Molecular studies on Beak and feather disease virus

Livio Edward Heath

Thesis presented for the degree of
DOCTOR OF PHILOSOPHY

Department of Molecular and Cell Biology, Faculty of Science,
University of Cape Town.

February, 2006

Certification by Supervisor

In terms of paragraph GP9 of the regulations for the degree of Doctor of Philosophy at the University of Cape Town, I certify that I approve of the inclusion in this thesis of the material already published, or submitted for publication by the candidate Livio Edward Heath

Signed by candidate

Signature removed

Edward P. Rybicki, PhD
Professor of Virology
Department of Molecular and Cell Biology, Faculty of Science
& Institute of Infectious Disease and Molecular Medicine
University of Cape Town

Signed by candidate

Signature removed

Anna-Lise Williamson, PhD
Professor of Medical Virology
Division of Medical Virology, Department of Clinical laboratory Sciences,
Faculty of Science & Institute of Infectious Disease and Molecular Medicine
University of Cape Town

February 2006

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Acknowledgements

I would like to express my heartfelt appreciation to everybody who was instrumental in the completion of this project. A special thanks to the following people:

Prof. Ed Rybicki and Prof. Anna-Lise Williamson for allowing me to be a part of their distinguished research groups and for their continued support and encouragement. Special thanks for allowing me the freedom to expand the project into areas of research that was not always directly applicable to our primary objectives.

Our collaborators at the University of the Free State, Prof. Rob Bragg and Kulsum Kondiah for productive and enthusiastic discussions regarding many aspects of BFDV biology.

Birdlife South Africa and the Cape Parrot Working Group for facilitating the productive interaction between the aviculturists, conservation agencies and academia. Special thanks to Prof. Mike Perrin, Dr. Tiawanna Taylor, Steven Evens, Kate Henderson, Claire Patterson, Craig Whittington-Jones and Kobus Pienaar.

Dr. Chris Kingsley, Dr. Lesley Kenmuir, Dr. Michelle Barrows, Ben Minnaar, William Horsfield and Ralph Correia for kindly providing the blood samples used during the course of the project.

Dr. Louise Warburton for shearing the samples and stories she collected during the time spent in Zambia.

Daniel Haydon for his assistance and insight into the aspects of selection discussed in this thesis.

Di James not only for the countless hours spent sequencing full length BFDV genomes, but more importantly for her friendship and positive outlook on life that seem to rub off on everybody around her.

Thanks to all my fellow students and researches at the University of Cape Town for their support and valued friendship. Special thanks to Darren, Eric, Arvind, Dionne, Allison, James, Thomas, Brian, Ziyaad, Richard, Juliet and Tracey.

To my family whose support and inspiration over the years has shaped me into the human being I am today. For that I am eternally indebted to all of you. A loving thanks to my father, mother, brother and Jeanette.

To Antoinette for never doubting in my ability, for her encouragement, indulgence and unwavering love.

Finally, I thank God for blessing me with the ability and opportunity to, through science, make a difference in the world. *Soli Deo Gloria*

Chapter 1

Literature Review

Contents

1.1 Introduction

Despite the tremendous variation in size, shape and colour, parrots are probably one of the most recognizable groups among the eight thousand species of birds living today. Some of the more obvious characteristics include their disproportionately large heads and hooked bills, short legs and the zygodactyl feet (Rowley & Coller, 1997). The skeletal remains of birds are extremely fragile and the scarcity of fossilized remains limits our knowledge regarding their historical origins, evolution and distribution. However, parrots are thought to be an ancient lineage with Australia being the likely center of their radiation (Juniper & Penn, 1998). The oldest evidence of parrot-like fossils dates as far back as the Miocene, approximately 40 million years ago. Today wild parrots are mainly found in tropical areas of the southern hemisphere and are most prevalent in Australasia and South America. Interestingly, significantly fewer species occur in Africa and Asia (Forshaw, 1977). The reasons for the low representation of the group on these continents are unclear, since environmental conditions often mirror those of the Australasia and South America.

Although subdivision of the group has proven difficult, the order *Psittaciformes* is currently subdivided into two families. The family *Cacutuidae* consists of the cockatoos while all other parrots are included in the family *Psittacidae*. Cockatoos are distinguishable from other parrots by several anatomical features, such as the presence of a gall bladder and the absence of blue and green colours in their plumage. The most striking feature of the cockatoos is their crests which they proudly display during social interaction. Despite the difficulty of assigning members of the order to specific subfamilies, dividing them into genera and species is relatively simple. There are currently six defined genera within the family *Cacutuidea* with a further 78 defined within the family *Psittacidae,* encompassing a total of 351 species (Rowley & Coller, 1997).

Parrots are one of the most threatened groups of animals on earth on earth with more than 90 species at risk of global extinction (Juniper & Penn, 1998). The human encroachment and industrial development during the last century has had a devastating impact on parrots worldwide. The situation in Latin America is particularly alarming where nearly 30% of the endemic species are considered to be at risk (Rowley & Coller, 1997). One of the major contributing factors to the demise of wild parrot populations is the loss and fragmentation of their habitat. Of particular concern is the rapid loss of tropical forests (Low, 1984). Economic gains to

be had from intensive logging and land-clearing for agricultural purposes often supercedes environmental concerns to the detriment of local parrot populations.

The high level of intelligence, potential for tameness, and beauty of parrots all contribute to the increasing popularity of parrots as pets. Despite the existence of well-established commercial breeding programs, large numbers of birds are taken from the wild each year to supplement the formal pet trade (Beissinger, 2005). Rare species are especially vulnerable, since unscrupulous collectors and breeders are willing to pay handsomely for these birds. Many governments have recognized the need to curb the exploitation of wild parrots and have put in place legislation to regulate the sustainable use of wild birds (Juniper & Penn, 1998). Intergovernmental agreements, such as the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES) establish a framework by which the international exploitation of threatened species can be controlled. However, many countries lack the basic capacity to enforce legislation, resulting in the continued exploitation of the birds.

It is not only due to human intervention that so many species of parrots find themselves on the brink of extinction. As with all other higher organisms parrots are susceptible to a multitude of diseases. The potential effects of these diseases on parrot populations range from inconsequential to potentially devastating. Parrots are affected by bacterial, fungal and viral pathogens and are especially vulnerable to communicable diseases due to their gregarious nature (Warburton & Perring, 2002). Wild and captive populations are equally affected by disease. With the constant movement of birds across geographical borders through trade, there is an increasing risk of spreading the disease into new areas and populations.

Psittacine beak and feather disease (PBFD) is the most common disease in parrots, affecting more than 60 psittacine species worldwide. The disease was first described in 1975 by Ross and Perry in several species of Australian cockatoos, but reports of a disease in avian species closely resembling PBFD can be traced back as far as the turn of the twentieth century (Pass & Perry, 1984;Ritchie & Carter, 1995). PBFD is endemic to Australia, where it threatens at least one species with extinction (Bassami *et al.*, 2001). The disease has since been diagnosed in parrots from the South Pacific, Americas and Africa (Todd *et al.*, 2001a). The global distribution of the

disease has been attributed to an ever-increasing international trade in pet birds (Woods & Shivaprasad, 1997).

1.2 PBFD and Beak and feather disease virus

1.2.1 The disease

The onset of PBFD is characterized by the development of lethargy, depression and severe anaemia in affected birds. Clinical symptoms generally progress to weight loss, feather dystrophy and loss, and in severe cases, deformities of the beak and claws (Pass & Perry, 1984) (Figure 1.1). A peracute form of the disease occasionally occurs in neonatal birds and is often accompanied by severe pneumonia resulting in sudden death (Todd *et al.*, 2001a). PBFD manifests itself in young birds commonly during their first moult and is characterized by interpulp haemorrhaging and necrosis of the feather follicles.

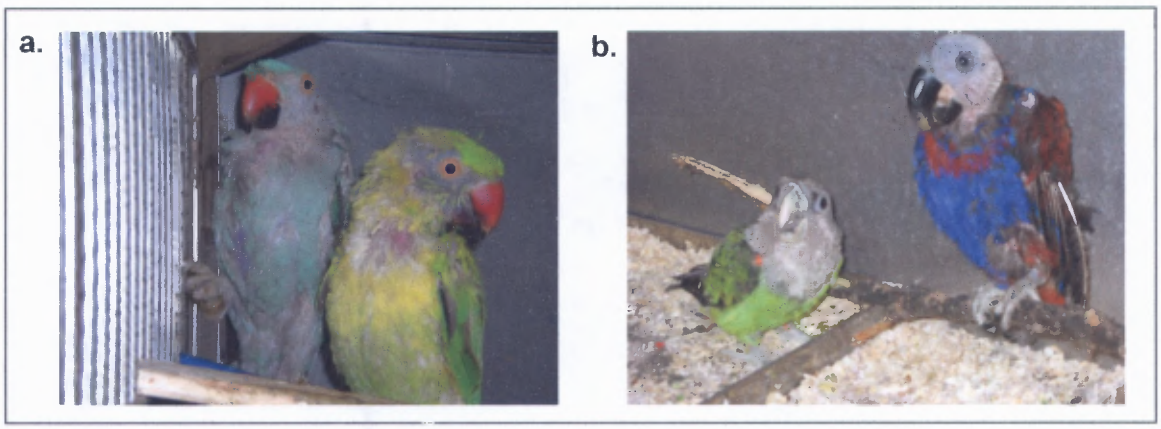

Figure 1.1 Examples of clinical symptoms typically associated with chronic cases of psittacine beak and feather disease. (a) Two ring-neck parakeets exhibiting typical feather discoloration and loss. (b) A Brown-headed Parrot (*Poicephalus cryptoxanthus*) and Eclectus parrot (*Eclectus roratus*) in the advanced stages of the disease. (Photographs courtesy of Prof. R Bragg, University of the Free State, South Africa).

Histological examination of feather follicles is routinely used to confirm clinical disease. PBFD is associated with the development of lesions in feather shafts and pulp, epidermal hyperplasia and hyperkeratosis (Todd, 2000;Todd *et al.*, 2001a;Latimer *et al.*, 1991;Pass & Perry, 1984). Infiltration of heterophils, plasma cells and macrophages into affected areas results in the development of suppurative inflammation (Todd *et al.*, 2001a). During the advanced stages of the disease necrotic lesions occur in the thymus and bursa of affected birds. Microscopically, basophilic intranuclear and intracytoplasmic inclusion bodies can be seen in tissue sections of the

feathers, beak, thymus, bursa and Kupffer cells of the liver (Figure1.2) and are considered to be indicative of the disease (Pass & Perry, 1984;Todd, 2000).

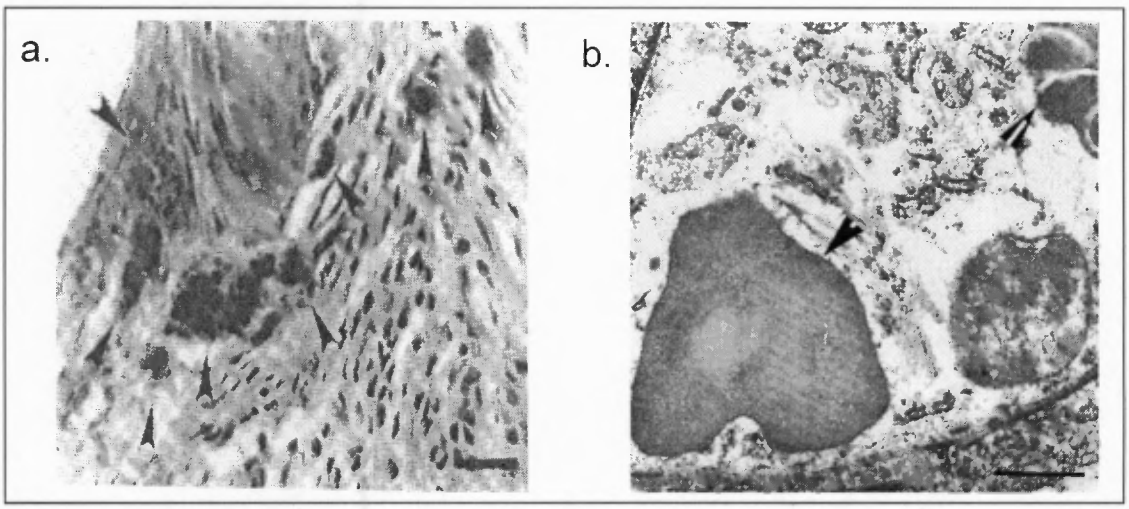

Figure 1.2 Histopathology associated with PBFD. (a) Basophilic intranuclear and intracytoplasmic inclusion bodies in macrophages (Reprinted from Kiatipattanasakul-Banlunara *et al.*, 2002). (b) Electron micrograph of cytoplasmic inclusion bodies of infected epithelial cells (Courtesy of Dr. W. L. Steffens and Dr. K Latimer, The University of Georgia, United States of America).

The disease is progressive with most affected birds developing a chronic form of the disease. The clinical outcome of chronic infections is heavily dependent on the specific species involved as well as the age at which the bird is first infected (Raidal *et al.*, 1993d;Pass & Perry, 1984). PBFD is commonly associated with immunodeficiency-related diseases caused by depletion of lymphoid tissue (Latimer *et al.*, 1991) with damage to the lymphoreticular tissue being most pronounced in the bursa of Fabricius and the thymus (Todd, 2000). Birds frequently succumb to secondary infections, such as peritonitis, chlamydiosis, and mycotic ventriculitis (Todd, 2000). However, in isolated cases affected adult birds recover completely without any apparent long-term effect.

1.2.2 The etiological agent of PBFD

Although a disease resembling PBFD was first described as early as 1907, the etiological agent of the syndrome remained elusive for the better part of a century (see Ritchie and Carter, 1995). This was largely due to the fact that the pathological manifestation of the disease was not always consistent and seemed to be heavily dependent on the species involved as well as the stage during

development at which infection occurred. Histologic and electron microscopic characterization of the pathology associated with the disease did, however, shed some light on the question. Affected follicles and epidermal cells were typically inflamed and necrotic, suggesting the involvement of an infectious agent in disease development.

In 1989 Ritchie *et. al.* (Ritchie *et al.*, 1989) reported on the successful isolation of a previously uncharacterized virus from the feathers of birds showing clinical signs of PBFD. Based on its physical characteristics, the virus was considered to be a member of a new family of pathogenic animal viruses and subsequently named *Beak and feather disease virus* (BFDV) (Todd *et al.*, 2000). The morphology of BFDV was consistent with the intranuclear and intracytoplasmic inclusion bodies located in the basophilic layers of follicular epithelium considered to be indicative of the disease (Figure 1.3). Moreover, inoculation of neonatal budgerigars with virus preparations produced clinical as well as histologic legions generally associated with the disease, confirming that the virus was indeed the etiological agent of PBFD (Ritchie *et al.*, 1989).

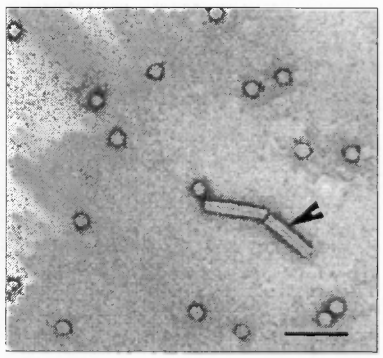

Figure 1.3 Electron micrograph of BFDV purified from the feathers follicles of infected cockatoos. Tobacco mosaic virus was included for comparative purposes. (Courtesy of Dr. W. L. Steffens and Dr. K Latimer, The University of Georgia, United States of America).

1.2.3 Classification and genome organization of BFDV

The International Committee of Viral Taxonomy (ICTV) currently classifies BFDV as a member of the family *Circoviridae,* a group of animal viruses characterized by having a covalently closed circular singe-stranded DNA genome encapsidated in icosahedral, non-enveloped virions (Biagini *et al.*, 2005). Circoviruses are the smallest known autonomously replicating viruses, with genomes as small as 2000 nucleotides in size. Members of the family are further divided into genera based on their specific genome organization and host range (Figure 1.4). There are

currently only two recognized genera within the family *Circoviridae*, namely *Gyrovirus* and *Circovirus*. The genes of the circoviruses have been named in accordance with the convention adopted for the plant geminiviruses, where genes encoded on the virion strand are labelled "V" whilst genes encoded on the complementary virus strand are labeled "C" (Todd, 2000).

Figure 1.4 The genomic arrangement of circoviruses and other related replicons. The origins of replication are indicated by the stem loop symbol. Analogous genes are coloured the same. The genes of the circoviruses have been named in accordance with the convention adopted for the plant geminiviruses, where ORFs on the virion strand are labeled "V" whilst those encoded on the complementary virus strand are labeled "C". CP = capsid protein, Rep = Replication-associated protein, AP = apoptotis-associated protein, MP = movement protein. The unabbreviated versions of the virus names are summarized in Table A.1 (see Appendix A).

Chicken anaemia virus (CAV) is the only member species of the genus *Gyrovirus*. This virus causes anaemia, atrophy of the thymus and haemorrhaging in young chickens (Crowther *et al.*, 2003;Goryo *et al.*, 1985;Todd *et al.*, 1995). The negative-sense genome of CAV encodes three major proteins all of which are transcribed from the complementary strand (Noteborn *et al.*, 1992;Phenix *et al.*, 1994). The CAV capsid protein, encoded by the largest of the three open

reading frames (C1), is unique amongst circovirus capsid proteins in that it is not only responsible for the encapsidation but is also directly involved in the replication of the viral genome (Koch *et al.*, 1995). The functions of the remaining two gene products encoded by C2 and C3 have not yet been clearly defined, but they are believed to contribute to virion assembly and pathogenesis, respectively (Douglas *et al.*, 1995).

In 1997 a previously uncharacterized virus, now designated *Torque teno virus* (TTV), was identified in the plasma of a Japanese patient suffering from non-A-G hepatitis (Nishizawa *et al.*, 1997). The discovery of the circular nature of its genome led to it being tentatively assigned to the family *Circoviridae*. During subsequent studies into the prevalence of TTV, a second virus closely resembling it was discovered (Takahashi *et al.*, 2000). Despite possessing a genome organization similar to that of TTV, this virus did not share significant sequence homology with TTV and was thus designated *TT-like mini virus* (TTMV). The natural host range of TTV is however not restricted to humans, since isolates have been found in non-human primates (Okamoto *et al.*, 2000) as well as several domesticated animal species, including dogs, cats, pigs and chickens (Leary *et al.*, 1999). The genomes of both these TTV and TTMV contain two major open reading frames, designated C1 and C2 (Okamoto *et al.*, 1999;Mushahwar *et al.*, 1999). The transcriptional product of C1 is analogous to the capsid protein of CAV and possesses several motifs conserved in proteins involved in rolling circle replication. The gene product of C2 has several features in common with C2 of CAV and is thought to also be involved in viral replication (Biagini *et al.*, 2000; Biagini, 2004). TTV and TTMV have recently been reclassified as members of a novel, floating genus called *Anellovirus* (Biagini *et al.*, 2005).

The genome organization of the generic circoviruses is strikingly similar to that of the plant-infecting geminiviruses and nanoviruses (Figure 1.4). *Porcine circovirus* (PCV) and BFDV are the only formally recognized members of the genus *Circovirus*. PCV was first isolated from pig kidney cell cultures and was considered to be non-pathogenic (Tischer *et al.*, 1982). However, in 1997 a PCV isolate was implicated in post-weaning multisystemic wasting syndrome (PMWS), a disease of young pigs closely resembling clinical conditions associated BFDV-infections (Hamel *et al.*, 1998;Ellis *et al.*, 1998). It was subsequently shown that a second genetic and antigenically distinct subtype of the virus is the causal agent of PMWS (Hamel *et al.*, 1998), resulting in the clear taxonomical separation between the non-pathogenic PCV1 and pathogenic PCV2 (Allan *et*

al., 1998). In recent years a number of novel circovirus-like viruses have been identified in non-psittacine avian species. These include *Columbid circovirus* (CoCV) (Todd *et al.*, 2001b), *Goose circovirus* (GCV) (Todd *et al.*, 2001b), *Canary circovirus* (CaCV) (Phenix *et al.*, 2001), *Duck circovirus* (DuCv) (Hattermann *et al.*, 2003). These viruses are genetically distinct from BFDV, but based on the similarities of their genome organization with other circoviruses have been classified as tentative members of the genus *Circovirus* (Mankertz *et al.*, 2000;Todd *et al.*, 2001b;Hattermann *et al.*, 2003). In terms of their relatedness to other circoviruses, these viruses cluster into genetically distinct lineages. CoCV and CaCV appear to be most closely related to BFDV (Phenix *et al.*, 2001), with GCV and DuCV almost equidistantly related to BFDV and PCV (Hattermann *et al.*, 2003). In addition to the tentative members of the family several avian circovirus-like infections in doves (Raidal & Ridddoch, 1997), gulls (Twentyman *et al.*, 1999), ostriches (Eisenberg *et al.*, 2003), finches (Shivaprasad *et al.*, 2004) and ravens (Stewart *et al.*, 2006) have recently been identified based on histopathology associated with disease.

Circoviruses possess ambisense genomes with the two major open reading frames (V1 and C1) encoded on opposite strands of the replicative double-stranded DNA intermediate (Todd, 2000). These encode the replication-associated (Rep) and capsid (CP) proteins from the virion and complementary strands, respectively (Meehan *et al.*, 1998;Niagro *et al.*, 1998a). Recently, a protein thought to be unique to PCV2 was found to be encoded from a gene located on the complementary strand (Liu *et al.*, 2005). The protein was shown to induce caspase-dependent apoptosis in PCV permissive cells. Since this protein has not yet been formally named, I will henceforth refer to it as the apoptosis-associated protein (AP). In addition to the genes described above, several smaller putative open reading frames have been predicted *in silico* (Niagro *et al.*, 1998b;Bassami *et al.*, 1998;Bassami *et al.*, 2001). It is, however, unclear whether any of these putative open reading frames are actively transcribed during productive infections.

1.2.4 Evolution and genetic diversity

Although the evolutionary origin of circoviruses remains unclear, analysis of their genomic sequences has highlighted some interesting aspects regarding the evolutionary history of these viruses. Striking similarities between circoviruses and plant-infecting ssDNA viruses, including geminiviruses and nanoviruses, strongly suggest that these viruses originate from a common

ancestor (Figure 1.5). Moreover, amino acid sequence motifs within the replication associated proteins of both the vertebrate- and plant-infecting ssDNA viruses share highly conserved spatial and sequence relationships with motifs found in protein homologues from a number of ssDNA prokaryotic replicons, suggesting that at least a portion of their genomes are of prokaryotic origin (Ilyina & Koonin, 1992;Koonin & Ilyina, 1993;Koonin & Ilyina, 1992).

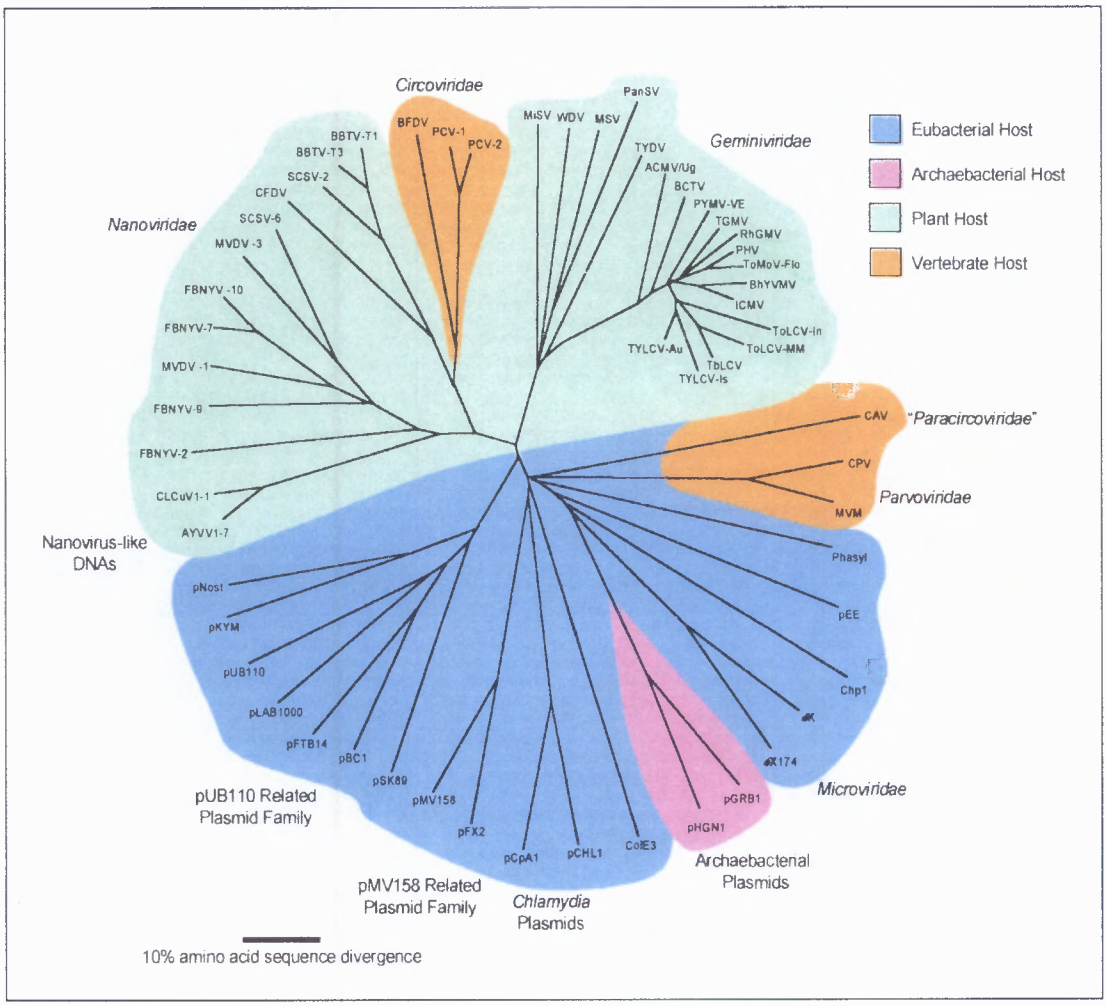

Figure 1.5 The evolutionary relatedness of the rolling circle replication (RCR) domains within the replication-associated proteins of several ssDNA replicons. The unrooted dendogram is based on a previously published alignment of ~80 amino acid spanning thethree RCR domains conserved across all ssDNA replicons which uses this mechanism of DNA replication (Koonin and Ilyina, 1993). The unabbreviated versions of the virus names are summarized in Table A.2 (see Apendix A).

Phylogenetic analysis has been used to suggest that circoviruses are most closely related to nanoviruses and that the circovirus lineage emerged only after the nanoviruses diverged from the common ssDNA virus ancestor (Gibbs & Weiller, 1999). Interestingly, the nanoviruses cluster

into two distinct lineages with circovirus sequences placed within only one of these lineages, suggesting that the latter evolved from a nanovirus only after the major diversification of the nanoviruses. Since all nanoviruses infect plants, the postulated evolution of circoviruses from this group would represent a major interspecies transmission event across phyla (Gibbs & Weiller, 1999).

The evolutionary history of circoviruses is further complicated by intervirus recombination. Although homology between the N-terminal region of the replication-associated proteins of geminiviruses, nanoviruses and circoviruses is easily detectable, the C-terminal region of the circovirus Rep apparently shares a common origin with the 2C-protein of the caliciviruses (Gibbs & Weiller, 1999). Caliciviruses are a small group of nonenveloped viruses with positive-sense ssRNA genomes which only infect vertebrates (Clarke & Lambden, 2000), suggesting that the recombination event occurred after the interspecies transmission event involving the ancestral nanovirus (Gibbs & Weiller, 1999). Interestingly, the 2C-protein contains a short glycine-rich region that includes a phosphate-binding loop (P-loop) similar to that found in the N-terminal region of the nano- and geminiviruses. Although the ancestral nanovirus was most probably capable of replicating in its new host, the replacement of its native P-loop by that from a calicivirus through recombination is thought to have improved the fitness of the recombinant offspring (Gibbs & Weiller, 1999).

Although members of the family *Circoviridae* share the same genome structure and probably the same evolutionary origin, these viruses are genetically distinct. Replication of single-stranded DNA genomes is inherently prone to high mutation rates due to the susceptibility of the genome to spontaneous deamination (Ritchie *et al.*, 2003;Lindahl & Nyberg, 1974). This is clearly evident when one considers the intertypic and intratypic variation between the different genera of circoviruses.

Initially, only limited diversity had been suggested to exist between different isolates of BFDV (Johne & Muller, 1998). However, as the number of completely sequenced BFD viruses has increased, so too has the realization that significant levels of genetic diversity between BFDV isolates exists. Bassami *et al.* (2001) (Bassami *et al.*, 2001) showed that the overall nucleotide identity of BFDV isolates in Australia could be as low as 84% and that isolates grouped into distinct genetic clusters. A subsequent study that included isolates from New Zealand confirmed

these findings, and furthermore suggested that the distinctive clustering pattern of BFDV strains was due to a genotypic association between the viruses and their hosts (Ritchie *et al.*, 2003). However, with the inclusion of isolates from a wider geographical distribution, the association with specific psittacine species became less apparent. Instead, a correlation between the genetic characteristics of certain isolates and atypical disease development emerged (Raue *et al.*, 2004). Differences in the clinical manifestations of the disease in a wide range of psittacine species are well established, but have generally been attributed to host factors rather that genetic variation between BFDV isolates (Kock *et al.*, 1993;Ritchie *et al.*, 1990).

TTV and TLMV exhibit an even wider range of diversity, with the divergence between isolates often exceeding 50% (Biagini, 2004). This has resulted in the definition of several genotypes of TTV and TLMV, respectively (Biagini *et al.*, 1999;Biagini *et al.*, 2001a). Infection of individual hosts by more than one distinct genotype appears to be common- place, with viral populations often resembling a quasi-species (Jelcic *et al.*, 2004). Moreover, recent studies have suggested that intertypic as well as intratypic recombination may contribute significantly to the evolution TTV and TLMV (Worobey, 2000;Biagini *et al.*, 2001b). Contrary to BFDV and the anelloviruses, significantly lower levels of divergence have been reported between different strains of CAV (4%) and PCV (1-6) (Todd, 2000). Birds diverged earlier from a common ancestral line than higher vertebrates such as pigs, with the parrots being one of the most ancient lineages (Juniper & Penn, 1998). This may account for differences in the level of intratypic diversity within each of the genera as well as the width of natural host range of the respective circoviruses.

1.3 Molecular biology of Circoviruses

Researchers have only recently begun to dissect the molecular biology of circoviruses and their associated diseases. Since the avian circoviruses cannot be propagated in cell culture, knowledge of the molecular biology of these viruses is limited. In contrast, PCV readily grows in several of the tissue culture systems typically used by molecular biologists (Hattermann *et al.*, 2004). As a result most of the insight into the molecular biology of circoviruses is based on the biology of PCV (Todd, 2004). However, common features shared between the members of the genus suggest that different circoviruses employ similar replication strategies, and specific aspects

relating to the molecular biology of PCV may be broadly applicable to all circoviruses (Mankertz *et al.*, 2004).

1.3.1 Cell tropism and viral entry

Although circoviruses are known to exhibit a widespread tissue distribution, their precise target cells have not yet been definitively identified. Histological lesions associated with BFDV infections are predominantly found in the developing feather shaft and pulp, but viral antigens can be detected in a wide range of organs including the bursa of Fabricius, spleen, liver, kidney, brain, crop and intestine (Smyth *et al.*, 2001;Ramis *et al.*, 1994). Basophylic inclusions are often found in epithelial cells, lymphoid tissue and macrophages (Latimer *et al.*, 1991;Todd, 2000). PCV exhibits a similar cell tropism during neonatal infections, with antigens easily detectable in cardiomyocytes, hepatocytes and macrophages (Sanchez *et al.*, 2001). However, the cellular targets of PCV gradually change during development from foetal to early postnatal life. Postnatally, antigens are restricted macrophages, monocytes and to a lesser extent lymphocytes circulating in the different organs (Sanchez *et al.*, 2003;Sanchez *et al.*, 2004;Gilpin *et al.*, 2003). Macrophages play a critical role in the host's cellular immune response to viral infection, and their apparent involvement in the infection process is consistent with the depletion of lymphoid tissue and immunosuppression commonly associated with circovirus infections (Nielsen *et al.*, 2003).

In addition to macrophages, PCV antigens can occasionally been found in dendritic cells. However, the infection of dendritic cells appears to be abortive with no sign of productive replication of the virus (Vincent *et al.*, 2003). Internalized viral particles persist within the affected cells without the loss of infectivity or the modulation of cell surface molecules. This interaction of PCV with dendritic cells led to speculation that circoviruses possibly enter the cell *via* endocytosis (Vincent *et al.*, 2003). Misinzo *et al.* (2005) (Misinzo *et al.*, 2005) recently confirmed that PCV preferentially enters target cells by clathrin-mediated endocytosis and furthermore showed that endosomal acidification is needed for the establishment of a productive infection. Viral particles rapidly attach to a yet unidentified cell-surface receptor, with attachment reaching saturation within 15 minutes. This is followed by the gradual time-dependent internalization of attached particles. The endocytotic process and the subsequent trafficking steps

are still poorly understood, but appears to be facilitated by the redistribution of actin cytoskeleton (Misinzo *et al.*, 2005). Actin is one of the most abundant cytosolic proteins and polymerization of its monomeric isoform results in the establishment of the morofilament network in all eukaryotic cells (da Costa *et al.*, 2003). Interestingly, disruption of the cytoskeleton had a greater effect on the efficacy of infection than the specific inhibition of clathrin-mediated endocytosis, suggesting that additional actin-dependent processes may be involved in circovirus cell entry. Macropinocytosis and caveolae-mediated endocytosis were however, excluded as alternative mechanisms responsible for circovirus internalization (Misinzo *et al.*, 2005).

Circoviruses lack an autonomous DNA polymerase and rely on host cell polymerases in order to replicate their own genome (Meerts *et al.*, 2005). Since host cell DNA synthesis occurs exclusively in the nucleus, the viral DNA needs to be relocated to this cellular compartment before a productive infection can be established. The exact mechanism by which the viral DNA is released into the cell and subsequently relocated to the nucleus is largely unknown. In the case of the related geminiviruses, viral DNA transport is known to be mediated by one or more of the proteins associated with the infectious virion (Boulton *et al.*, 1993;Liu *et al.*, 1999;Qin *et al.*, 1998). These proteins are actively targeted to the nucleus and are able to shuttle between the nucleus and the cytoplasm. The capsid protein of PCV2 has similarly been shown to localize to the nucleus (Liu *et al.*, 2001b). In light of this, the capsid proteins of PCV and BFDV are expected to interact with the packaged viral DNA in a similar way to that of the geminiviruses. It is however unclear whether this process involves intact viral particles or disassociated capsid monomers tightly associated with the viral DNA.

Some insight into these processes may be gained from the life cycle of the Adeno-associated virus (AAV). These ss-DNA viruses enter host cells by receptor-mediated endocytosis, analogous to PCV (Seisenberger *et al.*, 2001;Bartlett *et al.*, 2000). The release of AAVfrom the endosomes, and by analogy circoviruses, is believed to take place at the late endosomal stage and is dependent on the acidification of the endosomal vesicles (Bartlett *et al.*, 2000). The transport of intact viral capsids into the nucleus of infected cells is a very inefficient process and although virions rapidly accumulate in the perinuclear region, only a fraction of intact particles cross into the nucleus of cells (Lux *et al.*, 2005). This suggests that uncoating of ss-DNA viruses take place

before or during nuclear entry, resulting in delivery of naked DNA to the nucleus (Tavassoli *et al.*, 2005).

1.3.2 Viral transcription and translation

Despite the simplicity of the genome organization of circoviruses, these viruses display surprisingly complex transcription patterns. Up to twelve transcripts are produced during PCV replication in permissive cells (Cheung, 2003b;Cheung, 2003a). Although PCV1 and 2 encode equivalent protein entities, some quantitive differences do exist with respect to specific RNAs (Cheung, 2003a). The current knowledge regarding the transcription patterns of BFDV is extremely limited.

