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1 **Evolution of Rabbit haemorrhagic disease virus (RHDV) in the**  
2 **European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula**

3

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27 **Abstract**

28 To date information on rabbit haemorrhagic disease virus (RHDV) in Spain and  
29 Portugal has been scarce, although the disease is endemic and continues to have a  
30 considerable impact on species conservation and hunting industry. We analysed  
31 RHDVs obtained between 1994 and 2007 at different geographic locations in  
32 Portugal (40 samples), Spain (3 samples) and France (4 samples) from wild European  
33 rabbits (*Oryctolagus cuniculus*) that succumbed to the disease. Phylogenetic analyses  
34 based on partial VP60 gene sequences allowed a grouping of these RHDVs into three  
35 groups, termed “Iberian” Groups IB1, IB2 and IB3. Interestingly, these three Iberian  
36 groups clustered separately, though not far from earlier RHDVs of Genogroup 1  
37 (containing e.g. strain “AST89”), but clearly distinct from globally described RHDV  
38 strains of Genogroups 2-6. This result, supported by a bootstrap value of 76%, gives  
39 rise to the hypothesis that the virus evolved independently since its introduction to  
40 wild rabbit populations on the Iberian Peninsula, with the Pyrenees acting as a natural  
41 barrier to rabbit and hence to virus dispersal. No differences were observed in RHDV  
42 sequences obtained from geographic regions where the rabbit subspecies *O. c. algirus*  
43 prevails compared with those obtained from *O. c. cuniculus*.

44

45 **Key words:** RHDV; Phylogeny; Iberian Peninsula; *oryctolagus cuniculus*; wild  
46 rabbit

47

## 48 **1. Introduction**

49 Rabbit haemorrhagic disease (RHD) outbreaks on the Iberian Peninsula were first  
50 described in 1988 and the disease is endemic since (Anon., 1989; Arguello Villares *et*  
51 *al.*, 1988). Wild rabbit abundance has declined over 30% since, raising conservational  
52 concerns and having a negative economic impact on the hunting industry (Alves and  
53 Ferreira, 2004; Moreno *et al.*, 2007; Villafuerte *et al.*, 1995). The European rabbit  
54 that nowadays is distributed in many continents originated from the Iberian  
55 Peninsula, where two well separated subspecies exist, *O. c. cuniculus* in the northeast  
56 and *O. c. algirus* in the southwest, which form a contact zone in the central region  
57 (Branco *et al.*, 2000; Geraldès *et al.*, 2006; Monnerot *et al.*, 1994) (Fig. 1). The  
58 subspecies *O. c. algirus* is endangered due to various factors, but, most importantly,  
59 due to high mortality rates of RHD epizootics (Alves and Ferreira, 2004; Moreno *et*  
60 *al.*, 2007).

61 Surprisingly, the available genetic information on rabbit haemorrhagic disease virus  
62 (RHDV) in Spain and Portugal is scarce (Boga *et al.*, 1994; Nowotny *et al.*, 1997;  
63 Parra and Prieto, 1990), although many studies have been conducted in other  
64 countries (e.g. Forrester *et al.*, 2007; Le Gall-Reculé *et al.*, 2003; Le Gall *et al.*, 1998;  
65 Matiz *et al.*, 2006; Moss *et al.*, 2002; Nowotny *et al.*, 1997). These phylogenetic  
66 analyses focus predominantly on sequences located on the viral capsid protein VP60  
67 gene, and although inferences could be made in some studies, linking RHDV genetic  
68 groups to variables such as the years of sampling, geographic origin, or virulence of  
69 strains, these relations were not always observed and therefore cannot be generalized.

70 Our objectives were to characterise RHDV strains obtained from wild rabbits  
71 presumed to have died from RHD in different years and geographic locations in the  
72 Iberian Peninsula as well as to characterise RHDV strains obtained from rabbits in  
73 distribution areas of *O. c. algirus*.

