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1 **Phylogenetic analysis of SRLV sequences from an arthritic**
2 **sheep outbreak demonstrates the introduction of CAEV-**
3 **like viruses among Spanish sheep**

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18

19

20 **Abstract**

21 Small ruminant lentiviruses (SRLV) cause different clinical forms of disease in sheep and
22 goats. So far in Spain, Maedi visna virus-like (MVV-like) sequences have been found in both
23 species, and the arthritic SRLV disease has never been found in sheep until a recent outbreak.
24 Knowing that arthritis is common in goats, it was of interest to determine if the genetic type
25 of the virus involved in the sheep arthritis outbreak was caprine arthritis encephalitis virus-
26 like (CAEV-like) rather than MVV-like. Alignment and phylogenetic analyses on nucleotide
27 and deduced amino acid sequences from SRLV of this outbreak, allowed a B2 genetic
28 subgroup assignment of these SRLV, compatible with a correspondence between the virus
29 genetic type and the disease form. Furthermore, an isolate was obtained from the arthritic
30 outbreak, its full genome was CAEV-like but the pol integrase region was MVV-like.
31 Although its LTR lacked a U3 repeat sequence and had a deletion in the R region, which has
32 been proposed to reduce viral replication rate, its phenotype in sheep skin fibroblast cultures
33 was rapid/high, thus it appeared to have adapted to sheep cells. This outbreak study represents
34 the first report on CAEV-like genetic findings and complete genome analysis among Spanish
35 small ruminants.

36

37 **Keywords:** small ruminant lentiviruses, ovine, caprine, arthritis, outbreak

38

39

40

41 **1. Introduction**

42 Spanish SRLV partial genetic sequences have been described recently (Grego et al., 2002;
43 Reina et al., 2006), even though SRLV disease had been detected in Spanish sheep long ago,
44 mainly causing pneumonia and mastitis in sheep and arthritis in goats. Recently, a disease
45 characterized by carpal joint synovitis, together with interstitial mastitis and/or pneumonia has
46 been detected among sheep from a farm in the Aragonese region of Spain (Biescas, 2006).

47 In this study, SRLV sequences from a unique Spanish SRLV ovine arthritis outbreak were
48 obtained and analyzed genetically and a complete viral genome sequenced, revealing a
49 phylogenetic proximity to CAEV-like prototypes B2. The results suggest a link between viral
50 genetic type and disease form, independently of the in vitro phenotype of the isolate.

51

52 **2. Material and Methods**

53 **2.1 Animals and samples**

54 SRLV-seropositive (Elitest, Hyphen Biomed) arthritic adult sheep of the Rasa Aragonesa
55 breed were used. Animals (Nos. 44, 84, 258, 449, 496 and 497) belonged to flocks with high
56 infection prevalence. EDTA-blood mononuclear cells (PBMC) and bronchoalveolar lavage
57 (BAL) were obtained. Tissues from mammary gland, brain, lung and articular joint were
58 collected and embedded in RNAlater (Qiagen).

59

60 **2.2 Virus isolation and phenotyping**

61 Synovial fluid (SF) from both carpal joints (left and right) of the arthritic sheep No. 496 was
62 incubated with sheep choroid plexus (SCP) and foetal ovine synovial membrane (FOS) cells.
63 Culture supernatants were used to further infect skin fibroblast cells (SFC) and then titrate the
64 virus (isolate 496) according to RT-activity of supernatants (Reverse Transcriptase Assay

65 chemiluminescent, Roche). The correlation between known (EV1 strain) TCID₅₀ and RT
66 activity was determined. The phenotype of isolate 496 was identified as low, medium or high
67 (titres of less than 10⁶, from 10⁶ to 10⁷ and higher than 10⁷, respectively); and as rapid,
68 intermediate or slow (when syncytia appeared within a week, a few weeks or over a month,
69 respectively). RT activity was determined at days 0, 1, 2, 3, 5, 6, 7, 8, 9, 21 and 42 post-
70 inoculation (p.i.).

71

72 **2.3. PCR procedures**

73 Genomic DNA was extracted from PBMC, tissue samples and infected SFC with QIAamp®
74 DNA Blood Mini Kit (QIAGEN). PCRs were performed using Pfu DNA polymerase
75 (Biotools) and 1 µg DNA. Partial amplification of LTR (300 nt) and *gag* (490 nt) regions
76 from outbreak sequences was performed with previously described primers (Reina et al.,
77 2006); *pol* (1.2 kb) and *gag-pol* (1.8 kb) PCR were done as previously described (Shah et al.,
78 2004). Table 1 shows the set of primers used for complete sequencing of the arthritic isolate
79 (Table 1). RNA from FOS, GSM (goat synovial membrane) cells and SFC infected *in vitro*
80 with isolate 496 was extracted with RNeasy® Mini Kit (QIAGEN) for *rev* transcripts.
81 Reverse transcription was done with SuperScript™II (Invitrogen) using oligo-dT.
82 Amplification of *rev*-encoding regions was performed using adapted primers originally
83 described by Gjerset et al. (2006).

