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NO EVIDENCE OF BOTTLENECK IN THE POSTGLACIAL RECOLONIZATION OF EUROPE BY THE NOCTULE BAT (*NYCTALUS NOCTULA*)

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Abstract.—During the Pleistocene, the habitat of the noctule bat (*Nyctalus noctula*) was limited to small refuge areas located in Southern Europe, whereas the species is now widespread across this continent. Using mtDNA (control region and ND1 gene) polymorphisms, we asked whether this recolonization occurred through bottlenecks and whether it was accompanied by population growth. Sequences of the second hypervariable domain of the control region were obtained from 364 noctule bats representing 18 colonies sampled across Europe. This yielded 108 haplotypes that were depicted on a minimum spanning tree that showed a starlike structure with two long branches. Additional sequences obtained from the ND1 gene confirmed that the different parts of the MST correspond to three clades which diverged before the Last Glacial Maximum (18,000 yrC^{14} BP), leading to the conclusion that the noctule bat survived in several isolated refugia. Partitioning populations into coherent geographical groups divided our samples (ϕ_{CT} = 0.17; P = 0.01) into a group of highly variable nursing colonies from central and eastern Europe and less variable, isolated colonies from western and southern Europe. Demographic analyses suggest that populations of the former group underwent demographic expansions either after the Younger Dryas (11,000–10,000 yrC^{14} BP), assuming a fast mutation rate for HV II, or during the Pleistocene, assuming a conventional mutation rate. We discuss the fact that the high genetic variability (h = 0.69–0.96; π = 0.006–0.013) observed in nursing colonies that are located some distance from potential Pleistocene refugia is probably due to the combined effect of rapid evolution of the control region in growing populations and a range shift of noctule populations parallel to the recovery of forests in Europe after the last glaciations.

Key words.—Bottleneck, control region, mitochondrial DNA, ND1, *Nyctalus noctula*, Pleistocene, population structure.

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Contraction and expansion of habitats are common events in a species' life history. This is exemplified by glaciation events (Hewitt 1996). During glacial maxima, populations retreat into small refuge areas, whereas larger territories are recolonized when the climate becomes milder. Following theoretical (Nei et al. 1975) and simulation (Ibrahim et al. 1996) results, classical models predict that, because of population bottlenecks, the colonization of new territories would lead to a loss of genetic variability (Hewitt 1996). The degree of genetic polymorphism is thus supposed to decrease with increasing geographic distance from potential Pleistocene refugia. This view is supported by empirical data (Sage and Wolff 1986; Lagercrantz and Ryman 1990; Merilä et al. 1996, 1997; but see Eber and Brandl 1997), and the argument that ancestral populations harbor the highest genetic variability has also been used to support the hypothesis of an African origin of human populations (e.g. Vigilant et al. 1991; Cavalli-Sforza et al. 1994; Tishkoff et al. 1996; Jorde et al. 1997; but see Templeton 1993; Templeton et al. 1995). The process of habitat contraction and expansion also implies demographic variations (Hewitt 1996). Geographic expansion often implies demographic expansion, and theoretical studies have shown that population growth has a strong effect on the pattern of genetic polymorphism of populations (Avise et al. 1984; Slatkin and Hudson 1991; Rogers and Harpending 1992; Templeton et al. 1995; Nee et al. 1996). In this regard, mismatch distribution analysis is of particular interest because it provides the opportunity to estimate the magnitude and age of population growth (Rogers and Harpending 1992). Empirical studies have recognized patterns compatible with population growth in a range of taxa for which a process of (re)colonization had been hypothesized or documented (*Birgus latro*, Lavery et al. 1996; humans, Rogers 1995; Comas et al. 1996; *Carduelis chloris*, Merilä et al. 1997; *Microtus agrestis*, Jaarola and Tegelström 1996). However, methodological complications including wide confidence intervals on the parameters estimated (Eller and Harpending 1996; Merilä et al. 1997, their table 3) and inaccurate sequence evolution models (Bertorelle and Slatkin 1995; Aris-Brosou and Excoffier 1996) render the detection of population growth difficult.

We used mtDNA genetic variability analyses to investigate population structure and history in the noctule bat (*Nyctalus noctula*), a migratory bat species that is common in Europe. At least for the period of nursing, this species roosts mainly in hollow trees (Gaisler et al. 1979), and its current range corresponds to the distribution of deciduous and mixed coniferous-deciduous forests (see Ahlén and Gerell 1989; Strelkov and Iljin 1992), which strongly indicates a dependence on forest habitats. According to Bennett et al. (1991) the main area of mixed and deciduous forest refugia at the Last Glacial Maximum (LGM, 18,000 yrC^{14} BP) was located in the western Balkans, with secondary refugia being situated in midaltitudes of the Italian mountains and the Alps. During the following period (16,000–11,000 yrC^{14} BP), the climate became milder and the forests recovered extensively in Eastern Europe (Huntley 1990), but not as much in Southwestern Europe (Turner and Hannon 1988). The cold climate of the

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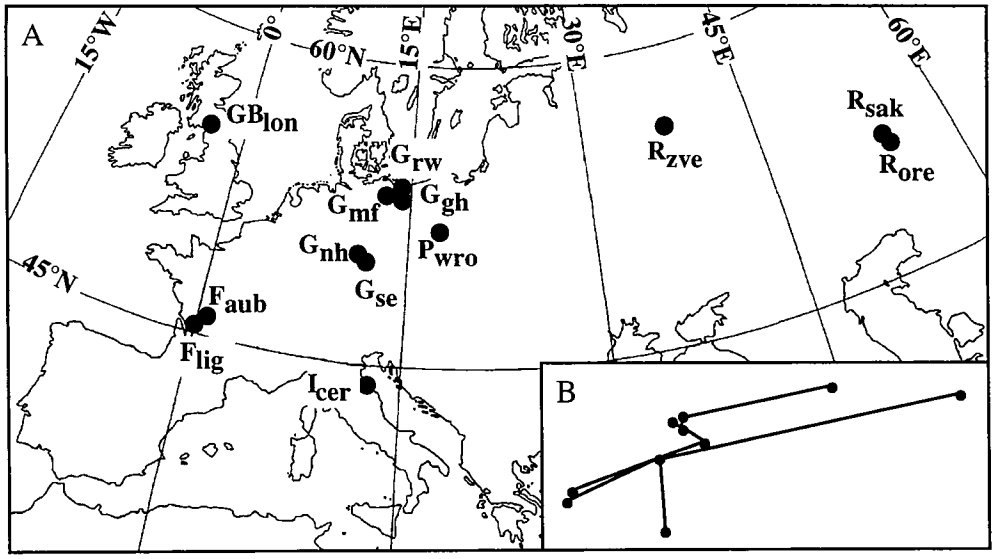

FIG. 1. (A) Location of the sampled nursing colonies (see Appendix 1 for an explanation of abbreviations). (B) Minimum spanning tree of these populations constructed from a matrix of pairwise ϕ_{ST}s. GB_{lon} has not been taken into account in this analysis and each of the two pairs G_{nh}/G_{se} and R_{ore}/R_{sak} was considered as one population (see text for an explanation).

