



ORIGINAL ARTICLE

Mitochondrial DNA signatures at different spatial scales: from the effects of the Straits of Gibraltar to population structure in the meridional serotine bat (*Eptesicus isabellinus*)

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The meridional serotine bat *Eptesicus isabellinus* is found in North Africa and the Iberian Peninsula. We analyzed the genetic structure of *E. isabellinus* at two different geographic scales to reveal the historical and ecological patterns that have shaped its populations. The role of the Straits of Gibraltar as an isolating barrier between African and Iberian populations is evaluated and the degree of genetic structure and female-mediated gene flow was assessed at a local scale between neighboring colonies. Populations of *E. isabellinus* from Iberia and northern Morocco show little genetic divergence and share mtDNA haplotypes, indicating that the Straits of Gibraltar are neither

an impediment to dispersal nor a cause of genetic differentiation. Our results also suggest that *E. isabellinus* may have dispersed from western Andalusia into northern Morocco after the last glacial period. At a smaller geographic scale, the colonies studied showed high variation in genetic variability and structure, indicating that no female-mediated gene flow is present. This pattern is consistent with a described pattern of independent endemic viral circulation of the bat rabies virus EBLV-1, which was found when studying rabies dynamics in the same serotine bat colonies.

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Introduction

Advances in population genetics have revealed that historical processes (for example, colonization or isolation in refuges) underlie current ecological processes (for example, breeding structure or gene flow). The scale at which historical and ecological factors interact is attracting increasing attention from ecologists and geneticists (Linhart, 1999). Recent evidence indicates that the relative importance of these processes in shaping observed patterns of genetic variation depends on the geographic scale at which they are studied.

Historically, populations in temperate zones have responded to Pleistocene climatic oscillations by retreating to suitable refugia during cold periods followed by a northward recolonization as climatic conditions improve (Coope, 1994). These contraction/expansion cycles generally leave a signature, traceable both phylogenetically and in populations' genetic structures. At a European scale the southern peninsulas (Iberia, Italy and the Balkan Peninsula) have acted as the main refugia for western Palearctic biotas (Taberlet *et al.*, 1998; Hewitt, 1999). Other areas in North Africa and along the Black Sea have also been suggested as Palearctic refugia (Leroy

and Arpe, 2007). In this context, understanding the importance of the Gibraltar and Messina straits in either connecting or isolating African and European biomes is essential for confirming the relevance of North African refugia for present western Palearctic biotas. The signatures left in the populations' genetic structures can also help answer these historical questions and it is predicted that populations from recolonized territories will generally show a relative lack of genetic variability and structure due to recurrent bottlenecks and loss of ancestral lineages. In contrast, older populations occupying Pleistocene refugia will have experienced less dramatic environmental and population changes (Hewitt, 1996; Pinho *et al.*, 2007).

At an ecological scale, meta-population theory describes current populations as groups of more or less genetically related individuals that are spatially divided from other groups by geographic features such as streams, mountains or marine barriers (Hanski, 1999). Geographic characteristics affect the structuring of genetic variability by limiting or enhancing migration and gene flow among populations. These effects would vary in accordance with the particular life history and ecological characteristics of each given species.

In relation to other small mammals, bats are extraordinary for their long lifespan and very slow reproduction rates. Despite their ability to fly, they show relatively high levels of genetic structuring, probably related to their diversified social organization systems: for example, *Myotis bechsteinii*, *Myotis myotis*, *Macroderma gigas*

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and *Mystacina tuberculata* (Worthington Wilmer *et al.*, 1999; Kerth *et al.*, 2000; Lloyd, 2003; Ruedi and Castella, 2003). The exceptions are the few migratory species, whose populations are typically poorly subdivided for example, *Tadarida brasiliensis* and *Pteropus* sp. (Webb and Tidemann, 1996; Russell *et al.*, 2005). In sedentary bat species, the geographic structure of genetic variation seems to be closer to that of large mammals or birds than to similar-sized small mammals (Ditchfield, 2000). This genetic partitioning is determined by an array of factors including dispersion, historical events and/or extrinsic barriers to gene flow (Burland and Worthington Wilmer, 2001).

The meridional serotine bat *Eptesicus isabellinus* is a North African species that has been recently discovered in the south of the Iberian Peninsula, where its distribution is allopatric with the very similar *E. serotinus* that occurs in the northern half of Iberia and throughout most of Europe (Ibáñez *et al.*, 2006).

Both *Eptesicus* species have a crucial role as reservoirs of the most common European bat rabies strain EBLV-1 (Pötzsch *et al.*, 2002), whose circulation dynamics and pathogenesis are currently being investigated (Vázquez-Morón *et al.*, 2008). Understanding this virus' population dynamics, critical for designing appropriate epidemiological control measures for rabies, requires an understanding of the regional structure, population dynamics and genetic connectivity present in bat host populations.

