Molecular characterisation of African swine fever viruses from Nigeria (2003-6) recovers multiple virus variants and reaffirms CVR epidemiological utility

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Nucleotide sequences have been submitted to the Genbank nucleotide sequence database and are accessible under accession numbers GQ427180-GQ427194

Abstract

Samples collected from wild and domestic suids in Nigeria, over a three year period (2003-6), were evaluated for African swine fever (ASF) virus genome presence by targeting three discrete genome regions, namely the 478 bp C-terminal *p72* gene region advocated for genotype assignment, a 780 bp region spanning the 5'ends of the *pB125R* and *pB646L* (*p72*) genes and the hypervariable central variable region (CVR) encoded within the *9RL* ORF (*pB602L*). ASF virus (ASFV) presence was confirmed in 23 of the 26 wild and domestic pigs evaluated. No evidence of ASF infection was found in two warthogs from Adamawa State however, one bushpig from Plateau State was positive. Nucleotide sequences of the 478 bp and 780 bp amplicons were identical across all ASFV-positive samples sequenced. However, five discrete CVR variants were recovered, bringing the total number identified to date, from Nigeria, to six. The largest of the CVR variants, termed 'Tet-36' was identical to a virus causing outbreaks in neighbouring Benin in 1997, indicating a prolonged persistence of this virus type. Co-circulation of three tetramer types (Tet-36, Tet-27 and Tet-20) was found in Plateau State in July 2004, whilst in Benue State, two tetramer types (Tet-20 and Tet-21) were present in August 2005. Despite simultaneous field presence, individual co-infection was not observed. This study has reaffirmed the epidemiological utility of the CVR genome region for distinguishing between geographically and temporally constrained genotype I viruses, and has revealed the presence of multiple ASFV variants in Nigeria.

Introduction

African swine fever (ASF) is a highly lethal and economically important viral disease of domestic pigs that causes an acute hemorrhagic fever and for which no effective vaccine is available. The disease is classified as notifiable by the World Organization for Animal Health (OIE) and is caused by a DNA arbovirus belonging to the genus *Asfivirus* and family *Asfarviridae* [1]. ASF virus (ASFV) has a double-stranded DNA genome of between 170 and 190 kb in size, which is variable due to deletions and insertions occurring within the terminal regions of the genome and within a coding gene region within the central region of the genome [1-3], termed the central variable region (CVR).

The virus is maintained in an ancient sylvatic cycle involving warthogs and soft ticks of the genus *Ornithodoros* [4], in endemic areas in Africa. A pig-tick cycle from which warthog involvement is apparently excluded is also known to occur in some areas of East Africa, whilst in West Africa, virus cycling occurs in the absence of sylvatic host involvement and appears to be perpetuated by domestic pigs alone [4].

Bush pigs (*Potamochoerus porcus*) and warthogs (*Phacocoerus africanus*) which harbour unapparent infections are considered to be the two most important African wild suid hosts of the disease in endemic regions [5]. In spite of the presence of representatives of both genera in some affected countries in West Africa [5], neither species appears to play an equivalent role in the epidemiology of the disease in this region of Africa to which ASF has been introduced. The possibility of spill-over from domestic pigs to both bush pigs and warthogs in Nigeria has however been raised following recent molecular confirmation of ASF virus genome presence in both species [6,7].

Since its first description in Kenya by Montgomery in 1921 [8], ASF has since been reported in nearly all southern, eastern and central African countries where it is endemic [4]. Whilst the precise timing of the introduction of the virus to the historically ASF-free West African region is not known, the first West African isolate made from a domestic pig in Senegal dates back to 1959 (Dakar/59) and has been typed as a genotype I virus based on partial *p72* gene sequencing [9]. Following an 11-year period of relative quiescence, ASFV reappeared in the Cameroon in 1982 and again in 1985 [9, 10, 11], prior to the widespread outbreaks that affected the West African region from the late-1990s onwards, and that continue to this day. Outbreaks were initially reported in Cote d'Ivoire in 1996 [12] and subsequently confirmed in several countries along the West African coast, north of the equator, including Cameroon, The Gambia, Senegal, Benin, Togo, Nigeria and the Cape Verde islands between 1997 and 1998 [4].

In Nigeria, the first documented outbreak of ASF in domestic pigs occurred between 1997 and 1998 [13]. A second wave of the disease erupted in mid-1998 which affected several states in the central, eastern and southern regions of Nigeria and which resulted in numerous pig mortalities [14]. In 2001, an outbreak occurred in Ibadan, South west Nigeria, in which 306 pig farms and 91 % of the 31,916 affected pigs succumbed to the disease [15]. ASF has since maintained a presence in the main pig rearing areas of Nigeria and has started occurring in predictable, periodic waves in north-central Nigeria. This cyclical occurrence of clinical disease which had hitherto not been experienced, together with perennial low pathogenicity ASF presence in asymptomatic domestic pigs [16] and lack of stringent control measure may eventually lead to a state of endemic stability of ASF in Nigeria.

