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REVIEW

Molecular bases of genetic diversity and evolution of the immunoglobulin heavy chain variable region (*IGHV*) gene locus in leporids

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Abstract The rabbit has long been a model for studies of the immune system. Work using rabbits contributed both to the battle against infectious diseases such as rabies and syphilis, and to our knowledge, of antibodies' structure, function, and regulated expression. With the description of rabbit Ig allotypes, the discovery of different gene segments encoding immunoglobulins became possible. This challenged the "one gene–one protein" dogma. The observation that rabbit allotypic specificities of the variable regions were present on IgM and IgG molecules also led to the hypothesis of Ig class switching. Rabbit allotypes contributed to the documentation of phenomena such as allelic exclusion and imbalance in production of allelic gene products. During the last 30 years, the rabbit Ig allotypes revealed a number of unique features, setting them apart from mice, humans, and other mammals. Here, we review the most relevant findings concerning the rabbit *IGHV*. Among these are the preferential usage of one *VH* gene in *VDJ* rearrangements, the existence of trans-species polymorphism in the *IGHV* locus revealed by serology and confirmed by sequencing *IGHV* genes in *Lepus*, the unusually large genetic distances between allelic lineages and the fact that the antibody repertoire is diversified in this species only after birth. The whole genome sequence of a rabbit, plus re-sequencing of additional strains and related genera, will allow further evolutionary investigations of antibody variation. Continued research will help define the roles that genetic, allelic, and population diversity at antibody loci may play in host-parasite interactions.

Keywords Leporids · Rabbit · *IGHV* · Evolution · Immunoglobulin repertoire

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Historic perspective

Studies of the rabbit immune system have greatly contributed to our knowledge of the structure, function, and regulation of antibodies. During the late nineteenth and early twentieth centuries, the use of rabbit in immunological research was crucial to the development of the rabies vaccine by Louis Pasteur and to the battle against syphilis (reviewed in Dubiski 1987). In the first half of the twentieth century, the foundations of molecular immunology were laid with almost exclusive use of the rabbit. The antigenic polymorphism of serum components was described in this

species as early as 1902, although the antigens involved were characterized only 50 years later (see Kelus and Gell 1967). In 1956, Oudin (1960) demonstrated and defined allotypy of immunoglobulins in the rabbit. Allotypes were proven to be hereditary traits in rabbit by Dubiski et al. (1959) prior to the establishment of the two major linkage groups "*a*" and "*b*", corresponding to the H and light chain loci, respectively, and even before the heterodimeric structure of the antibody molecule was clearly established.

The existence of genetic markers of the different gene fragments encoding the antibody H chain was unique to this species and the rabbit Ig allotypes challenged the "one gene–one protein" dogma. Indeed, Todd (1963) and others found that rabbit allotypic specificities of the variable regions were present on both IgM and IgG molecules, suggesting that the same *VH* gene segment can be translocated to different constant region genes. This observation opened the road to confirmation of the concept of multiple "germline" *VH* gene segments that can be joined to a limited number of genes encoding the different heavy chain constant regions (Dreyer and Bennett 1965), and led to the hypothesis of Ig class switching (Kearney et al. 1976; van der Loo et al. 1979; reviewed in Severinson et al. 1982). Hamers and co-workers (1966) reported cis-expression of the *VH* and *CH* genes by using allelic markers on both protein domains. The markers in the CH2 domain were re-discovered by Dubiski (1969) and renamed the e14 and e15 allotypes. Mage and co-workers (1971) reported the first of a number of crossing-over events observed by laboratories during breeding. Findings of genetic recombination between the genes controlling the rabbit *VH* and *CH* (estimated recombination frequency of 0.1%; (Mage 1979; Kelus and Steinberg 1991)) confirmed the model of Dreyer and Bennett (1965).

Documentation of the current *V-D-J-C* model therefore originated through studies of the rabbit allotypes. In addition to genetic linkage of *VH* and *CH* genes, and the expression of apparently identical *VH* regions on different classes of Ig, it was through studies of rabbit immunoglobulin markers that phenomena such as allelic exclusion and imbalance in production of allelic gene products were described (e.g., Davie et al. 1971; Loor and Kelus 1978; Schmale et al. 1969; Wolf et al. 1971). During the last 30 years, the rabbit Ig allotypes revealed a number of unique features, setting them apart from mice, humans, and other mammals. Among these are the preferential usage of one *VH* gene in *VDJ* rearrangements, the evidence for the existence of trans-species polymorphism in the *IGHV*, *IGHG*, and *IGKC1* loci, the unusually large genetic distances between the allelic lineages and the fact that the antibody repertoire is diversified in this species only after birth.

Lagomorph taxonomy

The order Lagomorpha comprises two families: Ochotonidae and Leporidae. The family Ochotonidae includes 30 species restricted to the genus *Ochotona* (Pikas). The family Leporidae can be divided into two groups: hares and rabbits. According to Chapman and Flux (1990), the hare group encompasses a single genus, *Lepus*, whereas the rabbit group includes ten genera (*Brachylagus, Bunolagus, Caprolagus, Nesolagus, Oryctolagus, Pentalagus, Poelagus, Pronolagus, Romerolagus,* and *Sylvilagus*) and 25 species (Alves and Hackländer 2008). There is not a consensus estimate of the divergence time between rabbits and hares. Analyses of several nuclear and mitochondrial markers suggest that the genera *Oryctolagus* and *Lepus* diverged between 6 and 20 Mya (Biju-Duval et al. 1991; Halanych and Robinson 1999; Matthee et al. 2004) (Fig. 1), although fossil data suggests an earlier divergence (between 2.5 and 3.5 Mya)) (Lopez-Martinez 2008).

