

POSTGLACIAL DISPERSAL OF THE EUROPEAN RABBIT (*ORYCTOLAGUS CUNICULUS*) ON THE IBERIAN PENINSULA RECONSTRUCTED FROM NESTED CLADE AND MISMATCH ANALYSES OF MITOCHONDRIAL DNA GENETIC VARIATION

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Abstract.—Nested clade analysis was applied to cytochrome *b* restriction site data previously obtained on 20 natural populations of the European rabbit across the Iberian Peninsula to test the hypothesis of postglacial dispersal from two main refugia, one in the northeast and the other in the southwest. Apart from historical fragmentation that resulted in geographic discontinuity of two distinct mitochondrial DNA (mtDNA) clades A and B, patterns of haplotype genetic variability have been shaped mostly by restricted gene flow via isolation by distance. The distribution of tip versus interior haplotypes suggests that dispersal occurred from both the southwestern and northeastern groups. Dispersal from the southwest had a north and northwest direction, whereas from the northeast it had mostly a western and southern orientation, with subsequent overlap in a southeastern-northwestern axis across the Iberian Peninsula. The analysis of the pairwise mismatch distribution of a 179–181-bp fragment of the mtDNA control region, for seven of those populations, further supports the idea that major patterns of dispersal were in the direction of central Iberia. Additionally, rabbit populations do not show signs of any significant loss of genetic diversity in the recent past, implying that they maintained large population sizes and structure throughout the ice ages. This is congruent with the fact that the Iberian Peninsula was itself a glacial refugium during Quaternary ice ages. Nonetheless, climatic oscillations of this period, although certainly milder than in northern Europe, were sufficient to affect the range distributions of Iberian organisms.

Key words.—European rabbit, Iberian Peninsula, mismatch distribution, mitochondrial DNA, nested clade analysis, *Oryctolagus cuniculus*, postglacial dispersal.

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The origin of the European rabbit (*Oryctolagus cuniculus*) is in southwestern Europe (reviewed by Corbet 1994), and until the Middle Ages its natural range was restricted to the Iberian Peninsula and southern France (Callou 1995). Human mediated colonization led to the rabbit spreading across central and northern Europe and the British Isles, and today it can be found throughout the world (Flux 1994). Two divergent genetic entities were found in the rabbit by blood protein (Ferrand 1995; Branco and Ferrand 1998; Branco et al. 1999), immunoglobulin (van der Loo et al. 1991, 1999) and mitochondrial DNA (mtDNA) variation (Biju-Duval et al. 1991; Monnerot et al. 1994; Branco et al. 2000). At the mtDNA level, there are two distinct parapatric lineages in the Iberian Peninsula. Lineage A is typical of the southwest and lineage B of the northeast, overlapping in a central region along a northwest-southeast axis (Branco et al. 2000). Visual inspection of how the main phylogeographic pattern overlays with geography led to the interpretation that rabbit populations where both maternal lineages were observed represent the overlapping region of two diverging groups that came recently into contact (Branco et al. 2000). As in many other organisms (Hewitt 1996; Taberlet et al. 1998), long-term isolation, triggered by Quaternary climatic oscillations, was responsible for the formation of two geographic races within the rabbit species. During the last postglacial period, rabbit recolonized formerly unsuitable territory culminating in secondary contact across the Iberian Peninsula. Yet, important questions remain to be answered, namely concerning the evo-

lutionary processes within each group underlying their geographic expansion.

Progress in analytical statistics allows the testing of hypotheses on evolutionary processes acting on populations. From salamanders (Templeton et al. 1995) to African bovids (Templeton 1998) and humans (Templeton 1997, 1999; Hammer et al. 1998), nested clade analysis (NCA) has been shown to be a useful statistical tool for the interpretation of phylogeographic data. Likewise, the distribution of pairwise nucleotide site differences (or pairwise mismatch distribution) has been extensively used not only to infer the demographic history in humans (e.g., Rogers and Harpending 1992; Marjoram and Donnelly 1994; Rogers 1995; Rogers and Jorde 1995; Harpending et al. 1998; Excoffier and Schneider 1999), but also on evolutionary studies of many other species (e.g., Lavery et al. 1996; Goldberg and Ruvolo 1997; Petit et al. 1999; Bernatchez 2001).

In the present work, we apply the NCA to rabbit cytochrome *b* gene restriction data to test statistically the hypothesis, raised from the interpretation of the described phylogeographic pattern, of an old fragmentation event followed by range expansion and overlap (Branco et al. 2000). We also examine the evolutionary events leading to that recolonization.

