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Genetic characterisation of African swine fever viruses from recent and historical outbreaks in Sardinia (1978–2009)

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Abstract Three discrete regions of the African swine fever virus (ASFV) were analysed in the genomes of a wide range of isolates collected from wild and domestic pigs in Sardinia, over a 31-year period (1978–2009). The analysis was conducted by genotyping based on sequence data from three single copy ASF genes. The *E183L* gene encoding the structural protein p54 and part of the gene encoding the p72 protein were used to delineate genotypes, before intra-genotypic resolution of viral relationships by analysis of tetramer amino acid repeats within the hypervariable central variable region (CVR) of the *B602L* gene. The data revealed that these isolates did not show significant variation in their p72 and p54 sequence when compared between different isolates showing a remarkable genetic stability of these genome regions. In particular, the phylogeny revealed that all the Sardinian isolates belong to the same largest and most homogeneous p72 genotype I together with viruses from Europe, South America, the Caribbean and West Africa, and p54 genotype Ia which comprises viruses from Europe and America. The analysis of *B602L* gene revealed a minor difference in the number of tetramer repeats, placing the Sardinian isolates into two clusters, accordingly to their temporal distribution, namely sub-group III and sub-group X, this latter showing a deletion of 12 tetramer repeats located in the centre of the array. The genetic variation of this fragment suggests that one sub-group could be derived from the other supporting the hypothesis of a single introduction of ASFV in Sardinia.

Keywords African swine fever virus · Sardinia · Molecular epidemiology · p54, p72, pB602L (CVR) sequencing

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Introduction

African swine fever (ASF) is an acute, highly contagious and often fatal 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. The primary reservoir of the virus is probably soft ticks of the genus *Ornithodoros* and ASFV is the only known arbovirus with a DNA genome. Wild African suids, most importantly warthogs and bush pigs, can be infected but do not exhibit clinical symptoms. ASF has been reported from most countries in sub Saharan Africa, where the virus is maintained either through a sylvatic cycle involving ticks associated with wild suids or in a domestic cycle with or without tick involvement. The disease spread outside Africa to the Iberian Peninsula, initially to Portugal in 1957 and 1960, and subsequently to several other countries in Europe and Latin America, but the virus has been eradicated from all of these, apart from Sardinia, where since its introduction in 1978, disease remains enzootic. Since the mid 1990's Sardinia was the only non-African region with the presence of infection. In 2007, ASF has caused a serious outbreaks in Georgia, subsequently spreading to adjacent countries

including Russia, which appears to have originated in south east Africa [1].

The causative agent is a double-stranded DNA virus classified within the *Asfarviridae* family, genus *Asfivirus* [2]. The ASFV genome is between approximately 170 and 193 kbp in size, depending on the isolate. Sequence analyses of virus genomes [3–5] have established that the central region 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 repeat sequences (TRS) located at loci both within coding regions and in intergenic regions between genes [6, 7]. Although p72 is useful for classification of major genotypes [8, 9], higher resolution definition of virus relationships in geographical areas where isolates are closely related to each other requires analysis of additional genes. Several regions containing variable TRS located in the rather conserved CVR of ASFV genome have been identified showing the most variable locus within the *B602L* gene [10, 11].

Sequence analysis of variable genome regions has been extensively used for molecular epidemiological studies of ASFV isolates [11–13]. A combined p72–CVR approach has been successfully used to investigate the field heterogeneity of viruses causing recent and historical outbreaks in eastern and southern Africa [12–15]. Recent studies have demonstrated the value of full p54 gene sequencing for providing additional, intermediate resolution when typing of ASFV viruses [16]. By combining p72, p54 and pB602L (CVR), a high level resolution approach is achieved for viral discrimination [16, 17].

The main aim of this study is to investigate by extensive sampling and genetic characterisation of ASFV isolates the relatedness of outbreaks occurred between 1978 and 2009 in Sardinia to contribute to the clarification of the epidemiological situation over years. For this purpose we applied genotyping to a wide range of field isolates collected from wild and domestic pigs over a 31-year period, based on partial sequencing of p72, full p54 gene sequencing, and sequencing of the CVR of the *B602L* gene. This study constitutes the first detailed assessment of the molecular epidemiology of ASF in Sardinia.

