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Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes

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Abstract Complete sequencing of *p54*-gene from 67 European, American, and West and East African Swine Fever virus (ASFV) isolates revealed that West African and European ASFV isolates classified within the predominant Genotype I according to partial sequencing of p72 were discriminated into four major sub-types on the basis of their p54 sequences. This highlighted the value of p54 gene sequencing as an additional, intermediate-resolution, molecular epidemiological tool for typing of ASFV viruses. We further evaluated p54-based genotyping, in combination with partial sequences of two other genes, for determining the genetic relationships and origin of viruses responsible for disease outbreaks in Kenya. Animals from Western and central Kenya were confirmed as being infected with ASFV using a *p72* gene-based PCR assay, following outbreaks of severe hemorrhagic disease in domestic pigs in 2006 and 2007. Eleven hemadsorbing viruses were isolated in macrophage culture and genotyped using a combination of full-length *p54*-gene sequencing, partial *p72*-gene sequencing, and analysis of tetrameric amino acid repeat regions within the variable region of the *B602L* gene (CVR). The data revealed that these isolates were identical in their p72 and p54 sequence to viruses responsible for ASF outbreaks in Uganda in 2003. There was a minor difference in the number of tetrameric repeats within the *B602L* sequence of the Kenyan isolates that caused the second Kenyan outbreak in 2007. A practical implication of the genetic similarity of the Kenyan and Ugandan viral isolates is that ASF control requires a regional approach.

Keywords ASFV · Epidemiology · Genotyping · p72 · p54 · CVR

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Introduction

African swine fever (ASF) is a viral disease of domestic pigs that causes a range of clinical syndromes varying from acute to chronic disease and apparently asymptomatic animals that are carriers of the virus. Virulent strains induce acute hemorrhagic disease, with symptoms, including high fever, hemorrhages in the skin and internal organs, and typically death in 3–10 days.

The causative agent is a double-stranded DNA virus classified within the *Asfarviridae* family, genus *Asfivirus* [1]. ASF has been reported from most countries in Sub-Saharan Africa, where the virus is maintained either through a sylvatic cycle involving warthogs (*Phacochoerus aethiopicus)* and soft ticks in the genus *Ornithodoros* or in a domestic cycle that involves pigs of local breeds, with or without tick involvement [2–5]. The wildlife reservoir is unlikely to be eliminated in the near future, making the disease very difficult to eradicate. Since there is no

currently available control measure other than diagnosis and slaughter, the disease poses a serious constraint to the development of both smallholder and industrial pig industries in Africa. Although the virus was first described by Montgomery from Kenya in 1921, there has subsequently been relatively little detailed research on the viruses involved in outbreaks in the East African region.

The ASFV genome consists of a linear double-stranded DNA molecule of 170–190 kbp with terminal inverted repetitions and hairpin loops [6, 7]. A high degree of variability in genome size and restriction fragment patterns is observed when different ASFV isolates are compared. Restriction enzyme site mapping [8] and sequence analysis of virus genomes [9] have established that the central region of the ASFV genome is relatively conserved but large length variations occur at the termini, particularly within 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome. Many of the length variations are associated with the loss or gain of copies within multigene families. In addition, smaller length variations are associated with the number of tandem repeats located at loci both within coding regions and in intergenic regions between genes [10–16]. ASFV molecular polymorphism has recently been investigated by partial sequencing of the gene encoding the major capsid protein p72, and 22 distinct genotypes have so far been defined [17, 18]. Isolates from Europe, the Caribbean, S. America, and W. and C. Africa are closely related to each other. In contrast, isolates from S. and E. Africa are more diverse probably because multiple introductions have occurred from wild suids and associated argasid tick vectors into domestic pig populations in these regions. Although p72 is useful for classification of major genotypes, higher resolution definition of virus relationships to uncover epidemiological relationships in regions where isolates are closely related to each other requires analysis of additional genes. Several regions containing variable tandem repeat sequences (TRS) located in the, generally more conserved, central region of the ASFV genome have been identified showing the TRS identified in the *B602L* gene (CVR) as the most variable locus [14, 18–22]. The tetramer amino acid repeats within the late-expressed p54 ASFV structural protein have been shown to exhibit variation in copy number in viral passages in cell culture [22]; however, the sequence of this gene has not previously been systematically employed for molecular discrimination of ASFV virus isolates. We report the first extensive use of p54 sequencing for molecular epidemiological studies of ASF viral isolates and demonstrate that virus isolates classified within the West African and European p72 genotype I can be discriminated. We have also applied genotyping based on p54, and partial sequencing of *p72* and *B602L* gene to viruses collected during recent outbreaks in Kenya that occurred in 2006 and 2007. The genotyping strategy employed involved sequencing of the 3′ end of gene encoding the p72 protein [23] and the full-length *p54*-gene to place isolates into major subgroups, followed by subtyping through analyzing the TRS in the CVR of the ASFV genome. This constitutes the first detailed assessment of the genotypic variability of ASFV field isolates from an active outbreak in East Africa

Materials and methods

Collection of samples

In May 2006, an outbreak of virulent hemorrhagic disease suspected to be caused by the ASF virus was reported from the Kenya–Uganda border district of Busia resulting in nine deaths. Quarantine was imposed on the movement of pigs and pork products within Busia municipality to avoid further spread of the disease. However, in October and November 2006, the disease reappeared in Busia and in neighboring Kisumu, respectively. In January 2007, a further outbreak was reported from Eldoret (Uasin Gishu district) bordering Busia with a total of 875 suspected cases resulting in 525 deaths. The disease spread rapidly from Western to central Kenyan districts, with new outbreaks reported in Nakuru and also Kiambu (Fig. 1). Following these outbreaks, a collection of samples (Table 1) from pigs with clinical signs of hemorrhagic disease were