Younger Dryas (11,000–10,000 yrC^{14} BP) probably led the forests from Eastern Europe to extinction (Bennett et al. 1991). During this period, forest refugia were in Spain, Italy, the Balkans, and the midaltitudes of mountain ranges such as the Alps and the Caucasus (Huntley 1990). It was only from 10,000 yrC^{14} BP that the forests recovered their previous range. The lack of *Nyctalus noctula* subfossils in Pleistocene cave deposits (except in one cave of the Balkans; Popov and Ivanova 1995) and the fact that all species of the genus *Nyctalus* are associated with forests (Nowak 1991) do not support the idea that the noctule could have been a cave-dwelling species during cold periods. Therefore, we can assume that the ecology of the noctule bat has not changed since the LGM, and its geographic distribution is likely to have followed the evolution of the distribution range of the mixed and deciduous forests during the last 20,000 years. In particular, noctule populations must have been restricted to quite small refugia during the LGM and the Younger Dryas, after which they must have undergone a dramatic demographic and geographic expansion to reach their current distribution.

In this study, we addressed the following: (1) Did the postglacial geographic expansion lead to a loss of genetic variability in the noctule? (2) Is the pattern of genetic variability observed in the noctule consistent with a recent demographic expansion from southern refugia? To answer these questions, we sequenced two regions of the mitochondrial DNA that show different divergence rates. A rapid evolving region, the hypervariable domain II (HV II) of the control region, was used to estimate the genetic variability, the population structure, and the demographic history of European populations of the noctule bat. A more conserved region, the ND1 gene, was used to characterize the major clades in the phylogeny of European noctules and to calibrate the divergence rate of HV II. This information was further used to make inferences about the possible number and location of Pleistocene refugia.

MATERIALS AND METHODS

Sample Collection

A total of 364 bats from 18 colonies was sampled. Small tissue samples were taken from 13 nursing colonies (Fig. 1) and from five winter colonies. The average number of bats sampled per colony was 20.2 (SD = 9.35). The nursing colonies (also referred to as "populations") consisted only of adult females and their offspring. Samples from these colonies were taken exclusively from adult females to avoid sampling first order relatives. Samples from winter and nursing colonies were used to assess the overall sequence polymorphism of the noctule bat in Europe, whereas the analysis of population genetic and demographic parameters was conducted only with samples from nursing colonies. The name, abbreviation, coordinates, sample size, and year of sampling of the sampled colonies are given in Appendix 1.

Tissue samples consisted of a piece of wing membrane taken from the plagiopatagium with a sterile biopsy punch of 4 mm diameter (Worthington Wilmer and Barratt 1996) and were preserved in 80% ethanol. The samples obtained from the Russian colonies R_{ore} and R_{sak} were taken from museum specimen preserved in alcohol (collection of V. Iljin) at the Zoological Institute of the Russian Academy of Science in St. Petersburg. The samples obtained from the winter colony of Vaux-le-Pénil were taken from living animals after the tree where they roosted had fallen. The samples obtained from Altkirch, Munich, Vienna, and Kiel were taken from dead animals found in their roosts. Other samples were taken from living animals captured as they were leaving their roosts.

DNA Isolation

Genomic DNA was isolated from wing membrane samples following a salt/chloroform procedure modified from Miller et al. (1988) by adding one step of chloroform/isoamylalkohol (24/1) extraction to their protocol.

Control Region Amplification and Sequencing

The control region of vertebrates consists of two hypervariable domains (HV I and HV II) and a conserved central domain. The hypervariable domain I, located near $tRNA^{Pro}$, is characterized in the noctule bat by R1 repeats (Fumagalli et al. 1996) consisting of four to nine 81bp repeats (Wilkinson et al. 1997). The evolution of such tandem repeat arrays is not yet well understood and, thus, the polymorphism of this region is not considered here. Instead, we have chosen to analyze the polymorphism of HV II. This part of the control region is located near $tRNA^{Phe}$.

All samples were amplified using the primers ER63 (L16517; Fumagalli et al. 1996) and ER88 (H607; Worthington Wilmer et al. 1994). A description of the primers used in this study is given in Appendix 2. The amplifications were carried out in a volume of 25 μl containing 2 μg/μl BSA, 2.5 mM $MgCl_2$, 1 μM each primer, 0.25 mM each dNTP, 0.5 unit Goldstar Polymerase (Eurogentec, Belgium), and its buffer (75 mM Tris-HCl). The amplifications, which were performed in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT), consisted of 40 cycles of 93°C for 30 sec, 45°C for 30 sec, and 72°C for 90 sec. PCR products were cleaned with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

All PCR products were then cycle sequenced with the internal primer ER86 using the Thermosequenase sequencing kit (Amersham, Uppsala, Sweden) on a GeneAmp PCR System 9600 (Perkin Elmer). The reactions were carried out in 75% of the volume recommended by the supplier with 0.375 μM primer. The sequencing consisted, after 3 min of denaturation, of 30 cycles of 95°C for 20 sec and 70°C for 45 sec, followed by 10 cycles of 95°C for 30 sec and 70°C for 15 sec. The sequences were run on 6% Sequagel®XR gels (National Diagnostics, Atlanta, GA) in a LI-COR DNA sequencer (model 4000L, Li-Cor, Lincoln, NE).

