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Population Structure of a Cave-Dwelling Bat, *Miniopterus schreibersii*: Does It Reflect History and Social Organization?

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Abstract

Many colonial bat species make regional migrations, and the consequent gene flow may eliminate geographic genetic structure resulting from history of colonization. In this study, we verified that history and social organization have detectable impacts on the genetic structure of *Miniopterus schreibersii*, a cave-dwelling bat with high female philopatry. After studying all known nursing colonies in Portugal, we concluded that there is a significant geographic structure and that the overall pattern is similar for mitochondrial and nuclear DNA. Both pairwise Φ_{ST} and F_{ST} were significantly correlated with geographical distance, suggesting that isolation by distance is relevant for both mitochondrial and nuclear markers. However, structuring of mitochondrial DNA was much more marked than that of nuclear DNA, a consequence of the strong female philopatry and a bias for male-mediated gene flow. Wintering colonies were more genetically diverse than nursing colonies because the former receive individuals from distinct breeding populations. Haplotype diversity of the northern colonies, the more recent according to population expansion analyses, is only about half of that of the central and southern colonies. This is most likely a consequence of the colonization history of *M. schreibersii*, which presumably expanded northward from the south of the Iberian Peninsula or North Africa after the last glacial age.

Key words: *male-mediated gene flow, Mediterranean, migratory, Miniopterus, philopatry*

Migratory species usually show low levels of regional genetic differentiation, as a consequence of high gene flow across their geographic ranges (Hartl 2000). Schreibers' bat *Miniopterus schreibersii* (Kuhl, 1817) is a migratory bat species that almost invariably inhabits underground roosts, predominantly caves, and abandoned mine galleries. *Miniopterus schreibersii* makes frequent migratory movements of several hundred kilometers, which potentially span a territory of the size of Portugal (Rodrigues and Palmeirim 2008). Under these circumstances, gene flow can hide any geographic genetic structure caused by historical events or restrain the establishment of any structuring due to social organization.

Like many other cave-dwelling bats, *M. schreibersii* is a social species that forms large colonies throughout most of the year, which in Portugal can include up to 20 000 individuals (Palmeirim and Rodrigues 1995). Males and females migrate extensively across hundreds of kilometers, and during the mating season, animals from different nursing populations share roosts, so intercolony fertilizations are to be expected. However, females almost always return to the colony where they were born to give birth, whereas males are somewhat less attached to their birth sites (Palmeirim and Rodrigues 1995). This social organization probably affects the genetic structure of the population of *M. schreibersii* as happens with other bat species. Strong female philopatry may result in a marked structure at the maternally transmitted mitochondrial DNA (mtDNA), whereas biparentally inherited nuclear markers may not evince strong structuring due to generalized male-mediated gene flow (Petit and Mayer 1999; Worthington Wilmer et al. 1999; Castella et al. 2001; Petit et al. 2001). Moreover, hibernation colonies often congregate individuals from different nurseries (Serra-Cobo et al. 1998; Rodrigues and Palmeirim 2008). Consequently, if social organization is relevant for genetic

structuring of *M. schreibersii* populations, nurseries and hibernacula may present different genetic compositions.

Miniopterus schreibersii was once considered the mammal species with the widest natural distribution, ranging from southern Eurasia to Africa, Australia, and the Solomon Islands (Nowak 1994; Rodrigues 1999). However, recent phylogenetic studies have split the species and suggest that *M. schreibersii* is probably restricted to Europe (from western Turkey) and North Africa (Appleton et al. 2004; Tian et al. 2004; Miller-Butterworth et al. 2005; Bilgin et al. 2006).

In Europe, this species shows Mediterranean climatic preferences, occurring mostly in the south of the continent, including all the Iberian Peninsula. However, during the glacial periods of the Pleistocene, northern Iberia was certainly too cold for this species, which may have survived in the south of the Peninsula, as suggested by the fossil record of the Würm (Telles Antunes 1993). The range of *M. schreibersii* certainly expanded northward after the last glacial period. Such range expansions are often important in shaping the genetic structure of populations, especially in species with low mobility (Hewitt 1996), but their impact in highly mobile species, like *M. schreibersii*, is less predictable.

We have studied the colonies of *M. schreibersii* in Portugal for more than 2 decades, so we have located virtually all of them. Therefore, in this study, we were able to obtain a particularly complete picture of the geographic patterns in the genetic structure of this species in a relatively large territory, using both mtDNA control region sequences and biparentally inherited microsatellite markers.

Our aim is to determine if social organization and historical events have detectable impacts on the genetic structuring of the populations of cave-dwelling bats at countrywide scales, using *M. schreibersii* in Portugal as a model. We studied the influence of social organization on the genetic structure of the populations by determining if 1) separate colonies are genetically distinct, 2) the stronger philopatry of the females results in a more marked structure at the mtDNA than at the nuclear DNA (nDNA), and 3) hibernation colonies are genetically more diverse than nurseries. In addition, we determined if genetic structure reflects the history of expansion of *M. schreibersii* in the Iberian Peninsula.

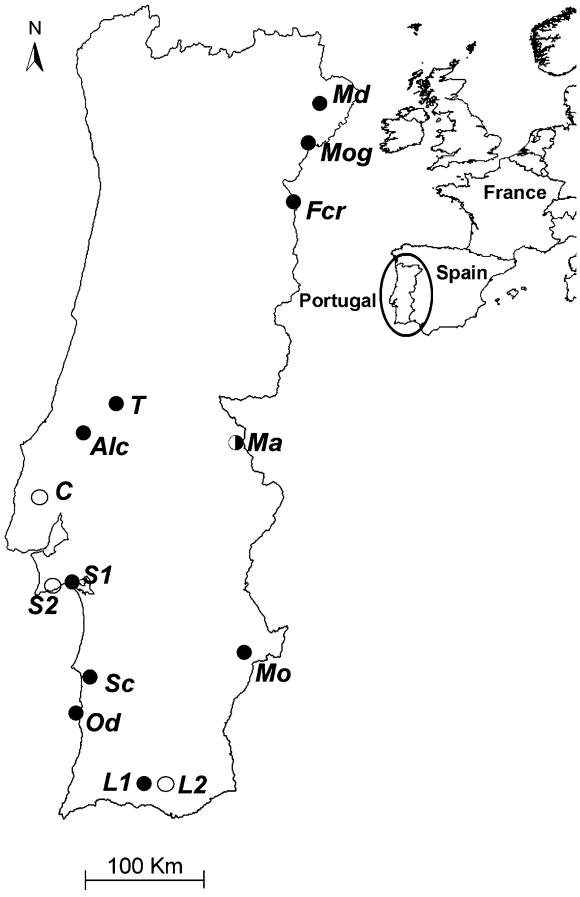

Figure 1. General location of sampling sites of tissues from adult bats. Filled circles correspond to nurseries (Md, Miranda do Douro I; Mog, Mogadouro II; Fcr, Figueira de Castelo Rodrigo I; T, Tomar I; Alc, Alcanena I; S1, Sesimbra I; Mo, Moura I; Sc, Santiago do Cacém; Od, Odemira I; and L1, Loulé I), and open circles correspond to hibernacula (C, Cadaval; S2, Sesimbra II; L2, Loulé II); Marvão I (Ma) is occupied all year being both a nursing and a hibernation roost. Roost codes according to Palmeirim and Rodrigues (1992).