The genus *Oryctolagus* is monospecific (*Oryctolagus cuniculus*). In the Iberian Peninsula, where the fossil record suggests the European rabbit originated (Pages 1980; Lopez-Martinez 1989, 2008; Corbet 1994), two morphologically differentiated subspecies of European rabbit have been distinguished: *O. cuniculus algirus* and *O. cuniculus cuniculus* (Cabrera 1914). These two subspecies diverged ~1.8 Mya (reviewed in Carneiro et al. 2009). *O. c.*

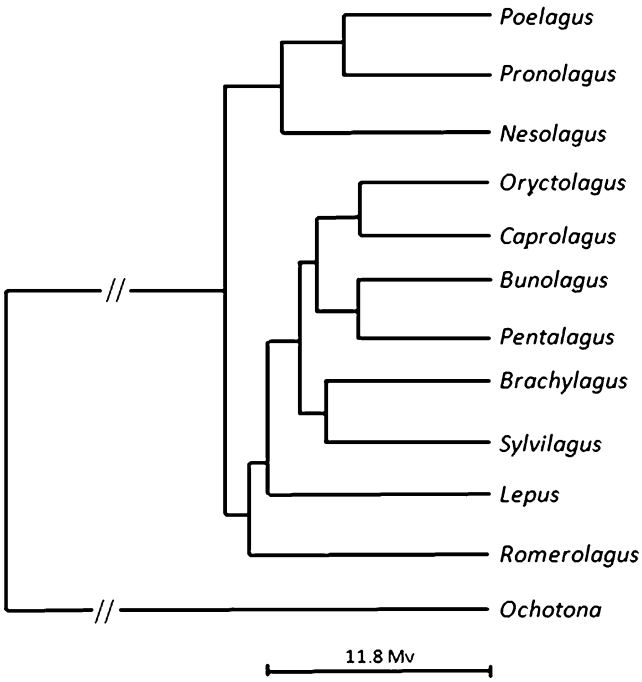

Fig. 1 Evolutionary topology reflecting the relationships within the Lagomorpha group based on a molecular super matrix (adapted from Matthee et al. 2004)

algirus inhabits the southwestern Iberian Peninsula, while *O. c. cuniculus* is present in the northeastern Iberian Peninsula. *O. c. cuniculus* later expanded its range north towards France, likely after the last glacial peak (Queney et al. 2001), where it still remains present. The Pleistocene Ice ages forced many temperate species to retreat into southern refugia, leading to high levels of diversity and endemism in these areas (Hewitt 1996). The Iberian Peninsula was one of these refugia in Europe (Taberlet et al. 1998). This is reflected in the significantly lower genetic diversity of the wild French *O. c. cuniculus* populations compared to the Iberian populations (van der Loo et al. 1991, 1999; Queney et al. 2001; Esteves et al. 2004; Ferrand and Branco 2007; Surridge et al. 2008; Carneiro et al. 2011). The European rabbit gene pool has been manipulated through a recent single domestication event of French origin, and therefore, all domestic rabbits belong to subspecies *O. c. cuniculus* (reviewed in Ferrand and Branco 2007; Carneiro et al. 2011). Today, through mediated anthropic dispersal, the European rabbit is present in Continental Europe, England, Australia, New Zealand, North and South America, and North Africa.

For other leporids, the study of immunoglobulin genes is mainly restricted to the genera *Lepus* and *Sylvilagus* (Cazenave et al. 1977; Teherani and Mandy 1976a, 1976b; Teherani et al. 1979; Teherani et al. 1982; Bouton and van der Loo 1997; Esteves et al. 2002a, 2005, 2006). *Lepus* is a polytypic, cosmopolitan genus, comprising 24 to 30 currently recognized species (Corbet and Hill 1980; Flux and Angermann 1990; Alves and Hackländer 2008), that, like *Sylvilagus*, most probably originated in North America (Lopez-Martinez 2008; Matthee et al. 2004). *Lepus* species that have been used in immunogenetic studies include *Lepus americanus*, *Lepus timidus*, *Lepus granatensis*, and *Lepus europaeus*. *L. americanus* is distributed throughout North America and occupies a basal position in the *Lepus* group (Matthee et al. 2004). *L. granatensis* is endemic to the Iberian Peninsula, covering the whole peninsula except the Northeast where it is replaced by *L. europaeus*, which spans a wide range throughout Europe, with several introduced populations in different regions, like South America, Australia, New Zealand, and several islands including Barbados, Reunion, and Falklands (reviewed in Alves and Hackländer 2008). *L. timidus* is a Palearctic relict species with an extensive distribution area in Europe and Asia, from Ireland and Scandinavia in the west, across Siberia, Mongolia, and China to the northern islands of Japan in the east. *Sylvilagus* is restricted to the American continent and comprises 17 species (reviewed in Alves and Hackländer 2008). Within this genus, the most studied species is *Sylvilagus floridanus*, a species that occupies Southern and Western North America.

