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Classical swine fever in Sardinia: epidemiology of recent outbreaks

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SUMMARY

A variable region of the gene encoding the major glycoprotein (E2) of Classical Swine Fever Virus (CSFV) was sequenced from 12 Sardinian isolates which had been obtained from three geographically distinct regions of the Island. Phylogenetic analysis of these viruses and others characterized in previous studies [1, 2] indicated that (*a*) the Sardinian viruses were all members of the common European subgroup 2·3 and were clearly distinct from live vaccines recently used in this area; (*b*) they could be resolved into four distinct groups in accordance with the region or date of isolation; (*c*) in at least two regions wild boar/domestic swine contact was implicated in virus spread; (*d*) the oldest isolate (1983) and some of the recent isolates were possibly introduced from mainland Italy. In addition, this study has wider implications for the interpretation of CSFV variation. We have been able to demonstrate that small variations within this region of the virus genome (possibly less than 2·7% or five nucleotide substitutions) can be used to separate isolates into groups that precisely fit their geographical distribution. This finding is especially important for deducing the epidemiological relationships between multiple outbreaks caused by similar viruses that occur in close proximity.

INTRODUCTION

CSFV is the causative agent of Classical Swine Fever (CSF), a serious viral disease affecting pigs in various parts of the world. The European Community has an eradication programme for this disease which no longer permits routine use of vaccines, relying instead upon restricting the movements of pigs in areas surrounding outbreaks, forwards and backwards tracing of virus spread, and slaughter of all affected herds. Although the incidence of the disease has been much reduced, outbreaks in Germany, Holland and Belgium in the last few years have resulted in large-scale slaughtering of pigs, whilst Italy and Austria have had sporadic but less economically serious outbreaks. Several mechanisms of virus spread have been proposed to explain the recurrence of the disease. These include virus reintroduction (*a*) via the importation of infected pigs or wild boar (either alive or as pig products) from Eastern Europe, or (*b*) from a CSFV reservoir present within indigenous wild boar populations accompanied by contact between wild boar and domestic swine, again either directly or via infected meat. Both theories have been suggested as causes of outbreaks in the Tuscany region of Italy, where it is believed that imported meat introduced a virus which was subsequently maintained in a wild boar population [1]. In Sardinia, there is a large wild boar population, and contact between wild boar and domestic pigs is often facilitated by extensive farming practices and/or inadequate fencing. In the Nuoro province of Eastern Sardinia, domestic pigs are allowed to forage freely with wild boar. Sardinia was

