Biochemical Genetics, Vol. 41, Nos. 11/12, December 2003 (© 2003)

Biochemical and Population Genetics of the Rabbit, *Oryctolagus cuniculus*, Carbonic Anhydrases I and II, From the Iberian Peninsula and France

M. Branco[1,2,4] **and N. Ferrand**[1,3]

Received 14 August 2002—Final 18 December 2002

*Available studies on the biochemical and electrophoretic characterization of European rabbit (*Oryctolagus cuniculus*) carbonic anhydrases I and II (CAI, CAII) show contradictory results about their relative electrophoretic mobility and substrate specificity. After positive identification of carbonic anhydrase activity by CO_2 hydration, the differential esterase activity of CAI and CAII toward β-napththyI acetate and flourescein diacetate,respectively, were used to identify the banding patterns corresponding to each locus. Electrophoretic and hybrid isoelectric focusing analyses of the CAI and CAII loci in 1 domestic and 19 wild rabbit populations led to the recognition of genetic polymorphism at the CAI locus and of extensive variability at the CAII locus. Four and nine alleles at the CAI and CAII loci, respectively, are described. The geographic distribution of genetic variability is consistent with the existence of two evolutionary groups within* O. cuniculus.

KEY WORDS: carbonic anhydrases; genetic polymorphism; specific enzyme detection; European rabbit; *Oryctolagus cuniculus*; hybrid isoelectric focusing.

INTRODUCTION

The carbonic anhydrases are a family of zinc metalloenzymes that catalyze the reversible hydration of CO_2 in the reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. So far,

[1] Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Universidade do Porto, Campus Agrário deVairão, 4485-661 Vairão, Portugal.
[2] Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCLM), 13005 Ciudad Real, Spain.
[3] Departamento de Zoologia e Antropologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal.
[4] To whom correspondence should be addressed; e-mail: msbranco@fc.up.pt.

0006-2928/03/1200-0391/0

11 enzymatically active enzymes have been discovered (Hewett-Emmett, 2000; Parkkila *et al*., 2001; Tashian, 1992; Hewett-Emmett and Tashian, 1996), which are present in a variety of tissues where they participate in several physiological processes.

Genetic polymorphism of rabbit (*Oryctolagus cuniculus*) carbonic anhydrase II (CAII) was described in domestic animals by Bernoco (1970), using starch gel electrophoresis. Additional studies confirmed this result both in domestic and wild rabbit populations, with the recognition of two common alleles, *CAII*1* and *CAII*2* (Arana and Zaragoza, 1986; Daly, 1981; Richardson *et al*., 1980). Rabbit carbonic anhydrase I (CAI) was identified as having no genetic variation and, migrating anodal to CAII (Richardson *et al*., 1980; Arana and Zaragoza, 1986).

Results on relative electrophoretic mobility of rabbit CAI and CAII were found to be highly contradictory when compared to their biochemical characterization by McIntosh (1970), Walther *et al*. (1977), and Skipski and Scott (1980). According to these authors, in contrast to most mammals, the chromatographic analysis clearly demonstrates that rabbit CAI migrates cathodally relative to CAII, leading to a similar expectation when they are studied with electrophoretic techniques. Walther *et al*., (1977) explicitly mention that the identification of both forms of carbonic anhydrase cannot be done solely on the basis of electrophoretic mobility, but it also requires an analysis of substrate specificity.

The rabbit species is known to have originated in the Iberian Peninsula (Callou, 1995; Lopez-Martinez, 1989). It is composed of two independent evolutionary groups that can be related to the accepted subspecies, *Oryctolagus cuniculus cuniculus* and *Oryctolagus cuniculus algirus. O. c. algirus* is found in the Iberian southwest (SW) and *O. c. cuniculus* is present in the Iberian northeast (NE) and in France (FR). Populations introduced by humans, both wild and domestic, and all domestic breeds are *O. c. cuniculus* (Ferrand, 1995). The two evolutionary groups are clearly identified by protein (Branco, 2000; Branco and Ferrand, 1998; Ferrand, 1995) and immunoglobulin (van der Loo *et al*., 1991, 1999) genetic variation as well as by mitochondrial DNA nucleotide polymorphism (Monnerot *et al*., 1994; Branco *et al*., 2000, 2002). Because of an ancient allopatric separation, both evolutionary groups originated within the Iberian Peninsula where they currently form a secondary contact zone across a southeast–northwest axis (Branco, 2000; Branco *et al*., 2002).

In the present study, we make a complete reevaluation of the separation and detection methods of rabbit CAI and CAII, taking into consideration the high sulphydryl reactivity of CAII and the differential esterase activity of the two enzymes toward specific substrates. Additionally, we describe, for the first time, genetic polymorphism at the CAI locus in the rabbit, the allele frequency variation of *CAI* and *CAII* loci, and their linkage disequilibrium, across the natural range of the rabbit (Iberian Peninsula and France) and in one Portuguese domestic stock of

mixed-breed origin, and discuss the contribution of these loci to our understanding of rabbit evolutionary history.