Materials and Methods

Sampling

We obtained tissue samples from 407 adult females at 11 of the 12 nursing colonies of *M. schreibersii* known in Portugal (Figure 1), during the late nursing season (15–25 July), when all the juveniles are already flying. Nursing colonies consist mainly of females and their young, which sometimes form separate clusters. Bats were caught by hand inside the daytime roosts or with harp traps during the emergence. Most samples were collected in 2004, but a few obtained in 1998 were also used. The sample sizes of 1998 were too small to allow meaningful comparisons with those of 2004, but the samples of the 2 years were pooled because it is very Luísa Rodrigues and Jorge M. Palmeirim, unlikely that any significant changes took place between those years. This is so because *M. schreibersii* can live for more than 19 years (Avril 1997) and the generation time is about 5 years (i.e., the average age of reproducing females; Luísa Rodrigues and Jorge M. Palmeirim, unpublished data). In addition, the colonies are large and their numbers, which were monitored yearly, remained stable throughout the sampling period.

During the winter of 2004, we also sampled 96 adult individuals (45 males and 51 females) at 4 of the 15 known hibernation colonies, which harbor both males and females of all age groups. Differences in sample sizes are mainly due to the difficulty of capturing bats in some of the studied colonies.

Age was determined by the ossification of the carpal joints, development of nipples and testis (e.g., Dwyer 1963a; Baagøe 1977), and the patterns of molt (Dwyer 1963b). Tissue samples were obtained from a nonlethal sterile biopsy punch of the wing membrane, as is usually done to

get tissue samples from bats (Worthington Wilmer and Barratt 1996), and preserved in 100% ethanol.

DNA Extraction and Amplification

Genomic DNA was extracted from wing punches following a salt–chloroform procedure modified from Miller et al. (1988) by adding one step of isoamyl alcohol (24/1) extraction to the original protocol. The precipitated DNA was resuspended in 100 µl of sterile water.

Mitochondrial "D-loop" was amplified in 25 µl polymerase chain reaction (PCR) volumes, as described by Miller-Butterworth et al. (2003), using the primers C and E (Wilkinson and Chapman 1991). Products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA). For mtDNA population differentiation, 518 base pairs of the control region were sequenced with the same primer E (Wilkinson and Chapman 1991) in a subsample of 312 individuals ($n_{\text{nurseries}}$ = 230 adult females; $n_{\text{hibernacula}}$ = 82 males and females).

We amplified the microsatellite loci in 10 µl PCR volumes, using the 5 primer pairs *Mschreib1–5*, described by Miller-Butterworth et al. (2002). We also amplified the locus *ncam* (Moore et al. 1998), but it revealed to be monomorphic. The PCR conditions were optimized in order to use the Multiplex PCR Kit (Qiagen), which facilitates the simultaneous amplification of several loci. The multiplex PCR profile consists of an initial denaturation and hot DNA polymerase activation at 95 °C for 15 min, followed by 30 cycles of the series: 95 °C for 30 s, annealing temperature (55 °C for *ms1* and *ms3*, 58 °C for *ms2* and *ms4*, and 53 °C for *MS5*) for 90 s, 72 °C for 60 s; a final amplification step of 10–20 min at 72 °C was performed. After detecting the polymorphic loci, primers were labeled with Beckman dyes and PCR products were run in a CEQ 2000XL-Beckman Coulter equipment. Allele calling was performed using a CEQ 8000 Genetic Analysis System.

Genetic Diversity within Colonies

mtDNA sequences were aligned and edited with SEQUENCHER 3.0 (Gene Codes Corp.). Haplotypes were connected on a network obtained using the 95% parsimony criterion implemented in Network 4.0.01 (Röhl 2003). Mitochondrial diversity was represented by haplotype (h) and nucleotide (π) diversities calculated in Arlequin 2.0 (Schneider et al. 2000). According to the classical models of range expansion, a postglacial expansion from southern refugia may result in a northward decrease in genetic polymorphism (Hewitt 1996, 2000). We investigated the existence of such a geographic trend in the levels of gene diversity of nurseries applying a bootstrap analysis to the haplotypes (P value was calculated by counting the number of times when the number of haplotypes of the northern colonies [Md, Mog, and Fcr; Figure 1] was inferior to the average and dividing by 10 000, the number of resampling procedures).

For the nuclear markers, we first tested the existence of deviations of observed versus expected frequencies of allele size differences among and within genotypes, to identify errors due to stuttering, large allele dropout, or null alleles with MICRO-CHECKER 2.2 (van Oosterhout et al. 2004). To investigate intracolonial genetic variability in the microsatellite data, we calculated allelic richness (A), allele frequencies, expected heterozygosity (H_E), and tested for Hardy–Weinberg equilibrium (HWE) using the Markov chain method. These calculations were done using the Excel Microsatellite Toolkit (Park 2001) and Arlequin 2.0 (Schneider et al. 2000). As for the mitochondrial markers, we tested the existence of a geographic trend in the levels of nuclear diversity applying a bootstrap analysis to the alleles. All calculations were corrected for sample size differences.

To test the hypothesis that hibernation colonies are genetically more diverse than nurseries, we compared mitochondrial genetic diversity (h and π) between hibernacula and nurseries using bootstrap. To control any bias caused by the fact that no males were sampled in nurseries, we performed 2 analyses: one with females only and one pooling males and females.

Differentiation among Nursing Colonies

The genetic differentiation among colonies was measured with pairwise F_{ST} (based on the infinite allele model) and R_{ST} (based on the stepwise mutation model) using ARLEQUIN. A permutation test available in the software Spagedi v. 1.2g (Hardy and Vekemans 2002) was carried out to check the influence of stepwise-like mutations versus drift on the genetic differentiation. Allele size at each locus was randomly permuted among allelic states (2000 permutations) simulating R_{ST} values (pR_{ST}) and 95% confidence intervals (CIs), under the null hypothesis that population differentiation is not affected by differences in allele sizes (Hardy et al. 2003).

