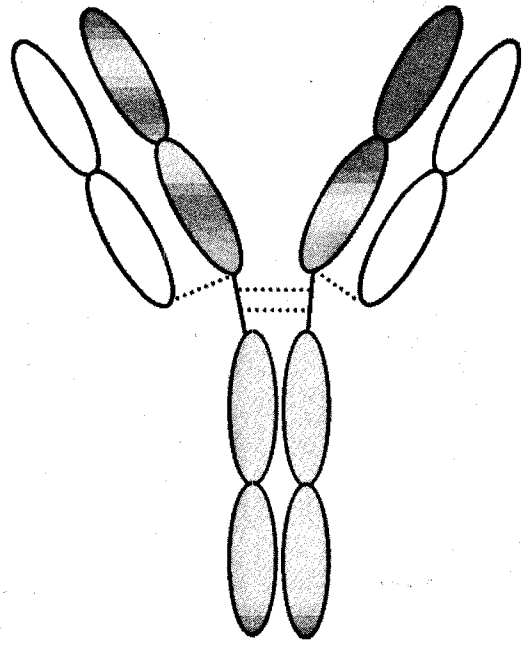
Pedro José de Castro Esteves

Molecular and population genetic analysis of polymorphism at the antibody loci *IgGCH2* and *IgVH* in lagomorphs



Faculdade de Ciências
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Dissertação apresentada à Faculdade de Ciências da Universidade do Porto para obtenção do grau de Doutor em Biologia

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À minha filha Mariana e aos meus pais

Declaração

Na elaboração desta dissertação e nos termos do nº 2 do artigo 8 do decreto-lei nº 388/70, foi feito o aproveitamento total dos resultados de trabalhos já publicados ou submetidos para publicação, que fazem parte integrante de alguns capítulos da presente dissertação. Em todos estes trabalhos, o candidato participou na obtenção, análise e discussão dos resultados, assim como na redacção dos manuscritos.

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Summary

Rabbits present a variety of allelic variants at different loci that encode the different constituents of the antibody molecule. The structure of the alleles are well described for domestic rabbit, but, except for the C_{K1} L chain locus, have not been studied in wild populations. By sequence determinations of "novel" allelic variants, we have substantially contributed to a better knowledge of these immunological polymorphisms. In these studies we also included species of other lagomorph genera, namely *Lepus*, *Sylvilagus* and *Ochotona*. The comparison of serological and DNA sequence data allowed a better comprehension of the evolutionary processes that underlie the unusual divergence patterns of these polymorphisms.

As a first step we have studied a polymorphism that is both simple and recent. It concerns the genetic variation at the second domain of Ig Gamma H chain (*IgGCH2*) or *e* locus, a domain that mediates important Ab effector functions, and is a known target of pathogen attack. We sequenced the *IgGCH2* domain of 11 different lagomorph species: one of *Oryctolagus*, eight of *Lepus* (*europaeus*, *granatensis*, *timidus*, *americanus*, *capensis*, *calotis*, *californicus* and *castroviejoi*) and two of *Sylvilagus* (*floridanus* and *cunicularis*). The analyses of the obtained data revealed a "hotspot" of variability at the amino acid position H309, where six different triplets were observed, corresponding to five different amino acids (Met, Lys, Val, Ala and Thr). The extension of this analysis to other mammal groups (*Primata, Metatheria, Rodentia, Carnivora, Perissodactyla* and *Artiodactyla*) also revealed high variability levels at this position. We could present indirect evidence that this variation is due to diversity enhancement selection. Furthermore, based on the sequence data, we have developed a PCR/RFLP methodology that distinguishes the rabbit *IgGCH2* alleles *e14* and *e15,* which is a useful alternative to the currently used serological methods.

A major part of our work concerns the *IgVH* locus. In contrast to the *e* locus the V_H polymorphism is complex and ancient. The VH region diversity has been thoroughly studied in the domestic rabbit. Three serologically defined allotypic lineages are described, the so-called V_Ha allotypes *a1, a2* and *a3*. These allotypes are inherited as mendelian alleles because of the preferential usage of the V_H1 gene segment in the *VDJ* rearrangements. We have extended these studies to wild rabbit. The *IgVH1* locus was serologically analysed in

18 Iberian wild rabbit populations (402 specimens). Besides the previously described alleles (*a1*, *a2*, *a3* and *a3v*), we found two more alleles, *a1v* and *"a-blank"*, that are endemic to the Iberian Peninsula. One of these alleles (*"a-blank"*) was found to be present in all Iberian populations. This allotype was however more frequent in populations of the subspecies *O. c. algirus*. Sequencing of V_H gene expressed in wild rabbit specimens typing *"a-blank"* allotype showed that these rabbits use preferentially V_H genes that, although clearly related to the known V_Ha genes, define a new major allotypic lineage, called here *a4*.

The large sequence difference between V_H1 alleles has suggested lineage coalescence times of 50 Million years, which is much longer than the time of separation between rabbits and hares. We have tested the trans-species nature of the polymorphism via sequence determination of V_H gene segments that are expressed in specimens of *Lepus* (*L. europaeus* and *L. granatensis)*. The results revealed the existence in this genus of two distinct V_H lineages, which we called *a2L* and *nL*. Phylogenetic analysis showed that the *nL* lineage was not closely related to this group nor to any other group described so far. In contrast, the lineage *a2L* clusters within the rabbit V_Ha lineages, being closely related to rabbit allele *a2*, with which it forms a monophyletic group designated as "V_Ha2-like". These results support the trans-species polymorphism hypothesis, implying that very long lineage persistence times can at least in part explain the high inter-allelic divergence.

The data concerning the V_H1 locus may seem contradictory. On one hand, the association of specific alleles with subspecies markers suggests that the large differences between V_Ha locus alleles may have accumulated after the separation of the subspecies. On the other hand, the confirmation of the trans-specific nature of V_H1 polymorphism supports the hypothesis of very long lineage persistence times. However, we have also shown that evolutionary rates can differ significantly among lineages, which allows the reconciliation of the apparent contradictions mentioned above. The different lines of evidences obtained in this study suggest therefore that allelic lineages have been maintained throughout successive speciation steps while evolutionary rates can differ among allelic lineages.

We further extended our knowledge of the V_H gene segment to other lagomorph species (genera *Sylvilagus* and *Ochotona*) by sequencing germline V_H gene segments. The comparison with other vertebrates suggests that in lagomorphs all V_H genes belong to group C. Our data allow the definition of four clusters within lagomorphs (V_Hn, V_HnL, V_Ha and V_HP). While some groups only contain V_H genes of one species (V_HnL and V_HP), some V_H genes from different species cluster together. This suggests that lagomorph V_H gene

segments evolved under the model of birth-and-death evolution (i.e. some V_H gene segments diverged after duplication while other were maintained with high homology, explaining the grouping of V_H genes from different species).

The hypothesis that at least four allotypic lineages have been maintained during 50 Million years of lagomorph speciation raises the question of the minimal size of founder populations. This question is addressed in the study of the genetic composition of the population of Porto Santo. The rabbit was introduced in this Atlantic island during the XVth century. Several historical documents suggest that a single pregnant female was released on this island, which was followed by a sudden increase in population numbers. By using a set of 34 markers (mtDNA, microsatellite, protein and immunogenetic loci) we showed that the Porto Santo rabbit population originated from Southwestern Portugal, and had, as expected, the signature of a recent bottleneck. A detailed analysis of the genetic data reported was in strong support of the hypothesis that one single pregnant female founded the contemporary Porto Santo rabbit population.

Resumo

Os diferentes *loci* que controlam a biossíntese dos anticorpos apresentam no coelho uma grande variedade alélica. No que diz respeito ao coelho doméstico, a estrutura desses alelos encontra-se bem estudada. Porém, até à data esses marcadores não foram caracterizados em populações de coelho bravo, com excepção do *locus* C_{K1} da cadeia leve. Através da determinação da sequência codificante de novos alótipos detectados na Península Ibérica, contribui-se substancialmente para um conhecimento mais alargado destes polimorfismos imunológicos. Nesta caracterização foram também incluídos outros lagomorfos, nomeadamente pertencendo aos géneros *Lepus*, *Sylvilagus* e *Ochotona*. A comparação de dados serológicos com a determinação das sequências nucleotídicas permitiu uma compreensão mais ampla dos processos evolutivos que são responsáveis pelos padrões de divergência pouco usuais encontrados nestes polimorfismos.

Numa primeira fase, estudou-se um polimorfismo que é simultaneamente simples e recente. Trata-se da variação genética do segundo domínio da região constante da cadeia pesada das imunoglobulinas da classe Gamma (*IgGCH2* ou *CHγ2*, também denominado como "locus *e*"). Este domínio desempenha um papel preponderante nas múltiplas funções dos anticorpos e é um dos alvos preferenciais do ataque de agentes patogénicos. Sequenciou-se o domínio *IgGCH2* em onze espécies diferentes: oito do género *Lepus* (*europaeus*, *granatensis*, *timidus*, *americanus*, *capensis*, *calotis*, *californicus* e *castroviejoi*) e duas do género *Sylvilagus* (*floridanus* e *cunicularis*), para além do coelho (género *Oryctolagus*). A análise dos dados obtidos revelou a existência de um "hotspot" de variabilidade na posição aminoacídica H309, onde foram observados cinco resíduos diferentes (Met, Lys, Val, Ala e Thr), ao passo que nas restantes posições a variabilidade se revelou praticamente inexistente. A extensão desta análise a outros grupos de mamíferos (*Primata, Metatheria, Rodentia, Carnivora, Perissodactyla* e *Artiodactyla*) demonstrou que este fenómeno não está limitado apenas aos lagomorfos. Foi possível mostrar de forma indirecta que esta variabilidade é promovida por selecção, que actua favorecendo a diversidade nesta posição em particular. Os dados obtidos relativamente à variação deste marcador na Península Ibérica confirmaram a tese de que o polimorfismo *e14/e15* do coelho doméstico, ausente das populações Ibéricas, se fica a dever a uma mutação recente de um gene ancestral *e15*, provavelmente de origem Ibérica. Com base na determinação das

sequências de DNA subjacentes a esta mutação, foi ainda possível desenvolver uma metodologia de PCR/RFLP que permite distinguir os dois alelos referidos, constituindo assim uma alternativa útil aos métodos serológicos normalmente usados.

Uma parte importante deste trabalho foi consagrada ao estudo da variação alélica do locus *IgVH*, que codifica a porção variável da cadeia pesada dos anticorpos. Contrariamente ao marcador anteriormente abordado (*IgGCH2*), no qual o polimorfismo se limita à variação de um só aminoácido, a variabilidade deste locus é complexa e filogeneticamente muito antiga. Este polimorfismo foi extensivamente estudado no coelho doméstico, onde foram descritas três linhagens alotípicas (*a1, a2* e *a3*). Estes alótipos comportam-se como alelos mendelianos devido ao facto de os coelhos utilizarem preferencialmente apenas um gene V_H durante o rearranjo *VDJ*, nomeadamente o gene V_H1. Com este trabalho, ampliou-se o estudo deste polimorfismo a populações Ibéricas de coelho bravo. Nestas populações foram detectados, para além dos já descritos (*a1, a2, a3* e *a3v*), dois novos alótipos (*a1v* e "*a-blank*"), um dos quais ("*a-blank*") está presente em todas as populações Ibéricas e parece estar associado à subespécie *O. c. algirus*. A determinação da sequência do RNA mensageiro demostrou que os indivíduos com o alótipo *"a-blank"* utilizam quase exclusivamente um gene V_H que, embora filogeneticamente relacionado com os genes V_Ha do coelho doméstico, define uma nova linhagem alotípica que se designou por *a4*.

Baseando-se nas elevadas distâncias que separam os diferentes alelos V_H1, alguns autores sugeriram um tempo de coalescência das linhagens da ordem dos 50 milhões de anos, o que é muito superior ao tempo de separação entre *Lepus* e *Oryctolagus*. Para testar a hipótese da natureza trans-específica deste polimorfismo, determinou-se a sequência nucleotídica dos segmentos V_H expressos em espécies do género *Lepus* (*L. europaeus* e *L. granatensis*). Nestas espécies foram detectadas duas linhagens, designadas por *a2L* e *nL*. As análises filogenéticas demonstram que a linhagem *nL* não se agrupa dentro do grupo V_Ha nem de qualquer outro grupo de genes V_H descrito até à data. Pelo contrário, a linhagem *a2L* é aparentada com a linhagem *a2* do coelho e as distâncias entre o alelo V_H-*a2* e os seus homólogos V_H-*a1,-a3* e *-a4* são notoriamente mais elevadas do que as distâncias detectadas entre as linhagens *a2* e *a2L*. Estes resultados apoiam a hipótese do polimorfismo dos genes V_Ha ser trans-específico, uma vez que implicam que as várias linhagens tenham iniciado a sua divergência antes da separação das duas espécies.

Os resultados relativos ao locus V_H1 aparentam ser contraditórios. A associação de diferentes alelos com subespécies distintas não se coaduna, aparentemente, com tempos de coalescência das linhagens que são claramente anteriores à data da especiação. No entanto, foi possível demonstrar que as taxas de evolução não são as mesmas para todas as linhagens, o que poderá em parte ajudar a conciliar as duas observações.

Adicionalmente, alargou-se a análise através da sequenciação de genes V_H da linha germinal de espécies pertencentes aos géneros *Sylvilagus* e *Ochotona*. A comparação das sequências obtidas com as de outros vertebrados sugere que todos os genes V_H dos lagomorfos pertencem ao grupo C, onde se repartem por quatro subgrupos: V_Hn, V_HnL, V_Ha e V_HP. Apesar de os subgrupos V_HnL e V_HP conterem apenas sequências pertencentes a um só género (*Lepus* e *Ochotona*, respectivamente), os outros agrupamentos reúnem genes de espécies e géneros diferentes. Este cenário sugere que os genes V_H dos lagomorfos seguem um modelo de evolução das famílias multigénicas denominado "birth-and-death" (ou seja, certos genes sofreram duplicação e divergiram desde então, enquanto outros mantêm um elevado grau de homologia).

A hipótese da manutenção de um número de linhagens alotípicas igual ou superior a quatro por um período de pelo menos 50 milhões de anos levanta a questão do tamanho mínimo das populações fundadoras. Esta questão é abordada no estudo da composição genética da população de Porto Santo. O coelho foi introduzido nesta ilha atlântica no século XV. Diversos documentos históricos sugerem que essa introdução consistiu na libertação de uma só fêmea prenha, à qual se sucedeu um rápido aumento do efectivo populacional. O estudo de uma bateria de 34 marcadores moleculares (incluindo loci proteicos, microssatélites, marcadores imunogenéticos e de DNA-mitocondrial) revelou que a população de Porto Santo se originou a partir do Sudoeste de Portugal e resulta de um recente efeito de fundador. Uma análise mais detalhada dos dados genéticos demonstra que as observações estão de acordo com a tese que propõe que esta população tenha sido fundada por uma só fêmea prenha.

Résumé

Les différents loci contrôlant la biosynthèse de l'anticorps, sont chez le lapin, munis d'un large éventail d'allèles. La structure de ces allèles est bien connue, en ce qui concerne le lapin domestique. Par contre ces allèles n'ont pas été étudiés dans des populations sauvages à l'exception près du locus C_{KI} de la chaîne L. Nous avons contribué de manière substantielle à une meilleure connaissance de ces polymorphismes d'anticorps, en déterminant les séquences codantes d'un nombre d'allotypes nouvellement découverts dans des populations ibériques. Nous avons étendu ces études à d'autres espèces de lagomorphes, notamment des espèces appartenant aux genres *Lepus*, *Sylvilagus* et *Ochotona*.

Dans un premier temps, nous avons étudié un polymorphisme qui peut être qualifié de simple et récent. Il s'agit de la variation génétique du deuxième domaine de la partie constante de la chaîne H de l'anticorps de la classe Gamma (*IgGCH2*, ou $CH_\gamma 2$, aussi connue sous le nom de "locus *e*"). Ce domaine joue un rôle prépondérant dans les multiples fonctions d'anticorps, et est une des cibles préférées d'agents pathogènes. Nous avons séquencé le domaine *IgGCH2* dans onze espèces différentes: huit du genre *Lepus* (*europaeus*, *granatensis*, *timidus*, *americanus*, *capensis*, *calotis*, *californicus* et *castroviejoi*), deux du genre *Sylvilagus* (*floridanus* et *cunicularis*), en plus du lapin de garenne (genre *Oryctolagus*). Ceci a permit de constater une variation "hotspot" à la position H309, où cinq différents résidus ont été observés (Met, Lys, Val, Ala et Thr), tandis que la variation était quasiment inexistante dans le restant du domaine. En incluant d'autres ordres de mammifères (*Primata, Metatheria, Rodentia, Carnivora, Perissodactyla* et *Artiodactyla*) l'étude montrait que ce phénomène n'est pas limité aux seuls lagomorphes. On a pu indirectement établir que cette variabilité est promue par une force déterministe qui favorise la diversité ou le changement à cette position particulière. Nos données sur la variation à l'intérieur de la Péninsule Ibérique ont confirmé la thèse que le polymorphisme *e14/e15* du lapin domestique, absent de la Péninsule, est dû à une mutation récente d'un gène ancestral e15, probablement d'origine ibérique. La connaissance des séquences d'ADN sous-jacentes à cette mutation, a rendu possible la mise au point d'une méthode de PCR/RFLP, permettant la détection des allèles *e14* et *e15* du lapin, sans le recours d' antisérum spécifique.

Une partie plus importante de nos travaux est consacrée à l'étude de la variation allélique au locus *IgVH*, qui gouverne la partie variable de l'anticorps. Notons d'emblée, que cette variabilité concerne les régions charpentes (FR), et non pas les régions qui sont impliquées dans la reconnaissance de l'antigène (CDR). Contrairement au polymorphisme précédant ($CH_\gamma 2$), qui se limite à la variation à un seul résidu, ce polymorphisme est complexe (à savoir les allèles diffèrent par des substitutions d'acide aminé multiples), et phylogénétiquement très ancien (plus ancien que les espèces). Ce polymorphisme a été étudié en détail dans les lapins domestiques, où on distingue trois lignées allotypiques (*a1*, *a2*, *a3*). Leur transmission mendélienne est due au fait que le lapin, lors du réarrangement *VDJ*, n'emploie en substance qu'un seul gène V_H, c'est à dire le gène V_H1. Nous avons élargit ces connaissances aux populations ibériques, où nous avons mis en évidence deux allotypes nouveaux, dont un semble être associé à la sous-espèce *O. c. algirus*. Un de ces allèles se distingue par l'absence de réactions croisées ("*a-blank*"). Le séquençage de l'ARN messager a permis de conclure que les lapins "*a-blank*" utilisent quasiment exclusivement un gène V_H, qui s'apparente phylogénétiquement aux V_H1 du lapin domestique, et constitue une nouvelle lignée allotypique, appelée "*a4*".

Les grandes distances séparant les différents allèles V_H1 ont été interprétées comme une indication du fait que leur coalescence pourrait se situe dans un temps très éloigné (50 million d'années, d'après M. Nei). Nous avons évalué cette hypothèse par l'étude des gènes V_H exprimés dans des lièvres (*Lepus europaeus* et *Lepus granatensis*). On a su établir dans ces espèces deux lignées de V_H, nommé *a2L* et *nL*. Il apparaît de façon claire, que la lignée *a2L* du lièvre s'apparente à la lignée *a2* du lapin. Les distances entre les allèles V_H-*a2* de ses homologues V_H1-*a1*, -*a3* et -*a4* sont nettement plus importantes que les distances entre les lignées *a2* et *a2L*. Ceci démontre que les gènes V_H qui, dans le lapin d'aujourd'hui se comportent comme des allèles mendéliens, ont débuté leur divergence dans une période qui est antérieure à la séparation des deux espèces.

Ces données concernant le locus V_H1 semblent contradictoires. L'association de différents allèles avec des sous-espèces distinctes ne s'accorde pas aisément avec des temps de coalescence des lignées qui sont nettement plus longs que les temps de spéciation. Nous avons néanmoins pu établir que les vitesses d'évolution ne sont pas les mêmes dans les différentes lignées, ce qui pourrait aider à réconcilier les deux observations.

Nous avons étendu ces études aux gènes V_H qui sont enfuis dans les génomes (sans être exprimés) des espèces appartenant aux genres *Lepus*, *Sylvilagus*, et *Ochotona*. Tous

s'apparentent au group C, où ils se répartissent en quatre sous-groupes (V_Hn, V_HnL, V_Ha et V_HP). Tandis que toutes les séquences des groupements V_HnL et V_HP n'appartiennent qu'à une seule espèce, dans d'autres groupements, différentes espèces sont réunies. Ceci est conforme à un modèle d'évolution des familles multigéniques dénommé "birth and death" (c'est à dire certains gènes se répandent par duplication et divergent et se perdent en partie, pendant que d'autres maintiennent un degré plus important d'homologie).

L'hypothèse qu'un nombre de lignées allotypiques, supérieur ou égal à quatre, a pu se maintenir durant 50 millions d'années, pose la question de la taille minime des populations fondatrices. Cette question clé est traitée dans l'étude détaillée de la composition génétique de la population de l'île de Porto Santo. Le lapin a été introduit dans cette île de l'Atlantique durant le XV ième siècle de notre ère. Les documents historiques indiquent qu'une seule femelle en gestation a été lâchée, évènement suivi peu de temps après par une démographie galopante. Notre étude de 34 marqueurs génétiques confirme l'existence d'un fort effet fondateur. Une analyse plus en profondeur démontre que les observations sont en parfait accord avec la thèse d'une seule femelle fécondée fondatrice.

Introduction

This work is situated at the meeting point of two ongoing research projects focusing on the extraordinary degree of genetic variation in European rabbit (*Oryctolagus cuniculus*). These are the phylogeographical studies of this variation and the evolutionary history of the complex immunoglobulin polymorphisms.

There are two subspecies of the European rabbit (*Oryctolagus cuniculus*): *O. c. algirus* and *O. c. cuniculus*. Both subspecies are present in the Iberian Peninsula where they are separated by a secondary contact zone along a Northeast-Southwest cline. The genetic diversity observed at nuclear and mitochondrial markers revealed that the level of population diversity was much higher in Iberia, than in the more recent area of distribution, Continental Europe (North of Pyrenean Mountains), Great Britain and overseas. The domestication of rabbit is recent (Middle Ages) and all domestic breeds are apparently derived from *O. c. cuniculus* populations. In this context, the wide variety of genetic markers makes the rabbit an ideal model species for theoretical genetic investigations regarding speciation phenomenon, hybridisation and introgression, etc., and for evaluating methods of evolutionary inference based on diversity patterns of autosomal, maternal (mtDNA) and paternal (Y chromosome) markers. The introduction of rabbits to numerous islands also provides favourable means for studies of the consequences of population bottlenecks.

Most of the polymorphisms mentioned above are in essence selectively neutral. Historically, the first polymorphisms studied in rabbits were those of the antibody genes. Their highly unusual patterns of gene diversity at both the molecular and the population genetic level revealed that these polymorphisms are adaptive, and maintained by strong selective forces. These polymorphisms have been mainly studied in laboratory breeds and in populations of feral rabbits from the recent distribution range. While we have a good understanding of the mechanisms of antibody gene organisation, ontogeny and expression of the antibody, we know little about aspects of population diversity of antibody loci. The evolutionary origin of rabbit variable region allotypes remains enigmatic, and has been qualified as "an evolutionary puzzle".

We expected that new insights concerning their biological importance could be obtained by studies of the wild populations from the native area of distribution. We have furthermore approached the evolution of these Immunoglobulin (Ig) polymorphisms by cloning and sequencing of Antibody (Ab) genes expressed in the subspecies *O. c. algirus*, and of *Lepus* species. We have also contributed to an evaluation of the hypothesis of multi-allele trans-species polymorphism, by determining the minimal size of founder populations by studying the rabbits of the Atlantic Island of Porto Santo.

We have subdivided our work into five chapters:

1. Essentials
2. Genetic variation at the constant region of the IgG antibody in leporids
3. Studies of gene diversity at the Heavy chain variable region gene locus (*a* locus or *IgVH* locus) in leporids
4. Evaluation of the bottleneck effect in the Porto Santo wild rabbit population.
5. Conclusions

The abstracts of these chapters are presented below:

CHAPTER 1

Essentials

This chapter reviews in short a number of topics that may help understanding of the following chapters. They concern the immune system, antibody structure, function and genomic organisation. The processes by which diversity at the antibody level is generated, are explained in some detail. We give a brief account on the Lagomorph's evolutionary history and systematics and survey the known genetic diversity of the rabbit (for protein loci, mitochondrial DNA, microsatellites and immunological markers). The role of the rabbit in immunological research is presented in a historical perspective.

CHAPTER 2

Genetic variation at the constant region of the IgG antibody in leporids

We developed a PCR/RFLP approach for studies of the single amino acid polymorphism (SAP) of the constant region of the rabbit Ig Gamma chain (*IgGCH2* or *e*-locus). It offers a convenient alternative to the arcane serological methods in use. Sequences of the *CH2* exon, which harbours the SAP, were determined for different genetic variants for eleven Leporid species (genera *Oryctolagus*, *Lepus* and *Sylvilagus*). The variability at amino acid position 309 (79 in IMGT numbering) was studied in different leporid species and compared to that in some groups of mammals. These studies have revealed the high variability at one particular amino acid position (i.e. H309).

CHAPTER 3

Studies of gene diversity at the Heavy chain variable region gene locus (*a* locus or *IgVH* locus) in leporids

The preferential usage of the rabbit V_H1 gene, together with its extensive allelic variation, suggests that different allotypic lineages may generate different antibody repertoires. A key question is whether the distribution of the different allelic forms in rabbit can be influenced by environmental factors (such as microbial flora or disease agents) or only depends upon evolutionary history. A major objective of this work was to initiate the phylogeographic approach of the allele diversity of the rabbit *IgVH* gene clusters in the subspecies *O. c. algirus*.

A second question concerns the length of persistence time of V_Ha allotypic lineages, and their number. We have therefore determined DNA sequences, both for expressed (cDNA) and germline V_H genes. These were obtained from specimens of *O. c. algirus* and of other Lagomorph species (*Lepus*, *Sylvilagus* and *Ochotona*). This allowed the reconstruction of likely phylogenetic relationships. Our studies added new facts that contribute to a better understanding of the evolution of the V_H gene cluster in rabbits. Our results show that the divergence between at least some allelic V_Ha lineages of rabbit was anterior to the *Lepus-Oryctolagus* split; i.e. that the polymorphism is older than the species.

The hypothesis of a multi allele trans-species polymorphism raises the question of the minimal population size of founder populations, which is addressed in Chapter 4.

CHAPTER 4

Evaluation of the bottleneck effect in the Porto Santo wild rabbit population

In this study we show that for rabbit, the size of founder populations can be extremely limited. Indeed, our study of the Porto Santo population demonstrate that successful populations can be founded by a single pregnant female, making it appear as highly unlikely that four or more V_Ha lineages have been maintained over millions of years. However, the importance and implications of this study reaches far beyond this particular issue.

One of the most important aspects of the natural history of the rabbit is anthropogenic introduction. This species was introduced to the Atlantic Island of Porto Santo in the XVth century and is described in several historical documents. The rabbit of Porto Santo is referred to by eminent scientists (e.g. Darwin and Hæckel) as an example of morphological divergence since its introduction in the island 600 years ago. In this work three immunoglobulin loci (V_H1 or *a*, C_K1 or *b and CH2* or *e*) and 31 additional "neutral" markers (mtDNA, protein loci, microsatellites) were studied, which contributed to: 1) determine the phylogeographic origin of this population; 2) understand the effects of a severe bottleneck on the population genetic structure; 3) confirm the hypothesis that the Porto Santo population was founded by one single pregnant female.

CHAPTER 1

Essentials

Basic Immunology

Vertebrates have a variety of mechanisms that provide protection against infectious pathogens and other agents (viruses, bacteria, protozoa, infectious and infected cells, foreign cells, etc.). Among these defence mechanisms the immune system is probably the most efficient and certainly the most elaborated. It can be innate or adaptive. The adaptive immune response is highly specific for a particular pathogen. Whereas the innate response does not change on repeated exposure to a given infectious agent, the adaptive response improves ("adapts") at each successive encounter with the same pathogen.

The first step of immune response is the recognition of foreign antigens. The adaptive immune system uses two distinct types of molecules in this process, the T cell antigen receptor (TCRs), which are expressed by mature T lymphocytes, and the immunoglobulins (Ig) or antibodies (Ab), which are produced by B lymphocytes. The immune system generates cells that can recognize an enormous range of antigens but each lymphocyte (B or T cell) is genetically capable of recognizing only one particular antigen. After an antigen binds to the few cells that can recognize it, these cells are stimulated to proliferate and mature. Some of them, called memory cells, are maintained in the plasma and constitute the immunological memory of particular antigens.

The second step of immune response is to destroy the pathogens by what is often called the effector system. There are numerous ways to eliminate the pathogens, such as: a) neutralization (antibodies can combat certain pathogens just by binding to them), b) phagocytosis (antibody is important in activating complement, or acting as an opsonin to promote ingestion by phagocytes that will digest the pathogen), c) cytotoxic reaction (where the target cell is recognized, either by a specific antibody bound to the cell surface or by specific TCRs). The cytotoxic cells can destroy the target cell or can induce the target cell to self-destruction (apoptosis).

The immune system is vital for survival. A deficiency in any part of these systems leaves the individual exposed to a greater risk of infection. These deficiencies can be hereditary or acquired (e.g. acquired immunodeficiency syndrome or AIDS). Particular ways by which the system can fail are autoimmunity, where the immune system lost the capacity to recognize the body's own tissues as "self" and reacts against them (e.g. rheumatoid arthritis and pernicious anaemia) and hypersensitivity, where the immune reactions may cause more damage than the pathogen or antigen itself (e.g. molecules on the surface of pollen grains).

The cells involved in the immune response are organized into tissues and organs referred to as the lymphoid system. The major lymphoid organs and tissues are classified as primary (central) or secondary (peripheral). The primary tissues in the mammals are the thymus, for T cells, and bone marrow and foetal liver, for B cells. In these organs the lymphocytes differentiate from the lymphoid stem cells, proliferate and mature into functional cells. Then the lymphocytes migrate into peripheral or secondary lymphoid organs that comprise the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT). They provide an environment in which lymphocytes can interact with antigens. Immune responses generated in these secondary lymphoid tissues require phagocytic macrophages, antigen-presenting cells, and mature T and B lymphocytes.

Structure and function of Antibodies

The antibodies are glycoproteins with typical tetrameric Y-shaped form composed of two distinct types of polypeptide chain. They consist of two identical heavy (H) chains of 50-77 Kilo Daltons (KD) that are structurally distinct for each Ig class or subclass, and two identical light (L) chain of 25 KD that are common to all classes. Two distinct domains are

distinguished, each possessing characteristic functions: a variable (V) region located at the amino-terminal part, which exerts recognition functions and a constant (C) region at the carboxyl-terminal part assuring an effector function. A single disulfide bond connects the L and H chains. The connection between the two H chains is made by one or more disulfide bounds. The Ig molecule is bivalent with two identical sites for potential binding to antigen formed by the amino terminal regions of the H-L chain pair. The schematic representation of one Ig molecule is presented in Figure 1.1.

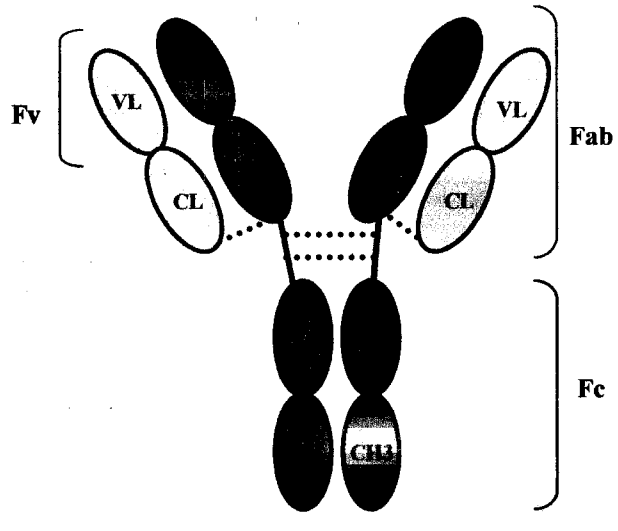

Figure 1.1. Schematic representation of a human IgG molecule. The Fv part is composed of the H and L chain V domains (VH and VL) and make up the antigen-binding sites. The Fv and the H chain CH1 and CL domains constitute the Fab part. The C-terminal constant domains of the H chain (CH2-CH3) form the Fc region of the molecule. The hinge region joins Fc and Fab. The inter-chain disulfide bonds are represented by dotted lines.

Each Ig chain is composed of domains with about 110 amino acid residues each. The H chain contains four to five domains (VH, CH1-CH4), while the L chain comprises only two (VL, CL). The amino acid sequence of the amino-terminal segments varies among antibodies, while the carboxyl-terminal domains are much more conserved. The unit composed of the paired H and L chain variable regions (VH and VL), is named Fv (variable Fragment), whereas the fragment containing the whole L chain, the variable (VH) and the first constant (CH1) domains of the H chain is called Fab (antigen-binding Fragment). Finally, the C domains (CH2-CH3, or CH2-CH3-CH4) form the Fc (crystallisable Fragment) part of the Ig, which interacts with effector molecules and cells.

The antigen-binding sites

Of the domains composing the Fab region (VL, VH, CL and CH1), only the V domains (VL, VH) are involved in antigen binding. The amino acid sequence comparisons within both VH and VL domains revealed in each domain the existence of three hypervariable regions. These are called complementarity-determining regions (CDRs) because they interact with the antigen. The regions separating the CDRs are relatively invariable and are referred to as framework regions (FRs). (see Figure 1.2).

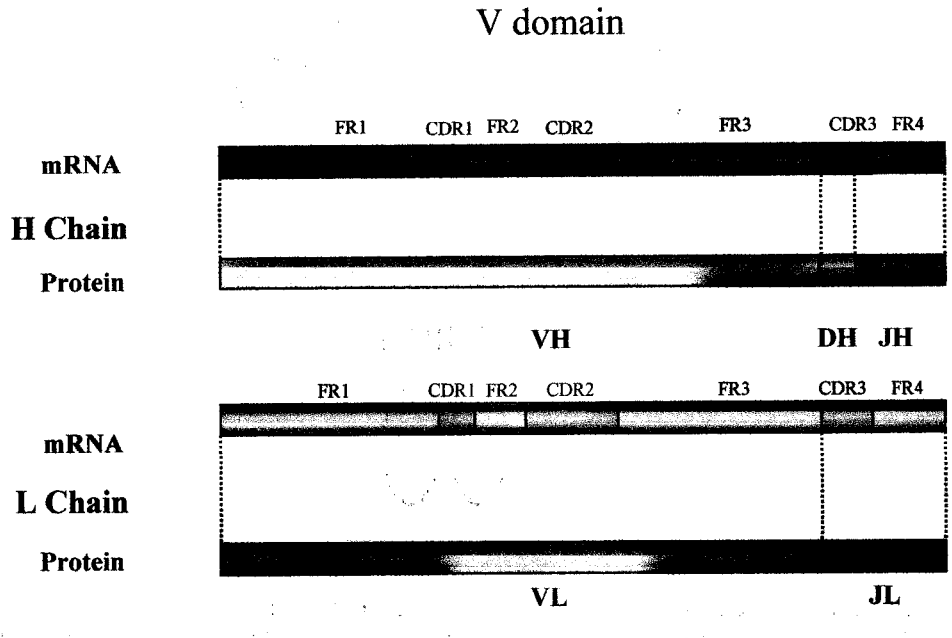

Figure 1.2. Schematic representation of the Heavy (H) and Light (L) chain. FR – Framework; CDR – Complementary Determining Region; V – Variable region; D – Diversity region; J – Joining region.

The contribution to antigen binding of the different CDRs in VH and VL domains varies from one antigen-antibody complex to another. Any residues in any of the six hypervariable regions can be actively involved in the molecular interaction with the antigen (see Padlan, 1994). For this reason, the definition of CDR and each FR region are subject to debate. There are different proposals. Most in use are the numbering suggested by Kabat *et al.* (1991) and the unique numbering of IMGT (the international ImMunoGeneTics database http://imgt.cines.fr, Lefranc, 2001). These are presented in Figure 1.3. We adopt the former convention, which is most in use among rabbit immunologists.

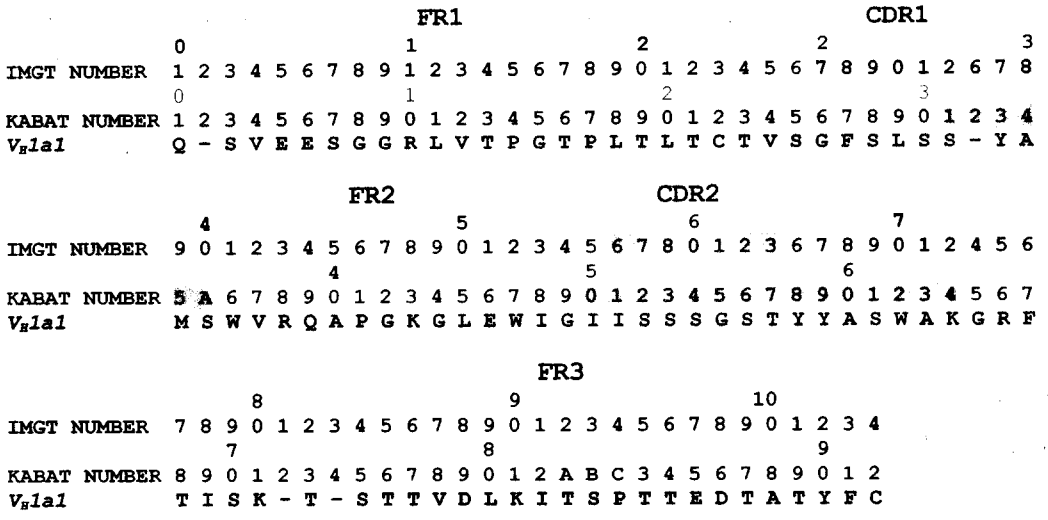

Figure 1.3. Amino acid boundaries delineating the sub-domains of the H chain of rabbit *V_H1a1* according to Kabat *et al.* (1991) and the unique numbering of IMGT (Lefranc, 2001). The CDRs are grey-shaded.

The overall shapes of the antigen-binding sites created by the juxtaposition of the CDR of the H and L chains vary greatly from clefts to grooves to flat surfaces. In general, small antigens such as haptens or oligo-peptides bind to clefts or pockets lying between the H and L chain V domains, whereas larger protein antigens bind on extended planar surfaces formed by binding sites of antibodies (Webster *et al.*, 1994; Padlan, 1996).

Fc region

The Fc fragment exposes a variety of epitopes that mediates effector functions, such as complement fixation. The docking of antibody onto Fc receptors of macrophages can either initiate a cascade of reactions leading to the lyses of the foreign cells, or trigger phagocytosis of the antibody-antigen complexes. Each of the five classes of Igs described in the mammals, IgA, IgG, IgD, IgM and IgE are characterized by distinct effector functions. However, all of these classes share the Fab region.

The Fc region of IgA, IgG and IgD consists of CH2-CH3 dimers, while in the case of IgM and IgE is composed of CH2-CH3-CH4. The binding sites of IgG for Fc receptors are localized in the CH2 region (e.g. Tao *et al.*, 1993; Idusogie *et al.*, 2000). In response to the destructive potential of the Fc region, many kinds of microorganisms have developed proteins that are able to bind to, or proteolytically cleave, the Fc part, thus preventing it from functioning. The blocking of IgG functions by staphylococcal protein A and streptococcal protein G are good examples of these strategies (Deisenhofer, 1981). The

rabbit is particular by having no IgG subclasses. The unique CH2 domain of this species shows a genetic polymorphism that will be studied more in detail in this thesis (*IgGCH2* or *e*-locus).

Hinge region

The hinge region joins the Fc and Fab regions and allows some degree of flexibility between the antigen-binding and effector components of the antibody. Hinge regions are rather variable between classes, both in length and in composition, and are less conserved in evolution. Structurally, hinges are extended segments of dimeric peptides held together by one or more disulfide bridges. It has been shown that the removal of inter-chain disulfide bridges of the hinge abrogates the antibody's effector functions (Coloma *et al.*, 1997). The hinge is the part of an Ig which is most exposed to proteolytic attack. As an example, the IgD immunoglobulins, with their long charged hinge are very susceptible to proteolysis, which may explain their short serum half-life (Spiegelberg, 1977). In the rabbit, 13 IgA isotypes show a high degree of amino acid divergence in the hinge region. The presence of 13 IgA isotypes, each with different patterns of resistance to the various IgA proteases, could provide enhanced protection to the rabbit when exposed to multiple bacterial IgA proteases (Burnett *et al.*, 1989). The IgG hinge region of the rabbit shows two serological allotypes, *d11* and *d12*, which are associated by a Met/Thr interchange at position 209 (Prahl *et al.*, 1969).

Heavy-Light domains interactions

The H and L chain components of V regions interact by hydrogen bonds (VH-VL), whereas a disulfide bridge covalently links CH1 and CL. The VH-VL association is exclusively mediated by non-covalent interactions. Residues from CDR as well as FR regions participate in this association and the affinity between the two domains is high.

Pairing of monomeric H and L chains obtained from different individuals, or even from different species, was observed under physiological conditions (i.e. between rabbit L chains and human H chains; Kulics *et al.* 1983, reviewed in Frazer and Capra, 1999).

Most of the residues that are important in VH-VL contacts are hydrophobic and well conserved throughout vertebrates (see Hsu and Steiner, 1992). The contribution of the different FRs and CDRs to the VH-VL interaction can vary considerably. The H-CDR3 and

L-CDR3 interaction accounts for more than 60% of the total of the CDR interactions. For the FR-FR interactions more than half of the hydrophobic core contacts involve FR2 of one chain and FR4 of the other (Padlan, 1994). The remaining interactions involve essentially FR2 and FR4 of the L chain with the CDR3 of the H chain while only FR2 of the H chain interacts with the CDR3 of the L chain (Chothia *et al.*, 1985).

Immunoglobulin classes

Five distinct classes of Ig molecule are recognized in higher mammals, namely IgA, IgD, IgE, IgG and IgM. The H chains that define these five classes are designated by α, δ, ε, γ and μ, respectively. The sequence difference between these H chains identifies the various isotypes and determines their functional activity. The number and location of inter-chain disulfide bonds, the number of attached oligosaccharide moieties, the number of C domains, and the length and sequence of the hinge region are characteristics of each isotype. There is also heterogeneity within classes. For example, in human and mice there are four subtypes of IgG antibodies that have different physical properties.

IgE and IgG were not identified in reptiles, amphibians or birds and are probably unique to mammals. In fact, in birds and reptiles, antibodies of the IgY isotype exhibit the functional characteristics of both IgE and IgG, whereas the amphibian *Xenopus laevis* expresses the IgX isotype that shows similar tissue distribution as mammalian IgA (Mussmann *et al.*, 1996). Lower vertebrates, such as fish, amphibians and reptiles possess fewer Ig isotypes than mammals. Some produce monomeric and polymeric IgM, truncated and full-length versions of the immunoglobulin polypeptide (Hsu, 1994; Warr, 1995). In addition, new isotypes have been identified in sharks. Indeed, the NAR (New Antigen Receptor), are homodimers consisting of two identical H chains, containing a variable and five constant CH1 type domains. Such homodimeric Igs seem to be restricted to chondrichthyes (Greenberg *et al.*, 1995), with the notable exception of the H chain antibodies of *Camelidae* (Nguyen *et al.*, 2000). The evolutionary trends of diversifying antibody classes suggest that different isotypes serve specific biological functions. B cells can switch from secreting IgM to other isotypes like IgD, IgE or IgG (Ig class switch, see below).

Genomic organization of Ig genes

An important feature of the immunological system is the fact that the antibody-encoding genes do not exist as such in the germline. The combinatorial rearrangement of the gene segments and the diversity obtained in the segment joining process, together with the diversification process of gene conversion and somatic hypermutation, contribute largely to the impressive capacity of the immune system to generate diversity (see Figure 1.4).

Three clusters of genes, localized on separate chromosomes, code for the different Ig molecule chains (Tonegawa, 1983). These are the gene families coding for H, K-L and λ-L chains, respectively. Each cluster can be composed by gene families encoding constituents of the V and C domains. The germline organization of L and H chains gene segments of higher vertebrates are schematically represented in Figure 1.5.

Heavy Chain

Variable region

Three types of germline elements encode the Ig H chain V region domain: V_H (Variable), D_H (diversity) and J_H (Joining) segments. For each chain there are several tandem repeats of *V* segments, which are composed of two exons (Leader and *V*-exon) separated by an intron of about 100-bp.

The human H chain gene complex has been localized on the chromosome 14 and consists of 123 V_H segments, classified into seven families, 27 functional D_H genes and nine J_H segments (six are functional) (see Matsuda *et al.*, 1998). The mouse Ig H-chain locus contains about 100-200 V_H genes classified into 15 families, a cluster of four J_H genes and 13 D_H gene segments (see Almagro *et al.*, 1997).

The FR1, CDR1, FR2, CDR2 and FR3 are encoded by the V_H gene segment, while the CDR3 is generated from the *D* and *J* exons. J_H exon encodes part of the CDR3 and the entire FR4 region.