MATERIAL AND METHODS

A total of 907 blood samples were collected from 16 populations covering most of the Iberian Peninsula, plus three French populations and one Portuguese domestic stock of mixed-breed origin (Fig. 1). Blood samples were collected and stored as described elsewhere (Ferrand *et al*., 1988).

Electrophoresis and Isoelectric Focusing

For agarose gel electrophoresis, haemolysates were incubated with a 120 mM solution of dithiothreitol (DTT) in a 1:1 (v/v) proportion for 1 h at 37°C. For isoelectric focusing separation, haemolysates were incubated with a 40 mM

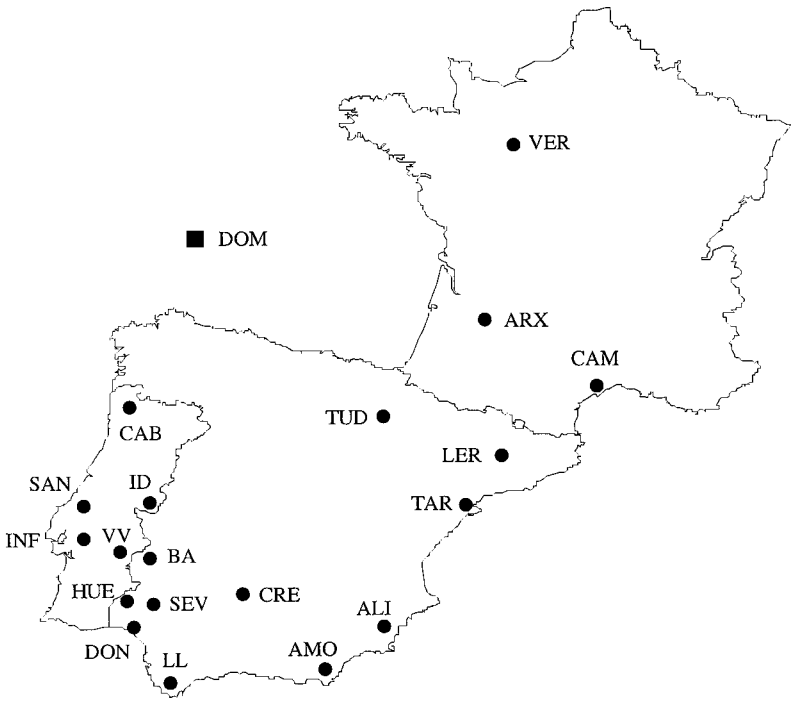

Fig. 1. Sampling sites for the 20 rabbit populations. CAB—Cabreira, ID—Idanha, SAN—Santarém, INF—Infantado, VV—Vila Viçosa, BA—Badajoz, CRE—Ciudad Real, AMO—Amoladeras, DON—Doñana, HUE—Huelva, SEV—Sevilha, LL—Las Lomas, ALI—Alicante, LER—Lérida, TAR—Tarragona, TUD—Tudela, CAM—Camargue, VER—Versailles, ARX—Arjuzanx, DOM—Portuguese domestic stock of mixed-breed origin.

solution of iodoacetic acid (IACH) in a 1:1 (v/v) proportion for 1 h at 37°C, and distilled water was added to a final dilution of 1:10.

Electrophoretic separation of CAI and CAII was carried out in an agarose gel (1% w/v, EEO, SERVA) using 0.095 M sodium barbital/0.048 M HCl (pH 9.2) as bridge buffer. A 1:1 dilution of the bridge buffer was used as gel buffer. Gels were run for 2 h with a voltage gradient of 17 V/cm, at 8°C. To compare the results obtained with this methodology and those previously described, starch gel electrophoresis (15% w/v, SIGMA) was carried out according to Hopkinson *et al.* (1974).

Specific detection of CO_2 hydration was used for a clear identification of CAI and CAII positions in agarose gels, but a correct analysis of banding patterns was not possible due to poor resolution. CAI and CAII, in both agarose and starch gels, were identified through their differential esterase activity toward different substrates (Harris and Hopkinson 1976). Thus, β-napthtyl acetate and fluorescein diacetate were used to detect CAI and CAII banding patterns, respectively, following Harris and Hopkinson (1976) protocols. Phenotypic survey of populations was carried out by protein staining with Coomassie R-250.

Hybrid isoelectric focusing (HIEF) separation of CAII was carried out in a 6.50–7.50 immobilized pH gradient in polyacrylamide gel ($T = 5\%$, $C = 3\%$, $247 \times 112 \times 0.5$ mm) constructed as indicated in the Application Note Nr 324 of LKB, and reduced by 2/3 the amount of immobilines. Immobiline reduction led to less protein adsorption to the matrix components and a better contrast of banding patterns. Immobiline gels were dehydrated overnight at 45°C and rehydrated in a 20% (w/v) saccarose, 1.33% (v/v) 6–8 Ampholine (LKB) solution for 2 h at room temperature. Prefocusing was carried out using a 1.33% (v/v) 6–8 Ampholine (LKB) solution as electrodes setting limits at 1500 V, 4 mA, 12 W. After 30 min, an ionic front visible by the inclusion of bromophenol traces to the cathode, reached the anode, at which time electrodes were replaced by 0.01 M glutamic acid and 0.01 M sodium hydroxide as anode and cathode solutions, respectively. Ten μL of the haemolysate solution was applied 1.5 cm from the anode in a silicone strip. Focusing was performed at constant voltage setting limits at 1500 V, 4 mA, 12 W (30 min), 3000 V, (2 h), and 5000 V (2 h). CAII banding patterns were visualized by a general protein staining with Coomassie R-250.