When comparing differentiation of loci with different levels of variations, such as microsatellites and mtDNA data, the standardized measure of genetic differentiation G'_{ST} (Hedrick 2005) is quite useful. With this measure, the magnitude is the proportion of the maximum differentiation possible for the level of population homozygosity observed, a value that can be used universally with different loci. G'_{ST} was calculated by dividing the observed F_{ST} value by its theoretical maximum, F_{max}, using the program RECODEDATA v. 0.1 (Meirmans 2006).

To define geographically homogenous groups of populations, we performed a spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) with mitochondrial and nuclear markers, using the program SAMOVA 1.0. SAMOVA determines the partitioning of populations that maximizes the Φct value when a certain number of groups are specified (Dupanloup et al. 2002). It was used to identify the most likely number of groups within the data set from repeated analyses, specifying 2–11 groups, and to choose the partitioning of populations that maximizes the Φct value. We also analyzed the geographical association between individual multilocus genotypes with a factorial correspondence analysis using Genetix (Belkhir et al. 1996).

Sex-Biased Gene Flow

We investigated sex-biased gene flow by testing if F_{IS}, F_{ST}, mean assignment index (AI), and AI variance differed

significantly between the 2 sexes (Goudet et al. 2002). The sex responsible for most of the gene flow is expected to have positive F_{IS} (demonstrating a positive fit to Hardy–Weinberg expectations), lower F_{ST} (as the allelic frequencies for individuals of the dispersing sex should be more similar), lower mean AI (indicating the presence of genotypes less likely than average), and larger variance of AI (demonstrating the mixture of common genotypes with rarer ones). All calculations were conducted in FSTAT 2.9.3.2 (Goudet 1995).

Population Expansion

The existence of more than 1 haplotype within the same colony may result from a founder event by multiple lineages, mutations gathered over time, or several colonization events. Sudden expansions possibly result in a unimodal distribution of pairwise differences (Slatkin and Hudson 1991; Rogers and Harpending 1992). In contrast, if present populations resulted from several colonization events, then a multimodal mismatch distribution is expected. We explored these contrasting scenarios with mismatch analysis implemented in ARLEQUIN (Harpending 1994). The overall validity of the estimated demographic model was tested through the Harpending (1994) raggedness index and by comparing the distribution of the sum of squared differences (SSD) between the observed and the expected mismatch distribution (Schneider et al. 2000). Time since expansion (t) was calculated from the demographic expansion parameter estimates (Rogers and Harpending 1992) with a calibrated rate of divergence of about 20% per million years (Petit et al. 1999).

Additionally, tests of nonneutral evolution were performed for mtDNA: R^2 (Ramos-Onsins and Rozas 2002) and Fu's Fs (Fu 1997) were computed using DnaSP (Rozas et al. 2008). The first statistic is based on the distribution of mutation frequencies, whereas the latter is based on haplotype distribution. Both have low values, where low-frequency mutations are in excess, as expected in the sequence of a selective sweep or a population expansion. Statistical significance was evaluated by coalescent simulations (10 000 replicates) in DnaSP.

Results

Mitochondrial and Nuclear Polymorphism

A stretch of 518 bp from the mtDNA control region was sequenced in 312 individuals (GenBank accession numbers: GQ149027–GQ149065). The alignment of the sequences resulted in 24 variable sites defining 39 distinct haplotypes (Table 1). Of the observed substitutions, 19 were transitions, 4 were transversions, and 2 single base pair deletions or insertions. Genotyping the total of 503 bats at the 5 microsatellite loci scored 56 distinct alleles. No null alleles or large allele dropout were identified. No pair of loci was significantly associated at the colony level after Bonferroni corrections (Rice 1989), and no departures from HWE (Guo and Thompson 1992) were detected, except for locus *Mschreib 4* in Alcanena and Marvão populations. Independent segregation of the alleles at the 5 loci was therefore assumed in subsequent analyses. Allele frequencies of the 5 microsatellite loci are presented as Supplementary Material.

The parsimony network of haplotypes showed a star-like shape (Figure 2). The central haplotype was the most abundant and was found in all colonies (both nursing and hibernation). A second haplotype was shared by several nursing and hibernation colonies but none located in the North-East (NE; Md, Fcr, and Mog). The remaining haplotypes were specific to one or few colonies within the same geographical region. Some of the haplotypes at the tips of the branches of the net had less parsimonious alternative links not shown in Figure 2. We indicate the links connecting haplotypes that belong to the same colony or group of colonies (Crandall and Templeton 1993).

The cave at Marvão harbors both a nursing and a hibernation colony. Haplotypes found there in winter were again found during nursing and were shared with several nursing colonies from central and southern Portugal. Sesimbra II and Cadaval wintering caves shared haplotypes with the nurseries Alcanena, Tomar, and Sesimbra I. The hibernation colony Loulé II shared haplotypes with the nurseries of Loulé I and Odemira.

Genetic Diversity within Colonies

Molecular variability of the nursing and hibernation colonies is shown in Table 2. Haplotype diversity of mtDNA in the northern nursing colonies (Md, Fcr, and Mog) was approximately half of the diversity held by the southern nursing colonies. A northward decrease in nucleotide diversity was also evident. This north–south decline in both haplotype and nucleotide diversities was shown to be significant ($P < 0.01$). For the nuclear markers, mean allele richness was 5.97 and mean H_E was 0.64. Alleles were roughly evenly distributed among the 15 colonies sampled. A few low-frequency alleles were specific to one or a few colonies. A north–south decline in allele richness and in H_E was also shown to be significant ($P < 0.01$). Resampling using just females or both males and females showed that hibernacula had significantly higher haplotype and nucleotide diversities values than nurseries ($P < 0.01$).