84

85 **2.4. Cloning, sequencing, alignment and phylogenetic analysis**

86 Amplicons were cloned by triplicate into pGEMT-easy® vector (Promega) and then
87 sequenced using BigDye® Terminator v3.1 chemistry on a 3730 DNA Analyzer (Applied-
88 Biosystems). Multiple alignments were made with ClustalX and PHYLIP (Phylogeny
89 Inference Package version 3.5c) programs and trees produced by the Neighbour Joining

90 method with Kimura's correction, using 1000 bootstrap confidence limits. Results with over
91 500 bootstraps were considered highly likely.

92 GenBank MVV and CAEV prototype sequences were included: EV1 (S51392), SAOMVV
93 (M31646), KV1514 (M10608), P1OLV (AF479638), 85/34 USA (AY101611), Swiss Goat
94 Isolate (AY445885), CAEVCo (M33677), CAEV Gansu (AY900630) and CAEV1GA
95 (AF322109). The phylogenetic tree on the 1.2 kb *pol* segment included previously described
96 Spanish sequences (Reina et al., 2006), one from sheep (Ov1) and two from goats (C1 and
97 C3). This tree and the tree based on the 1.8 Kb *gag-pol* segment include sequences with a
98 four-digit numeric assignment (Shah et al., 2004). The *gag-pol* tree also includes [B2]
99 sequences from Leroux et al. (1995) and Grego et al. (2002) (GenBank accession numbers:
100 U35808, U35679 and U35680, and AY265456, respectively).

101 Partial sequences from the six animals under study and the whole genome sequence of isolate
102 496 were given GenBank accession numbers: FJ187802-FJ187812 for *gag* amplicons;
103 FJ187813-FJ187820 for LTR amplicons; and FJ195346 for complete genome.

104

105 **3. Results**

106 **3.1. Sequence similarity and phylogenetic analysis**

107 Sequences from short LTR (300 nt) and *gag* (490 nt) amplicons (Reina et al., 2006) from the
108 tissues of the arthritic sheep involved in the study (brain was not found infected) were CAEV-
109 like and highly similar to each other. An isolate, 496, was obtained from sheep No. 496 and
110 its genome (9136 nt) fully sequenced, being CAEV-like according to sequence similarity
111 analysis (Table 2) and belonging to the B2 genetic sub-type, using current phylogenetic
112 classification (Fig. 1).

113

114 **3.2. Biological properties of the isolate**

115 Productive infection (RT-positive supernatants and presence of CPE) was first observed in
116 FOS and SCP cells after 3 days p.i. with SF from carpal joint of animal No. 496. Supernatants
117 of these cultures were used to infect and titrate the virus (496) in SFC cultures. These cells
118 formed syncytia and yielded RT-positive supernatants within 2-4 days p.i. (Fig. 2). The virus
119 titre reached a value close to 10^8 TCID₅₀/ml at 5 to 9 days p.i. Thus, the phenotype of isolate
120 496 was rapid in FOS and SCP cells, and rapid/high in SFC.

121

122 **3.3. Comparative analysis of the genetic regions and proteins of the isolate**

123 ***LTR region***

124 The isolate lacked a LTR repeat present in some MVV-like isolates (37-53 nt) or CAEV-like
125 isolates (70 nt) (Fig. 3). Although the AP-4 site and the TATA-box were conserved, the AP-1
126 site distribution was similar to that of CAEVCo. However, distal to TATA box, 496 had one
127 AP-1 site, whereas CAEVCo had two sites (one of them corresponding to the repeat), and the
128 MVV-like sequence KV1514 had 4 sites (two belonging to the repeat). Moreover, 496 LTR
129 presented only one highly conserved AML (vis) site, lacking the AML sequence close to the
130 TATA box present in MVV-like sequences. The distribution of CAAAT, GAS and TAS sites
131 was similar to that of CAEVCo, except that 496 lacked the 70 nt repeat that also contains
132 these sites. In addition, the R region of 496 presented a deletion, similar to that found in
133 CAEVCo, other highly pathogenic strains such as SAOMVV and some strains from sub-
134 clinically infected sheep (Angelopoulou et al., 2008) (Fig. 3).

