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Sequencing of *Sylvilagus VDJ* genes reveals a new *VHa* allelic lineage and shows that ancient *VH* lineages were retained differently in leporids

Ana Pinheiro · José Melo-Ferreira · Joana Abrantes · Nicola Martinelli · Antonio Lavazza · Paulo C. Alves · Christian Gortázar · Pedro J. Esteves

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Abstract Antigen recognition by immunoglobulins depends upon initial rearrangements of heavy chain *V*, *D*, and *J* genes. In leporids, a unique system exists for the *VH* genes usage that exhibit highly divergent lineages: the *VH*a allotypes, the *Lepus* sL lineage and the *VH*n genes. For the European rabbit (*Oryctolagus cuniculus*), four *VH*a lineages have been described, the a1, a2, a3 and a4. For hares (*Lepus* sp.), one *VH*a lineage was described, the a2L, as well as a more ancient sL lineage. Both genera use the *VH*n genes in a low frequency of their *VDJ* rearrangements. To address the hypothesis that the *VH* specificities could be associated with different environments, we sequenced *VDJ* genes from a third leporid genus, *Sylvilagus*. We found a fifth and equally divergent *VH*a lineage, the a5, and an ancient lineage, the sS, related to the hares' sL, but failed to obtain *VH*n genes. These results show that the studied leporids employ different *VH* lineages in the generation of the antibody repertoire, suggesting that the leporid *VH* genes are subject to strong selective pressure likely imposed by specific pathogens.

Keywords *IGHV* · Leporids · Antibody diversification · *VH* allotypes · Evolution

A. Pinheiro · J. Melo-Ferreira · J. Abrantes · P. C. Alves · P. J. Esteves (✉)
CIBIO Centro de Investigação em Biodiversidade e Recursos Genéticos, InBio Laboratório Associado, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
e-mail: pjesteves@cibio.up.pt

A. Pinheiro · P. C. Alves · P. J. Esteves
Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal

A. Pinheiro · C. Gortázar
SaBio - IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain

N. Martinelli · A. Lavazza
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Via Antonio Bianchi 7/9, 25124 Brescia, Italy

P. C. Alves
Wildlife Biology Program, College of Forestry and Conservation, University of Montana, Missoula, MT 59812, USA

P. J. Esteves
Centro de Investigação em Tecnologias da Saúde, IPSN, CESPU, 4585-116 Gandra, Portugal

Introduction

For the generation of the primary antibody repertoire, most mammals express a varying number of rearranged *VDJ* genes (Flajnik 2002). The European rabbit (*Oryctolagus cuniculus*) has a unique system in that, despite having more than 200 *VH* genes (Ros et al. 2004), it predominantly expresses just one *IGHV*, the most D-proximal *VH1* gene, to generate 80–90 % of its antibody repertoire (Knight and Becker 1990; Knight 1992). The immunoglobulin (Ig) molecules derived from the *VH1* express the so-called *VH*a allotypic markers (e.g. Kindt 1975; Margolies et al. 1977), with three serological lineages having been described in the domestic rabbit, the a1, a2 and a3. These are highly divergent (±20 % amino acid sequence differences), with the allelic specificities of a1 and a2 being correlated with several amino acid differences in framework regions (FR) 1 and 3 (Tonnelle et al. 1983; Mage et al. 1984; Knight and Becker 1990). A fourth equally divergent allotypic lineage, the a4, was described in wild European rabbit Iberian populations (Esteves et al. 2004). The remaining 10–20 % of Ig molecules are encoded by the *VH*n genes, *VH*x, *VH*y and *VH*z (Kim and Dray 1973; Horng et al. 1976; Roux 1981), that

map at least 100 Kb upstream of *VH1* (Mage et al. 2006) and do not express *VH*a allotype-specific determinants.

