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Genetic diversity comparison of the DQA gene in European rabbit (*Oryctolagus cuniculus*) populations

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Abstract The European rabbit (*Oryctolagus cuniculus*) natural populations within the species native region, the Iberian Peninsula, are considered a reservoir of genetic diversity. Indeed, the Iberia was a Pleistocene refuge to the species and currently two subspecies are found in the peninsula (*Oryctolagus cuniculus cuniculus* and *Oryctolagus cuniculus algirus*). The genes of the major histocompatibility complex (MHC) have been substantially studied in wild populations due to their exceptional variability, believed to be pathogen driven. They play an important function as part of the adaptive immune system affecting the individual fitness and population viability. In this study, the MHC variability was assessed by analysing the exon 2 of the DQA gene in several European rabbit populations from Portugal, Spain and France and in domestic breeds. Twenty-eight DQA alleles were detected, among which 18 are described for the first time. The Iberian rabbit populations are well differentiated from the French population and domestic breeds. The Iberian populations retained the higher allelic diversity with the domestic breeds harbouring the lowest; in contrast, the DQA nucleotide diversity was higher in the French population. Signatures of positive selection were detected in four codons which are putative peptide-binding sites and have been previously detected in other mammals. The evolutionary relationships showed instances of trans-species polymorphism. Overall, our results suggest that the DQA in European rabbits is evolving under selection and genetic drift

Keywords European rabbit (*Oryctolagus cuniculus*) · MHC-DQA · Population genetics · Genetic diversity

The GeneBank accession numbers for the new sequences of the DQA exon 2 determined in this study are: KR534620, KR534621, KR534622, KR534623, KR534624, KR534625, KR534626, KR534627, KR534628, KR534629, KR534630, KR534631, KR534632, KR534633, KR534634, KR534635, KR534636 and KR534637.

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Introduction

The effectiveness of innate and adaptive immune responses, and hence the fitness of individuals and populations, is driven by pathogen exposure history and therefore by the immunogenetic repertoire of the molecules that recognise the pathogenic antigens. The major histocompatibility complex (MHC) is a multigene family of receptors that encode, among others, cell-surface class II glycoproteins that bind extracellular pathogenic antigens and present them to T lymphocytes to initiate the immune response. Within the vertebrate's genome, the MHC complex contains some of the most polymorphic functional loci being the variability of the MHC molecules correlated with the diversity of T lymphocyte receptors which in turn determine the resistance of an organism to a pathogen (Piertney and Oliver 2005; Ujvari and Belov 2011). The unique level of genetic polymorphism found in MHC genes, when compared with other protein-coding genes, occurs predominantly at functional important sites, i.e., residues

involved in peptide binding (peptide-binding regions, PBR) such as those encoded by exon 2 of class II α and β chains, (reviewed in Piertney and Oliver 2006). Several reports have evidenced that MHC diversity is maintained by balancing selection through pathogen-mediated selection (reviewed in Bernatchez and Landry 2003; Piertney and Oliver 2006). Another important feature of MHC polymorphism is its trans-species mode of evolution (Klein et al. 1998).

The European rabbit (*Oryctolagus cuniculus*) (Leporidae, Lagomorpha) originated in the Iberian Peninsula, where two subspecies are found: *Oryctolagus cuniculus algirus* and *Oryctolagus cuniculus cuniculus* (Cabrera 1914; Corbet 1994; Lopez-Martinez 1989). These two morphologically differentiated subspecies evolved during the Pleistocene when two distinct refuges in the Iberian Peninsula were occupied by the European rabbit (Ferrand and Branco 2007). *O. c. algirus* inhabits the southwestern Iberian Peninsula, while *O. c. cuniculus* is present in the northeastern Iberian Peninsula; through anthropogenic dispersal, the species expanded to the Continental Europe, England, Australia, New Zealand, North and South America and North Africa (Corbet 1994). A single origin of domestication from French wild populations was initiated within the last 1500 years and led to levels of genetic diversity in the domestic rabbits significantly lower than those found in the wild populations (Carneiro et al. 2011, 2014), reviewed in (Ferrand and Branco 2007; Geraldes et al. 2005; Hardy et al. 1995). In the last decades, the European rabbit populations, both in its native and non-native ranges, have suffered an alarming decline mainly as a consequence of habitat loss and the emergence of viral diseases (myxomatosis and rabbit haemorrhagic disease), reviewed in (Abrantes et al. 2012, 2013b; Esteves et al. 2015; Lopes et al. 2014a). In this context, the European rabbit provides an interesting system for the study of the dynamics of host–pathogen interactions and their effect on the evolution of immune genes. In addition, the European rabbit has been extensively studied as a laboratory model for human diseases in both biomedical and fundamental research (Carneiro et al. 2011). Indeed, further to its use in the early understanding of the genetics of antibody synthesis, it has been recently used in the new smallpox vaccines and antiviral drugs research (Adams et al. 2007) and in HIV vaccine research (Chen et al. 2013). However, our current knowledge of rabbit immunogenetics lags somewhat behind that of other model species, such as the mouse, particularly at important loci such as the MHC.

The MHC class II region of the European rabbit, also referred to as the rabbit leukocyte antigen (RLA), contains several clusters; the main clusters are the DP, DQ and DR and each has genes coding for an α and a β chains (A and B genes, respectively) which are grouped together. The exons 2 of DQA and DQB genes have attracted most attention since they encode for highly polymorphic peptide-binding sites. The exon 2 of DQA has further been shown to be under positive selection (Amills et al. 2008; Bryja et al. 2006; Cutrera and Lacey 2007; Surridge et al. 2008). Interestingly, the rabbit DQA has been reported to appear as a single copy (Fain et al. 2001; LeGuern et al. 1985; Sittisombut and Knight 1986) in contrast with the majority of vertebrates that carry multiple copies of classical MHC loci (Flajnik and Kasahara 2001). The most recent study concerning the European rabbit MHC class II focused on the exon 2 of the DQA locus and compared its diversity in several leporid species (Surridge et al. 2008). To date, 19 RLA-DQA alleles have been described.

