  ................................................I.......................................................................................I.............
[A] Sw/Hokkaido/67/96(H5N3)     ..............................................S.....................................................G...................................I.............
[A] Sw/Hokkaido/4/96(H5N3)      ..............................................S.....................................................G...................................I.............
[A] Sw/Hokkaido/51/96(H5N3)     ..............................................S.....................................................G...................................I.............
[A] Go/Guangdong/3/97(H5N1)     .....D...........................I...........AS.A.D..................R...........K..................H................S................................
[A] Go/Guangdong/1/96(H5N1)     .....D...........................I...........AS.A.D..................R...........K..................H................S................................
[D] Dk/Hokkaido/299/04(H5N3)    ..............................................S.I.....................................................................................................
[D] Dk/Mongolia/500/01(H5N3)    ..............................................S.I.....................................................................................................
[D] Dk/Mongolia/596/01(H5N3)    ..............................................S.I.....................................................................................................
[D] Dk/Hokkaido/193/04(H5N3)    ..............................................S.I.....................................................................................................
[D] Ma/Sweden/80/02(H5N9)       ......................................................................................................................................................
[D] Ma/Sweden/52/02(H5N9)       ......................................................................................................................................................
[D] Ma/Sweden/40/02(H5N6)       ......................................................................................................................................................
[D] Ma/Sweden/49/02(H5N9)       .....................................................................N.............Y..................................................................
[D] Ma/Sweden/28/02(H5N9)       .....................................................................R................................................................................
[D] Ma/Sweden/31/02(H5N2)       .....................................................................R................................................................................
[D] Dk/ZA/811/04(H5N1)          ..............................................K.............................V.....D...S...T.......Q...K..........................................NGE..
[D] Ma/Sweden/21/02(H5N2)       .........I.................................................................................................................T..........................
[D] Dk/Hokkaido/447/00(H5N3)    ......................................................................................................................................................
[D] Dk/Hokkaido/69/00(H5N3)     ......................................................................I...............................................................................
[D] Ma/Sweden/64/02(H5N2)       ......................................................................................................................................................
[D] Ma/Sweden/58/02(H5N3)       ............................................................................V................................................N........................
[B] Tl/Italy/3931/2005(H5N2)    ...........................................................N.....X....................................................................I...............
[B] Ma/Sweden/7/02(H5N2)        ................................................................R...................................S.................................................
[B] Ma/Sweden/2/02(H5N2)        ................................................................R...................................S.................................................
[B] Ma/Sweden/39/02(H5N3)       ......................................................................................................................................................
[B] Ma/Netherlands/3/99(H5N2)   ...................A..................................................................................................................................
[B] Tl/Italy/3812/2005(H5N3)    ......................................................................................................................................................
[B] Ma/Denmark/64650/03(H5N7)   ......................................................................................................................................................
[B] Ma/Italy/3401/2005(H5N1)    ................................................I.......................................E.............................................................
[B] Os/ZA/N227/04(H5N2)         .......K.......................................................................F.....................................................Y................
[B] A.pl/Chany Lake/9/03(H5N3)  .......K....F..G....................................W............................K....................................................................
[B] Ma/Bavaria/1/2005(H5N2)     ......................................................................................................................................................
Consensus                       NGKLCSLNGVKPLILRDCSVAGWLLGNPMCDEFLNVPEWSYIVEKDNPVNGLCYPGDFNDYEELKHLLSSTNHFEKIQIIPRSSWSNHDASSGVSSACPYNGRSSFFRNVVWLIKKNNAYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQ
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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

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                                210       220       230       240       250       260       270       280       290       300       310       320       330       340       350
                                .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
[C] Ck/Italy/367/97(H5N2)       ..................................................V.................................................................R...................RR--KK........
[C] Ck/Italy/8/98(H5N2)         ..................................................V.................................................................R...................RR--KK........
[C] Gf/Italy/330/97(H5N2)       ..................................................V.................................................................R...................RR--KK........
[C] Ck/Italy/312/97(H5N2)       ..................................................V.............................E.......................................................RR--KK........
[A] Ck/Italy/9097/97(H5N9)      ......................I......................M......................................................T............................P.....K.----.........
[A] Tk/England/50-92/91(H5N1)   .E......................A..............................................................................L.........................P......KR--K.........
[A] Dk/Malaysia/F119-3/97(H5N3  .........................................................................G...............................................G...............----.........
[A] Dk/Miyagi/54/76(H5N3)       .........................................................................................................................................----.........
[A] Dk/Hong Kong/698/79(H5N3)   ..............................R.................................................E.K......................................................----.........
[A] Dk/Hong Kong/342/78(H5N3)   ......................................I......................................I...........................................................----.........
[A] Go/Hong Kong/23/1978(H5N3)  .........................................................................................................................................----.........
[A] Dk/Potsdam/1402-6/86(H5N2)  .........................................................................................................................................----.........
[A] Sw/Hokkaido/67/96(H5N3)     .....................................................................................................K...................................----.........
[A] Sw/Hokkaido/4/96(H5N3)      .........................................S...........................................................K...................................----.........
[A] Sw/Hokkaido/51/96(H5N3)     .........................................................................................................................................----.........
[A] Go/Guangdong/3/97(H5N1)     ..........I..........L..........................................................E........................................N..........T....RRRKK........
[A] Go/Guangdong/1/96(H5N1)     ..........I..........L..........................................................E........................................N..........T....RRRKK........
[D] Dk/Hokkaido/299/04(H5N3)    .........................................................................N......E........................................................----.........
[D] Dk/Mongolia/500/01(H5N3)    .........................................................................................................................................----.........
[D] Dk/Mongolia/596/01(H5N3)    ....................................................................V....................................................................----.....V...
[D] Dk/Hokkaido/193/04(H5N3)    ................................................................................E........................................................----.........
[D] Ma/Sweden/80/02(H5N9)       ....................................................................................................................................I....----.........
[D] Ma/Sweden/52/02(H5N9)       ....................................................................................................................................I....----.........
[D] Ma/Sweden/40/02(H5N6)       ...........................................................................T........................................................I....----.........
[D] Ma/Sweden/49/02(H5N9)       ...........................................................................T........................................................I....----.........
[D] Ma/Sweden/28/02(H5N9)       ....................................................................................................................................I....----.........
[D] Ma/Sweden/31/02(H5N2)       ...........................................................................................................R.............................----.........
[D] Dk/ZA/811/04(H5N1)          .........................................L..........K....................N...............................................N...............----.........
[D] Ma/Sweden/21/02(H5N2)       ................................................................................E........................................................----.........
[D] Dk/Hokkaido/447/00(H5N3)    ................................................................................K........................................................----.........
[D] Dk/Hokkaido/69/00(H5N3)     ................................................................................K........................................................----.........
[D] Ma/Sweden/64/02(H5N2)       ..........I..................L...........................................................................................................----.........
[D] Ma/Sweden/58/02(H5N3)       ..........I.....................................................................E........................................................----.........
[B] Tl/Italy/3931/2005(H5N2)    ............I..............................................................V.............................................................----.........
[B] Ma/Sweden/7/02(H5N2)        ..............................................R..........................................................................................----.........
[B] Ma/Sweden/2/02(H5N2)        ..............................................R..........................................................................................----.........
[B] Ma/Sweden/39/02(H5N3)       .................................................................................................................K........K..............----.........
[B] Ma/Netherlands/3/99(H5N2)   .........................................................................................................................................----.........
[B] Tl/Italy/3812/2005(H5N3)    .............-........I.............................-....................................................................................----.........
[B] Ma/Denmark/64650/03(H5N7)   M.....................................I..................................................................................N.............K.----.........
[B] Ma/Italy/3401/2005(H5N1)    .........................................................................................................................................----.........
[B] Os/ZA/N227/04(H5N2)         .........................................................................................................................................KRRKK........
[B] A.pl/Chany Lake/9/03(H5N3)  ................................................................................D........................................................----.........
[B] Ma/Bavaria/1/2005(H5N2)     .........................................................................................................................................----.........
Consensus                       TKLYQNPTTYVSVGTSTLNQRSVPEIATRPKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSAIMKSGLEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSDRLVLATGLRNVPQRE----TRGLFGAIA
```

Fig 3.3 continued

```
                                 360       370       380       390       400       410       420       430       440       450       460       470       480       490       500
                                .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
[C] Ck/Italy/367/97(H5N2)       .................................................................................................................................................
[C] Ck/Italy/8/98(H5N2)         .................................................................................................................................................
[C] Gf/Italy/330/97(H5N2)       .................................................................................................................................................
[C] Ck/Italy/312/97(H5N2)       .................................................................................................................................................
[A] Ck/Italy/9097/97(H5N9)      ................................................................................................................E................................
[A] Tk/England/50-92/91(H5N1)   .................................................................................................................................................
[A] Dk/Malaysia/F119-3/97(H5N3  .................................................................................................................................................
[A] Dk/Miyagi/54/76(H5N3)       ..................................................................................Y....................................K...R.....................
[A] Dk/Hong Kong/698/79(H5N3)   ..................................................................................N....................................K...R.....................
[A] Dk/Hong Kong/342/78(H5N3)   .......................................................................S.........................................Q.....K...R.....................
[A] Go/Hong Kong/23/1978(H5N3)  .......................................................................................................................K...R.....................
[A] Dk/Potsdam/1402-6/86(H5N2)  ............................................................................................................R..............R.....................
[A] Sw/Hokkaido/67/96(H5N3)     .................................................................................................................................................
[A] Sw/Hokkaido/4/96(H5N3)      .................................................................................................................................................
[A] Sw/Hokkaido/51/96(H5N3)     .................................................................................................................................................
[A] Go/Guangdong/3/97(H5N1)     .................R......................V...................R..............Q....L................................................................
[A] Go/Guangdong/1/96(H5N1)     ........................................V...................R..............Q.....................................................................
[D] Dk/Hokkaido/299/04(H5N3)    ......................................N......Y............................................................................................D......
[D] Dk/Mongolia/500/01(H5N3)    ........................................................G.................................................................................D......
[D] Dk/Mongolia/596/01(H5N3)    ..........................................................................................................................................D......
[D] Dk/Hokkaido/193/04(H5N3)    ......................................N.................G.................................................................................D......
[D] Ma/Sweden/80/02(H5N9)       .........................................................T.......................................................................................
[D] Ma/Sweden/52/02(H5N9)       ...........................................................E.....................................................................................
[D] Ma/Sweden/40/02(H5N6)       ........................................................G........................................................................................
[D] Ma/Sweden/49/02(H5N9)       .......................................................................D.........................................................................
[D] Ma/Sweden/28/02(H5N9)       .................................................................................................................................................
[D] Ma/Sweden/31/02(H5N2)       .................................................................................................................................................
[D] Dk/ZA/811/04(H5N1)          ....................KR............P.............................Y................................................................................
[D] Ma/Sweden/21/02(H5N2)       ..........I.......................................................................G..............................................................
[D] Dk/Hokkaido/447/00(H5N3)    ................................................TN..............................................................E................................
[D] Dk/Hokkaido/69/00(H5N3)     .................................................N..............................................................E................................
[D] Ma/Sweden/64/02(H5N2)       .................................................................................................................................................
[D] Ma/Sweden/58/02(H5N3)       .....................................................................T...........................................................................
[B] Tl/Italy/3931/2005(H5N2)    ...........................................................................-................................R....................................
[B] Ma/Sweden/7/02(H5N2)        ...................................................................K.............................................................................
[B] Ma/Sweden/2/02(H5N2)        ...................................................................K...............-.............................................................
[B] Ma/Sweden/39/02(H5N3)       .................................................................................................................................................
[B] Ma/Netherlands/3/99(H5N2)   .................................................................................................................................................
[B] Tl/Italy/3812/2005(H5N3)    ........................................................A........................................................................................
[B] Ma/Denmark/64650/03(H5N7)   ......................................................................................................L..........................................
[B] Ma/Italy/3401/2005(H5N1)    ................................................T................................................................................................
[B] Os/ZA/N227/04(H5N2)         ...........------.........S...T...HQ.........Y..........K.A...............R...A......P...........................................................
[B] A.pl/Chany Lake/9/03(H5N3)  ............................................................................................................................D.........P....D.....
[B] Ma/Bavaria/1/2005(H5N2)     ..................................................................................N..............................................................
Consensus                       GFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGITNKVNSIIDKMNTQFEAVGKEFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESV
```

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:

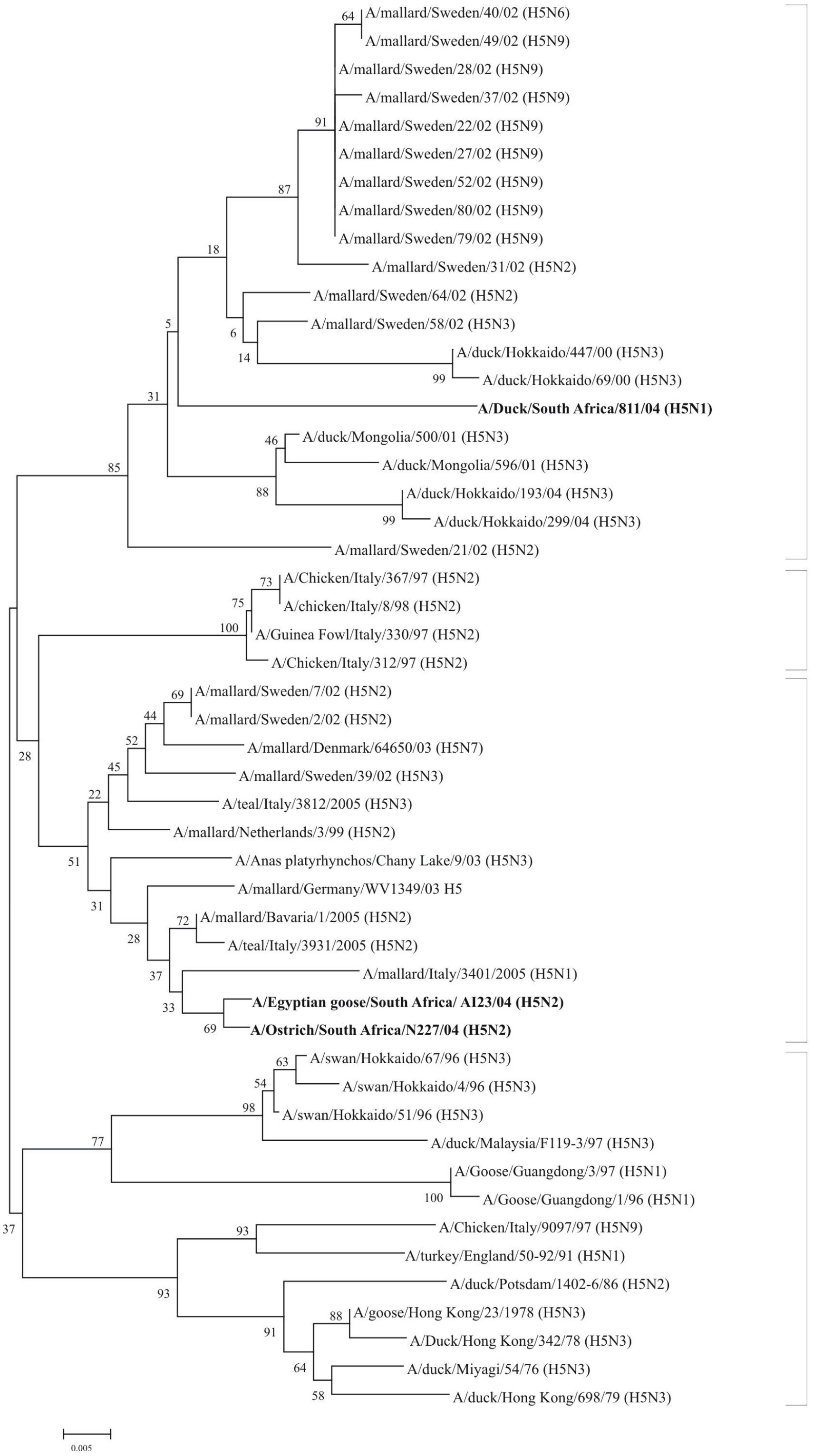

Figure 3.4 Phylogenetic tree inferred from a 373-nt multiple sequence alignment of the H5 hemagglutinin genes including that of A/Egyptian Goose/AI23/04 (H5N2) (in boldface). Sub-lineages A to D are indicated, corresponding to Fig 3.2.

