

Molecular epidemiology of African swine fever in East Africa

By

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Summary

African swine fever (ASF), a hemorrhagic disease of domestic pigs, is caused by a large enveloped icosahedral arbovirus of the *Asfivirus* genus in the family *Asfarviridae*. It has a linear, covalently close-ended, double stranded DNA (dsDNA) genome, 170 – 190 kbp in size. Warthogs (*Phaecochoerus aethiopicus*), bushpigs (*Potamochoerus porcus*) and soft ticks of the *Ornithodoros* genus are natural reservoirs of ASF virus (ASFV) in Africa. Serological differentiation between field strains is not possible and as no vaccine is available, control relies on stamping out and strict zoosanitary practices. In order to identify possible sources of virus and prevent further introductions, ASFVs need to be differentiated on genotype basis. Genotyping of East African ASFV isolates was achieved in this study by characterization of the C-terminal region of the major capsid protein gene, *p72*. Thirteen genotypes were identified among East African sylvatic and domestic hosts. Of significance was the recovery of an apparently geographically restricted homogenous domestic pig cycle in the region, as well as the presence of viruses of the ESACWA genotype, formerly believed to be confined to Europe, South America, the

Caribbean Islands and West Africa. Due to the conserved nature of the *p72* gene, intra-genotypic relationships of viruses recovered from a homogeneous domestic pig-associated lineage, genotype VIII, could not be resolved. In an attempt to address this short-coming, genetic characterization of the Central Variable Region (CVR) within the *9-RL* open reading frame (ORF) of the central conserved region of ASFV genome was conducted. Eight groups were resolved, some of which confirmed prolonged periods of circulation of a particular virus type, whilst others confirmed transboundary transmission of the disease. The complexity of ASF epidemiology in East Africa exemplified by the presence of a sylvatic and domestic pig cycle, multiple genotypes within countries and identical genotypes shared by multiple countries, was revealed. The East African ASFV molecular epidemiological database created in this study will be of great assistance to veterinary officials implementing prevention and outbreak control strategies.

Scientific publications emanating directly from this dissertation

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LIST OF ABBREVIATIONS

aa	Amino Acid
ASF	African swine Fever
ASFV	African Swine Fever Virus
BP	Base Pair
CI	Consistency Index
CO ₂	Carbon Dioxide
ConA	Concanavalin A
CSF	Classical Swine Fever
CVR	Central Variable Region
DR	Direct Repeat
DR2	Dominican Republic
DRC	Democratic Republic of Congo
DsDNA	Double Stranded DNA
E	East
EDD	Exotic Diseases Division
ELISA	Enzyme Linked Immunosorbent Assay
ESACWA	Europe South America Caribbean and West Africa
FAO	Food and Agriculture Organization
FITC	Fluorescein Isothiocyanate
G	Gamma Shape Distribution Parameter
HI	Homoplasy Index
HPL	Hairpin Loop
HTLV	Human T Cell Leukemia Virus
I	Invariable Sites
IAH	Institute for Animal Health
IFA	Indirect Fluorescent Antibody
INF	Interferon
IP	Infected Premises
LTR	Long Terminal Repeat
MDV	Mareks Disease Virus

ME	Minimum Evolution
MGF	Multigene Family
ML	Maximum Likelihood
MP	Maximum Parsimony
NA	Not Applicable
ND	Not Done
NJ	Neighbor-Joining
NK	Not Known
OIE	Office International des Epizootis
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
OVI	Onderstepoort Veterinary Institute
PBMC	Pig Bone Marrow Culture
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemeagglutinin
RC	Rescaled Consistency Index
RFLP	Restriction Fragment Length Polymorphism
RI	Retention Index
RPM	Rotations Per Minute
SLA	Swine Lymphocyte Antigen
STR	Short Terminal Repeat
SW	South West
TBR	Tree Bisection Reconnection
TIR	Terminal Inverted Repetition
UGA	Uganda
VP	Virus Protein
VR	Variable Region
VSV	Vesicular Stomatitis Virus
WRL	World Reference Laboratory
ZK	Zambian Kwacha

Chapter 1

Literature review

1.1 INTRODUCTION

African swine fever (ASF) is a viral disease of domestic pigs responsible for a wide variety of clinical symptoms. In the typical and acute form, the lymphoreticular endothelial cells are affected resulting in widespread hemorrhages. Morbidity and mortality classically approach 100 %. In sub-acute cases pigs lose condition and die of pneumonia. Chronically, survivors are characterized by emaciation, stunted growth, hemorrhagic necrosis of the skin overlying bony protuberances, followed by abscessation and deep ulceration (Plowright *et al.*, 1994).

1.2 Etiological agent

The etiological agent, African swine fever virus (ASFV), presently constitutes the only member of the genus *Asfivirus* within the family *Asfarviridae* (Dixon *et al.*, 2000). The mature virion consists of a nucleoprotein core structure, 70 – 100 nm in diameter, surrounded by an icosahedral capsid, 172 – 191 nm in diameter, and a lipid containing envelope (Dixon *et al.*, 2000).

1.2.1 Nucleic acid

ASFV genome is a linear, covalently close-ended, double-stranded DNA (dsDNA) molecule, 170 – 190 kbp in size (Blasco *et al.*, 1989). The genome has a central conserved region approximately 125 kb long, and highly variable left and right end sequences, characterized by inverted complementary tandem repeats, of about 35 kb and

15 kb respectively (Wesley and Tuthill, 1984; Gonzalez *et al.*, 1986; Blasco *et al.*, 1989 a, b). Genes are encoded on both DNA strands and are generally closely spaced. Short tandem repeat arrays are observed at several intergenic locations (Fig.1.1). This nucleic acid molecule can encode 150 to 200 proteins (Salas, 1999). The complete nucleotide analysis of the Spanish isolate BA71V, whose genome structure is depicted in Fig.1.1, has a genome 170,101 nucleotides in length and contains a G-C content of 38.95 % (Yañez *et al.*, 1995).

1.2.2 Proteins

Sequence analysis of the entire genome of the Spanish isolate, BA71V (Yañez *et al.*, 1995) revealed that this isolate contains 151 major open reading frames (ORF). There are many genes that code for proteins that are required for virus replication in cells, and these proteins are suspected to be at least 90 in quantity (Salas, 1999). More than 100 virus-induced proteins have been detected in infected Vero cells (Salas, 1999). The complex virus particle is formed by proteins in excess of 50, including those with structural functions and enzymes packaged for use early in infection. Genes encoding 15 of the structural proteins have been characterized, with two of these encoding polyproteins that are processed into four and two virion proteins each (Simon-Mateo *et al.*, 1993,1997; Andres *et al.*, 2001).

Approximately 54 structural proteins of molecular weights ranging from 10 000 to 150 000 have been resolved by two-dimensional gel electrophoresis analysis (Salas, 1999). Virus attachment proteins p12 and p24 have been identified in the external region of the extra-cellular virions, and proteins p150, p37, p34 and p14, products of polyprotein pp220, are located in the core shell (Salas, 1999). The capsid protein, p72, is located on the surface of unenveloped intracellular virions.

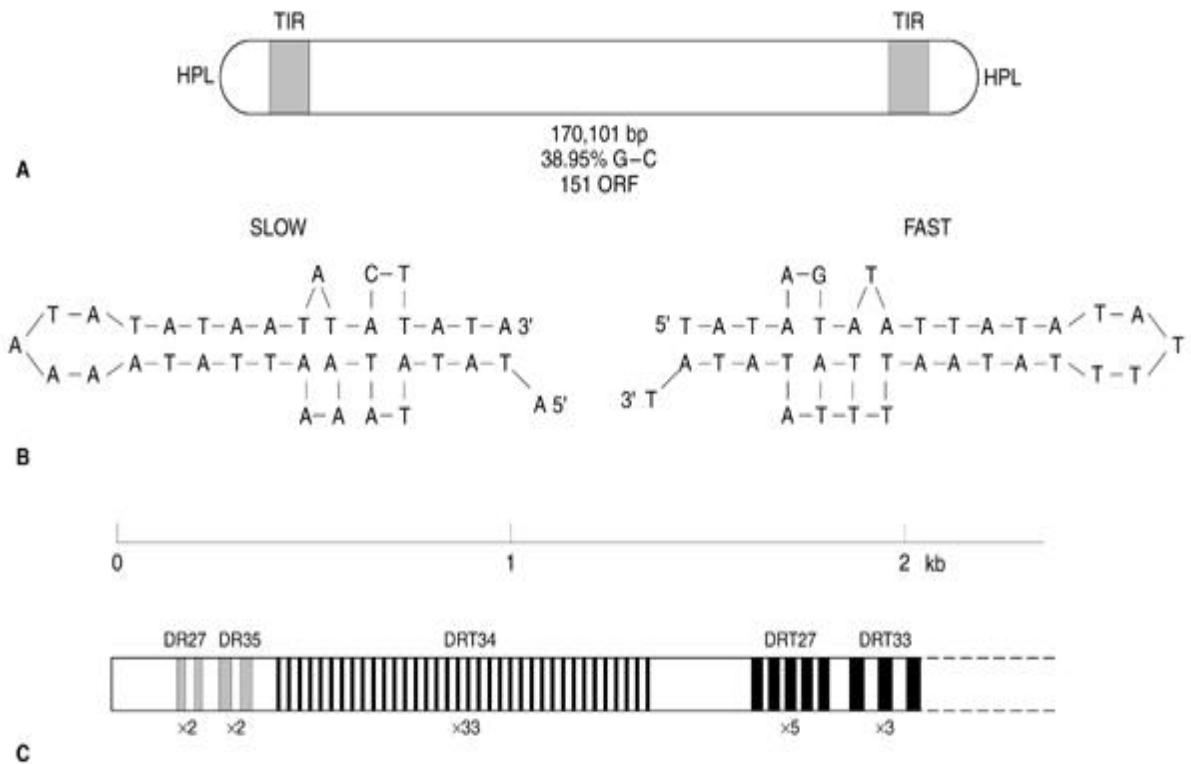


Fig 1.1 Structure of the ASFV genome. (A) ASFV DNA (BA71V strain). HPL, hairpin loop; TIR, terminal inverted repetition. (B) Predicted secondary structure of the 37 nucleotide-long fast and slow components in the HPLs. (C) Structure of the TIR. The TIR is 2134 nucleotides long and consists of unique sequences (in white) interspersed with two types of direct repeats (DR) and three sets of direct repeats in tandem (DRT). Copyright © 1999 Academic Press (Salas, 1999).

A DNA dependent RNA polymerase and other proteins involved in activities such as polyadenylation, methylation and capping of RNA are contained in the ASFV particles (Salas, 1999; Dixon *et al.*, 2000). A protein kinase, two nucleotide triphosphate phosphohydrolases, an acid phosphatase and a deoxyribonuclease active on single-stranded DNA, are present in the virions (Salas, 1999; Dixon *et al.*, 2004). A further four enzymes are encoded by the genome, and these may play roles in regulating virus replication or modulating host cell function (Salas, 1999).

A large percentage of the genome encodes multigene families (MGF), which occur at the left and right termini of the genome, and render different ASFV isolates highly variable genetically (Almendral *et al.*, 1990; De La Vega *et al.*, 1990; González, *et al.*, 1990; Rodriguez *et al.*, 1994; Yozawa *et al.*, 1994; Zsak *et al.*, 2001). Five MGF have been identified thus far. Proteins encoded by MGF 530 and 360 have been proven to specify macrophage host ranges and promote survival of infected cells (Tulman & Rock, 2001; Zsak *et al.*, 2001). These proteins also interfere with host interferon response (Afonso *et al.*, 2004). In the tick *Ornithodoros porcinus porcinus*, MGF360 genes are significant tick host range determinants and they are required for efficient virus replication and generalization of infection (Burrage *et al.*, 2004).

The early expressed 92 – 156 amino acid (aa) long UK protein with four to ten tandem repeats, determines the virulence of the strain (Zsak *et al.*, 1996,1998). Also thought to be associated with virulence is the NL gene, which is located next to the UK gene in the right variable region of the genome (Zsak *et al.*, 1996,1998; He *et al.*, 1998).

The remaining genes encode proteins that play an important role in virus survival and transmission between hosts (Dixon *et al.*, 1994; Yanez *et al.*, 1995; Tulman and Rock, 2001). Of these, a few have been identified as role players in the evasion of the host defense mechanism. Viral protein A238L (5EL) inhibits host NF_κB and calcineurin (Powell *et al.*, 1996; Miskin *et al.*, 1998; Revilla *et al.*, 1998). The transcriptional activation in infected macrophages of genes dependent on NF_κB is inhibited as a result, and so is the activation of the nuclear factor of activated T cells (NFAT) transcription factor family regulated by calcineurin. Virus protein 5EL evades and modulates or interferes with the host immune response, including suppression of immune responses *in vitro* (Powell *et al.*, 1996; Miskin *et al.*, 1998). The 8DR protein, also called CD2v, is a virus adhesion protein involved in virus spread and impairment of lymphocyte function (Borca *et al.*, 1998; Tulman and Rock, 2001; Dixon *et al.*, 2004). Two anti-apoptosis proteins are encoded by the ASFV genome, and these ensure progeny virus production in infected cells (Afonso *et al.*, 1996; Revilla *et al.*, 1997; Nogal *et al.*, 2001). Host protein

synthesis shut-off is inhibited by ASF114L protein that shares some sequence similarity with the Herpes simplex virus-encoded neurovirulence factor ICP34.5 (He *et al.*, 1997, 1998; Goatley *et al.*, 1999). Protein 4jR and the ASFV ubiquitin conjugating (UBCv) enzyme are thought to modulate host gene transcription (Bulimo *et al.*, 2000; Goatley *et al.*, 2002).

However, the purpose of approximately 50 % of the genes that are found in the ASFV genome remains unknown (Tulman and Rock, 2001). The quest for factors and mechanisms by which the virus interacts with its hosts, especially those determining virulence and host range, nevertheless continues.

1.2.3 Lipids and carbohydrates

The envelope contains lipids, including the glycolipids, and carbohydrates have only been identified in the latter molecules (Dixon *et al.*, 2000).

1.2.4 Replication

Entry into the host cell is by receptor-mediated endocytosis (Alcami *et al.*, 1989; Bernades *et al.*, 1995). Virus proteins p12, p30, p54 and p72 play important roles in the binding and entry processes (Borca *et al.*, 1994, Gomez-Puertas *et al.*, 1996, 1998). A mechanism involving fusion of the virus envelope and that of the endocytic pits is responsible for virus release into the cytoplasm (Valdeira *et al.*, 1998). DNA first replicates in the nucleus of infected cells and continues in the cytoplasm at a later stage (Garcia-Beato *et al.*, 1992; Rojo *et al.*, 1999). Pulse chase experiments have demonstrated that these DNA fragments are precursors of the mature cross-linked viral DNA (Rojo *et al.*, 1999). The synthesis of mRNA and its processing ensues in the cell cytoplasm, a process mediated by enzymes contained in the viral core (Dixon *et al.*, 2004). The transcript is polyadenylated at the 3' end while the 5' terminal is capped, and encodes enzymes involved in mRNA transcription and DNA replication (Dixon *et al.*, 1994, 2000). Peak DNA replication is achieved 8 hours post infection, a period within

which the head to head concatameric forms which are presumably the replicative intermediate forms are found in the cells. Self-priming mechanisms may mediate the proceeding DNA replication process. Late genes are expressed after the onset of DNA replication and synthesis of some early genes, continues throughout the infection. Post translational modification of some virus proteins in the form of proteolytic cleavage, phosphorylation, glycosylation and myristylation, takes place (Yáñez *et al.*, 1995). Productive infection requires the cell nucleus, while virus morphogenesis occurs in the fibrin and membrane rich perinuclear area, often surrounded by Golgi apparatus and numerous ribosomes (Garcia-Beato *et al.*, 1992; Rojo *et al.*, 1999). Virus is released by cell destruction or budding through the plasma membrane (Dematos and Carvalho, 1993).

1.3 Physico-chemical properties of ASFV

African swine fever virions have a buoyant density of 1.095 g/cm³ in Percoll and 1.19 – 1.24 g/cm³ in CsCl. S_{20W} is approximately 3 500. The virions are sensitive to irradiation, chemicals like ether, chloroform and deoxycholate, and infectivity is destroyed by disinfectants such as 1 % formaldehyde in 6 days, 2 % NaOH in 1 day and paraphenylphenolic substances which are very effective (Dixon *et al.*, 2000). Heat inactivation is possible at 60⁰C within 30 minutes, but the virus may survive a number of years at 2⁰C or 4⁰C. ASF viruses have been shown to be resilient to desiccation and putrefaction, and some survive at pH 4 or 13 and high relative humidity (Plowright *et al.*, 1967; Donaldson and Ferris, 1976; Dixon *et al.*, 2000).

1.4 Pathogenesis

Following oral or respiratory infections of domestic pigs with ASFV, the virus penetrates the tonsils or the dorsal pharyngeal mucosa, proceeds to the mandibular or retropharyngeal lymphnodes, and disseminates through viraemia (Plowright *et al.*, 1994). The virus has a predilection for antigen presenting phagocytic cells of the

lymphoreticular system, where it causes cytolysis and hyperplastic changes in acute and subacute cases, respectively (Boulanger *et al.*, 1966). It is also associated with 90 % of the erythrocytes, and lymphopenia is observed (Plowright *et al.*, 1968). Hemorrhage, oedema and effusions are preceded by thrombocytopaenia, impaired vascular integrity, coagulopathy and dysfibrinogenaemia (Moulton *et al.*, 1975; Edwards and Dodds, 1985; Anderson *et al.*, 1987). Deposition of immune complexes in kidneys, lungs and skin is observed in chronically infected pigs (Slauson and Sanchez Vizcaino, 1981). Wildlife vertebrate reservoirs display a more subtle viral course and the infection remains subclinical (Anderson *et al.*, 1998; Oura *et al.*, 1998). In *Ornithodoros* ticks, initial ASFV replication occurs in phagocytic digestive cells of the midgut epithelium, followed by undifferentiated midgut cells (Kleiboeker *et al.*, 1998, 1999). Replication in the midgut epithelium is essential for a generalised and persistent infection following a *per os* infection (Kleiboeker *et al.*, 1999). Dissemination of virus infection from midgut to other tick tissues occurs through the basal lamina of the midgut into the hemocoel. Secondary sites of virus replication include hemocytes (type I and II), connective tissue, coxal gland, salivary gland, and reproductive tissue. Significant mortalities have been observed in ticks that fed on infected blood (Endris *et al.*, 1992 a, b; Rennie *et al.*, 2000), while low mortalities until after the first oviposition have also been documented (Kleiboeker and Scoles, 2001).

1.5 Pig immune response to ASFV infection

1.5.1 ASFV interactions with porcine macrophages

The most crucial event of ASFV infection is the infection and lysis of the cells of the mononuclear – phagocytic system independent of isolate virulence (Wadley and Wilkinson, 1977; Martins *et al.*, 1988). Macrophage phagocytic and chemotactic functions are substantially decreased as a result (Martins *et al.*, 1988). Splenic swine lymphocyte antigen (SLA) expression is differentially modulated even though Gonzalez-Juarrero *et al.* (1992) could not find a clear-cut relationship between modulation of SLA

class I and class II by isolates of different virulence. Prostaglandin PGE₂, known for its immunosuppressive effect, was shown to be released from alveolar macrophages, quantities of which seemingly correlated positively with the virulence of the virus (Anderson *et al.*, 1987). Some isolates were proven to cause the depression of lectin driven lymphocyte proliferation and IgG secretion, in an experiment utilizing crude supernatants of pig bone marrow cultures (PBMC) infected with the attenuated ASFV (Spain E77) and a multiple passaged Ugandan isolate (Wadley, 1982). It was however, proven that lectin driven lymphocyte proliferation and IgG secretion depression was not virulence dependent but caused by soluble inhibitors of 40 to 80 kDa (Gonzalez *et al.*, 1990). Clearly, basic macrophage function related to non-specific protective mechanisms and antigen presenting capacities are seriously damaged and when coupled with the production of immune mediators, the course of the host immune response during ASFV infection is strongly influenced.

1.5.2 Functional integrity of pig lymphocytes and ASFV infection

Many studies on the functional integrity of the swine immune system during infection with ASFV were based on measurement of peripheral blood leukocyte cultures, T and B lymphocyte quantification, and lymphoproliferative responses to mitogens and virus or viral antigens.

While some investigators noticed negligible differences in blood components of swine inoculated with different ASFV isolates (Knudsen *et al.*, 1987 b; Genovesi *et al.*, 1988), some observed lymphocytopenia early post infection. The latter was attributed to B-lymphocytes by Wadley and Wilkinson (1980) or to a decrease in T-lymphocytes by Sanchez-Vizcaino *et al.* (1981).

Conflicting results have been obtained from studies of lymphocyte function of ASFV infected pigs based on proliferation responses to mitogens, concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). Some authors recorded a correlation between lymphocytopenia and a decrease in lymphoproliferative response

(Wadley and Wilkinson, 1980; Sanchez-Vizcaino *et al.*, 1981), but others observed a strong decrease in lymphoproliferative response when one virus isolate was used, and a clear enhancement of response when the pigs were inoculated with different strains (Martins and Leitão, 1994).

In vitro studies of porcine lymphocyte response to the same mitogens as above also yielded contradicting results. Wadley (1982) observed a marked decrease in response to Con A and PHW after lymphocyte incubation with attenuated virus strains, and an enhanced response with virulent isolates. Knudsen *et al.* (1987 b) noticed an unimpaired lymphoproliferative response to PHA, Con A and PWM when inoculated with a wide dosage range of a moderately virulent strain. Gonzalez *et al.* (1990) have on the contrary, recorded strong inhibition of PHA responses by eleven virus isolates at low dosages independent of virulence.

Despite ASFV ability to modulate the host immune response, pigs surviving experimental inoculations often resist challenge with homologous strains irrespective of their virulence (Coggins *et al.*, 1968; DeBoer *et al.*, 1969; Hamdy and Dardiri, 1984).

