Molecular Immunology 112 (2019) 182–187

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Sequencing of VDJ genes in *Lepus americanus* confirms a correlation between *VH*n expression and the leporid species continent of origin

Ana Pinheiro[a,*], Patricia de Sousa-Pereira[a,b,c], Tereza Almeida[a,b], Catarina C. Ferreira[d,e], Josée-Anne Otis[d], Melanie R. Boudreau[d], Jacob L. Seguin[d], Dennis K. Lanning[f], Pedro J. Esteves[a,b,g]

[a] CIBIO Centro de Investigação em Biodiversidade e Recursos Genéticos, InBio Laboratório Associado, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
[b] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4169-007, Porto, Portugal
[c] Max von Pettenkofer-Institute for Virology, Ludwig-Maximilians-University Munich, Munich, Germany
[d] Department of Biology, Trent University, Peterborough, Ontario, Canada
[e] UFZ - Helmholtz Centre for Environmental Research, Department of Conservation Biology, Leipzig, Germany
[f] Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, United States
[g] Centro de Investigação em Tecnologias da Saúde, IPSN, CESPU, 4585-116, Gandra, Portugal

ARTICLE INFO

Keywords:
Leporids
Lepus americanus
IGHV
Adaptive selection

ABSTRACT

Leporid VH genes used in the generation of their primary antibody repertoire exhibit highly divergent lineages. For the European rabbit (*Oryctolagus cuniculus*) four *VH*a lineages have been described, the a1, a2, a3 and a4. Hares (*Lepus* spp.) and cottontail (*Sylvilagus floridanus*) express one *VH*a lineage each, the a2L and the a5, respectively, along with a more ancient lineage, the *Lepus* spp. sL and *S. floridanus* sS. Both the European rabbit and the *Lepus europaeus* use a third lineage, *VH*n, in a low proportion of their *VDJ* rearrangements. The *VH*n genes are a conserved ancestral polymorphism that is being maintained in the leporid genome.Their usage in a low proportion of *VDJ* rearrangements by both European rabbit and *L. europaeus* but not *S. floridanus* has been argued to be a remnant of an ancient European leporid immunologic response to pathogens. To address this hypothesis, in this study we sequenced *VDJ* rearranged genes for another North American leporid, *L. americanus*. Our results show that *L. americanus* expressed these genes less frequently and in a highly modified fashion compared to the European *Lepus* species. Our results suggest that the American leporid species use a different *VH* repertoire than the European species which may be related with an immune adaptation to different environmental conditions, such as different pathogenic agents.

1. Introduction

Immunoglobulin gene diversity in the European rabbit (*Oryctolagus cuniculus*) has been well studied. In the early 1960's, serological surveys in domestic breeds defined three allotypic immunoglobulin heavy chain variable region (IGHV) lineages, the so-called VHa allotypes a1, a2 and a3 (Dray et al., 1963; Oudin, 1960). VH allotypic markers have not been found in other species, and rabbit IGHV allotypes were puzzling for many years since they are inherited in a Mendelian fashion and homozygous VHa rabbits were found to express distinct *VH* genes. Some of these were devoid of VHa allotype-specific determinants (the so-called VHa negative or VHn, (VHx, VHy, and VHz); Horng et al., 1980). The rabbit derives 80–90% of circulating immunoglobulin (Ig) molecules from the *VH1* gene, the most D-proximal *VH* gene (Knight, 1992; Knight and Becker, 1990), despite having more than 200 *VH* genes (*e.g.* Ros et al., 2004). Since VH1 expresses the VHa allotypic markers (*e.g.* Kindt, 1975; Margolies et al., 1977) this explains the Mendelian inheritance of *VH*a genes. The remaining 10–20% of Ig molecules are encoded by the *VH*n genes (Kim and Dray, 1973; Roux, 1981) which are localized at least 100 Kb upstream of *VH*1 (Mage et al., 2006). A fourth and equally divergent allotypic lineage, a4, was described in wild European rabbit Iberian populations that had no cross-reaction to rabbit anti a-locus allo-antisera (Esteves et al., 2004).

Studies on *IGHV* gene diversity have been extended to some other leporids. Early serologic analysis of *Lepus americanus* showed cross-reaction with rabbit anti-a2 and anti-a3 antisera (reviewed in van der

* Corresponding author.
E-mail address: ana.pinheiro@cibio.up.pt (A. Pinheiro).

https://doi.org/10.1016/j.molimm.2019.05.008
Received 6 November 2018; Received in revised form 12 March 2019; Accepted 27 May 2019