Data Analysis

Allele frequencies of *CAI* and *CAII* were obtained by direct gene counting. Linkage disequilibrium between *CAI* and *CAII* for the unknown gametic phase was analyzed through a likelihood-ratio test and maximum likelihood haplotype frequencies were estimated, using Arlequin 2.0 software package (Schneider *et al.*, 2000). *CAI/CAII* haplotypes were constructed considering only *CAII* electromorphs *CAII*1* and *CAII*2*.

RESULTS

Identification of CAI and CAII Specific Activity After Electrophoretic Separation

Figure 2 shows the results of specific CAI and CAII esterase detection in the same starch gel. β-naphthyl acetate is rapidly hydrolysed by CAI, and CAII has strong esterase activity towards fluorescein diacetate (Harris and Hopkinson, 1976). Figure 3 shows the results of fluorescein diacetate detection followed by general protein staining in an agarose gel. Both figures show that, in the rabbit, CAI has a cathodal position relative to CAII conforming with the chromatographic and electrophoretic characterization carried out by Walther *et al*. (1977) and Skipski and Scott (1980), respectively.

Good resolution is obtained with both starch and agarose media. Electrophoretic separation in agarose gel takes 2 h, while in starch gel it takes 16 h, therefore population survey of CAI and CAII was carried out in agarose gels. Phenotyping was carried out using fluorescein diacetate for CAII detection prior to a general protein staining to read CAI phenotypes, thus permitting further confirmation of CAII phenotypes (Fig. 3).

Description of CAI Genetic Polymorphism

Phenotyping of 907 samples from 1 domestic and 19 wild populations led to the identification of four electromorphs in locus *CAI* (Fig. 4). Observed allele

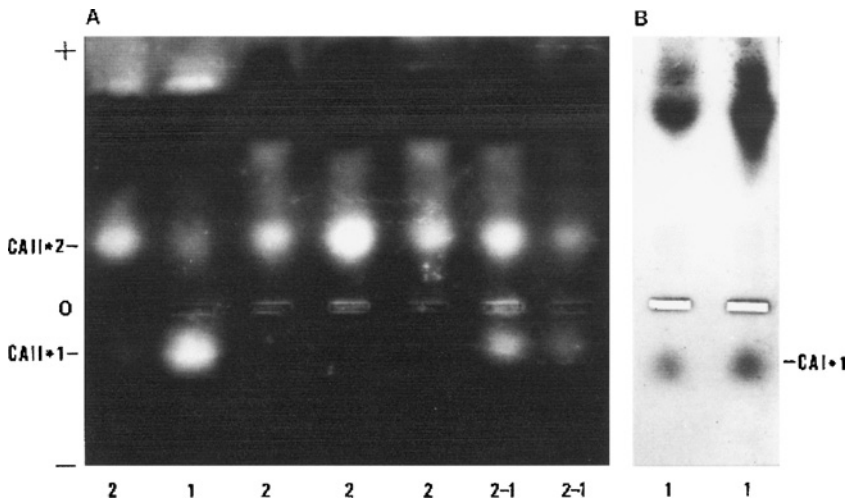

Fig. 2. Electrophoretic patterns of CAII (A) and CAI (B) in starch gel detected with fluorescein diacetate and β-naphthyl acetate, respectively.

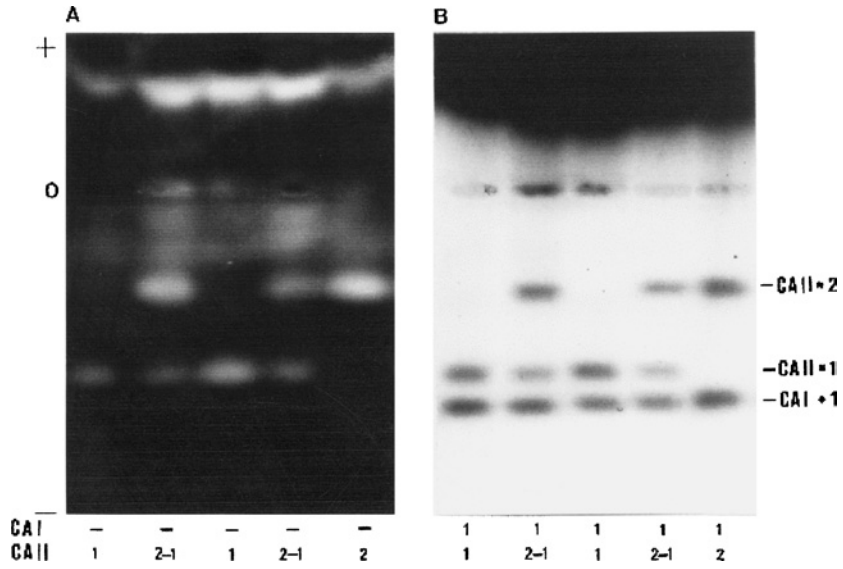

Fig. 3. Banding patterns of CAII and CAI observed after electrophoresis in agarose gel. (A) Fluorescein diacetate was used to detect CAII followed by (B) detection of both CAI and CAII with general protein staining.