1.5.3 Humoral immunity

The fact that ASFV infects, replicates in and destroys cells of the mononuclear phagocytic system irrespective of their virulence (Wardley and Wilkinson, 1977) has intrigued scientists for a number of years. Initially scientists could not demonstrate neutralizing antibodies against ASFV during infection (Wardley *et al.*, 1983), but subsequently found that there were some protective measures taking place in the host immune system. These were antibodies which reduced virus multiplication, delayed the onset of clinical signs, and allowed better survival rates (Wadley and Wilkinson, 1985; Wadley *et al.*, 1987).

In vitro studies have shown that anti ASFV antibodies reduce viral infectivity and viral yields from porcine macrophages (Malmquist, 1963; Knudsen *et al.*, 1987a) and mediate

lysis of infected fibroblast cells by neutrophils and by the classical pathway of complement activation (Norley and Wadley, 1982, 1983a). These observations assist in demonstrating the protective role of anti-ASFV antibodies in infected swine.

Neutralizing antibodies against viral proteins p72, p54, p30 and p12 have recently being confirmed (Zsak *et al.*, 1993; Gómez-Puertas *et al.*, 1996). However, Neilan *et al.* (2004) showed that neutralizing antibodies to these ASFV proteins are not sufficient for antibody-mediated protection. Previous studies have also indicated that even though there are some neutralizing antibody responses to ASFV infection, these tend to appear on days 10 – 12 and 12 – 14 post infection, a time when a pig would have already died from a virulent strain of ASFV (Wardley *et al.*, 1987).

1.5.4 Cellular response

Experimental models formed the basis of investigating potential cellular effector mechanisms involved in swine protection against African swine fever. Scientist found results which strongly suggested a strong correlation between activation of Natural killer (NK) cell activity and the virulence of the strain (Mendoza *et al.*, 1991; Martins and Leitao, 1994). These cells have not been shown to kill ASFV infected macrophages but the interferon (IFN) they produce may be significant since *in vitro* studies have shown inhibition of ASFV replication in monocyte/macrophage and cell lines by IFN- γ and IFN- α (Esparza *et al.*, 1988; Paez *et al.*, 1990).

Cellular responses to infection have yielded controversial results which led scientist to believe that the virulence of a strain may be the determining factor in that regard, even though cytotoxic T-lymphocytes could be playing a significant role in the initial stages of infection (Wardley *et al.*, 1987; Martins and Leitao, 1994). It is due to this poor understanding of the host – virus immune interaction that a vaccine against the disease is presently unavailable (Tulman and Rock, 2001).

1.6 Laboratory diagnosis of ASF

Field diagnosis of ASF should be confirmed in the laboratory since the displayed clinico-pathological signs are not pathognomonic for ASF. The recommended diagnostic techniques are focused on antigen, antibody and genomic DNA detection, and on virus isolation from clinical samples. Where possible, it is highly recommended that antigen detection tests be conducted simultaneously with antibody detection in serum or tissue fluids to avoid a delay in detecting infection by a virus of low virulence (<http://www.oie.int/animalhealthcode>).

1.6.1 Virus isolation

Malmquist and Hay (1960) described the hemadsorption and cytopathic effects of ASFV on swine bone marrow and leukocyte (buffy coat) cultures, properties distinguishing this virus from that causing Classical Swine Fever (CSF). Primary swine leukocyte and bone marrow cultures have since been used in the diagnosis of ASF. Attempts to grow the virus on continuous cell lines, especially on cells other than the reticulo-endothelial system resulted in the virus losing some of its characteristics (Malmquist, 1962). Recently, however, Weingartl *et al.* (2002) described a continuous cell line of swine alveolar macrophages on which ASFV was successfully grown and isolated without any adverse effects on the virus. The success of this cell line after validation trials would provide a cheaper and more convenient method of ASFV isolation.

1.6.2 Fluorescent Antibody Test (FAT)

A positive ASFV diagnosis can be made by virus/antigen detection in the acute stage or antibody demonstration in the latent or chronic stages of the disease. The former test could be preceded by virus growth on primary leukocyte cultures to concentrate the antigen more thus ensuring the success of the test (Botija, 1970). Impression smears or cryostat sections of tissues or leukocyte cultures of suspected ASFV infected animals are fixed on glass slides and stained with fluorescein isothiocyanate (FITC) conjugated anti-

ASFV immunoglobulins (Bool *et al.*, 1970). An ultraviolet microscope with barrier and exciter filters is used to view slides.

1.6.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a rapid, highly sensitive and specific technique that employs oligonucleotide primers in a series of thermally controlled cycles to amplify a highly conserved region of the ASFV genome (Steiger *et al.*, 1992). The test is useful where work on live viruses is undesirable or prohibited, samples have undergone putrefaction, or the virus has been chemically disinfected prior to testing. Through this technique, ASFV DNA can be detected and characterized without the need to isolate the virus (Steiger *et al.*, 1992; Agüero *et al.*, 2003; Bastos *et al.*, 2003).

1.6.4 Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA for the detection of antibodies against ASFV in convalescing swine sera using non-infectious soluble antigen was described (Hamdy and Dardiri, 1979). It combines the specificity of antibodies with the sensitivity of a simple photometric enzyme assay system by conjugating the antibodies with an enzyme. Results could be read visually or by spectrophotometer at 405 nm wavelength. The test was found to be extremely sensitive, and detected 5 ng of antibodies to soluble antigen.

1.6.5 Immunoblotting

Immunoblotting combines the resolution of gel electrophoresis (SDS-PAGE) with the specificity of immunochemical detection. The technique is especially useful in identifying a specific ASFV antigen in a complex mixture of antigens (Oviedo *et al.*, 1997; Barderas *et al.*, 2000). This diagnostic method is as sensitive as ELISA but has the following additional advantages:

1. Detergents do not affect adsorption of proteins on membranes
2. Fewer samples are required

3. Both antigenic and electrophoretic properties of the antigen are characterized simultaneously

4. Immobilized antigens of interest can be excised and used in inoculation of animals to raise monospecific antibodies

1.6.6 Indirect Fluorescent Antibody (IFA) test

ASFV infected cells are smeared and fixed on glass slides, test sera added onto the slide in various dilutions and a fluorescent antibody or protein A conjugate added finally to detect the reaction under an ultraviolet light microscope (Bool *et al.*, 1970). The technique is advocated for use as a confirmatory test in sera from ASF free zones for which ELISA has yielded positive results (<http://www.oie.int>).

1.6.7 Counter immunoelectrophoresis (immunoelectro-osmophoresis) test

This test employs electrophoresis equipment with slide frames and a gel cutter, and a 500-volt constant current power supply. It has a low sensitivity and is thus recommended for screening large groups of pigs, not individual sera (<http://www.oie.int>). Test antiserum and positive antigen are loaded on a glass slide in a gel on one side, while positive and negative control antisera and antigens are loaded on the other side as well. The samples are electrophoresed and the slides are examined under an indirect light source for specific precipitation lines after staining and destaining procedures (Pan *et al.*, 1972).

1.7 Socio-economic impact of ASF

The catastrophic effect of this disease on pig production, both at household and commercial levels in Africa poses serious socio-economic problems and threatens food security. These are best described in a report on the Cameroonian experience by Nana-Nukechap and Gibbs (1985). Following the 1982 outbreak in the western Cameroonian

province where 63 % of the country's 1.1 million total pig population occurs, 3, 762 pig farmers reported pig mortalities. The 54, 432 pigs reported dead were believed to be an underestimation as farmers failed to disclose their losses. A loss of \$36, 000 in accumulated stocks was declared by one farmer, while feed manufacturers estimated their losses at \$ 260, 000 in lost sales. Many farm workers lost their jobs as a result of the continued deaths that forced employers to reduce the labor force. Socially, pig eating at ceremonies was completely abandoned. Another West African country to suffer economically was Cote d'Ivoire, where the 1996 epizootic resulted in the death of 135,000 pigs in Abidjan (el Hicheri *et al.*, 1998). This was equivalent to 29 % of the pig population, of which 80 % came from the commercial sector.

In East Africa, the 1989 outbreak in an ASF endemic Zambia involving a commercial property cost 421,238 Zambian Kwacha (ZK) in control measures. It is estimated that the cost would have been at least ZK14, 917,500 (US\$1,415,323) if the control measures had not been effected (Samui *et al.*, 1996). The ASF outbreak in Malawi which spanned from 1989 to 1990, claimed 31,000 pigs (45 %) from a population of 70,000, either from mortalities or stamping out (Edelsten and Chinombo, 1995). In affected villages this accounted for 83 % of the pigs present.

For countries outside the African continent, ASF control and eradication also resulted in severe economic losses. The cost of ASF eradication in Malta and the Dominican Republic was a 100 % depopulation of their pigs, while 400 000 pigs in Cuba were lost before elimination of the disease (Plowright *et al.*, 1994). The Iberian Peninsula spent approximately \$100 (US) million/year to control both endemic ASF and new outbreaks (Wardley *et al.*, 1987). In a model designed for the assessment of the economic impact an ASF outbreak would have in the U.S, Rendleman and Spinelli (1999) showed that such an incident would cost \$4 500 billion both in trade embargoes and eradication.

1.8 Control and eradication

Current control measures are based on rigorous prevention of contact between domestic pigs and the free living reservoirs, quarantine of affected areas, stamping out the ill and in-contact pigs, prevention of movement of pigs and pig products through the infected areas and the application of chemical disinfectants in infected swine habitats (Plowright *et al.*, 1994). Europe successfully eradicated the disease in 1990, with the exception of Sardinia, (there was also an outbreak in Portugal in 1999) the only ASF endemic place outside Africa (Wardley *et al.*, 1987; Dixon *et al.*, 1999).

Identification of in-contact pigs in Spain does not only rely on the history and visual presence of ticks but a more reliable serological assay technique for antibodies (ELISA) against *Ornithodoros erraticus* salivary gland antigens (Canals *et al.*, 1990) is employed. This test has been validated for use under field conditions. Although the implemented measures are effective in curbing the spread of the disease in an outbreak, a detailed epidemiological investigation remains crucial for tracing the possible origin of the disease and preventing further introductions.

1.9 Epidemiology

1.9.1 Sylvatic cycle

ASF is indigenous to the African continent where it is thought to occur in one of three possible cycles: (i) an ancient sylvatic cycle, (ii) a pig/tick cycle and (iii) a more recently described domestic pig cycle (Penrith *et al.*, 2004). In countries south of the Sahara the sylvatic cycle seems to play an important role, with warthogs (*Phaenoceros aethiopicus*), bushpigs (*Potamochoerus porcus*), and possibly giant forest hogs (*Hylochoerus meinertzhageni*) acting as natural subclinical vertebrate reservoirs of ASFV (Plowright *et al.*, 1969; Pini and Hurter, 1975; Thomson, 1985). Argasid ticks of the *Ornithodoros moubata* complex are invertebrate hosts and vectors, mechanically

transmitting the virus to domestic and wild suids during feeding (Plowright *et al.*, 1969; Pini and Hurter, 1975; Thomson, 1985). Efficient infection and primary replication occurs in phagocytic and undifferentiated cells of the midgut epithelium, whereas secondary replication and generalization occurs in hemocytes, coxal and salivary glands, reproductive tissues, with viral persistence occurring in all tissues. Midgut replication and escape of ASFV is extremely important for long-term maintenance of infection in ticks (Kleiboeker *et al.*, 1999). The virus is transsexually, transovarially and transtadially transmitted in these ticks, thereby ensuring that the virus can be maintained within the tick population even in the absence of an infected blood meal (Plowright *et al.*, 1970; Plowright *et al.*, 1974).

1.9.2 Pig/tick cycle

This cycle involves domestic pigs and ticks of the *Ornithodoros* genus that inhabit pig pens (kholas) and crevices in huts inhabited by human beings. The West–Central Malawian situation best describes this particular cycle (Haresnape *et al.*, 1988; Haresnape and Wilkinson, 1989).

1.9.3 Domestic pig cycle

The present domestic pig cycle is thought to have resulted from viruses of reduced virulence that evolved in a few domestic pigs after surviving acute forms of ASF (Plowright *et al.*, 1994). Inapparent to subclinical infections have been recorded in this cycle (Haresnape, 1984; Costa, 1990; Plowright *et al.*, 1994; Allaway *et al.*, 1995; Eldelstein and Chinombo, 1995). Pigs infected with such virus can pass veterinary inspections and transmit ASFV if slaughtered and fed to their naïve counterparts (Wardley *et al.*, 1987).

1.9.4 Historical trans-continental spread of ASF

Movement of pigs and pig products between countries, and from infected to non-infected areas within countries is suspected to be responsible for transmission of the disease. The virus was introduced into countries outside the African continent *via* the feeding of ASF infected meat products to pigs, as is believed to have been the case with the first European ASF outbreak affecting Portugal in 1957 (Manso-Ribeiro *et al.*, 1958). ASF spread repeatedly in Europe, from the Iberian Peninsula by illicit movements of infected pigs and pig products to invade France (1964, 1967, 1977), Madeira (1965, 1974, 1976), Italy (1967, 1980), Malta (1978), Sardinia (1978), Belgium (1985) and Holland (1986). Outside Europe, Cuba was infected, probably from Europe, in 1971. In 1977 – 1978 the disease resurfaced in the Iberian Peninsula, followed by its appearance in Brazil and the Dominican Republic in 1978, Haiti in 1979 and again in Cuba in 1980 (Plowright *et al.*, 1994). The source of infection of the 1982 epidemic in Cameroon is suspected to have been Europe or the Americas, since the Cameroon, Dominican Republic and Haiti isolates were found to be genetically indistinguishable (Wesley and Tuthill, 1984).

In addition to *Ornithodoros porcinus* found in Africa, a number of African, North American, Central American, Caribbean and European ticks of the *Ornithodoros* genus have been shown to be potential vectors of ASFV (Groocock *et al.*, 1980; Mellor and Wilkinson, 1985; Endris *et al.*, 1991,1992; Hays, 1996; Kleiboeker and Scoles, 2001; Boinas *et al.*, 2004). The fact that these ticks not only act as mechanical vectors but can support ASFV growth, maintain and transmit the infection to naïve domestic pig populations, makes ASF a threat to pigs worldwide.

1.9.5 Current situation in sub-Saharan Africa

1.9.5.1 Epidemiology of ASF in West Africa

Involvement of a sylvatic cycle in the epidemiology of ASF in West Africa is not evident, despite the distribution of warthogs throughout the Savanna belt from Cameroon to

Senegal and the presence of *tampans* (*Ornithodoros erraticus*) in Senegal, north of the ASF endemic area (Penrith *et al.*, 2000). Ticks of the *Ornithodoros* genus are absent in Nigeria, and attempts to isolate ASFV from the free-ranging Nigerian dwarf pigs and warthogs have failed (Scott and Hill, 1966). The western province of Cameroon and the north and south-western parts of the country, where 70 % of the country's pig population is reared are the regions most frequently affected by ASF outbreaks. *Ornithodoros porcinus* and warthogs do not however occur in these regions (Nana – Nukechap and Gibbs, 1985; Ekue and Wilkinson, 1990). It is therefore deduced that pig-to-pig infections (pig cycle) or the feeding of contaminated pork products are the mechanism involved in the outbreaks.

1.9.5.2 Epidemiology of ASF in East Africa

Information on the history and current ASF status in the East African countries under study is incomplete. Even though the O.I.E lists the disease situation in these countries from 1990 to date in its Handistatus (that can be viewed on the website <http://www.oie.int>), very few publications have focused on ASF in Burundi, DRC, Mozambique, Tanzania and Uganda (Edelstein, 1989; Edelstein *et al.*, 1991; Jere, 1991). Incidents of disease in Kenya following Montgomery's (1921) study are limited and the current situation is insufficiently articulated. In Malawi and Zambia nevertheless, extensive studies have been conducted and the course of disease from as early as 1912 to date, has been reported.

Two thirds of the entire pig population is concentrated in the central region in Malawi, and in the eastern part in Zambia, with the remaining third found in the Southern part in both countries (Sumption, 1992). Pig meat production constitutes 7 % in Zambia and 30 % in Malawi (Wilkinson *et al.*, 1988; F.A.O, 1988). ASF outbreaks have been reported on regular basis in the neighbouring districts of Zambia (Northern Rhodesia) and Malawi, in Chipata and Mchinji (Wilkinson *et al.*, 1988; Wilkinson, 1989). The latter area was declared enzootic for ASF as early as 1933 (Turnbull, 1933).

1.9.5.2.1 Malawi

In the 1980s, the Mchinji area experienced a considerable number of outbreaks which were confirmed in the Central Veterinary Laboratory, Lilongwe, by Haresnape and colleagues (Haresnape, 1984; Haresnape *et al.*, 1985, 1987). Wild pigs were implicated in the first report of the disease in East Africa, and these wild suids, especially warthogs, and *Ornithodoros* ticks, are present, in many of the localities where ASF outbreaks occur in the region. ASFV specific antibodies and viraemias were detected, and virus was isolated from some of these reservoirs, confirming the active roles they played in outbreaks (Montgomery, 1921; Plowright, 1977; Haresnape, 1984; Thomson, 1985; Dixon and Wilkinson, 1988; Haresnape *et al.*, 1988; Wilkinson *et al.*, 1988). Despite the presence of sylvatic reservoirs in East Africa, the location of ASF outbreaks, distribution of warthogs and to a lesser extent bushpigs, dispersal of argasid ticks in warthog burrows and pig sties, and the isolation of ASFV from argasid ticks inhabiting these burrows seldom have a positive correlation (Plowright, 1977; Allaway *et al.*, 1995). The latter prompted researches to suggest that there could also be a pig-to-pig cycle playing a role in some parts of the region, especially in Malawi.

To corroborate the presence of a domestic pig cycle, investigations into the extent of ASF outbreaks in Malawi were carried out and revealed a wider distribution than was previously documented. The role played by wild pigs and *Ornithodoros* ticks could not be established (Haresnape, 1984). ASF disease surveys in the same country also identified enzootic localities in which the sylvatic hosts were irrelevant (Haresnape *et al.*, 1985, 1987). An ASF enzootic situation in domestic pigs was thus reported for the first time in Mchinji, and the enzootic area was later extended to include Ntcheu and Dedza with the discovery of seropositive ASF survivor pigs at least nine months after the outbreaks.

The disease spread to the southern region of Malawi decimating 86 % of the entire pig population in the region, with damages estimated at £500 000 (Sumption, 1992). Matson (1960) estimated that 12000 of the 19000 pigs died from the 1959 – 1960 outbreaks in the

same area. Serological studies of pigs for antibody against ASFV in two areas of southern Malawi correspondingly suggested circulation and transmission of ASFV among domestic pigs only (Allaway *et al.*, 1995). The disease did not persist in surviving pigs post the outbreaks.

Studies of ASFV infected *Ornithodoros* ticks collected from villages in the ASF enzootic area of Malawi following outbreaks of the disease in domestic pigs, deduced two important facts: 1) The circulation of virus between pigs and ticks. 2) The possibility of *O. porcinus* becoming an ASFV reservoir and initiating new outbreaks by transmitting the virus to susceptible pigs during feeding (Haresnape *et al.*, 1988; Haresnape and Wilkinson, 1989). These findings thus supported the existence of a pig/tick cycle in that country.

1.9.5.2.2 Zambia

ASF has been described in the Eastern Province of Zambia since 1912 and the first confirmation of the disease was made in this country in 1953, preceding that made in Malawi in 1962 (Cox and Hess, 1962; DeTray, 1963; Wilkinson *et al.*, 1988). The eastern Province is now considered to be enzootic for ASF. Zambia nonetheless reports fewer ASF outbreaks than Malawi, all occurring within the enzootic area. This could be a result of the 400 km distance between this area and the Central Veterinary Research Laboratory in Lusaka, as compared to the 90 km distance between the enzootic area in Malawi and the laboratory in Lilongwe, resulting in the former laboratory receiving fewer samples (Sumption, 1992). Non-commercial farmers were usually affected by repeated ASF outbreaks in the Eastern Province of Zambia, until 1989 when an outbreak occurred on a commercial property in central Zambia for the first time. The property suffered losses amounting to 421,238 Zambian Kwacha (ZK) from pig deaths and control measures in the form of depopulation and a ban on exporting pig products (Samui *et al.*, 1996). A survey of the distribution of ASF virus in *Ornithodoros* ticks in Zambia was carried out by virus isolation on samples collected from animal burrows in National Parks and Game

Management Areas in northern, eastern, central and southern Zambia. ASFV was isolated from ticks in all areas examined (Wilkinson *et al.*, 1988).

1.9.5.2.3 Regional considerations

The prevalence and incidence of ASF in East Africa varies greatly between countries and is related to the efficacy of the veterinary measures, type of husbandry system practised and the socio - political stability within countries. Fewer pigs are intensively reared in both Malawi and Zambia than are free roaming. Although both types of pig farming have experienced outbreaks, the source of infection in the two systems seems to differ. Swill feeding and scavenging are the biggest causes of ASFV introduction in free ranging pigs, while movement of infected pig material from one place to the other is responsible for disease occurrences in intensive pig farms. Both countries have a large number of small-scale pig owners with unregistered farms. A fall in pig meat price, saturation of local markets and increased roadside and uncontrolled illegal sales of pig meat, is frequently accompanied by ASF outbreaks (Sumption, 1992). The outbreaks are perpetuated by cross border trading with common tribal groups, the presence of peri-domestic ticks of the *O. moubata* complex, poaching of warthogs and bushpigs, and the inadequate standards and infrastructure of veterinary services. The veterinary services often lack transport, equipment, training and a well-developed disease control policy that reflects the current ASF situation (Sumption, 1992).

Despite the limitations detailed above, it is known that all three virus cycles play an active role in ASF epidemiology in East Africa, however information on the domestic pig and pig/tick cycles is based exclusively on studies conducted in Malawi. While proof of existence of a regional domestic pig cycle is still outstanding, the ravaging effects of ASF on small scale communal and bigger commercial farmers is undisputed (Table 1.1).