74

## 75 **2. Materials and Methods**

### 76 **2.1. Samples**

77 In Portugal, a total of 65 wild rabbits that were found dead during known epidemics  
78 of RHD were collected and immediately stored at -20°C. The animals were collected  
79 from different geographic areas in different years (Fig. 1). Thirty-six samples were  
80 collected between 1994 and 1997 for a study aiming to determine the presence of  
81 RHDV as cause of death in wild rabbits. These had previously tested positive by  
82 antigen capture ELISA (Capucci *et al.*, 1991). These and the remaining liver samples  
83 (collected 2004 - 2007) were processed in the Laboratory of the University of Porto.

84 Seven RHDV sequences were obtained from wild rabbits from Spain and France  
85 (Table 1; Fig. 1). The sequences of the two Spanish RHDVs collected in Alicante and  
86 Albacete in 2004 were kindly provided by Dr. Ramon Soriguer (CSIC). The sequence  
87 from the Toledo specimen was obtained in the Laboratory of the University of Porto.

88 It was the only nested RT-PCR positive sample out of 39 hunted wild rabbits  
89 collected in 1994. The four recent RDHV sequences from France corresponded to  
90 three strains (“2000-08”, “2001-23” and “2002-20”) that had been collected in 2000,  
91 2001 and 2002, respectively, in the Department “Pyrénées-Orientales”, and to strain

92 “2005-01” that was obtained in 2005 in the Department “Manche”. These sequences  
93 were obtained in the Laboratory of AFSSA, Ploufragan.

94

## 95 **2.2. RT-PCR and sequencing**

96 RNA was extracted from liver homogenates and cDNA was synthesized using  
97 random priming and M-MLV reverse transcriptase (Invitrogen). Nested PCR was  
98 used to amplify partially the RHDV VP60 capsid protein gene (Moss *et al.* 2002),  
99 corresponding to positions 6157 to 6703 of strain AST89 (GenBank accession  
100 number Z49271). The amplicons of 547bp were directly sequenced in both directions  
101 employing the nested PCR forward and reverse primers.

102

## 103 **2.3. Sequence analysis**

104 From Portugal only the 40 non-identical sequences were further analysed. For  
105 phylogenetic analysis, different methods (minimum evolution, maximum parsimony,  
106 maximum likelihood and neighbour-joining) were applied using the software package  
107 MEGA3.1 (Kumar *et al.*, 2004). The obtained trees showed similar clustering of the  
108 sequences. The neighbor-joining tree was presented as considered adequate for  
109 comparing relatively short sequences (Takahashi and Nei, 2000). The nucleotide  
110 substitution model of Kimura-2-parameter was used, and the reliability of the tree  
111 was tested by bootstrap analysis of 1000 replicates. The obtained sequences were  
112 compared with published homologues from GenBank database (NCBI). Eighteen  
113 sequences representing previously described RHDV genogroups were selected and

114 included (Table 1). These sequences were grouped into Genogroup1 to 6, adapted to  
115 the classification used by Le Gall-Reculé *et al.* (2003). The Italian non-pathogenic  
116 rabbit calicivirus “RCV” (GenBank accession number X96868) was used to root the  
117 tree. The amino acid sequences and the consensus sequences of each group were  
118 deduced and aligned.

119

## 120 **Results**

121 The phylogenetic tree estimated by the neighbor-joining method (Fig. 2) shows the  
122 clusters formed by RHDV strains from the Iberian Peninsula and the south of France,  
123 which have been termed “Iberian” Groups IB1 to IB3, together with the clusters  
124 containing “known” RHDV strains representing Genogroup 1, Genogroup 2,  
125 Genogroups 3-5 and Genogroup 6 antigenic variants or “RHDVa” strains (Capucci *et*  
126 *al.*, 1998). The Groups IB1 and IB2 contain sequences collected between 1994 and  
127 1997, whereas Group IB3 includes more ones collected between 2000 and 2007. Each  
128 group contains sequences from different geographic locations, except Group IB1, that  
129 contains five sequences obtained in 1994 and 1997 from Santarém district of central  
130 Portugal. Group IB2 contains strains collected between 1994 and 1997 from northern  
131 and central Portugal as well as the virus obtained in 1994 from Toledo, Spain. Group  
132 IB3 includes the more recent (2004-2007) Portuguese strains collected in the southern  
133 province Algarve (2004-2005) as well as in the North (2006-2007). Also included in  
134 Group IB3 are the sequences from three French RHDVs that had been identified  
135 between 2000 and 2002 in the Department “Pyrénées-Orientales”, and the two Spanish