Rabbit preferentially expresses *IGHV1*

The rabbit *IGHV* allotypes are highly divergent and behave as Mendelian alleles (Oudin 1956, 1960; Dubiski et al. 1959; Dray et al. 1963; Kim and Dray 1972; Mage et al. 1984). Serological surveys of domestic rabbits defined three allotypic lineages, the so-called *VHa* allotypes *a1*, *a2* and *a3*. *VH* allotypic markers have not been found in other species, and the Mendelian behavior of rabbit *IGHV* allotypes was puzzling for many years, as rabbit serological data revealed a complex situation where homozygous *VHa* rabbits were found to express distinct *VH* genes. Some of them were devoid of *VHa* allotype-specific determinants (the so-called *VHa* negative or *VHn*, alias *VHx*, *VHy*, and *VHz*; Horng et al. 1976). The *VHa*-positive molecules (i.e., displaying motifs of *VHa*-characteristic determinants) also showed variation which revealed the existence of multiple *VHa*-related gene fragments (Brezin and Cazenave 1975; Mage et al. 1976; van der Loo et al. 1977). This was later confirmed by genomic mapping of the rabbit *VH* region which revealed the existence of over 200 *VH* elements in the germline (Currier et al. 1988; Gallarda et al. 1985; Ros et al. 2004). This may, in part, explain why for many years, it was so difficult to understand the presence of allelic allotypic markers on most serum immunoglobulins of all classes (and probably the skepticism of researchers less acquainted with serological methods).

An important contribution to understanding the mechanisms underlying preferential *VDJ* rearrangement in the rabbit was the study of a rabbit strain, called “Alicia”, detected as a mutant during breeding at the Basel Institute, Switzerland (Kelus and Weiss 1986). The mutant rabbits derived from a *VH1a2* parental rabbit in which a 10 Kb segment of genomic DNA containing the *VH1* gene was deleted (Knight and Becker 1990; Allegrucci et al. 1990; Ros et al. 2004). In contrast to normal individuals of the *a2* lineage, the young homozygous *ali*/*ali* mutant Alicia rabbits produced only trace amounts of *a2* molecules, and their serum contained mostly Ig resulting from rearranged genes not encoding *VHa* allotype-associated epitopes (*VHa* negative or *VHn* genes) (Kelus and Weiss 1986; DiPietro et al. 1990; Chen et al. 1993). As the genes encoding the *VHn* allotypes map at least 100 Kb upstream of *VH1* (Mage et al. 2006), it is intriguing that *VDJ* rearrangements of these appeared to be more prevalent than *VDJ* rearrangements of the *D*-proximal functional *VHa* positive *VH4* gene in young Alicia rabbits. Further analysis showed that the *VH4* gene is in fact the predominantly rearranged *VH* gene found in the bone marrow of young Alicia rabbits, but for some reason most *VH4*-utilizing B-cells are eliminated. Thus, the immunoglobulins of the young rabbits are produced by B-cells that

utilize *VHn*. As the young "Alicia" rabbit ages, the proportions of B cells expressing serological *a2* specificities increase (Kelus and Weiss 1986; DiPietro et al. 1990; Allegrucci et al. 1990; Chen et al. 1993; Pospisil et al. 1995). Analysis of nucleotide sequences of the promoter region showed that more than 80% of the *VDJ* rearrangements in older Alicia rabbits utilize either the functional *VH4* or *VH7* genes localized upstream of *VH1*. The *VH4* and the *VH7* genes have 7 (out of 11) specific nucleotides associated with the allotype *a2*, while the other nucleotides that characterize *a2* are gained through somatic gene conversion using *VH9* or a *VH9*-like germline gene as donor (Sehgal et al. 1998; Zhu et al. 1999).

The study of this mutant rabbit strain showed that the Mendelian inheritance of the *VHa* allotypes in normal rabbit is explained by the preferential usage of only one *VH* gene in *VDJ* rearrangements, i.e., the *D*-proximal *VH* gene segment *VH1* (see Fig. 2), which is deleted from the "ali" genome (Knight and Becker 1990; Knight 1992; Allegrucci et al. 1990; Ros et al. 2004). Despite having more than 200 *VH* genes (Currier et al. 1988; Gallarda et al. 1985; Ros et al. 2004), over 50% are "non-functional" and, apart from *VH1*, an even smaller fraction seems to encode a-positive proteins (Ros et al. 2004). About 80% to 90% of circulating Ig molecules are derived from the *VH1* gene and express the *VHa* allotypic markers (e.g., Kindt 1975; Margolies et al. 1977). The *VH* regions of the remaining 10–20% of Ig molecules are encoded by the *VHn* genes (Kim and Dray 1973; Roux 1981), which are localized at least 100 Kb upstream of *VH1* (Mage et al. 2006). Thus far, the basis for the preferential usage of the *VH1* gene in *VDJ* rearrangements remains unanswered. Enhanced chromatin accessibility of this region of the DNA may be responsible (Mage et al. 2006). Furthermore, we can speculate that the retention and occasional usage of the *VHn* genes in 10–20% of *VDJ* rearrangements may represent an evolutionary relic of Lagomorphs ancestral immunologic response to pathogens.