Table 1.1 Summary of ASF disease situation in East African countries for the past 10 years according to the O.I.E and Promed – mail records

Country	1996	1997	1998	1999	2000	2001	2002	2003
Burundi						+		
DRC			-			+		?*
Eritrea		-	-	-		
Ethiopia	1993	1993	1993	1993	1993	1993		
Kenya	11/94	11/94	11/94	11/94	11/94	+		
Madagascar	0000	0000	+	+	+	+		
Malawi	+	+	+	+	+	+		
Mozambique	+()	+()	+()	+	+	+	+	
Swaziland	0000	0000	0000	0000	0000	0000		
Tanzania	...	-	-	-	-	+	+	
Uganda	+	+	+	+	+	+	+*	+
Zambia	12/95	+()	+()	+	+	+	?*	

0000 Disease never reported; - Disease not reported (date of last outbreak not known); Date (month/year) of the last reported occurrence of the disease in previous years; ? Disease suspected but presence not confirmed; + Reported present or known to be present; +? Serological evidence and/or isolation of the causal agent, but no clinical signs of disease; () Disease limited to specific zones; ... No information available; * Promed – mail records; DRC-Democratic Republic of Congo

1.9.6 Molecular Characterisation of East African ASF viruses

Malawi and Zambia have suffered many ASF outbreaks since their first confirmed cases of the disease in the mid 1900s. A need arose to study the epidemiology of the disease at a molecular level to determine the following: 1) The genetic relationship of the viruses causing outbreaks in domestic pigs; 2) The genetic relationship between viruses of wildlife and those of domestic pig origin; 3) The genetic diversity of ASFV from *Ornithodoros* ticks; 4) The potential route of spread of viruses causing outbreaks (Dixon

and Wilkinson, 1988; Blasco *et al.*, 1989; Sumption, 1992; Bastos *et al.*, 2003, 2004; Lubisi *et al.*, 2005). All these studies were aimed at acquiring a better knowledge of ASF epidemiology, with the ultimate aim of curbing future spread or re-introductions.

Molecular biological tools like the restriction fragment length polymorphism (RFLP), where the virus genome is cut into pieces of various sizes by restriction endonucleases and electrophoresed to determine the size of the resulting DNA fragments, was earlier used for viral epidemiological classification (Blasco *et al.*, 1989; Dixon *et al.*, 1989; Sumption, 1992).

The RFLP method when applied to ASFV isolates from Malawi and Zambia revealed eight variable regions (VR) of the genome, designated VR1 to VR8 respectively (Sumption, 1992). VR1, 2 and 3 are located approximately:

- 1) At a region proximal to the first *NeoI* enzyme restriction site from both the left and right genome terminal hairpin loop and about 1.2 kb in size. It is thought to be the terminal inverted repeat (TIR) sequence consisting of unique sequences interspersed with a total of 1650 bp of repeat sequences, comprising 42 tandem direct repeats of 34bp, five repeats of 24 bp and three of 33 bp.
- 2) Between 7.3 kb and 17.6 kb from left terminal end.
- 3) Between 36 kb and 46 kb from left terminus of the Lil20/1 virus.

Within the central region of the genome, previously described as conserved (Blasco *et al.*, 1989), lies VR4, located 90 – 92 kb from the left terminus. VR 5, 6 and 7 are located within the right genome terminus, approximately 22.5 kb, 12.8 kb to 17.7 kb and 10.8 kb to 12.2 kb from Lil20/1 genome's right terminus. In the right genome terminus, 7 kb from VR7 and 1.1 kb from VR1, spanning 1.2 kb to 3.5 kb from the right terminus, is the location of VR8.

Sumption (1992) preferred the *BamHI* restriction enzyme to other restriction enzymes in determining diverse ASFV relatedness due to the fact that the results were reproducible and yielded more fragments which could assist in determining relationships between closely related isolates. *BamHI* a, b (or a'b) and c were fragments from the left genome

terminus, while *BamHI* t, u, v and w were fragments from the right terminus. All but one fragment yielded size differences of no more than 0.82 kb between the viruses compared. The combined fragment, *BamHI* a'b however, displayed significant size differences of up to 9.1 kb between isolates, and was thus used for comparison of the different isolates from East, West and South Africa (Sumption, 1992).

More recently, viral DNA is amplified by polymerase chain reaction (PCR) and the amplified product's nucleotide sequences are used to group viruses (Bastos *et al.*, 2003, 2004; Lubisi *et al.*, 2005). Of the ten major genotypes shown to occur on the African continent, five were of East African origin (Bastos *et al.*, 2003).

1.9.7 Phylogenetic tools and considerations in ASF molecular epidemiology

Nucleotide sequence data of a conserved genome region, the *p72* gene can be used to discern ASFV phylogenetic relationships (Bastos *et al.*, 2003). This data is amenable to both distance and character-based methods of analysis. Uniformity of results between methods is crucial for the confidence bestowed to the discerned phylogeny (Kim, 1998). Constancy of results is however not always easily attainable due to the complexity of the data which includes the number of operational taxonomic units (OTU), transition/transversion and nucleotide composition bias, unequal nucleotide substitution rates and the presence of homoplasy. A five-point criterion system, based on power, efficiency, consistency, robustness and falsifiability (Penny *et al.*, 1992) is used to judge each method's performance given a data set. Power determines the amount of data a method needs to draw a phylogenetic tree, while efficiency measures the speed with which a tree is drawn as data is applied. Consistency assesses a method's ability to infer the correct tree with increasing data, and robustness looks into the ability to infer a correct tree even if its assumptions are violated. Falsifiability assesses the method's discriminating ability (Huelsenbeck, 1995; Penny *et al.*, 1992).

The different methods of phylogenetic inference commonly used are neighbor-joining (NJ), minimum evolution (ME), maximum likelihood (ML) and maximum parsimony

(MP). NJ method (Saitou and Nei, 1987) creates distance data on the basis of pairwise comparisons and is related to the cluster analysis of Sattath and Tversky (1977). Another distance method, ME, uses an optimality criterion, resulting in various optimal and sub-optimal solutions, of which the tree with the shortest total sum of branch length is assumed the best estimate of the phylogeny (Edwards and Cavalli-Sforza, 1963). MP methods are cladistic in principle, and utilize character data and optimality criteria to infer phylogenies (Eck and Dayhoff, 1966; Fitch, 1971). ML methods of phylogenetic tree inference are evolutionary in principle, and utilize all the sequence data under analysis to formulate a probabilistic model of evolution with application of known statistical methods (Felsenstein, 1981).

In the quest for accurate phylogenetic construction, the methods used to prescribe the standards of performance of the tree building techniques are also of great importance. Simulation methods, on which most method comparisons are based, known phylogenies, statistical approaches and congruence studies are employed to set the standards by which methods are expected to perform given a certain data set (Huelsenbeck, 1995; Sourdiss and Nei, 1988; Lin and Nei, 1991; Kuhner and Felsenstein, 1994). Many biases such as the selection of parameters that describe the simulated tree, evolutionary model, poor method implementation and the acquisition of multiple and equally good solutions (Tanaka *et al.*, 1997; Takezaki, 1998; Oberste *et al.*, 1999; Servant *et al.*, 2002) occur in the quest for uncovering the true phylogenetic relationships and therefore have to be approached with caution.

All the methods discussed above have different strengths and limitations, and different requirements for input data, in order to perform maximally. Methods are only superior to others when all their assumptions are satisfied and the questions asked are within their capability and the amount of data they process is not overwhelming their search capabilities. Selection of one particular method should therefore be based on the research question asked, the number of taxa examined and the type and nature of the data (e.g amount of base pair substitutions, transition/transversion (si/sv) ratios and the number of nucleotides). Knowledge of each available method's principles and assumptions and how

it would perform under the given circumstances is important. Care should be taken to scrutinise the models used to assess the performance of a particular method since results of such models often seem to match pre-existing preferences of the particular investigators (Hillis, 1995). The availability of computer software to perform the desired phylogenetic analysis is also a factor of consideration when choosing a method. Software such as Farris' Hennig 86, MEGA by Kumar, Tamura and Nei, Swofford's PAUP, Felsenstein's PHYLIP, and many more are available for the researcher to analyse data (Swofford *et al.*, 1996). It should however be noted that real data hardly, if ever, fulfil any method's ideal assumptions and the investigator should be familiar with available corrective measures in order to acquire optimum results from the methods employed.

1.10 OBJECTIVES

The review of the literature clearly indicates that ASF is endemic in Africa and continues to cause great economic losses. The surface glycoproteins do not allow these viruses to be distinguished on a serological basis; thereby hindering serologically based epidemiological studies. It has also been seen that East Africa has all the epidemiologically significant factors for ASF, while the West African situation lacks some of these important factors. The primary objective is therefore to obtain a regional ASF epidemiological picture for utilization in the control of outbreaks.

The aim of this study is two fold:

- ◆ To genetically characterize African swine fever viruses of diverse species, geographical and temporal origins from East Africa, in order to trace the possible origins of outbreak strains.
- ◆ To genetically characterize intra-genotypic relationships within a large homogeneous domestics pig genotype so that a finer resolution of the origins of outbreak viruses can be obtained.

Chapter 2

p72 genotyping of African swine fever viruses from East Africa

SUMMARY

African swine fever (ASF) a lethal, viral hemorrhagic disease of domestic pigs, first reported from East Africa in 1921, is still widespread in this region. In order to assess field heterogeneity at the regional level, nucleotide sequences corresponding to the C-terminal end of the *p72* gene were determined for 77 ASF viruses of diverse temporal and species origin occurring in eight East African countries. The number of sites that were not completely conserved across all East African sequences characterised in this study was 15.8 % and 13.4 % on nucleotide and amino acid level, respectively. Phylogenetic analysis of a homologous 404 bp region revealed the presence of thirteen East African genotypes, of which seven appear to be country specific. An East African, pig-associated, homogeneous virus lineage linked to outbreaks in Mozambique, Zambia and Malawi over a 23 year period was demonstrated. In addition, genotype I (ESACWA) viruses were identified in East African sylvatic hosts for the first time which is significant as this genotype was previously thought to be restricted to the West African region where it occurs only in domestic pigs. The presence of discrete epidemiological cycles in East Africa and recovery of multiple genotypes affirms the epidemiological complexity of ASF in this region.

Parts of the results presented here have been accepted for publication in *Archives of Virology*

2.1 INTRODUCTION

The epidemiology of ASF in East Africa is complex. Not only is there evidence for a sylvatic cycle, but a domestic pig cycle and a pig-tick cycle have also been described for Malawi (Haresnape, 1984, Haresnape *et al.*, 1985, 1987, 1988; Plowright *et al.*, 1994). Despite the presence of all three cycles no extensive molecular database comprising ASFV strains from different host species is available which would assist in clarifying the epidemiology of the disease in this region.

Of the 30 plus proteins contained in ASFV particles ranging in molecular weight from 10 to 150 kDa, the major polypeptide, virus protein 72 (VP72) has a weight of 72 kDa (Carrascosa *et al.*, 1985). Through the availability and use of ASFV specific monoclonal antibodies, Sanz *et al.* (1985) discovered that other ASF viral proteins changed following passage in macrophages while the protein VP72 remained stable. Analysis of the binding properties of these monoclonal antibodies using cloned field isolates demonstrated the antigenic stability of this protein (Sanz *et al.*, 1985). Based on these discoveries and the findings of Tabarés and co-workers (1980) that VP72 was the major immunogen in natural infections, the protein was ear-marked for use in newly improved diagnostic tests. For this purpose, the gene encoding VP72 had to be identified for expression and further characterization of this protein. Oligonucleotide probes deduced from amino acid sequences of tryptic peptides obtained from purified protein VP72 were used to map the encoding gene (López-Otín *et al.*, 1990). An open reading frame (ORF) coding for 646 amino acids corresponding to a protein of approximately 73 kDa was revealed, assuring investigators of the genomic area being the correct *p72* gene location. Further validation of the antigenic stability of VP72 at molecular level involved *p72* gene comparison of one virus from the Dominican Republic (DR-2), two isolates from Spain (BA71-V and E70) and one Ugandan virus (UGA). Results revealed a 95.5 to 100% sequence homology at nucleotide, and a 97.8 to 100% identity at amino acid level (Yu *et al.*, 1996).

In addition to VP72's potential as an efficient antigenic protein in serological assays, the *p72* gene's capability to resolve phylogenetic relationships among field and outbreak

ASFV was postulated and explored (Bastos *et al.*, 2003). Virus clusters similar to those identified by restriction enzyme digestion analysis (Wesley and Pan, 1982; Blasco *et al.*, 1989) were obtained when comparing different ASFV isolates on the basis of VP72 nucleotide sequences. The gene's proficiency in resolving virus relatedness was significant as its surface glycoproteins do not allow differentiation into serological types (Dixon *et al.*, 2000).

Nucleotide and amino acid variability plots of ASFV complete gene showed the central and C-terminal regions to be rich in non-synonymous mutations (Bastos *et al.*, 2003). The latter region was also found to be more variable than the former making it better suited to distinguishing between field strains. Furthermore, the region was surrounded by conserved nucleotide sequences, allowing the design of oligonucleotide primers complementary to these flanking sites. This led to the C-terminal being chosen over the central region for phylogenetic studies using the *p72* PCR-sequencing approach (Bastos *et al.*, 2003).

In an attempt to validate the potential of partial *p72* genotyping for resolving ASFV relationships, Bastos and co-workers (2003) tested isolates from Africa, the Caribbean Islands, Europe and South America, including the same isolates previously genotyped by restriction fragment length polymorphism (RFLP) (Blasco *et al.*, 1989), or a geographical representative thereof. Ten lineages were resolved with partial *p72* gene sequencing, and five of these corresponded with those previously identified by RFLP analysis. Of these, genotype I comprising viruses from Europe, South America, the Caribbean and West Africa (and termed the ESACWA genotype), represents the most widespread and homogeneous genotype identified thus far.

The corroboration of *p72* genotyping's ability to differentiate ASFV isolates on a genetic level in a more rapid and less labor-intensive manner, and the potential to apply the method directly to clinical specimens, without first isolating the virus resulted in this approach being favored over RFLP to resolve ASFV relationships from East Africa.

2.2 MATERIALS AND METHODS

2.2.1 Study area and samples

For the purpose of this study, East African countries are defined as those occurring east of longitude 20°00E and south of latitude 5°00N, but excluding Namibia, South Africa, Botswana and Zimbabwe. This area encompasses the following eight countries: Burundi, Democratic Republic of Congo, Kenya, Malawi, Mozambique, Tanzania, Uganda and Zambia. A total of 77 viruses of diverse species and temporal origin from these eight East African countries were sequenced and characterized in this study, with the majority of viruses being supplied by the World Reference Laboratory, Institute for Animal Health (IAH), Pirbright. Additional strains were isolated at the Onderstepoort Veterinary Institute (OVI), Agricultural Research Council (ARC) from clinical material supplied by the respective departments of veterinary services. Twenty-five ASF viruses representative of each of the ten previously identified *p72* genotypes (Bastos *et al.*, 2003) were included for phylogenetic analysis purposes, bringing the total number of viruses used in this study to 102 (summarized in Table 2.1). In order to place the results of this study in context with the RFLP studies performed previously with isolates from the East African region (Sumption 1992), a summary of the viruses used in the latter study, together with the main findings, have been summarized in Table 2.2.

2.2.2 Pig primary cell culture preparation

2.2.2.1 Peripheral blood leukocyte culture

The Malmquist and Hay (1960) method of swine macrophage preparation was employed, with a few modifications. Where peripheral blood leukocyte cells were used, whole blood from a juvenile pig was collected by injecting the pig with 20 % Sodium pentobarbitone intra-cardially and exsanguination by severance of the Axillary blood vessels followed shortly thereafter. A 1 litre (L) vessel containing 1 ml of Heparin Sodium (5000i.u/ml)

was used to collect the blood. For the prevention of microbial and fungal growth, an antimicrobial cocktail and antifungal agent consisting of Penicillin – 4 % Benzylpenicillin (Sigma), Streptomycin – 6.4 % streptomycin sulphate (Sigma), Neomycin - 7.5 % Neomycin-sulphate (Merck) and Fungizone – 5 mg Amphotericin B (Bristol-Myers Squibb) in 1 ml quantities, were added to the blood. Iron dextran – 10 % (BDK) was added to the blood in a 1:10 ratio. The blood was then aliquoted in 100 ml Erlenmeyer flasks and incubated at 37°C for 20 minutes until the red blood cells formed a sediment. The white blood cell rich supernatant was pipetted out and collected into a plastic centrifuge bottle, where it was diluted 1:1 with wash buffer. The wash buffer consisted of 1L phosphate buffered saline (PBS), 0.4 % Penicillin, 0.64 % Streptomycin, 0.75 % Neomycin and 10 % normal bovine serum (NBS). Centrifugation at 2000 rotations per minute (rpm) for 4 minutes ensued. This was the first wash step. At the end of centrifugation the supernatant was discarded and a second wash step followed. At the third wash step, the cell rich sediment was re-suspended in 100 ml of 0.82 % Ammonium chloride and centrifuged as above. Wash buffer was again used for the fourth and final wash step. The clear white blood cells were suspended in growth medium constituting the following: 42 % distilled water, 42 % Earle's medium (Appendix A), 0.4 % HEPES (UniLab), 13 % swine serum, 0.52 % penicillin, 0.832 % streptomycin and 0.975 % neomycin. The cells were grown in 100 µl volumes on 96 well plates (Nunc). The medium was changed after 48 hours (day 2 post preparation) and fresh growth medium containing 0.5 % red blood cells was added. The primary cultures were stored for a minimum of 7 days, or a maximum of 11 days. Growth medium was changed for the third time on day 6 post preparation in instances where cultures were stored for the maximum period.

2.2.2.2 Bone marrow cultures

The same pig that was euthanased for primary white blood cell cultures had two long bones removed from its body and placed in a litre of wash buffer prepared as in 2.2.2.1. Forceps were used to remove the bones from the wash buffer and these were placed on a stainless steel tray. Flesh and cartilage were excised from the bones with a scalpel blade

and the clean bones were immersed in 300 ml of fresh wash buffer and incubated at 37°C for 20 minutes. After incubation, bone-cutting forceps were used to crush the bones into small fragments and these were collected in a 1 L conical flask containing 250 ml of wash buffer. The conical flask was incubated at 37°C and gently shaken for 90 minutes. Butter muslin was placed over funnels through which the crushed bones were sieved and the cell rich filtrate collected into centrifuge containers. The filtrate was centrifuged at 1000 rpm for fifteen minutes. After the removal of the supernatant, the sediment was washed with 0.82 % ammonium chloride solution and centrifuged at 1000 rpm for 15 minutes. This was followed by two extra washing steps with wash buffer, followed by suspension of the sediment in growth medium as detailed in section 2.2.2.1. The cell rich suspension was poured into a boat, and a multichannel pipette was used to transfer 100 µl of the suspension into each well of a 96 well microtitre plate. The plates were stored in a carbon dioxide (CO₂) incubator at 37°C, and growth medium was changed after 48 hours. The primary bone marrow cultures were stored for a maximum of 7 days.

2.2.3 Virus isolation

Inoculum containing 10 % (w/v) of sample material in PBS was processed in a series of 10 fold dilutions. Rows of wells were inoculated with the sample material at decreasing dilutions, in 50 µl quantities per well. Plastic plate sealers, followed by the lids, were used to cover the plates. The cells were examined daily for cytopathogenic effect or haemadsorption. Virus from positive wells was harvested by freezing the plates at -80°C for at least 30 minutes to lyse the cells. After thawing, a syringe with a sharp needle was used to pierce the plastic sealers and collect the contents from each positive well, and these were pooled in a 20 ml bottle. Centrifugation at 15000 rpm for 10 minutes followed. The virus rich supernatant was removed and stored at -80°C.