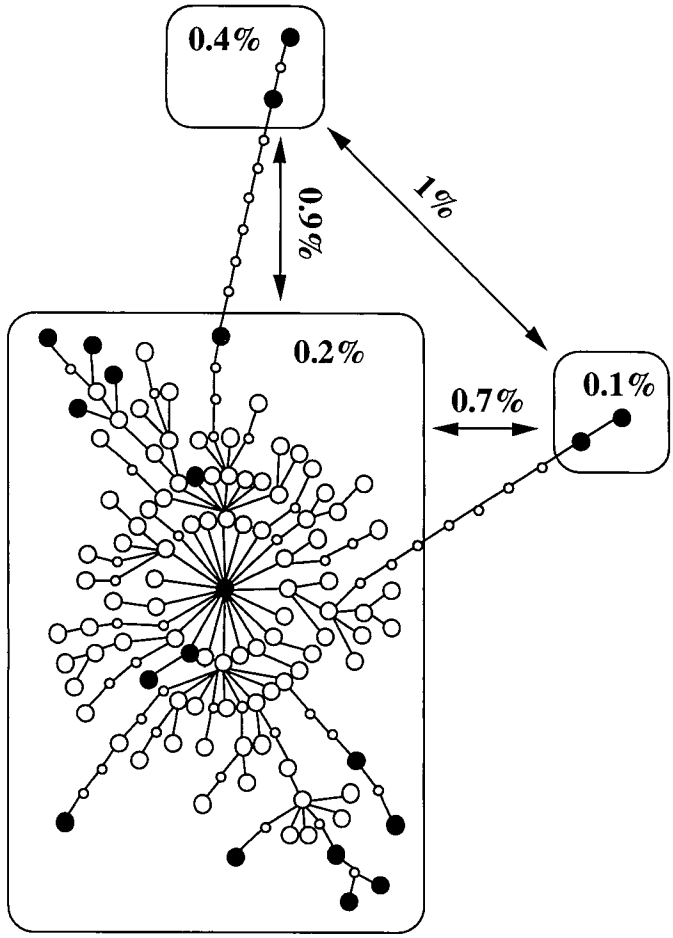

FIG. 2. A minimum spanning tree of the 108 haplotypes found by sequencing the control region (HV II) of 364 European noctule bats. Each line between two circles corresponds to one mutation. Big circles are for observed haplotypes, whereas small ones are for haplotypes which were inferred from the minimum spanning tree but were not observed in the sample. Filled circles are haplotypes for which the ND1 sequence was determined. The numbers in the boxes indicate the maximum sequence divergence found by sequencing ND1 within a box and the numbers outside the boxes indicate the average divergence between boxes as estimated by the mean of distances between ND1 haplotypes belonging to two different boxes.

ND1 Amplification and Sequencing

The ND1 gene was sequenced in 20 individuals who carried different haplotypes in the control region (Fig. 2). The PCR conditions were the same as for the amplification of the control region, except for the following: the mix contained 2 mM $MgCl_2$ and 0.4 unit polymerase, and the annealing temperature was 50°C. The primers used for amplifying the ND1 gene were ER65 and ER66 (A. Janke, pers. comm.). After amplification, the PCR products were cleaned and sequenced in the same conditions as those described for the control region, except for the annealing temperatures of two sequencing primers (see below). Sequences were obtained with three specific internal primers: ER70, ER89, and ER175. For the primer ER89, the sequencing consisted, after 3 minutes of denaturation, of 30 cycles of 95°C for 20 sec, 62°C for 30 sec, and 72°C for 15 sec, followed by 10 cycles of 95°C for 30 sec and 70°C for 15 sec. The same was used for the primer ER175 with an annealing temperature of 40°C. The sequences were run on 4.5% Sequagel®XR gels (National Diagnostics) in a Li-cor DNA sequencer (model 4000L).

Sequence Polymorphism

The sequences were aligned manually in MacDNASIS Pro (vers. 3.5, Hitachi Software, San Francisco, CA). Indels were removed at that stage and discarded from our analysis. Genetic distances between haplotypes were simply computed as the number of pairwise differences. The resulting distance matrix was used to construct a minimum spanning tree (MST, Prim 1957) among all observed haplotypes.

Because the samples of nursing and hibernating colonies are not independent, we excluded the hibernacula from fur-

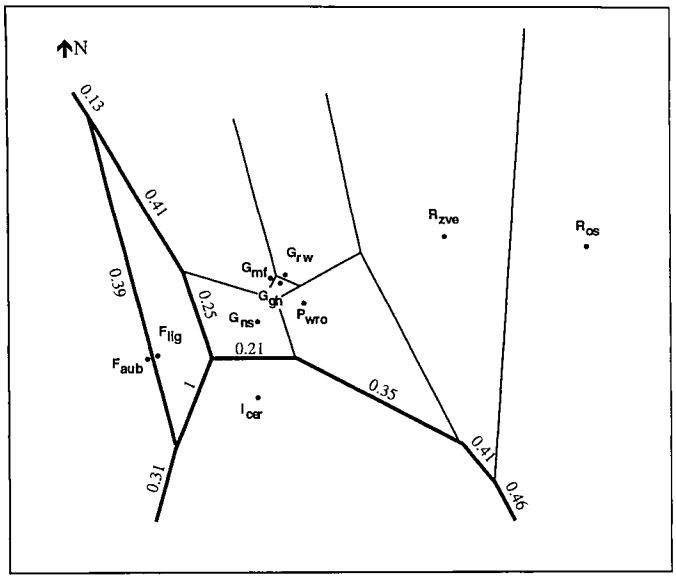

FIG. 3. Result of the partitioning of 10 nursing colonies of the noctule bat into four groups by applying Monmonier's algorithm. All Voronoi polygons are shown, with thick lines corresponding to the boundaries between the four regions. The pairwise ϕ_{ST}s associated with the edges belonging to the boundaries are also reported. The relative geographic location of the colonies is conserved.

ther analyses. GB_{lon} was also removed from the statistical analyses because its sample size was too small (n = 3), thus, the remaining analyses were made with twelve nursing colonies. Within colony variation was estimated with gene (h) and nucleotide (π) diversity, which are equivalent to heterozygosity at the haplotype and nucleotide level respectively. They were computed following Nei (1987, his formulas 8.4 and 10.5, respectively) with the software package Arlequin vers. 1.1 (Schneider et al. 1997).

Population Structure

Past events as well as current gene flow are important in determining how genetic variability is partitioned among populations. Unless these parameters and their relative importance are known, it is difficult to predict the genetic structure of populations. We did not attempt to define a genetic structure a priori. Instead, we used an algorithm defined by Monmonier (1973) to define boundaries between genetically homogeneous geographic groups, and therefore the genetic structure to test. The principle of Monmonier's algorithm is as follows: (1) The samples are first located on a map according to their relative geographic position; (2) Voronoi polygons (Voronoi 1908; Watson 1981) are drawn around each population point (Fig. 3). These polygons define regions where any point is geographically closer to the contained population than to any other population. Moreover, the edges of these polygons are orthogonal to straight lines connecting two neighboring populations. The set of these orthogonal lines connecting all neighbor populations is often called a Delaunay network, and it is implicitly embedded into the Voronoi polygon representation; (3) Genetic distances between neighboring populations, as defined by the Delaunay network, are then associated to each edge of the Voronoi polygons; (4) The edge with the largest associated distance is selected. It is used to start or extend a boundary; (5) The edges directly adjacent to the growing boundary are examined in turn, and the one with the largest associated distance is selected to extend the boundary; (6) Steps (4) and (5) are repeated until a growing boundary meets another boundary or reaches the edge of the area under study; and (7) Steps (4) to (6) can be repeated for delineating as many boundaries as required.

Genetic distances were calculated as pairwise ϕ_{ST}s with the software Arlequin vers. 1.1 (Schneider et al. 1997). As shown by Michalakis and Excoffier (1996), ϕ_{ST} is here strictly equivalent to the fixation index θ_w defined by Weir and Cockerham (1984). The genetic structure defined by Monmonier's algorithm was tested and quantified by a hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) performed with Arlequin. The significance of the fixation indices was tested using a nonparametric permutation scheme using 10,000 permutations.