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136 strains “Albacete” and “Alicante” collected in 2004.

137 All described RHDV sequences from Portugal, Spain and the South of France  
138 represented in Groups IB1, IB2 and IB3 formed, together with Genogroup 1  
139 sequences, a distinct cluster separated from all previously described RHDV  
140 genogroups, supported by a bootstrap value of 76%. Group IB2 and IB3 viruses share  
141 a common ancestor with Genogroup 1 viruses (bootstrap 82%). Group IB1, which  
142 contains five sequences from central Portugal (bootstrap 97%), separated before the  
143 known RHDV Genogroup 1. The recent French strain “2005-01” obtained in the  
144 Department “Manche” clustered within Genogroups 3-5, close to another French  
145 strain collected in 2000 (“00-13”).

146 The grouping of the RHDVs was supported by the bootstrap values as well as a  
147 comparison of nucleotide proximities. Nucleotide similarities of 97%, 98%  
148 (excluding strain “1996-08”) and 99-94% were observed within groups IB1, IB2 and  
149 IB3, respectively. Lower values were observed between groups and genogroups.  
150 Group IB2 strain “1996-08” displayed a higher genetic distance proportionally to all  
151 other strains, but was still closer to RHDVs of its own group IB2 (96-95%) than to  
152 Genogroup 1 strains (94%) or others (less than 94%).

153 The comparison of the group consensus sequences of the deduced RHDV VP60  
154 polymorphic amino acid composition showed that, in comparison to Genogroup 1,  
155 Group IB1 presents three replacements: A<sub>2176</sub>N, N<sub>2178</sub>S and S<sub>2194</sub>N. The strains  
156 contained in Groups IB2 and 3 are characterised by five amino acid replacements  
157 when the respective group consensus sequences are compared to those of Genogroups

158 1 to 6: R<sub>2062</sub>Q, L<sub>2152</sub>P, T<sub>2163</sub>I, A<sub>2176</sub>T and V<sub>2230</sub>I. The more recent Iberian and French  
159 RHDV strains of Group IB3 are further characterised by the residues P<sub>2069</sub>N, N<sub>2071</sub>G  
160 and I<sub>2092</sub>V, which is not a feature of the other described strains.

161

#### 162 **4. Discussion**

163 In Spain and Portugal, rabbit mass mortality due to RHD was observed in the late  
164 1980s and early 1990s, coinciding with the observation of the epidemic in other  
165 European countries, suggesting that the origin of the virus was the same.  
166 Accordingly, Genogroup 1 sequences were obtained from RHD outbreaks in 1989 not  
167 only in Spain (Boga *et al.*, 1994; Parra and Prieto, 1990), but also in France (“France  
168 SD”) and Germany (“Eisenhuettenstadt”). No genetic information has ever been  
169 published on more recent RHDVs circulating in wild rabbits in Spain, and none ever  
170 from Portugal. Here, forty-seven RHDV strains from wild rabbits (*Oryctolagus*  
171 *cuniculus*) obtained between 1994 and 2007 at different geographic locations in  
172 Portugal, Spain and France (Fig. 1) were characterised based on their partial VP60  
173 gene sequences and grouped into three groups, termed “Iberian” Groups IB1, IB2 and  
174 IB3. All these “Iberian” Groups sequences formed, together with those of Genogroup  
175 1, a distinct cluster separated from all other described RHDV genogroups. Group IB2  
176 and IB3 viruses share a common ancestor with Genogroup 1 viruses, whereas Group  
177 IB1 strains separated before Genogroup 1 RHDV.