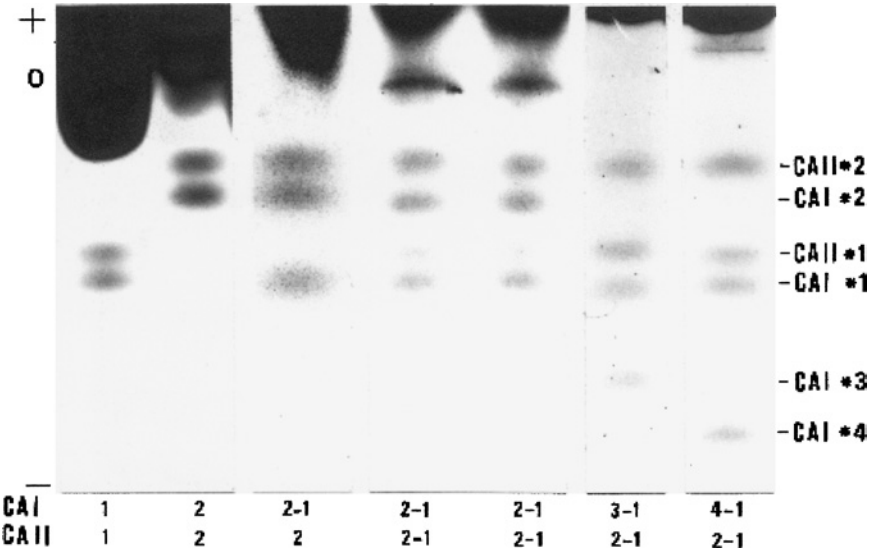

Fig. 4. Phenotypes of CAI and CAII determined by general protein staining after electrophoretic separation in agarose gel.

frequencies of the *CAI* locus are described in Table I. *CAI*1* is the most common allele, it is present in all populations and fixed in most of the Iberian south-west, *CAI*2* is typical of wild rabbits from the Iberian northeast and from France, *CAI*3* and *CAI*4* are low frequency private alleles for Idanha and Las Lomas, respectively. The domestic stock was found to be monomorphic for *CAI*1*.

Genetic Polymorphism of CAII: Electrophoresis and Hybrid Isoelectric Focusing

Phenotyping of 846 samples from 1 domestic and 19 wild populations by electrophoretic separation led to the recognition of the two electromorphs, *CAII*1* and *CAII*2* (Fig. 4), previously described (Bernoco, 1970). Sample treatment with DTT and IACH showed that CAII has a reactive sulphydryl residue. Comparison of reduced and alkylated banding patterns, after agarose or starch gel electrophoresis, show that the difference between *CAII*1* and *CAII*2* is in one charged residue (bands of oxidized *CAII*1* coincide with *CAII*2* reduced ones). Therefore, conclusive interpretation of CAII phenotypes is only possible when there is complete reduction or alkylation of the sulphydryl residue.

Hybrid isoelectric focusing separation of CAII revealed a high level of heterogeneity within both electromorphs, *CAII*1* and *CAII*2*, with five and four alleles, respectively. Figure 5 shows that gene products within *CAII*1* and *CAII*2* form two well-separated groups in a 6.5–7.5 pH interval. *CAII*1A* and *CAII*2A* show the largest separation, whereas *CAII*1B*, *CAII*1C*, *CAII*1D*, and *CAII*1E* cluster around *CAII*1A*, and *CAII*2B*, *CAII*2C*, and *CAII*2D* are close to *CAII*2A*. The fact that these alleles are not detected when conventional electrophoresis is used, suggests that what distinguishes them are substitutions of neutral amino acids or charged ones with altered pK values. Hybrid isoelectric focusing separation of CAII also confirmed the presence of a second cysteine in the CAII molecule, in agreement with Walther *et al*. (1977). This second sulphydryl residue is much less reactive, as the band corresponding to the molecule with both cysteines alkylated is only observed when incubated with IACH at a concentration of at least 120 mM (results not shown).

