



## **Small Ruminant Lentivirus genotype E is widespread in Sarda Goat**

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1     **Small Ruminant Lentivirus genotype E is widespread in Sarda Goat**

2

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16

## 17    **Abstract**

18    The highly divergent SRLV genotype E has recently been characterized in Italy as  
 19    a low pathogenic caprine lentivirus in the Roccaverano breed. The availability of  
 20    a genotype specific diagnostic test based on a comparative assay, using a  
 21    combination of genotype specific recombinant antigens allows a wide serosurvey  
 22    in other goat populations. The island of Sardinia still has the highest small  
 23    ruminant population of any Italian region and crossbreeding has been limited to  
 24    goats, mainly with the Maltese breed.

25    A serological survey was carried out on sheep flocks and goat herds, using  
 26    individual sera as well as a bulk milk-adapted procedure. Genotype E was  
 27    identified in more than 50% of goat herds and none of the sheep flocks thus  
 28    supporting the idea that this genotype is specifically associated with the goat  
 29    species. The full length proviral sequence of a Sardinian isolate revealed and  
 30    confirmed the deletion of dUTPase subunit and the absence of both *vpr* gene and  
 31    the 71 bp repeat of the LTR. Genetic similarity of this isolate with the prototype  
 32    strain Roccaverano was no more than 84%, supporting the designation of two  
 33    subtypes within genotype E. Nevertheless, in vitro properties of the Sardinian  
 34    strain were different from those of the Roccaverano strain in terms of ability to  
 35    infect synovial membrane and produce syncytia. Remarkable differences in the  
 36    HV1 and HV2 of the *env* gene were recorded, with the Sardinian isolate  
 37    displaying sequence motif more similar to arthritic strains. Data presented suggest  
 38    diffusion of genotype E is wider than previously thought.

39

40 Keywords: **small ruminant lentivirus / genotype e / pathogenic subtype / sarda**

41 **goat.**

42

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## 43    **Introduction**

44    Small ruminant lentiviruses (SRLV) are a group of viruses displaying different  
 45    genetic, antigenic and biological properties in their natural hosts. These viruses  
 46    cause slow progressive multi systemic diseases involving joints, mammary  
 47    glands, brain and lungs. Beside the Maedi Visna Virus (MVV) and Caprine  
 48    Arthritis Encephalitis Virus (CAEV), prototypes of genotype A and B  
 49    respectively, additional genotypes C, D and E have been described (Reina *et al.*,  
 50    2009a; Shah *et al.*, 2004). The latter has so far been identified in the Roccaverano  
 51    goat, an endangered Italian breed. Full length genome analysis of the prototype  
 52    strain *Roccaverano* revealed unusual genetic organization with natural deletions  
 53    of the dUTPase subunit of the *pol* gene and the absence of *vpr* gene (previously  
 54    characterized as *tat* gene) (Reina *et al.*, 2009a). We proposed the designation of  
 55    low pathogenic caprine lentivirus to characterize this viral cluster for two main  
 56    reasons: i) reduced viral load and disease progression have been observed using  
 57    CAEV molecular clones artificially deleted for the same gene or gene subunit; ii)  
 58    the arthritic clinical index in a Roccaverano flock infected with genotype B is  
 59    significantly higher than that found in a flock infected with genotype E (personal  
 60    observations). A recent study has indicated that, due to antigenic diversity of *gag*  
 61    encoded proteins among genotypes A, B or E, distribution of E-like infection in  
 62    other small ruminant population would require a specific antigen design. To  
 63    address this problem a comparative assay was proposed using the recombinant  
 64    P16 (matrix) and P25 (capsid antigen) fusion protein from both B and E  
 65    genotypes. This test was able to selectively detect genotype E infected animals,