Neutrality Tests

Selective neutrality tests were performed to check whether the pattern of sequence polymorphism in populations conformed to the neutral Wright-Fisher model. Fu (1996, 1997) has recently developed statistical tests that are based on the comparison of rare and frequent mutations, which occur in the recent and the old part of the genealogy, respectively (Fu and Li 1993). After an episode of population growth, coalescence theory predicts that external branches (the new part of the genealogy) are elongated (Slatkin and Hudson 1991; Donnelly and Tavaré 1995), and that there is an excess of low frequency mutations as compared to the neutral model. Because we expect the noctule bat colonies to have undergone population expansion, we chose to perform two tests, F and F_S, that were designed to detect an excess of low frequency mutations (Fu and Li 1993; Fu 1997). However, genetic hitchhiking and background selection can also lead to an excess of low frequency mutations. Interestingly, power analyses have shown that the two tests are not equally sensitive to the different alternative hypotheses: F_S is particularly sensitive to demographic expansion and genetic hitchhiking, whereas F is more sensitive to background selection (Fu 1997). Both tests were computed online using the computer programs that are available from Fu (http://hgc.sph.uth.tmc.edu/fu).

Demography

The sample distribution of the number of pairwise differences (the mismatch distribution) between mtDNA HV II sequences was used to infer the parameters of a recent and sudden population expansion (Rogers and Harpending 1992). Instead of using the method developed by Rogers and Harpending (1992) under the infinite site model, we used an extension based on a more realistic mutation model (Schneider and Excoffier 1999) that allowed for a finite sequence length consisting of a Kimura 2-parameter mutation model (Kimura 1980) with 95% of substitutions being transitions and with mutation rates following a Gamma distribution of shape parameter α. The shape parameter α of a Gamma distribution of mutation rates (Kocher and Wilson 1991; Tamura

and Nei 1993; Wakeley 1993) was estimated by the method of moments (Johnson and Kotz 1973) as $\alpha = m^2/(s - m)$, where m and s are, respectively, the mean and variance of the number of substitutions per site. The mean and variance of the number of mutations per site, as calculated on a neighbor-joining tree (Saitou and Nei 1987) using MacClade (vers. 3.0, Maddison and Maddison 1992) were 0.427 and 1.698, respectively. These figures lead to a value of 0.14 for the shape parameter α of a Gamma distribution of mutation rates, in keeping with values found for the human mtDNA control region (α = 0.11–0.47, Tamura and Nei 1993; Wakeley 1993), which indicates a strong heterogeneity of mutation rates among sites in this region.

Under this new model, the probability that two sequences differ at S positions is given by

$$F_S^{\alpha}(\theta_1, \theta_0, \tau) = \sum_{i=S}^{\infty} F_i^{\infty}(\theta_1, \theta_0, \tau) H_m^{\alpha}(S, i) \quad (1)$$

where $\theta_0 = 2N_0u$, $\theta_1 = 2N_1u$, N_0, and N_1 being the size of the population before and after the expansion, respectively, u is the total mutation rate of the sequence, and $\tau = 2ut$, where t is the expansion time expressed in generations. $F_i^{\infty}(\theta_1, \theta_0, \tau)$ is the probability that two sequences differ by i mutations in a population after an expansion of parameter θ_0, θ_1, and τ as derived by Li (1977) and Rogers and Harpending (1992),

$$F_i^{\infty}(\theta_1, \theta_0, \tau) = F_i(\theta_1) + \exp\left(-\tau\frac{(\theta_1 + 1)}{\theta_1}\right) \sum_{j=0}^{i} \frac{\tau^j}{j!} [F_{i-j}(\theta_0) - F_{i-j}(\theta_1)], \quad (2)$$

where $F_i(\theta) = \theta^i/(\theta + 1)^{i+1}$ (Watterson 1975) is the probability that i mutations have occurred in the ancestry of two random sequences in a stationary population.

The parameter $H_m^{\alpha}(S, i)$ in equation (1) is simply the probability that i mutations lead to S differences in a sequence of length m according to the mutation model described above. Note that there is no closed form expression for $H_m^{\alpha}(S, i)$ but a recurrence equation is provided in Schneider and Excoffier (1999) to compute those quantities.

Equation (1) is used to obtain the expected mismatch distribution which is fitted to the observed distribution by a generalized least-square method (Schneider and Excoffier 1999). Monte-Carlo simulations based on the coalescent algorithm described by Hudson (1990) were then used to generate random samples following the estimated demographic parameters. The validity of the estimated demographic model was assessed by comparing the sum of squared deviations between the observed and the estimated mismatch distribution (SSD_{obs}) to the SSDs obtained for each simulated data (Schneider and Excoffier 1999). Approximate confidence intervals for the mismatch distribution and for the estimated parameters were obtained by estimating new sets of parameters from each of these simulated samples.

Results

Sequence Polymorphism in the Control Region

The second hypervariable segment (HV II) of the control region was sequenced in 364 bats. Although the PCR product was about 1 kb long, we were only able to sequence 339 bp because of the presence of 6 bp repeats (or R2 repeats, Fumagalli et al. 1996) in the middle of the domain. The 6 bp motif (CGCATA) was found to be repeated a minimum of 50 times (data not shown). Nevertheless, a total of 108 haplotypes defined by 63 point mutations (58 transitions, 3 transversions, and 2 insertions) were found. Raw data are available at http://www.biologie.uni-erlangen.de/zoo2/mayer.html and the consensus sequence, which corresponds to the haplotype H12, was submitted to GeneBank and can be found under the accession number AF054869. Removing the two insertions had no effect on the number of haplotypes. The relationships between haplotypes were resolved on a minimum spanning tree (Fig. 2). We chose to select the links connecting haplotypes that belong to the same colony and not those connecting haplotypes found in different colonies (Excoffier and Langaney 1989; Crandall and Templeton 1993). Overall, these alternative connections would not change the general topology of the tree (data not shown), which clearly shows a starlike structure with two long branches. The haplotypes of the star-like part of the MST were found in all sampled nursing colonies, whereas the haplotypes of the two long branches were found only in one colony of Central Europe (G_{mf}, where one individual carried one of these haplotypes) and two colonies of Western Europe (GB_{lon}, n = 1; F_{aub}, n = 7).

Sequence Polymorphism of ND1

The use of three internal primers generated overlapping sequences spanning over a total of 906 bp of the ND1 gene read on both strands. The 20 samples were chosen for sequencing according to their position in the minimum spanning tree generated from HV II sequences; we sequenced the four haplotypes which belonged to the two long branches and some central as well as the most divergent haplotypes of the starlike part of the MST (Fig. 2). The total number of variable positions was 15 (14 transitions and 1 transversion, see http://www.biologie.uni-erlangen.de/zoo2/mayer.html). They defined seven ND1 haplotypes (GeneBank accession numbers AF065103 to AF065109) that were used to measure the divergence within and between the three groups of haplotypes found in the control region defined on Figure 2. Overall, the within group divergence (0.1–0.4%, Fig. 2) was low compared to the average divergence observed between the groups of haplotypes (0.7–1%).