In this study, we investigated the genetic diversity and evolutionary history of the exon 2 of the DQA locus in three wild rabbit populations from Portugal, Spain and France and from two domestic breeds representing different genetic backgrounds with the following aims: (1) to investigate the occurrence of new DQA alleles and compare their distribution among the assessed European rabbit populations, (2) to compare the genetic diversity and the occurrence of trans-species polymorphism and (3) to investigate the action of selective forces and recombination at the DQA locus. This is, to our knowledge, the first population genetics study carried on rabbit MHC.

Materials and methods

DNA sequencing and cloning

Exon 2 DQA sequences were obtained from 40 *O. cuniculus* individuals from wild populations and domestic breeds. The wild populations were Portuguese (*O. c. algirus*, 10 individuals); Spanish (*O. c. cuniculus*, 10 individuals) and French (*O. c. cuniculus*, 10 individuals) and the domestic breeds were Lièvre belge (5 individuals) and Bélier français (5 individuals). Despite the low number of samples from each population, this study can provide a first insight concerning the overall picture of the variation of the genetic diversity between several European rabbit populations. Genomic DNA was extracted using an EasySpin Tissue Kit (Citomed) according to the manufacturer's recommendations. DQA exon 2 (nucleotide positions 82–330 of the DQA gene), encoding the peptide binding region was amplified using the primers 5′-TCATCAGCTGACCACGTTGG and 5′-AGGAGGAAAGATGTTGTCCAC described in (Fain et al. 2001). Amplifications were carried out by PCR in a total volume of 10 μl containing approximately 50 ng of genomic DNA, 0.45 μM of each primer, 2.5 U Taq DNA polymerase, 0.2 nM dNTPs and 1.5 mM $MgCl_2$ (5 μl 2× reaction mix, Taq PCR Master Mix Kit, QIAGEN). Cycling parameters consisted of an initial denaturation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for

30 s and a final extension step at 60 °C for 10 min. PCR amplicons of the expected size were purified with Exo/Sap and sequenced in both directions with the amplification primers and using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City) according to the manufacturer's protocol. All sequences obtained in this work are available in the GenBank (accession numbers: KR534620-KR534637). Sequences were analysed and aligned with BioEdit software (Hall 1999). Haplotype inference was performed with PHASE implemented in DnaSP v. 5.10 (Librado and Rozas 2009) with the 'recombination' model (−MR0) and 1000 iterations after 100 burn-ins. Only haplotypes with $p>0.90$ were considered as inferred reliably. For heterozygous individuals who failed in the haplotype reconstruction, the PCR amplicons were cloned and sequenced. For this, PCR products were cleaned with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and cloned directly using the pGEM®-T Easy Vector Systems (Promega) following the manufacturer's protocols. DNA inserts from at least three white positive colonies were PCR amplified using pUC/M13 primers (Promega) and sequenced using the pUC/M13 forward or reverse primers in the same conditions previously described.

Population genetic analysis

For allele identification, the obtained exon 2 DQA allelic sequences were aligned in BioEdit using ClustalW multiple alignment (Thompson et al. 1994) and compared with other leporid sequences available in GenBank. The allelic numbers for the new rabbit sequences were assigned according to the guidelines of (Klein et al. 1990). The distribution of allelic frequencies globally and per population, the number of effective alleles (A_e) and the expected heterozygosity (eHet) were estimated using GENALEX v. 6.2 (Peakall and Smouse 2012) and the allele richness (A_R) was obtained using FSTAT version 2.9.3.2 (Goudet 1995). The level of differentiation between the four studied rabbit populations was estimated directly from the aligned sequences using a matrix of pairwise differences between haplotypes to perform a standard AMOVA as implemented in ARLEQUIN 3.5.2.2 (Excoffier et al. 2005). We selected this method since, contrasting with other F_{ST} estimators such as G_{ST}, G'_{ST} and D, it takes the information on the evolutionary relationships between haplotypes into account, standardisation is not required and estimates are independent from the mutation rate (Meirmans and Hedrick 2011).

Sequence-level allelic diversity values, such as the nucleotide diversity (π), the number of segregating sites (S), the average number of nucleotide differences (k) and the Watterson's mutation parameter (θ_w), were estimated using DnaSP v. 5.10. To evaluate if the observed differences between nucleotide diversity were statistically significant, we performed a jackknife resampling analysis. For that, we estimated all the possible π values per population excluding one individual and obtained four distributions of nucleotide diversities (one per population). Then, we compared these distributions using a Mann–Whitney test as implemented in the base package in R.

In order to perform comparative analysis of the evolutionary history of the exon 2 DQA in leporids, we have established two datasets: (1) the 28 European rabbit DQA sequences, detected in this study (Fig. 1) and (2) a 75 DQA sequences dataset comprising several leporid species available in GenBank (in Electronic supplementary material, ESM 1). Since intragenic recombination has been suggested to play an important role in determining variability at MHC loci (Schaschl et al. 2006), we firstly looked for evidence of recombination in the detected DQA sequences or in the larger dataset. Evidence for recombination was assessed by the GARD tool which uses a genetic algorithm to search a multiple-sequence alignment for putative recombination break points, on the Data Monkey web server (http://www.datamonkey.org) and by four different programs implemented in RDP v.3.44 beta package (Martin et al. 2010): (1) RDP, (2) GENECONV, (3) Maxi Chi2 and (4) BootScan, using the default settings. No evidences of recombination were detected in the DQA sequences dataset used in the phylogenetic analyses. The software MEGA6 (Tamura et al. 2013) was used to construct the neighbour-joining (NJ) trees either of the detected rabbit DQA sequences (28 sequences) or the larger leporid DQA dataset (75 sequences). The NJ tree of the larger dataset was rooted using human and sheep DQA sequences (GenBank accession numbers NM002122 and AY312383, respectively) and the length of the sequences was restricted to 243 bp (complete exon 2, 249 bp) which corresponds to the common part of the included sequences. The best nucleotide and amino acid substitution models were chosen according to the Bayesian Information Criterion and used to compute evolutionary distances by the Kimura 2-parameter method through 1000 bootstrap replicates. MEGA6 was also used to compute pairwise and overall mean nucleotide and amino acid distances. Phylogenetic relationships among the European rabbit exon 2 DQA haplotypes found in our study were further examined by performing a network analysis using NETWORK 4.6.1.3 (Fluxus Technology Ltd; www.fluxus-engineering.com) by a median-joining (MJ) algorithm using the default parameters.