Allele frequencies for the *CAII* locus are described in Table I. All populations were found to be polymorphic for this locus. *CAII*1A* is the most common allele in the Iberian northeast, in France and in the domestic stock and *CAII*2A* predominates in the Iberian southwest. Alleles other than *CAII*1A* and *CAII*2A* were only found in Iberian wild rabbits. *CAII*1B* is a low frequency allele detected in three southwestern locations (Infantado, Ciudad Real, and Sevilha). *CAII*1C* is typical of the southwest being found in most populations, with low frequencies as well. *CAII*1D* and *CAII*1E* are low frequency private alleles of Tudela and Badajoz, respectively. *CAII*2B* was observed in Vila Viçosa, Doñana, Sevilha and Tudela with frequencies between 0.01 and 0.07. *CAII*2C* is the only group *CAII*2*

Table I. Allele Frequencies of CAI and CAII Loci in One Domestic and 19 Wild Rabbit Populations[a]

	Populations[b]																			
	Iberian peninsula																France			
Alleles	CAB	ID	SAN	INF	VV	BA	CRE	AMO	DON	HUE	SEV	LL	ALI	LER	TAR	TUD	CAM	VER	ARX	DOM
*CAI*1*	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.98	0.81	0.96	0.86	0.71	0.45	1.00
*CAI*2*														0.02	0.19	0.04	0.14	0.29	0.55	
*CAI*3*		0.02																		
*CAI*4*												0.03								
N	20	52	65	55	35	15	20	11	37	163	18	63	31	26	24	37	29	63	43	100
*CAII*1A*	0.31		0.15	0.03	0.03	0.03	0.29	0.40	0.29	0.23	0.20	0.25	0.27	0.68	0.58	0.53	0.46	0.60	0.34	0.83
*CAII*1B*				0.05			0.03				0.02									
*CAII*1C*		0.15	0.04	0.02	0.09	0.06				0.06	0.05									
*CAII*1D*																0.16				
*CAII*1E*						0.03														
*CAII*2A*	0.64	0.84	0.82	0.71	0.87	0.88	0.68	0.50	0.70	0.71	0.64	0.75	0.73	0.32	0.42	0.28	0.54	0.40	0.66	0.17
*CAII*2B*					0.01				0.01		0.07					0.03				
*CAII*2C*	0.05	0.01		0.18							0.02									
*CAII*2D*								0.10												
N	29	43	57	49	35	16	19	10	47	54	22	54	13	19	12	16	28	135	43	145

[a] Screened by agarose gel electrophoresis and hybrid isoelectric focusing.
[b] Populations abbreviated as in Fig. 1.

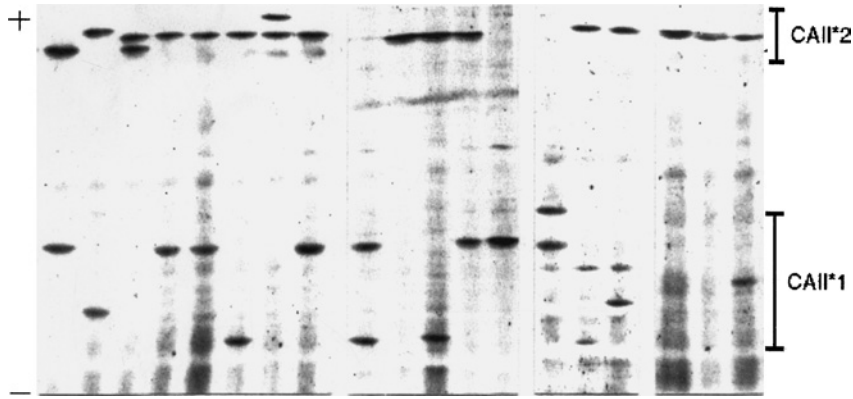

Fig. 5. Phenotypes of CAII determined by general protein staining after hybrid isoelectric focusing separation in polyacrylamide gel in a 6.5–7.5 pH gradient. From left to right, 2B1A, 2C1B, 2AB, 2A1A, 2A1A, 2A1C, 2AD, 2A1A, 1AC, 2A, 2A1C, 2A1A, 1A, 1AD, 2A1C, 2A1B, 2A, 2A, 2A1E.

allele detected in Idanha and was also found in Cabreira, Infantado, and Sevilha. *CAII*2D* was only detected in Amoladeras.

Linkage Disequilibrium Between *CAI* and *CAII*

Significant linkage disequilibrium of the unknown gametic phase was detected in Tarragona, Arjuzanx, Camargue, and Versailles. Maximum likelihood estimates of *CAI*1/CAII*1*, *CAI*1/CAII*2*, *CAI*2/CAII*1*, and *CAI*2/CAII*2* haplotype frequencies are described in Table II.

Table II. Maximum Likelihood Estimates of Haplotype Frequencies for the Pair of Loci CAI/CAII in Four Wild Rabbit Populations[a]

	Populations			
Haplotypes	Tarragona	Camargue	Versailles	Arjuzanx
n	20	29	64	43
*CAI*1/CAII*1*	0.625	0.448	0.585	0.337
*CAI*1/CAII*2*	0.250	0.414	0.134	0.116
*CAI*2/CAII*1*	0	0	0.009	0
*CAI*2/CAII*2*	0.125	0.138	0.272	0.547

[a]Calculations were made using Arlequin 2.0 software package (Schneider *et al*., 2000).

DISCUSSION

Comparison With Previously Published Results

An evaluation of several studies focusing on the biochemical and population genetics of rabbit carbonic anhydrases I and II (e.g. Arana and Zaragoza, 1986; Arana *et al*., 1989; Daly, 1981; Richardson *et al*., 1980; Skipski and Scott, 1980; Walther *et al*., 1977) revealed a number of contradictions.

