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Review

# African swine fever virus replication and genomics

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ABSTRACT

African swine fever virus (ASFV) is a large icosahedral DNA virus which replicates predominantly in the cytoplasm of infected cells. The ASFV double-stranded DNA genome varies in length from about 170 to 193 kbp depending on the isolate and contains between 150 and 167 open reading frames. These are closely spaced and read from both DNA strands. The virus genome termini are covalently closed by imperfectly base-paired hairpin loops that are present in two forms that are complimentary and inverted with respect to each other. Adjacent to the termini are inverted arrays of different tandem repeats. Head to head concatemeric genome replication intermediates have been described. A similar mechanism of replication to Poxviruses has been proposed for ASFV. Virus genome transcription occurs independently of the host RNA polymerase II and virus particles contain all of the enzymes and factors required for early gene transcription. DNA replication begins in perinuclear factory areas about 6 h post-infection although an earlier stage of nuclear DNA synthesis has been reported. The virus genome encodes enzymes required for transcription and replication of the virus genome and virion structural proteins. Enzymes that are involved in a base excision repair pathway may be an adaptation to enable virus replication in the oxidative environment of the macrophage cytoplasm. Other ASFV genes encode factors involved in evading host defence systems and modulating host cell function. Variation between the genomes of different ASFV isolates is most commonly due to gain or loss of members of multigene families, MGFs 100, 110, 300, 360, 505/530 and family p22. These are located within the left terminal 40 kbp and right terminal 20 kbp. ASFV is the only member of the *Asfarviridae*, which is one of the families within the nucleocytoplasmic large DNA virus superfamily.



**Contents**



## 1. Aetiology

African swine fever is caused by a large, icosahedral DNA virus, African swine fever virus (ASFV), that is the only member of the *Asfarviridae* family. ASFV replicates predominantly in the

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cytoplasm and is a member of the nucleocytoplasmic large DNA viruses (NCLDV) superfamily (Dixon et al., 2005, 2008; Iyer et al., 2006; Yutin et al., 2009). Based on analysis of their sequences and gene repertoires these families are proposed to share a common ancestor. Recently it was proposed that the term NCLDV is not appropriate for all the viruses included in the superfamily and that instead a new viral order the Megavirales is defined (Colson et al., 2012).

## 2. Genome structure

The ASFV genome is a linear double-stranded DNA molecule that ranges in length between isolates from about 170 to 193 kbp (Chapman et al., 2008; de Villiers et al., 2010; Yanez et al., 1995). ASFV encodes for between 151 and 167 open reading frames (ORFs) and the differences in genome length and gene number are largely due to gain or loss of ORFs from the multigene families (MGF) encoded by the virus. Some smaller length variations occur as the result of variation in numbers of short tandem repeats either within genes or intergenic regions (Dixon et al., 1990; Lubisi et al., 2007).

The complete sequences of 12 ASFV isolates have been determined (Chapman et al., 2008; de Villiers et al., 2010; Yanez et al., 1995). The first complete sequence determined was of the tissue culture adapted BA71V isolate and the genes were named based on *Eco*R I restriction enzyme fragmentation, gene orientation left or right (L or R), and number of amino acids encoded. Other nomenclatures used the position from the left end of each restriction enzyme fragment. The large variation in copy numbers of genes from different multigene families between isolates caused confusion and more recently a nomenclature was proposed which is based on that of the BA71V isolate for unique genes and names genes within each multigene family according to their family name and position from the left genome termini (Chapman et al., 2008). The gene families encoded are named according to the average number of amino acids in the proteins encoded by each family, the direction they are read in and position in that family from the left genome end. The families include MGF 100, 110, 300, 360 and 505/530. In addition, related genes encoding an early membrane protein, p22, which is close to the left genome terminus are present in some isolates in 1 or 2 copies close to the right genome end.

The genes encoded by the genome are closely spaced and encoded on both DNA strands with no clear bias for coding of genes on either strand across the complete genome. However, in some genomic regions, the orientation of neighbouring genes is the same (see Fig. 1). For example, these include regions encoding MGFs which have evolved by gene duplication. As expected for a virus that is transcribed in the cytoplasm, genes do not contain introns and splicing of transcripts does not occur. There is also no evidence for virus encoded micro-RNAs. Upstream of each gene is a short sequence containing the promoter which is recognised by the viral RNA polymerase complex. Promoter sequences are generally short and A+T rich and they are recognised by virus-encoded transcription factors specific for the different stages of virus gene expression; early, intermediate and late gene classes have been defined. These are expressed in a cascade with early gene expression occurring from partially uncoated cores using enzymes and other factors packaged in virus particles (Almazan et al., 1992, 1993; Kuznar et al., 1980a; Rodriguez et al., 1996; Salas et al., 1981). Some genes have transcripts that belong to multiple gene classes and these transcripts initiate from different positions relative to the start codon (Rodriguez et al., 1996). Mapping of critical residues within the late promoter for the B646L gene which encodes the major capsid protein p72 showed the promoter function is within −56 and +5 relative to the translation initiation codon. Two essential regions at −18 to −14 and −4 to +2 were identified. A TATA sequence is present in promoters of several late genes and mutation of this reduced transcriptional activity (Garcia-Escudero and Vinuela, 2000). The transcription termination signal consists of a sequence of at least 7Ts but requires up to 10Ts for strong termination (Almazan et al., 1992). These can be found at a considerable distance (up to several kbp) downstream from the translation termination codon and as a consequence transcripts can extend through downstream ORFs. However there is no evidence to suggest that downstream ORFs are translated from these transcripts and it is thought that the mRNA that is translated for each ORF initiates in the promoter regions immediately upstream from genes.

The genome termini are covalently cross-linked and are present in two forms that are inverted and complimentary to each other. The sequence of the hairpin loops of the tissue-culture adapted BA71V isolate has been shown to be 37 nucleotides long and incompletely base-paired such that extrahelical bases are present (Gonzalez et al., 1986). Adjacent to these terminal loops are inverted repeats consisting of several arrays of tandem repeats varying in sequence. In the BA71V isolate the terminal inverted repetitions (TIR) are about 2.1 kbp long and consist of three different types of tandem direct repeats and two types of direct repeats. These are interspersed with unique sequences (Yanez et al., 1995). Variation is observed in the sequence and number of direct repeats from genomes of different isolates. The structure of the genome termini resembles that of Poxvirus genomes although the sequences are different. In the genomes of Poxviruses sequences adjacent to the hairpin loops are essential for both replication and resolution of the concatemeric replication intermediates (Du and Traktman, 1996; Stuart et al., 1991) but this has not been investigated in the ASFV genome. The ASFV genome consists on average of approximately 61–62% A+T nucleotides (Yanez et al., 1995).