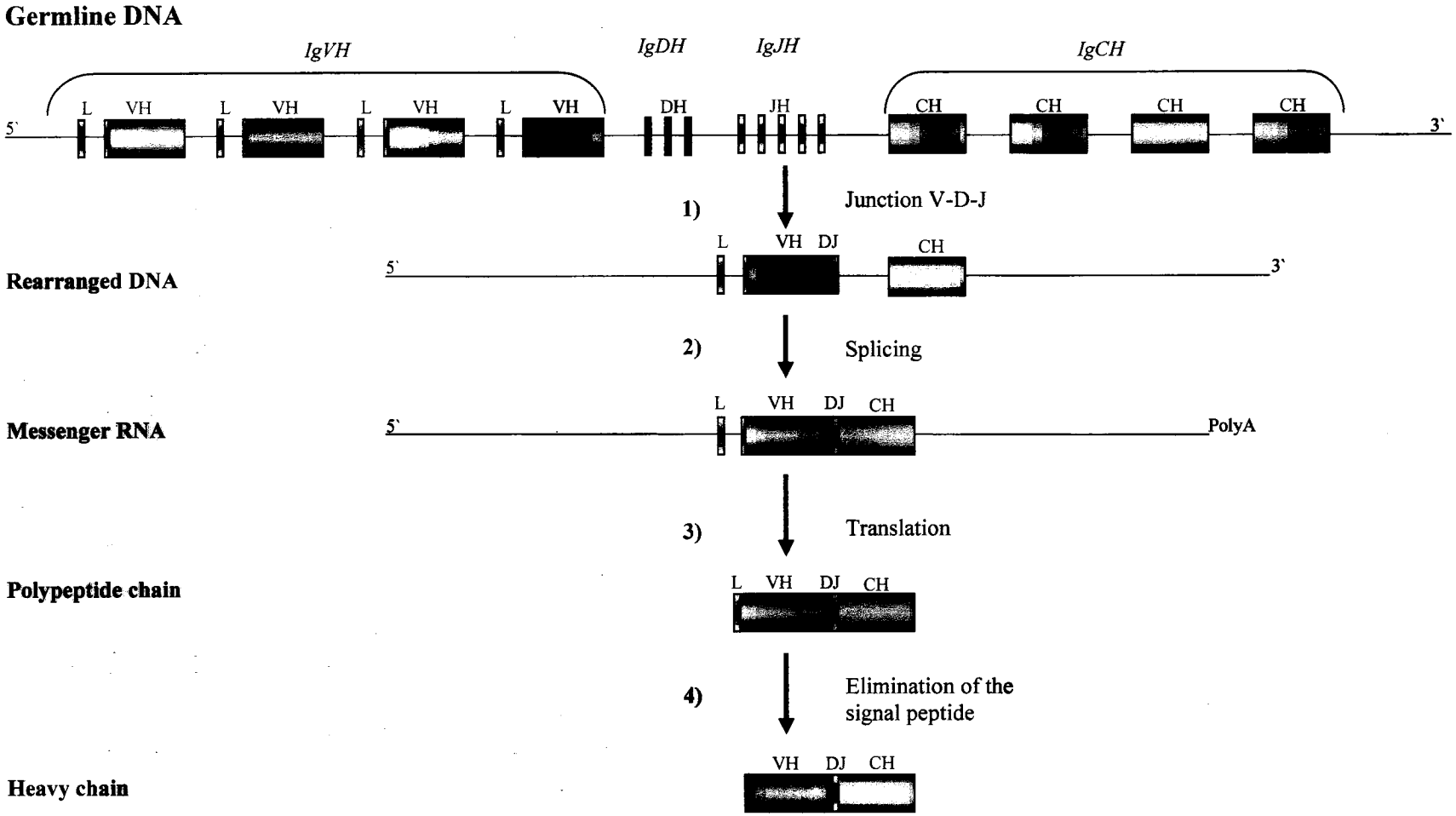

Figure 1.4: Synthesis of an immunoglobulin. 1) At the DNA level, one *IgDH* gene is joined to one *IgJH* gene, with deletion of the intermediary DNA, to create a partially rearranged *D-J* gene. 2) In a second step, one of the *IgVH* gene is joined to *D-J*, with the deletion of the intermediary DNA, to generate a completely rearranged *IgVH-D-J* gene. This rearrangement is transcribed with the *IgCH* gene into a *IgVH-D-J-C* pre-messenger RNA, the RNA sequences corresponding to the introns and to the non used *IgJH* genes are excised by splicing, and a mature messenger, which comprises the spliced regions, and the 5`and 3` untranslated sequences, is obtained. 3) The messenger RNA is translated into a polypeptide chain by the ribosome. 4) The signal peptide is cleaved off by a peptidase following the entry of the polypeptide chain in the endoplasmic reticulum, and a mature heavy chain is produced.

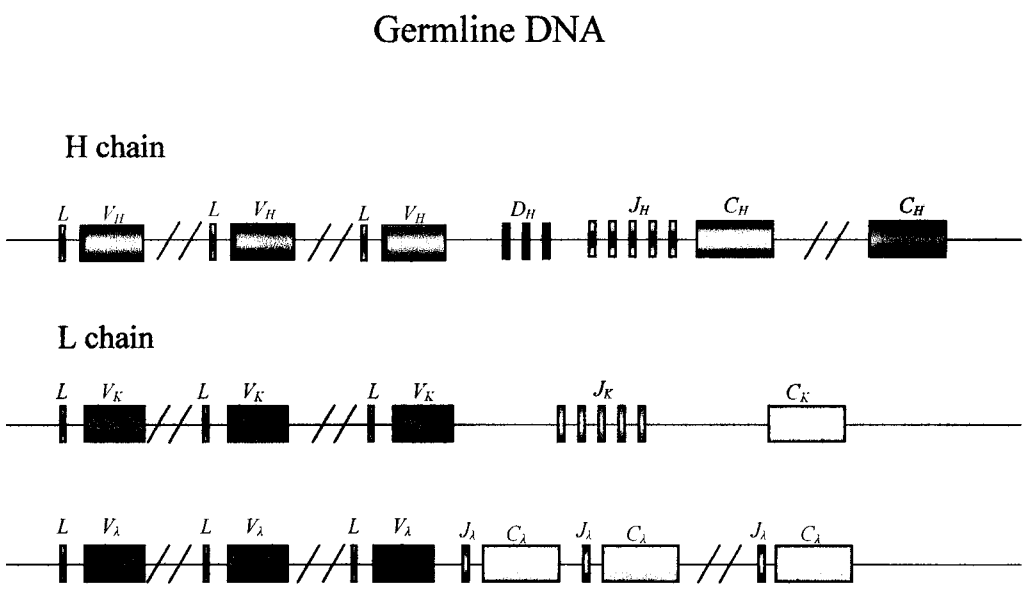

Figure 1.5. Schematic representation of the localisation in the human Ig molecule of the gene segments that compose the H and L chain. H chain: *L* – Leader, *V* – Variable, *D* – Diversity, *J* – Joining and *C* – Constant gene segments. Kappa (K) and Lambda (L) L chains: *L* – Leader, *V* – Variable, *J* – Joining and *C* – Constant gene segments.

Constant region

The number of genes encoding the H chain constant regions can vary between species. For example, in the mouse it is composed of eight *C* genes whereas in the human this gene region consists of nine functional genes. The rabbit shows an unusual organization of the H chain *C* gene loci, since it possesses only one C_γ gene (see Hamers, 1987) and 13 C_α genes (Burnett *et al.*, 1989; Knight and Winstead, 1997). Neither the unicity of the C_γ gene, nor the extensive duplication of the C_α genes has been observed in any other mammalian species. The expansion of C_α genes is found throughout the order *Lagomorpha* and suggests that the C_α genes began to duplicate in a common ancestor (Burnett *et al.*, 1989).

Heavy chain genomic organization in different vertebrate species

The gene organization of immunoglobulins varies extensively among the different classes of vertebrates (see Figure 1.6). In lower vertebrate such as sharks, the basic unit of repeats is *V-D-D-J-C*, and linked gene groups $(V\text{-}D\text{-}D\text{-}J\text{-}C)_n$ are repeated several times in throughout the chromosomes. In the genome of higher vertebrates the gene segments for the different components of the Ig chains are linked together (*V*n-*D*n-*J*n-*C*n).

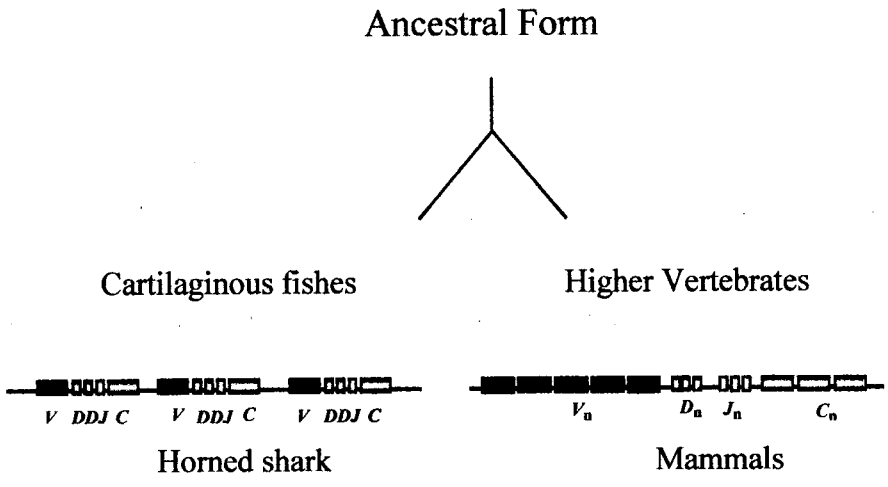

Figure 1.6. Genomic organization of immunoglobulins H chain genes. Cartilaginous fishes have the $(V_H\text{-}D_H\text{-}D_H\text{-}J_H\text{-}C_H)$n type organization including germline joined genes, whereas most higher vertebrates, have the (V_H)n-(D_H)n-(J_H)n-(C_H) type gene organization. *V* – Variable segment gene; *D* – Diversity segment genes; *J* – Joining segment gene; *C* – Constant segment gene.

Light Chain

Two Ig L chain isotypes (K and λ), that are functionally indistinguishable, exist as separate gene loci in mammals.

Variable region

Two types of germline elements encode the Ig V region domain of both Kappa (K) and Lambda (λ) L chains: V_L (Variable) and J_L (Joining) segments. The structure of V_L segments is identical to that described above for V_H.

In the mouse, 95% of the L chains are of the K type and only 5% are of the λ type (Cotner *et al.*, 1978). 140 V_K and five J_K genes, localized on chromosome 6, have been identified. The V_K genes were subdivided into 18 gene families (Kirshbaum *et al.*, 1998). The λ locus is localized in chromosome 16 and is composed by 3 *V* segments and four *C* segments (e.g. Sanchez *et al.*, 1991).

In the human only 60% of immunoglobulins are of the K type (Fu *et al.*, 1979). There are 76 V_K genes, from which 32 are potentially functional, and five J_K genes, localized on the chromosome 2 (Malcolm *et al.*, 1982). Human V_λ genes have been grouped into 10

families. There have been identified 70 genes, from which 37 are functional (e.g. Stiernholm *et al.*, 1994).

Constant region

The mouse C_L gene loci are composed of a single C_K gene and four C_λ. Each of the C_λ is associated with its own J_λ segment. The human CK also consists of only one gene, while the C_λ locus is composed of seven J_λ-C_λ segments (Figure 1.5)

Rabbit uses almost exclusively the K chain, of which two isotypes, K1 and K2, are described (Benammar and Cazenave, 1982; Heidmann and Rougeon, 1983). The majority of rabbit V_K genes encodes an extra cysteine at position 80 (see for review Mage, 1987). K1 chain is more expressed than K2, and differs from all other known Ig chains by a supplementary disulphide bond between the VK and the CK domain. This is performed by cysteines at positions 80 (V_K) and 171 (C_{K1}). A schematic representation of rabbit partial *K* chain locus is represented in Figure 1.7.

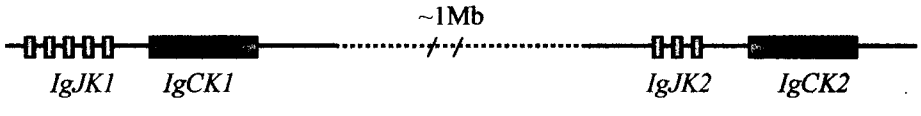

Figure 1. 7. Schematic representation of rabbit K Light chain isotypes 1 and 2. J_K–Joining K chain genes; C_K – Constant K chain genes.

Immunoglobulin class switch

During B cell differentiation into memory or Ab secreting cells, the isotype of the Ig H chain changes from μ to γ, ε or α. This important mechanism allows changing the biological effector function of the Ab without changing its specificity. These Ig class switches occur within the cell. It was shown that the same cell that previously secreted IgM could start the secretion of IgG (van der Loo *et al.*, 1979). During the process of class switching C_H genes are replaced through a site-specific DNA recombination process, which takes place between switch regions (S) located upstream from the C_H genes. Switch recombination is accompanied by intramolecular DNA deletion, letting the switched H chain gene on the chromosome (Storb *et al.*, 1981).

Generation of antigen-binding repertoire

The efficiency of the immune system is in part due to the potential of generating a bewildering variety of receptor molecules. This potential is such that it can match the wide variety of antigens and even anticipate antigen variation. The generation of primary repertoires and Ig gene diversification can occur by many different mechanisms. Furthermore, the anatomical site of primary repertoire diversification varies among species. Indeed, studies in mice and humans identified bone marrow and foetal liver as the site of B cell genesis and development (Claman *et al.*, 1966; Nuñez *et al.*, 1996), whereas the bursa of Fabricius is the site for B cell lymphopoiesis in birds (Cooper and Lawton, 1972). In sheep and cattle, ileal Peyer's patches serve as the main site of B cell diversification (Reynolds and Morris, 1983; Reynolds, 1987). While diversification occur in the bovine spleen (Lucier *et al.*, 1988). In the rabbits, B cell diversification takes place in the appendix (e.g. Archer *et al.*, 1963; Cooper *et al.*, 1968).

Germline and combinatorial diversity

The large number of inherited *V*, *D*, and *J* gene segments available for generating the V domain of the Ig chain contributes substantially to antibody diversity. This is well established in the case of mice and humans, where the B cells express different receptor molecules due to numerous functional *V*, *D*, and *J* segments.

Combinatorial diversity results from the random selection for recombination of inherited *V*, *D* and *J* segments in the case of H chain and *V*, and *J* segments in case of L chain. The large primary repertoire produced by the somatic juxtaposition of the different segments is further increased by the random choice of individual H and L chain V domains. In theory, there are $>10^7$ possible combinations.

Junctional diversity

Each H and L chain are the outcome of chromosomal rearrangements involving the V_H and V_L gene segments (*V(D)J* joining). *V* exons are flanked at the 3'end by a recombination signal sequence (RSS) which consists of conserved heptameric (CACAGTG) and nonameric (ACAAAAACC) sequences separated by a spacer sequence of 23-bp in the H and λ L chain and of 12-bp in K L chain (see Figure 1.8). Each *D* exon is flanked on both

sides by an RSS with a short signal element spacer of 12-bp. The J_H and J_K exons possess at its 5`end an RSS with the long spacer of 23-bp, while J_λ possesses a spacer of 12 bp. Recombination takes place between two heptamer-nonamer pairs, and can occur only when one pair is separated by a 12-bp spacer, and the other pair is separated by a 23-bp spacer, (12-23 spacer-rule; for review see Akamatsu *et al.*, 1994). The *V(D)J* type recombination is determined by the recombination-activating proteins, RAG1 and RAG2. It has been shown that these proteins are involved in the cleavage reaction recognizing the RSS and in the efficient joining of coding ends (Ramsden *et al.*, 1997; Agrawal *et al.*, 1998).

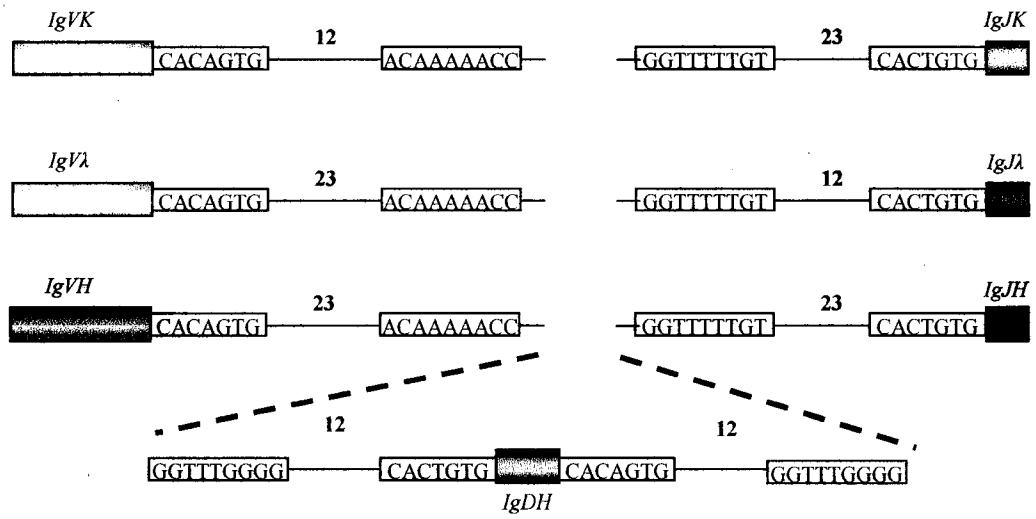

Figure 1.8. Schematic representation of *IgV*, *D* and *J* gene recombination signals. The conserved heptameric and nonameric sequences that flank each of the segments are represented in boxes. See details in the text.

Much of the antibody diversity is generated from the imprecise joining of *V*, *D* and *J* gene segments. This somatic process deletes and sometimes inverts short DNA segments in lymphocytes, and has so far been observed in most of the major lineages of jawed vertebrates, including cartilaginous fishes. This random loss or gain of nucleotides increases specifically the diversity of the third hypervariable region (CDR3). Two types of mechanisms are responsible for the incorporation of additional nucleotides at the coding ends: a) introduction of palindromic nucleotide sequences, that incorporate a few extra nucleotides reversed in orientation to the original coding end; b) n-region addition, that consists of a random insertion of non-template nucleotides between the coding ends of H chain gene segments, and is catalysed by terminal deoxynucleotidyl transferase (TdT) (Landau *et al.*, 1987; Meier and Lewis, 1993).

Post-rearrangement diversification

Although these two mechanisms are able to generate an extensive repertoire of possible Ag-binding sites, several species possess, or use, only a limited number of functional germline *V* segments. For example birds, together with a number of mammal species (rabbit, sheep, pig and bovine), have less extensive germline repertoires than mice and humans and consequently use strategies of primary repertoire development that overcome this limitation. In these cases, the primary antibody repertoire is diversified after *V(D)J* rearrangement by two mechanisms: somatic hypermutation and gene conversion.

Somatic hypermutation is characteristic of Ig genes and affects the affinity and the specificity of the antibody repertoire. The mechanism responsible for mutations is targeted to rearranged *V* gene segments, both active and inactive, introducing immediate point mutations at a rate of about 10^{-4} to 10^{-3} per base pair per cell generation, which is clearly higher than the normal frequency of random mutations. The general pattern of somatic mutations is not random, since replacement mutations are often over-represented in the CDRs, whereas silent mutations are concentrated in the FRs. Like any other mechanism introducing mutations, there are preferred sequences and pronounced hotspots for somatic hypermutation (Betz *et al.*, 1993; Reynaud *et al.*, 1995; Belessi *et al.*, 2001). Indeed, hotspot clusters have been observed in the CDRs 1, 2, and 3 regions as well as in the J-C region intron (Motoyama *et al.*, 1991; González-Fernandez *et al.*, 1994; Lanning and Knight, 1997).

Gene conversion is a non-reciprocal transfer of genetic information. In the particular case of Ig diversification, it involves the replacement of segments of the V_H gene engaged in *V(D)J* joining, with homologous sequences from upstream *V* genes. Gene conversion is the major mechanism of the Ab diversification in the chicken, where a set of pseudogenes are used as donors and the unique rearranged V_H/V_L-*(D)J* acts as acceptor (Reynaud *et al.*, 1985). The diversification takes place in the Bursa of Fabricius and in the spleen upon immunization (Arakawa *et al.*, 1996, 1998). A similar mechanism is found in the rabbit H chain loci, where the rearrangement involves a single V_H gene that is diversified mainly through gene conversion using other upstream V_H genes (Becker and Knight, 1990; Knight and Crane, 1994). Somatic hypermutation also takes place throughout the entire *VDJ* genes (Short *et al.*, 1991; Weinstein *et al.*, 1994). The rabbit's diversification process is explained

in more detail in chapter 3. In sheep, the B cell diversification occurs before birth in early development in the ileal Peyer's patches as a result of somatic hypermutation and gene conversion (Reynaud *et al.*, 1991). Both processes are used to diversify the Ag-binding repertoire in cattle (Parng *et al.*, 1996; Lucier *et al.*, 1998). Gene conversion mechanisms are possibly the main source of Ab repertoire diversification in swine (Sun and Butler, 1996).

Rabbit taxonomy and genetics

Taxonomy of Lagomorpha group

The order LAGOMORPHA was proposed by Gridley (1912) to distinguish the lagomorphs from the rodents. The phylogenetic position of the lagomorph group is not consensual. According to fossil data the lagomorphs are associated with rodents (e.g. Novaceck, 1992; Meng *et al.*, 1994), and classified within the super-order GLIRES. Based on molecular data, two conflicting evolutionary scenarios have been proposed for the mammals, placing the lagomorphs in different phylogenetic positions. For some authors the rodents group separated 110 Million years (My) ago, before the great mammalian radiation (90 My) and in this case the lagomorphs are more related to the primates and artiodactyls (Graur *et al.*, 1996; Kumar and Hedges, 1998; Nei *et al.* 2001). Other authors cluster the rodents with the lagomorphs and propose that the artiodactyls diverged 95 My ago, before the separation of the primates from the ancestral of rodent/lagomorphs (Huchon *et al.*, 1999; Liu and Miyamoto, 1999; Adkins *et al.*, 2001; Murphy *et al.*, 2001; Springer *et al.*, 2003).

The order LAGOMORPHA is divided into two families: OCHOTONIDAE and LEPORIDAE. The family OCHOTONIDAE is restricted to the genus *Ochotona* (Pikas) that includes 25 extant species. These species are of small body size, possess 26 teeth and are adapted to alpine environments. The family LEPORIDAE can be divided into two groups: hares and rabbits. They have well-developed posterior members, elevated eyes, long ears and 28 teeth. According to Chapman and Flux (1990), the hare group encompasses a single genus, *Lepus,* comprising 29 species, whereas the rabbit group includes 10 genera (*Brachylagus*, *Bunolagus*, *Caprolagus*, *Nesolagus*, *Oryctolagus*, *Pentalagus*, *Poelagus*, *Pronolagus*, *Romerolagus* and *Sylvilagus*) and 25 species. The genus *Oryctolagus* is monospecific (*O. cuniculus*) while for genus *Sylvilagus* 13 species are described. The genus *Ochotona* is present in Northern Asia and West North America, the genus *Oryctolagus* is present in Continental Europe, North Africa (Morocco and Algeria), England, islands over the world, Australia, New Zealand, and South America. The genus *Lepus* is distributed in Holartic, Indomalayan and Afro-tropical regions (except tropical forest) and the genus *Sylvilagus* is restricted to the American continent (Corbet, 1994).

Paleontological data

The lagomorph fossil data are scarce and fragmented. Some authors suggested that the lagomorphs appeared in Asia during the later Eocene (approximately 45 My ago) (Erbajeva, 1981). The leporids probably separated from ochotonids during the Oligocen or Upper Eocene (30-40 My ago) (Dawson, 1981). The family LEPORIDAE developed and differentiated in North America and Asia (Dawson, 1981; Lopez-Martinez, 1998) and arrived in Europe during the great migration of the upper Miocene (around 20 My ago) with posterior radiation (Lopez-Martinez, 1989).

Molecular data

Recently, the study of 19 nuclear and three mitochondrial genes from 42 representative species of all extant orders of placental mammals confirmed previous estimates that the genus *Ochotona* diverged from the rabbit at 50-55 My ago (Springer *et al.*, 2003). The divergence time between rabbits and hares is not consensual. The cytochrome *b* analyses suggest that the genera *Oryctolagus*, *Lepus* and *Sylvilagus* diverged 6-8 MY ago (Biju-Duval *et al.*, 1991). On the other hand the study of 12S rRNA indicated that the most recent common ancestral lived 12.2-16.3 My ago (Halanych and Robinson, 1999). Polychlorinated biphenylbinding protein gene (PCB) studies pointed out a time of divergence between *Oryctolagus* and *Lepus* of 20 My (Su and Nei, 1999).

The genetic diversity of European rabbit

In the Iberian Peninsula there are two morphologically differentiated subspecies of rabbit (*Oryctolagus cuniculus)*: *O. c. algirus* and *O. c. cuniculus* (Cabrera, 1914). In view of the small and fragmented fossil record, it seems that the European rabbit was probably originated in the Iberian Peninsula during the medium Pleistocene and then expanded towards Southern France (Pages, 1980; Lopez-Martinez, 1989; Corbet, 1994). The Phoenicians 3000 years ago carried out what might be the first human-mediated geographical expansion of the rabbit species by introducing it into Mediterranean Islands (Balearic Islands, Corsica, Sardinia, Tunisian islands) (Vigne, 1988; Hardy *et al.*, 1994). Today, the rabbit is present in Continental Europe, England, Australia, New Zealand, South America, and North Africa (Morocco and Algeria).

Genetic variation in wild and domestic rabbit populations has been studied in detail. We give an overview of molecular genetic studies performed that involved (a)

mitochondrial DNA (mtDNA), (b) protein variation, (c) immunological markers, and (d) microsatellites.

Mitochondrial DNA (mtDNA)

The study of the mtDNA molecule (cytochrome *b*) in rabbit populations revealed two major maternal lineages, one that is present in wild populations from Southwestern Iberian Peninsula, called lineage *A*, and other, called lineage *B*, present in the Northeast of Iberian Peninsula, in all domestic breeds and in all wild populations of Continental Europe (north of Pyrenean Mountains), Great Britain and overseas. These lineages showed 4% of nucleotide divergence, which suggests that, using the average rate of evolution of mammalian mtDNA, these two groups diverged in the Pleistocene (about 2 million years) (Biju-Duval *et al.*, 1991). Later studies of more French and Spanish wild rabbit populations led to the suggestion of two refugia that separated two MY ago, one in the Southwest of the Iberian Peninsula, and another probably located in Northern Spain (Monnerot *et al.*, 1994). Branco *et al.* (2000, 2002) showed the existence of a contact zone in the Iberian Peninsula suggesting a post-glacial expansion from the Southwestern refugium to North, and from the Northeastern to South or West.

Genetic variation of proteins

The first works that used the genetic variation of proteins in the characterisation of rabbit populations confirmed the British origin of the feral rabbits of Australia (Richardson *et al.*, 1980). The study of wild rabbit populations from the Iberian Peninsula, Azores (Atlantic islands) and France, using 16 polymorphic markers confirmed the hypothesis that the rabbit was originated in the Iberian Peninsula (Ferrand, 1995). The same author also suggested the existence of two glacial refugia in which the long-term geographical isolation gave rise to two different rabbit subspecies. One refugium was located in Southwestern Iberian Peninsula, where subspecies *O. c. algirus* originated. The other refugium was localized in Northeastern Spain and Southern France, where the rabbit *O. c. cuniculus* originated. The study of numerous protein markers in wild rabbit populations from the Iberian Peninsula revealed a broad and gradual transition between the Southwestern and Northeastern groups of populations, suggesting the absence of intrinsic barriers to gene flow between these two evolutionary units (Branco, 2000).

Rabbit Ig allotypes

The first genetic studies at the protein level were done on laboratory rabbits (Oudin 1956). By immunizing one rabbit with the antibody of another rabbit, Oudin discovered the Ab allotypes. At a time that the Ig molecule was believed to be monomeric, rabbit genetics showed that the Ab was encoded by two independently segregating loci, called *a* locus and the *b* locus. The *a* locus was identified as the locus encoding the H chain variable region (*IgV$_H$a*-locus), while the *b* locus encodes the constant part of the L chain (*IgCK*1 locus). The variability at both the *a* and the *b* locus is characterized by extremely large distances between alleles. The largest distances exist among *b* locus alleles (up to 35% of amino acid differences; note that the CK regions of human and chimpanzee are identical).

While the *a* and *b* markers concern the Fab region, genetic variation was also observed at the Fc region (H chain constant region). In contrast with the rabbit "complex" allotypes, these markers resemble the Ig allotypes described in species like human or mouse, and relate to single amino acid substitutions. One of them, the *e*-locus, of the *CH2* exon of the IgG class, was studied in this work.

The very large divergence at the C_{K1} locus, which was confirmed as a structural single-gene locus, makes it instrumental for evolutionary studies of the rabbit species. In domestic breeds, wild and feral rabbit populations from the most recent distribution range of the species (Continental Europe North of the Loire river, Great Britain, Australia, Kerguelen islands), four alleles can be distinguished by serological methods at the *b* locus, the so-called *b4*, *b5*, *b6* and *b9* (van der Loo and Arthur, 1986; van der Loo, 1987, 1993). The study of this locus in Iberian populations by serological methods revealed at least 16 alleles (Cazenave *et al.*, 1987; van der Loo *et al.*, 1991, 1999). This high number of alleles is in accordance with the hypothesis that the rabbit originated in the Iberian Peninsula.

Cytonuclear disequilibrium was observed in wild rabbit populations from the Iberian Peninsula. The alleles *b4* and *b5* of *IgCK1* that comprise over 90% of the gene pool in the more recent area of distribution of the species were also predominant in areas of mtDNA type *B* prevalence within the Iberian Peninsula. However, in areas of mtDNA lineage *A* prevalence, the *b4* and *b5* allotypes are rare or absent, the majority of the alleles are serologically related to *b4* and *b5*, but are distinct "endemic" variants (van der Loo *et al.*, 1999).

The DNA sequence comparison of the rabbit *IgCK1* alleles revealed that substitutions rates were significantly larger at amino acid replacement sites than at synonymous sites (van

der Loo and Verdoodt, 1992), while non-coding regions were more conserved than exons (Akimenko *et al.*, 1986). This unusual form of allelic diversity is therefore due to forces favouring variation at the protein level. Furthermore, population genetic studies revealed a generalised excess of heterozygous individuals at *b* locus but not at other loci (van der Loo and Arthur, 1986; van der Loo, 1987). Linkage disequilibrium between the *b* locus and *e locus* (van der Loo *et al.*, 1987, 1996; van der Loo, 1993) strongly suggested that overdominance-type selection is occurring at this locus, because these loci segregate independently.

Microsatellite markers

In the rabbit, 27 polymorphic microsatellite loci have been described (van Haeringen *et al.*, 1997; Mougel *et al.*, 1997; Surridge *et al.*, 1997; Ferrand *et al.*, 2000). In contrast to the results obtained in the above-mentioned markers, the use of nine microsatellite loci in the genetic characterization of rabbit populations from the Iberian Peninsula did not confirm the occurrence of two well-separated groups. Indeed, these loci revealed severe allele-size homoplasy, which underestimates the divergence time between the main groups of populations in Iberia. However, for the same set of markers it was detected a significant difference in allelic diversity between the French and the Iberian Peninsula populations (Queney *et al.*, 2001).

Domestication

The rabbit is the only domesticated mammal of Western Europe origin. This process began late in the Middle Ages, probably in southern France monasteries (Robinson, 1984; Callou, 1995). The genetic characterisation of 12 domestic breeds through the study of mtDNA, microsatellites, immunological and protein markers, (Bolet *et al.*, 1999; Queney *et al.*, 2002; Esteves *et al.*, unpublished; van der Loo *et al.*, unpublished) showed that only a very small fraction of the species diversity is present in the domestic breeds. The results obtained in this study are congruent with previous publications on the immunoglobulins polymorphism (van der Loo *et al.*, 1991) and genetic variation of proteins (Ferrand, 1995) and consolidate the viewpoint that domestic breeds originated from France.

The rabbit in immunological research

The use of rabbit in immunological research is very old. In the nineteenth century, Louis Pasteur used the rabbit spinal cord to prepare rabies vaccine (Pasteur *et al.*, 1881). The study of rabbit was also crucial in the battle against syphilis (Wassermann *et al.*, 1906; Parodi, 1907). In the first half of the twentieth century, the foundations of molecular immunology have been 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). Studies on laboratory rabbit have greatly contributed to our knowledge of the structure, function and regulation of antibodies. In 1956, Oudin demonstrated and defined allotypy of immunoglobulins in the rabbit. Three years later Dubiski *et al.* (1959) showed that the rabbit allotypes were hereditary traits and in 1960, Oudin established the two major linkage groups "*a*" and "*b*" corresponding to the H chain and the K light chain loci, respectively. Hamers and Hamers-Casterman (1965) discovered allotypes of the Ig constant region (*e* locus). The existence of genetic markers of the different gene segments was – and to a large extent still is – unique to this species. The rabbit Ig allotypes allowed challenging the "one gene-one protein" dogma. Indeed, Todd (1963) and others found that in rabbit allotypic specificities of the variable regions presented by IgG or IgM molecules were identical, suggesting that a same V_H gene segment can be translocated to different constant region genes.

This observation lead to the hypothesis of the Ig class switch (Severinson *et al.*, 1982), which opened the road to the concept of multiple "germline" V_H gene segments that can be joined to a limited number of genes encoding the different H constant regions (Dreyer *et al.*, 1965). In 1962, Hamers and co-workers showed the cis-expression of the V_H and C_H genes, by using the associated genetic markers (i.e. V_Ha or *a* locus and *CH2* or *e* locus). Later, Mage *et al.* (1971) confirmed this model by documenting a number of crossing-over events between the genes controlling the rabbit variable and constant heavy chain. Mage (1979) and Kelus and Steinberg (1991) estimated a crossing-over frequency of 0.1%.

The rabbit allotype model is therefore at the origin of the current *V-D-J-C* model. It was through studies of rabbit immunoglobulin markers that phenomena such as allelic exclusion, allelic imbalance in gene expression, genetic linkage of V_H and C_H genes, and the expression of apparently identical VH regions on different classes of Ig were described.

However, the use of the rabbit in several immunological fields was restricted by technical difficulties (e.g. failure to induce rabbit myeloma tumors, and the absence of inbred strains). Nevertheless, the rabbit is still important in transplantation and ophthalmologic research. Rabbit antibodies are more stable than those from rodents and are therefore often used in the production of high affinity antibodies or of anti-idiotype vaccines. During the last 25 years the rabbit Ig allotypes revealed a number of unique features (described in chapters 2 and 3), setting them apart from the allotypes found in mice and human. Most important are the highly unusual large genetic distances between the allelic lineages, which can reach 35% of amino acid divergence between *IgCK1* (*b* locus) alleles.

It appeared that this unusual degree of allotype diversity is the outcome of selection (van der Loo, 1993). The detection of selection on polymorphic genes in natural populations is particularly difficult. The rabbit unites a number of characteristics that make it an ideal species for studies of the biological importance of population diversity at the Ig loci (van der Loo 1987): 1) the rabbit Ig allotypes are well characterized at the genetic, structural and genomic level; 2) the rabbit is a popular laboratory animal and a game species with the physiology and ecology well studied; 3) the population dynamics of this species (high mortality and birth rates), makes it more likely that the genotype dependent components of mortality can be recorded at the population genetic levels; 4) the rabbit is a recent world wide distributed species with a documented and interesting history of epizootic diseases (myxomatosis and hemorrhagic viral diseases).

Another unusual feature of the rabbit immune system resides in the fact that the antibody repertoire is diversified in this species after birth. The primary Ab repertoire of rabbit results from the preferential rearrangement of a very limited number of *V* gene segments (i.e. those that encode the allotypic markers). The B cells are developed in the fetal liver and bone marrow and migrate to appendix and other gut-associated lymphoid organs (GALT) where are diversified by somatic hypermutation and/or somatic gene conversion-like mechanism. In the rabbit this process only occurs at 1-2 months of age (Cooper et al., 1968; Weinstein *et al.*, 1994) and is not developmentally regulated like it appears to be in other species, such as chicken, sheep and cattle. It has been shown that the Ab repertoire diversification requires interaction of exogenous factors, such as the intestinal microflora (Lanning *et al.*, 2000a, 2000b; Sehgal *et* al., 2002) (for details see chapter 3).

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CHAPTER 2

Genetic variation at the constant region of the IgG antibody in leporids

Introduction

Rabbits possess only one IgG class

IgG antibodies are synthesised at a high level and constitute up to 75% of serum immunoglobulins. Four subclasses are produced in the human (IgG1, IgG2, IgG3, IgG4), and in the mouse (IgG1, IgG2a, IgG2b, IgG3). The choice of an IgG subclass is not random and different antigenic stimuli induce the production of different IgG subclasses. For example, in the mouse, protein antigens stimulate predominantly IgG1 responses while carbohydrate antigens induce substantial IgG3 responses (Perlmutter *et al.*, 1978; Slack *et al.*, 1980; Scott and Fleischman, 1982). In addition, the selection of an IgG subclass is strongly influenced by the numerous cytokines produced by Th1 and Th2 cells (helper T cells). It is therefore surprising that in the rabbit no subclasses of IgG antibodies have been identified.

Indirect evidence for adaptive variation at the rabbit IgG CH2 domain (*e*-locus)

Structural evidence

Epitopes at the IgG CH2-CH3 interface play, in conjunction with the Fc receptors, a crucial role in the processing of immunoglobulins including their transplacental transport (Johanson *et al.* 1981, Ghetie and Ward 1997). A major antibody function is the recruitment of the complement pathway, which requires binding of the CH2 domain to C1q, a constituent of the first complement. Mapping by site-directed mutagenesis showed that in human, several amino acids of the CH2 region participate in complement binding (Idusogie *et al.*, 2001). The CH2-CH3 interface is also the target of pathogens (Deisenhofer, 1981; Kim *et al.*, 1999), and different lines of evidence suggest that isotype and allotype variation at this region can be adaptive (Dubiski and Good, 1972; Granoff *et al.*, 1984).

The rabbit *IgGCH2* or *e* locus is localised on the chromosome 17 (Medrano and Duttrillaux, 1984) and exhibits two serological markers, *e14* and *e15* (Hamers and Hamers-Casterman, 1965; Mage, 1981). These alleles are inherited as codominant Mendelian alleles

and are correlated with an Alanine-Threonine replacement at amino acid position 309 (79 in the IMGT numbering) (Appella *et al.*, 1971). This position is adjacent to the canonical histidine at 310, which is known to affect interaction with a variety of Fc receptors (West and Bjorkman, 2000). X-ray crystallography reveals direct contact between the side chains of *IgG*-Leu309 of the rat Fc receptor (Burmeister *et al.*, 1994). The *e15* allotype was found in species of genera *Oryctolagus*, *Lepus*, *Sylvilagus*, *Romerolagus* and *Ochotona* (Cazenave *et al.* 1977; van der Loo and Hamers-Casterman, 1977). A number of species seem to be fixed for this allele, while the *e14-e15* polymorphism was observed in the Mexican volcano rabbit (*Romerolagus diazi*) (van der Loo and Hamers-Casterman, 1979, 1981) and in cottontail rabbit (*Sylvilagus floridanus*) (Teherani and Mandy, 1976; Cazenave *et al.*, 1977). Both alleles were absent in mountain hare (*Lepus timidus*).

Population genetic evidence

In the most recent distribution range of the species, all wild and feral rabbit populations studied were polymorphic, and allele frequencies of *e14* were very similar among major geographical regions ($p_{e14} \approx 20\%$, van der Loo, 1993; van der Loo *et al.*, 1996). However, all Iberian wild rabbit populations studied so far showed the fixation of *e15* (N. Ferrand and W. van der Loo, unpublished data cited in van der Loo and Arthur (1985), and van der Loo (1987)). The absence of the polymorphism in the original distribution area is puzzling. Indeed, the *e14* allele is among the rare genetic variants that are not present in Iberian populations. Strong linkage disequilibria (LD) were observed between alleles of *e* locus and other H chain loci; i.e. *d* locus (H chain Hinge region) and *a* locus (V_H genes). The allotype *d11-e14* has not been reported so far (Mage *et al.*, 1977; van der Loo, 1987), while the *a-e* haplotype *a3-e14* has not been observed in most domestic breeds (see Mage *et al.*, 1977). The *a-e* and *d-e* LD can be explained by the close linkage between these H chain loci and a rather recent origin of the *e14* variant (<5000 years). More surprising was the observation of a highly significant LD between the *e* locus and alleles of the L-chain constant region (*b* locus), because both loci are on different chromosomes (van der Loo *et al* 1987, 1996). This LD was observed within and between populations from all areas where the interacting loci were polymorphic (Australia, Great Britain, Continental Europe North of the Loire river). The observation of LD (or non-random association) between alleles of independently segregating loci in natural populations is considered one of the most reliable indicators of selection (Franklin and Lewontin, 1970; Lewontin, 1974), and, as a matter of fact, had never been documented for protein loci in mammalian populations before.

The population genetic data suggest therefore that the *b* and *e* locus polymorphisms are maintained by epistatic selection pressure. van der Loo and co-workers have presented population genetic evidences for the hypothesis that the extremely large interallelic distances among *b* locus alleles is due to overdominant selection (some 10% heterozygosity excess; van der Loo and Verdoodt 1992; van der Loo 1993). van der Loo (1996) observed furthermore that the heterozygosity excess at one Ig locus tends to be higher if heterozygosity at another Ig locus is low (higher order LD), and introduced the concept of "compensatory overdominance". This author speculated that the emergence of *e14-e15* polymorphism in the recent species range might have to do with the sharp reduction in allele diversity at the *b* locus observed in this region (of more than 16 *b* locus alleles occurring in the original range, only four are present in the recent range).

We have conducted an evolutionary study at the molecular level, which confirms the existing evidence that the *e* locus polymorphism is maintained by selection. Previous molecular studies have been limited to the relationships between serology and protein variation at *IgGCH2* domain (Appella *et al.* 1971; Aggarwal and Mandy, 1976; Cazenave *et al.*, 1977; Teherani *et al.* 1979, 1982). Until the present work, no nucleotide data on lagomorph *IgGCH2* were available, except for three sequences of domestic rabbit (Martens *et al.*, 1982; Bernstein *et al.*, 1983; Martens *et al.*, 1984).

Article 1

P.J. Esteves, P.C. Alves, N. Ferrand and W. van der Loo (2002).

Restriction fragment alleles of rabbit IGHG genes with reference to the rabbit *IgGCH2* or *e* locus polymorphism. *Animal Genetics* **33**: 309-311.

SHORT COMMUNICATION

Restriction fragment alleles of the rabbit *IGHG* genes with reference to the rabbit *IGHGCH2* or *e* locus polymorphism

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Summary

Among domesticated mammals, rabbit (*Oryctolagus cuniculus*) is the only species possessing not more than one subclass of immunoglobulin (IgG) antibodies. The rabbit *IGHGCH2* or *e* locus presents two serologically defined alleles, the *e14* and *e15* allotypes, which are correlated with amino acid variation at the IgG *CH2–CH3* interface. Genetic studies, while revealing the adaptive value of this polymorphism, have relied so far entirely upon allo-antisera. Here we show how these alleles can be distinguished by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) methods. The proposed PCR–RFLP approach allows the monitoring of *IGHG* locus diversity in rabbit.

Abbreviations: IgG, gamma class immunoglobulin; IGHG, IgG immunoglobulin heavy chain gamma constant region; IGHGCH2, second domain of IGHG; bp, base pair; aa, amino acid; PCR, polymerase chain reaction, RFLP, restriction fragment length polymorphism

Keywords allotypes, antibody, *e* locus, *IGHGCH2* locus, PCR–RFLP, polymorphism, rabbit.

In conjunction with FcRn receptors, epitopes at the IgG *CH2–CH3* interface play a crucial role in the processing of IgGs and their transplacental transport (Johanson *et al.* 1981; Gethie & Ward 1997). They are also the target of pathogens (Kim *et al.* 1999), and different lines of evidence suggest that genetic variation in this region is adaptive (van der Loo *et al.* 1987, 1996). Unlike other mammals, rabbit possesses only one subclass of IgG antibodies. Two serologically defined alleles, the *e14* and *e15* allotypes, were found to be correlated with an alanine–threonine exchange at amino acid (aa) position 309 (Appella *et al.* 1971; Mage 1981; for IMGT numbering see http://imgt.cines.fr). Position 309 is adjacent to the canonical histidine at 310, which is known to affect interaction with a variety of Fc receptors (West & Bjorkman 2000).

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Each of more than 6000 specimen of European rabbit showed either the *e14* or the *e15* allotype, or both (van der Loo 1993 unpublished data). In the more recent distribution range of the rabbit species (Continental Europe North of the Loire river, Great Britain, Australia, Kerguelen Islands), all wild and feral rabbit populations were polymorphic at the *IGHGCH2* locus (van der Loo *et al.* 1996). Allele frequencies were very similar among regions ($p_{e14} \approx 20\%$, van der Loo 1993). However, in a survey of gene diversity in seven domestic breeds, the mean *e14* frequency was below 5% (G. Bolet *et al.* unpublished data). Although highly reliable, serological methods depend upon good allo-antisera, which are difficult to obtain. We show here how the *e15* allotype, or its absence, can be detected by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products. We also present for the first time the *CH2* nucleotide sequence as obtained with genomic DNA of *e14* rabbits.

Serological analyses was performed by immunodiffusion, in the presence of *e14* or *e15* positive control sera as previously described (van der Loo 1993). Genomic DNA was

obtained by Qiagen extraction of tissues (including blood clots) of specimen that were also studied serologically. The RFLP analysis was performed on PCR products. These were obtained using oligonucleotide primers, constructed according to the sequence of rabbit *IGHGCH2* gene (EMBL databank accession number L29172). The primers were F3: (5′GTGAGTCCCATCTAGCCTCAC3′) and R2: (5′GGGCCCAT GGTGTAGACCTT3′). The primers F3 and R2 are homologous to positions (705–724) and (1256–1236) of the L29172 sequence, respectively. The PCR amplification and DNA sequencing protocols were routine, and can be obtained upon request.

The PCR products were 553–557 base pair (bp) in length. They were digested with *Tha*I prior to electrophoresis. All PCR products obtained (19 rabbits studied) showed a *Tha*I site (i.e. GCGC) that comprises a shared Arg301(CGC) codon (T1 in Figs 1 and 2). A second shared *Tha*I site (T3) was found in the 3′ intron region, 130 bp downstream of T1. A third *Tha*I site (T2), which cuts the 130 bp fragment into 106 and 24 bp fragments, was exclusively found in PCR products obtained with *e15* rabbits (15 sequences). This site depends upon the allotype-specific Ala_{309}(GCG) codon and is absent in *e14* rabbits, which instead feature Thr_{309}(ACG). As expected, PCR products of heterozygous *e14/e15* rabbits showed partial digestion of the *Tha*I 130 bp fragment and had either G or A nucleotides at first position of codon 309 (cf. Table 1).

This shows that, although the rabbit allo-antisera remain the safest shortcut for detecting either Ala_{309} or Thr_{309} at the *CH2–CH3* interface of IgG, PCR–RFLP can be helpful in population studies, allowing the monitoring of the *IGHGCH2* diversity in breeding programmes.

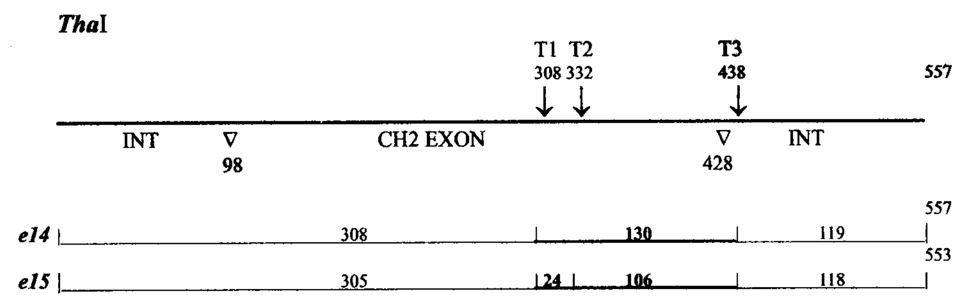

Figure 1 *Thal* restriction patterns of the PCR products of the *IGHGCH2* exon. Products include the 5′ primer *F3* (20 bp), the 3′primer R2 (20 bp), the entire *CH2* exon (330 bp) and parts of the flanking introns. ∇ indicate the exon–intron boundaries; ↓ indicate the potential restriction sites T1, T2, T3.

```
                |     20      |      40      |      60      |      80      |     100      |     120      |     140      |
           |Int->                                                                                         ∇CH2-exon ->
Orcu_e14g: GTGAGTCCCATCAGCCTCACCCTCTAGCCCCAGCCCGGGGAGCCCCACCCCAGGGTGCCCCCCAGGTGTTGACTCTTCCCCGTCTCTCCCACTGCAGCCCCTGAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCCCAAAACCCAAG
Orcu_e14r:                                                                                                  .....................................................
Orcu_e15g  ...................................................-...........-.-......................TTT..................T................T........T..............

              160      |     180      |     200      |     220      |     240      |     260      |     280      |     300

Orcu_e14g: GACACCCTCATGATCTCACGCACCCCCGAGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACATGGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCG CTACGGGAGCAGCAGTTCAACAGC
Orcu_e14r: ......................................................................................................................................................
Orcu_e15g: ..........................................................G...........................................................................................

                |     320      |     340      |     360      |     380      |     400      |     420      |     440      |
              ThaI-1          ThaI-2                                                                          ∇Int->   ThaI-3
Orcu_e14g: ACGATCCGCGTGGTCAGCACCCTCCCCATCACGCACGAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCA CAACAAGGCACTCCCGGCCCCCATCGAGAAAACCATCTCCAAAGCCAGAGGTGGGAGC CGCGGGCTGGAAACA
Orcu_e14r: ...............................................................................................................................
Orcu_e15g: ...............................G.............................................................................................................G.G..

              460      |     480      |     500      |     520      |     540      |                       EMBL ACC   References

Orcu_e14g: GGGCAGGCAGCTCCCACGGCCCGAGGCCTCCGCCCGGGAGTGGACCCTGTGCTGTCCGCTGTCCCCACAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCCC: 553    DS50323    This paper
Orcu_e14r:                                                                                                            :        K007 52    Bernstein et al. (1983)
Orcu_e15g: ............................-...A..........................................................................: 557    L29172     Martens et al. (1984)
```

Figure 2 *Orcu_e15g* is part of the L29172 sequence obtained by Martens *et al.* (1984) with genomic DNA of an *e15* rabbit and includes the *IGHGCH2* exon; *Orcu_e14r* (EMBL Accession K00752; Bernstein *et al.* 1983) was obtained with mRNA of an *e14* rabbit and features the only published *e14* sequence. Because it lacks the introns, we show the sequence (DS50323) as obtained in this study with genomic DNA of four different *e14* rabbits '*Orcu_e14g*' (no variation was observed). '-': Indels; '.': identity with *Orcu_e14g*; bold italic: the primers F3 and R2; bold underlined: *Thal* restriction sites. The exon (nucleotide position 98-427) is translated in the third reading frame, the *CH2* protein domain starting with a Proline encoded by (CCT, 100–102).