Additionally, we do a mismatch distribution analysis (MDA) to refine the inferences drawn from the NCA concerning the most recent expansion events. This is done by surveying the sequence variation of an hypervariable region of the mtDNA in a few populations located in the centers of

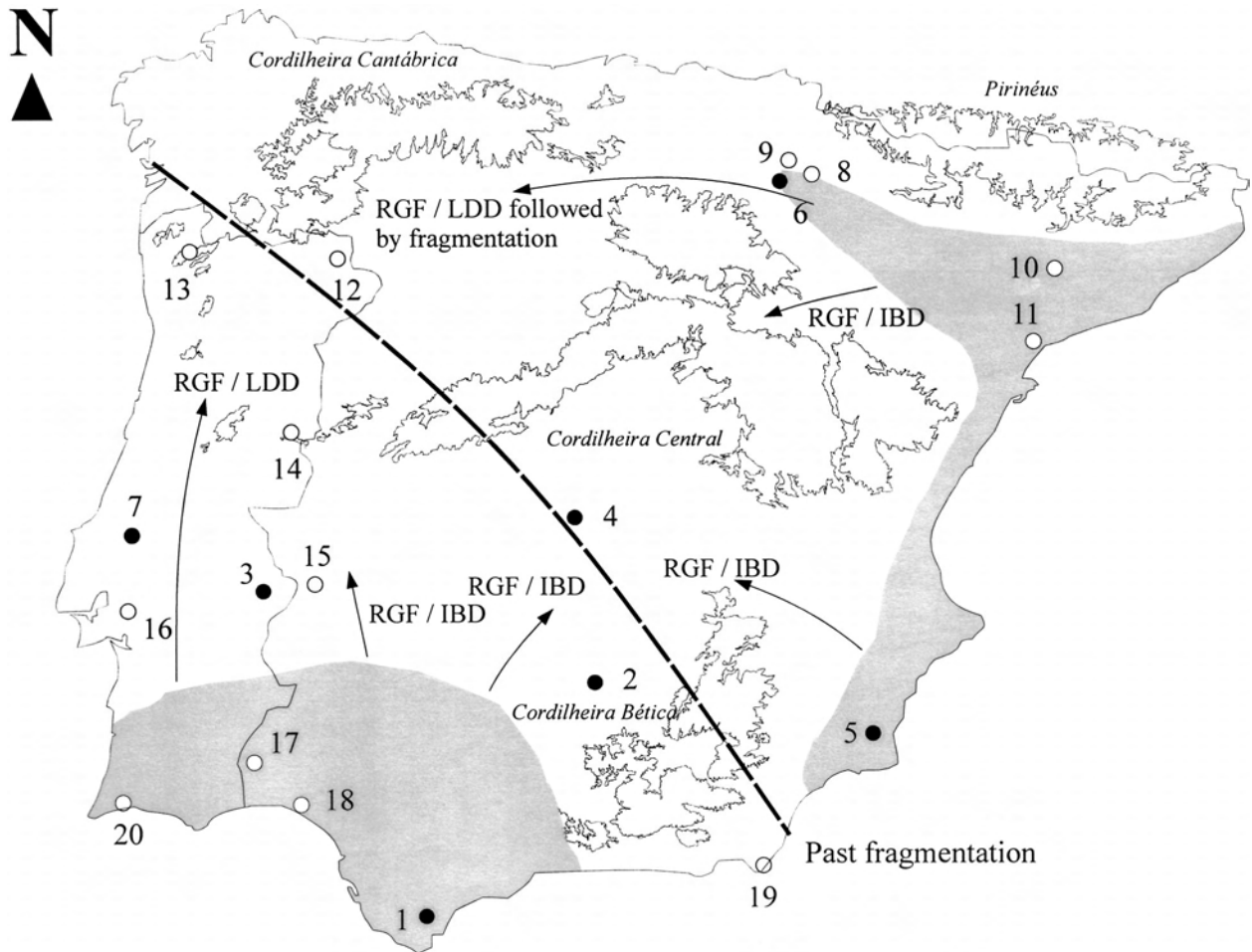


FIG. 1. Sampling sites. Samples from locations in black were sequenced for the mitochondrial DNA control region during the present work. (1) Las Lomas; (2) Ciudad Real; (3) Vila Viçosa; (4) Toledo; (5) Alicante; (6) Peralta; (7) Santarém; (8) Tudela; (9) Caparrosa; (10) Lérida; (11) Tarragona; (12) Bragança; (13) Cabreira; (14) Idanha; (15) Badajoz; (16) Infantado; (17) Huelva; (18) Doñana; (19) Amoladeras; (20) Portimão. Location numbers are given in parentheses in the text following the location name. Significant inferences from the geographical analysis of rabbit cytochrome *b* gene haplotype tree are also shown. Fragmentation is represented by a thick dashed line, restricted gene flow (RGF), isolation by distance (IBD), and long-distance dispersal (LDD) are represented by arrows. Fragmentation occurred in the early history of the rabbit generating two distinct groups. Afterward gene flow restricted by isolation by distance has predominated. In the colonization of the northwest, there was some long-distance dispersal followed by fragmentation from the northeast. Shaded areas represent putative glacial refugia.

expansion (southwest and northeast) and along the main dispersal routes. A trend for decreasing modes of mismatch distributions in direction of central Iberia is expected either from the southwest and the northeast.

MATERIALS AND METHODS

Figure 1 shows the sampling sites. Sampling and DNA methodologies for assaying genetic variation of cytochrome *b* gene by RFLP are given in Branco et al. (2000).