Variation within Nursing Colonies

The number of haplotypes varied greatly among colonies (Table 1), with two colonies fixed for two different haplotypes. Gene diversity ranged from 0 to 0.96 (median = 0.92) and nucleotide diversity varied from 0 to 0.014 (median = 0.011). There was a positive and significant correlation between the gene diversity and latitude (r = 0.78; P = 0.003, Fig. 4), but this effect disappeared when the two fixed populations were removed from the analysis. There was no significant correlation between nucleotide diversity and latitude (r = 0.53, P = 0.07, Fig. 4).

TABLE 1. Genetic diversity in nursing colonies of the noctule bat. Sample size (n), number of haplotypes (n_h), gene diversity (h), and nucleotide diversity (π) with their standard deviation (SD) are given.

Nursing colony	n	n_h	h	SD	π	SD
F_{aub}	23	4	0.68	0.06	0.014	0.008
F_{lig}	17	1	0	—	0	—
I_{cer}	18	1	0	—	0	—
G_{gh}	43	20	0.93	0.02	0.012	0.006
G_{mf}	24	15	0.94	0.03	0.012	0.007
G_{rw}	14	9	0.93	0.04	0.013	0.007
G_{nh}	22	10	0.9	0.04	0.013	0.007
G_{se}	21	11	0.93	0.03	0.01	0.006
P_{wro}	29	17	0.96	0.02	0.009	0.005
R_{zve}	18	9	0.91	0.04	0.012	0.007
R_{sak}	12	9	0.95	0.05	0.007	0.005
R_{ore}	9	4	0.69	0.15	0.006	0.004

Population Structure

The computation of pairwise ϕ_{ST}s showed that R_{ore} and R_{sak} did not significantly differ in haplotype composition and shared the same pattern of relationships with the other colonies. Therefore they were pooled into a single colony (R_{os}). The same situation applied to G_{nh} and G_{se}, which were also pooled into one sample (G_{ns}). A minimum spanning tree built from the pairwise ϕ_{ST} values calculated for the 10 remaining samples showed a radiating structure with a center situated in Central Europe (Fig. 1). Successive application of Monmonier's algorithm separated the 10 colonies into 4 regions which were genetically highly divergent (Fig. 3, Table 2, ϕ_{CT} = 0.17, P = 0.01). The first three regions consisted each of only one colony located in Western and Southern Europe. The fourth region included all the colonies from Central and Eastern Europe. Whereas the overall standardized variance among colonies was very high (ϕ_{ST} = 0.23, $P < 0.0001$), the variance among colonies within the groups (here the variance among the colonies of Eastern and Central Europe) was much lower but still significant (ϕ_{SC} = 0.06, $P < 0.0001$), confirming the homogeneity of this group.

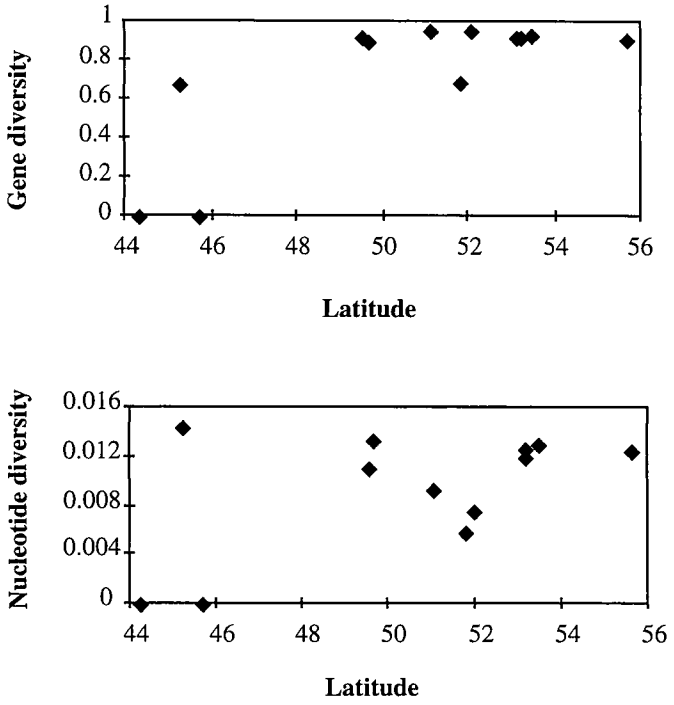

FIG. 4. Gene diversity and nucleotide diversity observed in 12 nursing colonies plotted against latitude. Gene diversity and latitude are significantly correlated (r = 0.78; P = 0.003), whereas nucleotide diversity and latitude are not (r = 0.53, P = 0.07).

TABLE 2. Results of a hierarchical Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) carried out on the population structure defined by Monmonier's algorithm (see text and Fig. 3). The AMOVA was performed with Arlequin (vers. 1.1, Schneider et al. 1997).

Source of variation	Percentage of variation	Fixation index	P-value
Among groups	17.32	Φ_{CT} = 0.17	= 0.01
Among populations within groups	5.33	Φ_{SC} = 0.06	< 0.0001
Within populations	77.35	Φ_{ST} = 0.23	< 0.0001

Neutrality Tests

Departure from neutral expectations are expected if populations have experienced different modes of selection, demographic fluctuations, or population subdivision. We computed two tests that can detect the effect of population growth, that is, an excess of low frequency mutations. However, the two tests yielded contrasting results. Fu's F-statistics, which is more sensitive to background selection, was never significant, whereas Fu's F_S statistics, which is more sensitive to genetic hitchhiking and population growth, was found to be significant in five out of eight samples (Table 3). It is worth noting that F_S is negative in all populations but one; the colony F_{aub} showed a high positive value.

Demography

Six of the eight nursing colonies that were genetically variable had a mismatch distribution that fitted well with a model of population expansion (Fig. 5). The exceptions were G_{rw} and F_{aub}, which had a bimodal mismatch distribution. The estimated demographic parameters of each population (Table 4) were then used as input in a coalescent simulation program to generate DNA sequences (Schneider and Excoffier 1999) for which new sets of parameters were computed. Approximate 95% confidence intervals (CI) were generated after 5000

TABLE 3. Results of Fu's neutrality tests.

Nursing colony	n	F_S	F
F_{aub}	23	6.477	1.386
G_{gh}	43	−7.264**	−0.861
G_{mf}	24	−5.516*	−1.502
G_{rw}	14	−0.828	−1.123
G_{ns}	43	−7.885**	0.530
P_{wro}	29	−9.797**	−1.093
R_{zve}	18	−1.002	−0.477
R_{os}	21	−5.317**	−0.954

* $P < 0.01$; ** $P < 0.005$.