Studies on the *IGHV* locus diversity for other leporids are limited to a few data obtained for some *Lepus* species. Serological analysis conducted for *Lepus americanus* showed cross-reaction with rabbit anti-a1, anti-a2 and anti-a3 antisera (van der Loo 1987). As for *Lepus europaeus* and *Lepus granatensis*, a serological analysis of several populations revealed two phenotypes: partial reaction to anti-a2 antisera and no reaction to any rabbit antiserum (Esteves 2003). Sequencing of rearranged *VH* genes for *Lepus* specimens, some of which expressed a2-cross-reacting serum proteins, proved that the a2 polymorphism is trans-specific. The *Lepus* a2L lineage showed some of the most outstanding characteristics of the rabbit *VH*a genes such as the presence of five out of 11 amino acid residues that characterize the allotype *VH*a2 (Mage et al. 1984), and being more closely related to the rabbit *VH1*-a2 allele than *VH1*-a2 was to its allelic counterparts *VH1*-a1 and *VH1*-a3 (Esteves et al. 2005). A second lineage, the sL, apparently a *Lepus*-specific lineage, groups distinctively apart from all other *VH* lineages present in rabbit, showing as distinctive characters the ancestral $_{70}$SVK$_{72}$ motif and residues A_{10} and K_{95} (IMGT numbering, Lefranc et al. 2003) (Esteves et al. 2005). More recently, it was shown that, like rabbits, *Lepus* uses the *VHn* genes in 5–10 % of its *VDJ* rearrangements revealing that the antibody repertoire is subject to selective pressure and that the low-frequency usage of *VHn* genes could be a remnant of an ancient leporid immunologic response to pathogens (Pinheiro et al. 2011, 2013).

Considering that evidence suggests that the leporid *VH* lineages used to generate the antibody repertoire are subject to selective pressure, in this study, we addressed the hypothesis that the *VH* lineages represented in the antibody repertoire could be different in species that inhabit different environments due to exposure to different pathogens. Family Leporidae comprises 11 genera, among which *Sylvilagus* occupies an intermediate position between *Oryctolagus* and *Lepus* (Alves and Hackländer 2008), having diverged from *Oryctolagus* 10 million years ago and from *Lepus* 12 million years ago (Matthee et al. 2004). *Oryctolagus* and the two *Lepus* species, *L. europaeus* and *L. granatensis*, for which *VDJ* genes nucleotide sequences have been obtained, inhabit the Eurasian continent while *Sylvilagus* is a Native American leporid (Chapman et al. 1980; Flux and Angermann 1990). Starting in 1966, eastern cottontails were introduced in northern Italy through a series of massive releases for hunting purposes; nowadays, eastern cottontails are widespread in this region (Vidus-Rosin et al. 2008). Herein, we sequenced *Sylvilagus floridanus VDJ*-rearranged genes.

Material and methods

Samples, amplification and sequencing of rearranged VDJ genes

Spleen samples were collected from four eastern cottontail (*S. floridanus*) specimens collected in Pistoia Province, Italy. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), following first-strand complementary (c)DNA synthesis using the iScript cDNA Kit (Bio-Rad, Hercules, CA, USA). *VDJ*-rearranged genes were PCR amplified using primers VH (5′GGAGACTGGGCTGCGCTG GCTTCTCCTGGT3′; Esteves et al. 2005) and JH2 (5′TGAG GAGACGGTGACCAGGGTGCCT3′; Pinheiro et al. 2013). PCR amplification conditions were as follows: 4 min at 95 °C followed by 35 cycles at 95 °C (45 s), 62 °C (45 s) and 72 °C (60 s), with a final extension at 72 °C (5 min). PCR products were purified (MinElute PCR Purification Kit, Qiagen, Hilden, Germany) and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI, USA).

Phylogenetic analyses

To perform phylogenetic analyses, available sequences for leporid *VDJ* genes were taken from GenBank. Used sequences include rabbit germ line *VH1* and *VH4* genes of a1, a2 and a3 allotypes; rabbit cDNA *VH* gene sequences representative of allotypes a1, a2, a3, a4.1 and a4.2; rabbit germ line and rabbit and *Lepus* cDNA *VH* gene sequences of *VHx*, *VHy* and *VHz* and *Lepus* cDNA *VH* gene sequences representative of lineages a2L, sLe and sLg. *VH* genes of the human *VH*3 family were used as an outgroup. These were aligned with sequences obtained in this study using CLUSTAL W (Thompson et al. 1994) as implemented in BioEdit software (Hall 1999) and the amino acid sequences inferred. Accession numbers for all sequences are given in Table 1.

The computer program jModelTest v2.1.1 (Darriba et al. 2012) was used to assess the fit of our dataset to 88 models of sequence evolution considering the corrected Akaike information criterion (AICc). The phylogenetic relationships were analysed in a Bayesian inference (BI) framework, specifying as optimal mutation model K80+G, retrieved from jModelTest. BI analyses were performed using BEAST v1.7.4 (Drummond and Rambaut 2007). The BEAUti application, part of BEAST package, was used to create the input file for *BEAST. Posterior phylogenies were determined using an uncorrelated lognormal relaxed clock (Drummond et al. 2006) and the Yule tree prior. All parameter priors were set to the defaults. Three independent runs of 50×10^6 generations were performed, sampling trees and parameters every 5×10^3 generations, and concatenated using LogCombiner.