A fragment of the mtDNA control region (corresponding to 15507–15685 of the complete mtDNA sequence, Genbank accession number AJ001588) was studied by direct sequencing of 139 individuals from six Iberian locations (Alicante [5], Ciudad Real [2], Las Lomas [1], Santarém [7], Toledo [4], and Vila Viçosa [3]; see Fig. 1). This segment was amplified with primers Pro1 (5'-CCACCAT-

CAGCACCCAAAGCT-3') and NC4 (5'-ATGGCCCT-GAGGTAAGAACC-3'). Sequences were obtained manually with Amersham (Paris, France) cycle sequencing kit (US 79750) using an internal primer (NC5, 5'-CTTTAA-TAAACTCAAGTACTTC-3'). Data from the seventh Iberian location of Peralta 6 ($n = 27$) and primers were obtained from Mougel (1997). Genbank accession numbers are Z83347, Z83356–Z83359, Z83362, Z83363, Z83365, and Z83367.

Data Analysis

The NCA tests the association between the phylogenetic and geographic positions of haplotypic variants. The first step of the NCA is to build a minimum spanning tree of haplotypes including all the one-step mutations. The network described in Branco et al. (2000) was converted into a nested series of

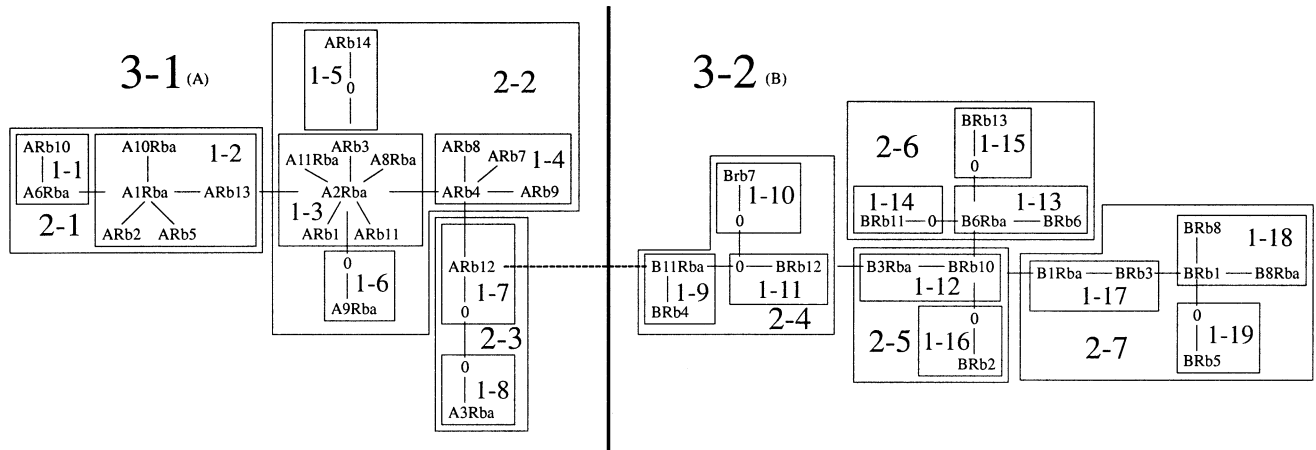


FIG. 2. Nested design for the mitochondrial DNA haplotypes found in *Oryctolagus cuniculus* populations. Haplotypes are designated as in Branco et al. (2000). Zeros indicate undetected intermediate haplotype states. Each solid line represents one mutational step. The thick dashed line represents a minimum of 14 mutational steps between the two main groups. The boxes indicate one- and two-step nesting levels (1- x and 2- x), where x is the number assigned to the clade. A thick vertical line separates the two three-step clades, 3-1 and 3-2, which coincide with the formerly designated clades A and B, respectively.