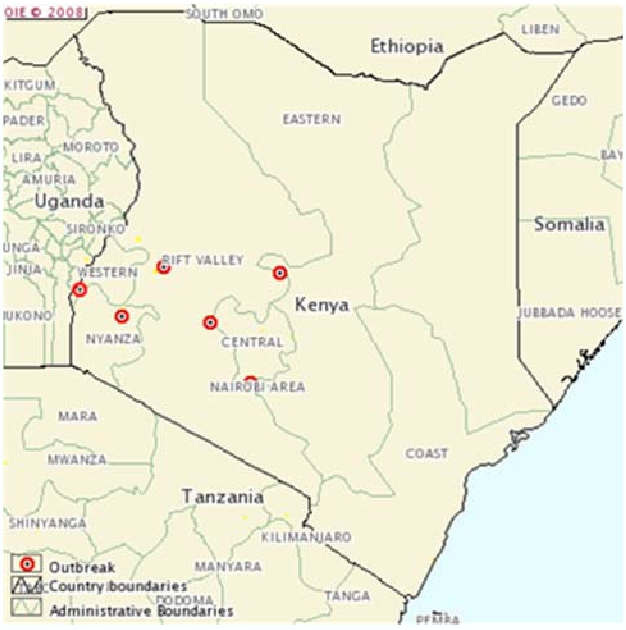

Fig. 1 Map of Kenya showing location of the 2006–2007 ASF outbreaks that were reported to the OIE and from which isolates were obtained and genotyped. Specific localities are indicated by red circles

Table 1 Samples submitted to CISA-INIA from ASF outbreaks in Kenya in 2006–2007

Animal number	Tissue	Source (District)	Date of onset of the outbreak
P9	Lymph node	Busia, WESTERN	10/05/2006
P10	Lung	Busia, WESTERN	10/05/2006
P11	Lung, spleen	Busia, WESTERN	10/05/2006
P31	Liver, Kidney, spleen	Busia, WESTERN	10/05/2006
P43	Lung, liver	Busia, WESTERN	10/05/2006
P9	Liver, Lung, Spleen, Intes.	Busia, WESTERN	13/10/2006
P49	Serum (1)	Kisumu, NYANZA	30/11/2006
P8	Sera (1–8)	Kapseret/Eldoret, Uasin Gishu, RIFT VALLEY	12/01/2007
P3	Spleen, LN gastric, Kidney		
P2	Liver, heart, lung	Kiambu CENTRAL	11/01/2007
P4	Spleen	Nakuru RIFT VALLEY	23/01/2007
P7	Sera (1–5)		

collected by Department Veterinary services staff and submitted to the Animal Health Research Centre (CISA-INIA), Valdeolmos, Spain (EU ASF Reference Laboratory), for ASF diagnosis and molecular characterization.

ASF diagnosis

Nucleic acid extraction and genomic DNA amplification

DNA was extracted directly from serum or 10% suspensions of ground tissues using a nucleic acid extraction kit (Nucleospin/Machery-Nagel–Cultek) following the manufacturer's procedures. A PCR assay using the ASF diagnosis primers PPA1/PPA2, which generates an amplicon of 257 bp within the p72 protein [24], was used to confirm the presence of ASFV DNA. The PCR products were analyzed by electrophoresis through 2% agarose gels and the specificity of the amplicons obtained was confirmed using the *BsmAI* [24] restriction endonuclease.

Virus isolation

Primary leukocyte cultures were used for the isolation of samples recovered from naïve domestic pigs as previously described [25]. Briefly, cells were seeded into 96-well tissue culture grade microtiter plates (200 µl; 300,000 cells per well) in homologous swine serum, and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Three day cultures were infected at a multiplicity of infection (moi) 1:10 with serum or 10% suspensions of ground tissues supplemented with 5 µg/ml gentamicine sulfates (BioWhittaker) and incubated for 24 hours at 37°C. After inoculation, a preparation of 1% homologous red blood cells in buffered saline was added to each well. The plates were examined for hemadsorption over a 6-day period. The samples were blind passaged three times.

ASF molecular characterization

Viruses

In addition to the 11 new isolates obtained from Kenya (Table 2), 56 ASF virus isolates from Africa, Europe, and Latin America available in the ASFV collection held at CISA Madrid, were characterized in this study. In addition, isolates representative of geographical localizations with published sequences available in GenBank were selected for this study. The geographical origins, sample source, and collection date of these ASF virus isolates are summarized in Table 3.

Genomic amplification and nucleotide sequence determination

A series of primers derived from several genes was used to PCR amplify specific regions of the Kenyan ASF isolates. The C-terminal region of the p72 protein, located between positions 86793 and 88733 on the Ba71V ASFV genome, was amplified using primers p72-U/D as previously described [23]. The primer pairs ORF9L-F/ORF9L-R were used to amplify the central variable region (CVR) located in the *B602L* gene [26]. The complete gene encoding the p54 protein, located between positions 145413 and 145964 on Ba71V ASFV genome, was amplified using the primers PPA722 (5′ CGAAGTGCATGTAATAAACGTC 3′, binding site 145342–145364) and PPA89 (5′ TGTAATTTC ATTGCGCCACAAC 3′ binding site 145997–146017). Primer binding sites and the predicted product size of p54 protein was based on the Ba71V ASFV genome (Accession No. U18466). Conditions for the PCR assays were as follows: 10–50 ng of sample DNA, 1× PCR buffer II (50 mM KCl, 10 mM Tris–HCl), 2.5 mM $MgCl_2$, 0.2 mM concentrations of the four deoxynucleoside triphosphates