In this study, we analyze sequences from the Control Region (CR, a fast-evolving mitochondrial marker) to test the hypothesis that dispersion is enhanced in a bat such

as *E. isabellinus* that flies in open areas, and that poor genetic structuring between populations is due to active gene flow across a wide area. We investigated these hypothetical genetic/spatial associations at two different geographic scales: (1) between Iberian and geographically disjunct African populations to evaluate the role of the Straits of Gibraltar as an isolating barrier; and (2) among maternity colonies at local scales to assess the degree of genetic structure and gene flow caused by female movements. Finally, we discuss the results in light of the rabies circulation pattern recently described (Vázquez-Morón *et al.*, 2008) between these bat colonies.

Materials and methods

Sampling

We sampled around 20 individuals from each of 10 *E. isabellinus* maternity colonies (populations) in Spain and two in Morocco (Figure 1). Colonies were grouped into four regions: western Andalusia (WAND), with bats from five populations in Huelva and three in Seville Provinces (between 4.7 and 105 km apart); eastern Andalusia (EAND), with bats from two colonies in Granada Province (more than 200 km east of WAND); northern Morocco (NMO), with bats from Oulad Ali Mansour (directly across the Straits from Spain); and southern Morocco (SMO), with bats from Oued Tanit, Assafied, near Agadir (several hundreds kilometers inland and at the southernmost point of this species' range).

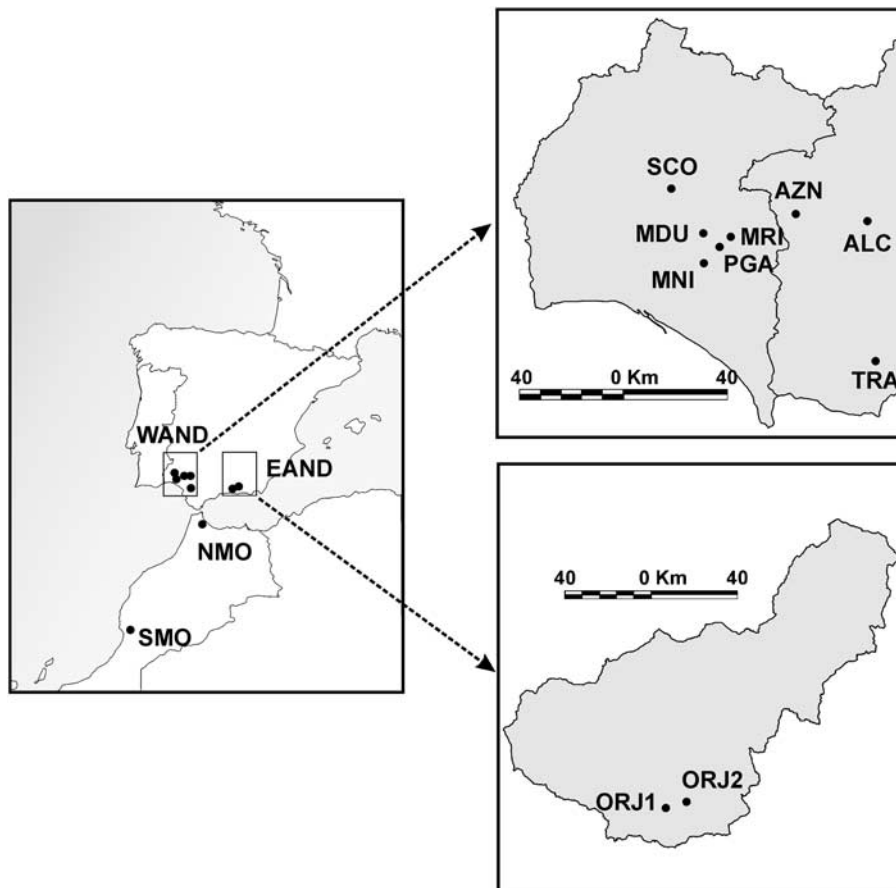


Figure 1 Geographic location of the studied colonies of *Eptesicus isabellinus*.

DNA extraction, PCR and sequencing

Total DNA was extracted from wing biopsies following the standard phenol/chloroform protocols (Sambrook *et al.*, 1989) with a few modifications. Isolated DNA was resuspended in 40 μ l of TE buffer (1 M TRIS (pH 8), 0.5 M EDTA (pH 8), H₂O Milli-Q). A fragment of the mitochondrial CR corresponding to its first hypervariable region (HVI) was amplified using primers H-15926 (5'-TGAATTGGAGGACAACCAGT-3') and CSBF-R (5'-GTTGCTGGTTTCACGGA GGTAG-3') (Wilkinson and Chapman, 1991). Polymerase chain reaction (PCR) mastermix was prepared to 50 μ l as the final reaction volume, which included 2 μ l of DNA extract, 1 μ l of each primer (10 μ M), 1 μ l of MgCl₂ (50 mM), 0.16 μ l of dNTP (25 mM) and 0.5 U of Taq polymerase (Bioline Inc., London, UK). Thermocycling consisted of a 4 min initial denaturation at 94 °C, followed by 39 cycles of 60 s at 94 °C, 90 s at 47 °C, and 120 s at 72 °C and a final extension of 10 min at 72 °C. Amplified PCR products were subjected to electrophoresis through a 0.8% agarose gel to check molecular size. All PCR products were purified and sequenced in both directions using the appropriate primers in an ABI 3100 automated sequencer (Applied Biosystems Corp., Foster City, CA, USA) following the manufacturer's protocols. Sequences were aligned and edited using Sequencher 4.5 (Gene Codes Corp., Ann Arbor, MI, USA).