135

136 ***Gag region***

137 The 496 gag-p17 sequence presented two conserved motifs within the immunodominant
138 epitope and a seven amino acid deletion (Grego et al., 2005; Pisoni et al., 2005) found in other
139 CAEV-like and not in MVV-like sequences. In the 496 gag-p25 protein, two major epitopes

140 and a major homology region were quite conserved (Fluri et al., 2006; Rosati et al., 1999). In
141 the gag-p14 protein, a CAEV-like one-amino acid deletion at the N-terminal, and a deletion at
142 the carboxy-terminal sequence, absent from CAEVCo, but present in some MVV-like
143 sequences, was observed (not shown).

144

145 ***Pol region***

146 Significantly, a stretch of 17 amino acids in the *pol* integrase DNA binding domain of isolate
147 496 was MVV-like (PGDWEGPTQVLWKGEA), although 496 had a CAEV-like sequence
148 in other *pol* sub-regions (not shown).

149

150 ***Env region***

151 The sequence of the 496 predicted signal peptide was similar in length to other CAEV-like
152 sequences and 9-10 amino acids shorter than MVV-like sequences. The predicted mature 496-
153 SU protein included 22 cysteine residues, conserved among all SRLV, as well as 19 potential
154 N-glycosylation sites. Like in CAEVCo, V1-V4 domains (Mordasini et al., 2006; Valas et al.,
155 2000) were conserved, unlike the variable region of SU5. The TM subunit presented 4
156 potential N-glycosylation sites conserved in all SRLV. One of the immunodominant epitopes,
157 widely used in ELISA diagnosis, was also detected in isolate 496 and had a CAEV-like (HQ)
158 sequence.

159

160 ***Rev, vif and tat regions***

161 Three *rev* transcripts (562 nt, 483 nt and 437 nt) were obtained from SFC and two (562 nt and
162 437 nt) from FOS and GSM cells. Amplicon sequencing revealed the presence of four *rev*
163 exons: 1) in the LTR region; 2) at the 3' end of *pol*; 3) at the 5' end of *env*; and 4) at the 3'
164 end of *env*. The longest amplicon was integrated by exons 1, 3 and 4 and its putative open

165 reading frame (ORF) corresponded to a deduced protein sequence of 134 amino acids,
166 analogous to that described in the CAEV Gansu strain (Gazit et al., 1996). Alignment of this
167 sequence with other SRLV-Rev protein sequences revealed a low similarity with known
168 SRLV isolates (Table 2), although the functional N-terminal, the basic (arginine-rich tract),
169 and the Nuclear Export Signal domains (Abelson and Schoborg, 2003) were present in the
170 496 Rev sequence. The 496 Rev responsive element (RRE) was located in the TM region like
171 in previously described sequences (Saltarelli et al., 1994). The RNA secondary structure of
172 the putative RRE domain was predicted to form a stable stem-loop similar to that of CAEV-
173 like sequences (Kalinski et al., 1991) (not shown).

174 Tat and Vif proteins (CAEV-like; Table 2) of 496 isolate presented an 8 nt overlap between
175 *tat* and *vif* ORFs, like most of the CAEV-like isolates (Gjerset et al., 2006).

176

177 **4. Discussion**

178 The appearance of a focus with a new form of SRLV disease (arthritis) in the history of
179 Spanish sheep prompted us to isolate and investigate the genetic characteristics of the virus
180 involved. SRLV amplicon sequences from the outbreak including those of isolate 496, were
181 close to each other and to the CAEV-like prototype CAEVCo, although a small genetic
182 variability was found within the host, like in previous SRLV studies (Pisoni et al., 2007).
183 Interestingly, isolate 496 showed a high replication rate in vitro regardless of the ovine or
184 caprine origin of the cells used, a phenotype commonly found in sheep and not in goat
185 isolates (Barros et al., 2005). The original goat virus may thus have adapted to the new ovine
186 host, acquiring genetic properties that confer the rapid/fast phenotype.

187 SRLV from the outbreak belonged to the B2 CAEV-like subgroup, in contrast with the
188 previously described sequences in Spain from sheep and goats, which belonged to A and D

189 MVV-like groups (Reina et al., 2006). Thus, there appears to be an agreement between the
190 phylogenetic group to which strain 496 was assigned, and the clinical form observed.

