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CHAPTER FIVE[1]

PHYLOGENETIC ANALYSIS OF PIGEON PARAMYXOVIRUSES ISOLATED IN SOUTH AFRICA

ABSTRACT

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

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

5.1 INTRODUCTION

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

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

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

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

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

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5.2 MATERIALS AND METHODS

5.2.1 Viruses

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

5.2.2 RNA Extraction

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

5.2.3 RT-PCR

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

5.2.4 DNA sequencing and phylogenetic analysis

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

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

Table 5.1 South African Pigeon Paramyxovirus isolates analysed in this study

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

5.3 RESULTS

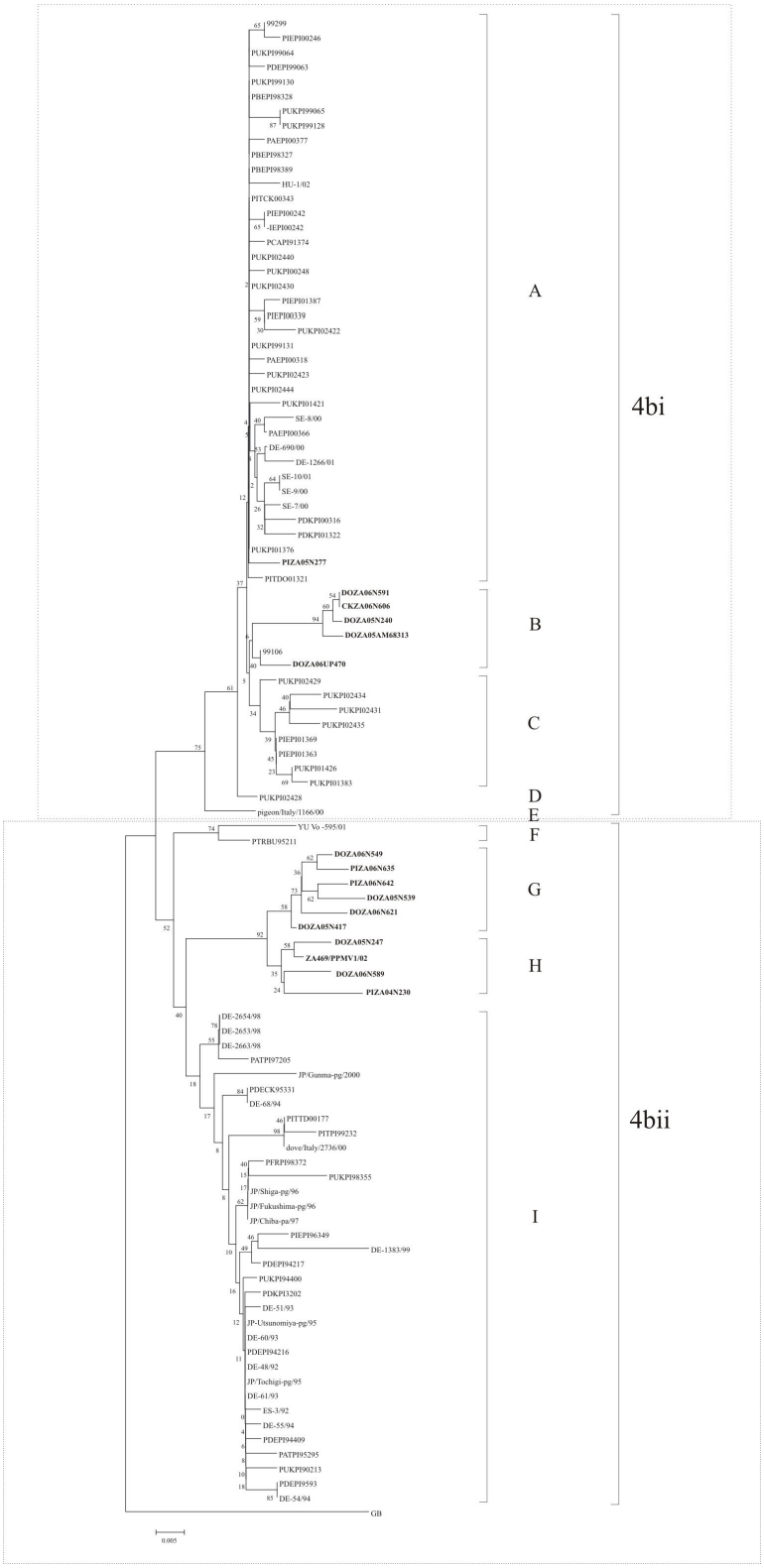

Figure 5.1(a) Dendogram inferred from the 374 bp region of the 3' end of the fusion protein (F) gene of PPMV-1 strains isolated in South Africa (boldface) and strains from Genbank. Enlargements of the regions representing subgroups 4bi and 4bii are presented separately in Figs 5.1(b) and 5.1(c) respectively.