Phylogeographic inferences

Phylogenetic relationships between haplotypes were first inspected using maximum parsimony (MP) and Bayesian posterior probability optimality criteria. Previously, the best-fitting substitution model was selected by the Akaike information criterion (AIC) implemented in Modeltest 3.06 (Posada and Crandall, 1998). Under MP, trees were obtained after a heuristic search with an initial tree obtained by step-wise addition (random input order) of the taxa, followed by a complete tree-bisection-reconnection branch swapping. This process was repeated 100 times. Topologies were obtained by differential weighting of transversions based on the maximum likelihood (ML) estimates of the Ts/Tv ratio value. The robustness for each topology was then assessed through bootstrapping (Felsenstein, 1985) using 2000 replicates. MP analyses were performed using the software PAUP* version 4.0b10 (Swofford, 2001). The Bayesian inference was obtained using MrBayes version 3.01b (Huelsenbeck and Ronquist, 2001) with random starting trees without constraints. Five simultaneous Markov chains were run for 2 000 000 generations and trees were sampled every 100 generations. Resulting burn-in values were determined empirically after tree likelihood scores reached stationary values. The values for model parameters were treated as unknown variables to be estimated in each analysis. Two separate analyses were run to ensure that trees converged on the same topology and similar parameters. Net genetic distances within the defined geographic regions were estimated under the selected model using MEGA 3.1 software (Kumar *et al.*, 2004). Relationships between haplotypes were also represented by a statistical parsimony network (Crandall and Templeton, 1999) obtained with the software TCS version 1.21 (Clement *et al.*, 2000).

Population structure and demographic inferences

Polymorphism was characterized for populations and regions using the following descriptors: haplotype

diversity, segregating sites, nucleotide differences and nucleotide diversity, estimated with DnaSP 4.10.9 (Rozas *et al.*, 2003) and Tajima's *D* statistic (1989) to test for neutrality of mutations within populations. Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) on the 12 colonies defined as populations and the four regions was used to explore the distribution of the genetic variability. ϕ_{st} , an F_{st} analogue for mitochondrial DNA, was used to analyze the degree of structuring between colonies. Restricted gene flow between two populations can be inferred if the genetic distance, measured as ϕ_{st} , is significantly greater than zero. The AMOVA computations and ϕ_{st} values between colonies were calculated using the program Arlequin version 2.0 (Schneider *et al.*, 2000). In a related approach, an isolation-by-distance test was used to determine if there was any association between genetic and geographic distances. This was carried out by means of a linear regression of pair-wise geographic distances and genetic distances between colonies. The logarithms of geographic distances between colonies were coupled with genetic distances standardized as $(1 - \phi_{st}) / \phi_{st}$ (Rousset, 1997) for this analysis. To improve understanding of migration patterns, we then analyzed the genetic relationships between groups under coalescent theory using the software Migrate 3 (Beerli, 2008). Markov chains Monte Carlo (MCMC) sampling designs were used to obtain ML and Bayesian between-population (BP) estimates of migration rates (in each direction). For the ML approach, the parameters *Q* (effective population size) and *M* (migration rate) scaled by the mutation rate per site were estimated simultaneously using estimates from the F_{st} matrix as starting values. These new estimates for *Q* and *M* and the empirical ts/tv value ($\kappa = 13$) were used for consecutive final runs. The final MCMC sampling strategy consisted of 10 short chains (sampling 500 000 genealogies) and three long chains (sampling 5 000 000 genealogies) with a burn-in period of 10 000 trees, and an adaptive heating regime with four parallel chains and standard initial relative temperatures of 1, 1.2, 1.5 and 6.0. For the Bayesian approach, we used uniform distributions as priors and we obtained posterior probabilities for the parameters based on 5000 samples from a probability landscape defined by 25 000 000 visited genealogies of which the first 200 000 were disregarded (burn-in). The robustness of estimates was assessed by examining convergence in different independent runs of the MCMC.

Finally, we investigated the geographic structure of genetic variation by means of a nested clade phylogeographic analysis (NCPA), which examines geographic association between haplotypes and suggests possible causal processes. This is considered a powerful phylogeographic tool despite recent controversy about its performance (Panchal and Beaumont, 2007; Petit, 2008; but see Templeton, 2008). NCPA examines geographic structuring of haplotypes, with a null hypothesis of a random association between geographic and genetic information (Templeton, 1998). If nonrandom associations are found, NCPA helps indicate whether contemporary (restricted gene flow) or historical forces (past population fragmentation, range expansions or long-distance colonization) best explain the association. We used this approach as corroborating evidence (Garrick *et al.*, 2008) and to reevaluate the association between

distance and genetic flow, a Mantel test (1000 permutations) was used, as implemented in the program GenALEX version 6.3 (Peakall and Smouse, 2005). A nested design was built from the statistical parsimony network following Templeton and Sing (1993) and GeoDIS version 2.0 (Posada *et al.*, 2000) was used to test the associations between clades and geographic distances. The most recent inference key was used to interpret results (Templeton, 2004).