In the case of PCV2, the majority of the RNAs are transcribed from the virion sense DNA with only the RNA encoding the CP and AP transcribed from the complementary sense DNA strand (Cheung & Bolin, 2002;Cheung, 2003b). CP is the first viral protein expressed and can be detected as early as 6 hours post-infection (Meerts *et al.*, 2005). This is somewhat surprising since transcription of the *CP* can only proceed once the double-stranded replicative intermediate DNA has been formed. The promoter and transcriptional start site of the *CP* are located within the *Rep*. This RNA is posttranscriptionally spliced resulting in the expression of the ~30kDa CP (Nawagitgul *et al.*, 2000a). During the early stages of infection, PCV CP localizes to the perinuclear region of the nucleus. The intracellular localization of the protein is directed by a bipartite nuclear localization signal situated at the N-terminus of the protein (Liu *et al.*, 2001b). As the infection progresses, the CP becomes exclusively localized to the nucleus. Forty-eight hours post-infection the nucleus becomes condensed, at which point the nuclei are devoid of CP (Meerts *et al.*, 2005). Changes in the subcellular localization of the CP could be indicative of the different roles the protein may play throughout the circovirus lifecycle.

Several co-linear transcripts are produced from the *Rep* of PCV. The full-length RNA is translated into Rep. A cluster of additional Rep-associated RNAs (*rep'*, *rep3a*, *rep3b*, *rep3c*) are generated by the posttranscriptional splicing of the full-length RNA (Mankertz & Hillenbrand, 2001;Cheung, 2003c). Analysis of the levels of Rep-associated transcripts revealed significant changes in the relative ratio of the full-length and spliced *Rep* transcripts over the course of PCV

infection (Mankertz & Hillenbrand, 2001). Although equivalent amounts of the respective transcripts are produced shortly after the establishment of a productive infection, *rep`* becomes the predominant RNA species during the latter stages of infection.

The Rep is highly conserved among different circoviruses and contains three motifs typical of proteins involved in the initiation of rolling circle replication of ssDNA genomes, as well as a P-loop associated with dNTP-binding (Figure 1.6). Rep and Rep' share a common N-terminus. However, the C-terminal moiety of Rep' differs from that of Rep, since splicing of the full-length transcript results in a frame shift in the C-terminal region of Rep'. This region of Rep' is in turn homologous to the putative MYB transcription factor of *Arabidopsis thaliana* (Cheung, 2003c;Cheung, 2003a).

The replication-associated proteins can only be detected during the latter stages of circovirus infections and then only in the nucleus of infected cells (Meerts *et al.*, 2005). The nuclear localization of both Rep and Rep' has recently been confirmed by the expression of each protein in *trans* fused to red and green fluorescent proteins, respectively (Mankertz *et al.*, 2004). The karyophillic nature of these proteins is somewhat surprising, since no nuclear localization signal has been described for either. Interestingly, Rep and CP appear to be co-localized in most cases where PCV antigens are found in the nucleus. This fact has lead to speculation that the CP interacts with Rep in order to cross the nuclear membrane (Meerts *et al.*, 2005). Although the proposed interaction between the CP and Rep is merely speculative, Rep and Rep' have been shown to interact to form both homomultimeric and heteromultimeric complexes when co-expressed in PCV-permissive cells (Mankertz *et al.*, 2004).

The *Rep* promoter (P_{rep}) of PCV is located directly adjacent to the start site of the gene and overlaps the intergenic region and origin of replication (Mankertz & Hillenbrand, 2001;Mankertz *et al.*, 2003). It contains two putative interferon-stimulated response elements suggesting that its activity may be regulated by cytokines. However, *in vitro* stimulation of P_{rep} using either human IFN-γ or TNF-α does not result in any measurable activity (Mankertz & Hillenbrand, 2001). Interestingly, P_{rep} is negatively regulated by the expression of Rep, but not Rep'. Repression of the promoter activity is brought about by the interaction of Rep with the first two of four conserved hexamer repeats adjacent to the origin of replication (Mankertz & Hillenbrand, 2001).

These hexamer repeats have been mapped as the essential part of the minimal binding site (MBS) of both Rep and Rep' (Steinfeldt *et al.*, 2001). However, Rep` is less sensitive to alterations of the MBS compared to Rep, suggesting distinct sequence requirements for each of these proteins (Mankertz *et al.*, 2004).

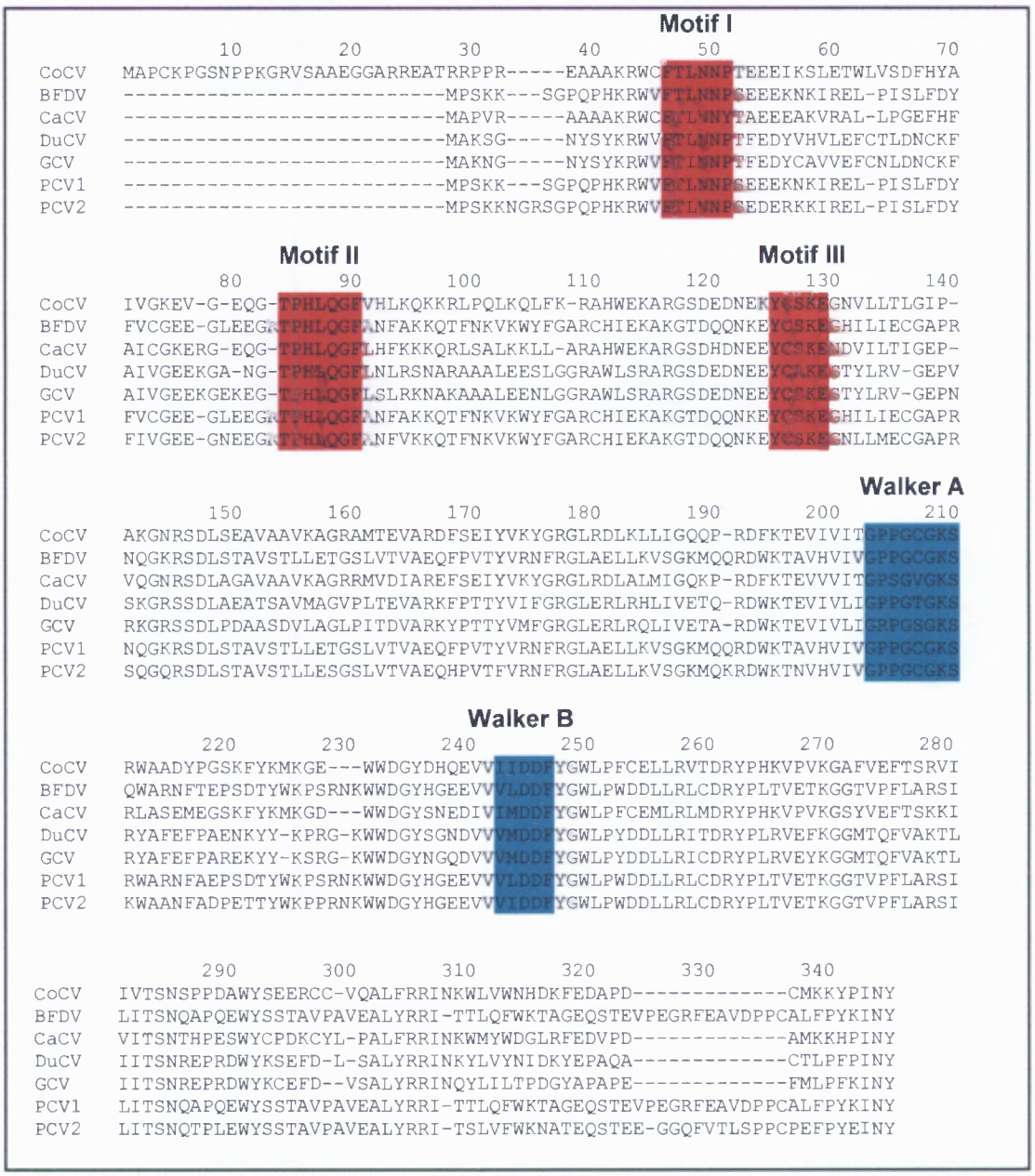

Figure 1.6 Multiple alignment of the replication-associated proteins of the all known circoviruses; Porcine circovirus (PCV), Beak and feather disease virus (BFDV), Columbid circovirus (CoCV), Goose circovirus (GCV), Canary circovirus (CaCV) and Duck circovirus (DuCv). Motifs I, II and III which are typically found in rolling circle replication associated proteins, as well as the Walker boxes for dNTP-binding, are boxed in red and green, respectively (Adapted from Mankertz *et al.*, 2004).

In addition to the CP- and Rep-associated RNA transcripts, three transcripts are produced from different promoters inside the *Rep* (Cheung, 2003c;Cheung, 2003a). Two of these transcripts (*ns1* and *ns2*) do not appear to code for functional proteins, since they do not contain any recognizable initiation codons (Cheung, 2003a). The third RNA (*ns0*) encodes a 23-amino acid protein that does not share significant homology with any known protein. Recently a novel viral protein (AP) was detected during productive infections of PCV2 in permissive cells (Liu *et al.*, 2005). This protein is encoded by a gene located on the complementary DNA strand. The gene is highly conserved among different PCV2 strains, but shares only limited homology with the corresponding region of nonpathogenic PCV1 isolates. The protein can readily be detected in the nucleus and to a lesser extent in the cytoplasm of PCV2-infected cells. It is not involved in viral replication, but inactivation of AP does delay the initiation of replication. Moreover, AP was shown to induce caspase-dependent apoptosis through the activation of capsase-8 followed by activation of the effector caspase-3 pathway (Liu *et al.*, 2005). Interestingly, CAV is also known to induce apoptosis as part of its natural life cycle (Jeurissen *et al.*, 1992). Apoptosis of CAV-infected cells are facilitated by the nonstructural protein VP3 and are thought to be directly responsible for the immunosuppressive nature of the virus (Noteborn *et al.*, 1994). PCV AP may similarly be responsible for the depletion of B-lymphocytes associated with PMWS.

1.3.3 Viral replication

Due to structural and sequence similarities shared by geminivirus and circovirus replication-associated proteins, circoviruses are believed to replicate in a manner similar to the geminiviruses. In addition to the three motifs within the Rep that are generally associated with the rolling circle replication mechanism (RCR), the origin of replication of circoviruses closely resembles that of the geminiviruses (Cheung, 2004) (Figurer 1.7). The origin of replication typically contains a stem-loop structure flanked by iterated sequences similar to the iterons described in geminivirus genomes (Niagro *et al.*, 1998b). The nonanucleotide sequence at the apex of the stem-loop structure is highly conserved across all viruses known to replicate via RCR (Figure 1.7 b).

Replication is initiated by the binding of the Rep to the origin of replication and the cleavage of the closed circular ssDNA genome between the seventh and eighth nucleotide of the

nonanucleotide sequence. This is subsequently followed by the initiation of the virus-strand DNA synthesis and synthesis of the double-stranded replicative intermediate DNA (Palmer & Rybicki, 1998). In the case of PCV both Rep and Rep' are essential for PCV replication (Mankertz & Hillenbrand, 2001). This is unusual since all other examples of RCR require only one multifunctional protein (Hanley-Bowdoin *et al.*, 2000). Interestingly, the generic mastreviruses of the family *Geminiviridae* also produce two isoforms of the replication-associated protein, Rep and RepA, through alternative splicing. However, only Rep is essential for viral DNA synthesis (Laufs *et al.*, 1995;Gutierrez, 1999;Gutierrez, 2000). It is currently not known whether *Rep* of the avian circoviruses directs the synthesis of more than one isoform of Rep, similar to PCV (Mankertz *et al.*, 2004).

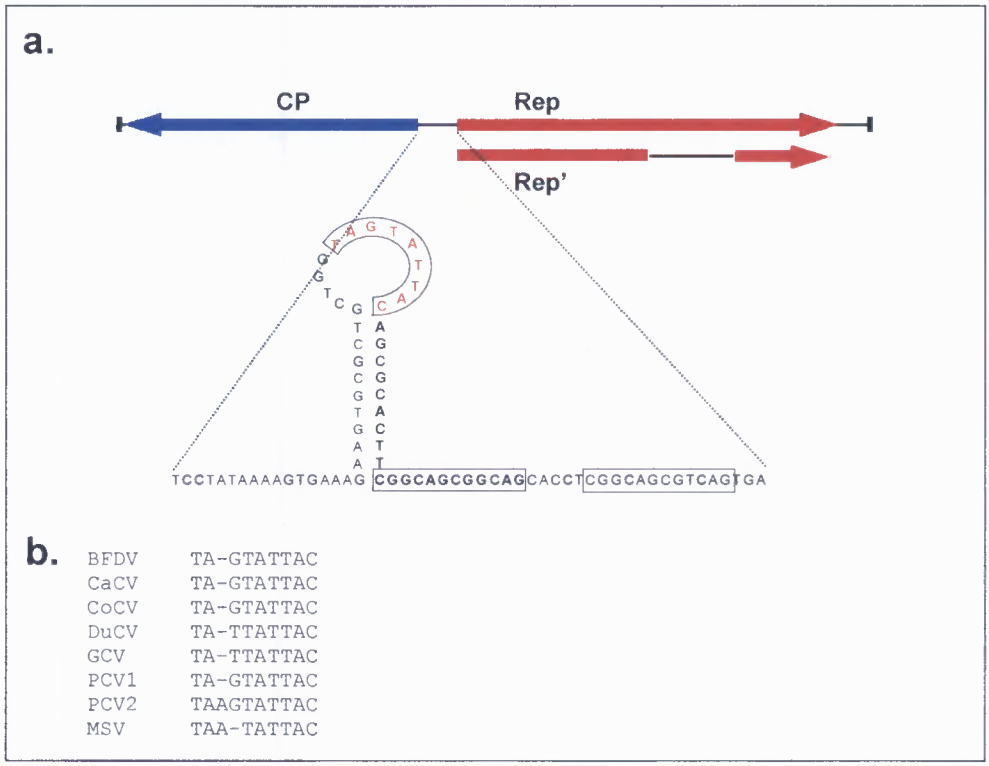

Figure 1.7 Comparison of the putative origin of replication of the circoviruses and a representative geminivirus. (a) A graphical representation of the origin of replication of Porcine circovirus 1. The highly conserved nanonucleotide sequence at the apex of the stem-loop structure is indicated by the red boxed text. The iterated sequences are indicated by the boxed text, with the minimal binding sites of the replication-associated proteins shown in bold (Adapted from (Mankertz *et al.*, 2004). (b) Comparison of the nanonucleotide sequence at the apex of the stem-loop structure of the circoviruses and a representative geminivirus *(Maize streak virus*; MSV).

Besides its role in encapsidation of the viral genome, the CP of the geminiviruses is known to influence the replication process (Stenger *et al.*, 1991;Saunders *et al.*, 1991). The involvement of

CP in the replication cycle is generally ascribed to a stochastic effect brought about by the binding of CP to newly synthesized viral ssDNA, since deletion or inactivation of the *CP* results in a reduction of ssDNA accumulation during the late stages of the infection process (Briddon *et al.*, 1989;Qin *et al.*, 1998;Padidam *et al.*, 1999a). Moreover, interaction between Rep and CP of the geminivirus Mung bean yellow mosaic Indian virus has recently been shown to down-regulate the nicking and ligation activity of Rep (Malik *et al.*, 2005). The interaction involves the specific motif within Rep responsible for oligomerisation of the protein. The oligomerisation motif has also been implicated in several other protein-protein interactions, including interactions with various host factors (Bagewadi *et al.*, 2004). Anecdotal evidence regarding the co-localisation of PCV Rep and CP suggest these proteins directly interact with each other and that the CP of circoviruses may be involved in controlling viral replication (Meerts *et al.*, 2005).

1.3.4 Encapsidation and viral spread

The final stage of the viral life cycle involves the encapsidation and spread of newly synthesized viral DNA. Oligomerisation of the CP subunits into mature virions is thought to be initiated by the accumulation of ssDNA and interaction of the basic N-terminal region of the protein with viral DNA. Viral particles can often be seen in the nucleus of feather epithelium of birds suffering from PBFD, suggesting that assembly occurs within the nucleus. Whereas the capsids of PCV and CAV are composed of a single protein, BFDV virions purified from the feathers of affected birds appear to contain two proteins (Ritchie *et al.*, 1989;Ritchie *et al.*, 1990). The size of only one of these proteins corresponds to the size of product predicted to be transcribes from the putative CP gene. The identity and origin of the second protein remains unclear, but it is in all likelihood simply a partially proteolysed remnant of the native CP (Todd, 2000). Despite this, the three-dimensional structure of the BFDV virion more closely resembles that of PCV of than CAV. PCV and BFDV virions appear to consist of 12 flat pentameric units. In contrast the pentagonal subunits of CAV are trumpet-shaped. All three viruses do however, consist of 60 units of the monomeric CP arranged in a T = 1 symmetry (Crowther *et al.*, 2003). The exact mechanism by which virus particles are transported out of the nucleus and released from infected cells remains unclear, but may involve apoptosis and general cell death.

1.4 Diagnosis of PBFD

Although the distinct clinical features of chronic PBFD facilitate the diagnosis of the disease by veterinary examination alone, other possible causes for feather deformities often complicate the diagnosis of less well-established BFDV infections. Conditions that mimic the clinical signs of PBFD include infection by *Chlamydia*, *Mycoplasma*, mycobacterium and several viral agents, whilst feather deformities caused by non-infectious factors such as malnutrition and stress can also be misconstrued as signs of PBFD. For this reason clinical diagnosis of the disease must be confirmed by the detection of viral antigens or DNA (Latimer *et al.*, 1991;Todd, 2000)

Histological examination is routinely used to confirm clinical diagnosis. Lesions occur in fast-dividing cell types such as epidermal cells associated with developing feathers and lymphoid tissue (Pass & Perry, 1984;Gerlach, 1994). Basophilic, eosinophilic and amphophilic cytoplasmic and nuclear inclusion in haematoxylin-stained tissue sections are indicative of the disease. However, only cytoplasmic inclusions are considered to be definitive of BFDV infection, since adenovirus, polyomavirus and herpesvirus infections may lead to the formation of similar nuclear inclusions (Ramis *et al.*, 1994).

Definitive diagnosis of viral disease has traditionally relied upon the isolation of the causal agent. However, since all attempts to cultivate the BFDV in tissue culture have so far been unsuccessful, researchers are forced to employ alternative methods for the definitive diagnosis of PBFD. Detection of the virus or virus-specific antibodies is achieved by the haemagglutination (HA) and haemagglutination inhibition (HI) assays, respectively (Raidal *et al.*, 1993d). However, differences in the agglutination ability of erythrocytes obtained from different sources limit the reliability of these assays (Sanada & Sanada, 2000;Ritchie *et al.*, 1991;Sexton *et al.*, 1994). Recently, an enzyme-linked immunosorbent assay (ELISA) was developed for the detection of virus-specific antibodies present in the sera of BFDV-infected psittacines (Johne *et al.*, 2004). Similar methods have been successfully implemented for both PCV (Walker *et al.*, 2000) and CAV (Todd *et al.*, 1999) and make use of recombinantly expressed capsid protein. Although different BFDV strains have been shown to be antigenically similar (Ritchie *et al.*, 1990), the wide range of genetic variation of BFDV may limit the applicability of these tests.

PBFD can also be diagnosed by the detection of BFDV DNA by *in situ* hybridization or PCR. *In situ* hybridization (ISH) techniques make use of sequence-specific DNA probes to identify nuclear inclusions associated with selected viral infections and have successfully been used to distinguish between polyomavirus, adenovirus, Pacheco's disease and PBFD in affected pisttacines (Ramis *et al.*, 1994). However, ISH is cumbersome and time-consuming and is generally not used as a routine method for diagnosing PBFD. In response to a growing demand for a quick and reliable technique for the detection of BFDV, Ypelaar *et al.* (1999) (Ypelaar *et al.*, 1999) developed a universal PCR assay for precisely this purpose. The technique exploits the complete conservation of motifs within *Rep* to amplify a 717 base pair fragment of the BFDV genome and is able to consistently distinguish between birds showing signs of PBFD and clinically normal birds (Ypelaar *et al.*, 1999). However, a major shortcoming of this technique is that it fails to detect virus in subclinically infected individuals. Technical advances in PCR methodology have resulted in the development of new tools to be used for the diagnosis of BFDV infections (Raue *et al.*, 2004). This second generation of PCR-based techniques is less time consuming and at least a hundred-fold more sensitive than the conventional methods (Raue *et al.*, 2004). However, the initial cost of acquiring the required infrastructure currently limits the broad application of this technology.

1.5 Control and treatment of PBFD

BFDV is highly infectious and environmentally stable (Warburton & Perring, 2002). Although there are some indications that the virus is vertically transmitted via the eggs, horizontal transmission accounts for the vast majority of infections (Ritchie & Carter, 1995). It has recently been shown that in the case of PiCV, viral DNA is most often detected in the respiratory organs, including the trachea, pharynx and lungs (Duchatel *et al.*, 2006). This suggests that transmission of the circoviruses from one individual to another occurs primarily through direct contact or inhalation of aerosolized virus (Ritchie & Carter, 1995). Feather dust is most likely a major source of contamination. Strategies for the control and management of PBFD generally rely on good husbandry and minimizing the risk of exposure (Gerlach, 1994). However, the long-term success of these strategies is limited by the resilience of the virus under extreme environmental conditions, as well as the complexities introduced by subclinical infection often associated with

adult birds. Prophylactic and therapeutic vaccination offer an attractive alternative to current control strategies.

Exploratory studies of the effectiveness of an inactivated BFDV vaccine have yielded promising results in two instances (Raidal *et al.*, 1993d;Ritchie *et al.*, 1992). In both studies vaccinated birds not only seroconverted, but appeared to be protected against virus challenge. The vaccines were produced by chemically inactivating virus harvested from infected birds. However, in the continued absence of an *in vitro* culturing system, the large-scale implementation of inactivated vaccines is impractical. Moreover, residual infectivity of vaccine preparations has been implicated in the death of vaccinated birds (Raidal *et al.*, 1993d). The threat of improperly inactivated vaccine preparations and the lack of a reliable infectivity assay, further limit the commercial application of such vaccines.

The success of inactivated vaccines does however justify investigations into the use of alternative vaccine strategies. Production of subunit vaccines through the use of recombinant DNA technology circumvents several of the problems associated with inactivated vaccines. The coat protein of circoviruses is a major constituent of the infectious viral particle and is therefore a likely target of immune surveillance. In line with this, several immunodominant epitopes have been identified for both BFDV and PCV capsid proteins (Mahe *et al.*, 2000;McNeilly *et al.*, 2001;Truong *et al.*, 2001;Lekcharoensuk *et al.*, 2004). Isotyping of monoclonal antibodies isolated following immunization of mice with purified BFDV indicated that both IgG and IgM were produced in response to the inoculum. However, none of the purified antibodies had any HI activity (Ritchie *et al.*, 1992). In the case of PCV2, several B-cell epitopes were found to be distributed across the CP. Three linear immunodominant epitopes were mapped to amino acids 65 to 87, 113 to 147 and 157 to 183, whilst amino acids 47 to 63 and 165 to 200 were shown to be involved in the formation of conformation-specific epitopes (Lekcharoensuk *et al.*, 2004;Mahe *et al.*, 2000).

Recombinant subunit vaccines have successfully been tested for CAV, as well as PCV. Inoculation of chickens with recombinant CAV C1 and C2, expressed in insect cells, was shown to induce neutralizing antibodies (Koch *et al.*, 1995). Interestingly, protection against challenge was vertically transferred from breeders to their progeny. Vertical transmission of protection via

maternally-derived antibodies has also been shown to occur when psittacines are immunized using inactivated vaccines (Raidal *et al.*, 1993d;Ritchie *et al.*, 1992). Interestingly, immunization of special-pathogen-free (SPF) piglets with recombinant PCV2 CP was sufficient to completely inhibit viral replication after challenge (Blanchard *et al.*, 2003). This is in contrast to CAV where both the VP1 and VP2 are needed to mount an effective immune response (Noteborn *et al.*, 1998).

In recent years the use of DNA vaccines, naked DNA carrying the genes encoding immunogenic proteins, has become increasingly popular (Kurath, 2005). Many studies have demonstrated the ability of DNA vaccines to elicit a protective immune response against different viral agents, including PCV2 (Blanchard *et al.*, 2003;Kamstrup *et al.*, 2004). Although PCV DNA vaccines have been shown to elicit a humoral immune response, they generally require multiple inoculations and are easily outperformed by conventional subunit vaccines (Blanchard *et al.*, 2003).

Attenuated live vaccines suitable for mass administration represent a cost-effective alternative for the vaccination of high-density livestock (Todd, 2000). Attempts to generate attenuated versions of CAV by repeated passage have yielded limited success (Todd *et al.*, 1995;Todd *et al.*, 1998). However, the threat of attenuated strains regaining pathogenicity through genetic drift is likely to limit the commercial application of these vaccines. Recently, a novel approach to viral attenuation has been explored in the case of PCV. This approach involves placing the CP of the pathogenic PCV2 into the genetic background of a non-pathogenic PCV1 isolate (Fenaux *et al.*, 2003). Fenaux *et al.* (2003) (Fenaux *et al.*, 2003) reported that the chimeric DNA clone was infectious when injected into SPF pigs, and that the resulting chimeric virus was attenuated in pigs. The chimeric virus was furthermore capable of inducing protective humoral immunity against PCV2 infection (Fenaux *et al.*, 2003;Fenaux *et al.*, 2004). Although not all vaccinated animals seroconverted, all were protected against challenge, supporting earlier reports that that protective immunity may also involve a cell-mediated immune response (Darwich *et al.*, 2003). A similar strategy could possibly be used in order to circumvent the inability of BFDV to grow in tissue culture.

1.6 Project aims

The breeding and export of psittacine birds is a substantial global industry, with North America, the Far East and Europe being the major target markets. In South Africa alone, the annual turnover of the industry is estimated to be in the order of US$ 30 million. PBFD is a major problem facing bird breeders and, in South Africa, approximately 10-20% of birds in commercial breeding programs are lost to the disease annually (Heath *et al.*, 2004;de Kloet & de Kloet, 2004). This equates to a loss of ZAR 24 million in annual revenue. An effective vaccine against BFDV would therefore be invaluable to breeders, and accordingly was one of the major aims of this project.

An integral part of the development of any new vaccine is estimating the genetic diversity of the pathogen in question. Significant genetic diversity often translates into high levels of antigenic variation among different isolates, which could negatively impact the broad application and effectiveness of a type-specific vaccine. Although different serotypes of BFDV have not yet been identified, isolates from Australia are known to exhibit a wide range of genetic diversity (Bassami *et al.*, 2001;Ritchie *et al.*, 2003). However, little is known about the diversity of isolates affecting birds in southern Africa. The first objective of the work described here was to establish what level of diversity exists among isolates implicated in recent epidemics of PBFD among commercial breeders in South Africa. At the time this work was initiated it was believed that a genotypic association between the viruses and their hosts existed. The validity of this association was tested through the comparative analysis of the African isolates and the previously described BFDV genotypes.

The CPs of other ssDNA viruses are not only responsible for the encapsidation of the viral genome, but are also known to be involved the replication cycle of the virus through direct interactions with both viral and host proteins. Although the CP of BFDV appears to be homologous to that of other ssDNA viruses, the specific role it plays in the replication cycle of the virus has never been explored. I undertook to investigate the biological activity of the BFDV CP, placing specific emphasis on the subcellular distribution of the protein and its role in transporting the viral genome across the nuclear envelope. I subsequently expanded the study to include investigation into the interaction of the CP with the replication-associated protein and its possible role in the replication cycle of the virus.

Since it is not possible to grow BFDV in cell culture, the use of recombinant DNA technology in the development of an effective vaccine appears to be inevitable (Todd, 2000). The coat protein of BFDV is a major constituent of the infectious viral particle and is therefore the likely target of immune surveillance. In light of this, the BFDV CP was chosen as the target for the development of a candidate recombinant vaccine. The suitability of recombinant DNA technology for the production of immunodominant antigens as subunit vaccines is well established. However, the appropriateness of any given expression system can be heavily influenced by the characteristics of the antigen to be expressed. The CP of circoviruses contains several motifs responsible for the biological activity of the proteins, which are known to impede expression in heterologous systems (Johne *et al.*, 2004). In addition, differences in the way protein is posttranscriptionally processed are likely to influence its antigenicity. The final objective of the work described here was to compare the immunogenicity of the BFDV CP expressed using prokaryotic and eukaryotic expression systems, respectively.

Chapter 2

Genetic diversity of Beak and feather disease virus in Southern Africa

Contents

Abstract

Psittacine beak and feather disease (PBFD), caused by Beak and feather disease virus (BFDV), is the most significant infectious disease in psittacines. PBFD is thought to have originated in Australia but is now found worldwide including Africa, where it threatens the survival of the indigenous endangered Cape Parrot and vulnerable Black-Cheeked Lovebird. We investigated the genetic diversity of putative BFD viruses from southern Africa. Feathers and heparinized blood samples were collected from 26 birds, representing 10 psittacine species, all showing clinical signs of PBFD. DNA extracted from these samples was used for PCR amplification of the putative BFDV coat protein gene (*CP*). The nucleotide sequences of the *CP* of 22 unique BFDV isolates were determined and compared with the previously described sequences of BFDV isolates from Australasia and America. Phylogenetic analysis revealed 11 BFDV lineages with the southern African isolates, representing at least 5 distinctly unique genotypes: 11 complete genome sequences were therefore determined, representing at least one of every distinct lineage. The nucleotide diversity of the southern African isolates was calculated to be 5.5% and is comparable to that found in Australia and New Zealand. BFDVs in southern Africa have, however, diverged substantially from viruses found in other parts of the world, as the average distance between the southern African isolates and BFDV-AUS ranged from 8.3% to 10.8%. In addition to point mutations, recombination was found to contribute substantially to the level of genetic variation amongst BFDVs, with evidence of recombination in all but one of the genomes analyzed.

A shorter version of this chapter was published as:

Heath, L., Martin D.P., Warburton, L., Perrin, M., Horsfield, W., Kingsley, C., Rybicki, E. P. and Williamson, A. Evidence of unique genotypes of Beak and feather disease in Southern Africa. *Journal of Virology* **78**: 9277-9284

2.1 Introduction

Psittacine beak and feather disease (PBFD) was first described in various species of Australian cockatoos in 1975 by Ross Perry, and has since been recognized as the most significant infectious disease in psittacine birds (Raidal *et al.*, 1993d). The International Committee for the Taxonomy of Viruses (ICTV) currently classifies all strains of Beak and feather disease virus (BFDV), the etiological agent of PBFD, as members of a single species and does not recognize the existence of any subtypes within the genus (Todd *et al.*, 2001a). However, the variability of clinical signs often associated with the disease has raised the question whether genetic diversity in BFDV may be responsible for the differences in the pathological manifestations of the disease.

Traditionally most of the research on BFDV has focused on isolates from Australasia. These isolates were found to be genetically diverse and grouped into four distinct phylogenetic clusters (Bassami *et al.*, 2001). Phylogenetic analysis of isolates from New Zealand revealed a similar clustering pattern and an apparent genotypic association with specific psittacine species (Ritchie *et al.*, 2003). The proposed host specificity of viral genotypes is based on the phylogenetic characteristics of a group of closely related viruses found only in lorikeets. These viruses differ significantly from other BFDV isolates and monophyly of the group is well supported. Recently, an atypical form of PBFD predominantly affecting African Grey parrots has been reported in parts of Europe (Schoemaker *et al.*, 2000). Strains associated with this form of the disease tend to cluster separately (Raue *et al.*, 2004). There is, however, no evidence of adaptive selection within the BFDV populations and the subdivisions are most likely the result of genetic drift of viral genotypes in different parrot populations.

PBDFV is a major problem among bird breeders in South Africa, where approximately 10 to 20% of the South African psittacine breeding stocks are lost due to the disease each year (Heath *et al.*, 2004;de Kloet & de Kloet, 2004). The incidence of PBFD in endemic parrot populations in southern Africa remains largely unknown, but at least two threatened species, the endangered Cape Parrot (*Poicephalus robustus)* and the vulnerable Black-Cheeked Lovebird (*Agapornis nigrigenis)*, are affected by the disease (Warburton & Perring, 2002). In this chapter we report on the genetic diversity of viruses associated with PBFD in southern Africa, and discuss the possible

Table 2.1 List of virus sequences determined from PBFD-affected birds originating from diverse geographical and host origins in southern Africa.

Host species	*Common name*	*Origin*	*Isolate*	*Genbank Acc. number*
Psittacus erithacus	African Grey parrot	South Africa	AFG3-ZA	AY450443
P. erithacus	African Grey parrot	Gauteng, South Africa	AFG4-ZA	AY450435
P. erithacus	African Grey parrot	KwaZulu-Natal, South Africa	AFG5-ZA	DQ397817
Cacatua alba	White Cockatoo	Gauteng, South Africa	UC1-ZA	AY450436
C.alba	White Cockatoo	Western Cape, South Africa	UC2-ZA	AY450450
Pionites leucogaster	White bellied Caique	Gauteng, South Africa	WBC1-ZA	AY450434
Ara macao	Scarlet macaw	South Africa	SM1-ZA	AY450451
Poicephalus rufiventris	African Red-bellied parrot	KwaZulu-Natal, South Africa	ARB1-ZA	AY450452
P. rufiventris	African Red-bellied parrot	KwaZulu-Natal, South Africa	ARB2-ZA	AY450448
P. rufiventris	African Red-bellied parrot	KwaZulu-Natal, South Africa	ARB3-ZA	AY450449
P. rufiventris	African Red-bellied parrot	KwaZulu-Natal, South Africa	ARB4-ZA	AY450440
Poicephalus gulielmi massaicus	Jardine parrot	KwaZulu-Natal, South Africa	GJP2-ZA	AY450447
P. g. fantiensis	Jardine parrot	KwaZulu-Natal, South Africa	LJP1-ZA	AY450446
Poicephalus gulielmi massaicus	Jardine parrot	KwaZulu-Natal, South Africa	GJP1-ZA	AY450441
P. g. gulielmi	Jardine parrot	KwaZulu-Natal, South Africa	BWJ1-ZA	AY450445
P. g. massaicus	Jardine parrot	KwaZulu-Natal, South Africa	GJP2-ZA	AY450447
P. g. fantiensis	Jardine parrot	KwaZulu-Natal, South Africa	LJP1-ZA	AY450446
P. g. gulielmi	Jardine parrot	KwaZulu-Natal, South Africa	BWJ1-ZA	AY450445
P. g. fantiensis	Jardine parrot	KwaZulu-Natal, South Africa	LJP2-ZA	AY450444
Poicephalus rueppellii	Rüppell's parrot	KwaZulu-Natal, South Africa	RP1-ZA	AY450439
Poicephalus robustus	Cape parrot	KwaZulu-Natal, South Africa	CPA8-ZA	AY450437
P. robustus	Cape parrot	KwaZulu-Natal, South Africa	CPA7-ZA	AY450438
P. robustus	Cape parrot	KwaZulu-Natal, South Africa	CPA10-ZA	DQ397818
P. robustus	Grey-headed parrot	Zambia	GHP1-ZAM	DQ397816
Agapornis nigrigenis	Black-cheeked Lovebird	Zambia	BCL1-ZAM	AY450442
Deroptyus accipitrinus	Hawk-head parrot	Gauteng, South Africa	HHP1-ZA	DQ397815

impact of the disease on conservation efforts directed towards protecting indigenous African parrots.

2.2 MATERIALS AND METHODS

2.2.1 Virus isolates

Feathers and heparinized blood samples from 26 psittacines suspected of suffering from PBFD were submitted by referring veterinarians throughout southern Africa. The birds either exhibited clinical signs of the disease or were known to have been in contact with diseased birds. Details of the samples are summarized in Table 2.1.

2.2.2 Determination of BFDV Diversity

DNA was extracted (Sambrook *et al.*, 1989) from the samples detailed in Table 2.1 and used for PCR amplification of the entire BFDV genome or parts thereof. The full-length BFDV genome was amplified by inverse PCR from individual blood samples using the primers 5' **GGATCC**A(G/T)CCGGTTCTGGC(G/A) 3' and 5' **GGATCC**CACTACAAAGGAGGACCC 3' (complementary sequences corresponding to the *BamHI* endonuclease restriction site are indicated in bold type). These partly complementary opposing sense primers were designed to straddle the *BamHI* endonuclease recognition site situated within the *CP,* taking into account all genetic diversity so far described for BFDV. Amplification of the circular BFDV genome would result in a linear representation of the entire genome. PCR products were purified using High Pure PCR purification columns (Roche) and cloned into the pGEM®-T-Easy plasmid (Promega). Recombinant clones were end-sequenced with commercially available M13 primers (Perkin-Elmer) using the DYEnamic ET Dye terminator cycle sequencing kit and analyzed on the MegaBACE 500 automated sequencer (Amersham Bioscience).