Table 1 Vh gene sequence accession numbers for sequences used in the phylogenetic analysis

Sequence identification	Accession number
Rabbit germline sequences	
VH1a1, VH4a1	M93171, M93181
VH1a2, VH4a2	M93172, M93182
VH1a3, VH4a3	M93173, L03846
Vhx, Vhx2	L03846, M19706
Vhy, Vhy2	L03890, L03874
Vhz	AF264469
Rabbit cDNA sequences	
a1_Ocun1-Ocun4	AF029933, AF029934, AF029938, AF029940
a2_Ocun1-Ocun4	L03849, L03851, L03853, L03856
a3_Ocun1-Ocun4	AF029923, AF098235, AF264434, AF264435
a4.1_I- IV, a4.2_I- III	AY207979- AY207982, AY208042, AY208047, AY207967
Vhx_Oca, Vhx_Oca2	AY207986, AY208045
Vhy_Oca	AY208006
Hare cDNA sequences	
sLe1_397, sLe1_400, sLe2_408, sLe2_412, Le8.1, Le8.12	AY288451, AY288453, AY288459, AY288462, KF460076, KF460085
sLg15_640-642, sLg15_644	AY288464- AY288467
a2_Le1.396, a2_Le1.401, a2_Lg19C83, a2_Lg19C86, Le5.13, Le6.1, Le8.16	AY288450, AY288454, AY288491, AY288494, KF460042, KF460056, KF460087
VHx Le5.2, Le5.17,	KF460034, KF460046
VHy-VHz Le6.17, Le6.24, Le8.11	KF460070, KF460075, KF460084,
Human VH3 family sequences	
Hsap1- Hsap4	M99666, M99672, M99679, M99682
Current study	
Sfl1	KM275500- KM275522
Sfl2	KM275523- KM275542
Sfl3	KM275543- KM275568
Sfl4	KM275569- KM275585

Convergence was checked using Tracer v1.5 (Rambaut and Drummond 2007), and summary trees were generated with TreeAnnotator v1.5.4, part of the BEAST package.

Genetic distances between *VH* genes among species were calculated using the "compute net average distance between groups" option of MEGA5.0 software (Tamura et al. 2011). This option corrects for variance due to differences within groups, as expected in VH-rearranged genes which are somatically diversified.

Results

A total of 86 *VDJ* gene sequences were obtained for the four studied *S. floridanus* individuals. Phylogenies were estimated with the whole *VH* sequence and excluding the CDRs, yielding similar clustering of the analysed sequences. Thus, we present here the phylogeny obtained with the whole *VH* sequence (Fig. 1). Clusters of the previously described rabbit and hare lineages are shown with good support on the phylogenetic tree: the *VHn* lineage (1.00 posterior probability), the sL lineage (1.00 posterior probability) and the *VHa* cluster (0.96 posterior probability) that includes the *VH*a1, *VH*a2, *VH*a2L, *VH*a3 and *VH*4 lineages (1.00, 1.00, 0.99, 1.00 and 1.00 posterior probabilities, respectively). The *S. floridanus* sequences obtained in this study fall into two groups that include only *Sylvilagus VH* sequences: (i) a cluster that is not related to any previously described leporid lineages, the sS lineage (1.00 posterior probability), and (ii) a second group that clusters with the rabbit *VH*a lineages, designated here as a5 lineage (1.00 posterior probability). This a5 lineage is further subdivided into two groups, the a5.1 and a5.2 variants (0.98 and 0.95 posterior probabilities, respectively).

Of the 86 obtained sequences, 46 were classified as a5.1, 26 were classified as a5.2 and 14 sequences were classified as sS. These three groups appear to have an allelic distribution as each one of the analysed individuals has sequences belonging to two of the groups: the Sfl1 and Sfl4 specimens have sequences that were classified as a5.1 and sS (13–9 and 11–5) and the Sfl2 and Sfl3 specimens have sequences that were classified as a5.1 and a5.2 (2–17 and 17–9).

