Chapter 7

The evolutionary history of the European rabbit (*Oryctolagus cuniculus*): major patterns of population differentiation and geographic expansion inferred from protein polymorphism

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Abstract
The patterns of population differentiation and geographical expansion of the European rabbit (*Oryctolagus cuniculus*) remain largely unknown. Using gene frequency data for 20 polymorphic protein loci (102 alleles), we investigated the evolutionary history of the rabbit through the analysis of 13 representative populations and the use of both the neighbor-joining (NJ) and the unweighted pair-group method with arithmetic mean (UPGMA) trees. We also conducted a separate analysis comparing one domestic and one wild population with previously published results. Our data indicate that an ancient split separated southwestern Iberian populations from all others, including domestic breeds, and that this division may have corresponded to the emergence of the subspecies *O.c. algirus* and *O.c. cuniculus*. Separation times between the two major groups of populations were estimated with Nei's genetic distance and were found to be highly discrepant with the mtDNA divergence estimate. The southwestern Iberian populations (*algirus* group) are more polymorphic than northern populations (*cuniculus* group), the latter displaying more than simply a subset of southern alleles. These results are thus compatible with the isolation of a marginal population or with a smaller long-term population size in the north. The high degree of genetic differentiation between the two subspecies allows the reconstruction of rabbit geographical expansion. France, Britain and other European countries, as well as Australia, were colonized by animals belonging to the *cuniculus* group, from which domestic breeds are exclusively derived. In contrast, Azorean island populations represent an expansion of the *algirus* group and show evidence of a strong bottleneck effect.

Keywords: protein polymorphism, phylogenetic trees, major groups of populations, geographical expansion, European rabbit, *Oryctolagus cuniculus*

Introduction

The European rabbit *Oryctolagus cuniculus* is a fascinating species for a variety of reasons. First, it constitutes one of the most remarkable geographical expansions of a mammal. Originally restricted to the Iberian Peninsula

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and the Mediterranean area of France (Callou 1995), it was probably introduced into North Africa during historical times (Dobson 1998), arrived in Britain around the 11th century, and later expanded into most of central and northeastern Europe (19th and 20th centuries). It was introduced into Australia, New Zealand, Chile and Argentina, as well as more than 800 islands throughout the world. This very successful process of transport and colonization began with the first contacts made by Mediterranean navigators (*ca.* 1000 years BC, e.g. the Balearic Islands, Spain, or Zembra, Tunisia) and still continues today (Flux & Fullagar 1992). Second, this successful colonizer now occupies regions with a remarkable diversity of ecological contexts, from subtropical to sub-Antarctic climates and, dependent on the various communities to which it is now adapted is viewed as a pest (Williams *et al.* 1995), a biological invader (Barrett & Richardson 1986) or a key species on which a variety of threatened predators depend to survive (Rogers *et al.* 1994; Villafuerte 1994). Third, throughout history man has expressed curiosity for the rabbit: Latin and Greek historians like Varro, Strabo or Pliny the Old, described the maintenance of animals in closed parks (*leporaria*), the introduction of animals on islands, ferret hunting or the consumption of rabbit embryos by humans. This perpetual interest in the rabbit culminated with western Europe's only successful attempt at animal domestication – a feat of ethnozoological significance. Today, the rabbit is an important laboratory animal for biomedical research (Weisbroth *et al.* 1974), serving as a highly valued model for research on the mammalian immunological system (Dubiski 1987; van der Loo 1993; Su & Nei 1999) and for human arteriosclerosis (Beaty *et al.* 1992). Further, rabbits are severely affected by epizootics like myxomatosis and the rabbit viral hemorrhagic disease (RVHD), offering opportunities for studying host/parasite coevolution (Lewontin 1970; Anderson & May 1982; Langman 1989) and its effects on the genetic diversity of wild populations (Queney *et al.* 2000).

The fossil record of the rabbit is very scarce: a single tooth found in the region of Granada (Andalusia, Spain) marks the appearance of the genus *Oryctolagus* six mya (Lopez-Martinez 1989). Other more complete fossil remains have been attributed to two species with intermediate characteristics between present day rabbits and hares: *O. laynensis* and *O. lacosti*. The first species seems to have occupied the whole of Spain about 2-3 mya, while the second reached southern France and existed about 1.8 to 2 mya (Lopez-Martinez 1989). *O. cuniculus* appears 900 000 years ago in southern Spain, but much later in southern France (approx. 300 000 years ago). More recent evidence is provided by archaeozoological research: in Portugal, the rabbit is the most abundant mammal in Mesolithic sites from the Tejo and Sado valleys (Lentacker 1986; Arnaud 1987), and remarkable bone ornaments in the shape of rabbits date to the second half of the 4th millenium BC (Leisner 1983). Early introductions of rabbits to some Mediterranean islands are also documented in archaeozoological findings, like Menorca, in the Balearics (1400-1300 BC,

Reumer & Sanders 1984) and Zembra (end of the Neolithic to the 3rd century, Vigne 1988). The situation is less clear in the Mediterranean region of France where the rabbit seems to have maintained a fundamental role in the human diet until the end of the Palaeolithic (Pages 1980). Subsequently, rabbits may have been extirpated from this region only to be reintroduced during the Roman period or, alternatively, may have persisted in small refugia from where recolonization followed. A recent analysis of all archaeozoological evidence obtained in France south of river Loire seems to favor this last hypothesis (Donard 1982; Callou 1995).

Differentiation of rabbit ecto-parasites may provide insights on coevolutionary processes between hosts and parasites and has deserved some attention in the past (Beaucournu 1980). Three specific rabbit fleas were found to be especially important in the analysis of rabbit evolution: *Xenopsylla cunicularis*, *Odontopsyllus quirosi* and *Caenopsylla laptevi*. The first genus, *Xenopsylla*, contains a high number of species that mainly occur in tropical Africa where they parasitize Gerbilidae rodents (Beaucournu & Launay 1990). In contrast, *X. cunicularis* is specific to the rabbit, almost exclusively occurring in the Iberian Peninsula and southern France. Launay & Beaucournu (1982) suggest that *X. cunicularis* results from the capture and derivation of an ancestral flea that parasitized those rodents, whose fossorial habits are similar to those of the rabbit. This hypothesis is supported by the fact that Gerbilidae fossils, contemporary with *Oryctolagus* were found in the Iberian Peninsula (Launay & Beaucournu 1982). Additionally, the occurrence of *X. cunicularis* in wild rabbit populations from North Africa was interpreted as a secondary event resulting from a recent expansion of the rabbit (Launay & Beaucournu 1982). The other species, *O. quirosi* and *C. laptevi*, can be each divided into two distinct subspecies that correspond to different groups of rabbit populations. Accordingly, *O. q. quirosi* and *C. l. ibera* were found in the central and southern Iberian Peninsula, whereas *O. q. episcopalis* and *C. l. relicta* are limited to the French Mediterranean basin (Beaucournu & Launay 1990). Taken as a whole, ecto-parasitological evidence allowed a more precise definition of hypotheses arising from paleontological and archaeozoological data because i) the origin of *O. cuniculus* is unequivocally placed in the European side of the Mediterranean basin, and ii) two main rabbit population groups are identified, one in the Iberian Peninsula and the other in southern France, their separation being probably caused by Quaternary glaciations.