191 The presence of LTR repeats, of 71 nt in CAEV-like and 37-57 nt in MVV-like isolates, and
192 an increased number of sites proposed to regulate viral activity, appears to affect the strain
193 phenotype and tropism (Barros et al., 2004; Murphy et al., 2007; Oskarsson et al., 2007),
194 leading to enhanced promoter activity and a rapid/high lytic phenotype. Alternatively,
195 deletion of these repeats has been reported to abolish the activity of the promoter in SCP cells
196 in some SRLV infections (Oskarsson et al., 2007). The absence of both repeats from isolate
197 496 LTR indicates that these duplications are not essential for a fast/high phenotype.

198 Conservation of GAS and TAS motifs, as well as AP-4 site, TATA-Box and PolyA-signal
199 confirmed previous findings (Barros et al., 2004), strongly suggesting that maintenance of
200 these sites is essential to the virus. Deletion of one of the two CAAAT sites of LTR may
201 result in viruses which are non-replicative in SCP cells (Oskarsson et al., 2007). Here we
202 describe sequences from a virus fully replicative in SCP cells lacking the CAAAT in the U3
203 repeat region of LTR. However, in the 496 sequence there were two extra CAAAAT sites,
204 both of them downstream the CAAAT site, which may help to explain the high replication
205 capacity of this isolate.

206 A 13-14 nt deletion has been found previously in the R region of the SRLV-LTR from
207 asymptomatic sheep and not in SRLV of pneumonia-affected sheep (Angelopoulou et al.,
208 2008). Interestingly, in our study this deletion was found in sequences from the clinically
209 affected sheep involved, regardless of the sample source, suggesting that factors besides this
210 deletion are involved in the appearance of clinical symptoms.

211 In contrast with the remaining regions of the 496 genome, the integrase DNA binding domain
212 of this isolate was MVV-like. Whether this is related to the MVV-like phenotype (rapid/high)
213 of isolate 496 observed in vitro is unknown. This phenotype is also compatible with the

214 appearance of a high number of affected animals in this outbreak, considering that a high viral
215 load may enhance the development of clinical signs (Zhang et al., 2000).

216 In the SU5 immunodominant epitope, the 496 sequence presented a two amino acid deletion
217 compared with CAEVCo and three amino acid substitutions compared with a SRLV
218 consensus sequence (Mordasini et al., 2006). In the immunodominant TM epitope (Bertoni et
219 al., 1994) the two amino acid inversion (HQ being CAEV-like) could be of diagnostic value
220 in this outbreak.

221 The features of the CAEV-like highly replicative isolate from the arthritic sheep outbreak
222 under study highlights a possible correspondence between genotype and phenotype and
223 provides further evidence of controversy in this research area within the field of SRLV.

224

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229

230 **Conflict of interest**

231 There is no conflict of interest.

232

233

234

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- 315

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316 Table 1. Primers used for sequencing the complete genome of isolate 496.

317

Designation	Sequence (5'-3')	Orientation	Location	Region	Size (nt)	Source
env-CAEV1	GGA GCT TTG GCA GAA CTC AC	Fw	8979-8998	<i>env</i>	530	This work
LTR	CCA CGT TGG GCG CCA GCT GCG AGA	Rv	455-432	LTR		Zanoni et al., 1992
LTR	TGA CAC AGC AAA TGT AAC CGC AAG	Fw	152-176	LTR	1260	Zanoni et al., 1992
CAEV-Oslo	GGC ATC ATG GCT AAT ACT TCT AA	Rv	1412-1390	<i>gag</i>		Rimstad et al., 1993
Wie5	CTT GTG TCC GAG GAT TTT GA	Fw	1324-1343	<i>gag</i>	596	Dr. D. Klein ^{p/c}
Wie3	CGC TTG TGC TAA TAA CTG CAT	Rv	1920-1900	<i>gag</i>		Dr. D. Klein ^{p/c}
WieRC	AAG ATG CAA GCA TGC AGG GAC	Fw	1858-1878	<i>gag</i>	1293	This work
P27	TTC TCC GGG TGT AGT TCA AAT C	Rv	3151-3130	<i>pol</i>		Shah et al., 2004
Pol I	TGGATGATATYATAYATAGGRAGTG	Fw	2989-3012	<i>pol</i>	1158	This work
Pol II	AAATCATAYCCWGCATCTTC	Rv	4147-4128	<i>pol</i>		This work
P29	GGT GCC TGG ACA TAA AGG GAT TC	Fw	4025-4047	<i>pol</i>	1174	Shah et al., 2004
P35	GCC ACT CTC CTG RAT GTC CTC T	Rv	5199-5178	<i>pol</i>		Shah et al., 2004
Pol II	GGACAAGCCCTATGGATATWT	Fw	5068-5088	<i>pol</i>	1150	This work
Tat	GGTTACATAGYCTRCAHCCACAD	Rv	6218-6196	<i>tat</i>		This work
5084	CAA MGA TGG CTH GCW ATG C	Fw	6092-6110	<i>tat</i>	939	Valas et al., 1997
SU1	TCT GTR CAD GAD ACW GCT YTT GC	Rv	7031-7009	<i>env</i>		This work
SU2	GCA GGR AVM ATM AGR GGA AGA TT	Fw	6874-6896	<i>env</i>	939	This work
567	CCA CAT WGT TGT CCA ATT HGT DTT	Rv	7813-7789	<i>env</i>		Mordasini et al., 2006
SU3	GAT GYA AYT GYW CAM GRT CAG G	Fw	7682-7703	<i>env</i>	747	This work
TM1	GCT TCY ACY CKA GCH ACT C	Rv	8429-8411	<i>env</i>		This work
TM2	TRG CHA AAG GVR TAM GMA TC	Fw	8381-8400	<i>env</i>	846	This work
LTR RC	GCG GTT ACA TTT GCT KTG TCA	Rv	9227-9206	LTR		This work