## 3. Genome replication

Early studies established that ASFV DNA replication occurs in perinuclear cytoplasmic viral assembly sites. Enzymes required for DNA replication are expressed immediately following virus entry into the cytoplasm from partially uncoated core particles. Early transcription utilises virus encoded transcription and transcript processing enzymes packaged in the core (Kuznar et al., 1980b; Salas et al., 1983). Following the onset of DNA replication in the cytoplasm at about 6 h post-infection a shift in pattern of virus gene transcription occurs (Salas et al., 1986). DNA replication intermediates consisting of head to head concatemers have been detected by pulsed-field electrophoresis and by restriction enzyme digestion and blotting followed by hybridisation with probes from the genome termini. Concatemeric genomes of double unit length were detected (Rojo et al., 1999). This contrasts with poxvirus replication intermediates which can be multimers. These concatemers are resolved into unit length genomes with terminal crosslinks and packaged into virus particles (Enjuanes et al., 1976; Gonzalez et al., 1986).

An early stage of ASFV DNA replication has also been detected in the nucleus by *in situ* hybridisation and by radioactive labelling. This nuclear phase of viral DNA replication peaks at about 6 h post-infection and declines to almost zero by 12 h post-infection (Garciabeato et al., 1992b; Rojo et al., 1999; Tabares and Sanchezbotija, 1979). Analysis of the size of genome fragments synthesised in the nucleus by radioactive-labelling, showed that they were relatively short and not chased into higher molecular weight or genome length fragments. In contrast, DNA synthesis in the cytoplasm was detected in short fragments which could be chased into higher molecular weight fragments. Viral DNA in the nucleus is detected in proximity to the nuclear membrane and this DNA has been suggested to bud through the nuclear membrane into

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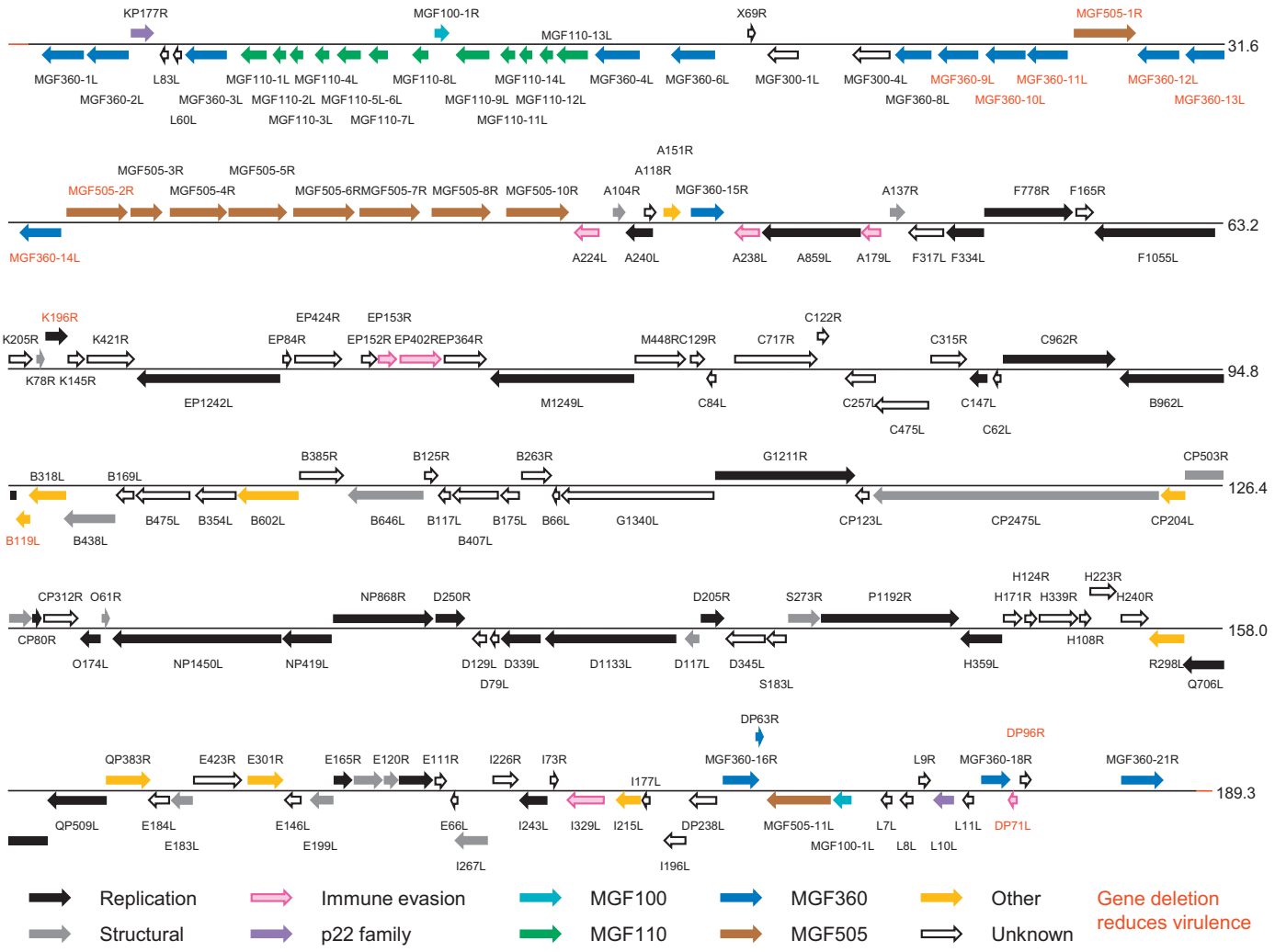


**Fig. 1.** Genome organisation of the African swine fever virus genome. The organisation of open reading frames (ORFs) on the genome of the virulent ASFV isolate Georgia 2007/1 is shown. ORFs are shown as arrows to indicate their size and direction they are read. The colours indicate ORFs with known functions. Black indicates ORFs encoding enzymes and factors involved in genome replication, repair or transcription. Grey indicates ORFs encoding structural proteins. Pink indicates ORFs encoding proteins involved in evading the host defences. Turquoise, blue, green, brown and mauve indicate members of multigene families. ORFs encoding proteins with other predicted functions are shown in yellow. ORFs encoding proteins of unknown function are shown in white. Red text indicates ORFs whose deletion reduces virus virulence.

the cytoplasm. Recent reports indicate that ASFV infection disrupts nuclear organization from an early stage of infection. Amongst the changes observed was increased lamin A/C phosphorylation by 4 h post-infection, followed by the disassembling of the lamina network in proximity to sites of virus replication. At later time points, lamin and other nuclear envelope markers were found in the cytoplasm of the infected cells. Other nuclear proteins were redistributed including RNA polymerase II, the splicing speckle SC-35 marker, and the B-23 nucleolar marker from 4 h post-infection (Ballester et al., 2010, 2011).

The role of the early nuclear phase of DNA replication is not known. Early studies showed that virus replication did not take place in enucleated cells (Ortin and Vinuela, 1977), however the precise role that the nucleus plays during viral replication remains unclear. It is possible that the nucleus may provide transcripts or other factors required for virus replication or that an early stage of virus DNA replication in the nucleus is required.

