Genetic variation of the second exon of *ELA-DRB* genes in Argentine Creole horses*

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Summary

Genetic variation in the equine leucocyte antigen-*DRB* (*ELA-DRB*) second exon was investigated using polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism (RFLP) of PCR products (PCR-RFLP) and deoxyribonucleic acid (DNA) sequencing. Eight distinct PCR-RFLP patterns could be identified in the studied Argentine Creole (AC) horses. The number of observed patterns per individual ranged from four to six, thus confirming the presence of multiple *DRB* copies in AC horses. Three PCR-RFLP alleles and three new sequences were identified. The estimated rates of synonymous and non-synonymous substitutions among *ELA-DRB* exon 2 sequences were higher within the antigen recognition site (ABS) than on the non-ABS. Phylogenetic analysis showed that the nucleotide sequences clustered in two main groups, while some sequences were not included in either group. Finally, the identification of the number of alleles per animal, the phylogenetic and segregation analyses allowed us to explain the number of *ELA-DRB* loci. However, it was not possible to identify specific alleles with specific loci.

Keywords Argentine Creole horses, *ELA-DRB*, MHC, PCR-RFLP, polymorphism.

Introduction

Class II major histocompatibility complex (*MHC*) products are highly polymorphic cell-surface molecules involved in the initiation of the immune response to foreign antigens. These molecules are heterodimers constituted by α and β chains encoded by closely linked A and B genes. The polymorphic sites of the class II genes are mainly located in exon 2, which codes for the first extracellular domain or the antigen binding site (ABS). Most class II genes show large genetic variation among and within species in both the number of loci and alleles (Bontrop *et al.* 1999; Lewin *et al.* 1999).

There appear to be multiple *DRB* loci in horses. Fraser & Bailey (1996) proposed the existence of as many as three *DRB* copies in domestic horses, *Equus caballus*. However, Hedrick *et al.* (1999) proposed the presence of only two *DRB* genes in *E. przewalski*. To date, 11 equine leukocyte antigen-*DRB* (*ELA-DRB*) exon 2 sequences have been reported for *E. caballus* (Gustavsson & Andersson 1994; Fraser & Bailey 1996) and six additional ones for *E. przewalski* (Hedrick *et al.* 1999). Considering the similarity between alleles described to date and the absence of a specific typing method for *ELA-DRB* genes, it has been difficult to assign sequences to specific loci in domestic horses.

In this study we analysed the polymorphism of *ELA-DRB* genes in Argentine Creole (AC) horses using restriction fragment length polymorphism of polymerase chain reaction products (PCR-RFLP) and deoxyribonucleic acid (DNA) sequencing methods, in order to identify additional

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ELA-DRB sequences in domestic horses. The number of alleles per animal, phylogenetic and segregation analyses allowed us to address two fundamental questions: the number of *ELA-DRB* loci and the assignment of *ELA-DRB* sequences to specific loci.

Materials and methods

Blood samples and DNA isolation

Blood samples from 27 AC horses belonging to three paternal half-sib families were used. Each family consisted of a sire, four dams and their respective offspring. Genomic DNA from blood lymphocytes and recombinant plasmid DNA isolation was performed by the DNAzol® method and Concert® Rapid Plasmid Purification System (Gibco, BRL-Life Technologies, Rockville, MD, USA), respectively.

PCR amplification

The oligonucleotide primers DRB2a and DRB2b developed by Fraser & Bailey (1996) were used for amplification of the *ELA-DRB* exon 2 by PCR. The DRB2a primer includes the first four bases of exon 2 and the DRB2b primer includes the last nine bases of exon 2 (Fig. 1). The amplification reactions were those previously reported (Albright-Fraser *et al.* 1996), with the exception of a final concentration of $MgCl_2$ (2 mM) in the reaction buffer.

RFLP analysis

Twelve microlitres of PCR product from AC genomic DNA samples or recombinant plasmid DNA were digested with 2.5 units of *Hae*III, *Rsa*I or *Msp*I. Restriction fragments were resolved by gel electrophoresis on 8% (19:1) acrylamide–bis acrylamide gels, according to van Eijk *et al.* (1992) and visualized with ethidium bromide staining. An *Msp*I digest of pBR322 was used as size marker (New England BioLab Inc., Beverly, MA, USA).