66 based on different reactivity against homologous antigen (Reina *et al.*, 2009b).  
 67 Since only few infected flocks have been recorded to date in the Piedmont region  
 68 (North-West Italy), it is difficult both to speculate on the distribution of genotype  
 69 E infection in other countries and to assess if genotype E might be present in other  
 70 goat populations displaying different biological behaviour (i.e. virulence). In Italy,  
 71 several local goat populations have been subjected to unplanned crossbreeding  
 72 with imported breeds to increase milk production. The introduction of B1 subtype  
 73 (CAEV-like strains), especially from France, is commonly believed to have  
 74 occurred through importation of French Alpine and Saanen breeds in the early  
 75 eighties (Grego *et al.*, 2007). Pathogenic strains such as those belonging to  
 76 subtype B1, tend to spread horizontally among adult animals. For this reason,  
 77 even if more than 50 goat breeds are currently farmed, B1 strains appeared to be  
 78 widespread in many regions. Local breeds with limited crossbreeding or  
 79 introduction represent a good starting point to investigate the presence of  
 80 genotype E. The Sarda goat lives only in Sardinia and represents more than 20%  
 81 of the Italian goat population (about 300,000 heads). Crossbreeding has mainly  
 82 occurred in the past with the Maltese breed, while introduction of B1 infected  
 83 goats has been limited (Ajmone-Marsan *et al.*, 2001; Sechi *et al.*, 2007). In the  
 84 present study, a large number of small ruminant flocks were tested using genotype  
 85 E and B comparative ELISA assay. While sheep flocks were negative to genotype  
 86 E, surprisingly, more than 50% of the goat herds resulted positive, suggesting that  
 87 genotype E infection is widespread in the Sarda goat. Genome analysis of a  
 88 Sardinian viral isolate revealed a similar genome organization within genotype

89 and moderate pathogenic behaviour in vitro. Different viral evolutionary strategies  
90 in the two different sized populations and potential genotype E reservoir in other  
91 countries are also discussed.

92

## 93 **Materials and Methods**

### 94 *Blood (serum and DNA) and milk samples*

95 Caprine herds or ovine flocks were selected randomly among the Sardinian  
96 population, involving the most populated areas on the island.  
97 Individual whole blood from 20 ovine flocks and from 30 caprine herds were  
98 initially collected and serum was stored at -20°C until ELISA testing. Buffy coats  
99 and milk were obtained from 21 samples belonging to three of the caprine herds  
100 and DNA was extracted using DNA blood minikit (Qiagen). Following this  
101 preliminary serological survey, 186 bulk milk samples were collected from  
102 additional caprine or mixed herds and subjected to milk-adapted ELISA (Fig. 1).  
103 For both sera and bulk milk, appropriate positive and negative controls were  
104 included in each test, including samples from three caprine herds characterized in  
105 a previous study and known to be infected with genotype B, genotype E and both  
106 genotypes (Reina *et al.*, 2009b).

107

### 108 *ELISA comparative assays*

109 A previously described ELISA test was used to serotype samples, consisting of  
110 P16-25 recombinant antigen derived from genotypes B (strain IT-Pi1) and E  
111 (strain Roccaverano) (Reina *et al.*, 2009a). Briefly, ELISA microplates



112 (Immunomaxi TPP) were coated with 100 ng of each recombinant antigen and  
 113 water as negative control. After drying and blocking steps, serum samples were  
 114 applied at 1/20 dilution and plates incubated at 37° for 1h. Following the washing  
 115 step, peroxidase labelled Mab anti-sheep/goat IgG was applied and plates  
 116 incubated as above. After the final wash, development was carried out using  
 117 ABTS and plates were read at 405 nm. Net absorbances were obtained by  
 118 subtracting the absorbance of negative antigen from the absorbance of each  
 119 recombinant antigen. Cut-off value was defined as percentage of reactivity  $\geq 20\%$   
 120 of the absorbance of positive control included in each plate.  
 121 Bulk milk samples were tested using the same P16-25 ELISA and a previously  
 122 described subunit ELISA (sub-ELISA) (Reina *et al.*, 2009b) in which microplates  
 123 were coated with 200 ng/well of the immunodominant epitope of capsid antigen  
 124 derived from genotype B (sequence KLNEEAERWRRNNPPPP) and E (sequence  
 125 KLNKEAETWMRQNPQPP). Since both peptides were expressed as GST fusion  
 126 protein, an equimolar amount of GST was used as negative control. Net  
 127 absorbances were obtained by subtracting the absorbance against GST antigen  
 128 from that of each recombinant subunit. Milk samples were used at 1/2 dilution in  
 129 both assays and procedures were carried out as above. For P16-25 ELISA, a  
 130 standard curve was generated using two fold dilutions of bulk milk sample into  
 131 negative bulk milk, the former obtained from a caprine herd with known  
 132 seroprevalence and known to be infected only with E genotype. Cut off was  
 133 defined as the absorbance level of the dilution corresponding to 20% prevalence  
 134 and included in each plate.