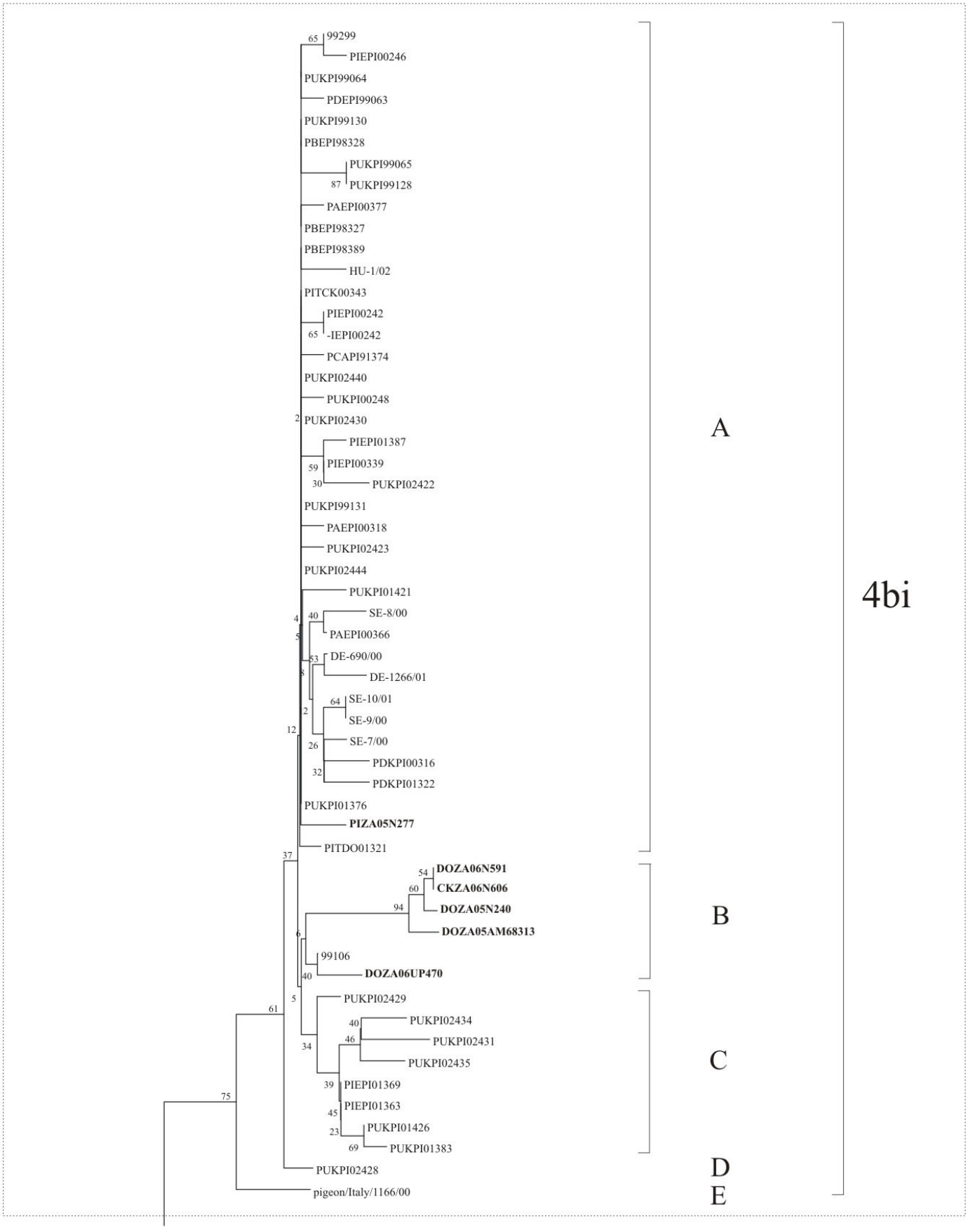

Figure 5.1(b) Enlargement of Fig 5.1(a) depicting Subgroup 4bi phylogeny

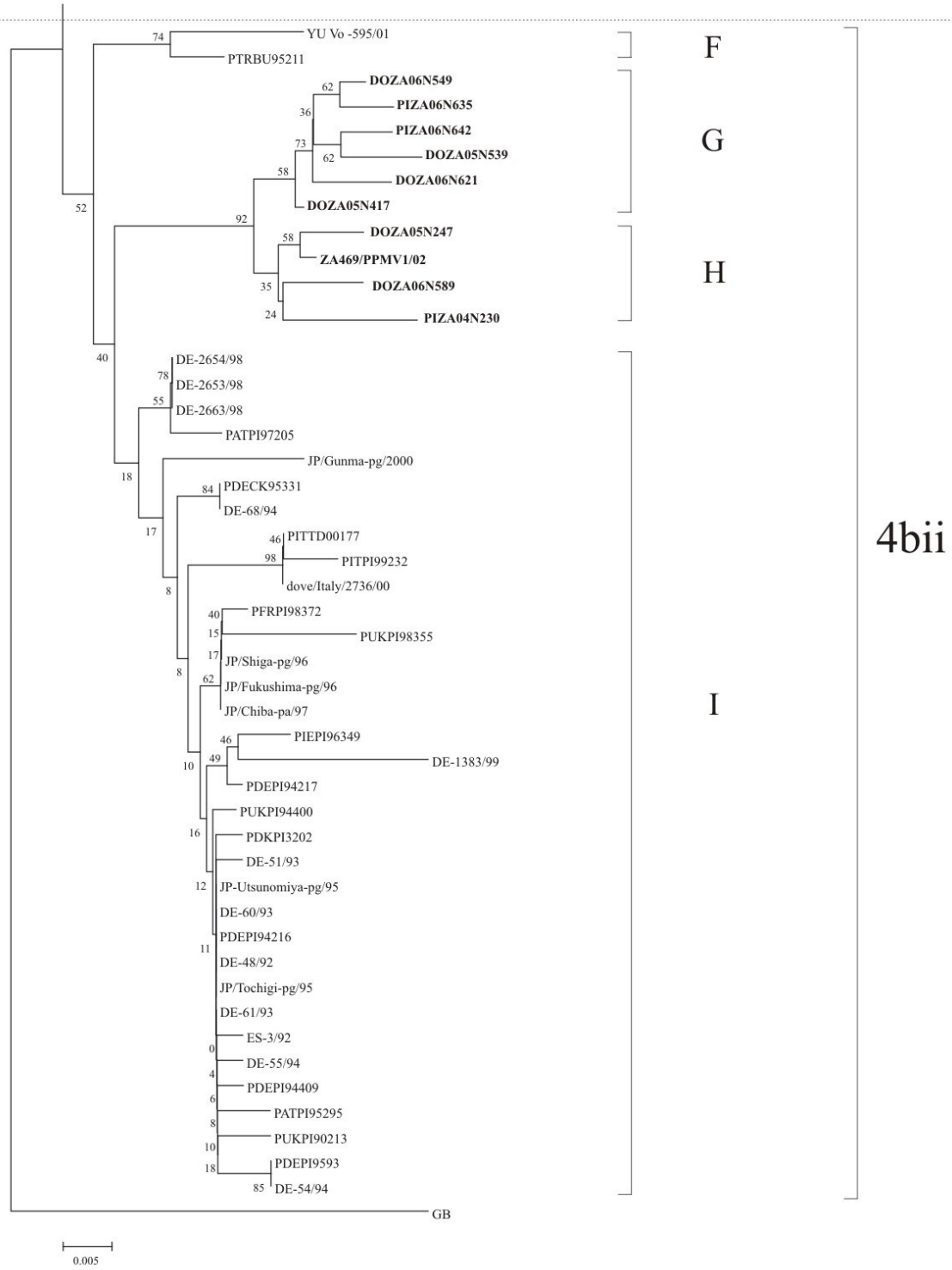

Figure 5.1(c) Enlargement of Fig 5.1(a) depicting Subgroup 4bii phylogeny

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

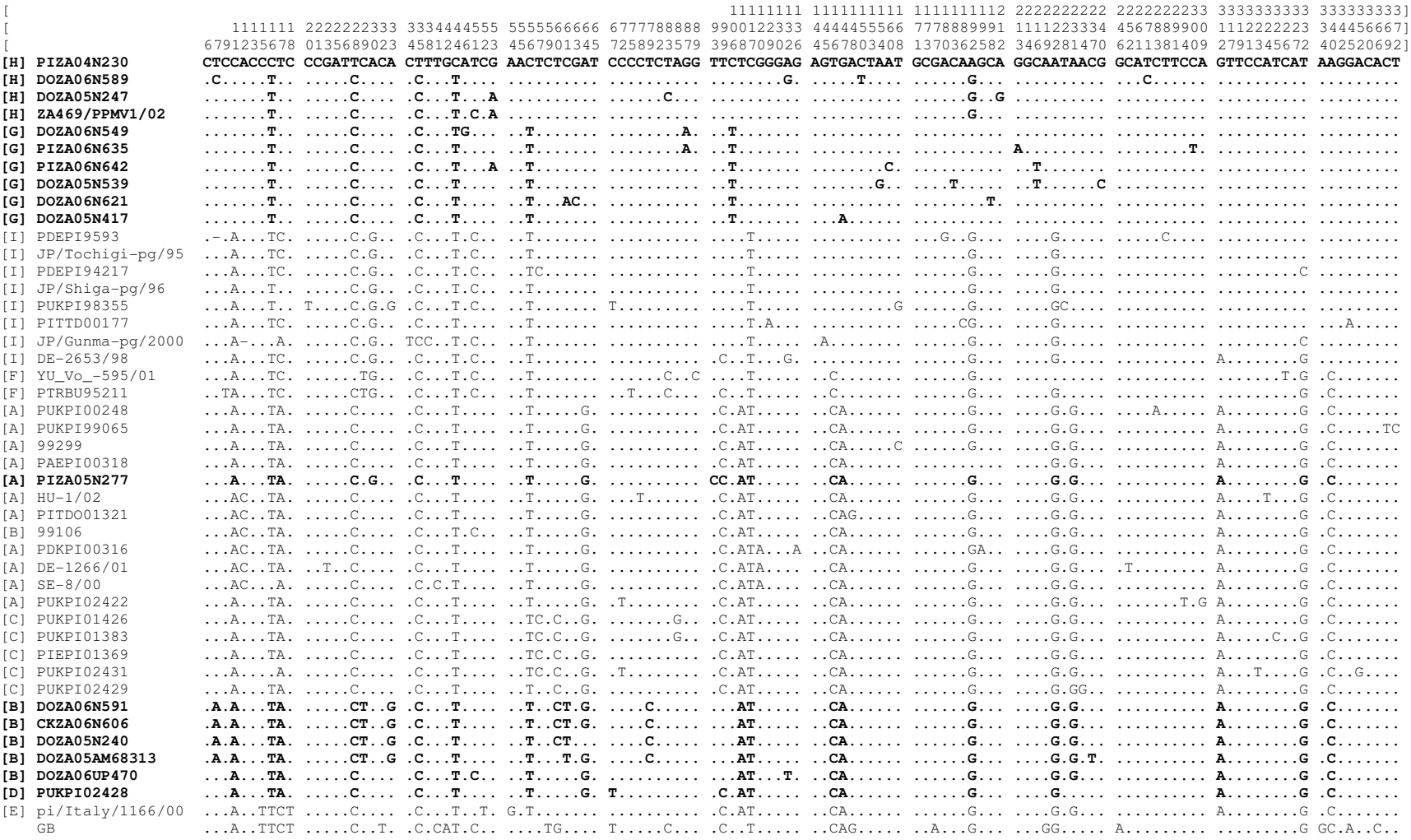

Figure 5.3 Nucleotide sequence alignment of variable sites within the 374 nt region of the 3' end of the fusion (F) protein. The South African isolates (in boldface) are compared with selected representatives of sub-lineages A to I.

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

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

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

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

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

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

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

5.4 DISCUSSION

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

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

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

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

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