Table 1 Correspondence of rabbit *IGHGCH2* allotypes with *Tha*I restriction sites and DNA sequences of PCR fragments.

Serology		PCR–RFLP			DNA sequencing		
e14	*e15*	T1	T2	T3	$G(CGC)_{301}$	$(GCG)_{309}C$	GCGC
+	–	+	–	+	+	$(ACG)_{309}C$	+
+	+	+	+/–	+	+	$(^{G}/_{A}CG)_{309}C$	+
–	+	+	+	+	+	$(GCG)_{309}C$	+

+: Positive or present; –: negative or absent; ±: partial digestion; $^{G}/_{A}$: G and A nucleotides at this site. The *e15* allotype is clearly correlated with the presence of *Tha*I restriction site T2, implying the presence of Ala_{309}.

Acknowledgements

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Genetic diversity at the *IgGCH2* region in *Oryctolagus cuniculus*

Until this work only three sequences of rabbit CH2 domain were available in Genbank. These sequences were obtained from rabbits serologically determined for the genetic markers *d* and *e* locus as *d12-e15* ("*e15*" in Figure 2.1), *d11-e15* ("*e15A*") and *d12-e14* ("*e14*"). Genbank accession numbers are L29172 (Martens *et al.*, 1984), M16426 (Martens *et al.*, 1982), and K00752 (Bernstein *et al.*, 1983), respectively. We compared the published rabbit CH2 sequences to those obtained in this study and with other leporid sequences. The phylogenetic tree shown in Figure 2.1 was constructed by applying the Neighbor-Joining (Saitou and Nei, 1987) and the p-distance methods provided in the software program MEGA 2 (Kumar *et al.*, 2001) to the nucleotide sequences listed in Table 2.1.

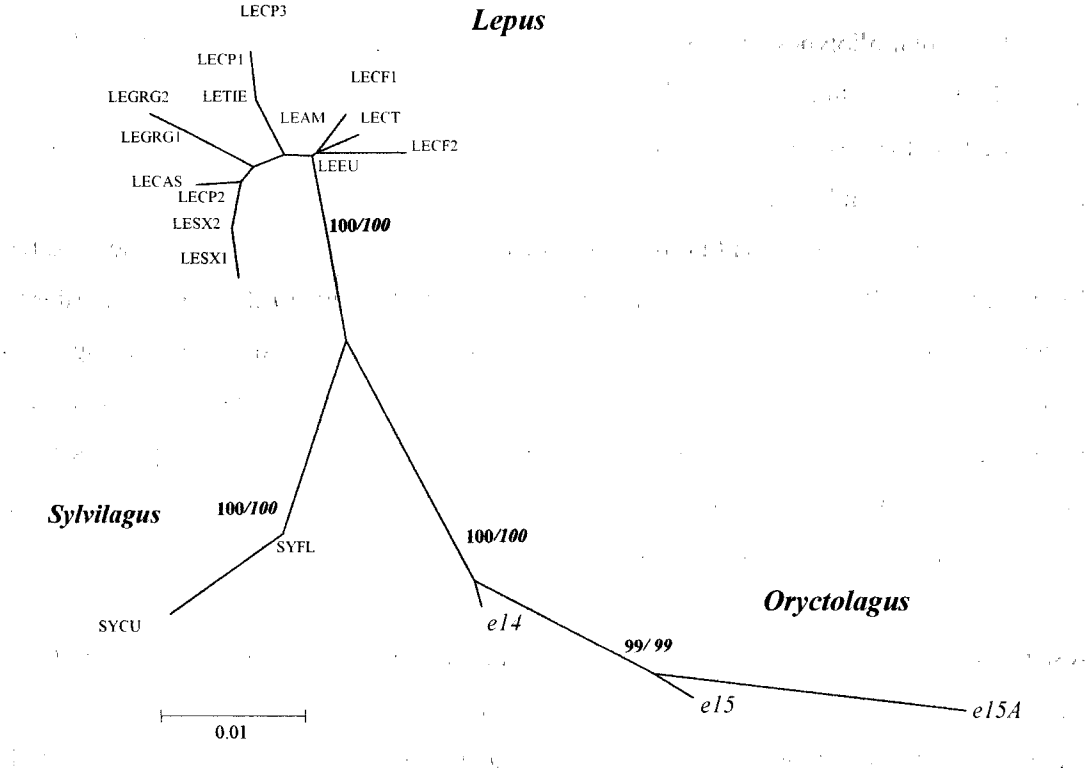

Figure 2.1: NJ tree of *IgGCH2* exon of three leporid genera using nucleotide sequences The bootstrap (italic) and the confidence probability values are indicated. Sample codes are shown in Table 2.1.

Table 2.1
Sequences obtained for *IgGCH2* in *Lepus* and *Sylvilagus* species

Species	Sample code	localisation	Genbank (accession number)
Lepus			
L. granatensis	LEGR1	Iberian Peninsula	AJ295217
L. granatensis	LEGR2	Iberian Peninsula	
L. timidus	LETI	Scotland	AJ295216
L. capensis	LECP1	Morocco	AJ295218
L. capensis	LECP2	Morocco	
L. americanus	LEAM	North America	
L. californicus	LECF	Mexico	AJ295219
L. castroviejoi	LECS	Iberian Peninsula	
L. callotis	LECT	Mexico	AJ295220
L. europaeus	LEEU	France	AJ295221
L. saxatilis	LESX1	South Africa	AJ295222
L. saxatilis	LESX2	South Africa	
Sylvilagus			
S. floridanus	SYFL	North America	
S. cunicularis	SYCU	Mexico	AJ295223

The rabbit allotypes *e14* and *e15* differ in three amino acid positions (268 Q/E, 309 T/A, 311 Q/E). Within the *e15* allotype only synonymous substitutions were observed. Compared to the two published *e15* sequences, the *e14* sequence is the closest to the common node of rabbit CH2, which would suggest that this is the oldest allele. However, the fact that *e14* is only present in the most recent area of distribution of the species and the LD with the *a3 VH* marker are in contradiction with an older age of this allele. In order to understand this discrepancy, we study this marker for wild rabbit individuals belonging to both subspecies (*O .c. algirus* and *O. c. cuniculus*) from different Iberian populations, and for domestic rabbit specimens. A total of 30 *IgGCH2* gene nucleotide sequences were determined and their amino acid sequences inferred. The comparison of the sequences obtained in this work (rabbit and other leporids) with published rabbit sequences allowed the establishment of nucleotide and amino acid consensus sequences (Figure 2.2 and 2.3, respectively). The wild rabbits, as expected by their allotype *e15*, showed Ala309 (GCG). All sequences obtained from domestic rabbit (allotypes *d11-e15*, *d12-e15* and *d12-e14*) were identical, except in the position 309 (data not shown). The study of wild rabbit populations revealed 6 new polymorphic positions (nucleotide positions: 23 C/T, 60 C/T, 78 C/T, 167 C/A, 178 C/T; 204 G/A) of which two were associated with amino acid substitutions (amino acid positions: 240 S/F, and 282 A/D) (see Figures 2.2 and 2.3).

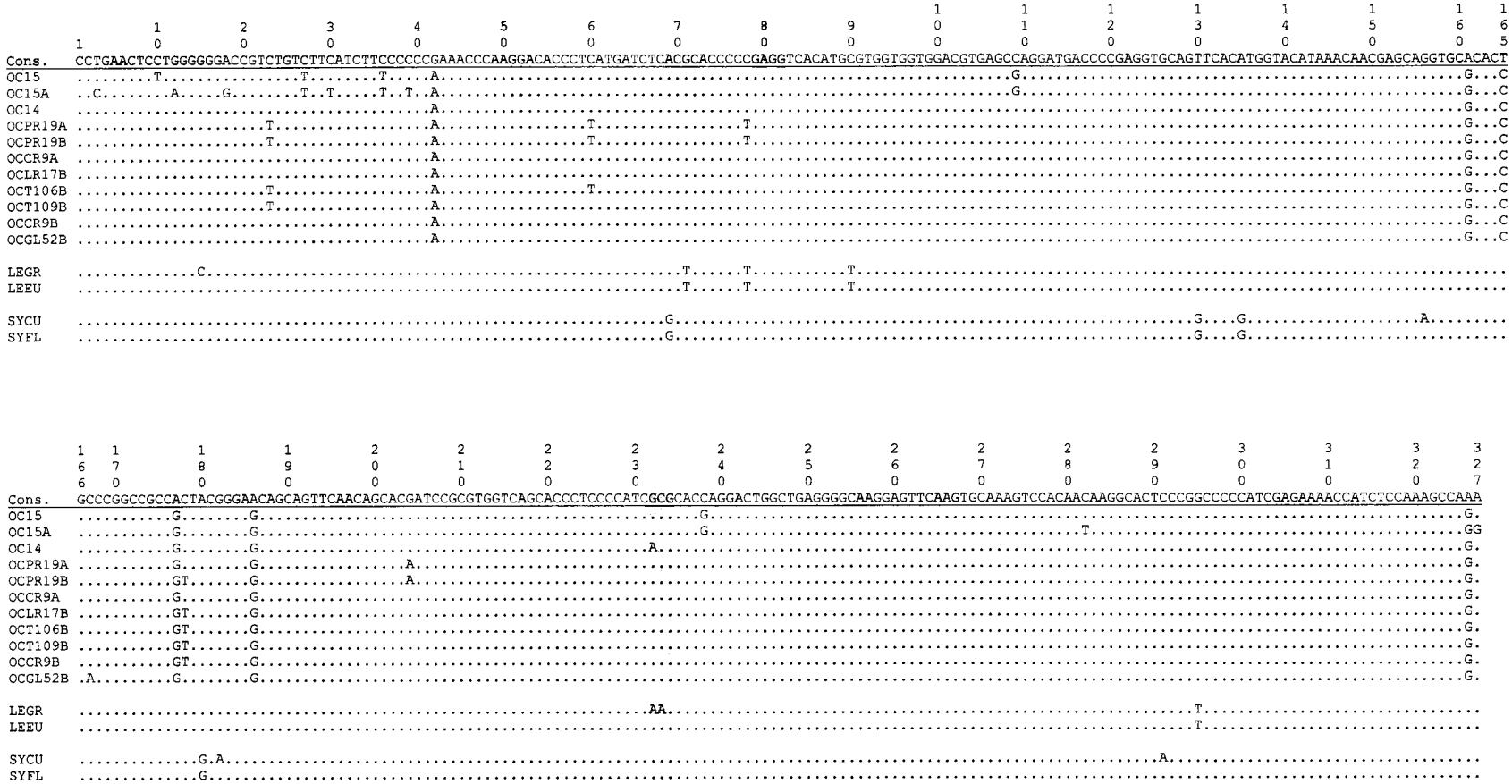

Figure 2.2. DNA sequences of *IgGCH2* obtained in wild rabbits from Iberian Peninsula. The comparison of these sequences with those obtained in *Lepus* and *Sylvilagus* allowed the establishment of a consensus sequence (Cons.). The codon that encodes position H309 is grey-shaded.

```
          2       2         2         2         2         2         2         3         3         3         3         3
          3       4         5         6         7         8         9         0         1         2         3         4
          2345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
Cons.     PELLGGPSVFIFPPKPKDTLMISRTPEVTCVVVDVSQDDPEVQFTWYINNEQVHTARPPLREQQFNSTIRVVSTLPIAHQDWLRGKEFKCKVHNKALPAPIEKTISKAK
ORCU15    ....................................E................R.........................E............................R
ORCU15A   ....................................E................R.........................E............................R
ORCU14    .....................................................R.......................T..............................R
OCPOR19A  .......F.............................................R......................................................R
OCPOR19B  .......F.............................................R......................................................R
OCLER17B  .....................................................R......................................................R
OC106B    .......F.............................................R......................................................R
OC109B    .......F.............................................R......................................................R
OCCRE9A   .....................................................R......................................................R
OCCRE9B   .....................................................R......................................................R
OCGAL52B  .....................................................R.D....................................................R

LEGRG1    .......................L.....................................................K....................S..........
LEEU      .......................L..........................................................................S..........

SYCU      ...........................................V................Q................................................
SYFL      ...........................................V.................................................................
```

Figure 2.3. Amino acid sequences as inferred from the nucleotide sequences displayed in figure 2.2. The consensus sequence is represented. Dots indicate identity with the consensus sequence. Bold indicates the new amino acids detected in wild rabbit specimens.

Compared to the published ORCU*e15* and ORCU*e15a* sequences, the *e15* sequences obtained in the Iberian rabbits showed more similarity to the domestic *e14* sequence. This suggests that *e14* derived from an *e15* lineage present in the Iberian Peninsula. The larger diversity among *e15* sequences could indeed also be explained by the much longer history of this lineage. However, the highly divergent sequences ORCU*e15* and ORCU*e15a*, which were obtained in a laboratory breed, are surprising. It is possible that they represent lineages of *e15* not yet observed in the Iberian Peninsula. Another possibility is that mutant recruitment rate increased in that particular domestic breed. Indeed it has been suggested that positive selection may cause increased rates of synonymous substitution in gene regions that are closely linked to sites under selection (e.g Kreitman and Hudson, 1991). However, the possibility of a cloning mistake cannot be discarded. More data are needed to evaluate these different possibilities.

The phylogeny of *IgGCH2*

The *IgGCH2* gene phylogram was constructed by the addition of all published *IgCH2* sequences to our data. The accession numbers for all sequences used are shown in Table 2.2. The NJ tree of nucleotide sequences (Figure 2.4) was obtained using the p-distance method in the program software MEGA 2 (Kumar *et al.*, 2001). Five major clearly separated clusters were obtained: 1) the lagomorph clade, which includes *Oryctolagus* (rabbit), *Lepus* (hares) and *Sylvilagus* (cottontail); 2) the primate clade, that encompasses *Homo sapiens* (human), *Macaca* sp. (macaque) and *Pan troglodytes* (chimpanzee); 3) the ferungulate clade, composed by artiodactyls (*Bos taurus* (cattle), *Sus scrofa* (swine), *Ovis aries* (sheep), *Camelus dromedarius* (camel), *Llama glama* (Llama)); carnivores (*Canis familiaris* (dog), *Felis catus* (cat) and *Mustela vison* (vison)) and perissodactyls (*Equus caballus* (horse)); 4) the rodent clade with *Mus musculus* (mouse), *Rattus norvegicus* and *Rattus rattus* (rat); and 5) the outgroup, marsupials: *Monodelphis* (opossum) and *Trichosurus* (possum). The lagomorph group is composed of three well-separated clades: a) *Oryctolagus*; b) *Sylvilagus*; c) *Lepus*.

Table 2.2

Accession numbers for each CH2 sequence used. The sample code and the IgG class are indicated.

species	IgG subclass	Sample code	Accession number
Pan troglodytes	1	PT1	X61311
Canis familiaris	1	CF1	AF354264
	2	CF2	AF354265
	3	CF3	AF354266
	4	CF4	AF354267
Rattus norvegicus	1	RANV1	M28670
	2A	RANV2A	M28669
	2B	RANV2B	M28671
Rattus rattus	2C	RARA2C	X07189
Homo sapiens	1*1	HS1 1	J00228
	1*2	HS1 2	Z17370
	2*1	HS2 1	J00230
	2*2	HS2 2	AJ250170
	3*1	HS3 1	M12958
	3*9	HS3 9	AJ390242
	3*12	HS3 12	AJ390252
	3*14	HS3 14	AJ390254
	3*17	HS3 17	AJ390262
	3*19	HS3 19	AJ390276
	4*1	HS4 1	K01316
	4*2	HS4 2	AJ001563
Equus caballus	1*1	EC1 1	AJ302055
	1*2	EC1 2	AJ300675
	2	EC2	AJ302056
	3	EC3	AJ312379
	4	EC4	AJ302058
	5	EC5	AJ312380
	6	EC6	AJ312381
Mus musculus	1*1	MM1 1	J00453
	1*2	MM1 2	L35252
	2A*1	MM2A 1	V00825
	2A*2	MM2A 2	X16997
	2A*3	MM2A 3	J00479
	2B*1	MM2B 1	V00763
	2B*2	MM2B 2	J00461
	2B*3	MM2B 3	V00799
	2C*1	MM2C 1	J00479
	2C*2	MM2C 2	X16998
	3	MM3	X00915
Bos taurus	1*1	BT1 1	X16701
	1*3	BT1 2	S82409
	2*1	BT2 1	M36946
	2*3	BT2 3	S624073
	3*1	BT3 1	U63638
Ovis aries	1	OA1	X69797
	2	OA2	X70983
Felis catus	1*1	FC1 1	AB016711
	1*2	FC1 2	AB016710
Camelus dromedarius	1A	CD1A	AJ421266
	2A	CD2A	AJ131945
	3	CD3	Z48947
Monodelphis	*1	MD 1	AF035195
Thricosurus	*1	TV 1	AF157619
	*2	TV 2	AF191648
Mustela vison		MV	L07788
Llama glama		LG	AF305955
Sus scrofa	1	SS1	U03778
	2A*1	SS2A 1	U03779
	2A*2	SS2A 2	M81769
	2B	SS2B	U03780
	3	SS3	U03781
	4	SS4	U03782
	5	SS5	M81771
Macaca mulata	1	MAMU1	AF045537
	2	MAMU2	AF045539
	3	MAMU3	AF045538
Macaca fascicularis	1	MAFA1	AF045536
Cricetulus migratorius		CM	U17166

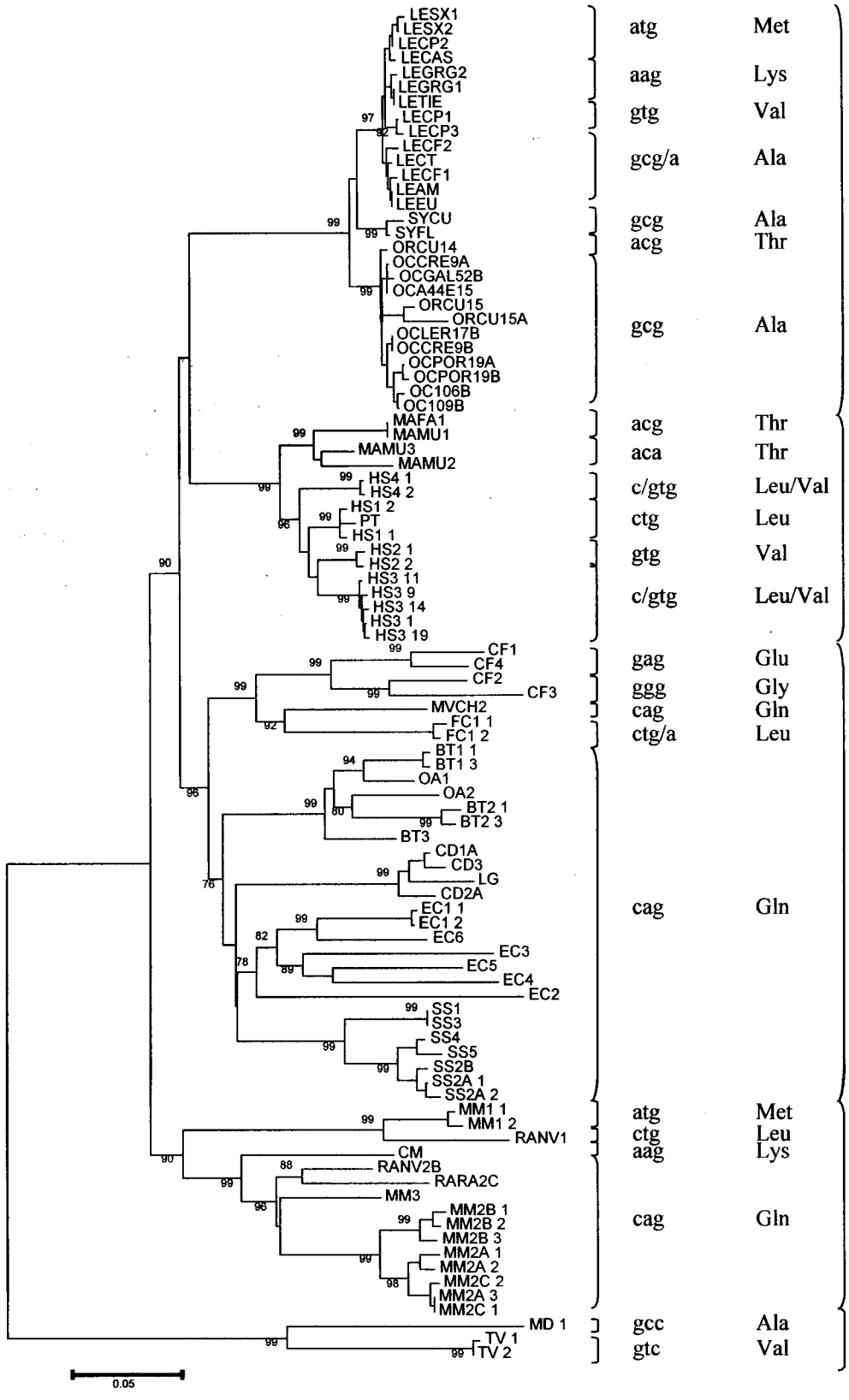

Figure 2.4. Neighbor-Joining tree for CH2 genes sequences obtained in species belonging to lagomorphs, primates, ferungulates and rodents groups. Marsupial sequences determine the root of the tree. Numbers represent the bootstrap values when 1000 replicate samplings were done. For each sequence the nucleotide triplet and the corresponding amino acid observed at position H309 are shown.

Analyses of molecular data have lead to two conflicting hypothesis regarding the phylogenetic position of lagomorphs among mammals (Figure 2.5). According to the first hypothesis the Rodents branched apart 110 My ago, before the great mammalian radiation (90 My). In that case, the lagomorphs are more related to the primates and artiodactyls (Kumar and Hedges, 1998; Nei *et al.* 2001) than to rodents. Under the second hypothesis artiodactyls diverged 95 My ago, before the separation of the primates from a rodents/lagomorph branch (Murphy *et al.*, 2001; Springer *et al.*, 2003).

The gene tree of the *IgGCH2* exon is concordant with the classification of the major phylogenetic groups proposed by Kumar and Hedges (1998) and Nei *et al.* (2001). Indeed, in the gene tree, rodents form a well-defined group apart from the other groups (lagomorphs, ferungulates and primates). There are some incongruences within the ferungulate group, since the swine, which belongs to the artiodactyls seems to be more related to the horse, which belongs to the *Perissodactyla* group, than to other *Artiodactyla* species like cattle or sheep. However, it is important to remind that we are only working a small portion of DNA, which does not allow inferring phylogenetic relationships. We only infer the most likely phylogeny for this particular gene.

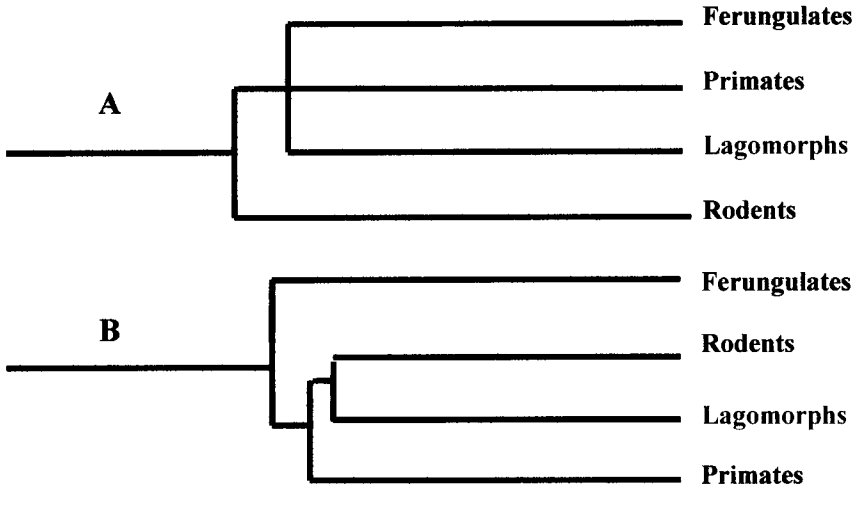

Figure 2.5: Two different evolutionary scenarios proposed for the relationship between the major mammal groups. A: Models based in the phylogenetic trees: A - obtained by Kumar and Hedges (1988); B – obtained by Springer *et al.* (2003). The *IgGCH2* gene tree conforms to model A.

Article 2

P.J. Esteves, P.C. Alves, N. Ferrand and W. van der Loo (2002).

Hotspot variation at the CH2-CH3 interface of Leporid IgG antibodies (*Oryctolagus*, *Sylvilagus* and *Lepus*). *European Journal of Immunogenetics* **29** (6): 529-536.

Short Communication

Hotspot variation at the *CH2–CH3* interface of leporid IgG antibodies (*Oryctolagus, Sylvilagus* and *Lepus*)[1]

P. J. Esteves,*†‡ P. C. Alves,*† N. Ferrand*† & W. van der Loo‡

Summary

The European rabbit (*Oryctolagus cuniculus*) is the only species known to express only one subclass of gamma class immunoglobulin (IgG) antibodies. The rabbit *IGHGCH2* (second domain of the gene region encoding the IgG heavy chain constant region) or *e* locus presents two serologically defined alleles, the *e14* and *e15* allotypes. These are correlated with amino acid variation at position 309, which is located within the target region of the neonatal FcRn receptor. The *e14* and *e15* markers were also observed in other lagomorph species. Population genetic research has indicated that polymorphism at this locus is sustained by selection. We present here the *IGHGCH2* exon sequences for 12 species of rabbit and hare (genera *Oryctolagus, Sylvilagus* and *Lepus*). The inferred amino acid sequences reveal that, despite an overall sequence identity of 97%, five different residues can occur at position 309. As for *Oryctolagus*, the *e15* allotype was always associated with the presence of an Ala309 codon. In all but one case, this codon defined an allotype-specific *Tha*I restriction site. The potential of PCR/*Tha*I restriction fragment length polymorphism (RFLP) analyses for studying *IGHGCH2* variation within and between populations is emphasized.

Introduction

In conjunction with FcRn receptors, epitopes at the gamma class immunoglobulin (IgG) *CH2–CH3* interface play a crucial role in the processing of IgGs and their transplacental transport (Johanson *et al.*, 1981; Gethie & Ward, 1997). The *CH2–CH3* interface is also the target of pathogens (Deisenhofer, 1981; Kim *et al.*, 1999), and various lines of evidence suggest that isotype and allotype variation in this region can be adaptive (Dubiski & Good, 1972; Granoff *et al.*, 1984). Unlike in other mammals, there are no subclasses of IgG antibodies in the European rabbit (*Oryctolagus cuniculus*). The rabbit IgG *CH2* domain presents two alleles that are distinguishable by serological markers, the *e14* and *e15* allotypes (Hamers & Hamers-Casterman, 1965; Mage, 1981). In the modern distribution range of the European rabbit, highly significant allele associations ('linkage disequilibrium') have been observed between the *IGHGCH2* locus (the second domain of the gene region encoding the IgG heavy-chain constant region) and other *IG* loci, including the *IGKC1* or *b* locus of the light-chain constant region (van der Loo *et al.*, 1987, 1996; van der Loo, 1993). The polymorphism was found to be correlated with an alanine-threonine replacement at amino acid position 309 (Appella *et al.*, 1971). Position 309 (position 79 in the International Immunogenetics Database (IMGT) numbering system) is adjacent to His310, which is known to affect interactions with a variety of Fc receptors (Medesan *et al.*, 1997; West & Bjorkman, 2000).

The *e15* allotype has been found in species of the genera *Oryctolagus*, *Lepus*, *Sylvilagus*, *Romerolagus* and *Ochotona* (Cazenave *et al.*, 1977; van der Loo & Hamers-Casterman, 1979): A number of species seem to be fixed for this allele (P. J. Esteves and W. van der Loo, unpublished data), while the *e14–e15* polymorphism has been found in the Mexican volcano rabbit (*Romerolagus diazi*) and in the cottontail rabbit (*Sylvilagus floridanus*). Both markers were absent in the mountain hare (*Lepus timidus*). The relationship between serology and protein variation at IgG *CH2* domains has been studied by amino acid sequencing of tryptic peptides in various lagomorph species (Appella *et al.*, 1971; Aggarwal & Mandy, 1976; Cazenave *et al.*, 1977; Teherani *et al.*, 1979, 1982). In contrast, the DNA sequence repertoire for the lagomorph IgG *CH2* domain consists of only four sequences, all obtained from domestic rabbits (*Oryctolagus cuniculus*). In this paper we present the *CH2* exon sequences of the *IGHG* locus for 12 leporid species. We also show how restriction fragment length polymorphism (RFLP) analysis of PCR products could replace serology in population genetic studies.

[1] The nucleotide sequence data described in this paper have been submitted to the EMBL nucleotide sequence database and have been assigned the accession numbers AJ295216, AJ295217, AJ295218, AJ295219, AJ295220, AJ295221, AJ295222 and AJ295223.

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Figure 1. DNA sequences of F3-R2/PCR products obtained with genomic DNA of, respectively, *Lepus granatensis* (Legr_*eng*), *Lepus americanus* (Leam_*e15*), *Lepus timidus* (Leti_*eng*), *Lepus capensis* (Lecp_*eng*), *Lepus europaeus* (Leeu_*e15*), *Lepus castroviejoi* (Lecs_*eng*), *Lepus saxatilis* (Lesx_*eng*), *Lepus calotis* (Lect_*e15*), *Lepus californicus* (Lecf_*e15*), *Sylvilagus floridanus* (Syfl_*e15*) and *Oryctolagus cuniculus* (Orcu_*e14* and Orcu_*e15*), where extentions '_*e15*', '_*e14*' and '_*eng*' refer to the serological allotypes *e15*, *e14*, and *e*-negative, respectively. Polymorphisms: Y = C and T; R = A and G; S = G and C. '.' indicates identity with Legr_*eng*; '*' indicates insertions/deletions; '–' indicates no clear data. The primers F3 and R2 are shown in bold italic, and the *Tha*I restriction sites in bold underlined.

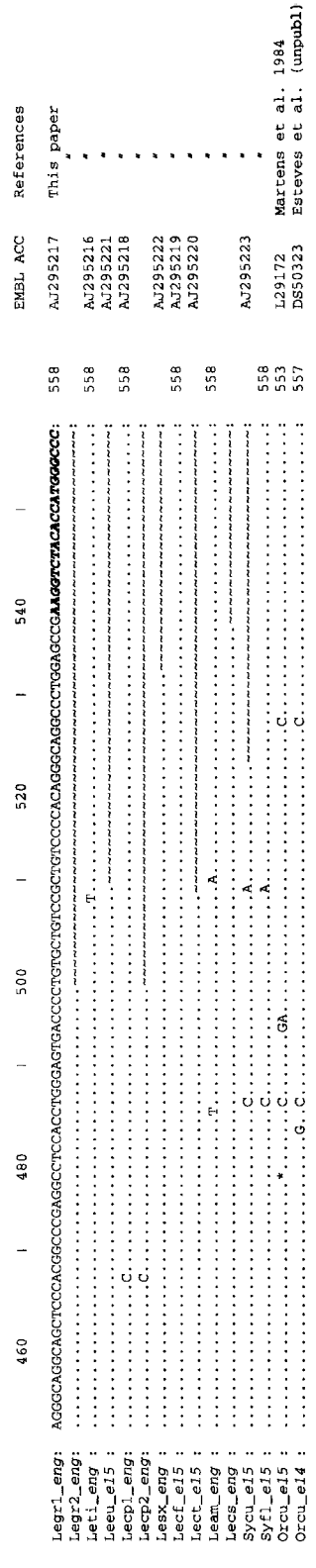

Figure 1. *Continued*

Materials and methods

Tissue and blood samples were taken from wild specimens collected in the Iberian Peninsula (*Lepus granatensis* and *Lepus castroviejoi*), France (*Lepus europaeus*), Scotland (*Lepus timidus*), Morocco (*Lepus capensis*), Mozambique (*Lepus saxatilis*), Mexico (*Sylvilagus cunicularis*, *Lepus calotis* and *Lepus californicus*), and the USA (*Sylvilagus floridanus* and *Lepus americanus*). Allotypes were determined in gel precipitation with allotype-specific alloantisera, as previously described (van der Loo, 1993). The DNA was extracted from liver and/or blood clots using a Qiagen extraction kit (Qiagen, Vienna, Austria). The *CH2* exon regions were amplified by PCR using oligonucleotide primers designed according to the genomic DNA sequence of *Lepus granatensis* (P. J. Esteves, unpublished data). The primers were F3 (5′gtgagtcccatTagcctcac3′) and R2 (5′gggcccatggtgtagacctt3′). The PCR amplification protocol can be obtained upon request. Sequences were determined by automated sequencing following the Big Dye Terminator Cycle Sequencing protocol (Perkin Elmer, Warrington, UK) using the primers F3 and R2. For RFLP analysis, the PCR products were digested with restriction enzyme *Tha*I (New England Bio Laboratory (Beverly, MA)), according to the manufacturer's instructions. Sequence diversity was estimated using MEGA version 2.1 (Kumar *et al.*, 2001) as well as DNASP version 3.51 (Rozas & Rozas, 1999).

Results and Discussion

As none of the *Sylvilagus* or *Lepus* specimens studied here displayed the *e14* allotype in their serum, the DNA data presented for these species are for two serologic phenotypes: *e15*-positive and *e14*-negative on the one hand ('*e15*'), and *e15*-negative and *e14*-negative ('*eng*') on the other.

Sequences including the entire *CH2* domain exon were determined for a total of 25 specimens. In Fig. 1, the unique sequences are displayed for each species. Results obtained with heterozygous *e14/e15* rabbits confirmed that heterozygous nucleotide positions were very clearly revealed in the sequencing profiles (note: our *e15* rabbits did not show the deletions occurring in the 5′ intron region of the L29172 sequence reported by Martens *et al.*, 1984). In Fig. 2, we show the inferred amino acid sequences, together with data obtained with tryptic digests, as published in Teherani *et al.* (1982). Ignoring the usual Gln-Glu and Asn-Asp ambiguities, there was good agreement between the peptide data and the genomic data. There were, however, conflicting results for the presence of Arg(285) in hares (HA-1 and HA-3). As His285 was found in each of the 48 amplified genomes of *Lepus* and *Sylvilagus*, and occurs most frequently in other mammalian species, the assignment of Arg(285) to *Lepus* and *Ochotona* species may reflect the difficulty of amino acid sequencing. If this is the case, the hare specimen studied by Teherani *et al.* (1982) probably belongs to *Lepus californicus*. HA-1 and HA-3 could indeed be homozygous for one of the two alleles (Lecf_*e15**1 and Lecf_*e15**2) present in the specimen studied here.

EU numbering	2 3 2345678901	2 4 2345678901	22 55 2345678901	2 6 2345678901	2 7 2345678901	2 8 2345678901	2 9 2345678901	3 0 2345678901	3 1 2345678901	3 2 2345678901	3 3 2345678901	n[a]
PCR DNA sequences												
Leeu_*e15*	PELLGGPSVF	IPPPKPKDTL	MISLTPEVTC	VVVDVSQDDP	EVQFTWYINN	EQVHTARPPL	REQQFNSTIR	VVSTLPIAHQ	DWLRGKEFKC	KVHNKALPSP	IEKTISKAK	
Leam_*e15*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2
Lect_*e15*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2
Lecf*1_*e15*	.....	.....	.....	.....	.....	K.....	.....	...I....	.....	.....	.....	1
Lecf*2_*e15*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1
Leti_*eng*	.....	.....	.....	.....	.....	.....	.....	.....K.	.....	.....	.....	4
Legr1_*eng*	.....	.....	.....	.....	.....	.....	.....	.....K.	.....	.....	.....	20
Legr2_*eng*	.....	.....	.....	.....	.....S.	.....	.....	.....K.	.....	.....	.....	2
Lecp1_*eng*	.....	.....	.....	...Y....	.....	.....	.....	.....V.	.....	.....	.....	2
Lecp2*1_*eng*	.....	.....	.....P.	...Y....	.....	.....	.....	.....V.	.....	.....	.....	1
Lecp2*2_*eng*	.....	.....	.....	.....	.....	.....	.....	.....M.	.....	.....	.....	1
Lecs_*eng*	.....	.....	.....	.....	.....	.....	.....	.....M.	.....	.....	.....	4
Lesx*1_*eng*	.....	.....	.....	.....	.....	...R....	.....	.....M.	.....	.....Q.	.....	1
Lesx*2_*eng*	.....	.....	.....	.....	.....	...R....	.....	.....M.	.....	.....	.....	1
Sycu_*e15*	.....	...R....	.....	...V....	.....	Q.....	.....	.....	.....	.....A.	.....	2
Syfl_*e15*	.....	...R....	.....	...V....	.....	.....	.....	.....	.....	.....A.	.....	2
Orcu_*e14*	.....	...R....	.....	.....	...R....	.....	.....	.....T.	.....	.....A.	.....R	
Orcu_*e15*	.....	...R....	.....	.....	...R....	.....	.....	.....	.....	.....A.	.....R	
Tryptic peptides (Teherani et al. 1982)												
CT-12		..V.....	.....	...V....D.		.....D....	.....	.....	...D....	.....		
HA-3		.....	.....E...	.....	.E.R.....	.....D....	.....	.....	...D....	.....		
HA-1		.....	.....	.....D.	K..R.....	.....	...I....	...S....	...D....	.....		
LT		.....	.....E...	...			.....K.	....				
PIKA		.....S.	.....E...	.....D.	...R.	.....L.	.....	...K....		.....	..R..	

Figure 2. Amino acid sequences, as inferred from nucleotide sequences displayed in Fig. 1. Dots indicate identity with sequence Leeu_*e15*. Data obtained from tryptic digests are from Teherani *et al.* (1982): CT-12: *Sylvilagus floridanus*; HA-1 and HA-3, unspecified *Lepus* spp. from Kansas (USA); LT: *Lepus timidus* (Ireland); PIKA: *Ochotona rufescens*. Italics suggest that data might be incorrect. 'N' indicates that the *cis-trans* configuration between alleles (n*1 and n*2) was not resolved. n[a], number of genes sequenced.

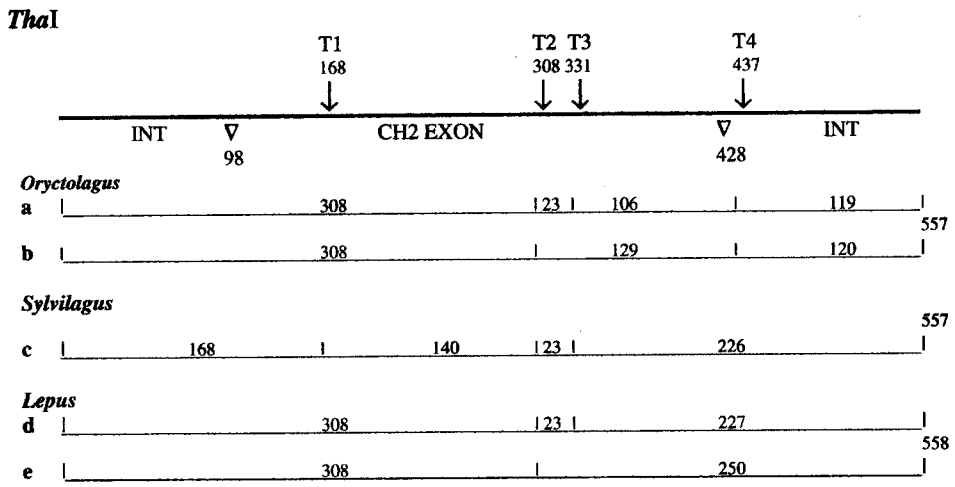

Figure 3. *Tha*I haploid restriction patterns inferred from RFLP analysis of F3-R2/PCR products. The full length of the PCR product is 557–558 bp, and includes the 5′ primer (20 bp), the 3′primer (20 bp), the entire *CH2* exon (330 bp), and parts of the flanking introns. '∇' indicates the exon–intron bounderies. '↓∏' indicates the potential restriction sites T1–T4. The nucleotide sites are numbered according to the sequence obtained for *Lepus granatensis* (AJ295217, Fig. 1). The patterns a and b correspond to *Oryctolagus cuniculus* allotypes *e15* and *e14*, respectively. c: *Sylvilagus* spp. showing allotype *e15* (*S. cunicularis* and *S. floridanus*); d: *Lepus* spp. showing allotype *e15* (*L. europaeus, L. calotis, L. californicus* and *L. americanus*); e: *Lepus* spp. that are negative for *e15* and *e14* markers (*L. timidus, L. granatensis, L. castroviejoi, L. capensis* and *L. saxatilis*), and *Lepus californicus* displaying a Ala(GCA) codon at amino acid position 309.

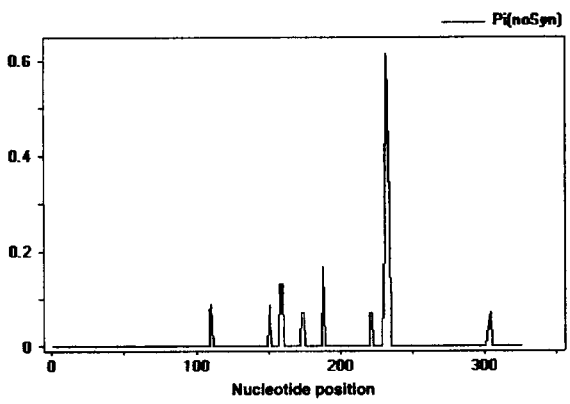

Figure 4. Non-synonymous nucleotide diversity among *Lepus IGHGCH2* coding regions, shown in a sliding window plot of non-synonymous diversity among exon sequences of *Lepus* spp. displayed in Fig. 1, using the DnaSP program (Rozas & Rozas, 1999), with window size = 2, step = 1. The value of Pi(noSyn) for the total sequence was 0.01.

Amino acid differences were observed at 13 sites, seven of which showed within-species variation, while five could define species-specific differences. Only position 309 was polymorphic in more than one species. This site can be occupied by five different amino acid residues. The *e15* allotype was always associated with a Ala309 codon. This codon contributes to a *Tha*I restriction site (C[G'CG], '*Tha*I-3' in Fig. 1), except for one allele of *Lepus californicus* (Lecf_*e15*), where Ala(309) was encoded by a GCA. Because all PCR products share the *Tha*I site related to the Arg(301) codon ([CG'C]G; '*Tha*I-2'), the presence of Ala309(GCG) was marked by a 23-bp fragment in RFLP analysis. This fragment was diagnostic for the *e15* allotype in all species studied. In Fig. 3 we show the different haploid *Tha*I restriction patterns as inferred from RFLP and sequence data. Two restriction sites were species-specific: one was specific to *Oryctolagus cuniculus* and was created by a nucleotide deletion within the 3′ intron region ('*Tha*I-4'), and the second was only found in *Sylvilagus* spp. and involved the Ser254Arg255 codons ([TCG]'[CGC], '*Tha*I-1'). Interestingly, Teherani *et al.* (1982) reported Val255 in tryptic peptides of *Sylvilagus floridanus* IgG (CT-12). Position 255 is situated close to the canonic Ile253, which, like His310, is known to interact with FcRn (Kim *et al.*, 1999). As *e14*-positive cottontail rabbits have been found (Cazenave *et al.*, 1977), it appears that, in this species, *Tha*I RFLP may detect amino acid variation at positions 255 and 309.

Although the rabbit allo-antisera provide the safest shortcut for detecting either Ala309 or Thr309 at the IgG *CH2–CH3* interface, they cannot detect new variants. In contrast, PCR-RFLP combined with sequence determination has allowed us to discover new polymorphisms in *Lepus* and *Sylvilagus* species. Whereas the *Tha*I-3 site always implies a GCG codon at position 309, five different 309 codons were associated with its absence (i.e. ACG, GTG, ATG, AAG and GCA). In previous studies we had observed that sera of *L. europaeus* always presented the *e15* allotype, whereas this marker was absent in all 30 specimens of *L. timidus* and *L. granatensis* studied (Schröder *et al.*, 1987; P. J. Esteves and W. van der Loo, unpublished data). As expected, this dichotomy is reflected in the *Tha*I restriction patterns (not shown). PCR-RFLP methods can therefore help to overcome the need for allotype-specific antisera for measuring gene flow between the different *Lepus* species in areas of range

overlap (cf. Schröder *et al.*, 1987). In fact, the spread of *e*-allotype negative genes within *L. europaeus* will be better monitored by RFLP than by serology. An adapted version of the PCR-RFLP method described here could also contribute to species determination for tissue samples and fossil remains deposited in field and museum collections.

Considering the very limited degree of sequence variation among the IgG *CH2* domains of leporids, it is interesting that position 309 was polymorphic within each genus. Despite the 97% average amino acid sequence similarity, five different residues were observed at this particular position, namely, Ala, Thr, Lys, Val, and Met. Amino acid sequence diversity (π) at 309 was 30 times larger than the mean diversity of the entire sequence (π_{309} = 0.742, π_{CH2} = 0.024; MEGA2 software: distances > sequence diversity > model > amino acid > p-distances). Figure 4 highlights the 'hotspot' variability among *Lepus* sequences, where position 309 accounts for some 40% of the total amino acid diversity. It is remarkable that human *IGHG3* and *IGHG4* show allotypic variation, which is caused by a Val309Leu mutation (Brusco *et al.*, 1998; Dard *et al.*, 2001). X-ray crystallography has revealed direct contact between the side-chains of *IGHG*-Leu309 of the rat FcRn receptor (Burmeister & Bjorkman, 1994), while population genetic studies have indicated that gene diversity at this site can be adaptive in the rabbit (van der Loo *et al.*, 1987, 1996). Within this context, it seems that the reported 'hotspot' variability at position 309 may have to do with diversity enhancement selection. A better knowledge of the sequence variation, as well as more advanced methods for measuring allele distribution in populations, could contribute to the understanding of the biological meaning of gene diversity in the antibody constant region.