Signatures of positive selection are inferred if the ratio of non-synonymous (dN) over synonymous (dS) substitutions is statistically higher than the value observed under neutrality ($dN/dS=\omega=1$). To detect such signatures in individual codons, two ML frameworks were used: the HyPhy package implemented in the Data Monkey Web Server and CODEML implemented in PAML version (Delport et al. 2010; Yang 1997). In the Data Monkey Web Server, the

Assigned Haplotype		30 40 50 60 70 80 90 100 110	Acession No	Population
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1	*Orcu DQA*18*	DHVGAYGMELYQYYGPSGQYTHEFDGDEQFYVDLDKKETVWRLPEFSKFTSFDPQGGLREIATAKYNLNNLMKRTNSTAAVN	EU686523	DB, FR
2‡	*Orcu DQA*20*	.....INVC.S.....I.....A.GN....R...DL.I.DS.....	KR534620	DB
3	*Orcu DQA*07*	.....INVC.S.....I.....R.....A.GN....R...DL.I.DS.....	EU686527	DB, FR, PT
4	*Orcu DQA*13*	....S..INV..S.....E.....S..M.RE.....A.GN...I....DIMI..S.....	EU686525	DB, SP
5	*Orcu DQA*01*	....S..INV..S.....I...S...R.A.....A.GN.....DIII..S.....	EU427436	DB, PT
6	*Orcu DQA*14*	....S..INV..S.....F.....E.....S..M.RE.....A.GN...T....DIMI..S.....	EU686532	DB, SP, PT
7‡	*Orcu DQA*21*	.....INV..S.....N.....S..M.RE.R.....A.GN.....DI.I..S.....	KR534621	FR
8	*Orcu DQA*03*	....S..INV..S.....F.....I.....R.A.....A.GN...I....DIII..S.....	EU686529	FR
9	*Orcu DQA*11*	.....INV..S.....I.....R.....A.GN.....DIMI..S.....	EU686530	FR, SP
10	*Orcu DQA*17*	....S..INV..S.....E.....S..M.RE.R.....A.GN.....T.DIII..S.....	EU686528	FR, SP
11‡	*Orcu DQA*22*	.....INF..S.DL.....G....I.....A.....A..N..SGRLT.DVM...S.....	KR534622	FR
12‡	*Orcu DQA*23*	....S..INF..S.DL.....A.....T.....	KR534623	FR, PT
13‡	*Orcu DQA*24*	....S..INV..S.....I.....R.....A.GN.....DI.I..S.....	KR534624	SP
14‡	*Orcu DQA*25*	.....INV..S.....I.....R.L.....A.GN.....DI.I..S.....	KR534625	SP
15‡	*Orcu DQA*26*	....S..INV..S.....F.....E.....S..M.RE.R.....A.GN.....DIMI..S.....	KR534626	SP
16‡	*Orcu DQA*27*	....S..INV..S.....E.....S..M.RE.....A.GN...T....DL.I.HS.....	KR534627	SP
17	*Orcu DQA*02*	....S..INV..S.....I.....R.A.....A.GN...E....DIII..S.....	EU686533	SP
18‡	*Orcu DQA*28*	....S..INV..S.....I...S...R.A.....A.GN.....DI.I.CS.....	KR534628	SP
19‡	*Orcu DQA*29*	....S..INV..S.....I...S.....R....H....D.....DI.I.CS.....	KR534629	SP
20‡	*Orcu DQA*30*	....S..INV..S.....I.....R.....A.GN...I....DI.I..S.....	KR534630	PT
21‡	*Orcu DQA*31*	....S..INV..S.....E.....S..M.RE.R.....A.GN...I....DIMI..S.....	KR534631	PT
22‡	*Orcu DQA*32*	.....INV..S.....F.....I.....R.L.....A.GN...I....DIVI..S.....	KR534632	PT
23‡	*Orcu DQA*33*	....S..INV..S.....F.....I.....R.L.....A.GN...I....DIVI..S.....	KR534633	PT
24‡	*Orcu DQA*34*	....S..INV..S.....E.....N.....S..M.RE.R.....A.GN.....DI.I..S.....	KR534634	PT
25‡	*Orcu DQA*35*	....S..INV..S.....E.....N.....S..M.RE.R.....A.GN.....DIII..S.....	KR534635	PT
26‡	*Orcu DQA*36*	....S..INV..S.....E.....S..M.RE.R.....A.GN...T....DI.I..S.....	KR534636	PT
27‡	*Orcu DQA*37*	....S..INVC.S.....I.....R.....A.GN....R...DL.I.HS.....	KR534637	PT
28‡	*Orcu DQA*16*	....S..INV..S.....E.....S..M.R..R.....A.GN...I....DIMI..S.....	EU686535	PT