Differentiation among Colonies

Global multilocus pairwise R_{ST} estimates among colonies were not significantly higher than mean pRST (Table 3). Applied to each locus, none of the tests were also significant. This shows that population differentiation of the Schreibers' bat is not strongly affected by allele size or stepwise mutations. Hardy et al. (2003) point out that in such cases, F_{ST} is a more adequate measure than RST for estimating population differentiation, so we only report F_{ST} pairwise results (Table 4). In mtDNA, genetic differentiation was generally moderate to low within the same geographical region. At the nDNA level, this differentiation was usually weak, but most F_{ST} values were significantly different from zero (Table 4). The correlation between geographical

Table 1. Variable nucleotide positions within the 518-bp sequence of the D-loop analyzed in 312 specimens of *Miniopterus schreibersii*

Haplotypes	Position (bp)																							
	1	3	20	21	31	32	67	68	77	79	83	85	91	93	95	174	223	245	246	310	464	477	485	492
M1 (174)	T	T	G	//	//	T	G	A	C	A	G	G	G	A	T	C	T	T	G	T	T	A	G	A
M2 (1)	A	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M3 (2)	A	A	·	·	·	·		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
M4 (1)	A	A	·	·	·	·		·	·	·	·	T	·	·	·	·	C	·	·	·	·	·	·	..
M5 (1)	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
M6 (1)	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M7 (1)	A	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
M8 (1)	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·
M9 (1)	·	G	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M10 (1)	·		C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M11 (1)	·		·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M12 (1)	·	·	·	A	·	·	·	·	·	·	A	·	·	·	·	·	C	·	·	·	·	·	·	·
M13 (16)	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M14 (1)	·	·	·	·	C	·	·	·	·	·	·	·	·	G	·	·	·	·	A	·	·	·	·	·
M15 (1)	·	·	·	·	C	·	·	·	·	·	A	·	·	G	·	·	·	·	·	·	·	·	·	·
M16 (1)	·	·	·	·	C	·	·	·	·	·	·	T	·	·	·	·	C	·	·	·	·	·	·	·
M17 (11)	·	·	·	·	C	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	A	·
M18 (1)	·	·	·	·	C	·	·	·	·	·	A	·	·	·	·	·	C	·	·	·	·	·	·	·
M19 (1)	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
M20 (2)	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·
M21 (7)	·	·	·	·	·	C	·	·	·	·	·	·	·	G	·	·	·	·	A	·	·	·	·	·
M22 (1)	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	G	·	·	·	·	·	·
M23 (1)	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M24 (1)	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
M25 (1)	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M26 (1)	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	T	·	·	·	·	·	·	·	·
M27 (14)	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·
M28 (2)	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	C	·	·	·	·	·	·	·
M29 (6)	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	C	·	·	·	·	G	·	·
M30 (17)	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	C	·	·	·	·	·	·	·
M31 (1)	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·
M32 (4)	·	·	·	·	·	·	·	·	·	·	·	·	A	·	C	·	·	·	·	·	·	·	·	·
M33 (2)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
M34 (1)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·
M35 (6)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	G	·	·
M36 (1)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·
M37 (24)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·
M38 (1)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
M39 (1)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	G

// correspond to deletions or insertions. The sample size for each haplotype is indicated between brackets.

distance and the genetic differentiation, investigated by Mantels' test, was significant for both Φ_{ST} ($r = 0.38$; $P < 0.01$) and F_{ST} ($r = 0.33$; $P < 0.05$).

The standardized genetic differentiation at the mtDNA $\left(G'_{ST}=0.517\right)$ implies that the F_{ST} value reaches more than 50% of its theoretical maximum, whereas the same measure calculated for microsatellites corresponds to only 10% of the maximum theoretical value $\left(G'_{ST}=0.103\right)$. This indicates that Schreibers' bat populations are about 5 times more structured at mitochondrial markers than at nuclear ones. When pairwise comparisons are made for colonies, the difference in G'_{ST} between the 2 types of markers is also evident (Table 5), with some pairwise comparisons being up to 100 times higher for haplotypes than for microsatellite genotypes.

In SAMOVA, Φ_{CT} approach a maximum as the number of groups increased, reaching 95% of the maximum value with 4 groups (Figure 3a, Table 6), both for the mitochondrial and nuclear data sets. When more groups are defined, the Φ_{CT} value increases but some groups begin to consist of single colonies. Though mitochondrial and nuclear data showed the same trend, the percentage of variation among groups was much higher in the mitochondrial data set, revealing a stronger structure at this female-inherited marker.

A similar pattern was shown by the factorial correspondence analysis. However, in this case, Marvão, which appears as a separate unit in the 4-group SAMOVA, is here particularly close to the Central-West (CW) colonies (Figure 3b).

Sex-Biased Dispersal

Tests for F_{IS} and mean AI were significant ($P < 0.05$) confirming the expectation that males are the key dispersing sex. Globally, males presented a positive F_{IS} against the negative value of the females (male $F_{IS} = 0.0765$; female