Genetic diversity at the heavy chain variable region *IGHV* locus (a locus)

In the domestic rabbit, the three serologically defined allotypic lineages, *VHa* allotypes *a1*, *a2*, and *a3*, are highly divergent (±20% amino acid sequence differences). The allelic specificities of *a1* and *a2* are correlated with several amino acid differences in framework regions 1 (positions 5, 8, 11, 13, 14, 17, and 18) and 3 (positions 74, 76, 79, 80, 83, 84, 96, and 97) (Fig. 3) (Tonnelle et al. 1983; Mage et al. 1984; Knight and Becker 1990). Serologically distinct *VHa* allotypes were discovered in wild rabbits and named VHa100-VHa109 by Cazenave and coworkers (reviewed in Cazenave et al. 1987). Partial protein sequences of purified antibodies of the VHa100 type (Tonnelle et al. 1983), suggest these are related to the more recently described and fully sequenced VHa allele(s) corresponding to the *a4* lineage (Esteves et al. 2004). Studies by double immunodiffusion of sera from domestic breeds and wild rabbits of Continental Europe (North of Pyrenees Mountains), Great Britain and overseas showed the presence of allotypes *a1*, *a2* and *a3*, as well as a rare allotype with partial reaction against *a3*-specific antiserum, found in the French populations. In all studied populations, the gene frequencies of *a1* and *a3* were similar (±40%) and higher than *a2* (±20%) (van der Loo 1993). Interestingly, these allelic frequencies reflect the "pecking order" of their relative expression in heterozygous animals (Mage et al. 1967; Lummus et al. 1967). Quite surprisingly, the study of wild rabbit populations from the Iberian Peninsula belonging to the subspecies *O. c. algirus* and *O. c. cuniculus* revealed a different picture with a much higher prevalence of the "a-blank" allele. Also, a gradient in the gene frequencies of this allotype was noted, decreasing from southwestern to northeastern populations (Fig. 4) (Esteves et al. 2004). Sequencing of rearranged *VH* genes expressed in *O. c. algirus* rabbits that were typed as "a-blank" showed that these rabbits preferentially use *VH* genes that, although

Fig. 2 Rabbit *IGHV* (regions from VH haplotype (a2 F-I)) for which nucleotide sequence is available from sequenced, assembled BAC clones. Depicted are *V* genes, *D* and *J* regions and also *Cμ*. *VH* functional genes are represented in white rectangles, *VH* possible pseudogenes in *gray* and *VH* pseudogenes in *black*, identified according to criteria described in Ros et al. (2004). The *D*-proximal *VH* gene, *VH1*, used in the majority of *VDJ* rearrangements is indicated. Identified above are the non-overlapping BAC clones 38A2 and 225P18 (GenBank accession numbers AY386694 and AY386697), and 219D23 (GenBank accession number AY386695). The 3′ end of BAC 225P18 overlaps the 5′end of BAC 219D23 (adapted from Ros et al. 2004)

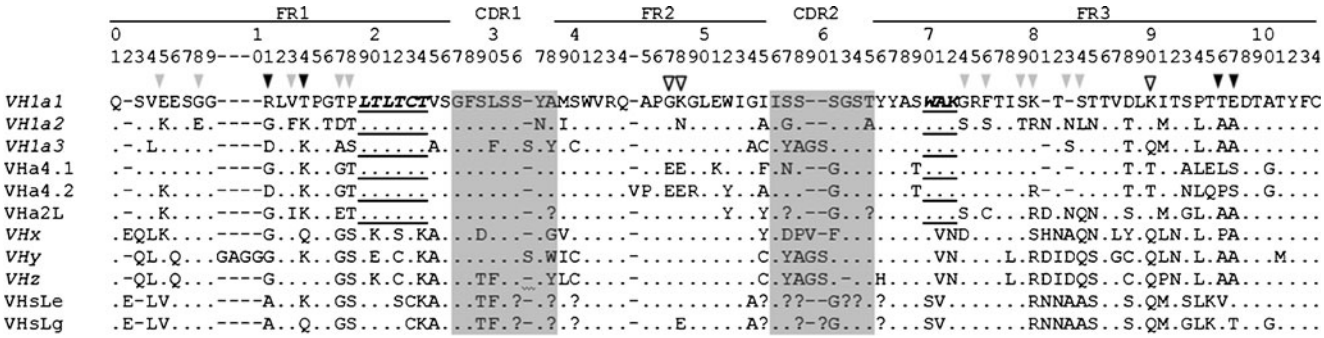

Fig. 3 Amino acid sequences of European rabbit (*O. cuniculus*) proteins (encoded by *VH1a1-a3*, VHa4.1–4.2, *VHx*, *VHy*, *VHz*) and hare (VHa2L, VHsLe, VHsLg) VH genes. Germline elements are *italicized* (Accession numbers: *VH1a1*-M93171; *VH1a2*-M93172; *VH1a3*-M93173; *VHx*-L03846; *VHy*-L03890; *VHz*-AF264469.). VHa4.1–4.2, VHa2L, VHsLe and VHsLg represent consensus protein sequences obtained by Esteves et al. 2004, 2005. Framework regions (FR1, FR2, FR3) and each CDR (CDR1 and CDR2) are defined according to the IMGT numbering system (also shown). CDRs are *shaded*. The amino acids that are characteristic of *a1*, *a2* and *a4* lineages are marked with *black*, *gray*, and *white arrows*, respectively. Hallmark rabbit VHa residues are highlighted in *bold* and *underlined*. Note that the *Lepus* VHa2L shares these residues, as well as five of the VH1a2 lineage characteristic amino acids. *Dots* indicate identity with *VH1a1*, *dashes* represent indels and *question marks* represent ambiguous positions in consensus sequences