Loo, 1987). For *Lepus europaeus* and *Lepus granatensis*, two serological phenotypes were described: partial reaction to anti-a2 antisera and no reaction to any rabbit antiserum (Esteves, 2003). Sequencing of rearranged VH genes showed that, in the generation of their antibody repertoire, these two *Lepus* species use three *VH* lineages: i) the a2L lineage, a trans-specific polymorphism to the rabbit VH1-a2 allele; ii) the sL lineage, an ancestral and apparently *Lepus*-specific lineage showing distinctive characters (Esteves et al., 2005); and iii) also, like European rabbits, the *VHn* genes in 5–10% of *VDJ* rearrangements (Pinheiro et al., 2013). Interestingly, for *Sylvilagus floridanus*, the expression of *VHn* was not observed and only two lineages were found among rearranged *VH* genes: a fifth and equally divergent *VH*a lineage, a5, and an ancient lineage, sS, related to the hares' sL (Pinheiro et al., 2014). Taken together these findings suggest that the leporid *VH* genes are subject to strong selective pressure likely imposed by specific pathogens (Pinheiro et al., 2014, 2016). The *VHn* genes are a conserved ancestral polymorphism that is being maintained in the leporids genome and their usage in a low proportion of *VDJ* rearrangements by both European rabbit and *L. europaeus*, but not *S. floridanus*, has been argued to be a remnant of an ancient European leporid immunologic response to pathogens (Pinheiro et al. 2013, 2014).

In this study, we addressed the hypothesis that the pathogen community of different continents may impose different selective pressures and favour the expression of *VHn* only in European species. The family Leporidae comprises 11 genera, which include *Oryctolagus*, *Lepus* and *Sylvilagus* (Alves and Hackländer, 2008); *Sylvilagus* diverged from *Oryctolagus* 10 million years ago and *Lepus* diverged from both *Oryctolagus and Sylvilagus* 12 million years ago (Matthee et al., 2004). Ancestral *Lepus* originated in North America and afterwards expanded into Eurasia; the dispersal events resulted in a rapid radiation of the genus (Wu et al., 2005). *Oryctolagus* and the *Lepus* species, *L. europaeus*, for which a low proportion of the *VHn* expression has been observed, inhabit the Eurasian continent while *Sylvilagus* is a Native American leporid (Chapman et al., 1980; Flux and Angermann, 1990). Here, to test our hypothesis we sequenced rearranged *VDJ* genes from another Native American leporid, *L. americanus* (Chapman et al., 1980; Flux and Angermann, 1990).

2. Material and methods

2.1. Samples, amplification and sequencing of rearranged VDJ genes

Spleen samples from *Lepus americanus* specimens were collected by local hunters. Samples were collected immediately post-mortem, placed in RNAlater and stored at -20 °C for further analysis. In total, we sampled 9 specimens, 8 in Canada (5 - LamKYU, Lam1675, Lam28961, Lam28997, Lam191030 - in the Yukon and 3 – Lam1, Lam2, Lam3 - in Quebec) and 1, LamINEZ, in the United States (Montana). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, followed by first-strand cDNA synthesis with the SuperScriptTM III Reverse Transcriptase Kit (Invitrogen) using 1 µg RNA. Rearranged VDJ genes were PCR amplified using primers VH (5′GGAGACTGGGCTGCGCTGGCTTCTCCT GGT3′; Esteves et al., 2005) and JH2 (5′TGAGGAGACGGTGACCAGGG TGCCT3′; Pinheiro et al., 2013). PCR amplification was performed using the PCR Master Mix (Promega) with annealing temperature of 62 °C for 45 s and 1 min extension, for 35 cycles. PCR products were purified (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel, Germany) and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI, USA). For each specimen, thirty clones were selected. Sequencing was performed on an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems).

2.2. Phylogenetic analyses

Of the sequences obtained in this study, those containing insertions and deletions (indels) or stop codons in the *V* and *J* segments were eliminated from the dataset. Additionally, redundant sequences were also eliminated. The remaining 216 unique *VDJ* gene sequences thus obtained were aligned with available sequences for leporid *VDJ* genes taken from GenBank using CLUSTAL W (Thompson et al., 1994) as implemented in BioEdit v7.2.5 (Hall, 1999) and the amino acid sequences inferred. Used sequences include rabbit germ line VH1 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* and *S. floridanus* cDNA VH gene sequences representative of lineages a2L, a5, sL and sS. VH genes of the human VH3 family were used as outgroup. Accession numbers for all sequences are given in Table 1.

The evolutionary relationships between leporid VH amino acid sequences were analysed in a Bayesian inference (BI) framework using MrBayes v3.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). All priors were set to the defaults and the MCMC chains were run for $60*10^6$ generations, sampling every 6000 generations and discarding the first 25% trees. The amino acid evolution model was not *a priori* defined; instead MrBayes searched the best fitting model during

Table 1
Accession numbers for VH gene 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
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	AY207979- AY207982
a4.2_I- III	AY208042, AY208047, AY207967
Vhx_Oca, Vhx_Oca2	AY207986, AY208045
Vhy_Oca	AY208006
Lepus 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_Lg1983, a2_Lg1986, 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,
S. floridanus cDNA sequences	
a5 Sfl1.26, Sfl3.10	KM275513, KM275544
sS Sfl1.14, Sfl4.31	KM275503, KM275580
Human VH3 family sequences	
Hsap1- Hsap4	M99666, M99672, M99679, M99682
Sequences in the current study	
LamKYU	MK145040-MK145042, MK145044-MK145067
Lam1675	MK144837-MK144852, MK144854-MK144860, MK144862, MK144864
Lam28961	MK144914-MK144928, MK144930-MK144937, MK144939-MK144942
Lam28997	MK144943-MK144952, MK144954-MK144958, MK144960-MK144970
Lam191030	MK144866-MK144868, MK144870-MK144872, MK144874-MK144889
Lam1	MK144890-MK144900, MK144902-MK144908, MK144910-MK144913
Lam2	MK144971-MK144984
Lam3	MK144985-MK145011
LamINEZ	MK145012-MK145039