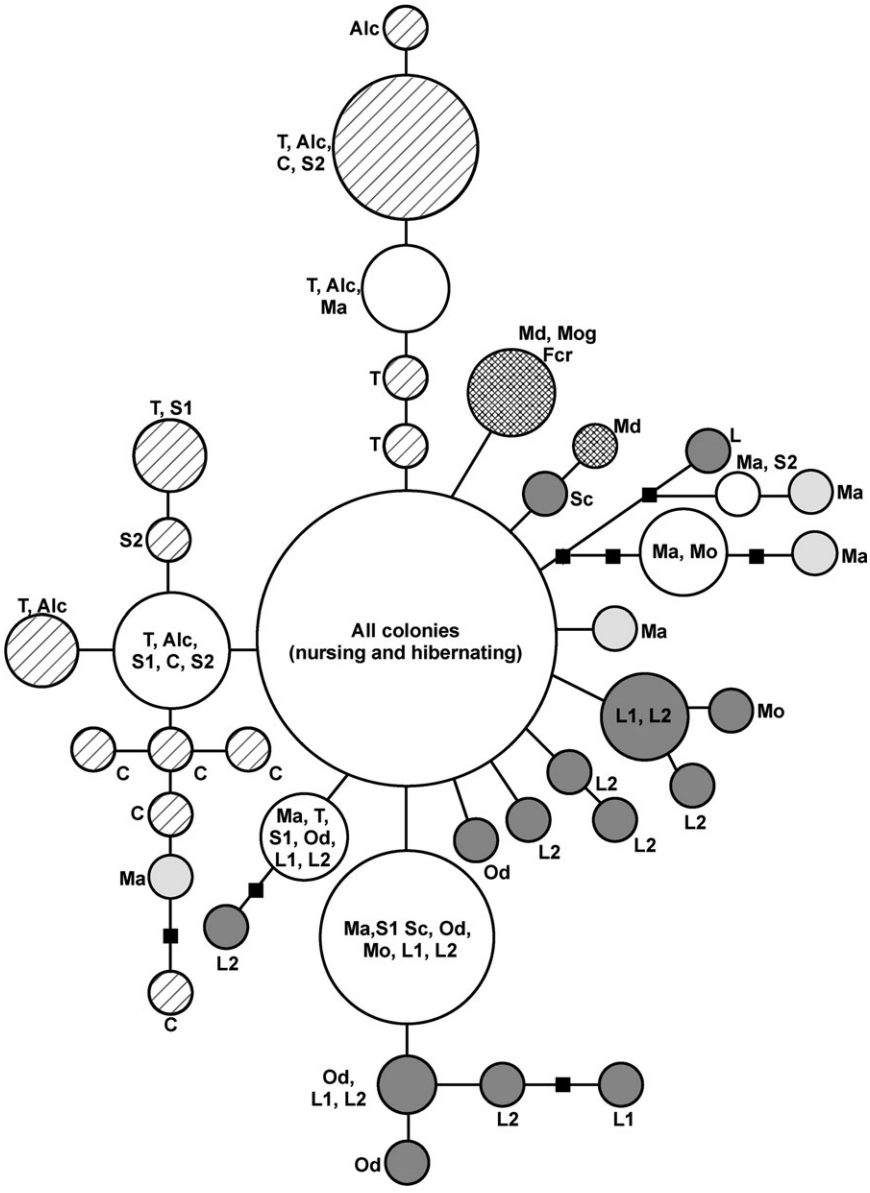

Figure 2. Parsimony network of the 39 haplotypes (circles) found by sequencing 518 bp of mtDNA D-loop of 312 Schreibers' bats. The area of circles is proportional to the frequency of the haplotypes. Abbreviated population names under each haplotype indicate their location (Ma, Marvão I; Md, Miranda do Douro I; Mog, Mogadouro II; Fcr, Figueira de Castelo Rodrigo I; T, Tomar I; Alc, Alcanena I; S1, Sesimbra I; Mo, Moura I; Sc, Santiago do Cacém; Od, Odemira I; L1, Loulé I; C, Cadaval; S2, Sesimbra II; and L2, Loulé II). White circles correspond to haplotypes found in multiple locations, spotted circles to haplotypes found in colonies located in the North-East of Portugal, circles with oblique line pattern to haplotypes found in colonies located in the Central-West, light gray circles to haplotypes found in Marvão (CE), and dark gray circles to haplotypes found in colonies located in the South. Small squares represent missing (or unsampled) haplotypes, and the line between 2 circles corresponds to a single mutation.

$F_{IS} = -0.0004$), lower F_{ST} (male $F_{ST} = 0.0351$; female $F_{ST} = 0.0408$), lower mean AI, and a slightly larger variance of AI (male AI = −0.0806, varAI = 3.9499; female AI = 0.3938, varAI = 3.1428).

Population Expansion

As colonies showed low differentiation within the same geographical region, we chose to treat as populations the 4 groups revealed by the SAMOVA. This approach minimizes the possible effects of dispersal between colonies, which is more likely to occur within the same region. The hypothesis of expansion is not rejected by the SSD tests (all P values $>$ 0.05). The Harpending raggedness index was nonsignificant in all nursing colonies and in the 4 groups (Table 7). The combined data set showed unimodal smooth curves, with single major peaks at around 1, 1, and 4 pairwise differences

Table 2. Sample size, year of sampling, and molecular variability of the 11 nursing colonies and the 4 hibernation colonies of *Miniopterus schreibersii* studied

Colony	Sample size	Year of sampling	Mitochondrial variability			Nuclear variability	
			nh	$h \pm$ SD	$\pi \pm$ SD	A $\pm$ SD	$H_E \pm$ SD
Nursing							
Md	40	1998/2004	4	0.38 ± 0.13	0.0009 ± 0.0009	4.8 ± 2.74	0.58 ± 0.15
Mog	17	1998/2004	2	0.40 ± 0.11	0.0015 ± 0.0013	4.0 ± 1.58	0.59 ± 0.13
Fcr	46	1998/2004	3	0.35 ± 0.12	0.0007 ± 0.0008	5.6 ± 2.77	0.57 ± 0.13
Alc	39	1998/2004	6	0.73 ± 0.07	0.0031 ± 0.0021	5.6 ± 2.07	0.56 ± 0.11
T	24	1998	8	0.87 ± 0.04	0.0031 ± 0.0021	5.8 ± 1.79	0.60 ± 0.08
S1	24	1998/2004	4	0.66 ± 0.07	0.0016 ± 0.0013	4.8 ± 2.17	0.60 ± 0.10
Ma	46	1998/2004	6	0.81 ± 0.05	0.0043 ± 0.0028	6.2 ± 2.39	0.61 ± 0.10
Mo	52	1998/2004	7	0.69 ± 0.09	0.0026 ± 0.0019	6.4 ± 2.51	0.69 ± 0.10
Sc	55	1998/2004	4	0.61 ± 0.06	0.0015 ± 0.0013	7.0 ± 3.16	0.68 ± 0.10
Od	20	2004	6	0.71 ± 0.07	0.0019 ± 0.0016	4.2 ± 2.28	0.61 ± 0.11
L1	44	1998/2004	7	0.79 ± 0.06	0.0026 ± 0.0019	7.2 ± 3.96	0.70 ± 0.10
Hibernation							
C	24	2004	9	0.84 ± 0.08	0.0023 ± 0.0018	6.6 ± 2.41	0.71 ± 0.09
S2	24	2004	6	0.77 ± 0.06	0.0030 ± 0.0021	7.4 ± 2.07	0.67 ± 0.09
Ma[a]	24	2004	7	0.69 ± 0.11	0.0047 ± 0.0025	6.6 ± 1.82	0.70 ± 0.10
L2	24	2004	11	0.95 ± 0.03	0.0043 ± 0.0028	7.0 ± 3.39	0.74 ± 0.10

Abbreviations: nh, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; A, allele richness per locus; SD, standard deviation. Abbreviations as in Figure 1.

[a] Ma is both a nursing and a hibernation roost.

between sequences in CW, South (S), and Central-East (CE), respectively (Figure 4). The NE group reached the maximum value around 0. These results may suggest that expansion of the regional populations during the late Pleistocene, starting at about 100 000 years in Marvão (CE), the oldest population, and at about 20 000 years in the more recent NE population (Table 7). However, CIs for θ_0 and θ_1 are very large, leading to some overlap between them and consequently challenging the model of expansion. In fact, although R^2 and Fu's *Fs* values seem to indicate an excess of low-frequency mtDNA alleles, consistent with an expansion scenario, most of these values were nonsignificant at $P = 0.05$ (Table 7).

Discussion

Miniopterus schreibersii Shows Clear Population Subdivision and Male-Biased Gene Flow

Mitochondrial and nDNA structure revealed similar geographic patterns. Both pairwise Φ_{ST} and F_{ST} were significantly correlated with geographical distance, suggesting that isolation by distance is relevant for the studied mitochondrial and nuclear markers.