Based on previously published sequence data (Bassami *et al.*, 2001), three additional primers (5' GTATCGCCTGATGTGACGTCTG 3', 5' CTGGACATTGTGGCGAGAGAC 3' and 5' GACCGTTACCACCATAAAGTG 3') were designed for sequencing of the remainder of the genome. At least two clones for each isolate were independently sequenced to ensure the validity of the data. For isolated cases where it was not possible to generate full-length clones, the Rep and CP genes were amplified by PCR using primer sets wtCP-F/wtCP-R (5'

Table 2.2 List of Beak and feather disease virus reference sequences used in this study.

Host species	*Common name*	*Origin*	*Isolate*	*Genbank Acc. number*
Psephotus haematogaster	Bluebonnet	Australia	BB-AUS	AF311295
Trichoglossu haematodus	Rainbow Lorikeet	Australia	LK- AUS	AF311299
T. haematodus	Rainbow Lorikeet	New Zealand	RL2-NZ	AY148294
T. haematodus	Rainbow Lorikeet	New Zealand	RL5-NZ	AY148293
T. haematodus	Rainbow Lorikeet	New Zealand	RL6-NZ	AY148300
T. haematodus	Rainbow Lorikeet	New Zealand	TH1-NZ	AY518913
Melopsittacus undulatus	Budgerigar	New Zealand	BG3-NZ	AY148301
Cacatua leabeateri	Major Mitchell's Cockatoo	Australia	MMC- AUS	AF311300
C. galerita	Sulphur-crested Cockatoo	Australia	BFDV-AUS	AF080560
C. galerita	Sulphur-crested Cockatoo	Australia	SCC1- AUS	AF311302
C. galerita	Sulphur-crested Cockatoo	Australia	SCC2- AUS	AF311301
C. galerita	Sulphur crested Cockatoo	New Zealand	SCC2-NZ	AY148286
C. galerita	Sulphur crested Cockatoo	New Zealand	SCC3-NZ	*AY148287*
C. galarita	Sulphur crested Cockatoo	USA	SSC3-USA	AY518916
C. tenirostitus	Longbill Corella	New Zealand	LC1-NZ	AY148289
C. tenuirostris	Eastern long-billed Corella	Australia	ELBC-AUS	AF311297
C. moluccensis	Salmon-crested Cockatoo	USA	CM1-USA	AY518923
C. leadbeaterii	Major Mitchell's Cockatoo	Saidi Arabia	CL1-SA	AY518922
C. galerita	Sulphur crested Cockatoo	New Zealand	SCC5-NZ	AY148285
C. heamaturopygia	Red-vented Cockatoo	Saidi Arabia	CH1-SA	AY518921
Psitteuteles goldiei	Goldie's Lorikeet	New Zealand	GL-NZ	AY148298
Aratinga solstitialis	Sun Conure	Spain	AS1-SP	AY518916
L. chlorocercus	Yellow-bib Lorikeet	New Zealand	YBL1-NZ	AY148292
Poicephalus senegalensis	Senegal parrot	USA	PS1-USA	AY518925
Coracopsis vasa	Vasa parrot	United Kingdom	CV1-UK	AY518932
Ara militaris	Military Macaw	USA	AM1-USA	AY518931
Eolophus roseicapillus	Galah	Israel	ER01-ISR	AY518930
E. roseicapillus	Galah	Australia	Galah- AUS	AF311298
Amazona autumnalis	Red-lored Amazon	United Kingdom	AAT1-UK	AY518924
Poicephalus senegalensis	Senegal parrot	USA	PS1-USA	AY518925
Coracopsis vasa	Vasa parrot	United Kingdom	CV1-UK	AY518932
Eclectus rotatus	Eclectus parrot	USA	ER1-USA	AY518917
Amazona aestiva	Blue-fronted Amazon	Germany	AA1-GER	AY518903
A. aestiva	Blue-fronted Amazon	Germany	AA2-GER	AY518911
Ara militaris	Military Macaw	USA	AM1-USA	AY518931

GCGGCCGCATGCTGTGGGGCACCTCTAACTGC 3', 5' CTCGAGTCTTTATTAAG TACTGGGATTG 3') and wtRep-F/wtRep-R (5' AGATCTAGTCCGTCCAAGGAGGGATCTG 3', 5' AAGCTTCTAATAATTGATGGGG TGGGCGAG 3'), respectively. These primers were designed from analysis of published sequence data (not shown). Amplified products were cloned into the pGEM®-T-Easy plasmid (Promega) and sequenced with commercially available M13 primers.

2.2.3 Detection of recombination and positive selection

All nucleotide sequences determined in this study have been submitted to GenBank under the accession numbers indicated in Table 2.1 and aligned to published BFDV sequences (Table 2.2) using ClustalX (Thompson *et al.*, 1997).

The multiple sequence alignments of the complete BFDV genome sequences, as well as of the major ORFs of the virus, were used to determine the phylogenetic relationship of the viruses. Phylogenetic reconstruction was performed using the maximum likelihood method implemented in Phylip ver. 3.6. Bootstrap values were calculated using the neighbour-joining method based on the two parameter corrected distance matrix in MEGA 2.1 (Kumar *et al.*, 2001). The phylogenetic trees were rooted with sequences from non-psittacine avian circoviruses.

Detection of potentially recombinant sequences, identification of likely parent sequences, and localization of possible recombination break points was done using RDP (Martin & Rybicki, 2000), Geneconv (Padidam *et al.*, 1999b), Maxchi (Smith, 1992) and bootscanning (Salminen *et al.*, 1995) methods implemented the RDP software package. Wherever possible the origin of the recombinant regions was identified. Whenever a particular recombinant region's origin was identified, the isolate named is the closest known relative of the parental virus of that region. Base frequencies, nucleotide diversity and tranversion/transition ratios (ti/tv) were calculated using either PAUP*4.1 (Swofford, 2001) or MEGA 2.1 where applicable. The average number of synonymous changes per synonymous site (π_s) and non-synonymous changes per non-synonymous site (π_{ns}) along all ORFs were calculated using DNASP 3.1 (Rozas & Rozas, 1999). The distribution of synonymous and non-synonymous substitutions along the viral genes was determined using the average number substitutions per codon position (site). The significance of

Table 2.2 Continued

Host species	*Common name*	*Origin*	*Isolate*	*Genbank*
Eos reticulata	Blue-streak Lorikeet	New Zealand	BSL1-NZ	AY148296
Eos reticulata	Blue-streak Lorikeet	New Zealand	BSL2-NZ	AY148297
Psittacus erithacus	African Grey parrot	United Kingdom	PE1-UK	AY521238
P. erithacus	African Grey parrot	Germany	PE2-GER	AY518920
P. erithacus	African Grey parrot	Germany	PE3-GER	AY521236
P. erithacus	African Grey parrot	Germany	PE4-GER	AY518928
P. erithacus	African Grey parrot	Germany	PE5-GER	AY521237
P. erithacus	African Grey parrot	Germany	PE6-GER	AY518915
P. erithacus	African Grey parrot	Germany	PE7-GER	AY518902
P. erithacus	African Grey parrot	Portugal	PE8-POR	AY518910
P. erithacus	African Grey parrot	India	PE9-IND	AY518906
P. erithacus	African Grey parrot	Japan	PE10-JAP	AY518905
P. erithacus	African Grey parrot	United kingdom	PE11-UK	AY518904
P. erithacus	African Grey parrot	Germay	PE12-GER	AY518914
P. erithacus	African Grey parrot	Portugal	PE13-POR	AY518901
P. erithacus	African Grey parrot	Germay	PE14-GER	AY518899
P. erithacus	African Grey parrot	Portugal	PE15-POR	AY518912
P. erithacus	African Grey parrot	USA	PE16-USA	AY518927
P. erithacus	African Grey parrot	Puerto Rico	PE17-POR	AY518926
Psittacula krameri	Rose-ringed Parakeet	USA	PK1-USA	AY518900
P. kramerii	Rose-ringed Parakeet	USA	PK2-USA	AY518929
P. kramerii	Rose-ringed Parakeet	USA	PK3-USA	AY521234
Agapornis roseicollis	Rosy-faced Lovebird	Australia	LB- AUS	AF311296
A. roseicollis	Rosy-faced Lovebird	United Kingdom	AR1-UK	AY521235
A. roseicollis	Rosy-faced Lovebird	United Kingdom	AR2-UK	AY518918
A. roseicollis	Rosy-faced Lovebird	USA	AR7-USA	AY518908
A. roseicollis	Rosy-faced Lovebird	USA	AR8-USA	AY518907
A. roseicollis	Rosy-faced Lovebird	United Kingdom	AR3-UK	AY518909
A. roseicollis	Rosy-faced Lovebird	United Kingdom	AR5-UK	AY518919

clustering in the distribution was tested with a permutation test using a sliding window ranging between 5 and 20 sites per window. Sites under positive selection were identified using likelihood-based models described by Yang *et al.* (Yang *et al.*, 2000), which assign each site to one of a number of estimated values of non-synonymous and synonymous substitution ratios (ω). The nomenclature used herein to describe each of the models is consistent with that proposed by Yang *et al.* (Yang *et al.*, 2000). The results from nested models were compared using a likelihood-ratio test. Twice the likelihood-ratio difference was compared with a χ^2 distribution having a degree of freedom equal to the difference in the number of parameters between the respective models.

2.3 RESULTS

2.3.1 Phylogenetic relationships and recombination

The complete nucleotide (nt) sequences of 11 putative BFDV isolates, obtained from diverse psittacine species at different geographical locations in southern Africa, were determined and aligned to previously published sequences. All southern African isolates displayed the same basic genome structure as previously described for BFDV (Niagro *et al.*, 1998a), including the positions of the ORFs and the stem-loop structure located between the *Rep* and *CP*. The genome sizes ranged from 1987 to 2002 nucleotides (CPA10-ZA = 1987, AFG4-ZA, UC1-ZA and WBC1-ZA = 1988 nt; AFRG3-ZA = 1993 nt; BCL1-ZAM = 1996 nt; GJP1-ZA, RP1-ZA, ARB4-ZA, CPA7-ZA and CPA8-ZA = 2002 nt). The mean nucleotide diversity of the southern African isolates was calculated to be 0.055 ±0.003 (n = 11) and the average distance between the southern African isolates and BFDV-AUS ranged from 8.3% to 10.8%.

The phylogenetic relationship of the 11 southern African isolates with 15 previously published BFDV isolates is shown in Figure 2.1 (a). As previously reported, the BFDV isolates were grouped into clearly defined genetic clusters or lineages. For descriptive purposes we have tentatively defined a genotype as any group of sequences that is at least 5% divergent from the next closest lineage. The southern African isolates were restricted to 5 out of the 10 apparent BFDV genotypes.

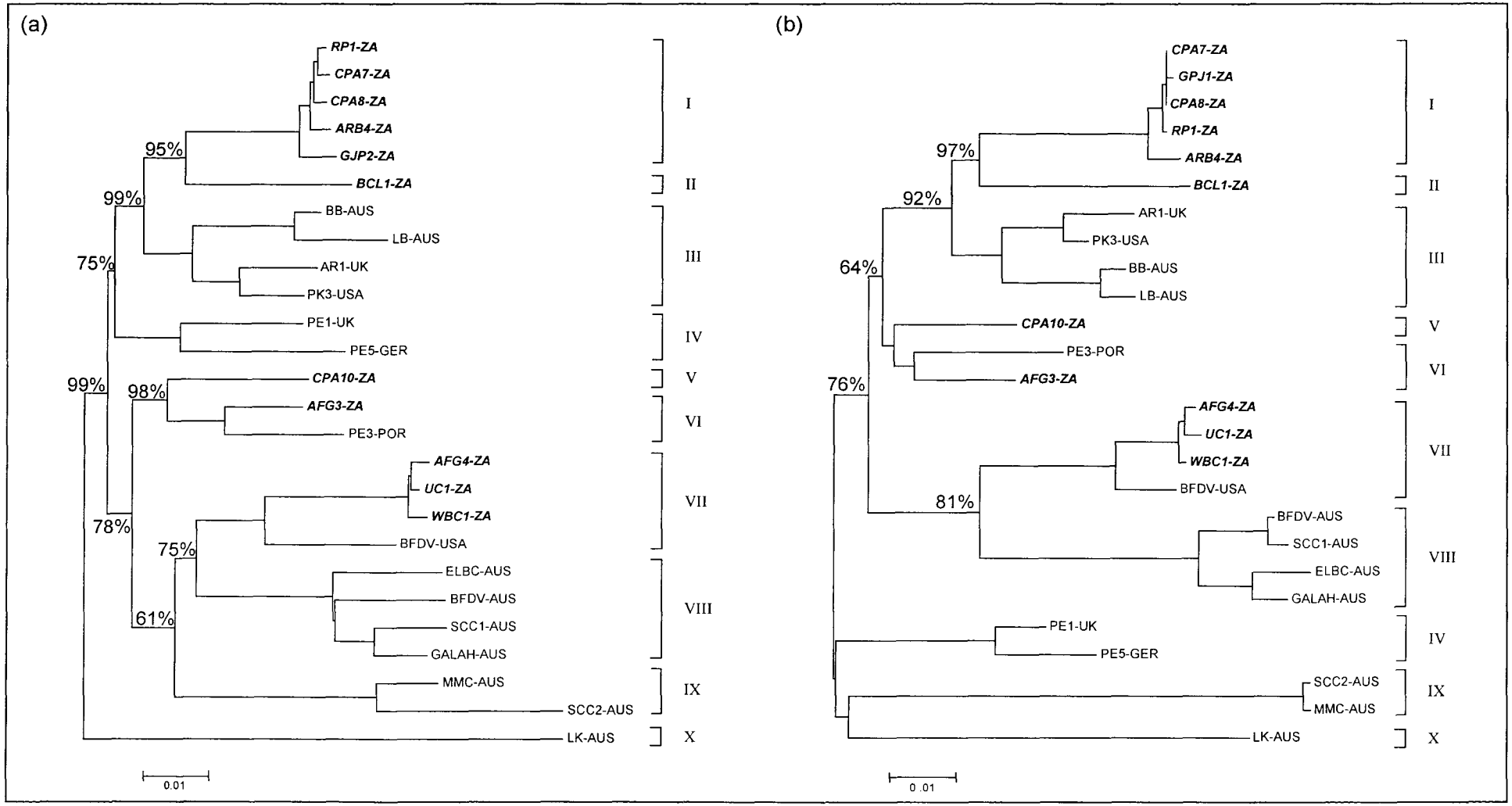

Figure 2.1 Maximum likelihood trees depicting the phylogenetic relationship of 20 BFDV isolates (a) ignoring recombination between isolates and (b) with major recombinant regions removed from individual sequences. The full-length tree was rooted using 3 non-psittacine avian circoviruses (Canary circovirus, AJ30633; Colimbid circovirus, AJ252610; Goose circovirus, AJ304456). Virus sequences determined during this study are printed in italicized bold type. Only the sub-tree containing the BFDV isolates are shown. Bootstrap values were calculated using the neighbour-joining method based on the two parameter corrected distance matrix. The major virus genotypes are bracketed and defined by the roman numerals I-VIII.

Three southern African isolates (AFG4-ZA, WBC1-ZA, UC1-ZA) were found to be closely related to a genomic consensus sequence derived from pooled BFDVs from the USA (GenBank accession number AF 071878).AFG3-ZA clustered with PE3-POR, a strain isolated from the same species (*Psittacus erithacu*) from Portugal. The remainder of the southern African isolates clustered as 3 distinct genotypes.

The relative positions of some viruses within the phylogenetic tree were found to vary when different parts of the genome were used as the basis of the analyses. This suggested that recombination may contribute significantly to the evolution of BFDV. A multiple alignment of the full-length BFDV genomes was used in conjunction with four independent recombination detection programs to detect potential recombination events. The results of the recombination analysis are shown in Figure 2.2. Evidence of recombination was found in all but one of the sequences analyzed: no recombinant regions were identified within the genome of isolate BCL1-ZAM. Recombination events were distributed across the entire genome, but were more frequent within the *Rep*. Inter-lineage, as well as intra-lineage recombination were detected. Most notably, two putative recombination events were detected within the LK-VIC sequence, each involving a second sequence outside the *LK* lineage (*LK* lineage previously described by Ritchie *et al.* 2003 (Ritchie *et al.*, 2003)). Both events were statistically well supported, and identified MMC-WA and GJP1-ZA-like viruses as potential minor parents.

It has long been recognized that recombination can drastically affect the accuracy of phylogenetic inference. In the case of BFDV isolates analyzed here, the branching pattern of the maximum likelihood tree constructed from an alignment with the obvious recombinant regions removed (Figure 2.1(b)), remained largely unchanged relative to that of a tree constructed from an alignment containing the recombinant regions. All 10 tentative genotypes remain clearly defined and well supported by significant bootstrap values. The branch lengths were, however, affected. In such cases where the evolutionary history is affected by recombination, it would be more accurate to describe the relationships in terms of reticulate evolution or, alternatively, the possible inaccuracy of phylogenetic estimates for samples with several underlying phylogenies can be addressed by defining data partitions (such as genes) and performing separate analyses on each partition (Wiens, 1998).

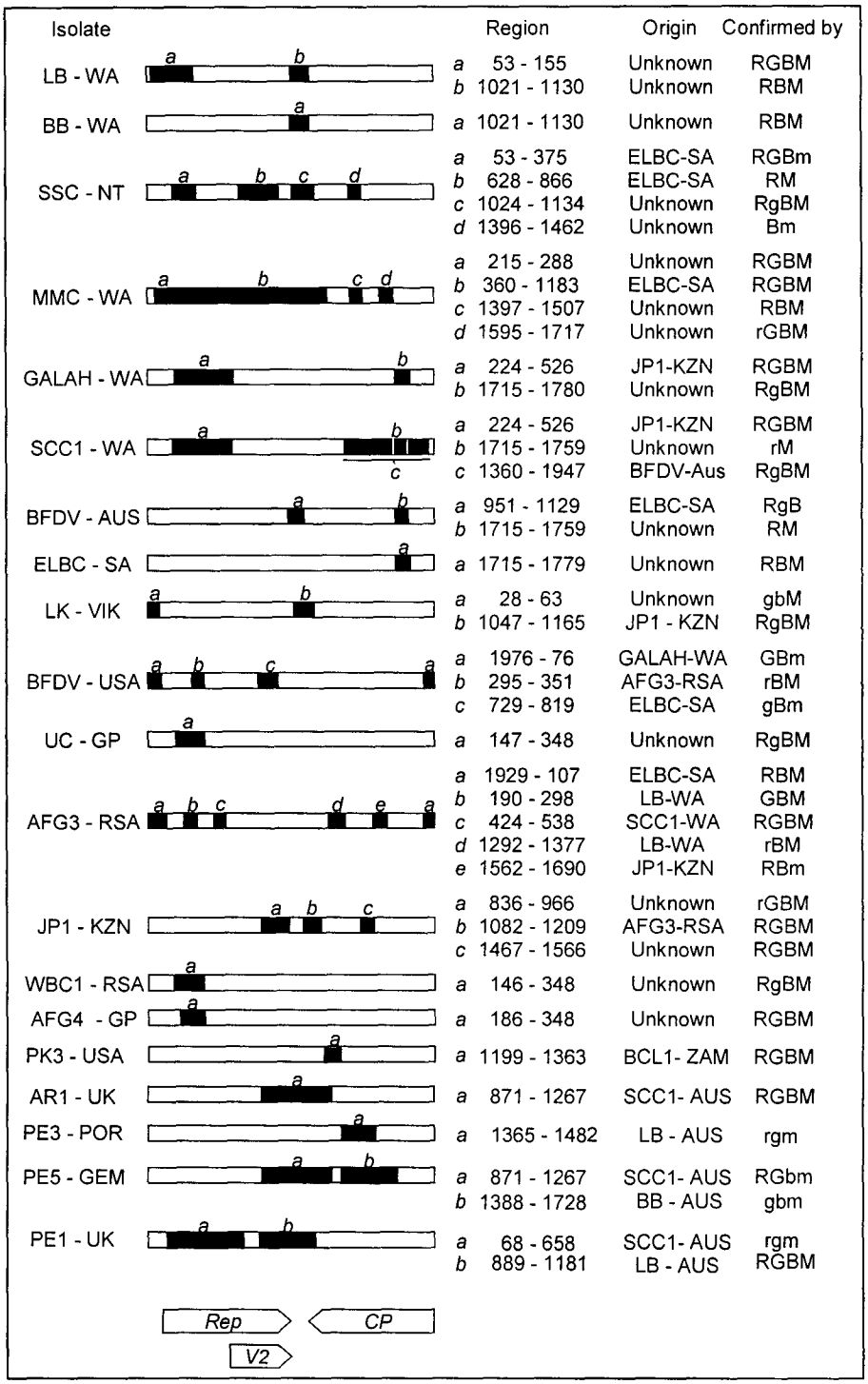

Figure 2.2. Putatively recombinant regions detected within BFDV sequences. Region coordinates are nucleotide positions relative to the origin of viron strand replication. *Rep* = replication associated protein gene. *CP* = coat protein gene. *V2* = second virion sense ORF. Wherever possible the origin of the recombinant regions has been identified. Whenever a particular recombinant region's origin is identified, the isolate named is a close relative of the parental virus of that region. Recombinant regions and parental viruses were identified using the RDP (R), GENECONV (G), bootscan (B) and MaxChi (M) methods. Uppercase letters indicate that a region was identified by a method with greater than 99% certainty (99% bootstrap support in the case of bootscan). Lowercase letters indicate that a region was identified by a method with between 95 and 99% certainty (between 95 and 99% bootstrap support in the case of bootscan).

2.3.2 The capsid protein

The capsid protein genes of 11 additional southern African BFDV isolates were amplified by PCR, sequenced and aligned to previously published sequences. Of the 702 nucleotides constituting the gene, 352 (50.07%) were conserved across all taxa. At an amino acid level this means 106 of a total of 236 amino acids (44.98%) were completely conserved. The mean nucleotide diversity (π) was calculated to be 0.110 ±0.007 (n = 71). The sequences displayed unequal base frequencies for each of the 3 codon positions and the rate of substitution was significantly biased towards transitions (ti/tv = 1.92).

The distribution of nucleotide substitutions along the *CP* is shown in Figure 2.3. The distribution of non-synonymous sites showed no evidence of clustering irrespective of the window size, whilst the synonymous profile contains a single significant mutational "cold spot" (i.e. gap) centered at position 25 (window size = 15, P = 0.026). Interestingly, the position of the cold spot corresponds to one of two putative bipartite nuclear localization signals situated between positions 15-32 and 23-40 respectively (data not shown). The presumably vital role of this region of the CP in nuclear transport and in assembly may severely limit the number of permissible mutations.

The ratio of non-synonymous to synonymous substitutions (dN/dS) across the entire CP gene was estimated at 0.455, suggesting that the gene was not under positive selective pressure. Calculating dN/dS values across an entire gene may, however, be too insensitive to detect positive selection acting on only a few sites, resulting in the underestimation of the overall selective pressure exerted on the protein (Nielsen, 1997). This problem can be partially overcome by codon-based methods implemented within a maximum likelihood framework which allow for heterogeneous ratios among sites (Nielsen & Yang, 1998). Analyses of the *CP* data set using models M3 (discrete) and M8 (beta&ω), implemented in the codeml component of the PAML program package, provided consistent evidence of positive selection in the gene. Approximately 8% of sites were estimated to be under positive selection (M3; $p_2 = 0.083$, $\omega_2 = 2.91$).

Individual sites under positive selection were identified by a Bayesian approach. Sixty-six sites with P(ω>1) > 0.5 were identified by model M3. Only 37 sites were identified by model M8, all included in the set identified under the discrete model. When more stringent criteria (P(ω>1) >

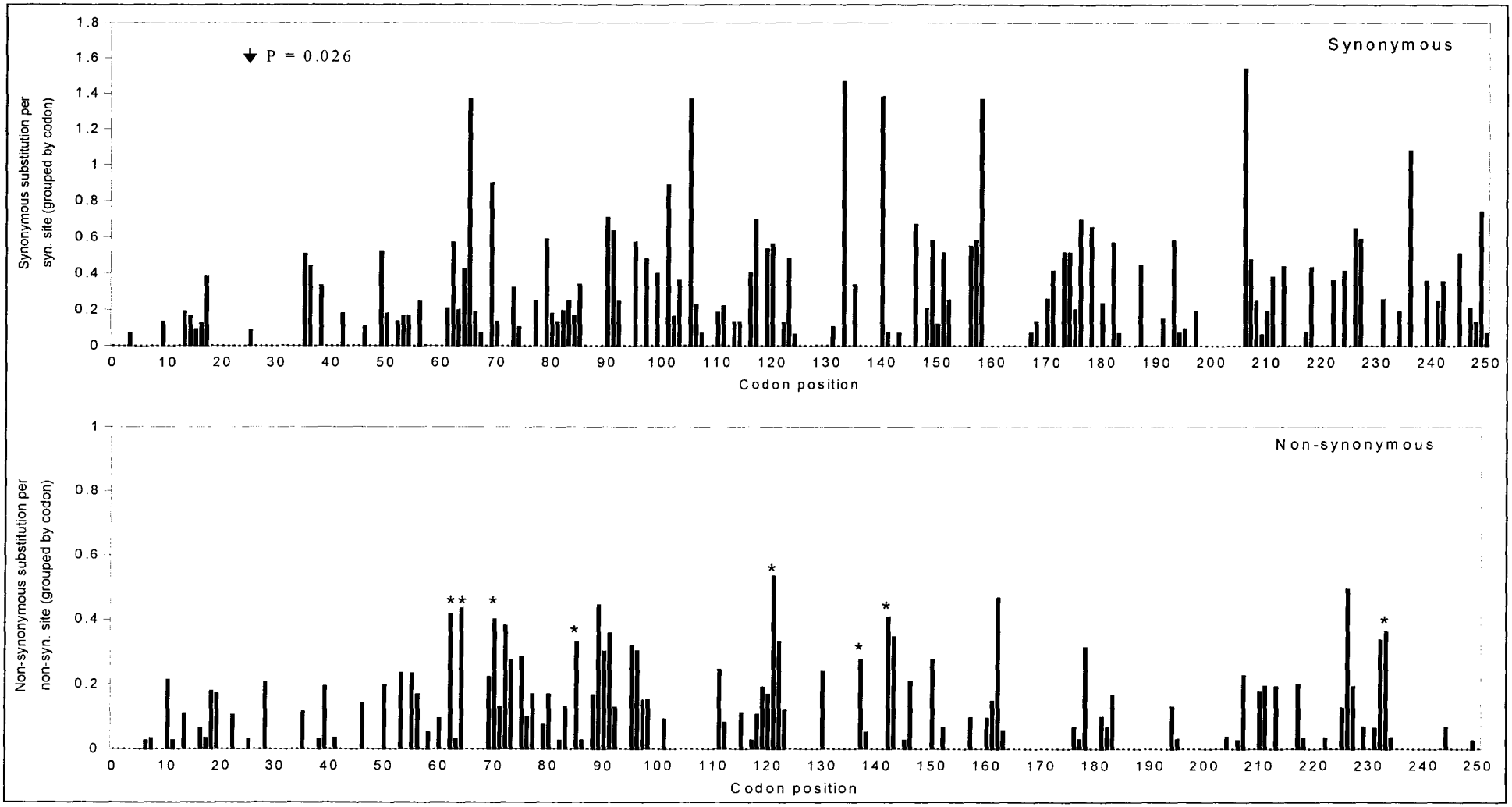

Figure 2.3. Distribution of nucleotide substitutions along the BFDV capsid protein gene. The Y-axis indicates the number of substitutions per site averaged over al possible pairwise sequences. The synonymous mutational cold spot centered at position 25 is indicated by the arrow. Non-synonymous substitutions identified as being under positive selection are marked by an asterisk (*).

0.95) were used only nine sites (sites 62, 64, 69, 70, 85 121, 137, 142 and 233) common to both models were identified.

Significantly high recombination rates can lead to an overestimation of substitutions, since the likelihood models for detection of positively selected sites rely on the phylogenetic relationship among sequences (Haydon *et al.*, 2001). To test whether recombination affected the identification of sites under positive selection, the analyses were repeated using a modified data set in which the major recombinant regions within individual sequences were removed. Estimates of the parameters using the revised tree topology under the M3 model did not differ significantly from those presented above (Table 2.3). The identification of sites under positive selection was also not influenced by recombination within the data sets (results not shown).

Table 2.3 Likelihood values and parameter estimates for the BFDV capsid protein.

Model Code	*n**	*ℓ*‡	*dN/dS*	*Estimates of parameters*
Ignoring recombination				
M3 (3 categories)	29	-3379.21	0.534	$p_2 = 0.083$ $\omega_2 = 2.91$
M7	29	-3399.08	0.365	$p = 0.102$ $q = 0.177$
M8	29	-3381.51	0.549	$p_0 = 0.84579$ $p = 0.23812$ $q = 0.84027$ $p_1 = 0.154$ $\omega = 2.356$
Recombinant regions removed				
M3 (3 categories)	28	-3383.78	0.537	$p_2 = 0.057$ $\omega_2 = 3.44$
M7	28	-3398.29	0.392	$p = 0.09449$ $q = 0.14654$
M8	28	-3384.28	0.558	$p_0 = 0.90511$ $p = 0.15727$ $q = 0.33863$ $p_1 = 0.09489)$ $\omega = 2.85629$

* Number of sequences in the data set.
‡ The log likelihood of the fitted model.

A maximum likelihood tree depicting the phylogenetic relationship of the *CP* sequences is shown in Figure 2.4 (a). The topology of the *CP* phylogeny closely resembles that reconstructed from the full genome sequences with 11 clearly defined genetic clusters. The southern African isolates were restricted to lineages I, II, V, VI and VII. The additional cluster consisted of a single isolate from the United Kingdom (CV1-UK). This isolated was originally considered to be a member of genotype VIII (de Kloet & de Kloet, 2004). However, it is our opinion that this isolate is sufficiently diverged for it to represent a unique genotype. Recent analyses of BFD viruses from Europe, the United Kingdom and the United States of America indicated a possible association

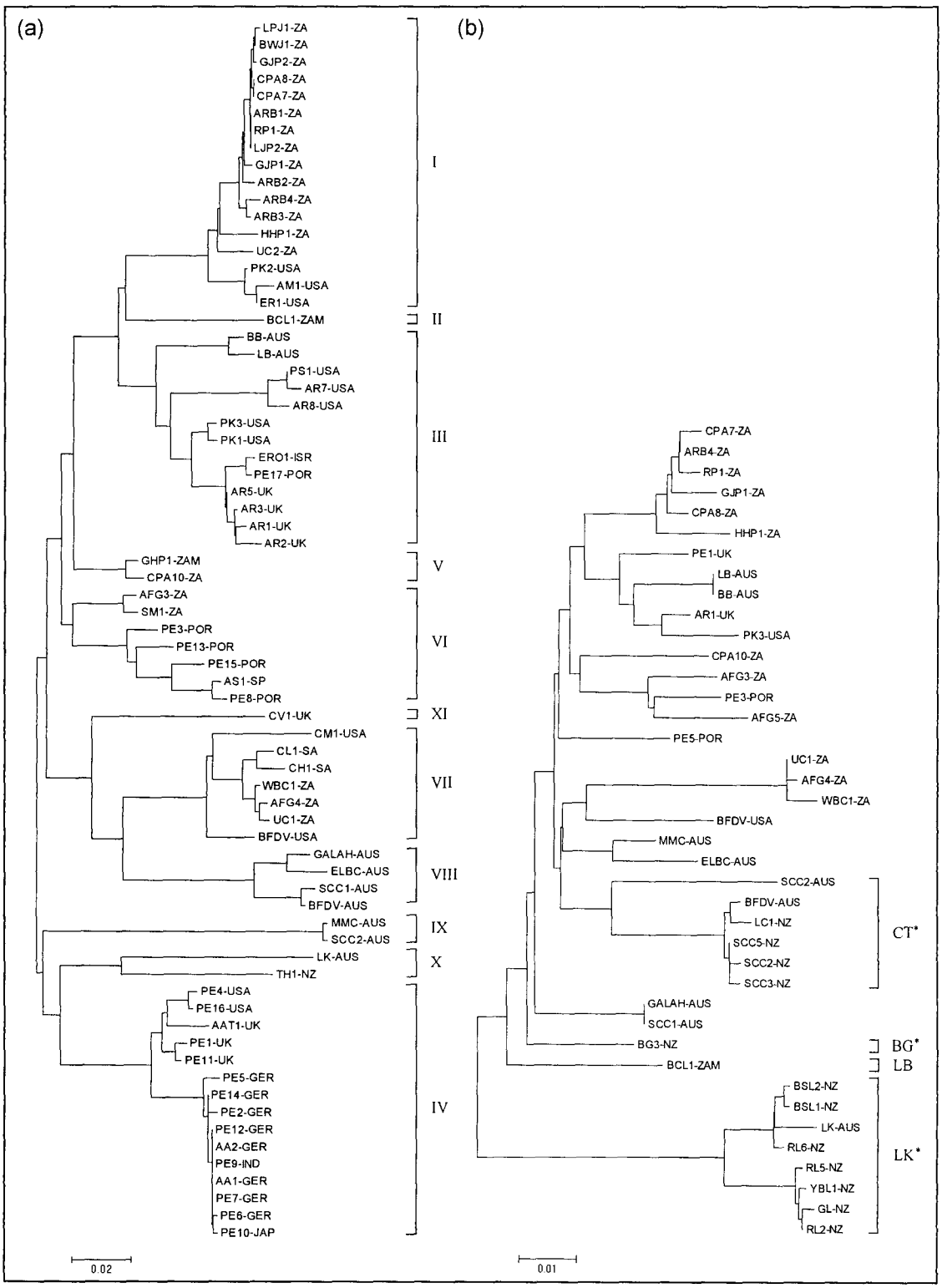

Figure 2.4. Maximum likelihood trees depicting the phylogenetic relationship of (a) the capsid and (b), Rep genes of BFDV, respectively. Lineages supported by significant bootstrap values are bracketed. The *CT, LB*, and *LK* lineages within the *Rep* phylogeny were previously described by Ritchie *et al.* (2003) and refer to the group of host species associated with the genotype: *CT* = cockatoos; *BG* = budgerigar; and *LK* = lorikeets. The LB lineage described herein similarly refers to the host species associated with the genotype:*LB* = lovebird.

between certain genotypes and specific host species (de Kloet & de Kloet, 2004). The specificity is however not absolute as strains generally associated with a certain species can on occasion infect distantly related hosts. Southern African lineages could not be associated with specific psittacine species. At least 3 southern African genotypes consist of closely related viruses isolated from species representative of both families *Psittacidae* (parrots) and *Cacatuidea* (cockatoos) within the order *Psittaciformes*.

2.3.3 The replication associated protein

A portion of the *Rep* of 40 isolates, corresponding to nt positions 198 to 759 of the BFDV genome AF080560, was aligned. As with the *CP*, unequal base frequencies were observed within *Rep* for all 3 codon positions, and transitions were favored above transversions. The genetic variation within *Rep* was, however, found to be considerably less than the variation within the *CP* (π = 0.066 ±0.006; n = 37). The ratio of non-synonymous and synonymous substitution across *Rep* was estimated at 0.17. The discrete model (M3) fitted the data well with less than 1% of ω>1. The beta mixture models (M8-M11) did not fit the data any better than the simple beta model (M7), the parameter estimates of which suggested a highly skewed L shape for the ω distributions. These results suggest that there are no obvious sites within *Rep* that are under positive selection. Removing recombinant regions within individual sequences did not significantly affect the outcome of the analyses (data not shown).

The phylogenetic relationships of the *Rep* sequences are shown in Figure 2.4 (b). The topology of the maximum likelihood tree based on the *Rep* sequences differed slightly from that of the tree based on the *CP* phylogeny. In addition to the 3 lineages described by Ritchie *et al.* (Ritchie *et al.*, 2003), only one additional lineage was supported by significant bootstrap values. This fourth lineage (Lovebird, *LB*) was identified from a single sequence isolated from a Black-cheeked Lovebird, sampled in the mid-Machile River area in Zambia. The *Rep* sequence of this isolate (BCL1-ZAM) differed by at least 5% from all other sequences.