The similarities in ASFV genome structure to that of Poxviruses and the presence of replication intermediates consisting of head to head genome concatemers indicates that ASFV may share a similar replication model to vaccinia virus (Baroudy et al., 1982; Moyer and Graves, 1981). According to this model, replication is thought to initiate with the introduction of a single strand nick in the genome near to one or both termini. The exposed 3′ OH group acts as a primer for DNA polymerase and DNA synthesis proceeds towards the genome termini. This generates an intermediate in which termini of nascent and template strands are self-complementary and fold-back to form a self-priming hairpin structure. A DNA primase (D5) encoded by vaccinia virus may play a role in initiation of DNA replication or lagging strand DNA synthesis and has led to suggestions of a modified model for poxvirus DNA replication model (De Silva et al., 2007). A putative DNA primase (C962R) encoded by ASFV may play a similar role as the poxvirus DNA primase. Consistent with this is the demonstration that large ASFV DNA fragments synthesised in the cytoplasm are chased into mature cross-linked DNA. This led to the suggestion of a model for *de novo* initiation of replication (Rojo et al., 1999). The mature head to head concatemeric intermediates are resolved to unit length terminally cross-linked genomes, and packaged into mature virus particles in the cytoplasmic factory sites. The mechanisms for genome encapsidation have not been elucidated for ASFV. Data from electron microscopy indicates that the viral DNA begins to condense into a pronucleoid and is then inserted, at a single vertex, into an "empty" particle. Further maturation of the viral particle, including closure of the narrow

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**Table 1**
Genes encoded by ASFV. Genes which encode proteins involved in DNA replication, repair, nucleotide metabolism, transcription and other enzymatic activities or host defence evasion are shown. The gene nomenclature is shown in the central column and predicted molecular weight in the Benin 97/1 genome is shown on the right. Asterisks (*) indicate those for which functional data is available.

| Gene function | Gene name | Predicted protein size (kDa) |
|---|---|---|
| **Nucleotide metabolism, transcription, replication and repair** | | |
| Thymidylate kinase | A240L | 27.8 |
| Thymidine kinase | K196R | 22.4 |
| dUTPase* | E165R | 18.3 |
| Ribonucleotide reductase (small subunit) | F334L | 39.8 |
| Ribonucleotide reductase (large subunit) | F778R | 87.5 |
| DNA polymerase family B | G1211R | 139.8 |
| DNA topoisomerase type II* | P1192R | 135.5 |
| Proliferating cell nuclear antigen (PCNA)-like | E301R | 35.3 |
| DNA polymerase X-like* | O174L | 20.3 |
| DNA ligase* | NP419L | 48.2 |
| AP endonuclease class II* | E296R | 33.5 |
| RNA polymerase subunit 2 | EP1242L | 139.9 |
| RNA polymerase subunit 6 | C147L | 16.7 |
| RNA polymerase subunit 1 | NP1450L | 163.7 |
| RNA polymerase subunit 3 | H359L | 41.3 |
| RNA polymerase subunit 5 | D205R | 23.7 |
| RNA polymerase subunit 10 | CP80R | |
| TFIIB like | C315R | |
| Helicase superfamily II | A859L | 27.8 |
| Helicase superfamily II | F1055L | 123.9 |
| Helicase superfamily II | B962L | 109.6 |
| Helicase superfamily II | D1133L | 129.3 |
| Helicase superfamily II | Q706L | 80.4 |
| Helicase superfamily II | QP509L | 58.1 |
| Transcription factor SII | I243L | 28.6 |
| Guanylyl transferase* | NP868R | 29.9 |
| PolyA polymerase large subunit | C475L | 54.8 |
| FTS J-like methyl transferase domain | EP424R | 49.3 |
| ERCC4 nuclease domain | EP364R | 40.9 |
| Lambda-like exonuclease | D345L | 39.4 |
| VV A2L-like transcription factor | B385R | 45.3 |
| VV A8L-like transcription factor | G1340L | 155.0 |
| VV VLTF2-like late transcription factor, FCS-like finger | B175L | 20.3 |
| DNA primase | C962R | 111.3 |
| **Other enzymes** | | |
| Prenyltransferase* | B318L | 35.9 |
| Serine protein kinase* | R298L | 35.1 |
| Ubiquitin conjugating enzyme* | I215L | 24.7 |
| Nudix hydrolase* | D250R | 29.9 |
| **Host cell interactions** | | |
| IAP apoptosis inhibitor* | A224L | 26.6 |
| Bcl 2 apoptosis inhibitor* | A179L | 21.1 |
| Inhibitor of host gene transcription* | A238L | 28.2 |
| C-type lectin-like* | EP153R | 18.0 |
| CD2-like. Causes haemadsorption to infected cells* | EP402R | 45.3 |
| Similar to HSV ICP34.5 neurovirulence factor | DP71L | 8.5 |
| Nif S-like | QP383R | 42.5 |
| Phosphoprotein binds to ribonucleoprotein-K | CP204L | 30.0 |

**Table 2**
Genes encoding structural proteins and other proteins involved in virus morphogenesis. Genes which encode known virus structural proteins and other proteins involved in virus morphogenesis are indicated. The gene name is indicated in the central column and predicted molecular weight in the right column.

| Gene function | Gene name | Predicted protein size (kDa) |
|---|---|---|
| **Structural proteins and proteins involved in morphogenesis** | | |
| P22 | KP177R | 20.2 |
| Histone-like | A104R | 11.5 |
| P11.5 | A137R | 21.1 |
| P10 | A78R | 8.4 |
| pA151R. Component of redox pathway | A151R | 17.5 |
| P72 major capsid protein. Involved in virus entry | B646L | 73.2 |
| Sulfhydryl oxidase componet of redox pathway | B119L | 14.4 |
| P49. Required for formation of vertices in icosahedral capsid | B438L | 49.3 |
| Chaperone. Involved in folding of capsid. Not incorporated into virions | B602L | 45.3 |
| ERV 1-like. Involved in redox metabolism* | B119L | 14.4 |
| SUMO-1-like protease. Involved in polyprotein cleavage | S273R | 31.6 |
| pp220 polyprotein precursor of p150, p37, p14 and p34. Required for packaging of nucleoprotein core | CP2475L | 281.5 |
| P32 (P30) phosphoprotein. Involved in virus entry | CP204L | 23.6 |
| pp62 (pp60) polyprotein precursor of p35 and p15 | CP530R | 60.5 |
| P12 attachment protein | O61R | 6.7 |
| P17. Required for progression of precursor membranes to icosahedral intermediates | D117L | 13.1 |
| J5R. Transmembrane domain | H108R | 12.5 |
| P54 (j13L). Binds to LC8 chain of dynein, involved in virus entry. Required for recruitment of envelope precursors to the factory | E183L | 19.9 |
| J18L. Transmembrane domain | E199L | 22.0 |
| P14.5. DNA-binding. Required for movement of virions to plasma membrane | E120R | 13.6 |
| E248R (k2R). Possible component of redox pathway required disulphide bond formation Structural protein | E248R | 27.5 |
| XP124L. Multigene family 110 member Contains KDEL ER retrieval sequence and transmembrane domain | MGF 110-4L (XP124L) | 14.2 |
| EP402R. Similar to host CD2 protein Required for binding red blood cells to infected cells and extracellular virus particles Glycoprotein inserted into external virus envelope | EP402R | 45.3 |

opening in the icosahedron, gives rise to "intermediate" particles, where the nucleoprotein core undergoes additional consolidation to produce the characteristic mature or "full" virions (Brookes et al., 1998a). Repressing expression for the gene encoding the pp220 polyprotein, which encodes the major components of the virus core shell, results in formation and egress of empty virus particles (Andres et al., 2002).