The SAMOVA and the factorial correspondence analysis yielded similar results in terms of the structuring of the populations. The small difference between these analyses is most likely due to the fact that Marvão shares several haplotypes and alleles with both the CW and S colonies causing a slight variation in the output of the 2 algorithms. In fact, although SAMOVA maximizes the proportion of total genetic variance due to differences between groups of populations, the factorial correspondence analysis searches the independent (orthogonal) directions where the inertia is maximal, in the hyperspace that has as many dimensions as alleles over all variables.

The observed structuring at the mtDNA level was much more marked than that for nDNA, as revealed by the results of the SAMOVA analysis. Additionally, the tests for sex-biased dispersal suggested that males are responsible for more gene flow than females. In fact, according to Goudet et al. (2002), sex bias needs to be very intense (larger than 80:20) to be detected by these methods. However, these results should be interpreted with caution because these tests are mainly indicated for nonoverlapping generations, which is not the case in our study.

The marked mtDNA structure that we detected can be explained by the strict female philopatry in this species. In fact, outside the nursing season, females make extensive movements and often stay in roosts of various nurseries but return to the cave in which they were born to give birth (Palmeirim and Rodrigues 1995). Exceptions to this rule seem so rare (Palmeirim and Rodrigues 1995) that female lineages are likely to remain associated with a single nursing

Table 3. Mean F_{ST}, R_{ST}, and permutated pR_{ST} (95% CI in parentheses) values of genetic differentiation among the Schreiber's bat populations measured at 5 microsatellite loci

Locus	F_{ST}	R_{ST}	pR_{ST} (95% CI)
Ms1	0.073	0.092	0.063 (0.009 to 0.121)
Ms2	0.021	0.046	0.025 (−0.002 to 0.068)
Ms3	0.015	0.001	0.016 (−0.004 to 0.050)
Ms4	0.021	0.011	0.021 (−0.001 to 0.044)
Ms5	0.053	0.019	0.049 (−0.008 to 0.135)
All loci	0.038	0.026	0.038 (0.007 to 0.081)

No significant comparisons (observed > expected) were found.

Table 4. Pairwise genetic differentiation among the nursing colonies of the Schreiber's bat for mtDNA Φ_{ST} (below diagonal) and for nDNA F_{ST} (above diagonal) data set

	Md	Mog	Fcr	Alc	T	S1	Ma	Mo	Sc	Od	L1
Md	—	-0.01^{+}	0.01	**0.09**	**0.05**	**0.06**	**0.03**	**0.03**	**0.03**	**0.04**	0.04
Mog	0.06^{+}	—	0.01^{+}	0.09	**0.05**	0.06	0.03	0.01^{+}	0.02	0.03^{+}	0.03^{+}
Fcr	0.01^{+}	0.04^{+}	—	**0.11**	**0.06**	**0.09**	**0.05**	**0.05**	**0.05**	**0.06**	0.06
Alc	0.23	0.25	0.25	—	0.04^{+}	0.01^{+}	0.01^{+}	**0.04**	**0.07**	**0.07**	**0.07**
T	0.20	0.23	0.24	0.04^{+}	—	0.02^{+}	0.02	**0.04**	**0.08**	**0.08**	0.07
S1	**0.33**	**0.37**	**0.39**	0.30	0.14	—	0.01^{+}	**0.02**	**0.06**	**0.07**	**0.04**
Ma	**0.20**	**0.23**	**0.23**	0.27	0.24	0.31	—	0.02	**0.04**	**0.04**	0.04
Mo	**0.30**	**0.33**	**0.33**	**0.32**	0.31	**0.43**	0.17	—	0.01	0.01^{+}	0.01^{+}
Sc	0.20	0.23	0.25	0.26	0.26	**0.39**	0.19	0.01^{+}	—	0.01^{+}	0.006
Od	0.22	0.25	0.27	0.25	0.25	**0.38**	0.17	0.01^{+}	0.02^{+}	—	0.02^{+}
L1	0.14	0.18	0.20	0.25	0.19	0.24	0.17	0.07^{+}	0.05^{+}	0.04^{+}	—

All P values < 0.01, except those marked with +, which are nonsignificant at $\alpha = 0.01$. In bold: significant ($P < 0.01$) F_{ST} values after a sequential Bonferroni correction. Different shadings indicate different regions from darker northern to lighter southern populations. Abbreviations as in Figure 1.

colony for many generations, resulting in the structure observed in the mtDNA.

Because females are philopatric and the structuring of the mtDNA is more marked than that of nDNA, we conclude that males are responsible for most of the gene flow. Female philopatry and male-mediated gene flow are characteristics of many mammal species (Greenwood 1980) and have been shown for several bat species such as *Macroderma gigas* (Worthington Wilmer et al. 1999), *Nyctalus noctula* (Petit and Mayer 1999, 2000; Petit et al. 2001), *Myotis myotis* (Castella et al. 2001; Ruedi and Castella 2003), *Myotis bechsteinii* (Kerth et al. 2002), and *Plecotus auritus* (Veith et al. 2004). Ringing studies have demonstrated that *M. schreibersii* males are also philopatric, although not nearly as much as females (Palmeirim and Rodrigues 1995). However, male-mediated gene flow occurs because males carry their genes to the mating roosts, where they mate with females from foreign colonies. Because females then return to their home nursery, this flow is virtually always from the nursery of the male to that of the female. This species is dependent on underground roosts throughout the yearly cycle, and as referred before, males and females from different nursing colonies gather in some of these roosts to mate (Palmeirim and Rodrigues 1995), suggesting widespread gene flow, and consequently, a near panmictic situation at the scale of the Portuguese territory. However, we found a clear structure at the nDNA, even though it is quite weak. How to explain the persistence of this structure at the nuclear level?

Females show a strong philopatric behavior, and contributing to half of the nuclear genome, some level of structure in this genome would indeed be expected, though reduced due to gene flow through the males. A complementary explanation is related to the spatial distribution of roosts. In fact, recent mining activities created a continuous network of roosts for these species in the Portuguese territory. Only a few of these roosts are used by nursing colonies but many are used as temporary shelters (Luísa Rodrigues and Jorge M. Palmeirim, unpublished data), where presumably mating takes place during the autumn. However, in the past, before the spread of underground mining activities, the availability of suitable roosts was far more discontinuous. In Portugal, the great majority of potential roosts were concentrated in 2 large and 3 small limestone areas (Figure 3a). The genetic units revealed in this study are, to some extent, coincident with these limestone areas, which are separated by vast regions without

Table 5. Estimated pairwise standardized G'_{ST} values (Hedrick 2005) among nursing colonies of the Schreiber's bat for mtDNA (below diagonal) and for nDNA (above diagonal) data set