```
                               230      240       250       260       270       280       290       300
                                .|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
[B] E.goose/ZA/AI23/04(H5N2)    ................................................................................
[B] Os/ZA/N227/04(H5N2)         ................................................................................
[B] Ma/Denmark/64650/03(H5N7)   .............I..................................................................
[B] Ma/Sweden/2/02(H5N2)        .....................R..........................................................
[B] Ma/Netherlands/3/99(H5N2)   ................................................................................
[B] Ma/Italy/3401/2005(H5N1)    ................................................................................
[B] Ma/Bavaria/1/2005(H5N2)     ................................................................................
[B] Ma/Sweden/39/02(H5N3)       ................................................................................
[B] Tl/Italy/3931/2005(H5N2)    ..................................................V.............................
[B] T/Italy/3812/2005(H5N3)     ...........................X....................................................
[B] Ma/Sweden/7/02(H5N2)        .....................R..........................................................
[B] A.pl/Chany Lake/9/03(H5N3)  .......................................................D........................
[A] Ck/Italy/9097/97(H5N9)      ....................M......................................................T....
[A] TK/England/50-92/91(H5N1)   ..............................................................................L.
[A] Dk/Miyagi/54/76(H5N3)       ................................................................................
[A] Dk/Potsdam/1402-6/86(H5N2)  ................................................................................
[A] Sw/Hokkaido/51/96(H5N3)     ................................................................................
[A] Sw/Hokkaido/67/96(H5N3)     ............................................................................K...
[A] Sw/Hokkaido/4/96(H5N3)      ................S...........................................................K...
[A] Dk/Malaysia/F119-3/97(H5N3) ................................................G...............................
[A] Go/Guangdong/3/97(H5N1)     .......................................................E........................
[A] Go/Guangdong/1/96(H5N1)     .......................................................E........................
[D] Dk/Hokkaido/193/04(H5N3)    .......................................................E........................
[D] Ma/Sweden/58/02(H5N3)       .......................................................E........................
[D] Ma/Sweden/21/02(H5N2)       .......................................................E........................
[D] Dk/Hokkaido/299/04(H5N3)    ................................................N......E........................
[D] Dk/ZA/811/04 (H5N1)         ................L..........K....................N...............................
[D] Ma/Sweden/80/02(H5N9)       ................................................................................
[D] Dk/Mongolia/596/01(H5N3)    ...........................................V....................................
[D] Dk/Mongolia/500/01(H5N3)    ................................................................................
[D] Ma/Sweden/31/02(H5N2)       ................................................................................
[D] Ma/Sweden/64/02(H5N2)       ....L...........................................................................
[D] Ma/Sweden/52/02(H5N9)       ................................................................................
[D] Ma/Sweden/28/02(H5N9)       ................................................................................
[D] Dk/Hokkaido/447/00(H5N3)    .......................................................K........................
[D] Dk/Hokkaido/69/00(H5N3)     .......................................................K........................
[C] Ck/Italy/367/97(H5N2)       .........................V......................................................
[C] Ck/Italy/8/98(H5N2)         .........................V......................................................
[C] Gf/Italy/330/97(H5N2)       .........................V......................................................
[C] Ck/Italy/312/97(H5N2)       .........................V.............................E........................
Consensus                       IATRPKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSAIMKSGLEYGNCNTKCQTPMGAINSSMPFH
```

```
                                 310      320       330       340       350
                                .|....|....|....|....|....|....|....|....|..
[B] E.goose/ZA/AI23/04(H5N2)    ........................P.......----........
[B] Os/ZA/N227/04(H5N2)         ................................KRRKK.......
[B] Ma/Denmark/64650/03(H5N7)   ................N.............K.----........
[B] Ma/Sweden/2/02(H5N2)        ................................----........
[B] Ma/Netherlands/3/99(H5N2)   ................................----........
[B] Ma/Italy/3401/2005(H5N1)    ................................----........
[B] Ma/Bavaria/1/2005(H5N2)     ................................----........
[B] Ma/Sweden/39/02(H5N3)       ........K........K..............----........
[B] Tl/Italy/3931/2005(H5N2)    ................................----........
[B] T/Italy/3812/2005(H5N3)     ................................----........
[B] Ma/Sweden/7/02(H5N2)        ................................----........
[B] A.pl/Chany Lake/9/03(H5N3)  ................................----........
[A] Ck/Italy/9097/97(H5N9)      ........................P.....K.----........
[A] TK/England/50-92/91(H5N1)   ........................P......KR--K........
[A] Dk/Miyagi/54/76(H5N3)       ................................----........
[A] Dk/Potsdam/1402-6/86(H5N2)  ................................----........
[A] Sw/Hokkaido/51/96(H5N3)     ................................----........
[A] Sw/Hokkaido/67/96(H5N3)     ................................----........
[A] Sw/Hokkaido/4/96(H5N3)      ................................----........
[A] Dk/Malaysia/F119-3/97(H5N3) ................G...............----........
[A] Go/Guangdong/3/97(H5N1)     ................N..........T....RRRKK.......
[A] Go/Guangdong/1/96(H5N1)     ................N..........T....RRRKK.......
[D] Dk/Hokkaido/193/04(H5N3)    ................................----........
[D] Ma/Sweden/58/02(H5N3)       ................................----........
[D] Ma/Sweden/21/02(H5N2)       ................................----........
[D] Dk/Hokkaido/299/04(H5N3)    ................................----........
[D] Dk/ZA/811/04 (H5N1)         ................N...............----.-......
[D] Ma/Sweden/80/02(H5N9)       ...........................I....----........
[D] Dk/Mongolia/596/01(H5N3)    ................................----.....V..
[D] Dk/Mongolia/500/01(H5N3)    ................................----........
[D] Ma/Sweden/31/02(H5N2)       ..R.............................----........
[D] Ma/Sweden/64/02(H5N2)       ................................----........
[D] Ma/Sweden/52/02(H5N9)       ...........................I....----........
[D] Ma/Sweden/28/02(H5N9)       ...........................I....----........
[D] Dk/Hokkaido/447/00(H5N3)    ................................----........
[D] Dk/Hokkaido/69/00(H5N3)     ................................----........
[C] Ck/Italy/367/97(H5N2)       ...........R...................--RRKK.......
[C] Ck/Italy/8/98(H5N2)         ...........R...................--RRKK.......
[C] Gf/Italy/330/97(H5N2)       ...............................--RRKK.......
[C] Ck/Italy/312/97(H5N2)       ...............................--RRKK.......
Consensus                       NIHPLTIGECPKYVKSDRLVLATGLRNVPQRE----TRGLFGAI
```

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}→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.

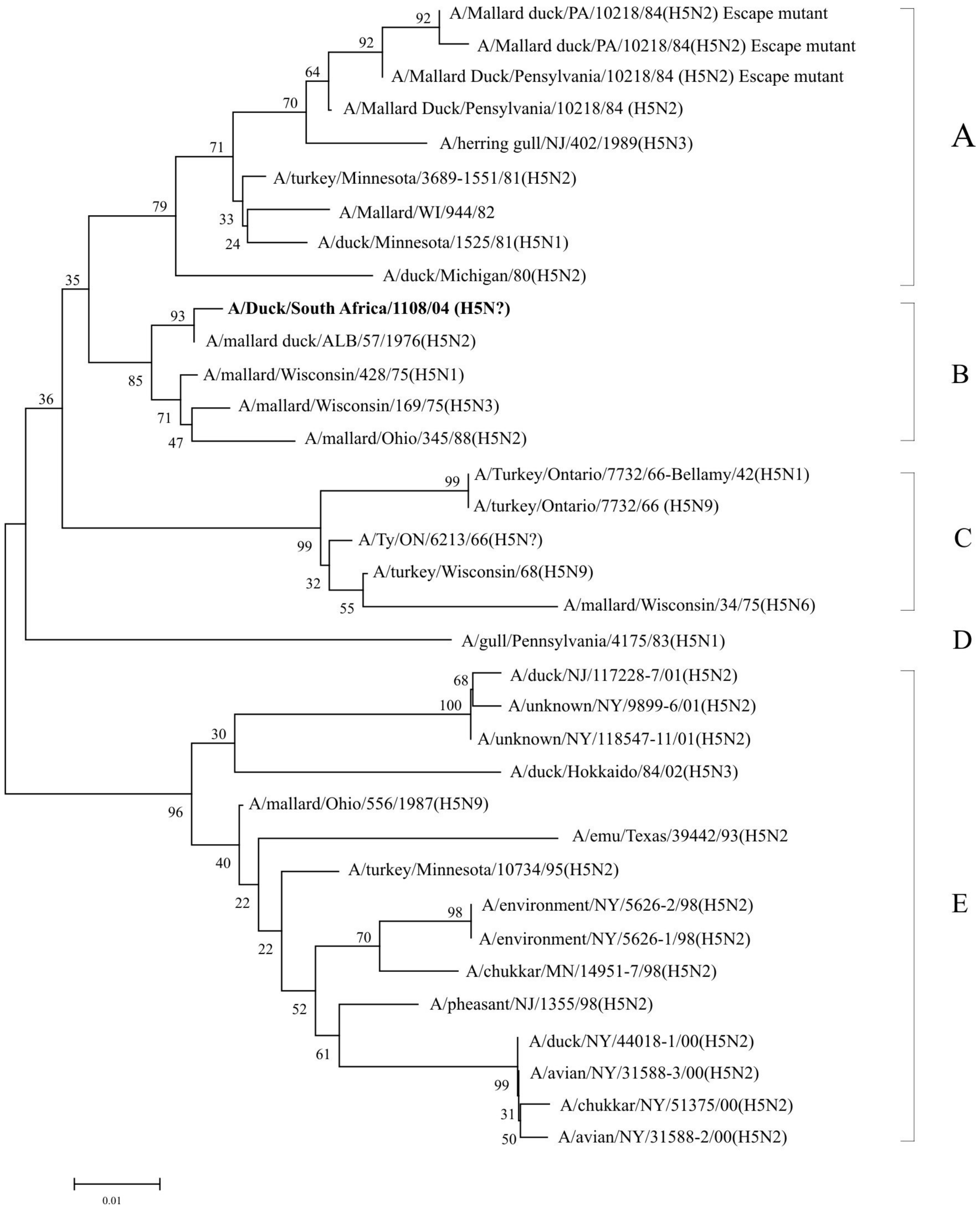

Figure 3.6 Phylogenetic trees inferred from a 308-nt multiple sequence alignment of a partial H5-type virus detected by RT-PCR in A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to E are indicated.