135

136 *PCR, sequencing and phylogenetic analysis*

137 DNA was extracted from individual blood and milk samples and used to amplify a  
 138 partial region of the *gag* gene (Grego *et al.*, 2007). Briefly, DNA was analyzed by  
 139 a nested PCR designed to amplify a 1.3 kb fragment in the first round and a 0.8 kb  
 140 fragment in the second one. The result of the nested amplification was sequenced  
 141 directly using an ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Monza,  
 142 Italy). Nucleotide sequences were aligned using Clustal X algorithm, in respect of  
 143 the amino acidic coding frame and were compared to SRLV homologous  
 144 sequences available on GenBank.

145 Genetic similarity was expressed as nucleotide and aminoacid diversity (Nei,  
 146 1987), or mean proportion of differences among sequences. Taken into account  
 147 the peculiar genomic organization of isolates within genotype E, the amount of G  
 148 to A transitions was analyzed to investigate the possible role of genome deletions  
 149 on the viral mutation rate. The evaluation of the amount of G-to-A substitutions  
 150 was carried out using hand made functions in R computer software (R  
 151 Development Core Team 2007), available upon request.

152 Selective pressure was evaluated calculating the ratio ( $\omega$ ) of non synonymous  
 153 substitutions per non synonymous sites ( $d_N$ ) and the number of synonymous  
 154 substitutions per synonymous sites ( $d_S$ ); evaluation of selective pressure was  
 155 performed considering the overall number of substitutions and analyzing  
 156 mutations at site-specific level (SNAP [www.hiv.lanl.gov](http://www.hiv.lanl.gov)).

157 In order to describe the phylogenetic relationships among new and SRLV  
158 reference sequences, we created a dataset including samples belonging to the  
159 genotype E published previously (Grego *et al.*, 2007) and sequences from A, B  
160 and C genotypes as outgroups. The phylogenetic tree was created evaluating the  
161 best model of molecular evolution (ModelTest software, (Posada and Crandall,  
162 1998)) and using Bayesian heuristic approaches (MrBayes software, (Ronquist  
163 and Huelsenbeck, 2003)).

164

#### 165 *Virus isolation and genome sequencing*

166 An uncharacterized viral isolate, which had been previously obtained by co-  
167 culture of the buffy coat with a primary culture of choroid plexus cells, was traced  
168 back in our laboratory as a frozen supernatant. It had been isolated in a Sardinian  
169 caprine herd, found reactive against genotype E antigen in the present study, from  
170 an adult animal suffering unspecific arthritis. DNA extracted from the buffy coat  
171 of the same animal was also available. Viral isolate (hereafter named *Seui*) was  
172 cultured on caprine foetal synovial membrane (CFSM) and analyzed for syncytia  
173 formation, immunocytochemistry, RT activity (Cavidi) and PCR. DNA from  
174 infected cells was used to obtain the complete genome sequence of the *Seui*  
175 isolate using primers described in Table 1. Rev transcripts analysis was carried  
176 out by RT-PCR with primers already described (Gjerset *et al.*, 2006).

177

#### 178 *Nucleotide accession numbers*

179 Nucleotide sequences of partial *gag* fragments and the complete genome of strain  
 180 *Seui* were submitted to the GenBank database and given accession numbers  
 181 GQ428519-36 and GQ381130 respectively.

182

## 183 **Results**

### 184 *P16-25 ELISA and sub-ELISA*

185 Serological test was conducted on a total of 504 animals from 19 goat herds  
 186 (n=309) and 19 sheep flocks (n=195) and only goats showed the presence of  
 187 genotype E infection in Sardinia, reaching absorbance values comparable to those  
 188 of the positive controls used. Serum P16-25 ELISA was able to serotype the  
 189 infection and although some animals reacted against both antigens (genotype B  
 190 and E), most reacted in a type specific manner against genotype E antigen (Fig  
 191 2A).

192 Following this preliminary screening, we used bulk milk from a total of 186 goat  
 193 herds to estimate the real prevalence of genotype E within the Sarda goat  
 194 population. Based on milk adapted P16-25 ELISA, serotyping was not always  
 195 possible due to highly reactive samples, which reached a saturation level against  
 196 both B and E derived antigens. Although titration of highly reactive samples may  
 197 has overcome this drawback, subunit-ELISA was able to serotype most milk  
 198 samples but sensitivity was obviously lower than that obtained by P16-25 ELISA.

199 When both methods were merged, the estimation of 74% of SRLV  
 200 seroprevalence was found in the Sardinian goat herds. Among these, 19.41% were  
 201 infected with genotype B, 44.12% with genotype E and 10.59% were infected by

both genotypes or not characterised. Finally, 18.24% of the flocks were found negative by both assays. Results clearly indicate that genotype E is widely distributed on the island of Sardinia, reaching a prevalence twice the levels found for genotype B.