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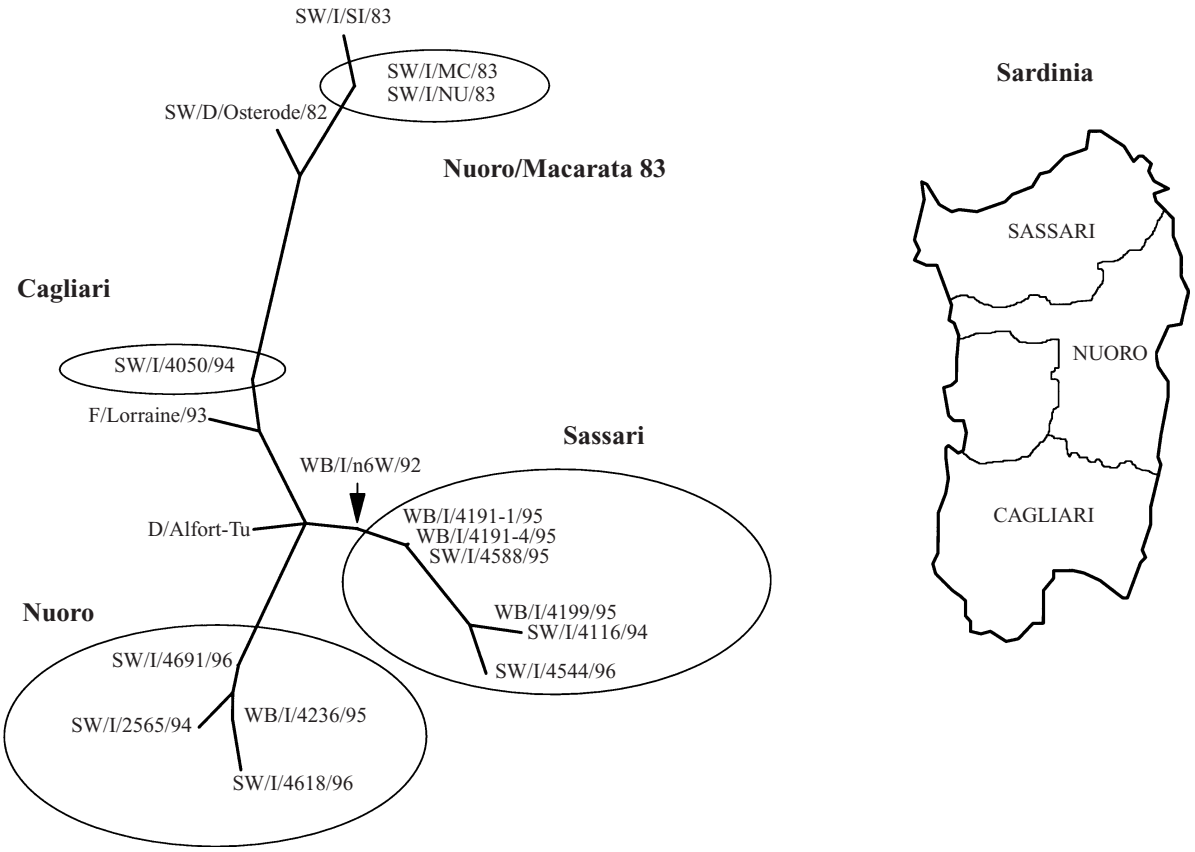

Fig. 1. Unrooted Maximum Likelihood tree of the Sardinian and other closely related isolates. The four Sardinian groups are circled and labelled with the province of isolation. The virus name indicates (where known) whether the isolate was from wild boar (WB/) or domestic swine (SW/). The country of origin is indicated by I/ (Italy), D/ (Germany) of F/ (France). The isolate name (e.g. /Osterode) is then followed by the date of isolation (e.g. 82). The strain Alfort–Tu is added as a reference. The map of Sardinia shows the location of the provinces discussed.

thought to be free of CSF after a period of vaccination in the 1970s. Vaccination remained compulsory, but incompletely enforced up until December 1991. There were no recorded cases of CSF between 1980 and 1983 [3]. However, in 1983 an outbreak of the disease was detected in Nuoro, followed by many more outbreaks in this region and in the northern province of Sassari. A single outbreak also occurred in the southern province of Cagliari.

We have previously demonstrated [2] that, of available methods, sequence comparisons of the 5′ region of the E2 gene produced the greatest ability to discriminate between closely related isolates of CSFV. Using this approach we determined the relationships between some recent Sardinian isolates from both wild boar and domestic pigs. The concordance between phylogenetic segregation and geographical virus distribution suggests that this method of virus characterization may be useful in determining the relationships between other multiple outbreaks caused by closely related CSF viruses.

METHODS

Viruses

Viruses were obtained from tissue homogenates from wild boar and domestic swine and were passaged no more than twice in PK-15 cells prior to RT-PCR and sequencing. The viruses have been coded to show their isolation dates, their province of isolation and the type of animal infected (wild boar or domestic swine) and are shown in Figure 1. For example, SW/I/SI/83 was isolated from a domestic pig (swine), in Italy (Siena province) in 1983.

RT–PCR and sequencing

RT–PCR was carried out on QIAamp viral RNA kit (Qiagen Ltd) purified RNA using the same conditions and primers previously described [2]. One hundred and ninety nucleotides of the E2 PCR products were sequenced using the *fmol* sequencing system (Promega) and primers described in the same publication.