178 In geographically neighbouring France, virulent RHDVs of two distinct Genotypes (1  
179 and 2) were initially present between 1987 and 1990, but were subsequently replaced

180 by other RHDV strains (Le Gall-Reculé *et al.*, 2003). Despite the lack of data  
181 between 1989 and 1994, none of the RHDV sequences from Spain and Portugal  
182 clustered within Genogroup 2, suggesting that only Genogroup 1-like virulent RHDV  
183 strains predominated initially and that these were subsequently replaced by strains  
184 here grouped as IB2 and IB3, indicating that RHDV could have evolved separately in  
185 the Iberian Peninsula since. Alongside the nucleotide sequence analysis, the observed  
186 amino acid polymorphisms suggest that the substitutions observed in Groups IB2 and  
187 IB3 seem to have become fixed around 1994 and are still present in RHDVs  
188 circulating in wild rabbits in the Iberian Peninsula, but not elsewhere. It is therefore  
189 tempting to speculate that the Pyrenees may act as a natural barrier, constraining wild  
190 rabbit and hence virus dispersal and evolution, similar to what was observed on  
191 Lambay island or in New Zealand (Forrester *et al.*, 2007; Forrester *et al.*, 2003).

192 Phylogenetic, serological and epidemiological studies related to RHD have led to the  
193 hypothesis that attenuated or avirulent forms of the virus have been circulating in  
194 Europe before the 1980s (Forrester *et al.*, 2003; Moss *et al.*, 2002; Nowotny *et al.*,  
195 1992; Rodák *et al.*, 1990). The drastic reduction in wild rabbit numbers observed on  
196 the Iberian Peninsula has been historically unprecedented, suggesting that, if any  
197 RHDV-like viruses were circulating in wild rabbit populations at that time, they must  
198 have been highly host-adapted but not cross-protective. Among the 39 wild rabbits  
199 obtained from hunters in 1994 from the Toledo region, only one tested positive by  
200 nested RT-PCR (2-3% of the total). Also, more recently in 2006, liver samples  
201 obtained from 30 healthy wild rabbits hunted in 2006 in Pancas (Lisbon district)

202 tested negative by nested RT-PCR (data not shown). This data is different from the  
203 high percentage (40-60%) of PCR positive healthy wild rabbits found in New  
204 Zealand (Forrester *et al.*, 2003), suggesting that in the Iberian Peninsula RHDV-like  
205 viruses are either not present or are present at a very low incidence. Climatic  
206 differences together with potentially circulating avirulent “RHDV-like” strains in  
207 cooler and more humid countries (Cooke, 2002) may explain the observed  
208 differences.

209 The categorisation of the samples obtained in Portugal between 1994 and 1997 in two  
210 distinct groups (IB1 and IB2) was quite surprising, as all were collected during the  
211 same time period, and because the five RHDVs that formed Group IB1 originated  
212 from the same district (Santarém) as others that grouped within IB2 (RHDV strains  
213 “1994-10”, “1995-01”, “1995-10”, “1995-11”), suggesting that two different RHDVs  
214 were circulating concomitantly in the presence of disease and mortality. The  
215 alignment of the amino acid sequence group consensus sequences further strengthens  
216 this hypothesis, as Group IB1 differed in eight positions when compared to Group  
217 IB2, but in only three when compared to Genogroup 1. Bearing in mind, that RHDV  
218 infection can perpetuate in rabbit holdings due to virus persistence (Gall *et al.*, 2007;  
219 Moss *et al.*, 2002), i.e. without repeated introduction of the virus from the  
220 environment, the question arises whether the detected subgroups are typical for wild  
221 rabbits of this region. Our data seem to support this hypothesis, as e.g. shown by the  
222 proximity of all recent wild rabbit RHDVs collected on the Iberian Peninsula and  
223 South of France forming Group IB3. This does not necessarily mean that the same

224 RHDV circulate in wild and farmed or pet rabbits. We characterised a RHDV from an  
225 outbreak that occurred in January 2007 in a commercial rabbit farm in the North of  
226 Portugal, which was classified as an antigenic variant RHDVa, based on partial VP60  
227 gene sequence analysis (own observations) and on antigenic characterisation using a  
228 panel of monoclonal antibodies performed by the OIE Reference Laboratory (Dr. L.  
229 Capucci).