Lack of discernible ASF serotypes has necessitated that field strains be grouped genetically into genotypes instead. This was previously achieved using a restriction fragment length polymorphism (RFLP) approach [10,17] but has been largely replaced by rapid PCR-based methods, such as C-

terminus *p72* genotyping which recovers the same major groupings as RFLP analysis [9]. It was the *p72* gene sequencing approach that revealed that ASF viruses causing outbreaks in Nigeria between 1998 and 2000 belong to *p72* genotype I, or the ESACWA genotype which derives its name from the four ASF-free regions that it has made incursions into *viz.* Europe, South America, the Caribbean and West Africa [9]. The partial *p72* gene region that is advocated for virus genotyping [9], is characterised by extreme intra-genotypic homogeneity for pig-associated genotypes such as the ESACWA or genotype I rendering this gene region of little use for tracing the origin and course of outbreaks [9,18]. Intra-genotypic size and sequence variability in the central variable region (CVR) of the *9RL* ORF (also termed *pB602L*) has however been demonstrated for genotype I viruses [3,18,11]. Whilst alternative genes with greater intra-genotypic resolution capabilities than *p72* have been identified [11,19], these alternative genome targets are of limited use for geographically and temporally constrained viruses such as those causing outbreaks in Kenya from 2006-7 [19]. Indeed, for the three genome regions compared in the Kenyan study, only the CVR recovered more than one virus variant [19], and it therefore remains the genome target of choice when attempting to determine the origin and map the spread of closely related virus. It was for this reason that, following genotype confirmation by *p72* gene characterisation, that the CVR was used to investigate ASF outbreaks occurring between 2003 and 2006 in the main pig producing areas of Nigeria (Fig. 1), in which sustained and intermittent outbreaks had been recorded and mortality rates had varied considerably.

Materials and Methods

Study area and samples

Nigeria is bordered by four countries, namely Benin, Niger, Chad and Cameroon, and comprises 36 states and the Federal Capital Territory (FCT. The main pig producing states in Nigeria are concentrated in the south-western, central and eastern parts of the county (Fig. 1). In this study, 46 samples were collected from 26 suids at 15 sampling sites, in seven states over a 3 year period (May 2003 – May 2006) and were primarily of domestic pig origin (Table 1). Samples collected from Kaduna, Plateau and Benue states were in response to extensive and prolonged outbreaks, which lasted for several months and involved institutional farms, backyard piggeries and free-roaming pigs. Samples from Enugu, Ogun, Lagos and Edo states were from isolated outbreaks that had no apparent links to previous outbreaks and which were characterised by high mortality rates. In contrast, the previously mentioned epidemics in Kaduna, Plateau and Benue states were characterised by high morbidity, lower mortality and a longer course of disease. In addition to domestic pig samples, multiple samples were taken from two warthogs (*Phacochoerus* sp.) captured in Adamawa state located in North-East

Nigeria, a region not known for pig farming. Samples were also taken in Plateau state, from a bushpig (*Potamochoerus* sp.) and from five hard tick (*Rhipicephalus* sp.) specimens collected from domestic pigs sampled at regional pig market, in Katsit, Kaduna State (Table 1).

ASFV genome detection and molecular characterisation

DNA was extracted using a modified guanidine-thiocyanate method as described previously (9) and was used as template for amplification of the C-terminus 478 bp *p72* gene region spanning positions 1460-1937, using the prescribed ASF genotyping primers [9]. Amplicons of the latter gene region were purified using the High Pure PCR Product Purification kit according to manufacturer specifications (Roche), cycle sequenced with BigDye v3.1 (Applied Biosystems) and run on the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). In addition, the CVR of the *9RL* ORF was amplified using flanking primers and methods described previously [20], and sequenced with each of the primers in separate reactions. A 780bp genome region corresponding to the 5' end of the *p72* gene and inclusive of the 5'end of the neighbouring *B125R* gene, was also evaluated in this study for its epidemiological potential. Intra-genotypic variability of this genome region relative to that of the CVR and C-terminal *p72* gene regions was evaluated following amplification and sequencing of one representative of each of the Nigerian CVR variants with primers AB-FL1: GCATGCGCAGATATTCTTCT and AB-IR: CCGTTGCGAGGAAACGTTTG which were designed specifically for this study. These primers amplified the 780bp target under the same thermal cycling conditions used for C-terminal *p72* gene amplification [9]. All sequences generated in this study were submitted to Genbank under accession numbers GQ427810-GQ427914.