Materials and methods

Virus isolates used in this study

For the purpose of this study, pig macrophage cell culture isolates and clinical specimens obtained from 36 ASF Sardinian outbreaks were selected and sequenced. Eighteen additional sequences of other representative Sardinian ASFV isolates characterised previously [16] were acquired from the GenBank database. The viruses analysed, collected during the period 1978–2009, were all from domestic pig with the exception of four samples collected from wild boar. Geographical origin, sample source and collection date are summarized in Table 1.

Extraction and genomic DNA amplification

Viral DNA was extracted directly from 200 µl aliquots of cell culture isolates or of tissue sample homogenates by using the commercial QIAamp DNA Mini Kit (QIAGEN), following the manufacturer procedures. A PCR assay using the ASF diagnosis primers PPA1/PPA2 which generates an amplicon of 257 bp within the p72 protein [18] was used to confirm the presence of ASFV DNA.

Our genetic study is based on analysis of three independent target regions of the Sardinian ASFV isolates represented by the p72, p54 and pB602L (CVR) genome regions. The partial p72 genotyping was achieved by PCR using primers p72-D and p72-U which amplify a 478 bp C-terminal region of the p72 gene [8]. The complete gene encoding the p54 protein was amplified using the primers PPA722 and PPA89 flanking a 676 bp DNA fragment [16]. The CVR located in the *B602L* gene was amplified using the primer pairs ORF9L-F/-ORF9L-R flanking a variable in size DNA fragment [12]. Conditions for PCR assays were as previously described [16].

Nucleotide sequencing and analysis

Amplification products of the expected size were identified against a molecular weight marker, following electrophoresis on a 1.5% agarose gel. Bands of correct size were excised and purified by means of a QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer specifications and used as templates for cycle sequencing reactions with Big Dye version 3.1 (Applied Biosystems). Precipitated products were run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Both the sense and antisense strands were sequenced performing six independent reactions for each isolate.

Sequence data were analyzed using the program SeqMan II from the DNASTAR package and subsequently with Clustal X version 1.83, in respect of the amino acidic coding frame and were aligned with homologous sequences available on GenBank. Editing was performed by BioEdit Sequence alignment Editor. The phylogenetic trees were inferred with the MEGA program version 3.1 [19] using the neighbour-joining algorithm method and the Kimura-2-parameter model of sequence evolution. Nodal support was evaluated by 10,000 bootstrap replications. Sequence