```
                              250       260       270       280       290       300       310       320       330       340
                             ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.....
[A] Ma/PA/10218/84[H5N2]     ................................T......G....N.........................................P.......-........
[A] MA/Pennsyl/10218/84[H5N2].......................................G....N.........................................P.......-........
[A] MA/Pennsyl/10218/84[H5N2]............................................N.................................................-........
[A] gull/NJ/402/1989[H5N3]   ............................................N.................................................-........
[A] Tk/Min/3689-1551/81[H5N2]............................................N.....................F...................P.......-........
[A] Ma/WI/944/82             ......S.....................................N.................................................-........
[A] Dk/Min/1525/81[H5N1]     .................................M..........N.................................................-........
[A] Duck/Michigan/80[H5N2]   ...S...............................S........NA.......T........................................-........
[B] Ma/Wisc/169/75[H5N3]     ...K...............................R..........................................................-........
[B] Ma/Wisc/428/75[H5N1]     ...K..........................................................................................-........
[B] Ma/Ohio/345/88[H5N2]     ...K............................T..................................................G..........-........
[B] Dk/SA/1108/04[H3N8][H5N?]...K...E......................................................................................-........
[B] MA/ALB/57/1976[H5N2]     ...K..........................................................................................-........
[C] TK/ON/7732/66-Bellamy/42 ......H............................R..........................................N.......P......RKK......K
[C] TK/ON/7732/66[H5N9]      ......H............................R..........................................N.......P......RKK......K
[C] Ty/ON/6213/66[H5N?]      ..............................G....R..................................................P.......-........
[C] Tk/Wisc/68[H5N9]         ...................................R..........................................................-........
[C] Ma/Wisc/34/75[H5N6]      ..............................G....R...............L..................................P.......-........
Gull/Pennsylvania/4175/83    ...K...................................K......................................E...............-.K......
[D] Un/NY/118547-11/01[H5N2] ...................................R..........................................................-........
[D] Dk/NJ/117228-7/01[H5N2]  .V.................................R..........................................................-........
[D] Un/NY/9899-6/01[H5N2]    ...................................R........................................................G.-........
[D] Tk/Min/10734/95[H5N2]    .......P..............................................................................P.......-........
[D] Emu/Texas/39442/93[H5N2] .....S.S..........................I...................................................P.......-........
[D] Ma/Ohio/556/87[H5N9]     ..............................................................................................-........
[D] Ql/Oregon/20719/86       ......................................MK......................................................-........
[D] Env/NY/5626-2/98[H5N2]   ...................................................M..........................................-........
[D] Env/NY/5626-1/98[H5N2]   ...................................................M..........................................-........
[D] Chkkr/MN/14951-7/98[H5N2].......................................G.................N............................P.......-........
[D] Ph/NJ/1355/98[H5N2]      ..............................N...............................................N...............-........
[D] Chkkr/NY/51375/00[H5N2]  .......T...............................D..............................K.......................-........
[D] Av/NY/31588-2/00[H5N2]   .......T..........................I....D......................................................-........
[D] Av/NY/31588-3/00[H5N2]   .......T...............................D......................................................-........
[D] Dk/NY/44018-1/00[H5N2]   .......T...............................D......................................................-........
[D} Dk/Hokkaido/84/02[H5N3]  .........................................................................................I..K.-........
Consensus                    TILRPNDAISFESNGNFIAPEYAYKIVKKGDSAIMKSELEYGNCDTKCQTPVGAINSSMPFHNVHPLTIGECPKYVKSDKLVLATGLRNVPQRE-TRGLFGAI
```

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

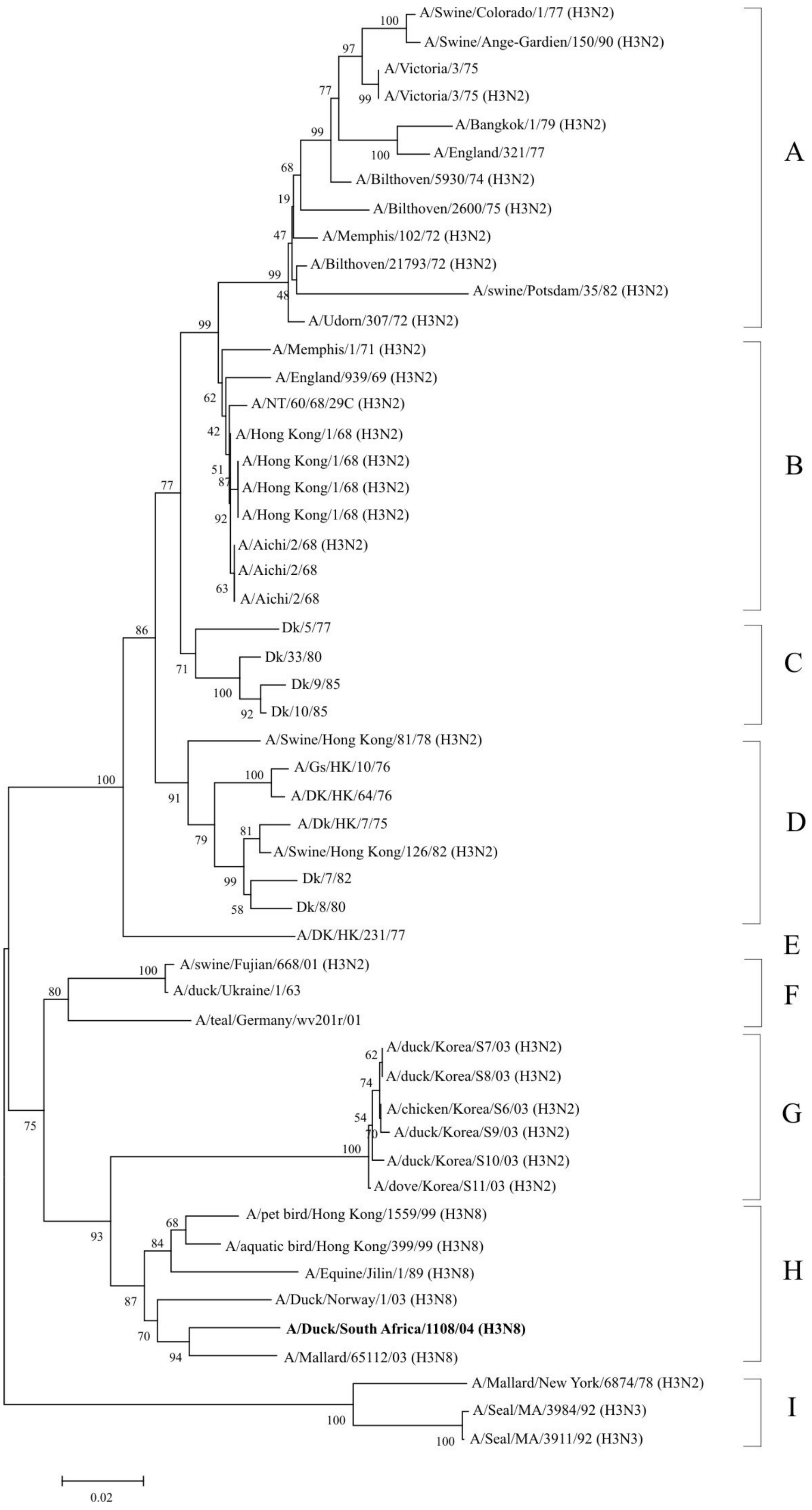

Figure 3.8 Phylogenetic tree inferred from a 1104-nt multiple sequence alignment of the H3 genes of A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to I are indicated.

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

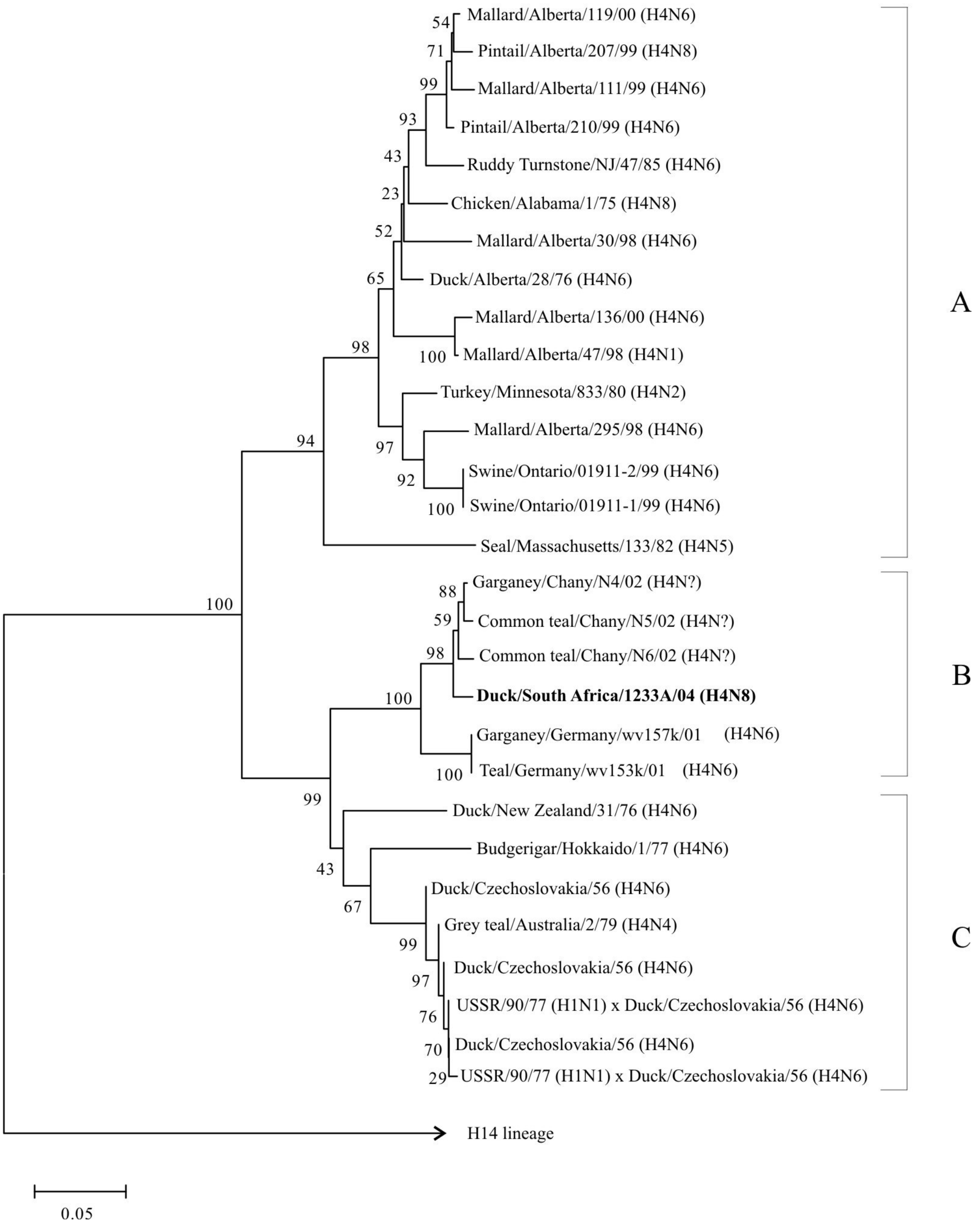

Figure 3.9 Phylogenetic trees inferred from a 459-nt multiple sequence alignment of the H4 genes of A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A, B and C are indicated.

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

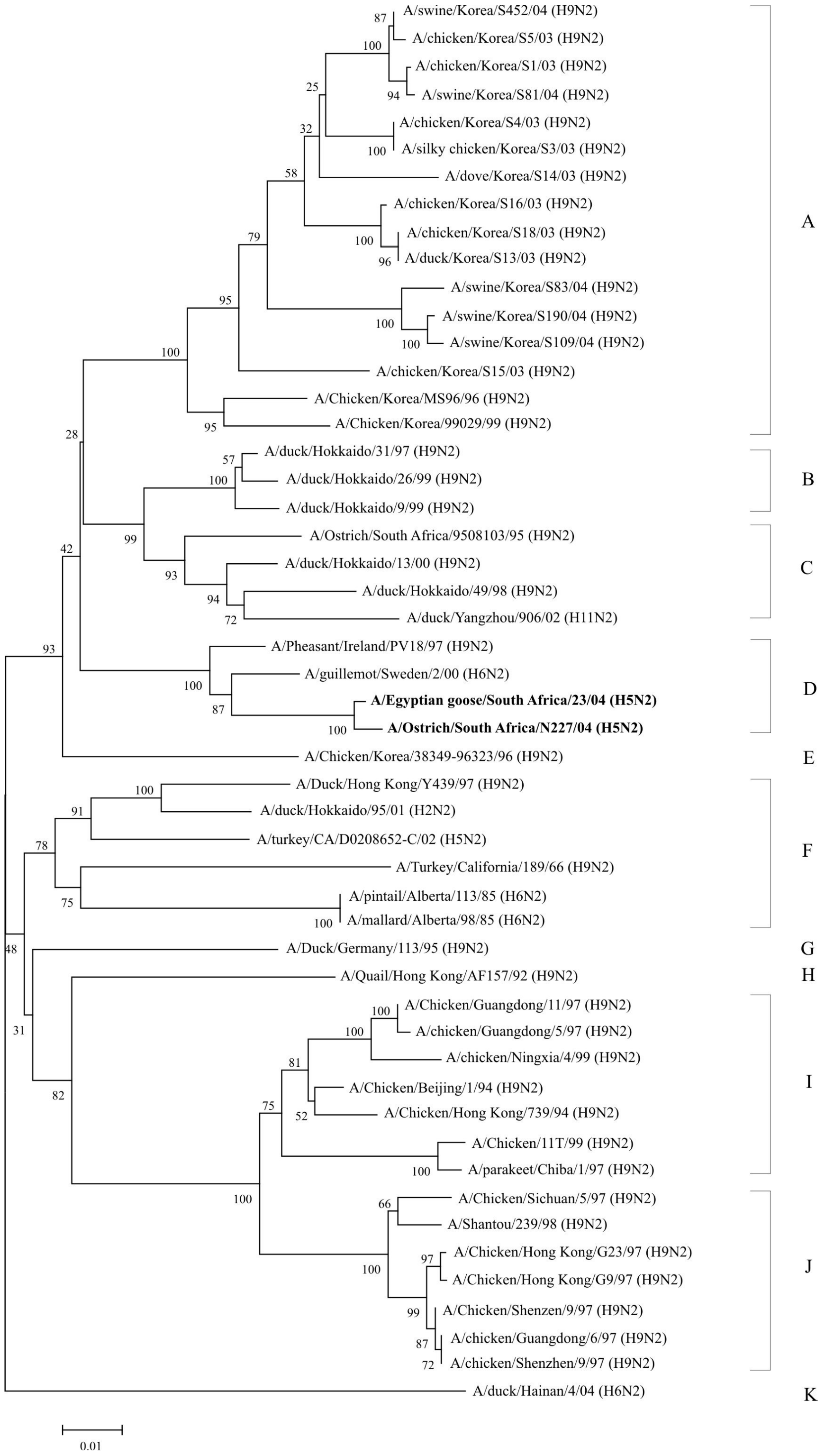

Figure 3.10 Phylogenetic tree inferred from a 1073-nt multiple sequence alignment of the NA (N2) genes of the outbreak strain A/Ostrich/South Africa/N227/05 (H5N2), A/Egyptian

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.