Table 2.1 Summary of the African swine fever viruses characterized in this study and those sourced from other studies and included for comparative and phylogenetic purposes

Virus name	Country of origin	Town / District	Year of isolation	Species of origin	GenBank Accession number
BAN 91/1	Malawi	Bangula, Lower Shire	1991	<i>Sus scrofa</i>	AY351501
#BRAZIL 79	Brazil	NK	1979	<i>Sus scrofa</i>	AF302809
Bartlett II	Kenya	Timau	1959	<i>Phaecochoerus aethiopicus</i>	AY351532
#BUR 1/84	Burundi	Mushasagitega	1984	<i>Sus scrofa</i>	AF449463
#BUR 2/84	Burundi	Mushasagitega	1984	<i>Sus scrofa</i>	AF449464
#BUR 90/1	Burundi	Muyinga	1990	<i>Sus scrofa</i>	AF449472
BUR 90/3	Burundi	Muyinga	1990	<i>Sus scrofa</i>	AY351525
#BOT1/99	Botswana	Sherwood	1999	<i>Sus scrofa</i>	AF504886
#CAM/82	Cameroon	Guzang North West Province	1982	<i>Sus scrofa</i>	AF301544
CHG 88/1	Zambia	Chaguza, Katete, Eastern Province	1988	<i>Sus scrofa</i>	AY351552
CHJ 89/1	Zambia	Chiphanje, Petauke, Eastern Province	1989	<i>Sus scrofa</i>	AY351519
CHK 89/2	Zambia	Chikuwe, Chipata, Eastern Province	1989	<i>Sus scrofa</i>	AY351526
CHM 88/1	Zambia	Chambula, Petauke, Eastern Province	1988	<i>Sus scrofa</i>	AY351520
#Dakar59	Senegal	Dakar	1959	<i>Sus scrofa</i>	AF301538
Davis	Kenya	Nanyuki	1959	<i>Phaecochoerus aethiopicus</i>	AY351527
⁶ DED 89/1	Malawi	Chiphazi, Dedza District	1989	<i>Sus scrofa</i>	AY351502
DED 91/1	Malawi	Mtenden Campus, Dedza	1991	<i>Sus scrofa</i>	AY351503
#Dedza	Malawi	Chilikum-Wera Dedza	1986	<i>Sus scrofa</i>	AF449479
Doig	Kenya	Kiganjo	1957	<i>Phaecochoerus aethiopicus</i>	AY351528
⁶ DOWA	Malawi	Moya, Dowa	1986	<i>Sus scrofa</i>	AY351509
Gasson	Kenya	Nanyuki	<1961	<i>Sus scrofa</i>	AY351529
GUL 88/1	Zambia	Gulumule, Katete, Eastern Province	1988	<i>Sus scrofa</i>	AY351521
Hinde I	Kenya	Nanyuki	1954	<i>Sus scrofa</i>	AY351530
#HINDE II	Kenya	Nanyuki	1959	<i>Sus scrofa</i>	AF449480
#JON 89/13	Zambia	Jonase Petauke	1989	<i>Sus scrofa</i>	AF449469

⁴ KAB 6/2	Zambia	Livingstone game park, south Zambia	1983	Tick *	AY351522
KAC 91/2	Malawi	Kachendere Seminary, Chisengu, Mchinji	1991	<i>Sus scrofa</i>	AY351504
[#] KAL 88/1	Zambia	Kaliyoyo-Chipate	1988	<i>Sus scrofa</i>	AF449468
KANA 89/1	Zambia	Kangwero farm 17, Katete, Eastern Province	1989	<i>Sus scrofa</i>	AY351523
[#] KAV 89/1	Zambia	Kavundula Katete	1989	<i>Sus scrofa</i>	AF449470
Killean I	Kenya	Nanyuki	1959	<i>Phaecochoerus aethiopicus</i>	AY351550
Killean II	Kenya	Nanyuki	1959	<i>Phaecochoerus aethiopicus</i>	AY351551
Killean III	Kenya	Nanyuki	1959	<i>Phaecochoerus aethiopicus</i>	AY351531
Kimakia I	Kenya	UK	1961	<i>Potamochoerus porcus</i>	AY351533
Kimakia II	Kenya	UK	1961	<i>Potamochoerus porcus</i>	AY351534
KIRT 89/2	Tanzania	Kiriwira	1989	Tick *	AY351511
KIRT 89/3	Tanzania	Kiriwira	1989	Tick *	AY351512
KIRT 89/4	Tanzania	Kiriwira	1989	Tick *	AY351513
KIRW 89/1	Tanzania	Kiriwira	1989	<i>Phaecochoerus aethiopicus</i>	AY351514
KLI 88/2	Zambia	Kalinda, Petauke, Eastern Province	1988	<i>Sus scrofa</i>	AY351553
[#] KWH/12	Tanzania	Kiriwira	1968	<i>Phaecochoerus aethiopicus</i>	AF301546
⁶ LIL 89/1	Malawi	Mlozi, Lilongwe District	1989	<i>Sus scrofa</i>	AY351505
LIL 90/1	Malawi	Kafere diptank, Lilongwe	1990	<i>Sus scrofa</i>	AY351510
[#] LISBON/57	Portugal	Lisbon	1957	<i>Sus scrofa</i>	AF301537
⁷ LIV 5/40	Zambia	Livingstone Game Park, South Zambia	1982	Tick *	AY351536
⁷ LIV 5/4	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY351537
LIV 9/31	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY351538
LIV 9/35	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY351539

¹ LIV 10/11	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY351535
⁷ LIV 12/17	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY351524
⁷ LIV 13/33	Zambia	Livingstone Game Park, South Zambia	1983	Tick *	AY494560
LUS 93/1	Zambia	Nawande farm, Lusaka district, Lusaka Province	1991	<i>Sus scrofa</i>	AY351563
[#] MAD 1/98	Madagascar	NK	1998	<i>Sus scrofa</i>	AF270705
Magadi w/hog 1	Kenya	Magadi	1959	<i>Phaecochoerus aethiopicus</i>	AY351548
Magadi w/hog 9	Kenya	Magadi	1959	<i>Phaecochoerus aethiopicus</i>	AY351565
¹ MAL 2002/1	Malawi	Mpemba Quarantine Camp	2002	<i>Sus scrofa</i>	AY494553
[#] MALAWI/197 8	Malawi	NK	1978	<i>Sus scrofa</i>	AF270707
MAN 89/2	Zambia	Mangulu, Katete, Eastern Province	1989	<i>Sus scrofa</i>	AY351562
⁶ MCH 89/1	Malawi	Kachebere Seminary, Mchinji	1989	<i>Sus scrofa</i>	AY351506
⁶ MCH 89/3	Malawi	Chisikwa diptank, Lilongwe District	1989	<i>Sus scrofa</i>	AY351507
⁶ Mchinji 075	Malawi	Mchinji	1987	<i>Sus scrofa</i>	AY351508
⁷ MFUE 6/1	Zambia	Mfue, Luangera National Park	1982	Tick *	AY351561
[#] MOZ/94/1	Mozambique	Maputo	1994	<i>Sus scrofa</i>	AF270711
⁵ MOZ 2001/1	Mozambique	Zambezi, Quilemane	2001	<i>Sus scrofa</i>	AY351516
⁵ MOZ 2002/1	Mozambique	Northern Nampula region	2002	<i>Sus scrofa</i>	AY351517
⁵ MOZ 2002/2	Mozambique	Northern Nampula region	2002	<i>Sus scrofa</i>	AY351518
MPI 89/1	Zambia	Mpima Seminary, Kabwe, Central Province	1989	<i>Sus scrofa</i>	AY351540
MPO 89/1	Zambia	Mpoka, Petauke, Eastern Province	1989	<i>Sus scrofa</i>	AY351541

MZI 92/1	Malawi	Euthini, Mzinda District, north Malawi	1992	<i>Sus scrofa</i>	AY351543
#NDA/1/90	Malawi	Nadula	1990	<i>Sus scrofa</i>	AF449473
NGE 92/1	Malawi	Ngerenge diptank, Karonga District	1992	<i>Sus scrofa</i>	AY351544
NKZ 88/1	Zambia	Nyankonzi, Petauke, Eastern Province	1988	<i>Sus scrofa</i>	AY351554
NYA1/2	Zambia	Kalumo	1986	Tick *	AY351555
PHW 88/1	Zambia	Phwata, Chipata, Eastern Province	1988	<i>Sus scrofa</i>	AY351567
#RSA/1/98	South Africa	Potgietersrus	1988	<i>Sus scrofa</i>	AF302818
#RSA/1/99	South Africa	Soutpansberg	1999	<i>Phaecochoerus aethiopicus</i>	AF302818
SAL 92/1	Malawi	Chiripa diptank, Salima District	1992	<i>Sus scrofa</i>	AY351546
SIY 91/2	Malawi	Sinyala diptank, Lilongwe	1991	<i>Sus scrofa</i>	AY351566
#SPEC 265	Mozambique	Maputo	1994	<i>Sus scrofa</i>	AF270210
⁷ SUM 14/11	Zambia	Sumbu National Park	1983	Tick *	AY351542
² TAN/1/01	Tanzania	Dar Es Salaam	2001	<i>Sus scrofa</i>	AY494552
² TAN/2003/1	Tanzania	Arusha	2003	<i>Sus scrofa</i>	AY494550
² TAN/2003/2	Tanzania	Arusha	2003	<i>Sus scrofa</i>	AY494551
TEN 89/1	Zambia	Tenesi, Petauke, Eastern Province	1989	<i>Sus scrofa</i>	AY351556
#TENGANI/60	Malawi	Tengani	1960	<i>Phaecochoerus aethiopicus</i>	AF301541
THY 90/1	Malawi	Comforzi farm, Thyolo District	1990	<i>Sus scrofa</i>	AY351545
TMB 89/1	Zambia	Tembo, Petauke, Eastern Province	1989	<i>Sus scrofa</i>	AY351557
Trench	Kenya	Mweiga	1959	<i>Phaecochoerus aethiopicus</i>	AY351547
#UGA/1/95	Uganda	Malindue	1995	<i>Sus scrofa</i>	AF449475
#UGA/3/95	Uganda	Simbale	1995	<i>Sus scrofa</i>	AF449476
⁴ UGA2003/1	Uganda	Maria Village, Masaka District	2003	<i>Sus scrofa</i>	AY351564
YEL88/4	Zambia	Yelani, Petauke, Eastern Province	1988	<i>Sus scrofa</i>	AY351558
³ ZAM01/1	Zambia	Lusaka	2001	<i>Sus scrofa</i>	AY494554
³ ZAM01/2	Zambia	Kafue	2001	<i>Sus scrofa</i>	AY494555
³ ZAM01/3	Zambia	Mazabuka	2001	<i>Sus scrofa</i>	AY494556

³ ZAM01/4	Zambia	Namwala	2001	<i>Sus scrofa</i>	AY494557
³ ZAM01/5	Zambia	Monze	2001	<i>Sus scrofa</i>	AY494558
³ ZAM02/1	Zambia	Kyiundi Ranch	2002	<i>Sus scrofa</i>	AY494559
Zaire	DRC	NK	NK	NK	AY351515
ZAW 88/1	Zambia	Gulumule, Katete, Eastern Province	1988	<i>Sus scrofa</i>	AY351559
[#] ZOM/2/84	Malawi	Zomba	1984	<i>Sus scrofa</i>	AF449471
ZON 88/1	Zambia	Zondola, katete, Eastern Province	1988	<i>Sus scrofa</i>	AY351560

Virus supplied by: ¹ Dr. Klauz Lorenz (Divisional Veterinary Officer, Blantyre Agricultural Development Division, Malawi; ² Dr. J.I.G Masambu, ADRI-TEMEKE, Dar-es-Salaam, Tanzania; ³ Chief Research Officer, Virology Laboratory, Central Veterinary Laboratories, Lusaka, Zambia; ⁴ Food and Agriculture Organization (FAO), Department of Livestock, Health and Entomology, Uganda; ⁵ The National Veterinary Institute, Mozambique. Viruses previously characterized by RFLP analysis in the studies of Sumption et al. 1990 and Dixon & Wilkinson 1988, are denoted by the superscript numbers '6' and '7', respectively. NK: Not known, * Indicates *Ornithodoros* ticks collected from warthog burrows; [#] Indicates reference isolates (Bastos *et al.*, 2003), DRC: Democratic Republic of the Congo.

Table 2.2 ASFV isolates characterized and grouped by Sumption (1992) using *Bam HI* RFLP

Group	Virus	Country	Location	Isolation year	Origin	Passage details	
						Pig	PBMC
I	KAV89/1#	Zambia	Kavundulo, E. Province	1989	Pig	NK	NK
	KON83	Malawi	Mchinji-Southern Province	1983	Tick	1	0
	Lil20/1	Malawi	Kamende-Mchinji	1983	Tick	NK	
	MOZ60#	Mozambique	NK	1960	Pig	NK	5
	RHO63	Zambia	E. Province	1963	Pig	NK	NK
II	ANG70#	Angola	NK	1970	Pig	3	NK
	BUR84/1#	Burundi	Mushasha-Butega	1984	Pig	0	0
	CAM82#	Cameroon	NK	1982	Pig	2	0
	KAT67	DRC	Katanga	1967	NK	NK	NK
	NAM86	Namibia	Grootfontein	1986		0	2
III	MOZ79#	Mozambique	Beira	1979	Pig	0	1
	Tengani62	Malawi	Tengani	1962	Pig	2	0
IV	HINDE54	Kenya	Hinde Ranch	1954	Pig	NK	NK
	LIV13/33*	Zambia	Livingstone Game Park	1983	Tick (warthog)	0	1
V	TAN87	Tanzania	Mbeya-SW Tanzania	1987	Pig	2	0
VI	MFUE6/1*	Zambia	Mfue, Luangera National Park	1982	Tick (warthog)	0	1
VII	SUM14/11*	Zambia	Sumbu National Park	1983	Tick (warthog)	0	1

PBMC: Pig Bone Marrow Culture SW: South West NK: Not Known E: East DRC: Democratic Republic of Congo

* Isolates common to those characterized in this study. # Isolates common to those characterized in previous *p72* genotyping studies (Bastos *et al.*, 2003, 2004)

2.2.4 Extraction of viral DNA

DNA was extracted from known virus cultures or tissue sample homogenates using a silica/guanidium-based nucleic acid extraction method (Boom *et al.*, 1990). For each virus or homogenized diagnostic specimen, a 100 µl aliquot was transferred to a 1.5ml labeled Eppendorf tube. A milliliter of L6 (Appendix B) lysis buffer was added to 40 µl of silica (Appendix C) and vortexed until completely dissolved. 940 µl of the lysis buffer-silica mixture was added to each specimen, vortexed and allowed to stand at room temperature for 5 minutes. At 1 minute intervals the samples were inverted. The tubes were later vortexed again and centrifuged at 15000 rpm for 15 seconds, and the supernatant discarded in a 10 M NaOH waste bottle. The L2 wash buffer (Appendix D) was then added in quantities of 900 µl per sample, vortexed, and centrifuged for a few seconds. The supernatant was pipetted off in the above-mentioned waste bottle. A wash step with 900 µl of 70 % ethanol was performed and finally 900 µl of acetone was added as the final cleaning step. After discarding the acetone supernatant, the silica pellets were then dried by placing on a heating block pre-set at 56°C, with the eppendorf tube lid open, for 20 minutes approximately. 1X TE (Tris-Acetate) was prepared (Appendix E), 30 µl of which was added to the dry silica pellet. The tube was vortexed and incubated for 2 minutes on the heating block with lid closed. A 10 minutes centrifugation step followed, and the supernatant was finally transferred to a clean 1.5 ml labeled eppendorf tube. The DNA was stored at -70°C until further use.

2.2.5 Genomic amplification of viral DNA

Two separate polymerase chain reactions (PCR) were performed: -

1. Diagnostic PCR for confirmation of the presence of ASF viral nucleic acid, where a 278 bp region corresponding to the central portion of the *p72* gene was amplified using ASF diagnostic primers ASF-1 (5'ATGGATACCGAGGGAATAGC3') and ASF-2

(5'CTTACCGATGAAAATGATAC3') described in Chapter 2.1.12 of the 2000 edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines.

2. A PCR for molecular epidemiological analysis, where the C-terminal region of the *p72* gene was amplified using epidemiological primers P72-U (5' GGCACAAGTTCGGACATGT3') and P72-D (5' GTACTGTAACGCAGCACAG3'), as described previously (Bastos *et al.*, 2003).

For each of these PCRs, genomic amplification was performed in a 50 µl volume in the presence of 200 µM dNTPs (Roche), 0.4 µM of each primer, 2.5 U of thermostable *Taq* DNA polymerase (Roche) and 3 µl of DNA extract. The template was amplified following 40 cycles of denaturation at 96°C for 12s, annealing at 53°C for 20s and extension at 70°C for 30s.

2.2.6 Agarose gel electrophoresis

Amplification products were loaded on a 1.5 % agarose gel and run against a 100 bp DNA ladder (Promega). Once sufficient electrophoretic separation was obtained, products were visualized by UV irradiation and ethidium bromide staining. The gels were photographed in order to obtain a hard copy record of the results.

2.2.7 Purification of PCR products

ASFV genome regions targeted by PCR for phylogenetic analysis were excised from the agarose gel and purified using either Qiagen (Qiagen) or Nucleospin (Machery-Nagel) PCR purification kits according to the manufacturer's specifications.

2.2.8 Nucleotide sequencing and alignment

The nucleotide sequence of the purified products was determined by automated cycle sequencing with Big Dye version 3.1 by running in a 47 centimeter capillary on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

All nucleotide sequences generated by the epidemiological primers in this study were aligned using the DAPSA program (Harley, 1994).

2.2.9 Phylogenetic analysis of the *p72* gene

A homologous region of 404 nucleotides was used for phylogenetic inference. The methods employed were either distance-based where a matrix was used to cluster closely related taxa, alternatively, character data and an optimality criterion was used to infer a phylogeny. The methods included neighbor-joining (NJ), minimum evolution (ME), maximum parsimony (MP) and maximum likelihood (ML). Model Test (Posada and Crandall, 1998) was used to identify the model of substitution that best fitted the data at hand.

2.2.9.1 Neighbor-joining (NJ)

The NJ tree was drawn in MEGA v2.1 (Kumar *et al.*, 2001). The nucleotide substitution model of Tamura and Nei (1993) was employed which takes transition/transversion (si/sv) bias within a dataset into account when calculating distances. 10 000 bootstrap replications were performed to determine the level of support for each node in the resulting mid point rooted phylogenetic tree.

2.2.9.2 Minimum evolution (ME)

An ME tree was drawn in MEGA 2 (Kumar *et al.*, 2001)) employing the Tamura and Nei model (1993) and specifying a gamma distribution shape parameter (G) of 0.8033 from the Akaike Information Criterion (AIC) in modeltest (Posada and Crandall, 1998). Mid - point rooting was applied to the tree.

2.2.9.3 Maximum likelihood (ML)

From Model Test, the TrN +I+G nucleotide substitution model selected under the Akaike Information Criterion was used for ML analysis in PAUP*. Base frequencies for A, C, G and T were 0.2690, 0.2344, 0.1929 and 0.3037, respectively. In addition, proportion of invariable sites (I) of 0.5647 and a gamma distribution shape parameter (G) of 0.8033 were specified.

2.2.9.4 Maximum parsimony (MP)

Unweighted and weighted parsimony analyses were explored. For the unweighted parsimony analysis, all characters were unordered and had equal weights. 330 characters were constant and of the variable characters, 24 were parsimony uninformative and 50 were parsimony-informative. Heuristic search option was in effect. Starting trees were obtained via stepwise addition, the closest method was used for addition of sequences, and 1 tree was held at each step during stepwise addition. Branches were swapped using the tree-bisection-reconnection (TBR) algorithm. 200 bootstrap replications were performed to assess nodal support

Successive weighting using the rescaled consistency indices (RC) was also performed. The weighted MP search settings were similar to those stated above with the following exceptions: 383 characters had a weight of 1 and 21 characters had a weight other than 1.

2.3 RESULTS

2.3.1 Data statistics

Preliminary exploration of the nucleotide sequence data of the 102 OTUs (Operational Taxonomic Units) in MEGA v1.0 indicated that the four nucleotides do not occur at the same frequency. An average percentage composition of 29.1 % thiamine (T), 28.2 %

adenine (A), 24.1 % cytosine (C) and 18.6 % guanine (G) was observed. On average, a si/sv ratio of 4.1 was detected, clearly indicating a transitional bias. From the aligned amino acid sequences, 134 codons in length, it was shown that the majority of mutations were non-synonymous and occurred at the third base position. Of the 404 nucleotides, 333 were conserved, 71 were variable, 49 were parsimony informative and 22 were singletons (Fig 2.1). On amino acid level, 114 of the 134 codons were completely conserved and 20 were variable. Among the variable sites, 6 were parsimony informative and 14 were singletons.

2.3.2 Model Test and data statistics

The TrN nucleotide substitution model, invariable sites and gamma distribution were the chosen parameters for use in inferring phylogeny. The log likelihood score was $\ln L$ 1272.4698. The nucleotide base frequencies of the data under this model of nucleotide substitution differed from that calculated directly from the data and was as follows: A = 0.2690, C = 0.2344, G = 0.1929 and T = 0.3037. The proportion of invariable sites (I) was 0.5502 and the gamma distribution shape parameter was 0.8033.

2.3.3 Phylogenetic analysis

2.3.3.1 Minimum Evolution (ME)

The ME analysis with MEGA2, using the Tamura-Nei model and specifying a gamma distribution shape parameter of 0.8033 was consistent and yielded reliable results. Thirteen East African genotypes were discerned on the 1 % genetic distance basis specified previously for distinguishing different *p72* genotypes (Bastos *et al.*, 2003) (genotype XVI divided into 2 genotypes), and on the 55 % or higher bootstrap support values. 13 East African *p72* genotypes, together with the three reference viruses of non-East African genotypes (III, IV and VII), brought the total number of identified *p72*

genotypes to 16 (Fig. 2.2). Similar results were obtained with the neighbor-joining method.

2.3.3.2 Maximum Likelihood

When using the Model Test parameters and a starting NJ tree in ML analysis, the resulting phylogeny had a branching pattern that corresponded well with that obtained by ME analysis (results not shown).

2.3.3.3 Maximum Parsimony

82 unweighted parsimony trees were recovered at the end of the heuristic search, where 20344797 rearrangements were tried. All trees had a length of 114, and a consistency index (CI), retention index (RI) and re-scaled consistency index (RC) of 0.728, 0.969 and 0.706, respectively was obtained. The sum of minimum possible lengths was 83, while the sum of maximum possible lengths was 1086. Mean tree length was 114. A 50 % majority-rule consensus tree was obtained.

Successive weighting with the RC yielded an MP tree 72.52599 in length, and resulted in 6 equally parsimonious trees (Fig 2.3). As with the ML, ME and NJ methods, 13 East African and 3 non East African genotypes were discerned with MP. No difference in topology was noted between unweighted and reweighted parsimony analyses.

2.3.3.4 Interpretation of phylogenetic analysis

As trees with comparable topology were obtained with all methods of phylogenetic inference, the ME tree (Fig. 2.2) which was obtained using an optimality criterion and which co-incides with the method used to identify the 10 major p72 genotypes (Bastos *et al.* 2003), will be used to discuss the phylogenetic relationships of the 102 OTUs used in this study.

A total of sixteen *p72* genotypes were consistently recovered (Fig. 2.2), of which thirteen occur within the East African region (Fig. 2.4). Genotypes I - X correspond to those identified previously (Bastos *et al.*, 2003), whilst *p72* genotypes XI - XVI are reported here for the first time and are therefore regarded as novel (Table 2.3).

Genotype I (also referred to as the ESACWA genotype), initially identified in pig isolates from Europe, South America, the Caribbean islands and West Africa (Bastos *et al.*, 2003), was found in this study to be present in East African domestic pigs and sylvatic hosts such as bushpigs and ticks. Similarly, genotype V, X and XII viruses were recovered from both domestic pigs and from wild vertebrate and invertebrate hosts, indicating that the argasid tick vector moves readily between wild and domestic vertebrates within the regions in which these genotypes occur (Fig. 2.2). These four genotypes I, V, X and XII were shown to be present in three, one, four and two East African countries, respectively (Fig. 2.4), with some of these genotypes having a field presence of more than four decades (Table 2.3).

Genotypes XI, XIII and XIV appear to be associated exclusively with a sylvatic cycle as these viruses, which were collected in Zambia between 1983 and 1986, were all of tick (from warthog burrow) origin. Genotypes II, VI, VIII, IX, XV and XVI comprised exclusively domestic pig strains. Of these, II and VIII were confined to three countries each, and circulated for between 10 and 20 years in the field, genotype IX caused two temporally unrelated outbreaks in domestic pigs in 1995 and 2003, while the remaining genotypes were restricted to one country each and were associated with a single epizootic (and sometimes a single virus).