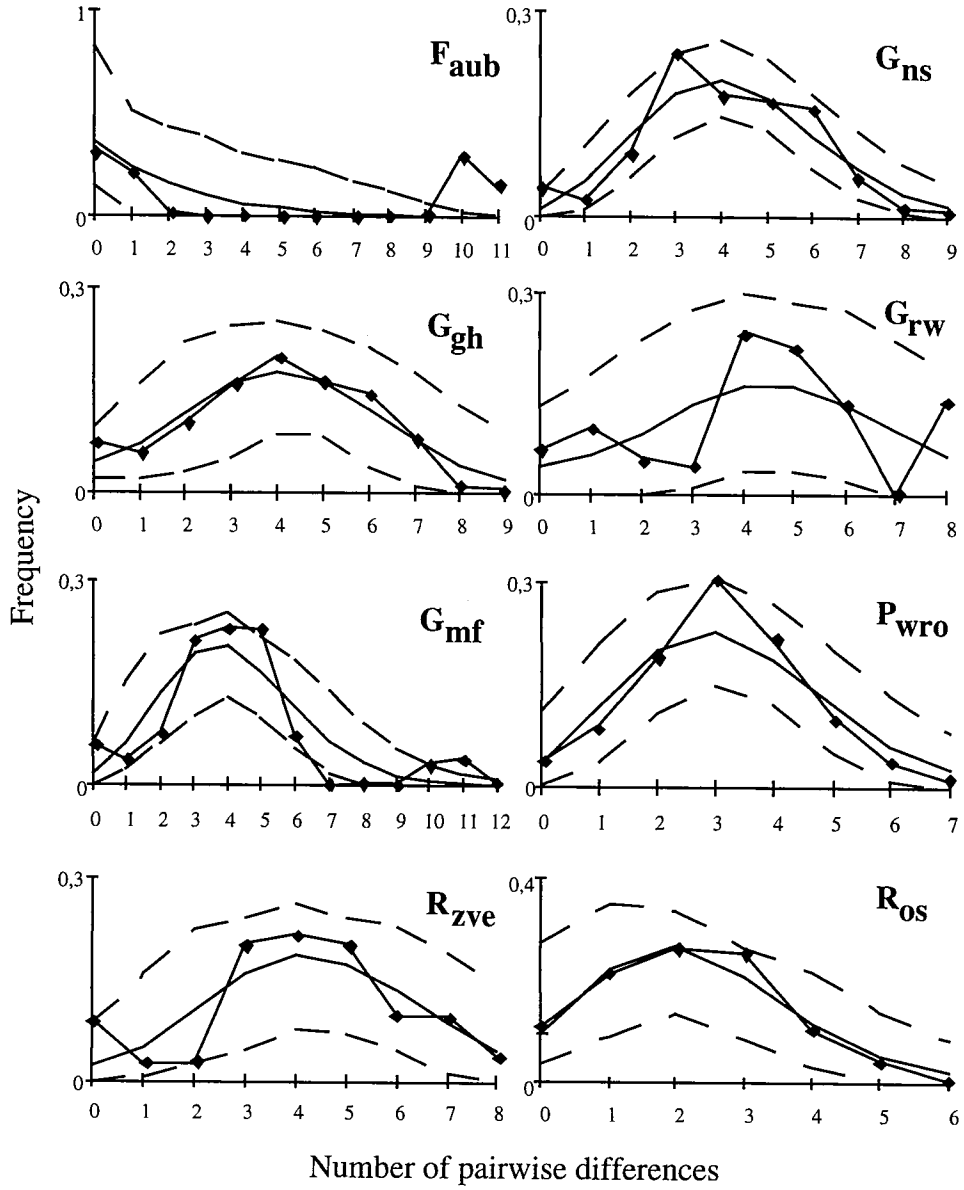

FIG. 5. Distribution of the number of pairwise differences in eight nursing colonies of the noctule bat. Diamonds represent the observed data, the line for the model fitted to the data, and dashed lines are the 2.5 and 97.5 percentile values of 1000 simulated samples.

TABLE 4. Observed (S) and 95% CI of simulated (95% CI Si) number of polymorphic sites as well as estimated demographic parameters (with their 95% CI) in eight nursing colonies of the noctule bat. τ, θ_0, and θ_1 are the age of the expansion, the population size before the expansion, and the population size after the expansion, respectively, all expressed in units of mutational time. $P(SSD_{obs})$ is the probability of observing by chance a less good fit between the observed and the mismatch distribution for a demographic history of the population defined by the estimated parameters τ, θ_0, and θ_1.

Nursing colony	S	95% CI Si	τ	θ_0	θ_1	$P(SSD_{obs})$
F_{aub}	12	1–12	5.4 (1.6–10.8)	1.8 (0–1.6)	1.8 (0.2–51.2)	0.059
G_{gh}	25	21–39	5.2 (2.7–7.8)	0 (0–2.3)	25.2 (11–3800)	0.478
G_{mf}	22	25–43	4.5 (2.0–5.7)	0 (0–2.6)	1733 (36.3–5558)	0.011
G_{rw}	20	11–27	5.8 (2.8–9.6)	0 (0–2.7)	23.8 (8.9–3878)	0.081
G_{ns}	22	40–60	4.7 (2.3–6.1)	0 (0–2.6)	3402 (54.9–7809)	0.023
P_{wro}	17	24–41	3.5 (1.7–4.6)	0 (0–1.8)	4510 (101–8373)	0.122
R_{zve}	16	17–34	5.2 (2.6–7.6)	0 (0–2.7)	59.1 (14–3949)	0.083
R_{os}	12	10–26	2.5 (0.7–3.6)	0 (0–1.9)	143 (4.9–4554)	0.441

simulations for the mismatch distribution and the number of polymorphic sites. The estimations of θ_0 and θ_1, and the P value of the SSD_{obs} statistic reveals that the pattern of polymorphism observed in F_{aub} can indeed be explained under a population stationarity model. Thus, for this population a model of sudden expansion does not seem compatible with the data. For the five colonies for which the model of population expansion was judged aceptable by the SSD test (G_{gh}, G_{rw}, P_{wro}, R_{zve}, and R_{os}), the observed number of polymorphic sites were compared to the 95% CI generated by the simulations (Table 4): only G_{gh}, G_{rw}, and R_{os} had observed values consistent with the simulated values, meaning that the parameters estimated by the model were accurate enough to account for the observed polymorphism only in these colonies.