Historically, the first genetic evidence contributing to our knowledge of rabbit history came from the discovery of immunoglobulin allotypes (Mage *et al*. 1973). Extensive serological studies developed in domestic breeds revealed genetic polymorphism at the IgKC1, IgVH1 and IgGCH2 loci (van der Loo 1987; van der Loo *et al*. 1987; Cazenave *et al*. 1987). When wild rabbit populations from central Europe (especially France, England, Holland and Belgium) and Australia were investigated, the most remarkable result was

the high degree of genetic identity with domestic populations: no new alleles were described and the allelic distribution profiles were very similar (Curtain *et al*. 1973; van der Loo 1987; van der Loo *et al*. 1987). However, these results also revealed the existence of a significant and systematic linkage disequilibrium between the IgKC1 and IgGCH2 loci in all populations, indicating the probable occurrence of selective mechanisms promoting particular genotype combinations (van der Loo *et al*. 1987). The extension of this investigation to wild rabbit populations from the Iberian Peninsula showed a completely different scenario: the loci were found to harbor much higher variation with seven to eight new alleles in IgKC1 (Cazenave *et al*. 1987; van der Loo *et al*. 1991) and at least 10 new alleles in IgVH1 (Cazenave *et al*. 1987), but no polymorphism was described for IgGCH2. Overall the results obtained so far clearly support an Iberian origin of the rabbit, but are not informative in the discrimination of animals outside of Iberia. Moreover, difficulties resulting from the application of serological techniques in the definition of immunological loci (Nei 1975), the population and molecular evidence for the occurrence of strong selective mechanisms involved in the structural divergence of alleles (van der Loo 1987, 1993; van der Loo & Verdoodt 1992) and also the fact that they often constitute transspecific polymorphisms (Cazenave *et al*. 1987; van der Loo *et al*. 1999; Su & Nei 1999; Esteves *et al.* 2005) raises doubts about the feasibility of obtaining a reliable phylogenetic tree of rabbit populations.

Monnerot and coworkers followed a different approach by investigating patterns of sequence variation in rabbit mitochondrial DNA (mtDNA) (see Monnerot *et al*. 1994 for a review). The results show the occurrence of two very divergent mtDNA lines (4%) (Biju-Duval *et al*. 1991), indicating that an ancestral molecule may have existed more than 2 mya, long before the first known fossils of *O. cuniculus*. One of these mtDNA lineages (type A) is circumscribed to southwest Iberia, while the other (type B) occurs in northern Spain, France, England, the rest of Europe, Australia and all domestic breeds (Biju-Duval *et al*. 1991; Monnerot *et al*. 1994). Recently, a comprehensive survey of Iberian wild rabbit populations showed that the two mtDNA lineages are essentially allopatric, with a very limited overlap along a northwest-southeast gradient that divides the peninsula (Branco *et al*. 2000). This investigation also confirmed that Iberian rabbits are characterized by high levels of inter- and intra-population variability, while French rabbits do not show intra-population polymorphism (Monnerot *et al*. 1994). While these data are compatible with an older age of southwestern Iberian populations and a more recent occupation of southern France, important aspects associated with the use of the single mtDNA gene tree may limit the usefulness of this genetic system for understanding rabbit evolution (Nei 1987; Pamilo & Nei 1988).

The principal aim of the present study was to survey the natural range of the species for the presence of main patterns of population differentiation and,

secondarily, to compare populations from introduced and natural ranges in the context of the documented history of rabbit expansion (Thompson & King 1994). Previous studies with rabbit electrophoretic polymorphisms were of little use because a very limited number of both populations and markers were investigated (Richardson *et al*. 1980; Hartl 1987; Arana *et al*. 1989; Peterka & Hartl 1992). When population-level phenomena are investigated, hypotheses must be tested across many loci (Pamilo & Nei 1988; Zhivolovsky & Feldman 1995). This strategy has been particularly effective for reconstructing the natural history of *Drosophila melanogaster* (Singh & Rhomberg 1987), and the house mouse *Mus musculus* (Boursot *et al*. 1993), as well as gaining an understanding of modern human origins (Nei & Roychoudhury 1982, 1993; Cavalli-Sforza *et al*. 1988, 1994; Nei 1995; Chikhi *et al*. 1998). We have therefore used gene frequency data for 102 alleles corresponding to 20 polymorphic loci to perform a phylogenetic analysis of a set of representative rabbit populations and propose, for the first time, a global scale scenario for the evolutionary history of *O. cuniculus*.

Materials and methods

Sampling

The sampling was designed to include representative populations in the natural range of the species (Iberian Peninsula and southern France), but avoiding hybrid populations occurring in central Iberia (Branco *et al*. 2000), because they are known to violate the fundamental principles of phylogenetic reconstruction (Felsenstein 1982; Nei 1987). Accordingly, we sampled seven populations from the southwestern Iberian Peninsula (Huelva, Doñana National Park, Las Lomas, Vila Viçosa, Santarém, Badajoz, and Idanha), one population from northeastern Spain (Tudela), and one population from the French Mediterranean region (Camargue National Park). In addition, we included samples that are known to represent recent expansions of the species: northern France (Versailles) and two Azorean islands (S. Jorge and Flores) (Figure 1). Finally, a sample of Portuguese domestic rabbits was also collected. This option is justified because these animals are not heavily selected and are thought to be the closest representatives of the initial stages of rabbit domestication (Ferrand, unpublished results).

A total of 546 blood samples was collected during 1990-1995 in EDTA-tubes from the marginal ear vein or, alternatively, by direct cardiac puncture. Red cells were separated from sera by quick centrifugation and mixed (1:2) with a glycerolated Tris-citric buffer pH 8.0. Both red cells and sera were stored at -20^0 until the electrophoretic analysis was conducted.

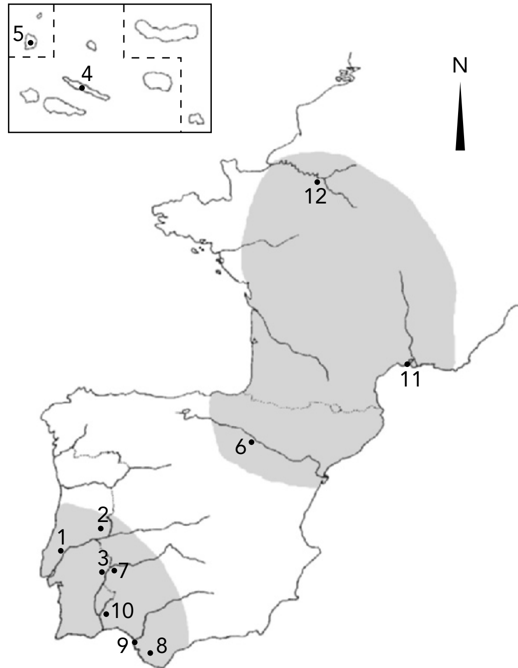

Figure 1. *Sampling locations of wild rabbit populations. 1 Santarém, 2 Idanha, 3 Vila Viçosa, 4 S. Jorge, 5 Flores, 6 Tudela, 7 Badajoz, 8 Las Lomas, 9 Doñana National Park, 10 Huelva, 11 Camargue, 12 Versailles.*

Protein analysis

The use of a limited number of electrophoretic systems to assay protein genetic variation is known to prevent the description of a considerable amount of hidden variability (Ramshaw *et al.* 1979; Brown *et al.* 1981), which severely limits phylogenetic inference (Lewontin 1991). However, the development of iso-electric focusing both in carrier ampholytes and immobilized pH gradients coupled with immuno-blotting detection techniques has substantially improved the ability to effectively discriminate electromorphs in their constituent alleles (Altland *et al.* 1987; Righetti *et al.* 1989; Righetti 1990). In this study, we chose a balanced strategy that included i) a detailed search for the adequate buffer systems and pH conditions for markers separated by conventional electrophoresis, ii) the analysis of denatured or chemically modified proteins when necessary, and iii) the use of high resolution iso-electric focusing systems whenever possible and sometimes in combination with conventional electrophoresis for the detection of subtypes.