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Table 1. *Percentage differences between the Sardinian viruses and other similar subgroup 2·3 isolates. Riems vaccine strain is included as a comparison. Boxed areas represent the Sardinian and mainland virus groups seen on Figure 1*

		% Nucleotide differences between Sardinian and other selected viruses																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SW/I/4618/96	1	0·0																			
SW/I/4691/96	2	1·1	0·0																		
WB/I/4236/95	3	0·5	0·5	0·0																	
SW/I/2565/94	4	1·1	0·5	0·5	0·0																
WB/I/4191-4/95	5	3·8	2·7	3·2	3·2	0·0															
WB/I/4191-1/95	6	3·8	2·7	3·2	3·2	0·0	0·0														
WB/I/n6W/92	7	3·2	2·1	2·7	2·7	0·5	0·5	0·0													
SW/I/4588/96	8	3·8	2·7	3·2	3·2	0·0	0·0	0·5	0·0												
SW/I/4544/96	9	5·4	4·3	4·9	4·9	1·6	1·6	2·1	1·6	0·0											
WB/I/4199/95	10	4·9	3·8	4·3	4·3	1·1	1·1	1·6	1·1	0·5	0·0										
SW/I/4116/94	11	5·4	4·3	4·9	4·9	1·6	1·6	2·1	1·6	1·1	0·5	0·0									
SW/I/MC/83	12	5·4	4·3	4·9	4·9	4·9	4·9	4·3	4·9	6·5	6·0	6·5	0·0								
SW/I/NU/83	13	5·4	4·3	4·9	4·9	4·9	4·9	4·3	4·9	6·5	6·0	6·5	0·0	0·0							
SW/I/SI/83	14	6·0	4·9	5·4	5·4	5·4	5·4	4·9	5·4	7·1	6·5	7·1	0·5	0·5	0·0						
SW/D/Osterode/82	15	4·9	3·8	4·3	4·3	4·3	4·3	3·8	4·3	6·0	5·4	6·0	1·6	1·6	2·1	0·0					
SW/I/4050/94	16	4·3	3·2	3·8	3·8	2·7	2·7	2·1	2·7	4·3	3·8	4·3	3·2	3·2	3·8	2·7	0·0				
F/Lorraine/93	17	4·3	3·2	3·8	3·8	2·7	2·7	2·1	2·7	4·3	3·8	4·3	4·3	4·3	4·9	3·8	1·1	0·0			
D/Alfort-Tu	18	3·2	2·1	2·7	2·7	1·6	1·6	1·1	1·6	3·2	2·7	3·2	4·3	4·3	4·9	3·8	2·1	2·1	0·0		
SW/D/Schweinfurt 242-2/93	19	4·3	3·2	3·8	3·8	1·6	1·6	1·1	1·6	3·2	2·7	3·2	5·4	5·4	6·0	4·9	3·2	3·2	2·1	0·0	
Riems Vaccine (C-Strain)	20	17·0	15·6	16·3	16·3	16·3	16·3	15·6	16·3	18·2	17·5	16·9	15·6	15·6	16·3	15·0	14·4	15·6	15·6	16·9	0·0

Computer analysis

Multiple alignments of nucleotide sequences were carried out using the PILEUP program of the GCG package [4] and phylogenetic analysis was performed using the Maximum Likelihood (DNAML) and Neighbour-joining program (NEIGHBOUR) in the PHYLIP package [5]. Sequence data from other viruses obtained in the previous studies were compared with these new data.