clearly related to the known *VHa* genes, defined a new major allotypic lineage, designated *a4*. The *a4* sequences display the rabbit *VHa* hallmark residues together with a number of unprecedented amino acid changes in FR2 and FR3, which may explain the lack of reactivity with known alloantisera (Fig. 3). The net protein distances between the VH-a4 and the VH-a1, a2, and *a3* lineages, was 20%, 29%, and 21%, respectively, similar to the net protein distances between VH-a1, a2, and a3 lineages. As such, a new *VHa* allele was found in Iberian *O. c. algirus* populations, constituting the fourth of the distantly related lineages of the rabbit *VHa*-locus, one of which, the *a4* lineage, seems to be endemic in the Iberian range (Esteves et al. 2004). The data for the *VHa* lineages also suggested that some lineages, namely *VHa a1*, *a2* and *a4*, showed higher evolutionary rates than the *VHa a3* and *VHn* lineages. This increased evolutionary rate may account for the accumulation of a considerable fraction of the amino acid differences characterizing the a4 lineage after the separation of the two *O. cuniculus* subspecies, explaining its association with subspecies *O. c. algirus* (Esteves et al. 2004).

Studies on the *IGHV* locus diversity for other leporids are limited to a few data obtained for some *Lepus* species, namely *L. americanus*, *L. europaeus*, and *L. granatensis*. For *L. americanus*, only serological analyses have been conducted; these showed cross-reaction with rabbit anti-a1, anti-a2, and anti-a3 antisera and the absence of individuals with no cross-reaction to rabbit antisera (De Poorter 1984). As for *L. europaeus* and *L. granatensis*, a serological analysis of several populations using allo-antisera against rabbit *a1*, *a2*, and *a3* allotypes revealed only two phenotypes: partial reaction to anti-a2 antisera and no reaction to any rabbit antiserum (Table 1) (Esteves 2003). Sequences of expressed *VH* genes were obtained for only five *Lepus* specimens of both species, three of which expressed *a2*-cross-reacting serum proteins, and these could be grouped into two different lineages (see below "The trans-specific polymorphism of leporids *IGHV*"). Extending the study of *IGHV* diversity to more *Lepus* individuals and species could unravel more *VH* lineages, as shown by the rabbit *VH1-a4* lineage found only in *O. c. algirus*.

The trans-specific polymorphism of leporid *IGHV*

Such large interallelic distances as those observed at the rabbit *VHa* locus can be the outcome of unusually long allele persistence times and/or increased mutant recruitment rates. The first indications that the different *VH1* polymorphisms could be trans-specific came from studies showing serological cross-reactivity of *Lepus* sera with rabbit *VHa*-allotype-specific allo-antisera (van der Loo et al. 1977; Horng et al. 1980; van der Loo 1987). Indeed, for *L. americanus* large-scale serological analysis, comprising several hundreds of specimens, showed cross-reaction with rabbit anti-a1, anti-a2, and anti-a3 antisera, revealing at least three alleles with hierarchical frequency distributions similar to those observed for feral rabbits (De Poorter 1984; van der Loo 1987). Su and Nei (1999) compared the extent of sequence divergence between the rabbit *a1*, *a2*, and *a3* allotypes with that between human and mouse *VH* gene sequences and concluded that assuming a "normal" mutation rate, the rabbit *VH1* polymorphism has persisted for about 50 My. Since the *Oryctolagus* and *Lepus* genera diverged 12 Mya (Matthee et al. 2004), the allelic lineages present in one leporid species should be more related to some of the alleles expressed in the other species than to their conspecific allelic counterparts. Serological cross-