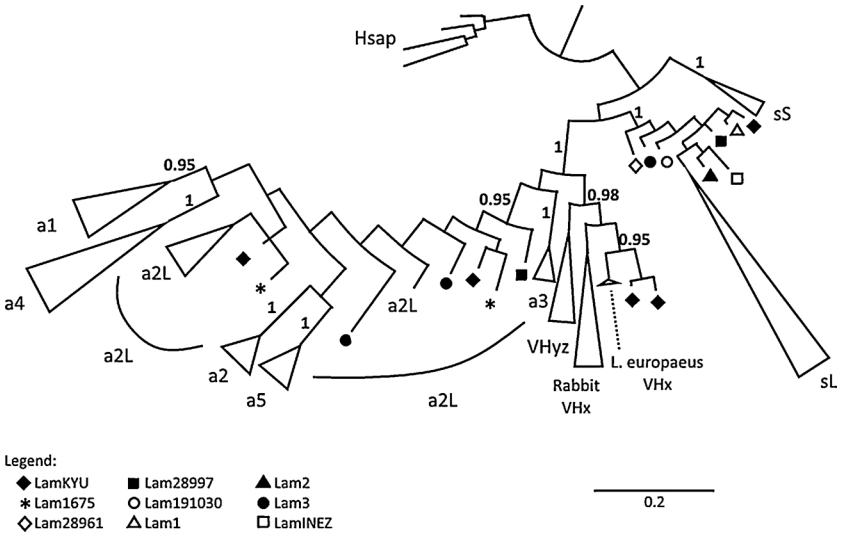

Fig. 1. Phylogenetic tree of leporid VH genes. Human VH clan 3 genes were used to root the tree. Some groups were collapsed for simplification; the sequences used in this analysis were all taken from GenBank, indicated in Table 1, and the consensus sequences obtained in the current study, as described in Material and Methods. The a1, a2, a3 and a4 groups include only European rabbit sequences, the a2L and sL groups include only hare sequences (*L. europaeus*, *L. granatensis* and *L. americanus*) and the a5 and sS groups include only *S. floridanus* sequences. The VHyz group includes European rabbit and *L. europaeus* sequences. *L. americanus* expressed VH genes obtained in this study for each individual are identified with symbols as indicated in the legend. BI posterior probabilities are depicted above each node.

the run. Convergence was checked using Tracer v1.6 (Rambaut et al., 2018) and the tree was visualized with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

The obtained BI trees showed both low node support and polytomies, a pattern interpreted as reflecting the high variability of VH gene sequences. To overcome this, a Neighbor-Joining (NJ) tree was obtained in MEGA6 (Tamura et al., 2013), using the p-distance, to group sequences according to their similarity. The NJ tree grouped the *L. americanus* sequences into four clusters: sL lineage similar sequences, *VH*a2-like sequences, *VH*a sequences showing signs of gene conversion with other lineages and *VH*n sequences. The sL lineage cluster included sequences of all *L. americanus* individuals except Lam1675; the a2 lineage cluster included LamKYU, Lam28997, Lam1675 and Lam3 sequences; the cluster of VHa sequences showing signs of gene conversion with other lineages included LamKYU, Lam1675 and Lam3 sequences and the VHn cluster included two sequences of LamKYU. Consensus sequences, calculated as the most frequent nucleotide residue at each position, were then obtained for each group of similar sequences and each specimen, except for the two LamKYU sequences that classified as Vhx. The following consensus sequences were obtained: LamKYU_consensus_a2Lx, LamKYU_consensus_sL, Lam1675_consensus_a2Lx, Lam1675_consensus_a2L, Lam28961_consensus_sL, Lam28997_consensus_a2L, Lam28997_consensus_sL, Lam191030_consensus_sL, Lam1_consensus_sL, Lam2_consensus_sL, Lam3_consensus_a2Lx, Lam3_consensus_sL and LamINEZ_consensus_sL. Consensus amino acid sequences and originating sequences are given in supplementary material 1. Additional *L. americanus* sequences included in the consensus dataset are LamKYU_15, LamKYU_cln25, LamKYU_cln29 and Lam3_2.

BI phylogenetic analyses were performed for the consensus sequences dataset using the specifications above except that MCMC chains were run for $10*10^6$ generations, sampling every 1000 generations.

2.3. Genetic distances

Genetic distances between VH genes among species were calculated using the “compute net average distance between groups” option of MEGA6.0 software (Tamura et al., 2013). This option corrects for variance due to differences within groups, as expected in rearranged VH genes which are somatically diversified.