```
                                      40        50        60        70        80        90        100       110       120       130       140       150       160
                                    ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
[F] A/Dk/HK/Y439/97(H9N2)          ..................IL............K.............................G.S..........A..............TLN....R.......N........................
[F] A/Dk/Hokkaido/95/01(H2N2)      ..................IL..........................................G.S.........................T.......................................
[F] A/Tk/CA/D0208652-C/02(H5N2)    ..................IP............................................G.............N...................................................
[F] A/Ma/Alberta/98/85(H6N2)       ........A....T....IV.................................I........D.S.................................................................
[F] A/Pt/Alberta/113/85(H6N2)      ........A....T....IV.................................I........D.S.................................................................
[F] A/Tk/Calif/189/66(H9N2)        ......T.A...I.....ILK.........................................D.G............................................N....................
[I] A/Ck/Beijing/1/94(H9N2)        ......N...........I...K.......................................GLG.............N.......T...........................................
[I] A/Ck/HK/739/94(H9N2)           ......N.P.........I...K.......................................GLG.............N.......T...........................................
[I] A/Ck/Guangdong/11/97(H9N2)     ......N...............K.......................................GLG.............K.......T...........................................
[I] A/Ck/Guangdong/11/97(H9N2)     ......N...............K.......................................GLG.............K.......T...........................................
[I] A/Ck/Ningxia/4/99(H9N2)        ......N........Y......K..................S....................GLG.............K.......T...........................................
[I] A/Ck/IR/11T/99(H9N2)           .....TN.....A.....I...K.......................................GLG.............N.......T...S.......................................
[I] A/Pt/Chiba/1/90(H9N2)          .....TN.....A.....I...K.......................................GLS.............N.......T...S.......................................
[J] A/Ck/Guangdong/6/97(H9N2)      .....IN...........I.D.........................................GLG.............E.......AL..T................A......................
[J] A/Ck/Shenzhen/9/97(H9N2)       .....IN...........I.D.........................................GLG.............E.......AL..T................A......................
[J] A/Ck/Shenzen/9/97(H9N2)        .....IN...........I.D.........................................GLG.............E.......A...T................A......................
[J] A/Ck/HK/G23/97(H9N2)           .....INS..........I.D...................................D.....GLG.............E.......A...T................A......................
[J] A/Ck/HK/G9/97(H9N2)            .....INS..........I.D.........................................GLG.............E.......A...T................A.Q....................
[J] A/Ck/Sichuan/5/97(H9N2)        .....INS..........IVD.........................................GLD.....................S...T................A......................
[J] A/Shantou/239/98(H9N2)         .....INS..........I.D.........................................GLG.....................S...T................A......................
[H] A/Ql/HK/AF157/92(H9N2)         ..D.Y........AL...V...........................................GLG.............N...........T...........I...........................
[G] A/Dk/DE/113/95(H9N2)           ..S...............I.G.........................................GSG...........................................................R.....
[A] A/Sw/KR/S452/04(H9N2)          ............A......V...S...................V......S............S..................................................................
[A] A/Ck/KR/S5/03(H5N2)            ............A......V...S...................V......S............S..................................................................
[A] A/Ck/KR/S1/03(H9N2)            ............A......V...S...................V......S............S..................................................................
[A] A/Sw/KR/S81/04(H9N2)           ............A......V...S...................V......S............S..................................................................
[A] A/Ck/KR/S4/03(H9N2)            ........L...A......V...S..........................S............S.................................................................I
[A] A/silky Ck/KR/S3/03(H9N2)      ........L...A......V...S..........................S............S.................................................................I
[A] A/Do/KR/S14/03(H9N2)           ............A......V...S..........................S..I.........S.................................................................I
[A] A/Ck/KR/S18/03(H9N2)           ......T.....A......V...S..........................S............S.................................................................I
[A] A/Dk/KR/S13/03(H9N2)           ......T.....A......V...S..........................S............S.................................................................I
[A] A/Ck/KR/S16/03(H9N2)           ......T.....A......V...S..........................S............S......................................G..........................I
[A] A/Sw/KR/S190/04(H9N2)          ....G..............VK..S.................D........S............S................R.................................................
[A] A/Sw/KR/S109/04(H9N2)          ....G..............VK..S.................D........S............S................R.................................................
[A] A/Sw/KR/S83/04(H9N2)           ....G..............VK..S.................D........S............S................R.................................................
[A] A/Ck/KR/S15/03(H9N2)           ...................V...S..........................S............S................R.................................................
[A] A/Ck/KR/MS96/96(H9N2)          .........S.........V..............................S............SD.................................................................
[A] A/Ck/KR/99029/99(H9N2)         .....RTS.S..A......V..............................G............SD.................................................................
[D] A/EG/ZA/AI23/04(H5N2)          ................D..V............A...............................DT................................................................
[D] A/Os/ZA/N227/04(H5N2)          ................D..V............A...............................DT................................................................
[D] A/Guillemot/SE/3/00(H6N2)      ................D.IV............A....L..........................D.................................................................
[D] A/Ph/IE/PV18/97(H9N2)          ...K............D..V............A...............................D.................................................................
[C] A/Dk/Hokkaido/49/98(H9N2)      ...G..............IL...D..I.......S...............S............SD.................................................................
[C] A/Dk/Yangzhou/906/02(H11N2)    .............M....ILK..D........A...............................D.................................................................
[C] A/Dk/Hokkaido/13/00(H9N2)      ..................IL...D........................................D.................................................................
[C] A/Os/ZA/9508103/95(H9N2)       ...G..............IL...D.............................I..........D.................................................................
[B] A/Dk/Hokkaido/31/97(H9N2)      ...G..............II...D........................................D.................................................................
[B] A/Dk/Hokkaido/26/99(H9N2)      ...G...L..........II...D........................................D..N..............................................................
[B] A/Dk/Hokkaido/9/99(H9N2)       ...G..............IV...D..........I.........L...................D....L............................................................
[E] A/Ck/KR/38349-96323/96(H9N2)   ..............T...IV..........R.................................D............................................R....................
[K] A/Dk/Hainan/4/04(H6N2)         ..D..N...K.........V............................................G...............S...........................................R.....
Consensus                          KQNECSIPSNNQVVPCEPMAEYRNWSKPQCQITGFAPFSKDNSIRLSAGGDIWVTREPYVSCSPNKCYQFALGQGTTLDNKHSNGTIHDRIPHRTLLMNELGVPFHLGTKQVCIAWSSSSCHDGKAWLHV
```

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

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                                    170       180       190       200       210       220       230       240       250       260       270       280       290
                                  ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
[F] A/Dk/HK/Y439/97(H9N2)         ...........K...N.................................................R...........................................V.....T..............
[F] A/Dk/Hokkaido/95/01(H2N2)     ...............N...........................I.....................R...........................................V.....V..............
[F] A/Tk/CA/D0208652-C/02(H5N2)   ......................................................................I......................................V....................
[F] A/Ma/Alberta/98/85(H6N2)      ...................I.....................S.......................R................V..........N...............V....................
[F] A/Pt/Alberta/113/85(H6N2)     ...................I.....................S.......................R................V..........N...............V....................
[F] A/Tk/Calif/189/66(H9N2)       .............H..............................................K....R....I......................N...............V....................
[I] A/Ck/Beijing/1/94(H9N2)       ............I..............K.....................................R................V..........E...............VLY..................
[I] A/Ck/HK/739/94(H9N2)          ............I..............K.....................................R................V..........E...............VLY...V..............
[I] A/Ck/Guangdong/11/97(H9N2)    ......K.....I..............K............................K........R................V..........E...............VLY........E.........
[I] A/Ck/Guangdong/11/97(H9N2)    ......K.....I..............K............................K........R................V..........E...............VLY........K.........
[I] A/Ck/Ningxia/4/99(H9N2)       ......K.....I..N...........K............................K........R................V..........E...............VLY........E.........
[I] A/Ck/IR/11T/99(H9N2)          ............I......T.......KS....................................R................V..........E...............VLY..V.........L.....
[I] A/Pt/Chiba/1/90(H9N2)         ............I......T.......KS....................................R................V..........E...............VLY..V...............
[J] A/Ck/Guangdong/6/97(H9N2)     ............I....I.........K.................A...................R....A...........V..........E...............VLY....N.............
[J] A/Ck/Shenzhen/9/97(H9N2)      ............I....I.........K.................A...................R....A...........V..........E...............VLY....N.............
[J] A/Ck/Shenzen/9/97(H9N2)       ............I....I.........K.................A...................R....A...........V..........E...............VLY....N.............
[J] A/Ck/HK/G23/97(H9N2)          ............I....I.........K.................A...................R....A...........V..........E...............VLY....N.............
[J] A/Ck/HK/G9/97(H9N2)           ............I....I.........K.................A...................R....A...........V..........E...............VLY....N.............
[J] A/Ck/Sichuan/5/97(H9N2)       ............I..N.I.........K.................A...................R................V..........E...............VLY....N.............
[J] A/Shantou/239/98(H9N2)        ............I....I.........K.....................................R................V..........E...............VLY...EN.N...........
[H] A/Ql/HK/AF157/92(H9N2)        ............I....................................................R................V.......Q..N................V........VG.........
[G] A/Dk/DE/113/95(H9N2)          .........................................D...................V...R......S.........V...............................................
[A] A/Sw/KR/S452/04(H9N2)         ..........................................................................Q......................................K................
[A] A/Ck/KR/S5/03(H5N2)           ..........................................................................Q......................................K................
[A] A/Ck/KR/S1/03(H9N2)           .............................................................V............Q.......................Y..............K................
[A] A/Sw/KR/S81/04(H9N2)          .............................................................V............Q.......................Y..............K................
[A] A/Ck/KR/S4/03(H9N2)           ..........................................................................Q.......................................................
[A] A/silky Ck/KR/S3/03(H9N2)     ..........................................................................Q.......................................................
[A] A/Do/KR/S14/03(H9N2)          ..........................................................................Q.......................................................
[A] A/Ck/KR/S18/03(H9N2)          ..S.......GC..............................................................Q............................................T..........
[A] A/Dk/KR/S13/03(H9N2)          ..S.......GC..............................................................Q............................................T..........
[A] A/Ck/KR/S16/03(H9N2)          ..........................................................................Q............................................T..........
[A] A/Sw/KR/S190/04(H9N2)         .................T...T.......................................V............Q.......................Y..............K................
[A] A/Sw/KR/S109/04(H9N2)         .................T...T.......................................V............Q.......................Y..............K................
[A] A/Sw/KR/S83/04(H9N2)          .................T...T....................................................Q......................................K................
[A] A/Ck/KR/S15/03(H9N2)          .................T...T.................................WT..................................H......................................
[A] A/Ck/KR/MS96/96(H9N2)         ..S............................................................................................................N.....H............
[A] A/Ck/KR/99029/99(H9N2)        ................................................................................K.................................................
[D] A/EG/ZA/AI23/04(H5N2)         ...............N.................................................R....I......................N....................................
[D] A/Os/ZA/N227/04(H5N2)         ...............N.................................................R....I....................H......................................
[D] A/Guillemot/SE/3/00(H6N2)     ...............N......................X..........................R....I...........................................................
[D] A/Ph/IE/PV18/97(H9N2)         ...............N.................................................R....I...Q.......................................................
[C] A/Dk/Hokkaido/49/98(H9N2)     ........................................................V.......VE.....................................R..............R...........
[C] A/Dk/Yangzhou/906/02(H11N2)   ......................................I.........................V.................................................................
[C] A/Dk/Hokkaido/13/00(H9N2)     ........................................................................................................................N.........
[C] A/Os/ZA/9508103/95(H9N2)      .................V...........................A..........................V......................K..................V...............
[B] A/Dk/Hokkaido/31/97(H9N2)     ...............G.V...............................................................................................S................
[B] A/Dk/Hokkaido/26/99(H9N2)     ......S........G.V...............................................................................................S................
[B] A/Dk/Hokkaido/9/99(H9N2)      ..S............G.V...............................................................................................S................
[E] A/Ck/KR/38349-96323/96(H9N2)  ......K..........I........A.........................G.............................................................................
[K] A/Dk/Hainan/4/04(H6N2)        .I.................A.......K.................A..........V.......AR...............................I...........VV...................
Consensus                         CVTGDDRNATASFIYDGMLVDSIGSWSQNILRTQESECVCINGTCTVVMTDGSASGRADTRILFIKEGKIVHISPLSGSAQHIEECSCYPRYPDVRCVCRDNWKGSNRPIIDINMADYSIDSSYVCSGLV
```