A total of 20 polymorphic loci was examined. Conventional electrophoresis was used for typing i) peptidases (PEPA, PEPB, PEPC, EC 3.4.11/13; and PEPD, EC 3.4.13.9) following Branco *et al*. (1999), ii) nucleoside phosphorylase (NP, EC 2.4.2.1), adenosine deaminase (ADA, EC 3.5.4.4) and phosphogluconate dehydrogenase (PGD, EC 1.1.1.44) according to the technique described by Amorim *et al*. (1982), iii) carbonic anhydrase I and the types of carbonic anhydrase II (CAI/CAII, EC 4.2.1.1) following the systems originally proposed by Branco & Ferrand (2003), iv) mannose-6-phosphate isomerase (MPI, EC 5.3.1.8) as described in Vieira & Ferrand (1995), v) galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12) following the methods of Siebert *et al*. (1980) and Ferrand (1995), and vi) transferrin (TF) (Ferrand *et al*. 1988). In addition, genetic variation in superoxide dismutase (SOD, EC 1.15.1.1) was routinely assayed in the same starch gel system used for peptidases (Ferrand, unpublished results). Extremely acid starch gel electrophoresis was employed in the analysis of the genetic polymorphisms of α- and β-globin genes (HBA and HBB, Ferrand 1989, 1990) after previous denaturation of hemoglobin. Iso-electric focusing in carrier ampholytes was used for typing i) albumin (ALB) (Ferrand & Rocha 1992), ii) acid phosphatase 3 (ACP3, EC 3.1.3.2) according to Branco & Ferrand (1998), iii) hemopexin (HPX) (Branco & Ferrand 2002), iv) vitamin-D binding protein (GC) (Ferrand 1995), v) glucose-phosphate isomerase (GPI, EC 5.3.1.9) following the technique established by Azevedo & Ferrand (unpublished results), and for the determination of subtypes in the loci ADA, GALT and TF (Ferrand 1995). Finally, hybrid iso-electric focusing was used for the separation of CAII subtypes (Branco & Ferrand 2003).

Samples were diluted sera or haemolysates for ACP3, ADA, ALB, GPI, MPI and SOD, as appropriate. When assaying GALT, NP, PGD and PEP's, samples were first reduced with dithiothreitol 120mM (5:1) for 1h at 37° C. The same procedure was used for the conventional separation of CAI/CAII, whereas for CAII subtypes samples were alternatively alkylated with iodoacetic acid 40mM in a 1:1 proportion. Globins (HBA and HBB) were obtained from diluted (1:5) haemolysates treated with acid acetone (Ferrand 1989). For GC, sera were treated with neuraminidase (*Clostridium perfringens*, SIGMA type V, 1.8 U/ml) in a 1:3 proportion for 18h at 37° C, while for HPX sera were diluted in distilled water in a proportion of 1:1. Finally, discrimination of TF alleles by IEF were made after partial purification with rivanol for two sets of samples: the first being iron-free due to an EDTA treatment, and the second being iron-saturated with ferric ammonium sulphate following (Zapolski & Princiotto 1980).

The recipes for enzyme staining of PGD, MPI, SOD, GALT, NP, ADA and GPI were adapted from Harris & Hopkinson (1976). Visualization of PEP's was done according to a procedure firstly described by Sugiura *et al*. (1977). CAI and CAII were previously identified by specific CO_2 hydration coupled with bromothymol blue detection; for routine assays, however, we used a stan-

dard Coomassie R-250 staining for CAI, and a fluorescein diacetate based detection method adapted from Harris & Hopkinson (1976) for CAII. Detection of ACP3 was made following Harris & Hopkinson (1976), but on nitrocellulose membranes according to an enzyme blotting procedure (Branco & Ferrand 1998). HBA, HBB, CAII subtypes and TF types were detected with a standard Coomassie R-250 staining, while for ALB and TF subtypes a standard Coomassie G-250 staining was used. Finally, both GC and HPX were detected after an immuno-blotting procedure described in Ferrand (1995).

Data analysis

Our set of 13 rabbit populations was analyzed for genetic distances using D_A, a modified Cavalli-Sforza distance suggested by Nei *et al.* (1983). These authors have shown that although not linearly related with time, D_A is more efficient in the separation of closely related populations, an assumption that is certainly verified for most of the samples used in this work. We therefore used the DISPAN package of Ota (1993). Phylogenetic reconstruction was done both with NJ and UPGMA methods. Although UPGMA trees are probably the most commonly used in the population genetics literature (Nei 1987), NJ trees are known to be more efficient than most other methods of phylogenetic reconstruction (Saitou & Imainishi 1989; Rzhetsky & Nei 1992). Reliability of nodes in the trees were assessed by bootstrapping loci (1000 replicates) using the DISPAN software, as above.

Various researchers have been involved in the investigation of rabbit evolution (Richardson *et al.* 1980; Arana *et al.* 1989; Peterka & Hartl 1992). Although differing in the number of loci and alleles used, these studies suggested that rabbit populations show low levels of differentiation and, notably, the last two concluded that the genetic distance between wild and domestic samples was not necessarily higher than that existing between two wild populations or between two domestic breeds. We therefore compared our results with those described earlier adopting the following strategy: i) selecting one wild rabbit population (Santarém) and the Portuguese domestics as representatives of our sampling, ii) constructing allele x population matrices for each of the works mentioned above but including the two Portuguese samples and accepting missing values for loci not investigated in our work, and iii) obtaining phylogenetic trees with and without the Portuguese samples following the exact procedures (genetic distances and reconstruction methods) adopted by the authors. Briefly, we used loci ADA, CAII, ES1 and PGD for comparisons made with the data of Richardson *et al.* (1980), ADA, CAII, DIA, ES1, ES3, HB, PGD and TF for comparisons made with the data of Arana *et al.* (1989), and ES1, GPI, MPI, PGD and PGM2 for comparisons made with the data of Peterka & Hartl (1992). Accordingly, we also typed the markers ES1, ES3, DIA and PGM2 for the two Portuguese samples (Santarém and domestic) in addition to the 20 electrophoretic polymorphisms ana-

lyzed in the present study (results not shown). Because of different separation techniques used by the various research groups, subtypes of ADA, CAII and TF were collapsed in conventional electromorphs for comparison purposes. Likewise, HB was treated as a single locus.

A hierarchical gene diversity analysis following the method described by Nei (1973, 1987) was done considering relevant levels of population subdivision: populations and subspecies (*O.c. algirus* and *O.c. cuniculus*). The Portuguese domestic stock was excluded from the analysis. In these conditions, total gene diversity (H_T) is partitioned into its components so that:

$$H_T = H_P + D_{PS} + D_{ST},$$

where H_P is average gene diversity within populations, D_{PS} is gene diversity between populations within subspecies and D_{ST} corresponds to diversity between subspecies. The relative importance of the three components are expressed in terms of G values (G_P, G_{PS}, and G_{ST}), that are obtained from the ratio of each component to H_T.