Phylogenetic analyses and mapping of genetic variants

Genotype assignment of viruses characterised from the ASF-positive samples was achieved by phylogenetic analysis of the C-terminal *p72* gene region in Mega 4 [21], as described previously [9]. For the CVR dataset, one virus representative of each of the 18 genotype I CVR amino acid sequence variants identified previously [11] was used to generate a reference sequence dataset (summarised in Table 2). In selecting the reference sequence for each CVR variant, preference was given to viruses of African origin. For those CVR variants that did not include viruses of African origin, priority was given to genotype I viruses for which both CVR and partial *p72* genotype-informative sequence data were available. The resulting reference amino acid tetrameric repeat dataset was complemented with one representative of each of the five unique CVR amino acid variants recovered from the samples included in this study (Table 2; Fig. 2). Gaps inserted for alignment purposes (Fig. 3) were treated as a 21st character state in the cladistic analyses performed in PAUP*4.0b10 [22], as described previously [20,23]. Sequences were coded [24] for the phenetic analyses which were performed in PAUP*4.0b10

[22], so that information contained in the gap regions could be retained. Nodal support for both the *p72* and CVR gene analyses was assessed following 10000 bootstrap replications. The origin and sampling date of each of the CVR variants recovered in this study were overlaid on a map of Nigeria in order to evaluate the spatial and temporal distribution of the variants (Fig. 1).

Results

ASFV genome detection

The expected 478 bp fragment corresponding to the C-terminal region of the *p72* gene was amplified in diverse samples taken from 22 of the 26 pigs sampled (Table 1). Non-specific amplification of bands ranging from 200bp to 300 bp in size was obtained with a small number of DNA extracts prepared from pig foetus and warthog samples, whilst no amplification occurred when DNA extracted from the *Rhipicephalus* sp. ticks was used as template (Table 1). All C-terminus *p72* gene positive samples amplified a single, discrete CVR amplicon ranging from 497 bp to 689 bp in size, with the CVR-FL1 and CVR-FL2 primers. Five size-discrete fragments were amplified, with the smallest, 497 bp in size, being identified in 10 of the 22 ASFV-positive suids and occurring in the Plateau, Kaduna and Benue States from July 2004 until September 2005. The next most abundant amplicon size was also the largest, viz. 689 bp, and occurred in six domestic pigs and in one bushpig sampled in the Plateau, Ogun and Lagos states between July 2004 and July 2006. Amplicons of 509 bp, 605 bp and 581 bp were temporally and geographically restricted and also less abundant. The 509 bp variant occurred in a single animal sampled in August 2005 in Benue State, the 605 bp variant was identified from two pigs sampled in Edo State in May 2006 and the 581 bp variant was present in three animals, of which two were sampled in Enugu State in 2003, and the third in Plateau State in July 2004 (Table 1). Representatives of each of the five size-discrete CVR variants selected for screening with the primers that target the *B125R-B646L* (*p72*) genome target, amplified the expected 780 bp fragment (results not shown).

Phylogenetic analyses

All virus genome segments amplified by PCR and characterised in this study were identical to each other across the homologous 404 nucleotide (nt) C-terminus *p72* gene region characterised (Fig. 2a). With the exception of Vict90/1, the Nigerian viruses also displayed 100% *p72* gene sequence identity to all other ESAC-WA (genotype) I viruses included in this study (Fig. 2a), thereby confirming their genotype I designation. Similarly, all Nigerian viruses were identical to each other across the 780 bp region spanning the 5'ends of the *B125R* and *B646L* (*p72*) genes, and to other West African genotype I viruses, such as Ben97/1 (Chapman et al. 2008) for which homologous data were available (results not

shown). In contrast, five size- and sequence-discrete CVR variants were obtained for the Nigerian 2003-6 viruses analysed, corresponding to virus variants containing between 20 and 36 tetrameric repeats (Fig. 3). For ease of reference, each unique CVR variant was assigned a haplotype name that reflected the size of the tetramer, viz. Tet-20, Tet-21, Tet-27, Tet-29 and Tet-36 (Table 1). Thus the taxon names of the Nigerian viruses characterised in this study are designated Nigeria (Tet-20), Nigeria (Tet-21), Nigeria (Tet-27), Nigeria (Tet-29) and Nigeria (Tet-36) in the *p72* and CVR phylogenies (Fig. 2). When comparing the *p72* and CVR gene phylogenies, 23 CVR variants (Fig. 2b) were recovered in contrast to just two *p72* gene variants (Fig. 2a). All five Nigerian CVR variants grouped together with Nigeria/01 (32), a virus variant bearing 32 tetramers and recovered from the 2001 outbreak, within a single monophyletic lineage with high levels of bootstrap support (95-97 %) in the CVR phylogeny (Fig. 2b). Other than the 100% sequence identity between Nigeria (Tet-36) and Benin97/1 (Tet-36), the remaining five Nigerian CVR variants displayed no links to other West African CVR variants recovered from Angola (Tet-14a) in 1970 and 1971, Cameroon/82 (Tet-23b) in 1982, Cape Verde (Tet-17) in 1997 and 1998, nor Benin97/3 (Tet-8).