Table 2 Kenyan isolates obtained in leukocyte culture from ASF outbreaks in Kenya in 2006–2007

Isolate name	Source (District)	Date of onset of outbreak	p72 gene Genbank accession no.	P72 genotype	p54 gene Genbank accession no.	p54 genotype	CVR Genbank accession no.	CVR Sub-group
Ken06.B1	Busia, WESTERN	10/05/2006	FJ154434	IX	FJ174441	IX	FJ174329	XXIV
Ken06.B2	Busia, WESTERN	10/05/2006	FJ154435	IX	FJ174442	IX	FJ174330	XXIV
Ken06.B3	Busia, WESTERN	10/05/2006	FJ154436	IX	FJ174443	IX	FJ174331	XXIV
Ken06.B4	Busia, WESTERN	10/05/2006	FJ154437	IX	FJ174444	IX	FJ174332	XXIV
Ken06.B5	Busia, WESTERN	10/05/2006	FJ154438	IX	FJ174445	IX	FJ174333	XXIV
Ken06.Bus	Busia, WESTERN	13/10/2006	FJ154439	IX	FJ174446	IX	FJ174334	XXIV
Ken06.Kis	Kisumu, NYANZA	30/11/2006	FJ154440	IX	FJ174447	IX	FJ174337	XXIV
Ken07.Eld1	Kapseret/Eldoret, Uasin	12/01/2007	FJ154441	IX	FJ174438	IX	FJ174335	XXIV
Ken07.Eld2	Gishu, RIFT VALLEY		FJ154442	IX	FJ174439	IX	FJ174336	XXIV
Ken07.Kia	Kiambu CENTRAL	11/01/2007	FJ154443	IX	FJ174437	IX	FJ238539	XXIV
Ken07.Nak	Nakuru RIFT VALLEY	23/01/2007	FJ154444	IX	FJ174440	IX	FJ174338	XXIV

(Roche Molecular Biochemicals), 0.2 μM concentrations of the primers, and 0.625 U of Taq Gold polymerase (Applied Biosystems), in a total volume of 100 μl. The PCR reactions performed in a Perkin–Elmer thermal cycles were (i) Denaturation for 5 min at 95°C; (ii) forty cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; (iii) Incubation for 10 min at 72°C.

Nucleotide sequencing and analysis

Amplicons of the predicted size were excised and purified by Quiaex gel extraction (QUIAGEN), cloned into a pGMT easy vector according the manufacturer's instructions, and sequenced using primers specific for the pGMT vector (SP6/T7) with an automated 3730 DNA sequence analyzer" (Applied Biosystems). The sequences obtained were aligned using CLUSTAL W package and phylogenetic analyses were conducted using MEGA version 4.0 [27]. Two datasets were generated for phylogenetic analyses, (i) *p72*-gene dataset comprising 67 taxa (404 characters) in which p72 nucleotide sequences generated in this study from Kenya and Uganda pig viruses were analyzed together with homologous sequences from at least 2 viruses representative of each of 22 p72 genotypes identified in a previous study [17], (ii) *p54* versus *p72*-gene dataset comprising 85 taxa in which p54 and p72 sequences generated in this study from Kenya pig viruses were compared with sequences generated from ASFV isolates held at CISA-INIA and with ASFV published sequences available in GenBank (Table 3). Unweighted pair-group arithmetic average (UPGMA), neighborjoining (NJ), and minimum evolution (ME) p72 and p54 trees were constructed employing the *p*-distance nucleotide substitution model as implemented in the MEGA v4.0 program. To determine the degree of statistical support for each node in the resulting p72 and p54 trees, data were re-sampled 1,000 times using the bootstrap method.

Results

ASF diagnosis

Genomic amplification and virus isolation

Nine samples from 5 pigs were received from an outbreak that occurred in Busia in May 2006. PCR was performed on organ biopsies. Following amplification, a single major band of approximately 260 bp was observed, in agarose gel electrophoresis, for 8 of 9 tissue samples analyzed. Only one liver sample (animal P31) was PCR negative. A second batch of 25 samples (tissues and sera), from 5 different locations (see Table 1), was received subsequently. Samples from all 25 animals comprising both sera and tissues were positive using the ASF diagnostic primers derived from the p72 gene. The specificity of the amplicon obtained was confirmed using *BsmAI* digestion (data not shown).

Virus isolation was performed using homogenized pooled tissues and also sera from each PCR-positive animal. Eleven ASFV strains with a hemadsorption pattern typical of virulent ASF viruses were isolated from the Kenyan animals after three passages in leukocytes (Table 2).

ASF molecular characterization

P72 genotyping of Kenyan ASFV isolates

In order to classify the Kenyan ASFV isolates obtained relative to previously defined major genotypes, the C-terminal end of the p72 protein was amplified and sequenced [4]. The

Table 3 Summary of the *African swine fever virus* isolates characterized in p54-*gene* versus p72-*gene* phylogenetic study excluding those from the Kenyan outbreaks which are described in Table 2