Measures of genetic variability (mean expected heterozygosity, H, mean number of alleles, n_a, and proportion of polymorphic loci, P, were obtained with the package BIOSYS, version 1.7 (Swofford & Selander 1989).

Results

Genetic distances and phylogenetic trees

The matrix of D_A genetic distances calculated after 20 polymorphic loci (102 alleles) for all 13 rabbit populations studied in this work is shown in Table 1, whereas the allelic frequencies are given in the appendix of this chapter (Table A1). The phylogenetic trees were constructed by applying both NJ (Saitou & Nei 1987) (Figure 2) and UPGMA (Sneath & Sokal 1973) (Figure 3) methods.

There are two major clusters of rabbit populations: A) all rabbit populations from the southwest of the Iberian Peninsula and the two samples from the Azorean islands of São Jorge and Flores, and B) the population from the northeast of Spain, the two French populations and the Portuguese domestic breed. The root of the NJ tree was found after evaluating the midpoint of the longest branch between two populations (Figure 2) (Farris 1972). This was necessary because the use of a closely related species as an outgroup (e.g. the Iberian hare, *Lepus granatensis*) is presently impossible due to the lack of adequate data. The complete separation of population groups A and B exhibits high bootstrap support in both the UPGMA (99%) and NJ (100%) trees. The major difference in the topology of the trees is associated with the position of the Azorean island populations: while in the UPGMA tree they correspond to the first split within group A (95% bootstrap value) and are separated from the southwestern populations, in the NJ tree they are clearly associated with

a central Portuguese population (Vila Viçosa) and well inside group A, showing long branch lengths. The explanation for this difference involving populations from the islands of São Jorge and Flores is based in the assumptions for each grouping method. It is well known that rabbits were introduced in the Azorean Islands by Portuguese navigators in the beginning of the 15th century, most likely corresponding to a genetic bottleneck followed by high genetic drift. This is equivalent to an acceleration of the evolutionary rate and implicates large genetic distances that may be properly analyzed by the NJ method (Saitou & Nei 1987; Nei & Roychoudhury 1993) but incorrectly so with a UPGMA tree.

Table 1. *Estimates of* D_A *distances for 13 representative rabbit populations based on gene frequency data for 20 polymorphic protein data. Values are multiplied by 100.*

Population		1	2	3	4	5	6	7	8	9	10	11	12	13
1	**Santarém**	-												
2	**Idanha**	8.00	-											
3	**Vila Viçosa**	3.27	7.47	-										
4	**S. Jorge**	7.37	11.48	6.31	-									
5	**Flores**	8.49	12.81	8.18	3.73	-								
6	**Tudela**	13.71	15.58	20.68	20.79	-								
7	**Badajoz**	6.43	7.95	5.53	9.12	9.95	16.34	-						
8	**Las Lomas**	6.01	8.58	6.34	10.18	12.42	14.51	7.03	-					
9	**Doñana**	5.88	7.18	5.56	9.81	11.24	13.63	9.04	5.93	-				
10	**Huelva**	4.62	7.71	4.44	8.05	9.45	12.91	6.48	5.03	4.77	-			
11	**Camargue**	16.40	19.31	18.15	20.84	22.82	9.66	18.63	16.91	16.55	15.82	-		
12	**Versailles**	17.63	20.47	19.75	22.23	21.85	11.23	19.53	18.42	17.79	16.48	2.87	-	
13	**Domestic**	19.44	21.78	22.36	25.67	24.20	10.27	20.48	20.77	20.59	19.32	5.35	3.31	-

Comparison with other datasets

Figure 4 shows the topology of the trees obtained with the data published by Richardson *et al*. (1980), Peterka & Hartl (1992) and Arana *et al*. (1989), and modified by the inclusion of two representative rabbit populations (Santarém and Portuguese domestic breed). In all three cases, the wild rabbit population from Santarém representing the A group as defined above is very different from all others and always corresponds to the first split in the tree. On the other hand, and most notably, Portuguese domestic rabbits are always included well within the main group of each tree – probably corresponding to the B group – and hardly distinguishable from a variety of other domestic breeds (e.g. New Zealand, Chinchilla, Californian and Spanish Giant), but also from wild populations of Australia, England, Austria, northern and southern France, and northern and central Spain. As the presence of missing values in the comparative allele x population matrices may influence the topology of trees (Nei & Roychoudhury 1993), the whole procedure was repeated again using only

common markers. No significant differences were obtained (results not shown).

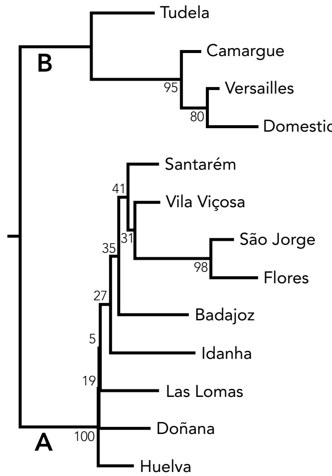

Figure 2**. NJ phylogenetic tree for 13 representative rabbit populations obtained from data in* D_A *values in Table 1. The bootstrap probabilities were obtained with Ota's (1993) computer program DISPAN. The two major groups of rabbit populations correspond to the subspecies* ***A) O.c. algirus *and* ***B)*** O.c. cuniculus.

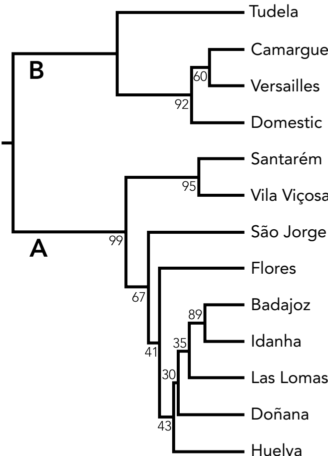

Figure 3**. UPGMA phylogenetic tree for 13 representative rabbit populations obtained from data in* D_A *values in Table 1. The bootstrap probabilities were obtained with Ota's (1993) computer program DISPAN. The two major groups of rabbit populations correspond to the subspecies* ***A) O.c. algirus *and* ***B)*** O.c. cuniculus.