In a model of population growth, the age of the expansion can be approximately estimated by the mode of the mismatch distribution τ expressed in units of mutational time (Rogers and Harpending 1992), as $\tau = 2ut$, where t is the expansion time expressed in generations, and u is the mutation rate for the whole sequence. The estimated values of τ were 5.2 for G_{gh}, 5.8 for G_{rw}, and 2.5 for R_{os} (Table 4). The mutation rate of HV II was calibrated using the data of the ND1 gene following the method of Quinn (1992). The idea is to compare the relative rate of evolution of two portions of DNA, one absolute rate being known. To do this estimation, we used the twenty individuals for which both HV II and ND1 sequences were available. A total of 32 substitutions (29 transitions and 3 transversions) among 337 bp was observed in the control region of the twenty noctule bats. Using a Kimura two-parameter model with gamma distribution of mutation rates ($\alpha = 0.14$), this corresponds to an estimate of 76.24 mutations having occurred along the tree. In the ND1 sequences of the same individuals, there were 15 substitutions (14 transitions and 1 transversion) among 906 bp. Assuming the same correction for multiple substitution, but with an α estimate of 0.24 (M. Ruedi, pers. comm.), the estimated number of mutations along the tree is 16.29. The HV II control region thus evolves approximately (76.24/337)/(16.29/906) = 12.6 times faster than the ND1 gene. The rate of divergence of the ND1 has been estimated to be 0.5–2% My^{-1} (Lopez et al. 1997) so that the rate of divergence of the hypervariable domain II of the control region of the noctule bat is 6.3–25.2% My^{-1}. These estimations are consistent with other estimations reported for the control region of different species (11.5–17.3% My^{-1} in humans, Vigilant et al. 1991; 10% My^{-1} in *Mus musculus*, Nachman et al. 1994; 8.3–14.3% My^{-1} in *Sorex*, Stewart and Baker 1994; 20.8% My^{-1} in *Chen caerulescens*, Quinn 1992). Using an average divergence rate of 20% My^{-1} results in expansion time estimations of 38,576 years (95% CI: 20,030–57,864 yr) for G_{gh}, 43,027 years (95% CI: 20,570–71,217 yr) for G_{rw} and 18,546 years (95% CI: 5,193–26,706 yr) for R_{os}.

For G_{gh}, G_{rw}, R_{os} and the other colonies except F_{aub}, the upper limit of the 95% confidence interval of θ_0 (the population size before the expansion calibrated by the mutation rate), was lower than lower limit of the 95% confidence interval of θ_1 (the population size after the expansion calibrated by the mutation rate) (Table 4), suggesting that the colonies underwent a demographic expansion, an expansion that is statistically supported for only three colonies (G_{gh}, G_{rw}, and R_{os}). However, these confidence intervals were too large to yield accurate estimates of the magnitude of the expansions.

Discussion

Within-Population Genetic Variability

Contrary to expectations, the answer to our first question was that genetic variability did not decrease with increasing latitude. In fact, the group of colonies from Central and Eastern Europe, which were the northernmost sampled populations, were also the most polymorphic. As the accumulation rate of new mutations depends on mutation rate as well as genetic drift, the recovery of genetic variability is facilitated when the mutation rate is high and when the effect of genetic drift is relaxed. Both conditions are met here since HV II is one of the fastest evolving DNA regions, and noctule populations could have undergone demographic expansion after the Younger Dryas if one admits a very fast evolutionary rate for HV II (see below). Studying the polymorphism of the control region in European populations of the greenfinch, Merilä et al. (1997) found that genetic variability decreased with latitude, even though these populations had grown. A decrease of genetic variability along colonization routes was also detected in studies of highly variable genetic markers in human populations (Vigilant et al. 1991; Tishkoff et al. 1996; Jorde et al. 1997). In both cases, the authors concluded that the (re)colonization process had been achieved through bottlenecks. This suggests that rapid accumulation of new mutations alone can not account for the pattern observed in the noctule.

As an additional explanation, we suggest that there could have been a whole range shift from a southern refuge area done quite progressively in parallel with the recolonization of the deciduous forest. Under this scenario, no strong bottleneck is required. This hypothesis is supported by several lines of evidence. First, almost all current nursing colonies are located north of potential refuge areas, although these regions are still covered by forests. Second, the analysis of pairwise ϕ_{ST}s showed that the eastern colonies were more related to colonies from Central Europe than to each other (Fig. 1). With gene flow being limited between colonies as indicated by the AMOVA (Table 2), this result might be the footprint of historical rather than actual factors. Furthermore the estimates of population expansion times τ indicated that the easternmost population was also the youngest one. Both results are consistent with the fact that the habitat of the noctule bat expanded eastwards after the Younger Dryas (Huntley 1990). Finally, the noctule bat is a highly vagile species which can migrate more than 1000 km between its summer and winter roosts (Strelkov 1969); it has no problem tracking its suitable environment (Hewitt 1996).

Another surprising result was that the southernmost colonies were also least polymorphic. Low genetic variability is expected either in populations where long-term population size is low or in post-bottleneck populations (see e.g., Harpending et al. 1998). In the former case, the colonies of I_{cer}, F_{aub} and F_{lig} would be remnant populations from the Younger Dryas. They would have survived in suboptimal ecological conditions; therefore, having low population size and much

lower genetic variability than the northern populations which live in better ecological conditions. One could thus expect that F_{aub} and F_{lig} should be more closely related to each other than they are to the other colonies. However, the analysis of pairwise ϕ_{ST}s shows that these populations are more closely related to Central European colonies than they are to each other (Fig. 1). Furthermore, they significantly differ from each other. Foundations of new colonies from a reservoir of genetic variability located in Central Europe might better explain this global pattern. Nursing colonies of the noctule bat are mainly distributed in regions characterized by a continental climate (north of the Alps and west of the Rhine). F_{aub}, F_{lig}, and I_{cer} are outside these boundaries and may be situated in suboptimal habitats. When individuals cross an ecological barrier toward less suitable habitats, the crossing may lead to lower survival rates and less reproductive success (known as the "edge effect", Lande 1988), and therefore to strong founder effects.

Historic Demography

The comparison of the two tests of neutrality (Table 3) and the star-like topology of the MST both indicate that the pattern of genetic variability observed in colonies of *Nyctalus noctula* is consistent either with genetic hitchhiking by an advantageous mutation or with population expansion (Maruyama and Birky 1991; Slatkin and Hudson 1991; Fu 1997). Choosing between the selective and the demographic hypothesis would require comparing the behavior of several unlinked loci (Tajima 1989). Unfortunately, we lack this information. When documented, the comparison of several datasets in taxa that expanded geographically sometime in the past is always in favor of the demographic explanation (Lavery et al. 1996; Tishkoff et al. 1996). This is, in particular, the case for the greefinch (Merilä et al. 1997), a passerine with similar dispersal abilities as the noctule and which underwent the same climatic changes during the last 20,000 years. Thus, the hypothesis of population growth will be favored in the rest of the discussion.

Beside F_{aub}, there was a good fit of observed mismatch distributions to the model of sudden population growth. We could indeed statistically show that three populations had experienced a sudden expansion. However, evaluation of our model revealed that it was unable to account for the diversity of four of seven populations. This can be due to either an inaccurate mutation model (Bertorelle and Slatkin 1995, Aris-Brosou and Excoffier 1996) or to a demographic history other than a simple sudden expansion, for example, if some colonies result from an admixture event (Marjoram and Donnelly 1994).