Isolate[a]	Country of origin	Host species	Year of outbreak	Town/ province	p72 gene Genbank accession no.	P72 genotype	Reference	P54 gene Genbank accession no.	P54 genotype	Reference
European										
Ali61	Spain	Pig	1961	Alicante	FJ154445	I	This study	FJ174384	Ia	This study
M61	Spain	Pig	1961	Madrid	FJ174345	I	This study	FJ174385	Ia	This study
Co61	Spain	Pig	1961	Cordoba	FJ174346	I	This study	FJ174386	Ia	This study
Co62	Spain	Pig	1962	Cordoba	FJ174347	I	This study	FJ174387	Ia	This study
Co68		Pig	1968	Cordoba	FJ238538	I	This study	FJ174388	Ia	This study
E70	Spain	Pig	1970	Pontevedra	AY578692	I	Zsak et al. 2005	FJ174389	Ia	This study
Ba71V	Spain	Vero cell adapted pig isolate	1971	Badajoz	FJ174348	I	This study	FJ174390	Ia	This study
Av71	Spain	Pig	1971	Avila	FJ174349	I	This study	FJ174391	Ia	This study
B74	Spain	Pig	1974	Barcelona	FJ174350	I	This study	FJ174393	Ia	This study
E75	Spain	Pig	1975	Lerida	AY578693	I	Zsak et al. 2005	FJ174394	Ia	This study
646	Spain	Pig	1969	Badajoz	FJ174351	I	This study	FJ174392	Ia	This study
Mu82	Spain	Pig	1882	Murcia	FJ174352	I	This study	FJ174395	Ia	This study
Z85	Spain	Pig	1985	Zaragoza	AF449465	I	Bastos et al. [23]	FJ174396	Ia	This study
Sa88	Spain	Pig	1988	Salamanca	FJ174353	I	This study	FJ174398	Ia	This study
Se88	Spain	Pig	1988	Sevilla	FJ174354	I	This study	FJ174397	Ia	This study
Hu90	Spain	Pig	1990	Huelva	FJ174355	I	This study	FJ174399	Ia	This study
Hu94	Spain	Pig	1994	Huelva	FJ174356	I	This study	FJ174400	Ia	This study
Ca78	Italy	Pig	1978	Sardinia	FJ174357	I	This study	FJ174401	Ia	This study
Nu81	Italy	Pig	1981	Sardinia	FJ174358	I	This study	FJ174402	Ia	This study
SS81	Italy	Pig	1981	Sardinia	FJ174359	I	This study	FJ174403	Ia	This study
Ori84	Italy	Pig	1984	Sardinia	FJ174360	I	This study	FJ174404	Ia	This study
Ori85	Italy	Pig	1985	Sardinia	FJ174361	I	This study	FJ174405	Ia	This study
Ss88	Italy	Pig	1988	Sardinia	FJ174362	I	This study	FJ174406	Ia	This study
Ori90	Italy	Pig	1990	Sardinia	FJ174363	I	This study	FJ174407	Ia	This study
Nu90.1	Italy	Pig	1990	Sardinia	AF302813	I	Bastos et al. [23]	FJ174408	Ia	This study
Nu91.3	Italy	Pig	1991	Sardinia	FJ174364	I	This study	FJ174409	Ia	This study
Nu91.5	Italy	Pig	1991	Sardinia	FJ174365	I	This study	FJ174410	Ia	This study
Nu93	Italy	Pig	1993	Sardinia	FJ174366	I	This study	FJ174411	Ia	This study
Ori93	Italy	Pig	1993	Sardinia	FJ174367	I	This study	FJ174412	Ia	This study
Nu95.1	Italy	Pig	1995	Sardinia	FJ174368	I	This study	FJ174413	Ia	This study
Nu96	Italy	Pig	1996	Sardinia	FJ174369	I	This study	FJ174414	Ia	This study
Nu97	Italy	Pig	1997	Sardinia	FJ174370	I	This study	FJ174415	Ia	This study
Ca97	Italy	Pig	1997	Sardinia	FJ174371	I	This study	FJ174416	Ia	This study
Nu98.3	Italy	Pig	1998	Sardinia	FJ174372	I	This study	FJ174417	Ia	This study
Nu98.8B	Italy	Pig	1998	Sardinia	FJ174373	I	This study	FJ174418	Ia	This study
Mal78	Malta	Pig	1978	Malta	AF301543	I	Bastos et al. [23]	FJ174419	Ia	This study
Lisbon57	Portugal	Pig	1957	Lisbon	AF301537	I	Bastos et al. [23]	FJ174420	Ib	This study