Results

Sequence analysis and variation

For 254 individuals of *E. isabellinus*, PCR products resulted in the amplification of the left domain of the CR, a fragment of variable length ranging from 600 to 900 bp. As for other mammals, this domain consists of an initial fragment followed by a motif repeated several times that varies between bat species (Wilkinson *et al.*, 1997) and populations of a given species (Wilkinson and Chapman, 1991). *E. isabellinus* samples typically showed a motif of 82 bp repeated from two to five times. Fifteen individuals from six populations showing uncertain sequence readings were disregarded and the remaining unambiguous 239 sequences were trimmed to a homologous fragment of 240 bp that included only the initial fraction of the HVI region. The repeats were disregarded because they showed an unclear pattern of evolution (Wilkinson and Chapman, 1991; Petit *et al.*, 1999). The selected fragment had 21 polymorphic sites, of which 17 were parsimony informative (GenBank accession numbers EU 183062–183081). Some haplotypes were quite abundant and widespread (for example, Eisa02 and Eisa03), although most were exclusive to just one or two populations. Two haplotypes (Eisa03 and Eisa07) were found on both sides of the Straits of Gibraltar and two colonies were fixed for the same Eisa02 haplotype.

Phylogeographic inferences

A TrN evolution model (Tamura and Nei, 1993) with an estimated proportion of invariable sites (I) = 0.613 and a uniform rate between sites was selected by the AIC as the best fit for our data. These parameters were used to set the priors in the posterior probabilities sampling for the Bayesian approach. The Bayesian and the MP phylogenetic reconstructions indicated an almost identical and quite poor geographic arrangement of haplotypes (Figure 2). Both approaches provided good evidence for a well-supported monophyletic group of unique haplotypes from southern Morocco (SMO) and a second group that included all northern Moroccan (NMO) and Iberian haplotypes (WAND and EAND). The genetic distance between NMO and WAND was only slightly larger (0.005) than the distance between the two Iberian regions (0.004). In contrast, the distance between the two African regions was three times larger (0.017). Relationships between haplotypes as revealed by the parsimony-based network were better defined (Figure 3). In this network, southern Moroccan (SMO) haplotypes were still highly differentiated from the rest, although eastern Andalusian (EAND) haplotypes clustered slightly apart from those from both western Andalusia (WAND) and northern Morocco (NMO). These last two regions were closely related and shared two haplotypes.

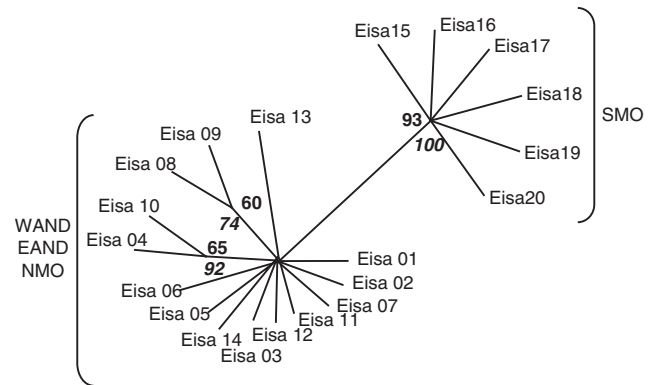


Figure 2 Phylogenetic relationships among haplotypes of *E. isabellinus* based on maximum parsimony (MP) and Bayesian approach (BP) using a Tamura–Nei sequence evolution model. Bootstrap values of maximum likelihood (ML) after 2000 replicates (above branches) and Bayesian posterior probabilities after sampling 20 000 trees (below).

Population structure and demographic inferences

Genetic structure and diversity values (Table 1) varied greatly between populations and even between populations located only a few kilometers apart (for example, 7.9 km between Puente Gadea and Molino del Duende). Tajima’s D statistics were not significant for any colony, suggesting that neither population subdivision, selection nor demographic fluctuations were causing any departure from neutral expectations.

Of the regions, SMO showed the highest values for almost all within-population genetic parameters, even though it had the smallest sample size (Table 1). Genetic haplotype diversity was highest in the southernmost population (SMO) (Table 1), despite the fact that it did not share haplotypes with any other population. The AMOVA results indicated shallow geographic structuring. In fact, most of the variation (44.93%) was explained by the ‘within-population’ factor, whereas ‘between-region’ variation accounted for only 14% of the total. Nevertheless, overall fixation indices were still significant for regions. Computation of the pair-wise ϕ_{st} values between populations showed that all but one of the comparisons were significantly higher than zero (Table 2), suggesting a hypothesis of restricted female-mediated gene flow between colonies. Migration rates between the four regions, as estimated by $4Nm$, also supported the idea of restricted gene flow, with values significantly smaller than one. The Mantel test did not show any association between genetic and geographic distances ($P = 0.6$). All estimates using both ML and BP methods of the migration parameter M indicated a consistently asymmetric between-region pattern of migration. All runs showed relatively low migration values within regions (low values driven to near 0 by the ML analysis). A number of low connection values were seen between the Andalusian regions (EAND and WAND) and between northern (NM) and southern Morocco (SM). Nevertheless, somewhat surprisingly relatively high migration rates were found to occur from Andalusia to northern Morocco, particularly from WAND (Table 3). The theoretical estimates consistently showed a pattern of gene flow from Iberia to North Africa across the Straits of Gibraltar.