Our comparative analysis of different electrophoretic and specific detection methods are consistent with the inversion of the relative electrophoretic mobilities of rabbit CAI and CAII relative to most mammalian species. In the rabbit, CAI is cathodal to CAII, according to the biochemical characterization of Walther *et al*. (1977) and Skipski and Scott (1980) using, respectively, chromatography and electrophoresis. Therefore, our results contradict the electrophoretic studies on rabbit CAI and CAII of Richardson *et al*. (1980), Arana and Zaragoza (1986) and Arana *et al*. (1989), because these authors assumed that CAI was anodal to CAII.

The high esterase activity of human CAI toward methylumbelipheryl acetate, demonstrated by Hopkinson *et al*. (1974), contrasts with a very low esterase activity of rabbit CAI (Skipski and Scott, 1980) (which we confirmed during the present work). Yet, Richardson *et al.* (1980) used this substrate in their population genetic studies of several wild rabbit populations, hindering the detection of CAI genetic polymorphism in several French populations included in their work. In fact, the enzyme detected by Richardson *et al*. (1980) is an esterase band anodal to CAII which is monomorphic in the rabbit (results not shown). CAII was detected with fluorescein diacetate, and thus Richardson *et al*. (1980) results are in agreement with our observations.

Because Arana and Zaragoza (1986) and Arana *et al*. (1989) used samples that had not been previously reduced or alkylated to phenotype both loci in the same gel using a protein-staining method, they assumed that CAI was anodal to CAII. Therefore, their banding patterns do not conform with those usually obtained with the electrophoretic method they used leading to the wrong interpretation for the genetic variability of CAI and CAII. Their incorrect identification of the electromorphs of both CAI and CAII becomes even more evident due to the contrasting allele frequencies they obtained, in both domestic and wild populations, when compared with our results and those of other authors (e.g. Bernoco, 1970; Richardson *et al*., 1980).

Analysis of Allele Frequency Variation Across the Studied Range

Geographic distribution of CAI genetic variation is characterized by a low level of polymorphism with a very common allele (*CAI*1*) fixed in most of the studied populations. In addition to *CAI*1*, only *CAI*2* shows a large geographic distribution including the Iberian northeast and France, although showing moderate

frequencies. Its complete absence from the Iberian southwest suggests that this is an allele private to the subspecies *O. c. cuniculus*. Assuming that domestic rabbits had their origin in populations of *O. c. cuniculus* (Ferrand, 1995), the absence of *CAI*2* from domestic stocks suggests that it was lost during domestication and thus can be useful to distinguish wild and domestic populations of this subspecies.

Allele frequency variation of the most common alleles of the CAII locus across Iberia and France conforms with the existence of two evolutionary groups within the rabbit species. *CAII*1A* increases in a northeast direction, while *CAII*2A* increases to the southwest. This pattern of allele frequency variation can be interpreted as the result of secondary contact and genetic admixture of those two previously separated groups.

Analysis of the mtDNA phylogeographic pattern across Iberia demonstrated that lineages A and B originated from a longterm allopatric separation, and that populations where both lineages exist, which are geographically intermediate between the SW and NE, are the result of recent geographic expansion and overlap of two diverging groups along a southeast–northwest axis across Iberia (Branco *et al*., 2000, 2002). The geographic distribution of mtDNA lineages is highly structured, contrasting with the allele frequencies of *CAII*1A* and *CAII*2A* that change gradually from one group into the other. Yet, the higher frequencies of *CAII*1A* and *CAII*2A* are respectively related to the predominance of mtDNA type B in NE and FR and of mtDNA type A in SW.

The genes coding for *CAI* and *CAII* are known to be tightly linked in the mammalian lineage (Tashian 1989, 1992). Although the genetic map of the rabbit is currently being developed (Korstanje 2000; Korstanje *et al*., 2001), there is no information about the location or linkage of the *CAI* and *CAII* genes.

In the present work, the detection of genetic polymorphism at the *CAI* locus allowed the testing of linkage disequilibrium (LD) in eight wild populations, but because of the high allele frequency of *CAI*1* in some of those populations, only Tarragona (NE Iberia), Arjuzanx (FR), Camargue (FR) and Versailles (FR) showed significant results (Table II). Still, the fact that it was found in all French populations studied is in agreement with the previous suggestion that France was recently colonized by wild rabbits from northeastern Iberia (Queney *et al*., 2001).

Do Allele Groups *CAII*1* and *CAII*2* Correspond to Independent Evolutionary Lineages?