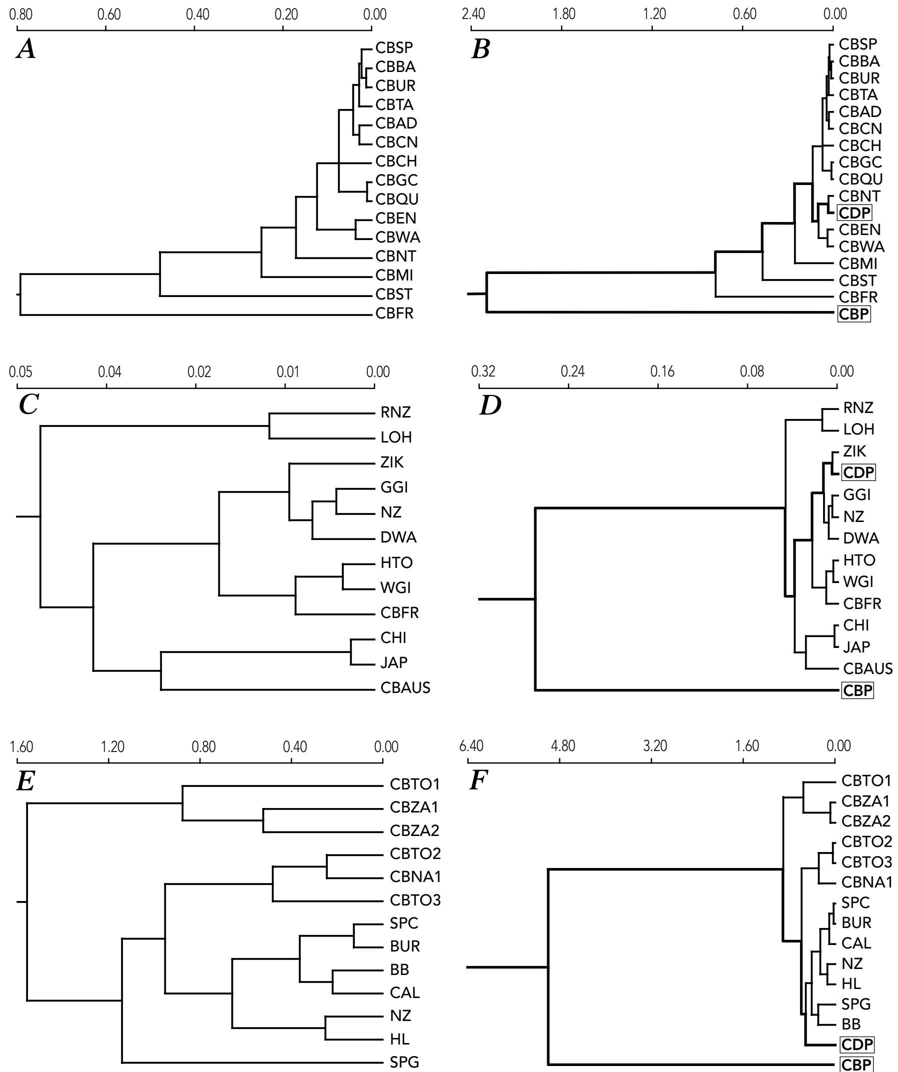

***Figure 4**. Comparison of the results described by Richardson* et al. *(1980), Peterka & Hartl (1992) and Arana* et al. *(1989) with the data presented in this paper. All dendrograms are UPGMA trees using Rogers' distance (**A** and **B**, Richardson* et al. *1980), Nei's distance (**C** and **D**, Peterka & Hartl 1992) and Cavalli-Sforza's distance (**E** and **F**, Arana* et al. *1989). Trees **B**, **D** and **F** include two Portuguese rabbit populations (Santarém - CBP - and domestic - CDP) that are highlighted. Wild rabbit populations are always preceded by 'CB', the other samples being from domestic breeds. For the abbreviations see the original references.*

Gene diversity analysis

The results of the gene diversity analysis are summarized in Table 2. It is clear that the major part of the total variability is found within populations (78.5%). The other fractions are asymmetrically distributed: 6.4% is attributable to differences between populations and 15.1% to differences between the subspecies *algirus* and *cuniculus*. When an intermediate level of population structure (regions: Portugal, Spain, France and Azores) is included in the analysis, only a very small fraction of the genetic variability is obtained, thus indicating that regions are not meaningful sources of genetic structuring (results not shown).

Table 2. *Absolute* (H_T) *and relative* (G*'s*) *gene diversities for 12 rabbit populations based on 20 protein polymorphic loci.*

	H_T	Relative distribution (%)		
		G_P	G_{SP}	G_{ST}
Twenty electrophoretic loci	0.345	78.5	6.4	15.1

G_P – within population; G_{SP} – between populations within subspecies; G_{ST} – between subspecies.

Table 3. *Genetic variability measures obtained in 13 rabbit populations based on 20 protein polymorphic loci.*

Population	N	n_a	P	H
***O.c. algirus* (continental populations)**				
Santarém	102.7 (13.3)	2.7 (0.2)	90.0	0.346 (0.042)
Idanha	25.1 (2.1)	2.4 (0.2)	80.0	0.264 (0.047)
Vila Viçosa	36.8 (1.4)	2.5 (0.2)	85.0	0.329 (0.043)
Badajoz	15.3 (0.6)	2.4 (0.2)	85.0	0.320 (0.049)
Las Lomas	35.5 (3.4)	2.5 (0.2)	85.0	0.344 (0.048)
Doñana	36.5 (8.9)	2.3 (0.2)	75.0	0.294 (0.054)
Huelva	57.2 (6.4)	2.7 (0.2)	90.0	0.305 (0.052)
***O.c. algirus* (insular populations)**				
São Jorge	50.9 (1.8)	1.6 (0.2)	50.0	0.227 (0.056)
Flores	51.2 (2.9)	1.6 (0.1)	55.0	0.226 (0.051)
O.c. cuniculus				
Tudela	15.6 (2.5)	1.8 (0.2)	50.0	0.200 (0.054)
Camargue	28.5 (1.2)	1.7 (0.1)	60.0	0.184 (0.051)
Versailles	53.2 (2.6)	1.7 (0.2)	50.0	0.218 (0.057)
Domestic	98.1 (11.7)	1.7 (0.2)	45.0	0.170 (0.053)

N – mean sample size per locus; n_a – mean number of alleles per locus; P – percentage of polymorphic loci; H - expected heterozygosity. Standard errors are indicated in parentheses.

Polymorphism, heterozygosities and allele diversities

The proportion of polymorphic loci (P), average heterozygosities (H) and mean number of alleles per locus (n_a) are presented in Table 3. These values must be interpreted taking into consideration that each of the 20 investigated loci are polymorphic in at least one population. There is a significant difference in the H values of both subspecies, varying between 0.294 and 0.346 for *O.c. algirus* and between 0.170 and 0.218 for *O.c. cuniculus* (continental populations). Azorean populations show intermediate values (0.226/0.227). For both P and n_a only two groups of populations are apparent: the first comprises the *algirus* group ($75.0\% < P < 90.0\%$, and $2.4 < n_a < 2.7$) while the second is formed by the *cuniculus* group and the Azorean populations ($45.0\% < P < 60.0\%$, and $1,6 < n_a < 1.8$).

Discussion

Evolutionary differentiation of *O. cuniculus*

We identify the major patterns of population differentiation during rabbit evolution using a set of representative samples and 20 polymorphic loci. Both NJ and UPGMA trees showed a very clear first split that is supported by a high bootstrap value (99% to 100%) and corresponding to two groups: A – rabbits of southwestern Iberia and Azorean Islands – and B – rabbits of northeast Spain, France and domestic breeds. When comparing data from previous authors (Richardson *et al.* 1980; Arana *et al.* 1989; Peterka & Hartl 1992) it is remarkable to observe i) the consistency of the first split that has occurred between Portuguese wild rabbits and all others, and ii) the fact that the Portuguese domestic stock is always deeply nested within group B populations, thus showing consistent close genetic relationships with rabbits from central and northern Iberia, the rest of Europe, Australia and all domestic breeds (Figure 4). This observation is much strengthened by the fact that both the number and type of electrophoretic systems varied considerably between those studies, confirming the deep divergence between the two groups that were already apparent in the phylogenetic trees (Figures 2 & 3). The comparison with mtDNA polymorphism show a clear correspondence between the two sets of data. Briefly, southwestern Iberian populations along with Azorean Islands show haplogroup A, while populations from northern Spain, France, the rest of Europe, Australia and domestic breeds present haplogroup B (Biju-Duval *et al.* 1991; Monnerot *et al.* 1994). Taken together, these results strongly suggest that group A populations correspond to the *algirus* subspecies, whereas group B populations correspond to the *cuniculus* subspecies.