	Md	Mog	Fcr	Alc	T	S1	Ma	Mo	Sc	Od	L1
Md	—	−0.026	0.031	0.201	0.113	0.150	0.080	0.079	0.071	0.105	0.113
Mog	0.020	—	0.020	0.214	0.111	0.153	0.079	0.038	0.062	0.076	0.085
Fcr	−0.049	−0.060	—	0.245	0.136	0.224	0.113	0.130	0.124	0.140	0.151
Alc	0.294	0.314	0.320	—	0.087	0.016	0.034	0.121	0.195	0.165	0.182
T	0.641	0.663	0.668	0.164	—	0.053	0.044	0.125	0.211	0.188	0.189
S1	0.612	0.638	0.637	0.460	0.390	—	0.026	0.071	0.176	0.187	0.120
Ma	0.668	0.704	0.701	0.632	0.655	0.747	—	0.047	0.110	0.094	0.125
Mo	0.706	0.710	0.718	0.684	0.807	0.819	0.659	—	0.025	0.029	0.006
Sc	0.362	0.385	0.383	0.363	0.634	0.672	0.642	0.054	—	0.002	0.018
Od	0.384	0.414	0.415	0.383	0.608	0.645	0.565	0.014	−0.095	—	0.047
L1	0.631	0.644	0.652	0.584	0.550	0.717	0.730	0.808	0.663	0.558	—

Different shadings indicate different regions from darker northern to lighter southern populations. Abbreviations as in Figure 1.

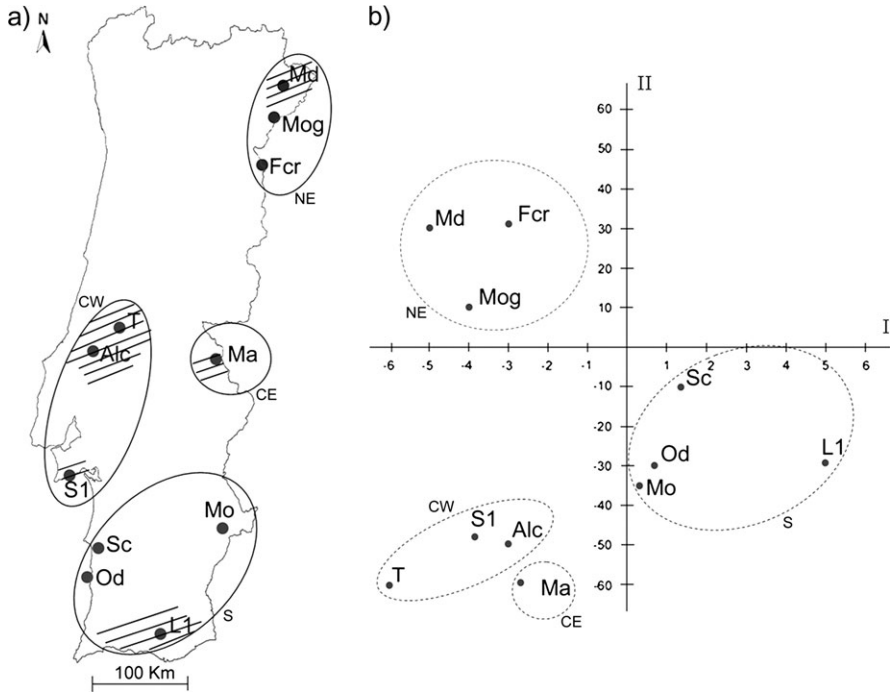

Figure 3. **a**) Map of the groups of populations identified by the SAMOVA, and (**b**) plot with the results of the factorial correspondence analysis of the populations, using the individual microsatellite genotypes. Shaded areas represent major limestone regions according to Ribeiro et al. (1987). Abbreviations as in Figure 1.

natural caves, even if we consider the bordering Spanish territory. Consequently, the observed geographic structure in nDNA markers may be a remnant of times when connectivity between populations was much lower than that existing today. The increased connectivity between populations of strict cave-dwelling bat species due to the creation of man-made roosts may enhance gene flow and, in the long term, reduce their geographic genetic structuring in the nDNA. At the mitochondrial level, this is less likely to occur due to the great philopatry of females to their birth colonies. Our results can, in the future, be used as a baseline reference to determine if this phenomenon is indeed occurring.

An alternative hypothesis to explain the observed nDNA geographic structuring would be the adaptation to regional conditions, as suggested by Miller-Butterworth et al. (2003), for the closely related *Miniopterus natalensis* in South Africa. There are indeed regional climatic variations in Portugal, but these are much less marked than those observed in South Africa, which even includes distinct biomes. Considering this, and the fact that geographic distances in Portugal are much smaller than those in South Africa, we believe that regional adaptations are unlikely to be responsible for the observed structuring.

Table 6. Comparison of mitochondrial and nuclear markers partitions of genetic variation (4 groups)

	Percentage of variation	
	mtDNA, Φ_{ST}	Microsatellites, F_{ST}
Among groups	0.170	0.030
Among colonies within groups	0.060	0.005
Within colonies	0.570	0.741
Total	0.800	0.776

Winter Roosts Are Genetically More Diverse because They Gather Bats from Multiple Nurseries

We found that the genetic structure of winter colonies is different from that of summer colonies and that the genetic diversity in hibernacula is greater than that found in nurseries. In fact, after the nursing season, the bats tend to abandon their nurseries and gather in mating and hibernation roosts (Rodrigues and Palmeirim 2008). A single hibernaculum may frequently harbor individuals from several nurseries, thus pooling the genetic diversity from distinct populations. Petit and Mayer (2000) obtained similar results when comparing summer and winter colonies of *N. noctula*.