Genetic distances between rabbit, hares and cottontail *VH* genes

The genetic distances between *S. floridanus* sS lineage and the a1–a4 lineages (0.12–0.16, Table 2) are identical to those between the hares sL lineage and the *VH*a1–*VH*a4 lineages (0.10–0.16, Table 2) or between the *VH*n and the *VH*a1–*VH*a4 lineages (0.08–0.15, Table 2). The *S. floridanus* a5 lineage genetic distances to other *VH*a lineages (0.06–0.10, Table 2) are identical to the genetic distances observed between *VH*a1 and *VH*a4 lineages (0.07–0.14, Table 2). The genetic distance between the a5.1 and a5.2 variants is lower than the genetic distance between a4.1 and a4.2 variants (0.03 and 0.06, respectively).

Characterization of expressed VH in *S. floridanus*

The inferred amino acid sequences were grouped according to the phylogenetic clustering and compared to *Lepus* cDNA and rabbit germ line and cDNA *VH* genes representative of *VH*n and *VH*a allotypes as well as sequences representative of lineages a2L and sL (Fig. 2).

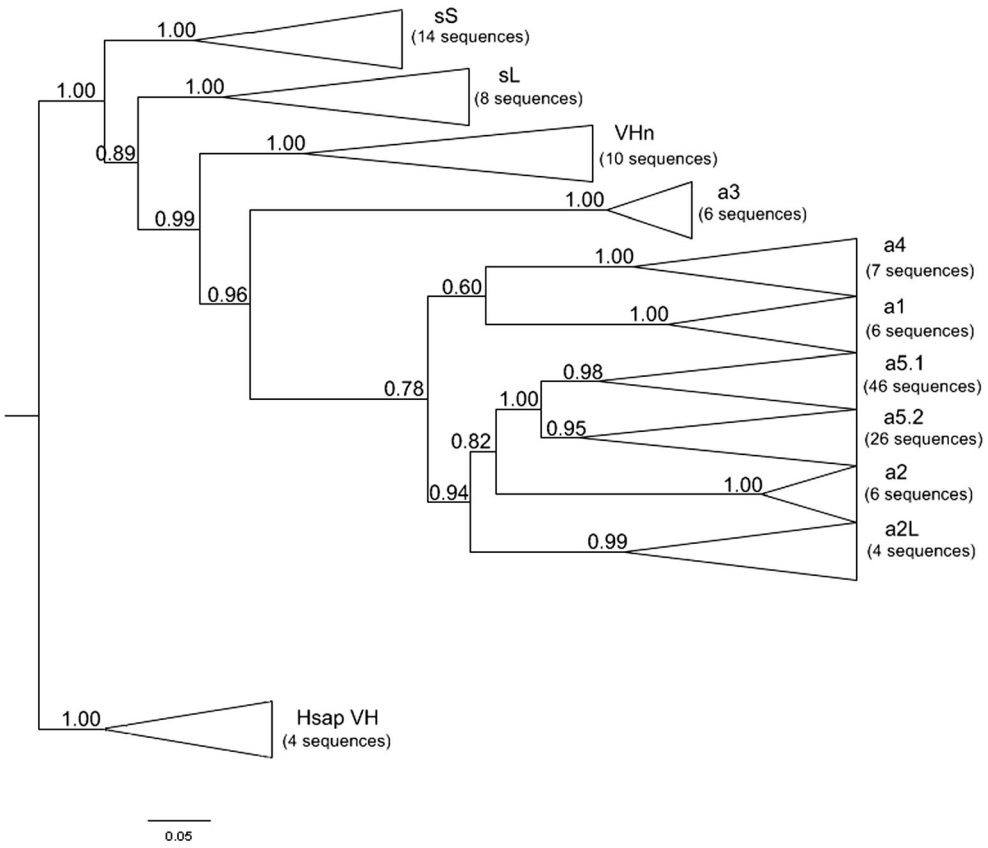


Fig. 1 Phylogenetic tree of leporid VH genes. Human VH clan 3 genes were used to root the tree. Groups were collapsed for simplification; the number of sequences in each group is indicated. The a1, a2, a3 and a4 groups include only European rabbit sequences, the a2L and sL groups include only hare sequences and the a5.1, a5.2 and sS groups include only *S. floridanus* sequences. The VHn group includes European rabbit and hares sequences. BI posterior probabilities are depicted in front of each node