Table 1 Description of the ASFV isolates used in this study

Isolate	Year	Province	Host species	GenBank accession number			Reference
				p72	p54	CVR	
Ca78	1978	Cagliari	Pig	FJ174357	FJ174401	AM259433	[11, 16]
56/Ca/78	1978	Cagliari	Pig	FR686761	FR686763	FR686765	This study
57/Ca/79	1979	Cagliari	Pig	FR686762	FR686764	FR686766	This study
Nu81	1981	Nuoro	Pig	FJ174358	FJ174402	AM259435	[11, 16]
Ss81	1981	Sassari	Pig	FJ174359	FJ174403	AM259436	[11, 16]
Ori84	1984	Oristano	Pig	FJ174360	FJ174404	AM259437	[11, 16]
Ori85	1985	Oristano	Pig	FJ174361	FJ174405	AM259397	[11, 16]
Ss88	1988	Sassari	Pig	FJ174362	FJ174406	AM259438	[11, 16]
Ori90	1990	Oristano	Pig	FJ174363	FJ174407	AM259406	[11, 16]
Nu90.1	1990	Nuoro	Pig	AF302813	FJ174408	AM259453	[11, 16]
Nu91.3	1990	Nuoro	Pig	FJ174364	FJ174409	AM259455	[11, 16]
Nu91.5	1990	Nuoro	Pig	FJ174365	FJ174410	AM259456	[11, 16]
Nu93	1993	Nuoro	Pig	FJ174366	FJ174411	nd	[16]
Ori93	1993	Oristano	Pig	FJ174367	FJ174412	nd	[16]
Nu95.1	1995	Nuoro	Pig	FJ174368	FJ174413	AM259457	[11, 16]
Nu96	1996	Nuoro	Pig	FJ174369	FJ174414	AM259458	[11, 16]
Nu97	1997	Nuoro	Pig	FJ174370	FJ174415	AM259459	[11, 16]
Ca97	1997	Cagliari	Pig	FJ174371	FJ174416	AM259460	[11, 16]
1/Nu/97	1997	Nuoro	Pig	FR668398	FR681812	FR681789	This study
2/Og/97	1997	Ogliastra	Pig	FR668399	FR681813	FR681790	This study
3/Og/98	1998	Ogliastra	Pig	FR668400	FR681814	FR681791	This study
Nu98.3	1998	Nuoro	Pig	FJ174372	FJ174417	AM259452	[11, 16]
Nu98.B	1998	Nuoro	Pig	FJ174373	FJ174418	AM259439	[11, 16]
4/Ol/02	2002	Olbia	Pig	FR668401	FR681815	FR686538	This study
5/Ca/02	2002	Cagliari	Pig	FR668402	FR681816	FR686539	This study
Ca04.1	2004	Cagliari	Pig	FR668270	FR668271	FR668272	This study
Nu04.3	2004	Nuoro	Pig	FR668262	FR668247	FR668255	This study
Nu04.4	2004	Nuoro	Pig	FR668263	FR668248	FR668256	This study
Nu04WB	2004	Nuoro	Wild boar	FR668269	FR668254	FR676962	This study
Nu04.6a	2004	Nuoro	Pig	FR668264	FR668249	FR668257	This study
Nu04.6b	2004	Nuoro	Pig	FR668265	FR668250	FR668258	This study
Ss04.10	2004	Sassari	Pig	FR668266	FR668251	FR668259	This study
11/Og/04	2004	Ogliastra	Pig	FR668403	FR681817	FR681792	This study
13/Nu/04	2004	Nuoro	Pig	FR668404	FR681818	FR681793	This study
16/Og/04	2004	Ogliastra	Pig	FR682502	FR681819	FR681794	This study
18/Nu/04	2004	Nuoro	Pig	FR677326	FR681820	FR681795	This study
22/Nu/04	2004	Nuoro	Pig	FR668405	FR681821	FR681796	This study
23/Or/04	2004	Oristano	Pig	FR668406	FR681822	FR681797	This study
24/Or/04	2004	Oristano	Pig	FR668407	FR681823	FR681798	This study
25/Nu/04	2004	Nuoro	Pig	FR668408	FR681824	FR686540	This study
26/Ss/04	2004	Sassari	Pig	FR668409	FR681825	FR686541	This study
30/Ol/04	2004	Olbia	Pig	FR668410	FR681826	FR686542	This study
Ss05.3a	2005	Sassari	Pig	FR668267	FR668252	FR668260	This study
Ss05.3b	2005	Sassari	Pig	FR668268	FR668253	FR668261	This study
36/Ss/05	2005	Sassari	Pig	FR668411	FR681827	FR686543	This study
41/Og/07	2007	Ogliastra	Wild boar	FR668413	FR681829	FR681799	This study
42/Og/07	2007	Ogliastra	Wild boar	FR668414	FR681830	FR681800	This study

Table 1 continued

Isolate	Year	Province	Host species	GenBank accession number			Reference
				p72	p54	CVR	
43/Og/07	2007	Ogliastra	Wild boar	FR668415	FR681831	FR681801	This study
46/Ca/08	2008	Cagliari	Pig	FR668416	FR681832	FR681802	This study
47/Ss/08	2008	Sassari	Pig	FR668417	FR681833	FR681803	This study
48/Ss/08	2008	Sassari	Pig	FR668418	FR681834	FR681804	This study
51/Nu/09	2009	Nuoro	Pig	FR668419	FR681835	FR686545	This study
52/Nu/09	2009	Nuoro	Pig	FR668420	FR681836	FR686546	This study
53/Nu/09	2009	Nuoro	Pig	FR677327	FR681837	FR686547	This study

nd not done

data from the current report were deposited in the EMBL nucleotide sequence data library (Table 1).

Results

P72 genotyping

In order to classify the Sardinian viruses in one of the 22 p72 genotypes as so far identified [14], sequences obtained after amplification of the C-terminal end of p72 gene were compared with ASFV sequences available in GenBank comprising representatives of each of the 22 p72 genotypes. The phylogenetic analysis established that all the Sardinian viruses were placed in the largest and most homogeneous p72 genotype I together with viruses from Europe, America and West Africa [8] (Fig. 1). Alignment and translation of sequences obtained from Sardinian isolates revealed that the C-terminal end of p72 gene was completely conserved between the sequences compared (data not shown).