O. cuniculus is currently treated as consisting of two subspecies (Lopez-Martinez 1989), although controversy exists (Sharples *et al.* 1996). The recog-

nition of subspecies was initially based mainly on size and characteristics of fur and led Cabrera (1914) to state that *algirus* is distributed in the Iberian Peninsula and North Africa while *cuniculus* occupies the rest of Europe and Australia. Difficulties in using morphological characteristics to define subspecies pushed Sharples *et al.* (1996) to contest this subdivision, suggesting instead a continuous gradient across the Iberian Peninsula. In a more extreme position, Gibb (1990) suggested that autochthonous rabbit populations exist only in Iberia, with all others being feral and derived from domestic animals released in different places. Our results combined with mtDNA variation as well as with immunoglobulin polymorphism (van der Loo *et al.* 1991, 1999) provide solid evidence in favor of two major population groups that have been evolving independently for a long period of time, thus conforming with a modern concept of subspecies (Avise & Hamrick 1997). However, it is also clear that the distribution boundaries settled by Cabrera (1914) for the subspecies *algirus* and *cuniculus* do not coincide with those of the present genetic analysis. This is because the morphological classification of rabbit subspecies has severe limitations and the genetic changes affecting those traits are not necessarily in direct relation to phylogeny. As a whole, the genetic evidence now presented allows a more accurate definition of the distribution areas of *algirus* and *cuniculus*.

The age of separation between the two rabbit subspecies is a more difficult issue to address. In the present study we used D_A, a distance measure that is not linear with time preventing the calculation of a divergence time between *algirus* and *cuniculus* (Nei *et al.* 1983). We therefore used the allelic frequency data of Table A1 together with a set of 10 monomorphic loci to have a random sample of the genome and calculate Nei's genetic distance (D_N) between the two subspecies (results not shown). This value (0.11) is well placed among other genetic distances described for subspecies and compiled by Nei (1987), and maybe used in the formula $t = 5 \times 10^6 \times D_N$ to calculate the divergence time (Nei 1978). However, this formulation assumes that only one-fourth of the amino acid substitutions in proteins are detectable by electrophoresis, which may not be appropriate in our study due to the extensive use of various buffers and pH values, high resolution iso-electric focusing systems and chemical modification of proteins (see material and methods). If we tentatively admit that detectability is 50%, then the formula for estimating t becomes $2.5 \times 10^6 \times D_N$. When both calculations are made, we obtain a window for the divergence time *algirus-cuniculus* that varies between 275 000 and 550 000 years. This is in sharp contrast with the 2 myr estimated by Biju-Duval *et al.* (1991) on the basis of mtDNA RFLPs of the whole molecule and recently confirmed with cytb RFLP data (Branco *et al.* 2000). Two different hypotheses may explain this discrepancy. The first is that divergence time maybe grossly underestimated due to the possible role of natural selection in keeping allelic frequencies constant across protein loci, thus preventing differentiation of isolated populations. Karl & Avise (1992) studied oysters from the Pacific

and the Atlantic with a set of allozyme loci and found very little differentiation of the two groups. However, the very same populations show a deep divergence when studied at the DNA level, both with mtDNA and nuclear markers. If this is the case of our study, then genetic distances based on protein polymorphism are not useful for the estimation of the time since the two subspecies diverged. Alternatively, it maybe noted that mtDNA represents a gene tree rather than a population tree (Pamilo & Nei 1988), and thus the divergence time of 2 myr relates to the separation of two molecules but does not necessarily corresponds to the split of *algirus* and *cuniculus*. In this case, the coexistence of two highly divergent mtDNA haplotypes in the rabbit species maybe be due to ancestral polymorphism and/or capture of one of the types. In natural populations several similar situations have been described for a variety of species including the house mouse, voles and tree frogs (see Avise 1994 for a revision). In this respect, we can speculate that around 2 mya at least two other rabbit species occurred in the Iberian Peninsula: *O. lacosti* and *O. laynensis* (Lopez-Martinez 1989). In any case, only the sequencing of different nuclear genes may resolve this issue in the future.

Partitions of genetic diversity

The distribution pattern of genetic diversity as measured by P, H and n_a indicate that *algirus* populations are characterized by a higher genetic variability than that observed for *cuniculus* populations. This results from the fact that southwestern Iberian rabbits harbor a higher number of polymorphic loci (for example GALT, GC, NP, PEPA, PEPD and TF) and also because there is a higher number of alleles per locus especially associated with the occurrence of many private alleles (for example GALT, GC and TF). These results are compatible with an Iberian origin for the species and also suggest that the separation and long geographic isolation between the two subspecies may have led to an asymmetrical partition of genetic diversity within *O. cuniculus*. It is thus possible that the populations now identified as *cuniculus* result from the isolation of a marginal population that would represent only a small fraction of the total genetic diversity of the rabbit. A different possibility may be associated with long-term population size after fragmentation. It has been proposed that Quaternary glaciations in Iberia recurrently left two main refugia: one large area in the southwest and a second, much smaller, in the eastern Mediterranean coast (Cooper *et al.* 1995; Comes & Abbott 1998; Branco *et al.* 2000). If the availability of refugia and habitats would correspond to population numbers, then it is to be expected that *algirus* show higher levels of genetic variability than *cuniculus*.

When a gene diversity analysis is conducted it is observed that the major part of the total variability was found within populations. However, a considerable fraction of that variability (G_{ST} = 15.1%) may be attributed to differences between *algirus* and *cuniculus*, confirming the extension of the genetic

differentiation between the two subspecies. If compared with results obtained in studies of other species (see Nei 1987 for a review), our values are generally higher and explain well the consistency of the first split of rabbit populations. Other components of genetic diversity are less important, but it may be stressed that G_{PS} (differences between populations within subspecies) is always higher than the component attributed to differences between regions (Portugal, Spain, France and Azores; see results section). When only southwestern Iberian populations are analyzed (results not shown) this difference is even more important, a fact that may have a biological meaning due to the social structure that characterizes rabbit populations (Ferrand & Branco, unpublished results).

Geographic expansion

Today, the rabbit shows a continuous distribution in Europe, including the Iberian Peninsula. In this region, the geographic expansion of both *algirus* and *cuniculus* groups of populations led to the establishment of a relatively narrow hybrid zone based on mtDNA analysis (Branco *et al.* 2000). These authors show that this zone follows a northwest-southeast direction that divides the Iberian Peninsula and where populations exhibit both maternal lines in similar frequencies. On the contrary, preliminary data on nuclear markers in the same populations suggest that introgression is much more important (M. Branco & N. Ferrand, unpublished results), which maybe expected because i) effective population size of nuclear markers is higher than mtDNA, and ii) males disperse more than females (Kunkele & Von-Holst 1996).

The evidence presented in this work also suggests that wild rabbits from France are the result of a recent colonization originating in the northeast of Spain and associated with a relatively well-marked bottleneck. In fact, genetic variability of French populations is a clear subset of that observed in northeastern Spain and may be explained by the difficulties in overcoming the geographical barrier of the Pyrenees. Alternatively, this process may have been due to human transportation, but the two hypotheses are not testable due to a complete lack of other sources of information. A third possibility is associated with the fact that rabbits have been present in the Mediterranean region of France for at least 300 000 years (Pages 1980). Notwithstanding a progressive decrease in rabbit abundance based on archaeozoological data, Donard (1982) suggests that relict populations may have persisted close to the Mediterranean and were at the origin of recolonization. In the light of the genetic evidence now described, this hypothesis seems very unlikely. From southern France rabbits expanded to the north, arriving in Britain during the 11th century and progressing to the northeastern limits of their range during the 19th and 20th centuries (Flux 1994).