^{p/c}: Personal communication.

318 Table 2. Similarity (%) between the amino
319 acid sequences of isolate 496 and those of
320 VMV-like and CAEV-like prototypes.
321

Protein compared	VMV-like prototypes	CAEV-like prototypes
Gag	70.8-72.4	91.0-92.1
Pol	74.0-76.6	81.5-84.2
Env	55.9-59.3	75.8-76.8
signal-peptide	24.6-26.8	56.0-58.0
mature SU	64.4-68.7	76.9-77.0
TM	54.1-57.0	81.4-83.7
Vif	45.6-53.4	76.4-77.2
Tat	43.6-46.3	78.1-80.4
Rev	18.5-24.3	50.7-52.2

322
323

324

325 **Figure captions**

326 Fig. 1. Nucleotide phylograms involving isolate 496 nucleotide sequences. (a) complete
327 sequence phylogram assigning isolate 496 to a CAEV-like phylogenetic group; the phylogram
328 involves complete GenBank SRLV sequences, VMV-like (EV1, SAOMVV, P1OLV, KV1514
329 and 85/34 USA and a Swiss Goat isolate) and CAEV-like (CAEV1GA, CAEV Gansu and
330 CAEVCo). (b) 1.2 kb *gag-pol* segment (Shah et al., 2004) and (c) 1.8 kb *pol* segment (Shah et
331 al., 2004) phylograms indicating that 496 belongs to the phylogenetic sub-type B2. 496 and
332 previous study (Reina et al., 2006) isolates are encircled. Bootstrap values are indicated.
333 Phylogenetic subtypes are within brackets.