The differences in the topology of the trees are partly due to recombination within the *Rep.* Posada and Crandall (Posada & Crandall, 2002) demonstrated that the effect of recombination on phylogenies is dependent upon the relatedness of the sequences involved and the size of the

recombinant region. In cases where recombination occurred between closely related taxa or when recombination was ancient, the phylogeny under which the majority of the sites were evolved will generally be recovered. However, when the recombinational break-point divides the region in two parts of similar length, an inaccurate phylogeny underlying the data will most likely be inferred (Posada *et al.*, 2002). This is most evident in our data when one considers the relative positions of isolates MMC-WA and SCC-NT within each of the respective trees. These isolates represent a unique lineage within the *CP* phylogeny (lineage IX), which is not represented in the tree based on the *Rep* sequences. As can be seen in Figure 2.2, the genomes of both isolates are recombinant for large portions of the *Rep*. The frequent occurrence of recombination within *Rep*, with recombinant tracts often exceeding 50% of the gene's length, will most likely seriously compromise the reliability of BFDV phylogenies and phylogenetic inferences based on this gene alone.

2.4 DISCUSSION

It is evident that indigenous psittacine species in southern Africa are at risk from psittacine beak and feather disease, and that to ignore the impact of BFDV on both captive and wild populations of African parrots might severely hamper efforts to conserve this natural heritage. As part of an integrated approach to the management of the threat posed by BFDV, we have investigated the genetic diversity of putative BFDV isolates in southern Africa.

The level of genetic diversity amongst southern African BFDV isolates is similar to that which has been described in Australia and New Zealand. The southern African isolates have apparently, however, diverged substantially from viruses found in other parts of the world, and cluster into 3 unique genotypes. Assuming the disease is not endemic in Africa, the existence of these clearly defined subpopulations would suggest that BFDV was introduced into southern Africa on at least 3 separate occasions. In addition to these genotypes, two groups of southern African isolates were found to be closely related to viruses isolated in Europe (Genotype VI) and North America (Genotype VII), respectively. The fact these genotypes are almost exclusively associated with African species suggest that they represent additional genotypes that evolved in Africa and subsequently spread to other parts of the world.

The level of divergence between the genotypes in Africa and isolates found in other parts of the world does, however, suggest that the occurrence of BFDV on the African continent is not due to recent introductions, and that Australian and African BFDV populations have possibly diverged sufficiently to produce regionally distinct lineages. The existence of African genotypes is further supported by variations in certain sequence elements unique to a group strains isolated from African species bred in Europe (de Kloet & de Kloet, 2004). The exclusive association of these elements with African species would suggest that they evolved subsequent to the dissemination of the ancestral strain throughout the world. This draws in to question the theory that BFDV arose in Australia. It could be argued that the similar level of genetic diversity of BFDV isolates in Africa relative to most of the Australian lineages, and the clear separation of African from Australasian lineages, indicates that these groups of viruses diverged at an very early stage in their evolutionary history and may in fact represent a Gondwanaland-type of distribution associated with continental drift as has been postulated for geminivirus divergences (Rybicki, 1994). The existence of the BFDV lineage exclusively associated with Lorikeets and thus far found only in Australasia, adds an interesting dimension to this theory: this group of viruses could represent the "true Australian variant" of BFDV, with all other BFDV isolates evolving from a common ancestor which possibly originated in Africa and subsequently spread to Australasia.

With the constant movement of birds across geographical borders through trade, there is an increasing risk of spreading the disease into new areas and populations. Coupled to this is the risk of generating unique viruses through recombination between established virus populations and newly introduced viruses. Recombination has been well documented as a key strategy for generating diversity in both RNA and DNA viruses (Chenault & Melcher, 1994b;Chenault & Melcher, 1994a;Lai, 1992) and appears to contribute substantially to the level of genetic variation amongst BFDVs. We found evidence of recombination in all but one of the viral genomes analyzed in this study. Putative recombination events between viruses from Africa and Australia presumably occurred prior to the geographical isolation. Alternatively, the generation of recombinant genotypes may have occurred due to the mixing of viral populations infecting avian species in captivity.

BCL1-ZAM is the only isolate that appear to represent a pure genotype and was isolated from a Black-cheeked Lovebird in the Machile River area during the 2000 breeding season (Warburton & Perring, 2002). The Black-cheeked Lovebird is exclusively found in a highly localized range in south-west Zambia with the geographically isolated northern population largely confined to Kafue National Park (Dodman *et al.*, 2000). It is highly unlikely that this population has had any contact with psittacines from outside their range. The failure to detect any recombinant regions within the genome of the Zambian isolate, further suggests that separate and unique southern African genotypes may exist.

Mixed infections have recently been shown to occur for BFDV (Albertyn *et al.*, 2004;de Kloet & de Kloet, 2004). The frequency of recombination detected amongst isolates suggests that it is a common feature of the disease. The putative recombination event between LK-VIC and viruses outside the *LK* lineage further implies that the speculative genotypic association of viral sequences and specific psittacine hosts does not exclude mixed infection involving diverse lineages. Several putative recombination events were detected for which the possible parent sequences could not be identified. This suggests that not all circulating BFDV genotypes were represented in our data sets and that the full breadth of BFDV diversity is not accurately represented by the isolates described thus far.

The relative contribution of mutation, reassortment and recombination to genetic diversity within viral genomes varies depending on the characteristics of the particular virus and gene products involved (Holland & Domingo, 1998;Keese & Gibbs, 1993). Interestingly, we found no evidence of sites under diversifying selective pressure within the *Rep*. The functional role of Rep may limit the number of non-synonymous substitutions that would not adversely affect its performance. This is reflected by the high proportion of the sites within Rep with $\omega < 1$ (M3; $p_0 = 0.647$, $\omega_0 = 0.00737$), suggesting that the protein might be under purifying selective pressure. However, most of the detectable recombination events in our dataset have occurred within *Rep*. These results suggest that recombination might contribute substantially more to the level of variation within *Rep* than genetic drift. This situation is similar to that found in plant infecting geminiviruses (Padidam *et al.*, 1999b). There is substantial phylogenetic evidence suggesting that BFDV-like circoviruses are descendants of a recombinant virus that inherited the 5' portion of its *Rep* and its origin of virion sense replication from a plant-infecting nanovirus or geminivirus (Gibbs &

Weiller, 1999;Niagro *et al.*, 1998b). Recombinational exchange of *Rep* fragments between massively diverged or even unrelated virus lineages suggests that this gene and its product may have uniquely modular properties - a suggestion that is supported by the *Rep* recombinants detected in this and a geminivirus recombination study (Padidam *et al.*, 1999b).

The coat protein of BFDV is a major constituent of the infectious viral particle and is therefore a likely target of immune surveillance. This assumption is supported by the varying ω ratios of individual sites across the gene. Comparison of relative fixation rates of synonymous and non-synonymous mutations revealed statistically significant evidence that the capsid protein is under positive selection pressure. The distribution of positively selected sites in structural and immunogenic proteins of various viruses, such as *Foot and mouth disease virus* and HIV, has been shown to correspond to known monoclonal antibody epitopes (Haydon *et al.*, 2001). The hypervariability of specific sites within the *CP* of BFDV could, similarly, be the result of immune evasion. Different serotypes of BFDV have not yet been identified, but the possibility that antigenically distinct subgroups of BFDV could exist should not be ignored in the design of vaccines against the virus.

The genetic diversity of the global virus population, compounded by the difficulty to effectively curb the illegal trade in wild-caught birds, has serious implications for the control of the PBFD in southern Africa. Continued monitoring of both wild and captive populations will be a central feature of our efforts to understand BFDV epidemiology and effectively address the threat posed by the virus to endangered parrot populations. Since all the putative genotypes of BFDV are clearly defined by the relationships between their capsid proteins sequences, molecular characterization of this gene can potentially be used to trace the origin of PBFD outbreaks at a global level. The PCR primers described in this chapter could be a useful tool in this regard. The effects of recombination on direct phylogenetic reconstruction, or phylogenetically based analyses should however not be ignored if accurate inferences about the evolutionary history of the viruses are to be made.

Chapter 3

Interaction of the Beak and feather disease virus capsid and replication-associated proteins

Content

Abstract

Circoviruses are dependent on the replication machinery of the host cell for *de novo* DNA synthesis. Accordingly, the viral DNA needs to cross both the plasma membrane and the nuclear envelope before replication can occur. Here, I report on the subcellular distribution of the respective BFDV proteins and test the hypothesis that the CP is responsible for transporting the viral genome, as well as Rep, across the nuclear envelope. The intracellular localization of the BFDV CP was found to be directed by one or more of three bipartite nuclear localization signals (NLS) situated between the residues 16 to 56 at the N-terminus of the protein. Moreover, a DNA binding domain was also mapped to the N-terminus of the protein and falls within the region containing the putative NLSs. The ability of BFDV CP to bind DNA, coupled with the karyophilic nature of this protein strongly, suggests that it may be responsible for nuclear targeting of the viral genome, presumably through the formation of a nucleoprotein complex. Interestingly, expression of BFDV Rep in insect cells revealed that it is restricted to the cytoplasm. However, the subcelullar distribution of BDFV Rep and CP when co-expressed in insect cells, strongly suggests that the nuclear localization of Rep is facilitated by an interaction with the CP. This report highlights the possible involvement of CP in the replication of BFDV.

A shorter version of this chapter was published as:

Heath, L., Williamson, A. and Rybicki, E. P. The Capsid Protein of Beak and Feather Disease Virus Binds to the Viral DNA and Is Responsible for Transporting the Replication-Associated Protein into the Nucleus. *Journal of Virology.* **80**.

3.1 Introduction

Circoviruses lack an autonomous DNA polymerase and are dependent on the replication machinery of the host cell for *de novo* DNA synthesis (Meerts *et al.*, 2005). Since DNA synthesis occurs exclusively in the nucleus, the viral DNA needs to cross both the plasma membrane and the nuclear envelope before a productive infection can be established. Entry of PCV into the cell has been shown to occur *via* clathrin-mediated endocytosis and undoubtedly involves the capsid protein (Gilpin *et al.*, 2003;Misinzo *et al.*, 2005). The exact mechanism by which the viral DNA is released into the cell and subsequently relocated to the nucleus is however unknown.

Viral replication is dependent upon cellular enzymes expressed during S-phase and will only commence after the host cell has passed through mitosis. This, coupled with the fact that the viral DNA is by itself unable to penetrate the nucleus, suggests that it is included in the daughter-nucleus at the completion of mitosis (Tischer *et al.*, 1987). However, in the case of the plant-infecting geminiviruses viral DNA transport is mediated by the capsid protein (CP), and either the nuclear shuttle protein (NSP) or the movement protein (MP) depending on the particular geminivirus (Boulton, 1991;Liu *et al.*, 1999;Qin *et al.*, 1998;Liu *et al.*, 2001a;Palanichelvam *et al.*, 1998;Palanichelvam *et al.*, 1998). Both the CP and NSP are actively targeted to the nucleus and are able to shuttle between the nucleus and the cytoplasm. The capsid protein of PCV 2 has also been shown to localize to the nucleus (Liu *et al.*, 2001c;Liu *et al.*, 2001b). The intracellular localization of the PCV CP is directed by a bipartite nuclear localization signal situated at the N-terminus of the protein (Liu *et al.*, 2001b). The karyophilic nature of this protein suggests that the CP of circoviruses may, like the geminivirus CP, be involved in DNA translocation.

A prerequisite for the involvement of proteins in the translocation of viral DNA is that they need to be directly or indirectly associated with the viral genome. In line with this, the CP and NSP of geminiviruses have been shown to bind cooperatively to ssDNA as well as to dsDNA in a sequence-independent manner (Liu *et al.*, 1997;Liu *et al.*, 1999). The interaction between structural proteins and viral DNA has furthermore been shown to be a requirement for effective capsid assembly of several DNA viruses (Liu *et al.*, 2001a;Johne *et al.*, 2004). The capsid proteins of PCV and BFDV are expected to interact in a similar way with the packaged viral

DNA. In this chapter, I report on investigations designed to determine the subcellular distribution of the BFDV capsid protein and to test the hypothesis that the CP is responsible for transporting the viral genome across the nuclear envelope.

3.2 Methods and materials

3.2.1 Virus, cells and sera

All bacterial plasmids were maintained in *E. coli* DH5α (Invitrogen). Recombinant baculoviruses cultivated in *Spodoptera frugiperda* Sf-21 cells, grown in TC-100 Insect medium (Highveld Biological) supplemented with 10 % foetal bovine serum, 50 µg/ml neomycin, 69.2 µg/ml penicillin G, and 100 µg/ml streptomycin.

BFDV-positive serum, used for immunoblotting and cytochemistry, were collected from an African Grey parrot (AFG5-ZA) showing clinical signs of the disease. A heparinized blood sample were taken from the arterial vein of the left wing and sent to the University of Cape Town for further analysis. The blood components were separated by centrifugation after which the serum fraction was removed and stored at –70°C.

3.2.2 Generation of recombinant baculoviruses

The 747-nt fragment of the BFDV genome comprising the putative capsid protein open reading frame (nucleotides 1234 - 1980 of genome AY450443), was amplified by PCR using primers wtCP-F (5' **GCGGCCGC**ATGCTGTGGGGCACCTCTAACTGC 3') and wtCP-R (5' **CTCG AG**TCTTTATTAAGTACTGGGATTG 3', sequences engineered to create endonuclease restriction sites are indicated in bold type). The PCR product was ligated with the pGEM®-T Easy plasmid (Promega) resulting in plasmid pGM.wtCP.

N-terminal truncated CP open reading frames (ORFs) were generated by amplifying varying lengths of the wildtype (wt) *CP* with primers ΔN25-F (5' **GCGGCCGC** ATGCACATCAGGCGATACC 3'; 25 amino acid N-terminal deletion), ΔN40-F (5' **GCGGCC GC**ATGCGCTTCTCAACCAATAG 3', 40 amino acid N-terminal deletion), or ΔN56-F (5' **GCGGCCGC**ATGAAACGCCAATTCAAATTCC 3'; 56 amino acid N-terminal deletion) used in conjunction with primer wtCP-R. The PCR products were ligated with plasmid pGEM®-T

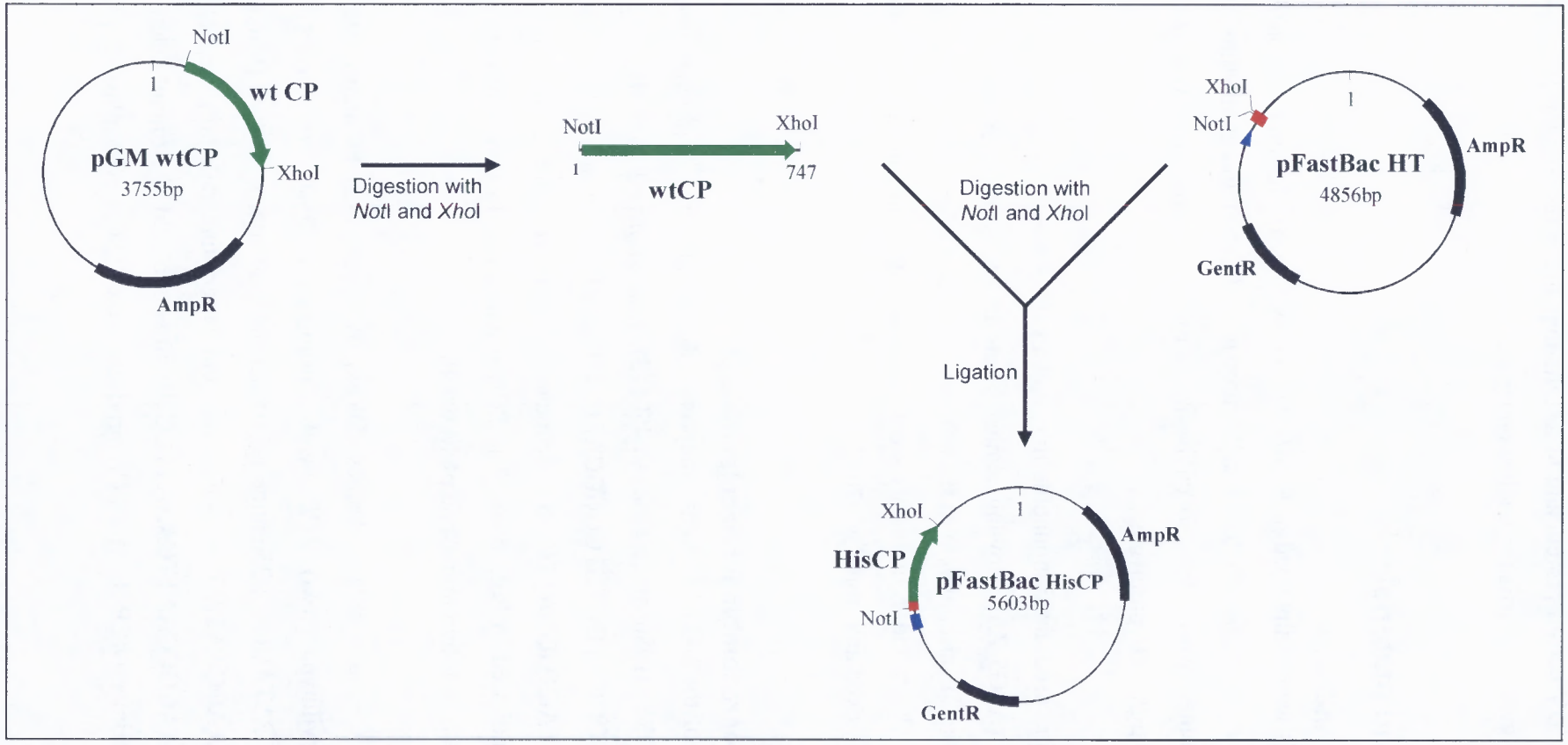

Figure 3.2 Construction of pFastBac donor plasmids used to generate recombinant baculovirus expressing BFDV CP in insect cells. The CP (green box) was cloned in-frame with a His_6 affinity tag and TEV protease recognition site (red box) positioned at the N-terminus of the ORF. The blue box represents the polyhedron promoter. The black boxes represent relevant antibiotic resistance genes.

Easy resulting in recombinant plasmids pGM.CP-ΔN25, pGM.CP-ΔN40, and pGM.CP-ΔN56, respectively. In addition to the wildtype and N-terminal truncated CP ORFs, a fifth CP ORF was constructed in which the 64 carboxy-terminal residues were removed. This was achieved by digesting plasmid pGM.wtCP with *Pst*I and re-ligating the larger fragment, yielding plasmid pGM.CP-ΔC64. The integrity of the ORFs was verified by sequence analysis. Figure 3.1 shows the truncated ORFs in relation to the wt *CP*.

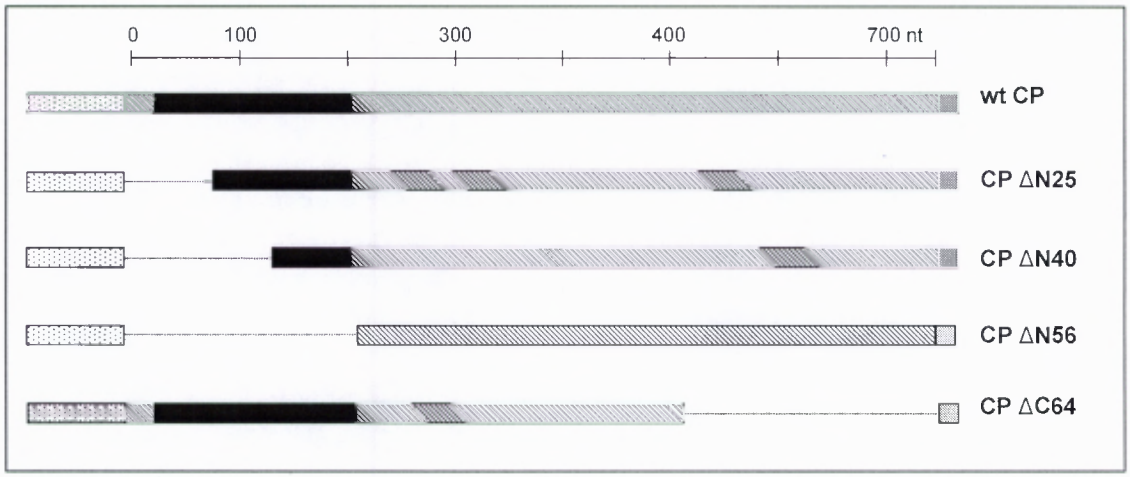

Figure 3.1 Schematic representation of the wildtype BFDV capsid protein and truncated open reading frames (ORFs). The ORFs (striped boxes) had a His_6 affinity tag and the TEV protease recognition site (dotted boxes) fused to their N-termini. The section of each ORF corresponding to the putative nuclear localization signals is indicated by solid black boxes. Dashed lines represent the deleted sections in each of the truncated variants, whilst the solid gray boxes represent the termination codon native to the expression cassette.

The Bac-to-Bac® baculovirus expression system (Invitrogen) was used for the expression of the various forms of the BFDV capsid protein. The strategy for constructing of the recombinant donor plasmids is indicated in Figure 3.2. The *Not*I-*Xho*I fragment was excised from each of the respective plasmids and ligated with pFastBac™ HT donor plasmid that had been linearised using the same enzymes. The pFastBac™ HT donor plansmid is designed to express a 6 histidine sequence (His_6) and the Tobacco etch virus (TEV) proteinase cleavage site fused to the N-terminus of the gene of interest. *E. coli* DH10Bac cells (Gibco BRL), containing a helper vector which facilitates the transposition of the recombinant fragment into the baculovirus shuttle vector (bacmid) were transformed with recombinant donor plasmids. Sf-21 cells were transfected with purified recombinant bacmid DNA using Cellfectin (Invitrogen) according to the manufacturer's

protocols. After 4 days of incubation, the culture media of the transfected cells were collected and stored at 4°C.

3.2.3 Expression and purification of recombinant proteins

High-titre seed virus stock was prepared by a single passage of infecting Sf-21 cells with a multiplicity of infection (MOI) of 0.1. Recombinant proteins were extracted from cells infected at a MOI of 5. Infected cells were harvested 60 hours post-infection (p.i.), suspended in cells lysis buffer (10mM Tris-HCl pH 7.8, 50 mM KH_2PO_4, 300 mM NaCl, 1 mM beta-mercaptoethanol, 40 mM imidazole) and subsequently lysed by sonication. Cell debris was removed by centrifuging the lysate at 10 000 x *g* for 30 minutes. The supernatant was further clarified by passing it through a 0.45 µm acetate filter (Osmonics) before loading it onto a HisTrap™ HP affinity chromatography column (Amersham Biosciences). The column was washed with 20 ml washing buffer (40 mM Tris-HCl pH 7.5, 20% glycerol, 100 mM KCl, 1 mM beta-mercaptoethanol, 40 mM imidazole), and proteins were eluted in 5 ml elution buffer (40 mM Tris-HCl pH 7.5, 20% glycerol, 100 mM KCl, 300 mM imidazole). Purified proteins were dialysised against dialysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM DTT, 10% glycerol) and analyzed by SDS-PAGE and immunoblotting.

3.2.4 SDS-PAGE and immunochemical staining

Samples were diluted in sample treatment buffer (Sambrook *et al.*, 1989), heated to 80 °C for 10 minutes, and subjected to electrophoresis on 12% denaturing SDS-polyacrylamide gels. Proteins were stained with PageBlue 83 (BDH Chemicals Ltd.). The purity and concentration of the recombinant protein was determined by densitometric analysis using GeneTools sofware (Syngene, Synoptics Ltd.). For immunoblotting, proteins were transferred onto nitrocellulose membranes (NitroBind, Osmonics Inc.) using a Trans-Blot® semi-dry transfer cell (Biorad). The membrane was soaked in a 5% (w/v) bovine serum albumin (BSA) solution for 1 hour, before incubation with Tetra-His™ mouse monoclonal IgG_1 (1:1000, Qiagen) or sera from BFDV-infected psittacines (1:500). Bound antibodies were revealed using polyclonal rabbit anti-IgY sera (1:500, Rybicki and von Wechmar, 1981) followed by the appropriate alkaline-phosphate anti-rabbit conjugate (1:1000, Sigma) or alkaline-phosphate anti-mouse conjugate depending on the primary antibody used. After equilibration in Tris-buffered saline, bound antibodies were

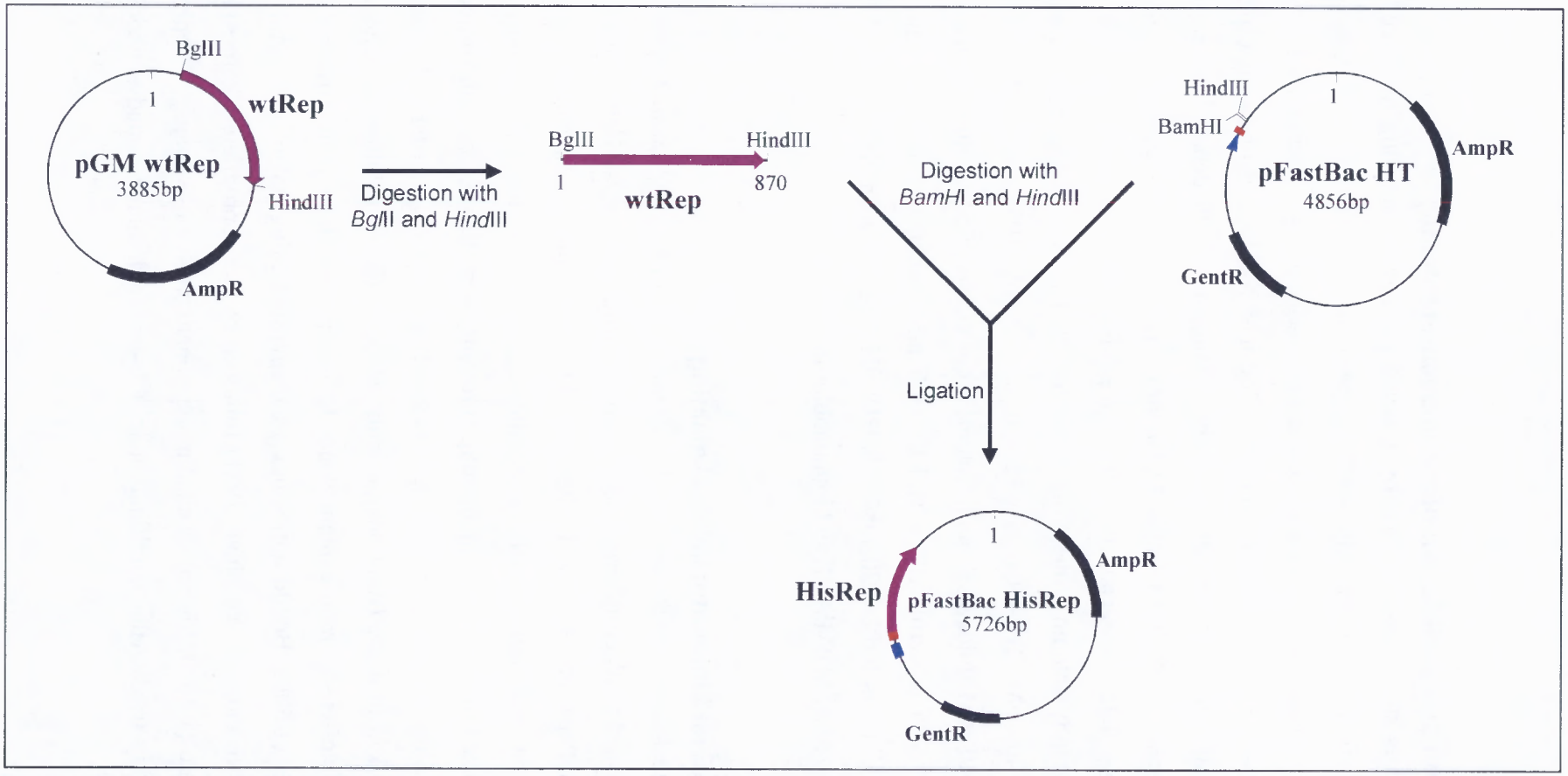

Figure 3.3 Construction of pFastBac donor plasmids used generate recombinant baculovirus expressing BFDV Rep in insect cells. The wildtype Rep (purple box) was cloned in-frame with a His_6 affinity tag and TEV protease recognition site (red box) positioned at the N-terminus of the ORF. The blue represent the polyhedron promoter. The black boxes represent relevant antibiotic resistance genes.

detected with Nitro-Blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt substrate (Roche).

3.2.5 Immunocytochemistry

For indirect immunofluorescence, Sf-21 cells were seeded onto glass slides and infected with recombinant baculovirus at a MOI of 0.1. The cells were fixed and permeabilised at 60 hours post-infection, blocked in 5 % BSA, and incubated with Tetra-His™ mouse monoclonal IgG_1 (1:250, Qiagen) for 3 hours. After being washed, cells were incubated with secondary fluorescein isothiocyanate conjugated anti-mouse IgG (1:500, Sigma). Cellular membranes and nuclei were stained with 1 % Evans Blue (BDH Chemicals Ltd.) and 10 μg/ml 4,6-Diamidino-2-phenylindole (DAPI, Sigma), respectively. Fluorescence was observed with a Nikon diaphot inverted epifluorescence microscope. Images were captured using the Zeiss Axiocam digital camera system.

3.2.6 DNA binding assay

The ability of each variant of the BFDV CP to bind single- and double-stranded DNA was assessed by electrophoretic mobility shift analysis. Purified protein was diluted to 0.5 μg/ml in binding buffer (100 mM Tris-HCl pH 8, 300 mM KCl, 25 mM $MgCl_2$, 20% glycerol, 500 μg/ml BSA) and added to 100 ng of M13mp18 single-stranded DNA (Amersham Bioscience), pUC18 plamis DNA (New England Biolabs Inc.) or pBFDV plasmid DNA. The pBFDV plasmid DNA consisted of the entire BFDV genome (AY450443) cloned into the pGEM®-T-Easy plasmid (see Section 2.2.2 for details). Plasmids were ourified from *E. coli* DH5α using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturers instructions. To confirm that the observed retardation of the DNA fragment was indeed caused by bound proteins, duplicate samples were treated with 1 μg /ml proteinase. The samples were incubated at 37 °C for 1 hour after which they were subjected to electrophoresis on 0.8 % agarose gels. DNA was stained with 0.5 mg/ml ethidium bromide and visualized by UV illumination.

3.2.7 Co-expression of BFDV Rep and CP

The 870-nt fragment of the BFDV genome comprising the Rep protein open reading frame (nucleotides 130 - 999 of the genome AY450443), was amplified by PCR using primers wtRep-F

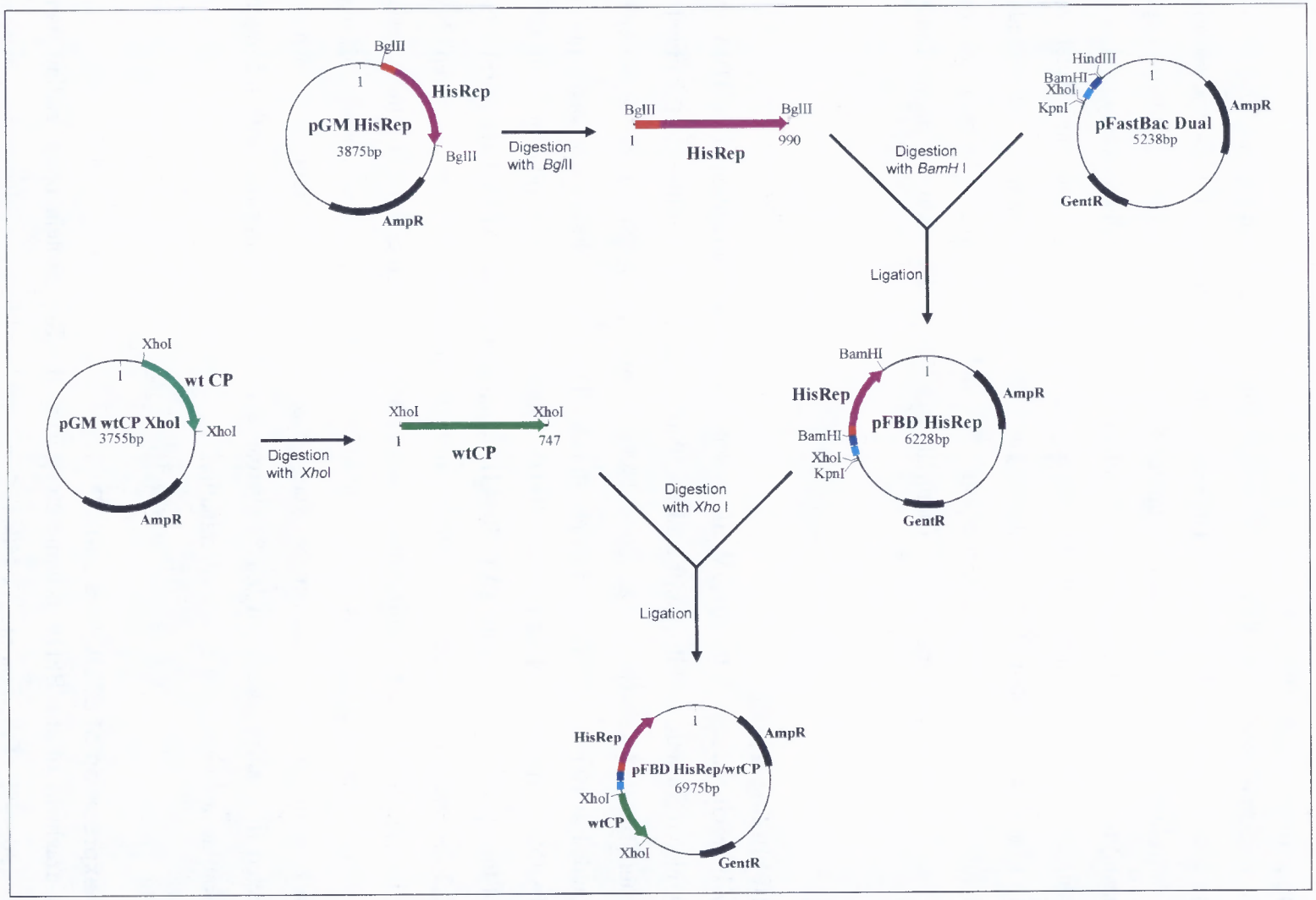

Figure 3.4 Construction of pFastBac Dual donor plasmid used to generate recombinant baculovirus co-expressing BFDV Rep and CP in insect cells. The His_6 -tagged *Rep* (red and purple boxes) was placed under control of the polyhedron promoter (dark blue box). The wildtype *CP* (green box) was placed under control of the p10 promoter (light blue box). The black boxes represent relevant antibiotic resistance genes.

(5' **AGATCT**ATGCCGTCCAAGGAGGGATCTG 3') and wtRep-R (5' **AAGCTTC**TAATAA TTGATGGGGTGGGCGAG 3'). The PCR product was ligated with into the pGEM®-T-Easy plasmid resulting in plasmid pGM.wtRep. The *Bgl*II/*Hind*III fragment was excised from the plasmid pGM.wtRep and ligated with the pFastBac™ HT donor plasmid that had been linearised using the same enzymes (Figure 3.3).

The strategy for constructing of the recombinant donor plasmids containing both *Rep* and *CP* is indicated in Figure 3.4. An extended fragment, including the His_6 affinity tag coding regions and TEV proteinase recognition site, was amplified by PCR using primers FB.HT-F (5' **AGATCT** ATGTCGTACCATCACCATCACCATC 3') and FB.HT-R (5' **AGATCT**TTATGATCCTC TAGTACTTCTCGA 3'). The PCR product was ligated with pGEM®-T-Easy plasmid resulting in plasmid pGM.HisRep. The 1085-nt fragment was excided from the plasmid pGM.HisRep using *Bgl*II and placed under control of the polyhedron promoter of the pFastBac Dual ™ donor plasmid that had been linearised with *BamH*I, yielding plasmid pFBD.HisRep/O .

Plasmid pGM.wtCP was modified to include an *Xho*I restriction endonuclease recognition site at the 5' end of *CP*, yielding plasmid pGM.wtCP-*Xho*I. This was achieved by PCR mutagenesis using primers wtCP-F-*Xho*I (5' **CTCGAG**ATGCTGT GGGCACCTCTAAC 3') and wtCP-R. The *Xho*I-*Xho*I fragment of was excised and placed under control of the p10 promoter of plasmid pFBD.HisRep/O, resulting in plasmid pFBD.HisRep/wtCP.

An identical strategy was used to generate a recombinant donor plasmid containing a His6-tagged *CAT* and BFDV wt CP. Recombinant baculoviruses expressing the various proteins were produced as described in 3.2.2

3.2.8 Immunoprecipitation assay

An immunoprecipitation assay was used to confirm that CP and Rep directly interact when co-expressed in insect cells. Insect cells were seeded into 25 cm^3 culture dishes and infected with recombinant baculovirus at a MOI of 0.1. Infected cells were harvested 60 hours p.i., suspended in phosphate buffered saline (pH 7.4) and lysed by sonication. Proteins were immunoprecipitated using 1 µl Tetra-His™ mouse monoclonal IgG_1 as previously described (Theron & Nel, 1997).