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Variability at position H309 among mammalian species

The nucleotide triplet and the inferred amino acid observed at position H309 (79 IMGT numbering) for each sequence are presented in Figure 2.4. Figure 2.6 shows the non-synonymous nucleotide diversity of the *IgGCH2* coding regions within mammalian groups (*Primata*, *Rodentia*, *Lagomorpha*, *Carnivora* and *Perissodactyla/Artyodactyla*). In the primates and carnivores, like in the lagomorphs, the highest π (the average number of nucleotide differences per site) is observed at nucleotides encoding H309. In the lagomorphs six different triplets are observed, corresponding to five different amino acid residues (Met, Lys, Val, Ala and Thr). Four different triplets, coding for amino acids Val, Leu, and Thr, are present in the primates. In the carnivores, there are five different triplets and four amino acids (Glu, Gly, Gln, and Leu) and in the rodents, four different triplets for four amino acids (Met, Leu, Lys and Gln). In the *Perissodactyla/Artiodactyla* group, the Gln triplet CAG is fixed at position 309.

The occurrence of positive selection at the amino acid level was tested by the differences between non-synonymous and synonymous substitutions (Hughes and Nei, 1988, 1989). Table 2.3 shows the values of dS (Synonymous distance) and dN (Non-synonymous distance) at position 309 and the average of these two measures in all remaining positions. The estimation of the distances was performed in the computer program MEGA 2 using Nei-Goijobori method (p-distance). It shows that the dN/dS ratios are higher at postion H309, whereas on the average, in the remaining part of the exon, the opposite situation prevails.

Table 2.3

Values of dS (Synonymous distance) and dN (Non-synonymous distance) obtained at position 309 and in the average of all remaining positions for several mammalian groups. The standard error values are in brackets.

Mammalian groups	all positions			H309		
	dS	*dN*	*dS-dN*	*dS*	*dN*	*dS-dN*
Marsupials	0.197 (0.04)	0.112 (0.02)	0.085 (0.05)	----	0.333	-0.333
Rodents	0.259 (0.03)	0.122 (0.01)	0.137 (0.03)	----	0.324	-0.324
Artyodactyls/Perissodactyls	0.297 (0.03)	0.130 (0.02)	0.167 (0.03)	----	----	----
Carnivores	0.288 (0.04)	0.103 (0.02)	0.185 (0.04)	0.411	0.517	-0.106
Primates	0.107 (0.01)	0.035 (0.01)	0.072 (0.02)	0.184	0.318	-0.134
Lagomorphs	0.068 (0.01)	0.014 (0.004)	0.054 (0.02)	0.090	0.394	-0.304

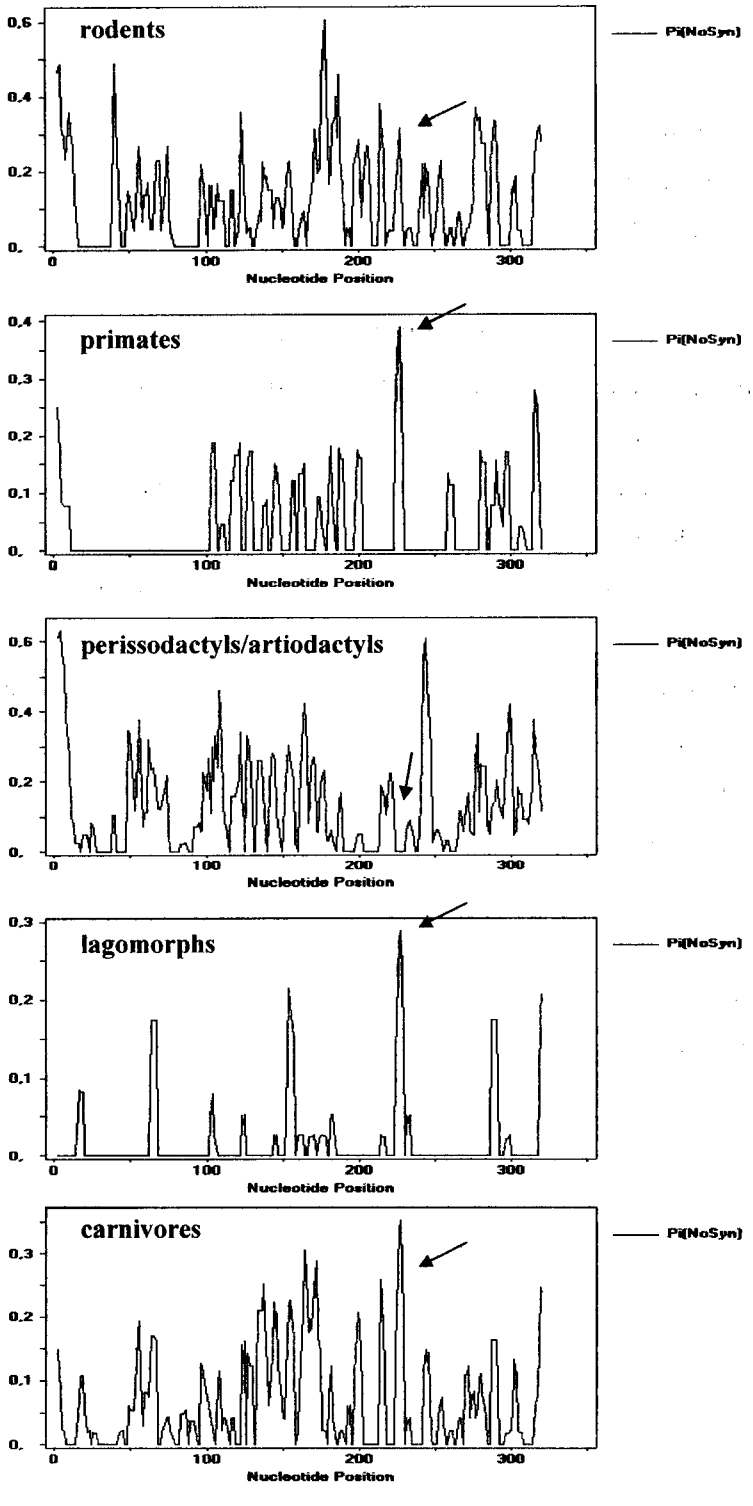

Figure 2.6. Non-synonymous nucleotide diversity (Pi) values obtained in *IgGCH2* locus shown in a sliding window (window size = 3, step = 1) for several mammalian groups: rodents – *Rattus*, *Mus and Cricetulus;* primates – *Pan*, *Homo* and *Macaca;* perissodactyls/artiodactyls – *Equus*, *Bos*, *Ovis*, *Camelus*, *Llama* and *Sus;* lagomorphs – *Oryctolagus*, *Sylvilagus* and *Lepus;* carnivores – *Canis*, *Mustela* and *Felis*. The analyses were performed using the program DnaSP (Rozas and Rozas, 1999). The arrows indicate the Pi values obtained at position H309.

These results seem to indicate that the "hotspot" variability at position 309 may have to do with diversity enhancement selection. However, this position does not seem to have the same importance for all groups, since it is fixed in the *Artiodactyla/Perissodactyla* group.

It can also be considered as fixed in the Iberian rabbits, which may be in contradiction with the adaptive value of the polymorphism at this coding region. It is therefore important to emphasize the population genetic evidence that the *e14-e15* polymorphism is only adaptive in situations where diversity at the V_H and/or C_{K1} loci is reduced (compensatory overdominance; van der Loo *et al.*, 1996).

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CHAPTER 3

Studies of gene diversity at the Heavy chain variable region gene locus (*a* locus or *IgVH* locus) in leporids

Introduction

The VH region diversity is well characterized in the domestic rabbit. Three serologically defined allotypic lineages exist, the so-called V_Ha allotypes *a1, a2* and *a3*. These 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). Except for chicken, V_H allotypic markers have not been found in other species, probably because each individual genome disposes of a large number of V_H gene segments. The genomic mapping of the V_H gene segments showed that the rabbit uses in the *VDJ* rearrangements almost exclusively the *D*-proximal V_H gene segment (V_H1) (see Figure 3.1), which can explain the allelic inheritance of the V_H allotypes in this species (Knight and Becker, 1990; Knight, 1992). About 80 to 90% of circulating Ig molecules are derived from the V_H1 gene and express the V_Ha allotypic markers (e.g. Kindt, 1975; Margolies *et al.*, 1977). The VH regions of the remaining 10-20% are encoded by the V_Ha-negative genes (V_Hx and V_Hy) (Kim and Dray, 1972; Kim and Dray, 1973; Roux, 1981), which are localized at least 50Kb upstream of V_H1 (Knight and Becker, 1990). These three alleles 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, 10, 12, 13, 16 and 17) and 3 (positions 65, 67, 70, 71, 74, 75, 84 and 85) (see Figure 3.2) (Tonnele *et al.*, 1983; Mage *et al.*, 1984; Knight and Becker, 1990).

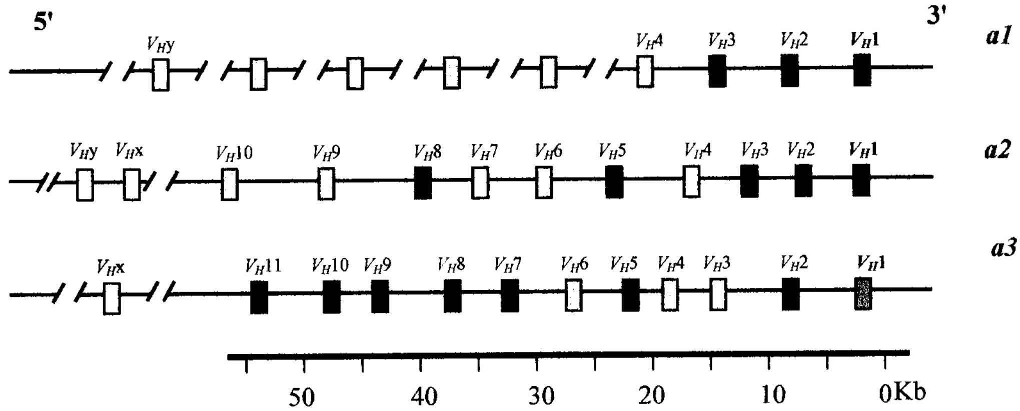

Figure 3.1. Genomic organisation of V_H genes in haplotypes: V_H-*a1*, -*a2* and -*a3* (Tunyaplin and Knight, 1995). Each haplotype was estimated to have more than 100 V_H genes in a 750-Kb region. Only genes with known genomic locations are shown. V_H1a1, V_H1a2 and V_H1a3 are indicated with red, blue and green boxes, respectively and the remaining functional V_H genes are presented in grey boxes. The putative pseudogenes are represented by black boxes. White boxes represent V_Hx and V_Hy. The genomic maps are approximate.

```
        0                         1           2                   3
        1 2 3 4 5 6 7 8 9 A B C D 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
                                        FR1
VH1a1   Q - S V E E S G G - - - - R L V T P G T P L T L T C T V S G F S L S
VH1a2   . - . . K . . E . - - - - G . F K . T D T . . . . . . . . . . . . .
VH1a3   . - . L . . . . . - - - - D . . K . . A S . . . . . . . A . . . . F .
VHx     . E Q L K . . . . - - - - G . . Q . . G S . K . S . K A . . . . D F .
VHy     . - Q L . Q . . . G A G G G . . K . . G S . E . C . K A . . . . . . .
VHz     R - Q L . H . . . - - - - G . . Q . R G S . K . C . K A . . . . T F .

        3                   4                   5                     6
        1 2 3 4 5 A 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 A B 4 5 6 7 8 9 0 1
            CDR1             FR2                           CDR2
VH1a1   S - Y A M S W V R Q A P G K G L E W I G I I S S - - S G S T Y Y A S
VH1a2   . - N . I . . . . . . . . . . . . . . A . G . - - . . . A . . . . .
VH1a3   . S . Y . C . . . . . . N . . . . . . . C . Y A G S . . . . . . . .
VHx     . - . G V . . . . . . . . . . . . . A Y . D P V - F . . . . . . . .
VHy     . S . W I C . . . . . . . . . . . . . C . Y A G S . . . . . . . . .
VHz     . - . Y . C . . . . . . . . . . . . . C . Y A G S . . . A . . . . .

        6             7                   8
        2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 A B C 3 4 5 6 7 8 9 0 1 2
                                 FR3
VH1a1   W A K G R F T I S K - T - S T T V D L K I T S P T T E D T A T Y F C
VH1a2   . . . S . S . . T R N . N L N . . T . . M . . L . A A . . . . . . .
VH1a3   . . . . . . . . . . - . . . . . . T . Q M . . L . A A . . . . . . .
VHx     . V N D . . . . . S H N A Q N . L Y . Q L N . L . A A . . . . . . .
VHy     . V N . . . . L . R . I D Q S . G C . Q L N . L . A A . . . M . Y .
VHz     . V N . . . . L . R . I D Q S . G C . Q L N . L . A A . . . M . Y .
```

Figure 3.2. Amino acid sequences of rabbit germline V_H genes: V_H1a1, V_H1a2, V_H1a3, V_Hx and V_Hy. The amino acids that are characteristic of allotypes *a1* and *a2* are marked with black and grey arrows, respectively. The borders of each framework (FR1, FR2 and FR3) and each CDR (CDR1 and CDR2) are defined according to Kabat *et al.*, (1991) numbering. Dots (.) indicate identity with V_H1a1 and dashes (-) represents indels (insertions or deletions).

An important contribution to the understanding of the mechanisms that drive the rearrangement process in the rabbit was the study of a rabbit strain, called "Alicia", raised in the Basel Institute, Switzerland. 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 contains mostly Ig encoded by V_Hn genes (Kelus and Weiss, 1986; Di Pietro *et al.*, 1990; Chen *et al.*, 1993). The "mutant" rabbits derived from a V_H1a2 parental rabbit in which a 10 Kb segment of genomic DNA, including the V_H1 gene, was deleted (Knight and Becker, 1990). The study of this "mutant" rabbit strain showed that the allelic inheritance of the allotypes in normal rabbit is explained by the preferential usage of only one V_H gene, i.e. the V_H1 gene which is deleted from "ali" genome (Knight and Becker, 1990). The level of B cells that show the allele *a2* specificities increases with age in the "Alicia" rabbit (Kelus and Weiss, 1986; Di Pietro *et al.*, 1990; Alegrucci *et al.*, 1990; Chen *et al.*, 1993; Pospisil *et al.*, 1995). The analysis of nucleotide sequences of the promoter region showed that more than 80% of the *VDJ* rearrangements utilise either the functional V_H4 or V_H7 genes localized upstream of V_H1. The V_H4 and the V_H7 have 7 (out of 11) specific nucleotides associated to the allotype *a2*, while the other nucleotides that characterise *a2* are gained through somatic gene conversion using V_H9 or a V_H9-like germline gene as donor (Shegal *et al.*, 1998; Zhu *et al.*, 1999).

The V_H genes in the rabbit are localized in the chromosome 17 (Medrano and Dutrillaux, 1984). Despite having, like human and mouse, more than 100 V_H genes (approximately 50 % are functional) (Gallarda *et al.*, 1985; Currier *et al.*, 1988), only a small fraction of them have been sequenced (see Figure 3.1).

Population genetic studies

The study by double immunodiffusion of domestic breeds and wild rabbits of Continental Europe (North of Pyrenean Mountains), Great Britain and overseas showed the presence of allotypes *a1*, *a2* and *a3* only. However, in the French population of Versailles, an allotype with partial reaction against *a3*-specific antiserum was observed, having a gene frequency of less than 5% (van der Loo, 1987, 1993). In all the populations studied the gene frequencies of *a1* and *a3* were similar (≅40%) and higher than *a2* (≅20%). These allelic frequencies reflect the "pecking order" of their relative expression in heterozygote animals (Mage, 1967; Lummus *et al.*, 1967). An excess of homozygotes was detected mainly due to *a2* homozygotes (van der Loo, 1982, 1987; van der Loo and Arthur, 1986).

Radioimmunoassay studies performed in wild rabbit populations from the Iberian Peninsula showed nine variants, *a100* to *a105*, *a107*, *a108* and *a109* (Cazenave *et al.*, 1974; Brézin *et al.*, 1979; Brézin and Cazenave, 1980; Haouas *et al.*, 1987, 1989; Haouas and Benammar-el Gaaied, 1994). The partial amino acid sequence of allotype *a100* showed a close relationship with the allotype *a3* (Tonnelle *et al.*, 1983).

Indications that the V_H1 polymorphism can be trans-specific

The serological analyses of 660 specimens of *Lepus americanus* (snowshoe hare) belonging to 27 populations showed cross-reaction against rabbit anti-*a1*, -*a2* and -*a3* antisera. These allotypes were called *am1*, *am2* and *am3*, respectively (De Poorter, 1984). These names reflect the chronology of their discovery (van der Loo, personal communication). Some analogies with the results that had been described in the rabbit were observed, such as the existence of a less frequent allele (*am3*) with an average gene frequency of 14% and with significant homozygous excess (van der Loo, 1987) (as observed in the rabbit populations for allele *a2*). Su and Nei (1999) considered these data as evidence for the trans-specific nature of the V_H1 polymorphism. Furthermore, these authors compared the extent of sequence divergence between the rabbit allotypes *a1*, *a2* and *a3* with that between human and mouse V_H gene sequences and concluded that, assuming a "normal" mutation rate, the V_H1 polymorphism has persisted for about 50 My. Since the age of separation between rabbit and *Lepus* is estimated between 13.1 and 29 million years ago (Halanych and Robinson, 1999; Su and Nei, 1999), the allelic lineages present in one species should be more related to some of the alleles expressed in the other species than to their allelic counterparts.

The diversification of Ab primary repertoire in the rabbit

Among the vertebrate species, two different strategies to generate the primary Ab repertoire have been adopted. 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 a number of mammal species (rabbit, sheep, pig and bovine), have less extensive germline repertoires than mice and humans and consequently use strategies of primary repertoire development

that overcome this limitation. In these cases, the primary antibody repertoire is post-rearrangement diversified by two mechanisms: gene conversion and somatic hypermutation.

In chicken, gene conversion is the major mechanism of the Ab binding site diversification. A set of pseudogenes are used as donors and the unique rearranged $V_H/V_L(D)J$ acts as acceptor (Reynaud *et al.*, 1985). In sheep, B cell diversification occurs in early development in the ileal Peyer's patches and results from somatic hypermutation rather than gene conversion (Reynaud *et al.*, 1991). Both processes are used to diversify the Ag-binding repertoire in cattle (Parng *et al.*, 1996; Lucier *et al.*, 1998). Although it is not yet completely proven, gene conversion mechanisms are possibly used in the Ab repertoire diversification in swine (Sun *et al.*, 1996).

Becker and Knight (1990) showed that the rearrangement process in the rabbit involves preferentially a single V_H gene that is diversified mainly through gene conversion using other upstream functional V_H genes (Becker and Knight, 1990; Knight and Crane, 1994). Somatic hypermutation also takes place, distributing several point mutations throughout the entire *VDJ* genes (Short *et al.*, 1991; Weinstein *et al.*, 1994). In this species B cells are generated, proliferate and develop in the bone marrow and in the foetal liver (lymphopoiesis is absent in the adult (Crane *et al.*, 1996)), and migrate to appendix and 0other gut-associated lymphoid tissues (GALT) where they are intensely diversified. The V_H genes upstream of V_H1 (V_H4a1, φV_H2a1, φV_H3a1, V_H4a2, φV_H3a2, V_H7a2, V_H9a2, etc...) contribute to the expressed V_H1 diversification by gene conversion. They evolved to some extent in concert, which resulted in the presence of the allotypic motifs on other V_H gene segments of the chromosome (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 Ab repertoire diversification only happens 1-2 months after birth (Cooper *et al.*, 1968; Weinstein *et al.*, 1994). The experiment of surgically removing the appendix and *sacullus rotundus* on the day of birth and the Peyer's patches at three weeks of age showed that GALT is essential in the generation of the primary antibody repertoire (Vadjy *et al.*, 1998). It has been shown that exogenous factors, such as the intestinal microflora, are also required for the Ab repertoire diversification (Lanning *et al.*, 2000a, 2000b; Sehgal *et al.*, 2002).

Evolution of V_H gene family

The study of V_H genes from several vertebrate species showed that V_H genes from the same species could belong to different groups. V_H gene sequences have been classified into five different groups (A-E). Those of cartilaginous fishes form a monophyletic group (E). V_H genes of bony-fish form the group D. Groups A, B and C include V_H gene sequences of bony-fish, amphibians, reptiles, birds, and mammal (Tutter and Riblet 1989; Schroeder *et al.*, 1990; Ota and Nei, 1994). The human and mouse possess V_H genes that are very diversified and may cluster with either A, B or C groups (polyphyletic), whereas V_H genes of birds, rabbits, horse, and artiodactyls (cattle, sheep, and swine) are monophyletic. Bird, rabbit and swine each form distinct clusters within group C, whereas horse, cattle and sheep each form distinct clusters within group B. Since V_H genes of different artiodactyl species sequences cluster within different groups (B and C), their common ancestor must have possessed V_H genes from both groups (Sitnikova and Su, 1998) (see Figure 3.3).

Monofunctional multigene families are generally believed to undergo processes of genetic exchange, like gene conversion and unequal crossover, which homogenizes the DNA sequences (Smith *et al.*, 1971; Smith, 1994; Zimmer *et al.*, 1980). This has been called "concerted" evolution. The cconcerted evolution model has been invoked to explain the evolution of V_H genes (Hood *et al.*, 1975; Ohta, 1983). However, the study of V_H gene families in human and mouse (Gojobori and Nei, 1984; Tutter and Ribblet, 1989), and the studies on a much longer evolutionary time scale, showed that the pattern of V_H 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), 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 V_H gene libraries are duplicated and can diverge functionally, while others become pseudogenes or/and 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) (see Figure 3.4).

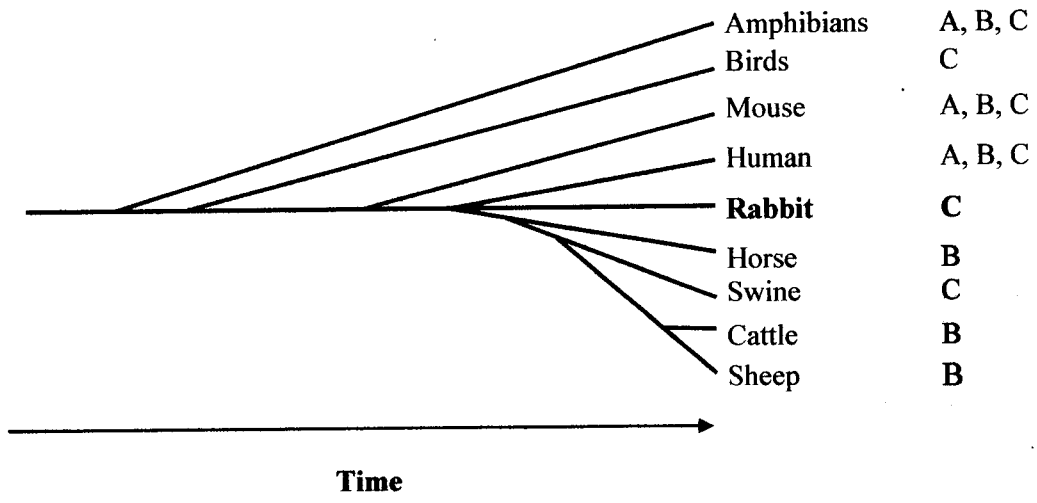

Figure 3.3. Schematic representation of V_H gene groups present in several tetrapod species.

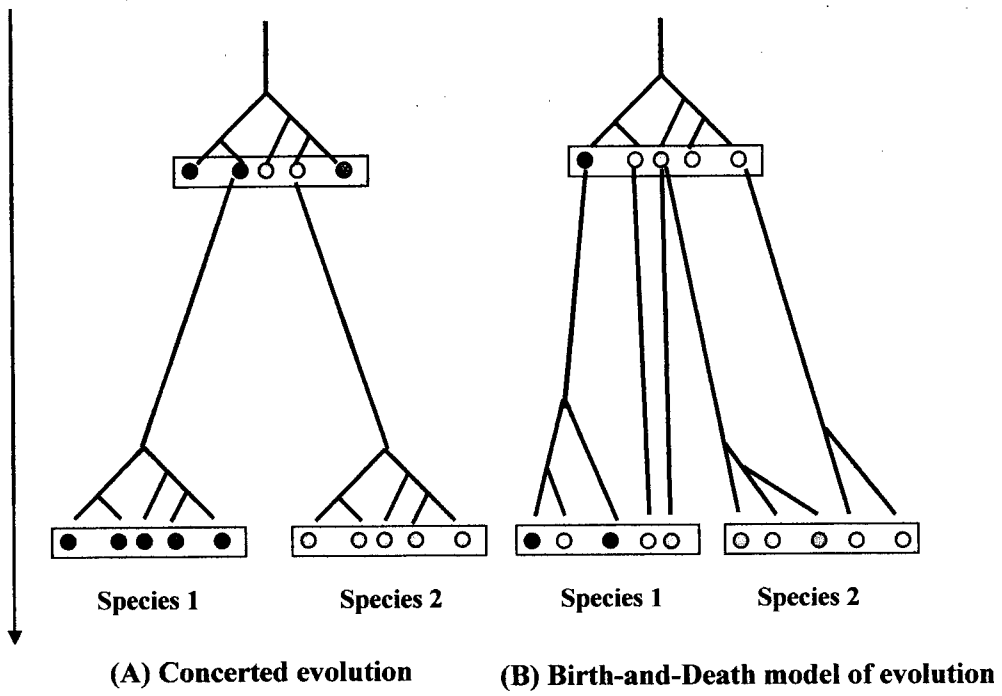

Figure 3.4. Schematic representation of two different models of evolution proposed to explain the evolution of monofunctional multigene families. Blue and red circles represent functional genes. Grey circles represent pseudogenes. In the concerted evolution model the genes have high homogeneity within species. In the Birth-and-Death model of evolution genes from one species can be more related to genes present in other species.

According to this model, the tetrapod ancestor possessed three V_H gene groups (A, B, and C) and library contraction events occurred in several lineages independently. In species that have inherited only one group of V_H genes, antibody diversification is mainly due to somatic hypermutation or gene conversion. It seems that the VH gene library contraction is associated with the recruitment of a specific organ for extensive somatic diversification (bursa of Fabricius in chicken; ileal Peyer's patches in sheep, cattle and probably swine and horse; appendix in rabbit).

Different hypotheses to explain the large inter-allelic differences among V_H1 genes

The large differences between alleles can be explained either by prolonged allele persistence times and/or increased evolutionary rates. If evolutionary rates among the allelic lineages are the same as in reference lineages (rate monotony), the genetic distance reflects the time of divergence between alleles. If on the contrary, evolutionary rates are increased, divergence time between alleles will be overestimated. The knowledge of the diversity of the rabbit IgV_H genes remains fragmentary and concerns only a small number of domestic breeds. These breeds are recent genetic isolates of the subspecies *O. c. cuniculus*, and retained only a fraction of the species diversity. To test the hypothesis of rate monotony we have used two different approaches: 1) a study of the patterns of V_H gene diversity in the Iberian Peninsula (subspecies *O. c. algirus* and *O. c. cuniculus*; 2) sequence determination of V_H genes in other *Leporidae*.

Material and Methods

Serological analyses

In this work, 402 specimens from 18 wild rabbit populations from the Iberian Peninsula belonging to the subspecies *Oryctolagus cuniculus algirus* and *O. c. cuniculus* were analysed by double immunodiffusion tests according to van der Loo *et al.*, (1991). The serum of each sample was tested independently against allo-antisera specific for the allotypes *a1*, *a2* and *a3*, by immunodiffusion in 1% agar gel containing 2% polyethylene glycol. In each test a reference was included to monitor the degree of serological similarity of the sample being tested with samples from rabbits expressing the immunizing allotype. The reactions were recorded as: 1) *"1"* (identity reactions) in case of perfect fusion of the precipitation lines of test and reference sera with the antiserum; 2) *"p"* (cross-reactions) in case the reference line spurs over the test line (such spur implies that the tested serum displays only a part of the epitopes recognized by the allo-antiserum on the Ig molecules of the reference allotypes); 3) *"0"* in case of negative test reaction (i.e. absence of precipitation lines). Additionally, using the same test system, the serological analyses were performed in 147 specimens of three different *Lepus* species (*L. granatensis*, *L. europaeus* and *L. capensis*).

The allotype with the negative reaction when tested against allo-antisera specific for the allotypes *a1*, *a2* and *a3* (*000*) was called *"a-blank"*. The gene frequencies of the putative *"a-blank"* allele were calculated assuming Hardy-Weinberg equilibrium and using the formula:

$$(p + q)^2 = p^2 + 2pq + q^2,$$

where p^2 is the frequency x of homozygous *"a-blank"*; q corresponds to the sum of gene frequencies of the other alleles; the gene frequency of p (*"a-blank"*) is therefore calculated by $p = \sqrt{x}$, where x represents the relative frequency of *a*-negative individual samples.

Analysis of genetic diversity

For each population analysed the heterozygosity (Hs) was calculated using the formula:

$$H_S = 1 - \sum_{i=1}^{n} (p_i)^2$$

where p_i is the gene frequency observed at each allele in each population.

To measure the genetic differentiation between groups of populations F_{ST} values were calculated using the formula:

$$F_{ST} = 1 - \frac{\overline{H_S}}{H_T}$$

H_T is the total heterozygosity in the group of populations and is obtained with the mean allele frequencies over localities. $\overline{H_S}$ is the average of H_S values calculated for each population.

Cytonuclear disequilibria (*a* locus vs mtDNA) values were determined according to Asmussen and Arnold (1991).

Sequencing of *VDJ* gene segments and germline V_H gene segments

Two different types of tissues were used: liver, to obtain genomic DNA, and spleen, to extract total RNA, to be used as template for synthesizing cDNA. Standard DNA and RNA extraction methods were used.

Sequencing of VDJ gene segments expressed in Lepus *specimens*

The samples were collected from wild specimens in Alcochete, South of Portugal (17 specimens of rabbit belonging to the subspecies *O. c. algirus*, and seven specimens of *Lepus granatensis*) and in Austria (two specimens of *Lepus europaeus*). The amplification of the genes encoding the *IgVH* regions (Figure 3.5) was performed using the primers VHRPS and JH-B described by Zhu *et al.*, 1999. All PCR amplifications proceeded for 30 cycles (each cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s). PCR fragments with approximately 500 bp in length were gel-purified and then cloned and sequenced.

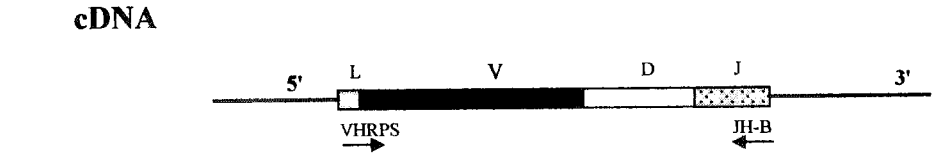

Figure 3.5. Schematic representation of the gene fragments that were amplified: Leader (L), Variable (V), Diversity (D) and Joining (J) segments of the Heavy chain; locations of primer homology are indicated (5' primer VHRPS and 3' primer JH-B).

Sequencing of germline V_H gene segments

Sequences from single specimens of *Ochotona princeps* (Pika), *Sylvilagus floridanus*, *Lepus americanus*, *Lepus granatensis*, and *Oryctolagus cuniculus algirus* were obtained. Germline V_H genes were PCR amplified with a 5' primer, VHRPS (localized in the leader segment), and a 3' primer, localized in the end of the V segment (OaFR3: 5`-GAGCTCACCTGAGAGACGGTGACCA-3`) (see Figure 3.6) and proceeded for 30 cycles (each cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 60s). PCR fragments with approximately 550bp in length were obtained by gel electrophoresis and then cloned and sequenced.

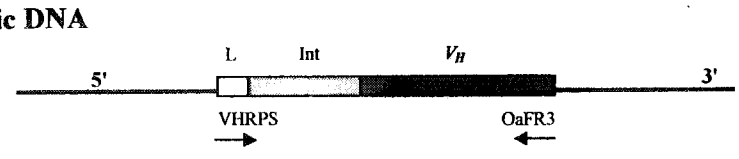

Figure 3.6. Schematic representation of the genomic DNA fragments that were amplified. This fragment comprises the complete Leader (L), Intron (Int) and Variable (V_H) gene segments of Heavy Chain. Regions of primer homology are indicated by horizontal arrows.

Phylogenetic analysis

The sequence alignments were made using the pileup program (GCG software), followed by visual adjustments. To construct a phylogenetic tree we used sequences obtained in this work and sequences available in the Genbank (accession numbers are presented in table 3.1): 1) Genbank rabbit germline V_H genes; 2) rabbit cDNA V_H gene sequences representative of each allotype; 3) hares cDNA V_H gene sequences; 4) other functional germline V_H gene sequences obtained in this work; 5) Genbank V_H gene sequences classified in the same group of the rabbit, the group C: human (class 3), mice, camel and domestic duck. As outgroup we used Genbank V_H gene sequences classified in

the group B: human (class 4), mouse and cattle. The reliability of the trees was examined by the bootstrap (Felsenstein, 1985) and the interior-branch (Rzhetsky and Nei, 1992; Sitnikova, 1996) methods, which produced the bootstrap probability (BP) and confidence probability (CP) values, respectively, for each interior branch in the tree. All data analyses were conducted by using the computer program MEGA (Kumar *et al.*, 2001).

Table 3.1

Accession numbers for each V_H gene used. The sample code and the major V_H gene group to which each sequence belongs are indicated.

Species	**V_H gene identification**	**Genbank (accession number)**
Mammals		
Oryctolagus cuniculus	V_H1a1 (group C)	M93171
	V_H1a2 (group C)	M93172
	V_H1a3 (group C)	M93173
	V_H4a1 (group C)	M93181
	V_H4a2 (group C)	M93182
	V_H4a3 (group C)	M93183
	V_H7a2 (group C)	U51027
	V_H9a2 (group C)	U51029
	V_Hx (group C)	L03846
	V_Hy (group C)	L03890
	V_Hz (group C)	AF264469
Homo sapiens	IgV_H3 (group C)	M99666
	"	M99672
	"	Z18900
	"	M99675
	"	M99679
	"	M99682
	IgV_H4 (group B)	X05713
	"	L10088
	"	M29811
Mus musculus	V_H12 (group B)	M22439
	V_H13 (group C)	X55935
Camelus dromedarius	(Group C)	AF000604
Bos taurus	174 (group B)	U55174
	172 (group B)	U55172
	170 (group B)	U55170
	168 (group B)	U55168
	166 (group B)	U55166
	164 (group B)	U55164
Birds		
Anas platyrhynchos	42M (group C)	X65218
	31M (group C)	X65219

Results and Discussion

Genetic diversity in Iberian rabbit populations

Population genetic studies

In the serological analyses six different allotypes were distinguished in wild rabbit populations from the Iberian Peninsula (*a1 (100)*, *a2 (010)*, *a3 (001)*, *a1v (p00)*, *a3v (00p)* and *"a-blank" (000)*). Estimates of the gene frequencies of these alleles and heterozygosity values are shown in Table 3.2. The geographical distribution of alleles is presented in Figure 3.7.

In this work, we divided the Iberian wild rabbit populations into three groups: 1) Southwest, where the mitochondrial lineage *A* is predominant; 2) populations in the hybrid region; 3) Northeast, where the mtDNA lineage *B* is prevalent. The *"a-blank"* allele is present in all Iberian populations (except Alicante). However, the gene frequency of this allotype decreases from southwestern (average gene frequency of 0.56) to northeastern populations (average gene frequency of 0.19). The allele *a1* is present in all Iberian Peninsula but, in contrast to the *"a-blank"* allele, its gene frequency increases from Southwest populations (average gene frequency of 0.27) to Northeast populations (average gene frequency of 0.45). The same pattern of variability is observed in the alleles *a2* and *a3*, hat triplicate the gene frequency from the Southwest (0.04 and 0.06, respectively) to the Northeast (0.12 and 0.18, respectively). The rare alleles *a3v* and *a1v* do not show any particular geographical pattern (note that serological identity between "variant" allotypes among regions has not been tested).

In rabbit populations from the recent area of distribution (France, Great Britain, Australia) the allotype *"a-blank"* is absent, and the alleles *a1*, *a2* and *a3* are present in all populations (Table 3.2).

Table 3.2

Gene frequencies obtained in rabbit populations at *a* locus. The immunodiffusion reactions obtained against specific antiserum for the allotypes *a1*, *a2* and *a3*, respectively, are shown in brackets. For identification of Australian and British locations see van der Loo (1987).

	n	*a1* (100)	*a2* (010)	*a3* (001)	*a1v* (p00)	*a2v* (0p0)	*a3v* (00p)	*"a-blank"* (000)	*H*	Reference
Inside Iberian Peninsula										
Southwest										
Portimão	18	0,25	----	0,06	0,03	----	----	0,66	***0.497***	**This work**
Santarém	27	0,18	----	0,10	0,08	----	0,08	0,54	***0.631***	"
Alcochete	17	0.28	0.09	0.06	----	----	0.03	0.54	***0.617***	"
Idanha	18	0,33	0,14	----	0,03	----	----	0,50	***0.621***	"
Cabreira	19	0,22	0,07	0,13	0,03	----	----	0,55	***0.626***	"
Las Lomas	40	0,38	0,03	0,09	0,13	----	0,10	0,27	***0.747***	"
Huelva	23	0,13	0,02	0,06	0,10	----	----	0,69	***0.493***	"
Doñana	19	0,26	0,05	0,08	----	----	----	0,61	***0.551***	"
Ciudad Real	20	0,32	0,03	----	0,03	----	----	0,62	***0.511***	"
Infantado	17	0,29	0,03	----	0,03	----	----	0,65	***0.492***	"
Vila Viçosa	18	0.31	----	0.11	----	----	0.05	0.53	***0.608***	"
average unweighted	**236**	***0,27***	***0.04***	***0.06***	***0.04***	**—**	***0,02***	***0,56***	***0.604***	
Populations in hybrid zone										
Amoladeras	11	0,40	----	----	----	----	----	0,60	***0.480***	"
Toledo	22	0,26	----	0,26	----	----	----	0,48	***0.634***	"
Bragança	15	0,39	0,23	----	0,13	----	----	0,25	***0.716***	"
average unweighted	**48**	***0.35***	***0,08***	***0,09***	***0,04***	**—**	**—**	***0,44***	***0,666***	
Northeast										
Tarragona	20	0,49	0,08	0,08	0,10	----	----	0,31	***0.641***	"
Alicante	19	0,58	0,08	0,34	----	----	----	----	***0.542***	"
Navarra	59	0,40	0,14	0,06	0,10	----	0,05	0,25	***0.742***	"
Lérida	20	0,33	0,18	0,25	0,03	----	----	0,21	***0.751***	"
average unweighted	**118**	***0,45***	***0,12***	***0,19***	***0,06***	**—**	***0,01***	***0,20***	***0.709***	
total (18 populations)	***402***	***0.33***	***0.07***	***0.09***	***0.07***	**—**	***0,01***	***0.45***	***0.670***	
Outside Iberian Peninsula										
France										
Perpignan	30	0,40	0,20	0,40	----	----	----	----	***0,640***	**van der Loo, 1987**
Camargue	40	0,45	0,18	0,37	----	----	----	----	***0,628***	"
Versailles	35	0,37	0,21	0,40	----	----	0,02	----	***0.659***	"
Ile de France	147	0.39	0.21	0.40	----	----	----	----	***0.644***	"
average unweighted	**252**	***0,40***	***0,20***	***0,39***	**—**	**—**	***0,005***	**—**	***0,644***	
Australia										
BB	23	0.37	0.13	0.50	----	----	----	----	***0596***	"
BW	39	0.26	0.29	0.45	----	----	----	----	***0.646***	"
CH	83	0.49	0.02	0.49	----	----	----	----	***0.519***	"
CN	71	0.46	0.18	0.36	----	----	----	----	***0.626***	"
UN	21	0.44	0.16	0.40	----	----	----	----	***0.621***	"
UR	77	0.54	0.31	0.15	----	----	----	----	***0.589***	"
GC	575	0.58	0.09	0.33	----	----	----	----	***0.547***	"
average unweighted	**889**	***0.45***	***0.17***	***0.38***	**—**	**—**	**—**	**—**	***0.624***	
Great Britain										
DO	89	*0.43*	*0.03*	*0.54*	----	----	----	----	***0.523***	"
GL	22	*0.61*	*0.05*	*0.34*	----	----	----	----	***0.510***	"
HI	75	*0.45*	----	*0.55*	----	----	----	----	***0.495***	"
PO	117	0.42	0.23	0.35	----	----	----	----	***0,648***	"
average unweighted	**303**	***0.45***	***0.10***	***0.45***	**—**	**—**	**—**	**—**	***0,585***	
total (15 populations)	***1444***	***0.44***	***0.15***	***0.40***	**—**	**—**	***0.001***	**—**	***0.618***	
total (33 populations)	***1846***	***0.38***	***0.11***	***0.24***	***0.03***	**—**	***0.008***	***0.25***	***0.728***	

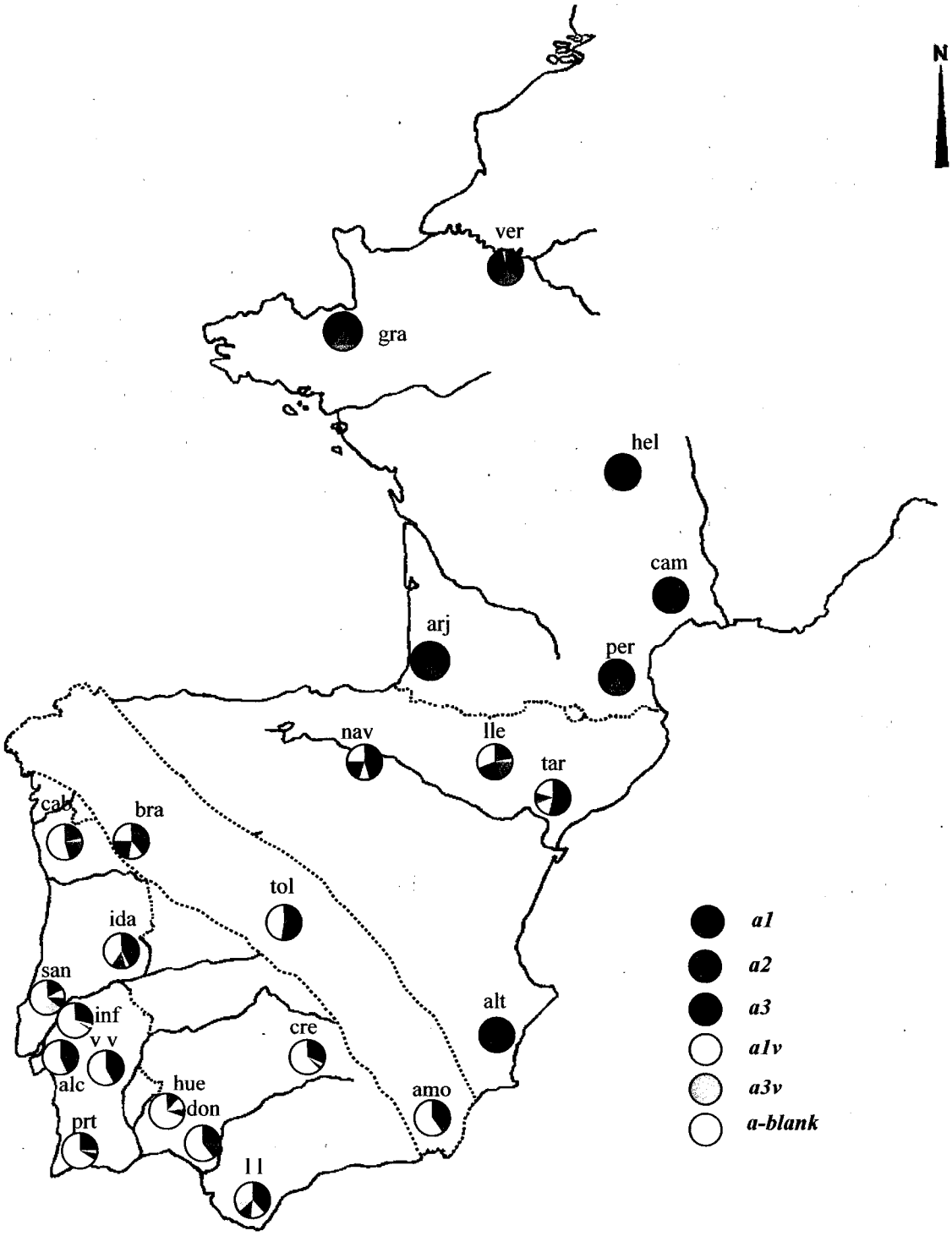

Figure 3.7. Geographic distribution of *a* locus alleles in wild rabbit populations of the Iberian Peninsula and France. Iberian Penisula populations: 1) Southwest: prt - Portimão; san - Santarém; alc - Alcochete; ida - Idanha; cab - Cabreira; ll - Las Lomas; hue - Huelva; don - Doñana; cre - Ciudad Real; inf - Infantado; v v - Vila Viçosa; 2) populations in the hybrid zone: amo - Amoladeras; tol - Toledo; bra - Bragança; 3) Northeast: tar-Tarragona; alt- Alicante; nav - Navarra; lle - Lerida. France: per - Perpignan; cam - Camargue; ver - Versailles; arj - Arjuzanx; gra - grax; hel - Helene. The hybrid zone is represented by a grey-shaded area.

Analysis of genetic diversity

Wright (1978) proposed a qualitative distinction of four classes of population differentiation based on F_{ST} values: low (<0.05), moderate (0.05-0.15) high (0.15-0.25) and very high (>0.25). The values of total F_{ST} obtained vary in the three different groups considered. It is moderate within the Iberian Peninsula populations (0.097), low among the populations from outside the Iberian Peninsula (0.039) and high over all populations (0.176). The F_{ST} values obtained for each allele in the three different groups are presented in table 3.3. Within the Iberian Peninsula the differentiation between populations is high for the *"a-blank"* allele and moderate for all the other alleles. Outside of Iberian Peninsula the F_{ST} values are low, except for the allele *a2* that is moderate. Using all populations, the differentiation between populations is very high for the blank allele, high for the *a3* allele and moderate for all the other alleles (*a1*, *a2*, *a1v* and *a3v*).

Table 3.3
F_{ST} values obtained at each allele

Populations	alleles			
	a1	*a2*	*a3*	*"a-blank"*
Inside Iberian Peninsula (18 populations)	0.053	0.078	0.110	0.152
Outside Iberian Peninsula (15 populations)	0.029	0.067	0.038	0
All populations (33 populations)	0.055	0.091	0.180	0.391

Cytonuclear disequilibria (*a* locus *vs* mtDNA)

Figure 3.8 highlights the positive correlation in population frequencies of mtDNA type *B* and the sum of alleles *a1* and *a2* (R^2=0.71).

For the calculus of the cytonuclear disequilibrium (D_T), it was not possible to calculate the value of D_S, because the samples used in this work were not the same that were used in the mtDNA study. Previous studies of the cytonuclear disequilibria in Iberian wild rabbit populations (van der Loo *et al.*, 1999) have shown that, as expected, within localities, equilibrium prevails between nuclear markers and maternal types (i.e. Ds =0). The value of D_T obtained in this work was therefore equivalent to interlocality covariances in gene frequencies.