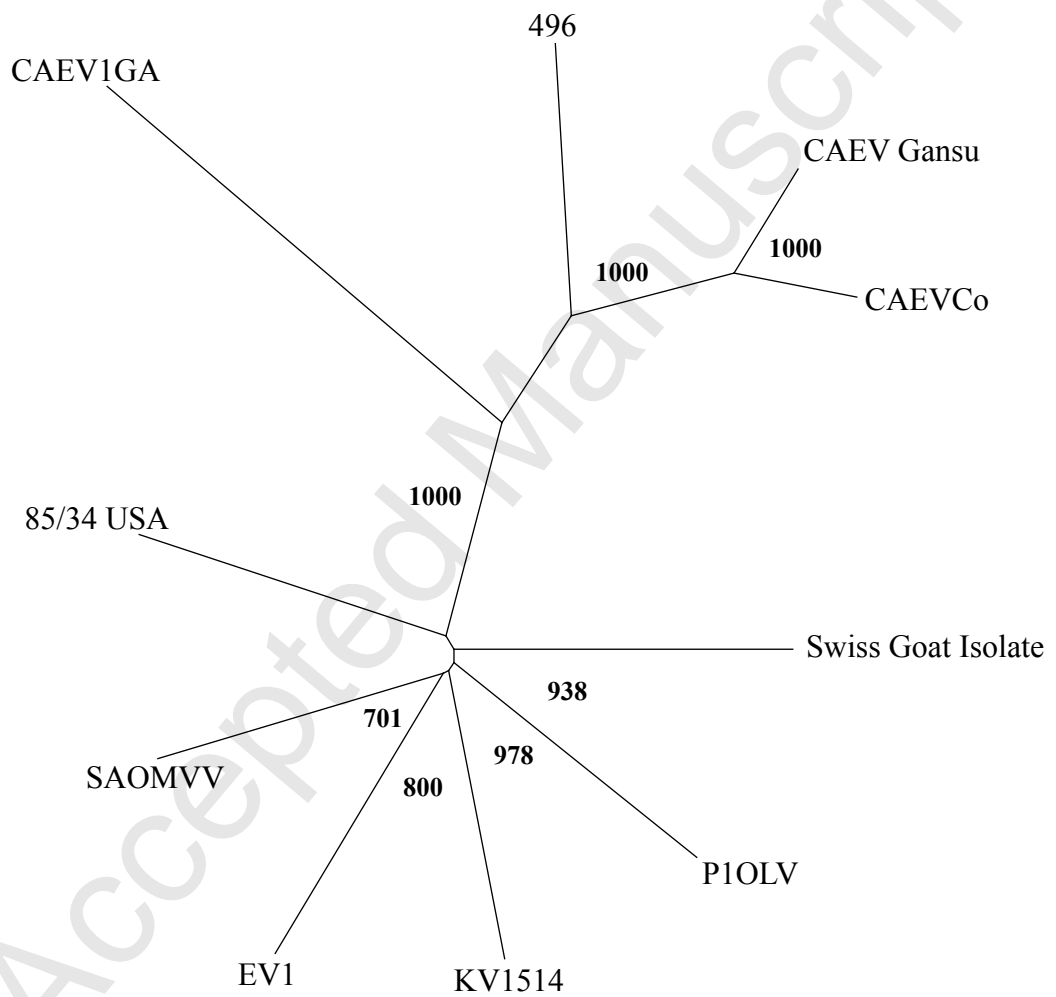
334

335 Fig. 2. Viral production of isolate 496 measured by reverse transcriptase (RT) activity in
336 culture supernatants of different cell types: Sheep choroid plexus (SCP), foetal ovine synovial
337 membrane (FOS) cells and heterologous (het) and autologous (aut) sheep skin fibroblast cells
338 (SFC).

339

340 Fig. 3. Alignment of U3-R sequences of the LTR region from SRLV isolates. Sequences are
341 aligned against KV1514 prototype. Dots indicate identity with KV1514 and dashes represent
342 gaps. The boundaries between U3, R and U5 are indicated by straight arrows. A bent arrow
343 indicates the start site of transcription. AP-1, AP-4 and AML(vis) motifs, the TATA box and
344 the polyadenylation signal are marked by boxes. TAS and GAS sites are underlined. The
345 polypurine tract region is marked with a horizontal line above the sequence. The grey boxes
346 represent the CAAAT sequences. Repeat regions are within brackets. The sequence of isolate
347 496 is in boldface.

a)



0.1

Fig. 1a. Glaria et al.

b)