Cloning and DNA sequencing

The *DRB* amplification products were cloned into dT-tailed pGEM-T easy vector (Promega, Madison, WI, USA). *Escherichia coli* competent cells (strain DH5αF′) were transformed with the recombinant plasmid and selected by ampicillin-white/blue screening.

Inserts from 40 positive clones for each animal were confirmed by PCR using the *DRB* primers mentioned above. Restriction fragment length polymorphism patterns were determined in each positive clone in order to define the putative *DRB* allele. Polymerase chain reaction products corresponding to different *DRB* alleles were chosen for DNA sequencing.

The DNA sequencing was performed with an Applied Biosystems 377 automated sequencer (Perkin-Elmer, Foster City, CA, USA) using ABI PRISM ready reaction dye-terminator (Commonwealth Biotechnologies Inc., Richmond, VA, USA). Three clones of each distinct RFLP pattern were sequenced in both directions using universal primers. Sequence was accepted if at least four of six reactions produced identical results at a given base.

As no locus designations are defined yet for horse *MHC DRB* genes, our sequence numbering continues sequentially from horse *DRB* sequences previously submitted, designating the alleles as follows: *LOCUS* *allele number (e.g. *DRB* *5).

Sequence analysis

The restriction sites analyses of reported DNA sequences were confirmed using the computer program Webcutter 2.0 (Max Heiman, Copyright 1997, Yale University in New Haven, CT, USA). The multiple alignment of the nucleotide and amino acid sequences of the *ELA-DRB* second exon were carried out using CLUSTAL W version 1.7 (Baylor College of Medicine, Human Genome Sequencing Center, Houston, TX, USA; Thompson *et al.* 1994), while the pairwise sequence alignment and identity calculation for DNA and protein sequences were estimated by means of BLAST 2 version blastn 2.0.8 (the Baylor College of Medicine Search Launcher; Altschul *et al.* 1997) and SIM (the Baylor College of Medicine Search Launcher; Huang & Miller 1991), respectively.

The equine *DRB* exon 2 sequences were subjected to phylogenetic analysis and neighbour joining trees were constructed from Jukes–Cantor distance and Kimura's two parameter estimates. The bootstrap statistical test was used to test the topology of the tree. The phylogenetic analysis and the rate of synonymous (d_S) and non-synonymous (d_N) substitutions per site according to Nei & Gojobori (1986), applying Jukes & Cantor's (1969) correction for multiple hits, were estimated using the MEGA program version 2.1 (Pennsylvania State University, PA, USA; Kumar *et al.* 1994).

Results

RFLP analysis

The oligonucleotide primers DRB2a and DRB2b amplified the equine *DRB* gene and gave a single product of the expected size (~276 bp). Genomic DNA typing of Creole horse samples showed eight PCR-RFLP defined alleles for the