clades by using the rules given in Templeton et al. (1987) and Templeton and Sing (1993). Starting at the tip haplotypes and moving one mutational step to the interior, all haplotypes are united in one-step clades. The next nesting level uses the one-step clades as units to form the two-step clades. This procedure goes on until the final nesting level, which consists of the entire haplotype tree (Fig. 2).

Geographic data for each nesting clade for all nesting levels are quantified into two distance statistics: the clade distance (D_C), which measures the geographic range of a clade, and the nested clade distance (D_N), which measures how a given clade is geographically distributed relative to its closest evolutionary sister clades (Templeton et al. 1995). Association between phylogenetic and geographic position is determined on the basis of the statistical significance of D_C and D_N by random permutation testing. In this step, the null hypothesis of a random geographic distribution for all clades within each nesting level is simulated based on the haplotype/clade frequencies and sample size per location. Testing association was done with Geodis version 2.0 software package (Posada et al. 2000). For those clades to which the null hypothesis is rejected, the biological causes for association are inferred using the inference key first published in Templeton et al. (1995; an improved version is available at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm).

Alignment of the 166 sequences was obtained using the Clustal X (ver. 1.3b) software package (Thompson et al. 1994). The final information for each individual was a string of 179–181 nucleotides (Table 1). Pairwise mismatch differentiation was obtained using the Arlequin 2.0 software package (Schneider et al. 2000).

RESULTS

The restriction site states of the 52 cytochrome *b* gene haplotypes found in the European rabbit, the frequency of each haplotype in the 20 Iberian sampling locations (Fig. 1; see Appendix 1) and the maximum parsimonious net-

work resulting from the application of statistical parsimony (Templeton et al. 1992) are described in Branco et al. (2000).

Figure 2 shows the nested design obtained using the rules given in Templeton et al. (1987) and Templeton and Sing (1993). The exact contingency analysis of 1000 permutations indicates strong associations between clades and geographic location (results not shown). Figure 3 presents the results of the NCA of geographic distances and describes the inferences drawn from the application of the key given in Templeton et al. (1995) to the statistical results (Appendix 2). It shows that both population structure and population history played an important role in determining the geographic associations of mtDNA haplotypes for the rabbit species.

The geographic associations inside clade A (from nesting level 1 to 3) are consistently explained by restricted gene flow via isolation by distance, with the one exception of nesting clade 1-4 where some long distance dispersal was also inferred. Conclusive results for clade B at the lowest nesting level were obtained only for the haplotypes nested within clade 1-18 where past fragmentation was inferred. For all other level one nesting clades, the outcome was inconclusive. Statistical power is recovered in clades of nesting level two where the inference of restricted gene flow via isolation by distance predominates. Some long-distance dispersal was diagnosed within clade 2-7. For clade 2-4, it was not possible to distinguish between fragmentation and isolation by distance. At nesting level 3, which corresponds to the total clade B, restricted gene flow via isolation by distance was also inferred to be the main cause of geographic association. At the highest nesting level, a fragmentation event is identified separating the species into a southwestern and a northeastern group.

A total of 53 different sequences, defined by 56 variable sites in a fragment of 179–181 nucleotides (15507–15685) of the control region, were found in 166 individuals from

seven locations (Table 1). As for the cytochrome *b* restriction site data, two distinct groups of sequences were detected that coincide with clades A and B (results not shown). We found little to no sharing of haplotypes between populations as only one A and three B type variant sequences appear in several locations (Santarém [7], Vila Viçosa [3], Alicante [5], Peralta [6], and Toledo [4]; Table 1). A decrease of the mean pairwise differences is observed from Las Lomas (1) to Toledo (4) and from Las Lomas (1) to Santarém (7) for clade A (Table 2). Within clade B, the mean pairwise difference is highest in Peralta (6), followed by Alicante (5) and Toledo (4), and null in Vila Viçosa (3; Table 2). Nucleotide diversity across populations followed the same trend of clinal variation and direction. One major peak is identified in the mismatch distributions (MD) of most populations (Fig. 4), and their relative positions from the right to the left of the graph can be related with geography.