## 4. ASFV encoded genes

The known functions of ASFV encoded genes are shown in Table 1 and of those involved in virus morphogenesis in Table 2.

### 4.1. Genes involved in DNA replication and repair

Enzymes and proteins, that are likely to be directly involved in replicating the virus DNA, include a DNA polymerase type B (G1211R) and a PCNA-like protein (E301R) which may function in clamping DNA polymerase to the DNA (Iyer et al., 2006; Yanez et al., 1995). The C962R gene encodes an NTPase that resembles the vaccinia virus D5 protein which is required for DNA replication and might function at the replication fork (Evans and Traktman, 1992; McFadden and Dales, 1979). D5 is classified within the AAA

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subfamily of superfamily III DNA helicases (Iyer et al., 2001, 2006). D5 also has motifs associated with DNA primases. In addition, the F1055L protein shares some similarity to a herpes virus UL-9 protein that is involved in binding to origins of replication and fused to a putative DNA primase (Iyer et al., 2001). These latter proteins are probably involved in initiating DNA replication.

Genes encoding putative enzymes involved in resolving concatemeric genome intermediates into unit lengths have been identified in the ASFV genome. One of these, an ERCC4-like nuclease (EP364R), is related to the principle Holliday junction resolvase, Mus81, of eukaryotes, and the virus also encodes a lambda type exonuclease (D345L) also likely to be involved in this process (Iyer et al., 2006). Other ASFV encoded proteins predicted to have roles in DNA replication or repair pathways include a putative DNA topoisomerase type II (P1192R) (Baylis et al., 1993; Garciabeato et al., 1992a) and DNA ligase (Hammond et al., 1992).

ASFV encodes enzymes involved in DNA repair and this may represent an adaptation to replication in the macrophage. The production of reactive oxygen species (ROS) by macrophages induce a wide spectrum of microbial damage including DNA lesions which have either miscoding properties or are blocks for DNA and RNA polymerases. The majority of lesions induced by ROS are repaired by a base excision repair pathway (BER). Mammalian BER pathways involve replacement of either a single nucleotide (short-patch) or up to 10 nucleotides (long patch). Three enzymes, a DNA polymerase type X (O174L), which is the smallest known, together with an ATP-dependent -DNA ligase (NP419L) and a class II apurinic/apyrimidinic (AP) endonuclease (E296R) comprise likely components of an ASFV minimalist DNA BER mechanism. The *in vitro* properties of the ASFV DNA polymerase X indicate that this enzyme acts during a short patch BER of damaged DNA (Beard and Wilson, 2001; Garcia-Escudero et al., 2003; Jezewska et al., 2006; Showalter et al., 2001). The repair of oxidative DNA lesions is often initiated with a bifunctional DNA glycosylase that has an associated lyase activity. This glycosylase removes a chemically modified base generating an abasic site with an incision at 5′ of the site leaving a 3′-blocking group. Since ASFV does not code for a DNA glycosylase, it is possible that the virus uses a cellular enzyme to initiate BER. When BER is initiated by a bifunctional glycosylase, as in the case of oxidative damage, the 3′-blocking group can be cleansed by the 3′-diesterase activities of the ASFV AP endonuclease, thus allowing pol X to fill in the gap, and the chain is then sealed by the virus DNA ligase. Alternatively, BER could be initiated by a monofunctional DNA glycosylase. In this case, the virus AP endonucleolytic activity will incise the DNA chain at the abasic site leaving a 5′-dRP blocking group, which in cellular BER is eliminated by the dRP lyase of DNA polymerase β. Since pol X lacks this activity, BER should proceed by the long-patch pathway, with the participation of pol X and/or the virus replicative DNA polymerase, the PCNA-like pE301R, the 5′–3′ exonuclease of pD345R and the DNA ligase. The AP lyase activity of pol X (Garcia-Escudero et al., 2003), led to the proposal of an alternative viral BER pathway initiated by a monofunctional DNA glycosylase, followed by incision of the chain at the abasic site by the pol X lyase, removal of the 3′-block by the AP endonuclease 3′-diesterase activities, filling in the gap by the polymerase activity of pol X and sealing the nick by the DNA ligase. The E296R protein was shown to be a redox sensitive enzyme with 3′–5′ exonuclease and 3′ repair diesterase activities and a strong preference for mispaired and oxidative base lesions at the 3′ termini of single strand breaks and thus well adapted to the repair of ROS-induced DNA strand breaks (Redrejo-Rodriguez et al., 2009). The requirement of the AP endonuclease for efficient replication in macrophages, but not tissue culture cells, supports the hypothesis that this BER repair system is an adaptation to virus replication in the macrophage cytoplasm (Lamarche and Tsai, 2006; Redrejo-Rodriguez et al., 2006).

The ASFV DNA polymerase X is the smallest DNA polymerase known (174 residues) and was the first DNA polymerase solution structure determined by nuclear magnetic resonance. The structure shows that the enzyme does not resemble a “hand” as has been described for other DNA polymerases since the “fingers” and “thumb” are missing and the topology of the “palm” is unique. Thus, it was predicted to lack the N-terminal lyase domain and duplex DNA binding subdomain of DNA polymerase B but to retain the catalytic and C-terminal subdomains involved in dNTP selection (Beard and Wilson, 2001; Showalter et al., 2001). Biochemical analysis of the ASFV DNA polymerase X suggests that it exhibits low fidelity (Showalter et al., 2001), in particular the enzyme showed a high propensity to form G:G mispairs. There are conflicting reports about this (Garcia-Escudero et al., 2003). The demonstration that fidelity of pol X depends on the redox state of the polymerase, with the reduced form of the enzyme being more accurate than the oxidised form (Voehler et al., 2009), provides a possible explanation for the discrepancy in observations. It has been suggested that the error prone nature of this repair polymerase might contribute to increase mutation frequency in the viral genome and that this may help to generate antigenic variants thereby facilitating virus survival (Showalter et al., 2001). For this hypothesis to be relevant, the ASFV DNA ligase should be low fidelity and capable of sealing nicks at 3′ mismatched base pairs. In support of this, an analysis of the catalytic efficiency of nick sealing, using all possible matched and mismatched base pair combinations, showed the ASFV DNA ligase is the lowest fidelity DNA ligase ever reported. Comparison of the mismatch specificity of the ASFV DNA polymerase X with that of ASFV DNA ligase suggests that the ligase may have evolved towards low fidelity for the purpose of generating the broadest spectrum of sealed mismatches (Lamarche et al., 2005). However, the *in vivo* relevance of this model remains to be determined.