Different authors have suggested that all populations outside Iberia would be feral and derived from animals domesticated in the Iberian Peninsula during

Roman occupation (Clutton-Brock 1987, 1992; Flux & Fullagar 1992; Flux 1994). Our data are not in agreement with this scenario and suggest instead that the hypothesis first proposed by Zeuner (1963) may be correct. According to this author, the geographical expansion of the rabbit is due to two independent processes, namely i) translocation of wild rabbits and ii) domestication. Translocation of rabbits in central Europe and Britain were common during the Middle Ages for hunting purposes and certainly allowed the escape of animals kept in *leporaria*. The allelic distributions of ALB and CAI clearly support this hypothesis: French populations of Camargue and Versailles, as well as a population from southern England (Ferrand & Rocha 1992), exhibit the allele ALB*2 in high frequencies, while the same does not happen in domestic breeds that show the fixation of ALB*1. On the other hand, CAI is polymorphic in wild *cuniculus* populations only (including Britain; Ferrand, unpublished results), thus implicating that the dispersion of the CAI*2 allele marks the expansion of wild rabbits in central Europe. Still associated with this is the colonization of Australia in the 19th century originating from a limited number of wild rabbits imported from Britain (Flux 1994). The available genetic evidence both at the nuclear (Richardson *et al.* 1980; Ferrand, unpublished results) and mtDNA levels are compatible with the historical documentation. The second process, domestication, did not take place earlier than the 15th century (Zeuner 1963), but it may have overlapped to some degree with translocation of wild animals, during the colonization of central and northern France, as suggested from the analysis of ancient DNA (Hardy *et al.* 1995). Releasing domesticated animals to the wild may have been common and certainly helped the final stages of rabbit colonization in Europe. Additionally, the recent invasion of important areas of South America (Chile and Argentina) has also been attributed to that practice, a hypothesis that is strongly supported by a genetic analysis of Chilean rabbits (Vieira 1993).

A remarkable aspect of rabbit geographical expansion is its presence in more than 800 islands throughout the world, where they were transported by man since historical times (Flux & Fullagar 1992). In this investigation, we have analyzed two Azorean populations (islands of São Jorge and Flores) and concluded that the colonization of the archipelago was achieved with rabbits belonging to the *algirus* group. Moreover, they show close genetic relationships with Portuguese continental populations (see Figure 2), thus being in agreement with historical documentation referring to its introduction in the period of Portuguese discoveries (15th century). It is probable that the colonization of other Atlantic islands (e.g. Madeira and Porto Santo, and the Canary Islands) happened during the same historical period, but probably involving other sources. In the Mediterranean, where human navigation started much earlier, the history of rabbit colonization was quite different. The introduction of the rabbit in Menorca (Balearic Islands) is dated between 1400 and 1300 BC (Reumer & Sanders 1984), while Zembra (Tunisia) was invaded between the end of the Neolithic and the 3rd century of our time (Vigne 1988).

This last case has been studied in detail by Ben Amor (1998) using protein polymorphism and mtDNA sequencing, and the comparison with our data and that described in Hardy *et al*. (1994) and Branco *et al*. (2000) strongly suggests an origin on the eastern Mediterranean coast of Spain. The arrival of the rabbit in North Africa dates probably to this period and very limited genetic data (Ferrand *et al*., unpublished results) do confirm this hypo-thesis and may suggest at least two different origins. Many more cases exist, that when studied will certainly show a mosaic of different histories. The patterns of population differentiation and distribution of genetic diversity described in this paper will help to unravel a number of interesting situations.

Our data may be summarized in a scenario that depicts an evolutionary history of rabbit populations at a global scale (Figure 5). This scenario should be viewed as a hypothesis to be tested in the future, when the introduction of hypervariable markers like microsatellites as well as the sequencing of multiple nuclear genes and mtDNA should help to better understand the evolutionary history of this species.

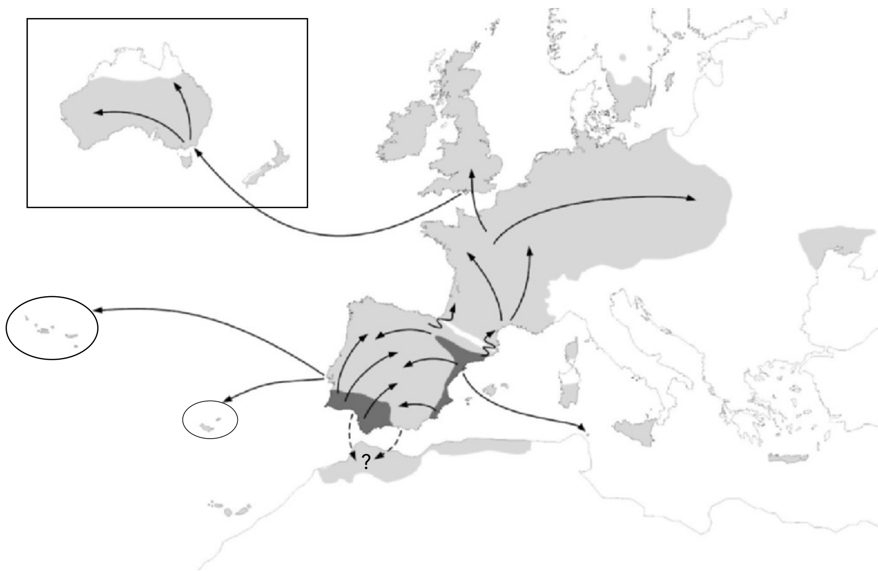

Figure 5. *A possible scenario for the origins and history of major groups of rabbit populations. This scenario summarizes the available paleontological, archaeozoological, historical and genetic data. Dark-gray areas represent the putative refugial areas for the two major groups of populations, and light-gray areas the present-day distribution of the rabbit in Europe, North Africa, some Mediterranean and Atlantic islands, Australia and New Zealand. Straight arrows represent both natural and human-mediated geographical expansions while broken arrows indicate restricted gene flow causing a large reduction in genetic diversity.*

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Appendix

Table A1. *Allele frequencies at 20 protein loci for the 13 European rabbit populations.*