206

#### 207 *PCR sequencing and phylogenetic analysis*

A total of 18 partial sequences (0.8 Kb) of the *gag* gene were obtained and analyzed. The mean nucleotide diversity among Sardinian samples was 9.915% (standard error of the mean 1.105%). Analyses on G to A transitions showed that the amount of this specific mutation was 24.75% (standard error of the mean = 0.60%) of the total number of substitutions and it is similar to that of Roccaverano cluster in Piedmont (Reina *et al.*, 2009a). The evaluation of selective pressure showed the presence of purifying selection ( $\omega = 0.032$ ).

Phylogenetic relationships among new Sardinian samples, sequences from Piedmont belonging to genotype E and reference sequences are described in the phylogenetic tree reported in Figure 3. Tree topology clearly indicates the divergence between Roccaverano and Sardinian clusters.

219

#### 220 *Complete genome sequencing and in vitro properties*

Sardinian genotype E (strain Seui) was able to infect synovial membrane as assessed by the presence of characteristic CPE, immunocytochemistry, RT activity and PCR.

224 DNA extracted from infected CFMS with *Seui* strain was used to amplify the  
 225 complete proviral genome in six steps (LTR, LTR-*gag*, *gag*, *gag-pol*, *pol*, and  
 226 *env*). Rev transcripts were successfully generated by RT-PCR. Since the complete  
 227 sequence was obtained by overlapping PCR fragments, it may not reflect the  
 228 sequence of a single provirus. However *env* sequences obtained from PBMC,  
 229 coculture and milk from the isolation's animal presented a divergence less than  
 230 1%. Furthermore, *Rev* sequences presented a divergence of 0.14% compared with  
 231 *env* sequence obtained from DNA indicating that the provirus sequence is  
 232 representative of a replication competent virus.

233 The mean nucleotide diversity between *Seui* and Roccaverano strain was 14.643%  
 234 (SEM: 1.104%). This result supports the definition of two different subtypes  
 235 within the genotype E, according to the previously proposed criteria (Shah *et al.*,  
 236 2004).

237 Proviral sequence revealed that the hallmarks of genotype E were confirmed in  
 238 the Sardinian isolate. Residual dUTPase subunit presented additional four amino  
 239 acids respect to Roccaverano strain. Differences were also observed in the hyper  
 240 variable regions of *env* gene (HV1 and HV2), the Sardinian isolate displaying  
 241 sequence motifs more similar to arthritic strains (Table 2). Long terminal repeats  
 242 included all the described enhancer elements already present in the Roccaverano  
 243 strain, except for the AP-4 site tandem repeat, a common feature of CAEV  
 244 isolates.

245

## 246 Discussion

247 As hypothesised in the previous study, the lack of a specific serological tool  
 248 allowed no speculation as to the distribution of genotype E in geographical  
 249 locations different from the one where it was initially described (Reina *et al.*,  
 250 2009b). Serological data from different goat herds, sequence analysis of specific  
 251 PCR products from three infected flocks and the full length proviral genome  
 252 sequence of a local strain demonstrate that genotype E infection is associated and  
 253 widely distributed in the Sarda goat, while Sarda sheep seems to harbour a  
 254 genotype B (CAEV-like) lentivirus, a common feature in Italian sheep population  
 255 (Grego *et al.*, 2002).  
 256 Sarda goat, unlike the Roccaverano breed, represents an important goat population  
 257 with economic significance at a local and national level. Moreover, since the  
 258 population size of the Sarda goat is not comparable to the Roccaverano breed, in  
 259 terms of average number of head per flock, farming system, management and  
 260 productive levels, the biological significance of genotype E as low pathogenic  
 261 caprine lentivirus needs to be redefined. The tree topology indicates a clear  
 262 divergence between Roccaverano and *Seui* strains, showing quite different clade  
 263 structures and features. These differences in the evolutionary pathway can be  
 264 justified by epidemiological and historical data. In fact, Roccaverano goats were  
 265 at risk of extinction in the early sixties, when people abandoned rural areas in  
 266 favour to towns, following industrial development. This social behaviour forced  
 267 the goat breed to pass through a bottleneck, and drove viral evolution to take  
 268 advantage, on one hand of reducing virulence and on the other hand of persisting  
 269 in a small population, limiting the transmission to lactogenic route. In Sardinia,