RESULTS

Comparison of the Sardinian viruses with sequences obtained in previous studies showed them all to be from subgroup 2·3, the group most commonly encountered in recent European outbreaks. All the viruses were distinct from the Chinese vaccine strain (subgroup 1·1) which had been used widely in Sardinia during the 1970s [3]. The greatest variation between the isolates was 6·5% between SI/I/NU/83 and either SW/I/4116/94 or SW/I/4544/96 (Table 1). Only three Sardinian viruses were identical in this region, these were the Sassari isolates SW/I/4588/96, WB/I/4191-1/95 and WB/I/4191-4/95 (Fig. 1). The latter two viruses were isolated from wild boar and the former from domestic swine. A similar close association of wild boar and domestic swine isolates was seen between the Sassari isolates SW/I/4116/94 and WB/I/4199/95 (0·5% variation). These two viruses were in turn, more closely related to isolate SW/I/4544/96 and the other aforementioned Sassari viruses than they were to any of the other isolates (Fig. 1). Similarly the Nuoro virus SW/I/4618/96 isolated in domestic swine in 1996 was very closely related to WB/I/4236/95 isolated from a wild boar in 1995 (0·5% variation). These two viruses were more closely related to the other two recent Nuoro isolates (SW/I/4691/96 and SW/I/4618/96) than they were to any other virus in the study. The remaining two Sardinian viruses were both isolated from domestic swine, one in the province of Nuoro SW/I/NU/83 (1983) and the other in Cagliari SW/I/4050/94 (1994). SW/I/NU/83 was distinct from other Nuoro viruses (equal or greater than 4·9% variation) but was identical to the Italian mainland isolate SW/I/MC/83 (1983). When the four Sardinian groups described above were compared to previously characterized viruses from subgroup 2·3 (Figs. 1, 2) the Sassari viruses were found to be most like WB/I/n6W/92 (0·5–2·1%), a mainland Italian virus isolated in 1992. The recent Nuoro viruses apparently formed a distinct group with greater than 2·1% variation when compared to all the other group 2·3 viruses. The Cagliari isolate was closely related (1·1%) to several Polish and a French virus (F/Lorraine/92). The isolates SW/I/NU/83 and SW/I/MC/83 were similar (1·6%) to SW/D/Osterode/82, a 1982 German isolate. The variation between the C-strain vaccine (Riems) and the Sardinian viruses was between 14·4% and 18·2%. Analysis of the nucleotide substitutions between the Sardinian viruses revealed 18 variable positions resulting in 5 positions of variation at the amino acid level. There appeared to be a clustering of nucleotide changes at the 3′ end of the region studied which corresponded to Tubingen–Alfort nucleotides 2658 to 2677 (GenBank accession no. J04358). The amino acid substitutions were too few to deduce any clustering.

DISCUSSION

The Sardinian isolates are clearly characterized as being within the same subgroup (2·3, Fig. 2) as the majority of isolates obtained from outbreaks of CSF in mainland Europe during the 1980s and 1990s. The close similarity of the Sardinian isolates to those from mainland Europe suggests a recent common origin for both, and argues strongly against the possibility that because of its island status, CSF persisted on Sardinia independent of one or more external virus introductions. The RT–PCR product amplified from the oldest isolate, SW/I/NU/83, is identical to that obtained from a virus from the Macerata province of central mainland Italy SW/I/MC/83 (Fig. 1), both viruses having been isolated in 1983 (the mainland isolate being obtained four months prior to the Sardinian virus). Assuming that this homology is not an artefact of cross-contamination, this suggests an introduction of this virus from mainland Italy. A less likely, alternative explanation is that the direction of spread was from Sardinia to the mainland as the result of an unrecognized reservoir of the disease persisting in the Sardinian population prior to 1983. The movement of pigs from Sardinia to the mainland at this time may have occurred but would have been illegal. The apparently inappropriate isolation dates could represent a delay in detection of the disease on the island. However, the high virulence of the 1983 isolates would make such a delay in detection unlikely. Compared to other known viruses, the Italian mainland and Sardinian isolates from 1983 are most closely related to the virus SW/D/Osterode/82 isolated in Germany in 1982. The genetic similarity in con-