The molecular phylogeny further revealed the presence of three distinct evolutionary groups (labelled A - C in Fig. 2.2). Viruses sharing a common evolutionary history fall within the following genotype clades:

- (A) Genotypes I - VII (80 % bootstrap support)
- (B) Genotypes VIII and XI - XVI (66 % bootstrap support)
- (C) Genotypes IX and X (100 % bootstrap support)

Levels of mean intra-genotypic variation ranged from 0 % (genotypes II, VI, IX and XVI) to 0.7 % (genotype XII), whilst mean inter-genotypic levels of variation ranged from 0.9 % (between genotypes V and VI, and XI and XII) to 8.0 % (between genotypes V and X). The maximum level of sequence divergence between any two isolates was 9.8 %.

Comparison of the findings of this investigation and that of Sumption (1992) displayed both similarities and differences. In the RFLP study *BamHI* restriction enzyme fragment patterns were generated in order to compare and characterize isolates from Zambia, Malawi and other African countries (Sumption, 1992; Table 2.2).

The first group consisted of isolates from Malawi and Zambia mainly, and one from Mozambique, MOZ60 (Table 2.2). This group corresponds well with *p72* genotype VIII since it involved identical isolates to those used in this study, or country and location representatives thereof (Fig. 2.2). However, the Mozambican isolate was classified within a different *p72* genotype (genotype V) by Bastos and co-workers (2004). The second group consisted of isolates from West Africa, Democratic Republic of Congo and Burundi. With the exception of the Burundi isolate, this group was identical to the ESACWA genotype (genotype I) by composition. Tengani 1962 and Mozambique 1979 constituted the third group. In this study and that of Bastos *et al.* (2004), Tengani60 and Malawi 2002/1, and Tengani60, Moz79 and Moz60 represented genotype V. LIV13/33 and HINDE54 comprised group IV. Contrary to RFLP, *p72* genotyping clustered LIV13/33 with the ESACWA isolates, while available viruses from HINDE formed part of genotype X. Similar to *p72* genotyping, tick isolate SUM14/11 formed a unique group, while MFUE 6/1 clustered with a pig isolate, MZI92/1, on the contrary. TAN87 also resulted in a unique restriction fragment pattern.

	111222	2355667778	8899900133	11111	1111122222	2222222222	2222223333	3333333333	3333333334
IC3/96 ¹	2679024457	9902031285	6713658025	4568900111	1233444456	667890123	4455566778	8888899990	
Lisbon ⁵⁷ ¹	TGCTATAGAA	GTCCAGTGA	GCGTGCATCA	TTGTCCCCAG	ACCTGTGCT	TGGCTCCAT	CGGCTCTTTA	TGGCTCTTA	
LIV10/11/ZAM/T(W)C.....G.....	
VICT90/1 ¹C.....G.....A.....	
LIV9/35/ZAM/T(W)T.....G.....	
LIV5/40/ZAM/T(W)T.....A.....	
LIV9/31/ZAM/T(W)T.....A.....	
NAM1/80 ¹C.....T.....	
ZAM021/PC.A.....T.....	
AF159503 ²TG.C.....T.....	
ZAM014/PC.A.....	
ZAM013/PC.A.....	
MAD1/98 ¹C.T.....A.....G.....	
BOT1/99 ¹C.T.....G.....A.....G.C.....C.....T.....C.....
RSA1/99W ¹C.T.....A.....G.....A.....G.C.....TT.....T.....
MOZ/94/1 ¹C.T.....C.....T.....A.....G.C.....C.....T.....
MAL2002/1/PC.T.....C.....T.....A.....G.....C.....T.....A.....
Tengani/60 ¹C.T.....C.....T.....A.....G.....C.....T.....A.....
MOZ/1960 ¹C.T.....C.....T.....A.....G.....A.....T.....
RSA1/98 ¹C.T.....A.....G.....G.TCT.....T.....T.....C.....
NYA1/2/ZAM/T(W)A.....C.T.A.....T.....T.....GT.....C.....T.....C.....
TAN/2003/2/PC.T.A.....T.....T.....T.....GTTC.....C.....T.....C.....
TAN101/PA.....C.T.A.T.....T.....T.....GTT.....C.....T.....C.....
SUM14/11/ZAM/T(W)A.....C.T.A.....T.....T.....G.C.C.....C.....T.....CGC.....
MZ1921/MAL/PA.....GC.T.A.....T.....T.....G.C.C.....C.....T.....TC.....
MFUE6/1/ZAM/T(W)C.....A.....GC.T.A.....T.....T.....G.C.C.....C.....T.....
KAB6/2/ZAM/T(W)A.....C.T.A.....T.....T.....T.....G.C.C.....C.....T.....
NDA1/90 ¹A.....C.T.T.....T.....T.....T.....G.T.C.....C.....TA.TC.....
SAL921/MAL/PT.TA.....C.T.A.....T.....T.....T.....G.T.C.....C.....TA.TC.....
TMB89/1/ZAM/PA.....C.T.A.....T.....T.....T.....G.T.C.....C.....TA.TC.....
PHW88/1/ZAM/PA.....C.T.A.....T.....T.....T.....G.T.C.....C.....TA.TC.....
JON/89/13 ¹A.....C.T.A.....T.....T.....T.....G.T.C.....C.....TA.TC.....
CHG881/ZAM/PG.....A.....C.T.A.....T.....T.....G.T.C.....C.....TA.TC.....
KLI882/ZAM/PA.C.....C.T.A.....T.....T.....T.....G.T.C.....C.....TA.TC.....
SIY91/2/MAL/PG.....A.....C.T.A.....T.....T.....G.T.C.....C.....TA.TC.....
UGA/1/95 ¹C.G.....T.CCT.....C.A.T.....T.....C.A.....T.....G.C.C.....A.C.....
MWHOG9/KEN/WC.G.....T.CCA.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
BARTLETT2/KEN/WC.AG.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
GASSON/KEN/PC.G.....T.CCAT.....C.T.A.T.G.....C.....A.....T.....T.....G.C.C.....
DOIG/KEN/WC.G.....T.CCAT.....C.T.A.T.G.....C.....A.....T.....T.....G.C.C.....
HindeI1/59 ¹C.G.....T.CCAT.....C.T.A.T.G.....C.....CA.....T.....T.....G.C.C.....
Uganda ⁴C.G.....T.CCAT.....C.T.A.T.G.....C.....A.....T.....T.....G.C.C.....
KILLEANI/KEN/WC.G.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
HINDEI/KENC.G.....T.CCAT.....C.T.A.T.G.....C.....A.....T.....T.....G.C.C.....
TRENCH/KEN/WCAG.....T.CCAT.....C.T.A.T.G.....C.....A.....T.....T.....G.C.C.....
DAVIS/KEN/WCT.G.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
MWHOG1/KEN/WC.G.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
UGA/3/95 ¹C.G.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....
KIRW89/1/TAN/WC.G.....T.CCAT.....C.A.T.G.....C.....A.....T.....T.....G.C.C.....

Fig. 2.1 Nucleotide sequence alignment showing the variable sites of the 49 unique sequences identified in this study and in previous studies. Dots indicate nucleotide sites identical to that of the master sequence, IC3/96. Superscript numbers 1 to 4 indicate those viruses included in the studies of Yu *et al.*, 1996, Odemuyiwa *et al.*, 2000, Bastos *et al.*, 2003 and Bastos *et al.*, 2004, respectively.

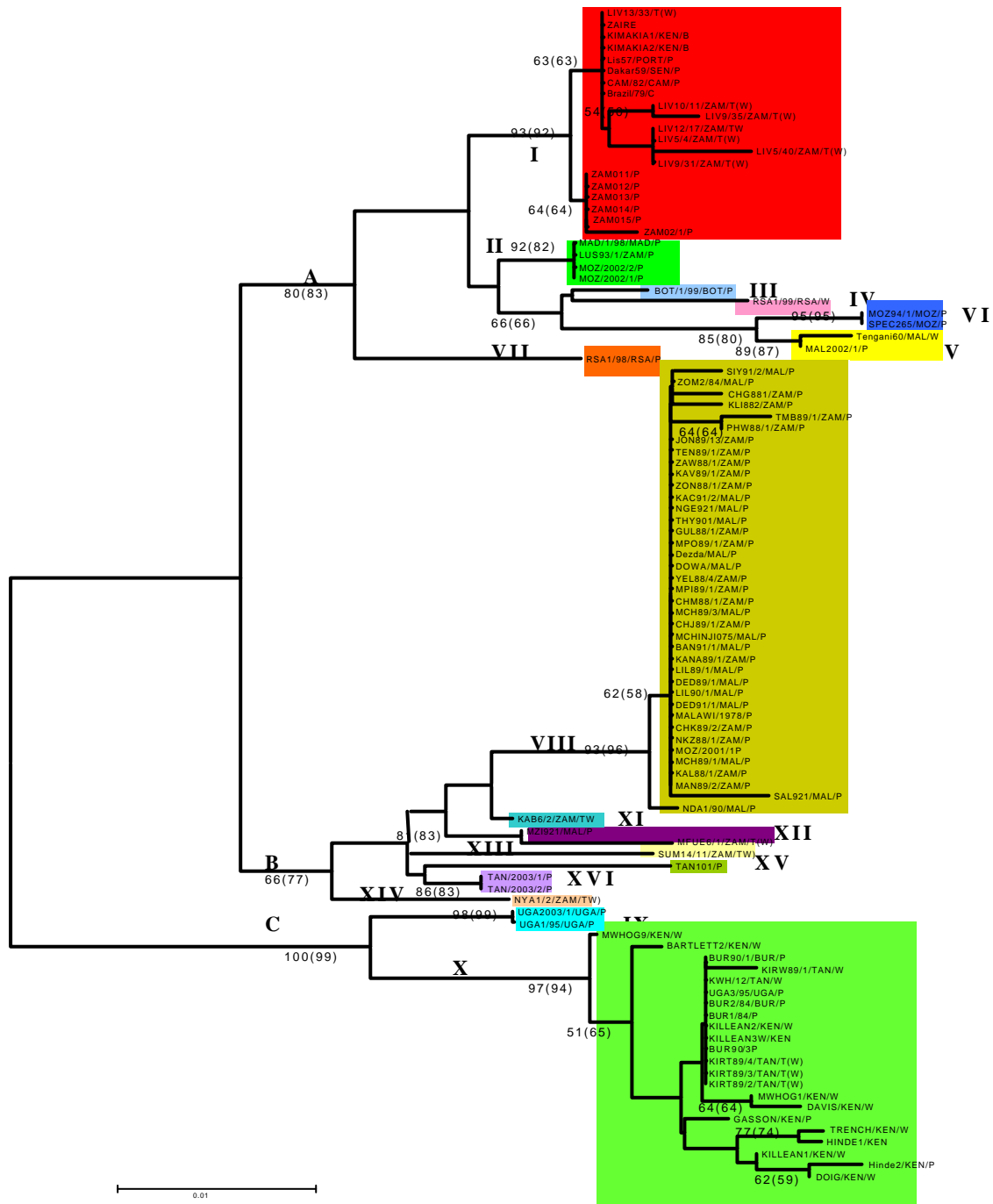
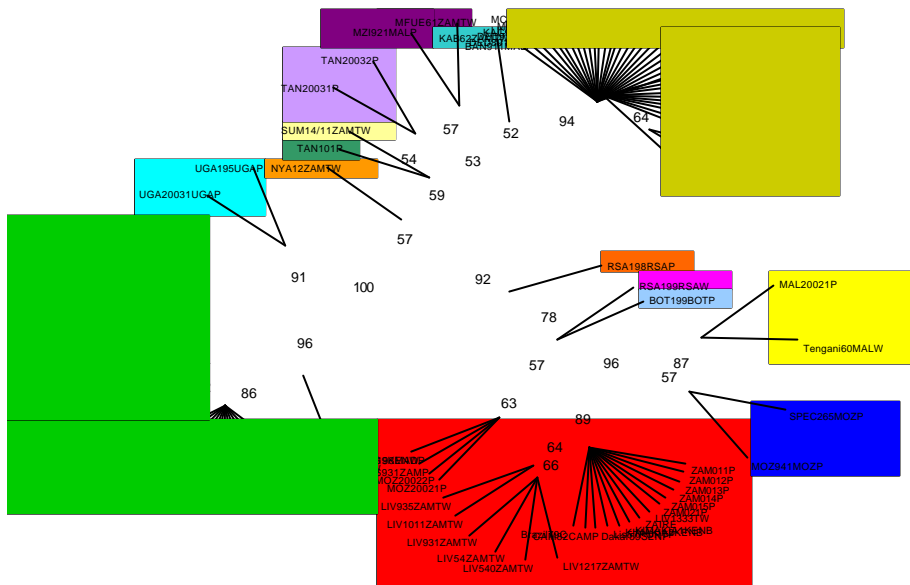


Fig. 2.2 Minimum evolution tree depicting the 16 ASFV *p72* genotypes (labeled I-XVI) and three main evolutionary lineages (labeled A – C). Bootstrap values > 50% and obtained by ME method are indicated next to the relevant node, whilst those obtained by NJ technique are indicated in brackets. All bootstrap values were obtained following 10 000 replications.



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Fig. 2.3 MP tree obtained with successive weighting with the RC showing p72 phylogenetic relationships of the 102 OTU used in this study. Bootstrap support of >50% are indicated next to the relevant nodes.

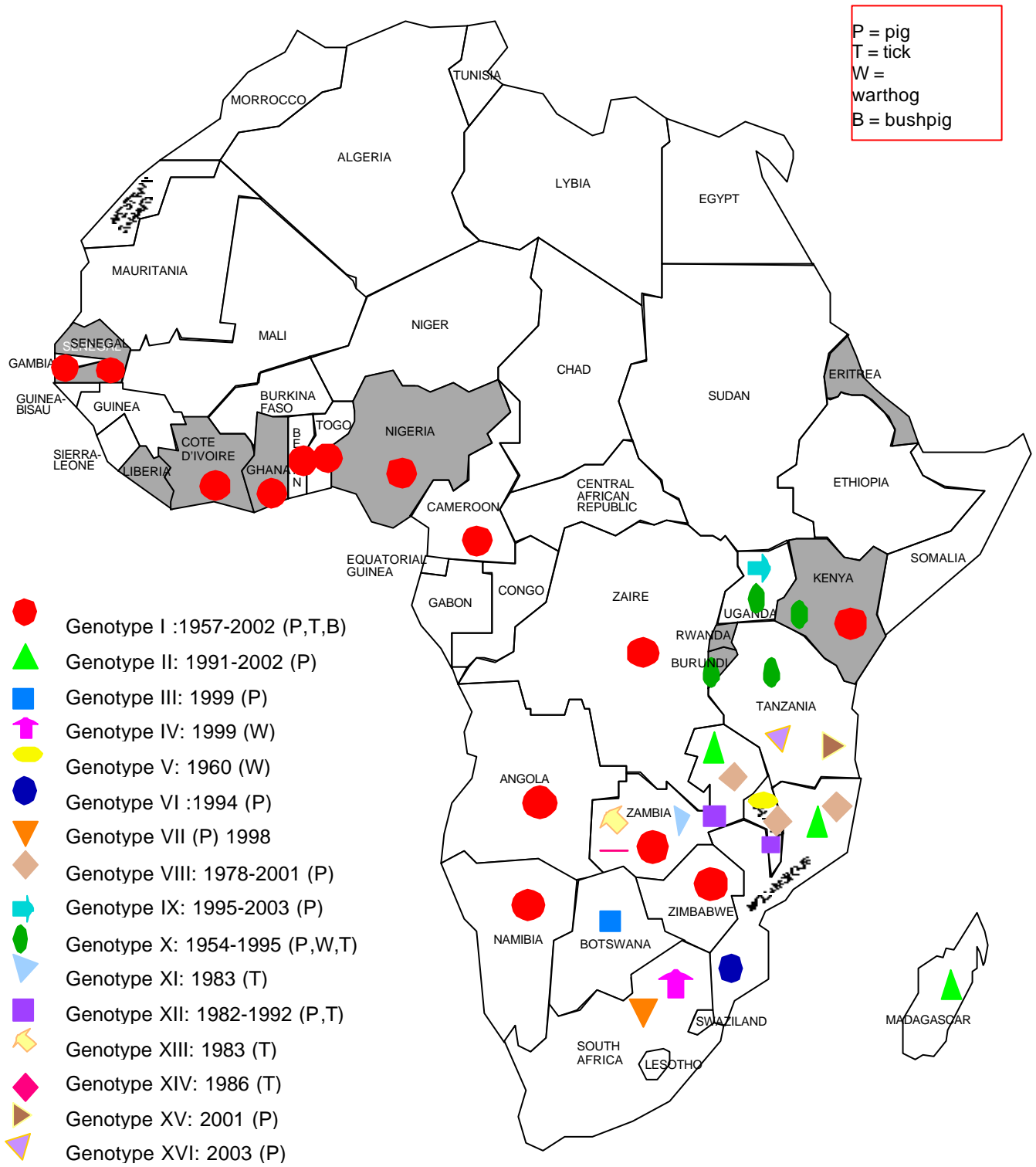


Fig. 2.4 Geographical distribution of the 16 major African Swine Fever virus genotypes identified by partial *p72* genotyping

Table 2.3 Summary of the distribution, field presence and intra-genotypic variation of the major African swine fever virus *p72* genotypes resolved in this study using 102 viruses and comparison with 39 virus sequences from previous studies (Lopez-Otin *et al.*, 1990; Yu *et al.* 1996; Odemuyiwa *et al.*, 2000; Bastos *et al.*, 2003, 2004). The isolates used for comparison were not included for phylogenetic analysis in this study.

Genotype	Representative countries	Presence in the field	Species affected	No. of viruses	No. of countries	Mean intragenotypic nucleotide variation
I	Zambia, Kenya, Zaire, Cameroon, Ghana, Senegal, Nigeria, Gambia, Benin, Côte d'Ivoire, Togo, Angola, Zimbabwe, Namibia, Portugal, Brazil, Spain, Sardinia, Malta, Holland, Belgium, Dominican Republic	1957 - 2002	Bushpig Domestic pig Tick (warthog)	57	22	0.2 %
II	Mozambique, Zambia, Madagascar	1991 - 2002	Domestic pig	5	3	0.0 %
III	Botswana	1999	Domestic pig	1	1	--
IV	Republic of South Africa	1999	Warthog	1	1	--
V	Malawi	1960 - 2002	Domestic pig Warthog	2	1	0.4 %
VI	Mozambique	1994	Domestic pig	3	1	0.0 %
VII	Republic of South Africa	1998	Domestic pig	1	1	--
VIII	Zambia , Malawi and Mozambique	1978 - 2001	Domestic pig	39	3	0.1 %
IX	Uganda	1995 - 2003	Domestic pig	2	1	0.0 %
X	Uganda, Burundi, Tanzania, Kenya	1954 - 1995	Domestic pig Tick (warthog) Warthog	22	4	0.6 %
XI	Zambia	1983	Tick (warthog)	1	1	--
XII	Malawi and Zambia	1982 -1992	Domestic pig Tick (warthog)	2	2	0.7 %

Genotype	Representative countries	Presence in the field	Species affected	No. of viruses	No. of countries	Mean intragenotypic nucleotide variation
XIII	Zambia	1983	Tick (warthog)	1	1	--
XIV	Zambia	1986	Tick (warthog)	1	1	--
XV	Tanzania	2001	Domestic pig	1	1	--
XVI	Tanzania	2003	Domestic pig	2	1	0.0 %

Table 2.4 Average p-distance (in %) between ASFV genotypes discerned by *p72* nucleotide sequencing

Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	1.1															
3	2.1	1.5														
4	2.4	1.8	1.3													
5	2.5	1.9	2.4	2.2												
6	2.7	2.1	2.0	1.8	0.9											
7	2.6	2.0	2.0	2.8	3.5	3.1										
8	4.0	3.9	4.4	5.3	4.9	5.6	3.9									
9	5.9	5.2	5.2	5.5	6.8	6.4	5.2	5.8								
10	6.7	6.4	5.9	6.2	8.0	7.6	6.5	7.0	2.5							
11	3.4	3.3	3.3	4.1	4.3	4.4	3.3	1.1	4.7	5.9						
12	3.8	3.7	3.7	4.5	5.2	4.8	3.7	1.9	5.0	6.3	0.9					
13	4.2	4.1	4.1	4.9	5.3	5.2	4.1	2.6	5.4	6.4	1.5	2.1				
14	2.9	2.8	3.3	4.1	4.3	4.4	3.3	2.6	5.2	6.4	2.0	2.3	2.8			
15	4.3	4.1	4.7	4.9	5.1	5.3	4.1	2.4	6.1	7.1	2.3	2.7	3.1	2.3		
16	3.2	3.1	3.1	3.9	4.6	4.2	2.5	1.8	4.4	5.7	1.3	1.6	2.0	1.8	1.5	

2.4 DISCUSSION

The presence of six more East African genotypes than was previously identified (Bastos *et al.*, 2003) was revealed by the molecular phylogeny. Genotypes I, V, X, XI, XIII and XIV are examples of viruses that are present within a sylvatic cycle (occurring either within eyeless tampans or sylvatic vertebrate hosts, or both), half of which have also caused outbreaks in domestic pigs. Genotype XII which comprised two viruses isolated 10 years apart from a tick and domestic pig may be an example of a pig-tick cycle but this requires confirmation by more intensive screening of sylvatic vertebrates within Malawi and Zambia. The pig-to-pig cycle is, however, classically exemplified by genotype VIII, which has been in active circulation for at least 23 years and is represented by 39 outbreak strains from three countries. A genetic feature of a domestic pig cycle appears to be a pronounced lack of genetic variation, as both genotype I in West Africa (where it has only been isolated from domestic pigs) and genotype VIII in East Africa have extremely low levels of intratypic variation (0.2 % and 0.1 %, respectively), with most isolates being identical to each other. This lack of genetic variation was also found following restriction enzyme profiling of some genotype VIII viruses from Malawi (Sumption *et al.*, 1990). Although sample sizes of the remaining genotypes are inadequate to permit speculation on the epidemiological cycles into which they may be classified, the results indicate that all three ASF epidemiological cycles appear to exist in East Africa. Wherever a sylvatic cycle is confirmed, higher levels of genetic variation are recovered (Dixon and Wilkinson, 1988; Bastos *et al.*, 2003).