Electrophoretic techniques are known to be inadequate for making inferences about genetic distances among alleles as relative positions after separation do not necessarily relate to their phylogenetic relationships (Antunes *et al*., 2002; Barbadilla *et al*., 1996; Ramshaw *et al*., 1979). In the case of locus *CAII*, several observations, related to electrophoretic behavior after HIEF separation as well as to the geographic distribution of alleles, lead us to hypothesize that the *CAII*1*

and *CAII*2* allele groups correspond to independent evolutionary lineages. All alleles revealed after HIEF separation have more or less restricted ranges, have low frequencies in Iberian populations and cluster around one of the most common alleles in the NE or in the SW (*CAII*1A* or *CAII*2A*). Thus, they not only form two well-separated groups in the 6.5–7.5 pH interval, but also define two geographically structured groups of populations. The scenario of two diverging lineages in *CAII* is concordant with what was previously described for the mtDNA (Branco *et al*., 2002), not only in the two lineage structure but also in their geographic distribution.

If, as we hypothesize, *CAII*1A* and *CAII*2A* represent the oldest forms of each lineage, we would expect that the derived forms would concentrate in the respective regions of origin. Yet, some of the variants of the *CAII*1* group appear in the southwest. Differential demographic histories of the SW and NE groups can explain those distributions. The Iberian SW harbors much better habitat for rabbit population establishment and growth relative to the Iberian NE (Villafuerte *et al*., 1998) thus favoring the acumulation of new variants that, in this case, occurred when lineage *CAII*1* was already present in the SW.

To test this hypothesis, the *CAII* gene will be studied by sequencing, to find which nucleotide substitutions are responsible for the alleles detected by HIEF separation. We also intend to examine the phylogenetic patterns of the *CAII* gene by the construction of phylogenetic trees in order to gain further insight into the evolutionary history of the European rabbit.

ACKNOWLEDGMENTS

Financial support was obtained from FCT (PRAXIS XXI BD/5007/95 PhD grant and SFRH/BPD/5610/2001 PostDoc grant to MB, and project grants POCTI/40280/BSE/2001 and POCTI/34151/99) and from Direção Geral das Florestas. We thank Armando Loureiro for his help in getting samples from domestic rabbits, Paulo Célio Alves and Helena Gonçalves for their help in wild rabbit sampling in Portugal, Sacramento Moreno and Rafael Villafuerte for their collaboration in sampling campaigns in Doñana National Park, and Marisa Azevedo and Rita Campos for their valuable support in the laboratory work. We also thank Agostinho Antunes and an anonymous referee for their comments and suggestions on the manuscript.

REFERENCES

Antunes, A., Templeton, A. R., Guyomard, R., and Alexandrino, P. (2002). The role of nuclear genes in intraspecific evolutionary inference: Genealogy of the transferrin gene in brown trout. *Mol. Biol. Evol*. **19:**1272–1287.

Arana, A., and Zaragoza, P. (1986). Estudios electroforeticosy geneticos de proteinas sanguineas de la especie *Oryctolagus cuniculus* L. (razas silvestre y comun español). *Genét. Ibérica* **38:**245–263.

Arana, A., Zaragoza, P., Rodellar, C., and Amorena, B. (1989). Blood biochemical polymorphisms as markers for genetic characteristics of wild Spanish and domestic rabbits. *Genetica* **79:**1–9.

Barbadilla, A., King, L. M., and Lewontin, R. C. (1996). What does electrophoretic variation tell us about protein variation? *Mol. Biol. Evol*. **13:**427–432.

Bernoco, D. (1970). Electrophoretic variants of carbonic anhydrase in rabbit red cells. *Atti. Ass. Genet. Ital*. **15**:227.

Branco, M. (2000). *Estrutura genética das populações de coelho europeu (*Oryctolagus cuniculus*) na Península Ibérica*, PhD thesis, Universidade do Porto, Porto.

Branco, M., and Ferrand, N. (1998). Genetic polymorphism of rabbit (*Oryctolagus cuniculus*) tissue acid phosphatases (ACP2 and ACP3). *Comp. Biochem. Physiol. B* **120:**405–409.

Branco, M., Ferrand, N., and Monnerot, M. (2000). Phylogeography of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula inferred from RFLP analysis of the cytochrome *b* gene. *Heredity* **85:**307–317.

Branco, M., Monnerot, M., Ferrand, N., and Templeton, A. R. (2002). Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested clade and mismatch analyses of mitochondrial DNA genetic variation. *Evolution* **56**(4):792–803.

Callou, C. (1995). Modifications de I'aire de répartition du Lapin (*Oryctolagus cuniculus*) en France et en Espagne, du Pléistocène à l'époque actuelle. État de la question. *Anthropozoologica* **21:**95–114.

Daly, J. C. (1981). Effects of social organization and environmental diversity on determining the genetic structure of a population of the wild rabbit, *Oryctolagus cuniculus*. *Evolution* **35**(4):689–706.

Ferrand, N. (1995). *Variação genética de proteínas em populações de coelho (*Oryctolagus cuniculus*). Anãlise da diferenciação subespecífica, subestruturação, expansão geográfica e domesticação*, PhD thesis, Faculdade de Ciências, Universidade do Porto, Porto.

Ferrand, N., Carvalho, G., and Amorim, A. (1988). Transferrin (Tf) polymorphism in wild rabbit, *Oryctolagus cuniculus*. *Anim. Genet*. **19:**295–300.

Harris, H., and Hopkinson, D. A. (1976). *Handbook of enzyme electrophoresis in human genetics*, North-Holland Publishers, Amsterdam.