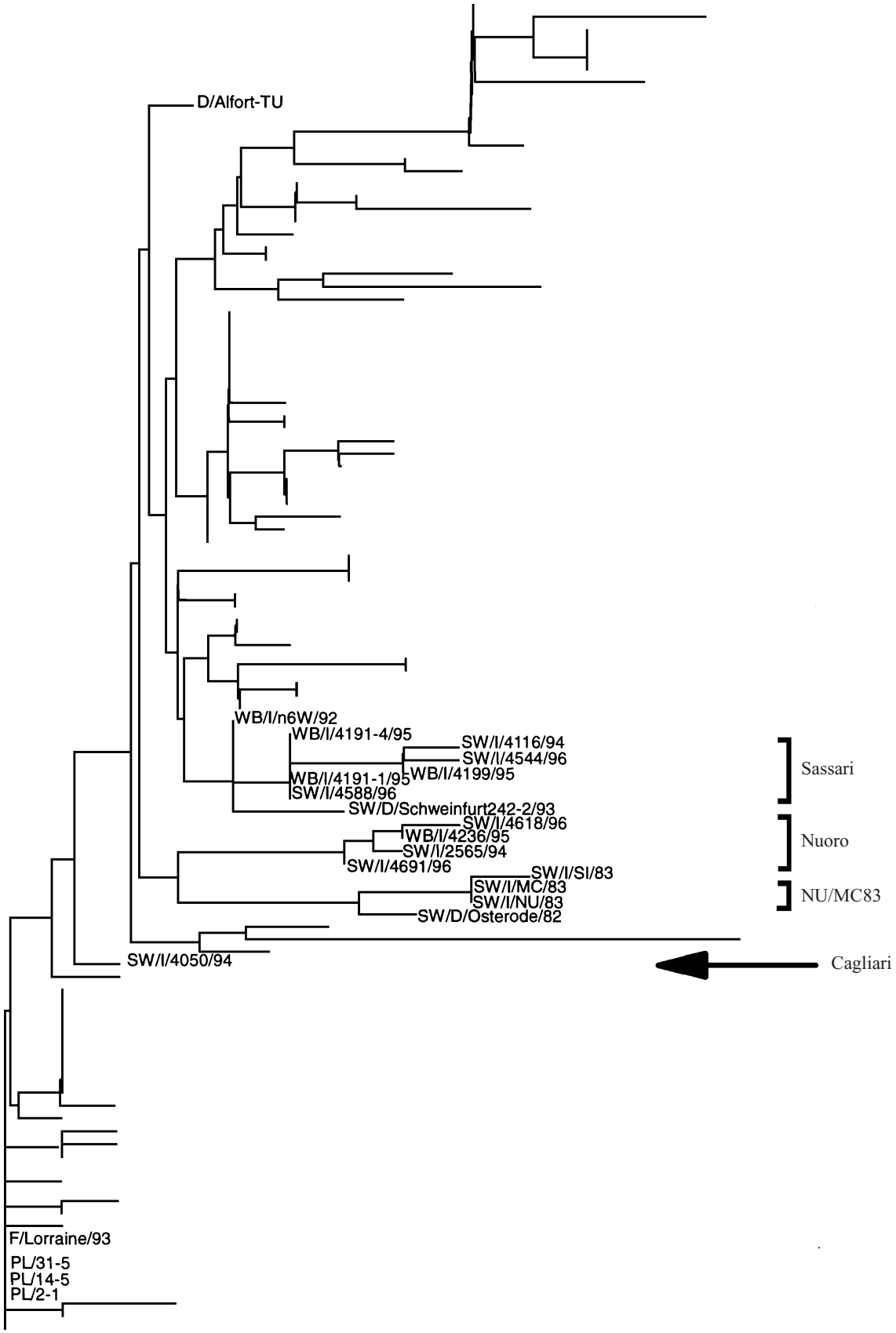

Fig. 2. Unrooted Neighbour-joining tree of subgroup 2·3 viruses. The Sardinian viruses are labelled with the province of origin. All viruses are named as in Figure 1. In addition, the Polish country code (/PL) is used.

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junction with the isolation dates and the existence of a thriving import trade in pigs from Germany suggests some sort of epidemiological link between these viruses. However, on its own, these data cannot be used to infer the direction of spread or the direct nature of any link.

Across the island of Sardinia, an average of 11% of wild boar shot between 1988 and 1992 were found to be seropositive to CSFV [3]. In the Nuoro region of Sardinia, where the same habitat is shared by wild boar and free-ranging domestic pigs, there is ample opportunity for exchange of CSFV between these two pig classes. In other parts of Sardinia, the opportunities for contact between domestic pigs and wild boar is less because of more intensive methods of pig husbandry [3]. The close genetic similarity of the Sassari wild boar isolates (WB/I/4191-1/95 and WB/I/4191-4/95) to the domestic pig isolate (SW/I/4588/96) does provide evidence for transmission of CSFV between wild boar and domestic pigs in this region of Sardinia. An equivalent similarity between the Nuoro isolates SW/I/4236/95 (wild boar) and SW/I/4618/96 (domestic swine) suggests that transmission between pigs and wild boar is not confined to the Sassari region. Similar relationships between wild boar and domestic swine isolates were obtained from a study of CSFV in Tuscany [1], which also provided evidence that CSFV could persist in wild boar for several years whilst periodically causing outbreaks in domestic pigs.