Due to the limiting pools of dNTPs present in cells, many large DNA viruses encode enzymes involved in nucleotide metabolism. These function to increase the precursor pools of dNTPs required for virus DNA replication. These enzymes are especially important for ASFV replication, since the main target cells for ASFV replication are non-dividing mature macrophages with low levels of dNTPs. This is evidenced by the virus-encoded thymidine kinase (K196R) and dUTPase (E165R) genes, both of which have been shown to be non-essential for virus replication in dividing tissue culture cells, but their deletion dramatically reduces virus replication in macrophages (Moore et al., 1998; Oliveros et al., 1999). The virus also encodes thymidylate kinase (A240L) and two subunits of ribonucleotide reductase (F134L, F778R) (Boursnell et al., 1991), both enzymes are involved in nucleotide metabolism.

### 4.2. Genes encoding enzymes and factors involved in mRNA transcription and processing

ASFV gene transcription does not require the host RNA polymerase II and it is therefore presumed that the virus encodes all the enzymes and factors needed to transcribe and process mRNAs. Virus core particles are transcriptionally active and therefore likely to contain all enzymes and factors for early mRNA synthesis (Salas et al., 1981, 1986; Santaren and Vinuela, 1986). Genes encoding proteins with similarity to six subunits of host RNA polymerase II complex have been identified (EP1242L, C147L, NP1450L, H359L, D205R, CP80R) as well as orthologs of the basal eukaryotic transcription factor TFIIB (C315R) and transcription elongation factor S-II (I243L) (Iyer et al., 2006). Other subunits of the ASFV RNA polymerase remain to be identified since the vaccinia virus (VACV) enzyme has nine subunits (Jones et al., 1987). ASFV is predicted to encode stage specific transcription factors and four putative factors G1340L, I243L, B175L and B385R, have been identified by comparison with sequences of the vaccinia virus factors VETF 82 kDa

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subunit (A8L), VITF-1 (E4L), VLTF-2 (A1L) and VLTF-3 (A2L), respectively. The VACV G8R gene encodes VLTF-1 and has recently been identified as a PCNA structural homologue (Da Silva and Upton, 2009), therefore it is possible that the ASFV PCNA homologue E301R may also play a role in late transcription. Of the six members of the helicase superfamily encoded by ASFV (A859L, F105L, B92L, D1133L, Q706L, QP509L), three are predicted to have roles in transcription based on their similarity with VACV enzymes.

ASFV transcripts are modified by addition of a 5′ cap and 3′ polyA tail. The gene encoding the mRNA capping enzyme (NP868R) encodes the three domains, triphosphatase, guanyl transferase and methyltransferase that are required for this function. A putative polyadenylation enzyme (C475L) has been identified by comparison with VACV (Iyer et al., 2006). A recent study showed that the VACV core polyadenylation enzyme also adenylates the 3′ end of micro RNAs (miRNA) resulting in their destabilisation. This is predicted to contribute to the down-regulation of miRNA expression during poxvirus infections. It remains to be determined if the ASFV counterpart has a similar function (Backes et al., 2012).

The ASFV encoded NUdix hydrolase (g5R, D250R) shares similarity in sequence to other enzymes in this family including the D9 and D10 VACV nudix hydrolases. The VACV D9 enzyme and the ASFV g5R NUdix hydrolases have been shown to cleave the mRNA cap when tethered to an RNA moiety, liberating m(7)GDP. The decapping activity of g5R was inhibited by excess uncapped RNA but not by methylated cap analogues (Parrish et al., 2009). This activity is proposed to be important for stage-specific switching of virus gene expression by causing destabilisation of mRNAs and reduced translation initiation. It may also be involved in the modulation of host mRNA expression. The ASFV g5R enzyme was also shown to degrade the inositol pyrophosphate diphosphoinositol pentakisphosphate and this function may be important in the viral replication cycle (Cartwright et al., 2002).

### 4.3. Genes encoding other enzymes

Other enzymes encoded by ASFV but without defined roles include a predicted FTS-J-like RNA methyltransferase (EP424R). Possibly this gene may stabilise rRNA in infected cells and help to prevent shut-off of protein synthesis (Iyer et al., 2006). The virus also contains a serine/threonine protein kinase (R298L) which is packaged into virus particles suggesting it may have a role early in infection (Baylis et al., 1993). ASFV encodes for an ubiquitin conjugating enzyme (I215L) that shuttles between the nucleus and cytoplasm and also binds to a host ARID DNA-binding domain containing protein. This suggests that it may have a role in regulating a host nuclear function (Bulimo et al., 2000; Hingamp et al., 1992), however, virus replication sites contain ubiquitinylated proteins and pI215L has also been shown to ubiquitinylate CP530R or one of its products *in vitro* (Hingamp et al., 1995), therefore the ASFV ubiquitin conjugating enzyme may have multiple roles in the infective cycle.

An ASFV encoded trans-prenyl transferase (B318L) catalyses the condensation of farnesyl diphosphate and isopentenyl diphosphate *in vitro* to synthesise geranylgeranyl diphosphate and longer chain prenyl diphosphates (Alejo et al., 1997). This viral enzyme is localised to cytoplasmic viral assembly sites, associated with precursor viral membranes derived from the endoplasmic reticulum (ER) suggesting that it may have a role in factory and/or virion assembly (Alejo et al., 1999).

The virus also encodes two enzymes involved in redox metabolism, a homologue of the bacterial NifS protein (QP383R) and an ERV1 homologue (B119L). The latter is likely to be part of a redox chain involved in virus assembly (Lewis et al., 2000; Rodriguez et al., 2006).