P54 genotyping

Previous studies have confirmed the p54 sequencing as a valuable additional genotyping method for molecular epidemiological studies of genotype I viruses, particularly in West Africa where this genotype predominates [16]. The nucleotide sequence analysis of the p54 gene showed that the highest heterogeneity found when different ASFV isolates are compared were due to the presence of tandemly repeated amino acid repeats [20]. In order to discern amongst ASFV Sardinian isolates included in this study, we studied the variation and distribution of these amino acid repeats using the primers PPA722/89 which flank the coding sequence of p54 protein. Amino acid sequences of the Sardinian viruses were identical across the 558 bp full length *p54*-gene (data not shown). Nucleotide sequence comparison with homologous available sequences within Genbank gave rise a high degree identity (98–100%) between Sardinian and European/American isolates. The phylogeny revealed that the Sardinian viruses cluster within the largest and most heterogeneous p54 genotype Ia previously defined by Gallardo et al. [16] together with viruses from Europe and America (Fig. 2).

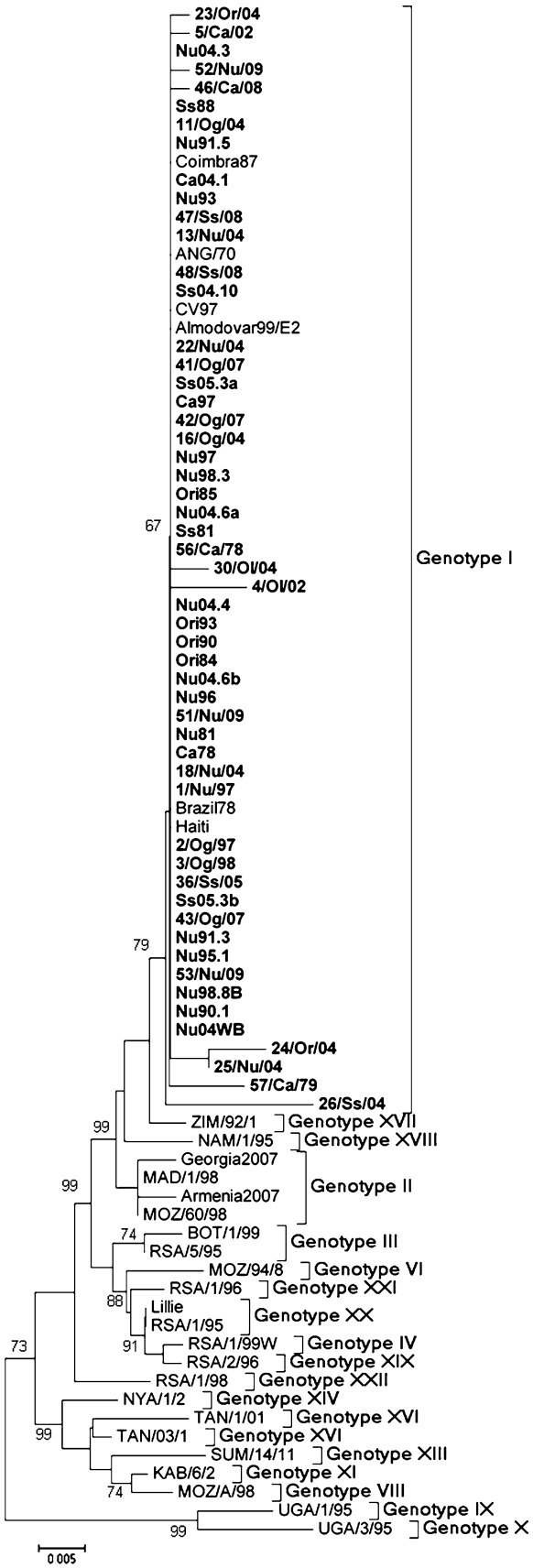
Analysis of TRS presented in B602L (CVR) gene

Several studies have reaffirmed the epidemiological utility of the CVR genome region within *B602L* gene for distinguishing between geographically and temporally constrained genotype I viruses [11, 13, 14, 21]. According to previous studies [12], our results confirmed that the Sardinian isolates could be sub-divided into two different subgroups temporally related.

Particularly, the analyses of the amino acid tetramer repeats sequences within the CVR of two Sardinian isolates from the period 1978–1979 characterised in this study, revealed the presence of 25 repeats identical to those included into the previously defined CVR sub-group III [11] which is represented by historical isolates from Spain, Portugal and Haiti and by all the Sardinian isolates prior 1990 with the exception of one isolate from 1998 (Nu98.B).