3. Results and discussion

The existence of IGHV allelic lineages is a feature unique to the European rabbit and other related leporids. Furthermore, each leporid seems to express its own set of VH allelic lineages: four of these lineages have been described for rabbit, a1-a4, (Esteves et al., 2004; Knight and Becker, 1990; Mage et al., 1984), two lineages, the a2L and sL, were described for the European *Lepus* spp., and the American *Sylvilagus floridanus* also seems to use two VH lineages, the a5 and sS (Esteves et al., 2005; Pinheiro et al., 2013, 2014). The *VH*n genes, also unique to leporids, are used in low proportion in the generation of the antibody repertoire by both the European rabbit and *Lepus europaeus* (Pinheiro et al., 2013), but seem to not be used by *S. floridanus* (Pinheiro et al., 2014), despite being present in the germline (Esteves, 2003). These findings suggest that the VH lineages which are rearranged in different leporid species are under selective pressure, possibly imposed by the pathogen community of the different continents. In fact, American and European leporids show varying susceptibilities to the same pathogens. The myxoma virus that causes a benign infection in Native American *Sylvilagus* leporids, such as *S. floridanus*, when introduced in Europe became pathogenic for the European rabbit. It has also been suggested that the *S. floridanus* introduction in Europe could have caused the emergence of two related Caliciviruses affecting the European rabbit and two hare species of Eurasian distribution (*L. europaeus* and *L. timidus*), but not *S. floridanus* (Esteves et al., 2015).

To address the hypothesis that the pathogen community of the two different continents may have imposed or still imposes different selective pressures and favour the expression of *VHn* only in European species, we sequenced rearranged *VDJ* genes from *Lepus americanus*, a Native American leporid. The BI phylogenetic tree using consensus sequences shows clusters of the previously described rabbit, *Lepus* spp. and *S. floridanus* lineages mostly with good support on the phylogenetic tree (Fig. 1): the *S. floridanus* sS lineage (1.00 posterior probability), the *Lepus* sL lineage (0.99 posterior probability,) the *VH*n lineages, *VH*x and *VH*yz, (0.98 posterior probability), and the *VH*a lineages *VH*a1, *VH*a2, *VH*a3, *VH*a4 and *VH*a5 (0.95, 1.00, 1.00, 1.00 and 1.00 posterior probabilities, respectively). The *Lepus VH*a2L lineage sequences group within the *VH*a1, *VH*a2, *VH*a4 and *VH*a5 cluster (0.95 posterior probability). *L. americanus* consensus sequences fall into these 3 groups: the sL lineage cluster, the VHa cluster, and a *Lepus* VHx cluster (1.00, 0.95 and 0.95 posterior probabilities, respectively). In fact, the *Lepus* VHx cluster groups *L. europaeus* and two *L. americanus* sequences. The calculated genetic distances (Table 2) are in agreement with the major clusters obtained in the phylogenetic tree and further support the classification of the two *L. americanus* sequences as VHx. These two sequences were obtained for specimen LamKYU that additionally has sequences classified as sL and a2L (23-3, respectively). As such, it seems that *L. americanus* may use the VHx genes in a low proportion of *VDJ* rearrangements (7%; this study) similarly to rabbit (10–20%; Kim and

Table 2
Genetic distances between leporids VH amino acid sequences.

Groups		VHa						VHn		sL	sS	*L. americanus*		
		a1	a2	a2L	a3	a4	a5	VHx	VHyz			a2L	a2Lx	Vhx
VHa	a2	0.22												
	a2L	0.16	0.15											
	a3	0.16	0.27	0.21										
	a4	0.14	0.26	0.19	0.20									
	a5	0.19	0.21	0.15	0.23	0.22								
VHn	VHx	0.20	0.31	0.19	0.18	0.25	0.26							
	VHyz	0.23	0.34	0.22	0.18	0.27	0.28	0.07						
sL		0.20	0.31	0.23	0.21	0.22	0.28	0.15	0.18					
sS		0.22	0.33	0.27	0.23	0.28	0.28	0.13	0.17	0.06				
L. americanus														
	a2L	0.11	0.16	0.04	0.16	0.16	0.15	0.16	0.16	0.17	0.20			
	a2Lx	0.16	0.23	0.08	0.16	0.22	0.19	0.10	0.11	0.15	0.17	0.06		
	VHx	0.27	0.37	0.24	0.22	0.31	0.33	0.09	0.15	0.20	0.23	0.21	0.14	
	sL	0.20	0.30	0.21	0.18	0.25	0.26	0.13	0.15	0.03	0.08	0.15	0.13	0.17

Dray, 1973; Roux, 1981) and *L. europaeus* (5–10%; (Pinheiro et al., 2013). As for the remaining studied hares, the totality of the sequences obtained for specimens Lam28961, Lam191030, Lam1, Lam2 and LamINEZ classified as sL while all Lam1675 sequences classified as a2L. For Lam 28997 and Lam3, sequences were obtained that classified as sL and as a2L (21-3 and 20-6, respectively).