DISCUSSION

Nested Clade Analysis: Characterizing the Evolutionary Process within Major Lineages

Application of the NCA approach to the rabbit cytochrome *b* gene haplotype tree yields a straightforward interpretation of past fragmentation that separates the species into southwestern (clade A) and northeastern (clade B) subgroups. Within these groups recurrent gene flow restricted by isolation by distance played the predominant role in shaping the present genetic structure of rabbit populations.

Within the southwestern region a consistent pattern of restricted gene flow is detected at all nesting levels, which may be explained by wavelike population movements whereby rabbits slowly spread northward with a decrease in genetic variability. Isolation by distance has been the main factor shaping the distribution of genetic variation through the entire time period marked by the diversification of clade A. The only exception is found along the western coast northward, from Infantado (16) to Cabreira (13) and Bragança (12), where some long-distance dispersal was detected within clade 1–4. The temporal juxtaposition of long-distance dispersal and the more widespread inference of restricted gene flow due to isolation by distance suggests at least two main routes of recolonization, one leading to central Iberia and another to the western coastal region.

For the northeastern region, the outcome of most level 1 nesting clades is inconclusive, possibly due to a small number of sampling locations in this region. Statistical power is recovered in clades of higher nesting levels where restricted gene flow due to isolation by distance predominates. There is an exception at nesting level 2, where some long-distance dispersal is inferred in clade 2–7. Nested within 2–7, clade 1–18 revealed fragmentation (Fig. 2). If rabbits carrying clade B mtDNA found refuge in the northeastern region during the last glacial maximum, when the ice retreated they would have started to move westward, with restricted gene flow and long-distance dispersal marking the first stages of northwestern recolonization. This was followed by progressive isolation and fragmentation due to the strong effect of geographic and ecological constraints. At present, fragmentation may reflect the near absence of rabbits at the foothills of Cordilheira

Cantábrica, a rather inhospitable region imposing a strong barrier to rabbit genetic exchange between the northwest and the northeast. Although rabbits have shown to adapt well to diverse ecological conditions, their native habitat is typically Mediterranean, where there is good warren building ground, abundance of herbaceous plants for food, and shrubs to hide from predators (Rogers and Myers 1979; Rogers et al. 1994). Northern Iberia is mountainous, rocky, and still largely forested, only supporting scattered and rather isolated small rabbit populations (Villafuerte et al. 1998). A history of smaller population sizes, as well as a stronger effect of structuring and fragmentation on the distribution of haplotypic variation in the northeast relative to the southwest had already been suggested by Branco et al. (2000), who examined the differences in branching patterns of clades A and B. The NCA not only confirms that interpretation, but also provides quantification and statistical significance to the biological inferences drawn from the observed patterns.

The overall results of the NCA imply that after long-term allopatric isolation, population advance has taken place in a wavelike fashion, mostly conditioned by geographic barriers and ecological constraints, from both the southwest and the northeast leading to range overlap in a southeast-northwest direction. Admixed populations tend to carry mostly tip haplotypes, whether from clade A or B, which is another indication that central Iberia was most recently colonized from both regions.

Mismatch Distribution Analysis: Differing Patterns of Dispersal among Lineages in the Postglacial Recolonization of Iberia

If central Iberia was most recently colonized from both the southwest and the northeast, decreasing MD modes are expected in its direction. By visual inspection of the MDs depicted in Figure 4, we can identify a main peak in most populations with relative positions from the right to the left of the graphs that can be related with routes of geographic expansion.

The MD pattern of lineage A populations suggests that, in the southwest, the center of expansion is the southernmost region. The shape of the main peak of Las Lomas (1) may be associated with a past episode of population growth, marking the beginning of the postglacial expansion in this region. In addition, gradients in population age, deduced from MD modes, consolidate NCA inferences of two main routes of geographic expansion: Dispersal occurred northward to Ciudad Real (2) and Toledo (4) and northwestward in direction of Vila Viçosa (3) and Santarém (7; Fig. 4). The drop in sequence divergence from the center of the distribution to the periphery conforms with these directions of geographic expansion (Table 2).

The MD pattern of lineage B populations shows that Vila Viçosa (3) is on the edge of the B expansion, drawing a east-west dispersal route from Alicante (5) to Toledo (4) and to Vila Viçosa (3). The absence of variation in Vila Viçosa (3) indicates that this is probably a very recent arrival. Other dispersal routes are not so obvious as for lineage A. Peralta (6) has the highest mean nucleotide pairwise difference, Alicante (5) has a slightly lower value,

TABLE 1. Extended.