In contrast, 34 ASFV isolates obtained from 1997 until 2009 were placed within CVR sub-group X. This sub-group characterized by the presence of 12 (32 isolates) or 13 (2 isolates) tetramer repeats comprises viruses collected from 1990 up to 1998 giving raise to a final Sardinian CVR subgroup which comprises a total of 43 isolates having a field presence of 20 years (1990–2009) (Table 2). A minor change due to the insertion of a single internally located tetramer repeat (CAST) was present in two isolates recovered from outbreaks in 2008 (46/Ca/08 and 47/Ss/08). Nevertheless the phylogenetic analysis established that these viruses do not form a new sub-group (data not shown).

Therefore sub-group X viruses contained repeat arrays from which 12 (2 isolates) or 13 (32 isolates) tetramer repeats were deleted compared to the sub-group III viruses (Fig. 3).



◀ **Fig. 1** Phylogenetic tree constructed using 404 nt from the ASFV p72 gene of 54 Sardinian isolates (*labelled* in *bold*) and of 29 reference strains retrieved from the GenBank database. Phylogenetic analysis was performed using the Neighbour-joining algorithm and the Kimura 2-parameter model of sequence evolution. The numbers on the major nodes indicate the bootstrap values in percentage, obtained from 10,000 replicates. *Bar* number of substitutions per site

Discussion

In Sardinia the disease appeared in 1978 in the southern part of the island, presumably introduced from the Iberian Peninsula via offal containing raw pork, which was subsequently fed to pigs [22]. The infection was successfully eradicated from this area, but in eastern-central Sardinia an enzootic area still persists in the province of Nuoro. In this mountainous area, with a surface of approximately a quarter of the whole island, traditional pig husbandry practices such as grazing free-range herds on vast communal land is considered a major obstacle to the disease eradication. ASF outbreaks outside this area have occurred sporadically in the other provinces. The wild boar (*Sus scrofa*) population that inhabits the hilly and mountainous areas that characterise the topography of Sardinia is susceptible to ASFV, shares its habitat with free-ranging pigs. Contacts and cross-breeding between wild and domestic animals are likely and ASFV transmission from wild to domestic pig can therefore occur. Nevertheless, wild boar probably play a negligible role as reservoir of ASFV as suggested by the outcomes of serological and virological investigations carried out according to the guidelines of the surveillance programmes running in Sardinia [23]. Further studies are needed to address the importance of wild boar in maintaining the disease. Moreover, the circulation of ASFV amongst pig farms cannot be ascribed to the arthropod host of the virus, because *Ornithodoros* species are absent from Sardinia, therefore a domestic pig-restricted cycle which is not reliant on the presence of the tick vector, is most likely to occur.

The inability of ASFV to induce neutralizing antibodies has hampered the prevention and control of the disease by vaccination. In the absence of effective vaccines, control of ASF in high-risk areas of Sardinia should be focused on municipalities at highest prevalence, particularly by rationalization of extensive pig farming, which is a critical step for disease eradication and for prevention of further infections. Delimiting pastures, as an example, could limit movements of free-ranging pigs and prevent contacts amongst herds of different area, reducing the risk of ASFV transmission.

The formulation of appropriate disease control strategies requires intensive epidemiological investigations that