We then grouped the *L. americanus* inferred amino acid sequences according to the phylogenetic clustering and compared these to *Lepus, Sylvilagus* and rabbit cDNA, as well as rabbit germline *VH* genes representative of *VH*n and *VH*a allotypes and sequences representative of lineages a2L, sL and sS (Fig. 2; Supplementary Table 1). The rabbit *VH*n sequences show the distinctive $_{70}$WVN$_{72}$ motif and Q$_3$, K/E$_{20}$ residues. The two *L. americanus* sequences that classified as *VH*x show the typical $_{70}$WVN$_{72}$ motif as well as the $_{83}$AQN$_{85}$ motif of rabbit *VH*x and *Lepus* VHn genes but have E$_3$ and T$_{20}$ instead of Q$_3$ and K/E$_{20}$, residues characteristic of the sL lineage. In fact, close inspection of these two sequences reveals these are products of recombination between sL and *VH*n genes, in which the FR1 is of the sL lineage genes and the remaining VH sequence is of *VH*n lineage (Fig. 2). This feature remains unobserved for other leporids *VH*n genes (Pinheiro et al., 2013). Although possible, it is highly unlikely that these sequences represent PCR chimaeras. This PCR methodology has been previously used to amplify leporids *VDJ* genes (Pinheiro et al., 2013, 2014) and no chimaera formation has been observed so far nor have we identified possible PCR chimaeras in the remaining analysed *L. americanus*. Thus, despite having found genes with *VH*n characteristics in the *L. americanus* rearranged *VDJ* genes, these were found for only one of the nine studied *L. americanus*. Using a similar sequencing effort *per* sample, Pinheiro et al (2013) found *VH*n genes in all studied *L. europaeus* showing no signs of gene conversion. Thus, comparing our present results to the *L. europaeus VH*n usage it seems that *L. americanus VH*n usage in VDJ rearrangements is much less frequent and, when used, the *VHn* genes undergo gene conversion events, despite being used in a similarly low proportion of the *VDJ* gene rearrangements. This shows that the *VH*n usage in *VDJ* rearrangements may in fact be under selective pressure since there seems to be a correlation between *VH*n expression and the

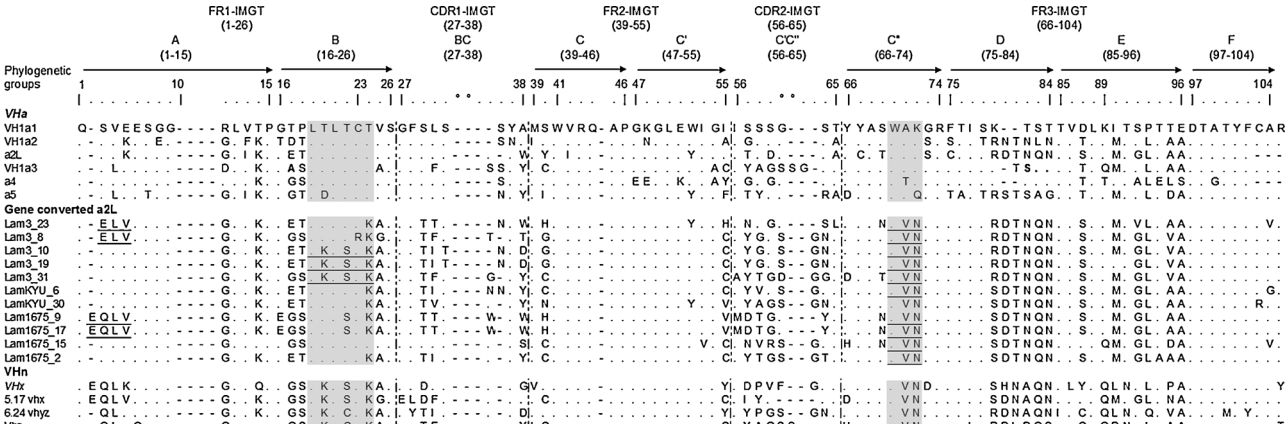

Fig. 2. Alignment of leporid *VH* protein sequences. Dashes (-) represent alignment gaps. Dots (·) indicate identity with the master sequence except at gap positions. The 'IMGT Protein display for V domain' header is shown (Lefranc et al, 2003). Shown are representatives of European rabbit VH1a1, VH1a2, VH1a3, Vhx and Vhz germline sequences (Genbank accession numbers M93171, M93172, M93173, L03846 and AF264469, respectively), European rabbit a4 and Vhy cDNA sequences (Genbank accession numbers AY207979 and AY208006), hare a2L and sL cDNA sequences (Genbank accession numbers AY288494 and KF460076) and *S. floridanus* a5 and sS cDNA sequences (Genbank accession numbers KM275513 and KM275580), as well as the sequences obtained in this study that classified as VHn (Genbank accession numbers MK145063 and MK145066) and the sequences classified as a2L that show signs of gene conversion (Genbank accession numbers MK144838, MK144845, MK144851, MK144853, MK144990, MK144992, MK145000, MK145003, MK145010, MK145045, MK145067). Highlighted in grey are the *VH*a $_{70}$WAK$_{72}$, *VH*n $_{70}$WVN$_{72}$ and sL/ sS lineages $_{70}$SVK$_{72}$ and *VH*a $_{19}$LTLTCT$_{24}$, *VH*n $_{19}$LKLS/CCK$_{24}$ and sL/ sS lineages $_{19}$LTLTCK$_{24}$ motifs. For the gene-converted a2L sequences, motifs obtained through gene conversion are underlined. For the *L. americanus* VHn sequences (LamKYU_29 and LamKYU_25), the sL FR1 sequence is underlined and the VHn sequence is double underlined.