270 viral evolution might have displayed a different behaviour, increasing or  
 271 maintaining a certain degree of both virulence and horizontal transmission. To  
 272 date it is difficult to speculate about the pathogenic role of genotype E for several  
 273 reasons. First of all, the genomic organization of the Sardinian isolate is similar to  
 274 the Roccaverano strain, lacking both dUTPase subunit and *vpr* gene. In other  
 275 SRLV models, dUTPase and *vpr* were specifically associated with an increased  
 276 viral load, tissue distribution and lesion severity, compared to the deleted  
 277 counterpart (Harmache *et al.*, 1998). In addition, the presence of other pathogens,  
 278 such as *Mycoplasma* spp, *Fusobacterium necrophorum*, *Bacteroides nodosus*,  
 279 which had consistently been reported in the Sarda goat, could lead to lentivirus-  
 280 induced overlapping clinical signs. Finally, the viral isolate used in this study had  
 281 originally been obtained from co-cultivation of peripheral blood mononuclear  
 282 cells with choroid plexus or synovial membrane cells, while viral isolates from  
 283 direct explantation of synovial membrane of arthritic goats are still unavailable for  
 284 genotype E. In vitro study, however, seems to attribute to the Seui strain a certain  
 285 degree of cytopathogenicity at least in terms of ability to infect synovial  
 286 membrane and syncytia formation, while replication of the Roccaverano isolate  
 287 in the same cell system is greatly reduced (personal observation; manuscript in  
 288 preparation). Cell tropism has been attributed to sequence variation in U3 region  
 289 of LTR, related to specific transcription factor binding sites, as well as variation in  
 290 the hyper variable (HV) regions of the *env* gene. We first analysed the structure of  
 291 viral enhancer elements and significant similarity was found between the two  
 292 strains, except for a genuine AP4 tandem repeat which is present in *Seui* as well as



293 several CAEV isolates, while a point mutation is present in one of the two  
 294 repetitions in the Roccaverano strain. In the *env* gene, amino acid motif in the  
 295 HV1 and HV2 regions were clearly different between strains, the Seui being more  
 296 similar to arthritic isolates. It should be noted that compartmentalization studies of  
 297 viral quasispecies revealed that different motifs in the HV1-2 regions of CAEV  
 298 are a normal finding in the same animal (Hotzel *et al.*, 2002) and arthritic related  
 299 sequences might have resulted from an in vitro adaptation of *Seui* isolate to  
 300 synovial membrane cells. For this reason we sequenced a PCR fragment  
 301 encompassing the HV region from PBMC of the same animal from which the  
 302 *Seui* strain was isolated and identical amino acid sequence was obtained.  
 303 Therefore, we suppose that higher, if any, in vivo virulence of the *Seui* strain  
 304 could be attributable to different cell tropism related to U3 and/or HV sequences.  
 305 Sequence analysis of the Sardinian strain *Seui* revealed 84% similarity with the  
 306 Roccaverano strain supporting the definition of the genotype E and, possibly two  
 307 subtypes, following the criteria recommended in the HIV field, where at least two  
 308 epidemiologically unlinked isolates should be sequenced in their entirety  
 309 (Robertson *et al.*, 2000). Divergence between genomes was not clearly  
 310 attributable to specific gene or gene fragment. Interestingly, a certain degree of  
 311 variability was found in the *pol* gene corresponding to residual dUTPase subunits.  
 312 This seems to confirm that dUTPase was lost during evolution and residual  
 313 sequence is not subjected to functional constrain except for frame conservation  
 314 and spacer function between RNaseH and Integrase subunits. On the contrary, the

entire ORF of *vpr* was absent in both strains and it is difficult to speculate if *vpr* gene has ever been present in genotype E.

If we assume that the Sarda goat is an ancient breed which came to Sardinia during the Mediterranean colonization of navigators from Middle East and, to the best of our knowledge, there has been limited introduction of improved breeds, we may also assume that SRLV genotype E has strictly been associated with the Sarda goat population, representing an excellent model to study a long lasting host-pathogen interaction and co-evolution. Moreover, phylogeographical partitioning of goat breeds suggests that the Sarda goat belongs to a West Mediterranean cluster, including French (Corse, Rove, Pyreneene) and Spanish (Brava, Verata, Payoya, Florida, Malagueña, Guadarrama) breeds (Canon *et al.*, 2006). Since serological tools adapted to bulk milk in this study proved to be very sensitive and dependable for the detection of the genotype E, a wider serological survey including these populations would be essential, in order to identify additional infection foci and evaluate more accurately the biological significance and impact of genotype E in SRLV control programs.