### 4.4. Genes encoding structural proteins and other proteins involved in assembly

The genes identified which encode structural proteins and other proteins involved in virus assembly are shown in Table 2. Seventeen genes which encode virion structural proteins have been identified and their location in the multi-layered virus particle defined (Dixon et al., 2005). For some of these proteins, construction of viruses expressing inducible copies of the genes has enabled their roles in virion morphogenesis to be studied. The polyprotein, pp220 (CP2475L) is cleaved to yield the mature virion proteins p150, p37, p14, p34 and polyprotein pp60 (CP530R), is cleaved to p35 and p15, by a virus-encoded SUMO-like protease (S273R) (Andres et al., 1997; SimonMateo et al., 1993, 1997). These proteins form the major components of the core shell of the virion. Surrounding the core shell, the icosahedral capsid is assembled on a single lipid envelope derived from the endoplasmic reticulum. The major virion capsid protein p72 is encoded by the B646L gene and its assembly in virions requires a virus encoded chaperone encoded by B602L. Expression of the B438L encoded protein p49 is required to form the vertices of the icosahedral capsid indicating that the protein itself may be localised in these positions. Several of the virion structural proteins contain transmembrane domains and are localised either on the internal or external virion membranes. Those localised in the internal membrane include envelope p17 (D117L), p54 or j13L (E183L) and probably j18L (E199L), j5R (H108R) (Brookes et al., 1998b; Rodriguez et al., 1994a; SimonMateo et al., 1995; Suarez et al., 2010; Sun et al., 1995, 1996). The CD2v protein (EP402R), which is required for the binding of red blood cells to infected cells and to extracellular virions, is present on the external membrane layer (Borca et al., 1998). The p22 (KP177R) (Camacho and Vinuela, 1991) and p12 (pO61R) (Alcami et al., 1992) proteins are also thought to be localised on this envelope. The virus encodes components of a redox pathway, including the pB119L (or 9GL), pE248R and pA151R proteins, of which the pA151R and pB119L proteins are non-structural (Rodriguez et al., 2006, 2009). Three proteins with experimental DNA binding properties are present in virions, p10 (K78R), p11.6 (A104R) and p14.5 (E120R) (Andres et al., 2001; Borca et al., 1996; MartinezPomares et al., 1997; Munoz et al., 1993) Protein E120R is present on the surface of intracellular virions and is required for transport of virions from the factory sites to the plasma membrane.

### 4.5. Genes encoding proteins involved in modulating host defences

As a macrophage-tropic virus, ASFV can manipulate both innate and adaptive immune responses by modulating macrophage functions and several ASFV proteins that interfere with host defences have been identified. These include the A238L protein which acts to inhibit transcriptional activation of host immunomodulatory genes. It does this by inhibiting activation of the transcriptional co-activator p300/CBP by several transcription factors, including NFκB, which interact with the N-terminus of p300/CBP. In addition, A238L inhibits the host calcineurin dependent pathways by directly binding calcineurin phosphatase (Granja et al., 2008; Miskin et al., 1998). The I329L protein acts as an inhibitor of Toll-like receptor 3 signalling pathways and members of the multigene families 360 and 530 have been implicated in inhibiting type I interferon induction and responses (Afonso et al., 2004; de Oliveira et al., 2011). The DP71L protein acts as a regulatory subunit of host protein phosphatase 1 (PP1) recruiting PP1 to dephosphorylate translation initiation factor eIF2α thereby preventing the global shut-off of protein synthesis induced by phosphorylation of PKR or other kinases such as PERK (Rivera et al., 2007; Zhang et al., 2010).

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Another strategy used by ASFV is to encode adhesion proteins that can modulate interactions of infected cells or extracellular virions with the extracellular environment. An ASFV encoded protein (EP402R or CD2v), which shares similarities in its extracellular domain with host CD2 protein, is required for binding of red blood cells to infected macrophages and extracellular virus particles. CD2V also has a role in the ASFV-induced inhibition of lymphocyte proliferation in response to mitogens (Borca et al., 1994, 1998). The EP153R protein is a type II transmembrane protein which contains a C-type lectin domain and resembles NK cell receptors such as CD69. This ASFV encoded protein has been shown to inhibit up-regulation of MHC Class I expression on the cell surface; EP153R also inhibits apoptosis (Hurtado et al., 2004, 2011). ASFV encodes other proteins, such as a Bcl2 and an IAP homologue that inhibit apoptosis and can thus prolong survival of infected cells to facilitate virus replication (Brun et al., 1996; Galindo et al., 2008; Nogal et al., 2001; Revilla et al., 1997).

### 4.6. Multigene families

Approximately 30% of the ASFV genome encodes sets of paralogous genes, MGF100, 110, 300, 360, 505/530 and p22, are each present in multiple copies per genome; the number of paralogs vary between different isolates (Aguero et al., 1990; Jones et al., 1987; Pires et al., 1997; Yanez et al., 1995; Yozawa et al., 1994). The virus MGFs are located within the left 40 kbp and right 20 kbp or the genome (Delavega et al., 1994; Rodriguez et al., 1993, 1994b; Yozawa et al., 1994). Generally members of MGFs are positioned adjacent to each other and read in the same direction indicating that they are evolved by gene duplication. Most MGF families have copies at each end of the genome as described for some poxvirus genes and these are proposed to have been transposed during genome replication and resolution. This large investment in coding for multiple copies of several MGFs implies that these offer a selective advantage to the virus. The large sequence divergence between individual genes, and the presence of additional functional domains within some copies of the MGF proteins, suggests that at least some of these genes have different functions. For example amongst the MGF110 members, two genes encode proteins which contain a KDEL endoplasmic reticulum (ER) retrieval sequence (XP124L, Y118L) (Netherton et al., 2004) and several encode proteins with predicted signal peptides and cleavage sites suggesting that they are secreted from infected cells. A subset of the MGF360 genes, encodes ankyrin protein interaction domains.

## 5. Variation between ASFV genomes

ASFV isolates collected from the wildlife cycle in East Africa and from outbreaks in domestic pigs in Africa, Europe and the Caucasus have been characterised by sequencing of individual genes and for 12 isolates, to date, complete genome sequences have been determined. Comparison of the partial sequence for the gene, B646L, encoding the major capsid protein p72 has been used to genotype isolates, and by this analysis, 22 genotypes have been defined. All of these genotypes are present in eastern and southern Africa where the ancient sylvatic cycle involving warthogs and soft ticks of the *Ornithodoros moubtata* species is present (Boshoff et al., 2007; Lubisi et al., 2005, 2007). The genetic diversity of isolates from this cycle is high, reflecting the long term evolution and selective constraints imposed by replication, in and transmission between, these hosts. It appears from analysis of isolates circulating in domestic pigs that spillover from the sylvatic cycle to domestic pigs is relatively rare and that most transmission in domestic pig populations, even in East Africa, are from pig to pig. Analysis of isolates circulating in domestic pigs identified that genotype I spread through central and western Africa and this genotype jumped to Portugal in 1957 and 1960 and from there to other European countries, including Sardinia, and also to the Caribbean and Brazil (Dixon and Wilkinson, 1988; Gallardo et al., 2011b; Lubisi et al., 2005, 2007; Nix et al., 2006). Until recently genotype I was the only one found in central and western Africa but genotype IX has now been identified in Republic of Congo (Gallardo et al., 2011b). This genotype is presumed to have spread from East Africa where it is the predominant genotype circulating in Uganda and Kenya (Gallardo et al., 2011a). Genotype II is one of the genotypes circulating in south-eastern Africa in pigs in Mozambique and Zambia and has also been introduced to Madagascar (1998), Mauritius (2007) and Georgia (2007) in the Caucasus (Chapman et al., 2011; Lubisi et al., 2009; Rowlands et al., 2008). From Georgia ASF has spread to neighbouring countries including the Russian Federation, Armenia, Azerbaijan and Ukraine (OIE WAHID).