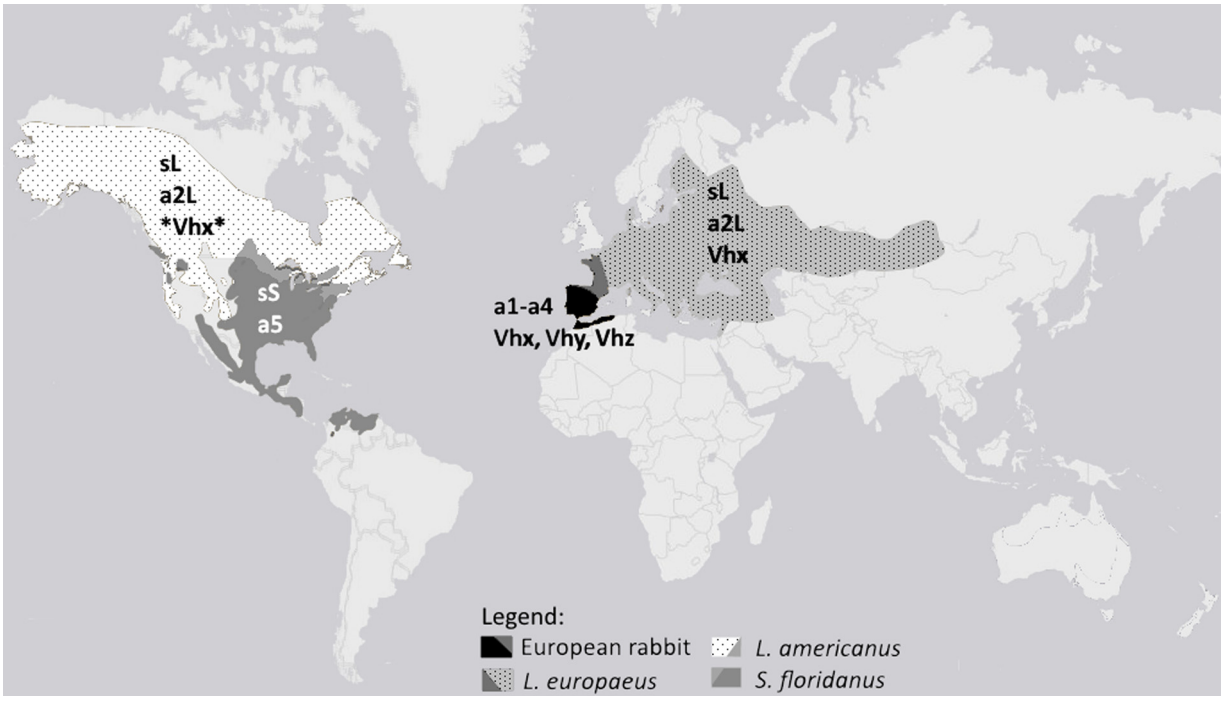

Fig. 3. Map of studied leporids distribution and respective VH lineages. The ancestral geographic distribution of the four leporid species for which the VH lineages have been studied is depicted: European rabbit (black), *L. europaeus* (speckled light grey), *L. americanus* (speckled white) and *S. floridanus* (dark grey). Areas of overlapping distribution are indicated in an intermediate shade. (distribution ranges were adapted from the IUCN Red List of Threatened Species. Version 2018-2. www.iucnredlist.org). The VH lineages that have been identified for each species are indicated (Dray et al., 1963; Esteves et al., 2004, 2005; Horng et al., 1980; Oudin, 1960; Pinheiro et al., 2013, 2014; this study).

continent of origin of the leporid species: European species, rabbit and *L. europaeus*, typically express the *VH*n genes in a similar low proportion of their *VDJ* rearrangements (Kim and Dray, 1973; Roux, 1981; Pinheiro et al., 2013), whereas North American species, *S. floridanus* and *L. americanus*, do not express the *VH*n genes or express gene-converted *VH*n genes very infrequently (Pinheiro et al., 2014; present study) (Fig. 3).