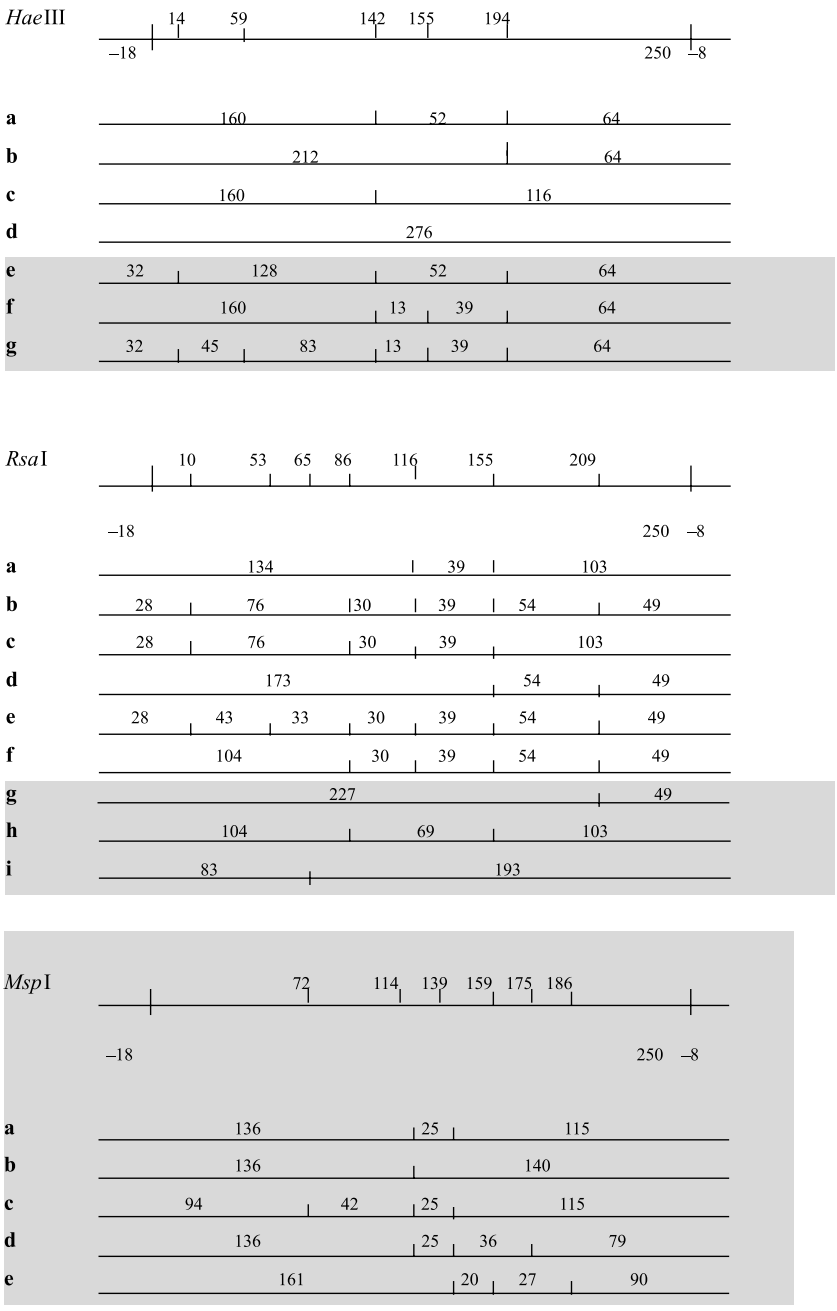

Figure 1 *Hae*III, *Rsa*I and *Msp*I restriction patterns of the polymerase chain reaction (PCR) product of *ELA-DRB* exon 2. The full length of the PCR product is 276 bp, that includes the 22 bp 5′ primer, 237 bp of *DRB* sequence and the 17 bp 3′ primer. The length of the restriction fragments (*Hae*III a–d, *Rsa*I a–f) were modified from Peral-García *et al.* (1999), taking into account the oligonucleotide primers used in the present work. 'I' indicates the boundary between exon 2 and flanking introns. Nucleotide positions of restriction sites are indicated above the site. Sizes for restriction fragments are indicated between the sites. The shaded lines represent the newly defined alleles.

second exon of *ELA-DRB* (Table 1 and Fig. 1). The amplification products of these horse samples were cloned and the restriction patterns observed in the cloned DNA segment corresponded to those previously detected in the digestion of PCR products from genomic DNA. Screening of clones indicated that the number of alleles in each individual ranged from four to six. The Mendelian inheritance of these alleles was verified by family segregation and the alleles detected in the offspring were present in either the dam or the sire.

The 11 *ELA-DRB* reported sequences were analysed for *Hae*III, *Rsa*I, *Msp*I and *Bst*YI restriction sites. These enzymes could distinguish all sequenced alleles (Table 1). The combined restriction enzyme analyses showed: (1) one to five restriction sites per sequence resulting in seven restriction patterns for *Hae*III; (2) one to six restriction sites per

Table 1 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) defined alleles and their correspondence to the *ELA-DRB* sequenced alleles.