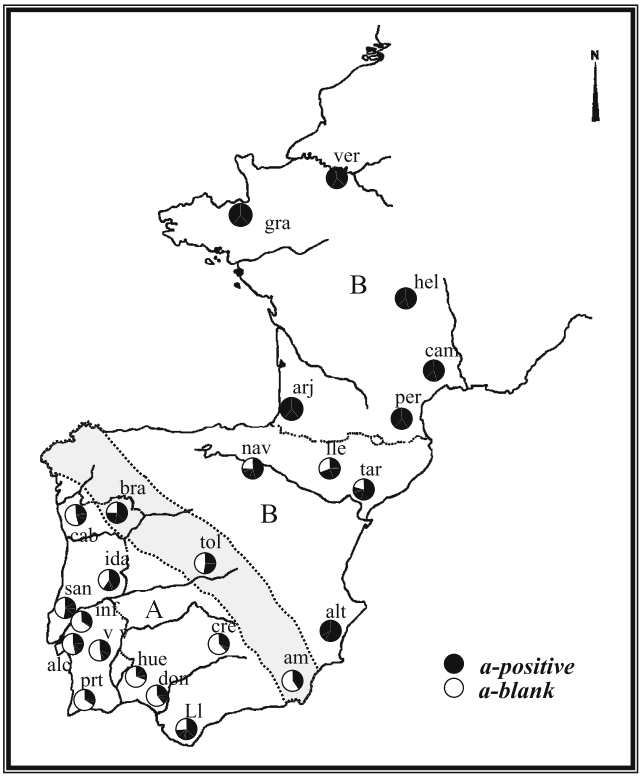

Fig. 4 Geographic distribution of *VHa* locus allotype frequencies, serologically determined for populations of European wild rabbit (*O. cuniculus*) from the Iberian Peninsula and France. The subspecies *O. c. algirus* occupies the southwestern part of Iberia, which is indicated by *A*. Rabbits of the Northwestern areas of Iberia and of the rest of Europe (indicated by *B*) belong to the subspecies *O. c. cuniculus*. The contact zone between the two subspecies is indicated in *gray*. The coloring of the disks reflects the relative allele frequencies per locality, analyzed as a two-allele locus with *a*-blank (*white*) and *a*-positive (*black*) alleles. Locality abbreviations are as follows: *prt* Portimão, *san* Santarém, *alc* Alcochete, *ida* Idanha, *cab* Cabreira, *Ll* Las Lomas, *hue* Huelva, *don* Doñana, *cre* Ciudad Real, *inf* Infantado, *vv* Vila Viçosa, *am* Amoladeras, *tol* Toledo, *bra* Bragança, *tar* Tarragona, *alt* Alicante, *nav* Navarra, *lle* Lerida, *per* Perpigan, *cam* Camargue, *ver* Versailles, *arj* Arjuzanx, *gra* Grax, *hel* Helene

reactivity can depend upon a small number of amino acid replacements, and allo-antisera may reveal plesiomorphic character states (i.e., which became antigenic in the recipient strain when they were altered), and so the hypothesis that the *VH1* polymorphism preceded the rabbit–hare split remained controversial until sequence data was obtained.

Focusing on this question, Esteves and co-workers (2005) sequenced rearranged *VH* gene products from five *Lepus* specimens of both *L. granatensis* and *L. europaeus* species, three of which expressed *a2*-cross-reacting serum proteins, and compared them to known rabbit *VH* sequences of different allotypes. Within the *Lepus VH* genes, two lineages were observed, one of which (a2L) showed some of the most outstanding characteristics of the rabbit *VHa* genes, furthermore possessing 5 out of 11 amino acid residues that characterize the allotype VHa2 (Mage et al. 1984). This lineage was only obtained from specimens showing rabbit *a2* antisera cross-reaction. A second lineage displayed more similarity to the rabbit *VHn* gene fragments (sL). Phylogenetic inference methods clearly placed the hare a2L sequences into the same monophyletic group with the rabbit a2 sequences (Fig. 5), thus showing that these sequences were more closely related to the rabbit *VH1-a2* allele than *VH1-a2* was to its allelic counterparts, *VH1-a1* and *VH1-a3*. Also, the genetic distance measured between different rabbit genomic *VHa* sequences was approximately 1.5 times larger than between the *Lepus* a2L consensus and genes of the rabbit a2 allotype. Together, these findings strongly suggest that the allotype split predates the *Oryctolagus–Lepus* species split, supporting previous evidence of the trans-species nature of the *VH1* polymorphism. The alternative interpretation that the evolutionary rate of the rabbit *a2* lineage has become significantly slower than at the other lineages, was not supported by the data, as the *a2* sequences were rather more derived compared to the other allotypes, in particular, the *a3* lineage.

The large inter-allelic differences at the *VH1* locus

Regarding the two different hypotheses invoked to explain the very large inter-allelic distances observed at the *IGHV1* locus, the results obtained seem to be contradictory. On the one hand, the association of specific alleles with subspecific markers suggests that the large differences between a4 and domestic alleles (a1, a2, and a3) may have accumulated after the separation of the subspecies, with different evolutionary rates among lineages. On the other hand, the confirmation of the trans-specific nature of the *VH1* polymorphism between *Oryctolagus* and *Lepus* supports the hypothesis that very long lineage persistence times have contributed to allelic divergence. The evidence obtained points to two major conclusions, (1) the allelic lineages can be maintained in the genome for a long time, and (2) mutation rates can differ between allelic lineages. The origin of the selection forces that regulate the *VH1* polymorphism is unknown. However, the requirement for exogenous factors, such as the intestinal microbiota, for diversification of the primary antibody repertoire in rabbits suggests that this mechanism might impose constraints on the evolution of the *VH1* alleles.

Diversification of the primary Ab repertoire in the rabbit

Among vertebrate species, two different strategies to generate the primary Ab repertoire have been adopted.

Table 1 Gene frequencies obtained in populations of *Lepus granatensis* and *L. europaeus* at IGHV

	Number	a1 (100)	a2 (010)	a3 (001)	a1v (p00)	a2v (0p0)	a3v (00p)	blank (000)
Lepus granatensis								
Portugal								
Bragança	13	–	–	–	–	0.08	–	0.92
Santarém	20	–	–	–	–	0.41	–	0.59
Idanha	20	–	–	–	–	0.29	–	0.71
Pancas	20	–	–	–	–	0.45	–	0.55
Aljustrel	20	–	–	–	–	0.55	–	0.45
Alcochete	7	–	–	–	–	0.08	–	0.92
Spain								
Granada	20	–	–	–	–	0.19	–	0.81
average unweighted	120	–	–	–	–	0.29	–	0.71
Lepus europaeus								
Lass (France)	20	–	–	–	–	0.5	–	0.5

Lepus individuals were tested for cross-reaction with rabbit anti-a1, anti-a2, and anti-a3 antisera. Serotype codes are shown in parentheses: *1* indicates complete identity with rabbit allo-antisera, *p* partial identity, and *0* no reaction (Esteves 2003)

Human and mouse preferentially use combinatorial rearrangements of a large number of *V*, *D*, and *J* gene segments. In contrast, several species possess or use only a limited number of germline *V* segments. Birds, together with several mammalian species (rabbit, sheep, pig, and bovine), use strategies of primary repertoire development that overcome this limitation. In these species, the primary antibody repertoire is diversified post-rearrangement by one or both of two mechanisms: somatic gene conversion and somatic hypermutation.