230 Phylogenetic analyses of RHDV strains are commonly based on sequences  
231 representing only a fragment of the capsid VP60 protein gene, however, discrepancies  
232 between authors in relation to the number of RHDV groups and subgroups and also in  
233 tree topology, warrant harmonisation of RHDV typing. Due to recombination events  
234 contributing to RHDV variability, it may be more appropriate to investigate the  
235 complete sequence encoding VP60, or ideally, the full length genome, rather than  
236 partial capsid gene sequences (Abrantes *et al.*, 2008; Forrester *et al.*, 2008).

237 In this study we report the genetic characterisation of RHDV strains which have been  
238 obtained in Portugal, where the rabbit subspecies *O. c. algirus* is prevalent, from  
239 locations in Spain within the contact zone, and from France where only *O. c.*  
240 *cuniculus* has been described. No significant epidemiological, clinical or pathological  
241 differences have been observed between the rabbit subspecies, suggesting that *O. c.*  
242 *algirus* is as susceptible to RHDV as *O. c. cuniculus*, and that the virus is equally  
243 virulent for both rabbit subspecies. No significant differences have been observed in  
244 RHDV partial VP60 gene sequences obtained from wild rabbit specimens in either  
245 region. To our knowledge this is the first genetic characterisation and molecular

246 epidemiology of RHDV sequences obtained from *O. c. algirus*.

247

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259

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349 **Figure captions**

350

351 **Fig. 1.** Map of the Iberian Peninsula and South of France displaying the geographic  
352 origin of the RHDV samples analysed in this study and the time period they were  
353 collected. The distribution areas of the wild rabbit subspecies *Oryctolagus cuniculus*  
354 *algerius* and *Oryctolagus cuniculus cuniculus* as well as the contact zone across the  
355 Iberian Peninsula are indicated.

356

357 **Fig. 2.** RHDV strains from Portugal cluster separately from known genogroups based  
358 on phylogenetic analysis of partial VP60 gene sequences. The neighbour joining tree  
359 was rooted with RCV. Bootstrap probability values above 75% for 1000 replicate  
360 runs are indicated at the nodes.

361

362

Figure 1.