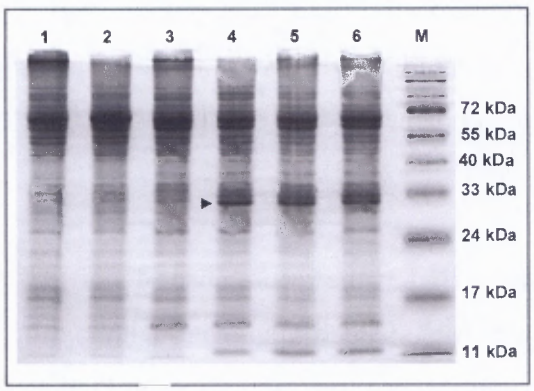

Figure 3.5 SDS-PAGE analysis of the wildtype BFDV CP expressed in insect cells at 0, 24, 48, 60 and 84 hours post-infection. Cell lysates were analysed on a 12% SDS-polyacrylamide Coommasie-stained gel. The recombinant protein is indicated by an arrowhead. Lane 1, uninfected cells; lane 2, HisCP at 0 h; lane 3, HisCP at 24 h; lane 4, HisCP at 48 h; lane 5, HisCP at 60 h; lane 6, HisCP at 84 h.

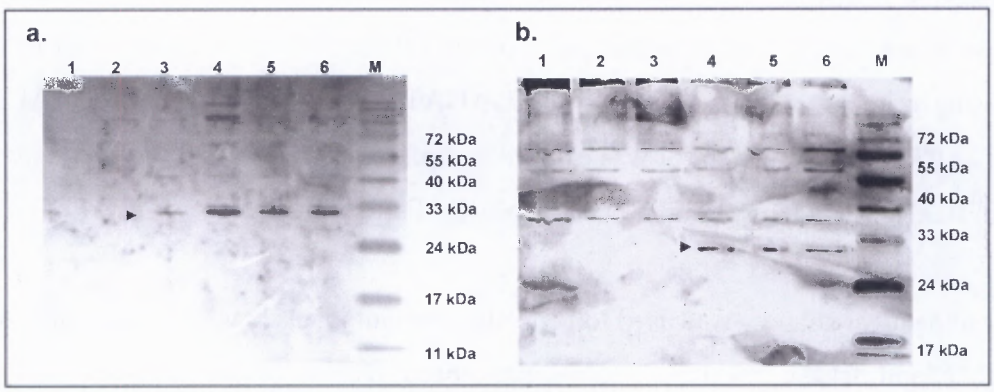

Figure 3.6 Western blot of the BFDV CP expressed in insect cells at 0, 24, 48, 60 and 84 hours post-infection. Cell lysates were analysed on a 12% SDS-polyacrylamide gel followed by immunochemical staining with (a)Tetra-His™ mouse monoclonal IgG_1 and (b) sera from BFDV-infected psittacines, respectively. The recombinant protein is indicated by an arrowhead. Lane 1, uninfected cells; lane 2, HisCP at 0 h; lane 3, HisCP at 24 h; lane 4, HisCP at 48 h; lane 5, HisCP at 60 h; lane 6, HisCP at 84 h.

The proteins were subsequently visualized using immunochemical staining as described in Section 3.2.4.

3.3 Results

Expression of recombinant BFDV protein

Recombinant baculovirus was constructed by placing the wildtype *CP* under control of the polyhedron promoter. Insect cells were infected with the recombinant virus and the expression of the BFDV capsid protein was monitored by SDS-PAGE over a period of 84 hours. The recombinant protein, approximately 32 kDa in size, could be detected as early as 24 hours p.i. (Figure 3.6 a). The apparent discrepancy between the predicted size of the wildtype CP (26 kDa) and the observed size of the recombinant protein is due to the presence of the His_6 affinity tag and the TEV proteinase recognition site, fused to the N-terminus of the CP. Expression levels reached a maximum 48 hours p.i. (10% of total cellular proteins), and the protein appeared to be stably expressed at this level for the remainder of the experiment (Figure3.5). The integrity of the recombinant protein was verified by immunoblotting. The protein reacted strongly with both the Tetra-His™ mouse monoclonal IgG_1, as well as sera from BFDV-infected psittacines (Figure 3.6 b).

Subcellular localization of the recombinant BFDV proteins

The capsid proteins of circoviruses contain a high proportion of basic amino acids at their N-termini (Niagro *et al.*, 1998b). The nuclear localization of the PCV CP is known to be directed by two NLSs located in this part of the protein (Liu *et al.*, 2001b). By analogy, the amino-terminal region of the BFDV capsid protein may similarly be involved in subcellular distribution of the protein. In an attempt to identify conserved motifs or domains located within this region of the protein, the deduced amino acid sequence was subjected to an online PROSITE database search (http://www.expasy.org/prosite/). Three partially overlapping bipartite nuclear localization signals (NLS) were identified, positioned between residues 16 to 56 (Figure 3.7).

NLSs facilitate the transport of proteins into and out of the nucleus through their association with the nuclear pore complexes. Unlike proteins that enter directly through membranes, proteins that translocate in and out of the nucleus are transported in their native folded form, often as

oligomeric complexes containing proteins and nucleic acids (Whittaker & Helenius, 1998). To test the functionality of the putative NLSs, we constructed a series of truncated capsid proteins, sequentially deleting N-terminal portions between position 1 to 50 (Figure 3.1). Similarly to the wildtype CP, the truncated proteins were expressed using recombinant baculovirus. The chloramphenicol acetyl transferase protein (CAT) was used for comparative purposes.

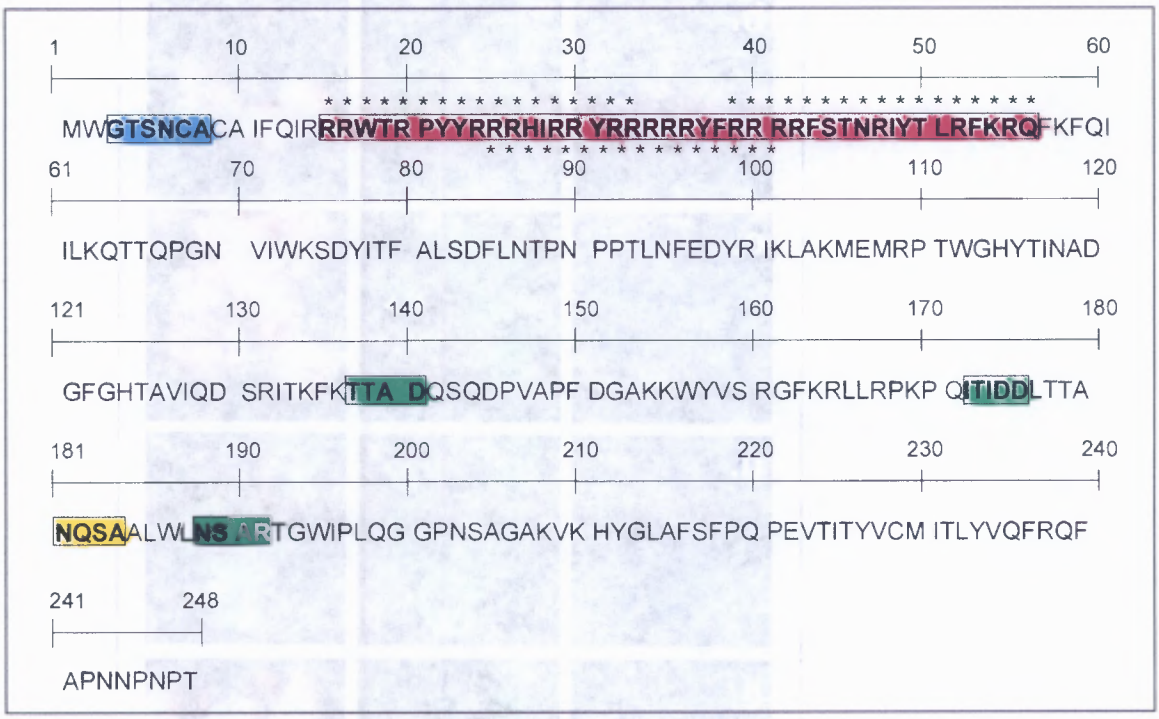

Figure 3.7 Schematic representation of conserved protein motifs within the BFDV CP. Residues potentially involved in nuclear localisation is boxed in red with individual bipartite nuclear localization signals indicated with asterisks (*). Also shown are the consensus patterns for putative posttranslational modifications, *N*-myristylation site (blue box), phosphorylation sites (green boxes) and *N*-glycosylation site (yellow box).

Insect cells were infected with recombinant baculoviruses expressing the individual BFDV CP variants. Recombinant proteins CP ΔN25 and CP ΔC64 were expressed at similar levels to the HisCP. However, expression of CP ΔN40 and CP ΔN56 was notably higher compared with the wt CP (Figure 3.8). Densitrometric analysis revealed that at least 24% of the total cellular protein consisted of the CP ΔN40, representing a 2½-fold increase in expression levels compared to HisCP.

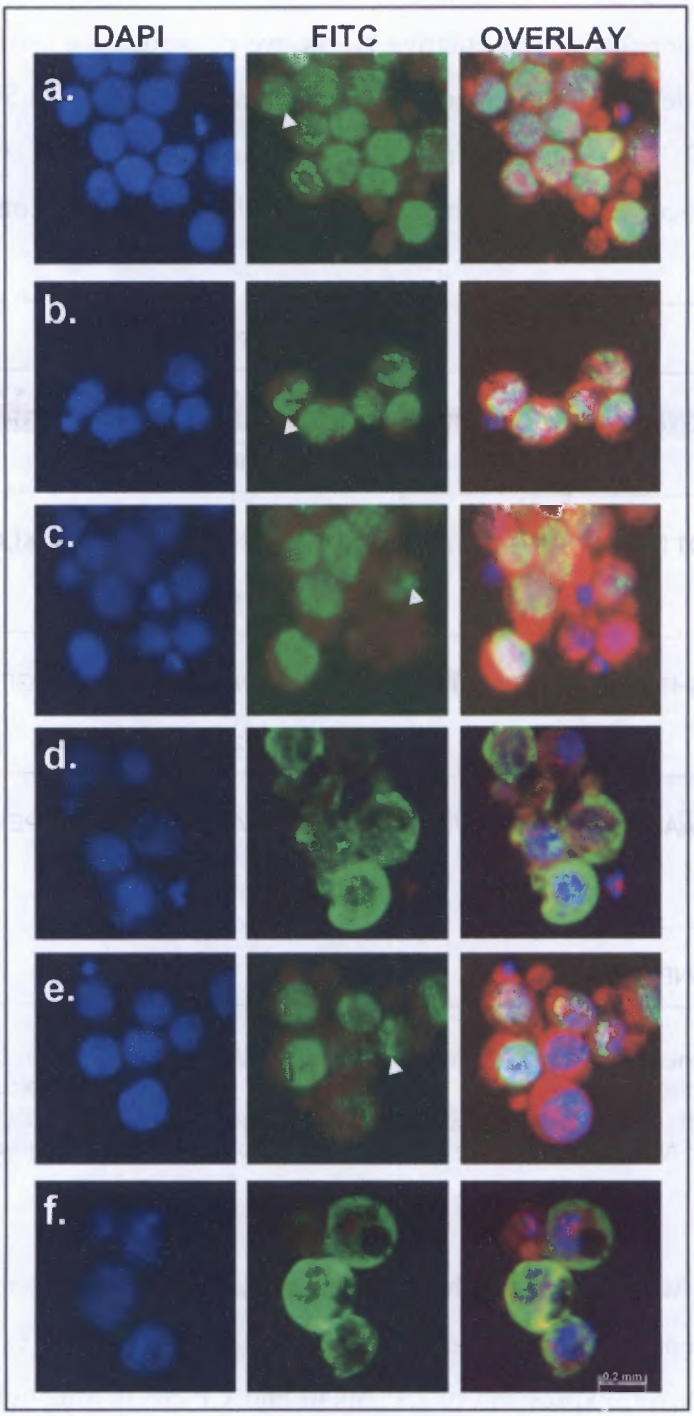

Figure 3.9 Subcellular distribution of BFDV CP. Sf-21 insect cells were infected with recombinant baculovirus and stained with monoclonal antibodies directed against the N-terminal His_6 affinity tag. Monoclonal antibody staining was followed by a fluorescein isothiocyanate-conjugated secondary antibody (green). DAPI stain was used to define nuclei (blue) whilst membranes were visualized with Evans Blue (red). Merged images (overlay) are also shown. Arrowheads indicate specific examples of the punctate perinuclear distribution of the relevant recombinant proteins. Panel (a), HisCP; panel (b), CP ΔN25; panel (c), CP ΔN40; panel (d), CP ΔN56; panel (e), CP ΔC64; panel (f), CAT.

At 60 hours p.i., localization of the proteins was determined by indirect immunofluorescence using the Tetra-His™ mouse monoclonal antibody (Figure 3.9). Full-length CP was observed exclusively in the nucleus of infected cells. Fluorescence was generally distributed evenly throughout the nuclei of infected cells, but occasionally appeared to be punctiformly associated with the perinuclear region. Partial deletion of the putative NLSs did not significantly alter the localization of the recombinant proteins, with ΔN25 and ΔN40 CPs similarly restricted to the nucleus. However, removal of the 56 amino-terminal residues completely abolished translocation of the recombinant protein into the nucleus resulting in the uniform distribution of the CP throughout the cytoplasm, with the subcellular distribution of CP ΔN56 closely resembling that of CAT. A construct carrying a specific deletion of the 64 carboxy-terminal residues clearly exhibited nuclear import, confirming that the karyophilic activity of the CP is associated with the N-terminal portion of the protein.

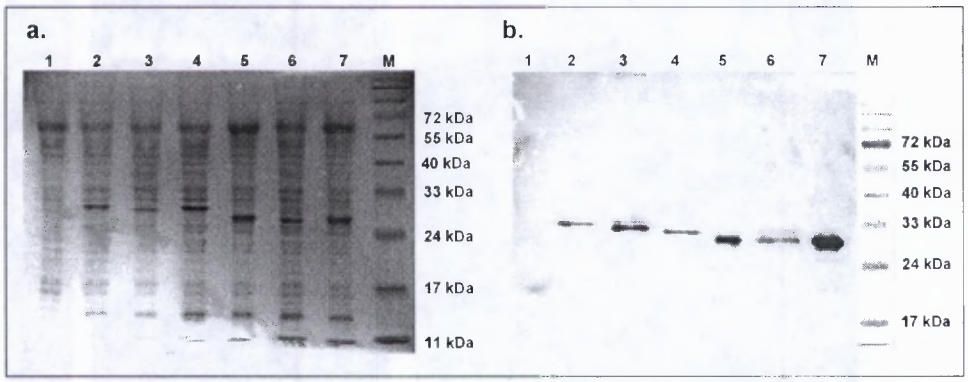

Figure 3.8 SDS-PAGE analysis (a) and western blot (b) of the wildtype BFDV CP and truncated variants expressed in insect cells at 60 hours post-infection. Cell lysates were analysed on a 12% SDS-polyacrylamide gel followed by Coomasie and immunochemical staining using Tetra-His™ mouse monoclonal IgG_1, respectively. Lane 1, uninfected cells; lane 2, HisCP; lane 3, CP ΔN25; lane 4, CP ΔN40; lane 5, CP ΔN56; lane 6, CP ΔC64; lane 7, CAT.

DNA binding ability of the recombinant BFDV proteins

It is very likely that the small size of the BFDV proteome has selectively favoured protein multifunctionality. Although the CP is predominantly responsible for encapsidasion of the viral DNA, once in the cell its role may not be limited to capsid formation. Protein-mediated nuclear transport of DNA has been described for several viral pathogens, including geminiviruses (Clever & Kasamatsu, 1991;Clever *et al.*, 1991;Palanichelvam *et al.*, 1998;Clever & Kasamatsu, 1991)

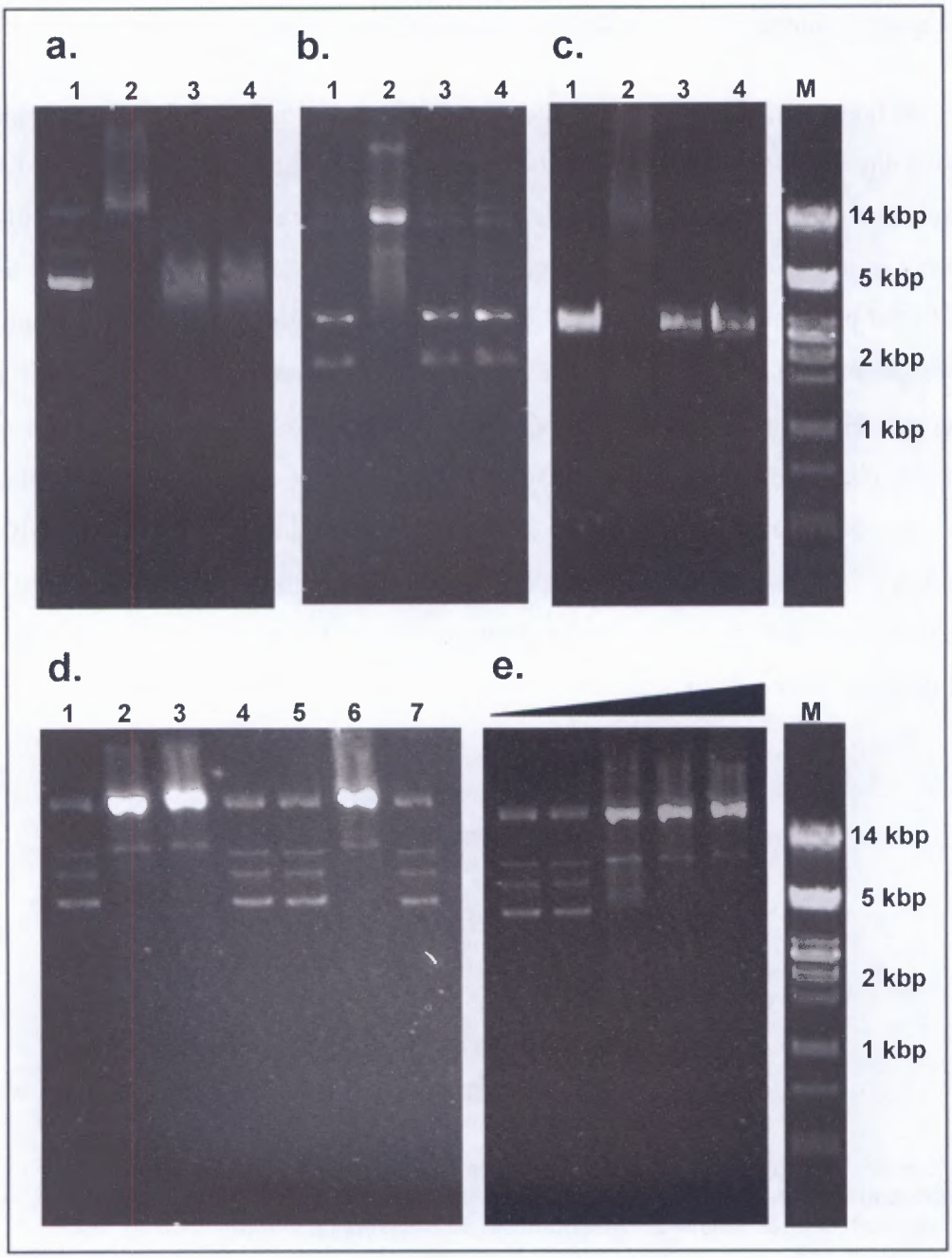

Figure 3.10 Electrophoretic mobility shift analysis of the interaction of recombinant BFDV CP with various DNA samples. For panels (a) plasmid preparations containing the BFDV genome, (b) plasmid preparations of pUC18 and (c) phageM13 ssDNA DNA samples were incubated in the absence of protein (lane 1), in the presence of purified HisCP (lane 2), and in the presence of purified chloramphenicol acetyl transferase protein (lane3), respectively. To confirm that the observed retardation of the DNA fragment were indeed caused by bound proteins, 1μg/ml proteinase K was added to the samples containing purified CP (lane 4). The interaction of each of the truncated variants of the BFDV CP with BFDV DNA was assed in a similar manner, panel (d) lane 1, DNA only, lane 2, HisCP; lane 3, CP ΔN25; lane 4, CP ΔN40; lane 5, CP ΔN56; lane 6, CP ΔC64; lane 7, CAT protein. Increasing amounts of HisCP were incubated with BFDV DNA, panel (e) lane 1, DNA only; lane 2, 10ng; lane 3, 20ng, lane 4, 50ng, lane 5, 500ng.

and may similarly be involved in the pathogenicity of BFDV. To test this hypothesis, I investigated the ability of CP to bind double- (ds) and single-stranded (ss) DNA molecules.

His_6-tagged recombinant proteins were purified by affinity chromatography under non-denaturing conditions using the HisTrap™ HP kit (Amersham Biosciences). The yield of purified recombinant protein varied between 9.5 and 12 mg/10^6 cells depending on the CP variant used, with purity approaching 85% for all preparations. The ability of the BFDV CP to bind DNA was assessed by its ability to retard the electrophoretic movement of ssDNA and dsDNA in a native agarose gel. Figure 3.10 a clearly shows that the purified wildtype protein strongly retarded ds plasmid DNA containing the full-length BVFV genome. This interaction appeared to be sequence non-specific, since pUC18 vector DNA was equally affected by the addition of the purified protein (Figure 3.10 b). Sequence non-specific binding of BFDV CP was further confirmed by its ability to retard single-stranded M13 phage DNA (Figure 3.10 c). Treatment of the reaction mixture with proteinase K completely abolished the binding in all cases, indicating that the retardation was indeed caused by the formation of a protein-DNA complex.

Mutational analysis of DNA-binding regions of several proteins has indicated that these regions often overlap with other functional domains (Matheny *et al.*, 1994;Braem *et al.*, 2002;Bruening *et al.*, 1996). To test whether the NLS and DNA-binding region of the BFDV CP overlap to any extent, the ability of the truncated CP variants to bind ds plasmid DNA containing the full-length BFDV genome was assayed (Figure 3.10 d). Deletion of the first 25 residues did not significantly alter the DNA-binding activity of the recombinant proteins, with CP ΔN25 exhibiting binding activity comparable to that of the full-length protein. However, the removal of an additional 15 residues (CP ΔN40) completely abolished the DNA binding capability of the protein. This is in contrast to its subcellular localisation characteristics, with CP ΔN40 being strictly localized to the nucleus. Deleting the 64 carboxy-terminal residues had no effect on the DNA binding activity of the protein.

To provide further insight into the specific mechanism responsible for the DNA binding characteristics of the BFDV CP, I investigated the effect of protein concentration on the binding activity. Gel mobility shift analysis of dsDNA incubated with increasing amounts of the purified protein, suggested that the interaction was cooperative in nature (Figure 3.10 e). The reaction

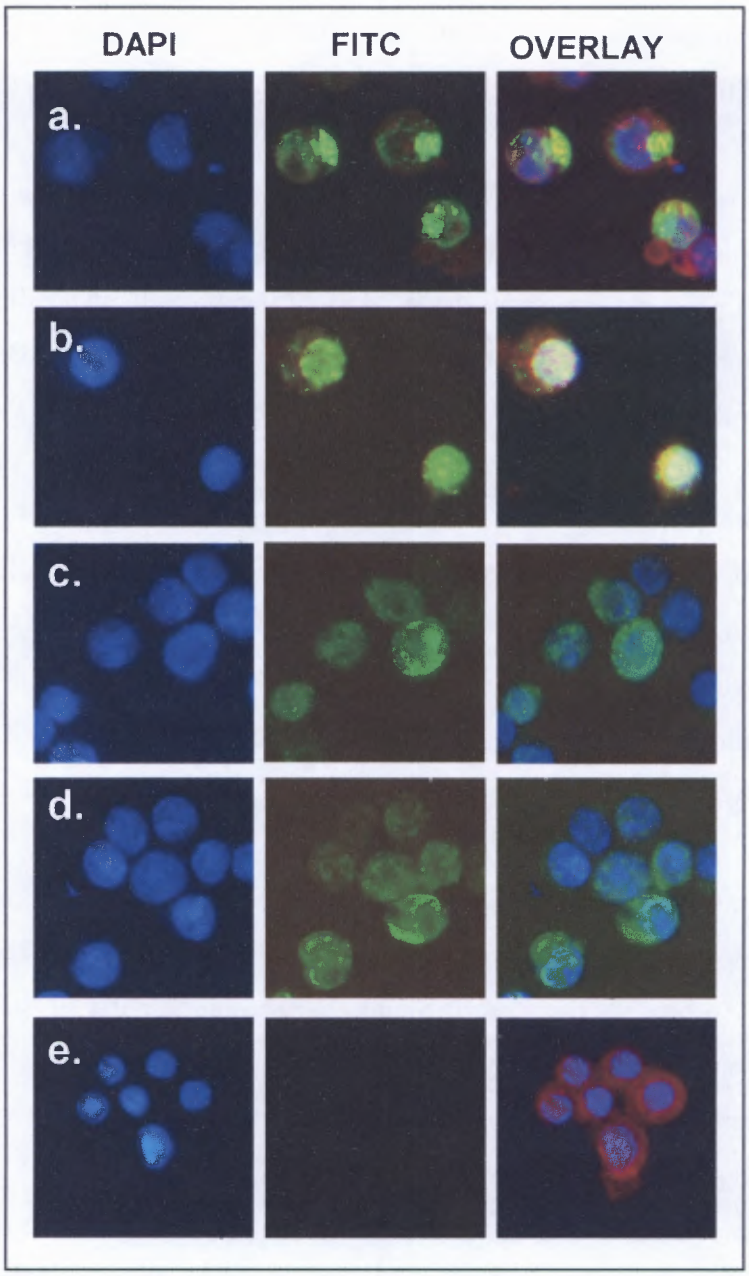

Figure 3.11 Subcellular distribution of BFDV Rep in the absence and presence of the wildtype CP, respectively. Sf-21 insect cells were infected with recombinant baculovirus and stained with monoclonal antibodies directed against the His_6 affinity tag fused to the N-terminus of Rep (HisRep) and CAT (HisCAT) proteins, repectively. Monoclonal antibody staining was followed by a fluorescein isothiocyante-conjugated secondary antibody (green). DAPI stain was used to define nuclei (blue) whilst membranes were visualized with Evans Blue (red). Merged images (overlay) are also shown. Panel a, HisRep; panel b, HisRep + wtCP; panel c, HisCAT; panel d, HisCAT + wtCP, panel e, wtCP.

appeared to be completely saturated at a protein concentration of 2.5 µg/ml. However, more detailed kinetic analyses are necessary to confirm these preliminary results.

Interaction between the BFDV CP and Rep proteins

It has recently been suggested that subcellular distribution of PCV CP is determined by an interaction with the viral Rep (Meerts *et al.*, 2005). However, this is unlikely to be the case with BFDV, since the CP is actively targeted to the nucleus even in the absence of the Rep. To investigate the possible protein-protein interaction between BFDV Rep and CP the respective proteins were co-expressed in insect cells using the baculovirus expression system (Figure 3.11).

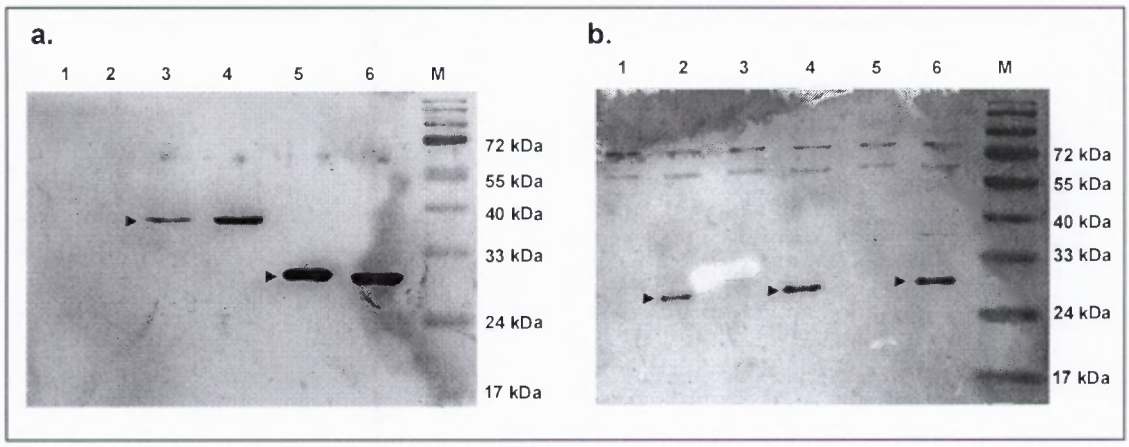

Figure 3.12 Western blot confirming the coexpression of the BFDV Rep and CP. Cell lysates were analysed on a 12% SDS-polyacrylamide gel followed by immunochemical staining with (a) Tetra-His™ mouse monoclonal IgG_1 and (d) sera from BFDV-infected psittacines, respectively. Lane 1, uninfected cells; lane 2, wtCP; lane 3, HisRep; lane 4, HisRep + wtCP, lane 5, HisCAT; lane 6, HisCAT + wtCP. The recombinant proteins are indicated by the arrowheads.

Indirect immunofluorescence assays done on cells infected with recombinant baculovirus expressing the HisRep only, clearly showed that the protein was exclusively localized in the cytoplasm, forming distinctive compact aggregates (Figure 3.12 a). When cells were infected with recombinant virus expressing both the Rep and CP, the localization of the Rep changed markedly, with the protein predominantly found within nucleus (Figure 3.12 b). In contrast to CP, Rep does not contain any recognizable NLSs. The proposed interaction appeared to be specific, since co-expression of the CP and CAT proteins did not result in the relocation of CAT to the nucleus (Figure 3.12 c & d).

The direct interaction between Rep and CP was further confirmed by coimmunoprecipitation. Crude lasyte of infected cells were immunoprecipitated with Tetra-His™ mouse monoclonal IgG_1 and analyzed by immunochemical staining. Western blotting was performed with an anti-His monoclonal antibody or sera from a BFDV-infected psittacine (Figure 3.13).

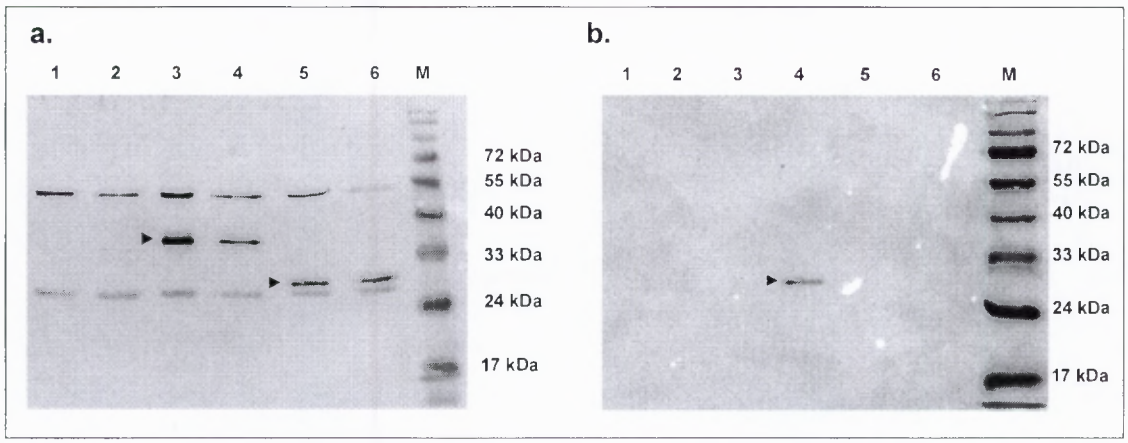

Figure 3.13 Coimmunoprecipitaion of BFDV Rep and CP expressed in insect cells. Proteins were immunoprecipitated with Tetra-His™ mouse monoclonal IgG_1. Samples were analysed on a 12.% SDS-polyacrylamide gel followed by immunochemical staining with (a) Tetra-His™ mouse monoclonal IgG_1 and (d) sera from BFDV-infected psittacines, respectively. Recombinant proteins are indicated by the arrowheads. Lane 1, uninfected cells; lane 2, wtCP; lane 3, HisRep; lane 4, HisRep + wtCP; lane 5, HisCAT; lane 6, HisCAT + wtCP.

The Tetra-His™ mouse monoclonal IgG1 coimmunoprecipitated the CP when it was expressed with HisRep. The CP was, however, not immunoprecipitated when expressed on its own or in with tagged CAT. These results are consistent with the observations made using the indirect immunofluorescence assays. Taken together, these results suggest that the structural protein of BFDV may be responsible for relocating the non-structural protein from the cytoplasm into the nucleus of infected cells through a direct protein-protein interaction.

3.4 Discussion

Circoviruses replicate exclusively in the nucleus and since they are able to infect terminally differentiated cells, the infection process is independent of the breakdown of the nuclear membrane during mitosis (Meerts *et al.*, 2005). The maximum pore size generally associated with the nuclear pore complex furthermore excludes diffusion as a possible mechanism for the entry of the viral genome into the nucleus. Nuclear import is more likely achieved through the

use of active nuclear transport systems native to the host cell. Karyophilic proteins often play a central role in the active nuclear import of DNA molecules (Hiscox, 2002). Protein-mediated nuclear transport of viral genomes has in fact been suggested for several DNA viruses, including SV40 (Hsieh & Griffith, 1988;Clever *et al.*, 1991;Yamada & Kasamatsu, 1993).

The process of viral nuclear import is often associated with the presence of a NLS located within the karyophilic protein. Classical NLSs can broadly be grouped into two classes based on their structure: (i) monopartite, consisting of a short continuous stretch of basic amino acids or (ii) bipartite, consisting of two basic regions separated by a short spacer region. Targeting of the protein to the nucleus is facilitated by an interaction between the viral protein and host nucleocytoplasmic transport machinery (Goldfarb *et al.*, 2004). NLSs are recognized, in the cytoplasm, by one or more members of the nuclear transport receptor family. The complex then relocates to the nuclear membrane and is subsequently translocated through the nuclear pore complex, delivering its cargo to the nucleus (Moroianu *et al.*, 1995). Although some variations on the theme do exist, most karyophilic proteins enter the nucleus through a direct interaction with nuclear transport receptors, such as importin α and β.

The BFDV capsid protein typically has a high proportion of arginine residues concentrated at the N-terminus of the protein. The concentrated localization of these amino acids is known to inhibit efficient mRNA translation in both prokaryotic and eukaryotic expression systems (Alexandrova *et al.*, 1995) and often indicates of the presence of NLSs (Whittaker & Helenius, 1998). In accordance with this, 3 partly overlapping putative bipartite NLSs were identified between residues 16 and 56 at the N-terminus of the BFDV CP. To test the functionality of the putative NLSs, I expressed, in addition to the wild-type CP, a series of CP variants each lacking specific residues potentially involved in effective nuclear localization. Our results show that the BFDV CP is indeed localized to the nucleus and that the nuclear localization is directed by one or more of the NLSs situated within the 56 N-terminal residues of the protein. This is in accordance with previously published data on the subcellular localization of PCV CP where two NLS motifs (^{12}R-H-R-P-R-S-H and ^{34}H-R-Y-R-W-R-R-K) situated in the first 41 residues of the protein were identified as being essential for nuclear targeting of the protein (Liu, 2001). It is important to note that although the NLSs are similarly positioned in both proteins, their exact composition does differ.

In 90% of karyophilic proteins for which both NLS and DNA binding regions have been identified, these regions actually overlap (Cokol *et al.*, 2000). This seems to be the case for the capsid proteins of circoviruses as well. Here I have shown that BFDV CP is capable of binding both ss and dsDNA in a cooperative manner. The DNA binding region was mapped to the N-terminus of the protein and falls within the region containing the three putative NLSs. The ability of the BFDV CP to bind DNA, coupled with the karyophilic nature of this protein, strongly suggests that it may be responsible for nuclear targeting of the viral genome, presumably through the formation of a nucleoprotein complex. This is similar to the role of CP in the related geminiviruses, where CP-mediated nuclear transport of Maize streak virus DNA is a prerequisite for the establishment of a productive infection of maize plants (Boulton *et al.*, 1989b). The exact structure and possible mechanisms regulating the formation of such a nucleocomplex are, however, unknown.