Nucleotide positions																																
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terns (Slatkin and Hudson 1991; Rogers and Harpending 1992; Harpending et al. 1998). Marjoram and Donnelly (1994) also state that geographic substructuring with restricted gene flow is among the factors that lead to such patterns, and in the NCA this was found to be the predom-

inant factor shaping rabbit genetic variability. In addition, the multimodal MDs of Peralta (6) and Alicante (5) support a stronger effect of geographic structuring and fragmentation in the northeast, when compared to the southwest, as suggested by NCA inferences.

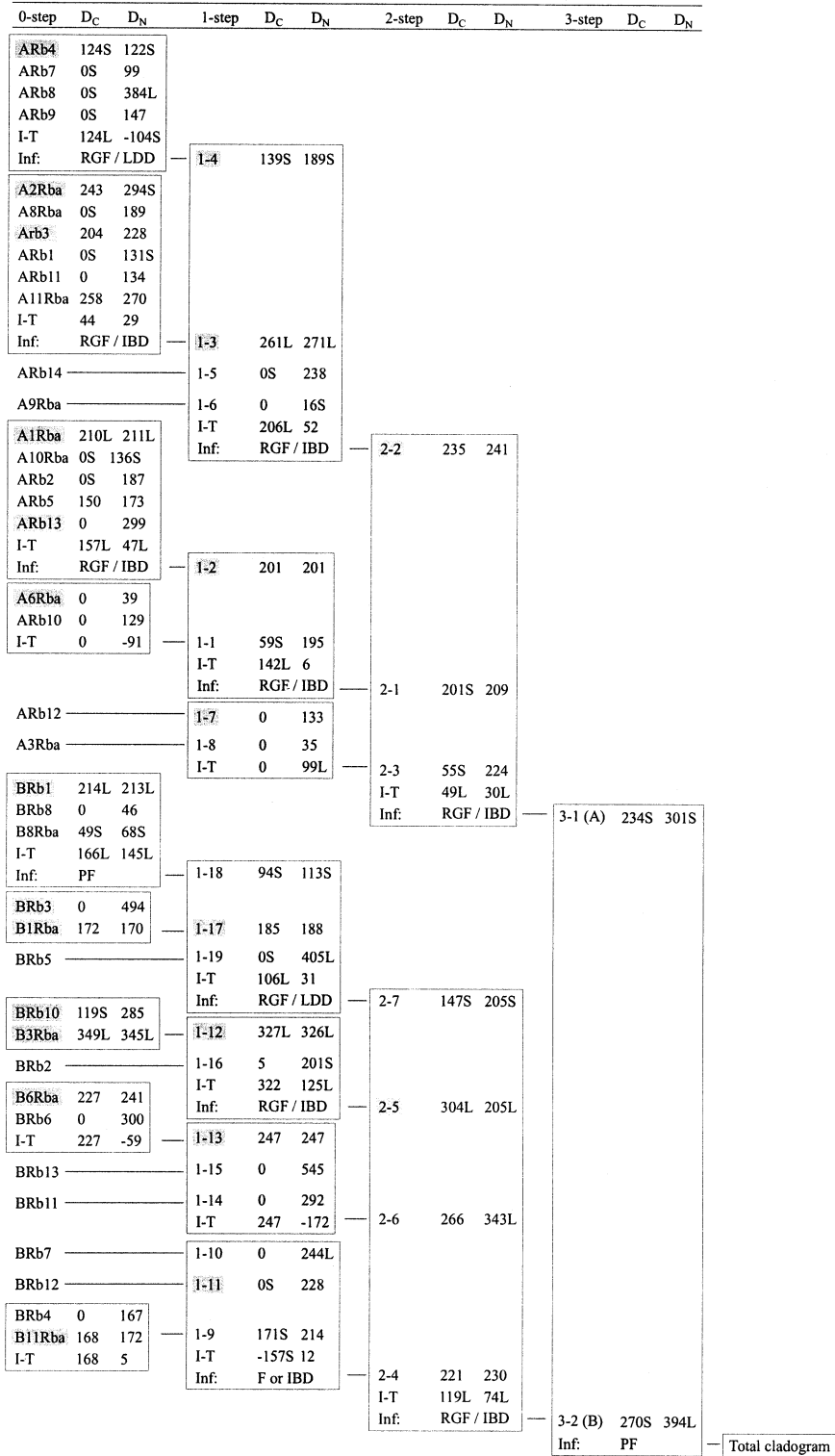


FIG. 3. Nested cladistic analysis of geographical distances. Clade (D_C) and nested clade (D_N) distances are given to the right of each haplotype designation. An S indicates that the distance is significantly small at the 5% level, and an L indicates that it is significantly large. For nested clades where the interior/tip status is known and for which both tips and interiors exist within the same nesting group, the clade name is shaded for interior clades. I-T gives the average difference between interior and tip clades within the nested group for clade and nested clade distances. Inferences are given to the left of each clade designation; RGF, restricted gene flow; IBD, isolation by distance; LDD, long-distance dispersal; F, fragmentation; PF, past fragmentation.

TABLE 2. Sequence data of a 179–181 nucleotide fragment (15507–15685) from the mitochondrial DNA control region. Sample size (*N*), number of different sequences found (*K*), number of variable nucleotide positions (*A*), mean nucleotide pairwise differences (*B*), and nucleotide diversity (*P*).

Location	Clade	<i>N</i>	<i>K</i>	<i>A</i>	<i>B</i>	<i>P</i>
Ciudad Real	A	16	9	21	6.82	0.038
Las Lomas	A	24	9	23	7.72	0.043