	RFLP patterns		
Sequence and accession number	*Hae*III	*Rsa*I	*Msp*I
*DRB**1 (L25644)	a	e	a
*DRB**2 (L25645)	c	f	a
*DRB**3 (L25646)	b	f	b
*DRB**4 (L77079)	a	a	a
*DRB**5 (L76978)	a	b	a
*DRB**6 (L76972)	d	c	b
*DRB**7 (L76973)	a	f	a
*DRB**8 (L76975)	a	d	a
*DRB**9 (L76974)	b	e	b
*DRB**10 (L76976)	a	d	c
*DRB**11 (L76977)	c	c	a
*DRB**12 (AF144564)	e	h	d
*DRB**13 (AF170067)	f	g	a
*DRB**14 (AY026311)	g	i	e

sequence resulting in nine restriction patterns for *Rsa*I; and (3) one to three restriction sites per sequence resulting in five restriction patterns for *Msp*I (Fig. 1). None of the sequences contained restriction sites for *Bst*YI. Consequently, 14 PCR-RFLP defined alleles could be detected by this method (Table 1).

Sequencing of *ELA-DRB*

In order to confirm the position of the predicted restriction sites from the reported sequences and determine the position of the unknown sites of the new observed restriction patterns, different clones were sequenced. They consisted of the clones that exhibited either the restriction patterns *Hae*III 'e', 'f' or 'g', *Rsa*I 'g', 'h' or 'i' and *Msp*I 'd' or 'e' (Fig. 1). Out of five PCR-RFLP defined alleles that were sequenced, two correspond to the *ELA-DRB**9 and *ELA-DRB**11 variants, while the other three represented new *ELA-DRB* alleles (*ELA-DRB**12, -13 and -14; Figs 2 and 3). These novel sequences showed three new *Hae*III (nucleotides 10, 55 and 151) restriction sites, two new *Msp*I (nucleotides 159 and 186) restriction sites and a new *Rsa*I restriction site (nucleotide 61). These data allowed us to explain all the observed restriction patterns.

DNA sequence analysis

Figure 2 shows DNA sequences for the *ELA-DRB* exon 2 new alleles *DRB**12, *DRB**13 and *DRB**14. The pair-wise comparisons between all the DNA sequences showed an identity ranging from 83 to 98%. Eighty-nine of the 237 nucleotide sites (37.5%) of the *ELA-DRB* exon 2 sequence were polymorphic.

At the predicted amino acid level, 42 of 83 sites (50.6%) were variable (Fig. 3). Among them, 14 of 16 sites (87.5%), corresponding to ABS, were variable. Variability for non-ABS involved 28 out of 67 sites (41.8%). Furthermore, 23 additional amino acid substitutions for *ELA-DRB* exon 2 were detected in the new reported sequences, neither present on *E. caballus* nor on *E. przewalski* reported sequences (Fig. 3).

The estimation of the average rate of synonymous and non-synonymous substitutions in the exon 2 of *ELA-DRB* showed similar values ($d_N = 0{\cdot}159$; $d_S = 0{\cdot}134$). For the ABS the rate of non-synonymous substitutions ($d_N = 0{\cdot}554 \pm 0{\cdot}241$) exceeded the number of synonymous substitutions ($d_S = 0{\cdot}126 \pm 0{\cdot}074$), larger than four units. In contrast, non-ABS exhibited more synonymous substitutions ($d_S = 0{\cdot}173 \pm 0{\cdot}036$) than non-synonymous ones ($d_N = 0{\cdot}089 \pm 0{\cdot}018$) with the d_N/d_S ratio of 0·065.

The *ELA-DRB* DNA sequences' tree constructed with the neighbour joining method from Jukes–Cantor distance estimates shows that there are at least two major allelic clusters (Fig. 4). The same relationships were found when the distances were calculated using the neighbour joining method from Kimura's two parameters. However, phylogenetic relationships among different alleles were not very clear. Group I consisted of four horse alleles, supported by bootstrap values of 69%, while alleles *DRB**4 and *DRB**5 clustered with group I but with low bootstrap value. Group II included four *ELA-DRB* sequences (99% bootstrap value), while *DRB**14 clustered with this group with low bootstrap value. The alleles *DRB**2, *DRB**10 and *DRB**12 located out of the main groups. Additionally, *E. przewalski* alleles were interspersed with *E. caballus* alleles (Fig. 4).

Discussion

The PCR-RFLP polymorphism of *ELA-DRB* exon 2 was first reported by Peral-García *et al.* (1999). In the present work we selected *Msp*I because, in combination with *Rsa*I and *Hae*III, it allowed us to distinguish all 14 reported sequences (Table 1).