Comparison of the sequences of the B646L gene and other individual genes revealed few if any changes in sequence from isolates collected from domestic pigs in Europe and Africa over periods of 40 years. Most genome variation was as the result of gain or loss of genes in the multigene families. In addition, variation in number of repeats within tandem repeats in non-coding regions was observed. Tandem repeats have been identified within the genes encoding the p54 structural protein encoded by E183L and the B602L virus encoded chaperone as well as in other proteins of unknown function.

Gene copy number is highest and most variable in MGF 360, which contains 22 paralogous genes in total. The number encoded by different genomes ranges between 11 in the low virulence Portuguese isolate OURT88/3 and tissue-culture adapted isolate BA71V to 18 in the Mkuzi isolate from Zululand 1979 and Kenya 1965 isolates. MGF 110 family includes 14 paralogs which vary in number from 5 in the Benin 97/1 and BA71V genomes to 12 in the Mkuzi isolate genome. Other MGF families have fewer members and are less variable in number between different genomes. For example, MGF 505/530 has 11 paralogs and 8–10 of these are present in all genomes, MGF 300 has four members with three or four present on different genomes and MGF 100 has three members varying between two and three copies. Comparison of the sequences of the MGF proteins shows that these can be quite divergent but share common family-associated motifs. Paralogous proteins (different copies of an MGF on an individual genome) are much more divergent than orthologs (same family member on different genomes). This is illustrated on Fig. 2 which compares the phylogeny of orthologs of MGF360-8L with paralogs of MGF360 from Georgia 2007/1. The orthologs of MGF360-8L share at least 86% identity between isolates whereas amino acid identity between paralogs on the Georgia 2007/1 genome can be as low as 12% (for example between MGF360-1L and 18R) or 15% (between MGF360-1L and 15R). These genes are presumed to have diverged following gene duplication events, but the time scale and any selection pressures leading to gene amplification is unknown. Comparison of MGF360 paralogs on the Georgia 2007/1 genome indicates that those present near the right genome end (MGF360-15R, MGF360-16R, MGF360-18R and MGF360-21R) are more divergent from each other (between 12% and 37% amino acid identity) and from MGF360 paralogs near the left genome end (see Fig. 2). One notable exception is the relatively close similarity between the MGF 360 genes present furthest left (MGF360-1L) with that furthest right (MGF360-21R) which share 69% identity. These are amongst the most closely related paralogs of MGF360 on the Georgia 2007/1 genome, suggesting that they were relatively recently duplicated by gene transposition from one genome end to the other. A few examples of recent gene duplications are evident.

The evolution of gene families in genomes of large DNA viruses is a common feature. Recent evidence shows that under strong

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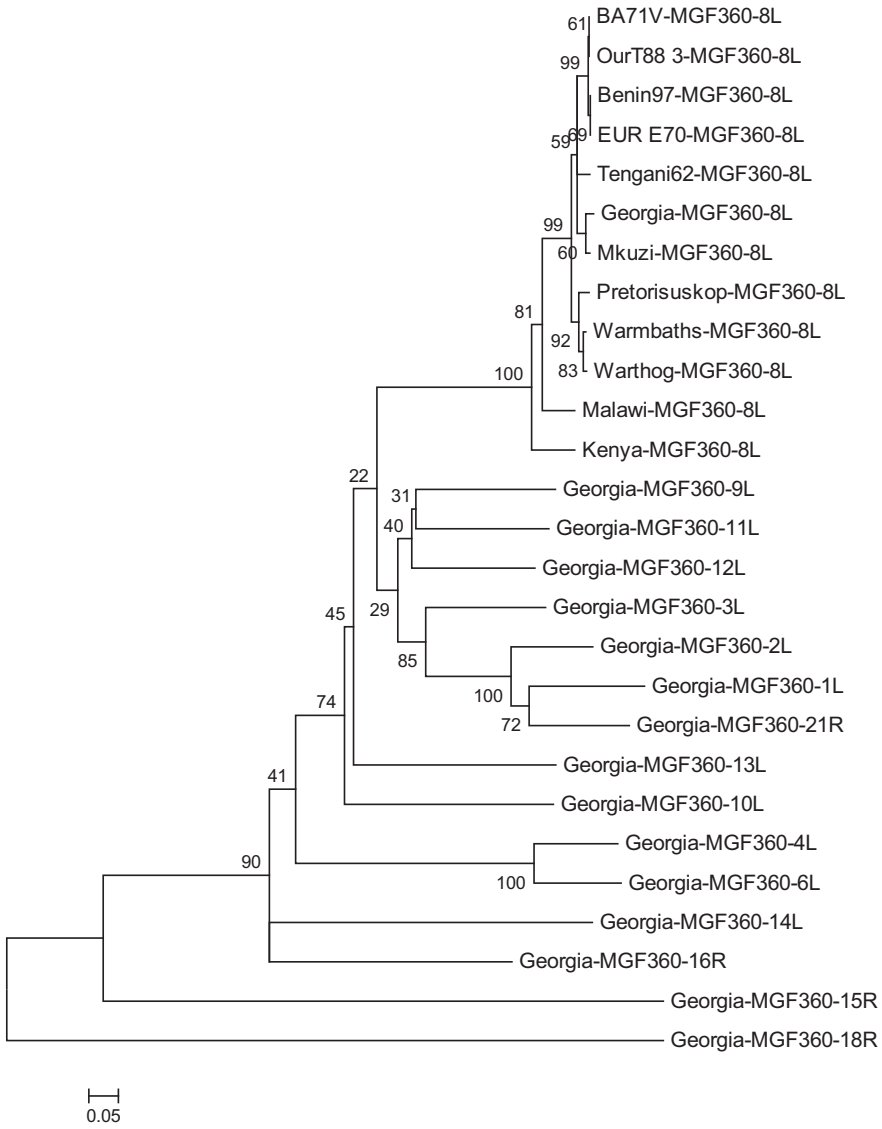


**Fig. 2.** Phylogeny of orthologs and paralogs of MGF360 genes. The evolutionary history of amino acid sequences of MGF360 encoded proteins was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 203 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

selection pressure during passage in culture poxviruses rapidly acquired fitness *via* recurrent amplification of a key anti-host defence gene facilitating the gain of a key amino acid substitution (Elde et al., 2012). Thus, it is possible that ASFV multigene families were acquired rapidly under a strong selection with subsequent divergence.

The OURT88/3 and BA71V isolates each have a large deletion of 8 or 10 kbp respectively compared to other genomes near the left genome end. These deletions are in similar but not identical genome positions. OURT88/3 lacks five MGF360 and two MGF 505/530 members and BA71V lacks five and a half MGF360 and two and a half MGF505/530 members (Chapman et al., 2008). Targeted deletion of six copies of MGF360 and two copies of MGF505/530 from the genome of the Pr4 isolate resulted in (1) increased expression of type I interferon and interferon response genes, (2) reduced replication in ticks and in macrophages, and (3) reduced virulence in pigs (Afonso et al., 2004; Burrage et al., 2004; Zsak et al., 2001). Uniquely the BA71V genome has a second large deletion of about 3 kbp near the right hand genome terminus that encodes five or six ORFs including one or two copies of a gene similar to that encoding the p22 early membrane protein (KP177L) at the left genome end and two copies of a gene encoding an unusual SH2 binding domain (Chapman et al., 2008).