Hewett-Emmett, D. (2000). Evolution and distribution of the carbonic anhydrase gene families. In Chegwidden, W. R., Carter, N. D., and Ewards, Y. H. (eds.), *Carbonic Anhydrase*: *New Horizons*, Basel, Birkhäusern verlag, pp. 29–76.

Hewett-Emmett, D., and Tashian, R. E. (1996). Functional diversity, conservation, and convergence in the evolution of the alpha-, beta- and gamma-carbonic anhydrase gene families. *Mol. Phylogenet. Evol*. **5**(1):50–77.

Hopkinson, D. A., Coppock, J. S., Mühlemann, M. F., and Edwards, Y. H. (1974). The detection and differentiation of the products of the human carbonic anhydrase loci, CAI and CAII, using fluorogenic substrates. *Ann. Hum. Genet*. **38:**155–162.

Korstanje, R. (2000). *Development of a genetic map of the rabbit*, PhD. thesis, University of Utrecht, Utrecht, The Netherlands.

Korstanje, R., den Bieman, M., Campos, R., Esteves, P. J., Lankhorst, A., van der Loo, W., van Zutphen, L. F. M., van Lith, H. A., and Ferrand, N. (2001). Genetic analysis and mapping of biochemical markers in an F2 intercross of two inbred strains, of the rabbit (*Oryctolagus cuniculus*). *Biochem. Genet*. **5/6:**169–178.

Lopez-Martinez, N. (1989). Revision sistematica y biostratigrafi ca de los lagomorphos (Mammalia) del neogeno y cuaternario de España. *Memorias del Museo Paleontologico de la Universidad de Zaragoza. Disputacion General de Aragon*.

McIntosh, J. E. A. (1970). Carbonic anhydrase isoenzymes in the erythrocytes and uterus of the rabbit. *Biochem. J*. **120:**299–310.

Monnerot, M., Vigne, J. D. Biju-Duval, C., Casane, D., Callou, C., Hardy, C., Mougel, F., Soriguer, R., Dennebouy, N., and Mounolou, J. C. (1994). Rabbit and Man: Genetic and Historic Approach. *Genet. Sel. Evol*. **26** (suppl. 1):167–182.

Parkkila, S., Parkkila, A. K., Rajaniemi, H., Shah, G. N., Grubb, J. H., Waheed, A., and Sly, W. S. (2001). Expression of membrane-associated carbonic anhydrase XIV on neurons and axons in mouse and human brain. *Proc. Nat. Acad. Sci. USA*. **98**(4):1918–1923.

Queney, G., Ferrand, N., Weiss, S., Mougel, F., and Monnerot, M. (2001). Stationary distributions of microsatellite loci between divergent population groups of the European rabbit (*Oryctolagus cuniculus*). *Mol. Biol. Evol*. **18**(12):2169–2178.

Ramshaw, J. A. M., Coyne, J. A., and Lewontin, R. C. (1979). The sensitivity of gel electrophoresis as a detector of genetic variation. *Genetics* **93:**1019–1037.

Richardson, B. J., Rogers, P. M., and Hewitt, G. M. (1980). Ecological genetics of the wild rabbit in Australia. II. Protein variation in British, French and Australian rabbits and the geographical distribution of the variation in Australia. *Aust. J. Biol. Sci*. **33:**371–383.

Schneider, S. D., Roessli, D., and Excoffier, L. (2000). Arlequin, Ver. 2.0: A software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Skipski, I. A., and Scott, W. N. (1980). Multiple forms of carbonic anhydrase in rabbit erythrocytes. *Comp. Biochem. Physiol. B* **65:**583–593.

Tashian, R. E. (1989). The carbonic anhydrases: Widening perspectives on their evolution, expression and function. *BioEssays* **10**(6):186–192.

Tashian, R. E. (1992). Genetics of the mammalian carbonic anhydrases. *Adv. Genet*. **30:**321–356.

van der Loo, W., Ferrand, N., and Soriguer, R. M. (1991). Estimation of gene diversity at the *b* locus of the constant region of the immunoglobulin light chain in natural populations of european rabbit (*Oryctolagus cuniculus*) in Portugal, Andalusia and on the Azorean islands. *Genetics* **127:**789–799.

van der Loo, W., Mougel, F., Sánchez, M. S., Bouton, C., Castien, E., Fonseca, A., Ferrand, N., Soriguer, R., and Monnerot, M. (1999). Cytonuclear disequilibria in wild populations of rabbit (*Oryctolagus cuniculus L.*) suggest unequal allele turnover rates at the *b* locus (*IGKCI*). *Immunogenetics* **49:**629–643.

Villafuerte, R., Viñuela, J., and Blanco, J. C. (1998). Extensive predator persecution caused by population crash in a game species: The case of red kites an rabbits in Spain. *Biol. Conservation* **84:**181–188.

Walther, H. C., Register, A. M., and Noltman, E. A. (1977). Chromatographic identification of a new subform of carbonic anhydrase in rabbit erythrocytes. *Arch. Biochem. Biophys*. **184:**319–327.