In the AC horses, we found eight PCR-RFLP defined alleles (Table 1) that included three newly reported *DRB* exon 2 sequences. The number of different variants detected within each genomic DNA amplification product that was cloned ranged from four to six. These data are in agreement with Fraser & Bailey (1996) who proposed the existence of three copies of the *DRB* gene in at least some domestic horses. In contrast, Fraser & Bailey (1996) found only two sequences in an Andalusian stallion and Hedrick *et al.*

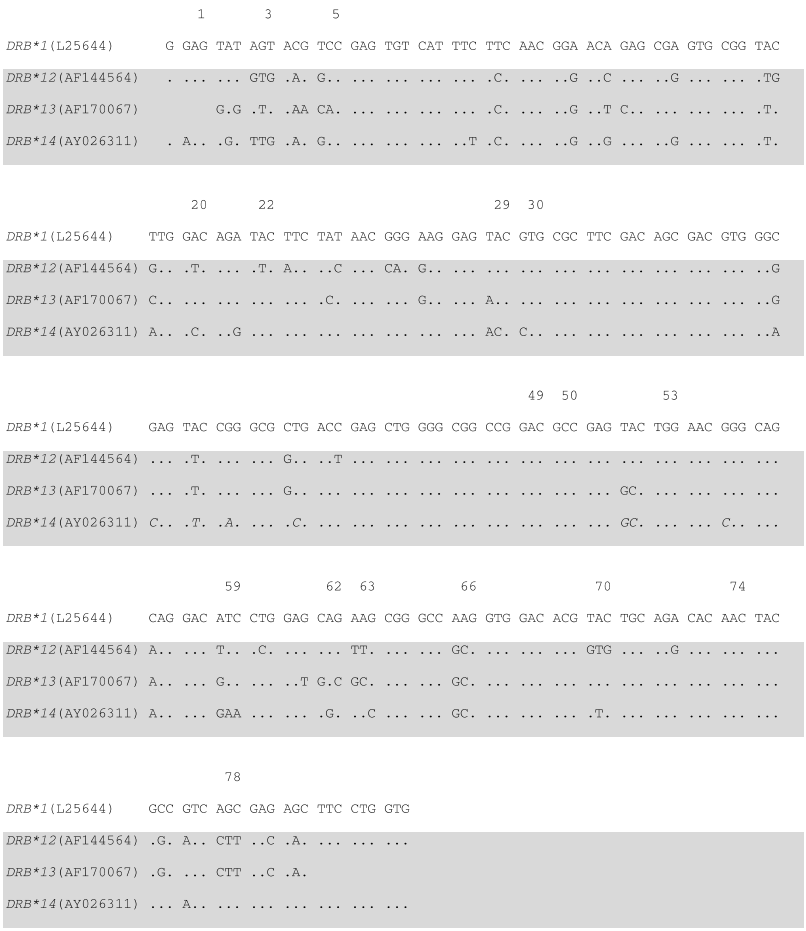

Figure 2 New *ELA-DRB* exon 2 DNA sequences. The sequence numbering continues sequentially from horse *DRB* sequences previously submitted (Gustavsson & Andersson 1994; Fraser & Bailey 1996). Identity to *DRB**1 is shown by a dot. The numbers indicate the predicted aminoacid positions of the contact antigen sites. GenBank accession numbers are presented in parentheses.

(1999) suggested the presence of two *DRB* genes in Przewalski's horses. These results suggested the possibility of haplotypes with variable number of *DRB* loci within and between horse species. This variation is not unexpected within a particular species and among closely related species, as this feature has been reported for many *MHC* genes (e.g. Bontrop *et al.* 1999; Lewin *et al.* 1999). Additional studies are needed to elucidate the number and composition of *DRB* loci within each equine haplotype.

In order to assign the DNA sequences to specific loci, we analysed the phylogenetic tree, the allele segregation and the number of synonymous and non-synonymous substitutions. The phylogenetic analysis showed that the *DRB* sequences clustered in two major allelic groups. These results could indicate two gene duplication events during the evolutionary history of horse *DRB* genes. The first duplication could have resulted in the two main allelic groups (referred to here as I and II) from a common ancestral gene. Another duplication event within group I could have divided it into two clusters. The phylogenetic tree could support the presence of three *DRB*-like genes. Additionally, *E. przewalski* alleles were intermingled with *E. caballus* alleles, sorted with the three groups of alleles (Fig. 4). The duplication of the *ELA-DRB* genes could have arisen before the divergence between *E. caballus* and *E. przewalski*, being an example of the trans-species polymorphism phenomenon (Klein 1987). These data could suggest the same scenario for the evolution of *DRB* genes in equine species.