Fig. 1 Amino acid alignment of the 28 detected exon 2 DQA European rabbit *Orcu* (*Oryctolagus cuniculus*) sequences. Amino acids are numbered according to the aligned protein translation given for the human and rabbit DQA gene in Ensembl (www.ensembl.org). The nomenclature for rabbit alleles follows that of Surridge *et al.* (2008). Ŧ denotes putative peptide binding sites (PBS) based on the HLA-DQA (Gouy de Bellocq et al. 2009). * denotes amino acid positions under positive selection with posterior probability of 99 % as identified by the BEB method in CODEML implemented in PAML (Yang et al. 2005). ‡ denotes the new DQA haplotypes detected in this study. DB, FR, SP and PT refer to the allelic sequences found in domestic breeds and wild French, Spanish and Portuguese rabbits populations, respectively

best-fitting nucleotide substitution model was first determined using the automated tool available. Sequences of the exon 2 DQA gene were analysed under six available models, single likelihood ancestor counting (SLAC), fixed-effect likelihood (FEL), internal fixed-effect likelihood (IFEL), mixed effects model evolution (MEME), random effect likelihood (REL) and fast unconstrained Bayesian approximation (Fubar). Details of the models can be found in (Kosakovsky Pond and Frost 2005; Murrell et al. 2012, 2013). In CODEML, the alignments were fitted to NSsites models where no codons could have a dN/dS ratio >1 (M0, M1a and M7) and to models that could incorporate a class of sites with a dN/dS >1 (M2a, M3 and M8). Details of the models can be found in (Yang et al. 2000, 2005). For each pair of nested models used in this study, the log-likelihood values were compared using a likelihood ratio test. We compared model M0 to M3 to test for dN/dS heterogeneity and both M1a to M2a and M7 to M8 to test for the presence of positive selection. Adaptive evolution was inferred if the model allowing positive selection estimates a value of ω greater than 1 and twice the difference in the log-likelihood values between nested models is greater than the chi-square critical value for the appropriate degrees of freedom. The comparison between models M0 and M3 was performed using four degrees of freedom, and in the comparisons between M1–M2 and M7–M8, two degrees of freedom were used. Finally, the Bayes empirical Bayes approach (BEB, (Yang et al. 2005)) was used to identify codons with $\omega>1$. Sites with posterior probabilities >0.95 were considered likely to have evolved under adaptive evolution. Furthermore, to detect population-level evidence of current selection, we calculated Tajima's D (Tajima 1989) and Fu & Li D* (Fu and Li 1993) using DnaSP v.5.10; these are tests based on allele frequency spectrum of nucleotide polymorphisms and details can be found in (Fu and Li 1993; Tajima 1989).

Results

Variation in MHC-DQA exon 2

A total of 28 exon 2 DQA alleles were detected among the 40 *O. cuniculus* individuals analysed, of which 18 represent new leporid DQA alleles (Table 1). These 18 novel DQA sequences coded for proteins differing by one or more amino acids and their authenticity was verified by repeated PCR and cloning. Nine sequences attributed to the *Orcu DQA*14* haplotype had a C/T synonymous substitution in position 246 and were assigned as *Orcu DQA*14* haplotype. Similarly, six sequences had a synonymous T/C substitution in position 243 of the *Orcu DQA*18* allele and were considered also as *Orcu DQA*18*. No insertions or deletions were detected. The amino acid alignments of the 28 detected exon 2 rabbit DQA sequences are displayed in Fig. 1 along with the assigned haplotype number; those of the larger leporid dataset are presented in ESM 1. Since 19 DQA alleles have

Table 1 RLA-DQA1 alleles detected in this study, GenBank accession numbers and its frequency in rabbits from the domestic breeds, French, Spanish and Portuguese rabbit populations

Group	Allele name	Accession number	Frequency
Domestic breeds	*Orcu DQA*07*	EU686527.1	0.600
	*Orcu DQA*18*	EU686523.1	0.250
	*Orcu DQA*13*	EU686525.1	0.050
	*Orcu DQA*01*	EU427436.1	0.050
	*Orcu DQA*20*‡	KR534620	0.050
France	*Orcu DQA*11*	EU686530.1	0.500
	*Orcu DQA*07*	EU686527.1	0.100
	*Orcu DQA*17*	EU686528.1	0.100
	*Orcu DQA*18*	EU686523.1	0.050
	*Orcu DQA*14*	EU686532.1	0.050
	*Orcu DQA*03*	EU686529.1	0.050
	*Orcu DQA*21*‡	KR534621	0.050
	*Orcu DQA*22*‡	KR534622	0.050
	*Orcu DQA*23*‡	KR534623	0.050
Spain	*Orcu DQA*14*	EU686532.1	0.200
	*Orcu DQA*17*	EU686528.1	0.150
	*Orcu DQA*13*	EU686525.1	0.050
	*Orcu DQA*02*	EU686533.1	0.050
	*Orcu DQA*24*‡	KR534624	0.200
	*Orcu DQA*25*‡	KR534625	0.100
	*Orcu DQA*26*‡	KR534626	0.100
	*Orcu DQA*27*‡	KR534627	0.050
	*Orcu DQA*28*‡	KR534628	0.050
	*Orcu DQA*29*‡	KR534629	0.050
Portugal	*Orcu_DQA*14*	EU686532.1	0.200
	*Orcu DQA*07*	EU686527.1	0.100
	*Orcu DQA*01*	EU427436.1	0.050
	*Orcu DQA*16*	EU686535.1	0.050
	*Orcu DQA*30*‡	KR534630	0.100
	*Orcu DQA*31*‡	KR534631	0.100
	*Orcu DQA*34*‡	KR534632	0.100
	*Orcu DQA*23*‡	KR534633	0.050
	*Orcu DQA*32*‡	KR534634	0.050
	*Orcu DQA*33*‡	KR534635	0.050
	*Orcu DQA*35*‡	KR534636	0.050
	*Orcu DQA*36*‡	KR534637	0.050
	*Orcu DQA*37*‡	KR534638	0.050

‡ Denotes the new DQA haplotypes detected in this study

already been described, the total number of European rabbit alleles increased to 37 being the new DQA alleles named from *Orcu DQA*20* to *Orcu DQA*37*. Pairwise distances between the different obtained nucleotide sequences ranged from 0.004 to 0.313 with a mean of 0.093. Amino acid distances ranged from 0.012 to 0.507 with a mean of 0.179.