Mapping of the CVR variants

When the CVR variants were overlaid on a map of Nigeria that captures pig density per state and sampling time (Fig. 1), it could clearly be seen that Tet-27 sampled in the southern state of Enugu in May 2003, maintained a field presence for over a year, being subsequently sampled in July 2004 in the centrally located Plateau state. In contrast, Tet-21 and Tet-29 were only sampled on a single occasion in Benue State in August 2005 and in Edo State in May 2006, respectively. The two most abundant tetrameric types, Tet-20 and Tet-36, predominated in the central and south-western states respectively, with Tet-20 being recovered between July 2005 and September 2005, and Tet-36 being present from July 2004 until July 2006. The 100% sequence identity of the seven viruses bearing the latter tetramer-type, to a virus linked to the 1997 ASF outbreaks in neighbouring Benin (Fig. 2b), indicates that Tet-36, which is identical in size to the Nigerian 1998, 1999 and 2000 viruses and to one of the 1996 Ivory Coast outbreak strains [18] has most likely persisted in Nigeria for more than eight years.

Discussion

Nucleotide sequencing of three discrete ASF viral genomic regions amplified from clinical samples taken from wild and domestic pigs in Nigeria between 2003 and 2006 confirmed that the outbreaks had been caused by genotype I viruses that were homogeneous across the C-terminus *p72* gene

region, and across the *B125R-B646L* (*p72*) genome target. Amplification and sequencing of the CVR recovered five discrete variants, of which one, Tet-36 was identical in size to the 1998 and 1999 Nigerian viruses [18], and identical in size and sequence to one of the two Benin CVR variants recovered from the 1997 outbreaks [18,11]. These results indicate a prolonged field presence of at least eight years, of this CVR variant in Nigeria. The Tet-20 and Tet-27 variants were both present for more than a year, whereas Tet-21 and Tet-29 were each only sampled on one occasion. Co-circulation of multiple tetramer types in two states, viz. in Plateau State in July 2004 and in Benue State in August 2005 was observed (Fig. 1). However, despite simultaneous field presence of three and two tetramer types, respectively, and the ability of the CVR PCR to detect mixtures of viruses bearing size-discrete CVRs [20], no evidence of individual co-infection was observed. This result is consistent with the lack of co-infection noted for individual pigs that were housed together in the same quarantine facility in Mozambique and that succumbed to the disease following exposure to two distinct ASF viral strains [20].

All five CVR variants clustered together with the Ben97/1 (Tet-36) variant and the Nigerian 2001 (Tet-32) variant within a single monophyletic lineage, confirming common ancestry for all of these virus variants. The two possible explanations consistent with the phylogeny are that (i) the related CVR variants evolved outside Nigeria and were introduced on separate occasions to this country, or (ii) following a single introduction to Nigeria in 1998, the virus persisted in the field evolving rapidly to give rise to numerous related and 'home-grown' CVR variants. The lack of an extensive CVR database for the West African region makes it impossible to determine which of the two is most plausible. However, the high levels of variation in the CVR recovered reaffirm the epidemiological utility of this genomic region and were extremely useful for tracking the spread of the virus within Nigeria. In particular, the establishment of Tet-36 in the south-west of Nigeria appears to be a consequence of the movement of Tet-36 variant infected pigs from the centre of Nigeria where infection with this variant type was already well-established in Plateau State in July 2004.

Previous studies have revealed the higher levels of CVR diversity in Europe than in West Africa [18,11]. One possible explanation put forward to explain this diversity was that attempts to attenuate virus for vaccine purposes may have inadvertently increased the levels of CVR variation in the field [18]. Prior to this study, Spain and Portugal were the most heterogeneous European countries, each having four CVR variants [11], whilst in Africa, Cote d'Ivoire and Benin were previously identified as being the most diverse, with each having two CVR variants [18,11]. The five variants identified from Nigeria in this study, together with the previously identified Tet-32 (2001) variant, brings the total number of variants in this country to six, and points to the possibility of an alternative explanation for

high levels of CVR diversity, *viz.* that ASFV presence in Nigeria has been for a sufficiently long period to allow for virus diversification, of put differently, that prolonged field persistence underlies CVR heterogeneity.

The detection and characterisation of ASF viruses from a wild suid, the red river hog (*Potamochoerus porcus*) in this study, verifies and expands on the first report of ASFV in a captive wildlife species that was based on molecular detection of viral genomic DNA [6]. Although we found no evidence of ASFV in the two warthogs sampled in northern Nigeria, elsewhere in the country, ASFV DNA was detected and amplified by PCR in a warthog [7], suggesting possible circulation in the traditional sylvatic vertebrate host, for which there was previously no evidence of involvement in West Africa. Together these results signal the possible establishment of a new cycle of ASF transmission and maintenance in Nigeria, which may complicate control and which has implications for the remainder of the West African region.

The historically broad distribution of genotype I viruses, and its current dominance in the West African region make it a genotype of particular relevance. Sampling and characterisation of currently circulating field strains, as well as historical viruses from this region of Africa to which ASFV was exotic, is required to fully understand virus spread and maintenance, with forms the basis of formulating effective region-specific control policies. The hypervariable CVR gene has been shown to be useful for revealing the transmission patterns of genotype I viruses, and will be no doubt play a crucial role in future, expanded studies on ASFV in West Africa.