The East African region is the most genotype rich, with thirteen *p72* genotypes being identified. This far exceeds West Africa, which only contains one genotype in the ten countries previously screened (Bastos *et al.*, 2003). Southern Africa is intermediate with at least eight genotypes (when Mozambique is excluded from the results) being identified thus far (Boshoff *et al.*, 2004). While many of the East African genotypes are apparently country specific (V, VI, IX, XI, XIII, XIV, XV and XVI), others (I, II, VIII, X and XII) are not restricted by national boundaries. In addition, most countries within the East African region have more than one genotype within their borders. Zambia is particularly

genotype rich with seven genotypes being identified, followed by Malawi, Mozambique and Tanzania with three each, and Kenya and Uganda with two each.

The ASFV introduced to Europe through illicit movement of pig products (Manso-Ribeiro *et al.*, 1958) and which is widespread throughout West Africa (Bastos *et al.*, 2003) may have its origins in East Africa as this study revealed it to be present in the natural sylvatic hosts in Kenya (Kimakia I and Kimakia II viruses) as far back as 1961. As the classical ASFV transmission mode involving a sylvatic cycle could not be proven in West Africa (Penrith *et al.*, 2004), it is likely that the disease was originally introduced from the East of the continent before becoming established in a domestic pig cycle in West Africa. The more widespread distribution of the ESACWA genotype identified in this study makes this genotype the most successful and extensively distributed genotype, being present in 22 countries described to date.

The low levels of intratypic genetic diversity within the large and homogeneous genotype VIII necessitates an investigation into a more variable gene region in order to clarify within genotype relationships. In addition, the possibility that genotype I, which was previously believed to be confined to West Africa, originated from East Africa should be confirmed through sequencing of an alternative and more informative gene region. By focusing on typing ASFV from domestic pigs in East and Central African countries, where genotype I is present in the sylvatic hosts, it may also be possible to trace the route of entry of this virus into West Africa.

Lineage A (Fig. 2.2) has an interesting evolutionary history since it constitutes the first European isolate, Lisbon57. This group consists of seven genotypes, involves all epidemiologically important ASFV hosts, and is found on at least three continents (Africa, Europe and South America). Links between East, Central and West Africa, and Europe demonstrated by genotype I in this study, make it the most widespread lineage of all the ASFV lineages identified in Africa and elsewhere. Lineage B constitutes seven East African genotypes, contains all epidemiologically important ASFV hosts, with the exception of bushpigs, and exemplifies all three ASF epidemiological cycles mentioned

by Penrith and co-workers (2004). Lineage C, which consists of two East African genotypes is basal to lineages A and B pointing to East Africa being the possible geographical origin of ASF viruses.

Comparison of the RFLP genotyping results of the study conducted by Sumption (1992), to those obtained with *p72* genotyping, clearly showed that most of the viruses clustered in comparable groups with the exception of MOZ60, BUR84/1 and LIV13/33 (Table 2.2; Bastos *et al.*, 2003; Lubisi *et al.*, 2005). The dissimilarities may have arisen from the following facts:

- 1) The two methods (RFLP and *p72* genotyping) focused on different parts of the genome, with RFLP characterizing differences in the left and right variable termini, whilst *p72* genotyping targets a gene coding for a major capsid protein;
- 2) The *BamHI* restriction fragments a'b spanned a region of at least 1.6 kb, but only a fraction of this was actually characterized since the endonuclease only recognized the 5' GGATCC 3' sequence (Stryer, 1988), thus the absolute genome size examined was less than the 404 bp region used for *p72* genotyping;
- 3) The genome area used in RFLP consisted of at least 2 variable regions as well, VR1 and VR2, while one gene of conserved length, *p72*, was the center of focus in the other method;
- 4) VR2 was found to be the most variable of all 8 VR located in the ASFV genome (Sumption, 1992), with deletions and insertions causing up to 9100 bp size differences between isolates, while the *p72* gene shows no size variation and is unaffected by passage on Monkey cells;
- 5) Even though the viruses displayed similar *BamHI* a'b restriction fragment patterns in the RFLP study, they may have had different nucleotide sequences which could have occurred naturally over time or with serial passages into different susceptible cells (Table 2.2). The latter differences may not necessarily affect the genome size but could alter virus classification into genotypes, as could have been the case with the grouping of MOZ60, BUR84/1 and LIV13/33.

The 9.1 kb size difference between *BamHI* a'b could also have been too large and too variable for genotyping viruses from diverse geographic locations, species of origin and temporal occurrence. On average, there is a 4.9 % nucleotide difference between *p72* genotypes V and VIII (RFLP group I) in which MOZ60 is classified using the two methods (Table 2.4 and Bastos *et al.*, 2004). Between *p72* genotype I (RFLP group II equivalent) where LIV13/33 clusters and X in which BUR84/1 and most HINDE viruses are categorized, an average of 6.7 % nucleotide difference exists (Table 2.4). Clearly, these differences are large and warrant classification into different genotypes as the more conserved *p72* gene characterization method has done. Perhaps the utilization of less variable *BamHI* fragments from the corresponding right terminal region could yield more agreeable results between the two methods.

Both the sylvatic and domestic pig cycles appear to play an important role in the epidemiology of ASF in East Africa. The existence of multiple genotypes within countries, trans-boundary distribution of genotypes between countries and regional genotype richness adds to the complexity of ASF epidemiology in East Africa. As genotyping in this study was based on partial characterization of the gene coding for the immunodominant protein VP72, future vaccination campaigns could utilize this information when formulating vaccine for specific countries, since immunizing pigs with antigens from viruses distantly related to those with which they are challenged offers less protection (Boinas *et al.*, 2004; Neilan *et al.*, 2004). These factors are important considerations that need to be taken into account for effective control of the disease in East Africa.

Chapter 3

Intra-genotypic resolution of *p72* genotype VIII virus relationships by Central Variable Region characterization

SUMMARY

Thirteen *p72* genotypes were identified within eight East African countries, of which one, genotype VIII, constituted a domestic pig cycle that was homogenous across the *p72* gene studied. In order to resolve within genotype relationships, sequencing of an amino acid tetrameric repeat region located within the *9-RL* Open Reading Frame (ORF), and termed the central variable region (CVR), was performed. Eight discrete genetic lineages, designated A-H were recovered by phylogenetic analysis of the aligned amino acid sequences. The largest CVR lineage (group A), comprised 25 isolates, from Zambia, Malawi and Mozambique with identical CVRs 84 amino acids (aa) in length. These group A viruses which caused outbreaks over a 23 year period indicate that the repeat region in these viruses with an extended field presence, is extremely stable. The next largest CVR cluster (G) comprised 10 viruses causing outbreaks over a 10 year period in Zambia, Malawi and Mozambique. Taken together, these results indicate that transboundary transmission of disease between the afore-mentioned three East African countries occurs readily. The remaining six intra-genotypic clusters comprised three or less viruses from outbreaks in individual counties, with a maximum circulation period of three years being identified. Intra-genotypic resolution by CVR has clearly been beneficial for identifying epidemiological links between different outbreaks caused by genotype VIII viruses, and is therefore advocated for clarifying viral relationships within homogenous *p72* genotypes that are associated with the domestic pig cycles.

3.1 INTRODUCTION

Virus genome characterization is increasingly being utilized in the quest for taxonomical classification, discerning evolutionary and epidemiological patterns, and differentiation on the basis of pathogenesis and virulence in the field of virology (Becker *et al.*, 1992; Gray *et al.*, 2001; Walker *et al.*, 2001; Costas, 2002; Shchelkunov *et al.*, 2002). Highly conserved genome regions, characterized by their low mutation rates, have been used successfully to group viruses into various genotypes (Vanderhallen *et al.*, 1999; Fenaux *et al.*, 2000; Bastos *et al.*, 2003). These regions, however, are often too stable for finer resolution of viruses within individual genotypes.

Genome regions that are variable to hyper variable have proven useful in the endeavors of scientists to determine close relationships or further resolve within genotype or serotype relationships (Bornkamm *et al.*, 1980; Sidagis *et al.*, 1997; Duffy *et al.*, 2002; Gao *et al.*, 2003; Gonz'alez-Candelas *et al.*, 2003; Zheng *et al.*, 2003). A considerable number of these regions are sequence repeats, usually in the form of long terminal repeats (LTR) (Rohn *et al.*, 1998; Chen *et al.*, 1999; Yamashita *et al.*, 1999) or short tandem repeats (STR) (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994; Tautz and Schlotterer, 1994; Field and Wills 1996, 1998).

The short tandem repeats, also called microsatellites, have been identified in viruses such as Herpes simplex, Epstein – Barr, Mengovirus, Vesicular Stomatitis (VSV), Hepatitis C, Human Respiratory Syncytial and Avian Influenza (Umene *et al.*, 1984; Duke *et al.*, 1990; Garcia - Barreno *et al.*, 1994; Yamada *et al.*, 1996; Barr *et al.*, 1997; Perdue *et al.*, 1997). To cite a few examples, Chen *et al.* (1999) as well as Yamashita *et al.* (1999) sequenced the LTR of Human T cell Leukemia virus (HTLV) type 1 to obtain finer resolutions of relationships within the serological type, and bring new insights into the possible origins of these viruses in America and Asia. The long terminal repeats of the cDNA of HIV type 1 viruses were used for subtyping (Harris *et al.*, 2003). To differentiate oncogenic and non-oncogenic strains of Marek's disease virus (MDV) type 1 by polymerase chain reaction DNA amplification, primers amplifying a region within the long inverted region

(Zhu *et al.*, 1992) and those amplifying tandem direct repeats of MDV 1 DNA were utilized (Silva, 1992).

Similar to the above viruses, ASFV from Malawi and Zambia have previously proven to be highly related phylogenetically (Sumption, 1992; Lubisi *et al.*, 2003). An exclusive study of isolates from outbreaks which occurred in Malawi between 1982 and 1989, and Zambia from 1986 to 1989, aimed at investigating the degree of relatedness of field virus isolates within and between the two countries using RFLP, was conducted by Sumption (1992). All *BamHI* restriction fragments were utilized to discern relationships of viruses within each country. Eighteen isolates from Malawi were compared and these resulted in 12 genotypes, consisting of 8 unique patterns and 4 groups of patterns formed by 10 isolates (Table 3.1). Of these genotypes, 7 comprising the Mchinji group, had 22 *BamHI* fragments, while the remainder had 23 fragments. The genome lengths, estimated from the summation of the fragment sizes, ranged from medium (178 kb) to large genomes (182 kb). Seven *BamHI* fragments, a,b,c,t,u,v and w were responsible for the variable mobility, except in seven genotypes where the fusion of fragments a and b into fragment a'b, reduced the fragment number to six. Large mobility variation was observed for fragments a and b or a'b, especially in isolate MCH87 which had a large combined a and b length of 33 kb, and isolate TIK82 with 36 kb. Dissimilar sizes and frequency of variation were recorded for fragments u which varied in size between 4.8 and 4.9 kb, t that showed a variation in length for 7 of the 12 genotypes discerned, and v which had a shorter length range of 2.75 to 2.9 kb. Apart from differences in the RFLPs of the above mentioned fragments and the loss of a *BamHI* site between a and b in the Mchinji viruses, loss of *BamHI* site between s and t fragments in the Dowa'86 isolate was another form of variation found among Malawi viruses. Fragments a - c were located within 50 kb from the left terminus of the genome while t - w were found within 25 kb from the right terminus. Fragments c and w showed similar size and frequency of variation among the isolates.

In the case of Zambia, 36 virus isolates resulted in 13 genotypes, with 6 viruses yielding unique restriction patterns while the remaining isolates formed 7 genotypes (Table 3.1).

All isolates had 22 *Bam*HI fragments, and the genome size ranged from short (172 kb) to medium (178 kb). Unlike the Malawi viruses, 5 fragments, a'b, c, t, v and w had variable mobility, with fragment a'b showing the largest mobility variation among the isolates. PHW88/1 and MAN89 had a'b fragment sizes of 33 kb and 31 kb respectively. Contrary to the Malawi virus *Bam*HI restriction fragments u, t and v which were responsible for dissimilar size and frequency of variation, minimal or no variation was observed in the t and v fragments, and the longer lengths of fragments caused the length variability among the Zambian isolates. RFLP was the sole criterion used in the comparison of Zambian viruses. Similar to the Malawian viruses, the variable regions were located in the 50 kb left terminal end (a - c) and the right terminal 25 kb (t - w). Fragments c and w in isolates from both countries showed similar size and frequency of variation.

Table 3.1 ASFV genotypes from Malawi and Zambia discerned by *Bam*HI restriction fragment pattern analysis (Sumption, 1992)

Malawi		Zambia	
Genotype	Virus	Genotype	Virus
1	TIK82	1	CHA86; CHGT88; KAZ88; ZAW88*
2	KON83	2	CHG88/1*; CHG88/2; ZON88/3; KAV89/1*; KAVT89/1; KAVT89/2; KAVT89/3; KAN/A89; KAN/B89
3	BON83	3	MWE88; NKZ88*
4	LIL20/1	4	PHW88*
5	NAD84; ZOM84*	5	NYA88*
6	DOWA86*; LIL89/3	6	CHS88; NYM88
7	DED86	7	KLI88*
8	MCH87; MCH87/2	8	KAL88*; KEF89/1; KEF89/6A; KEF89/6B; CHK89*
9	LIL89/1*	9	GUL88*
10	MCH89/1*	10	YEL88/5; CHM88/2; MPI89/1*
11	LIL89/2; DED89/1*; DED89/2; NTC88	11	MAN89*
12	MCH89/3*; TEN 89 (Zambia)	12	TEN89/1*; JON89/13*; CHJ89/1*; MPO89/1*
		13	TMB89*

*Isolates common to this study and that of Sumption (1992)

Sumption (1992) investigated relationships between ASFV from Malawi and Zambia in a number of different ways. One method involved the grouping of isolates according to increasing *BamHI* a and b or a'b fragment lengths, while another focused on fragment deletion characteristics in the left terminus of the genome. The first method resulted in 6 virus groups, while the second resolved 7 virus classes (Table 3.2). Five of the 23 genotypes however failed to cluster in the same genotypes when the two methods were compared.

Table 3.2 Classes of ASFV genotypes from Malawi and Zambia obtained by comparison of restriction fragment lengths (A) and deletion characteristics in the left terminus of the genome (B) (Sumption, 1992)

Reference genotype	A		B	
	Class	No. of genotypes within group	Class	No. of genotypes within group
CHA86 (Zambia)	I	11	VII	11
MAN86 (Zambia)	II	1	VI	1
BON83 (Malawi)	III	4	V	4
LIL20/1 (Malawi)	IV	5	IV	1
			III	4
KON83 (Malawi)	V	1	II	1
TIK82 (Malawi)	VI	1	I	1

Subgrouping of the shared genotype classes was also conducted, with groups assigned according to the number of *BamHI* restriction fragment pattern mobility differences. Virus sets which resulted from categorizing genotypes according to differences in the mobility of single *BamHI* restriction fragments, and in the mobility of two or less fragments are shown in Table 3.3.

Table 3.3 Genotype sets resolved by differences in the mobility of single *Bam*HI restriction fragments (I), and in the mobility of two or less fragments (II) (Sumption, 1992)

Set	Method	
	I	II
1	TEN89; TMB89; YEL88; MWE88; KAL88	TEN89; TMB89; YEL88; MWE88; KAL88; CHG88; GUL88
2	MCH87; BON83; MCH89/1	CHS88; TEN89; CHG88; KAL88
3	CHG88; GUL88	TMB89; MAN89
4	NYA88; KLI88	MAN89; BON83; KON83
5	PHW88; CHA86	NYA88; KLI88; CHA86
6	LIL20/1; LIL89/1	

Subgrouping within the 6 groups (I – VI) discerned by *Bam*HI a and b / a'b fragment size differences, focusing on single differences in fragment mobility, resulted in 3 subgroups within group I designated A – C, and one subgroup each for groups III and IV designated A (Table 3.4).

Apart from the left and right ends of ASFV genome, approximately 35 kb and 15 kb in size respectively, which vary primarily on the basis of length (Wesley and Tuthill, 1984; Blasco *et al.*, 1989 a, b), there is a central variable region (CVR) of up to 686 bp (Phologane *et al.*, 2005) within the 125 kb long central conserved region. This central variable region (CVR) occurs within a late viral gene, termed 9 – *RL* (Sumption *et al.*, 1990; Irusta *et al.*, 1996). The molecular basis of the CVR variation is due to variation in the number and type of tandemly repeated amino acid tetramers and analysis of this region has shown a high degree of variation among different ASFV isolates as well as between parental strains and their cell culture adapted strains (Irusta *et al.*, 1996). These

discoveries prompted an investigation into the possibility of using this gene for within genotype resolution of ASF viruses (Bastos *et al.*, 2004; Phologane *et al.*, 2005).

Table 3.4 ASFV genotypes and subgenotypes from Malawi and Zambia discerned by RFLP (Sumption 1992)

Virus	Country	Genotype	Group	Subgroup
MCH89/3	Malawi	1	I	
CHA86 ZAW88 CHGT88 KAZ88	Zambia	1	I	
CHS88	Zambia	6	I	
KEF89	Zambia	8	I	
TEN89	Zambia	12	I	A
YEL88 MPI88	Zambia	10	I	A
KAL88	Zambia	8	I	A
	Zambia	3	I	A
CHG88 KAV89 KAVT89/1-4	Zambia	2	I	B
KLI88	Zambia	7	I	C
NYA88	Zambia	5	I	C
MAN89	Zambia	11	II	
LIL89/2	Malawi	11	III	
PHW88	Zambia	4	III	
BON83	Malawi	3	III	A
MCH87/1-2	Malawi	8	III	A
MCH89/1	Malawi	10	III	A
NAD84	Malawi	5	IV	
DOW86	Malawi	6	IV	
DED86	Malawi	7	IV	
Virus	Country	Genotype	Group	Subgroup
LIL20/1	Malawi	4	IV	A
LIL89/1	Malawi	9	IV	A
KON83	Malawi	2	V	
TIK82	Malawi	1	VI	

Following the promising results that Bastos *et al.* (2004) obtained when differentiating intra-genotypic relationships of viruses of Mozambican origin, and the identification of at least four discrete CVR size classes for genotype VIII viruses (Phologane *et al.*, 2005), this gene was targeted for characterization in this study to attempt to resolve relationships within the homogeneous *p72* genotype discerned previously (Chapter 2) in East Africa.

3.2 MATERIALS AND METHODS

3.2.1 Viruses

All viruses which constituted genotype VIII on *p72* genotyping in chapter 2 (Fig. 2.1) were selected for CVR typing. Five additional genotype VIII viruses and one isolate from a different genotype were obtained from Bastos *et al.* (2004) bringing the total number of viruses used in this study to 45 (Table 3.5).

3.2.2 DNA extraction and CVR - PCR

The DNA extracted for *p72* genotyping purposes in the previous chapter was utilized in this investigation. PCR mixtures were prepared in which the CVR and flanking regions were amplified using the following primers with a forward and reverse orientation, respectively:

CVR-FL1 (5' TCG GCC TGA AGC TCA TTA G 3') and CVR-FL2 (5' CAG GAA ACT AAT GAT GTT CC 3') (Bastos *et al.*, 2004). All reactions were performed in a final volume of 50 μ l containing 200 μ M dNTPs (Roche), 0.4 μ M of each primer, 2.5 U of thermostable Taq DNA polymerase (Roche) and 3 μ l of DNA extract. The templates were amplified following 35 cycles of denaturation at 96 °C for 12 s, annealing at 52 °C for 20 s and extension at 70 °C for 25 s. These cycles were preceded by an initial incubation step of 96 °C for 20 s, and followed by an elongation step of 70 °C for 1 minute.

3.2.3 Agarose gel electrophoresis and molecular weight estimation

The amplified DNA products were loaded on a 1.5 % agarose gel and run against a 100 bp DNA ladder (Promega). Once sufficient electrophoretic separation was obtained, products were visualized by UV irradiation and ethidium bromide staining. The gels were photographed in order to obtain a hard copy record of the results from which to measure the distance migrated by the samples. A linear graph and standard curve were plotted using the log of the molecular weight (MW) of each fragment in the 100 bp ladder against the distance migrated (mm). The molecular weights of the CVR-PCR products were then determined by extrapolation of the standard curve, where the log of the molecular weights corresponding to the estimated distances that each of the CVR fragments had migrated, were read using Microsoft Excel.

3.2.4 PCR product purification and nucleotide sequencing

CVR products were excised from the agarose gel and purified using the Nucleospin kit (Machery-Nagel) according to the manufacturer's specifications. The nucleotide sequence of the purified product was determined by automated sequencing on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using a Big Dye version 3.1 kit and a 47 centimeter capillary.

3.2.5 Sequence alignment

Forward and reverse sequences were aligned with the DAPSA program (Harley, 1994) to obtain a final consensus sequence for each virus sample. The sequences were translated into amino acids and grouped into tetramers. Amino acid sequences generated in this way for the diverse viruses were aligned manually and gaps were inserted for alignment purposes.

3.2.6 Phylogenetic analysis of the CVR

A final dataset consisting of 124 characters/amino acids grouped according to tetrameric composition (Fig. 3.1) was used for phylogenetic inference. The gaps were treated as a 21st character state and coded according to the simple indel coding method specified by Simmons and Ochoterena (2000).

3.2.6.1 Neighbor-joining (NJ)

Neighbor-joining analysis was carried out on the uncoded dataset employing both the amino Poisson correction and p - distance methods in MEGA 2 (Kumar *et al.*, 2001). Pairwise deletion of gaps was specified for pairwise distance calculations and 10 000 bootstrap replications were used to test the nodes of the resulting phylogeny. Mean character distances were also determined for the coded dataset in PAUP* and used to generate an alternative neighbor-joining tree. In the latter approach, information contained within the gap regions was taken into account, rather than being excluded in each pairwise comparison.

3.2.6.2 Maximum parsimony analysis

The most parsimonious tree was sought using the heuristic search settings. Characters were weighted with gaps being down weighted by a factor of 0.25 (PAUP*), as specified previously (Bastos *et al.*, 2004). Starting tree(s) were obtained via stepwise addition, and subsequent taxa were added by the simple method. MOZ 1960 isolate was used as an outgroup taxon (Bastos *et al.*, 2004). One tree was held at each step during stepwise addition and branches were collapsed (creating polytomies) if maximum branch length was zero. Tree bisection-reconnection (TBR) was the branch-swapping algorithm selected and topological constraints were not enforced. The bootstrap method was employed for assessing nodal support, where 1000 replications were performed.