Table 3 continued

Isolate[a]	Country of origin	Host species	Year of outbreak	Town/ province	p72 gene Genbank accession no.	P72 genotype	Reference	P54 gene Genbank accession no.	P54 genotype	Reference
Lisbon60	Portugal	Pig	1960	Lisbon	AF301539	I	Bastos et al. [23]	X84889	Ic	Sun et al. [22]
NH/P68	Portugal	Pig	1968	NK	DQ028313	I	Unpublished	DQ028322	Ia	Unpublished
Mafra86	Portugal	Pig	1986	Mafra	DQ028312	I	Unpublished	DQ028321	Ia	Unpublished
Coimbra87	Portugal	Pig	1987	Coimbra	DQ028310	I	Unpublished	DQ028319	Ia	Unpublished
OURT 88/3	Portugal	Tick	1988	NK	AM712240	I	Complete genome	AM712240	Ia	Complete genome
Portalegre90	Portugal	Pig	1990	Portalegre	DQ028314	I	Unpublished	DQ028323	Ia	Unpublished
Barrancos93	Portugal	Pig	1993	Barrancos	DQ028307	I	Unpublished	DQ028318	Ia	Unpublished
Almodovar 99	Portugal	Pig	1999	Almodovar	DQ028306	I	Unpublished	DQ028315	Ia	Unpublished
Almodovar 99/E2	Portugal	Tick	1999	Almodovar	DQ028308	I	Unpublished	DQ028316	Ia	Unpublished
Almodovar 99/NE1	Portugal	Pig	1999	Almodovar	DQ028309	I	Unpublished	DQ028317	Ia	Unpublished
Fr64	Francia	Pig	1964	Francia	FJ174374	I	This study	FJ174421	Ia	This study
Georgia2007	Georgia	Pig	2007	NK	AM999764	II	Unpublished	AM999765	II	Unpublished
American										
Brazil78	Brasil	Pig	1978	Rio Jaineiro	FJ238537	I	This study	FJ238535	Ia	This study
Dom Rep	Dominican Republic	Pig	1978	Dominican Republic	AF302810	I	Bastos et al [23]	FJ238534	Ia	This study
Haiti	Haiti	Pig	1981	Port-au-Prince	FJ174375	I	This study	FJ238536	Ia	This study
African										
Kat67	Zaire	Pig	1967	Katanga	FJ174377	I	This study	FJ174423	Ib	This study
Ang72	Angola	Pig	1972	NK	FJ174378	I	This study	FJ174424	Ib	This study
IC96	Ivory Coast	Pig	1996	NK	FJ174379	I	This study	FJ174429	Ib	This study
CV97	Cape Verde	Pig	1997	NK	FJ174380	I	This study	FJ174427	Ib	This study
CV98	Cape Verde	Pig	1998	NK	FJ174381	I	This study	FJ174428	Ib	This study
Nig01	Nigeria	Pig	2001	NK	FJ174382	I	This study	FJ174426	Ib	This study
MZUKI/ 1979	South Africa	Tick	1979	Mkuzi	AY261362	I	Complete genome	AY261362	Id	Complete genome
Warmbaths	South Africa	Tick	NK	NK	AY261365	III	Complete genome	AY261366	III	Complete genome
Namibia	Namibia	Warthog	NK	NK	AY261366	IV	Complete genome	AY261366	IV	Complete genome
Tengani62	Malawi	Pig	1962	Nsanje	AY261364	V	Complete genome	AY261364	Va	Complete genome
Moz64	Mozambique	Pig	1964	NK	FJ174376	V	This study	FJ174422	Vb	This study
MwLil 20/1	Malawi	Tick	1983	Chalaswa	AY261361	VIII	Complete genome	FJ174425	VIII	This study
Ug03H.1	Uganda	Pig	2003	Hoima	FJ154428	IX	This study	FJ174431	IX	This study
Ug03H.2	Uganda	Pig	2003	Hoima	FJ154429	IX	This study	FJ174432	IX	This study
Ug03H.3	Uganda	Pig	2003	Hoima	FJ154430	IX	This study	FJ174433	IX	This study
Ug03P.4	Uganda	Pig	2003	Pallisa	FJ154431	IX	This study	FJ174434	IX	This study
Ug03P.5	Uganda	Pig	2003	Pallisa	FJ154432	IX	This study	FJ174435	IX	This study
Ug03P.6	Uganda	Pig	2003	Pallisa	FJ154433	IX	This study	FJ174436	IX	This study
Ug64	Uganda	Pig	1964	NK	FJ174383	X	This study	FJ174430	Xa	This study

Table 3 continued

Isolate[a]	Country of origin	Host species	Year of outbreak	Town/ province	p72 gene Genbank accession no.	P72 genotype	Reference	P54 gene Genbank accession no.	P54 genotype	Reference
Kenya1950	Kenya	Pig	1950	NK	AY261360	X	Complete genome	AY261360	Xb	Complete genome
Pr96/4	South Africa	Tick	1979	Kruger N. Park	AY261363	XX	Complete genome	AY261363	XXa	Complete genome
Lillie	South Africa	Tick	1979	NK	DQ250109	XX	Boshoff et al. 2006	X84888	XXb	Sun et al. [22]

NK Not known

[a] The number following the name of the virus isolate indicates the isolation year

sequences obtained were compared with additional sequences obtained in a separate study from six ASF isolates recovered from domestic pigs during a 2003 ASF outbreak that occurred in the Hoima and Pallisa districts of Uganda (numbered Ug03H1-3 and Ug03P4-6, respectively: previously unpublished data held at CISA-INIA). On sequencing p72 from the 11 Kenyan isolates and comparing with the Uganda isolates, it was found that ASF viruses collected from domestic pigs in Kenya were identical to those collected from the Ugandan outbreak in 2003. Alignment and translation of these sequences revealed that the gene sequence was completely conserved between the sequences compared (data not shown).

The p72 nucleotide sequences generated from Kenya and Uganda pig viruses were analyzed together with homologous sequences from at least 2 viruses representative of each of 22 (I-XXII) p72 genotypes identified in a previous study [17]. Trees of similar topology were recovered using UPGMA, NJ, and ME methods, confirming the presence of Kenyan viruses in genotype IX p72, together with the Uganda 2003 isolates. In the rooted ME tree presented in Fig. 2, genotypes were labeled I–XXII, according to the previous classification of p72 genotypes [17]. The p72 Genotype IX comprises a domestic pig-associated genotype isolated from two temporally distinct outbreaks in Uganda in domestic pigs in 1995 and 2003 (UGA/1/95GenBank accession no AF449475; UGA2003/1 GenBank accession no AY351564). Despite the very close genetic similarity of the Kenyan isolates to the viruses responsible for the 2003 outbreak in Uganda, the p72 genotype (number XVI) of the viruses that caused an outbreak in Northern Tanzania (Arusha district) in 2003 was different [18].