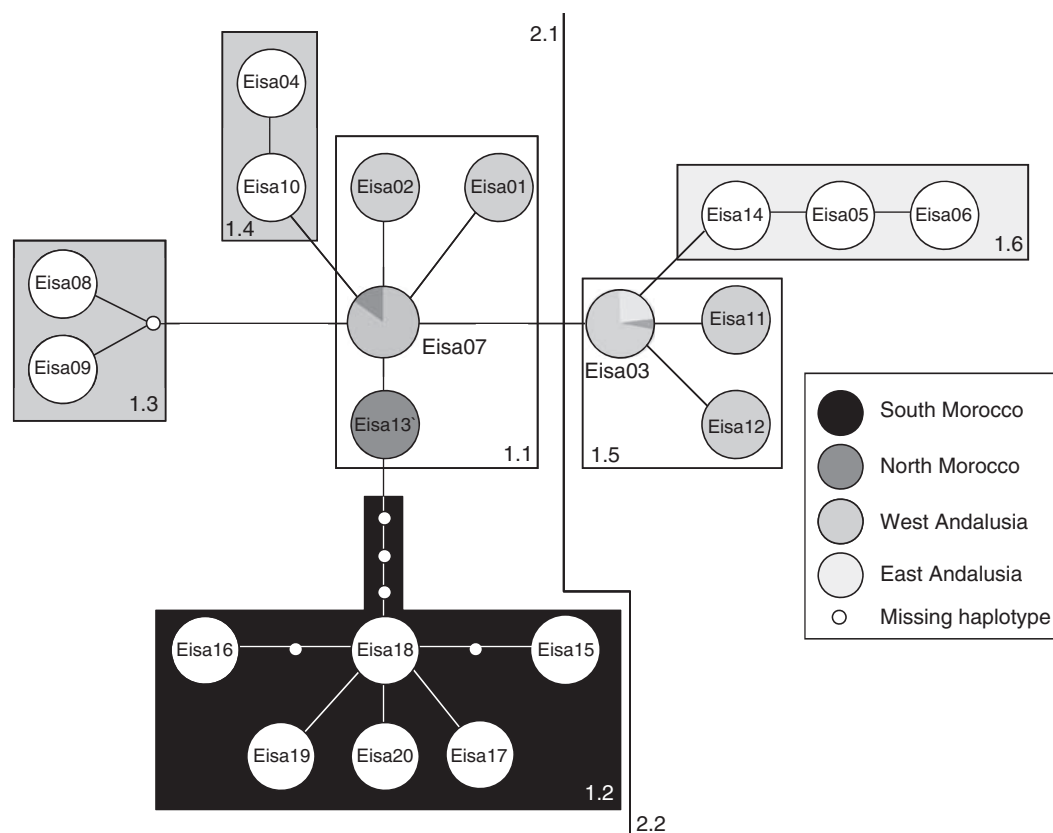


Figure 3 Nested clad design from the statistical parsimony network using 239 sequences of a 240 bp fragment of the hypervariable region (HVI) (Control Region, CR) of *E. isabellinus*. Circles are not proportional to the sampling size.

Table 1 Descriptive statistics of genetic diversity in a 240 bp long fragment of the mtDNA CR from 12 colonies of the meridional serotine bat *E. isabellinus*

Colony	Haplotype	Haplotype diversity	Segregating sites	Nucleotide differences	Nucleotide diversity	Neutrality test
	<i>N</i>	Hd	S	K	Pi	D
ORJ1 (21)	3	0.667	3	1.286	0.00536	1.910
ORJ2 (17)	3	0.581	2	1.044	0.00437	1.423
EAND (38)	4	0.696	3	1.6381	0.00687	—
TRA (22)	5	0.744	5	1.541	0.00646	0.367
ALC (19)	4	0.637	5	1.789	0.00746	0.780
AZN (19)	2	0.199	2	0.398	0.00166	0.780
MDU (19)	1	0	0	0	0	—
MRI (20)	2	0.479	1	0.478	0.002	1.262
MNI (17)	3	0.522	3	0.676	0.00282	-0.673
PGA (22)	3	0.506	4	1.259	0.00525	0.416
SCO (23)	1	0	0	0	0	—
WAND (161)	10	0.761	10	1.83075	0.00763	—
NMO (25)	3	0.157	2	0.233	0.00097	-1.214
SMO (15)	6	0.819	7	1.638	0.00683	0.780
Total (239)	20	0.852	21	2.567	0.0107	—

Colony names as in text followed by sample sizes in brackets.
Mean population values and totals are in bold.