A branch-and-bound search where characters were unordered and bore equal weights, was also attempted. The furthest option was chosen for addition of sequences and branches were collapsed (creating polytomies) if maximum branch length was zero. 'MulTrees' option in effect and topological constraints were not enforced.

3.3 RESULTS

3.3.1 CVR size and sequence differentiation

CVR product size differentiation on the basis of molecular weight estimates and number of amino acids are indicated in Table 3.5.

3.3.2 Data statistics from MEGA

Of the 124 sites utilized, 67 were constant, 57 variable, 29 parsimony informative and 28 were singletons. The dataset comprised the following 10 amino acids, arranged in descending order of percentage composition: 23.19 Asparagine, 21.18 % Alanine, 19.47 % Threonine, 14.57 % Serine, 9.52 % Aspartic acid, 5.58 % Isoleucine, 3.75 % Valine, 2.60 % Cysteine, 0.12 % Glycine and 0.02 % Glutamic acid. The remaining 10 possible amino acids did not occur within the CVR tetrameric repeat region.

3.3.3 Data and tree analysis in PAUP

Results of lengths and fit measures of all parsimony trees are summarized in Table 3.6

3.3.4 Maximum parsimony tree

On completion of the heuristic search, 24123 rearrangements were tried, and 6 discrete CVR clades were resolved. 249 bootstrap replications were performed from which a 50 % majority-rule consensus tree was obtained (Fig. 3.3).

Table 3.5 CVR product size estimates and amino acid sequence composition of *p72* genotype VIII viruses identified in this study and Bastos *et al.* (2004). The tetrameric repeats reflect tetrameric content only and do not represent the order in which the repeats occur within the CVR of each virus isolate.

VIRUS	Genotype VIII CVR lineage	Estimated size (bp)	Number of amino acids	Tetrameric repeat composition	Reference
CHJ89/1/ZAM/P	A	543	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
LIL90/1/MAL/P	A	535	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
DED91/1/MAL/P	A	539	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
MCHINJI075/MAL/P	A	547	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
DED89/1/MAL/P	A	540.5	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
LIL89/1MAL/P	F	650	120	(cast) ₃ (nadt) ₂ (nasi) ₄ (nvdt) ₇ (nast) ₉ (nadi) ₂ (nant) ₂ nani	This study
BAN91/1/MAL/P	A	581	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
CHM88/1/ZAM/P	A	511	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
THY90/1/MAL/P	A	514	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
MALAWI/1978/P	A	519.5	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MPO89/1/ZAM/P	A	569	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
KANA89/1/ZAM/P	A	542	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
KAC91/2/MAL/P	B	603	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study

MCH89/1/MAL/P	A	554	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
MOZ/2001/P	A	402	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
CHK89/2/ZAM/P	B	402	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
YEL88/4/ZAM/P	A	492	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
DOWA/MAL/P	F	641	120	(cast) ₃ (nadt) ₂ (nasi) ₄ (nvdt) ₇ (nast) ₉ (nadi) ₂ (nant) ₂ nani	This study
KAV89/1/ZAM/P	D	N/D	116	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₃ (nvdt) ₄ (nast) ₇ (nadi) ₂ (nant) ₂ nani nvgt	This study
KAL88/1/ZAM/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
ZON88/1/ZAM/P	E	641	124	(cast) ₂ (nadt) ₅ (sast) ₄ (nasi) ₃ (nvdt) ₄ (nast) ₇ (nadi) ₂ (nant) ₂ nani nvgt	This study
MPI89/1/ZAM/P	A	554	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
MCH89/3/MAL/P	C	600	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
GUL88/1/ZAM/P	D	621	116	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₃ (nvdt) ₄ (nast) ₇ (nadi) ₂ (nant) ₂ nani nvgt	This study
NGE92/1/MAL/P	A	529	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
NKZ88/1/ZAM/P	A	492	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
ZAW88/1/ZAM/P	B	512	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
DEDZA/MAL/P	A	N/D	56	(cast) ₁ (nadt) ₁ (sast) ₁ (nasi) ₁ (nvdt) ₂ (nast) ₃ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MAN89/2/ZAM/P	A	560	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
JON89/13/ZAM/P	A	N/D	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
TEN89/1/ZAM/P	A	533	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study

SAL92/1/MAL/P	A	536	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
ZOM2/84/MAL/P	A	N/D	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
KLI88/2/ZAM/P	D	702	116	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₃ (nvdt) ₄ (nast) ₇ (nadi) ₂ (nant) ₂ nani nvgt	This study
CHG88/1/ZAM/P	D	652	116	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₃ (nvdt) ₄ (nast) ₇ (nadi) ₂ (nant) ₂ nani nvgt	This study
SIY91/2/MAL/P	B	652	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
PHW88/1/ZAM/P	A	569	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
TMB89/1/ZAM/P	A	492	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	This study
NDA1/90/MAL/P	A	N/D	84	(cast) ₂ (nadt) ₃ (sast) ₂ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZCA98/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZB98/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZC98/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZ34/98/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZ62/98/P	B	N/D	92	(cast) ₂ (nadt) ₄ (sast) ₃ (nasi) ₂ (nvdt) ₃ (nast) ₄ (nadi) ₂ (nant) ₂ nani	Bastos <i>et al.</i> , 2004
MOZ1960 [#]	N/A	N/D	88		Bastos <i>et al.</i> , 2004

N/D: Not done; N/A: Not applicable; # Not belonging to genotype VIII

Table 3.6 Results of lengths and fit measures of all parsimony trees in memory (PAUP) deduced from CVR sequences with gaps down weighted by a factor of 0.25 and treated as a 21st amino acid state

Parameter	Value/Specification
Search method	Heuristic
Score of best tree(s) found	189
Sum of min. possible lengths	144
Sum of max. possible lengths	541
Length	201
Consistency Index	0.762
Retention Index	0.87
Re-scaled consistency Index	0.676

3.3.5 Neighbor-joining tree

A phenogram was obtained indicating 6 discrete CVR clusters for the uncoded dataset, supported by bootstrap values higher than 50 % on all but 2 branches. Branching patterns were similar to those identified by the parsimony method for four of the subgroups (C, D, E and F), while the remaining two (A and B) differed (Fig. 3.4). The NJ tree obtained using the coded data set (Fig. 3.2) and the distance between isolates measured as mean character differences however yielded eight well-resolved clusters (Fig. 3.5). Labeling of these clusters was done according to that used in the NJ tree obtained with the uncoded dataset. Subgroups B, C, E and F were identical, while A and D were different in terms of the number of constituent isolates, and subgroups G and H (Fig. 3.5) were unique for the coded dataset.

3.3.6 Interpretation of results

Conflicting results for two of the six clades recovered from uncoded NJ and weighted MP analyses were obtained. These lesser resolved phylogenies contrast markedly with the NJ tree obtained for the coded dataset, where eight well-resolved and well-supported clusters were obtained. As the NJ tree manages to resolve the two conflicting clades and because the cluster number corresponds with the eight discrete CVR size classes that were discerned by amino acid alignment (Fig. 3.1), the coded NJ tree (Fig. 3.5) is regarded as the better phylogenetic estimate on which to base epidemiological inferences.

The eight discrete intra-genotypic lineages obtained by NJ analysis of the coded dataset were designated A to H (Fig. 3.5). Tetrameric repeats, NAST, NVDT, NADT and to a lesser extent SAST, were most abundant in the CVR sequences (Table 3.5 and Figure 3.1). The largest CVR lineage (group A), with a CVR length of 84 amino acids (aa), comprised isolates from three countries (Zambia, Malawi and Mozambique). These were recovered from outbreaks over a 23 year period. Group B was characterized by Dedza, a 1986 virus from Malawi with a 56 aa long CVR. MCH 89/3, a Malawian isolate with a CVR length of 92 aa, was the sole member of group C. Lineage D was solely comprised of Zambian isolates with a CVR length of 116 aa, and a two year field presence. A single Zambian virus with a length of 124 aa represented group E. Two Malawian isolates with a CVR 120 aa in length constituted group F, and had a field presence of three years. Viruses from Zambia, Malawi and Mozambique, with CVR aa length of 92 and spanning a 10 year period, formed group G. CHG 88/1, a Zambian isolate with a CVR aa size of 116, constituted group H.

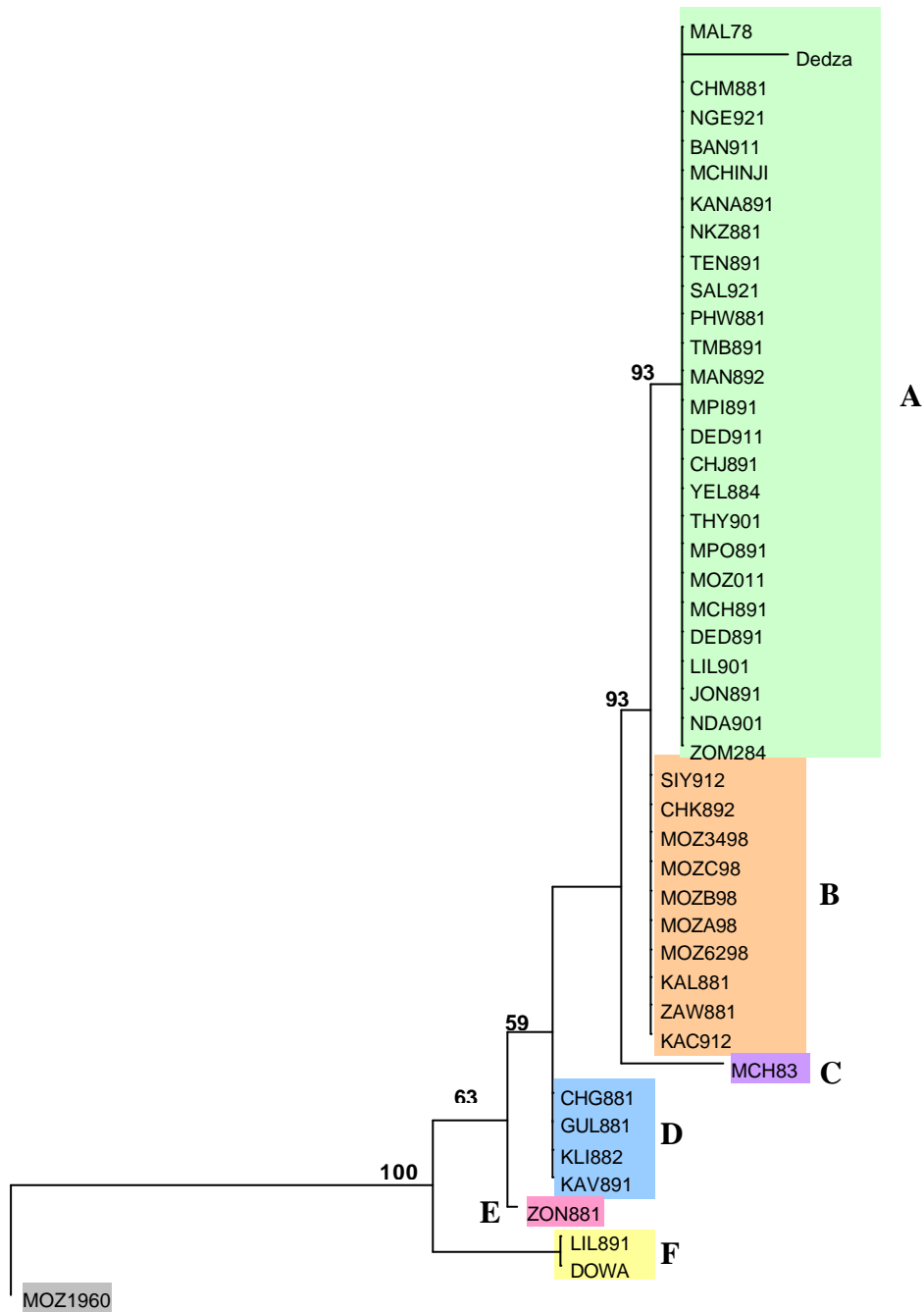


Fig. 3.3 Parsimony tree depicting 6 genotype VIII ASFV sub groups (denoted A-F) discerned by central variable region sequencing. Phylogeny was supported by 249 bootstrap replications

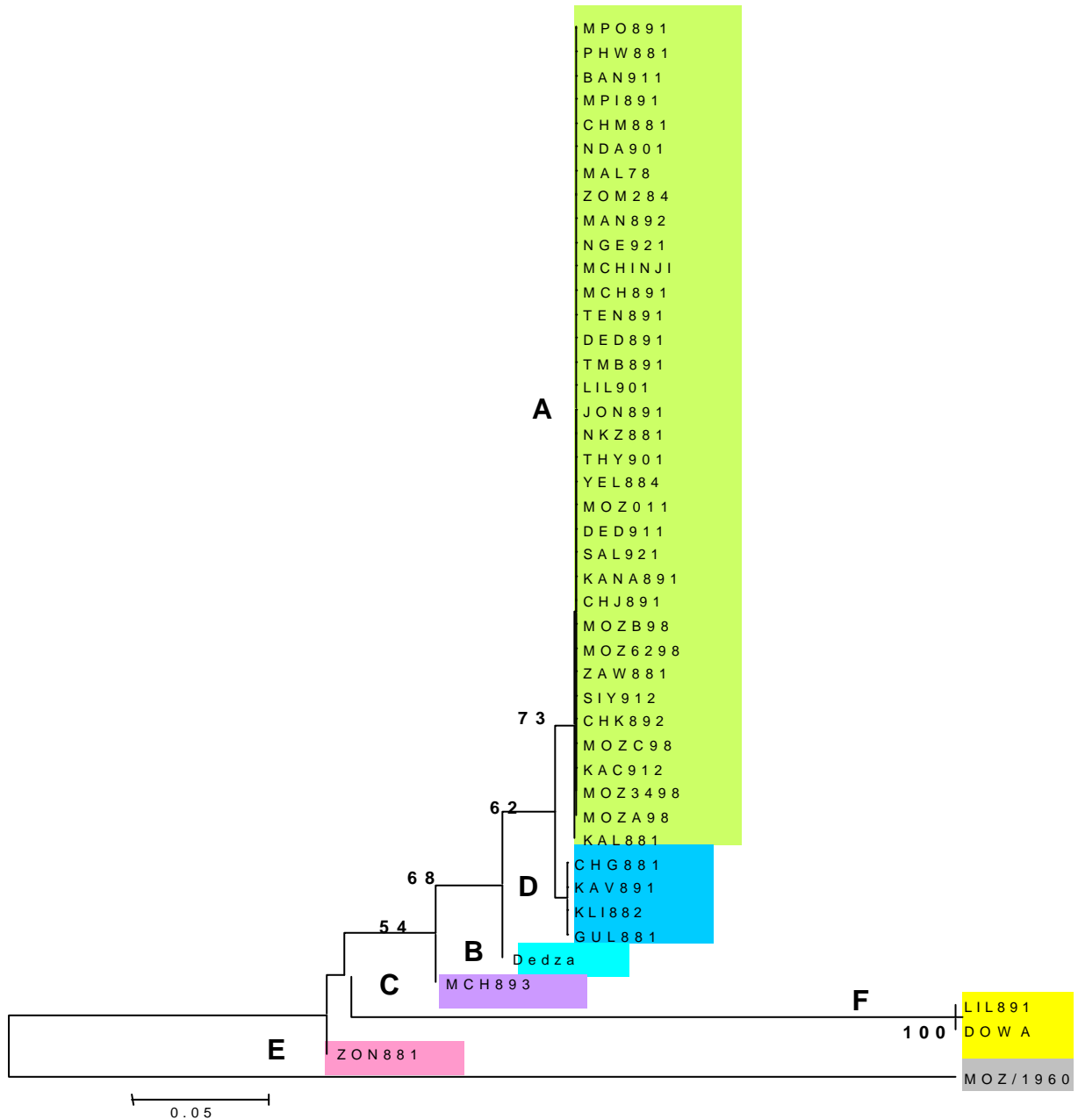


Fig. 3.4 Neighbor joining tree depicting phylogenetic relationships recovered when using uncoded CVR sequences of genotype VIII viruses from this and previous studies (Bastos *et al.*, 2003 and 2004). Nodal support is based on 10 000 bootstrap replications.

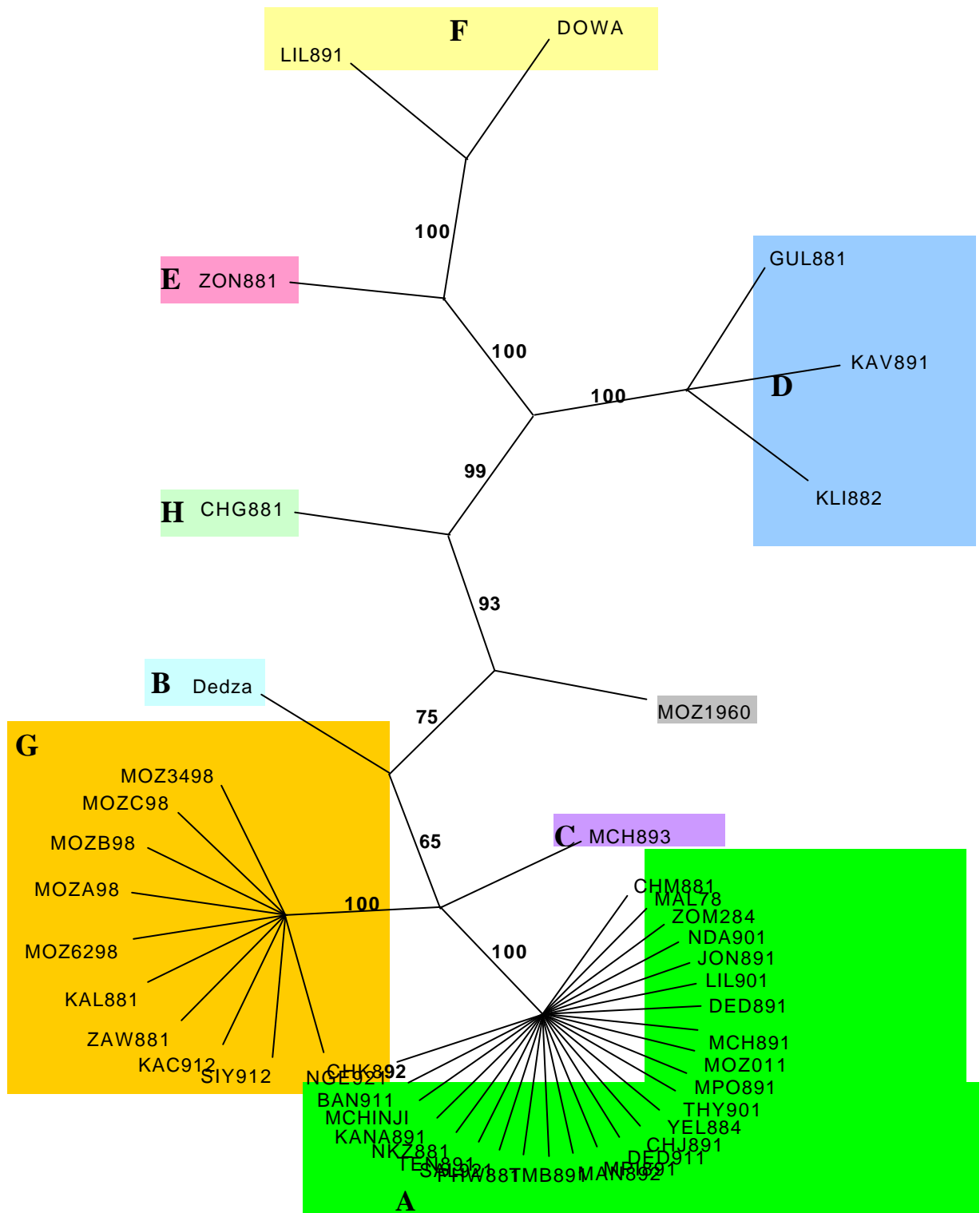


Fig 3.5 Neighbor joining tree depicting phylogenetic relationships of *p72* genotype VIII viruses using the coded CVR dataset. 1000 bootstrap replications were conducted.

3.4 DISCUSSION

The variable nature of the CVR allowed for the isolates in genotype VIII to be classified into eight discrete lineages. Despite the hypervariable nature of the repeat region, some of the unique CVR sequences uncovered appear to have remained unchanged for at least 23 years, as seen in isolates constituting lineage A.

The huge disparity observed in the estimated molecular weights of the CVR products in each lineage/sub-genotype were probably due to the electrophoresis conditions, perturbations in the gel and the interpolation method used to calculate these values (Gough and Gough, 1984; Maina *et al.*, 1984). This was supported by the fact that the reference strain, Malawi 1978, yielded different molecular weights when estimates were obtained from different agarose gels. Maina *et al.* (1984) found the following conditions to be important for DNA electrophoresis: electrophoresis apparatus must be extremely clean; care must be taken in pouring the gel so that the agarose cools evenly; all samples must be in the same buffer and of the same volume; the sample buffer must be of a salt concentration which is less than that of the running buffer; the standard lane should have at least 10 bands; and electrophoresis should be between 10 and 30 volts. Some of these conditions could however not always be met due to constraints in the laboratory such as lack of some equipment, and most importantly, performance of the electrophoresis on different days with different buffers, and the fact that a single gel could only accommodate a limited number of samples. Due to these error prone gel estimations, sizing on the basis of the number of amino acids empirically determined following sequencing was adopted (Table 3.5).

Generation of comparable sequence positions that provide the basis for the establishment of hypothesis of primary homology is dependent on alignment. With homologous sequences that differ in length (Fig. 3.1), insertion of gaps is required and unavoidable for the production of comparable positions for the entire sequences. Thus gaps, defined by Simmons and Onchoterena (2000) as single or contiguous sets of positions for which no bases are present in one or more sequences, bounded on either side by aligned nucleotides

or amino acids, become integral to the pattern like nucleotides and amino acids. The reliability of gaps as characters in phylogenetic inferences has been debated extensively, but their importance is undeniable and they continue to be used (Lloyd and Calder, 1991; Golenberg *et al.*, 1993; Bailey and Doyle, 1997; Lai *et al.*, 1997; Van Dijk *et al.*, 1999; Simmons and Onchoterena, 2000). Common complications with the use of gaps as characters in tree searches are the ambiguously aligned gaps, where these characters have more than one equally optimal alignment, as is possible with the CVR data. Gatesy *et al.* (1993) have however found that insertions and deletions may be unambiguous phylogenetic indicators despite the alignment ambiguity. Various investigators concluded that gap costs used in alignment may be incorporated into tree searches by coding these gaps as extra character states, a strategy which was adopted in this study in the quest for a true phylogenetic picture (Wheeler, 1994; Janies and Wheeler, 1998; Giribet and Wheeler, 1999). A decision to down weight gaps to 0.25 stemmed from the assumption that each gap, irrespective of length, was a result of a single mutational event, and also due to the ambiguity surrounding their alignment (Simmons and Onchoterena, 2000; Young and Healy, 2003). The simple indel coding proposed by Simmons and Onchoterena (2000) was applied (Fig. 3.2) because it was conservative, easy to implement and highly informative in determining a correct phylogeny.