Santarém	A	16	9	10	2.10	0.012
Toledo	A	18	7	16	3.91	0.022
Vila Viçosa	A	21	5	9	3.17	0.018
Alicante	B	19	4	11	4.69	0.026
Peralta	B	27	9	23	5.35	0.029
Toledo	B	15	6	8	2.86	0.016
Vila Viçosa	B	10	1	0	0.00	0.000

Patterns of Rabbit Evolution in the Iberian Peninsula

The major factors that shaped the recent evolution of the rabbit species on the Iberian Peninsula are synthesized in Figure 1. Overall, levels of genetic variability and population and regional differentiation suggest that rabbits have been able to maintain large populations throughout the Quaternary

glaciations. Notably, these observations argue against the conclusions of SurrIDGE et al. (1999) that myxomatosis erased from rabbit populations most traces of past historical events, and are good evidence that in the Iberian Peninsula they did not go through severe bottlenecks in their recent past. Yet, there is a remarkable difference in the level of genetic variation between southwest and northeast groups, with southwestern populations being generally more variable (Table 2). It is not only at the mtDNA level that a southwest to northeast loss of genetic variation is observed, as such a pattern has already been described for protein markers (Branco et al. 1999). Additionally, the current analyses also show that the geographic distribution of genetic variation is distinct (Fig. 1). Whereas genetic differentiation in the southwest shows an overall pattern of geographic continuity, within the northeast the scenario is of strong geographic subdivision.

Showing that two rabbit evolutionary lineages evolved within the Iberian Peninsula has important implications for our understanding of the major biogeographic patterns during the Quaternary ice ages in this region. It suggests that the Iberian Peninsula was not one large refugium, but that several refugial areas may have existed depending on the environ-