Considering that, for *L. americanus,* serologic cross-reaction with rabbit anti-a2 and anti-a3 antisera was observed (reviewed in van der Loo, 1987) we expected to identify the a2L lineage in the obtained *L. americanus* sequences, as well as a lineage related to the rabbit VHa3. Even though we sampled *L. americanus* across its geographic distribution no *L. americanus* sequences grouped in the rabbit *VH*a3 cluster on the phylogenetic tree (Fig. 1). Characteristic amino acids for the a1, a2 and a4 lineages have been described but none for the a3 lineage (reviewed in Pinheiro et al., 2011). Compared to the other *VH*a lineages, the a3 sequences are less derived (Esteves et al., 2005) and even share some characteristic residues with the *VH*n lineages, namely C_{40}, C_{55}, Y_{57} and S_{60}. The majority of the 42 sequences obtained in this study that classified as a2L have the *VH*a characteristic $_{19}$LTLTCT$_{24}$ of FR1 and $_{70}$WAK$_{72}$ of FR3 motifs as well as S_3, T_{18}, R_{80} and N_{83} residues, as previously described for the a2L lineage (Esteves et al., 2005; Pinheiro et al., 2013) but have E_5 instead of K_5 residues (Supplementary Table 1). Of these sequences, 11 show the *VH*n motif $_{70}$WVN$_{72}$ along with some residues characteristic of other lineages such as lineage sL E_3, lineage *VH*n Q_3, K_{20} and lineages *VH*a3 and *VH*n C_{55}, Y_{57} and S_{60}. Thus, a high percentage (27.5%) of the a2L sequences identified in this study share some of these residues with both the *VH*n and *VH*a3 lineages. The cross reaction to rabbit anti-a3 sera observed for *L. americanus* individuals (reviewed in van der Loo, 1987) may be due to this sharing of residues between a2L sequences and the *VH*a3 lineage.

Overall, our results indicate that although some features of leporid *VH* gene usage are genus-specific, in their in *VDJ* rearrangements all studied *Lepus* spp. characteristically use the sL and a2L lineages, *Sylvilagus* uses the sS and a5 lineages and rabbit uses the a1, a2, a3 and a4 lineages, other features seem to be the product of natural selection, such as the usage of the *VH*n genes. *VH*n genes are a conserved ancestral polymorphism that has been maintained in the leporid genome; their usage in a low proportion of the *VDJ* rearrangements by the European rabbit and European *Lepus* species has been argued to be a remnant of an ancient leporid immunologic response to pathogens (Pinheiro et al., 2013). During leporid evolutionary history the VHn genes must have been important in the immune response against some pathogens and, hence, their retention and occasional usage in 5–20% of *VDJ* rearrangements. The *VH*n were not expressed in *Sylvilagus* (Pinheiro et al., 2014) and our results showed that *L. americanus* expressed these genes less frequently and in a highly modified fashion compared to the European *Lepus* species. Collectively, our findings suggest that the American leporid species use a different *VH* repertoire than the European species, which may be related with an immune adaptation to different environmental conditions, such as different pathogenic agents.

Acknowledgements

The authors would like to thank to the local hunters for kindly providing the Canadian samples and to Paulo C. Alves for kindly providing the Montana sample. Funding was provided by the FEDER Funds through the Operational Competitiveness Factors Program − COMPETE and by National Funds through FCT - Foundation for Science and Technology within the scope of the project "POCI-01-0145-FEDER-029667". FCT also supported the doctoral fellowships of PSP and TA (ref. PD/BD/52602/2014 and SFRH/BD/108253/2015) and the post-doctoral fellowship of AP (ref. SFRH/BPD/117451/2016). CCF was supported by a Marie Curie Outgoing International Fellowship for Career Development (PIOF-GA-2013-621571) within the 7th Framework Programme of the European Union. This article is a result of the project NORTE-01-0145-FEDER-000007, supported by Norte

Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.05.008.

References

Alves, P.C., Hackländer, K., 2008. Lagomorph species: geographical distribution and conservation status. In: Alves, P.C., Ferrand, N., Hackländer, K. (Eds.), Lagomorph Biology. Springer, Berlin Heidelberg, pp. 395–405.

Chapman, J.A., Hockman, J.G., Ojeda, M.M., 1980. Sylvilagus floridanus. Mamm Species 136, 1–8.

Dray, S., Young, G.O., Nisonoff, A., 1963. Distribution of allotypic specificities among rabbit gamma-globulin molecules genetically defined at two loci. Nature 199, 52–55.

Esteves, P.J., 2003. Molecular and Population Genetic Analysis of Polymorphism at the Antibody Loci IgGCH2 and IgVH in Lagomorphs Faculdade De CIências. Universidade do Porto.

Esteves, P.J., Lanning, D., Ferrand, N., Knight, K.L., Zhai, S.K., van der Loo, W., 2004. Allelic variation at the VHa locus in natural populations of rabbit (Oryctolagus cuniculus, L.). J. Immunol. 172, 1044–1053.

Esteves, P.J., Lanning, D., Ferrand, N., Knight, K.L., Zhai, S.K., van der Loo, W., 2005. The evolution of the immunoglobulin heavy chain variable region (IgVH) in Leporids: an unusual case of transspecies polymorphism. Immunogenetics 57, 874–882.