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TABLE 1 Summary of the animals and samples screened for African swine fever virus genome presence

Animal ID	Date sampled	Nigerian State	Sample type/s used for DNA extraction & PCR	*p72* PCR	CVR PCR	Tetramer type
Katsit fetus 1	Jun/2005	Kaduna	Kidney, Liver, Heart	NS	-ve	--
Katsit fetus 2	Jun/2005	Kaduna	Liver	NS	497 bp	Tet-20
Katsit sow 1	Jun/2005	Kaduna	Placenta	+	497 bp	Tet-20
Warthog 1	Sep/2005	Adamawa	Messenteric L/N, Kidney	NS	-ve	--
Warthog 2	Sep/2005	Adamawa	Liver, Lung, Spleen	NS	-ve	--
Udi pig 1	May/2003	Enugu	Messenteric L/N, Parotid L/N, kidney	+	581 bp	Tet-27
Udi pig 2	May/2003	Enugu	Submandibular L/N, kidney	+	581 bp	Tet-27
Bushpig	Jul/2004	Plateau	Lung, Spleen, Submandibular L/N	+	689 bp	Tet-36
Ogun pig 1	Sep/2004	Ogun	Messenteric L/N	+	689 bp	Tet-36
Ogun pig 2	Sep/2004	Ogun	Messenteric L/N	+	689 bp	Tet-36
Makurdi pig	Aug/2005	Benue	Spleen, Tonsil	+	509 bp	Tet-21
Yandev pig	Aug/2005	Benue	Spleen, Lymphnode	+	497 bp	Tet-20
Abeokuta pig 1	Jul/2005	Ogun	Lymphnode, Liver	+	689 bp	Tet-36
Abeokuta pig 2	Jul/2005	Ogun	Lymphnode, Liver	+	689 bp	Tet-36
PACE pig 1	Sep/2005	Plateau	Liver	+	497 bp	Tet-20
PACE pig 2	Sep/2005	Plateau	Liver	+	497 bp	Tet-20
ECWA pig	Sep/2004	Plateau	Messenteric L/N	+	497 bp	Tet-20
Riyom pig	Jul/2004	Plateau	Messenteric L/N, spleen	+	581 bp	Tet-27
LID pig 7	Aug/2004	Plateau	Liver	+	497 bp	Tet-20
DD pig 4	Jul/2004	Plateau	Lung	+	497 bp	Tet-20
DD pig 1	Jul/2004	Plateau	Lung	+	497 bp	Tet-20
DD pig 3	Jul/2004	Plateau	Spleen	+	497 bp	Tet-20
Oke-Aro pig 1	Jul/2006	Lagos	Messenteric L/N	+	689 bp	Tet-36
Oke-Aro pig 2	Jul/2006	Lagos	Liver	+	689 bp	Tet-36
OJ pig 1	May/2006	Edo	Liver	+	605 bp	Tet-29
OJ pig 2	May/2006	Edo	Kidney	+	605 bp	Tet-29
Tick 1	Mar/2006	Plateau	Whole tick	-ve	-ve	--
Tick 2	Apr/2006	Plateau	Whole tick	-ve	-ve	--
Tick 3	Apr/2006	Plateau	Whole tick	-ve	-ve	--
Tick 4	Apr/2006	Plateau	Whole tick	-ve	-ve	--
Tick 5	May/2006	Plateau	While tick	-ve	-ve	--

NS: Non-specific amplification; +: amplification of the expected 478 bp fragment; -ve: Negative; bp: base pairs

TABLE 2 Summary of the reference sequences used for the comparative CVR and *p72* gene analyses