In most ssDNA virus infections, including circoviruses, the complete replication cycle and encapsidation occurs within the nucleus (Boulton *et al.*, 1989a). For the infection to spread, the viral genome has to be transported to the cell periphery. Therefore, viral DNA must be shuttled in and of the nucleus during the infection cycle. In addition to their presumptive role in the docking of the DNA-protein complex at the nuclear pore, the NLSs associated with the BFDV CP may also play a critical role during viral assembly. The events that lead to virion assembly within the nucleus are poorly defined, but the process is thought to involve an interaction of the capsid protein with DNA and histones (Liu *et al.*, 2001b;Li *et al.*, 2001). Proteins with exposed basic domains, such as that found at the N-terminus of the BFDV CP, tend to aggregate in the presence of polyanionic molecules like DNA. In fact, the interaction of the SV40 major structural protein (VP1) with the viral DNA has been shown to be an absolute requirement for effective virion formation (Li *et al.*, 2001).

Drawing an analogy from papovavirus virion assembly, the assembly of circoviruses could possibly be described as a two-step process, the first involving the relocation of CP from the cytoplasm to the nucleus followed by the sequential addition of CP to the viral minichromosome, resulting in the condensation and packaging of the viral DNA. However, in contrast to papovaviruses the protein-DNA interaction may not be absolutely essential for virion assembly. The capsid protein of PCV 2 has been shown to self-assemble into virus-like particles when

expressed in a heterologous system (Nawagitgul *et al.*, 2000a). Although these particles closely resembled the native virus, they often appeared to be less structurally ordered.

The replication-associated protein of all circoviruses is derived from the virion-sense ORF and is an absolute requirement for viral replication (Mankertz *et al.*, 2003). In this study I demonstrated that the BFDV Rep expressed on its own in insect cells is restricted to the cytoplasm. This is in contrast to the PCV Rep, which has recently been shown to be localized to the nucleus during active infection in PCV-permissive cells (Meerts *et al.*, 2005). The apparent affinity of the PCV Rep for the nucleus is somewhat surprising since, as is the case with the BFDV Rep, no NLS has been described for this protein. Interestingly, Meerts *et al.* (Meerts *et al.*, 2005) furthermore showed that during active infections, most cells where PCV antigens were found in the nucleus both CP and Rep were nuclear localized.

It is my contention that the nuclear localization of the BFDV Rep is facilitated by an interaction with the CP, which is responsible for trafficking it across the nuclear membrane. This is the converse of what seems to occur in the related PCV, where it is Rep that apparently facilitates trafficking of the CP into the nucleus (Meerts *et al.*, 2005). My hypothesis is strongly supported by the subcellular localization data on individually expressed and co-expressed BFDV protein. That it is directed protein-protein interaction, which is involved in this process, is evident from the immunoprecipitation data. An analogous interaction between of the CP and Rep of the geminivirus Mung bean yellow mosaic India virus (MYMIV) has recently been described. Rather than being implicated in nuclear trafficking, the MYMIV Rep-CP interaction results in both the down-regulation of replication initiation activity, and a general decrease in ssDNA accumulation and viral replication.

It is becoming increasingly clear that the involvement of the capsid protein in the life cycle of circoviruses reaches far beyond its role in encapsidation. It has recently been established that the subcellular localisation of PCV2 CP in permissive cells is dependent on the specific stage of infection (Meerts *et al.*, 2005). In addition to this, contrasting localization patterns have been observed in PCV non-permissive cells compared to that in permissive cell lines. In PCV-infected macrophages CP was exclusively found in the cytoplasm. Despite the ability of the virus accumulate within the cytoplasm of dendritic cells there was no evidence of viral replication in

these cells (Gilpin *et al.*, 2003). Meerts *et al.* (Meerts *et al.*, 2005) subsequently showed that in certain instances the viral antigens are targeted to the nucleus of macrophages, but that the localization is substantially delayed, resulting in a significant decrease in viral replication. The inability of the CP, and by association Rep, to translocate to the nucleus of these cells may directly account for the lack of viral replication.

The ability of the virus to replicate in an infected cell, however does not necessarily guarantee the establishment of a productive infection. Replication of PCV has been shown to occur in certain human cells, but these infections were found to be non-productive. In contrast to macrophages, PCV CP is localized to the nucleus of infected human and primate cell lines where it aggregates punctiformly (Hattermann *et al.*, 2004). Despite the presence of viral DNA in the supernatant from PCV-infected human cells, no virus particle could be detected, suggesting that viral assembly may be disrupted in these cells. The exact way in which viral assembly is perturbed is unclear. The ability of the circovirus CP to self-assemble is, however, not affected when expressed in insect cells, since PCV CP have been shown form virus-like particles in insect cells (Nawagitgul *et al.*, 2000b). It is unknown whether BFDV CP would similarly display the ability to self-assemble when expressed in these cells. This does, however, warrant further attention since it directly relates to the suitability of the recombinant protein as a possible vaccine candidate.

It should, however, be noted that the behavior of the BFDV proteins in insect cells may not necessarily reflect the functionality of the proteins during a natural infection. Although most recombinant proteins expressed in insect cells are generally correctly processed, are targeted to their appropriate cellular locations, and in most cases remain functionally active, slight differences in the inherent characteristics of specific cell types can dramatically influence their behavior. While the relevance of our findings to the natural state of affairs remains to be determined, they are strongly reminiscent of the behavior of the PCV proteome during productive infections of permissive cells. Adaptation of BFDV to tissue-cultured psittacine cells would undoubtedly answer all questions raised here. Unfortunately this has yet to happen.

In summary, I have shown that the capsid protein of BFDV is actively localized to the nucleus when expressed in insect cells. The CP is targeted to the nucleus by one or more of three bi-

partite nuclear localization signals located at the N-terminus of the protein. Moreover, this portion of the protein appears to also contain a DNA binding region, which facilitates the binding of CP to both single- and double-stranded DNA in a cooperative manner. It remains to be determined whether the NLSs and DNA binding region are functionally coupled or whether they act independently. In addition to the protein-DNA interaction, the results strongly suggest that CP directly interacts with Rep, enabling co-translocation of the latter into the nucleus. The precise regions involved in this interaction and the possible impact of the Rep-CP interaction on other aspects of the virus lifecycle, remain to be determined. Fine mapping of the regions involved in this interaction will however undoubtedly shed light on the precise role of CP in the replication cycle of the virus.

Chapter 4

The immunogenicity and antigenicity of the Beak and feather disease virus capsid protein

Content

Abstract

As the causal agent of PBFD, BFDV is currently one of the most significant pathogens of parrots. It is believed that the development and widespread use of an effective BFDV vaccine is a viable solution to the global PBFD problem. However, the inability of BFDV to be propagated in tissue culture systems and the threat posed by improperly inactivated vaccine preparations has severely hampered the large-scale application of an inactivated vaccine. Recombinant DNA technology offers an attractive alternative to use of inactivated vaccines and circumvents many of the problems associated with more classical methods of vaccine development. In this proof-of-principle study I demonstrate that it is possible to produce BFDV-specific antigens in both prokaryotic and eukaryotic expression systems, which could potentially be used as recombinant subunit vaccines. Immunization of chickens with the recombinant antigens resulted in a strong antigen-specific humoral response. Protein expressed in insect cells was found to be efficient in inducing broadly reactive antibodies, capable of recognizing antigen produced in heterologous systems. Although antibodies raised against protein expressed in *E. coli* were capable of effectively binding to the homologous protein, the antibodies were less efficient in binding to CP expressed in insect cells. These differences may reflect slight differences in the posttranscriptional processing and conformation of the antigens. I further describe the development of a novel indirect capture ELISA for the detection of BFDV-specific antibodies in psittacine sera. This assay can be used to test whether birds have been exposed to the virus and assess the prevalence of the disease. For comparative purposes the assay was adapted to make use of recombinant CP expressed in either insect or bacterial cells. Recombinant proteins expressed in insect cells appeared to increase the sensitivity of the assay compared to protein expressed in *E. coli*. An inverse relationship between the presence of BFDV-specific DNA and antibodies were found to exist. This would suggest that BFDV-infected birds develop a detectable humoral immune response only after the viraemic stage of the disease.

4.1 Introduction

4.1.1 Vaccination

In recent years vaccination has become the preferred and most cost-effective methods of combating infectious diseases encountered by the modern livestock-industry (Rogan & Babiuk, 2005). Successful vaccination programs do not only drastically reduce the incidence of the disease, but also limit the economic impact associated with sporadic outbreaks. Currently, the majority of licensed veterinary vaccines are produced using conventional technologies, such as attenuation and inactivation of whole pathogens. However, regulatory constraints have force the industry to develop specifically defined antigens free from pathogen-associated toxicity. This has in turn lead to the use of recombinant DNA technologies for the production of subunit vaccine (Ritchie *et al.*, 1992) (Raidal *et al.*, 1993c). Subunit vaccines circumvent the problem of residual toxicity in that they utilize only parts of the virus, rendering it non-infectious. This approach has been proven to be both effective and safe, and has successfully been used to combat several human and animal pathogens (Karlsson & Liljestrom, 1998).

Various expression systems are available for the production of subunit vaccines with the choice of an appropriate expression system largely dependent on the nature of the antigen being expressed. The expression of heterologous proteins using prokaryotic expression systems has been used extensively in the production of veterinary pharmaceuticals. Antigens produced in this manner include respiratory syncytial virus and feline leukaemia virus proteins expressed in *E. coli* (Martin-Gallardo *et al.*, 1993) (Marciani *et al.*, 1991) (Kensil *et al.*, 1991) (Murby *et al.*, 1995). However, size constraints imposed on gene insertions, lack of posttranslational modification and toxicity of some eukaryotic gene products often necessitates the use of alternative systems.

Viral expression systems are popular alternatives to bacterial. Vaccinia virus vectors have traditionally been used for this purpose (Rogan & Babiuk, 2005). However, insect cells expression systems are fast becoming the proffered method of expressing antigens via viruses. This system makes use of the *Autographa californica* nuclear polyhedrosis virus to produce recombinant proteins in permissive insect cell lines (Wu *et al.*, 2004). Several complex animal and human proteins, which require folding, subunit assembly and extensive posttranscriptional

modification, have successfully been expressed using the baculovirus system (Wu & Carstens, 1998). A major advantage of viral expression systems is that recombinant proteins are processed, modified and targeted to their appropriate cellular locations and in most cases remain functionally active (Wu *et al.*, 2004). This allows for a more pertinent immune response and a higher efficacy of the vaccine preparations.

4.1.2 Immunological response to BFDV

Although indirect transfer of virus occasionally involves contaminated objects or material, most often spread is affected by direct contact between infected animals actively excreting virus and susceptible host. It is for this reason that control strategies generally focus on prevention by limiting the exposure of healthy birds to virus-contaminated environments (Gerlach, 1994). However, due to the highly infectious nature of the virus quarantine measures often prove inadequate in preventing the spread of the disease (Ritchie & Carter, 1995). This has prompted researchers to investigate the use of vaccination as a supplementary control measure. In isolated instances birds exposed to the virus remain clinically normal and often present high anti-BFDV antibody titers. This would suggest that birds are able to mount an effective immune response directed towards the virus and is indicative of the potential usefulness of a BFDV vaccine (Ritchie *et al.*, 1991).

This apparent immunity can be experimentally reproduced by inoculating birds with β-propiolactone-inactivated virus. Vaccinees not only seroconvert, but are protected against challenge with live infectious virus (Ritchie *et al.*, 1992). The inactivated vaccine is produced by purifying live virus from infected birds. Although quantities sufficient for small-scale pilot studies of the virus can be harvested in the way, large-scale production for commercial application of the vaccine is impractical and economically not viable. Added to this is the lack of adequate *in vitro* assays to ensure that vaccine preparations do not contain any residual infectivity. The death of vaccinated birds has previously been linked to the use of the inactivated vaccine (Raidal *et al.*, 1993b). Although the residual infection of each vaccine preparation can be tested *in vivo* prior to commercial release, the threat of exposing healthy birds to the virus still limits the commercial application of this approach to the vaccine development.

The problems associated with the inactivated BFDV vaccine could largely be circumvented by the development of a subunit vaccine. The identification of a suitable target antigen is paramount to the success of a subunit vaccine. The BFDV capsid protein (CP) is the major constituent of the infectious viral particle and the most likely a predominant target of the immune response (Todd, 2000). The BFDV capsid consists of 60 copies of the CP arranged in 12 pentameric clusters to form a T=1 icosahedral structure (Crowther *et al.*, 2003). The CP is approximately 26 kDa in size and has a characteristic basic N-terminal region expected to interact with the DNA during viral maturation and assembly. Recombinantly expressed CP of PCV, a closely related circovirus, has been shown to self-assemble into virus-like particles resembling the native virion and is known to cross-react with sera from infected pigs (Liu *et al.*, 2001b). In this chapter I discuss the recombinant expression of the BFDV CP in both prokaryotic and eukaryotic cells. To explore the potential use of the recombinant protein as a vaccine, the immunogenicity of the purified protein was characterized in an animal model.

4.2 Methods and materials

4.2.1 Virus, Cells and animals

Due to its approximate equidistant phylogenetic relationship to all known BFDV genotypes (described in Chapter 2), isolate AFG3-ZA (AY450443) was used in the development of the recombinant subunit vaccines. This isolate was extracted from an African Grey parrot showing clinical symptoms of PBFD.

The recombinant baculoviruses were constructed and propagated as described in Section 3.2. Bacterial cultures were grown in Luria-Bertani medium, supplemented with 100 µg/ml amplicillin. All plasmids were maintained in *E. coli* DH5α (Invitriogen).

The Animal Research Review Committee of the University of Cape Town approved all animal protocols used in this study. Immunizations and general animal care were performed by trained animal technologists.

4.2.2 Expression of BFDV CPΔN40 in *E. coli*

An N-terminal truncated *CP* was generated by PCR and cloned into the pGEM®-T-Easy plasmid (Promega) as described in Section 3.2.2. This shortened version of the wildtype gene encodes a

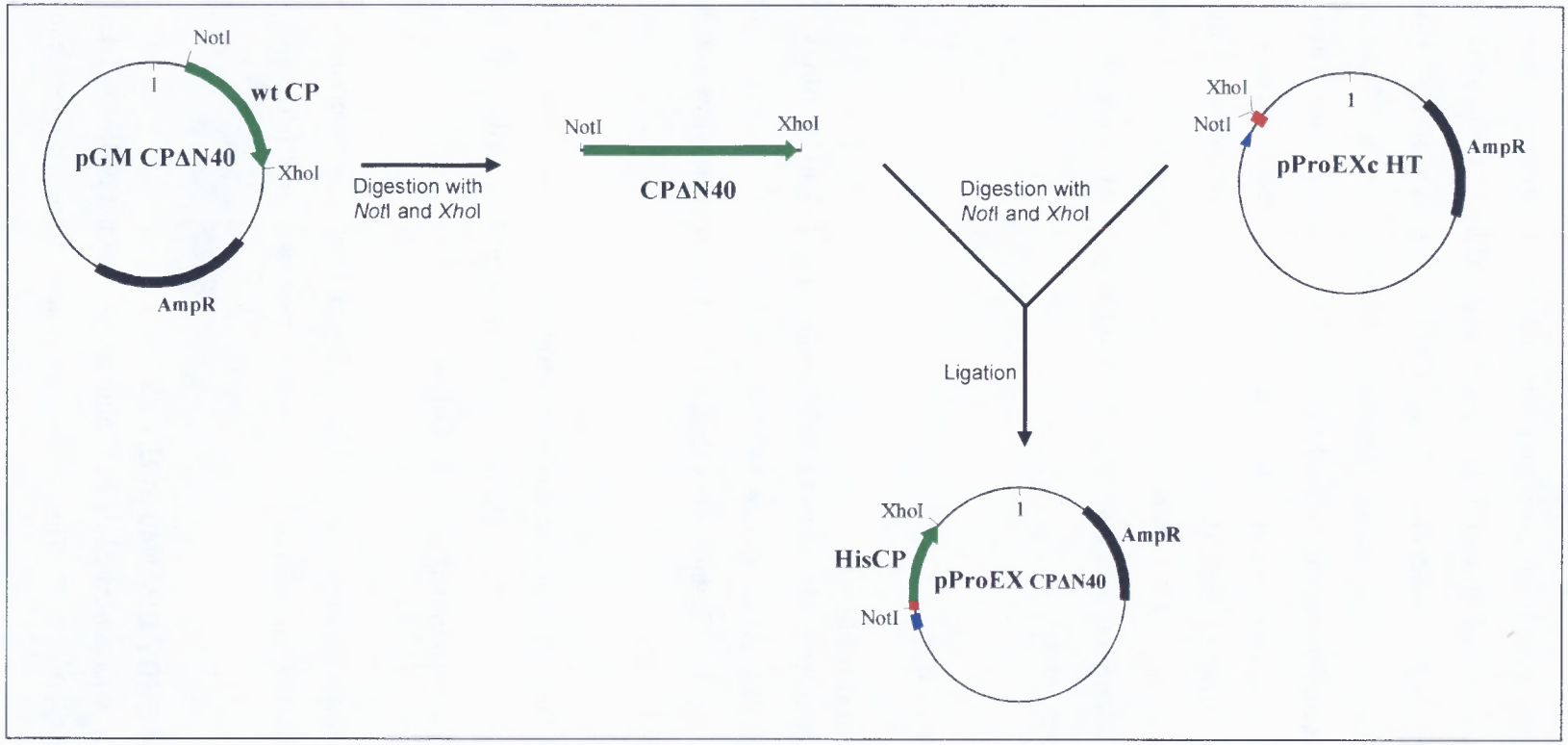

Figure 4.1 Construction of pProEX HT plasmids used to expres the BFDV CP in bacterial cells. The CP (green box) was cloned in-frame with a His6 affinity tag (red box) positioned at the N-terminus of the ORF. The blue box represents the promoter sequence. The black boxes represents relevant antibiotic resistance gene.

truncated CP starting at amino acid 40. This particular construct was chosen since it displayed significantly higher levels of expression in insect cells compared to the wildtype CP (See section 3.3.1). For expression of the CP ΔN40 protein in *Escherichia coli* (*E. coli*), the gene was excised from plasmid pGM.CP-ΔN40, using *Not*I and *Xho*I, and ligated with pProEXc™ HT plasmid that had been by linearised using the same enzymes (Figure 4.1). The integrity of the recombinant expression plasmid containing the gene of interest was verified by sequence analysis.

An overnight culture of *E. coli*, transformed with the recombinant plasmid, was used to inoculate fresh medium. The cell density was adjusted to an optical density at A_{590} of 0.1. The culture was incubated at 37 °C until the optical density reached 0.6 after which expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation 4 hours after induction, resuspended in 4 volumes of lysis buffer (20 mM Tris-HCl pH 7.8, 100 mM KCl, 300 mM NaCl, 10 mM beta-mercaptoethanol, 40 mM imidazole, 8 M urea) and lysed by sonication. The lysate was clarified by centrifugation and subsequently used for purification of the recombinant protein by HisTrap™ HP affinity chromatography (Amersham Biosciences) under denaturing conditions. The column was equilibrated with 2 volumes of lysis buffer before the lysate was loaded onto it. Unbound proteins were removed by washing the column with 10 ml wash buffer (40 mM Tris-HCl pH 7.5, 20% glycerol, 100 mM KCl, 1 mM beta-mercaptoethanol, 40 mM imidazole, 8 M Urea). Bound proteins were eluted in 5 ml elution buffer (40 mM Tris-HCl pH 7.5, 20% glycerol, 100 mM KCl, 300 mM imidazole, 8 M Urea). Purified proteins were refolded by dialysis against dialysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM DTT, 10% glycerol).

Proteins were purified from insect cells infected with recombinant baculuvirus expressing CP ΔN40 as described in Section 3.2.3. Proteins were analyzed by SDS-PAGE analysis and western blotting as described in Section 3.2.4.

4.2.3 Immunization of chickens

All animals were acclimatized for 1 week prior to the initial immunization. Two groups of twenty-four-week-old White Leghorn chickens (n = 3/group) were immunized intramuscularly with 50 μg purified CP ΔN40 produced in either insect or *E. coli* cells at day 0, 14 and 21. For

comparative purposes, a third group was immunized with an equivalent amount of chimeric Human papillomavirus type 16 (HPV-16) virus-like particles (VLPs) purified from baculovirus infected insect cells (Varsani *et al.*, 2003). Both antigens were emulsified in Freund's incomplete adjuvant.

4.2.4 Isolation of IgY from eggs

Eggs laid by the immunized hens were collected at weekly intervals and stored at 4 °C. Immunoglobulin Y (IgY) was extracted from the eggs as described by Polson (Polson *et al.*, 1980). Briefly, egg yolks were separated from the egg white and washed with dd.H_2O. The yolk sacks were punctured and two volumes of phosphate buffer (100mM NaH_2PO_4, pH 7.4) were added to the yolk. Crushed PEG 6000 was added to 3.5% (w/v) and dissolved by rotary inversion. The vetelin fraction and contaminating lipids were removed by centrifugation (5000 x g, 10 minutes) followed by filtering the supernatant though cotton wool. The concentration of PEG was adjusted to 12% by adding 8.5 % (w/v) PEG 6000 to the filtrate. The solution was mixed, centrifuged (12 000 x g, 10 minutes), and the pellet dissolved in phosphate buffer (equal volume to that obtained after filtration) The IgY was again precipitated with 12% (w/v) PEG 6000, before dissolving it in $1/10^{th}$ of the original egg yolk volume using phosphate buffer. All eggs were processed within 7 days of being laid. The concentration of total soluble protein was assayed using the D_c Protein Assay system (Bio-Rad). The purity and concentration of IgY in each preparations was determined by densitometric analysis using GeneTools software (Syngene, Synoptics Ltd.).

4.2.5 Antibody binding to denatured protein

Binding of antibodies to denatured BFDV CP was assayed by western blot. Crude lysates of cells expressing CP ΔN40 were separated on 12% denaturing SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (NitroBind, Osmonics Inc.) as described in Section 3.2.4. The membrane was soaked in a 5% (w/v) bovine serum albumin (BSA) solution for 1 hour, before incubation with purified IgY preparations (diluted 1:250 in blocking solution). Bound antibodies were revealed using polyclonal rabbit anti-IgY sera (1:500, University of Cape Town) followed by the goat anti-rabbit alkaline-phosphate conjugate (1:1000, Sigma). After

equilibration in Tris-buffered saline, bound antibodies were detected with Nitro-Blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt substrate (Roche).

4.2.6 Antibody binding to non-denatured protein

Binding of antibodies to non-denatured BFDV CP were assayed by indirect capture enzyme-linked immunosorbent assay (ELISA). Polysorp 96-well microtitre plates (Nunc) were coated with 0.01 µg Tetra-His™ mouse monoclonal IgG1 (Sigma) in carbonate buffer (50 mM Na_2CO_3, pH 9) for 12h at 25°C and blocked for 1 h with 5% non-fat dried milk in PBS (blocking solution). IgY preparations (diluted 1:250 in blocking solution) were assayed for binding to proteins in crude lysates of insect and *E. coli* cells expressing CP ΔN40. Crude lysates insect cells expressing CAT, as well as uninduced *E. coli* cells, were used as negative controls.

Bound antibodies were revealed using polyclonal rabbit anti-IgY sera (1:500, University of Cape Town) followed by alkaline-phosphate goat anti-rabbit conjugate (1:1000, Sigma). The substrate solution used was p-nitrophenyl phosphate (Sigma). After 15 minutes incubation, the optical density at 405 nm was measured using a PowerWave™ XS Universal Microplate Spectrophotometer (Bio-Tek®). IgY preparations were considered to be positive for BDFV-specific antibodies if the OD was > 4x that of pre-immunized eggs.

The endpoint titres were determined for preparations purified from eggs collected 8 weeks after the first imunization. Samples were serially diluted from 1:200 to 1:24800 and assayed as described above. Endpoint titres for each of the immunized groups were expressed as the reciprocal of the highest dilution where the OD was > 4x that of pre-immunized eggs.

4.2.7 Comparison of PCR and ELISA assay for the detection of BFDV

Sera from 25 psittacine birds (Table 4.1) were used to assess the applicability of the ELISA assay for diagnosing BFDV infections. Of the 25 samples, 14 were known to contain BFDV-specific DNA (see Chapter to for details). Together the isolates present in these samples represent all but one of the 5 southern African genotypes discussed in Chapter 2. In addition to this, 11 samples taken from birds tentatively diagnosed as being BFDV-free, but that had been exposed to BFDV-infected individuals were also assayed.

The sera were diluted serially starting at 1:50 in blocking solution and assayed for binding to CP ΔN40 as described above. The antibody titre of each sample was expressed as the reciprocal of the highest dilution where the OD was > 4x that of IgY preparations of pre-immunized eggs (diluted 1:200 in blocking buffer). The results were compared to that of the PCR testing. The PCR assay was performed using primer sets wtCP-F/wtCP-R and wtRep-F/wtRep-R as described in Section 2.2.2. The different data were compared by the Fisher's exact tests for count data using R 2.0.1.

4.3 Results

4.3.1 Expression of recombinant BFDV proteins in *E. coli*

The recombinant protein appeared to be insoluble when expressed in the bacterial cells and aggregated to form inclusion bodies. As a result it could only be purified under denaturing conditions. The concentration of the purified protein was estimated to be ~0.15 mg/ml (Figure 4.2 a). Densitometric analysis of Coomassie stained gels suggested that the fractions containing CP ΔN40 were only 45% pure. The integrity of the CP ΔN40 was confirmed by immunochemical staining (Figure 4.2 b). Interestingly, CP ΔN40 purified from *E. coli* appeared to have a slightly higher molecular weight compared to the same protein expressed in insect cells. This discrepancy in size may be due to difference in the posttranscriptional processing of the proteins.

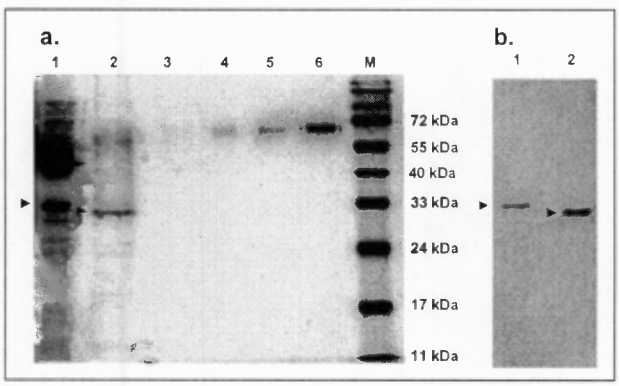

Figure 4.2 (a) SDS-PAGE analysis of BFDV CP ΔN40 used to immunize chickens. Proteins were purified by affinity chromatography. Samples were analyzed on a 12% SDS-polyacrylamide gel and by immunochemical staining. Lane 1, Proteins purified from E. coli cells; lane 2, Proteins purified from insect cells; lane 3, 0.1 µg BSA; lane 4, 0.5 µg BSA; lane 5, 1 µg BSA; lane 6, 2 µg BSA. (b) Immunochemical staining of proteins purified from E. coli cells (lane 1) and insect cells (lane 2). Proteins were stained using Tetra-His™ mouse monoclonal IgG1. The recombinant proteins are indicated by an arrowheads.

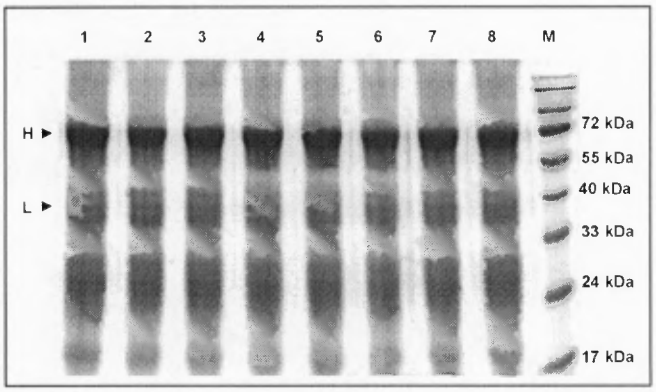

Figure 4.3 SDS-PAGE analysis of IgY preparations purified from eggs collected at weekly intervals p.im. Samples were analyzed on a 12% SDS-polyacrylamide gel. The heavy (H) and light (L) chains of the IgY are indicated by arrowheads. Lane 1, Pre-immune IgY; lane 2, 1 week p.im.; lane 3, 2 weeks p.im.; lane 4, 3 weeks p.im.; lane 5, 4 weeks p.im.; lane 6, 5 weeks p.im.; lane 7, 6 weeks p.im.; lane 8, 7 weeks p.im.

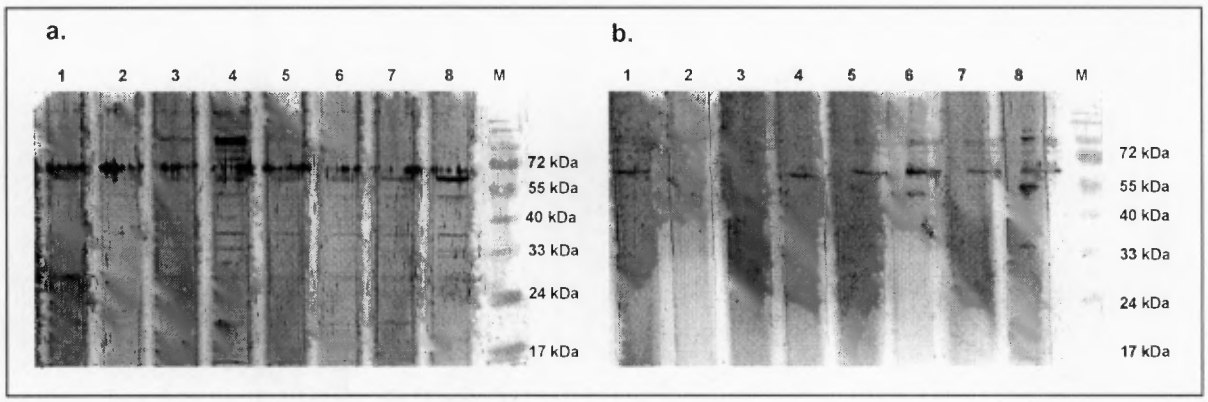

Figure 4.4 Western blot of BFDV CP expressed in (a) E. coli and (b) insect cells probed with IgY preparations purified from eggs collected at weekly intervals p.im. Samples were analyzed on a 12% SDS-polyacrylamide gel. Lane 1, Pre-immune IgY; lane 2, 1 week p.im.; lane 3, 2 weeks p.im.; lane 4, 3 weeks p.im.; lane 5, 4 weeks p.im.; lane 6, 5 weeks p.im.; lane 7, 6 weeks p.im.; lane 8, 7 weeks p.im.

4.3.2 Immunogenicity

In order to assess whether the use of different expression systems resulted in differences in the ability of the respective antigens to serve as a vaccine, chickens were immunized with proteins purified from insect and *E. coli* cells. Antibodies were purified from eggs collected at weekly intervals following immunization. A single egg yielded on average ~80mg of protein, with IgY constituting ~40 % of the soluble proteins (Figure 4.3).

The reactivity of the purified antibodies to denatured BFDV CP was assessed by western blotting. Although the antibodies reacted strongly with proteins from both the insect and bacterial cells, they appeared to be non-reactive to the denatured recombinant BFDV CP (Figure 4.4). This suggests that the immune response to the recombinant protein is predominantly directed towards conformational epitopes. However, the failure of the chicken antibodies to react with denatured protein is in contrast to the reactivity of the recombinant protein with sera from infected birds (see Chapter 3 for details). It is reasonable to assume that during the life cycle of the virus, infected birds are exposed to immature viral particles, which would not necessarily be present in the purified preparation of the recombinant protein. The exposure to partially folded proteins could result in the production of a broader range of antibodies, some of which are able to react with denatured proteins.

In order to determine whether the production of antibodies capable of binding to non-denatured proteins was stimulated, the kinetics of CP-specific antibody development in each of the groups of immunized chickens was evaluated using indirect capture ELISA. The use of a capture ELISA assay effectively circumvented the problem of non-specific background associated with the western blot analysis, since chickens immunized with HPV-16 VLPs produced in insect cells had negligible levels of reactive antibodies (Figure 4.5). Seroconversion was evident in all groups immunized with the recombinant BFDV CP with antibody titres steadily increasing over time (Figure 4.5). The antibodies appeared to be directed against the recombinant protein rather than the N-terminal His_6-tag as indicated by the fact that no response was detected when the captured CP ΔN40 antigen was substituted with purified His_6-tegged CAT protein.

Immunized chickens seroconverted only after the third immunization, suggesting the response to the recombinant is protein is dose-dependent. Interestingly, immunization of animals with CP

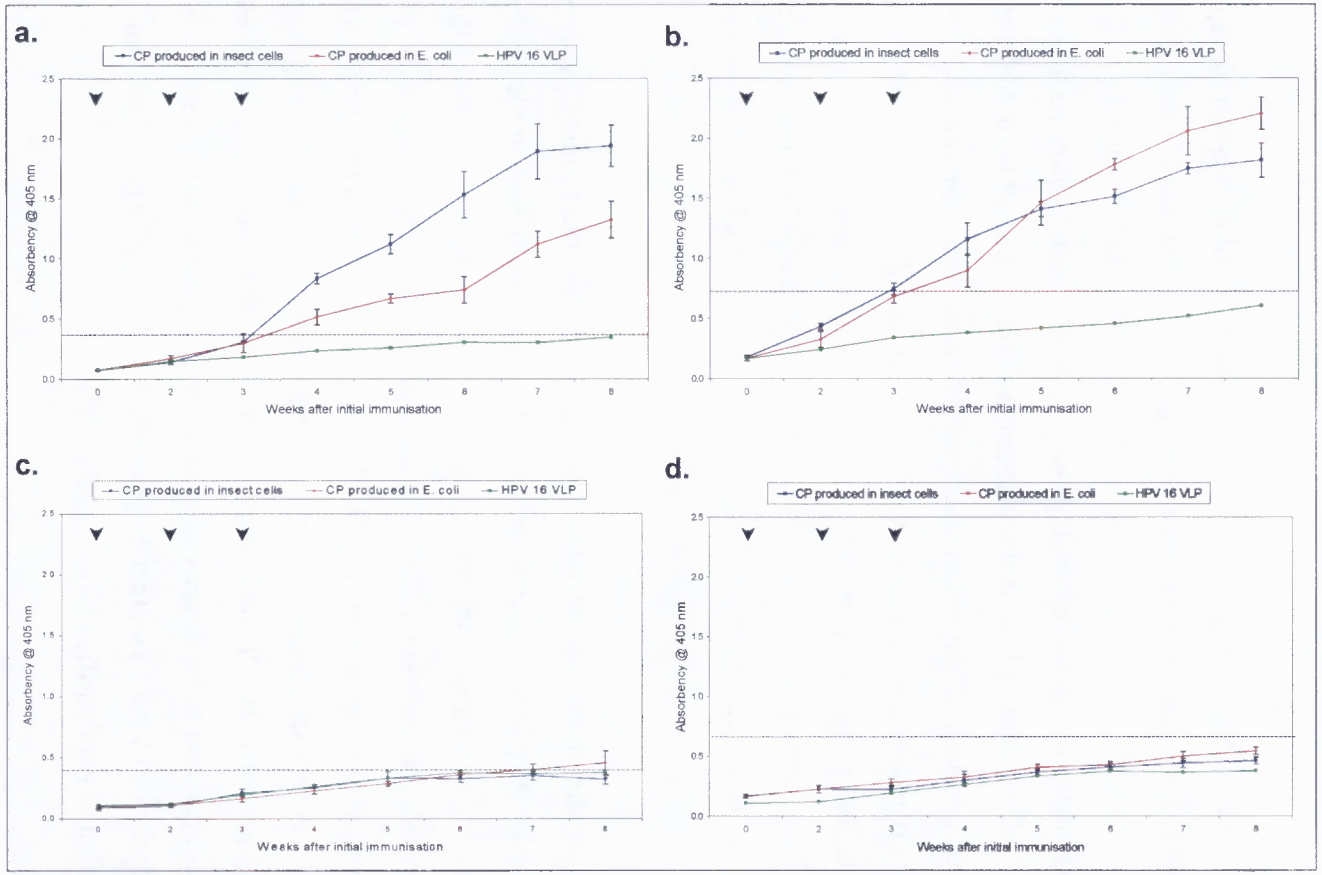

Figure 4.5. Antibody responses specific to BFDV CP induced following immunization of chickens with recombinant CP ΔN40 produced in insect and *E. coli* cells, respectively. HPV 16 VLP was included for comparative purposes. IgY preparations purified from eggs collected weekly p.im. were assayed in an indirect capture ELISA. Chickens were immunised and then boosted at 2 and 3 weeks (represented by arrowheads). Each data point represents the mean optical density (± standard deviation) of 2 animals. Preparations were assayed for binding to proteins captured from (a) insect cells expressing CP ΔN40, (b) *E. coli* cells expressing CP ΔN40, (c) insect cells expressing CAT, and (d) non-recombinant *E. coli* cells. Preparations with OD values 4x that of pre-immunized eggs (dotted black line) were considered positive for BFDV-specific antibodies.