The frequency of the nuclear allele *"a-blank"* and the mtDNA type are indicated in Table 3.4. The *"a-blank"* allele is significantly correlated to the maternal lineage *A* (cytonuclear disequilibrium D *(mtDNA –type A, "a-blank")* $= 0.06$; $X^2_1 = 17$).

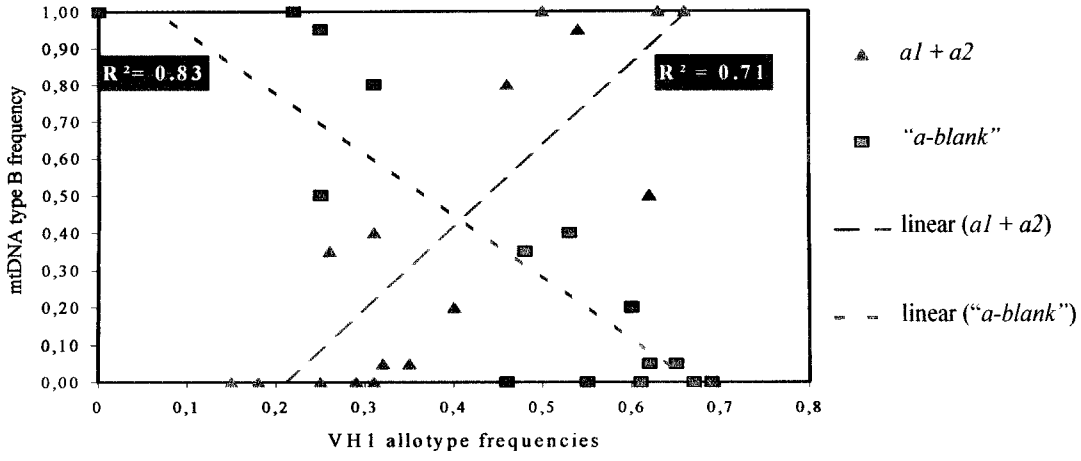

Figure 3.8. Frequency correlations between *a* locus alleles and mtDNA type *B*. R^2-value measured the correlations *"a-blank"*/mtDNA *B* and (*a1*+*a2*)/mtDNA *B*.

Table 3.4

Gene frequencies of *"a-blank"* allele and mtDNA type *A* in wild rabbit populations from Iberian Peninsula.

	n	***"a-blank"* allele**	mtDNA *A*
Inside Iberian Peninsula			
Southwest			
Portimão	18	**0.66**	1
Santarém	27	**0.54**	1
Alcochete	17	**0.54**	1
Idanha	18	**0.50**	1
Cabreira	19	**0.55**	1
Las Lomas	40	**0.27**	1
Huelva	23	**0.69**	1
Doñana	19	**0.61**	1
Ciudad Real	20	**0.62**	0.95
Infantado	17	**0.65**	0.95
Vila Viçosa	18	**0.53**	0.60
average unweighted	**236**	***0.561***	***0.95***
Population in hybrid zone			
Amoladeras	11	**0.60**	0.80
Toledo	22	**0.48**	0.65
Bragança	15	**0.25**	0.50
average unweighted	**48**	***0.443***	***0.65***
Northeast			
Tarragona	20	**0.31**	0.20
Alicante	19	**0.00**	0.00
Navarra	59	**0.25**	0.05
Lérida	20	**0.21**	0.00
average unweighted	**118**	***0.193***	***0.07***
average	***402***	***0.459***	**0.70**

Article 3

P.J. Esteves, D. Lanning, S.K. Zhai, N. Ferrand, K.L. Knight and W. van der Loo (submitted).

Allelic variation at the V_Ha locus in natural populations of rabbit (*Oryctolagus cuniculus*, L.)

Allelic variation at the $V_H a$ locus in natural populations of rabbit (*Oryctolagus cuniculus*, L.)

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Abstract

The large interallelic distances between the three rabbit Ig $V_H a$ lineages, *a1*, *a2*, and *a3*, have suggested that the persistence time of the $V_H a$ polymorphism could amount to 50 million years, which is much longer than that of *MHC* polymorphisms. Rabbit originated in the Iberian Peninsula where two subspecies coexist, one of which is confined to Southwestern Iberia (*O. c. algirus*). We studied the V_H loci in the original species range to obtain a better understanding of the evolutionary history of this unusual polymorphism. Serological surveys revealed that a majority of sera of the subspecies *algirus*, when tested with $V_H a$-locus specific alloantisera, showed either crossreactivity (*"a-positive"* variants) or no reaction at all (*"a-blank"*). Using RT-PCR, we determined 120 sequences of rearranged V_H genes expressed in seven *algirus* rabbits that were typed as either *"a-positive"* or *"a-blank"*. The data show that the V_H genes transcribed in *"a-positive"* rabbits are closely related to one or the other of the $V_H 1$ alleles of domestic rabbits. In contrast, *"a-blank"* rabbits were found to preferentially use V_H genes that, although clearly related to the known $V_H a$ genes, define a new major allotypic lineage, designated *a4*. The *a4* sequences have hallmark rabbit $V_H a$ residues together with a number of unprecedented amino acid changes in FR2 and FR3. The net protein distances between the V_H-*a4* and the V_H-*a1*, *a2*, and *a3* lineages, was 20%, 29%, and 21% respectively. We conclude that at least four distantly related lineages of the rabbit $V_H a$-locus exist, one of which seems to be endemic in the Iberian range.

Introduction

In rabbit, the gene locus controlling the variable region of the Ig heavy chain (*IGVH, or* V_H) is unusual by showing extensive allelic diversity. Initially, this was discovered by serological methods, which revealed that V_H regions of nearly all rabbit antibodies can be placed, on the basis of the allotypic motifs, into one of three groups, namely, *a*, *x*, or *y* (Kim and Dray, 1972, 1973; Roux *et al.*, 1981). Three "*a*" allotypic lineages have been described, and their Mendelian inheritance was established by extensive breeding studies (Oudin, 1960; Dray *et al.*, 1963; Kim and Dray, 1972). These so called *a1, a2*, and *a3* allotypes have characteristic amino acid sequence differences in FR1 (Kabat positions 5, 8, 10, 12, 13, 16, and 17) and FR3 (positions 65, 67, 70, 71, 74, 84 and 85; Mage *et al.*, 1984). These differences reflect allelic variation at the *D*-proximal V_H gene, V_H1, which accounts for approximately 80% of *VDJ* gene rearrangements (Knight and Becker, 1990). Unlike for other mammalian species, where V_H1, V_H2, V_H3, *etc.* refer to gene "families" with pronounced sequence similarity, in rabbit, V_H gene segments are numbered according to their relative position on the chromosome of a given allotype (Note: for convenience "V_H gene segments" will be written below "V_H genes"). Thus, V_H1-*a1* is the 3'-most V_H gene of the *a1* haplotype; V_H7-*a2* is immediately 5' of the V_H6-*a2* gene of the *a2* haplotype, *etc.* Only a small number of the more than 100 rabbit germline V_H genes have been sequenced (Gallarda *et al.*, 1985). Besides V_H1, other V_H genes have been identified that encode V_H1-associated "allotypic" motifs (including V_H4-*a1*, φV_H2-*a1*, φV_H3-*a1*, V_H4-*a2*, φV_H3-*a2*, V_H7-*a2*, V_H9-*a2*). They contribute to *VDJ* gene diversification by gene conversion (Becker and Knight, 1990) and evolved to some extent in concert with the neighboring V_H1 gene, defining therefore a haplotype polymorphism. The group of these genes will be indicated as "V_Ha" or "*a-positive*", to be distinguished from V_Hn genes, which do not encode V_H1-associated allotypic markers (V_Hx, V_Hy and V_Hz, or "*a*-negative"; Kelus and Weiss, 1986). The latter genes are situated more than 50 Kb upstream of the V_Ha genes and contribute to less than 20% of rabbit antibodies (Di Pietro *et al.*, 1990; Short *et al.*, 1991). In this study, the concept of "V_Ha-genes", which depends on serology, will be extended to "V_H genes showing significant monophyletic clustering with one or more known V_Ha-genes in phylogenetic inference analyses based upon DNA or protein sequences". Newly described V_H genes sharing derived character states with the V_Ha genes can then be classified as such, independent of serology. V_Ha genes of the *aj* haplotype will be designated as V_Ha-*aj*, where *j* = 1, 2, 3, 4, etc.

Rabbit Ig allotypes are among the first protein polymorphisms ever described (Kelus and Gell, 1967), but after 50 years of research, we still do not understand its *raison d'être*. The problem posed by the Mendelian inheritance of genetic markers at a multi-gene locus was resolved by the finding that rabbit preferentially uses the V_H1 gene (Knight and Becker, 1990), and further, by the mechanism of concerted evolution (Smith, 1976). However, neither preferential expression nor concerted evolution can explain how, or why, such large differences between allelic lineages have evolved (concerted evolution being a mechanism which tends to homogenise the gene pool). Large differences between alleles are believed to be the outcome of diversity enhancement selection, which can favor both prolonged allele persistence times and increased evolutionary rates. According to an evolutionary analysis by Su and Nei (1999), the large genetic distances between the three V_H1 alleles suggest allele persistence times of about 50My, which is an order of magnitude larger than average mammalian speciation times. Both prolonged persistence times and increased evolutionary rates imply that this population diversity must fulfill some crucial function (cf. Raman and Knight, 1992; Pospisil and Mage, 1998; Mage and Pospisil, 2000). However, the former hypothesis has specific and profound population genetic implications in regard to founder population sizes, which must always be large enough to contain each of the different alleles. In view of the importance of the questions raised by the rabbit IgH *a*-locus polymorphism for half a century, it might be surprising that data on the rabbit V_H genes remain fragmentary and concern only a small number of domestic breeds. These breeds are recent genetic isolates (<200 years) of the subspecies *O. c. cuniculus*, while the genus originated some 4-6 My ago on the Iberian Peninsula (Lopez-Martinez, 1989; Callou, 1995). Today, this area is inhabited by two subspecies, *O. c. cuniculus* and *O. c. algirus*, which, according to mitochondrial DNA data, could be separated by some 2 My (for details see Branco *et al.*, 2000, 2002).

Serological studies of Cazenave *et al.*, (1987) had already indicated that wild rabbits from the Iberian Peninsula can express V_Ha allotypes that differ from those occurring in domestic breeds. A large number of alleles would not be supportive of extremely long allele persistence times, because it is unlikely that many allelic lineages would be maintained throughout numerous speciation steps. However, the serological characterization suggested that the "wild-type" allotypes could represent more recent variations occurring within one of the 3 lineages of domestic rabbits (for convenience referred to as "domestic" allotypes). This was also supported by partial amino acid sequence of one of these allotypes (*a1*00), which showed a close relationship with the *a3* lineage (i.e. 4 a. a. differences in the FR

regions; Tonelle *et al.*, 1983). When we analysed V_H allotypes in wild rabbits from the Iberian Peninsula, a large fraction of samples from the Southwestern areas did not show any cross-reactivity with V_Ha-specific allo-antisera. As illustrated in Figure 1, this phenotype was correlated with genetic markers characteristic of the subspecies *O. c. algirus*, and in particular to the mtDNA-type *A* (Hardy *et al.*, 1995; Monnerot *et al.*, 1994). The cytonuclear disequilibrium between the cytotype *A* and the *"a-blank"* allotype (nucleotype), when estimated according to Asmussen and Arnold (1991), was highly significant ($X^2_1 = 36$; data not shown). Among the possible explanations were (1) these rabbits preferentially use V_Hx or V_Hy genes, possibly because the *D*-proximal V_H genes were reshuffled, damaged or deleted (*cf.* Kelus and Weiss, 1986; Knight and Becker, 1990) or (2) they express a V_H1 allotype that differs markedly from those occurring in domestic breeds.

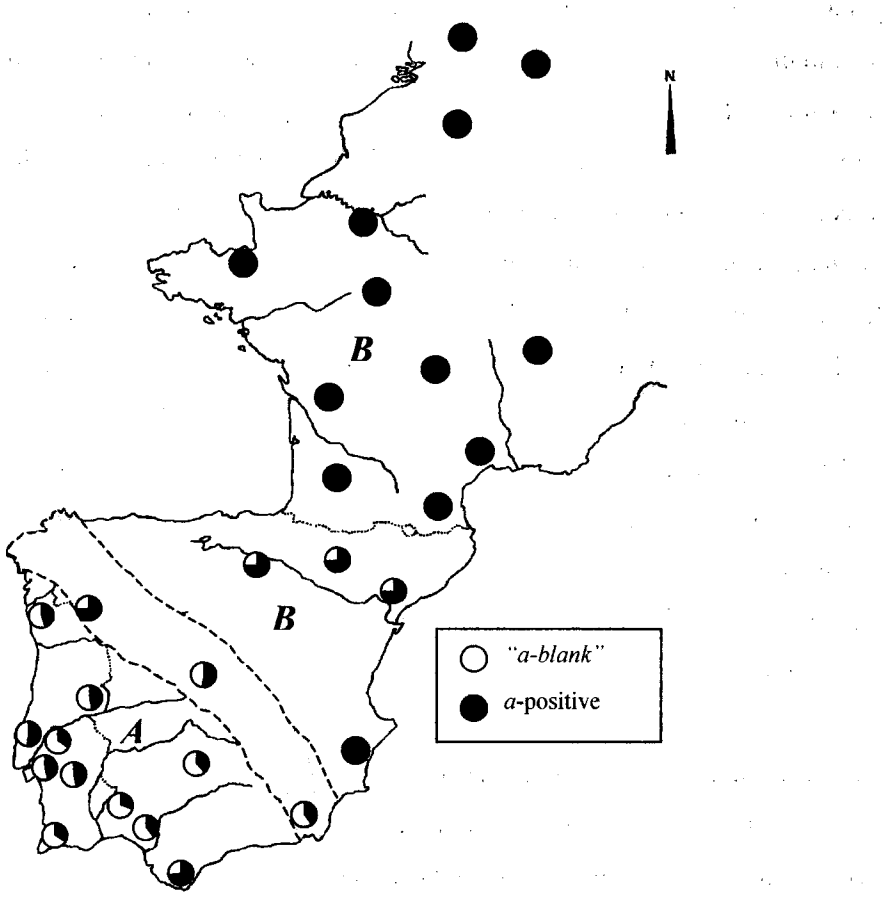


Figure 1 - Geographic distribution of V_Ha locus allotype frequencies determined for populations of European wild rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula, France, and Belgium. The subspecies *O. c. algirus* occupies the Southwestern part of Iberia, which is indicated by "*A*". Rabbits of the Northeastern 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 grey. The colouring of the disks reflects the relative allele frequencies per locality, analysed as *a*-allele locus with *"a-blank"* (white) and "*a*-positive" (black colouring) alleles.

We have sequenced the V_H genes expressed in eight wild specimens of the subspecies *O. c. algirus*, four of which were typed *"a-blank"*. The data show that these latter rabbits preferentially express V_H genes, which define a fourth, major lineage of V_H1 allotypes (hypothesis 2). The lack of cross-reactivity with allotype-specific alloantisera can be explained by the observed amino acid changes. At the same time, the sequences obtained from *"a-positive" O. c. algirus* rabbits suggest that variations have also occurred within the major V_H1 lineages.

Material and Methods

PCR amplification, cloning and nucleotide sequencing of rearranged VDJ genes

Spleen and serum samples were obtained from adult wild rabbits (*O. c. algirus)* that were collected within the military domain of Alcochete (Ribatejo, Portugal). Serum samples were analysed serologically by double immunodiffusion to determine a1, a2 and a3 *VH* allotypes. Total RNA was isolated from frozen spleen samples with TRIzol, following the manufacturer's instructions (GibcoBRL, Grand Island, NY). First strand cDNA was synthesized from 3μg of RNA using oligo(dT) as a primer (Krug and Berger, 1987). *VDJ* gene rearrangements were PCR-amplified using primers specific for conserved regions in the V_H leader and in the J_H gene segments (Zhu *et al.*, 1999). PCR amplifications proceeded for 30 cycles (each cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s). PCR products with approximately 500 bp in length were gel-purified and cloned into the pGEM-TEasy vector (Promega, Madison, WI). Nucleotide sequences of the cloned *VDJ* genes were determined using an automated ABI Prism 310 sequencer with Big Dye-labelled terminators (Perkin-Elmer Applied Biosystems, Foster City, CA).

Phylogenetic studies

The V_H gene nucleotide sequences were aligned using the computer program pileup (GCG package, Wilkinson, as provided by the Belgian EMBnet Node (BEN; *http://www.be.embnet.be*) and Clustal W (Thompson *et al.*, 1994) as provided by the freeware DAMBE package (Xia and Xie, 2001; *http://aix1.uottawa.ca/~xxia*). The alignments were corrected upon visual inspection using Genedoc (Nicholas and Nicholas 1997, *http://www.psc.edu/biomed/genedoc*). Phylogenetic analyses were performed using the MEGA 2.1 computer program (Kumar *et al.*, 2001; *http://www.megasoftware.net*).

Evolutionary distances between nucleotide or protein sequences were estimated using uncorrected p-distance. For nucleotide sequences, we also show results using Kimura's 2-parameter option. We prefer the former approach because it makes a minimal number of assumptions, and according to Nei and Kumar (2000), it gives better results when large numbers of relatively short sequences are compared. Phylogenetic trees were constructed using the neighbour-joining (NJ) method (Saitou and Nei, 1987). For the construction of the phylograms we included a sample of published diversified rabbit V_H gene sequences representing the three domestic allotypes (Accession numbers AF264476, AF264478-81, AF264493, AF264496, AF264499, AF264528, AF264544, AF264545, AF264546, AF264571-73, AF264577, AF264583, AF264585, AF264587, AF264589, AF264592, AF264595-96). The reliability of the phylogenetic tree was tested by determining the Bootstrap values (BP; Felsenstein, 1985) and Confidence Probability (CP; Rhezetsky and Nei, 1992; Sitnikova, 1996) values. For sequence pairs under comparison, alignment gaps were excluded from analysis (pairwise deletion option in MEGA). The molecular clock was tested by the relative rate test (Tajima, 1993), as provided in the MEGA package.

Identification of novel genetic variants

For discerning the germline V_H genes from groups of diversified V_H sequences, the "*algirus*" sequences were aligned with all known rabbit V_H germline genes. The "*algirus*" sequences often show some systematic variation that differentiates them from sequences obtained from domestic rabbits. For the vast majority of sequences of *"a-blank"* rabbits, this "unprecedented" variation clearly indicates novel variants of V_H genes in *algirus* rabbits. This is less obvious for some sequences of "a-positive" rabbits, where recurrent variation could either indicate "novel" genetic variants of the "domestic" allotypes, or the consistent use, for gene conversion events, of donor V_H genes present in both *algirus* and domestic rabbits. The latter possibility was evaluated by searching the Genbank database for rabbit sequences showing the same unexpected amino acid replacements. This was tested by submitting such variant sequences, or segments of them, to protein BLAST searches using the software provided by NCBI (*http://www.ncbi.nlm.nih.gov/blast*). The alignments for the 1000 most similar rabbit V_H gene sequences were edited under the "flat query-anchored with identities" option, and examined visually.

Results

Serological typing and sequence determination

cDNA was obtained from spleen and peripheral blood cells of eight adult wild rabbits: Rb07, Rb11, Rb27, Rb32, Rb36, Rb40, Rb43 and Rb60. According to their mtDNA (Monnerot *et al.*, 1994), as well as to other genetic markers (protein and microsatellite DNA; Branco, 2000; Queney *et al.*, 2001), these rabbits belong to the subspecies *O. cuniculus algirus* (data not shown). When tested with conventional allo-antisera, Rb43 typed "*a1/a2*" heterozygous, Rb07 and Rb36 "*a1* only" and Rb11 "*a3* only". Sera of Rb32 showed weak cross-reaction with *a3*-specific antisera (*a3*-variant 32). Sera of rabbits Rb27, Rb40 and Rb60 did not show any cross-reactivity (*"a-blank"*). Because of the prevalence of *"a-blanks"* in the study area (>20%), the *"a-positive"* rabbits (Rb07, Rb36, Rb11, Rb32) have a reasonable chance of being heterozygous for the *"a-blank"* genotype.

A total of 120 V_H sequences were obtained, of which 111 sequences are deposited in Genbank under accession numbers AY207941 to AY208048 and AY299401 to AY299403. Except AY208000 and AY208009, all submitted sequences encode unique amino acid sequences. Forty-four of them were expressed in rabbits typed as homozygous *"a-blank"*, while eleven more were obtained from a rabbit that appeared to be heterozygous for the *"a-blank"* allotype (Rb07). Table 1 lists, for each of the 8 serotypes, the number of sequences leading to unique amino acid sequences (110 sequences, of which 52 were classified *"a-blank"*, 55 *"a-positive"*, and 3 *"a-negative"*). Unless stated otherwise, we present and discuss the results at the level of the protein sequence.

Table 1
Number and allotypic classification of *VDJ* sequences obtained from 8 rabbits

Rabbit	Serotype	putative allotype		number of sequences [a]			
				V_Ha		V_Hx	V_Hy
		G1	G2	G1	G2		
Rb07	*a1 only*	*a1*	*a41*	1	9	1	0
Rb11	*a3 only*	*a3*	*a3v11*	5	4	0	0
Rb27	*"a-blank"*	*a41*	*a42*	10	4	0	0
Rb32	*a3v*	*a3v32*	*a3v32*	18-n	n	0	0
Rb36	*a1 only*	*a1v36*	*a1v36*	8-n	n	0	0
Rb40	*"a-blank"*	*a41*	*a42*	8	7	0	0
Rb43	*a1/a2*	*a1v36*	*a2*	3	16	0	1
Rb60	*"a-blank"*	*a42*	*a42*	14-n	n	1	0

[a] underlying unique aa. sequences

Inferring V_H genes expressed in O. c. algirus

The V_H regions of rearranged *VDJ* genes in adults are likely somatically diversified versions of the utilized genomic V_H genes. Sequence comparisons can help to infer the likely

sequences of these germline genes, as well as identify genes contributing to the V_H gene diversification by gene conversion.

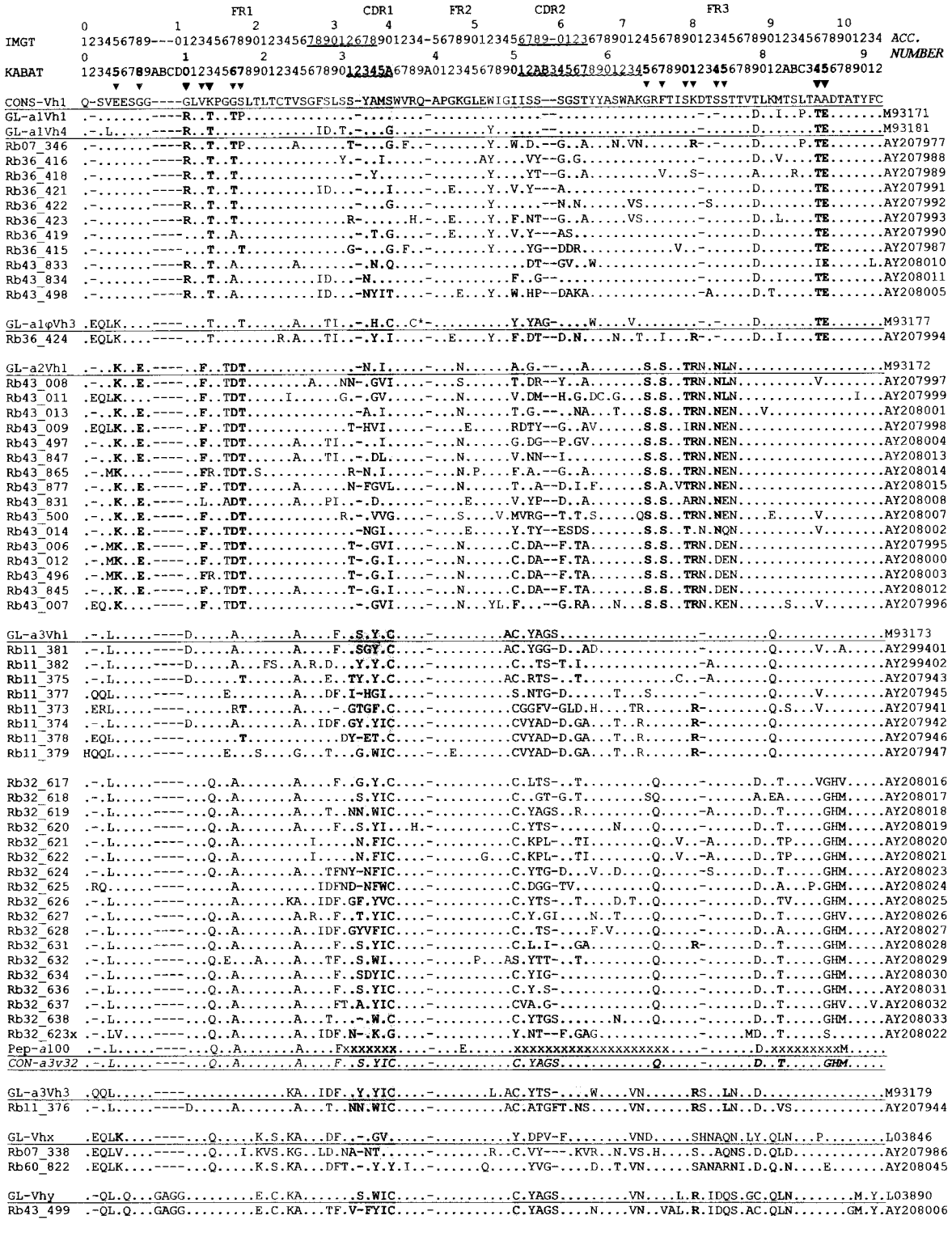
The "*O. c. algirus*" sequences were therefore aligned with all published rabbit genomic V_H sequences. Rabbit V_H sequences belong to the class C (Tutter and Riblet, 1989; Ota and Nei, 1994). For this reason we used, as outgroups in phylogenetic analysis, vertebrate V_H sequences that are separated by a relatively short branch from the root of class C sequences (Human V_H3, Camel V_H, and Duck V_H). Figure 2 displays the unique amino acid sequences inferred from the nucleotide data, grouped according to highest similarity to known rabbit germline genes. They are compared to a consensus sequence of rabbit V_Ha genes, in order to highlight the differences among the V_Ha allotypes. Residues are numbered according to IMGT numberings (*http://imgt.cines.fr*; Lefranc, 2001) or to Kabat *et al.*, (1991), the latter numbering being used throughout the text below. For groups of V_H sequences that show systematic differences from known genes (which is the case for the majority of sequences obtained from *"a-blank"* rabbits), consensus sequences are proposed. These likely represent the underlying germline sequence. The possibility that differences might be due to gene conversion with V_H genes also present in domestic breeds was examined by blast searches outlined in the Material and Methods section.

V_H genes used by "*a*-positive" *algirus* rabbits

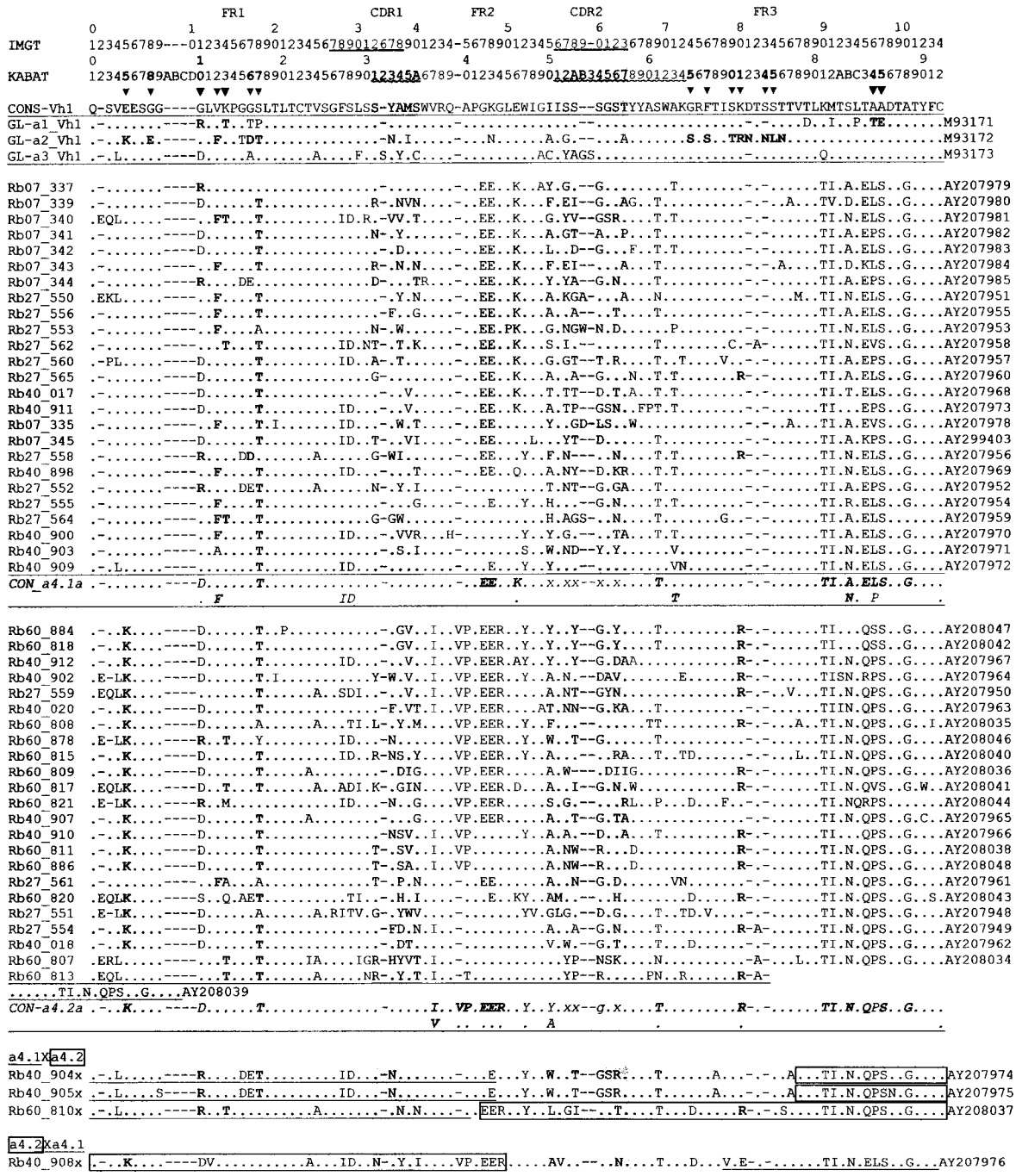
V_Ha-a1 genes expressed by Rb07, Rb36 and Rb43:

Rb07 appears heterozygous for the a1 allele and another allele that is also expressed in *"a-blank"* rabbits (see below). Whereas the *a1* sequence of Rb07 conforms to a "domestic" *V_H1-a1* gene, those expressed by Rb36 and Rb43 consistently show at positions 17, 74, 82, and 82C the residues that distinguish *V_H4-a1* from *V_H1-a1*. The second deletion in FR3 (position 74), which is characteristic of *V_H1-a1* and absent in *V_H4-a1*, is also absent in all *V_Ha-a1* sequences of these two rabbits, except for sequence Rb36-424. The latter sequence, however, shows more similarity with the pseudogene *V_H3-a1*, and is likely a product of gene conversion (Fig. 2A). One sequence from Rb07 (Rb07_332; Fig. 2A) is clearly derived from a *V_Hx*-like gene, while one sequence from Rb43 (Rb_499) is clearly derived from a *V_Hy*-like gene.

A



B


FR1 CDR1 FR2 CDR2 FR3
IMGT
KABAT
CONS-Vh1 Q-SVEESGG----GLVKPGGSLTLTCTVSGFSLSS-YAMSWVRQ-APGKGLEWIGIISS--SGSTYYASWAKGRFTISKDTSSTTVTLKMTSLTAADTATYFC
GL-a1_Vh1 M93171
GL-a2_Vh1 M93172
GL-a3_Vh1 M93173
Rb07_337 AY207979
Rb07_339 AY207980
Rb07_340 AY207981
Rb07_341 AY207982
Rb07_342 AY207983
Rb07_343 AY207984
Rb07_344 AY207985
Rb27_550 AY207951
Rb27_556 AY207955
Rb27_553 AY207953
Rb27_562 AY207958
Rb27_560 AY207957
Rb27_565 AY207960
Rb40_017 AY207968
Rb40_911 AY207973
Rb07_335 AY207978
Rb07_345 AY299403
Rb27_558 AY207956
Rb40_898 AY207969
Rb27_552 AY207952
Rb27_555 AY207954
Rb27_564 AY207959
Rb40_900 AY207970
Rb40_903 AY207971
Rb40_909 AY207972
CON_a4.1a
Rb60_884 AY208047
Rb60_818 AY208042
Rb40_912 AY207967
Rb40_902 AY207964
Rb27_559 AY207950
Rb40_020 AY207963
Rb60_808 AY208035
Rb60_878 AY208046
Rb60_815 AY208040
Rb60_809 AY208036
Rb60_817 AY208041
Rb60_821 AY208044
Rb40_907 AY207965
Rb40_910 AY207966
Rb60_811 AY208038
Rb60_886 AY208048
Rb27_561 AY207961
Rb60_820 AY208043
Rb27_551 AY207948
Rb27_554 AY207949
Rb40_018 AY207962
Rb60_807 AY208034
Rb60_813
TI.N.QPS..G....AY208039
CON-a4.2a
a4.1Xa4.2
Rb40_904x AY207974
Rb40_905x AY207975
Rb60_810x AY208037
a4.2Xa4.1
Rb40_908x AY207976

C

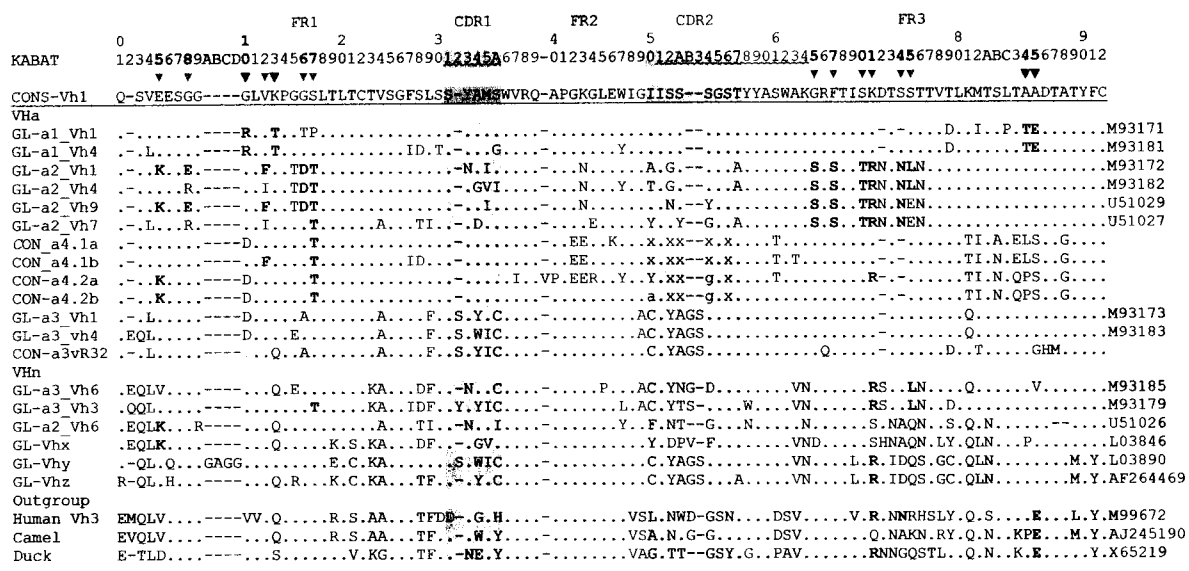


Figure 2 - *Alignment of unique protein sequences of V_H regions.* V_H regions shown are inferred from *VDJ* genes obtained from 8 wild Iberian rabbits and of published genomic rabbit V_H genes. Sequence names of the former refer to the donor rabbits (Rb07, Rb11, Rb27, Rb32, Rb36, Rb40, Rb43 and Rb60). They are compared to a consensus sequence of rabbit V_H1 gene segment (CONS-VH1) and grouped according to phylogenetic relationships, shown in Figure 3. Allotypic marker positions are indicated by▼ and for the *a1* and *a2* allotypes, respectively, and corresponding matches are highlighted in bold. Genbank accession numbers of underlying nucleotide sequences are included. Each *VDJ* gene cluster is compared to germline V_H gene(s) (marked GL-) of that cluster. For *VDJ* groups showing recurrent differences with known genomic genes, consensus sequences are proposed (indicated by CON-). Potential gene conversion tracts are enclosed in dashed boxes. Sequence names of suspected crossing-over products are marked by "x" (*a4.1* sequences are underlined and *a4.2* sequences are enclosed in boxes). Hypervariable parts of CDR's are shaded. **A:** Sequences showing significant clustering with one or more known genomic sequences. **B:** Sequences clustering apart from known genomic sequences. **C:** Comparison of inferred consensus sequences with relevant genomic sequences of rabbit and with class-C V_H sequences used as outgroups in phylogenetic trees presented in Figures 3 and 4.

V_Ha-a2 genes expressed by Rb43:

The majority (14/16) of *a2*-like sequences from Rb43 encodes a glutamic acid residue at position 75 (E75; Fig. 2A), where *V_H1-a2* encodes a leucine residue (L75). E75 has also been observed in *VDJ* gene sequences from domestic *a2* rabbits, although at much lower frequencies. As two tandem nucleotide are involved (GAG vs. CTG), this variation is probably not due to somatic hypermutation, but more likely due to somatic gene conversion involving E75-encoding donor genes, such as *V_H7-a2* or *V_H9-a2* (cf. Sehgal *et al.*, 1998). However, the high frequency of occurrence suggests that the V_H1-a2 gene of Rb43 could represent an E75-encoding *V_H1-a2* variant allotype. In that case, the L75 codons of the two remaining Rb43 sequences (Rb43_008 and Rb43_011; Fig. 2A) may represent gene conversion events involving L75-encoding donor genes, such as *V_H4-a2*.

V_Ha-a3 genes expressed in Rb11:

The amino acid variation among Rb11 sequences is similar to that of domestic *a3* rabbits (Fig. 2A). However, 4 sequences (Rb11_373, _374, _378 and _379) are atypical in containing arginine (R) residues at positions 64 and 71, where virtually all published *a3* sequences (>99%) contain lysine (K). The sequence of Rb11_376 between positions 63 and 78 could be the product of gene conversion between *V_H1-a3* and a gene homologous, or identical, to domestic rabbit pseudogene *V_H3-a3*.

V_Ha-a3 variant genes expressed in Rb32 (V_H1-a3v32):

Serological analysis shows that rabbit Rb32 expresses some, but not all, of the *a3* determinants. The Rb32 sequences differ from those of the *V_H1-a3* allotype at nine FR positions: two in FR1 (D10, Q13), and seven in FR3 (Q66, D79, K81, T82, G85, H86, M87). The amino acid differences at positions 10 (D/G) and 13 (Q/P) of FR1 have also been observed in domestic *a3* rabbits, although much less frequently, possibly due to gene conversion with *V_H6-a3* or a similar donor gene. In contrast, the substitutions in FR3 were never, or extremely rarely, observed in domestic rabbits. Among the 1000 rabbit *VH* sequences retrieved by a Protein Blast search using the FR3 region of Rb32_634 as query sequence (see M&M), none showed either the residues Q66 or H86 that are present in all but two of the R32 sequences. Residues T82, G85 and M87 each occurred at frequencies below 0.5%. D79 and K81 are frequently observed among *a1* and *a2* sequences, but not among *a3* sequences. Rb32 undoubtedly expresses a genetic variant of the *a3* lineage, which

we call "*a3v32*". Interestingly, residues G10, Q13, D79 and M87 have previously been reported in the partial amino acid sequence obtained by Tonelle *et al.*, (1983) from a wild rabbit typed "*a100*" (Fig. 2A). It is possible that *a100* and *a3v32* are different names for the same allotype.

V_H genes used by *"a-blank" algirus* rabbits define a fourth V_Ha allotypic lineage: V_Ha *-a4*

V_H genes expressed in "a-blank" rabbits belong to the V_Ha group

Except for one single sequence (Rb60-822), which is clearly a product of an "*a-negative*" V_Hx-like gene, all of V_H genes expressed in the *"a-blank"* rabbits (Rb27, Rb40, Rb60) appear to be derived from very similar germline genes. Visual inspection of the inferred protein sequences (Fig. 2B) shows that they are clearly more related to the V_Ha than to V_Hx or V_Hy genes. This conclusion is supported by a variety of phylogenetic inference programs conducted at both the nucleotide and protein levels and is independent of the CDR regions.

For the construction of the phylogram shown in Fig 3, we included a random sample of published diversified V_H sequences of domestic rabbit (see Material and Methods). The *"a-blank"* sequences form a monophyletic cluster with the V_H1-*a* genes, which clearly branches apart from the V_Hn genes (V_Hx, V_Hy and V_Hz). At the protein level, the *"a-blank"* sequences share a number of apomorphic (or "derived") characters with one or more of the "domestic" V_Ha genes. Examples are the V_Ha hallmark peptides (16)LTLTCT(23) of FR1, and (61)WAK(65) of FR3. These are unique to rabbit V_Ha genes and do not occur in the V_Hx, V_Hy or V_Hz sequences. The *"a-blank"* type shares a larger number of derived characters with the *a1* and *a2* allotypes than with the *a3* allotype. The two deletions between positions 72 and 74, which are characteristic of the V_H1-*a1* allotype, are also present in *each* of the 49 *"a-blank"* sequences, where we observe either (71)K-T-ST(76) (as in V_H1-*a1*) or (71)<u>R</u>-T-ST(76), confirming a close affiliation with the rabbit V_H1 genes.

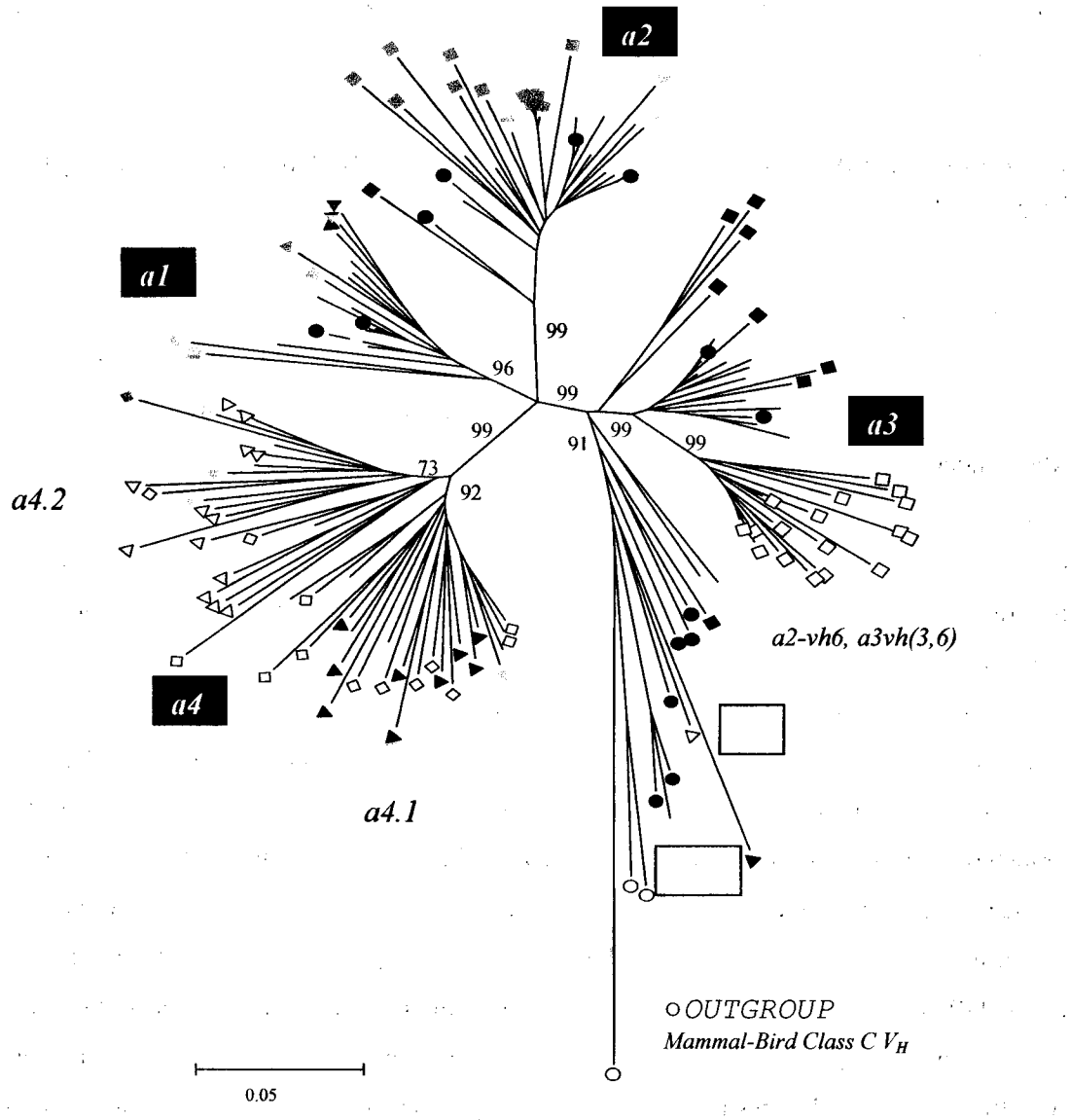


Figure 3 - *Phylogenetic neighbour-joining tree of rabbit V_H regions.* Based upon nucleotide p-distances. Germline genes are labelled by dots and comprise •: V_Ha genes (*a1-vh(1,4), a2-vh(1,4,7,9),* and *a3-vh(1,4)*); •: V_Hn genes (*x, y, z*); V_Ha pseudogenes (*a1-vh(2,3)*); ○: outgroup (*Hum V_H3, Camel V_H, Duck V_H*). Squares, triangles and diamonds indicate V_H regions of RT-PCR-amplified *VDJ* genes obtained from wild rabbits of subspecies *O. c. algirus*: ▲: Rb07; ■: Rb11;□: Rb27; □: Rb32; ▽: Rb36; : Rb40; : Rb43; □: Rb60. *VDJ* gene sequences representing domestic rabbits of the *a1, a2* and *a3* allotypes are not labelled (Accession numbers are displayed in M&M). Sequences reflecting possible crossing-over (marked by "x" in Fig. 2) were excluded from the analysis. CP values (1000 replicates) are shown at branch nodes if larger than 85, except for the *a4.2* clade. The tree illustrates the fact that the vast majority of *VDJ* genes expressed in rabbits Rb07, Rb27, Rb40 and Rb60 form a well-defined cluster that is monophyletic and equidistant from the clades formed by the V_Ha genes expressed in domestic rabbits of the *a1* and *a2* allotypes.

V_H "a-blank" defines a new V_H1 allele

The *"a-blank"* V_Ha cluster is well separated from the three clusters defined by the sequences expressed in *a1*, *a2* and *a3* rabbits, respectively. BP and CP values are highest when *"a-blank"* sequences, or their consensus sequence, are solely compared to genomic V_H genes (Fig. 4). Visual inspection of the protein sequences (Fig.2B) reveals that the *"a-blank"* sequences, although clearly belonging to the V_Ha genes, have unique features in common that set them apart from the *a1*, *a2* and *a3* alleles. The most consistent marker residues are found in FR3, where, for *each* of 49 *"a-blank"* V_Ha sequences, the V_Ha consensus residue K/Q(81) is replaced by T(81). The V_Ha consensus peptide (82C)TAA(85), which is TTE in the *a1* allotype, is replaced by ELS, or by QPS in most *"a-blank"* sequences.