The rabbit *VH*a sequences are characterized by the $_{19}$LTLTCT$_{24}$ of FR1 and $_{70}$WAK$_{72}$ of FR3 motifs as well as showing a deletion at position 2 (Mage et al. 1984). The 72 sequences obtained in this study that were classified as *VHa* have the deletion at position 2, and the majority either has the characteristic $_{70}$WAK$_{72}$ or a $_{70}$WAQ$_{72}$ motif. The $_{19}$LTLTCT$_{24}$ of FR1 is also present though the majority of the sequences have a D$_{20}$ substitution. These sequences further have as characteristics the motif $_{83}$SAG$_{85}$ and residue D$_{96}$, while also sharing the *VH*a2 I/F$_{13}$ and T$_{79}$ residues. Thus, these sequences have the rabbit *VH*a hallmark residues but have evolved some particular features which substantiate their inclusion in a new *VH*a lineage that we designate as a5. According to the phylogenetic tree, this a5 lineage has two variants, the a5.1 and a5.2, and amino acid differences exist between them that support their distinction. The $_{19}$LTLTCT$_{24}$ motif is more conserved in the sequences assigned as a5.2 while those that were classified as a5.1 have the D$_{20}$ as well as K$_{24}$ substitutions. In FR3, the $_{83}$SAG$_{85}$ D$_{96}$ motif is more conserved in the sequences that were classified as a5.1 while those identified as a5.2 have K$_{80}$, L$_{87}$, G$_{93}$ and E$_{97}$ residues that, although not a characteristic to this variant, are not present in the a5.1 variant.

The 14 sequences that were classified as a new lineage (sS) have the ancestral $_{70}$SVK$_{72}$, also present in the hares sL lineage, and the $_{2}$EQ$_{3}$ motif of rabbit *VH*x peptides. The FR3 amino acid sequence between residues 83 and 101 has nine substitutions that are characteristics to this lineage: $_{83}$DA$_{84}$, $_{87}$LE$_{88}$, D$_{93}$, $_{95}$KTT$_{97}$ and I$_{101}$.

Discussion

The generation of antibody repertoire in the European rabbit has been widely studied, and several particularities have been found. It seems to rely on a reduced number of *VH* genes: the *VH1*, which expresses the so-called *VH*a allotypic markers, and the *VH*x, *VH*y and *VH*z genes, or *VH*n genes, which do not have the *VH*a allotype specificities (Mage et al. 1984; Knight and Becker 1990). For the European rabbit, four highly divergent allelic lineages have been described for the *VH*a allotypes: a1, a2, a3 and a4 (Mage et al. 1984; Knight and Becker 1990; Esteves et al. 2004). In hares (*Lepus* sp.), the existence of a *VHa* lineage, a2L, with nucleotide and amino acid sequence similarity with the rabbit a2 lineage and which shows cross-reaction with sera against the rabbit a2 lineage has showed the trans-specific nature of the *VH*a2 polymorphism (Esteves et al. 2005). A more ancestral lineage, sL, presumably also derived from the *VH1* gene, was found to be expressed in hares (Esteves et al. 2005). More recently, Pinheiro and co-workers (2013) showed that hares and rabbits