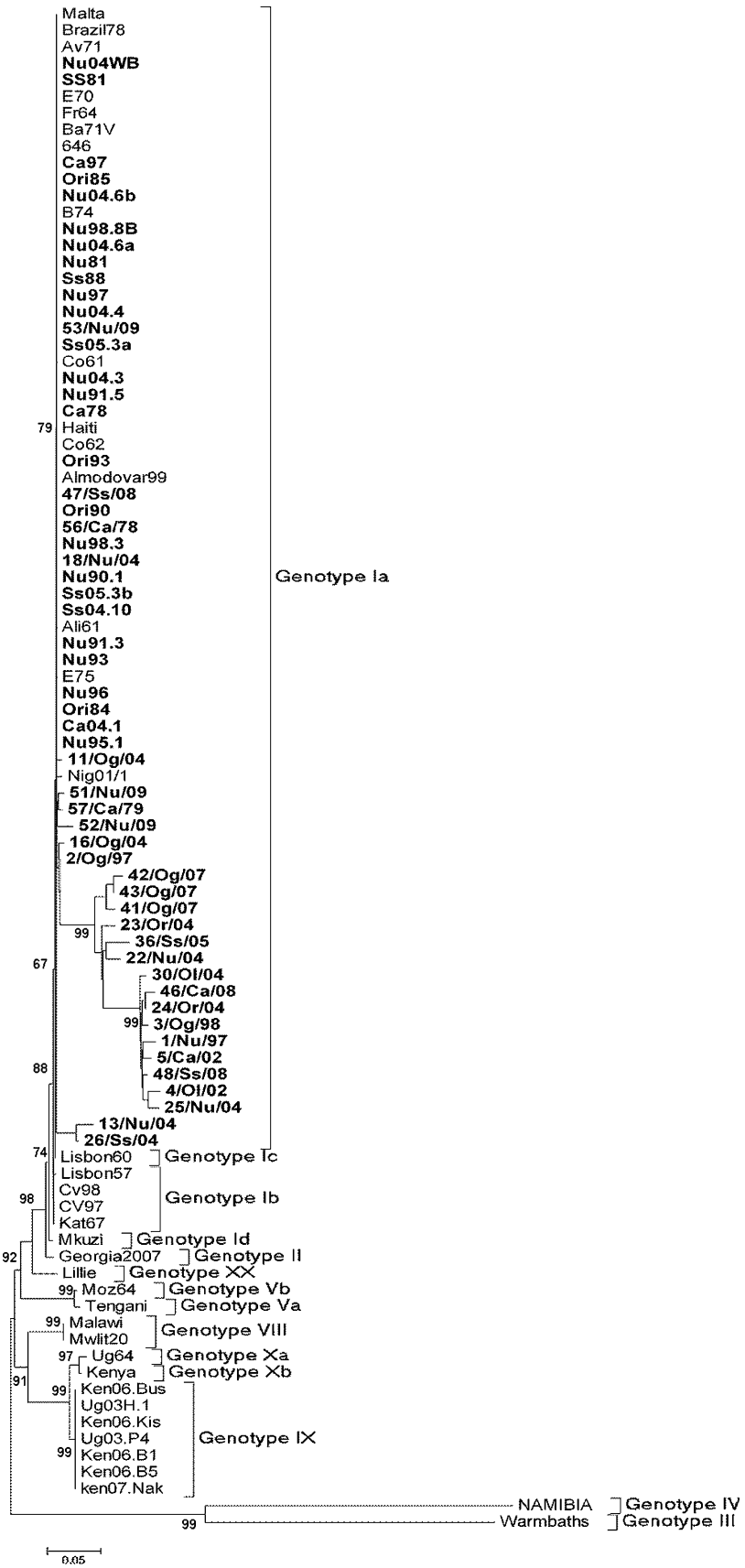


Fig. 2 Phylogenetic tree constructed using 552 nt from the ASFV p 54 gene of 54 Sardinian isolates (*labelled* in *bold*) and of 38 reference strains retrieved from the GenBank. Phylogenetic analysis was performed using the Neighbour-joining algorithm and the Kimura 2-parameter model of sequence evolution. The *numbers* on the major *nodes* indicate the bootstrap values in percentage, obtained from 10,000 replicates. *Bar* number of substitutions per site

Table 2 Amino acid tetramer sequence of ASFV isolates

Subgroups	Country	Isolates		P72 genotype	CVR amino acid sequence	No repeats
I	Spain	M 61	Co 62	I	-A--BNAAAAAA-BABNABNABNAB-----TD---FA	25
II	Spain	Co 61		I	-AAABNAAAAAAAACBNABNABNAB-----TD---FA	28
III	France	Fr 64		I	-A--BNAAAA----CBNABNABNAB-----TDBNAFA	25
	Portugal	Mon 84	Vis 86			
		Por 86	Coi 86			
		San 86	Tom 86			
		OurT91/1	Port 99			
	Sardinia	Ca78	Nur 79			
		Ori 84	Ss 81			
		Nu 81	Ori 85			
		Ss 88	Nu 98/8B			
		56/Ca/78	57/Ca/79			
	Spain	Ali 61	Mad 62			
		Av 71	Val 76			
		B 74	E 75			
		Ss 88	Zar 85			
		Mu 82	Se 88			
		Hu 94				
		646				
	Haiti	Haiti				
IV	Portugal	Our T88/1		I	-A--BNAAAA---CBNABTABNAB------TDB	21
V	Spain	Ba 71V		I	-A--BNAAAAAAACBNABNABNAB------TDBNAFA	28
	Portugal	Por 63				
VI	Malta	Malta 78		I	-A--BNAAAA---CBNABNABNABNABNABTDBNAFA	31
VII	Belgium	Bel 85		I	-A--BNA-------BNABNAB---------TDBNAFA	18
	Holland	Hol 86				
VIII	Portugal	Lis 60		I	-A--BNAAAA---CBNABNABNA------DTDBNAFA	25
IX	Spain	Co 68		I	-A--BNAAAAA--CBNABNABNA------BTDBNAFA	26
X	Sardinia	Nu90/1	Nu 90/2	I	-A--BN-AAA--------------------DBNAFA	12
		Ori 90	Nu 91/3			
		Nu 91/5	Nu 95/1			
		Nu 95/4	Nu 96			
		Ca 97	Nu 97			
		Nu 98/3	Nu 04.3			
		1/Nu/97	2/Og/97			
		3/Og/98	4/Ol/02			