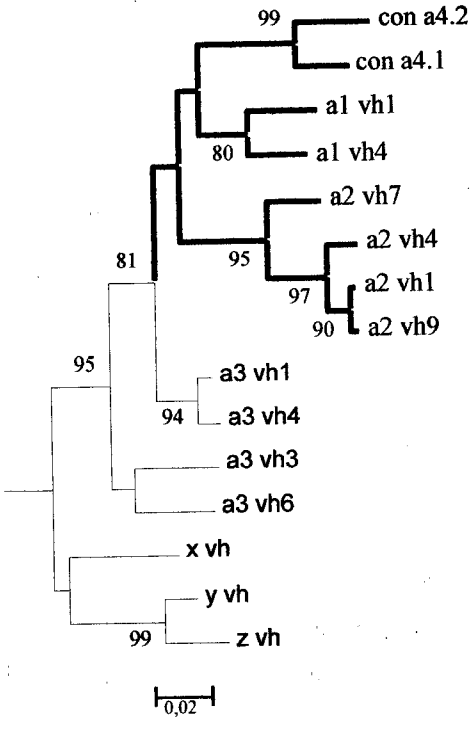


Figure 4 - *Phylogenetic neighbour-joining tree of rabbit V_H regions.* Based upon amino acid p-distances. The consensus sequences of the *a4* allotype subtypes *a4.1* and *a4.2* were inferred from the data presented in Figure 2B and compared to inferred amino acid sequences of published genomic rabbit V_H genes. BP values were obtained with 1000 bootstrap replicates.

While FR2 is highly conserved among vertebrate class-C V_H genes (including the rabbit V_H1 genes), the *"a-blank"* sequences present some unprecedented changes. In the majority of them, the canonical (41)GK/NG(45) is replaced by either EEG or EER. Quite remarkable is the change of A(40) into VP(40), due to a 3 bp insertion before the second

codon position of *A40(gct->g[tg c]ct)*. This was observed in 17 sequences, obtained with 3 different rabbits. Assuming that, like domestic rabbits, *"a-blank"* rabbits preferentially use the *D*-proximal V_H gene, we propose that the majority of the sequences obtained from *"a-blank"* rabbits are derived from a genuine allele of the V_H1 gene, which we call V_H1-*a4*. The phylograms in Figures 3 and 4 compare the "*a4*" sequences with the known functional genomic V_H sequences of rabbits. The pairwise distances between the *a4* sequence and the *a1*, *a2*, or *a3* sequences were similar to those between the latter three ("domestic") allotypes (Table 2), confirming that the *a4* lineage cannot be considered a subtype of one of the known allotypic lineages.

Table 2

Estimation of genetic distances between major V_Ha allotypic lineages.

A	B	Net distances between VDJ groups [a]				Pairwise distances between V_H genes [b]			
		a. a. pD[c]	s.e.[d]	nuc K2[e]	s.e.[d]	a. a. pD[c]	s.e.[d]	nuc K2[e]	s.e.[d]
a4.1	-a1	0.137	*0.030*	0.094	*0.018*	0.203	*0.045*	0.124	*0.024*
a4.2	-*a1*	0.158	*0.032*	0.103	*0.019*	0.241	*0.048*	0.129	*0.025*
a4.1	-*a2*	0.227	*0.042*	0.146	*0.024*	0.291	*0.051*	0.171	*0.030*
a4.2	-*a2*	0.226	*0.041*	0.143	*0.024*	0.278	*0.050*	0.154	*0.028*
a1	-*a2*	0.216	*0.041*	0.131	*0.023*	0.253	*0.049*	0.134	*0.026*
a4.1	-*a3*	0.141	*0.033*	0.085	*0.016*	0.215	*0.046*	0.114	*0.023*
a4.2	-*a3*	0.148	*0.033*	0.087	*0.017*	0.253	*0.049*	0.119	*0.024*
a1	-*a3*	0.108	*0.029*	0.061	*0.015*	0.177	*0.043*	0.080	*0.019*
a2	-*a3*	0.187	*0.038*	0.114	*0.021*	0.250	*0.048*	0.132	*0.025*

* Calculated using the corresponding option of the MEGA2 package

[a] Net distances were determined for the FR regions between allotypic clusters of *VDJ* sequences formed by the sequences as presented in the tree in Fig. 3 (including *VDJ* sequences of domestic rabbits); they are main distances between groups corrected for variation within groups

[b] Pairwise distances between genomic V_H sequences and between consensus sequences, as presented in the tree in Fig. 4

[c] amino acid p-distances; 93 aa

[d] standard error

[e] Kimura's 2 parameter nucleotide distances; 279 bp.

Allelic diversity among V_H1-a4 sequences?

The degree of diversity among *a4* sequences tended to be larger than currently observed among *a-positive* sequences of the same allotype in domestic rabbits (data not shown). This could have to do with the fact that our sample of *a4* sequences was obtained from 7 different haplotypes (1 heterozygous and 3 homozygous *a4* rabbits), *i.e.* cryptic allelic variation could account for some of this diversity. Phylogenetic clustering methods and visual inspection of the amino acid alignments indeed indicate that there might be at least two *a4* variants, *a4.1* and *a4.2*. The consensus sequences of these two variants are presented in Fig. 2B. Rb60 appears to be homozygous for one variant (*a4.1*). Rabbit Rb07, which is heterozygous *a1/a4*, appears to possess the *a4.2* variant, whereas Rb27 and Rb40 appear to be heterozygous *a4.1/a4.2*. The phylogram in Fig. 4 highlights the position of the *a4*.1 and *a4*.2 consensus protein sequences among known genomic V_H genes.

Gene conversion

There are strong indications that the putative *a4* variants are diversified by gene conversion events involving V_H donor genes that, in some cases, might be present in both lineages. At a number of positions, amino acid replacements requiring two or more nucleotide substitutions occur more often than expected if due to somatic hypermutation (examples are D/G10, 27ID/FS28, W/Y47, 71AK/VN72, N/A82b). Inspection of the sequence alignments indicates that in some cases variation could possibly be due to PCR crossing-over. Indeed, sequences Rb40_905, Rb40_901, and Rb27_561 show characteristics of *a4.1* and *a4.2* sequences in the 5' and 3' regions, respectively, while the opposite is true for Rb40_908 (Fig. 2). These sequences were not included in the phylogenetic analysis shown in Fig. 3. As these sequences were obtained from rabbits that express both subtypes, PCR crossing-over and/or gene conversion could be an additional source of variation. However, crossing-over was not observed between the *a1* and *a4* genes of Rb07 or between the *a1* and *a2* genes of Rb43. The presence or absence of the valine (position 39A) insertion in FR2 among the *a4.1* sequences indicates that more than one V_H gene contributes to this particular allotype. The sequence of Rb60_810 between position 1 and 17 could be a product of gene conversion of the putative a4.2 gene and a gene resembling the *vh4-a1* gene of domestic rabbit. We conclude that the V_Ha-*a4* sequences presented here are likely diversified by gene conversion. In Fig. 2B we have indicated frequent alternates of the consensus sequences.

Gene usage

In sharp contrast with our first hypothesis, the *"a-blank"* rabbits do not preferentially use V_Hn genes. In fact, of the 43 sequences obtained from rabbits Rb27, Rb40 and Rb60, 42 had the V_Ha signature, while only 1 was V_Hx-like. If indeed the number of PCR clones reflects gene usage, V_Hn genes were used significantly less frequently in *"a-blank"* rabbits than was previously observed in domestic rabbits. This pronounced bias in gene usage is apparently not a characteristic linked to the *"a-blank"* genotype, because it was also observed for the "*a-positive*" rabbits of the same population (55 V_Ha sequences *vs.* zero V_Hx and one V_Hy).

Allelic imbalance

Another characteristic of the rabbit V_Ha allotypes is "allelic imbalance" in gene expression or "pecking order" (Lummus *et al.*, 1967, Mage, 1967, Akimenko *et al.*, 1986). This refers to the fact that the different V_Ha alleles are not used to the same extent in heterozygous animals. Thus, heterozygous *a1/a2* rabbits consistently use *a1* genes more often than *a2* genes. We note that with Rb43, which typed *a1/a2* heterozygous, the ratio *a1/a2* was on the contrary 3/16. This could possibly have to do with the fact that the V_H1-*a1* of this rabbit differs from that in domestic breeds (see above). For rabbit Rb07, which is heterozygous *a1/a4*, the *a1/a4* ratio was 1/7, indicating that allelic imbalance, if occurring at all, would favour the expression of the *a4* allotype in the presence of the *a1* allotype.

Absence of V_Ha serological markers

In Fig 2, the V_Ha-allotypic residues (*i.e.* residues known to be associated with serological determinants of the allotype) are highlighted. The *a4* sequences present most of the "domestic" allotypic marker residues of FR1. These marker residues however are apparently not presented in combinations required for serological cross-reactivity. For example, while the *a2* marker residues K5, F12 and T17 (FR1 region) are frequently found among *a4* molecules, they never occur together. The same is true for the *a1*-associated FR1 residues R10 and T13, except for two Rb60 sequences (Rb60_810, Rb60_878), where we would expect the phenotypic expression of an *a1* determinant. The fact that rabbit Rb60 was typed *"a-blank"* suggests that the serum level of such molecules is too low for detection or, more likely, that a majority of the antibodies of our *a1*-specific antisera are directed against determinants in the FR3 region. Indeed, none of the marker residues of the *a1, a2,*or *a3* allotypes was observed in the FR3 region of the *a4* sequences.

Discussion

Proteins encoded by allelic genes show in general not more than 1-3% a. a. differences. Evolutionary theory offers two possible explanations for interallelic distances as large as those observed at the rabbit V_Ha locus: unusually long allele persistence times and increased mutant recruitment rates. The concept of a molecular clock is a powerful tool that evolutionists avoid abandoning. The fact that allo-antisera raised in rabbit distinguish three

$V_H a$ allotypes in populations of *Lepus americanus* suggests that both species have inherited the same three lineages, which could explain the large interallelic distances without invoking acceleration of evolution rates (Su and Nei, 1999).

The main objective of this study was to determine whether the study of natural populations in the original species range could contribute to a more accurate estimate of the genetic variation at this locus. We show here that there are at least four highly divergent lineages (Fig. 2). A correct estimation of the distance between the *a4* lineage and the other $V_H a$ lineages is hampered by the fact that only diversified sequences of the putative $V_H 1$-*a4* gene are available. However, the net distances between diversified V_H sequences of the four lineages are similar (Table 2). If we assume with Su and Nei (1999) that the $V_H a$ genes of different haplotypes evolved at a similar rate as other proteins (*i.e.* 1.4×10^{-9} n/y), then not three, but four, lineages have been maintained over numerous speciation steps (40-60 My). However, the present geographical distribution of the four allotypes in the native species range shows that the *a4* allele is associated with the maternal markers of the subspecies *O. c. algirus*, and did not pass the Pyrenean Mountains (Fig 1).

A detailed evolutionary analysis of the present data is beyond the scope of this paper. It is nevertheless interesting to compare the present observations with those made in a study of gene diversity at the *IGKC1*, or C_{K1} *b*-locus in Iberian rabbit populations (van der Loo *et al.*, 1999). The C_{K1} gene, which encodes the constant region of the Ig kappa1 L chain, is used in more than 95% of L chain rearrangements. Allelic differences at this locus are even larger than those at the $V_H a$ locus and contrast sharply with the very limited variation at the quasi-silent C_{K2} *bas* locus. The *b4* and *b5* C_{K1} alleles, which predominate in all populations of *O. c. cuniculus*, were less frequent in populations of *O. c. algirus*. These populations instead express genetic variants serologically related to the *b4* and *b5* alleles but differing from them at up to 15% of the a. a. residues. It was argued that the association of specific alleles with subspecies markers is in contradiction with extremely long allele persistence times.

The present results, showing that the $V_H a$ allotypes that prevail in *O. c. cuniculus* populations, are in part "replaced" in *O. c. algirus* populations by the related *a4* allotype (Fig. 1), mirrors to some extent the situation described for the *b*-locus. In the study mentioned, the molecular clock hypothesis was rejected for the C_{K1} lineages, and the data analysis furthermore indicated that, compared to the *b9* lineage, the *b4* and *b5* lineages showed both higher allele turnover rates and higher evolutionary rates.

Our data suggest that evolutionary modes might also vary among V_Ha lineages. In Fig. 4 we show a phylogenetic tree reduced to the genomic sequences and the *a4* consensus sequences. It strongly suggests that amino acid divergence was markedly increased in the V_Ha *a1*, *a2*, and *a4* lineages in comparison to that in the *a3* and V_Hn lineages. The statistical significance of these differences was confirmed by Tajima's relative rate tests (Table 3) and the Felsenstein ML ratio test (not shown). Because of the increased evolutionary rate in the V_Ha *a1*, *a2*, and *a4* lineages, allele persistence time could be largely overestimated. A considerable fraction of the amino acid differences characterizing the *a4* allele might indeed have accumulated after the separation of the subspecies, explaining its association with subspecies *O. c. algirus*.

Table 3

Tajima's relative rate test for rate constancy among rabbit V_H genes

Seq A V_H1-	Seq B V_H1-	Outgroup	Chi-sq	P[a]	Chi-sq	P
			279 nucleotides[b]		*93 amino acids*[b]	
a1	*a3*	Duck V_H	8.07	**0.005**	6.4	**0.011**
a2	"	"	8.05	**0.005**	7.1	**0.008**
a4.1	"	"	9.00	**0.003**	7.1	**0.008**
a4.2	"	"	9.00	**0.003**	8.1	**0.005**
x	"	"	0.93	0.336	0.0	1.000
y	"	"	0.50	0.480	0.1	0.815
a1	"	Human V_H	5.40	**0.020**	4.5	0.034
a2	"	"	7.35	**0.007**	4.6	0.033
a4.1	"	"	10.71	**0.001**	5.3	0.021
a4.2	"	"	8.91	**0.003**	4.6	0.033
x	"	"	1.29	0.260	1.7	0.197
y	"	"	0.12	0.730	1.1	0.285
a1	*x*	"	6.28	**0.009**	8.0	**0.005**
a2	"	"	9.76	**0.002**	8.0	**0.005**
a4.1	"	"	11.92	**0.001**	10.9	**0.001**
a4.2	"	"	11.11	**0.001**	8.9	**0.003**
a3	"	"	1.29	0.257	1.6	0.197

* The test was performed using the MEGA2 package, applied to the published genomic sequences and the consensus sequences obtained from the *a4.1* and *a4.2 VDJ* sequences

[a] Probability of rate monotony between SeqA and SeqB lineages vs. Outgroup. The hypothesis of identical in evolutionary rates between the V_H lineages *a3*, *x*, *y*, and, *z* can not be rejected (P>0.3). In contrast, evolutionary rates are shown to be significantly differ in the *a1*, *a2* and *a4* lineages compared to that in *a3*, *x* and *y* lineages (P<0.03).

[b] The hypervariable parts of the CDR regions were excluded in the analysis.

In view of the evidence suggesting a role for superantigens in the expansion of B cell lineages that present V_Ha allotypic motifs, (Raman and Knight, 1992, Pospisil and Mage, 1998, Mage and Pospisil, 2000) and of the gut microflora in Ab repertoire diversification (Cooper, 1968, Lanning *et al.*, 2000), another explanation for the correlation between the complex *Ig* allotypes (V_Ha and $C_{K1}b$) and subspecies markers should be considered. By occupying different habitats, each subspecies might indeed acquire a different intestinal microflora, which could favour habitat-specific frequency distributions of (pre-existing) V_Ha allotypes. Thus, the four V_Ha allotypes were perhaps present in the

common ancestor species, but the expansion of the *a4* allotypes were favoured by selection only in Southwestern regions.

As already mentioned, different authors have commented on the fact that allelic variation concerns residues that are liable to interact with superantigens (Pospisil and Mage 1998). These authors suggest that a relation might exist between the high allotypic variability and the low diversity at the FR3 regions, which results from preferential gene usage. Sehgal *et al.*, (1998) and Zhu *et al.*, (1999) invoked selection factors for explaining the "reconstruction" the *a2* allotypic motives in V_H*1-a2* knockout rabbits (i.e. *Alicia* strain). All 52 *a4* sequences here obtained share highly unusual residues at H81-H85 (or H90-H97 in IMGT numbering), suggesting that this region is not affected by gene conversion, or/and, that B cells presenting these motives are preferentially stimulated to expand. X ray crystallography shows that in human V_H*3* residues at this position interact with a protein *A* domain of *Staphylococcus aureus* (H81-H82b; Graille *et al.*, 2000).

Unlike FR3, the FR2 region is not expected to interact with superantigens. In fact, the allelic variation at the FR2 of V_H*a-a4* proteins affects residues known to interact with the V_L domain, and is highly conserved among vertebrate V_H genes. The replacements of glycine residues at positions H42 and H44 by charged residues are surprising. It is noteworthy that in the camel and lama, amino acid replacements in this molecular region were observed for the V_H*H* domains of the heavy chain antibodies (incuding a G/E exchange at H44), and have been associated with the loss of L chains (Nguyen *et al.*, 2002).

In conclusion, the presented data show that the study of wild specimens of the original species range can contribute significantly to our understanding of the evolution and biological meaning of the rabbit immunoglobulin polymorphisms.

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Genetic diversity in *Lepus* species

In order to evaluate the possibility of a trans-specific polymorphism of locus *a* in lagomorphs, we performed a serological analysis of several *Lepus* populations, using allo-antisera against rabbit *a1*, *a2* and *a3* allotypes.

Population distribution

The gene frequencies obtained are presented in Table 5. The frequency of *"a-blank"* allotype was calculated as for the rabbit populations. In the three different *Lepus* species studied only two phenotypes were observed, *a2v* and *"a-blank"*. This result differs from that obtained with *Lepus americanus*, where cross-reaction with rabbit anti- *a1*, *-a2* and *-a3* antisera and the absence of phenotype *blank* were observed (De Poorter, 1984).

Table 3.5

Gene frequencies obtained in populations of *Lepus granatensis*, *L. europaeus* and *L. capensis* at locus *a*. Serotype codes are shown in brackets.

	n	*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 unwheighted	120	----	----	----	----	*0.29*	----	*0.71*
Lepus europaeus								
Lass (France)	20	----	----	----	----	0.5	----	0.5
Lepus capensis								
Tetouan (Morocco)	7	----	----	----	----	0.47	----	0.53

Article 4

P.J. Esteves, D. Lanning, S.K. Zhai, N. Ferrand, K. L. Knight and W. van der Loo (submitted).

The evolution of the immunoglobulin heavy chain variable region (IgV_H) in Leporids: a case of trans-species polymorphism

The evolution of the immunoglobulin heavy chain variable region (*IgV_H*) in Leporids: a case of trans-species polymorphism

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Abstract

The European rabbit (*Oryctolagus cuniculus*) uses in *VDJ* gene rearrangements preferentially the *D*-proximal Ig heavy chain variable region gene segment, V_H1. Three lineages are distinguished at this locus: the a1, a2 and a3 allotypes, which are inherited as codominant Mendelian alleles. The distances between lineages are unusually large and sequence comparisons with human and mouse V_H gene segments have suggested that the V_H1 polymorphism has persisted for about 50 Million years (My). By consequence, it should predate the separation between the *Oryctolagus* and *Lepus* genera. To test the trans-generic nature of the polymorphism, we have sequenced V_H gene segments expressed in two European *Lepus* species (*L. granatensis* and *L. europaeus*). Two major lineages were observed, one of which was unrelated to the V_H1 alleles expressed in rabbit. In contrast, a second lineage, called *a2L*, was found to be more closely related to the rabbit V_H1-*a2* allele than the latter was to its allelic counterparts V_H1-*a1* and V_H1-*a3*, as would be expected in case the divergence between the V_Ha alleles preceded the separation of the *Lepus Oryctolagus* split.

Introduction

The rabbit (*Oryctolagus cuniculus*) immunoglobulin heavy chain variable region (IgV_H) possesses three serologically defined allotypes (*a1*, *a2*, *a3* V_Ha allotypes). The *a1* and *a2* allotypic specificities are correlated with several amino acid differences, which are located in framework regions 1 (FR1) and 3 (FR3) (Mage *et al.*, 1984; Knight and Becker, 1990). The V_H region allotypic specificities behave like Mendelian alleles (Oudin, 1956, 1960; Dubiski *et al.*, 1959; Dray *et al.*, 1963; Kim and Dray, 1972, 1973). These lineages differ indeed by some 20% amino acid differences. Both the allelic behaviour and the large genetic distances between the V_Ha allotypes is surprising. The allelic behaviour is explained by the preferential use of the *D* proximal V_H gene segment, V_H1, in *VDJ* gene rearrangements (Knight and Becker, 1990; Becker and Knight, 1990): Approximately 80 to 90% of circulating immunoglobulins are derived from this gene segment. The remaining 10 to 20% of serum Ig do not contain the V_Ha allotype-associated amino acids (Friedman *et al.*, 1994; Short *et al.*, 1991). These molecules are called V_H "*a*-negative" or V_Hn and are encoded by separate V_H genes, V_Hx, V_Hy, and V_Hz (Kim and Dray, 1972; Kim and Dray, 1973; Roux, 1981), which are located at least 50 Kb upstream of the V_H1 gene (Knight and Becker, 1990).

There are two mechanisms that could account for the large differences between V_Ha lineages. An acceleration of evolutionary rate and/or very long lineage persistence time. Su and Nei (1999) compared the extent of sequence divergence between the rabbit V_H1-*a1*, -*a2* and -*a3* gene segments with that between human and mouse V_H gene sequences. Assuming a normal mutation rate, they concluded that the V_H1 polymorphism in the rabbit lineage has persisted for about 50 My, which is much longer than the age of separation between the genera *Oryctolagus* and *Lepus* (Bouton and van der Loo, 1997; Halanych and Robinson, 1999; Su and Nei, 1999). If so, some allelic forms of one species are expected to be more closely related to alleles expressed in related species than to their allelic counterparts ("trans-species", or "trans-generic" polymorphism).

Besides the large interallelic distance, evidence for the trans-species nature of the V_H1 polymorphism relies mainly upon the fact that sera of *Lepus* species can show serological cross reactivity with rabbit V_Ha-allotype specific antisera (van der Loo *et al.*, 1977, Horng *et*

al., 1980, van der Loo, 1987). While it can allow the distinction of allotypic lineages in *Lepus* populations, it does not warrant phylogenetic clustering of lineages showing cross-reactivity with a same antiserum, nor does the absence of cross-reactivity preclude close relationship with rabbit V_H1 genes. Indeed, recently, a fourth allotype (*a4*) was described in wild rabbits of the subspecies *O. c. algirus*, that did not show any cross-reaction with conventional V_Ha specific antisera (*"a-blank"*; Esteves *et al.*, unpublished). These rabbits were found to express preferentially *VH* genes that are clearly monophyletic with V_H1 genes of domestic rabbits.

The phylogenetic relationships between V_H gene of rabbits and hares requires therefore the sequence determination in hares. We have determined DNA sequences of V_H genes expressed in specimens of two European *Lepus* species, that were typed either "V_Ha-*a2* postive", or "V_Ha blank". The data are in strong support of the trans-generic nature of the V_Ha polymorphism.

Material and Methods

Amplification, cloning and sequencing of rearranged VDJ genes

Spleen samples were collected from wild specimens in the Iberian Peninsula (*Lepus granatensis*, three specimens), and Austria (*Lepus europaeus*, two specimens). Each sample was serological analysed by double immunodiffusion test as previously described by van der Loo *et al.*, (1974). Total RNA was prepared using TRIzol according to the manufacturer's instructions (Life Technologies, Grand Island, NY). First strand cDNA was prepared from 3μg of RNA and synthesized using oligo (dT) primers (Krug *et al.*, 1987). *VDJ* gene rearrangements were PCR-amplified using primers specific for conserved regions in the V_H leader and in the J_H gene (Zhu *et al.*, 1999). All PCR amplifications proceeded for 30 cycles (each cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 60 s). PCR products approximately 500 bp in length were gel-purified and cloned into the pGEMT-Easy vector (Promega, Madison, WI). The nucleotide sequences were determined by automated sequencing following the Big Dye Terminator Cycle Sequencing protocol (Perkin-Elmer Applied Biosystems, Foster City, CA).

Evolutionary analyses

The phylogenetic tree shown in Figure 1, was constructed using the following sequences a) rabbit germline V_H genes (V_H1a1, V_H1a2, V_H1a3, V_Hx, V_Hy and V_Hz); b) rabbit

cDNA V_H gene sequences representative of each allotype (*a1*, *a2*, *a3* and *a4*); c) cDNA V_H genes obtained in this study in *Lepus granatensis* (*Lg*) and *Lepus europaeus* (*Le*) specimens. As outgroups, we used human V_H genes of the *VH3* family (accession number: M99672; M99679; M99682; M99666; X92206). These genes belong to the same phylogenetic group as the rabbit V_H genes, i.e. group C (Ota and Nei, 1994; Nei, 1997; Sitnikova and Su, 1998; Su and Nei, 1999). The alignments were made using the computer program "pileup" of the GCG© package (Wilkinson), as provided by the Belgian EMBnet Node (BEN; *http://www.be.embnet.be*) and Clustal W (Thompson *et al.*, 1994) as provided by the freeware DAMBE package (Xia and Xie, 2001), followed by visual adjustments. The reliability of the trees was examined by the bootstrap (Felsenstein, 1985) and the interior-branch (Rzhetsky and Nei, 1992; Sitnikova, 1996) methods, which produced for each interior branch in the tree the bootstrap probability (BP) and confidence probability (CP) values, respectively. All data analyses shown were conducted by using the computer program MEGA2 (Kumar *et al.*, 2001). We used the "pairwise deletion" option to compute evolutionary distances, but essentially the same results were obtained when the alignment gaps were eliminated. The V_H region of a rearranged *VDJ* gene is somatically diversified by gene conversion and somatic hypermutation. For this reason genetic distances between grouped V_H gene were estimated using the option "compute net average distance between groups" of the MEGA2 package, which corrects for distance components due to variance within groups.

Results

Serum samples Lg7 and Lg15 *Lepus granatensis* failed to show any cross-reactivity with conventional V_Ha-allotype specific alloantisera (V_Ha blank). In contrast, the serum of *Lepus granatensis* Lg19 and of *Lepus europaeus* Le1 and Le2 showed clear cross-reaction with anti-*a2* alloantisera raised in rabbits that were homozygous for the *a3* allele. These reactions were very similar to those obtained with hare sera that were described previously in van der Loo *et al.*, (1977).

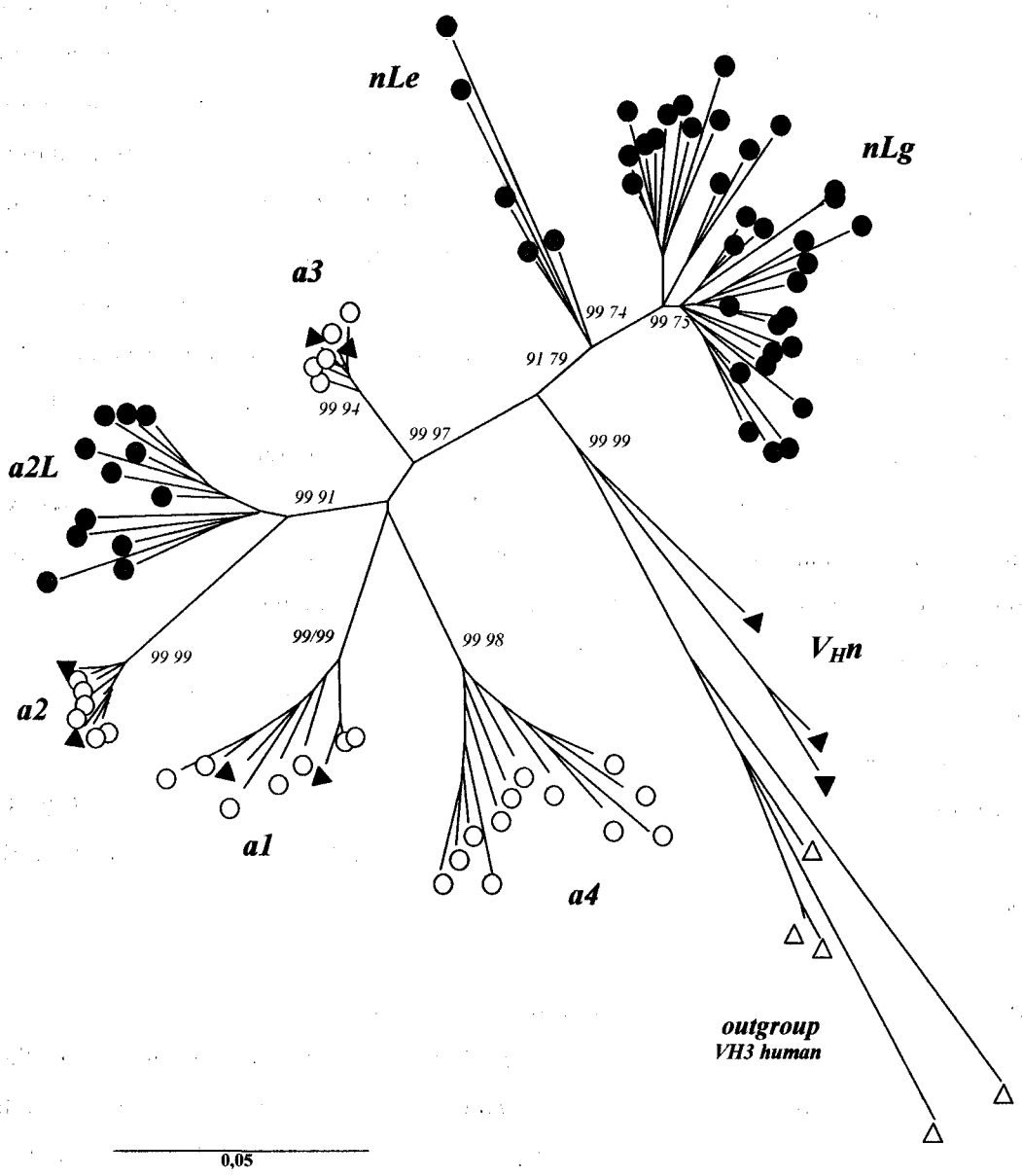

Figure 1: Neighbor-Joining tree for *Oryctolagus cuniculus*, *Lepus europeus* and *Lepus granatensis VH* genes. VH genes from human class 3 determine the root of the tree. Numbers represent the Confidence Probability (CP, in bold) and the bootstrap (BP, italic) values when 1000 replicate samplings were done. Expressed V_H genes (cDNA): ● *Lepus europaeus*; ● *Lepus granatensis*; ○ *Oryctolagus cuniculus*; Germline V_H gene sequences: *Oryctolagus cuniculus* allotypes *a1*:▲, *a2*: ▲, *a3*:▲ and V_Hn genes:▲; V_H3 human (outgroup):△.

Phylogenetic tree

A total of 57 V_H gene nucleotide sequences were determined and their amino acid sequences inferred. Their phylogenetic relationships to known rabbit V_H sequences were studied using different inference methods (Minimum Evolution, Maximum Parsimony, Maximum Likelihood). The trees obtained with these different methods showed similar clustering of the analysed sequences. The exclusion of the CDR regions in the analyses did not affect the general branching patterns. In Figure 1 we present therefore only the NJ tree (Neighbor-Joining; Saitou and Nei, 1987) of the entire nucleotide sequences constructed using the MEGA2 package (Options: pairwise deletions, uncorrected p-distances). We distinguish four major clusters: a) V_H *a*-negative cluster (V_Hn), which include the rabbit V_Hx, V_Hy and V_Hz gene segments, b) V_H*a-like* cluster, which include the rabbit V_H1 gene segments of the *a1, a2,* and *a3* allotypes, c) V_HnL cluster, which contains only *Lepus* derived sequences; d) outgroup (human V_H3 gene segments). Each of these four clusters are well supported by confidence values (CP=99) as well as by bootstrapping (BP>74). The V_H*a-like* group in turn consists of four clusters (CP=99 and BP>91). Three of these, the *a1*, *a3* and *a4* groups, are composed exclusively of rabbit sequences, whereas the fourth group, the "*a2-like*", is composed of sequences obtained from both rabbit and hare specimens. The *Lepus*-derived sequences of this cluster will be referred to as "*a2L*" sequences. The V_HnL group encompasses gene sequences from both *Lepus europaeus* and *Lepus granatensis*, and clearly branches apart from the clusters including the rabbit V_H1 sequences.

Characterisation of rearranged V_H gene segments in hare

The inferred protein sequences were aligned and grouped according to the phylogenetic tree. The *a2L* group consists of sequences obtained from specimens of both hare species studied, *Lepus europaeus* (Le1, Le2) and *Lepus granatensis* (Lg19). The V_HnL-like group can be divided into two subgroups, of which one *(nLg)* is composed of sequences obtained from *Lepus granatensis* (Lg7, Lg15, Lg 19), and one *(nLe)* from *Lepus europaeus* (Le1, Le2), respectively. We derived a consensus sequence of the amino acid sequences inside each group, i.e. Cons-*a2L*, Cons-*nLg* and Cons-*nLe* (Figure 2A). In Figure 2B we compare these consensus sequences with the inferred amino acid sequences of the V_H1a1, V_H1a2, V_H1a3, V_Hx, V_Hy and V_Hz gene segments, and with Cons-*a4.1* and Cons-*a4.2*, which are the consensus sequences of two variant forms of the allotype *a4* (Esteves *et al.*, unpublished).

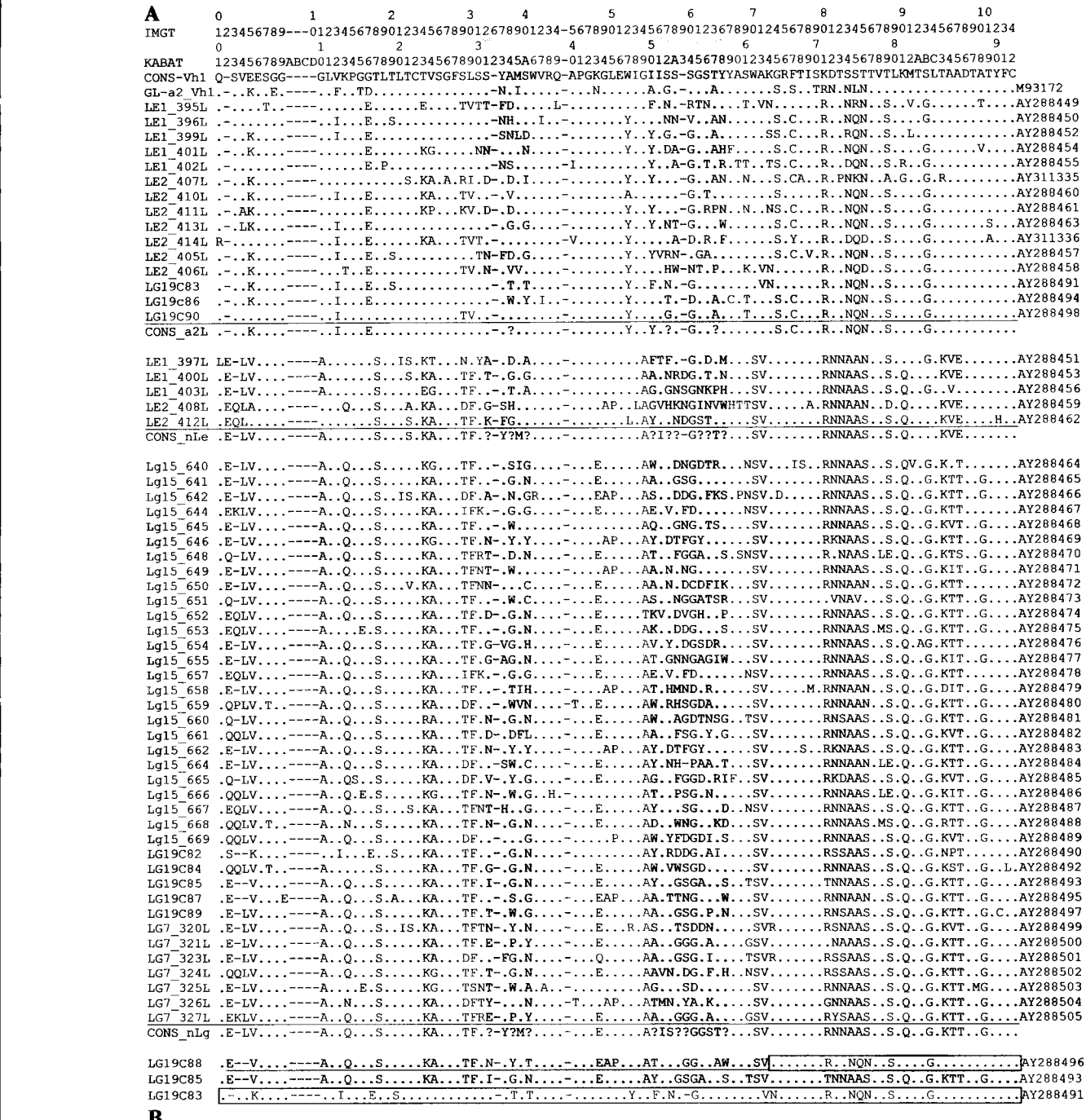

Figure 2. Alignment of inferred amino acid sequences of V_H genes. **A**: V_H gene sequences of *Lepus europaeus* and *Lepus granatensis* that form the *a2L*, n*Le* and *nLg group*. CONS_*a2L*, CONS_*nLe* and CONS_*nLg* are the established consensus sequences. LE1_*: *Lepus europaeus* 1; LE2_*: *Lepus europaeus* 2; Lg19_*: *Lepus granatensis* 19. **B:** Sequences of rabbit and *Lepus* V_H genes. CONS-vh1 is the established consensus sequence. GL-a1_Vh1, GL-a2_Vh1, GL-a3_Vh1: rabbit germline VH1gene of allotypes a1, a2 and a3, respectively; CONS a4.1, CONS a4.2: consensus sequence established for allotypes *a4.1* and *a4.2*; GL_Vhx, GL_Vhy, GL_Vhz: rabbit germline Vhx, Vhy and Vhz, respectively. The hypervariable region of CDRs are shaded and were excluded from the analyses. Dots (.) indicate identity with the consensus sequence; Dashes (-) represent indels.

The *a2L* sequences have the signature of V_Ha alleles. They share a number of apomorphic characters with the V_Ha genes, such as the V_Ha hallmark peptides 18LTLTCT23 of FR1, and 62WAK64 of FR3 (amino acid numbering of Kabat 1987). Furthermore, they possess five out of eleven amino acid residues that characterize the allotype V_Ha-*a2* (Mage *et al.*, 1984), *i.e.* two in the FR1: K5 and T17, and three in the FR3: S65, R71, and N74. Another feature that they have in common with the V_H*1-a2* sequence is the absence of deletions in the FR3 (positions 72 and 74). These shared features might explain the cross-reactivity observed in immunodiffusion tests. Among the leporid sequences shown, the *a2L* sequences are characterized by amino acids residues I12 and E16 (FR1), and C67 (FR3). I12 is also observed in the rabbit *VH4-a2* and *VH7-a2* gene segments. The lineages *nLg* and *nLe* differ at six positions: Q/K13, T/S21, E/K45, G/S80b, T/V84, T/E85. It is interesting that the two *nL* lineages differ at positions 84 and 85, because these positions vary among rabbit allotypes *a1*, *a2/a3*, *a4.1*, and *a4.2* and are known to define allotypic determinants (Mage *et al.*, 1984).

Net-distances between rabbit and hare V_H sequences

The amino acid p-distance between the different clusters of diversified V_H sequences, are presented in Table 1. Other distance methods essentially provided the same result. The net-distances between the (diversified) rabbit *a2* allotype sequences and *a1, a3, a4.1* and *a4.2* allotypes vary between 0.199-0.220, which is significantly larger than the net-distance observed between the rabbit *a2* sequences and the *Lepus a2L* sequences (0.121 +/- 0.028). This supports the phylograms suggesting that the *a2* and *a2L* lineages diverged after the separation of the major V_Ha lineages (Figure 1). The distance between the *a2L* sequences and the *VH* gene groups *nLg* and *nLe* was 0.206 and 0.179 respectively, which is of the same order as that observed between allotypic V_Ha lineages of rabbit.

Table 1

Distances between rabbit and hare V_H genes. Net distances were determined using the "uncorrected p-distance" of the MEGA2 package, applied to diversified rabbit and hare VH sequences representative of different clusters defined in Figure 1. The genetic distances are represented below and the standard deviations above the diagonal.

	a1	*a2*	*a3*	*a4.1*	*a4.2*	*a2L*	*nLg*	*nLe*	*n*
a1	----	0.039	0.035	0.031	0.030	0.033	0.041	0.038	0.040
a2	0.220	----	0.036	0.036	0.034	0.028	0.039	0.037	0.039
a3	0.153	0.208	----	0.031	0.028	0.030	0.033	0.032	0.030
a4.1	0.151	0.206	0.151	----	0.015	0.031	0.035	0.034	0.035
a4.2	0.163	0.199	0.149	0.050	----	0.028	0.032	0.031	0.031
a2L	0.177	0.121	0.167	0.162	0.148	----	0.032	0.031	0.022
nLg	0.264	0.307	0.183	0.214	0.190	0.206	----	0.014	0.014
nLe	0.243	0.271	0.169	0.219	0.194	0.179	0.039	----	0.016
n	0.249	0.272	0.143	0.243	0.214	0.140	0.186	0.163	----

Discussion

There are different ways to explain the unusually high allelic divergence observed in rabbit V_H1 polymorphism: very long lineage persistence time and/or an acceleration of the evolutionary rate within lineages. If the latter hypothesis is correct, the mutation rate is higher than that estimated under neutrality, and the time of divergence between the alleles will be overestimated. If we assume that evolutionary rates were normal, the allelic lineages must have existed before the separation of the *Lepus* and *Oryctolagus* genera took place. In that case, allelic forms in distinct species can be more closely related than are allelic forms in the same species. *MHC* allelic lineages are prime examples of this phenomenon, having persisted for more than 25 MY in humans and in the old world monkeys (Lawlor *et al.*, 1988; Fan *et al*, 1989; Mayer *et al.*, 1992; Klein *et al.*, 1993).

Oryctolagus originated in the Iberian Peninsula, where two subspecies (*O. c. cuniculus* and *O.c. algirus*) have coexisted with *Lepus* species for more than 6 My (Lopez-Martinez 1989, Callou 1995). In this study, we found two V_H lineages in *Lepus* species: one that is clearly part of the cluster formed by the rabbit V_Ha-like genes (*a2L*), and another that clearly is not (*nL*). Comparison of these lineages with the rabbit alleles (*a1*, *a2*, *a3*, *a4.1* and *a4.2*) revealed that the *a2L* lineage is more similar to the *a2* lineage than the latter is to its allelic counterparts *a1* and *a3* (see Figure 1 and Table 1). These results strongly suggest that the high allelic divergence of rabbit V_Ha alleles is at least in part due to prolonged lineage persistence times.

It seems that in V_Ha-positive typing hares, genes encoding *a2L* and *nL* proteins are equally expressed (14 *nL* sequences *vs.* 13 *a2L* sequences for *Lg19*, *Le1*, and *Le2*). V_HnL genes were expressed in all *Lepus* specimens, and were the only V_H genes detected in *Lg7* and *Lg15* forming a thigh cluster in phylogenetic analysis. In the absence of breeding studies, it would be highly presumptions to speculate about the allelic nature of the *a2L* and *nL* coding genes. Genomic mapping will furthermore be necessary for the understanding of the mechanism that regulates the expression of these V_H gene lineages. This does however not diminish the evidence for the hypothesis that the common ancestors of V_H1 alleles of modern rabbits have started their divergence long before the rabbit-hare split.

The origin and maintenance of V_Ha polymorphism is unknown. Su and Nei (1999) suggested that the presence of only three highly divergent alleles indicates that each might have some specific adaptive significance and proposed that the three known lineages (*a1*, *a2*

and *a3*) have been maintained by overdominant selection. 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), diversification of the primary antibody repertoire in rabbits is not developmentally programmed. It has been shown that Ab repertoire diversification in the rabbit requires interaction with exogenous factors, such as the intestinal microflora (Lanning *et al.*, 2000a; 2000b, Sehgal *et al.*, 2002). It is possible that the mechanism driving diversification of the primary antibody repertoire might impose constraints on the evolution of the $V_H a$ polymorphism.

This study shows how extending the studies on the rabbit $V_H a$ allotypes to other lagomorph species, can contribute to a better comprehension of the evolution and the biological meaning of this unusual polymorphism.

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Understanding the large inter-allelic differences at the V_H1 locus.

Evolution theory offers two possible explanations for interallelic distances as large as those observed at the rabbit IgV_H1 locus: unusually long allele turnover times, or increased mutant recruitment rates. The fact that allo-antisera raised in rabbit allowed to distinguish three V_H allotypes in populations of *Lepus americanus* has suggested that both species have inherited the same three lineages, which could explain the large interallelic distances without invoking acceleration of evolution rates (Su and Nei, 1999). In this case allelic forms in distinct species will be more related than are allelic forms in the same species.

In Article 3 we show that there are for the rabbit *IgVH1* locus not three but at least four highly divergent lineages in Iberian wild rabbit populations. The fourth lineage (*a4*) is associated with the maternal markers of subspecies *algirus* and did not cross the Pyrenean Mountains, which is in contradiction with extremely long allele persistence times. It suggests that the large differences between *a4* and other V_H1 alleles may have accumulated after the separation of the subspecies, estimated in 2 million years ago.

On the other hand, in Article 4 we observe the existence of a V_H lineage in *Lepus* species (*a2L*) that clusters with sequences of the rabbit *a2* lineage, which supports the hypothesis of very long lineage persistence time. However, the serological analyses indicate that some allelic lineages present in *L. americanus* could be absent in *L. granatensis*, *L. europaeus* and *L. capensis*, suggesting that in the *Lepus* speciation process some of these lineages were lost.

These two different lines of evidence suggest, on one hand, that allelic lineages can be lost stochastically and, on the other hand, that different mutation rates can be associated to each allelic lineage. The study of V_H genes of *Lepus americanus* could help to clarify this situation.

Evolution of V_H genes in the lagomorph group

Phylogenetic analyses

The lagomorph V_H gene sequences used in this analysis were obtained by "random" PCR sampling among more than 100 V_H gene segments. To construct the NJ tree present in Figure 3.9 only functional V_H genes were considered. The major vertebrate V_H genes classes B and C are well differentiated. Inside of class C three clusters are well supported: 1) lagomorph V_H genes (rabbits, hares, cottontails and pikas; 2) bird V_H genes; 3) other

mammals (camel, human and mice). Four clusters compose the lagomorph group. These are: a) the V_Ha group that encompasses *a1*, *a3*, *a4* and *a2-like* groups; b) V_HnL group that includes both expressed and germline V_H gene sequences of *Lepus granatensis*; c) V_Hn group, that contains germline V_H genes of *Oryctolagus*, *Sylvilagus*, *Ochotona*, *Lepus granatensis* and *L. americanus*; d) V_HP group, represented only by pika germline sequences. The V_Ha group contains three clusters (*a1*, *a3* and *a4*), which are composed exclusively by rabbit sequences. In contrast, the *a2-like* cluster includes sequences of three different Leporid genera, i. e. the rabbit *a2* allele, the lineage *a2L* of *Lepus granatensis* (cDNA) and genomic V_H gene sequences obtained in *Lepus americanus* and *Sylvilagus floridanus*.