The results of Petit and Mayer (2000) and Wilkinson and Fleming (1996) suggest that the simultaneous analyses of the population genetics of summer and winter colonies can reveal the migratory routes of bats. Our colonies did not differ enough in their nuclear structure to assign each individual to a specific nursery, so we could not use it to determine the origin of the animals found in winter caves. mtDNA was more informative because the nursing colonies were more distinct, but even then, it was not possible to be certain about the origin of most animals. The results suggest that summer and winter roosts may be separated by several

Table 7. Summary of values regarding the population expansion analyses

Population	τ (95% CI)	θ_0 (95% CI)	θ_1 (95% CI)	*t* (95% CI, in years)	Raggedness index	*P* value	R^2 (95% CI)	*Fs* (95% CI)
$NE_{(Md + Fcr + Mog)}$	0.89 (0.66–2.31)	0.00 (0.00–1.88)	0.67 (0.00–4005.67)	21 477 (15 927–55 743)	0.017	0.20	0.10^{ns} (0.04–0.25)	-1.14^{ns} (−2.28–1.99)
$CW_{(T + Alc + S1)}$	1.93 (0.69–3.52)	0.00 (0.00–2.23)	11.92 (1.29–7319.42)	46 573 (16 651–84 942)	0.032	0.55	0.12^{ns} (0.04–0.22)	-0.86^{ns} (−3.76–4.29)
$CE_{(Ma)}$	4.14 (1.14–11.86)	0.00 (0.00–12.17)	4.91 (0.57–4975.91)	99 903 (27 510–286 197)	0.129	0.25	0.18^{ns} (0.09–0.23)	0.54^{ns} (−3.15–4.36)
$S_{(Mo + Sc + Od + L1)}$	1.63 (0.89–2.23)	0.00 (0.00–1.10)	18.14 (3.089–5699.69)	39 334 (21 477–53 813)	0.021	0.90	0.05^{ns} (0.03–0.20	−4.80*(−3.89–4.59)

τ, parameter of time of expansion inferred from mismatch distribution; θ_0; mutation parameter before population expansion; θ_1, mutation parameter after expansion; time since expansion (*t*); raggedness index; and respective *P* values, R^2, and Fu's *Fs* (10 000 coalescent simulations)
$^*P < 0.05$; $^{ns}P > 0.1$.

hundred kilometers, but bats tend to hibernate in colonies located in the same region of their nurseries. A more complete sampling scheme that includes nursing, mating and hibernation roosts, and the analysis of more microsatellite loci would give a better picture of the migration patterns of *M. schreibersii*.

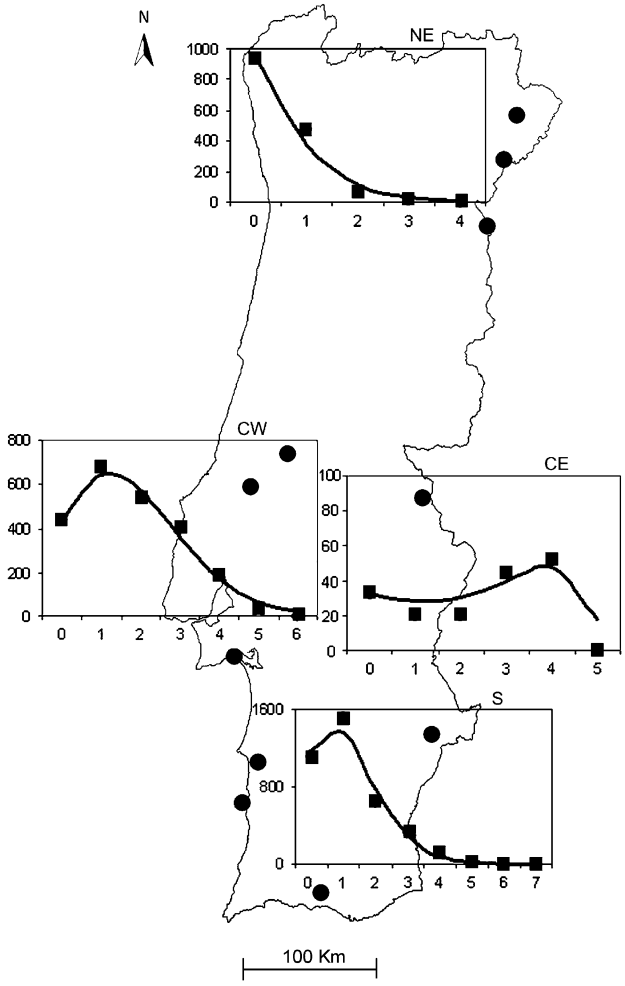

Figure 4. mtDNA control region mismatch distributions for groups of nursing colonies. *x* axis , number of pairwise differences between sequences; *y* axis, frequency; black squares indicate observed values; and lines show model distribution.

Postglacial Northward Expansion Is Reflected in Genetic Structure

Haplotype diversity of the northern colonies is only about half of that of the central and southern colonies. This is most likely a consequence of a recent northward expansion of *M. schreibersii*, and the expansion time of the northern population roughly coincides with the end of the last glaciation (10 000–18 000 years). In fact, *M. schreibersii* is a thermophilic species that would be incapable of living in the cool northern Iberia of glacial periods. A northward decline in genetic diversity has also been detected in other bat species such as *M. myotis* (Ruedi and Castella 2003) and *Plecotus austriacus* (Juste et al. 2004). These authors have also interpreted this pattern as a consequence of postglacial population expansions.

In spite of the mountainous terrain of northern Portugal, which could limit the amount of exploitable habitat, the population of *M. schreibersii* in the region and adjacent areas in Spain includes both nursing and hibernation colonies and is about as large as those of central and southern Portugal. This excludes the possibility that the observed lower diversity is a result of a smaller population size. In addition, mismatch distribution also indicates that the northernmost Portuguese colonies are the youngest ones. Estimates of population time expansion show very large CIs, and coalescent analyses returned overall nonsignificant values of the statistics indicating excess of low-frequency mutations and thus have to be interpreted with care. These estimates may be somewhat inaccurate not only due to sample size but also as a consequence of the migration of individuals into some colonies, after these became established.

It has been demonstrated that southern Iberia served as a glacial refuge for a variety of thermophilic organisms, such as the woodmouse, *Apodemus sylvaticus* (Michaux et al. 2003); the European rabbit, *Oryctolagus cunniculus* (Branco et al. 2002); the pygmy marbled newt, *Triturus pygmaeus* (Wallis and Arntzen 1989); and deciduous oak species, *Quercus* spp. (Brewer et al. 2002). The presence of *M. schreibersii* in fossil material from the Würm from southern Portugal (Telles Antunes 1993) and our estimates of expansion times suggest

that the species survived there during the Late Pleistocene. Our results strongly support that there was a postglacial northward expansion of *M. schreibersii*. However, due to the low precision of the estimates of expansion times, we should not discard the possibility that this expansion occurred regardless of the survival of the species in southern Iberia during the glacial periods. Indeed, a scenario of disappearance from Iberia, followed by a postglacial recolonization from North Africa across the Strait of Gibraltar, should also be considered. The sampling of North African colonies is essential to fully understand the history of postglacial colonization of the Iberian Peninsula by *M. schreibersii*.

In conclusion, both social organization and history have played a key role in shaping the present patterns of population structure in *M. shreibersii*. Northern colonies are less diverse as a result of the expansion history of *M. schreibersii* in the Iberian Peninsula, but the structuring and the maintenance of significant differentiation among the populations is probably the result of female philopatry, male-mediated gene flow, and ecological constraints.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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