Figure 3.11 continued

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                                   300       310       320       330       340       350       360       370       380       390
                                   ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
[F] A/Dk/HK/Y439/97(H9N2)          .....Y.....S.....................N...........................A.....L................--V........V.
[F] A/Dk/Hokkaido/95/01(H2N2)      ...........S.....................N...........................A........D...........C.SCV........V.
[F] A/Tk/CA/D0208652-C/02(H5N2)    ...........S.....................N..................................................--V..........
[F] A/Ma/Alberta/98/85(H6N2)       ...........S.....................N.....................K.....AI......T..............--V..........
[F] A/Pt/Alberta/113/85(H6N2)      ...........S.....................N.....................K.....AI......T..............--V..........
[F] A/Tk/Calif/189/66(H9N2)        .................................N.DM..........L.............A.P....................--V....RV....
[I] A/Ck/Beijing/1/94(H9N2)        ...........S...........A.........N...I......K............V...........I..P....SD.....--VS.........
[I] A/Ck/HK/739/94(H9N2)           ...........S...........A.........N...I......KE...........V...........I.......SD.....--VS.........
[I] A/Ck/Guangdong/11/97(H9N2)     ...........S...........A.........N...I......KE.L.........V...........I.......SD.....--VS......T..
[I] A/Ck/Guangdong/11/97(H9N2)     ........N..S...........A.........N...I......KE.L.........V...........I.......SD.....--VS......T..
[I] A/Ck/Ningxia/4/99(H9N2)        ...........S...K.......A.........N...I......KE...........V...........I.......SE.....--VS......I..
[I] A/Ck/IR/11T/99(H9N2)           ...........S...........G.........N..........K....A.......V...........I.......SD.L...--VY.........
[I] A/Pt/Chiba/1/90(H9N2)          ...........S...........G.........N..........K....A.......V...........I.......SD.....--VY......S..
[J] A/Ck/Guangdong/6/97(H9N2)      ........R..S...........A.........N...I......K........................I.......SD.S...--VS.........
[J] A/Ck/Shenzhen/9/97(H9N2)       ........R..S...........A.........N...I......K........................I.......SD.S...--VS.........
[J] A/Ck/Shenzen/9/97(H9N2)        ........R..S...........A.........N...I......K........................I.......SD.S...--VS.........
[J] A/Ck/HK/G23/97(H9N2)           ........R..S...........A.........N...I......K........................I.......SD.S...--VS.........
[J] A/Ck/HK/G9/97(H9N2)            ........R..S...........A.........N...I......K........................I.......SD.S...--VS.........
[J] A/Ck/Sichuan/5/97(H9N2)        ........R..S...........A.........N...I......KR................N......I.......SD.S...--VS.......V.
[J] A/Shantou/239/98(H9N2)         ........R..S...........A.........N.D.I......K.................I..............SDSS...--VS.........
[H] A/Ql/HK/AF157/92(H9N2)         ...........S.....................N..........N........................A..............--VL....V....
[G] A/Dk/DE/113/95(H9N2)           ...........S.......S.............D...........................N......................--V.......V..
[A] A/Sw/KR/S452/04(H9N2)          ...............K.......................................K..D.........................--...........
[A] A/Ck/KR/S5/03(H5N2)            ...............K...................H...................K..D.........................--.........
[A] A/Ck/KR/S1/03(H9N2)            .....D.........K.......................................K..D.........................--...........
[A] A/Sw/KR/S81/04(H9N2)           .....D.........K.......................................K..D.................G.......--...........
[A] A/Ck/KR/S4/03(H9N2)            ...............K.......................................K.........A.................I--....-......
[A] A/silky Ck/KR/S3/03(H9N2)      ...............K.......................................K.........A.................I--....-......
[A] A/Do/KR/S14/03(H9N2)           ...............K.......................................K..D.....Y.....T..A..AITTC...--P.....P.I..
[A] A/Ck/KR/S18/03(H9N2)           ...............K.............................................A......................--...........
[A] A/Dk/KR/S13/03(H9N2)           ...............K.............................................A......................--...........
[A] A/Ck/KR/S16/03(H9N2)           ...............K.............................................A......................--...........
[A] A/Sw/KR/S190/04(H9N2)          .....D.........K.......................................K..D.........................--...........
[A] A/Sw/KR/S109/04(H9N2)          .....D.........K.......................................K..D.........................--...........
[A] A/Sw/KR/S83/04(H9N2)           ...............K.......................................K..D........................C--...........
[A] A/Ck/KR/S15/03(H9N2)           ....................................................................................--E.......V..
[A] A/Ck/KR/MS96/96(H9N2)          ........G......K...........................N.......................................--...........
[A] A/Ck/KR/99029/99(H9N2)         ...............K....................................................................--.S.........
[D] A/EG/ZA/AI23/04(H5N2)          ...............K.........................................T..........................--V.......T..
[D] A/Os/ZA/N227/04(H5N2)          ...........K...K................................H........T..........................--V.......T..
[D] A/Guillemot/SE/3/00(H6N2)      ...............K...................D.....................T..........................--V......NT..
[D] A/Ph/IE/PV18/97(H9N2)          ...............K..............................................A.....................--V......NT.
[C] A/Dk/Hokkaido/49/98(H9N2)      ...............K.........................................................I..........--..........L
[C] A/Dk/Yangzhou/906/02(H11N2)    ...............K..............................................M.....................--..F.......L
[C] A/Dk/Hokkaido/13/00(H9N2)      ...............K..................................................................C.--.....T....L
[C] A/Os/ZA/9508103/95(H9N2)       ...............K....................................................................--..........L
[B] A/Dk/Hokkaido/31/97(H9N2)      ...............K...................S...............F................................--...........
[B] A/Dk/Hokkaido/26/99(H9N2)      ...............K...................S..............................................C.--...........
[B] A/Dk/Hokkaido/9/99(H9N2)       ...............K...................S..............................................C.--...........
[E] A/Ck/KR/38349-96323/96(H9N2)   ......E........K.......................................K......M.....................--......E....
[K] A/Dk/Hainan/4/04(H6N2)         ...........S.....................D...........R.........K.....I......................--V.........
             Consensus                          GDTPRNDDSSSNSNCRDPNNERGNPGVKGWAFDYGNDVWMGRTISKDSRSGYETFRVIGGWTTANSKSQVNRQVIVDNNNWSGY—AFSLLKAKAAS
```

3.3.2.2 Neuraminidase (N8) genes

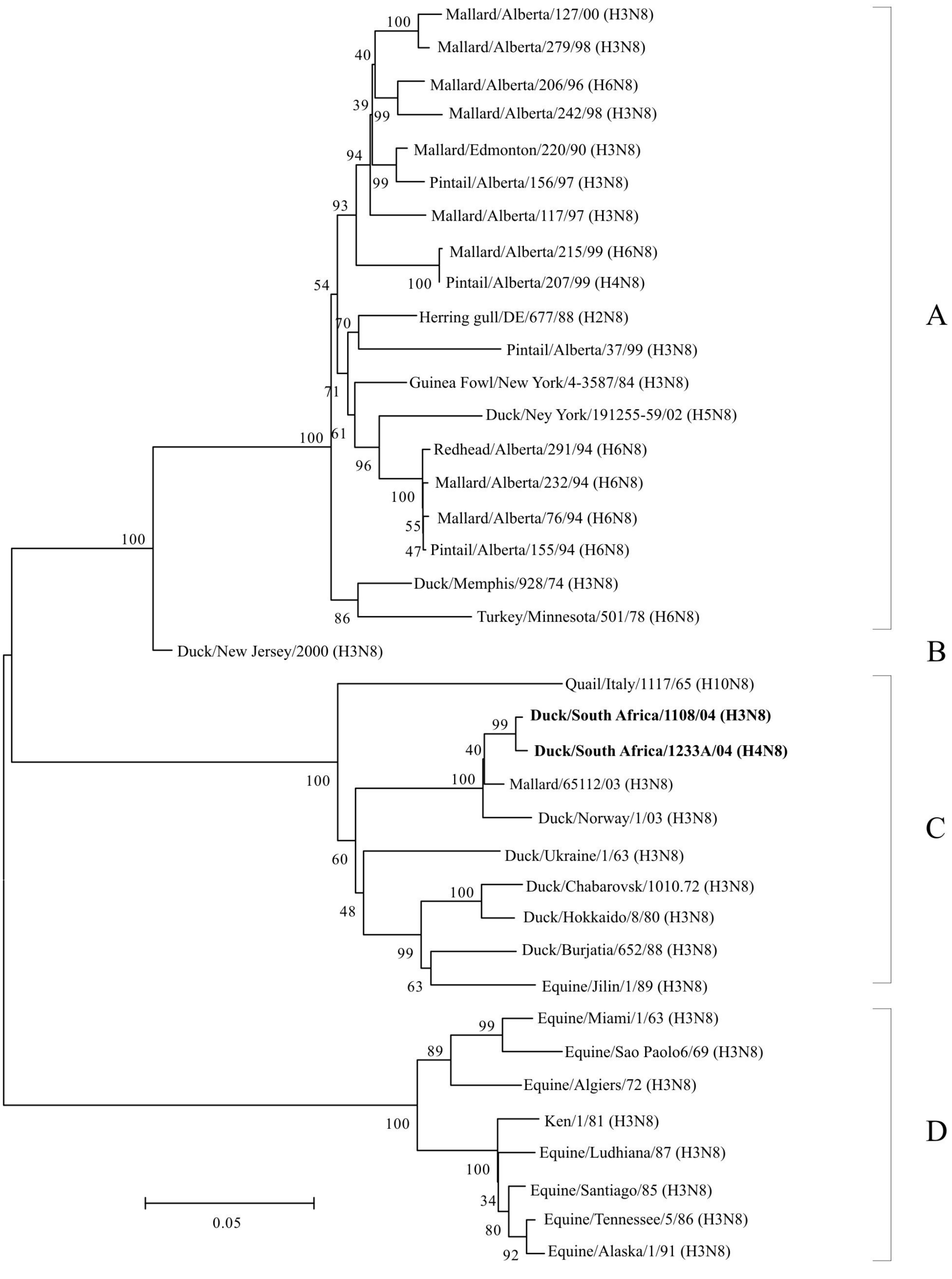

Figure 3.12 Phylogenetic tree inferred from a 1305-nt multiple sequence alignment of the N8 genes of A/Duck/South Africa/1233/04 (H4N8) and A/Duck/South Africa/1108/04 (H3N8) (in boldface) and related sequences. Sub-lineages A to D are indicated.

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

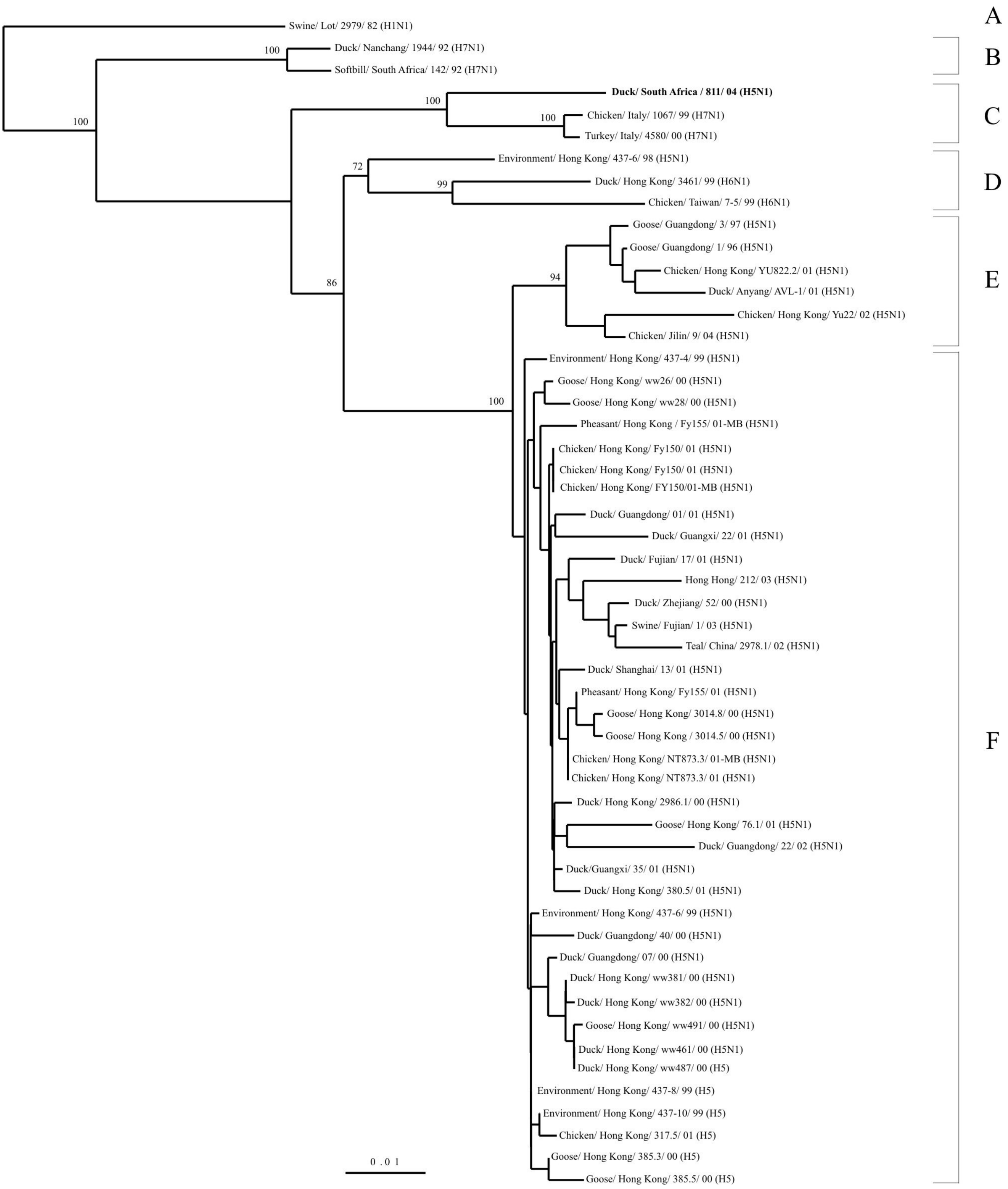

Figure 3.13 Phylogenetic trees inferred from a 1025-nucleotide multiple sequence alignment of the N1 genes of A/Duck/South Africa/811/04 (H5N1) (in boldface) and related sequences. Sub-lineages A to F are indicated.

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}.