Unfortunately no CSFV isolates from Sardinia were available from the period between 1984 and 1993. This makes it difficult to rule out the possibility that all of the recent Sardinian viruses have evolved from SW/I/NU/83. Indeed the evolution rates for this to have occurred (data not shown) would be comparable to those calculated for CSFV previously [2]. The area-specific grouping of the isolates made in 1994 and 1996 suggests that if they do have a common origin in SW/I/NU/83, this antecedent virus was initially able to spread throughout Sardinia, but later became restricted to isolated pockets of infection with subsequent divergence through independent evolution. Such an isolation event would be difficult to imagine. Another explanation would be that one or more of the three area-specific virus types may be the result of separate introductions to Sardinia which have been maintained as distinct populations by the severe geography and man-made barriers separating these regions coupled with the small territorial range of wild boar. This theory of geographical isolation is supported by serology and virus isolation studies which have shown that communes separating the major outbreaks in Nuoro and Sassari have wild boar populations which are consistently seronegative and uninfected [6]. Furthermore the similarity between the recent Sardinian isolates and SW/I/NU/83 is lower than that seen between these viruses and other groups from continental Europe. Assuming no convergent evolution, this suggests a closer epidemiological link with the mainland. The fact that each recent Sardinian group is more closely related to mainland viruses than it is to the other Sardinian groups again support the view that these groups are probably the result of independent introductions. The virus WB/I/n6W/92, a 1992 isolate from the Italian province of Massa, and the Sassari viruses, isolated in 1995 and 1996, are very similar, differing by a single nucleotide in this region. Likewise, the Polish and the F/Lorraine/93 virus isolated in France in 1993 differ from the 1994 Cagliari isolate by only two nucleotides. The close similarity between these viruses strongly supports the independent introduction theory. The recent Nuoro viruses seem more distinct but probably appear so as a result of the limited number of isolates examined rather than the presence of a divergent virus group.

This study has wider implications for the control of CSFV. Epidemiological studies provide a vital tool for eradication programmes and the techniques described in this paper have previously been helpful in attributing pathways of virus spread between outbreaks. However, problems are encountered when outbreaks caused by very similar viruses are investigated. In these cases, it is difficult to determine whether variation between the viruses from each outbreak is the result of recent local divergence (i.e. epidemiologically linked) or indicates a separate pathway of introduction (i.e. no epidemiological link). This difficulty has been a feature of phylogenetic analysis of subgroup 2·3 viruses which are responsible for many of the current European outbreaks. Phylogenetic trees based on these data do not always seem to predict the correct evolution. Thus, it can be seen that in Figure 2, some viruses are represented as precursors to isolates with an earlier isolation date! A possible explanation is that the rate of CSFV evolution is too slow, or reversion mutations occur too regularly to make accurate calculations of evolutionary history over periods of less than 5–10 years. Reversion mutation 'noise' can be reduced by sequencing a genetic region less prone to mutation.

Unfortunately, this strategy has been shown to result in lower resolution when closely related CSF viruses are compared [1, 2]. Despite the inability to predict the actual sequence of evolutionary events this technique is clearly capable of determining epidemiological links using a small degree of variation. This paper describes the interpretation of very small differences amongst a series of subgroup 2·3 CSFVs isolated in Sardinia. The results indicate that virus typing based on differences of as few as 5 nucleotides over the 190 bases sequenced (2·7%) can be used to deduce epidemiological information from subgroup 2·3 viruses. Further genetic comparisons of isolates obtained from a larger sequence of outbreaks would help to confirm our hypotheses regarding the speed with which virus variability accumulates.

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