Table 2 continued

Subgroups	Country	Isolates		P72 genotype	CVR amino acid sequence	No repeats
		5/Ca/02	11/Og/04			
		13/Nu/04	16/Og/04			
		18/Nu/04	22/Nu/04			
		23/Or/04	24/Or/04			
		25/Nu/04	26/Ss/04			
		30/Ol/04	36/Ss/05			
		41/Og/07	42/Og/07			
		43/Og/07	Ss05.3a			
		Ss05.3b	51/Nu/09			
		52/Nu/09	52/Nu/09			
		Ca 04.1	Nu 04.6b			
		Nu 04.6a	Nu 04.4			
		Nu 04WB	Ss04.10			
		46/Ca/08	47/Ss/08		-A--BNAAAA----------DBNAFA	13
XI	Dominican R.	Dom. Rep.		I	-A-BNAAAA-----CBNABNABN--------DBNAFA	22
XII	Brazil	Brazil 78		I	-A--BNAAAA----CBNA----------BTDBNAFA	19
XIII	Angola	Ang 70		I	-AA-BNA----------BTDBNAAAA	14
	Portugal	Lis 57				
XIV	Zaire	Kat 67		I	-AAAAAAAABNA----------BTDBNAAAAAAA	23
XV	Cape Verde	CV 97	CV 98	I	-AABNABNA----------BTDBNAAAA	17
XVI	Cameroon	Cam 82		I	-A-BNAAAA-----CBNA----------BTDBNAAAAANA	23
XVII	Zambia	Vict 90/1		I	---BNAAFN----------BTDBTAFF	14
XVIII	Benin	Ben 87/3		I	AAABNABA	8
XIX	Benin	Ben 97/6		I	-A-BNAAAA-----CBNAAAAA-CBNAAAAAA-CBNAAAACBNAFA	36
XX	Nigeria	Nig 01			-AAA----------CBNAAAAAACBNAAAAAAACBNAAAACBNA	32
XXI	S.Africa	Ten 60		V	-ABNBBVa	7
XXII	Mozambique	Moz 94/1		VI	-AAAA----------BABNABABNBTBA	17
XXIII	Mozambique	Moz 64			-A-----BNAAAaBNBNBABN-BABTAa	21
XIV	Uganda	Uga 95/1		IX	-AAA---BNABBNABBNABBaaBBNABNBA	26
XXV	Uganda	Uga95/3		X	-AAAAAABNaABA	12
XXVI	Kenya	Hind II		X	-AAA---BNAAAAAAAAAABA	17
	Burundi	Bur 84/1	Bur 90/1			
XXVII	Botswana	Bots 1/93		III	-BVSVSV---VNAAABAB	14
XXVIII	Zambia	Kal 88/1		VIII	-AVSVSVSVSVNAAAAAAACBNABNABNABT-	30

Table 2 continued

Subgroups	Country	Isolates	P72 genotype	CVR amino acid sequence	No repeats
XXIX	Malawi	Malwi Lil 20/1	VIII	-AVSVS---OVNA---ONOVVNVOVNAVNOVVN--OVOOV	31
XXX	Malawi	Malawi 78	VIII	-AVSVS-----BVVVAVNOVVNVVNVVNAANOVVDBOVOOV	34
XXXI	Malawi	Zom 64/2	VIII	-AVSVS------VVVAVNOVVNV----------OVOOV	21

Key A = CAST; a = CVST/CTST/CASI; B = CADT/CTDT; C = GAST/GANT; D = CASM; F = CANT; N = NVDT/NVGT; T = NVNT; H = RAST; S = SAST; O = NANI/NADI/NASI; V = NAST/NAVT/NANT/NADT. *Dashes* indicate gaps introduced for alignment purposes

benefit greatly from insights provided from retrospective analysis of the source and spread of recent and historical outbreaks [14]. Achieving this requires the availability of historical isolates and techniques capable of differentiating between strains [14]. The usefulness of the C-terminal end of the p72 gene for genotyping ASFV viruses and for inferring broad epidemiological relationships is well established [8, 9, 14], however, low levels of variation remains problematic and requires the characterisation of additional more variable genome regions to achieve a better level of resolution.