ΔN40 purified from insect cells resulted in the production of antibodies equally capable of binding to recombinant protein produced in either insect or *E. coli* cells. However, antibodies raised to CP ΔN40 purified from *E. coli* appeared to have a lower affinity for the heterologous protein, compared to that expressed in *E. coli*. This trend was also reflected in the endpoint titres of IgY preparations purified from eggs collected 8 weeks p.im. (Figure 4.6). The reciprocal of the endpoint titres of the group immunized with protein purified from insect cells were estimated to be in the order of 12000 in both cases. In contrast, the endpoint titres of the group immunized with protein purified from *E. coli*, as measured against proteins expressed in the same system, was at least two-fold higher compared to the titres measured against proteins produced in insect cells.

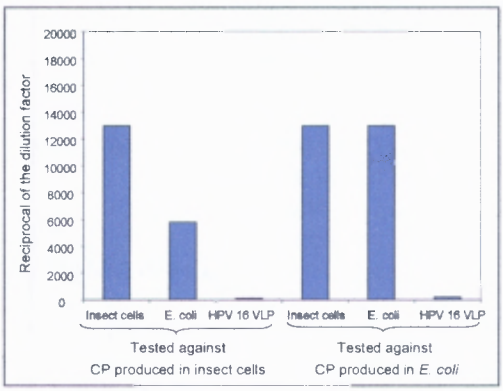

Figure 4.5 Endpoint antibody titres induced in chickens specific to BFDV CP after immunization with recombinant CP ΔN40 produced in insect and *E. coli* cells, respectively. IgY preparations purified from eggs collected 8 weeks p.im. were serially diluted and assayed using an indirect capture ELISA. Endpoint titres are expressed as the reciprocal of the highest dilution where the OD was >4x that of pre-immunized eggs. Each column represents the titre of pooled sample from 2 animals.

4.3.3 Comparison of PCR and ELISA assay for the detection of BFDV

In recent years PCR testing has replaced the haemagglutination (HA) and haemagglutination (HI) inhibition assays as the preferred method for routine diagnosis of BFDV infections (Todd, 2000). However, extensive genetic diversity associated with the virus may limit the sensitivity and accuracy of diagnosis based solely on PCR testing (Heath *et al.*, 2004;de Kloet & de Kloet, 2004;Raue *et al.*, 2004). It is for this reason that PCR technologies should be used in combination with serological methods, such as HA and HI for the conclusive diagnosis of PBFD (Khalesi *et al.*, 2005). However, the interpretation of HA and HI results are severely hampered by fact that

Table 4.1 List of samples tested for the presence of BFDV specific DNA and antibodies.

Host species	*Origin*	*Sample*	*PCR*	*ELISA* (insect cell)*	*ELISA* (E. coli)*
Psittacus erithacus	South Africa	**AFG3-ZA**[#]	+	-	-
P. erithacus	Gauteng, South Africa	**AFG4-ZA**	+	-	-
Cacatua alba	Gauteng, South Africa	**UC1-ZA**	+	-	-
C.alba	Western Cape, South Africa	**UC2-ZA**	+	-	-
Pionites leucogaster	Gauteng, South Africa	**WBC1-ZA**	+	+ (100)	+ (50)
Ara macao	South Africa	**SM1-ZA**	+	-	-
A. macao	South Africa	**SM2-ZA**	-	+ (50)	+ (50)
A. ararauna	Gauteng, South Africa	**BGM1-ZA**	-	+ (150)	+ (100)
Poicephalus rufiventris	KwaZulu-Natal, South Africa	**ARB1-ZA**	+	-	-
P. rufiventris	KwaZulu-Natal, South Africa	**ARB2-ZA**	+	+ (150)	+ (50)
Poicephalus gulielmi massaicus	KwaZulu-Natal, South Africa	**GJP1-ZA**	+	-	-
P. gulielmi massaicus	KwaZulu-Natal, South Africa	**GJP2-ZA**	+	-	-
Poicephalus robustus	Zambia	**GHP1-ZAM**	+	+ (50)	-
Poicephalus robustus	KwaZulu-Natal, South Africa	**CPA8-ZA**	+	-	-
P. robustus	KwaZulu-Natal, South Africa	**CPA10-ZA**	+	-	-
P. robustus	KwaZulu-Natal, South Africa	**CPA12-ZA**	-	+ (200)	+ (100)
P. robustus	KwaZulu-Natal, South Africa	**CPA13-ZA**	-	+ (250)	+ (100)
P. robustus	KwaZulu-Natal, South Africa	**CPA14-ZA**	-	+ (50)	-
P. robustus	KwaZulu-Natal, South Africa	**CPA15-ZA**	-	+ (100)	+ (50)
P. robustus	KwaZulu-Natal, South Africa	**CPA16-ZA**	-	+ (200)	+ (150)
P. robustus	KwaZulu-Natal, South Africa	**CPA20-ZA**	-	-	-
P. robustus	KwaZulu-Natal, South Africa	**GHP2-ZA**	-	-	-
Deroptyus accipitrinus	Gauteng, South Africa	**HHP1-ZA**	+	-	-
D. accipitrinus	Gauteng, South Africa	**HHP2-ZA**	-	-	-
D. accipitrinus	Gauteng, South Africa	**HHP3-ZA**	-	-	-

* The antibody titre of each sample expressed as the reciprocal of the highest dilution where the OD was > 4-times that of IgY preparations of pre-immunized eggs are shown in brackets.
[#] The recombinant BFDV protein was based on the CP of AFG3-ZA

that erythrocytes from psittacines are not readily available and differences the ability of the virus to agglutinate erythrocytes obtained from different sources (Sanada & Sanada, 2000).

To address this problem, I have developed an indirect capture ELISA for the detection of BFDV-specific antibodies in psittacine sera. For comparative purposes the assay was adapted to make use of recombinant CP expressed in either insect or bacterial cells. A total of 25 samples were tested for the presence of BFDV-specific antibodies using each of the respective antigens (Table 4.1). Of the 25 samples, 8 tested positive for BFDV-specific antibodies on both the ELISA assays, whilst an additional 2 samples tested positive only when CP ΔN40 expressed in insect cells were used as the target antigen. The assay was significantly less sensitive when antigen expressed in *E. coli* was used, with antiserum titres generally 2-fold lower compared to the insect cell ELISA.

The results of the ELISA assays were compared to that of PCR testing. Of the 25 birds only 3 tested positive for both BFDV-specific antibodies and DNA. Seven birds tested positive for antibodies only, whilst 11 others tested positive for BFDV DNA only. There was an inverse correlation between the PCR and ELISA results. Samples that tested positive by PCR were significantly less likely to test positive for the presence of antibodies ($P = 0.042$).

The phylogenetic relationship of the 14 isolates associated with the sera that tested positive by PCR is shown in Figure 4.7. There did not appear to be any correlation between genetic diversity and the ability of the serological assays to detect BFDV-specific antibodies. This is consistent with previous reports that antigenic serotypes of BFDV do not exist (Khalesi *et al.*, 2005). Recombinant CP of AFG3-ZA was chosen as the candidate vaccine based on its approximate equidistant phylogenetic relationship to all known BFDV genotypes. This choice seems to be justified, since antiserum from WBC1-ZA reacted strongly with the recombinant protein. The *CP* of AFG3-ZA and WBC1-ZA differ approximately 10% and are the two most distantly related isolates in our current testset. This is only slightly less that distance between AFG3-ZA and the most diverged isolates, MMC-AUS and SCC2-NZ ($\pi = 0.134$). Interestingly, serum taken from AFG3-ZA failed to react with either of the recombinant antigens. The phylogenetic relationship of the isolates tentatively associated with the sera samples that were PCR negative, but tested positive for the presence of BFDV-specific antibodies were undetermined.

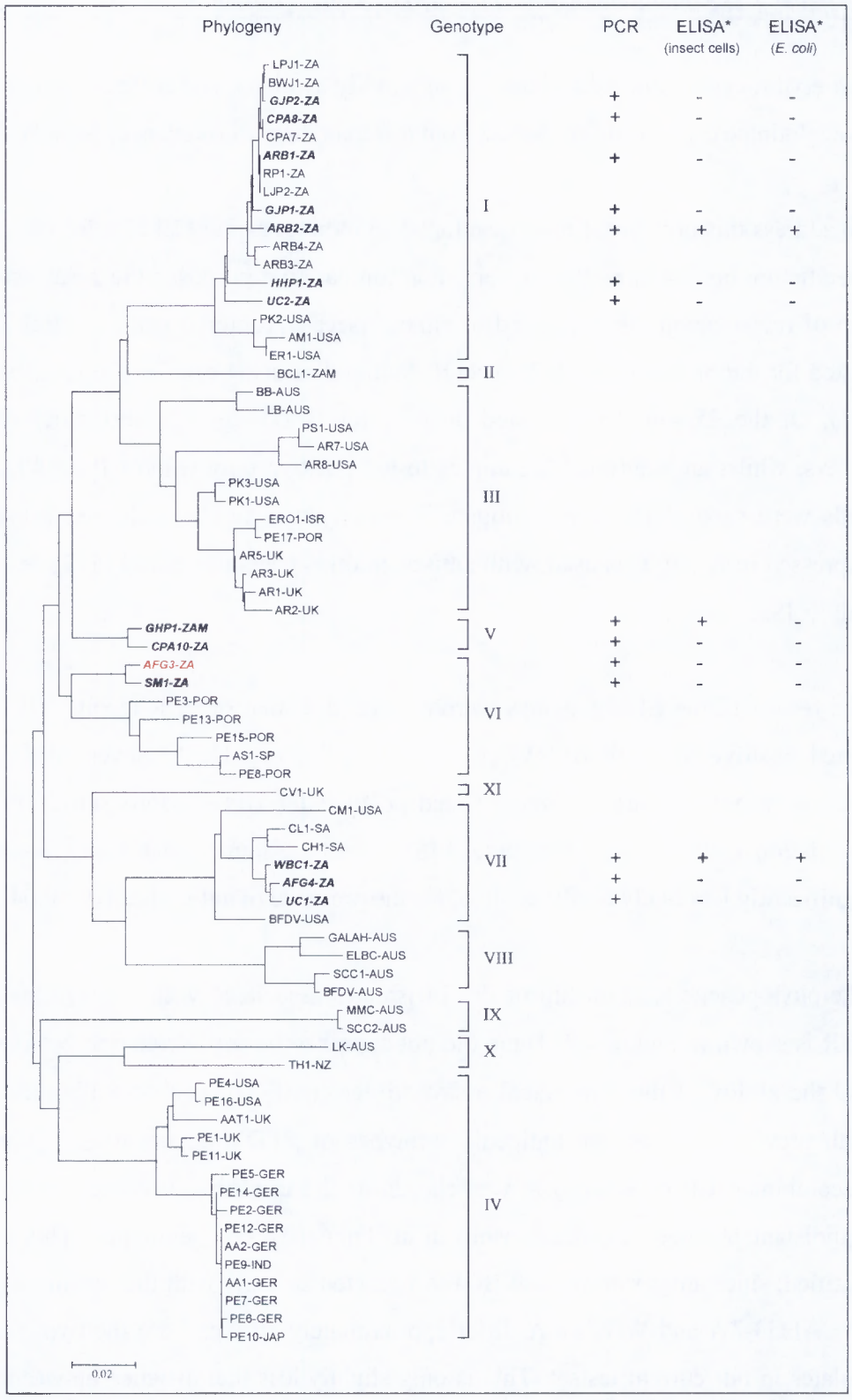

Figure 4.7 Maximum likelihood tree depicting the phylogenetic relationship of the BFD viruses used to assess the applicability of the ELISA assay for diagnosing BFDV infections. Viruses associated with serum samples used are printed in italicized type. Recombinant protein from AFG3-ZA (red) was used as the target antigen in the assay.

4.4 Discussion

Despite the fact that an inactivated BFDV vaccine has been shown to be effective in preventing infection, commercial application of the vaccine is still limited. This is mainly due to the impracticality of harvesting antigens from infected birds for commercial application. However, through the use of recombinant DNA technology the large-scale production of fully characterized immunogens are not only feasible, but also economically viable.

In Chapter 3, I reported on the expression of several variants of the BFDV CP in insect cells using recombinant baculovirus. The wildtype CP was shown to cross-react with sera from birds with clinical signs of PBFD. This suggests that the recombinant protein closely resembles the wildtype antigen in terms of antigenicity and cloud potentially be used as a recombinant subunit vaccine. I furthermore showed that the level of expression of the recombinant protein was significantly influenced by the high proportion of positively charged amino acids found at the N-terminus of the protein. Deletion of the first 40 codons of the wildtype gene resulted in a two-fold increase in the level of expression. In accordance with previously published results (Johne *et al.*, 2004), the expression of the BFDV CP in *E. coli* could be achieved only after the deletion of the first 40 codons. The majority of this truncated protein remained insoluble and could only be purified under denaturing conditions. A similar situation exists for the PCV CP with the yield of the truncated PCV CP reported to be at least 6 times higher than of the full-length protein when expressed in *E. coli* (Zhou *et al.*, 2005). It was further demonstrated that the first 47 amino acid residues at the N-terminus of the PCV 2 CP are not involved in the formation of conformational epitopes (Lekcharoensuk *et al.*, 2004). Removal of this portion of the protein should thus not influence the antigen to act as a subunit vaccine.

In this chapter the immunogenicity and antigenicity of the recombinant BFDV CP was tested. In order to establish whether recombinant CP would induce a humoral immune response, I compared the response to proteins expressed in *E. coli* and insect cells, respectively, in a suitable animal model. The avian immune system differs from that of mammals in various ways (Schade *et al.*, 1996). As a result the immunogenic characteristics of the BFDV CP in mammalian species, typically used to model the immunogenicity of candidate vaccines, may not necessarily accurately reflect the response to the protein during naturally acquired infections. Chickens are the only avian species that can readily be used as an alternative to mammalian systems (Larsson

et al., 1993) and are likely to be a reasonable model for predicting antibody responses in parrots. The use of chickens, or more specifically hens, has the added advantage that large quantities of the serum antibodies are transferred into the egg which can easily be purified from the yolk for use in downstream applications

Based on the results of the indirect capture ELISA, protein expressed in insect cells were found to be more efficient in inducing higher titres of reactive antibodies capable of recognizing antigens produced in homologous as well as heterologous systems. Although antibodies raised against the protein expressed in *E. coli* was capable of effectively binding to the homologous protein, they were less efficient in binding to CP ΔN40 expressed in insect cells. Differences in the reactivity of antibodies raised against protein produced in insect and *E. coli* cells may be indicative of a difference in the conformation adopted by the proteins in each of the systems. This is supported by the fact that the antibodies did not bind to denatured CP ΔN40 as shown by western blotting.

For a vaccine to be effective, it should elicit a complex mixture of antibodies, which closely resembles the response to the native virion during natural infections. Ideally, vaccination should result in the recognition of both linear and conformational epitopes required for neutralization of the infectious virion. The PCV CP has been shown to assemble into VLPs closely resembling infectious virions when expressed in insect cells (Liu *et al.*, 2001b). In addition, several monoclonal antibodies produced in response to the vaccination of mice with PCV 2 CP produced in *E. coli* is capable of neutralizing infectious virus *in virto* (Ju *et al.*, 2005b). Although it is currently unclear to what degree the antigens describe here resembles the native BFD virus, one could reasonably argue that the BFDV CP would in a similar way self-assemble into particles with a distinctive quaternary structure when recombinantly expressed. However, in contrast to CP ΔN40 produced in insect cells, which could readily be purified under native conditions, the recombinant proteins produced in *E. coli* could only be purified under denaturing conditions. Although the denaturing agents were removed by dialysis, the purified proteins may not have adequately refolded. This would result in the loss of conformational epitopes and a general lowering of the efficacy of the protein to act as an antigen and could account for the lower titre of antibodies capable of recognizing heterologous protein.

Although the majority of BFDV-infections occur in young birds, typically less than 3 years of age, older are occasionally also affected by the disease. In most cases these birds recover from the infections and subsequently act as carriers of the virus allowing the disease to persist longer in affected flocks. To detect such cases reliable and reproducible serological tests for monitoring BFDV infections in flocks are required. Current serological tests include HI and HA assays. These tests are however unreliable and impractical to for the purpose of routine diagnosis. To address this problem, an ELISA has been developed for the detection of BFDV-specific antibodies in psittacine sera (Johne *et al.*, 2004). The use of recombinant CP allows for the standardized application and interpretation of ELISA-based technologies. The results of an ELISA using recombinant protein expressed in *E. coli* for the detection of BFDV-specified antibodies in psittacine sera were found to correlate reasonably well with haemagglutination inhibition activity (Johne *et al.*, 2004). A major drawback of this technology is that it requires larges volumes of purified antigen. I have developed an indirect capture ELISA that eliminates the need for purifying the recombinant protein prior to its use in the assay. Recombinant proteins expressed in insect cells appeared to increase the sensitivity of the assay compared to protein expressed in *E. coli*. In both cases the presence of BFDV-specific antibodies correlated strongly with the absence of BFDV DNA. This is consistent with previous reports were the presence of HI antibodies was shown to inversely related to the presence of BFDV antigens (Khalesi *et al.*, 2005). Based on these results, it would appear that BFDV-infected birds develop a detectable humoral immune response only after the viraemic stage of the disease. This may be expected since BFDV infections typically result in severe damage to the bursa and the thymus (Latimer *et al.*, 1991;Khalesi *et al.*, 2005).

The inverse relationship between the presence of BFDV-specific antibodies and the virus holds significant implications for the development new diagnostics for the disease. The absence of a humoral response may make the use of serological based-techniques such as ELISA and HI obsolete for the detection of active and chronic BFDV infections. The early detection of BFDV-specific antigens or genetic material remains critical to the effective management of the disease during outbreaks. ELISA-based technologies may yet play a central role in this regard as is the case with the quantification of p24 antigen by ELISA in determining the viral load in HIV infected individuals (Saville *et al.*, 2001). I have shown here that it is possible to produce large quantities of BFDV-specific antibodies by immunizing chickens with recombinant proteins.

These antibodies can potentially be used to develop highly standardized ELISA-based assays for the detection of viral particles circulating in the blood of infected birds. The use of antibodies developed in chickens would circumvent concerns regarding the cross-reactivity of anti-psittacine IgY preparations, since thoroughly characterized anti-chicken antibodies are readily available.

An added advantage of these antibodies is their potential therapeutic use during the clinical stages of PBFD. Neutralizing antibodies prevent the attachment of viruses to host cells and have been shown to be effective as immunoprophylaxis as well as therapeutics when applied as passive immunization (Kweon *et al.*, 2000;Kim *et al.*, 2004). Passive immunization strategies could be of immeasurable value during outbreaks of the disease in a typical aviculture setting. Treatment of infected birds would aid their native immune system to effectively clear the virus. The establishment of flock immunity through passive immunization during the initial stages of an epidemic could drastically reduce the economic impact of an outbreak.

Taken as a whole, the results describes here suggests that recombinant BFDV CP could be used as a subunit vaccine. However, it is unclear whether a humoral response to the antigen would be sufficient to protect vaccinated birds against challenge with infectious virus. Some evidence suggests that cellular immunity may play a critical role in the early response to circovirus infections. Blanchard et al. (2003) showed that DNA vaccinated pigs were protected from challenge with PCV 2 despite the absence of PCV 2-specific antibodies. The presence of significant cellular immunity following vaccination was recently confirmed when the immunogenicity of a recombinant pseudorabies virus vaccine expressing a PCV Rep-CP fusion protein was tested (Ju *et al.*, 2005a). The protective immunity of the recombinant subunit vaccine describe here, can only be assessed through controlled infection experiments in suitable psittacine species.

Chapter 5

Concluding remarks

Since the isolation and characterization of BFDV, the number of characterized circoviruses from novel avian species has steadily increased. These viruses share remarkably similar pathologies, including the targeting the immune cells of infected individuals, which ultimately results in the complete suppression of the immune system. The likeness between these viruses extends to the molecular level, with all members of the family *Circoviridae* displaying similar organization and structure of their genetic material. Despite this each species are highly specialized with regards to its particular host range. This would suggest that although the basic biology is well conserved within the group, differences in their genetic make-up have far-reaching implications.

This is clearly evident if one considers the intertypic and intratypic variation between the different genera of the circoviruses. The work presented in Chapter 4 of this thesis indicates that this diversity is reflected in the phylogeny of the different BFD viruses. Despite the substantial dissemination of the different viral genotype throughout the world via the international pet trade, the southern African isolates have diverged substantially from viruses found in other parts of the world and separate into clearly defined lineages. The significant genetic distance between the African and Australian lineages suggest that the occurrence of BFDV on the African continent is not due to recent introductions, but rather that the disease agent has been circulating in wild African parrot populations for the better part of the virus's evolutionary history.

Although sightings of free-living African parrots exhibiting feather dystrophies generally associated with the disease are occasionally reported (Warburton & Perring, 2002), PBFD has only been confirmed in one southern African psittacine species. In 2000 blood from a Black-cheeked Lovebird chick from the mid Machile River region in Zambia tested positive for BFDV (Warburton & Perring, 2002). This species has a highly localized range in south-west Zambia and is considered to be Vulnerable and is classified as a CITES Appendix II species. The incidence of PBFD in this population is somewhat surprising, if one considers its geographical isolation. Phylogenetic characterization based on the *CP* confirmed that this BFDV isolate was genetically distinct, and it is thought to be representative of a true southern African BFDV genotype.

The incidence of BFDV in free-living African parrots were later confirmed when a Cape parrot tested positive for the disease. The Cape parrot is the only psittacine species endemic to South Africa and primarily inhabits afromontane forests in the KwaZulu-Natal and Eastern Cape region

of South Africa (Downs & Symes, 1998). Degradation of their natural habitat and persecution has lead to a drastic reduction of their numbers in the wild. The Cape parrot is currently classified as Endangered under IUCN/Birdlife International threat criteria, with only ~500 birds left in the wild (Warburton & Perring, 2002).

Small and localized populations such as that of the Cape parrot and Black-cheeked Lovebird are especially vulnerable to disease. Although complete eradication of BFDV through large scale vaccination of wild populations is hardly feasible, understanding the epidemiology of the disease in southern Africa will be of critical importance for the effective management of the threat posed by PBFD. Commercial breeding facilities are often found within the same areas as wild parrot populations and although direct contact between these populations is unlikely to occur, the transmission of BFDV from infected captive birds to wild parrots remains a concern. As part of an integrated approach to the management of the threat posed by the disease, the possible impact of the disease on the indigenous African parrots of South Africa should be considered.

It is unclear whether the genetic diversity between the different BFD viruses would translate into differences in antigenicity or pathogenicity, but incidences of PBFD involving the African genotypes generally follow typical patterns of disease development. In contrast African parrots in Europe have been reported to be dying of an atypical from of PBFD characterized by severe immuno-suppression and the absence of typical feather dystrophies commonly associated with BFDV infection (Raue *et al.*, 2004). This atypical disease development is not consistently associated with a particular genotype, but it is interesting to note that genetically similar strains have not yet been identified in South Africa. This would suggest that this species group may be particularly vulnerable to European or Australian genotypes of BFDV. Although the distributions of the different genotypes appear to be restricted at this time, it can potentially be altered by the movement of birds via the commercial pet trade. The introduction of new genotypes into naïve populations may lead to devastating epidemics.

Very little is currently known about the molecular biology of the avian circoviruses. It is generally assumed that the biology of BFDV is analogous to that of PCV (Mankertz *et al.*, 2004). However, the work presented in Chapter 3 highlights some unexpected differences in the behavior of the respective capsid proteins of the two viruses. As is the case with PCV, the BFDV

CP was found to be nuclear localized. In both cases the subcellular distribution is directed by bipartite nuclear localization signals situated at the N-terminus of the protein. In contrast to the PCV CP though, the BFDV CP was shown to directly interact with the Rep resulting in a redistribution of the latter into the nucleus. In this regard, the biological function of the BFDV CP more closely resembles that of its geminivirus counterparts. This observation is consistent with the popular line of thought that the circoviruses and the plant-infecting ssDNA viruses originated from a common ancestor. However, it is not clear why the CP of the different circoviruses would have functionally diverged. The development of a tissue culture system for the propagation of BFDV is paramount to expanding our knowledge of avian circovirus biology.

As the causal agent of PBFD, BFDV is currently one of the most significant pathogens of parrots affecting the global pet bird industry. It is believed that the development and widespread use of an effective BFDV is a viable solution to the global PBFD problem. Researchers have risen to the challenge and have successfully produced an inactivated vaccine capable of inducing protective immunity in vaccinated birds (Raidal *et al.*, 1993a) (Ritchie *et al.*, 1992). However, the inability of BFDV to be propagated in tissue culture systems and the threat posed by improperly inactivated vaccine preparations has severely hampered the large-scale application of this model vaccine.

Recombinant DNA technology offers an attractive alternative to use of inactivated vaccines and circumvents many of the problems associated with more classical methods of vaccine development. The work presented in Chapter 4 of this thesis indicates that it is indeed possible to produce BFDV-specific antigens in both prokaryotic and eukaryotic expression systems, which could potentially be use as recombinant subunit vaccines. Immunization of chickens, an animal model homologous to the intended target species, with the recombinant antigens resulted in a strong antigen-specific humoral response. However, differences in the reactivity of antibodies raised against protein produced in the respective systems were clearly evident. These differences may result from slight differences in the posttranscriptional processing of the proteins, which is reflected in the conformation of the antigens. Differences in the conformation of the recombinant antigens could ultimately affect the efficacy of the candidate subunit vaccines.

A major concern associated with the use of vaccines as an integral part of disease management, is the impact of antigenic variation among different isolates of the causal agent. In Chapter 2 the genetic variability amongst BFDV isolates are discussed. The *CP* was found to be hypervariable, suggesting that the evolution this gene is directed by positive selection brought about by immune surveillance. Despite this, no evidence of antigenic serotypes has been found to date (Khalesi *et al.*, 2005). This would suggest that a monovalent vaccine would be sufficient to address the antigenic diversity of the global BFDV population. However, controlled infection experiments are required to test the efficacy of the recombinant antigen to act as a vaccine in the natural host species of the virus.

Appendix A

Sequence Accession Numbers

Content

Table A.1 Genbank accession numbers of sequences used in the construction of Figure 1.4

Genus	*Name*	*Species*	*Accession*
Gyrovirus	*Chicken anaemia virus*	**CAV**	NC001427
Circovirus	*Porcine circovirus type1*	**PCV 1**	NC006266
	Porcine circovirus type1	**PCV 2**	Nc005148
	Beak and feather disease virus	**BFDV**	AF080560
	Canary circovirus	**CaCV**	AJ301633
	Columbid circovirus	**CoCV**	AJ252610
	Goose circovirus	**GCV**	AJ304456
	Duck circovirus	**DuCV**	AY228555
Anellovirus	*Torque teno virus*	**TTV**	NC002076
	TT-like mini virus	**TTMV**	NC002195
Mastrevirus	*Maize streak virus*	**MSV**	NC001346
Nanovirus	*Milk vetch dwarf virus*	**MCDV-C1**	NC003638

Table A.2 Genbank accession numbers of sequences used in the construction of Figure 1.5

ssDNA replicon	*Name*	*Species*	*Accession*
Geminiviridae	*African cassava mosaic virus*	**ACMV-UG**	AAF42737
	Beet curly top virus	**BCTV**	S28360
	Bhendi yeloow vein mosaic virus	**BhYVMV**	AF241479
	Indian cassava mosaic virus	**ICMV**	AF241478
	Miscanthus streak virus	**MiSV**	JQ1358
	Maize streak virus	**MSV**	A05158
	Panicum steak virus	**PanSV**	JQ1552
	Pepper hausteco virus	**PHV**	JQ2300
	Pepper yeloow mosaic virus	**PYMV-VE**	AF155806
	Rhynchosia golden mosaic virus	**RhGMV**	AAF44669
	Tobacco leaf curl virus	**TbLCV**	BAA34016
	Tomato golden mosaic virus	**TGMV**	P0356
	Tomato leaf curl virus (In)	**ToLCV-In**	CAA88229
	Tomato leaf curl virus (MM)	**ToLCV-MM**	AF206674
	Tomato mottle virus	**ToMoV-Flo**	AF241479
	Tobacco yellow dwarf virus	**TYDV**	D42452
	Tomato yellow leaf curl virus (Au)	**TYLCV-Au**	JQ1887
	Tomato yellow leaf curl virus (Is)	**TYLCV-Is**	AF239671
	Wheat dwarf virus	**WDV**	B24356
Circoviridae	*Chicken anaemia virus*	**CAV**	M81223
	Beak and feather disease virus	**BFDV**	AAC69861
	Porcine circovirus type 1	**PCV 1**	AF166528
	Porcine circovirus type 2	**PCV2**	AAC98885
Parvoviridae	*Canine parvovirus*	**CPV**	A29962
	Minute virus of mice	**MVM**	A29510

Table A.2 Continued.

ssDNA replicon	*Name*	*Species*	*Accession*
Nanoviridae	*Banana bunchy top virus (T1)*	**BBTV-T1**	BAA33981
	Banana bunchy top virus (T3)	**BBTV-T3**	CAA06791
	Coconut foliar decay virus	**CFDV**	A46353
	Faba bean necrotic yellow virus (10)	**FBNYV-10**	BAA34048
	Faba bean necrotic yellow virus (2)	**FBNYV-2**	CAB44020
	Faba bean necrotic yellow virus (7)	**FBNYV-7**	CAB44025
	Faba bean necrotic yellow virus (9)	**FBNYV-9**	CAB44027
	Milk vetch dwarf virus (1)	**MVDV-1**	BAA33980
	Milk vetch dwarf virus (3)	**MVDV-3**	BAA33982
	Subterranean clover stunt virus (2)	**SCSCV-2**	AAA68018
	Subterranean clover stunt virus (6)	**SCSCV-6**	AAA68022
Nanovirus-like DNA	*Cotton leaf curl virus DNA1*	**ClCuV1**	AJ132344
	Ageratum yellow vain virus DNA1	**AYVV1**	AJ138493
Microviridae	-	**ΦK**	P25244
	-	**Φ174**	A04239
pUB110 Related plasmids	-	**pBC1**	M64604
	-	**pFTB14**	S01098
	-	**pKYM**	M38574
	-	**pLAB1000**	B35390
	-	**pNost**	M81381
	-	**pSK89**	M37889
	-	**pUB110**	M19465
pMV185 Related plasmids	-	**pMV158**	S05981
	-	**pFX2**	X54310
Chlymidia plasmids	-	**pCcpA1**	X62475
	-	**pCHL1**	S02220

Table A.2 Continued.

ssDNA replicon	*Name*	*Species*	*Accession*
Archaebacterial plasmids	-	**pHGN1**	*S06780*
	-	**pPGRB1**	S10152
Unclassified Replicons	-	**ColE3**	S04456
	-	**Chp1**	JU0348
	-	**Phasyl**	S03290
	-	**pEE**	M81382

References

1. **Albertyn, J., Tajbhai, K. M. & Bragg, R. R. (2004).**Psittacine beak and feather disease virus in budgerigars and ring-neck parakeets in South Africa. *Onderstepoort J Vet Res* **71**, 29-34.

2. **Alexandrova, N., Niklinski, J., Bliskovsky, V., Otterson, G. A., Blake, M., Kaye, F. J. & Zajac-Kaye, M. (1995).**The N-terminal domain of c-Myc associates with alpha-tubulin and microtubules in vivo and in vitro. *Mol Cell Biol* **15**, 5188-5195.

3. **Allan, G. M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E. G., Ellis, J. A., Haines, D. M., Meehan, B. M. & Adair, B. M. (1998).**Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest* **10**, 3-10.

4. **Bagewadi, B., Chen, S., Lal, S. K., Choudhury, N. R. & Mukherjee, S. K. (2004).**PCNA interacts with Indian mung bean yellow mosaic virus rep and downregulates Rep activity. *J Virol* **78**, 11890-11903.

5. **Bartlett, J. S., Wilcher, R. & Samulski, R. J. (2000).**Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* **74**, 2777-2785.

6. **Bassami, M. R., Berryman, D., Wilcox, G. E. & Raidal, S. R. (1998).**Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. *Virology* **249**, 453-459.

7. **Bassami, M. R., Ypelaar, I., Berryman, D., Wilcox, G. E. & Raidal, S. R. (2001).**Genetic diversity of beak and feather disease virus detected in psittacine species in Australia. *Virology* **;279**, 392-400.

8. **Beissinger, S. R. (2005).***New World parrots in crisis*. Smithsonian Institution Press.

9. **Biagini, P. (2004).**Human circoviruses. *Vet Microbiol* **98**, 95-101.

10. **Biagini, P., Attoui, H., Gallian, P., Touinssi, M., Cantaloube, J. F., de Micco, M. P. & de Lamballerie, X (2000).**Complete sequences of two highly divergent european isolates of TT virus. *Biochem Biophys Res Commun* **19;271**, 837-841.

11. **Biagini, P., Gallian, P., Attoui, H., Cantaloube, J. F., de Micco, M. P. & de Lamballerie, L., X (1999).**Determination and phylogenetic analysis of partial sequences from TT virus isolates. *J Gen Virol* **80**, 419-424.

12. **Biagini, P., Gallian, P., Attoui, H., Cantaloube, J. F., Touinssi, M., de Micco, M. P. & de Lambellerie, X (2001a).**Comparison of systems performance for TT virus detection using PCR primer sets located in non-coding and coding regions of the viral genome. *J Clin Virol* **22**, 91-99.

13. **Biagini, P., Gallian, P., Attoui, H., Touinssi, M., Cantaloube, J., de Micco, M. P. & de Lambellerie, X (2001b).**Genetic analysis of full-length genomes and subgenomic sequences of TT virus-like mini virus human isolates. *J Gen Virol* **82**, 379-383.

14. **Biagini, P., Todd, D. & Bendinelli, M. (2005).** *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses.* London: Academic Press.

15. **Blanchard, P., Mahe, D., Cariolet, R., Keranflec'h, A., Baudouard, M. A., Cordioli, P., Albina, E. & Jestin, A. (2003).**Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. *Vaccine* **21**, 4565-4575.

16. **Boulton, M., Raineri, D. M., Davies, J. W. & Nester, E. W. (1993).**Identification of genetic factors controlling the ability of *Agrobacterium* to transfer DNA to maize. In *Advances in Molecular Genetics of Plant-Microbe Interactions*, 2 edn, pp. 73-78. Edited by Nester, E. W. & Verma, D. P. S. The Netherlands: Kluwer Academic Publishers.

17. **Boulton, M. I. (1991).***Molecular pathology of maize streak virus.* University of East Anglia.

18. **Boulton, M. I., Buchholz, W. G., Marks, M. S., Markham, P. G. & Davies, J. W. (1989a).**Specificity of *Agrobacterium*-mediated delivery of maize streak virus DNA to members of the Gramineae. *Plant Mol Biol* **12**, 31-40.

19. **Boulton, M. I., Steinkellner, H., Donson, J., Markham, P. G., King, D. I. & Davies, J. W. (1989b).**Mutational analysis of the virion-sense genes of maize streak virus. *J Gen Virol* **70**, 2309-2323.

20. **Braem, C. V., Kas, K., Meyen, E., biec-Rychter, M., Van, D., V & Voz, M. L. (2002).**Identification of a karyopherin alpha 2 recognition site in PLAG1, which functions as a nuclear localization signal. *J Biol Chem* **277**, 19673-19678.

21. **Briddon, R. W., Watts, J., Markham, P. G. & Stanley, J. (1989).**The coat protein of beet curly top virus is essential for infectivity. *Virology* **172**, 628-633.

22. **Bruening, W., Moffett, P., Chia, S., Heinrich, G. & Pelletier, J. (1996).**Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product. *FEBS Lett* **393**, 41-47.

23. **Chenault, K. D. & Melcher, U. (1994a).**Patterns of nucleotide sequence variation among cauliflower mosaic virus isolates. *Biochimie* **76**, 3-8.

24. **Chenault, K. D. & Melcher, U. (1994b).**Phylogenetic relationships reveal recombination among isolates of cauliflower mosaic virus. *J Mol Evol* **39**, 496-505.

25. **Cheung, A. K. (2003b).**Transcriptional analysis of porcine circovirus type 2. *Virology* **305**, 168-180.