```
                                40        50        60        70        80        90       100       110       120       130       140       150       160
                              ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
[A] Swine/Lot/2979/82(H1N1)   .............I...N.P.T....V.....................VA.Q....................I......V..................................................
[A] Dk/Nanchang/1944/92(H7N1) .................N.P...............................Q...............V....I.........................................................
[A] softbill/ZA/142/92(H7N1)  ..............E..N.P..............................KQ...............V....I.........................................T...............
[B] Ck/Italy/1067/99(H7N1)    .............---------------------------------.QS..Q..T.................I.........................................................
[B] Tk/Italy/4580/99(H7N1)    .............-------------------------GINTSL.C.QS..Q..T.................I.........................................................
[B] Duck/ZA/811/04(H5N1)      .................Y.P.S.............................QT.T..A..............I.........................................................
[D] Dk/HK/3461/99 (H6N1)      ...................PK..............................Q....................I..............I..........................................
[D] Ck/Taiwan/7-5/99(H6N1)    .......I.....-------------C........................Q....I...............I.........................................................
[D] Af.starl./UK/983/79(H7N1) .................N.P.T....T..................N...A.Q..S.................I.........................................................
[D] Aq.Bd/HK/m603/98(H11N1)   ...................P...............................Q..T.................IH........................................................
[D] Env/HK/437-6/99 (H5N1)    ..................................................................................................................................
[D] Ck/HK/YU22/02(H5N1)       ..M...................--------------------...............................H....S.................................................H.
[D] DK/Anyang/AVL-1/01(H5N1)  ..............E.......--------------------...............................H......................................................H.
[D] Gs/Guangdong/1/96(H5N1)   .........................................................................H......................................................H.
[D] Ck/HK/YU822.2/01(H5N1)    .........................................................................H......................................................H.
[D] Gs/Guangdong/3/97(H5N1)   .........................................................................H....................................................G.H.
[D] Env/HK/437-8/99 (H5N1)    ..................................................................................................................................
[D] Gs/HK/385.3/00(H5N1)      .....................................................M............................................................................
[D] Env/HK/437-10/99 (H5N1)   ..............................................H...................................................................................
[D] Ck/HK/317.5/01(H5N1)      ..................................................................................................................................
[D] Env/HK/437-4/99 (H5N1)    ........................................................................I.........................................................
[D] Gs/HK/ww26/00(H5N1)       ..............................................................................S...................................................
[D] Dk/HK/ww381/00(H5N1)      ..................................................................................................................................
[D] Dk/HK/ww382/00(H5N1)      ...................................................N..............................................................................
[D] Dk/HK/ww461/00(H5N1)      ..................................................................................................................................
[D] Dk/HK/ww487/00(H5N1)      ..................................................................................................................................
[D] Gs/HK/ww491/00(H5N1)      ..................................................................................................................................
[D] Gs/HK/ww491/00(H5N1)      ...........................................G......................................................................................
[D] Dk/Guangdong/40/00(H5N1)  ..................................................................................................................................
[D] Gs/HK/385.5/00(H5N1)      ........T............................................M.........................L..............L...................................
[D] Gs/HK/3014.8/00(H5N1)     .......I................................I.........................................................................................
[D] Gs/HK/3014.5/00(H5N1)     .......I................................I.........................................................................................
[D] swine/Fujian/1/03(H5N1)   .......I............................................T...........................................................................H.
[D] Dk/Zhejiang/52/00(H5N1)   ....................................................T...........................................................................H.
[D] teal/CH/2978.1/02(H5N1)   .......................I............................T...........................................................................H.
[D] Ck/HK/NT873.3/01-MB(H5N1) ..................................................................................................................................
[D] Ck/HK/NT873.3/01(H5N1)    ..................................................................................................................................
[D] Ph/HK/FY155/01(H5N1)      .......I.......................................L..................................................................................
[D] Ck/HK/FY150/01(H5N1)      ...............................................L..................................................................................
[D] Ck/HK/FY150/01-MB(H5N1)   ...............................................L..................................................................................
[D] Ck/HK/FY150/01(H5N1)      ...............................................L..................................................................................
[D] Gs/HK/ww28/00(H5N1)       ...................................G...........L..............P...............S...................................................
[D] Dk/HK/2986.1/00(H5N1)     ..................................................................................................................................
[D] Dk/Guangxi/35/01(H5N1)    ..............E......................................A............................................................................
[D] Dk/Shanghai/13/01(H5N1)   .......A............................................T.............................................................................
[D] Dk/Guangdong/01/01(H5N1)  ..................................................................................................................................
[D] Dk/HK/380.5/01(H5N1)      ..................................................................................................................................
[D] Dk/Fujian/17/01(H5N1)     ...................T............................................................................................................H.
[D] Gs/HK/76.1/01 (H5N1)      ...........................................................................................K.............L..N.E..M................
[D] Dk/Guangxi/22/01(H5N1)    ...............D.......S...........................................V....I.........................................................
[D] HK/212/03(H5N1)           ................................................................................................................................H.
[D] Dk/Guangdong/22/02(H5N1)  ....P......................................N..................................................................E...................
[D] Ph/HK/FY155/01-MB(H5N1)   ...............................................L..................................................................................
[D] Ck/Jilin/9/04(H5N1)       ..M..........----------------------GINAKL.....K.................................................................................H.
Consensus                     GNIISIWVSHSIQTGNQHQAEPCNQSIITYENNTWVNQTYVNISNTNFLTEKAVASVTLAGNSSLCPISGWAVYSKDNGIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPYR
```

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

Fig. 3.14 continued

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                                      170       180       190       200       210       220       230       240       250       260       270       280       290
                                ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
[A] Swine/Lot/2979/82(H1N1)     .........V.....................I....................................................I...........................................S.
[A] Dk/Nanchang/1944/92(H7N1)   ...............................I.............................N....................................................................
[A] softbill/ZA/142/92(H7N1)    ...............................I..................................................................................................
[B] Ck/Italy/1067/99(H7N1)      ................K..............I..................................................................................................
[B] Tk/Italy/4580/99(H7N1)      ...............................I..................................................................................................
[B] Duck/ZA/811/04(H5N1)        ...............................I...........................................V.......................S..............................
[D] Dk/HK/3461/99 (H6N1)        ...............................I...................................M..............................................................
[D] Ck/Taiwan/7-5/99(H6N1)      ...............................I..................................................................................................
[D] Af.starl./UK/983/79(H7N1)   ...............................I...........................V......................................................................
[D] Aq.Bd/HK/m603/98(H11N1)     ...............................I..................................................................................................
[D] Env/HK/437-6/99 (H5N1)      ..................................................................................................................................
[D] Ck/HK/YU22/02(H5N1)         .....................................................................................................M...........D................
[D] DK/Anyang/AVL-1/01(H5N1)    .....................................................................................................M...........D................
[D] Gs/Guangdong/1/96(H5N1)     .....................................................................................................M............................
[D] Ck/HK/YU822.2/01(H5N1)      .....................................................................................................M...........D................
[D] Gs/Guangdong/3/97(H5N1)     .....................................................................................................M............................
[D] Env/HK/437-8/99 (H5N1)      ..................................................................................................................................
[D] Gs/HK/385.3/00(H5N1)        ..................................................................................................................................
[D] Env/HK/437-10/99 (H5N1)     ..................................................................................................................................
[D] Ck/HK/317.5/01(H5N1)        ..................................................................................................................................
[D] Env/HK/437-4/99 (H5N1)      ..................................................................................................................................
[D] Gs/HK/ww26/00(H5N1)         ..................................................................................................................................
[D] Dk/HK/ww381/00(H5N1)        ...........................................S......................................................................................
[D] Dk/HK/ww382/00(H5N1)        ...........................................S......................................................................................
[D] Dk/HK/ww461/00(H5N1)        ...........................................S......................................................................................
[D] Dk/HK/ww487/00(H5N1)        ...........................................S......................................................................................
[D] Gs/HK/ww491/00(H5N1)        ...........................................S......................................................................................
[D] Gs/HK/ww491/00(H5N1)        ..................................................................................................................................
[D] Dk/Guangdong/40/00(H5N1)    ................................G.................................................................................................
[D] Gs/HK/385.5/00(H5N1)        ..................................................................................................................................
[D] Gs/HK/3014.8/00(H5N1)       .....................................................................................................V.................N..........
[D] Gs/HK/3014.5/00(H5N1)       ...........T.........................................................................................V............................
[D] swine/Fujian/1/03(H5N1)     ....................................................................................................R.............................
[D] Dk/Zhejiang/52/00(H5N1)     ....................................................................................................R.............................
[D] teal/CH/2978.1/02(H5N1)     ................................N...................................................................R.............................
[D] Ck/HK/NT873.3/01-MB(H5N1)   ..................................................................................................................................
[D] Ck/HK/NT873.3/01(H5N1)      ..................................................................................................................................
[D] Ph/HK/FY155/01(H5N1)        ..................................................................................................................................
[D] Ck/HK/FY150/01(H5N1)        ..................................................................................................................................
[D] Ck/HK/FY150/01-MB(H5N1)     ..................................................................................................................................
[D] Ck/HK/FY150/01(H5N1)        ..................................................................................................................................
[D] Gs/HK/ww28/00(H5N1)         ..................................................................................................................................
[D] Dk/HK/2986.1/00(H5N1)       ..................................................................................................................................
[D] Dk/Guangxi/35/01(H5N1)      ..................................................................................................................................
[D] Dk/Shanghai/13/01(H5N1)     ..................................................................................................................................
[D] Dk/Guangdong/01/01(H5N1)    ..................................................................................................................................
[D] Dk/HK/380.5/01(H5N1)        ..................................................................................................................................
[D] Dk/Fujian/17/01(H5N1)       .................................................................................................R..R.............................
[D] Gs/HK/76.1/01 (H5N1)        ........K.....................................................................................................................HN.S
[D] Dk/Guangxi/22/01(H5N1)      ...............................................................M..............S...................................................
[D] HK/212/03(H5N1)             ...................................................................M................................R.........A...................
[D] Dk/Guangdong/22/02(H5N1)    .....................................................................................V................V.....Q.................L...
[D] Ph/HK/FY155/01-MB(H5N1)     .....................................................................................................M............................
[D] Ck/Jilin/9/04(H5N1)         .............................................................................I.......................M...........D................
Consensus                       TLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASYKIFKIEKGKVVKSVELNAPNYHYEECSCYPDAG
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Fig. 3.14 continued

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                                      300       310       320       330       340       350       360       370
                               ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
[A] Swine/Lot/2979/82(H1N1)    ..................................I..................S.....V.......D...........S.
[A] Dk/Nanchang/1944/92(H7N1)  .....................................................S.....V...................S.
[A] softbill/ZA/142/92(H7N1)   .....................................................S.....V...................S.
[B] Ck/Italy/1067/99(H7N1)     ..M................................................M.S.....V...................S.
[B] Tk/Italy/4580/99(H7N1)     ..M................................................M.S.....V...................S.
[B] Duck/ZA/811/04(H5N1)       K.M..................................................S.....V...................S.
[D] Dk/HK/3461/99 (H6N1)       ..................................I..................S.....V...................S.
[D] Ck/Taiwan/7-5/99(H6N1)     ..................................I..................S...F.V.................NIS.
[D] Af.starl./UK/983/79(H7N1)  .....................................................S.....V...................S.
[D] Aq.Bd/HK/m603/98(H11N1)    .....................................................S.....V...................S.
[D] Env/HK/437-6/99 (H5N1)     ...........................................................V.....................
[D] Ck/HK/YU22/02(H5N1)        ...........................................................V.....................
[D] DK/Anyang/AVL-1/01(H5N1)   ...................................................M.L.....V.....................
[D] Gs/Guangdong/1/96(H5N1)    ...........................................................V.....................
[D] Ck/HK/YU822.2/01(H5N1)     ...........................................................V.....................
[D] Gs/Guangdong/3/97(H5N1)    ...........................................................V.....................
[D] Env/HK/437-8/99 (H5N1)     ...........................................................V.....................
[D] Gs/HK/385.3/00(H5N1)       ...........................................................V.....................
[D] Env/HK/437-10/99 (H5N1)    ...........................................................V.....................
[D] Ck/HK/317.5/01(H5N1)       ...........................................................V.....................
[D] Env/HK/437-4/99 (H5N1)     ...........................................................V.....................
[D] Gs/HK/ww26/00(H5N1)        ...........................................................V.....................
[D] Dk/HK/ww381/00(H5N1)       ..........................K................................V.....................
[D] Dk/HK/ww382/00(H5N1)       ..........................K................................V.....................
[D] Dk/HK/ww461/00(H5N1)       ..........................K................................V.....................
[D] Dk/HK/ww487/00(H5N1)       ..........................K................................V.....................
[D] Gs/HK/ww491/00(H5N1)       ..........................K................................V.....................
[D] Gs/HK/ww491/00(H5N1)       ..........................K................................V.....................
[D] Dk/Guangdong/40/00(H5N1)   .....................K...............................S.....V.....................
[D] Gs/HK/385.5/00(H5N1)       ...........................................................V.....................
[D] Gs/HK/3014.8/00(H5N1)      .................................................................................
[D] Gs/HK/3014.5/00(H5N1)      .................................................................................
[D] swine/Fujian/1/03(H5N1)    ..................................I..............................................
[D] Dk/Zhejiang/52/00(H5N1)    ..................................I..............................................
[D] teal/CH/2978.1/02(H5N1)    ..................................I..................S............Y.............P
[D] Ck/HK/NT873.3/01-MB(H5N1)  .................................................................................
[D] Ck/HK/NT873.3/01(H5N1)     .................................................................................
[D] Ph/HK/FY155/01(H5N1)       .................................................................................
[D] Ck/HK/FY150/01(H5N1)       .................................................................................
[D] Ck/HK/FY150/01-MB(H5N1)    .................................................................................
[D] Ck/HK/FY150/01(H5N1)       .................................................................................
[D] Gs/HK/ww28/00(H5N1)        ...........................................................V.....................
[D] Dk/HK/2986.1/00(H5N1)      .................................................................................
[D] Dk/Guangxi/35/01(H5N1)     .................................................................................
[D] Dk/Shanghai/13/01(H5N1)    .................................................................................
[D] Dk/Guangdong/01/01(H5N1)   ..I........................M.....................................................
[D] Dk/HK/380.5/01(H5N1)       ........S...................................................................R....
[D] Dk/Fujian/17/01(H5N1)      ........................K........................................................
[D] Gs/HK/76.1/01 (H5N1)       V...................I............................................................
[D] Dk/Guangxi/22/01(H5N1)     ..I................................................M.............................
[D] HK/212/03(H5N1)            ..........................R...........................K..........................
[D] Dk/Guangdong/22/02(H5N1)   .........................................................H.......................
[D] Ph/HK/FY155/01-MB(H5N1)    .................................................................................
[D] Ck/Jilin/9/04(H5N1)        ...........................................................V.....................
Consensus                      EITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVSPNGAYGIKGFSFKYGNGVWIGRTKSTNS
```

3.3.3 Matrix protein (M) genes

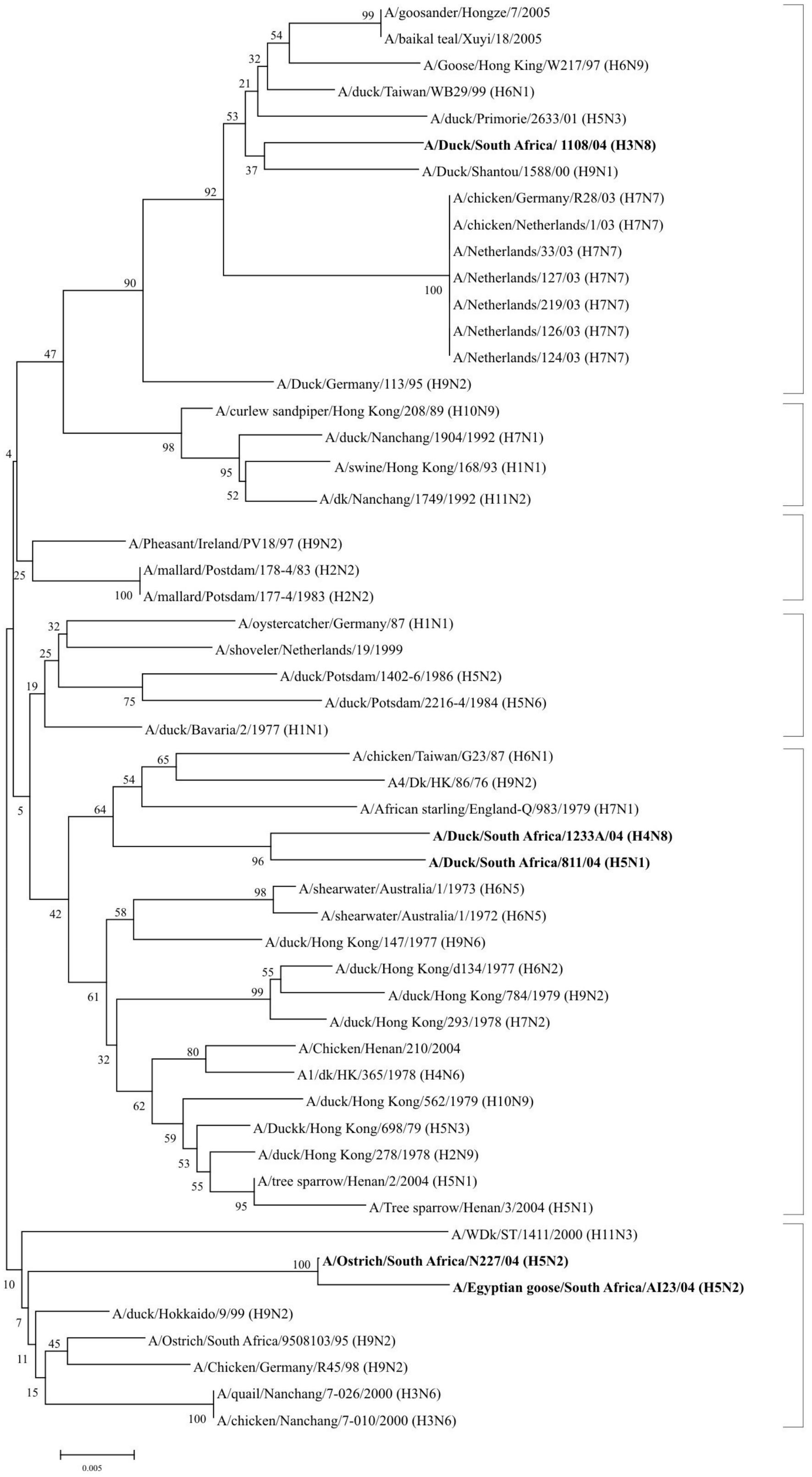

Figure 3.15 Phylogenetic tree inferred from a 674-nt multiple sequence alignment of the M genes of South African AI viruses isolated in 2004 (in boldface) and related sequences. Sub-lineages A to F are indicated.