Virus Isolate	Country (year of outbreak)	*p72* GAN	Reference	CVR GAN	Reference	CVR taxon label*
Lisbon/60	Portugal (1960)	AF301539	Bastos et al. [9]	AM259405	Nix et al. [11]	Portugal/60 (Tet-25a)
M61	Spain (1961)	FJ174345	Gallardo et al. [19]	AM259429	Nix et al. [11]	Spain/M61 (Tet-25b)
Co61	Spain (1961)	FJ174346	Gallardo et al. [19]	AM259431	Nix et al. [11]	Spain/Co61 (Tet-28a)
Por63	Portugal (1963)	N/A	--	AM259388	Nix et al. [11]	Portugal/63 (Tet-28b)
Kat/67	DRC (1967)	FJ174377	Gallardo et al. [19]	AM259461	Nix et al. [11]	DRC/Kat67 (Tet-23a)
Co68	Spain (1968)	FJ238538	Gallardo et al. [19]	AM259451	Nix et al. [11]	Spain/Co68 (Tet-26)
ANG/70	Angola (1970)	AF301542	Bastos et al. [9]	AM259411	Nix et al. [11]	Angola (Tet-14a)
ANG/72	Angola (1972)	FJ174378	Gallardo et al. [19]	AM259410	Nix et al. [11]	Angola (Tet-14a)
Val/76	Spain (1976)	AF449462	Bastos et al. [9]	AM259398	Nix et al. [11]	Spain/Val76 (Tet-25a)
Brazil/78	Brazil (1978)	FJ238537	Gallardo et al. [19]	AM259409	Nix et al. [11]	Brazil/78 (Tet-19)
Malta/78	Malta (1978)	AF301543	Bastos et al. [9]	AM259402	Nix et al. [11]	Malta/78 (Tet-31)
DomRep/79	Dominican Republic (1979)	AF302810	Bastos et al. [9]	AM259408	Nix et al. [11]	Dominican Republic/79 (Tet-22)
CAM/82	Cameroon (1982)	AF301544	Bastos et al. [9]	AM259413	Nix et al. [11]	Cameroon/82 (Tet-23b)
BEL/85	Belgium (1985)	AF449466	Bastos et al. [9]	AM259403	Nix et al. [11]	Belgium/85 (Tet-18)
Ourt/88/1	Portugal (1988)	AF302811	Bastos et al. [9]	AM259401	Nix et al. [11]	Portugal/88 (Tet-25c)
NUR/1/90	Sardinia (1990)	AF302813	Bastos et al. [9]	AM259453	Nix et al. [11]	Sardinia/90 (Tet-12)
VICT/90/1	Zimbabwe (1990)	AF449474	Bastos et al. [9]	AM259414	Nix et al. [11]	Vict/90 (Tet-14b)
CV97	Cape Verde (1997)	FJ174380	Gallardo et al. [19]	AM259462	Nix et al. [11]	Cape Verde (Tet-17)
CV98	Cape Verde (1998)	FJ174381	Gallardo et al. [19]	AM259463	Nix et al. [11]	Cape Verde (Tet-17)
Benin97/1	Benin (1997)	AF302816	Bastos et al. [9]	AM712239	Chapman et al. [25]	Benin97/1 (Tet-36)
Benin97/3	Benin (1997)	N/A	--	AM259415	Nix et al. [11]	Benin97/3 (Tet-8)
NIG/01	Nigeria (2001)	FJ174382	Gallardo et al. [19]	AM259464	Nix et al. [11]	Nigeria (Tet-32)
MOZ/1960	Mozambique (1960)	AF270708	Bastos et al. [20]	AY274465	Bastos et al. [20]	Mozambique/60

GAN: Genbank Accession Number; N/A: Not available; * CVR Tetramer number (and subtype, if more than one tetramer sequence variant is known) is indicated in brackets.