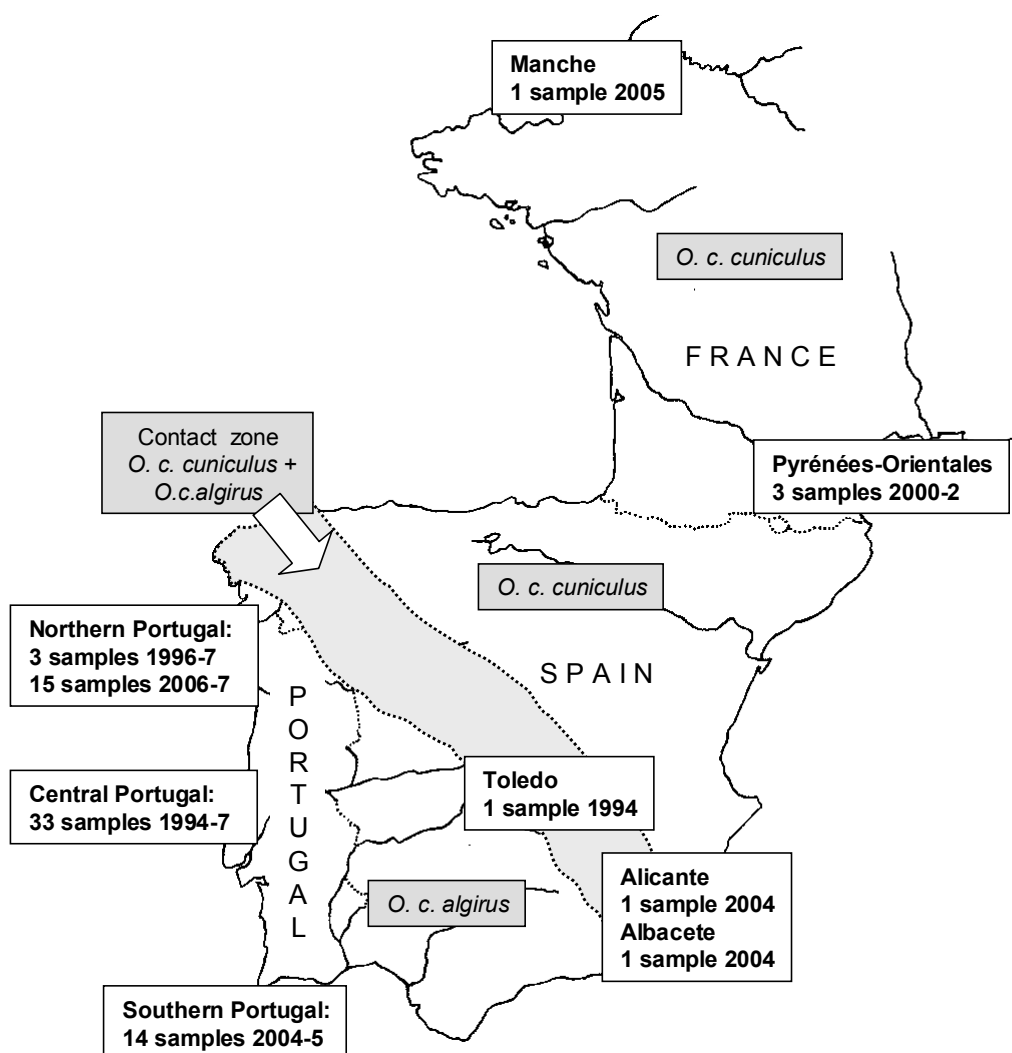
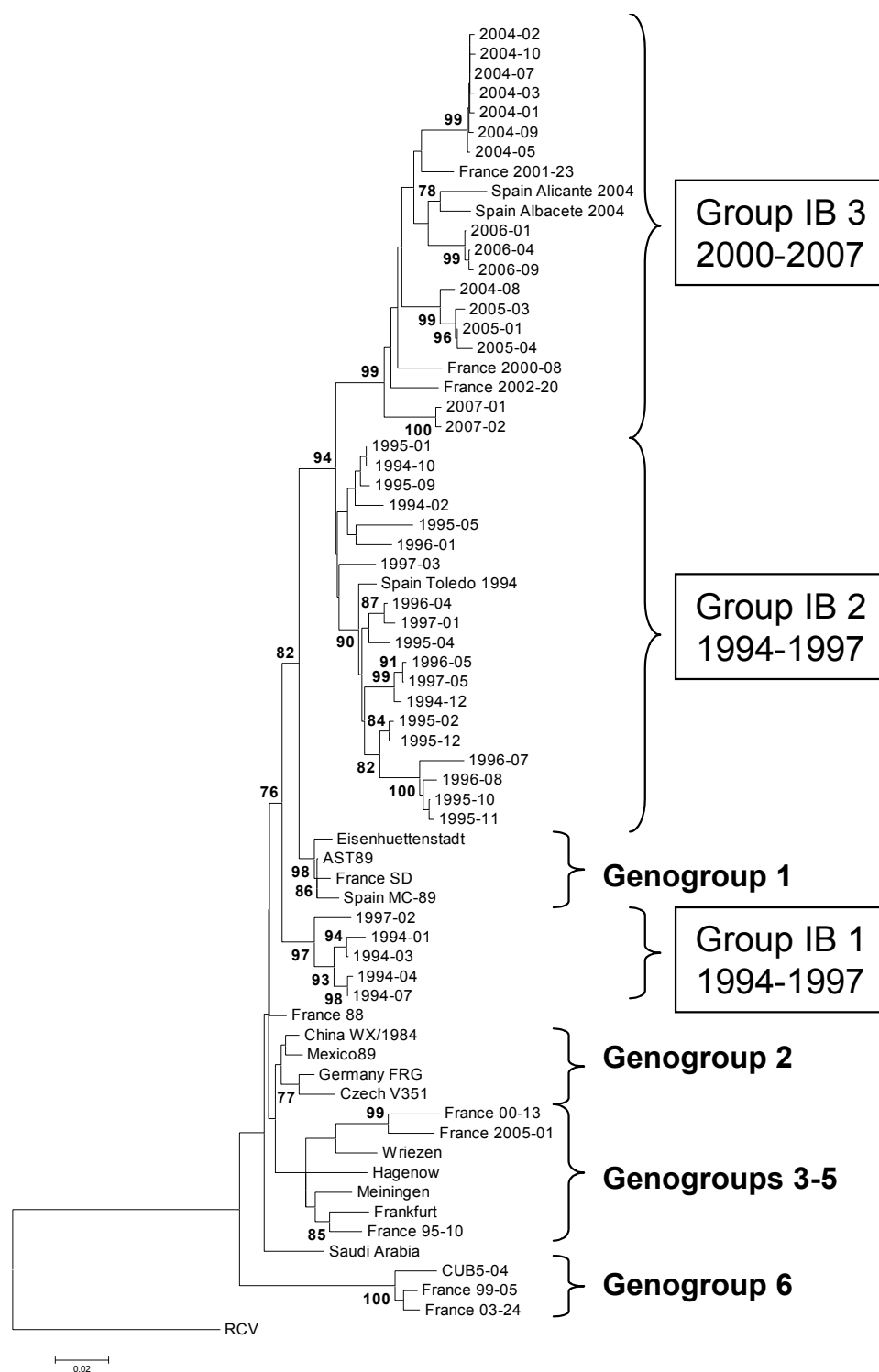