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

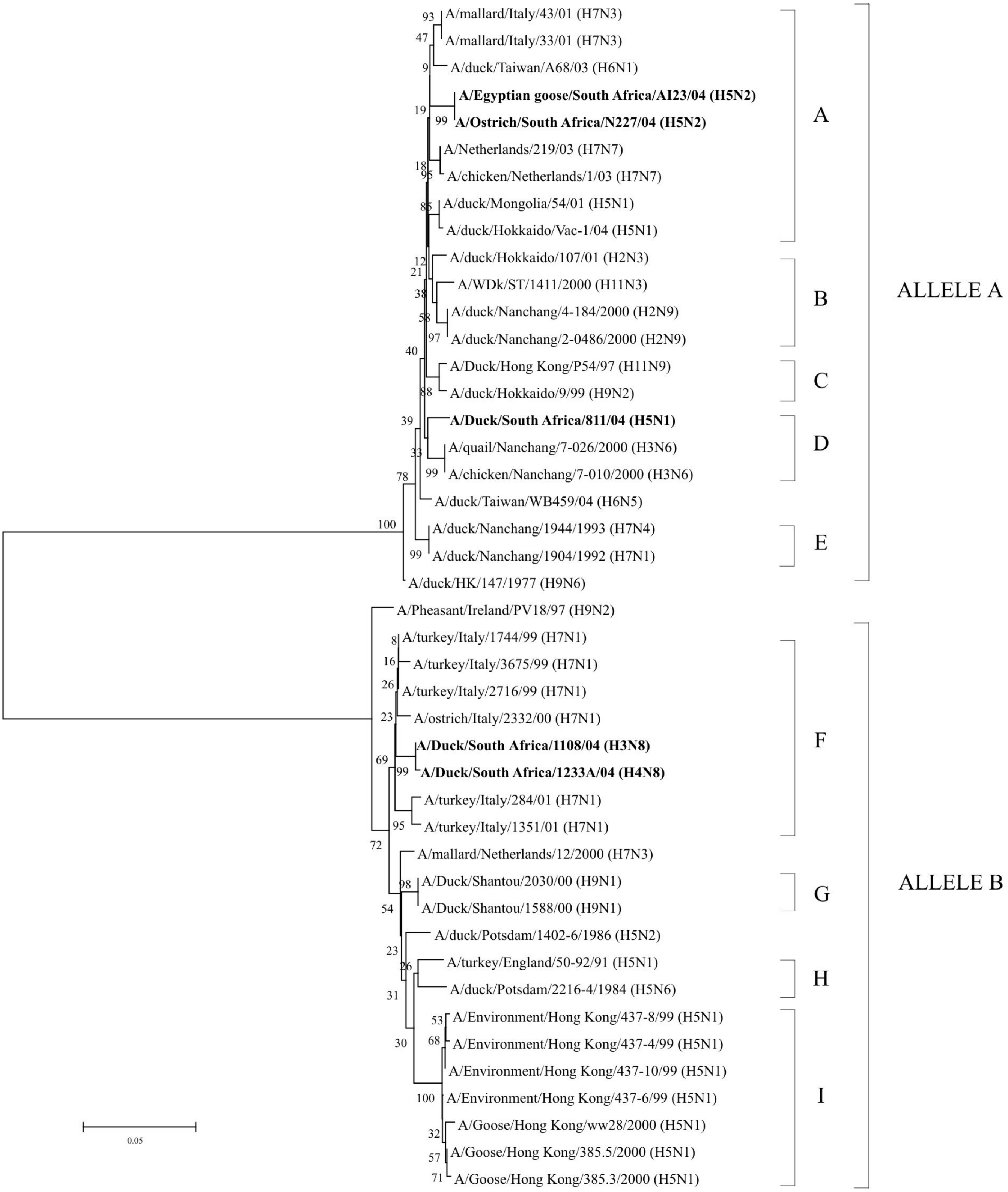

Figure 3.16 Phylogenetic tree inferred from a 675-nt multiple sequence alignment of the NS1 genes of South African AI viruses isolated in 2004 (in boldface) and related sequences. Alleles A and B and sub-lineages A to I are indicated.

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.

```
                                 10        20        30        40        50        60        70        80        90       100       110       120       130       140       150
                                 |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
[N] A/DK/HK/T37/97(H11N8)        ............................................................................T...............................K....................................T....
[N] A/DK/HK/T25/97(H11N8)        ............................................................................................................K....................................T....
[N] A/DK/HK/P185/97(H3N8)        ....................................................................R.......................................K..................N.................V....
[N] A/DK/HK/P169/97(H3N8)        ...................................................E...................................K....................K..................N.................V....
[F] A/EG/ZA/AI23/04(H5N2)        ................D..............................S......................................................................................................
[F] A/Os/ZA/N227/04(H5N2)        ................D..............................S....................---...............................................................................
[F] A/DK/Prim/2621/01(H5N2)      ......................................................................................................................................................
[A] A/CK/NL/1/03(H7N7)           ...........................................................................L...................................................V......................
[A] A/NL/033/03(H7N7)            ...........................................................................L........................................R..........V......................
[A] A/NL/219/03(H7N7)            ...........................................................................L..........................................................................
[A] A/CK/DE/R28/03(H7N7)         ...........................................................................L..........................................................................
[B] A/CK/Iran/11T/99(H9N2)       ......................................................................................................V...........I.N................N................
[C] A/WDk/ST/988/00(H4N9)        ............................................................................T.........................................................................
[D] A/WDk/ST/1411/00(H11N3)      ............................................................................T.........................................................................
[D] A/WDk/ST/1737/00(H6N8)       ............................................................................T.........................................................................
[E] A/TK/IT/220158/02(H7N3)      ........................................................................T..............................................K..............................
[E] A/TK/IT/214845/02(H7N3))     ........................................................................T..............................................K..............................
[E] A/MA/IT/43/01(H7N3)          ......................................................................................................................................................
[E] A/MA/IT/33/01(H7N3)          ......................................................................................................................................................
[H] A/Ck/HK/31.2/02(H5N1)        .............................................................K............LS.L.......................................T................................
[H] A/SCk/HK/YU100/02(H5N1)      ..........N..........................A....V................M.K.F..........LS.L.......................................T................................
[H] A/Ph/HK/sv674.15/02(H5N1)    .............................................................K............L..L.......................................T................................
[H] A/DK/Anyang/AVL-1/01(H5N1)   .............................................................K............L..L........................T..............T...E............................
[H] A/Gf/HK/38/02(H5N1)          .............................................................K..N.........L..L.......................................T................................
[H] A/Ck/HK/37.4/02(H5N1)        .............................................................K............L..L.......................................T................................
[H] A/DK/Shandong/093/04(H5N1)   .............................................................K............L..L.......................................T................................
[G] A/DK/Korea/S17/03(H6N1)      ........................................................................................................P.............................................
[I] A/DK/HK/P54/97(H11N9)        ...............................................................A......................................................................................
[I] A/DK/HK/P50/97(H11N9)        ........................................................D.............................................................................................
[I] A/AQ/HK/399/99(H3N8)         ............................................................................................................K.........................................
[I] A/CK/DE/R45/98(H9N2)         .............................................................................................L........................................................
[I] A/CK/Taiwan/7-5/99(H6N1)     ..................................................E.......V..................................L.........................T..............................
[J] A/DK/HK/Y264/97(H4N8)        .........................................................Q..............T......................................................T.G....................
[J] A/GO/HK/W217/97(H6N9)        K........................................................Q............................................................................................
[J] A/Dk/ST/5048/01(H3N8)        .................................................H....................................................................................................
[K] A/AQ/HK/M603/98(H11N1)       .................................................................K....................................................................................
[L] A/OS/ZA/9508103/95(H9N2)     .................................................................................................................................E....................
[M] A/DK/Nanchang/1944/93(H7N4)  .................................................................................................................................G....................
[Q] A/GO/HK/17/77 (H6N4)         ............................................................................T........................................D................................
[Q] A/DK/HK/289/78(H9N2)         ............................................................................T....................................................N....................
[Q] A/DK/HK/175/77(H6N1)         ......................................................................A...........................................................Q...........K.......
[Q] A/DK/Prim/695/76(H2N3)       ......................................................................................................................................................
[Q] A/swine/China/8/78           .................................................................................................................................E....................
[Q] A/OYS/DE/87(H1N1)            ......................................................................................................................................................
[Q] A/CK/Scotland/59(H5N1)       ................................................................................................................................S.....................
[Q] A/CK/HK/17/77(H6N4)          ..........................................................V.................S.........................................................................
[Q] A/DK/HK/717/79 (H1N3)        .....................................S........I.....................................................................................K.................
[Q] A/DK/HK/610/79(H9N2)         ......................................................................................................................................................
[P] A/CK/IT/330/97(H5N2)         .......................................................................................................................T..............................
[P] A/CK/IT/367/97(H5N2)         .......................................................................................................................T............G.................
Consensus                        QVDCFLWHVRKRFADQELGDAPFLDRLRRDQKSLRGRGSTLGLDIETATRAGKQIVERILEEESDEALKMTIASVPASRYLTDMTLEEMSRDWFMLMPKQKVAGSLCIRMDQAIMDKNIILKANFSVIFDRLETLILLRAFTEEGAIVGE
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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

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                                160       170       180       190       200       210       220       230
                                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|
[N] A/DK/HK/T37/97(H11N8)        ........................................................S..............
[N] A/DK/HK/T25/97(H11N8)        ........................................................S..............
[N] A/DK/HK/P185/97(H3N8)        ................................I.I.....................S..............
[N] A/DK/HK/P169/97(H3N8)        ................................I.......................S..............
[F] A/EG/ZA/AI23/04(H5N2)        .......................................................................
[F] A/Os/ZA/N227/04(H5N2)        .......................................................................
[F] A/DK/Prim/2621/01(H5N2)      .......................................................................
[A] A/CK/NL/1/03(H7N7)           .......................................................................
[A] A/NL/033/03(H7N7)            .......................................................................
[A] A/NL/219/03(H7N7)            .......................................................................
[A] A/CK/DE/R28/03(H7N7)         .......................................................................
[B] A/CK/Iran/11T/99(H9N2)       .......................................................................
[C] A/WDk/ST/988/00(H4N9)        .......................................................................
[D] A/WDk/ST/1411/00(H11N3)      .......................................................................
[D] A/WDk/ST/1737/00(H6N8)       .......................................................................
[E] A/TK/IT/220158/02(H7N3)      .......................................................................
[E] A/TK/IT/214845/02(H7N3))     .......................................................................
[E] A/MA/IT/43/01(H7N3)          .......................................................................
[E] A/MA/IT/33/01(H7N3)          .......................................................................
[H] A/Ck/HK/31.2/2002(H5N1)      .................................................G...S............T....
[H] A/SCk/HK/YU100/02(H5N1)      ...............................................D.G...S............T....
[H] A/Ph/HK/sv674.15/02 (H5N1)   ....L............................................G...S............T....
[H] A/DK/Anyang/AVL-1/01(H5N1)   .................................................G...S............T....
[H] A/Gf/HK/38/02(H5N1)          .................................................G...S............T....
[H] A/Ck/HK/37.4/02(H5N1)        .................................................G...S............T....
[H] A/DK/Shandong/093/04(H5N1)   ....................I............................G...S............T....
[G] A/DK/Korea/S17/03(H6N1)      .......................................................................
[I] A/DK/HK/P54/97(H11N9)        .......................................................................
[I] A/DK/HK/P50/97(H11N9)        .......................................................................
[I] A/AQ/HK/399/99(H3N8)         .......................................................................
[I] A/CK/DE/R45/98(H9N2)         ..........................................T............S...............
[I] A/CK/Taiwan/7-5/99(H6N1)     ...........N...........R.F.............................................
[J] A/DK/HK/Y264/97(H4N8)        ..................................................................T....
[J] A/GO/HK/W217/97(H6N9)        ..................................................................TG...
[J] A/Dk/ST/5048/01(H3N8)        .............................................N....................T....
[K] A/AQ/HK/M603/98(H11N1)       .................................................N.....................
[L] A/OS/ZA/9508103/95(H9N2)     .......................................................................
[M] A/DK/Nanchang/1944/93(H7N4)  .......................................................................
[Q] A/GO/HK/17/77 (H6N4)         .......................................................................
[Q] A/DK/HK/289/78(H9N2)         .......................................................................
[Q] A/DK/HK/175/77(H6N1)         .......................................................................
[Q] A/DK/Prim/695/76(H2N3)       .......................................................................
[Q] A/swine/China/8/78           .......................................................................
[Q] A/OYS/DE/87(H1N1)            ............................................................W..........
[Q] A/CK/Scotland/59(H5N1)       .......................................................................
[Q] A/CK/HK/17/77(H6N4)          .......................................................................
[Q] A/DK/HK/717/79 (H1N3)        .................................................N.....................
[Q] A/DK/HK/610/79(H9N2)         .......................................................................
[P] A/CK/IT/330/97(H5N2)         .....................................I........N........................
[P] a/CK/IT/367/97(H5N2)         .....................................I.................................
Consensus                        ISPLPSLPGHTDEDVKNAIGVLIGGLEWNDNTVRVSETLQRFAWRSSNEDGRPPLPPKQKRKMARTIESEV
```

3.3.4 Nucleocapsidprotein (NP) genes

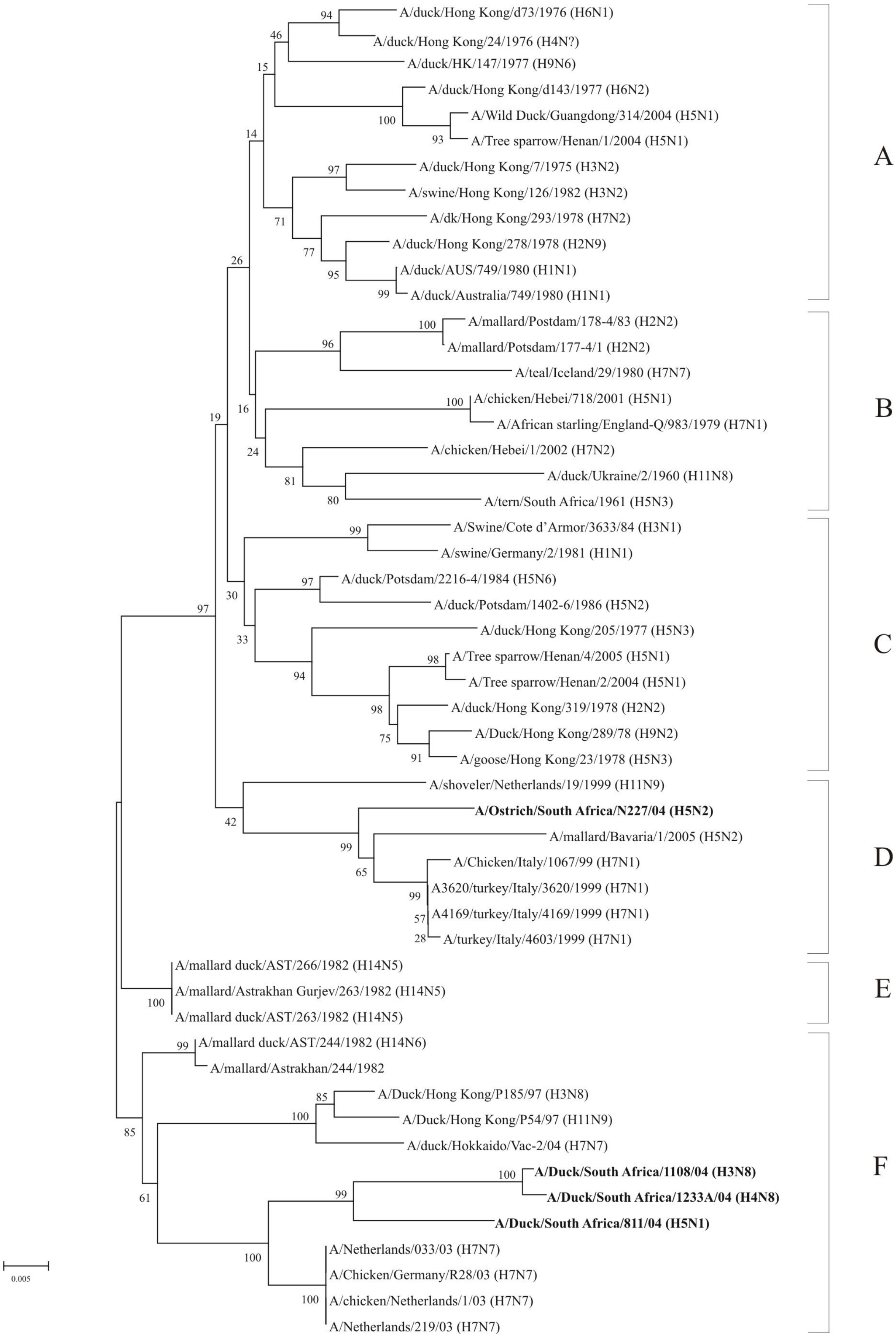

Figure 3.18 Phylogenetic tree inferred from a 776-nt multiple sequence alignment of the NP genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to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