Table 2 Genetic distances between leporid VH nucleotide sequences

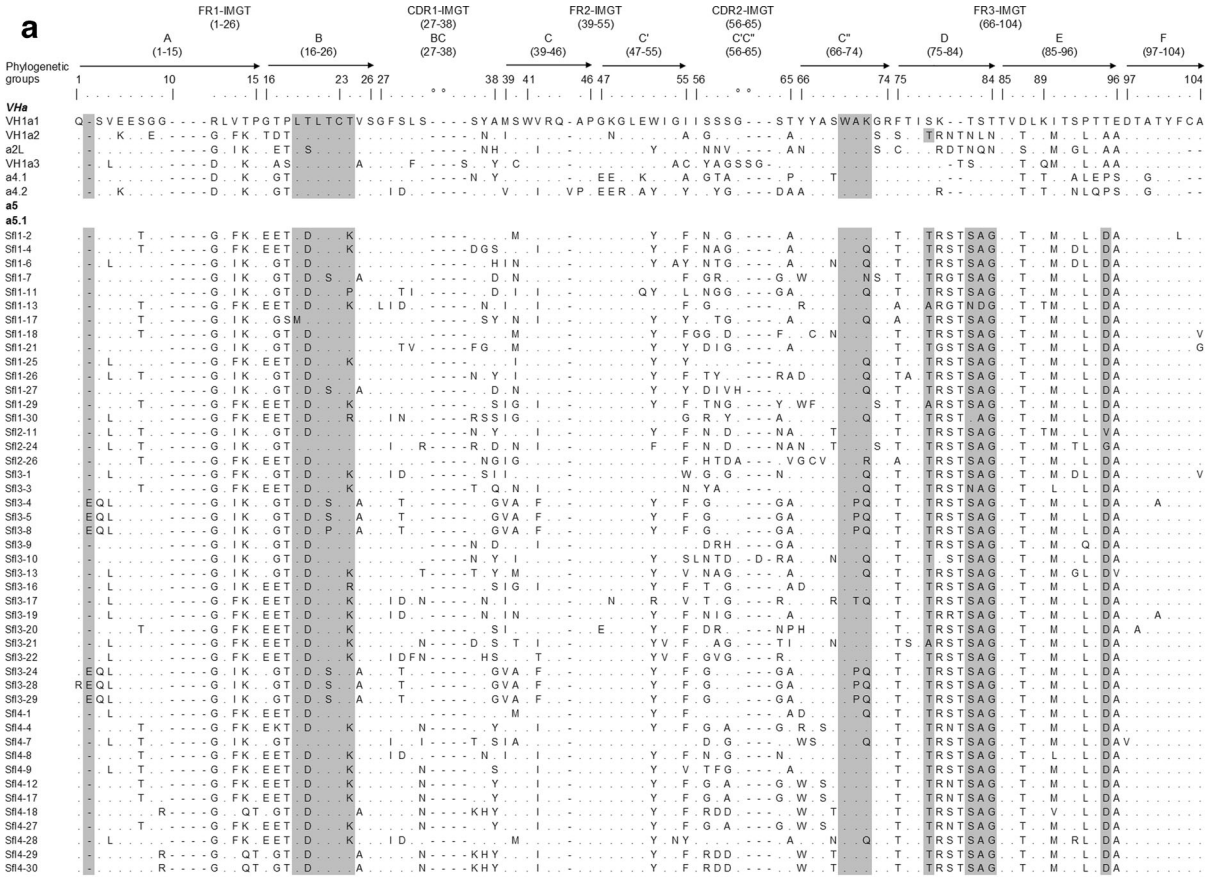
Groups			VHn	sL	sS	VHa									
						a1	a2	a2L	a3	a4	a4.1	a4.2	a5	a5.1	a5.2
VHn				*0.01*	*0.01*	*0.02*	*0.02*	*0.01*	*0.01*	*0.02*	*0.02*	*0.02*	*0.01*	*0.01*	*0.01*
sL			0.07		*0.01*	*0.02*	*0.02*	*0.02*	*0.01*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.01*
sS			0.09	0.05		*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.02*	*0.01*	*0.02*	*0.01*
VHa	a1		0.10	0.11	0.12		*0.02*	*0.01*	*0.01*	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*	*0.01*
	a2		0.15	0.16	0.16	0.11		*0.01*	*0.02*	*0.02*	*0.02*	*0.02*	*0.01*	*0.01*	*0.01*
	a2L		0.08	0.10	0.14	0.07	0.08		*0.02*	*0.01*	*0.02*	*0.02*	*0.01*	*0.01*	*0.01*
	a3		0.08	0.10	0.13	0.08	0.14	0.10		*0.02*	*0.02*	*0.02*	*0.01*	*0.01*	*0.01*
	a4		0.12	0.11	0.14	0.08	0.13	0.09	0.11		–	–	*0.01*	–	–
		a4.1	0.12	0.11	0.14	0.08	0.13	0.09	0.10	–		*0.01*	–	*0.02*	*0.02*
		a4.2	0.16	0.15	0.19	0.12	0.16	0.11	0.15	–	0.06		–	*0.02*	*0.02*
	a5		0.10	0.11	0.12	0.07	0.08	0.06	0.10	0.09	–	–		–	–
		a5.1	0.11	0.12	0.13	0.09	0.09	0.07	0.11	–	0.11	0.13	–		*0.01*
		a5.2	0.11	0.11	0.12	0.07	0.08	0.06	0.10	–	0.09	0.13	–	0.03	