331

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335

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400

**Figure 1.** Map of the Sardinia island, divided in municipalities. A) goat herds density. Grey level indicates goat herd density within the municipality: white = less than 1<sup>st</sup> quartile (3 herds); light grey = between 1<sup>st</sup> and 2<sup>nd</sup> quartile (11 herds); grey = between 2<sup>nd</sup> and 3<sup>rd</sup> quartile (15 herds); dark grey = more than the 3<sup>rd</sup> quartile. B) Municipalities including flocks tested with bulk milk analysis (grey). Circles = E positive flocks; squares = B positive flocks; triangles = coinfectd (or uncharacterised) flocks.

**Figure 2.** Net absorbance against E (x axis) and B (y axis) antigens. Dashed diagonal line represents equal reactivity versus both antigens. Vertical and horizontal dotted lines represent ELISA E and B cut-offs respectively.

A) Data from 19 ovine flocks and from 19 caprine herds. White circles: median absorbance of samples belonging to goat herds. White triangles: median absorbance of samples belonging to sheep flocks. Black squares: reactivity detected in herds previously characterized (Reina *et al.*, 2009b) infected with B (a), E (c) or both strains (b). Vertical and horizontal bars represent the variation (interquartile range) in the distribution of absorbances within flock against B and E antigens respectively.

B) Data from 186 goat herds tested using bulk milk sub-ELISA. Dots represent tested flocks.

**Figure 3.** Phylogenetic tree constructed by Bayesian analysis of 33 partial *gag* gene sequences (consensus alignment length: 525 bp). New sequences are

424 reported in bold. Genbank accession numbers are reported within brackets.

425 Posterior probabilities of clades are indicated above branches.

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**Table 1.** Nucleotide sequence of primer pairs.

Amplicon	Length (Kb)	Primer forward (5'→3')	Primer reverse (3'→5')
LTR	0.3	TGACACAGCAAATGTAACCGCAAG	CCACGTTGGGCGCCAGCTGCGAGA
LTR-GAG	0.8	TGACACAGCAAATGTAACCGCAAG	CCCTGGGGGCTGTGGATTCTG
GAG	1.3	TGGTGARKCTAGMTAGAGACATGG	CATAGGRGGHGCAGCGGCASCA
GAG-POL	2.6	AACCGGGTCATCTAGCAAGAC	CTATCCAGAGAATTTGCACGTCTTG
POL	0.8	GGTGCCTGGACATAAAGGGATTG	GCCACTCTCCTGRATGTCCTCT
ENV	3.0	ATGGACAAGAAGGACGGG	GTGGTTACATTTGCTATGTC
REV	0.5	TGCGGTCCTCGCAGGTGGC	TGAGGCGATCTCCACTCCATC

2

**Table 2.** Comparison of HV1 and HV2 *env* protein aminoacidic motifs between

Seui strain, different SRLV genotypes and the Roccaverano strain. Dots indicate

identity. HV1 and HV2 regions are highlighted in grey (Hotzel *et al.*, 2002).

Strain	Genotype	Ref	HV1	HV2
Seui strain	E2	This work	I-GNNTVIGNCSAQK	GHWTCKPRTKEGKTDSLVI-GGKK
PBMC colture	E2	This work	.....	.....
SM colture	E2	This work	.....	.....
CAEV Cork	B1	(Saltarelli <i>et al.</i> , 1990)	V...G.IT...TTN	NK...A..QRD...A....
CAEV-63	B1	(Hotzel <i>et al.</i> , 2002)	VDR.Q.IT...VTN	NK...A..QR...A....
Ov496	B2	(Glaria <i>et al.</i> , 2009)	V...G.IT...VTN	NK...A..WRG.MS...A...Q
K1514	A	(Staskus <i>et al.</i> 1991)	V...G.IT...VTN	NK...AA.R.GSRR...A.-RD
1GA	C	(Gjerset <i>et al.</i> , 2006)	I...S.LQ.Q.NRSN	R.YVN-----D
Roccaverano	E1	(Reina <i>et al.</i> , 2009a)	L.DAQG.....KEN	NQ.....QRGNR...V..GA-RR