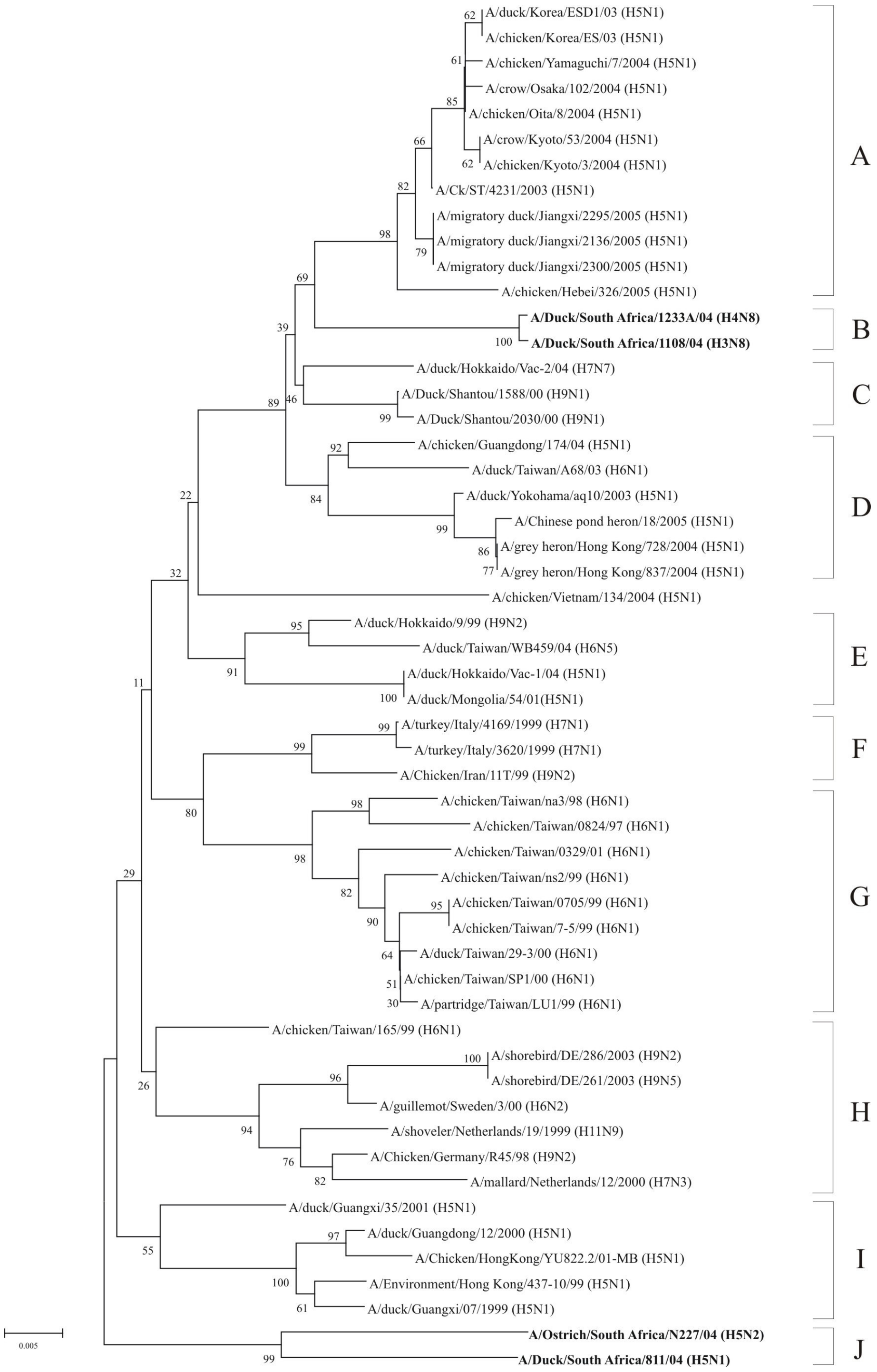

Figure 3.19 Phylogenetic tree inferred from a 695-nt multiple sequence alignment of the PA genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A to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

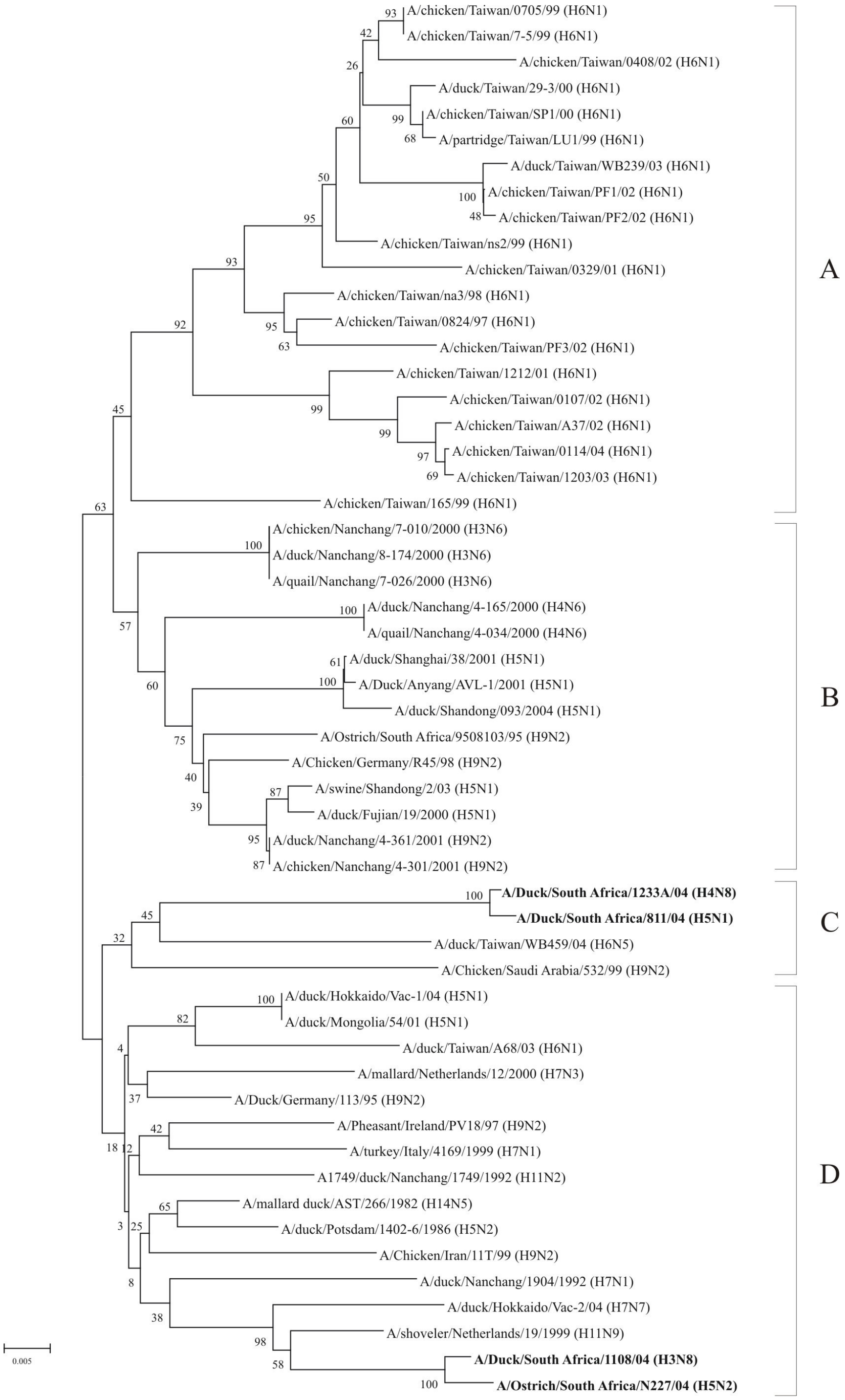

Figure 3.20 Phylogenetic tree inferred from a 753-nt multiple sequence alignment of the PB1 genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A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

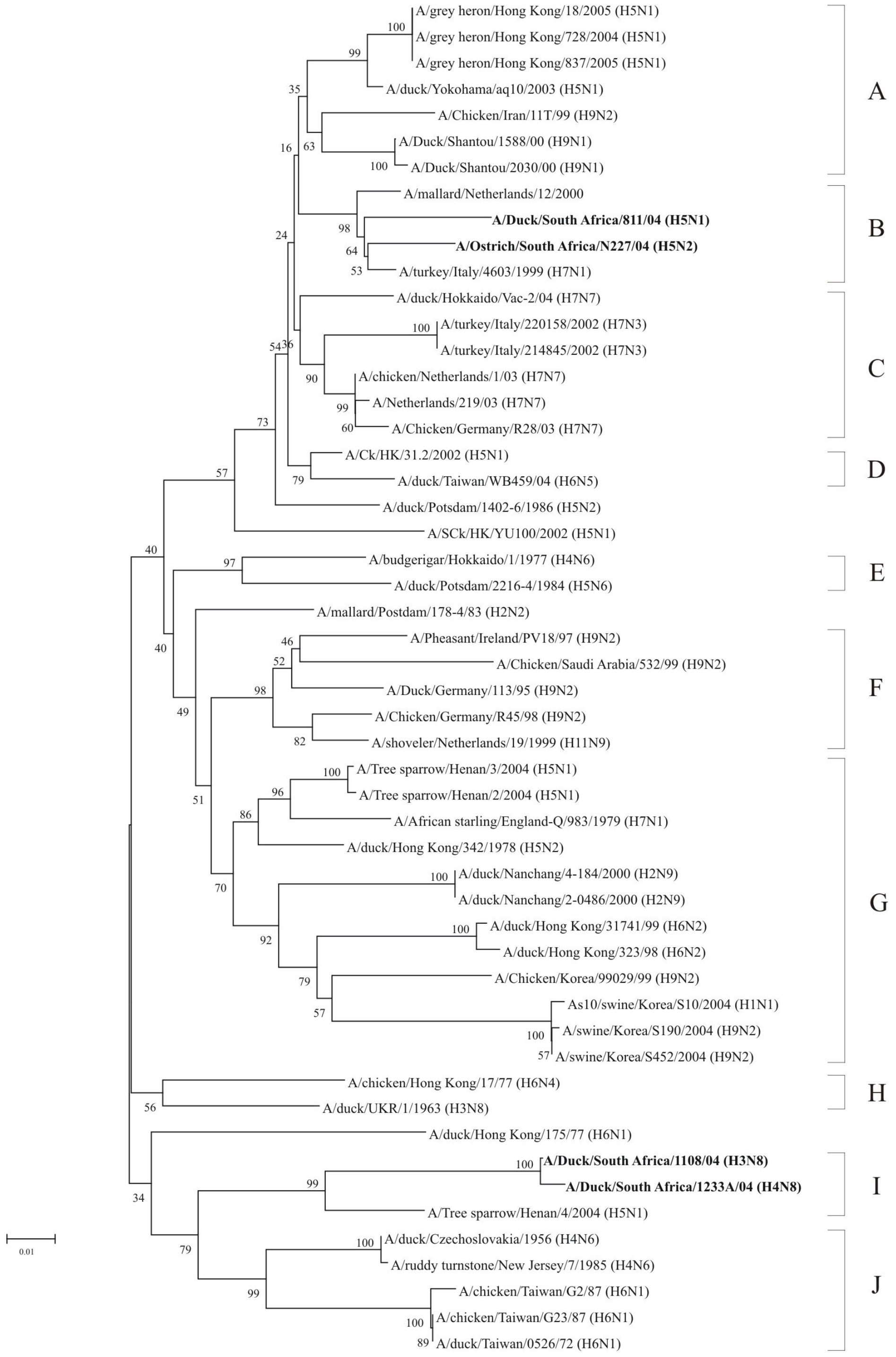

Figure 3.21 Phylogenetic trees inferred from a 726-nt multiple sequence alignment of the PB2 genes of A/Ostrich/South Africa/N227/04 (H5N2), A/Duck/South Africa/811/04 (H5N1), A/Duck/South Africa/1108/04 (H3N8) and A/Duck/South Africa/1233/04 (H4N8) (in boldface) and related sequences. Sub-lineages A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

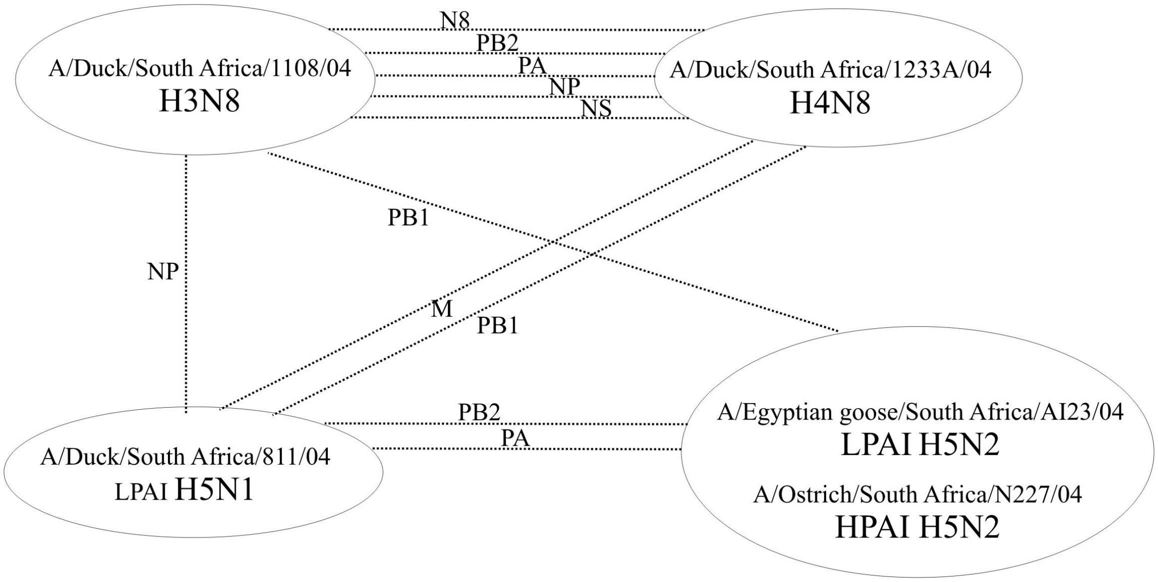

Figure 3.23. Reassortment between South African AIVs isolated in 2004.

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.