26. **Cheung, A. K. (2004).**Identification of an octanucleotide motif sequence essential for viral protein, DNA, and progeny virus biosynthesis at the origin of DNA replication of porcine circovirus type 2. *Virology* **324**, 28-36.

27. **Cheung, A. K. (2003c).**The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2. *Virology* **313**, 452-459.

28. **Cheung, A. K. (2003a).**Comparative analysis of the transcriptional patterns of pathogenic and nonpathogenic porcine circoviruses. *Virology* **310**, 41-49.

29. **Cheung, A. K. & Bolin, S. R. (2002).**Kinetics of porcine circovirus type 2 replication. *Arch Virol* **147**, 43-58.

30. **Clarke, I. N. & Lambden, P. R. (2000).**Organization and expression of calicivirus genes. *J Infect Dis* **181**, 309-316.

31. **Clever, J. & Kasamatsu, H. (1991).**Simian virus 40 Vp2/3 small structural proteins harbor their own nuclear transport signal. *Virology* **181**, 78-90.

32. **Clever, J., Yamada, M. & Kasamatsu, H. (1991).**Import of simian virus 40 virions through nuclear pore complexes. *Proc Natl Acad Sci U S A* **88**, 7333-7337.

33. **Cokol, M., Nair, R. & Rost, B. (2000).**Finding nuclear localization signals. *EMBO Rep* **1**, 411-415.

34. **Crowther, R. A., Berriman, J. A., Curran, W. L., Allan, G. M. & Todd, D. (2003).**Comparison of the structures of three circoviruses: chicken anemia virus, porcine circovirus type 2, and beak and feather disease virus. *J Virol* **77**, 13036-13041.

35. **da Costa, S. R., Okamoto, C. T. & Hamm_Alvarez, S. F. (2003).**Actin microfilaments et al.-the many components, effectors and regulators of epithelial cell endocytosis. *Adv Drug Deliv Rev*, 1359-1383.

36. **Darwich, L., Balasch, M., Plana-Duran, J., Segales, J., Domingo, M. & Mateu, E. (2003).**Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. *J Gen Virol* **84**, 3453-3457.

37. **de Kloet, E. & de Kloet, S. (2004).**Analysis of the beak and feather disease viral genome indicates the existence of several genotypes which have a complex psittacine host specificity. *Arch Virol* **149**, 2393-2412.

38. **Dodman, T., Katenekwa, V., Aspinall, D. & Stjernstedt, R. (2000).**Status and distrobution of the Black-cheeked Lovebird, Zambia. *Ostrich* **71**, 228-234.

39. **Douglas, A. J., Phenix, K., Mawhinney, K. A., Todd, D., Mackie, D. P. & Curran, W. L. (1995).**Identification of a 24 kDa protein expressed by chicken anaemia virus. *J Gen Virol* **76**, 1557-1562.

40. **Downs, C. T. & Symes, C. T. (1998).**Cape Parrots: Report on the second Cape Parrot Big Birding Day. *Psittascene* **10**, 5-7.

41. **Duchatel, J.P., Todd, D., Smyth, J.A., Bustin, J.C. and Vindevogel, H. (2006).** Observations on detection, excretion and transmission of Pigion circovirus in adult, young and embryonic pigions. *Avian pathol.* **35,** 30-34.

42. **Eisenberg, S. W., van Asten, A. J., van Ederen, A. M. & Dorrestein, G. M. (2003).**Detection of circovirus with a polymerase chain reaction in the ostrich (Struthio camelus) on a farm in The Netherlands. *Vet Microbiol* **95,** 27-38.

43. **Ellis, J., Hassard, L., Clark, E. & other authors (1998).**Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J* **39**, 44-51.

44. **Fenaux, M., Opriessnig, T., Halbur, P. G., Elvinger, F. & Meng, X. J. (2004).**A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. *J Virol* **78**, 6297-6303.

45. **Fenaux, M., Opriessnig, T., Halbur, P. G. & Meng, X. J. (2003).**Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. *J Virol* 77, 11232-11243.

46. **Forshaw, J. (1977).***Parrots of the world.* TFH publications Inc.

47. **Gerlach, H. (1994).**Circoviridae-psittacine beak and feather disease virus. In *Avian Medicine:Principles and Practice.*, pp. 894-903. Edited by Ritchie, B. W., Harrison, G. T. & Harrison, L. R.: Wingers Publishing Incorporation.

48. **Gibbs, M. J. & Weiller, G. F. (1999).**Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. *Proc Natl Acad Sci U S A* **96**, 8022-8027.

49. **Gilpin, D. F., McCullough, K., Meehan, B. M. & other authors (2003).**In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. *Vet Immunol Immunopathol* **94**, 149-161.

50. **Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T. & Adam, S. A. (2004).**Importin alpha: a multipurpose nuclear-transport receptor. *Trends Cell Biol* **14**, 505-514.

51. **Goryo, M., Sugimura, H., Matsumoto, S., Umemura, T. & Itakura, C. (1985).**Isolation of an agent inducing chicken anaemia. *Avian Pathol.* **14**, 483-496.

52. **Gutierrez, C. (1999).**Geminivirus DNA replication. *Cell Mol Life Sci* **56**, 313-329.

53. **Gutierrez, C. (2000).**DNA replication and cell cycle in plants: learning from geminiviruses. *EMBO J* **19**, 792-799.

54. **Hamel, A. L., Lin, L. L. & Nayar, G. P. (1998).**Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* **72**, 5262-5267.

55. **Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S. & Robertson, D. (2000).**Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Biochem Mol Biol* **35**, 105-140.

56. **Hattermann, K., Roedner, C., Schmitt, C., Finsterbusch, T., Steinfeldt, T. & Mankertz, A. (2004).**Infection studies on human cell lines with porcine circovirus type 1 and porcine circovirus type 2. *Xenotransplantation* **11**, 284-294.

57. **Hattermann, K., Schmitt, C., Soike, D. & Mankertz, A. (2003).**Cloning and sequencing of Duck circovirus (DuCV). *Arch Virol* **148**, 2471-2480.

58. **Haydon, D. T., Bastos, A. D., Knowles, N. J. & Samuel, A. R. (2001).**Evidence for positive selection in foot-and-mouth disease virus capsid genes from field isolates. *Genetics* **157**, 7-15.

59. **Heath, L., Martin, D. P., Warburton, L., Perrin, M., Horsfield, W., Kingsley, C., Rybicki, E. P. & Williamson, A. L. (2004).**Evidence of unique genotypes of beak and feather disease virus in southern Africa. *J Virol* **78**, 9277-9284.

60. **Hiscox, J. A. (2002).**The nucleolus--a gateway to viral infection? *Arch Virol* **147**, 1077-1089.

61. **Holland, J. & Domingo, E. (1998).**Origin and evolution of viruses. *Virus Genes* **16**, 13-21.

62. **Hsieh, C. H. & Griffith, J. D. (1988).**The terminus of SV40 DNA replication and transcription contains a sharp sequence-directed curve. *Cell* **52**, 535-544.

63. **Ilyina, T. V. & Koonin, E. V. (1992).**Conserved Sequence Motifs in the Initiator Proteins for Rolling Circle DNA Replication Encoded by Diverse Replicons from Eubacteria, Eucaryotes and Archaebacteria. *Nucleic Acids Res* **20**, 3279-3285.

64. **Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., zur, H. H. & de Villiers, E. M. (2004).**Isolation of multiple TT virus genotypes from spleen biopsy tissue from a Hodgkin's disease patient: genome reorganization and diversity in the hypervariable region. *J Virol* **78**, 7498-7507.

65. **Jeurissen, S. H., Wagenaar, F., Pol, J. M., van der Eb, A. J. & Noteborn, M. H. (1992).**Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. *J Virol* **66**, 7383-7388.

66. **Johne, R. & Muller, H. (1998).**Avian polymavirus in wild birds: genome analysis of isolates from Falconiformes and Psittaciformes. *Arch Virol* **143**, 1501-1512.

67. **Johne, R., Raue, R., Grund, C., Kaleta, E. F. & Muller, H. (2004).**Recombinant expression of a truncated capsid protein of beak and feather disease virus and its application in serological tests. *Avian Pathol* **33**, 328-336.

68. **Ju, C., Fan, H., Tan, Y., Liu, Z., Xi, X., Cao, S., Wu, B. & Chen, H. (2005a).**Immunogenicity of a recombinant pseudorabies virus expressing ORF1-ORF2 fusion protein of porcine circovirus type 2. *Vet Microbiol* **109**, 179-190.

69. **Ju, C. M., Chen, H. C., Fan, H. Y., Liu, Z. F. & Cao, S. B. (2005b).**[Recombination and expression of ORF1 and ORF2 gene of porcine circovirus type 2 and gene of pseudorabies virus]. *Sheng Wu Gong Cheng Xue Bao* **21**, 370-374.

70. **Juniper, T. & Penn, M. (1998).***Parrots: A guide to the parrots of the world.* Pica Press.

71. **Kamstrup, S., Barfoed, A. M., Frimann, T. H., Ladekjaer-Mikkelsen, A. S. & Botner, A. (2004).**Immunisation against PCV2 structural protein by DNA vaccination of mice. *Vaccine* **22**, 1358-1361.

72. **Karlsson, G. B. & Liljestrom, P. (1998).***Methods in Molecular Medicine*. Humana Press Inc.

73. **Keese, P. & Gibbs, A. (1993).**Plant viruses: master explorers of evolutionary space. *Curr Opin Genet Dev* **3**, 873-877.

74. **Kensil, C. R., Barrett, C., Kushner, N., Beltz, G., Storey, J., Patel, U., Recchia, J., Aubert, A. & Marciani, D. (1991).**Development of a genetically engineered vaccine against feline leukemia virus infection. *J Am Vet Med Assoc* **199**, 1423-1427.

75. **Khalesi, B., Bonne, N., Stewart, M., Sharp, M. & Raidal, S. (2005).**A comparison of haemagglutination, haemagglutination inhibition and PCR for the detection of psittacine beak and feather disease virus infection and a comparison of isolates obtained from loriids. *J Gen Virol* **86**, 3039-3046.

76. **Kim, D. K., Jang, I. K., Seo, H. C., Shin, S. O., Yang, S. Y. & Kim, J. W. (2004).**Shrimp protected from WSSV disease by treatment with egg yolk antibodies (IgY) against a truncated fusion protein derived from WSSV. *Aquaculture* **237**, 21-30.

77. **Koch, G., Van Roozelaar, D. J., Verschueren, C. A., van der Eb, A. J. & Noteborn, M. H. (1995).**Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus. *Vaccine* **13**, 763-770.

78. **Kock, N., Hangertner, P. U. & Lucke, V. (1993).**Variation in clinicaldisease and species susceptibility to psittacine beak and feather disease in Zimbabwean lovebirds. *Onderstepoort J Vet Res* **60**, 159-161.

79. **Koonin, E. V. & Ilyina, T. V. (1993).**Computer-assisted dissection of rolling circle DNA replication. *Biosystems* **30**, 241-268.

80. **Koonin, E. V. & Ilyina, T. V. (1992).**Geminivirus replication proteins are related to prokaryotic plasmid rolling circle DNA replication initiator proteins. *J Gen Virol* **73**, 2763-2766.

81. **Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001).**MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244-1245.

82. **Kurath, G. (2005).**Overview of recent DNA vaccine development for fish. *Dev Biol (Basel)* **121**, 201-213.

83. **Kweon, C. H., Kwon, B. J., Woo, S. R., Kim, J. M., Woo, G. H., Son, D. H., Hur, W. & Lee, Y. S. (2000).**Immunoprophylactic effect of chicken egg yolk immunoglobulin (Ig Y) against porcine epidemic diarrhea virus (PEDV) in piglets. *J Vet Med Sci* **62**, 961-964.

84. **Lai, M. M. (1992).**RNA recombination in animal and plant viruses. *Microbiol Rev* **56**, 61-79.

85. **Larsson, A., Balow, R., Lindahl, T. L. & Forsberg, P. (1993).**Chicken antibodies:Taking advantage of evolution-A Review. *Poultry Science* **72**, 1807-1812.

86. **Latimer, K. S., Rakich, P. M., Niagro, F. D., Ritchie, B. W., Steffens, W. L., Campagnoli, R. P., Pesti, D. A. & Lukert, P. D. (1991).** An updated review of psittacine beak and feather disease. *J Assoc Avian Vet* **5**, 211-220.

87. **Laufs, J., Jupin, I., David, C., Schumacher, S., Heyraud-Nitschke, F. & Gronenborn, B. (1995).**Geminivirus replication: genetic and biochemical characterization of Rep protein function, a review. *Biochimie* **77**, 765-773.

88. **Leary, T. P., Erker, J. C., Chalmers, M. L., Desai, S. M. & Mushahwar, I. K. (1999).**Improved detection systems for TT virus reveal high prevalence in humans, non-human primates and farm animals. *J Gen Virol* **80**, 2115-2120.

89. **Lekcharoensuk, P., Morozov, I., Paul, P. S., Thangthumniyom, N., Wajjawalku, W. & Meng, X. J. (2004).**Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. *J Virol* **78**, 8135-8145.

90. **Li, P. P., Nakanishi, A., Shum, D., Sun, P. C., Salazar, A. M., Fernandez, C. F., Chan, S. W. & Kasamatsu, H. (2001).**Simian virus 40 Vp1 DNA-binding domain is functionally separable from the overlapping nuclear localization signal and is required for effective virion formation and full viability. *J Virol* **75**, 7321-7329.

91. **Lindahl, T. & Nyberg, B. (1974).**Heat-induced deamination of cytosine residues in deoxyribonucleic acid. *Biochem* **13**, 3405-3410.

92. **Liu, H., Boulton, M. I. & Davies, J. W. (1997).**Maize streak virus coat protein binds single- and double-stranded DNA in vitro. *J Gen Virol* **78**, 1265-1270.

93. **Liu, H., Boulton, M. I., Oparka, K. J. & Davies, J. W. (2001a).**Interaction of the movement and coat proteins of Maize streak virus: implications for the transport of viral DNA. *J Gen Virol* **82**, 35-44.

94. **Liu, H., Boulton, M. I., Thomas, C. L., Prior, D. A., Oparka, K. J. & Davies, J. W. (1999).**Maize streak virus coat protein is karyophyllic and facilitates nuclear transport of viral DNA. *Mol Plant Microbe Interact* **12**, 894-900.

95. **Liu, J., Chen, I. & Kwang, J. (2005).**Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. *J Virol* **79**, 8262-8274.

96. **Liu, Q., Tikoo, S. K. & Babiuk, L. A. (2001b).**Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. *Virology* **285**, 91-99.

97. **Liu, Q., Willson, P., ttoh-Poku, S. & Babiuk, L. A. (2001c).**Bacterial expression of an immunologically reactive PCV2 ORF2 fusion protein. *Protein Expr Purif* **21**, 115-120.

98. **Low, R. (1984).***Endangered Parrots*. Sterling Publications.

99. **Lux, K., Goerlitz, N., Schlemminger, S. & other authors (2005).**Green fluorescent protein-tagged adeno-associated virus particles allow the study of cytosolic and nuclear trafficking. *J Virol* **79**, 11776-11787.

100. **Mahe, D., Blanchard, P., Truong, C., Arnauld, C., Le, C. P., Cariolet, R., Madec, F., Albina, E. & Jestin, A. (2000).**Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *J Gen Virol* **81**, 1815-1824.

101. **Malik, P. S., Kumar, V., Bagewadi, B. & Mukherjee, S. K. (2005).**Interaction between coat protein and replication initiation protein of Mung bean yellow mosaic India virus might lead to control of viral DNA replication. *Virology* **337**, 273-283.

102. **Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T. & Finsterbusch, T. (2004).**Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. *Vet Microbiol* **98**, 81-88.

103. **Mankertz, A., Hattermann, K., Ehlers, B. & Soike, D. (2000).**Cloning and sequencing of columbid circovirus (coCV), a new circovirus from pigeons. *Arch Virol* **145**, 2469-2479.

104. **Mankertz, A. & Hillenbrand, B. (2001).**Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. *Virology* **279**, 429-438.

105. **Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C. & Finsterbusch, T. (2003).**New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. *J Virol* **77**, 9885-9893.

106. **Marciani, D. J., Kensil, C. R., Beltz, G. A., Hung, C. H., Cronier, J. & Aubert, A. (1991).**Genetically-engineered subunit vaccine against feline leukaemia virus: protective immune response in cats. *Vaccine* **9**, 89-96.

107. **Martin, D. & Rybicki, E. (2000).**RDP: detection of recombination amongst aligned sequences. *Bioinformatics* **16**, 562-563.

108. **Martin-Gallardo, A., Fleischer, E., Doyle, S. A., Arumugham, R., Collins, P. L., Hildreth, S. W. & Paradiso, P. R. (1993).**Expression of the G glycoprotein gene of human respiratory syncytial virus in Salmonella typhimurium. *J Gen Virol* **74**, 453-458.

109. **Matheny, C., Day, M. L. & Milbrandt, J. (1994).**The nuclear localization signal of NGFI-A is located within the zinc finger DNA binding domain. *J Biol Chem* **269**, 8176-8181.

110. **McNeilly, F., McNair, I., Mackie, D. P., Meehan, B. M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S. & Allan, G. M. (2001).**Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. *Arch Virol* **146**, 909-922.

111. **Meehan, B. M., McNeilly, F., Todd, D. & other authors (1998).**Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* **79**, 2171-2179.

112. **Meerts, P., Misinzo, G., McNeilly, F. & Nauwynck, H. J. (2005).**Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages. *Arch Virol* **150**, 427-441.

113. **Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H. M. & Nauwynck, H. J. (2005).**Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. *J Gen Virol* **86**, 2057-2068.

114. **Moroianu, J., Hijikata, M., Blobel, G. & Radu, A. (1995).**Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc Natl Acad Sci U S A* **92**, 6532-6536.

115. **Murby, M., Samuelsson, E., Nguyen, T. N., Mignard, L., Power, U., Binz, H., Uhlen, M. & Stahl, S. (1995).**Hydrophobicity engineering to increase solubility and stability of a recombinant protein from respiratory syncytial virus. *Eur J Biochem* **230**, 38-44.

116. **Mushahwar, I. K., Erker, J. C., Muerhoff, A. S., Leary, T. P., Simons, J. N., Birkenmeyer, L. G., Chalmers, M. L., Pilot-Matias, T. J. & Dexai, S. M. (1999).**Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. *Proc Natl Acad Sci U S A* **96**, 3177-3182.

117. **Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D. & Paul, P. S. (2000a).**Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. *J Gen Virol* **81**, 2281-2287.

118. **Nawagitgul, P., Morozov, I., Sirinarumitr, T., Sorden, S. D. & Paul, P. S. (2000b).**Development of probes to differentiate porcine circovirus types 1 and 2 in vitro by in situ hybridization. *Vet Microbiol* **75**, 83-89.

119. **Niagro, F. D., Forsthoefel, A. N., Lawther, R. P., Kamalanathan, L., Ritchie, B. W., Latimer, K. S. & Lukert, P. D. (1998).**Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. *Arch Virol* **143**, 1723-1744.

120. **Nielsen, J., Vincent, I. E., Botner, A., Ladekaer-Mikkelsen, A. S., Allan, G., Summerfield, A. & McCullough, K. C. (2003).**Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). *Vet Immunol Immunopathol* **92**, 97-111.

121. **Nielsen, R. (1997).**Correlation between transepithelial Na+ transport and transepithelial water movement across isolated frog skin (Rana esculenta). *J Membr Biol* **159**, 61-69.

122. **Nielsen, R. & Yang, Z. (1998).**Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. *Genetics* **148**, 929-936.

123. **Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y. & Mayumi, M. (1997).**A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* **241**, 92-97.

124. **Noteborn, M. H., Kranenburg, O., Zantema, A., Koch, G., de Boer, G. F. & van der Eb, A. J. (1992).**Transcription of the chicken anemia virus (CAV) genome and synthesis of its 52-kDa protein. *Gene* **118**, 267-271.

125. **Noteborn, M. H., Todd, D., Verschueren, C. A. & other authors (1994).**A single chicken anemia virus protein induces apoptosis. *J Virol* **68**, 346-351.

126. **Noteborn, M. H., Verschueren, C. A., Koch, G. & van der Eb, A. J. (1998).**Simultaneous expression of recombinant baculovirus-encoded chicken anaemia virus (CAV) proteins VP1 and VP2 is required for formation of the CAV-specific neutralizing epitope. *J Gen Virol* **79**, 3073-3077.

127. **Okamoto, H., Kato, N., Iizuka, H., Tsuda, F., Miyakawa, Y. & Mayumi, M. (1999).**Distinct genotypes of a nonenveloped DNA virus associated with posttransfusion non-A to G hepatitis (TT virus) in plasma and peripheral blood mononuclear cells. *J Med Virol* **57**, 252-258.

128. **Okamoto, H., Nishizawa, T., Tawara, A., Peng, Y., Takahashi, M., Kishimoto, J., Tanaka, T., Miyakawa, Y. & Mayumi, M. (2000).**Species-specific TT viruses in humans and nonhuman primates and their phylogenetic relatedness. *Virology* **277**, 368-378.

129. **Padidam, M., Beachy, R. N. & Fauquet, C. M. (1999a).**A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. *J Virol* **73**, 1609-1616.

130. **Padidam, M., Sawyer, S. & Fauquet, C. M. (1999b).**Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**, 218-225.**Palanichelvam, K., Kunik, T., Citovsky, V. & Gafni, Y. (1998).**The capsid protein of tomato yellow leaf curl virus binds cooperatively to single-stranded DNA. *J Gen Virol* **79**, 2829-2833.

131. **Palmer, K. E. & Rybicki, E. P. (1998).**The molecular biology of mastreviruses. *Adv Virus Res* **50:183-234**, 183-234.

132. **Pass, D. A. & Perry, R. A. (1984).**The pathology of psittacine beak and feather disease. *Aus Vet J* **61**, 69-74.

133. **Phenix, K. V., Meehan, B. M., Todd, D. & McNulty, M. S. (1994).**Transcriptional analysis and genome expression of chicken anaemia virus. *J Gen Virol* **75**, 905-909.

134. **Phenix, K. V., Weston, J. H., Ypelaar, I., Lavazza, A., Smyth, J. A., Todd, D., Wilcox, G. E. & Raidal, S. R. (2001).**Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus Circovirus of the family Circoviridae. *J Gen Virol* **82**, 2805-2809.

135. **Polson, A., von Wechmar, M. B. & van Regenmortel, M. H. V. (1980).**Isolation of vial IgY antibodies from yolks of immunized hens. *Immunol Comm* **9**, 475-493.

136. **Posada, D. & Crandall, K. A. (2002).**The effect of recombination on the accuracy of phylogeny estimation. *J Mol Evol* **54**, 396-402.

137. **Posada, D., Crandall, K. A. & Holmes, E. C. (2002).**Recombination in evolutionary genomics. *Annu Rev Genet* **36:75-97. Epub;%2002 Jun 11.**, 75-97.

138. **Qin, S., Ward, B. M. & Lazarowitz, S. G. (1998).**The bipartite geminivirus coat protein aids BR1 function in viral movement by affecting the accumulation of viral single-stranded DNA [In Process Citation]. *J Virol* **72**, 9247-9256.

139. **Raidal, S. R., Firth, G. A. & Cross, G. M. (1993).**Vaccination and challenge studies with psittacine beak and feather disease virus. *Aust Vet J* **70**, 437-441.

140. **Raidal, S. R. & Ridddoch, P. A. (1997).**A feather disease in Senagal doves (Streptopilia senegalensis) morphology similar to psittacine beak and feather disease. *Avian Pathol* **26**, 829-836.

141. **Ramis, A., Latimer, K. S., Niagro, F. D., Campagnoli, R. P., Ritchie, B. W. & Pesti, D. A. (1994).**Diagnosis of psittacine beak and feather disease (PBFD) viral

infection, avian polyomavirus infection, adenovirus infection and herpesvirus infection in psittacine tissues using DNA in situ hybridization. *Avian Path* **23**, 643-657.

142. **Raue, R., Johne, R., Crosta, L., Burkle, M., Gerlach, H. & Muller, H. (2004).**Nucleotide sequence analysis of a C1 gene fragment of psittacine beak and feather disease virus amplified by real-time polymerase chain reaction indicates a possible existence of genotypes. *Avian Pathol* **33**, 41-50.

143. **Ritchie, B. W. & Carter, K. (1995).***Avian viruses: function and control.* Blackwell Publishing.

144. **Ritchie, B. W., Niagro, F. D., Latimer, K. S., Lukert, P. D., Steffens, W. L., Rakich, P. M. & Pritchard, N. (1990).**Ultrastructural, protein composition, and antigenic comparison of psittacine beak and feather disease virus purified from four genera of psittacine birds. *J Wildl Dis* **26**, 196-203.

145. **Ritchie, B. W., Niagro, F. D., Latimer, K. S., Steffens, W. L., Pesti, D. A., Campagnoli, R. P. & Lukert, P. D. (1992).**Antibody response to and maternal immunity from an experimental psittacine beak and feather disease vaccine. *Am J Vet Res* **53**, 1512-1518.

146. **Ritchie, B. W., Niagro, F. D., Latimer, K. S., Steffens, W. L., Pesti, D. A. & Lukert, P. D. (1991).**Hemagglutination bu psittacine beak and fetaher disease virus and the use of hemagglutination inhibition for the detection of antibodies against the virus. *Am J Vet Res* **52**, 1810-1815.

147. **Ritchie, B. W., Niagro, F. D., Lukert, P. D., Steffens, W. L., III & Latimer, K. S. (1989).**Characterization of a new virus from cockatoos with psittacine beak and feather disease. *Virology* **171**, 83-88.

148. **Ritchie, P. A., Anderson, I. L. & Lambert, D. M. (2003).**Evidence for specificity of psittacine beak and feather disease viruses among avian hosts. *Virology* **306**, 109-115.

149. **Rogan, D. & Babiuk, L. A. (2005).**Novel vaccines from biotechnology. *Rev Sci Tech* **24**, 159-174.

150. **Rowley, I. & Coller, N. J. (1997).**Class AVES,Order PSITTACIFORMES. In *Handbook of the birds of the world*, pp. 246-298. Edited by del Hoyo, J., Jordi, A. & Sargatal, J.: Lynx Edicions.

151. **Rozas, J. & Rozas, R. (1999).**DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174-175.

152. **Rybicki, E. P. (1994).**A phylogenetic and evolutionary justification for three genera of *Geminiviridae*. *Arch Virol* **139**, 49-77.

153. **Rybicki, E.P. and von Wechmar, M.B. (1981).** The serology of the bromoviruses. I. Serological interrelationships of the bromoviruses. *Virology* **109**, 391-402.

154. **Salminen, M. O., Carr, J. K., Burke, D. S. & McCutchan, F. E. (1995).**Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Res Hum Retroviruses* **11**, 1423-1425.

155. **Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).***Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

156. **Sanada, N. & Sanada, Y. (2000).**The sensitivities of various erythrocytes in a haemagglutination assay for the detection of psittacine beak and feather disease virus. *J Vet Med B Infect Dis Vet Public Health* **47**, 441-443.

157. **Sanchez, R. E., Meerts, P., Nauwynck, H. J., Ellis, J. A. & Pensaert, M. B. (2004).**Characteristics of porcine circovirus-2 replication in lymphoid organs of pigs inoculated in late gestation or postnatally and possible relation to clinical and pathological outcome of infection. *J Vet Diagn Invest* **16**, 175-185.

158. **Sanchez, R. E., Meerts, P., Nauwynck, H. J. & Pensaert, M. B. (2003).**Change of porcine circovirus 2 target cells in pigs during development from fetal to early postnatal life. *Vet Microbiol* **95**, 15-25.

159. **Sanchez, R. E., Nauwynck, H. J., McNeilly, F., Allan, G. M. & Pensaert, M. B. (2001).**Porcine circovirus 2 infection in swine foetuses inoculated at different stages of gestation. *Vet Microbiol* **83**, 169-176.

160. **Saunders, K., Lucy, A. & Stanley, J. (1991).**DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. *Nucleic Acids Res* **19**, 2325-2330.

161. **Saville, R. D., Constantine, N. T., Cleghorn, F. R., Jack, N., Bartholomew, C., Edwards, J., Gomez, P. & Blattner, W. A. (2001).**Fourth-generation enzyme-linked immunosorbent assay for the simultaneous detection of human immunodeficiency virus antigen and antibody. *J Clin Microbiol* **39**, 2518-2524.

162. **Schade, R., Staak, C., Hendriksen, C. & other authors (1996).**The production of avian (egg yolk) antibodies: IgY. *ATLA* **24**, 925-934.

163. **Schoemaker, N. J., Dorrestein, G. M., Latimer, K. S., Lumeij, J. T., Kik, M. J., van der Hage, M. H. & Campagnoli, R. P. (2000).**Severe leukopenia and liver necrosis in young African grey parrots (Psittacus erithacus erithacus) infected with psittacine circovirus. *Avian Dis* **44**, 470-478.

164. **Seisenberger, G., Ried, M. U., Endress, T., Buning, H., Hallek, M. & Brauchle, C. (2001).**Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* **294**, 1929-1932.

165. **Sexton, N., Penhale, W. J., Plant, S. L. & Pass, D. A. (1994).**Use of goose red blood cells for dectection of infections with psittacine beak and feather disease virus by haeagglutination and haemagglutination inhibition. *Aus Vet J* **71**, 345-347.

166. **Shivaprasad, H. L., Hill, D., Todd, D. & Smyth, J. A. (2004).**Circovirus infection in a Gouldian finch (Chloebia gouldiae). *Avian Pathol* **33**, 525-529.

167. **Smith, J. M. (1992).**Analyzing the mosaic structure of genes. *J Mol Evol* **34**, 126-129.

168. **Smyth, J. A., Weston, J., Moffett, D. A. & Todd, D. (2001).**Detection of circovirus infection in pigeons by in situ hybridization using cloned DNA probes. *J Vet Diagn Invest* **13**, 475-482.

169. **Steinfeldt, T., Finsterbusch, T. & Mankertz, A. (2001).**Rep and Rep' protein of porcine circovirus type 1 bind to the origin of replication in vitro. *Virology* **291**, 152-160.

170. **Stenger, D. C., Revington, G. N., Stevenson, M. C. & Bisaro, D. M. (1991).**Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proc Natl Acad Sci U S A* **88**, 8029-8033.

171. **Stewart, M. E., Perry, R. & Raidal, S. R. (2006).**Identification of a novel circovirus in Autralian ravens (Corvus coronoides) with feather disease. *Avian Pathol* **35**, 86-92.

172. **Swofford, D. L. (2001).**PAUP:Phylogeneticanalysis using parsimon and other methods: version 4.0b8. *Sinauer Associates, Sunderland USA.*

173. **Takahashi, K., Hijikata, M., Samokhvalov, E. I. & Mishiro, S. (2000).**Full or near full length nucleotide sequences of TT virus variants (Types SANBAN and YONBAN) and the TT virus-like mini virus. *Intervirology* **43**, 119-123.

174. **Tavassoli, M., Guelen, L., Luxon, B. A. & Gaken, J. (2005).**Apoptin: Specific killer of tumor cells? *Apoptosis* **10**, 717-724.

175. **Theron, J. & Nel, L. H. (1997).**Stable protein-RNA interaction involves the terminal domains of bluetongue virus mRNA, but not the terminally conserved sequences. *Virology* **229**, 134-142.

176. **Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).**The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876-4882.

177. **Tischer, I., Gelderblom, H., Vettermann, W. & Koch, M. A. (1982).**A very small porcine virus with circular single-stranded DNA. *Nature* **295**, 64-66.

178. **Tischer, I., Peters, D., Rasch, R. & Pociuli, S. (1987).**Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. *Arch Virol* **96**, 39-57.

179. **Todd, D. (2000).**Circoviruses: immunosuppressive threats to avian species: a review. *Avian Path* **29**, 373-394.

180. **Todd, D. (2004).**Avian circovirus diseases: lessons for the study of PMWS. *Vet Microbiol* **98**, 169-174.

181. **Todd, D., Conner, T. J., Calvert, V. M., Creelan, J. L., Meehan, B. & McNulty, M. S. (1995).**Molecular cloning of an attenuated chicken anaemia isolate following repeat cell culture passage. *Avian Pathol* **24**, 171-187.

182. **Todd, D., Conner, T. J., Creelan, J. L., Borghmans, B. J., Calvert, V. M. & McNulty, M. S. (1998).**Effects of multiple cell culture passages on the biological behaviour of chicken anaemia virus. *Avian Pathol* **27**, 74-79.

183. **Todd, D., Mawhinney, K. A., Graham, D. A. & Scott, A. N. (1999).**Development of a blocking enzyme-linked immunosorbent assay for the serological diagnosis of chicken anaemia virus. *J Virol Methods* **82**, 177-184.

184. **Todd, D., McNulty, M. S., Adair, B. M. & Allan, G. M. (2001a).**Animal circoviruses. *Adv Virus Res* **57:1-70.**, 1-70.

185. **Todd, D., McNulty, M. S., Mankertz, A., Lukert, P. D., Randles, J. & Dale, J. L. (2000).**Virus Taxonomy: Seventh report of the International Committee on Taxonomy of Viruses. pp. 299-302. Edited by Fauquet, C., Bishop, D. H., Carsten, E. B. & other authors: Academic Press.

186. **Todd, D., Weston, J. H., Soike, D. & Smyth, J. A. (2001b).**Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. *Virology* **286**, 354-362.

187. **Truong, C., Mahe, D., Blanchard, P., Le, D. M., Madec, F., Jestin, A. & Albina, E. (2001).**Identification of an immunorelevant ORF2 epitope from porcine circovirus type 2 as a serological marker for experimental and natural infection. *Arch Virol* **146**, 1197-1211.

188. **Twentyman, C. M., Alley, M. R., Meers, J., Cooke, M. M. & Duignan, P. J. (1999).**Circovirus-like infection in a southern black-back gull (*Larus dominicanus*). *Avian Pathol* **28**, 513-516.

189. **Varsani, A., Williamson, A. L., de Villiers, V. D., Becker, I., Christensen, N. D. & Rybicki, E. P. (2003).**Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J Virol* 77, 8386-8393.

190. **Vincent, I. E., Carrasco, C. P., Herrmann, B., Meehan, B. M., Allan, G. M., Summerfield, A. & McCullough, K. C. (2003).**Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. *J Virol* 77, 13288-13300.

191. **Walker, I. W., Konoby, C. A., Jewhurst, V. A., McNair, I., McNeilly, F., Meehan, B. M., Cottrell, T. S., Ellis, J. A. & Allan, G. M. (2000).**Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. *J Vet Diagn Invest* **12**, 400-405.

192. **Warburton, L. & Perring, T. M. (2002).**BBFDV bei frei lebenden Rußköpfchen in Sambia. *Papagein* **5**, 166-169.

193. **Whittaker, G. R. & Helenius, A. (1998).**Nuclear import and export of viruses and virus genomes. *Virology* **246**, 1-23.

194. **Wiens, J. J. (1998).**Combining data sets with different phylogenetic histories. *Syst Biol* **47**, 568-581.

195. **Woods, L. W. & Shivaprasad, H. L. (1997).***Diseases of Poultry*. Iowa State University Press.

196. **Worobey, M. (2000).**Extensive homologous recombination among widely divergent TT viruses. *J Virol* **74**, 7666-7670.

197. **Wu, X., Cao, C., Xu, Y. & Lu, X. (2004).**Construction of a host range-expanded hybrid baculovirus of BmNPV and AcNPV, and knockout of cysteinase gene for more efficient expression. *Sci China C Life Sci* **47**, 406-415.

198. **Wu, Y. & Carstens, E. B. (1998).**A baculovirus single-stranded DNA binding protein, LEF-3, mediates the nuclear localization of the putative helicase P143. *Virology* **247**, 32-40.

199. **Yamada, M. & Kasamatsu, H. (1993).**Role of nuclear pore complex in simian virus 40 nuclear targeting. *J Virol* **67**, 119-130.

200. **Yang, Z., Nielsen, R., Goldman, N. & Pedersen, A. M. (2000).**Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* **155**, 431-449.

201. **Ypelaar, I., Bassami, M. R., Wilcox, G. E. & Raidal, S. R. (1999).**A universal polymerase chain reaction for the detection of psittacine beak and feather disease virus. *Vet Microbiol* **68**, 141-148.

202. **Zhou, J. Y., Shang, S. B., Gong, H., Chen, Q. X., Wu, J. X., Shen, H. G., Chen, T. F. & Guo, J. Q. (2005).**In vitro expression, monoclonal antibody and bioactivity for capsid protein of porcine circovirus type II without nuclear localization signal. *J Biotechnol* **118**, 201-211.