Figure 2.



**Table 1. Genbank accession numbers of RHDV sequences included in the phylogenetic analysis.**

<b>RHDV Group</b>	<b>Strain</b>	<b>GenBank Reference</b>
<b>Group IB3</b>	2004-03	EU192134
	France_2001-23	AM746980
	Spain_Alicante_2004	AM884394
	Spain_Albacete_2004	AM884395
	2006-01	EF571322
	2006-04	EF571325
	2006-09	EF571330
	2005-01	EU192140
	France_2000-08	AJ319594
	France_2002-20	AM746981
	2007-01	EU192135
	<b>Group IB2</b>	1995-01
1994-02		EU192136
1997-03		EU192139
Spain_Toledo_1994		EU192137
1996-08		EU192138
<b>Group IB1</b>	1997-02	EU192133
	1994-07	EU192131
<b>Genogroup 1</b>	Eisenhuettenstadt	Y15440
	AST89	Z49271
	France_SD	Z29514
	Spain_MC-89	L48547
<b>Genogroup 2</b>	China_WX/1984	AF402614
	Mexico89	AF295785
	Germany_FRG	M67473
	Czech_V351	U54983
<b>Genogroups 3-5</b>	France_00-13	AJ495856
	France_2005-01	AM085133
	Wriezen	Y15427
	Hagenow	Y15441
	Meiningen	Y15426
	Frankfurt	Y15424
	France_95-10	AJ535094
<b>Genogroup 6</b>	CUB5-04	DQ841708
	France_03-24	AJ969628
	France_99-05	AJ302016
<b>Root</b>	RCV	X96868