The nested cladograms constructed for NCPA showed six first-level and two second-level nested clades (Figure 3). The clade containing the SMO samples (clade 1.2) grouped separately from all other groups, as in the phylogenetic analyses. There were significant associations between haplotypes and their geographic distributions in six clades, which included first-level clades 1.1, 1.5 and 1.6, second-level clades 2.1 and 2.2,

and the total cladogram (Figure 4). Inferences obtained from NCPA regarding the mechanism for the origin of nested clades were variable. For clades 1.1, 2.1 and 2.2, there was more than one explanation; for clades 1.5 and 1.6, a pattern of isolation-by-distance was supported. Finally, at the whole cladogram level, the analysis pointed toward a scenario of continuous range expansion (Figure 4).

Table 2 Pair-wise ϕ_{st} values (lower diagonal) and Nm values (upper diagonal) between colonies of *E. isabellinus*

	ORJ1	ORJ2	TRA	ALC	AZN	MDU	MRI	MNI	PGA	SCO	NMO	SMO
ORJ1	-	1.187	0.600	0.505	0.316	0.132	0.418	0.600	0.394	0.119	0.167	0.704
ORJ2	0.174	-	0.501	0.456	0.686	0.096	0.460	1.303	0.373	0.086	0.133	0.575
TRA	0.294	0.333	-	0.578	0.236	0.173	0.399	0.446	0.456	0.156	0.200	0.886
ALC	0.331	0.354	0.302	-	0.222	0.152	0.342	0.426	0.637	0.136	0.151	0.656
AZN	0.442	0.267	0.514	0.530	-	0.031	0.246	1.588	0.222	0.028	0.056	0.237
MDU	0.655	0.722	0.591	0.622	0.889	-	0.081	0.090	1.080	---	0.024	0.150
MRI	0.374	0.352	0.385	0.422	0.504	0.755	-	0.456	0.279	0.073	0.112	0.441
MNI	0.294	0.161	0.359	0.370	0.136	0.735	0.354	-	0.391	0.080	0.119	0.499
PGA	0.388	0.401	0.354	0.282	0.530	0.188	0.473	0.390	-	0.946	0.121	0.464
SCO	0.678	0.745	0.616	0.647	0.899	0.000	0.774	0.757	0.209	-	0.022	0.133
NMO	0.599	0.653	0.555	0.624	0.818	0.911	0.691	0.678	0.674	0.919	-	0.192
SMO	0.262	0.303	0.220	0.276	0.513	0.625	0.362	0.334	0.350	0.653	0.565	-

All values are significant ($P < 0.05$) except for the one depicted in bold type. In lighter gray are the Andalusian (EAND, WAND) colonies and in darker gray the Moroccan colonies (NM, SM).

Discussion

Phylogeographic inference and the role of the Straits of Gibraltar in *E. isabellinus*

Our analyses support the differentiation of the southern Morocco population from the other populations, but only show shallow genetic divergence between the populations in Andalusia and northern Morocco. Potentially, bats can colonize large areas with ease by dispersing from isolated refugia and overcoming geographic barriers. Our results indicate that the Straits of Gibraltar have not impeded movements or occasional dispersion events and therefore cannot be considered to be a factor in the genetic differentiation of *E. isabellinus* populations. On the other hand, the lack of shared haplotypes between the northern and southern Moroccan populations was a particularly unexpected result and indicates that the Middle and High Atlas mountains, as well as the highlands in between, represent an effective geographic barrier to this species. The Atlas mountains play a similar isolating role in other organisms such as lizards (Brown *et al.*, 2002; Harris *et al.*, 2002) and freshwater turtles (Fritz *et al.*, 2005). Alternatively, the differences between southern and northern Moroccan populations may indicate that these colonies were founded by individuals coming from different refugia. To fully understand the historical relationships of the marginal southern Moroccan population, more sampling of North African populations in countries such as Algeria, Tunisia and Libya is needed.

Although only a few kilometers wide (14 km), the Straits of Gibraltar seem to have acted as an important

Table 3 Migration rate (M) between regions

	WAND	EAND	NMO	SMO
WAND	—	248.78	0	0
EAND	0	—	0	0
NMO	1844.3	0	—	0
SMO	1531.5	979.5	—	703.5
	0	0	39.6	—
	79.5	26.35	109.5	—

Values are scaled by mutation rate per site. The table is to be read the following way: for a given population (rows) migration estimates are shown from the regions indicated in each column. Upper values are migration estimates from maximum likelihood and lower values are estimates from Bayesian posterior probabilities.

barrier preventing faunal contacts ever since their reopening at the start of the Pliocene about 5.3 million years ago (Dobson and Wright, 2000). Nevertheless, a