Analysis of sequence based data matrices by different methods, especially the treatment of gaps, has been demonstrated to affect the resulting phylogenetic picture (Simons and Mayden, 1977; Eernisse and Kluge, 1993; Vogler and DeSalle, 1994). CVR phylogenetic inference using NJ (uncoded dataset) and MP methods resulted in an equal number of groups, yet two of these possessed different branching patterns (Figs. 3.3 and 3.4). In distance methods, positions containing gaps are either completely deleted across all sequences under examination, a phenomenon called complete deletion, or these sites are only deleted in two sequences under comparison, termed partial deletion (Nei and Kumar, 2000). Unless a coded dataset is used and mean distances computed in PAUP*, a large proportion of the dataset is excluded with partial or complete deletions, and phylogenetic inference is only made on those positions where gaps are absent. The differences observed between the two NJ trees (Figs. 3.4 and 3.5) thus resulted from the fact that the

datasets analyzed for phylogenetic inference were different (unequal number of sites analyzed). Similar to NJ analysis with coded data, character-based methods completely retain data and all sites are taken into consideration (including gaps). The differences observed between the MP (Fig. 3.3) and NJ tree of the coded dataset (Fig. 3.5) is likely due to the manner in which the two methods handle data and draw trees. Due to the nature of the CVR data where gap insertion was integral to sequence alignment, the phylogeny discerned by the NJ method using the coded dataset where each character was included in the search and more clusters resolved, was adopted as the correct one (Fig. 3.5).

ME results obtained using *p72* genotyping indicated that Zambia was the most genotype - rich East African country with seven genotypes occurring within its borders (Figs. 2.2 and 2.4). Further analysis of genotype VIII viruses by CVR sequence characterization yielded eight groups designated A – H (Figure 3.5). The CVR phylogenetic results indicate that Malawi and Zambia had an equally high diversity of CVR lineages, having five each, followed by Mozambique with two. Group A, the largest and longest circulating lineage, consists of 25 viruses (Fig. 3.5), the most historical of which is a Malawian virus recovered from an outbreak in 1978. This could favour Malawi over Zambia as possibly being the original source of genotype VIII, from where it subsequently spread to Zambia and Mozambique. Interestingly, despite the variable nature of the CVR, this region was completely conserved across group A viruses that were recovered over a period of 23 years. Group B notably had the smallest CVR aa length of all isolates examined, and was represented by a single 1986 Malawian virus named Dedza. Groups B, C and F appear to be restricted to Malawi, whilst groups D, E and H seem to be confined to Zambia. It was notable that Lil89/1 forming group F had the tetramer SAST deleted from its CVR sequence, while group D, E and H isolates possessed a unique NVGT tetramer. More extensive sampling and sequencing of genotype VIII viruses may reveal the significance of these deletions and insertions.

The minimum positional homology could explain why viruses possessing the same types of tetramers both in equal or unequal quantities, formed different clusters, A, C and G (Figs. 3.1 and 3.5; Table 3.5).

Evidence of the existence of a domestic pig cycle in East Africa was previously presented by Haresnape and Wilkinson (1989). To corroborate this, no evidence of genetically unrelated virus isolates was found in the investigation of ASF outbreaks in Malawi and Zambia, and sylvatic hosts were trivialised in the enzootic status or as sources of ASFV in new outbreaks (Sumption, 1992). It was also shown that once a sylvatic isolate was established in the pig population, it developed low tick infectivity. Sumption (1992) speculated that the low infection rates of ticks challenged with virus from pigs by natural feeding, could have been due to lost sequences of significance in the production or functioning of viral proteins essential for tick infections. This could be the reason why domestic pig cycles like that forming *p72* genotype VIII are established.

RFLP using *BamHI* endonucleases has shown that pig viruses from Malawi and Zambia were very similar genetically, while isolates from ticks of the *O. moubata* complex were significantly different and showed great heterogeneity among themselves (Dixon and Wilkinson, 1988; Sumption, 1992). In contrast to these findings, in this study MFUE6/1, a tick isolate from a Zambian warthog, clustered with MZI92/1, a pig isolate from Malawi by *p72* genotyping. Similar to the *p72* gene and CVR, *BamHI* restriction sites were found to be highly conserved as the 1953 isolate from Zambia had 23 fragments similar in mobility to the fragment patterns of recent Malawi viruses, indicating high relatedness among viruses in these two countries (Sumption, 1992). Though conserved when compared with the left and right termini of the genome, the central region contains VR4, which, like VR5, VR8a and VR8b located towards the right termini, is characterised by the presence of tandemly repeated sequence arrays (Blasco *et al.*, 1989; Dixon *et al.*, 1990a, b; Sumption, 1992). The genome map location and the nature of the nucleotide sequences of VR4 corresponds to the CVR *9RL – ORF*'s location and composition (Sumption *et al.*, 1990; Irusta *et al.*, 1996).

Resolution of relationships between viruses from Malawi and Zambia by comparison of restriction fragment lengths of *Bam*HI a and b / a' b and deletion characteristics in the left terminus of the genome resulted in 6 and 7 classes of viruses, respectively (Table 3.2). These classification methods did not correspond with *p72* genotyping, which clustered all the isolates common to the two studies (This study and that of Sumption, 1992) into one genotype, genotype VIII, nor did they correspond with the 8 CVR subgroups in this study (Fig. 3.5; Table 3.7).

Table 3.7 Comparison of groups/subgroups of isolates common to this study and that of Sumption (1992) resolved by *p72* and CVR sequence, and *Bam*HI a and b/ a'b restriction fragment analysis

Virus	P72 genotype	CVR subgroup	BamHI a'b class	BamHI a'b subgroup
MCH89/3	VIII	C	I	-
ZAW88	VIII	G	I	-
TEN89	VIII	A	I	A
YEL88	VIII	A	I	A
MPI88	VIII	A	I	A
KAL88	VIII	G	I	A
NKZ88	VIII	A	I	A
CHG88	VIII	H	I	B
KAV89	VIII	D	I	B
KLI88	VIII	D	I	C
NYA88	XIV	-	-	-
MAN89	VIII	A	II	-
LIL89	VIII	F	III	-
PHW88	VIII	A	III	-
MCH89/1	VIII	A	III	A
DOW86	VIII	F	IV	-
LIL89/1	VIII	F	IV	A

Similar to the differences observed in genotyping viruses from different parts of Africa employing the two methods in chapter 2 (Fig. 2.2 and Table 2.2), the different sub/grouping results of Malawi and Zambia isolates could have been due to the following reasons:

- a) Classification based on two different areas of the genome i.e. VR1 found on the left and right termini and VR2 located on the left terminal side of the genome for *BamHI* a and b / a'b, and 9 - *RL ORF* located in the central part of the genome for CVR analysis.
- b) The different areas under study had different sizes, with up to 9 kb in *BamHI* a and b / a'b, and 300 bp/ 68 aa in CVR size differences observed.
- c) *BamHI* a and b / a'b fragments contain multigene families 110 and 360, while CVR contains only one gene, the 9 – *RL ORF*.

Comparison of the two methods would be more justified if both techniques targeted the same genomic area, and if an RFLP phylogeny was available which summarised all fragment data, rather than handling it individually as was done in Sumption (1992).

In conclusion, many ASF outbreaks occurring in Malawi and Zambia of different genotypes and CVR lineages attest to the fact that there are multiple, independent introductions from persisting virus reservoirs in these countries. CVR sequence characterisation can assist in resolving intra-genotypic relationships, thereby providing epidemiological insights into the origin and spread of outbreaks. Analysis of the CVR sequence further emphasised the complexity of the disease situation in East Africa where both sylvatic and domestic pig cycles occur and where the majority of genotypes have a trans-boundary distribution (Figs.2.2 and 2.5).

Chapter 4

General discussion

The devastating impact of outbreaks of African Swine Fever both in and outside the African continent has been cited in chapter 1. Since ASFV surface glycoproteins do not allow differentiation into serological types, assessment of field heterogeneity at the regional level in East Africa was determined for 77 viruses from eight countries, utilizing nucleotide sequences corresponding to the C-terminal end of the *p72* gene. The complex epidemiological picture in East Africa was exposed, as attested by the presence of 13 genotypes in the region (Figs. 2.2 and 2.5), multiple genotypes in many of the countries, common genotypes between and among countries, and the presence of an active sylvatic cycle. New insights into the probable origin of the domestic pig cycle in West Africa was revealed by the recovery of genotype I viruses in East Africa. Due to the conserved nature of the *p72* gene, intra-genotypic relationships of viruses recovered from a homogeneous domestic pig associated genotype (VIII), which appears to be confined to East Africa, could not be resolved. In order to resolve relationships within this genotype, sequencing of an amino acid tetrameric repeat region located within the *9-RL* Open Reading Frame (ORF), and termed the central variable region (CVR), was performed. This resolved eight discrete CVR lineages (Fig. 3.2) and allowed for identification of epidemiological links between outbreaks caused by viruses within the homogenous genotype.

According to Geering *et al.* (2001), risk analysis for ASF involves risk assessment, management and communication. Assessment involves the establishment of such a danger occurring, its likelihood of occurrence and potential consequences. When determining risk presence, information on the following issues is usually required: geographic distribution and incidence of the disease in the world; status of neighboring countries and if present, the closeness of the outbreak point to the border with the

inquiring country; dissemination of the disease and the role of various formites, biological and mechanical vectors; border and barrier security issues to prevent unlawful entry of viruses; political turmoil and civil unrests in neighboring countries; trade issues with infected countries; own ASF history, possibility of the disease laying dormant in domestic and wild hosts; unlawful livestock movement within the country and pig husbandry practices.

The molecular epidemiological database created in this study can undoubtedly assist any country trading or intending to trade in pigs and pig products with East African countries, in assessing its ASF risk. Clear presence in East African countries of infected sylvatic hosts (genotypes I, V, X, XI, XII, XIII, XIV) and domestic pigs (genotypes II, VI, VIII, IX, XV, XVI), number of recorded outbreaks involving these animal species and the socio-economic costs incurred from such occurrences in the past (Chapters 1 and 2), assist in clarifying the disease situation and incidence in the region.

The likelihood of ASF occurring and its potential socio-economic consequences are governed by factors such as: likelihood of disease establishment in a country due to presence of suitable biological hosts; wildlife management; ease of disease recognition in different parts of the country; the size, distribution and type of the pig industries; threat posed to food security and income generation from exports; possibility of control or eradication, and cost of such actions (Geering *et al.*, 2001). In the East and South African context, the likelihood of ASF establishment and far reaching economic and social consequences is very high due to the presence of all epidemiologically important factors for disease occurrence (Chapter1).

Following the confirmation of presence of danger, hazard management has to be implemented. If a concerned country intends preventing introductions or re-introduction, strict endorsement of the guidelines of the Office International Des Epizootis (OIE)'s International Animal Health Code with regards to the safe importation of domestic and wild pigs, pig meat and meat products, pig semen, embryos and ova, and other products containing pig tissues such as pharmaceuticals, must be applied.

Adequate quarantine services must be provided to stop food and other risk factors containing pig meat or products from being delivered into the country. These should ideally be placed at international airports, seaports and border crossing points. All risk factors seized should be destroyed and disposed off by deep burial or incineration. Swill feeding, especially feed from airplanes, ships and known ASF positive areas, should be abandoned and safer methods of feeding pigs be investigated. Containment of pigs is very important as large numbers of uncontrolled pigs constitute a high risk for ASF entry and spread. These pigs have access to carcasses and offal of other pigs that may have died from the disease. Freely roaming pigs are also more likely to have contact with wildlife reservoirs of ASFV. Epidemiological knowledge of the ASF situation of neighboring countries and those with whom business is conducted, such as the one generated in this study, can greatly influence the stringency placed on the above preventative measures. Farmers should also be informed of the dangers of careless disposal of carcasses, and of allowing pigs to range freely.

Eradication of the disease is ideal and possible, and mainly involves the following actions:

1. Zoning of the country into infected, surveillance and free zones.
2. Quarantine and movement control practices
3. Immediate slaughter of the infected and in contact animals
4. Safe carcass disposal
5. Strict and effective zoo-sanitary practices
6. Observation of safe re-stocking periods
7. Epidemiological surveillance for ASF

Zones are proclaimed geographic areas in which specific disease control actions are to be carried out. These areas are classified as infected, which immediately surround the infected premises (IP) or villages, surveillance or control, surrounding the infected zones, and ASF free zones encompassing the whole country. In infected zones, the prevention of further spread through quarantine and pig movement control, elimination of the source of infection by stamping out the infected and potentially infected pigs, safe disposal of

carcasses, decontamination of the premises and continued disease surveillance and epidemiological investigations are crucial. The entire infected zone should be under quarantine, and a complete ban on movement of pigs and their products should be implemented. Stamping out should involve all the pigs irrespective of their clinical appearance, and deep burial of carcasses is important to eliminate the source of ASFV. Contact area decontamination with effective chemical agents, or burning of informal pigsties is essential for reducing the risk of contamination of clean environments. Strict adherence to the OIE's recommended 40 days of destocking is advisable.

Disease surveillance and other epidemiological investigations should be conducted simultaneously with the above activities. For each infected herd, tracing backwards to determine the origins of any new pigs brought 3 – 4 weeks prior to the outbreak, which may have been the source of infection, and tracing forwards for destinations of pigs that may have left the IP shortly before or after the outbreak, is critical. With knowledge of the different ASFV genotypes and sub-genotypes of suspect countries of origin, it would be easier to identify the real source of the ASFV. Genotyping can also give an indication of the number of outbreak viruses the authorities are dealing with as results from previous studies have indicated that different viruses can co-circulate within what appears to be a single outbreak focus (Bastos *et al.*, 2003, 2004).

The molecular database will also be highly applicable when communicating the risk with all relevant stakeholders. It provides scientific proof of the presence of the risk and its origin and can be used to justify the management procedures proposed or implemented.

At present, there is no vaccine against ASF. However, protection of pigs that have survived infection with ASFV of mild virulence against challenge with homologous yet virulent strains has been observed (Coggins *et al.*, 1968; DeBoer *et al.*, 1969; Hamdy and Dardiri, 1984; Dixon *et al.*, 1999). Boinas *et al.* (2004) have also observed protection of pigs that have been infected with non pathogenic and non hemeadsorbing (HAD) viruses, against challenge with pathogenic HAD viruses isolated from the same farm. Following the discovery of neutralizing antibodies against ASFV structural proteins 30, 54 and 72,

ideas that the pig humoral immune response against ASFV depended on these proteins, were conceived (Neilan *et al.*, 2004). Development of neutralizing antibodies against the latter proteins was shown to be important, but not enough to protect pigs against homologous virulent isolates (Neilan *et al.*, 2004). No matter the kind of vaccination attempt, investigators all seem to agree that less effective protection is achieved when recovered pigs are challenged with a more distantly related isolate (Boinas *et al.*, 2004; Neilan *et al.*, 2004). Hypothetically, if an effective vaccine against African swine fever was to be developed, areas of known presence of infection would have to be zoned and the genotypes circulating identified so that each of these areas can use vaccines with relevant strains or antigens. The data generated in this study based on characterizing the gene coding for the immunodominant protein VP72, clearly indicates the number and distribution of ASFV genotypes and may therefore be extremely useful in future vaccine campaigns for the East African region.

Molecular typing of viruses is ideally aimed at providing clinically and biologically useful information about viruses, especially on tissue specificity, virulence and epidemiological issues. The extent to which virus molecular classification techniques have approached or achieved these aims varies. Genotyping techniques based on *p72* phylogenetic studies however, do not achieve all the aims of virus molecular typing. This is attested by viruses of different virulence, haemadsorption ability and species of origin, clustering together (Gonzague *et al.*, 2001; Fig. 2.2; Personal communications with diagnostic specimen suppliers).

Future studies should therefore aim at whole ASFV genome sequencing, and resolution of the functions of all the ORF identified, as is the trend in DNA virus relationship studies (Lukashov and Goudsmit, 2001). Once an extensive full genome sequence database is available, genomic regions targeted for sequence analysis will be determined by the objectives of the molecular typing exercise. Classification based on biological features like receptor usage, tissue tropism or virulence will be based on sequence information of genomic areas influencing these properties (Muir *et al.*, 1998).

In the near future however, the accuracy of the discerned phylogenetic relationships by a one-step method warrants investigation by full length genotyping. Although useful in determining the intra-genotype relationships, the CVR cannot currently be used independently of the *p72* genotyping, as it cannot resolve relationships across genotypes (Bastos *et al.*, 2004). A more variable gene than the *p72*, but less variable to the CVR should be identified for simultaneous genotype identification and within-genotype relationship resolution.

A future research aim is to attempt to trace the route of entry of genotype I viruses from East African into West Africa. In an attempt to address this, CVR sequences of eight of the *p72* genotype I viruses used in this study have been generated. A sister project where the gene coding for the structural protein p54 has been sequenced for 26 viruses from East, West and South Africa, including viruses used in this study, is also in progress. The aim of this project is to determine the potential of the *p54* gene to resolve ASFV relationships in a manner that is less conservative than the *p72* gene, but less variable than the CVR.

Disease surveillance operations are undoubtedly crucial for prevention, control and eradication. In cases of outbreaks or virus isolation from natural hosts of ASFV, a molecular epidemiological database should be available and constantly updated in order for a country to also update its outbreak contingency plans. Molecular epidemiological studies like the ASFV *p72* genotyping and intra-genotypic resolution by CVR provide a powerful tool for disease control and should be updated and improved on a regular basis.

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APPENDIX

A. Dextran 10% - PLASMA VOLUME EXPANDER

NaCl	9 g
Double Distilled Water	1000 ml
Dextran 150 (BDH Laboratory Supplies # 38108 2M)	10 g
NaCl 0.9 %	100 ml

Make 0.9 % NaCl by adding 9 g NaCl to 1000 ml double distilled water. Shake well and autoclave.

Add 10 g Dextran 150 to 100 ml NaCl and mix thoroughly.

B. WASH BUFFER

Phosphate buffered saline (PBS)	1 L
Penicillin	0.4 %
Streptomycin	0.64 %
Neomycin	0.75 %
Normal bovine serum (NBS).	1 %

Add all ingredients to 1 L PBS and mix thoroughly.

C. AMMONIUM CHLORIDE (0.82%)

KCl	0.37 g
NH ₄ Cl	8.2 g
Double distilled water	1 L

Mix the above constituents and add NaHCO₃ (4.4 %) to adjust pH to 7.4

Autoclave the solution.

D. HEPES

Hepes	23.8 g
Distilled water	100 ml

Mix, filtrate or autoclave, and store at +4°C.

E. EARLE'S SALINE X2

NaCl	13.6 g
KCl (Merck 104936) (UNILAB 504200)	0.8 g
MgSO ₄ (Merck 5886)	0.4 g
Glucose (Merck 8337)	2.0 g
NaH ₂ PO ₄ .2H ₂ O (UNILAB 758516)	0.31 g
CaCl ₂	0.4 g
NaHCO ₃ (Merck 6329)	4.4 g
Double Distilled Water	1L
1% Phenol Red solution	2 ml

Mix the above. Spin to dissolve and then filtrate with a 0.22 μ filter and store at +4°C

F. GROWTH MEDIUM

Distilled water	42 %
Earle's medium	42 %
Hepes (UniLab)	0.4 %
Swine serum (Highveld Biological)	13 %
Penicillin (Sigma)	0.52 %

Streptomycin (Sigma)	0.832 %
Neomycin (Merck)	0.975 %

Add all ingredients and mix.

G. SILICA

Double distilled water	100 ml
Silicon dioxide 99% (Sigma)	6 g
HCl(32% wt/vol) (Merck)	60 μ l

- Perform all steps in treated glass and wear gloves.
- Suspend 6 g of silicon dioxide 99 % (Sigma) in a 50 ml volume of sterile ddH₂O in a 50 ml glass cylinder and allow to sediment for 24 hours at room temperature.
- Remove 43 ml of the supernatant portion by suction with a pipette-aid.
- Add sterile ddH₂O to the measuring cylinder so that a final volume of 50 ml is obtained.
- Resuspend the silica pellet by vigorous shaking.
- Allow a second sedimentation for 5 hours at room temperature.
- Remove 44 ml of the supernatant and add 60 μ l of HCl (32 % wt/vol) (Merck) in order to adjust to pH 2.

H. L6 LYSIS BUFFER

Guanidine Thiocyanate (GuSCN- Promega)	60 g
Tris-HCL (0.1M)	50 ml
EDTA (0.2 M)	11 ml
Triton X-100 (Roche)	1.3 g

- Dissolve 60g of Guanidine Thiocyanate (GuSCN- Promega) in 50 ml of 0.1 M Tris-HCL (pH 6.4).
- Add 11 ml of 0.2 M EDTA solution and 1.3 g of Triton X-100 (Roche)

- Place at 50°C until completely in solution.
- Wrap in foil and store at room temperature.

I. L2 WASH BUFFER

Guanidine Thiocyanate (GuSCN- Promega)	60 g
Tris-HCL (0.1 M)	50 ml

Dissolve 60 g GuSCN (Promega) in 50 ml of 0.1 M Tris-HCL (pH 6.4) by placing at 50°C.

Wrap in foil and store at room temperature.

J. 1 x TE

Double distilled water (ddH ₂ O)	100 ml
Tris-HCl (1M)	1 ml
EDTA (1M)	1 ml

Add the 1M Tris-HCl (pH 7.4) and 1M EDTA (pH 8.0) to the ddH₂O and mix, then autoclave.