In chicken, somatic gene conversion is the major mechanism of diversification of immunoglobulin heavy and light chain variable regions. A set of pseudogenes is used as donors and the unique rearranged *V*(*D*)*J* gene acts as acceptor (Reynaud et al. 1985; 1989). In sheep, B-cell diversification occurs in early development in the ileal Peyer's patches and results from somatic hypermutation rather than somatic gene conversion (Reynaud et al. 1991; 1995). Both processes are used to diversify the antibody repertoire in cattle (Parng et al. 1996; Lucier et al. 1998).

Becker and Knight (1990) showed that the rearrangement process in the rabbit involves a single preferentially rearranged *VH* gene that is diversified mainly through somatic gene conversion, using other upstream *VH* genes as donors (Becker and Knight 1990; reviewed in Knight and Crane 1994). Somatic hypermutation also takes place, distributing point mutations throughout the entire *VDJ* gene (Short et al. 1991; Weinstein et al. 1994; Sehgal et al. 2000). In rabbits, B cells generated in the fetal liver and bone marrow subsequently migrate to the appendix and other gut-associated lymphoid tissues (GALT), where they proliferate and are intensively diversified. The *VH* genes upstream of *VH1* contribute to the expressed *VH1* diversification by somatic gene conversion. They evolved to some extent in concert, which resulted in the presence of the allotypic motifs on other *VH* gene segments of the Ig heavy chain locus (haplotype polymorphism).

In contrast to species such as chickens (e.g., Reynaud et al. 1989), sheep (Reynaud et al. 1995), and cattle (e.g., Lucier et al. 1998), the diversification of the primary antibody repertoire in rabbits is not developmentally programmed. It has been shown that the peripheral Ab repertoire is diversified between 4 weeks and 2 months after birth (Cooper et al. 1968; Weinstein et al. 1994). Surgical removal of the appendix and sacculus rotundus on the day of birth and the Peyer's patches at 3 weeks of age (Cooper et al. 1968) more recently showed that GALT is essential for the generation of the primary antibody repertoire (Vajdy et al. 1998). It has also been shown that exogenous factors, such as the intestinal microbiota, are required for Ab repertoire diversification in the rabbit (Lanning et al. 2000a, 2000b; Sehgal et al. 2002). The rabbit is a species with altricial young (naked and blind at birth). Other lagomorph species, such as hares (genus *Lepus*) have precocial young. The unique situation of post-natal diversification in rabbit could be related to the shortening of gestation time (4 weeks in rabbit and 6 weeks in hares). Considering the differences in reproductive biology between the genera *Oryctolagus* and *Lepus*, it would be interesting to investigate whether in *Lepus*, the Ab repertoire is diversified neonatally or during fetal life.

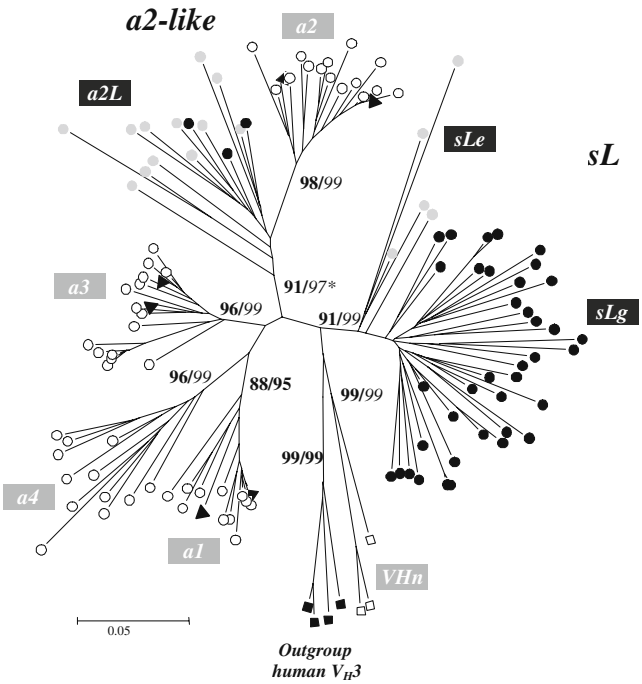

Fig. 5 Neighbor-joining tree for *Oryctolagus cuniculus*, *Lepus europaeus* (Le), and *Lepus granatensis* (Lg) *VH* genes, showing the transpecies polymorphism of VHa2. *VH* genes from human class 3 (VH3) determine the root of the tree. *Numbers* represent the bootstrap values (BP, in *bold*), and the confidence probability values (CP, in *italics*). Expressed VH genes (cDNA) are labeled by *black* (Le), *gray* (Lg), and *white* (O. cuniculus) *circles*. Germline *VHa* gene sequences (*VH1-VH4*) of rabbit allotypes a1, a2, and a3 are marked by *black triangles*. *White squares* indicate *VHn* germline genes. *Black squares* indicate human *VH3* germline genes. "With kind permission from Springer Science+Business Media: Immunogenetics, The evolution of the immunoglobulin heavy chain variable region (IgVH) in leporids: an unusual case of trans-species polymorphism., 57(11), 2005, 874, Esteves PJ, Lanning D, Ferrand N, Knight KL, Zhai SK, van der Loo W, Fig. 1"