		Population												
Locus	**Allele**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
ACP3	**1**	0.88	1.00	1.00	1.00	1.00	0.82	1.00	1.00	0.89	0.98	0.71	0.42	0.40
	2						0.18				0.02	0.29	0.58	0.60
	3	0.12								0.11				
	n	132	21	36	43	57	11	16	28	26	53	29	57	96
ADA	**1**	0.26	0.21	0.17		0.34	0.83	0.53	0.29	0.21	0.26	0.67	0.50	0.58
	2	0.04	0.10	0.03				0.03		0.12				
	3	0.70	0.62	0.76	1.00	0.66	0.13	0.44	0.71	0.65	0.73	0.31	0.35	0.18
	4		0.07											
	5											0.02	0.15	0.24
	6						0.04			0.02	0.01			
	7			0.04										
	n	89	21	36	50	54	12	16	28	26	53	29	58	77
ALB	**1**	0.65	0.91	0.51	0.52	1.00	0.62	0.75	0.44	0.44	0.39	0.34	0.83	1.00
	2	0.34	0.09	0.49	0.48		0.38	0.25	0.56	0.56	0.61	0.66	0.17	
	3	0.01												
	n	120	22	36	58	67	13	16	70	26	54	47	57	100
CAI	**1**	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.86	0.74	1.00
	2											0.14	0.26	
	3		0.02											
	n	55	21	36	47	49	12	16	29	26	52	29	57	100
CAII	**1**	0.15		0.03			0.46	0.03	0.38	0.28	0.23	0.45	0.59	0.83
	2	0.03	0.24	0.08				0.06		0.02	0.06			
	3						0.03							
	4							0.03						
	5	0.82	0.76	0.88	1.00	1.00	0.25	0.88	0.62	0.68	0.71	0.55	0.41	0.17
	6			0.01			0.04			0.02				
	n	58	21	36	58	50	12	16	29	25	54	29	59	145
GALT	**1**	0.70	0.91	0.81	1.00	1.00	0.63	0.59	0.72	0.84	0.93	1.00	1.00	0.99
	2			0.03										
	3	0.26	0.02	0.15			0.37	0.13	0.04		0.03			0.01
	4									0.08				
	5	0.04		0.01				0.13	0.22	0.02	0.04			
	6									0.06				
	7							0.15						
	8								0.02					
	9		0.07											
	n	74	21	36	50	50	12	16	27	26	53	29	44	99

Continued on next page

Table A1. *Continued.*

		Population												
Locus	**Allele**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
GC	**1**	0.92	0.90	0.79	1.00	1.00	1.00	0.91	0.83	0.96	0.82	1.00	1.00	1.00
	2	0.01												
	3	0.06	0.02	0.03				0.06			0.13			
	4	0.01	0.02	0.15						0.04	0.04			
	5			0.03				0.03						
	6								0.11					
	7								0.06					
	8										0.02			
	9		0.05											
	n	83	21	36	50	50	13	16	27	26	57	28	44	100
GPI	**1**	0.97	1.00	0.95	1.00	1.00	1.00	0.97	0.94	1.00	0.99	1.00	1.00	1.00
	2	0.03							0.06					
	3			0.01				0.03			0.01			
	4			0.04										
	n	37	21	57	50	47	56	16	63	33	37	18	58	47
HBA	**1**											0.97	0.89	0.76
	2	0.68	0.33	0.79	0.56	0.89	0.63	0.22	0.57	0.84	0.90	0.03	0.11	0.20
	3	0.26	0.67	0.21	0.44	0.11	0.12	0.78	0.43	0.16	0.10			0.04
	4	0.06					0.08							
	5						0.17							
	n	62	21	36	55	46	12	16	28	25	54	29	58	109
HBB	**1**	0.18		0.08			0.08	0.06			0.06	0.07	0.08	0.49
	2	0.82	1.00	0.92	1.00	1.00	0.92	0.94	1.00	1.00	0.94	0.93	0.92	0.51
	n	68	21	36	55	44	12	16	28	25	54	29	58	143
HBX	**1**								0.03					
	2										0.03			
	3	0.46	0.72	0.61	0.51	0.32	0.61	0.70	0.69	0.37	0.61	0.31	0.38	0.55
	4	0.13		0.10	0.49	0.68		0.10	0.04	0.28	0.19		0.12	0.02
	5	0.41	0.28	0.29			0.26	0.20	0.23	0.35	0.16	0.28	0.11	0.05
	6						0.13		0.01		0.01	0.39	0.39	0.38
	7											0.02		
	n	46	32	33	57	28	39	15	28	58	36	27	88	110
MPI	**1**	0.10	0.01	0.04	0.01			0.12	0.11		0.01	0.50	0.50	0.56
	2	0.57	0.91	0.58	0.47	0.50	1.00	0.13	0.81	0.82	0.80	0.50	0.50	0.39
	3													0.05
	4	0.20	0.01		0.23	0.32			0.02		0.05			
	5	0.08	0.02	0.25	0.29	0.18		0.75	0.06		0.12			
	6	0.05	0.04	0.13						0.18				
	7										0.01			
	8		0.01								0.01			
	n	159	49	24	46	49	12	4	27	25	42	25	25	66
NP	**1**	0.84	0.98	0.76	0.76	0.30	1.00	0.81	0.94	0.96	0.96	1.00	1.00	1.00
	2	0.13	0.02	0.24	0.24	0.70		0.19	0.06	0.04	0.04			
	3	0.03												
	n	257	23	36	47	70	12	16	27	26	53	29	57	87

Table A1. *Continued.*

		Population												
Locus	**Allele**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
PEPA	**1**	0.44	0.19	0.43	0.74	0.80	1.00	0.66	0.32	0.42	0.68	1.00	1.00	1.00
	2	0.56	0.81	0.57	0.26	0.20		0.34	0.55	0.58	0.32			
	3								0.13					
	n	62	21	35	23	53	12	16	28	26	53	27	42	48
PEPB	**1**	0.77	0.80	0.78	0.45	0.72	0.92	0.97	0.95	1.00	0.91	0.93	1.00	1.00
	2	0.23		0.15	0.55	0.28						0.07		
	3		0.04	0.07			0.08	0.03	0.05		0.07			
	4		0.16								0.02			
	n	124	23	36	57	59	12	16	30	26	54	29	54	23
PEPC	**1**	0.59	0.54	0.52	0.22	0.20	1.00	0.53	0.61	0.44	0.40	0.98	1.00	1.00
	2	0.29	0.15	0.41	0.67	0.68		0.22	0.30	0.15	0.23	0.02		
	3	0.04							0.02		0.06			
	4		0.24		0.11	0.12		0.22	0.02	0.14	0.10			
	5	0.06	0.07	0.07				0.03	0.05	0.27	0.18			
	6	0.02												
	7										0.03			
	n	133	23	35	55	62	12	16	30	26	54	29	54	44
PEPD	**1**	0.80	0.74	0.83	0.92	0.88	1.00	0.59	0.46	0.58	0.61	1.00	1.00	1.00
	2	0.14	0.17	0.15	0.08	0.12		0.38	0.45	0.36	0.18			
	3	0.06	0.09					0.03	0.07	0.02	0.16			
	4			0.02					0.02	0.04				
	5										0.02			
	6										0.03			
	n	56	23	36	56	56	12	16	29	25	54	29	51	24
PGD	**1**	0.34	0.71	0.36	0.59	0.44	0.88	0.47	0.54	0.77	0.39	0.98	0.60	0.98
	2												0.27	0.02
	3	0.63	0.29	0.64	0.41	0.56		0.53	0.46	0.23	0.61	0.02	0.13	
	4	0.03												
	5						0.12							
	n	236	21	36	46	70	12	15	26	26	53	29	44	160
TF	**1**	0.79	0.64	0.60	0.61	0.65	1.00	0.69	0.53	0.73	0.62	1.00	1.00	1.00
	2	0.01												
	3	0.20	0.05	0.40	0.39	0.35		0.31	0.34	0.27	0.29			
	4								0.01					
	5										0.07			
	6								0.08					
	7								0.04		0.02			
	8		0.07											
	9		0.24											
	n	117	21	36	58	48	13	16	65	26	52	25	49	150
SOD	**1**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.82	1.00	1.00	1.00	1.00	1.00
	2								0.18					
	n	85	55	48	57	14	12	16	63	202	176	22	50	238