Allelic diversity in the four groups of European rabbit, differentiation and population genetics

The number of alleles per population ranged from five in the domestic breeds to 13 in the Portuguese population and their frequencies varied among populations (Table 1). In the domestic breeds, we detected five DQA haplotypes; a new allele was found (*Orcu DQA*20*) and the most frequent allele was *Orcu DQA*07* (0.600; Table 1). In the French wild population, we detected nine DQA haplotypes, of which three were new: *Orcu DQA*21* to *Orcu DQA*23*, and *Orcu DQA*11* was the most frequent allele (0.500, Table 1). In the Spanish population, we detected 11 DQA haplotypes with 3 new haplotypes, *Orcu DQA*24* to *Orcu DQA*26*, and with no occurrence of a dominant haplotype. In the Portuguese population, we found 13 DQA haplotypes, of which 9 of them were new: *Orcu DQA*23* and from *Orcu DQA*30* to *Orcu DQA*37*, and with no occurrence of a dominant haplotype.

Table 2 shows the allelic diversity indexes and DNA sequence diversity values concerning the DQA locus of the four analysed populations. The Spanish and Portuguese populations have the higher number of DQA haplotypes, higher number of effective alleles and higher allelic richness, providing for the higher heterozygosity in these groups. In contrast, the DNA sequence diversities, S, π, k and θ_W are higher in the French rabbits and domestic breeds. Accordingly, the mean nucleotide and amino acid distances are also higher in these groups. For domestic rabbits, pairwise distances between DNA sequences ranged from 0.008 to 0.144 with a mean of 0.082. For the wild populations, the pairwise genetic distances ranged from 0.026 to 0.199 with a mean of 0.108 in the French wild rabbits; from 0.009 to 0.155 with a mean of 0.069 in the Spanish wild rabbits and from 0.004 to 0.223 with a mean of 0.081 in the Portuguese wild rabbits (Table 2). Differences in nucleotide diversity between domestic breeds and French populations were marginally significant ($p=0.059$), whereas the remaining population pairs showed significant differences in π ($p<10^{-4}$). Concerning the genetic differentiation among the four European rabbit groups, the calculated F_{ST} parameters clearly indicate the existence of significant population structure. Only the Portuguese and Spanish populations did not show any statistically significant genetic differentiation (Table 3). The estimates of Tajima's D and Fu and Li's D* were not statistically significant.

Recombination and selection analysis

No evidence of recombination occurring at the DQA sequences obtained in this work was detected either using GARD (in Data Monkey Web Server) or any method in RDP (Martin et al. 2010). Signatures of positive selection were detected on the leporid DQA sequences. Four codons

Table 2 Genetic diversity values for the RLA-DQA locus in the different rabbit groups

	Genetic diversity parameters									Nucleotide distance[a]	Amino acid distance[b]	Tajima's D	Fu and Li D*
Population	*n*	H	A_E	A_R	eHet	*S*	π	*k*	θ_W				
Domestic	10	5	2.33	5.00	0.570	42	0.0764	18.80	0.0820	0.082 (0.013)	0.172 (0.033)	−0.508	−0.508
French	10	9	3.51	9.00	0.715	54	0.0816	20.08	0.0867	0.108 (0.012)	0.241 (0.050)	−0.301	−0.067
Spain	10	11	8.70	10.0	0.865	30	0.0479	11.78	0.0458	0.069 (0.018)	0.145 (0.048)	0.212	0.051
Portugal	10	13	10.0	13.0	0.900	43	0.0586	14.82	0.0602	0.081 (0.018)	0.163 (0.044)	−0.124	−0.198

n sample size, *H* number of haplotypes, A_e number of effective alleles, A_R allelic richness, *eHet* expected heterozygosity, *S* number of segregating sites, π nucleotide diversity, *k* average number of nucleotide differences, θ_w Watterson's mutation parameter

[a] The number of nucleotide substitutions per site between the sequences were obtained by a bootstrap procedure (1000 replicates) being the standard error estimates shown in brackets. Analyses were conducted using the Kimura 2-parameter model (K2) for the domestic rabbits, the K2 with a gamma distribution shape parameter (G) for the French and Portuguese rabbits and the Jukes-Cantor model with G for the Spanish rabbits

[b] The number of amino acid substitutions per site between the sequences were obtained by a bootstrap procedure (2500 replicates) being the standard error estimates shown in brackets. Analyses were conducted using the Jones-Taylor-Thornton matrix-based model (JTT, with G for the French, Spanish and Portuguese wild populations)

[a,b] Analysis involved 5, 9, 11 and 13 sequences for the domestic breeds and French, Spanish and Portuguese wild populations, respectively

were considered to be significant positively selected codons: amino acid sites 33, 78, 92 and 99 (Tables 4 and 5).

Phylogenetic relationships among alleles and trans-species polymorphism

The phylogenetic relationships among the 28 *O. cuniculus* DQA alleles identified in this study (Fig. 1) and the larger dataset of leporid DQA alleles (ESM 1) were assessed by the construction of NJ trees. The NJ tree of the 28 rabbit DQA sequences show that the alleles fall into three clades (referred to as G_1, G_2 and G_3) with bootstrap support >95 % (Fig. 2). The clades G_1 and G_2 contain DQA haplotypes present in all the analysed European rabbit populations: G_1 contains 11 DQA haplotypes, mostly of Portuguese and Spanish origin (each 40.7 %); G_2 contains 15 haplotypes, of which the majority are DQA alleles from the French population and domestic breeds (30.7 and 35.9 %, respectively); G_3 contains the previously described *Orcu DQA*18* and the newly described *Orcu DQA*23* alleles which are present in the French and Portuguese rabbit populations and in the domestic breeds. In accordance with the NJ tree, the network also showed three clusters, each one containing the DQA haplotypes of the corresponding clade in the NJ tree (G_1, G_2 and G_3, Fig. ESM 2). For the larger dataset (75 DQA alleles) (ESM 1), the NJ tree also shows three clades (designated C_1, C_2 and C_3), in which each clade presents a mixture of alleles from several leporid species, supporting the trans-species polymorphism origin of MHC DQA alleles in leporids (Fig. 3).