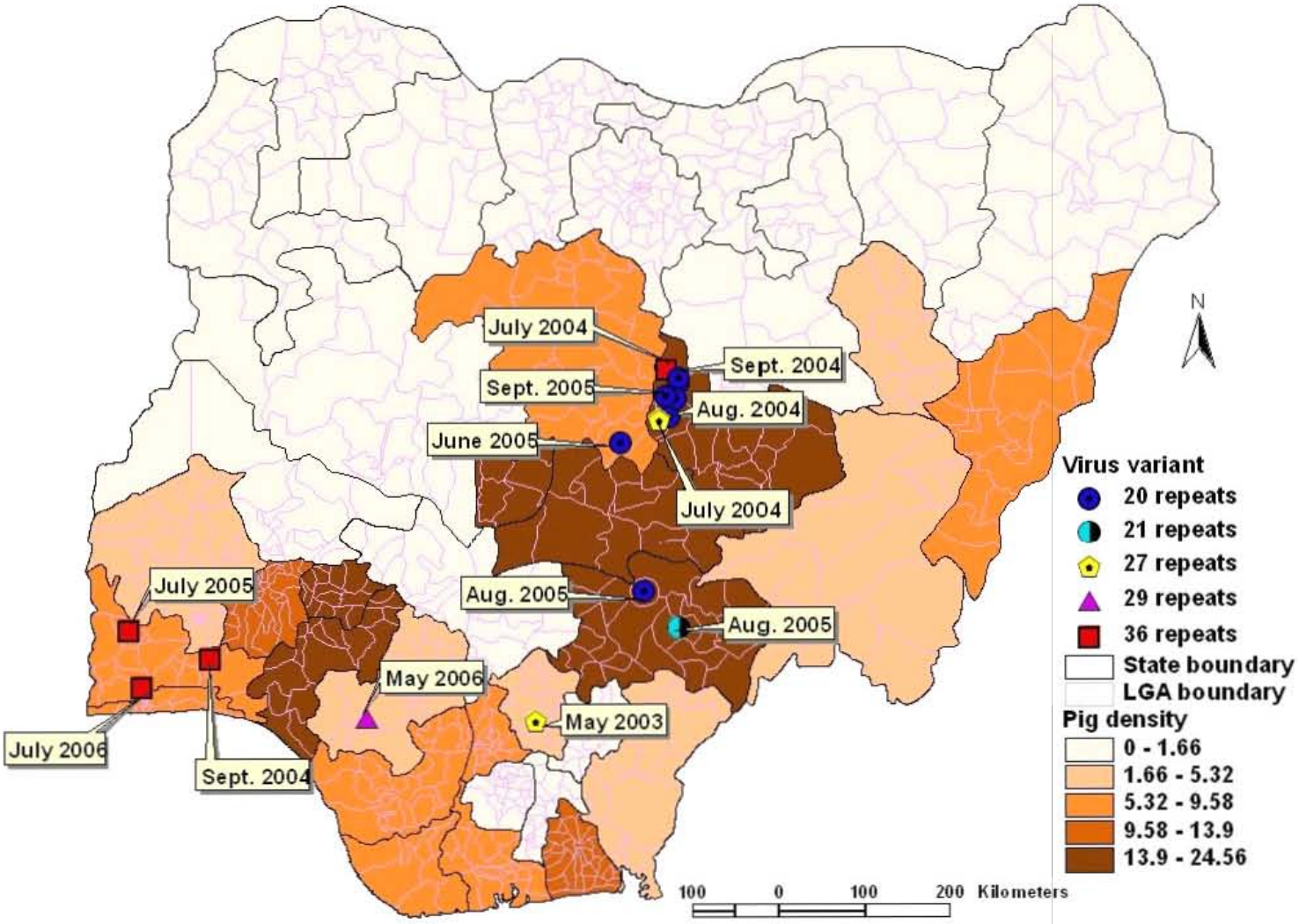

Fig. 1 Map depicting the temporal and geographical distribution of Nigerian CVR variants (May 2003-May 2006) identified in this study.

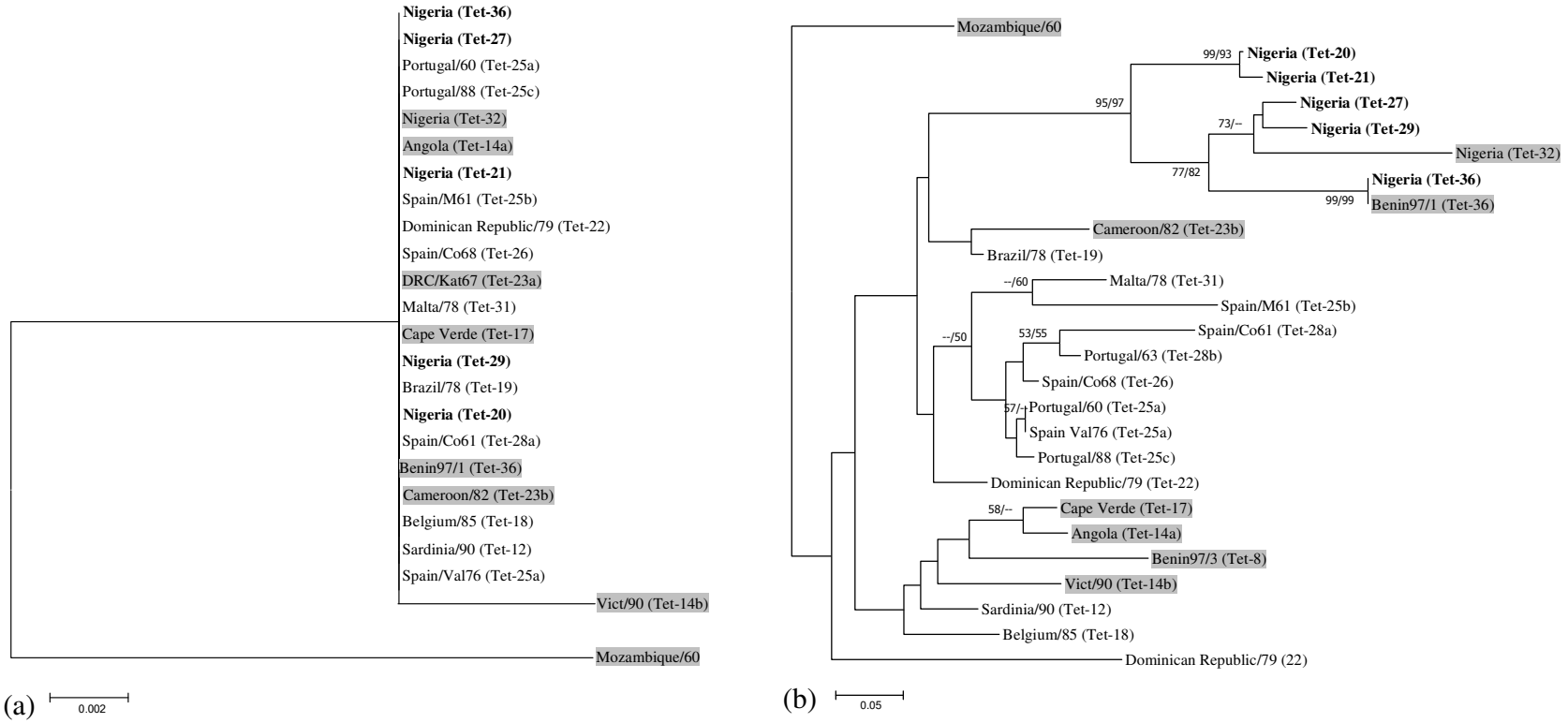

Fig. 2 Phylogenetic trees depicting (a) *p72* gene relationships and (b) CVR relationships of genotype I (ESACWA) viruses. Sequences generated in this study are indicated in bold. Viruses of African origin, characterised in previous studies, are denoted with grey shading. Bootstrap values in the CVR phylogeny are those ≥50% obtained from the 10000 bootstrap replicates. Nodal support from neighbour joining (NJ) and from parsimony (MP) is indicated NJ/MP on the relevant nodes, and '--' reflects bootstrap values < 50.