Characterization of lagomorph germline V_H *gene sequences*

The amino acid sequences of V_H germline genes obtained in this work were compared to other rabbit germline sequences available in Genbank and with the consensus sequences of *a4*, *a2L* and *nL* allelic lineages (see article 3 and 4) in the Figure 3.10.

These results suggest that only V_H sequences belonging to group C are present in the genome of lagomorph species. Probably, the loss of V_H genes from group A and B happened in an ancestral of the lagomorphs at least 50 My years ago, which is the time of divergence estimated between pika and rabbit (Springer *et al.*, 2003). The lagomorph V_H gene sequences define an independent group. Within this cluster the V_H gene sequences are separated into four major groups: V_Ha, V_Hn, V_HnL and V_HP. The V_H genes sequences from different species can be clustered in the same group and V_H gene sequences of the same species are sometimes clustered in different groups. It seems likely that in the lagomorphs, the duplication of V_H genes is in accordance with the model of birth-and-death evolution.

It has been shown that in the rabbit the diversification process is not developmentally regulated like it appears to be in other species, such as chicken, sheep and cattle, and requires interaction of exogenous factors, such as the intestinal microflora (Lanning *et al.*, 2000a; 2000b, Sehgal *et al.*, 2002). We speculate that the intestinal microflora is probably the mechanism that is responsible for the occurrence of highly divergent V_H gene sequences within the lagomorph group.

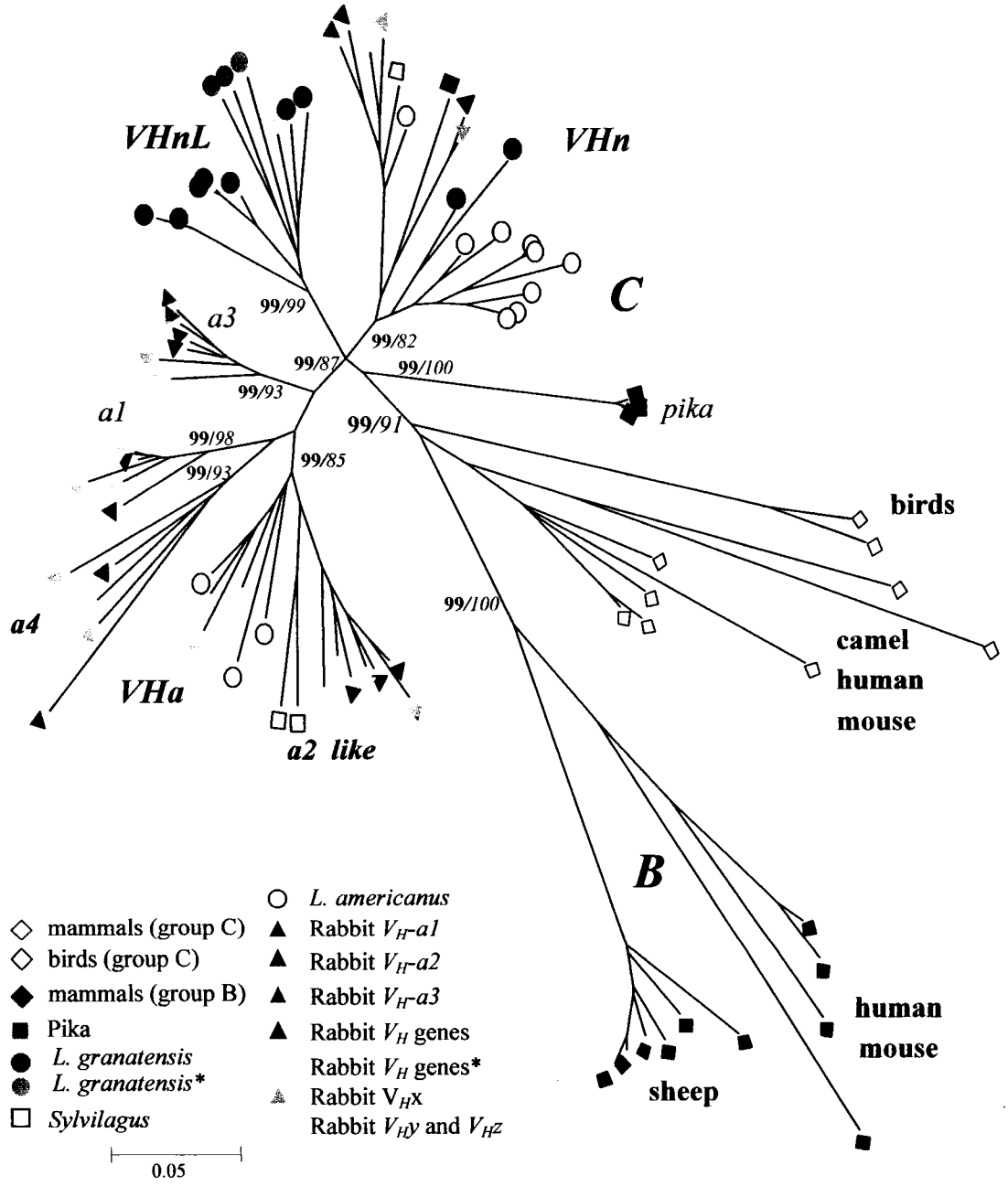

Figure 3.9. Neighbor-Joining tree for *Oryctolagus cuniculus*, *Lepus granatensis*, *Lepus americanus*, *Sylvilagus*, *Ochotona* and other mammals and birds V_H genes clustered in group C. V_H genes from mammals belonging to group B determine the root of the tree. Numbers represent the Confidence Probability (CP, in bold) and the bootstrap (BP, italic) values when 1000 replicate samplings were done. The expressed *VH* gene sequences (cDNA) are indicated by *.

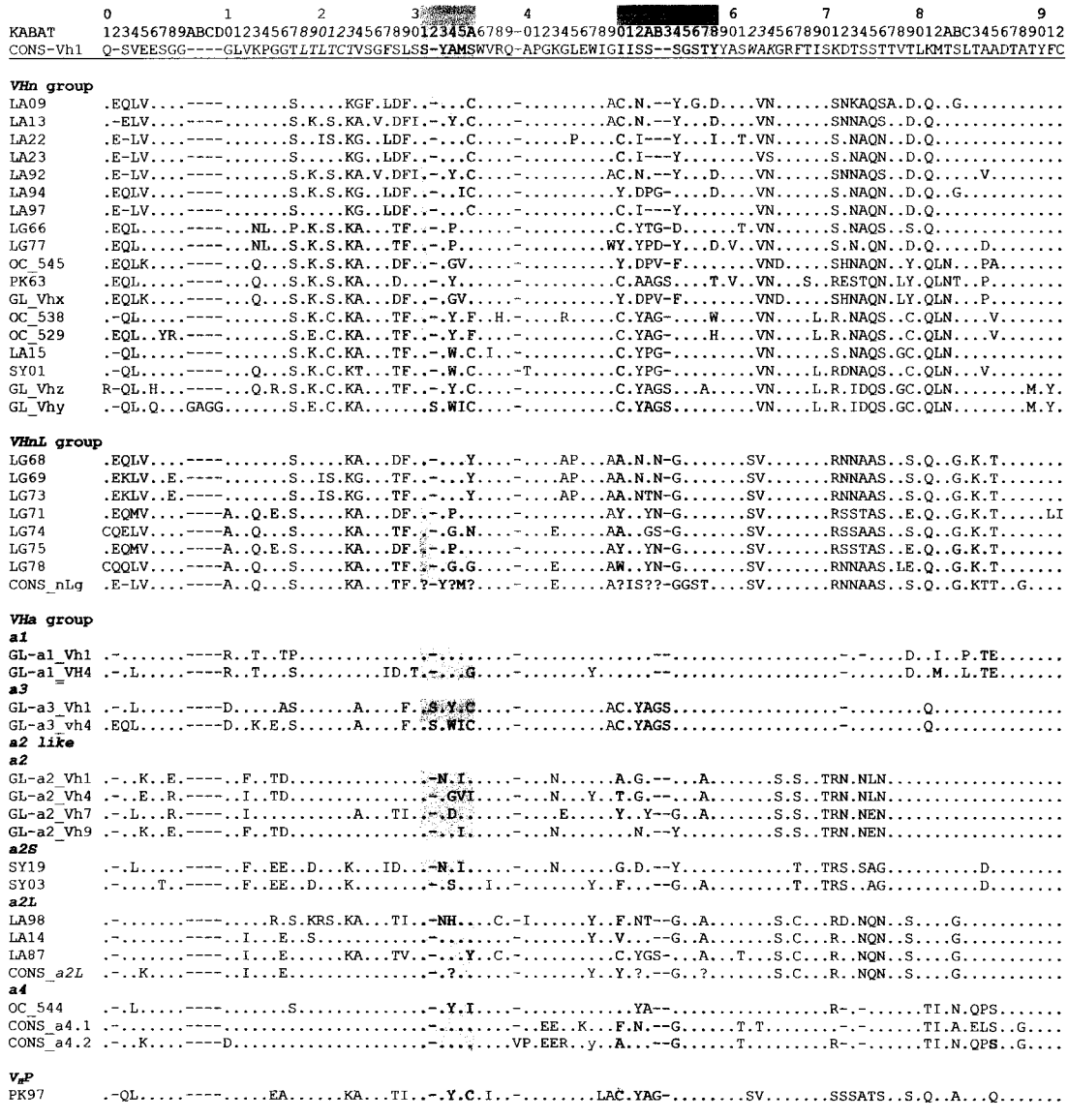

Figure 3.10. Alignment of lagomorph V_H gene sequences. The amino acid V_H regions are inferred from: 1) genomic V_H nucleotide sequences obtained in this work for *Oryctolagus* (OC), *Sylvilagus* (SY), *Lepus americanus* (LA), *Lepus granatensis* (LG) and *Ochotona* (PK); 2) genomic rabbit V_H nucleotide sequences (GL) published in Genbank. CONS_*a4.1*, *a4.2*, *nLg* and *a2L* are the consensus sequences of the lineages *a4.1*, *a4.2*, *nLg* and *a2L*, obtained from the V_H gene expressed (cDNA). They are compared to a consensus sequence of rabbit V_H1 gene segment (CONS-VH1) and grouped according to phylogenetic relationships, shown in Figure 3.9.

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CHAPTER 4

Evaluation of the bottleneck effect in the Porto Santo wild rabbit population

Introduction

The presence of rabbits in more than 800 islands all over the world points out the success of this species in the colonization of new environments (Flux and Fullagar, 1992). The reasons for this success are particular features such as high growth and reproduction rate, an efficient food utilisation (associated with coprophagy, i.e. the capacity to eat the faeces increasing nutritional efficiency), and the ability to shift between growth strategies ("r" or "K - selection") according to the carrying capacity of the environment. Other aspects are also fundamental in its success in islands, like the absence of competitors, few predators and inexistence of diseases.

A particularly successful introduction was documented for the Porto Santo, a small island of the Portuguese Madeira archipelago. Several historical documents suggest that a single pregnant female was released in this island by a Portuguese navigator, Bartolomeu Perestrelo, in 1419 (Cordeiro, 1717; Barros, 1778; Azurara, 1841; Frutuoso, 1873). This event was followed by a sudden increase in population numbers (Cadamosto, 1812; Frutuoso, 1873). Charles Darwin, in his book "*The variation of animals and plants under domestication*" (1868), described seven specimens brought from Porto Santo Island as remarkably different in appearance from the British rabbit. For Darwin, the rabbit of Porto Santo was a striking example of divergence in form since its introduction to the island. This opinion was also shared by Hæckel (1874) who considered the Porto Santo rabbit a distinct species named as *Lepus huxley*. Later studies, however, demonstrated by comparison with rabbits from the Iberian Peninsula that Porto Santo rabbits were clearly *Oryctolagus cuniculus* (Miller, 1912; França, 1913), although its domestic or wild origin was not clarified (Zeuner, 1963; Hemmer, 1980).

The derivation of populations from one single pregnant female has only been described in *Drosophila* (Prakashi, 1972; Nei *et al*, 1975). If the history of the Porto Santo rabbit is true, this would be the first time that such successful introduction is reported for a mammalian species. Porto Santo could thus be seen as a natural population in nearly "laboratory" conditions, which can be very useful in conservational genetics issues, because it represents the ideal situation where consequences of an extreme bottleneck event can be studied in mammals. Indeed, the study of founder effects has become increasingly important in population genetics, speciation theory and conservation biology. One of the issues raised here is the likelihood that four or more allelic lineages can persist over millions of years, as was proposed for *IgVH1* locus (Su and Nei, 1999).

Population bottlenecks are particularly important because they can increase the demographic stochasticity, the rate of inbreeding and the fixation of deleterious alleles, therefore reducing adaptive potential and increasing the probability of population extinction (e.g. Lande, 1994; O'Brien *et al.*, 1994; Newman and Pilson, 1997; Saccheri *et al.*, 1998)). In natural populations, for selectively neutral loci, the number of alleles and heterozigosity values result from equilibrium between mutation and genetic drift. This equilibrium depends therefore on two parameters: mutation rate and effective population size. When a strong bottleneck occurs there is a high decrease in the number of alleles and heterozigosity values. A potential test for detecting recent bottlenecks is to compare the expected gene diversity (in fact, the variance in gene frequencies) observed in the population (Ho) with that expected (He) from the observed number of alleles for each locus under a particular mutation model. Note that this heterozygosity excess should not be confused with the excess of heterozygotes. The former compares the observed heterozygosities, whereas the latter compares the number of heterozygotes with Hardy-Weinberg expectation. Theoretically, in non-bottlenecked populations a similar number of loci with slight excess or with a slight deficiency of Ho are expected. On the other hand, in a bottlenecked population the majority of loci show an excess of Ho (Nei *et al*, 1975; Chakraborty and Nei, 1977; Chakraborty et al., 1980; Maruyama and Fuerst, 1985; Cornuet and Luikart, 1996; Luikart and Cornuet, 1998).

Material and Methods

Assessment of genetic diversity

In this work, the Porto Santo rabbit population was studied by means of a battery of loci that had previously been shown to be polymorphic in Iberian rabbit populations. We used: 1) 20 protein loci (allozymes and plasmatic proteins), 2) three immunological markers; 3) nine microsatellite loci and 4) mtDNA markers, a 1120 bp-long fragment of cytochrome *b* and a fragment of approximately 370 bp length of the control region.

The samples (tissues, red cells and serum) utilized in this work were obtained in November 1995 and stored at -20ºC.

The different alleles obtained in a battery of 20 protein loci were separated by starch gel electrophoresis, agarose gel electrophoresis or isoelectric focusing and detected by general staining of proteins, specific enzymatic activity staining or by immunoblotting. In the study of immunological markers the serum of each sample was tested against antisera specific for particular allotype markers: (*b* locus: *b4*, *b5*, *b6*, *b9*; *a* locus: *a1*, *a2*, *a3*; *e* locus: *e14*, *e15*). The phenotypes were determined by immunodiffusion in 1% agar gel containing 2% polyethylene glycol. In each test a reference was included to monitor the degree of serological similarity of the sample being tested with samples from rabbits expressing the immunizing allotype. Typing of the microsatellites was achieved with multiple PCR, fluorescently labeled primers, and analyzed using an ABI 310 (Applied Biosystems) automated sequencer. The cytochrome *b* was amplified according to the methodology described by Branco *et al.* (2000) and was then digested with eight restriction enzymes (*Alu*I, *Bsa*I, *Dde*I, *Fok*I, *Hae*III, *Mse*I, *Taq*I, and *Tsp*509I). The fragments patterns were

obtained after electrophoresis on 3% Nusieve agarose gels. A control region fragment with approximately 400 bp was amplified according to the conditions given in Branco *et al.* (2002) and then sequenced.

The loci used and the methodologies followed are indicated in the Table 4.1.

Table 4.1
Protein, immunological, microsatellite and mtDNA loci analysed in this work.

Loci	Separation method	Reference
Nuclear		
Protein markers		
Acid phosphatase 3 (ACP3)	IEF	Branco and Ferrand (1998)
Adenosine deaminase (ADA)	SGE	Ferrand (1995)
Albumin (ALB)	IEF	Ferrand and Rocha (1992)
Anti-Thrombine III (ATIII)	IEF	Branco and Ferrand (2002)
Carbonic anhydrase I (CAI)	AGE	Branco and Ferrand (2003)
Carbonic anhydrase II (CAII)	AGE	Branco and Ferrand (2003)
Galactose-1-phosphate uridyl transferase (GALT)	SGE	Ferrand (1995)
Vitamin D binding region (GC)	IEF	" "
Hemoglobin alpha chain (HBA)	SGE	Campos *et al.* (2003)
Hemoglobin beta chain (HBB)	SGE	Campos *et al.* (2003)
Hemopexin (HPX)	IEF	Branco and Ferrand (2002)
Nucleoside Phosphorylase (NP)	SGE	Ferrand (1995)
Properdin factor B (BF)	IEF	Branco *et al.* (1998)
Peptidase A (PEPA)	SGE	Branco *et al.*. (1999)
Peptidase B (PEPB)	SGE	" "
Peptidase C (PEPC)	SGE	" "
Peptidase D (PEPD)	SGE	" "
Phosphogluconate dehydrogenase (PGD)	SGE	Ferrand (1995)
Superoxide Dismutase (SOD)	SGE	Ferrand (1995)
Transferrin (TF)	AGE/IEF	Ferrand *et al.* (1988)
Immunogenetic markers		
IGGHCH2	IDT	Kellus and Gell (1967)
IGVH1	IDT	" "
IGCK1	IDT	" "
Microssatellites		
Sat 2	CE	Queney *et al.* (2001)
Sat 3	CE	" "
Sat 4	CE	" "
Sat 5	CE	" "
Sat 7	CE	" "
Sat 8	CE	" "
Sat 12	CE	" "
Sat 13	CE	" "
Sat 16	CE	" "
Mitochondrial (mtDNA)		
cytochrome *b*	PCR/RFLP	Branco *et al.* (2000)
Control region	S	Branco *et al.* (2002)

IEF- Isoelectric Focusing
SGE- Starch Gel Electrophoresis
AGE- Agarose Gel electrophoresis
IDT- Immunodifusion Test
PCR/RFLP- Polymerase Chain Reaction/Restriction Fragment of Length Polymorphism
CE- Capilar Electrophoresis
S- Sequencing

Statistical tests to detect bottleneck situations

To test the occurrence of a bottleneck effect we followed the methodology described by Cornuet and Luikart (1996).

Assumptions and Principles

In order to use simulation tests to detect bottleneck events in natural populations it is necessary to have a representative sample of a well-defined population, under no immigration, population substructure and where loci are selectively neutral. All these situations can be generally detected through Hardy-Weinberg equilibrium. Loci that are not in accordance to Hardy-Weinberg equilibrium should be excluded or used only with caution. In our study, all loci (microsatellites and the protein markers) used for testing the bottleneck effect are in Hardy-Weinberg equilibrium.

The method described by Cornuet and Luikart (1996) calculates for each population sample and for each locus the distribution of the gene diversity expected from the observed number of alleles (k), given the sample size (n) under the assumption of mutation-drift equilibrium. This distribution is obtained through simulating the coalescent process of n genes, assuming mutation/drift equilibrium under different mutation models, the Infinite Allele Model (IAM), the Two-Phase Mutation Model (TPM) and the Stepwise Mutation Model (SMM). Under the IAM, a single mutation is allocated at a time and the resulting number of alleles is computed. The process is repeated until the latter reaches the observed number of alleles. Under the SMM, a Bayesian approach is used as explained in Cornuet and Luikart (1996). Briefly, the likelihood distribution of the parameter theta (= $4N_e\mu$) given the number of alleles (k) and the sample size (n) is evaluated as the proportion of iterations (in the simulation process) producing exactly k alleles for a varying set of thetas. Only gene diversities found in iterations producing exactly k alleles are considered. The way in which the coalescent process is simulated is unconventional due to the conditioning by the observed number of alleles. The phylogeny of the n genes is simulated according to Hudson (1990).

This enables the computation of the average (He) that is compared to the observed gene diversity (Ho) (see Nei, 1987) to establish whether there is a gene diversity excess or deficit at each locus. For the protein markers we choose to use the IAM, because the allozyme allele frequency distribution seems to fit this model (Nei *et al.* 1976; Chakraborty

et al, 1980; Luikart and Cornuet, 1998), but for the microsatellite loci we used the Two-Phase Model (TPM) (90% of Stepwise Mutation Model (SMM) and 10% of IAM). In IAM each mutation produces a new allele that is different from all existing ones (Kimura and Crow, 1964), while in SMM mutations change the state of an allele by one step forward or backward with equal probability (Ohta and Kimura, 1973). The TPM is intermediate to the SMM and IAM. Most microsatellite data sets fit the TPM better than the SMM or IAM (Di Rienzo *et al.*, 1994). The TPM recommendation for microsatellite consists of mostly one-step mutations, but a small percentage (5-10%) of multi-step changes (Luikart and Cornuet, 1998).

We used two statistical methods: the Standardized Differences Test (SDT) and the Wilcoxon Test (WT). The SDT is more powerful than the WT, but is parametric and requires at least 20 polimorphic loci in order to become valid. The Wilcoxon test is nonparametric, provides relatively high power and it can be used with as few as four polymorphic loci and any number of individuals (15-40 individuals and 10-15 polymorphic loci is recommended to achieve high power). Nevertheless, the Wilcoxon Test provides relatively high power and it can be used with as few as four or five polymorphic loci and any number of individuals. In these statistical tests, probability values lower than 0.05 reject the null hypothesis (mutation/drift equilibrium) in favor of the hypothesis of overall heterozygosity excess and a recent bottleneck effect. Additionally, we used a qualitative descriptor of the allele frequency distribution ("mode-shift" indicator), which discriminates bottlenecked populations from stable populations. However, this qualitative method is not a proper statistical test and cannot be used with confidence with samples of fewer than 30 individuals.

Results and Discussion

Assessment of genetic diversity

The gene frequencies obtained for each marker studied for the Porto Santo rabbit population are presented in Table 4.2.

Table 4.2
Allele frequencies at nuclear and mitochondrial loci for Porto Santo rabbit population. n is the sample size.

Markers	n	alleles	gene frequency
Nuclear			
Protein markers			
ACP3	59	1	1.00
ADA	64	1	0.64
		2	0.36
ALB	22	1	1.00
ATIII	20	A	1.00
CAI	22	1	1.00
CAII	22	2	1.00
GALT	15	1	0.57
		2	0.43
GC	20	1	1.00
HBA	59	2	0.48
		3	0.52
HBB	60	1	0.01
		2	0.99
HPX	20	F1	0.50
		F3	0.50
NP	66	1	0.50
		2	0.25
		3	0.25
BF	42	A	0.54
		B	0.06
		D	0.40
PEPA	61	1	0.21
		2	0.78
		3	0.01
PEPB	68	1	0.91
		2	0.09
PEPC	68	1	0.18
		2	0.28
		4	0.27
		8	0.28
PEPD	55	1	0.74
		2	0.26
PGD	64	A	0.45
		C	0.55
SOD	68	1	1.00
TF	20	1	1.00

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Markers	n	alleles	gene frequency
Immunologenetic markers			
IGGCH2	20	**e15**	1.00
IGVH1	20	**a2**	0.13
		a3	0.70
		a1v	0.17
IGCK1	20	b4wa	0.30
		b5wf	0.25
		b5wb	0.45
Microsatellites			
Sat2	32	**237**	0.23
		239	0.66
		241	0.11
Sat3	30	**146**	0.21
		154	0.79
Sat4	32	**227**	0.14
		231	0.21
		235	0.65
Sat5	32	**206**	0.14
		210	0.38
		214	0.20
		216	0.03
		222	0.25
Sat7	33	**185**	0.02
		187	0.54
		191	0.21
		193	0.23
Sat8	28	**152**	0.23
		154	0.20
		166	0.22
		168	0.35
Sat12	33	**126**	0.11
		130	0.21
		134	0.59
		138	0.09
Sat13	31	**112**	0.59
		114	0.05
		126	0.07
		128	0.29
Sat16	34	**109**	0.91
		115	0.09
Mitochondrial			
Cytochrome b	27	Arb4	1.00
Control region	7	**Pst**	1.00

Phylogeographic origin of Porto Santo rabbit population

Nuclear markers

Protein markers

The NJ tree (Saitou and Nei, 1987) (see Figure 4.1) was obtained using the genetic distance of Nei (Nei, 1978) calculated from the gene frequencies obtained for the rabbit population of Porto Santo as well as for a set of wild rabbit populations of different origins (Ferrand, 1995; Branco, 2000) in 20 protein markers. All analyses were conducted by using the computer program PHYLIP version 3.5 (Felsenstein, 1993). The tree clearly separates two groups (BP=100): one that is composed by Southwest Iberian populations, where Porto Santo is included, clustering with the Portuguese population of Idanha, and other that includes the populations from the Northeast. Previous studies using only sixteen polymorphic markers clustered the French (FR) and the domestic rabbit populations with the Northeast populations (Ferrand, 1995).

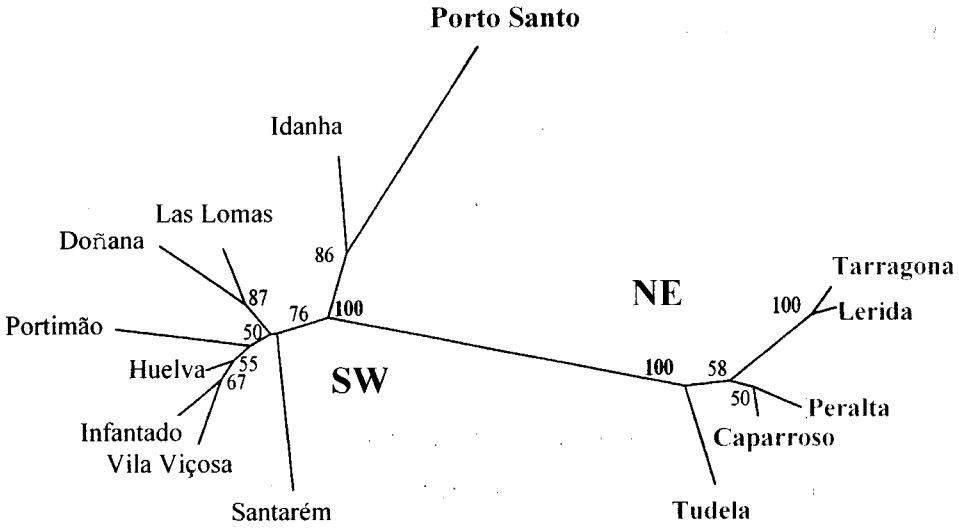

Figure 4.1. NJ tree obtained with Nei's genetic distances calculated using the gene frequencies of 20 polymorphic protein loci studied in Porto Santo and in Iberian Peninsula rabbit populations. The bootstrap values are indicated. SW- Southwest and NE- Northeast.

Immunogenetic markers

The gene frequencies obtained for *a*, *b* and *e* loci in Porto Santo were compared to the obtained in Southwest and Northeast of Iberian Peninsula and in French rabbit populations (van der Loo *et al.*, 1999). The alleles observed in Porto Santo for *b* locus are typical of Southwestern populations; for example the allele *b5wb* is the most frequent both in Southwestern populations and Porto Santo. On the other hand, allele *b4* is far out the most frequent in the Northeastern Iberian, in the French and in the domestic rabbit populations but is absent from Porto Santo (see Figure 4.2). The *a* and *e* loci are not informative in determining the origin of Porto Santo, since the *a1* and *a-blank*, highly represented in the wild rabbit populations, are both absent from Porto Santo and the *e* locus shows fixation for allele *e15*.

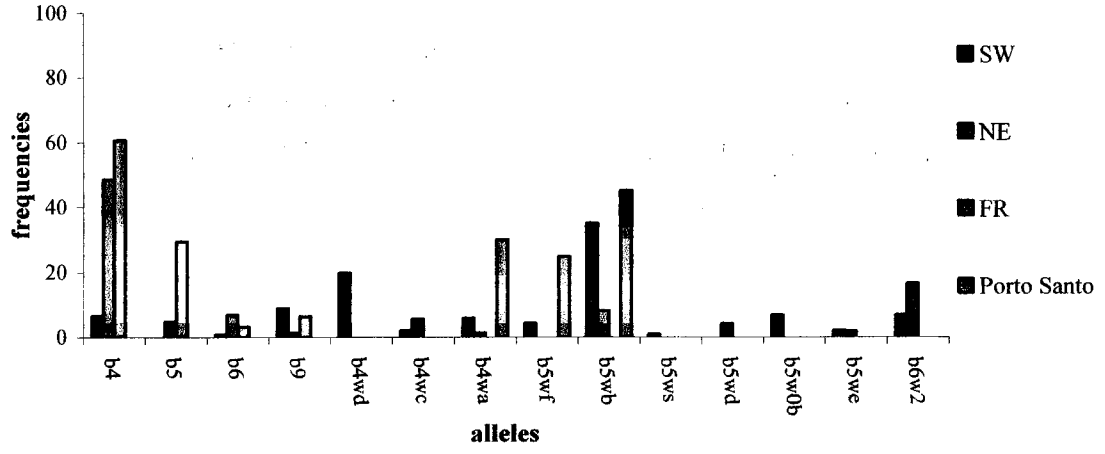

Figure 4.2. Gene frequencies obtained at *b* locus in rabbit populations from SW and NE of Iberian Peninsula, France and in Porto Santo.

Microsatellites

The gene frequencies obtained in Porto Santo as well as in a set of wild rabbit populations of different origins in 9 microsatellite markers (Queney *et al.*, 2001) were used to construct a NJ tree (see Figure 4.3) using the genetic distance of Nei (Nei, 1978). The tree clearly separates three groups: one that is composed by Southwest Iberian populations, where Porto Santo is included (clustering again with the Portuguese population of Idanha), other that includes the Iberian populations from the Northeast and a third group composed exclusively by French populations. Previous studies with this set of microsatellites showed that the French (FR) populations are closely related with the domestic breeds (Queney *et al.*, 2002).

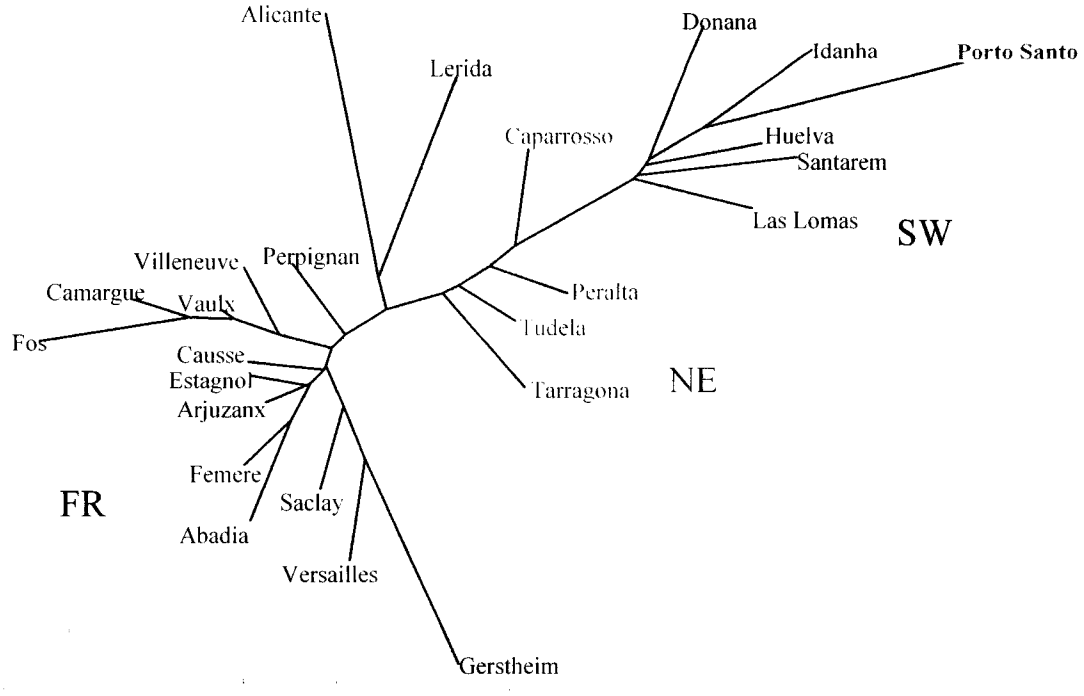

Figure 4.3. NJ tree obtained with Nei's genetic distances of calculated using the gene frequencies of 9 microsatellites studied in Porto Santo, the Iberian Peninsula and in French rabbit populations. SW - Southwest of Iberian Peninsula; NE - Northeast of Iberian Peninsula; FR - France.

Mitochondrial DNA

Cytochrome b

The restriction fragment profiles obtained in the Porto Santo population correspond to the mtDNA haplotype ARb4 (Branco, 2000; Branco *et al.*, 2000), which was only found in Portuguese populations, where it is the most common. The frequencies of each haplotype of mtDNA lineage *A* in Portuguese, Spanish and in Porto Santo populations are shown in Figure 4.4. In all wild rabbit populations from outside of Iberian Peninsula and in all domestic breeds studied so far only the mtDNA lineage *B* was detected. Consequently, this result confirms that the Porto Santo rabbit population does show closest relationships with the Portuguese populations and is clearly not derived from a domestic population.

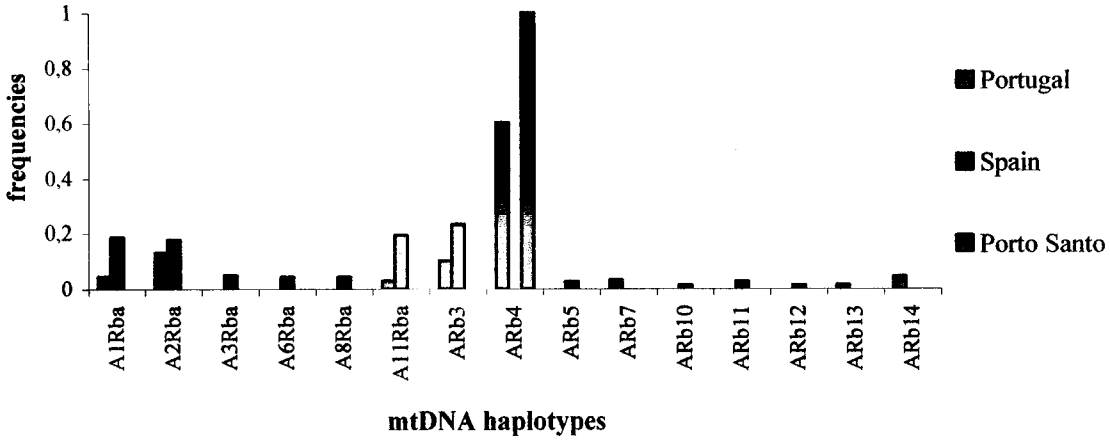

Figure 4.4. mtDNA haplotypes of lineage *A* obtained in rabbit populations from Portugal and Spain and in the island population of Porto Santo.

Control region

We sequenced a fragment with 373 bp of the control region in seven rabbits from Porto Santo. All of these sequences were identical. Part of this haplotype was compared to 53 different Iberian haplotypes defined by 56 variable sites in a fragment of 179-181 bp (Branco *et al.*, 2002). The NJ tree representing the relationships between these haplotypes is presented in figure 4.5. Although representing a haplotype that was not detected in the previously studied populations, the sequence found in Porto Santo, called here Pst, clusters within a group that is clearly of Portuguese origin.

Taken together, these results clearly show that founders of the Porto Santo rabbit population were drawn from Southwestern Iberian populations belonging to the subspecies *Oryctolagus cuniculus algirus*. The clustering with the Portuguese population of Idanha both for protein and microsatellite trees, the fixation of a mtDNA *A* type only present in the Portuguese populations and the fact that the control region haplotype found in Porto Santo is more related to the described in the Portuguese populations strongly suggest a Portuguese origin.

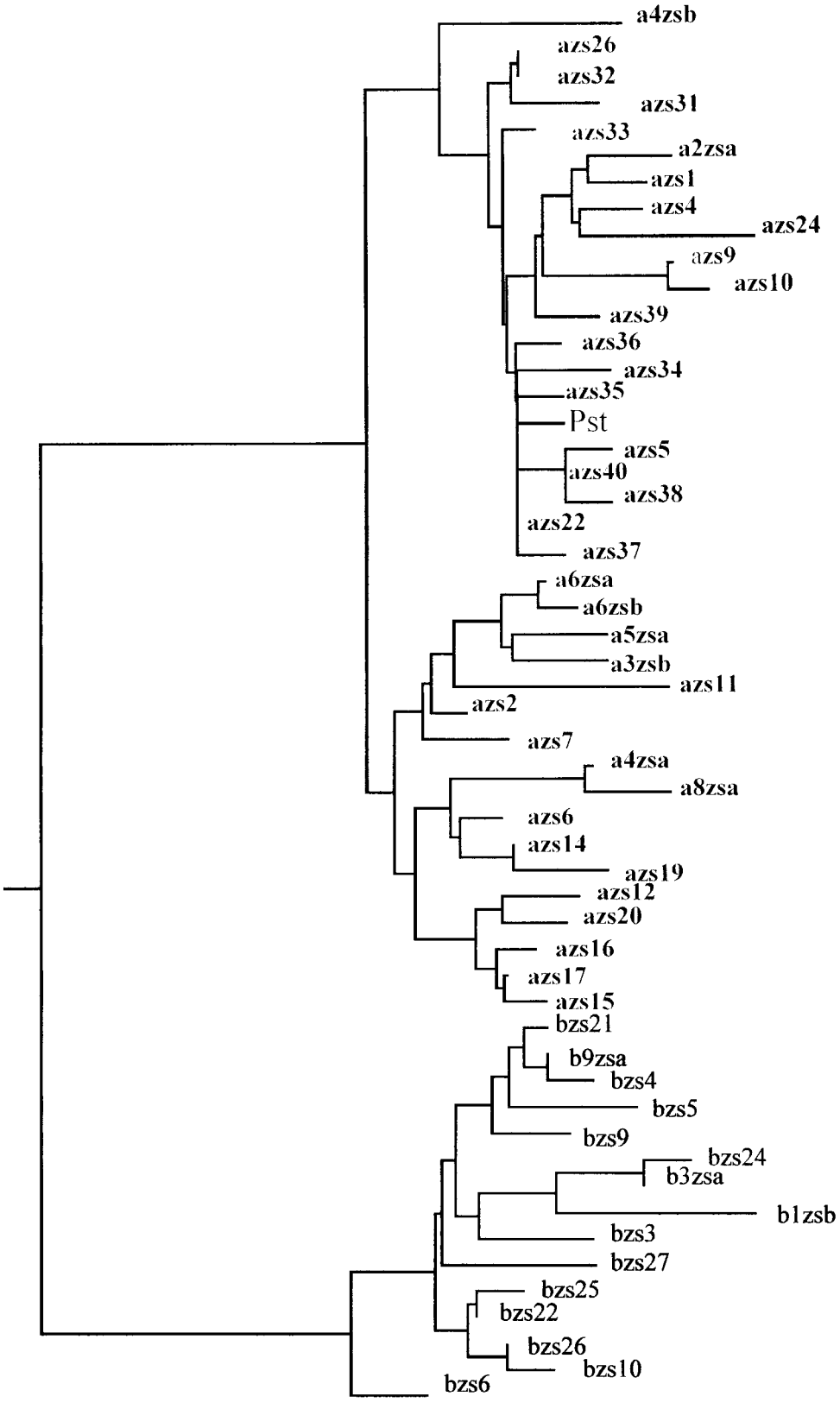

Figure 4.5. NJ tree for control region haplotypes obtained in wild rabbit populations. ■ haplotypes A obtained in Western Spanish populations; ■ haplotypes A obtained in Portuguese populations; ■ haplotypes B obtained in Eastern Spanish populations; ■ haplotype obtained in Porto Santo.

Article 5

P.J. Esteves, S. Weiss, J. Rocha, G. Queney, M. Branco, W. van der Loo and N. Ferrand (Submitted).

The "Rabbit-Eve" of Porto Santo: A 500 year-old mammalian population derived from a single female

The "Rabbit-Eve" of Porto Santo: A 500 year-old mammalian population derived from a single female.

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How many individuals does it take for establishing a viable mammal population? An early answer to this key question in evolutionary sciences, was proposed by Noah, who allowed for «one of each gender» on his ark (Genesis 6-9). Closer to us, Charles Darwin[1] reported how in 1419 AD Bartolomeu Perestrelo, by introducing a single pregnant rabbit doe into the island of Porto Santo (Madeira Archipelago, Portugal), founded a population, which within a few years was considered a plague[2,3]. Here we report genetic data that are in strong support of the Single Female Ancestor (SFA) hypothesis of the contemporary Porto Santo rabbits.

Of 38 cyt*b* haplotypes described in Iberian populations[4], one single haplotype occurred in Porto Santo, which was furthermore unique to Portugal. In addition, the sequencing of 350 base-pairs in the mtDNA hypervariable region from seven Porto Santo rabbits confirmed the occurrence of a single haplotype, in sharp contrast with the extreme degree of polymorphism resolved in continental populations[5]. The distinct allelic variation at nuclear loci confirmed Southwestern Iberia as the origin of Porto Santo rabbits[6]. We have estimated the allele frequencies at 30 loci that were polymorphic in this area (22 protein loci and eight microsatellites). In Porto Santo, all loci revealed four or fewer alleles, except one microsatellite locus, which had a fifth allele. Estimating 500 generations since the introduction, and a mean mutation rate of 5×10^{-4} per locus/per generation, the emergence of a new microsatellite allele at Porto Santo is expected and therefore concordant with four sets of founder chromosomes.

Of more relevance is the fact that our knowledge of the allele frequencies in the source population allows estimates of the expected number of loci that would retain a given number of distinct alleles, if either four (SFA), or more than four, chromosomes were drawn randomly from it. It appears that the distribution observed in Porto Santo is a perfect match of expectations under SFA. For microsatellites, where most loci display more than ten alleles in the founder region[7], the alternative hypothesis can be rejected ($P<0.01$). Remarkably, two of these microsatellites are closely linked in the mammalian casein gene cluster[8,9] and occur in complete linkage disequilibrium defining four haplotypes, while a sample of four southwestern rabbit populations exhibited a minimum of 80 haplotypes. For protein loci, however, where gene diversity is more limited, observations are in accordance with four to eight founding chromosomes.

We have therefore used an additional criterion. Because the founding event was immediately followed by exponential expansion[2,3], the initial allele frequencies might, to some extent, have been conserved. Under the SFA hypothesis, these frequencies should be multiples of 25% or quartiles. The observed distribution of allele frequency classes across loci most convincingly supports a single female founder (Figure 1).

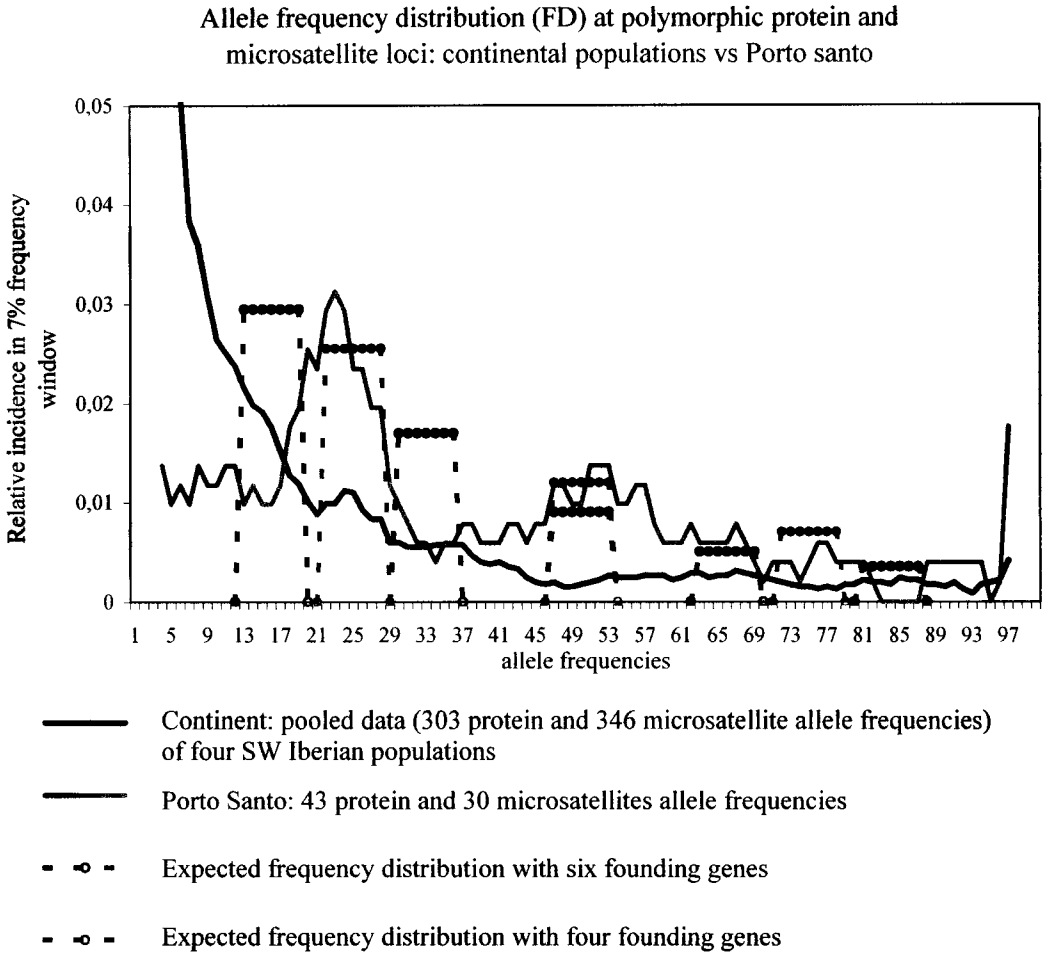

Figure 1. Distribution of allelic frequency classes for 8 microsatellite and 22 protein loci. Frequency classes are centered on the expected quartiles among four founding chromosomes, with the distribution tail split between rare (<12.5%) and frequent (>12.5%) alleles. Normal populations display a L-shaped frequency distribution whereas extreme bottlenecks are marked by intermediate frequencies. Several markers in our data reveal allele frequencies matching perfectly or nearly so expected quartiles (e.g. NP, n=66, 3 alleles at 0.50, 0.25, 0.25, HBA, n=59, 2 alleles at 0.52, 0.48, PGD, n=64, 2 alleles at 0.55, 0.45, PEPC, n=68, 4 alleles at 0.28, 0.28, 0.18, 0.27, PEPD, n=55, 2 alleles at 0.45, 0.55, GALT, n=15, 2 alleles, 0.57, 0.43, HPX, n=16, 2 alleles, 0.50, 0.50, sat3, n=31, 2 alleles 0.79, 0.21, sat8, n=30, 4 alleles 0.23, 0.22, 0.20, 0.35).

We provide therefore strong empirical support for the viewpoint that the rabbit population of Porto Santo was indeed founded by a single pregnant female, to our knowledge representing the only such wild mammalian population. As subsequent bottlenecks or introductions apparently did not occur, it provides a superb natural laboratory for testing theoretical expectations of allelic drift, evaluating mammalian mutation rates or studying natural selection. This report also suggests that gene diversity conveyed by one female can sustain long-term population viability.