Evolution of *VH* gene families

The study of *VH* genes from several vertebrate species showed that *VH* genes from the same species could belong to different groups. In tetrapods, the *IGHV* sequences have been classified into three clans, clans I, II, and III (Schroeder et al. 1990; Kirkham et al. 1992; Das et al. 2008). The human and mouse possess *VH* genes that are very diversified and may cluster with either clans I, II, or III (polyphyletic), whereas *VH* genes of chicken, rabbit, horse, and artiodactyls (cattle, sheep, and swine) are monophyletic. Chicken, rabbit, and swine VH genes each form distinct clusters within group III, whereas those of horse, cattle, and sheep each form distinct clusters within group II. Since *VH* genes of different artiodactyl species cluster within different groups (II and III), their common ancestor must have possessed *VH* genes from both groups (Sitnikova and Su 1998). Thus, the presence of *IGHV* genes of only one clan in several tetrapod species reflects an evolutionary loss of *IGHV* genes.

Monofunctional multigene families are generally believed to undergo processes of genetic exchange, like gene conversion and unequal crossover, which homogenize the DNA sequences (Smith et al. 1971; Smith 1974; Zimmer et al. 1980). This has been called "concerted" evolution. The concerted evolution model has been invoked to explain the evolution of *VH* genes (Hood et al. 1975; Ohta 1983). However, the studies of *VH* gene families in human and mouse (Gojobori and Nei 1984; Tutter and Riblet 1989), and studies on much longer evolutionary time scales, showed that the pattern of *VH* gene evolution could be better explained by the so-called "birth-and-death" model of evolution (Ota and Nei 1994; Nei et al. 1997; Sitnikova and Su 1998; Das et al. 2008), in which the number of genes in a family (or "library") are allowed to expand and contract. This model is similar to the "accordion model" of MHC evolution proposed by Klein et al. (1993) and postulates that depending upon the need to protect the host from ever-changing groups of parasites, some *VH* gene libraries are duplicated and can diverge functionally, while others become pseudogenes and/or are deleted from the genome. The end result of this process is a mixture of divergent and highly homologous groups of genes, and the maintenance of a substantial number of pseudogenes (Ota and Nei 1994; Nei et al. 1997). According to this model, the tetrapod ancestor possessed three *VH* gene groups (I, II, and III) and library contraction events occurred in several lineages independently. In species that have inherited only one group of *VH* genes, showing low levels of *IGHV* gene sequence variation, antibody diversification is mainly due to somatic hypermutation and/or somatic gene conversion. It appears that *VH* gene library contraction is associated with the development of a specific organ for extensive somatic diversification of the Ab repertoire (bursa of Fabricius in chicken; ileal Peyer's patches in sheep, cattle and probably swine and horse; appendix in rabbit).

Genome sequencing and future prospects

Whole genome sequencing (WGS) at ~7× coverage and assembly of a high-quality draft rabbit genome sequence was recently completed at the Broad Institute, Boston (OryCun2.0). The ENCODE Project, with ~1% of rabbit genomic sequence from a different, normal NZW animal, includes some genes of immunological interest but none of the regions with immunoglobulin heavy or light chain loci. The Thorbecke rabbit strain chosen by Broad for WGS was partially inbred. Although the rabbits accepted skin grafts, they were still segregating for the VHa

allotypes a1 and a2. The rabbit chosen for sequencing was heterozygous at the immunoglobulin heavy chain locus. The *IGH* locus is not assigned to a chromosome in the Broad assembly. The *IGHV* allele encoding a1 (VH1a1) is located in unplaced scaffold chrUn0742 and the *IGHV* allele encoding a2 (VH1a2) is located in unplaced scaffold chrUn0439. Unfortunately, there are missing traces or traces of low quality between the telomere and the *IGH*. Until new sequences are obtained, the current available traces from Broad OryCun2 may not allow further assembly. There are ~75 additional unplaced scaffolds that appear to contain *VH* genes.

Little is known about the evolution of the *IGHV* genes in the Lagomorph group. Thus far, only the rabbit *O. cuniculus* has been extensively studied and some sequences of *IGHV* genes have been obtained for two *Lepus* species, *L. europaeus* and *L. granatensis*. The study of these species has yielded some interesting results and led to new insights on the evolution of *IGHV* genes in the Lagomorph group. Also, the few data obtained so far for the Lagomorph *IGHG* hinge region and CH2 domain has shown some particular patterns of genetic diversity possibly linked to resistance against pathogens (Esteves et al. 2002a; Esteves et al. 2002b; Esteves et al. 2006). Extending our knowledge about both the *IGHV* genes and *IGH* constant regions to other Lagomorph species would certainly contribute further to our understanding of the evolution of *IGHV* genes and the roles that genetic diversity of immunoglobulins may play in host resistance to pathogens.

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