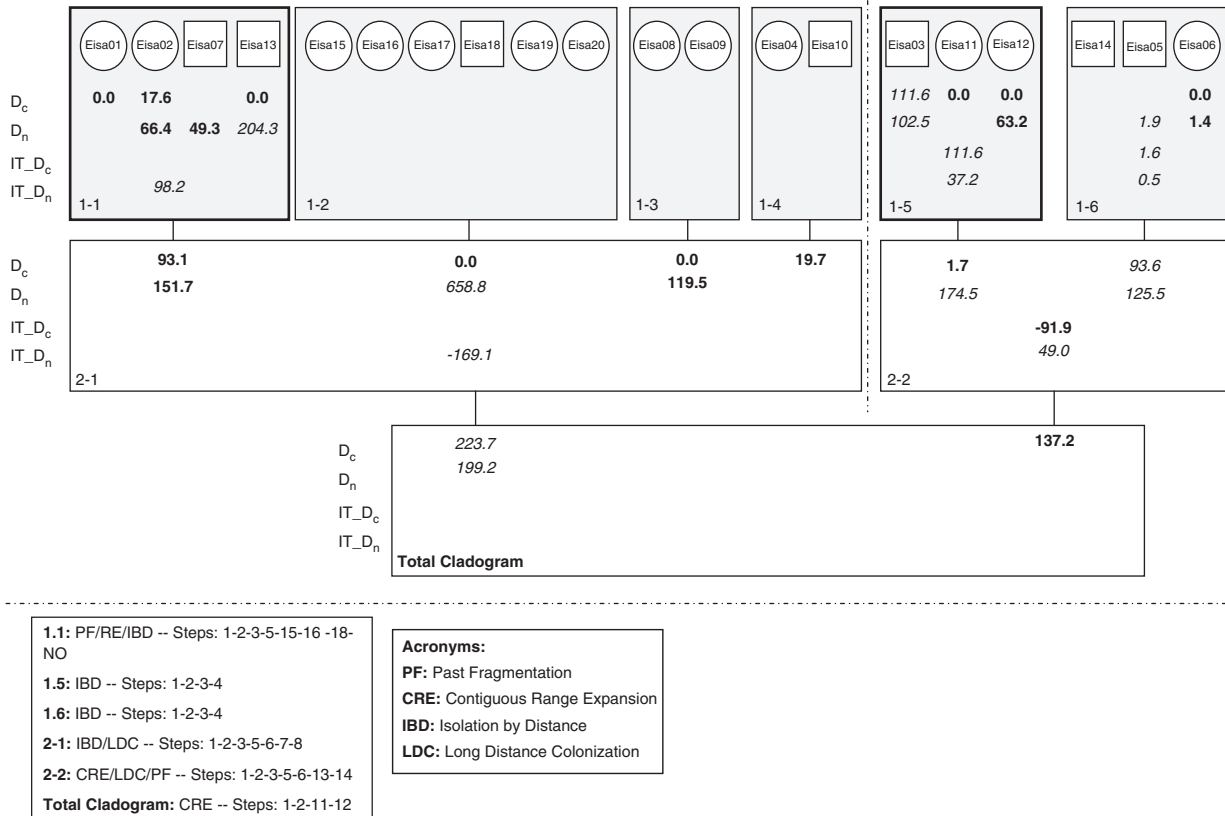


Figure 4 Nested clade phylogeographic analysis (NCPA) statistics and inferences: italicized values are statistically larger, and the values in bold are statistically smaller than expected by chance.

lack of differentiation between one side of the Straits of Gibraltar and the other has been found in birds (Guillaumet *et al.*, 2006), shrews (Brändli *et al.*, 2005), salamanders (Veith *et al.*, 2004) and probably even in worm lizards (Vaconcelos *et al.*, 2006). However, for the bats *Myotis myotis* (Castella *et al.*, 2000), *Myotis nattereri* (García-Mударра *et al.*, 2009), *Plecotus austriacus* (Juste *et al.*, 2004) and *Pipistrellus pipistrellus* (Hulva *et al.*, 2004), the Straits of Gibraltar seem to be an effective barrier and to have helped in promoting or maintaining lineages that are genetically highly differentiated.

The lack of evidence for any effect caused by the Straits of Gibraltar or for isolation-by-distance in *E. isabellinus* could be related to its preference for flying in open areas, although a similar lack of structuring for the Straits of Gibraltar has also been found in *Barbastella barbastellus* (Juste *et al.*, 2003), a bat with theoretically less powerful flight. The degree of permeability of the Straits of Gibraltar to bats may be the result of a complex equation in which life history traits are also important. A recent comparative approach including all bat species present on both Iberian and African sides of the Mediterranean has shown the absence of a clear pattern regarding the role of the Straits of Gibraltar on bat communities (García-Mударра *et al.*, 2009), and caution should be taken when drawing general conclusions (Dobson and Wright, 2000).