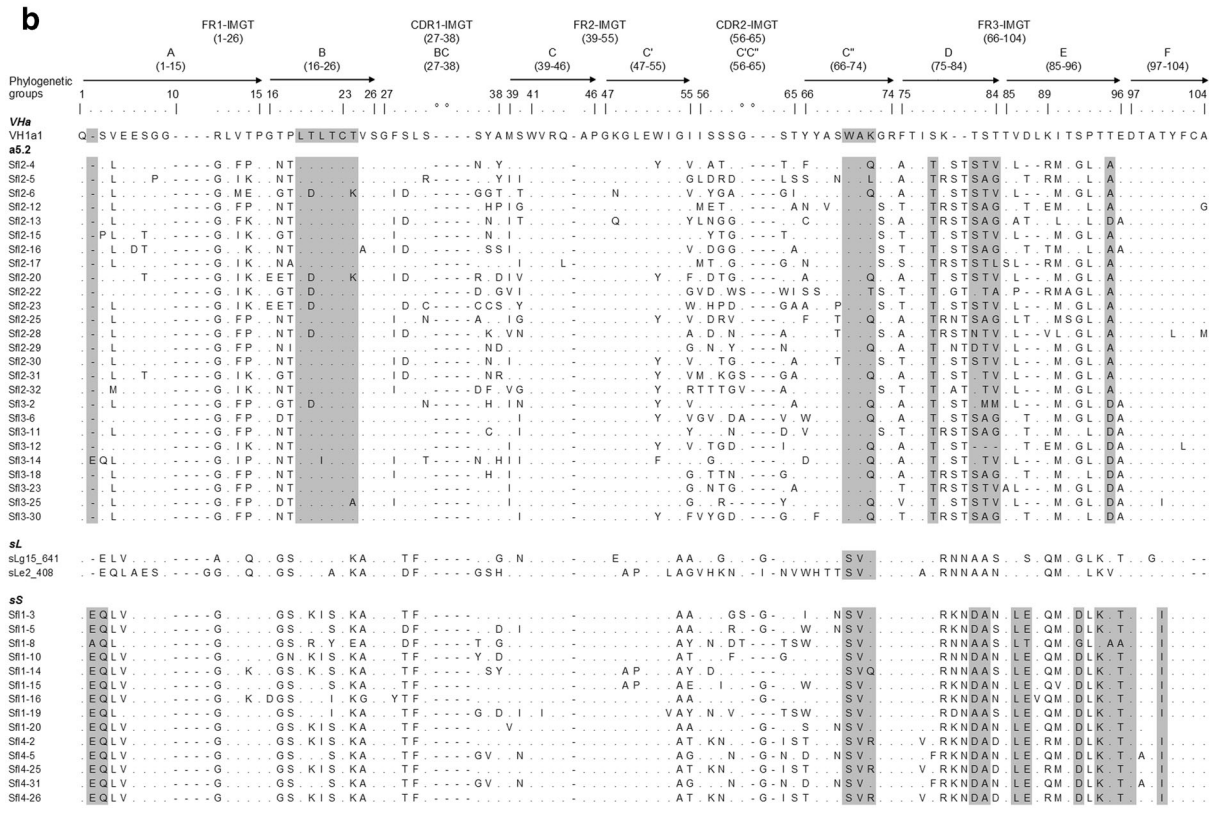
Genetic distances between groups of sequences, calculated using the p-distance, are shown below the diagonal. Standard error estimates are shown above the diagonal in italics. All ambiguous positions were removed for each sequence pair

share the low-frequency usage of the *VH*n genes in the generation of the antibody repertoire.

In this study, we observed *VH*-expressed genes for *S. floridanus* that have the rabbit *VHa* signature, lineage a5, as well as *VH*-expressed genes that belong to a more ancestral lineage, sS, that seems to be *S. floridanus* specific. In the phylogenetic tree, the *S. floridanus* a5 sequences group with rabbit and hare *VHa* lineages, clearly apart from the *S. floridanus* sS sequences, and the genetic distances also indicate that the a5 lineage is more similar to the *VHa* lineages than to the sS lineage. These results suggest that the *VHa* allotypes have an origin that pre-dates the leporid radiation. Some *VHa* lineages originated before the species split and are shared between species, such as the a2 and a2L lineages, whereas others apparently are species specific. As Esteves et al. (2004) argued, it is likely that evolutionary modes, and mutational rates, vary among *VHa* lineages having increased in the a1, a2 and a4 lineages, which would explain the large inter-lineage distances despite a more recent origin. An interesting aspect resides in the differences in number of *VHa* lineages found in each leporid species studied so far. Apparently, the rabbit has retained a greater diversity of *VHa* lineages, with at least four lineages (Mage et al. 1984; Knight and Becker 1990; Esteves et al. 2004), while hares and cottontail have just one lineage each (Esteves et al. 2005; Pinheiro et al. 2013; present study). This higher number of *VHa* lineages found in rabbit can be the result of a compensatory mechanism due to the loss of a more ancient lineage of *VH* genes, which would be the rabbit equivalent of the hares sL and cottontail sS. These ancient lineages, characterized by the motif $_{70}$SVK$_{72}$ and more highly divergent from the *VHa* lineages, can importantly contribute to the diversity of the antibody repertoire. Its loss may have imposed the diversification of the *VHa* lineages in rabbit. Contrarily to what is widely accepted, increasing evidence suggests the *VH* lineages which are rearranged in different leporid species are under different selective pressures, either driven by pathogens or by the self, though it is not evident how this selection operates (Pinheiro et al. 2013). It has also been shown that in rabbit, the diversification of the antibody repertoire requires the interaction with exogenous factors such as the gut microbiota (Lanning et al. 2000a, b; Sehgal et al. 2002), which may also exert selective pressure on the expressed *VH* lineages.