6

Figure1

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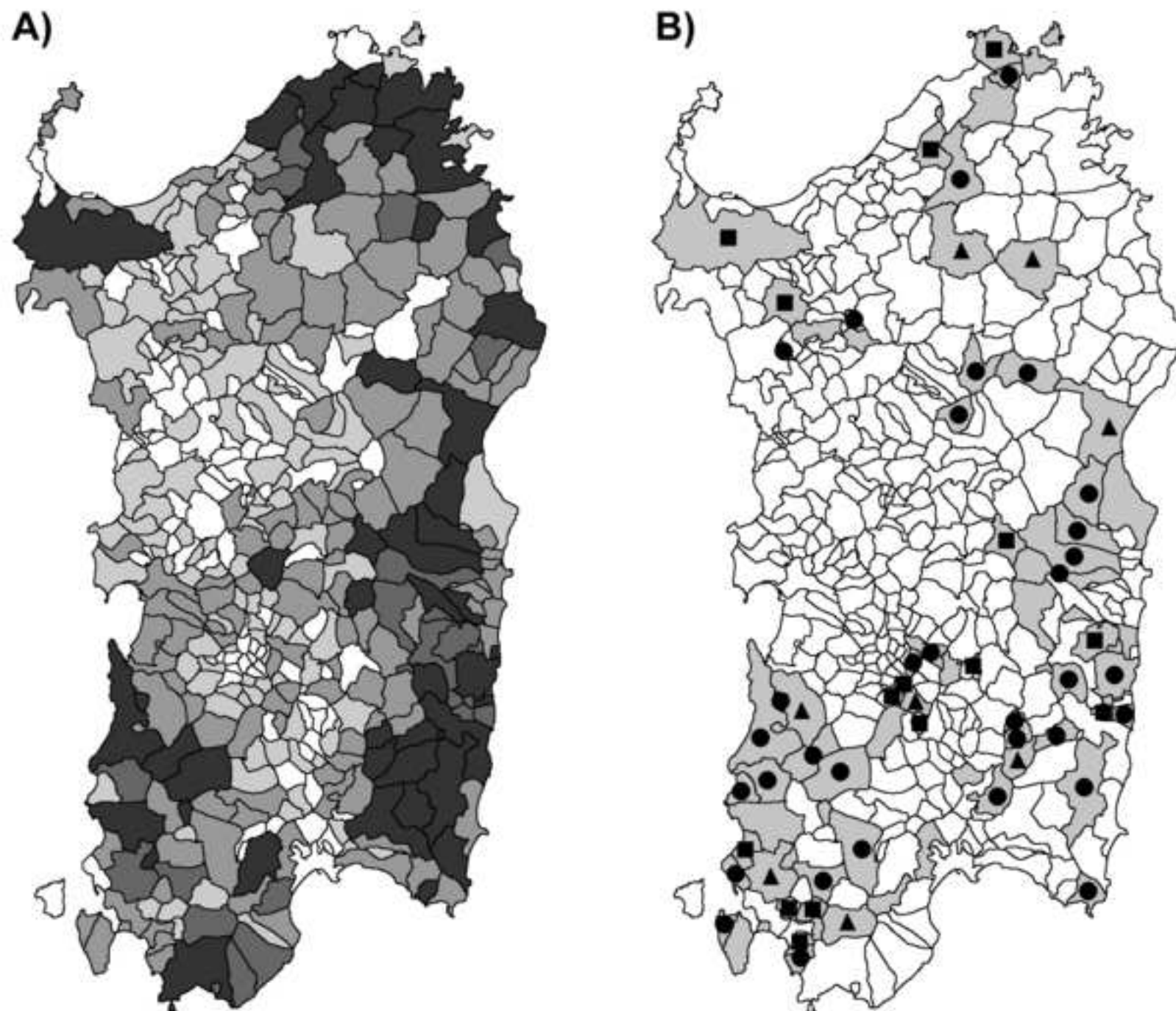