Esteves, P.J., et al., 2015. Emergence of Pathogenicity in Lagoviruses: Evolution from Pre-existing Nonpathogenic Strains or through a Species Jump? PLoS Pathog. 11, e1005087. https://doi.org/10.1371/journal.ppat.1005087.

Flux, J.E.C., Angermann, R., 1990. The Hares and Jackrabbits. In: Chapman JA, Flux JEC (eds) Rabbits, Hares and Pikas: Status Conservation Action Plan. International Union for Conservation of Nature and Natural Resources, Gland, Switzerland, pp. 61–94.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Horng, W.J., Papagiannes, E., Dray, S., Rodkey, L.S., 1980. Expression of cross-reacting determinants of the immunoglobulin heavy chain variable region a3 allotype in Oryctolagus and Lepus. Mol. Immunol. 17, 111–117.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Kim, B.S., Dray, S., 1973. Expression of the a, x, and y variable region genes of heavy chains among IgG, IgM, and IgA molecules of normal and a locus allotype-suppressed rabbits. J. Immunol. 111, 750–760.

Kindt, T.J., 1975. Rabbit immunoglobulin allotypes: structure, immunology, and genetics. Adv. Immunol. 21, 35–86.

Knight, K.L., 1992. Restricted VH gene usage and generation of antibody diversity in rabbit. Annu. Rev. Immunol. 10, 593–616.

Knight, K.L., Becker, R.S., 1990. Molecular basis of the allelic inheritance of rabbit immunoglobulin VH allotypes: implications for the generation of antibody diversity. Cell 60, 963–970.

Mage, R.G., Bernstein, K.E., McCartney-Francis, N., Alexander, C.B., Young-Cooper, G.O., Padlan, E.A., Cohen, G.H., 1984. The structural and genetic basis for expression of normal and latent VHa allotypes of the rabbit. Mol. Immunol. 21, 1067–1081.

Mage, R.G., Lanning, D., Knight, K.L., 2006. B cell and antibody repertoire development in rabbits: the requirement of gut-associated lymphoid tissues. Dev. Comp. Immunol. 30, 137–153.

Margolies, M.N., Cannon, L.E., Kindt, T.J., Fraser, B., 1977. The structural basis of rabbit VH allotypes: serologic studies on a1 H chains with defined amino acid sequence. J. Immunol. 119, 287–294.

Matthee, C.A., van Vuuren, B.J., Bell, D., Robinson, T.J., 2004. A molecular supermatrix of the rabbits and hares (Leporidae) allows for the identification of five intercontinental exchanges during the Miocene. Syst. Biol. (Stevenage) 53, 433–447.

Oudin, J., 1960. Allotypy of rabbit serum proteins. I. Immuno-chemical analysis leading to the individualization of seven main allotypes. J. Exp. Med. 112, 107–124.

Pinheiro, A., de Mera, I.G., Alves, P.C., Gortazar, C., de la Fuente, J., Esteves, P.J., 2013. Sequencing of modern Lepus VDJ genes shows that the usage of VHn genes has been retained in both Oryctolagus and Lepus that diverged 12 million years ago. Immunogenetics 65, 777–784.

Pinheiro, A., Lanning, D., Alves, P.C., Mage, R.G., Knight, K.L., van der Loo, W., Esteves, P.J., 2011. Molecular bases of genetic diversity and evolution of the immunoglobulin heavy chain variable region (IGHV) gene locus in leporids. Immunogenetics 63, 397–408.

Pinheiro, A., et al., 2014. Sequencing of Sylvilagus VDJ genes reveals a new VHa allelic lineage and shows that ancient VH lineages were retained differently in leporids. Immunogenetics 66, 719–726.

Pinheiro, A., Neves, F., Lemos de Matos, A., Abrantes, J., van der Loo, W., Mage, R., Esteves, P.J., 2016. An overview of the lagomorph immune system and its genetic diversity. Immunogenetics 68, 83–107.

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior summarization in bayesian phylogenetics using tracer 1.7. Syst. Biol. (Stevenage) 67, 901–904.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Ros, F., Puels, J., Reichenberger, N., van Schooten, W., Buelow, R., Platzer, J., 2004. Sequence analysis of 0.5 Mb of the rabbit germline immunoglobulin heavy chain locus. Gene 330, 49–59.

Roux, K.H., 1981. A fourth heavy chain variable region subgroup, w, with 2 variants defined by an induced auto-antiserum in the rabbit. J. Immunol. 127, 626–632.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic. Acids. Res. 22, 4673–4680.

van der Loo, W., 1987. Population genetical studies on the adaptive significance of the immunoglobulin allotypes in wild rabbit. In: Dubiski, S. (Ed.), The Rabbit in Contemporary Immunological Research. Longman Scientific & Technical, John Wiley, pp. 101–126.

Wu, C., Wu, J., Bunch, T.D., Li, Q., Wang, Y., Zhang, Y.P., 2005. Molecular phylogenetics and biogeography of Lepus in Eastern Asia based on mitochondrial DNA sequences. Mol. Phylogenet. Evol. 37, 45–61.