Table 3 Subpopulation differentiation analysis

	F_{ST}
Domestic/French	0.148**
Domestic/Spanish	0.346***
Domestic/Portuguese	0.302**
French/Spanish	0.126**
French/Portuguese	0.122**
Spanish/Portuguese	−0.0124
All	0.191***

Pairwise F_{st} values within population and overall fixation index

Asterisks denote significance tested with 9,999 permutations (*p<0.05, **p<0.01, ***p<0.001)

Discussion

In this study, we investigated the genetic diversity and evolutionary history at the exon 2 of the rabbit DQA locus within and among three European wild rabbit populations from Portugal, Spain and France and two domestic breeds. Among the 28 DQA detected alleles, 18 were new, taking the total number of DQA alleles described for this species to 37. These results show a substantial level of allelic polymorphism for the DQA locus, in line with that described for other small mammals such as the Australian bush rats (Seddon and Baverstock 1999) and water voles (Bryja et al. 2006).

Portuguese and Spanish European rabbit populations exhibited the greatest levels of DQA polymorphism since higher allelic diversity and heterozygosity levels were detected. Decreasing levels of polymorphism are observed towards the French population and domestic breeds. This is in agreement with the history of this species which originated from the Iberian Peninsula. Indeed, a pattern of higher diversity in this region compared to the rest of Europe and to domestic breeds is also observed for proteins, mitochondrial DNA, microsatellites and other nuclear markers (Abrantes et al.

Table 4 Phylogenetic tests of selection acting on codons of DQA exon 2 in the 28 detected allelic sequences of the European rabbit using the HyPhy package implemented in the Data Monkey Web Server

DQA gene	SLAC	FEL	IFEL	REL	MEME	FUBAR	Total number of sites
Positive selection	78, 92	33,78, 92	78, 92	33, 38, 44, 48, 72, 76, 78, 88, 92, 94, 98, 99, 102	33, 78, 92, 99	33, 78, 92, 98, 99	4 (33, 78, 92, 99)
Negative selection	56	56, 61	56	–	–	56	1 (56)

2013a; Branco et al. 2000; Carneiro et al. 2011; Esteves et al. 2004; Geraldes et al. 2006; Pinheiro et al. 2015; Queney et al. 2001; Surridge et al. 1999; van der Loo et al. 1999). The reduced genetic diversity in non-Iberian populations has been partially assigned to the founder effect that occurred when the species expanded its range north after the Pleistocene glaciations (Hewitt 2000; Taberlet et al. 1998). However, several studies indicate that the diversity of the DQA locus may not agree with this phylogeographic scenario (Koutsogiannouli et al. 2009; Stamatis et al. 2009), showing that the MHC genes combine high rates of mutation and recombination with a complex evolutionary mode that extends beyond historical demography and biogeography. In fact, the phylogeny of the 28 exon 2 *Orcu DQA* alleles showed a poor phylogeographic signal, a characteristic feature of some MHC loci (Klein et al. 1998), since alleles that belonged to a specific population did not group together (Fig. 2, ESM 2). Nevertheless, some DQA haplotypes can be considered representatives of each group, e.g., H1 and H3 are mainly represented in the domestic breeds and H9 in the French population, while others are shared between populations, e.g., H6 and H10 (Fig. 2, ESM 2). Both the NJ tree and the MJ network retrieved three clusters containing the same DQA haplotypes (G_1, G_2 and G_3, Fig. 2, ESM 2). In the MJ network, the considerable number of alternative more parsimonious connections found among the DQA alleles, mainly those in the core (ESM 2), show the intricate relationships within this genealogy. Moreover, the long branches exhibited by some haplotypes tips suggest that they are divergent alleles with high genetic distances (e.g., H1 and H11, ESM 2). No evidence of intragenic recombination occurring at the DQA sequences obtained in our study was detected. Moreover, although there are indications that intergenic recombination has contributed to the evolution of MHC class II loci in Canidae (concerning the DRB1 and DQB alleles) (Seddon and Ellegren 2002), we did not find any reports in the literature regarding this occurrence in DQA alleles.

Although the Iberian European rabbit populations have a higher allelic diversity, there was a greater sequence diversity in wild rabbits from France and domestic breeds, shown either in the higher values of sequence diversity parameters (number of segregating sites, nucleotide diversity, average number of nucleotide differences and Watterson's mutation parameter) or in the higher genetic distances (Table 2). The higher sequence diversity parameters exhibited in the domestic breeds and French population might be attributed to a compensatory mechanism to maintain a higher amount of diversity in the presence of a lower number of alleles to preserve the efficiency of pathogen recognition. Indeed, to explain the widespread occurrence of the *d11* and *e14* allotypes (localised in the hinge and in the second exon of the heavy chain constant region of rabbit IgG) (Appella et al. 1971; Esteves et al. 2002, 2006; Hamers and Hamers-Casterman 1965; Mage 1981; Pinheiro et al. 2014b; Prahl et al. 1969) only outside the Iberian Peninsula, van der Loo (1993) hypothesised "compensatory overdominance"' as a mechanism to compensate the lack of diversity shown by the non-Iberian populations at the *a* and *b* loci (*IGHV* and *IGKC*, respectively) when compared to the Iberian Peninsula populations (Esteves et al. 2004; Pinheiro et al. 2011; van der Loo 1993; van der Loo et al. 1999).