However, in disparity with the results reported by Hedrick *et al.* (1999), the relationships among the different *DRB* alleles showed by our phylogenetic tree are not so clear. The alleles *ELA-DRB**2, *10, *12 and *14 are not included in either group I or II, being intermingled between them (Fig. 4). The low resolution of *MHC* class I and class II allelic lineages have been explained in different ways: first, a high rate of recombination, which indicates interlocus recombination (or gene conversion). Secondly, this allelic classification was based on a single exon. Additional allelic classifications for *ELA-DRB* variants using other exon

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                        10         20         30         40         50         60         70         80
DRB*1(L25644)    EYSTSECHFF NGTERVRYLD RYFYNGKEYV RFDSDVGEYR ALTELGRPDA EYWNGQQDIL EQKRAKVDTY CRHNYAVSES FLV
DRB*2(L25645)    .AVKF..R.S .......F.E .R.H..E..A .......... .V........ .......... D.R..E.... .....G.IDG ...
DRB*3(L25646)    .LVKH....S ...Q...F.. .....RE... .......... .V.K...T.. ..#
DRB*4(L77079)     .T......S ...Q...F.. ........T. .......... .V........ ......K.F. DDA..A...L .....GI...
DRB*5(L76978)     ........S ...Q...F.. ...S..E.T. .......... .V........ ......K.V. DDA..A.... ..........
DRB*6(L76972)     ...F....S ...Q..L..H .L........ .....L.... .......RS. .......... ....,E...V .....G....
DRB*7(L76973)     LVKH....S ...Q...F.. .....RE... .......... .V........ ......K.V. DDA..A.... .....G..D.
DRB*8(L76975)     LVKH....S ...Q...F.. ...H....F. ........F. .V........ ......K.V. DDA..Q.... .....GI.D.
DRB*9(L76974)     ......... .......... .......... .......... .......RS. .......... .......... ..........
DRB*10(L76976)    LVKH....S ......X.VF .DV..RE.H. ........F. .V........ ......K.V. .R...A.... .....G.LDN
DRB*11(L76977)     ...F....S ...Q..L..H .L........ .....L.... .,........ .......... .....E...V .....X....
DRB*12(AF144564) ..VKA....S .......LVV .FI..QE... ........F. .V........ .....K.FP ..L..A...V .....GILDN ...
DRB*13(AF170067)  EIKH....S ...Q...F.. ...S..E.N. ........F. .V........ .A....K.V. DDA..A.... .....G.LDN
DRB*14(AY026311) KCLKA....S .......FMA ........TL .......QFQ .P........ .A..R.K.E. .RN..A...F .....I... ...
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Figure 3 Predicted amino-acid sequence of new *ELA-DRB* exon 2 alleles (shaded). Identity to *ELA-DRB**1 is shown by a dot. GenBank accessionnumbers are presented in parentheses. Bold residues are those which are thought to contact antigen (Brown *et al*. 1988). The '#' on *ELA-DRB**3 (L25646) indicates a stop codon. Italicized residues indicate the 20 new amino acids were located on putative antigen binding sites (ABS) (positions 1, 3, 5, 20, 29, 30, 38, 59, 63, 70) and on the non-ABS (positions 2, 18, 19, 23, 26, 40, 42, 52, 55, 60).

sequences or a complete coding sequence would be needed as this method is statistically more reliable than that based on a single exon. And finally, phylogenetic reconstruction based on only the peptide-binding region could be misleading because positive selection for polymorphism and/or convergent evolution apparently occurs in this region (Gu & Nei 1999).

The pair-wise comparisons of the similarity among the 14 *ELA-DRB* exon 2 nucleotide sequences showed that the similarity within groups I and II (94–98% and 90–98%) was higher than the identity values among them (83–91%). These results could represent additional evidence that would support the allelic relationships of the sequences that belong to the same group.