Estimations of the age of the expansions given by the analysis of the mismatch distributions largely exceeded the end of the the last European cold phase, some 10,000 years ago. This discrepancy could be due to the fact that we chose a relatively moderate divergence rate of 20% My^{-1} to make these inferences. Recently, two separate studies of the rate at which mutations occurred along a known pedigree structure in humans have suggested that the divergence rate of the control region might be as high as 250% My^{-1} (Howell et al. 1996; Parsons et al. 1997). The use of these new values would lead to the following estimations of time of expansion: 3,090 years (95% CI: 1600–4690 yr) for G_{gh}, 3,500 years (95% CI: 1660–5880 yr) for G_{rw} and 1480 years (95% CI: 360–2260 yr) for R_{os}, which would make these estimates of expansion times too recent. Using the lower limit of the 95% CI estimate of Parsons et al. (1997), which is 120% My^{-1}, still leads to expansion times which would be younger than the end of the Younger Dryas. Thus, the idea of a demographic expansion at the end of the Younger Dryas would only be supported if we appropriately calibrated our molecular clock for this purpose. In contrast with our results, studies of human mitochondrial DNA showed some agreement between archeological and genetic estimates of population expansion dates using standard mutation rates (Rogers 1995; Comas et al. 1996). An alternative to our hypothesis of post-Younger Dryas expansion would be to accept the hypothesis of an earlier Pleistocene expansion using the conventional mutation rate. This implies that there would have been no severe population bottleneck during the last ice age and no subsequent demographic expansion. Note that this scenario is compatible with the progressive range shift hypothesis discussed above.

Refugia

Taking into account the divergence rate of the ND1 gene (0.5–2% My^{-1}, Lopez et al. 1997) points to a separation between the three groups of haplotypes that is older than the Last Glacial Maximum (18,000 yC^{14} BP). A plausible explanation for the occurrence of different clades is that the noctule bat survived in different refugia during the last glaciations, and that these refugia were isolated from each other, as forest refugia were (Bennett et al. 1991). There is no clear indication of where the refugia of noctule populations were located, but one plausible area is the Balkans which were the main Quaternary forest refugia (Bennett et al. 1991) and from which the only Pleistocene remains of *Nyctalus noctula* are known (Popov and Ivanova 1995). In F_{aub} and in GB_{lon}, the presence of a haplotype that belongs to one of the two long branches of the MST together with haplotypes that belong to the star-like structure shown in Figure 2 suggests that at least another refuge area may have existed in Western Europe, and also that these colonies could have been formed by an admixture process.

The effect of Quaternary glaciations has been recognized and studied in different European taxa, showing the importance of the Balkans, Italy, and the Iberic peninsula as refuge areas (Hewitt 1996; Taberlet et al. 1998). However, reliable postglacial colonization routes have been identified only when an extensive sampling of the potential refugia was possible (see for example Taberlet and Bouvet 1994; Cooper et al. 1995; Dumolin-Lapègue et al. 1997). The actual distribution range of the noctule bat seems to be almost completely disconnected from its Quaternary distribution range, and our results on the population structure show that the southernmost colonies are more likely to result from recent colonizations coming from the North than to be relict representatives of Pleistocene populations. A good sampling of the potential refugia is therefore impossible in this species. Even if we could identify three clades for which distribution is consistent

with the existence of refugia in the Balkans and Southwestern Europe, it would then be difficult to reach definitive conclusions concerning the number and the location of refugia for the noctule bat.

Conclusions

Although not providing a definitive scenario for the postglacial history of European noctule bat populations, the pattern of mtDNA variability we observed suggest: (1) as more and more taxa are surveyed, compelling evidence is accumulating that the last glacial events have had a major effect on the genetic polymorphism of most species. At least at large geographic scales, this warrants the use of methods which do not make a priori assumptions on population structure because it is then difficult to predict the relative weights of current gene flow and past events in the partition of genetic diversity. Such methods comprise cladistic (i.e., Templeton et al. 1995) and phenetic (i.e., Monmonier's algorithm) approaches; (2) more reliable estimations of mutation rates are needed before any paleobiological scenario can be seriously tested; and (3) classical models of genetic changes consequent to ice ages predict that both contraction and expansion phases would lead to loss of diversity (Hewitt 1996). The noctule bat and the tephritid fly *Urophora cardui* (Eber and Brandl 1997) cases show that the expansion phase does not necessarily lead to reduced genetic diversity. Whether the absence of bottlenecks in (re)colonization processes is a more common phenomenon than previously believed clearly requires more empirical support.

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APPENDIX 1

Name, abbreviation, coordinates, year(s) of sampling, and sample size of the sampling locations.

Nursing colonies	Longtown	GB_{lon}	55°N/2.6°W	1996	3
	Aubeterre	F_{aub}	45.3°N/0.2°E	1995	23
	St-Jean Ligoure	F_{lig}	45.7°N/1.4°E	1995	17
	Cervia	I_{cer}	44.3°N/12.3°E	1996	18
	Große Heide	G_{gh}	53.2°N/13.7°E	1993	43
	Melzower Forst	G_{mf}	53.2°N/13.9°E	1992–1993	24
	Rittgartner Wald	G_{rw}	53.5°N/13.8°E	1993	14
	Neuhaus/Höchstadt	G_{nh}	49.7°N/10.8°E	1993	22
	Schloßgarten Erlangen	G_{se}	49.6°N/11°E	1993	21
	Wroclaw	P_{wro}	51.1°N/17°E	1996	29
	Zvenigorod	R_{zve}	55.7°N/36.8°E	1996	18
	Sakmara	R_{sak}	52°N/55.3°E	1995	12
	Orenburg	R_{ore}	51.8°N/55°E	1995	9
Winter colonies	Vaux-le-Pénil	Vlp	48.5°N/2.7°E	1997	20
	Altkirch	Alt	47.6°N/7.2°E	1995	10
	Munich	Mun	48.1°N/11.6°E	1996	24
	Vienna	Vie	48.2°N/16.4°E	1993	23
	Kiel	Kie	54.3°N/10.1°E	1988–1993	34

APPENDIX 2

Name, location(*), and sequence (5′ → 3′) of the primers.

ER63, L16517, CATCTGGTTCTTACTTCAGG, Fumagalli et al. 1996
ER88, H607, AGGACCCATCTAAGCATTTTCAGTG, Worthington Wilmer et al. 1994
ER86, L16558, GACATCACGATGGACTAATGACTAATC, this study
ER65, L2985, CCTCGATGTTGGATCAGG, A. Janke, pers. comm.
ER66, H4419, GTATGGGCCCGATAGCTT, A. Janke, pers. comm.
ER70, L3073, CAGACCGGAGTAATCCAGGTCGGTT, this study
ER89, H4272, CTCTATCAAAGTAACTCTTTTATCAGA, this study
ER175, L3664, GGCTGGGCCTCAAACTCNAAATA, this study

* L and H refer here to the light and heavy strands of the mitochondrial DNA, respectively, and the numbers correspond to the notation of Anderson et al. (1981).