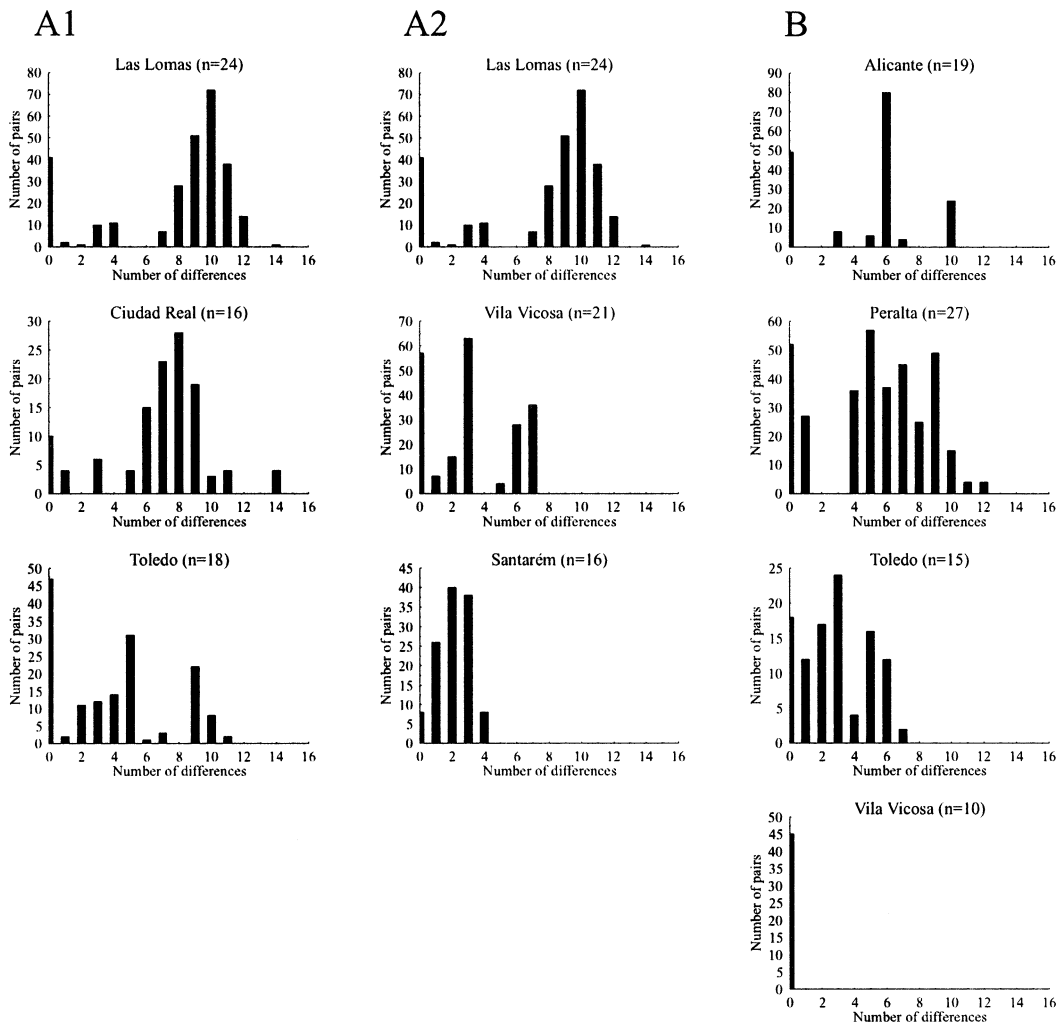


FIG. 4. Nucleotide pairwise difference distribution in seven locations.

mental limitations of each species, thus acting as a source of endemic differentiation (Bilton et al. 1998). The European grasshopper, *Chorthippus parallelus* (Cooper et al. 1995), and the Mediterranean ragwort, *Senecio gallicus* (Comes and Abbott 1998), displayed phylogeographic structures concordant with the scenario of southwest and northeast refugia now described for the rabbit. Nevertheless, the Iberian biogeographic patterns are not straightforward, as suggested by the clear ancestral subdivision detected in the golden-striped salamander, *Chioglossa lusitanica* (Alexandrino et al. 2000). Because this species is confined to the Iberian northwest, its phylogeographic structure is not immediately superimposable with the one we describe. It reflects, instead, a rather complex biogeographic history in the Iberian Peninsula where shared historical events in the evolutionary and ecological factors shaped a variety of intraspecific phylogeographies.

Therefore, in extending such studies to other Iberian species it is expected that a large variety of genealogical assemblages will come to light but, according to what has been done in other regions (reviewed by Avise 2000), only an enlarged regional phylogeographic survey will give us further insight on the major historical biogeographic factors that shaped the contemporary genetic architectures of Iberian organisms.

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APPENDIX 2

Inference chain on the results given in Figure 2. RGF, restricted gene flow; IBD, isolation by distance; LDD, long-distance dispersal; PF, past fragmentation; F, fragmentation.

Clade	Chain of inference	Inference
Haplotypes nested in 1-2	1-2-3-4 no	RGF with IBD
Haplotypes nested in 1-3	1-2-3-4 no	RGF with IBD
Haplotypes nested in 1-4	1-2-3-5-6-7 yes	RGF with LDD
Haplotypes nested in 1-18	1-2-3-4-9 no	PF
One-step clades nested in 2-1	1-2-3-4 no	RGF with IBD
One-step clades nested in 2-2	1-2-3-4 no	RGF with IBD
One-step clades nested in 2-4	1-2-3-4-9-10 no	F or IBD
One-step clades nested in 2-5	1-2-11-17-4 no	RGF with IBD
One-step clades nested in 2-7	1-2-3-5-6-7 yes	RGF with LDD
Two-step clades nested in 3-1	1-2-3-4 no	RGF with IBD
Two-step clades nested in 3-2	1-2-3-4 no	RGF with IBD
Total cladogram	1-2-3-5-15 no	PF