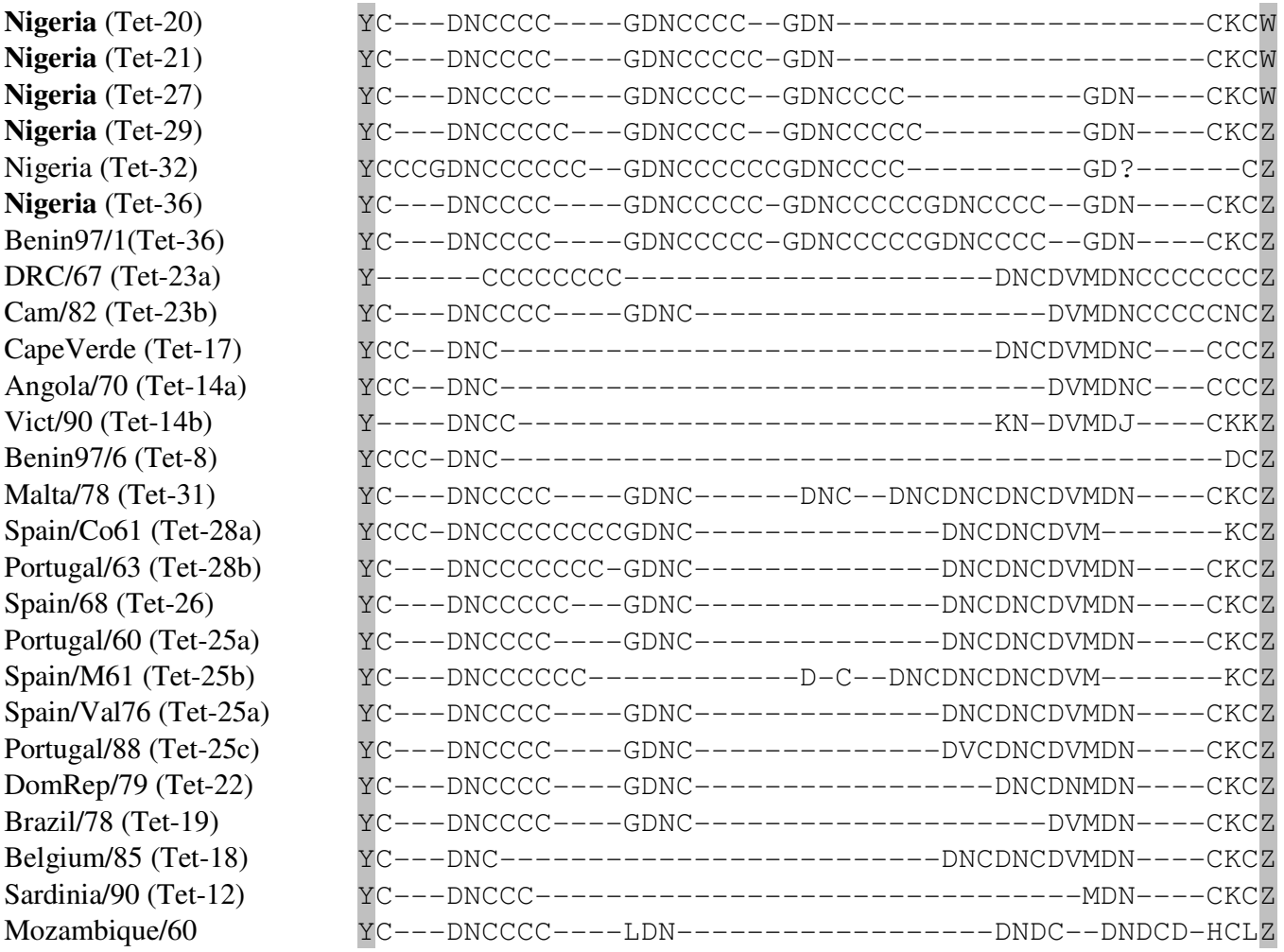

Fig. 3: Amino acid sequence alignment of the tetrameric repeats that constitute the central variable region (CVR) of the *9RL* (*B602L*) ORF. Single letter codes for each of the tetrameric repeats are the following: SAYT=Y; SADT=S; EYTD=Z; NAST=F; NVNT=V; EYSD=W; NEDT=H; CAST=C; NVNI=J; CADT=D; CANT=K; NVDT=N; CTST=L; GAST=G; NADT=P; CASM=M; NVGT=Q; CTNT=R; DVDT=T. Sequences generated in this study are indicated in bold and the CVR haplotype of each of the genotype I viruses reflects that number of tetrameric repeats, with 'a', 'b', and 'c' denoting different sequence variants. Grey shading indicates the tetramers flanking the CVR. Mozambique/60 was included for outgroup purposes.