Application of ASFV p54 gene sequencing for molecular epidemiological studies

Previous studies have demonstrated size variability in the *E183L* gene that encodes the ASFV p54 protein when different ASFV isolates were compared, due to the presence of tandemly repeated arrays [22]. With the aim of defining the potential of the *E183L* gene as an additional tool for ASFV molecular epidemiology, we have analyzed the complete sequence, following amplification with primers PPA89/PPA722, which flank the coding sequence. PCR products with an estimated size of 683 bp were generated from all eleven Kenyan isolates (data not shown) and sequenced directly. The sequences obtained were compared with p54 sequences generated from 56 ASFV isolates held at CISA-INIA and with 10 ASFV published sequences available in GenBank (Table 3) resulting in a final dataset comprising 85 taxa. The same taxa were used to infer a *p72*-gene phylogeny in order to compare the resolution capabilities of p54 versus p72. The NJ, UPGMA, and ME trees inferred from 552 nucleotides corresponding to the complete sequence of the *p54*-gene, produced fourteen well-supported p54 genotypes (Fig. 3b), while p72-based phylogenetic analyses using the same isolates resulted in the definition of nine p72 genotypes (Fig. 3a). Virus representatives of p72 genotype I that are homogeneous across the C-terminal region of the gene, formed four discrete sub-clusters in the *p54*- gene tree, designated "a," "b," "c," and "d." The largest and most heterogeneous p54 cluster, genotype Ia, comprised viruses from Europe and America. P54 genotype Ib was represented by viruses from West African countries, which included the Portuguese isolate Lisbon57, while the Lisbon60 and Mzuki viruses were placed in independent genotypes designed Ic and Id, respectively. In addition, viruses from East African countries that clustered together within three different p72 genotypes V, X, and XX, were each differentiated into two distinct genotypes through *p54*-gene characterization (these are labeled in Fig. 3b as Va, Vb, Xa, Xb, and XXa, XXb). The Kenyan isolates were classified in genotype IX together with the isolates from the Ugandan outbreak in 2003 and were also closely related to the Kenya 1950 and Ug64 ASFV isolates. Alignment and translation of the sequences

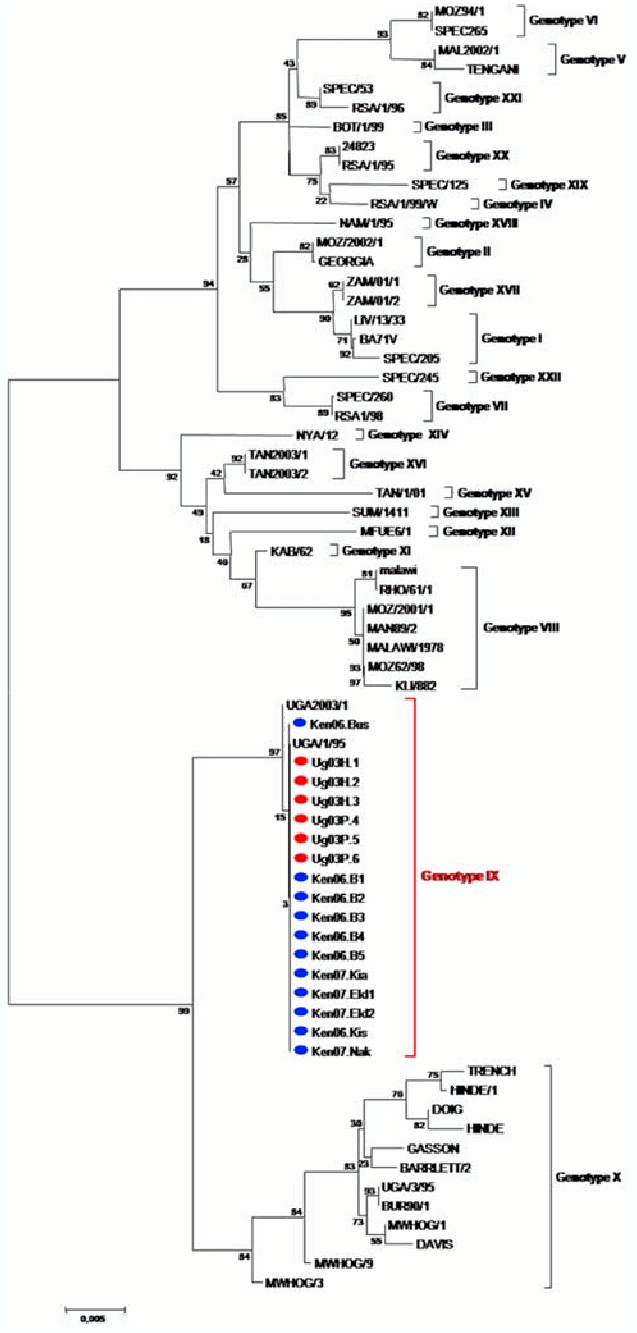

Fig. 2 Minimum evolution tree showing the 22 ASFV *p72* genotypes from African swine fever viruses (labelled I–XXII). Tree #1 from 100 minimum evolution trees (sum of branch length = 0.24309884) is illustrated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown adjacent to the nodes. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. A Neighbor-joining algorithm was used to generate the initial tree. The Kenyan and Ugandan viruses characterized in this study are marked with blue (●) and red (●) dots, respectively within genotype IX (marked in red)

generated in this study, revealed, as in the case of p72, no sequence variation between Kenyan isolates and isolates recovered from the previous Ugandan outbreaks in 2003.