Figure2

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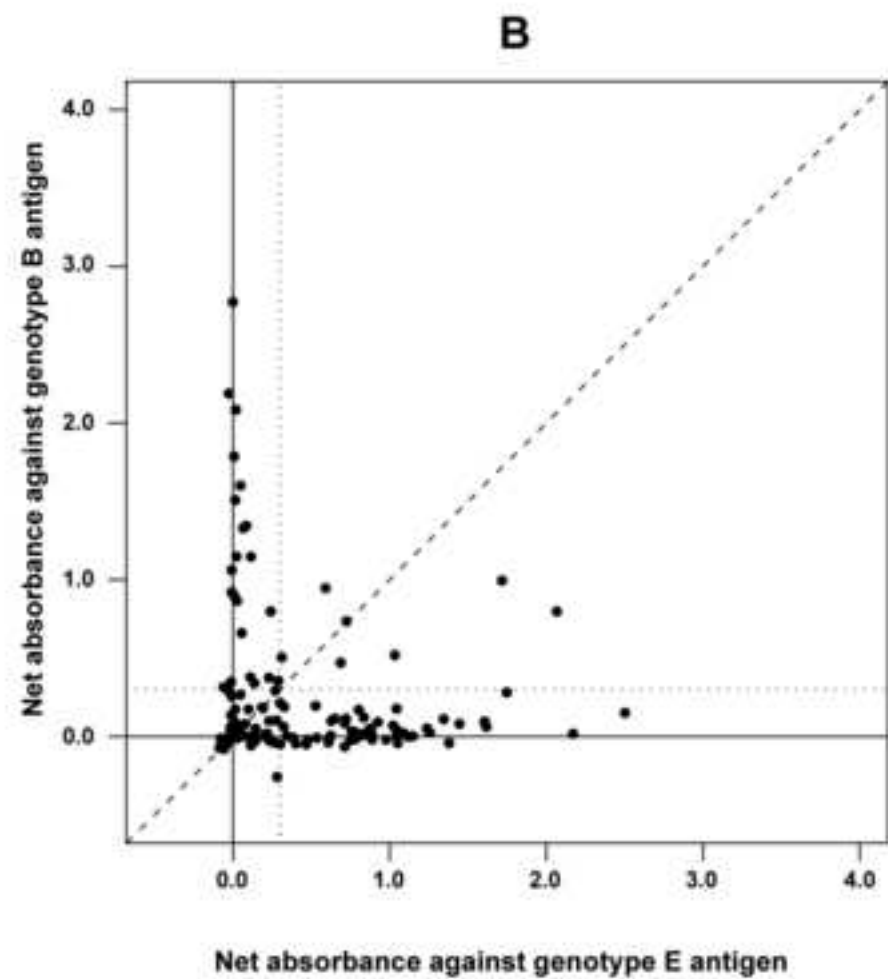
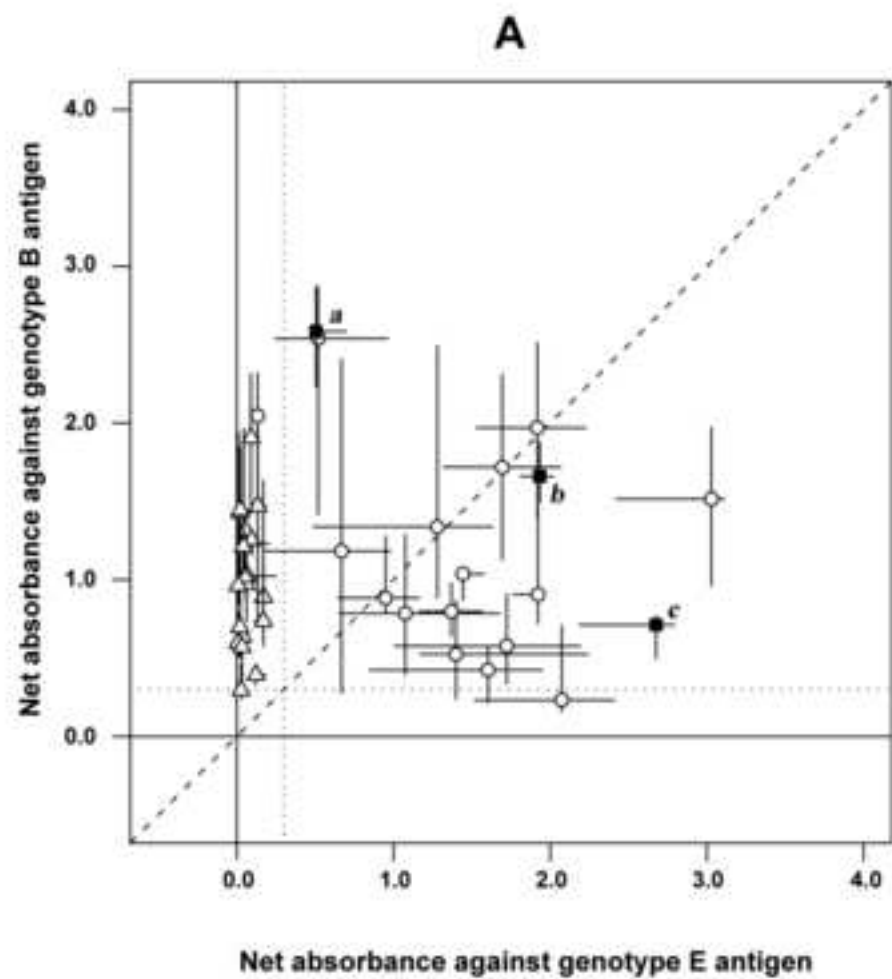


Figure 3  
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