Coalescence time between the exclusively North African haplotype (Eisa13) and the closest Iberian haplotype (Eisa07) can be inferred using the age in mutations ($\rho = 0.74$) and the mutation rate estimated for

this same mtDNA fragment for a related bat species (6.3–25% per million years; Petit *et al.*, 1999). The estimated divergence time for haplotypes on both sides of the Straits of Gibraltar is between 46 and 12 thousand years ago. According to the network, the distribution of the potentially most ancestral haplotypes (Eisa03 and Eisa07) was restricted almost exclusively to Spanish populations, which suggests that the refugium for this species was located in southern Iberia and that it was from here that it subsequently expanded into North Africa during favorable conditions along any of the Pleistocene climatic pulses. The theoretical migration estimates also suggest a pattern of dispersion from western Andalusia into North Africa. A similar north–south dispersion pattern has been suggested for lizards (Carranza *et al.*, 2006) and freshwater turtles (Fritz *et al.*, 2006). Southwest Iberia seems to have been an important refugium for a variety of species, from cork oaks (López de Heredia *et al.*, 2007), to fish (Carmona *et al.*, 2000) and aquatic invertebrates (Gómez *et al.*, 2000; Muñoz *et al.*, 2008). Despite the need for additional data, our results support the idea that the Iberian Peninsula and, in particular, southwestern Iberia acted as a refugium and genetic source region for both European (Taberlet *et al.*, 1998; Hewitt, 1999), and North-African biotas.

Genetic structure and gene flow among populations

On a smaller geographic scale, the populations studied show remarkable variation in their genetic diversity. The relatively young population at Trajano (TRA, WAND)

occupying the expansion joints of a road bridge built 30 years ago has one of the highest nucleotide diversities and the highest haplotype diversity of all Spanish populations. The creation of new shelter sites in a highly productive area such as the Guadalquivir marshes may have attracted females from nearby populations and have led to this high diversity. The remaining colonies show striking differences in their degree of genetic structure and variability and all ϕ_{st} pair-wise comparisons other than the monomorphic populations (MDU and SCO) were significant. These results indicate that at local geographic scale high levels of differentiation exist, thus suggesting that no female-mediated contacts between colonies exist. *E. isabellinus* maternity colonies typically only last from spring to late summer and by autumn bats have moved to other shelter sites, probably only few kilometers apart. It is expected that the colonies on the African side of the Straits will show similarly high levels of structure. However, difficulties in conducting fieldwork have thus far prevented us from assessing this question.

Molecular results agree with the strong philopatric pattern found in the field, in which females return every year to their birth colonies. No case of colony switching has ever been detected in the 10 years we have been banding in these colonies (1709 bats banded and 400 recaptured, Ibáñez *et al.*, unpublished data). Little is known about the process governing the establishment of new colonies, although they are probably founded by a small number of females. With no alleles being added to the population through the recruitment of females from other colonies, random genetic drift would cause the differences we found between colonies and even the fixation of some haplotypes within a few generations. A similar pattern of highly structured colonies has been found in the forest-dwelling bat, *Myotis bechsteinii* (Kerth *et al.*, 2000, 2002), although its colonies do not have large differences in genetic diversity, indicating that different mechanisms are at work here in colony formation. In this type of social structure usually only males disperse and they are therefore solely responsible for the gene flow between established colonies. mtDNA only gives partial information and further studies based on nuclear markers such as microsatellites are needed to detect the real gene flow between populations.

Geographic scale and genetic signature

Although it would seem that *E. isabellinus* is capable of dispersing throughout the entire study area, a process that would lead to a rapid coalescence of colonies, the amount of genetic differentiation observed within regions is markedly greater than between regions. These results, which seem counterintuitive and to contradict the species' ability to cross the Straits of Gibraltar, can only be explained by females' high philopatry. Contacts between different geographic regions take place through occasional dispersion events, most probably by males that homogenize the genetic diversity between populations and maintain the cohesion of species' genetic pool at a regional level. Geographic features—very often the same ones—tailor both historical and ecological processes, making it necessary to work at different scales to understand fully and be able to interpret the patterns occurring in the present. Our results show the impor-

tance of the spatial scale in distinguishing historical and ecological processes acting on observed genetic diversity.

Eptesicus genetic structure and rabies epidemiology

The colonies of *E. isabellinus* that were genetically studied between 1998 and 2003 for this paper were also banded and checked for rabies. Reverse transcriptase-PCR and serology testing found EBLV-1 rabies circulation to be present in nine of the colonies, all in the Iberian Peninsula. Each colony had a different temporal pattern of viral dynamics, suggesting that independent endemic viral circulation occurs in each colony (Vázquez-Morón *et al.*, 2008). Our findings of restricted gene flow between colonies of *E. isabellinus* based on the genetic analysis of its mtDNA agree with viral epidemiological results and also help explain them. With maternity colonies behaving as closed communities, a pattern of epidemiological circulation by fast-spreading viral waves—as is typical in other viruses—is unlikely to occur. In fact, given that *E. isabellinus* bats can survive after infection (Pérez-Jordá *et al.*, 1995; Echevarría *et al.*, 2001), vertical transmission within bat colonies (mother-offspring) would be the most likely way for the virus to spread, with only occasional transmission between colonies caused by individual interactions between females and males (for example, mating) at the end of the reproductive season and when maternity colonies disaggregate. Although EBLV-1 has not yet been found in Africa (only around 50 *E. isabellinus* tested to date), we predict that *Eptesicus* from Northern Morocco will also carry the rabies virus and that its strains will be very similar to the EBLV-1 rabies strains found in Andalusia.

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