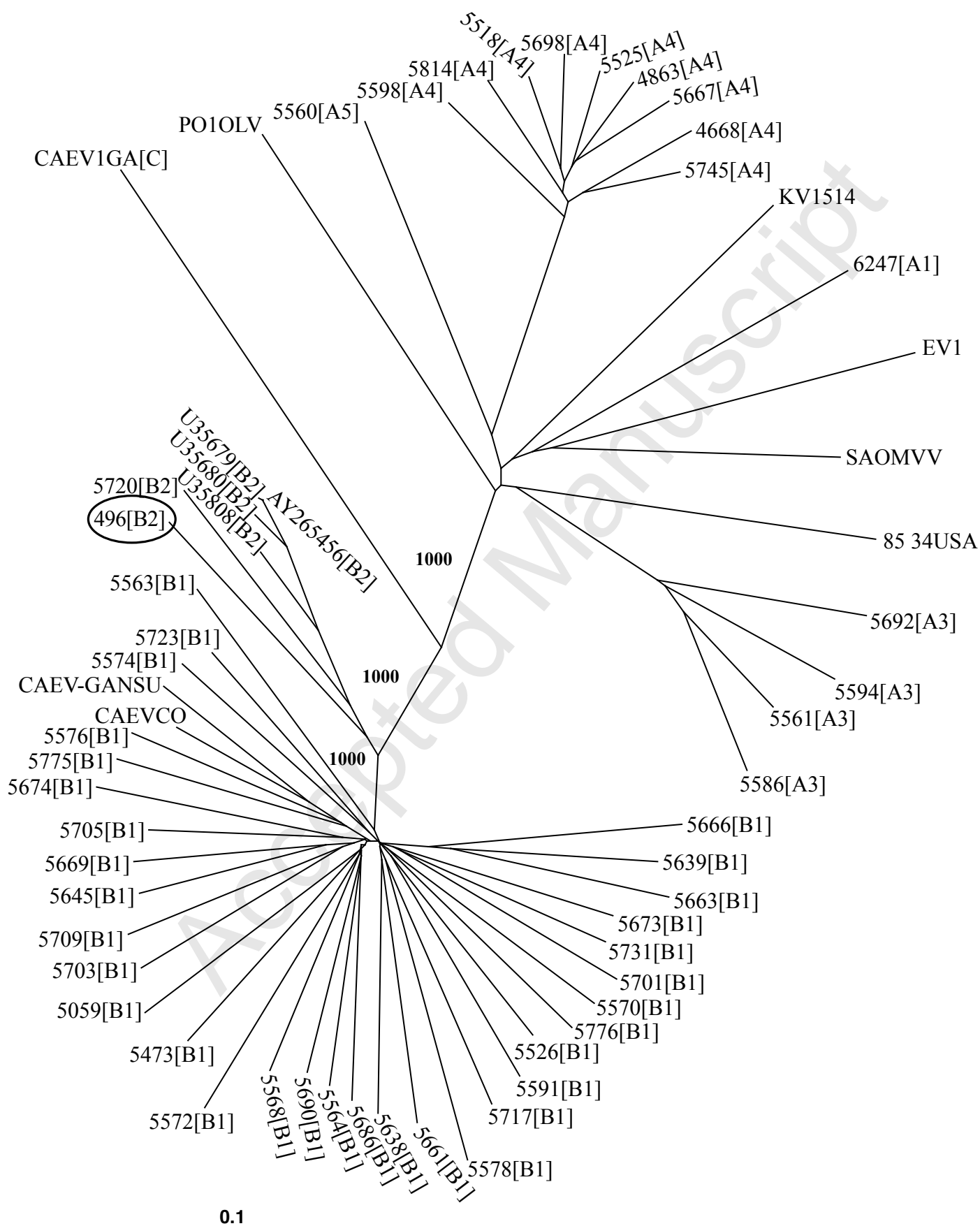


Fig. 1b. Glaria et al.

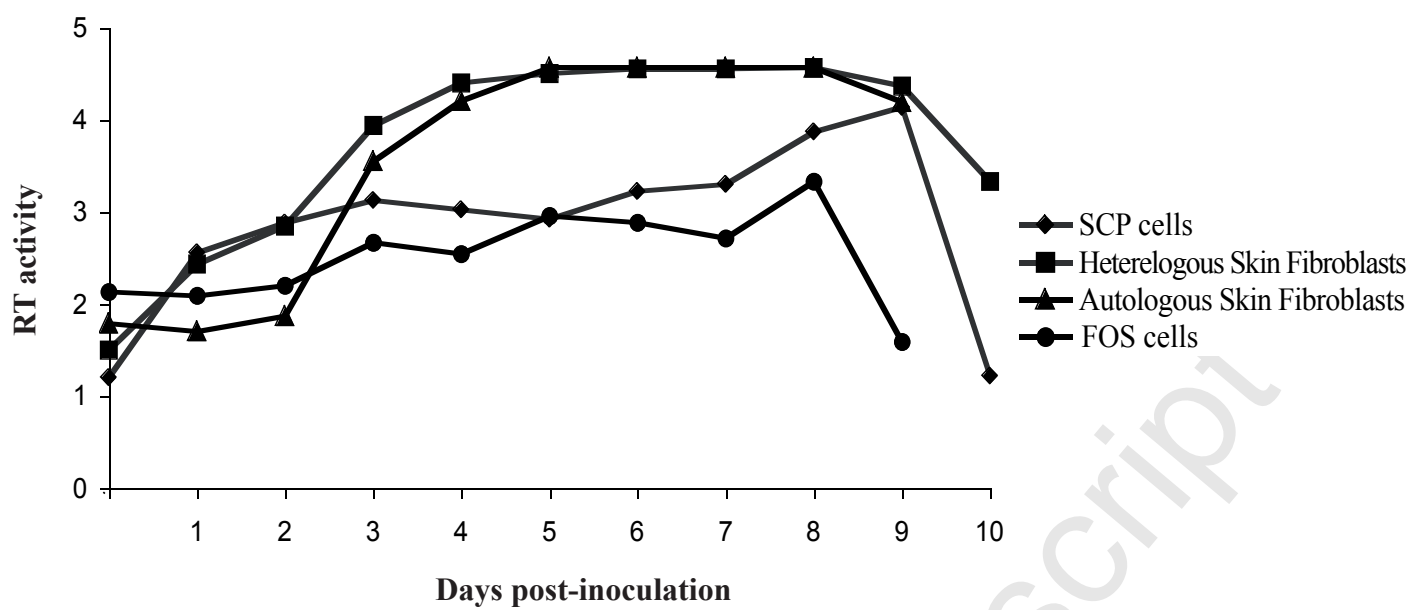


Fig. 2. Glaria et al.