Comparison of the sequenced genomes reveals 109 unique genes that are present in all 12; a further 16 members of the MGFs are also conserved in these 12 genomes. The conserved genes encode for the structural proteins, proteins involved in virus assembly, enzymes and other factors involved in nucleotide metabolism, DNA replication and repair, mRNA transcription and processing, several proteins involved in regulating host cell pathways, and several of unknown function. Of the remaining 26–42 ORFs, which are not conserved between all of the 12 ASFV isolates sequenced, about half are members of 5 MGFs (Chapman et al., 2008; de Villiers et al., 2010).

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Comparison of the concatenated amino acid sequences of proteins encoded by the 125 conserved ORFs, comprising 40,810 amino acids, shows that most of the sequenced isolates cluster in two main clades. The first group comprises isolates from West Africa and Europe belonging to genotype I. The Mkuzi 1979 and Georgia 2007/1 isolate also fall within this group but are more distantly related to the genotype I isolates. The second group comprises other isolates from East and southern Africa (Tengani 62, Warthog, Warmbaths, Pretoriskup 96). Two isolates, Malawi Lil 20/1 and Kenya 1950, are outliers from these groups. Interestingly, trees derived from analysis of individual protein alignments do not always match the phylogeny derived from the concatenated conserved proteins. For example for the CD2v (EP402R) protein sequence from the Georgia 2007/1 sequence clusters more closely with the Malawi Lil20/1 and Kenya 1950 isolates whereas the EP153R protein sequence from the Georgia 2007/1 isolate clusters more closely with the warthog isolate (Chapman et al., 2008; de Villiers et al., 2010). This suggests that past recombination events may have occurred, although there are no longer contiguous genome segments where recombination between genomes is evident. Persistently ASFV infections of warthogs and *Ornithodoros* spp. soft ticks over long periods likely provide opportunity for co-infections with different genotypes with subsequent recombination between genomes.

Comparison of synonymous and non-synonymous mutations at the genome level identified about 18 genes subject to selection pressure for diversification. These included members of MGF 360 (8L, 16R) and MGF 505/530 (4R, 7R, 9R), EP402R (CD2v), EP153R, several enzymes and the B602L chaperone. The major capsid protein did not have any sites under strong selection pressure and almost all mutations are synonymous indicating strong stabilising selection (de Villiers et al., 2010).

The advent of next generation sequencing will mean that many more complete genome sequences can be determined (Radford et al., 2012) enabling a much more comprehensive analysis of the relationship between different isolates. To date genome sequences are only available for 6 or the 22 genotypes identified by partial sequencing of the B646L major capsid protein.

## 6. Relationship to other virus families

ASFV is currently the only member of the *Asfarviridae* (standing for African swine fever and related virus) family. The *Asfarviridae* form one family within the nucleocytoplasmic large DNA virus (NCLDV) superfamily. The NCLDV comprise an apparently monophyletic class of viruses that infect a broad range of eukaryotic hosts. They share the feature of carrying out at least part of their replication cycle in the cytoplasm with varying dependence on the nucleus. The relative independence of the NCLDV's from the nucleus means that they must encode conserved proteins that mediate most processes essential for virus replication and transcription (Iyer et al., 2001, 2006). The first virus families proposed in the NCLDV included; the *Asfarviridae*; the *Poxviridae*, different members of which infect insects, reptiles, birds and mammals; the *Iridoviridae*, which infect insects and cold-blooded vertebrates; the *Phycodnaviridae* which infect algae (Iyer et al., 2001). The NCLDV were later expanded to other newly discovered virus families including the *Mimiviridae*, and *Marseillevirus* which infect *acanthamoeba* and the *Ascoviridae* which infect insects, mainly Noctuids. The *Mimiviridae* have the largest known genome of a virus (1180 kbp), other families in the NCLDV have genomes from 100 to 400 kbp (Iyer et al., 2006). Comparison of the genome sequences of different NCLDV families identified a core set of 9 genes that were present in all families and a variety of others that were present in more than one family. A reconstruction mapped about 40 genes to a putative common ancestor. Typically the NCLDV share additional hallmark genes not present in the other large DNA virus families such as a Superfamily 3 helicase fused to DNA primase, a packaging ATPase and a disulphide oxidoreductase involved in morphogenesis. In addition, individual NCLDV virus families encode unique genes acquired to facilitate replication in particular environmental niches (Iyer et al., 2006; Koonin et al., 2009; Koonin and Yutin, 2010; Yutin et al., 2009).

Unexpectedly, metagenomic sequencing projects of virus fractions from different environmental niches have identified gene fragments that share high sequence similarity to a variety of genes, including hallmark genes of the *Asfarviridae* family. These include samples from the marine environment and, surprisingly, from humans (Loh et al., 2009; Monier et al., 2008; Ogata et al., 2009; Yozwiak et al., 2012). Furthermore, the *Heterocapsa circularisquama* DNA virus (HcDNAV, genome ~356 kbp), that infects marine dinoflagellates (Dinophyceae), encodes a DNA polymerase B with sequence similarity to that of ASFV suggesting that it may be more closely related to the *Asfarviridae* than the *Phycodnaviridae* as was originally believed. However, additional genome sequencing is required to confirm this hypothesis (Ogata et al., 2009).

These very interesting findings suggest that additional members of the *Asfarviridae* family may soon be identified.

## 7. Conclusions and future prospects

Although basic information is available regarding the mechanisms of ASFV replication, many details of the process are lacking. In particular, the role of the nucleus, and the early stage of DNA synthesis that is thought to take place there, in the replication cycle requires further investigation. Recent work has defined many of the functions of the ASFV encoded enzymes involved in the base excision repair pathway, but some details are still unknown. This ASFV-associated BER pathway seems distinct from others since a virus encoded DNA glycosylase has not been identified.

A striking feature of the ASFV genome is its large number of multigene families. At least the MGF 360 and MGF 505/530 seem to have important roles in virus tropism, virulence and in suppressing the interferon response. However, almost nothing is known about how the encoded proteins function and the selection pressures leading to their amplification and deletion.

The advent of next generation sequencing will lead to increasing numbers of complete ASFV genome sequences becoming available (Radford et al., 2012). This will have multiple applications to improve our understanding of: the evolutionary relationship between different isolates; epidemiological tracing of disease outbreaks; definition at the molecular level the phenotypic differences between isolates; identifying selection pressures which lead to genome evolution.

Although ASFV is currently the only member of the *Asfarviridae* family closely related sequences have been identified from different environments by metagenomic sequencing studies; this suggests that new members of the family are on the horizon.

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