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Evaluating bottlenecks in wild rabbit populations

Genetic diversity

Different measures of genetic diversity (average number of alleles per locus (n_a), percentage of polymorphic loci (P) and the average of expected heterozigosity (He) were obtained using BIOSYS program version 1.7 (Swofford and Selander, 1989). The average values of the different measures of genetic diversity obtained in Iberian populations for protein loci (Ferrand, 1995; Branco, 2000), microsatellites (Queney, 2000; Queney *et al.*, 2001) and immunoglobulin markers (van der Loo *et al.*, 1999) and the values of genetic diversity obtained in Porto Santo studied in this work are presented in Table 4.3.

Table 4.3
Genetic diversity obtained in the SW, NE, FR, domestic and Porto Santo rabbit populations, for protein, microsatellites and two Immunoglobulin markers (*a* and *b* locus). n_a is the average number of alleles per locus; He is the average expected heterozygosity; P is percentage of polymorphic loci.

	SW			NE			FR			Porto Santo			Domestic		
Markers	n_a	He	P	n_a	He	P	n_a	He	P	n_a	He	P	n_a	He	P
Microsatellite	9.3	0.84	100	7.8	0.78	100	5.2	0.60	100	3.44	0.53	100	3.2	0.49	100
Protein	3.0	0.35	77.8	2.1	0.23	65	1.7	0.20	57	1.9	0.26	63	1.6	0.14	43
Immunogenetic	7.2	0.60	100	7	0.67	100	4	0.59	100	3	0.50	100	2.5	0.40	100

As expected, gene diversity was lower in Porto Santo than in most Iberian mainland populations. However, the diversity of protein markers in Porto Santo population is higher than the observed in wild rabbit populations from France or in the domestic breeds. In microsatellite markers the levels of diversity observed in Porto Santo are slightly lower than those observed in French populations, but higher that those obtained in the domestic breeds. Also in loci under selection, such as immunoglobulin markers, the level of diversity observed in Porto Santo is similar or higher than the obtained in wild populations outside the Iberian Peninsula and in the domestic populations (van der Loo *et al.*, 1991, 1999). These results show that the genetic diversity conveyed in this population is generally higher than most of the natural French populations and may eventually explain long-term population viability.

Statistical tests to detect bottleneck situations

Table 4.4 shows the probability values and the model-shift descriptor outcomes obtained in Portuguese island populations (Porto Santo, São Jorge and Flores), in five Southwest Iberian Peninsula populations (Las Lomas, Huelva, Doñana, Idanha and

Santarém), in three Northeast Iberian populations (Lerida, Alicante and Tudela) and in three French populations (Camargue, Perpignan and Versailles). Independent of the type of data, all statistical tests showed a situation of mutation-drift equilibrium for all continental populations, i.e. approximately 50% of the loci sampled are a slight excess (Ho>He) or deficiency (Ho<He) heterozygosity, and a bottleneck signature for island populations ($p<0.05$), the majority of loci have a heterozygosity excess (Ho>He) (see Figures 4.4 and 4.5). The "mode-shift" descriptor showed for all continental populations a normal L-shaped distribution while in all island populations a mode-shift distribution was observed.

Table 4.4

Probability values obtained in the bottleneck test, and the result obtained in the mode-shift descriptor for five Southwest, three Northeast and three island populations. IAM- Infinite Allele Model; TPM- Two-phase Mutation Model; WT- Wilcoxon test; SDT- Standardized difference test.

	Microsatellite			**Proteins**		
	TPM (90%)	**Mode-shift**	**Reference**	**IAM**	**Mode-shift**	**Reference**
Continental populations						
Southwest						
Donana	0.08 (WT)	Normal	1	0.07 (SDT)	Normal	2, 3
Huelva	0.75 (WT)	Normal	1	0.36 (SDT)	Normal	2, 3
Santarém	0.08 (WT)	Normal	1	0.06 (SDT)	Normal	2, 3
Las Lomas	0.15 (WT)	Normal	1	0.37 (SDT)	Normal	2, 3
Idanha	0.18 (WT)	Normal	1	0.74 (WT)	Normal	2, 3
Northeast						
Alicante	0.32 (WT)	Normal	1	0.41 (WT)	Normal	2, 3
Lerida	0.75 (WT)	Normal	1	0.34 (WT)	Normal	2, 3
Tudela	0.41 (WT)	Normal	1	0.40 (WT)	Normal	2, 3
France						
Camargue	0.25 (WT)	Normal	1	0.10 (WT)	Normal	Normal
Versailles	0.15 (WT)	Normal	1	0.07 (WT)	Normal	Normal
Perpignan	0.59 (WT)	Normal	1	**n. a.**	**n. a.**	**n. a.**
Island populations						
Porto Santo	0.006*(WT)	Shifted	This work	0.0004*(WT)	Shifted	This work
São Jorge	n. a.	n. a.	n. a.	0.0005*(WT)	Shifted	2
Flores	n. a.	n. a.	n. a.	0.0009*(WT)	Shifted	2

1) Queney *et al.* (2001);
2) Ferrand (1995);
3) Branco (2000).
n. a. - There is no data available

In the SW and NE rabbit populations the most frequent allelic classes are the rare alleles (0.00-0.10 and 0.10-0.20). In contrast, for Porto Santo the most frequent are classes with intermediate frequencies (0.20-0.30, 0.40-0.50 and 0.50-0.60, see Figures 4.4 and 4.5. These results revealed, as expected, that all insular rabbit populations showed the signature of a recent bottleneck and a "shifted mode" allelic distribution. In contrast, the continental rabbit populations are clearly under mutation/drift equilibrium and have a normal L-shaped allelic distribution. This indicates that natural rabbit populations have maintained the same level of genetic diversity even after multiple epizootic episodes of myxomatosis and haemorragic viral diseases.

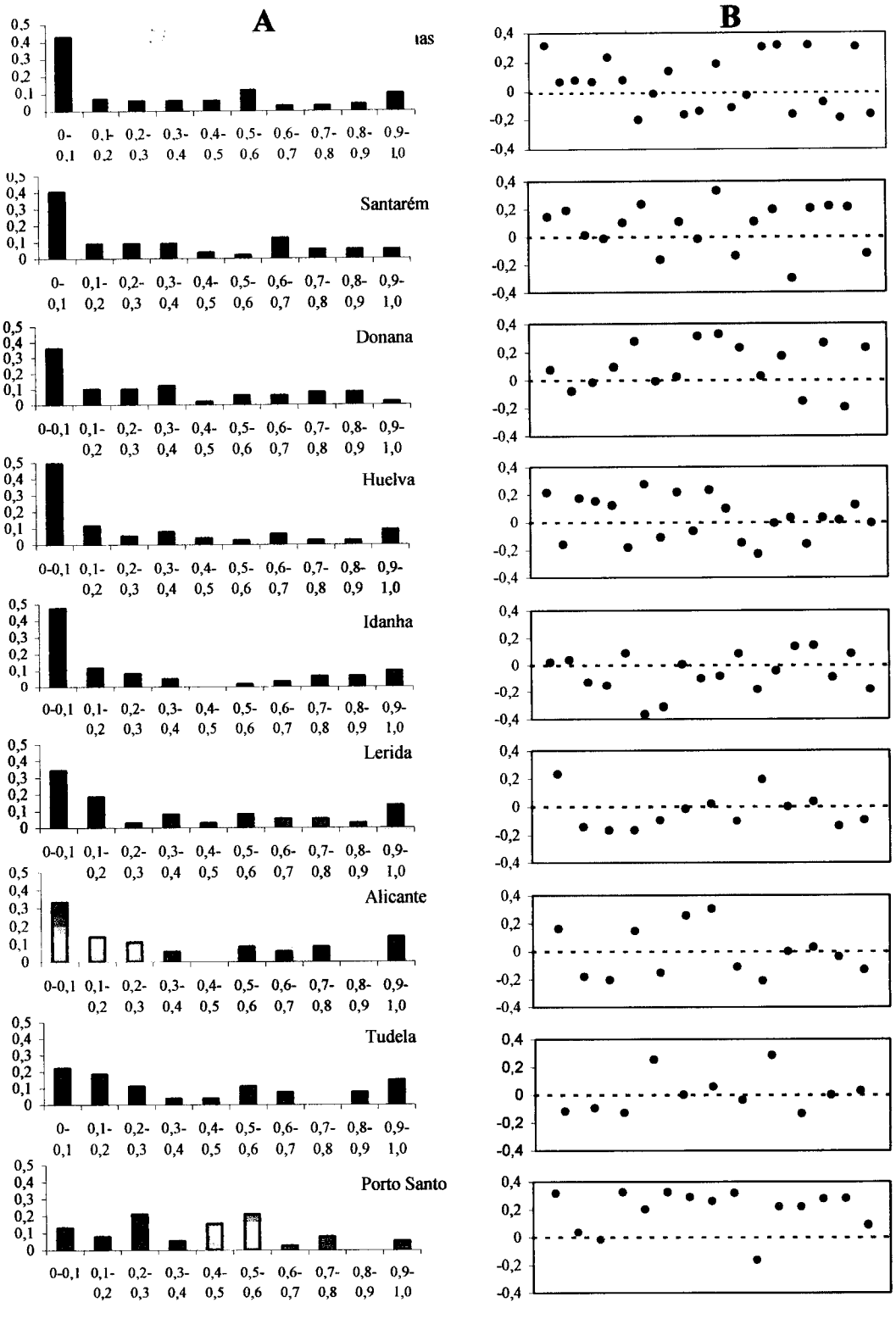

Figure 4.4. A- Distribution of allelic frequencies at selected protein loci in wild rabbit populations from Southwestern and Northeastern Iberia and the island of Porto Santo. **B-** Magnitude of heterozygosity excess observed at each polymorphic protein locus. Dots above the dashed line represent loci with heterozygosity excess; dots below are loci with heterozygosity deficiency.

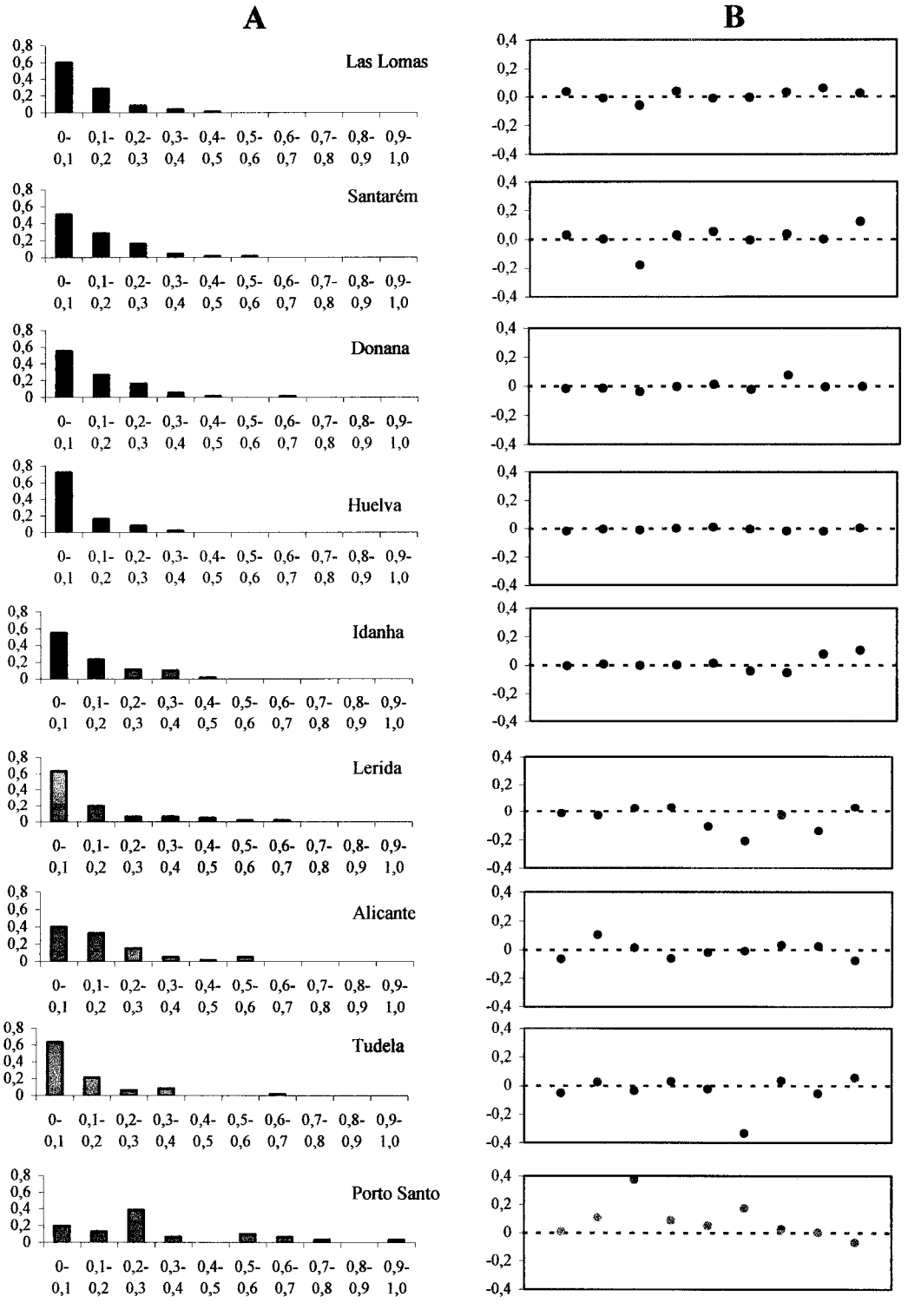

Figure 4.5. A- Distribution of allelic frequencies at nine microsatellite loci in wild rabbit populations from Southwestern and Northeastern Iberia and the island of Porto Santo. **B-** Magnitude of heterozygosity excess observed at each polymorphic microsatellite loci. Dots above the dashed line represent loci with heterozygosity excess; dots below are loci with heterozygosity deficiency.

The likelihood that one single pregnant female originated the Porto Santo rabbit population

In article 5 we provided strong empirical support for the viewpoint that the rabbit population of Porto Santo was indeed founded by a single pregnant female. The methodology used to estimate the expected number of loci that would retain a given number of distinct alleles, if either four, or more than four, chromosomes were drawn randomly from the original species range is given in the appendix 1. The likely allele frequencies in the population from which the founders of the Porto Santo population originated were inferred from the allele frequencies in four rabbit populations (Las Lomas, Doñana, Santarém and Huelva; SW of Iberian Peninsula). Data for protein markers were from Ferrand (1995) and Branco (2000), immunoglobulin markers from van der Loo *et al.* (1999) and microsatellites from Queney *et al.* (2001). The expected probability values under the hypotheses of four or six chromosomes founding the population for microsatellite and protein data are presented in appendix 2 (tables A1 and A2, respectively). Additionally, for microsatellites three different scenarios were considered: 1) no mutations; 2) assuming mutation at Sat5 and 3) mutations at Sat 5 and Sat 7 (appendix 2 see figure A1).

Implications for the trans-generic origin of the complex Ig allotypes

The explanation of the large inter-allelic distances at the V_H1 and the C_K1 loci by the trans-generic hypothesis has specific and profound implications with regard to sizes of founder populations, which must always be large enough to contain each of the different alleles. These numbers are at least four, for the V_H1 locus, and larger than six, for the C_K1 locus, suggesting average founder populations sizes of some 10 to 20 individuals. The demonstration that populations can be founded by just two individuals (four chromosomes) is not supportive for the trans-generic origin of the complex Ig allotypes, and it suggests, on the contrary, that lineages have a high chance of being lost during speciation steps.

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CHAPTER 5

Conclusions

In this work we studied two rabbit immunogenetic loci, the *IgGCH2* or *e* locus and the *IgVH1* or *a* locus. These loci both contribute to biosynthesis of an immunoglobulin H chain. However, they differ in complexity, in the level of diversity and in the phylogeographic patterns of distribution. The *IgGCH2* is a single gene locus whereas the *IgVH* gene locus is a multigene locus. The *IgCH2* polymorphism is restricted to a single amino acid substitution whereas large inter-allelic distances characterise the *IgVH1* polymorphism. The *IgGCH2* polymorphism has a recent age whereas the *IgVH1* is very ancient and persisted in the populations for long period of time. For the complex rabbit allotypes (*IgVH* or *a*-locus and *IgCK1* or *b*-locus) the initial evidence for the adaptiveness of variation is based upon large inter-allelic distances. In contrast, in the case of *IgGCH2* it is based upon population genetic evidence (see chapter 2), which indicated that the polymorphism at the *e*-locus is adaptive only through the lack of diversity at *a*- and *b*-loci.

Gene diversity at the *e* locus (*IgGCH2*) in *Oryctolagus cuniculus*

The geographical variation at this locus differs from that observed at other loci studied until now. Indeed, in the original species range, this polymorphism, defined by one amino acid substitution at position 309, is fixed for *e15* allele whereas it is polymorphic in all wild and feral rabbit populations studied ($p_{e14} \approx 20\%$) in the more recent area of the species distribution. The linkage disequilibrium with V_H locus implies that *e14* is a recent polymorphism (see chapter 2). However, the sequence data available prior to this work indicated that the *e14* allele was more ancestral than *e15*. Our data for Iberian wild rabbit specimens show that the allele *e14* can indeed be derived by a single nucleotide substitution from an *e15* lineage present in the Iberian Peninsula. The sequences obtained here also revealed six new polymorphic nucleotide positions, of which two were associated with amino acid substitutions. The occurrence of this polymorphism can be explained either by compensatory overdominance (i.e. it emerged in order to compensate the reduction of diversity at *a*- and *b*- loci in the populations from outside of Iberian Peninsula) or by a selective advantage of *e14* allele when exposed to a particular microorganism.

The sequences that we produced from other leporids and the analyses of CH2 sequences of other mammals, showed in general an excess of dN (non-synonymous substitutions) at this position, which confirms the population genetic evidence for adaptive variation.

Gene diversity at the *IgVH* in leporids

Genetic diversity in Iberian rabbit populations

In domestic breeds and in wild and feral populations from outside the Iberian Peninsula in essence three alleles (*a1*, *a2* and *a3*) contribute effectively to the *a* locus diversity. We analysed serologically 18 wild rabbit Iberian Peninsula populations which allowed us to distinguish different endemic alleles, one of which ("*a*-blank") was particular by failing to react with specific *a1*, *a2* and *a3* antisera. The frequency distribution suggests that the "*a-blank*" phenotype is associated with the subspecies *O. c. algirus*. This pattern is similar to that previously reported in the Iberian Peninsula at *IgCK1* or *b* locus, where the most common alleles (*b4* and *b5*) of *O. c. cuniculus* populations were rare or absent in *O. c. algirus* populations. These associations of Ig allotypes with subspecies could be explained by the fact that subspecies occupy different habitats, where there are exposed to different environmental factors. A bottleneck effect seems to be the most likely explanation for the fact that the most common alleles of the Iberian populations did not cross the Pyrenean Mountains.

Our data show that the rabbits serologically homozygous for the "*a-blank*" allele use preferentially V_H genes that, although clearly related to the known V_H1 genes, define a new major allotypic lineage, called here *a4*. We conclude that there exist at least four distantly related lineages at the rabbit *IgVH1* or *a* locus.

Genetic diversity in Lepus *species*

VH gene sequences of leporids (other than *Oryctolagus*) had never been obtained before this work. Here we sequenced *VH* genes that were expressed in specimens of two *Lepus*, *L. europaeus* and *L. granatensis* species. The results obtained show two *VH* lineages: one with the signature of V_Ha alleles (*a2L*), and another unrelated to these (*nL*). The comparison of these lineages with the rabbit alleles revealed that *a2L* is more similar to the rabbit *a2* than *a2* is to its rabbit allelic counterparts. This supports a trans-species polymorphism hypothesis, i.e. that very long lineage persistence times explain this high allelic divergence.

Are the allotypes described in Lepus *(*a2L *and* nL*) truly alleles?*

The V_H gene segments that encode the allotypes *a1*, *a2* and *a3* have been mapped in the most *D*-proximal region of the V_H cluster (i.e. V_H1). However, the genomic location of

these newly described lineages (*a4*, *a2L* and *nL*) is unknown. It seems likely that the lineages *a4* and *a2L*, which cluster with the rabbit allotypes *a1*, *a2* and *a3* in the $V_H a$-like group, are encoded by allelic variants of the $V_H 1$ locus. However, the *nL* sequences show more sequence similarity with the $V_H x$, and $V_H y$ gene segments of rabbit, which, in this species, are located more than 50 kb upstream of the $V_H 1$ locus. The preferential expression of the *nL* allotype in *Lepus* specimens could indicate: a) they are indeed encoded by the *D*-proximal V_H locus (i.e. the homologue of rabbit $V_H 1$). Such situation would suggest that V_H gene segments can be reshuffled; b) unlike in the rabbit, in hare species the preferential expression of V_H genes is not imposed by its *D*-proximal position. To evaluate the likelihood of these scenarios a genomic library of V_H gene segments must be constructed with amplification and sequencing of the most *D*-proximal V_H genes.

The role of intestinal microflora in the $V_H 1$ *polymorphism*

The origin of the selection forces that regulate the $V_H 1$ polymorphism is unknown. However, the requirements of exogenous factors, such as the intestinal microflora, for the diversification of the primary rabbit antibody repertoire in the rabbits, suggest that this mechanism might impose constraints on the evolution of the $V_H 1$ alleles. The rabbit is a species with altricial young (naked and blind at birth). Other lagomorph species, such as hares (genus *Lepus*) have precocial young. Considering the differences in reproductive biology between the genera *Oryctolagus* and *Lepus*, it might be interesting to investigate if in *Lepus* the Ab repertoire is diversified neonatally or during foetal life (rather than after two or three weeks like in *Oryctolagus*). The unique situation of post-natal diversification in rabbit could be related to the shortening of gestation times (4 weeks in rabbit and six weeks in hares).

The $V_H 1$ *large inter-allelic differences*

Regarding the two different hypotheses invoked to explain the very large interallelic distances observed at the *IgVH1 locus*, the obtained results seem to be contradictory. On 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 $V_H 1$ polymorphism between *Oryctolagus* and *Lepus* supports the hypothesis that very long lineage persistence times have contributed to allelic divergence. The different evidences obtained point 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.

For persistence of distinct lineages it is necessary that, during the several episodes of population expansion and retraction, the effective population size remains large enough to maintain these lineages. The study of Porto Santo showed that one population might survive with a gene pool reduced to four chromosomes. This makes it unlikely that four or more allelic lineages can be maintained for millions of years.

Evolution of V_H *gene in the lagomorph group*

Under the concerted evolution model, the multigene family genes show high homogenisation and all the genes of the same species are clustered together. In contrast, the multigene family genes evolving according to the birth-and-death model, when duplicated, can diverge functionally. The end result of this process is an admixture of both divergent and highly homologous groups of genes. The analyses of V_H gene segments obtained in several vertebrate species showed different evolutionary patterns. Indeed, the human and mouse possess V_H gene sequences that are very diversified and clustered within all groups, whereas V_H gene sequences from birds, rabbit, swine, sheep and cattle, are restricted to one V_H group. To understand the mechanisms that regulate the evolution of these genes, more species must be studied, in particular those that live in natural conditions and are submitted to changing environmental conditions.

We extended the knowledge of the V_H gene segment to other lagomorph species (genera: *Sylvilagus* and *Ochotona*). The phylogenetic analysis of these sequences suggests that only V_H genes belonging to group C are present in the genome of lagomorph species. Probably, the loss of V_H genes from groups A and B happened in a lagomorph ancestor at least 50 My ago, which is the estimated time of divergence between *Ochotonidae* and *Leporidae*. We speculate that in the lagomorph ancestor a particular family of V_H gene (class C) became more used in *VDJ* rearrangements and compensated at the same time the reduction of diversity by somatic diversification. In this way, the loss of all other V_H gene families from the genome became possible.

The lagomorph V_H gene group is divided into four clusters, V_Ha, V_Hn, V_HnL and V_HP. The V_H gene sequences from different lagomorph species can cluster within the same group and V_H gene sequences of the same species are sometimes clustered in different

groups (for example in both V_Ha and V_Hn group are present sequences of *Lepus*, *Oryctolagus* and S*ylvilagus).* These results suggest that the lagomorph V_H gene segments follow the model of birth-and-death evolution, i.e. during the V_H gene duplication process several genes diverged functionally while others maintained high homology. The intestinal microflora is probably a factor contributing to the occurrence of highly divergent V_H genes sequences within the lagomorph group.

Evaluating the bottleneck effect in the Porto Santo wild rabbit population

The data reported in this work support historical evidence that only one pregnant rabbit female was introduced to the Porto Santo Island. Introductions with a reduced effective size generally provoke a reduction in the number of alleles and in the heterozygosity values. This is more dramatic when only four chromosomes founded the population. The different parameters of genetic variability (He, P and n_a) obtained for protein, microsatellite and immunogenetic markers in Porto Santo show that, although the level of diversity in this island is lower than the genetic diversity detected in the Iberian populations, it is still higher than the natural French populations and domestic breeds. This relatively high level of diversity may explain why a population founded by only one pregnant female became established and survived until today and give some indications about the minimum level of diversity that is necessary for sustaining long-term population viability. It highlights the fact that it is not the size of the founder population that is important, but the amount of genetic variation it contains.

Appendix 1

Question: What is the number of possible rearrangements with p alleles taking out n chromosomes from one population?

The total number of possible rearrangements for each possibility is estimated by:

$$\alpha_n{}^p = p^n,$$

where p is the number of distinct categories (alleles) and n is the number of classes (chromosomes).

a) The probability of obtaining one allele is given by the formula:

$$P(X) = p^n,$$

where p is the frequency of allele X and n is the number of classes.

b) The probability of obtaining two alleles is given by the formula of the distribution of binomial probability:

$$P(X) = \frac{n!}{n\text{-}x!\times x!} \times \left[\left(q^{n-x}\right) \times p^x\right],$$

where, n is the number of classes, x is the number of copies of each distinct category; p and q are the frequencies of each category.

c) The probability to obtain three or more alleles is giving by the formula of distribution of multinomial probability

$$P(X_1, X_2, \ldots\ldots, X_k) = \frac{n!}{X_1! \times X_2! \ldots X_k} \times \left[\left(p_1{}^{X1} \times p_2{}^{X2} \times \ldots\ldots \times p_k{}^{Xk}\right)\right]$$

where, n is the number of classes, x is the number of copies of each distinct categories, p is the frequency of each category.

Hypothesis 1: The Porto Santo rabbit population was originated from only one pregnant female (four genes)

1. To take out four genes and to obtain only one allele (a)

Total number of rearrangements:

$$\alpha_4{}^1 = 1^4 = 1$$

a) fixation of one allele (all copies belong to the same category)*

$[\ a^4\ ]\quad 1\ *$

2. To take out four genes and to obtain two alleles (a, b):

Total number of possible rearrangements

$$\alpha_4{}^2 = 2^4 = 16$$

a) fixation of one allele (all copies belong to the same category)

$$\begin{bmatrix} a^4 \\ b^4 \end{bmatrix} \quad 2$$

b) to obtain only two alleles (categories *a* and *b*) *

$$\begin{pmatrix} 4a^3b \\ 6a^2b^2 \\ 4ab^3 \end{pmatrix} \quad 14\ *$$

$$\text{e.g. } P(3a,1b) = \frac{4!}{3! \times 1!} \times \left[\left(p_a{}^3\right) \times p_b\right] = 4ab^3$$

The sum of rearrangements obtained in the point a) and b) (2+14) correspond as expected to the total number of possible rearrangements 16.

3. To take out four genes and to obtain three distinct alleles (a, b, and c):

Total number of possible rearrangements

$$\alpha_4{}^3 = 3^4 = 81$$

a) fixation of one allele (all copies belong to the same category)

$$\begin{pmatrix} a^4 \\ b^4 \\ c^4 \end{pmatrix} \quad 3$$

b) to obtain only two alleles (categories *a* and *b; a* and *c; b* and *c*)

$$\begin{pmatrix} 4a^3b \\ 6a^2b^2 \\ 4ab^3 \end{pmatrix} \begin{pmatrix} 4a^3c \\ 6a^2c^2 \\ 4ac^3 \end{pmatrix} \begin{pmatrix} 4b^3c \\ 6b^2c^2 \\ 4bc^3 \end{pmatrix} \quad 3\times14 = 42$$

c) to obtain three distinct alleles (distinct categories *a, b,* and *c*) *

$$\begin{pmatrix} 12a^2bc \\ 12ab^2c \\ 12abc^2 \end{pmatrix} \quad 3\times12 = 36\ *$$

$$\text{e.g. } P(2a,1b,1c) = \frac{4!}{2!\times1!\times1!}\times\left[\left(p_a{}^2\right)\times p_b\times p_c\right] = 12a^2bc$$

The sum of rearrangements obtained in the point a), b), and c) (3+42+36) correspond as expected to the total number of possible rearrangements 81.

4. To take out four genes and to obtain four distinct alleles (a, b, c, and d):

Total number of possible rearrangements

$$\alpha_4{}^4 = 4^4 = 256$$

a) fixation of one allele (all copies belong to the same category)

$$\begin{pmatrix} a^4 \\ b^4 \\ c^4 \\ d^4 \end{pmatrix} \quad 4$$

b) to obtain only two alleles (categories: *a* and *b; a* and *c; a* and *d; b* and *c; b* and *d; c* and *d*).

$$\begin{pmatrix} 4a^3b \\ 6a^2b^2 \\ 4ab^3 \end{pmatrix} \begin{pmatrix} 4a^3c \\ 6a^2c^2 \\ 4ac^3 \end{pmatrix} \begin{pmatrix} 4a^3d \\ 6a^2d^2 \\ 4ad^3 \end{pmatrix} \begin{pmatrix} 4b^3c \\ 6b^2c^2 \\ 4bc^3 \end{pmatrix} \begin{pmatrix} 4b^3d \\ 6b^2d^2 \\ 4bd^3 \end{pmatrix} \begin{pmatrix} 4c^3d \\ 6c^2d^2 \\ 4cd^3 \end{pmatrix}$$

$$6 \times 14 = 84$$

c) to obtain three distinct alleles (categories: *a, b,* and *c; a, b, and d; a, c, and d; b, c, and d*).

$$\begin{pmatrix} 12a^2bc \\ 12ab^2c \\ 12abc^2 \end{pmatrix} \begin{pmatrix} 12a^2bd \\ 12ab^2d \\ 12abd^2 \end{pmatrix} \begin{pmatrix} 12a^2cd \\ 12ac^2d \\ 12acd^2 \end{pmatrix} \begin{pmatrix} 12b^2cd \\ 12bc^2d \\ 12bcd^2 \end{pmatrix}$$

$$4 \times 36 = 144$$

d) to obtain four distinct alleles (distinct categories: a, b, c, and d) *.

$$\begin{pmatrix} 24\ abcd \end{pmatrix} \quad 24\ *$$

$$\text{e.g. } P(1a,1b,1c,1d) = \frac{4!}{1! \times 1! \times 1! \times 1!} \times (p_a \times p_b \times p_c \times p_d) = 24abcd$$

The sum of rearrangements obtained in the point a), b), c), and d) (4+84+144+24) correspond as expected to the total number of possible rearrangements 256.

Hypothesis 2: The Porto Santo rabbit population was originated from three rabbits (six genes)

1. To take out six genes and to obtain only one allele (a):

Total number of possible rearrangements

$$\alpha_6^1 = 1^6 = 1$$

a) fixation of one allele (all copies belong to the same category) *

[a^6] 1*

2. To take out six genes and to obtain two alleles (a,b):

Total number of possible rearrangements

$$\alpha_6^2 = 2^6 = 64$$

a) fixation of one allele **(all copies belong to the same category)**

$$\begin{bmatrix} a^6 \\ b^6 \end{bmatrix} \quad 2$$

b) to obtain only two alleles (distinct categories a and b) *

$$\begin{pmatrix} 6a^5b \\ 15a^4b^2 \\ 20a^3b^3 \\ 15a^2b^4 \\ 6ab^5 \end{pmatrix} \quad 62 *$$

$$\text{e.g. } P(5a,1b) = \frac{6!}{5! \times 1!} \times \left[\left(p_a{}^5 \right) \times p_b \right] = 6a^5b$$

The sum of rearrangements obtained in the point a) and b) (2+62) corresponds as expected to the total number of possible rearrangements 16.

3. To take out six genes and to obtain three distinct alleles (a, b, and c):

Total number of possible rearrangements

$\alpha_6^3 = 3^6 = 729$

a) fixation of one allele (all copies belong to the same category)

$$\begin{pmatrix} a^6 \\ b^6 \\ c^6 \end{pmatrix} \quad 3$$

b) to obtain only two alleles (distinct categories: *a* and *b; a* and *c; b* and c).

$$\begin{pmatrix} 6a^5b \\ 15a^4b^2 \\ 20a^3b^3 \\ 15a^2b^4 \\ 6ab^5 \end{pmatrix} \quad \begin{pmatrix} 6a^5c \\ 15a^4c^2 \\ 20a^3c^3 \\ 15a^2c^4 \\ 6ac^5 \end{pmatrix} \quad \begin{pmatrix} 6b^5c \\ 15b^4c^2 \\ 20b^3c^3 \\ 15b^2c^4 \\ 6bc^5 \end{pmatrix} \quad 62\times3=186$$

c) to obtain three distinct alleles (distinct categories: *a, b*, and *c*) *

$$\begin{pmatrix} 30a^4bc \\ 60a^3b^2c \\ 60a^3bc^2 \\ 60a^2bc^3 \\ 60a^2b^3c \\ 90a^2b^2c^2 \\ 60ab^3c^2 \\ 60ab^2c^3 \\ 30ab^4c \\ 30abc^4 \end{pmatrix} \quad 540\ *$$

$$\text{e.g. } P(4a,1b,1c) = \frac{6!}{4!\times1!\times1!}\times\left[\left(p_a{}^4\right)\times p_b\times p_c\right] = 30a^4bc$$

The sum of rearrangements obtained in the point a), b), and c) (3+186+540) correspond as expected to the total number of possible rearrangements 729.

4. To take out six genes and to obtain four distinct alleles (a, b, c, and d):

Total number of possible rearrangements

$$\alpha_6{}^4 = 6^4 = 4096$$

a) fixation of one allele (all copies belong to the same category)

$$\begin{pmatrix} a^6 \\ b^6 \\ c^6 \\ d^6 \end{pmatrix} \quad 4$$

b) to obtain two alleles (categories a and b; a and c; a and d; b and c; b and d; c and d).

$$\begin{pmatrix} 6a^5b \\ 15a^4b^2 \\ 20a^3b^3 \\ 15a^2b^4 \\ 6ab^5 \end{pmatrix} \begin{pmatrix} 6a^5c \\ 15a^4c^2 \\ 20a^3c^3 \\ 15a^2c^4 \\ 6ac^5 \end{pmatrix} \begin{pmatrix} 6a^5d \\ 15a^4d^2 \\ 20a^3d^3 \\ 15a^2d^4 \\ 6ad^5 \end{pmatrix} \begin{pmatrix} 6b^5c \\ 15b^4c^2 \\ 20b^3c^3 \\ 15b^2c^4 \\ 6bc^5 \end{pmatrix} \begin{pmatrix} 6b^5d \\ 15b^4d^2 \\ 20b^3d^3 \\ 15b^2d^4 \\ 6bd^5 \end{pmatrix} \begin{pmatrix} 6c^5d \\ 15c^4d^2 \\ 20c^3d^3 \\ 15c^2d^4 \\ 6cd^5 \end{pmatrix}$$

$$6 \times 62 = 372$$

c) to obtain three alleles (categories: a, b, and c; a, b, and d; a, c, and d; b, c, and d).

$$\begin{pmatrix} 30a^4bc \\ 60a^3b^2c \\ 60a^3bc^2 \\ 60a^2bc^3 \\ 60a^2b^3c \\ 90a^2b^2c^2 \\ 60ab^3c^2 \\ 60ab^2c^3 \\ 30ab^4c \\ 30abc^4 \end{pmatrix} \begin{pmatrix} 30a^4bd \\ 60a^3b^2d \\ 60a^3bd^2 \\ 60a^2bd^3 \\ 60a^2b^3d \\ 90a^2b^2d^2 \\ 60ab^3d^2 \\ 60ab^2d^3 \\ 30ab^4d \\ 30abd^4 \end{pmatrix} \begin{pmatrix} 30a^4cd \\ 60a^3c^2d \\ 60a^3cd^2 \\ 60a^2cd^3 \\ 60a^2c^3d \\ 90a^2c^2d^2 \\ 60ac^3d^2 \\ 60ac^2d^3 \\ 30ac^4d \\ 30acd^4 \end{pmatrix} \begin{pmatrix} 30b^4cd \\ 60b^3c^2d \\ 60b^3cd^2 \\ 60b^2cd^3 \\ 60b^2c^3d \\ 90b^2c^2d^2 \\ 60bc^3d^2 \\ 60bc^2d^3 \\ 30bc^4d \\ 30bcd^4 \end{pmatrix}$$

$$4 \times 540 = 2160$$

*d) to obtain four distinct categories: a, b, c, and d**

$$\begin{pmatrix} 120a^3bcd \\ 120ab^3cd \\ 120abc^3d \\ 120abcd^3 \\ 180a^2b^2cd \\ 180a^2bc^2d \\ 180a^2bcd^2 \\ 180ab^2c^2d \\ 180ab^2cd^2 \\ 180abc^2d^2 \end{pmatrix} \quad (4x120)+(6x180)=1560\ *$$

$$\text{e.g. } P(3a,1b,1c,1d) = \frac{6!}{3!\times 1!\times 1!\times 1!} \times (p_a \times p_b \times p_c \times p_d) = 120abcd$$

The sum of rearrangements obtained in the point a), b), c), and d) (4+372+2160+1560) correspond as expected to the total number of possible rearrangements 4096.

Note: The formulas used to calculate the probabilities expected for Porto Santo under the two hypotheses considered above are marked with *.

Appendix 2

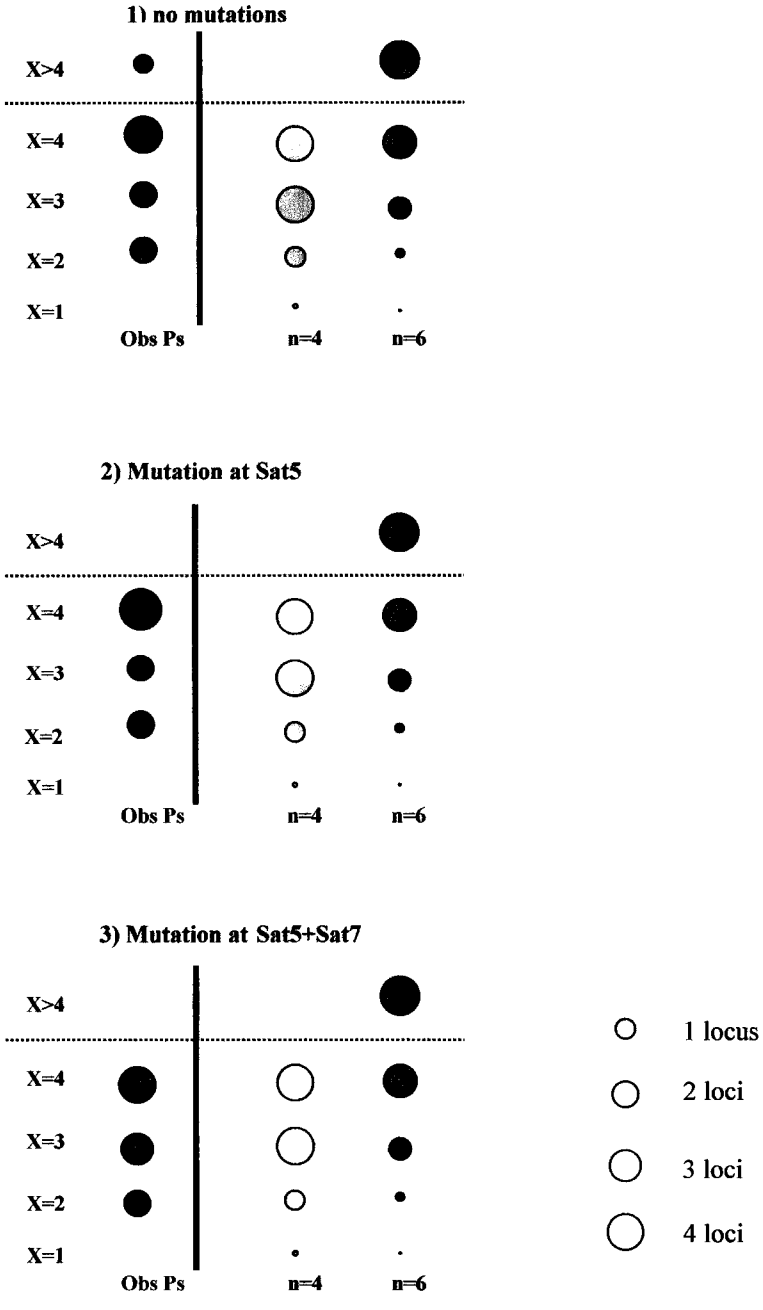

Figure A1: Three different scenarios considered to explain the results obtained at microsatellite markers in the Porto Santo rabbit population. 1) no mutations; 2) mutation at Sat5; 3) mutations at Sat5 and Sat7. The criteria used for considering the possibility of mutation at microsatellite loci combined a low allelic frequency ($p_i < 0.05$) with the close vicinity of putative mutants to major alleles (gene frequencies obtained in Sat5: 206-0.14; 210-0.38; 214-0.20; **216-0.03**; 222-0.25 and in Sat7: **185-0,02**; 187-0,54; 191-0,21; 193-0,23). X=1, 2, 3, 4, >4 are the number of alleles per locus observed in Porto Santo (Obs Ps) and expected under the hypothesis four (n=4) or six (n=6) founding chromosomes. The size of each circle corresponds to the number of loci observed and expected with 1, 2, 3, 4 or more than 4 alleles.

Table A1: Distribution values obtained and expected from the gene frequencies of 9 microsatellite for each class of alleles per locus (X=1, 2, 3, 4 or >4) under the hypotheses of 4 or 6 chromosomes founding the Porto Santo rabbit population. X is the number of alleles expected per locus.

	N=4				N=6				
Microsatellite	X=1	X=2	X=3	X=4	X=1	X=2	X=3	X=4	X>4
	expected								
Sat2	0,004	0,079	0,3917	0,5251	0,0002	0,008	0,0784	0,2838	0,6294
Sat3	0,0015	0,065	0,4110	0,5224	3,11E-05	0,004	0,0665	0,2979	0,6314
Sat4	0,01766	0,187	0,4739	0,3214	0,0022	0,045	0,2196	0,3910	0,3420
Sat5	0,0012	0,054	0,3813	0,5634	2,42E-05	0,003	0,0508	0,2504	0,6956
Sat7	0,0052	0,142	0,5018	0,3510	0,0002	0,019	0,1848	0,4339	0,3619
Sat8	0,0005	0,03	0,3079	0,6615	3,75E-06	0,001	0,0230	0,1747	0,8011
Sat12	0,0169	0,286	0,5291	0,1680	0,0015	0,075	0,3654	0,4206	0,1373
Sat13	0,0050	0,134	0,5193	0,3416	0,0002	0,017	0,1671	0,4270	0,3886
Sat16	0,0066	0,1689	0,5322	0,2923	0,0066	0,0263	0,2309	0,4609	0,2750
total	0,0587	1,1458	4,0485	3,7469	0,0111	0,1983	1,3869	3,1408	4,2627
	Observed Ps								
Mutation in Sat5	0	2	2	5	0	2	2	5	0
χ^2	0,0587	0,6367	1,0365	0,4190	0,0111	16,361	0,271	1,1005	4,2627
χ^2 total				2,1511					22,007
	Observed Ps								
Mutations sat5+Sat7	0	2	3	4	0	2	3	4	0
χ^2	0,0587	0,6367	0,2715	0,0170	0,0111	16,361	1,8761	0,2350	4,2627
χ^2 total				0,9841					22,746
	Observed Ps								
no Mutations	----	----	----	----	0	2	2	4	1
	----	----	----	----	0,0111	16,361	0,271	0,2350	2.497
									19.1[redacted]

Table A2: Distribution values obtained and expected from the gene frequencies of 20 protein and 2 immunological markers for each class of alleles per locus (X=1, 2, 3, 4 or >4) under the hypotheses of 4 or 6 chromosomes founding the Porto Santo rabbit population. X is the number of alleles expected per locus.

Proteins	N=4 X=1	X=2	X=3	X=4	N=6 X=1	X=2	X=3	X=4	X>4
	expected								
NP	0,76	0,2311	0,0054	--------	0,6671	0,3202	0,0127	--------	--------
PEPA	0,1087	0,7902	0,100	0,0008	0,0255	0,7902	0,1809	0,0034	0,00001
PEPB	0,6395	0,3291	0,031	0,0005	0,5114	0,4186	0,0679	0,0021	--------
PEPC	0,0685	0,4880	0,3980	0,0453	0,0155	0,2992	0,4735	0,1910	0,02092
PEPD	0,0916	0,6727	0,225	0,0107	0,0209	0,5734	0,3605	0,0438	0,00139
PGD	0,1226	0,8388	0,038	--------	0,0314	0,8969	0,0717	--------	--------
ADA	0,2105	0,6943	0,093	0,0022	0,0925	0,7273	0,1723	0,0079	0,00004
GALT	0,3882	0,4971	0,112	0,0026	0,2417	0,5261	0,2205	0,01149	0,00014
HBA	0,3332	0,6367	0,030	--------	0,1902	0,7488	0,0610	--------	--------
HBB	0,7651	0,2349	--------	--------	0,6692	0,3308	--------	--------	--------
ACP3	0,7642	0,2324	0,003	--------	0,6681	0,3240	0,0079	--------	--------
ALB	0,1243	0,8682	0,007	--------	0,0311	0,9549	0,0140	--------	--------
TF	0,18	0,7211	0,093	0,0015	0,0737	0,7496	0,1662	0,01018	0,00018
CAI	0,9683	0,0316	--------	--------	0,9529	0,0471	--------	--------	--------
CAII	0,2986	0,6474	0,054	0,0003	0,1608	0,7316	0,1065	0,00118	--------
GC	0,4211	0,4646	0,108	0,0067	0,2731	0,4966	0,2039	0,0255	0,00093
BF	0,0471	0,5012	0,416	0,0352	0,0071	0,2800	0,5769	0,12868	0,00723
HPX	0,0652	0,4492	0,420	0,0657	0,0153	0,2432	0,5077	0,20701	0,02676
SOD	0,8274	0,1726	--------	--------	0,7527	0,2473	--------	--------	--------
AT3	0,7032	0,2708	0,0253	0,0006	0,5897	0,3513	0,0559	0,00296	0,00004
IGHV1	0,0696	0,4213	0,3662	0,1428	0,0173	0,2281	0,4453	0,24263	0,06666
IGKC1	0,0167	0,2028	0,4805	0,2999	0,0019	0,0501	0,2489	0,4022	0,29697
Total	7,98	10,40	3,01	0,61	6,00[redacted]	10,3354	3,9542	1,2800	0,4213
	Observed Pst								
	8	8	5	1	8	8	5	1	0
χ^2	4,04029E-05	0,5525	1,3216	0,2416	0,6595	0,5276	0,2766	0,0612	0,42131
χ^2 total				2,1158					1,9464