Analysis of TRS presented in CVR on ASFV genome

The ASF *B602L* gene (CVR region) is a hyper-variable genetic marker that has been demonstrated to be useful for high-resolution discrimination of viruses that are identical according to their p72 and p54 genotypes. The *B602L* variable region contains twelve-base-pair repeats which encode 4 amino acids that vary in number and sequence when genomes of different isolates are compared [14, 26]. The p72 and p54 genes were useful for initial classification of the Kenyan isolates, but in order to achieve finer discrimination between the viruses the variation and distribution of these amino acid repeats was investigated using primers ORF9L-R/ORF9L-F [26]. An amplicon with an estimated size of between 300 and 350 bp was generated from all eleven isolates (data not shown). The PCR products were cloning into a pGMT vector and sequenced. All CVR nucleotide and translated amino acid sequences generated in this study were compared with 50 homologous amino acid sequences from viruses representing CVR sub-groups identified previously by Nix et al. 2006. As in the case of the p72 genotyping, the Kenyan isolates clustered with the Uganda isolate UGA95/1 (GenBank accession no AM259419) including in the CVR sub-group XXIV. Differences were identified mainly in the number of tetrameric amino acid repeats. When the tetrameric repeats within the Kenyan isolates were compared with those inside Ugandan sequences, the isolates from Busia obtained in May 2006 (Ken06.B1-5) were identical to those obtained from an outbreak that occurred in Eastern Uganda (Pallisa) in 2003. However, the analyses of isolates recovered from a second outbreak in Busia in October 2006 (Ken06.Bus) revealed a minor change due to the absence of a single internally located tetrameric repeat (CADT) and this difference was present within all isolates from subsequent outbreaks in the country (Fig. 4).

Discussion

Complete nucleotide sequencing of the *p54*-gene of 67 ASF viruses originating from Africa, Europe, South America, and the Caribbean included in this study, permitted the identification of 14 major viral genotypes. By comparing the sequencing results with those obtained previously by p72 analysis, it was shown that the viruses included within the homogeneous p72 genotype I, comprising viruses from West Africa, Europe, South America, and the Caribbean, were separated into four clearly distinct p54 genotypes allowing discrimination between West African isolates from Europe, South America, and the Caribbean. It is interesting to note that the Portuguese ASFV Lisbon57 isolate was identical to West African isolates confirming

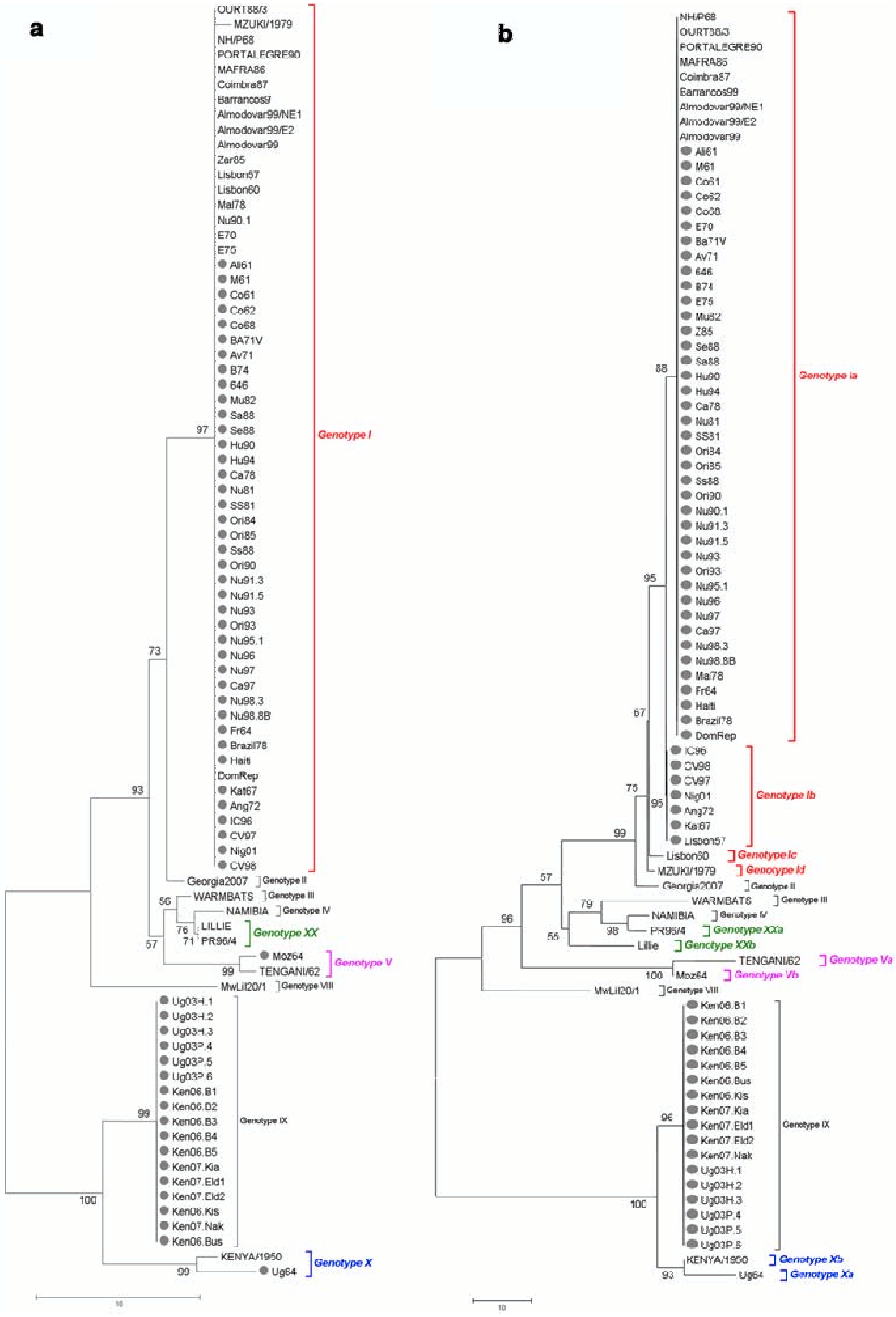

Fig. 3 Minimum evolution trees depicting **a** p72 protein and **b** p54 protein among 85 African swine fever virus isolates. The evolutionary history was inferred using the Minimum Evolution method (ME). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME trees were searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. A neighbour-joining algorithm was used to generate the initial trees after 1,000 replicates. Bootstrap values >50% are indicated next to the relevant node. All positions containing alignment gaps or where there were missing data were eliminated in the pair-wise sequence comparisons (pair-wise deletion option). The p72 genotype I and corresponding p54 sub-groups Ia, Ib, Ic, and Id are indicated in red. The p54 subgroups-within p72 genotypes V, X, and XX are highlighted in pink, green, and blue respectively. (●) indicates viruses characterized in this study