Sequencing of random germ line *VH* genes has shown that *VH*n genes are present in *S. floridanus* genome (Esteves 2003); thus, we hypothesized that, similarly to what was found for rabbit and hares (Pinheiro et al. 2013), *S. floridanus* would use these genes in a low percentage of *VDJ* rearrangements. Interestingly, despite the identical sequencing effort made for *Lepus* and *Sylvilagus* rearranged *VH* (circa 25 sequences per individual; Pinheiro et al. 2013; present study), we failed to recover rearranged *VDJ* genes with characteristics of rabbit and hares *VHn* genes in the studied *S. floridanus* specimens. *S. floridanus* may not use the *VHn* genes in the generation of the antibody repertoire or may do so in such a low percentage (under 4 %) that the sequencing effort would have to be greatly increased to allow its detection. The usage of *VH*n genes in low frequency both in rabbit (Kim and Dray 1973; Roux 1981) and hares shows that, in leporids, the *VH* lineages used to generate the antibody repertoire are subject to selective pressure (Pinheiro et al. 2013). The rabbit and hare species for which the expression


a
FR1-IMGT (1-26)
A (1-15)
B (16-26)
CDR1-IMGT (27-38)
BC (27-38)
FR2-IMGT (39-55)
C (39-46)
C' (47-55)
CDR2-IMGT (56-65)
C'C" (56-65)
FR3-IMGT (66-104)
C" (66-74)
D (75-84)
E (85-96)
F (97-104)
Phylogenetic groups
VHa
a5
a5.1


b
FR1-IMGT (1-26)
A (1-15)
B (16-26)
CDR1-IMGT (27-38)
BC (27-38)
FR2-IMGT (39-55)
C (39-46)
C' (47-55)
CDR2-IMGT (56-65)
C'C" (56-65)
FR3-IMGT (66-104)
C" (66-74)
D (75-84)
E (85-96)
F (97-104)
Phylogenetic groups
VHa
a5.2
sL
sS
VHn

◀ **Fig. 2** Alignment of leporid *VH* protein sequences. **a** European rabbit and hares VHa lineages and *S. floridanus* a5.1 lineage. **b** *S. floridanus* a5.2 and sS lineages, hares sL lineage and European rabbit and hares VHn lineage. *Dashes* represent alignment gaps. *Dots* indicate identity with the master sequence except at gap positions. The "IMGT Protein display for V domain" header is shown (Lefranc et al. 2003). Sequences are grouped into phylogenetic groups according to the tree in Fig. 1. Shown are representatives of European rabbit VH1a1, VH1a2, VH1a3, Vhx, Vhy and Vhz germ line sequences (GenBank accession numbers M93171, M93172, M93173, L03846, L03890 and AF264469, respectively); European rabbit a4.1 and a4.2 cDNA sequences (GenBank accession numbers AY207980 and AY207967) and hares a2L and sL cDNA sequences (GenBank accession numbers AY288450, AY288465 and AY288459). Also shown are all *S. floridanus* cDNA sequences obtained in this study that were grouped into the a5.1, a5.2 and sS lineages (for GenBank accession numbers please see Table 1). Highlighted in *grey* are the *VH*a deletion at position 2 and motifs $_{19}$LTLTCT$_{24}$ and $_{70}$WAK$_{72}$, and sL/sS sequences $_{70}$SVK$_{72}$ motif. Also highlighted are residue characteristics of a5 and sS lineages

of *VHn* has been shown, *L. europaeus*, are European leporids while *S. floridanus* is an American leporid (Chapman et al. 1980; Flux and Angermann 1990). Interestingly, two related caliciviruses affect the European rabbit and two hare species of Eurasian distribution (*L. europaeus* and *Lepus timidus*) but not cottontails (Abrantes et al. 2012; Lopes et al. 2013). Thus, we can put forward the hypothesis that the different continents' pathogen community may impose different selective pressures and favour the expression of *VH*n only in European species.

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