Signatures of selection were observed in our sample of 28 detected rabbit DQA alleles. Indeed, four codons were detected as under positive selection (Tables 4 and 5). Three of the four codons under positive selection (78, 92 and 99) are located in putative peptide-binding sites, based on the HLA-DQA

Table 5 Phylogenetic tests of positive selection acting on sites of the European rabbit DQA exon 2 using likelihood ratio statistics (2ΔlnL) for comparisons of different models of codon evolution and the Bayes empirical Bayes (BEB) approach

Population	Models compared	2ΔlnL	Significance[a]	Positively selected sites[b]
All detected individuals (28seq)	M0 (1 ratio) vs M3 (discrete)	75.43	$p<0.001$	Not allowed
	M1a (nearly neutral) vs M2a (selection)	48.19	$p<0.001$	33, *78*, *92*, *99*
	M7 (beta) vs M8 (beta and ω)	47.89	$p<0.001$	*33*, *78*, *92*, *99*

[a] In M0 vs M3 model, 4 degrees of freedom were used; in M1a vs M2a and in M7 vs M8 models, 2 degrees of freedom were used

[b] Sites inferred to be under selection with posterior probabilities ≥95 %; numbers in italics with posterior probabilities ≥99 %

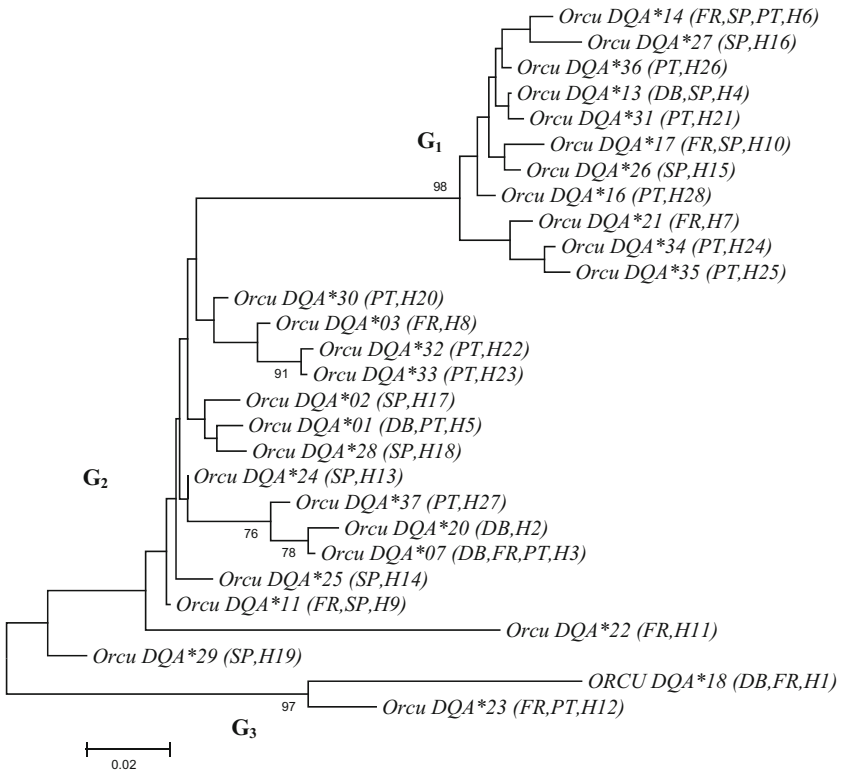

Fig. 2 Neighbour-joining tree of the 28 European rabbit DQA exon 2 sequences detected in this study with branch support provided by 1,000 bootstraps replicates. The evolutionary distances were computed using the Kimura 2-parameter method with a gamma distribution (shape parameter=0.17). DB, FR, SP and PT refer to the allelic sequences found in domestic breeds and wild French, Spanish and Portuguese rabbits populations, respectively. *H1-H28* corresponds to the assigned haplotypes (see Fig. 1). G_1, G_2 and G_3 refer to the three assigned clades with bootstrap support >95 %

(Gouy de Bellocq et al. 2009). These four codons under positive selection were also predicted in previous studies concerning leporids (Surridge et al. 2008), voles (Bryja et al. 2006) and wolves (Arbanasic et al. 2013; Galaverni et al. 2013) emphasising their important role in the interaction with antigens. This probably reflects an adaptation pressure to antigenic peptides resulting in the variability of some sites (PBR) and the neutrality of others in maintaining the integrity of the protein's structure and function (TCR-binding regions). Given the central role of MHC in the vertebrate immune system, it is generally accepted that pathogen-driven balancing selection is the most likely mechanism that explains the high level of MHC polymorphism.

In agreement to results previously reported for lagomorphs (Gouy de Bellocq et al. 2009; Koutsogiannouli et al. 2009; Surridge et al. 2008), our results also show that rabbits and hares share similar allelic sequences exhibiting trans-species polymorphism (Fig. 3) This is a MHC feature already reported in several mammals groups, such as primates (Otting et al. 2002), rodents (Cutrera and Lacey 2007; Edwards et al. 1997; Seddon and Baverstock 1999), canids (Seddon and Ellegren 2002) and ungulates (Hedrick et al. 2000), that may be an evidence of the need for a specific immune response to common or similar pathogens. Rabbit haemorrhagic disease virus (RHDV) and European brown hare syndrome virus are examples of similar pathogens that affect rabbits and hares, respectively. Although being mostly species-specific (Abrantes et al. 2012), RHDV infections in several *Lepus* species have been recently reported (Camarda et al. 2014; Lopes et al. 2014b; Puggioni et al. 2013). Although MHC class II genes are classically related with the presentation of antigenic peptides released from extracellular pathogens, a recent study suggests a link between class II MHC and viral infections (Deter et al. 2008). Moreover, there are other evidences in the leporids immune system that support this idea: trans-species polymorphism was also observed for two immunoglobulin genes, the *IGKC* and *IGHV* (Bouton and van der Loo 1997; Esteves et al. 2005; Pinheiro et al. 2011, 2013, 2014a).