that the most likely source of the infection for Europe was one of the African countries along the historical west-coast shipping route between Africa and Europe, stretching from Angola in the South to Senegal in the North. This confirms p54 sequencing as a valuable additional genotyping method for molecular epidemiological studies of genotype I viruses, particularly in West Africa where this genotype predominates. The higher level of resolution of the viral discrimination possible using *p54*-gene sequencing was confirmed by the separation of viruses within three additional clusters (V, X, and XX) that were homogeneous using p72.

According to data from the Office International des Epizooties, the last previous documented outbreak of ASF in Kenya was recorded in August 2001. However, the occurrence of regular outbreaks of ASF in neighboring Uganda and Tanzania since 2001 poses a threat to the pig industry in Kenya. In May 2006, the presence of a virulent

Fig. 4 Amino acid sequence alignment of the tetrameric tandem repeats identified within the central variable region (CVR) of gene *B602L* from Kenyan isolates associated with the 2006–2007 outbreaks. The sequences obtained were compared with CVR sequences from isolates recovered in Uganda in 2003 (Ug03) as well as with Ugandan CVR sequences available in GenBank (UGA95/1; Accession No. CAJ90783) belonging to B602L sub-group XXIV. The mutation that results in the absence of a single internally located tetrameric repeat (CADT) between the two groups of Kenyan isolates that were sequenced is marked in gray

hemorrhagic swine disease was notified in Busia district bordering Uganda, spreading from Western to Central Kenya that resulted in the death or slaughter of more than 600 swine. Sampling from different areas across the country in collaboration with the Kenyan Department of Veterinary Services resulted in confirmation of the disease as ASF by PCR amplification and subsequent virus isolation at the CISA-INIA EU Reference Laboratory. In order to clarify the epidemiological situation in any given geographic region, it is important that the relationships between contemporary field strains and those involved in past outbreaks, both locally and regionally be investigated in order to determine the source of the outbreak. Since serological methods for differentiating ASFV variants are unreliable, differentiation between viruses is dependent on genetic characterization methods, among which restriction fragment length polymorphism (RLPF) and partial p72 protein sequencing [8, 23] have been the most useful for identifying major ASFV genotypes. In order to ascertain the genetic relationships of the viruses that were responsible for the ASF outbreaks in Kenya in 2006–2007, eleven ASFV Kenyan isolates obtained after virus isolation were first genotyped by partial p72 gene characterization [23]. All of them were classified into the domestic pig-associated p72 genotype IX, together with viruses from both Western and Eastern Uganda recovered from domestic swine during outbreaks that occurred in 2003. The same result was obtained by sequencing the complete gene that encodes the ASFV p54 protein which revealed no differences between Kenyan and Ugandan isolates. However, the fact that the p72 genotype of the virus causing an outbreak that occurred in Northern Tanzania in 2003 differed from that of the Ugandan isolates confirms the complexity of the molecular epidemiology of ASFV in East Africa [18]. Although the p72 and p54 genes are useful for identifying the major genotypes, higher resolution discrimination of virus relationships enables more detailed dissection of the epidemiology. Therefore, sequences of the CVR characterized by the presence of TRS were generated from Kenyan isolates. As with the p72 and p54 genotyping data, comparison of amino acid tetrameric repeats located in CVR revealed that viruses associated with the Kenyan outbreak in Busia district in 2006 were identical to those obtained from outbreaks in Uganda in 2003. This indicates that the viruses responsible for these outbreaks are closely related. However, due to the lack of extensive genotyping of Kenyan and Ugandan viruses predating these outbreaks the ultimate source of this viral genotype is unclear. A deletion of one tetrameric repeat was observed from viruses recovered from a subsequent outbreak in the same district. This is consistent with the hypothesis that this outbreak was caused by a closely related, but mutated, form of the virus that had been circulating in the Kenya–Uganda border region. Contact between warthogs and domestic pigs is theoretically possible in the case of free-ranging smallholder domestic pigs kept in the communal lands, however, warthogs are rare in these regions of West Kenya and East Uganda. Bushpigs (*Potamochoerus larvatus*) are also present in these areas and it has previously been shown that Bushpigs experimentally infected with ASFV, although not exhibiting clinical symptoms, can transmit the virus to both domestic pigs and *Ornithodoros* ticks [28, 29]. The rapid spread of the virus among pigs and the peracute and acute forms of the disease suggest that the disease may have been maintained in the border region either in contaminated pork products, or live pigs that had become immune after surviving the first outbreak. The presence of a domestic pig-associated genotype causing ASF outbreaks in the border region between Kenya and Uganda and evidence of trans-boundary transmission between these countries indicates that a regional approach to disease control would be more efficient. More extensive

sampling and characterization of viruses transmitted by both domestic and sylvatic hosts from East Africa will be required in order to develop a sequence database enabling more accurate assessment of the origins of past and future outbreaks of ASF in the region.

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