This study represents a comprehensive attempt to resolve the intra-genotypic relationships of 54 p72 genotype I viruses causing outbreaks in Sardinia over a 31 year period to detect epidemiological links that may exist between outbreaks from different provinces of Sardinia. The combined p72, p54 and CVR approach was used to achieve optimal levels of discrimination of closely related virus isolates. Results indicate that genetic variation amongst Sardinian isolates is low. Particularly, the Sardinian viruses did not show any significant variation in their p72 and p54 genome regions characterised on amino acid level, confirming a remarkable genetic stability of these regions. In contrast, the analysis of the *B602L* gene revealed the presence of minor difference in the number of TRS placing the Sardinian isolates into two clusters accordingly to their temporal distribution, namely sub-group III, comprising viruses collected up to 1990 and one isolate from 1998, and sub-group X which comprises all except one of the isolates collected from 1990 until 2009. Interestingly, isolates recovered from wild boar are indistinct from viruses causing outbreaks in domestic pig. This data reaffirms previous findings in which the *B602L* gene has been showed as a genetic marker useful for high resolution discrimination of viruses that were identical according to their p72 and p54 amino acid profile [10, 11] and it has proved useful for resolving epidemiological complexities at the country [15], genotype [11] and regional level [14].

In conclusion, the viruses responsible for the ASF outbreaks in Sardinia showed a deletion of 12 or 13 tetramer repeats observed from all except one of the isolates obtained since 1990. This is consistent with the hypothesis that the ASF outbreaks occurring during the last two decades were caused by very closely related, but mutated forms of the virus that had been circulating from the beginning, supporting the assumption of a single introduction of ASFV in Sardinia.

On the other hand, such low levels of intra-genotype diversity of a wide range of field isolates collected over time remains problematic and requires further investigations into alternative and more informative gene regions to clarify the within-genotype relatedness by achieving finer discrimination amongst viruses so closely related. In the

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                 10        20        30        40        50        60        70        80        90       100       110
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
56/Ca/78 LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ca78     LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
57/Ca/79 LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu81     LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ss81     LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ori84    LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ori85    LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLADPERI
Ss88     LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ori90    LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu90.1   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu91/3   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu91/5   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu95/1   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu96     LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu97     LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ca97     LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
1/Nu/97  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLPDPERI
2/Og/97  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu98.3   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu98/8B  LHAQSAYTCASTCADTNVDTCASTCASTCASTCASTGASTCADTNVDTCASTCADTNVDTCASTCADTNVDTCASTCADTNVNTCASMCADTNVDTCASTCANTCASTEYTDLTDPERI
3/Og/98  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
4/Ol/02  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPARI
5/Ca/02  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPARI
Ca04.1   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu04.6b  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu04.4   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu04.6a  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu04WB   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Nu04.3   LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ss04.10  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
11/Og/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
13/Nu/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
16/Og/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
18/Nu/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPARI
22/Nu/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
23/Or/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
24/Or/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPARI
25/Nu/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
26/Ss/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
30/Ol/04 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
Ss05.3b  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
Ss05.3a  LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
36/Ss/05 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
41/Og/07 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
42/Og/07 LHTQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
43/Og/07 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPQRI
47/Ss/08 LHAQSAYTCASTCADTNVDTCASTCASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDFTDPERI
46/Ca/08 LHAQSAYTCASTCADTNVDTCASTCASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
48/Ss/08 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDFTDPERI
51/Nu/09 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
52/Nu/09 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
53/Nu/09 LHAQSAYTCASTCADTNVDTC~~~~ASTCASTCAST~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CASMCADTNVDTCASTCANTCASTEYTDLTDPERI
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Fig. 3 Amino acid sequence alignment of the tetramer tandem repeats identified within the central variable region of gene B602L (CVR) from Sardinian isolates collected during the period 1978–2009

future, this approach should be taken into account for effective control of the disease, making possible tracing isolates, enabling a more accurate assessment of the origin of outbreaks and in this way extending knowledge of virus evolution and epidemiology of ASF in Sardinia.

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