Overall, our results can be interpreted in the light of both neutral–selective forces, although it is rather difficult to assess their relative contributions. On the one part, either the reduced allelic diversity in the French population and in domestic breeds or the high genetic differentiation between groups is in line with a strong genetic drift. On the other part, our results evidence the occurrence of historical balancing selection (dN/dS>1) favouring amino acid changes mainly in the PBR sites. The maintenance of shared DQA haplotypes between rabbits and hares supports the idea of a strong evolutionary/selective pressure likely providing an appropriate regulated immune response which is also in line with the occurrence of historical balancing selection. On the relative contribution of neutral and adaptive forces shaping MHC variation, there are several reports that support genetic drift as playing a prominent role, e.g., (Eimes et al. 2011; Luo et al. 2012; Strand et al. 2012; Zeisset and Beebee 2014), mainly in small and fragmented

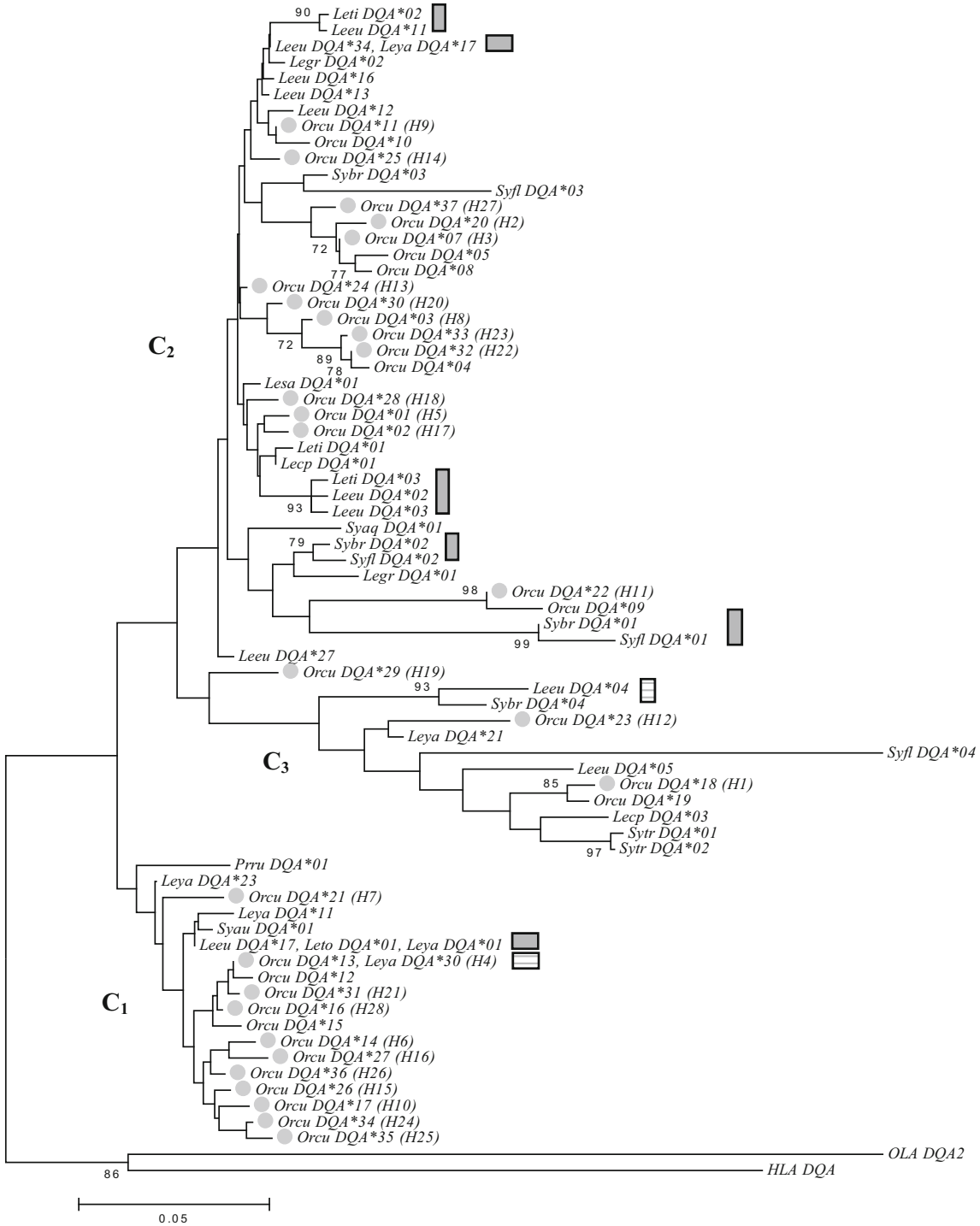

Fig. 3 Neighbour-joining tree of exon 2 DQA sequences of the 75 sequences leporid dataset with branch support provided by 1,000 bootstrap replicates using human (HLA) and sheep (OLA) DQA sequences (accession numbers NM002122 and AY312383) as outgroups. The evolutionary distances were computed using the Kimura 2-parameter method with a gamma distribution (shape parameter=0.50). Instances of trans-species and trans-generic polymorphism are indicated by *shadow and striped bars*, respectively. *Filled circles* DQA alleles found in this study. Species abbreviations are given in ESM 1 caption

populations in contrast with the paradigm that MHC polymorphism is mainly maintained by pathogen-mediated balancing selection, reviewed in (Bernatchez and Landry 2003; Piertney and Oliver 2005; Ujvari and Belov 2011).

In summary, we characterised the DQA locus in wild and domestic European rabbits and identified 18 new alleles that further support the high diversity at this locus. This diversity is higher in the Iberian wild populations and decreases in the

French wild population; the lowest diversity is found in the domestic breeds. Recombination, regarded as an important evolutionary mechanism for the generation of diversity, does not seem to be responsible for the detected DQA high allelic diversity in the European rabbit. Given the central role of MHC in the vertebrate immune system, it is likely that balancing selection may be pathogen driven. Nonetheless, neutral forces may have a role in shaping DQA allelic diversity. Adaptive immune diversity at the domestic breeds and French population might be maintained by higher levels of genetic variation in the presence of a lower number of alleles. Instances of trans-species evolution were observed. Future studies are warranted to investigate the link between the European rabbit immunogenetic diversity and pathogen community structure to better understand the mechanisms underlying adaptation.

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