Figure 2 Glaria et al



Fig. 3. Glaria et al.

c)

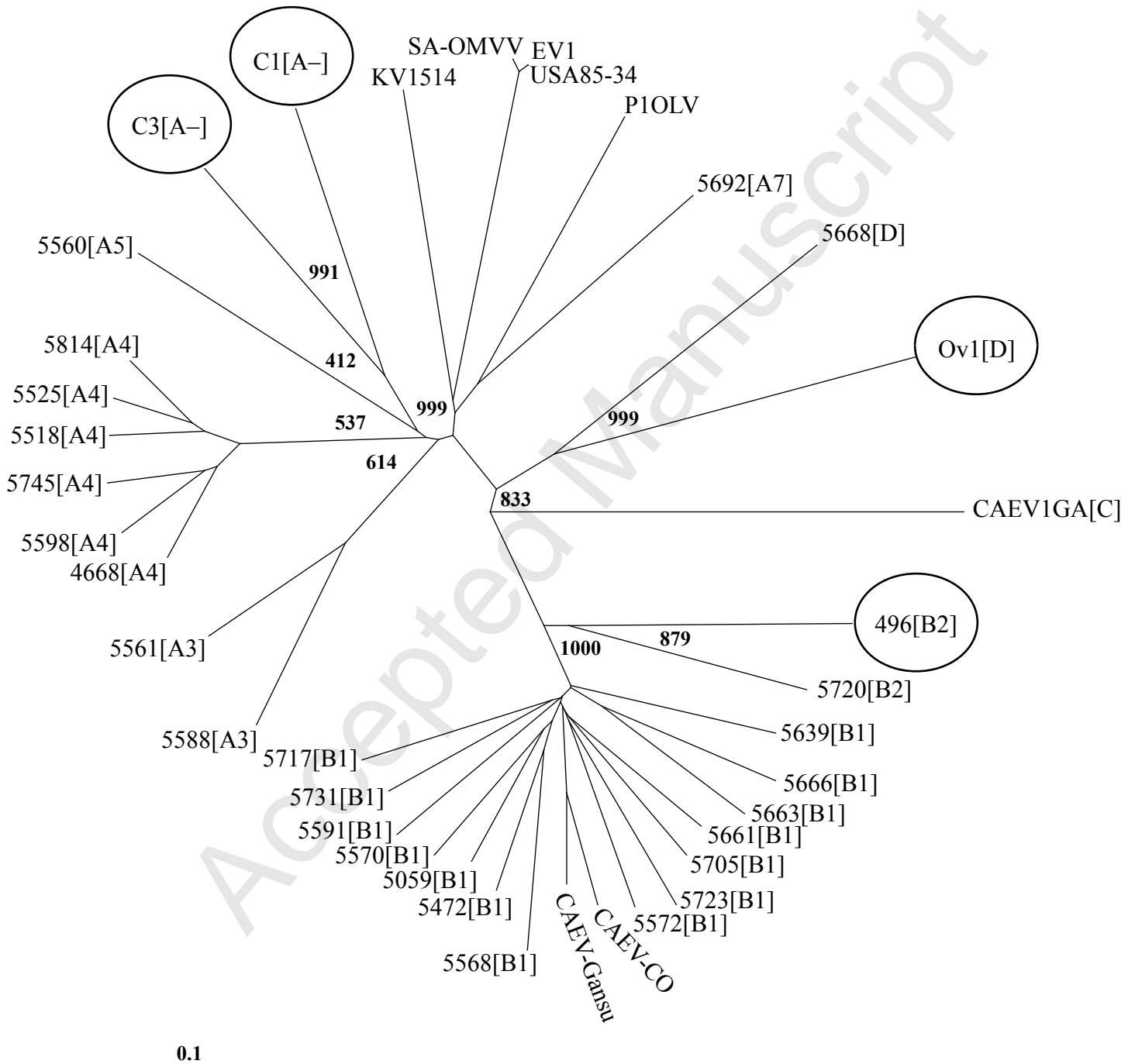


Fig. 1c. Glaria et al.