The segregation of the different PCR-RFLP defined alleles in the tested horses were verified, showing that the alleles observed in the offspring were present in at least one of the parents. However, these alleles have not been assigned to a single locus so it is not possible to know whether these alleles are in heterozygous or homozygous state. In addition, the joint analysis of the phylogenetic tree and the segregation data showed that the alleles detected on each animal

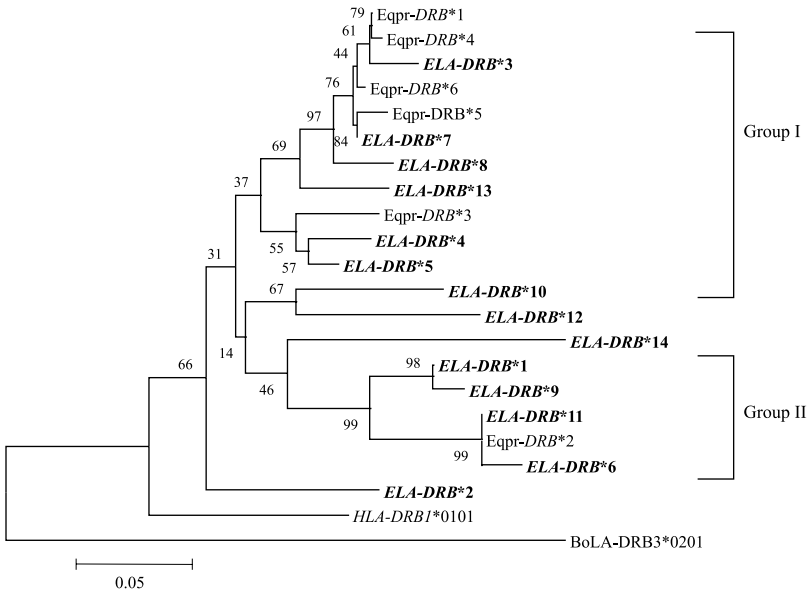

Figure 4 Phylogenetic tree of the 19 equine *DRB* exon 2 sequences constructed by means of the neighbour joining method from Jukes–Cantor distance and Kimura's two parameter estimates. The bootstrap confidence levels are shown in the figure. Additional *DRB* sequences include *HLA-DRB1**0101 (Marsh & Bodmer 1991) and *BoLA-DRB3**0201 (Sigurdardóttir *et al*. 1991).

were distributed throughout the entire tree. For example, one offspring exhibited two alleles that corresponded to group I (*ELA-DRB**3 and *7), two alleles from group II (*ELA-DRB**9 and *11) and two alleles that corresponded to the intermingled sequences (*ELA-DRB**10 and *12), supporting the presence of at least three *DRB* copies in AC horses. Fraser & Bailey (1996), analysing homozygous by descent individuals, predicted that *ELA-DRB**4, *5 and *6 were the allelic pairs to *ELA-DRB**7, *8 and *9, distribution that fits with our tree (Fig. 4).

The analysis of predicted amino acid changes among sequences clearly showed similar rates of non-synonymous and synonymous substitutions in *ELA-DRB* exon 2. However, as expected, the d_N/d_S ratio within the ABS is greater than within the non-ABS. This feature was common to almost all species, including domestic animals, analysed to date. Comparison of rates of non-synonymous and synonymous substitutions provided strong evidence that *MHC* polymorphism is maintained by positive selection (Hughes & Nei 1988). As the 14 *ELA-DRB* sequences belong to different loci, some of which could be pseudogenes, these sequences could be under distinct selective processes. Therefore, if the sequences clustered within groups I and II, all corresponded to expressed loci, we would expect a higher d_N/d_S ratio within each group. Nevertheless, this analysis did not show a clear pattern, failing to add novel data that support the reliability of the clusters of the *ELA-DRB* sequences.

In conclusion, the PCR-RFLP and DNA sequencing technique provided evidence of polymorphism in exon 2 of *ELA-DRB* genes in AC horses. The presence of more than two alleles for each animal showed that the primers employed in this work are not specific for a unique *DRB* locus. Assignment of allelic and non-allelic relationships of *ELA-DRB* sequences would allow the development of specific set of primers for typing purposes.

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