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Mitochondrial DNA variation and population structure of the island endemic Azorean bat (*Nyctalus azoreum*)

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Abstract

The Azorean bat *Nyctalus azoreum* is the only endemic mammal native to the remote archipelago of the Azores. It evolved from a continental ancestor related to the Leisler's bat *Nyctalus leisleri* and is considered threatened because of its restricted and highly fragmented distribution. We studied the genetic variability in 159 individuals from 14 colonies sampled throughout the archipelago. Sequences of the D-loop region revealed moderate but highly structured genetic variability. Half of the 15 distinct haplotypes were restricted to a single island, but the most common was found throughout the archipelago, suggesting a single colonization event followed by limited interisland female gene flow. All *N. azoreum* haplotypes were closely related and formed a star-like structure typical of expanded populations. The inferred age of demographic expansions was consistent with the arrival of founder animals during the Holocene, well before the first humans inhabited the Azores. Comparisons with a population of *N. leisleri* from continental Portugal confirmed not only that all *N. azoreum* lineages were unique to the archipelago, but also that the current levels of genetic diversity were surprisingly high for an insular species. Our data imply that the Azorean bat has a high conservation value. We argue that geographical patterns of genetic structuring indicate the existence of two management units.

Keywords: Chiroptera, colonization, D-loop, genetic structure, island, mismatch analysis

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Introduction

Remote archipelagos have played a significant role in the study of speciation processes. Islands are also the focus of conservation efforts because insular endemic species are the most vulnerable to extinction (Frankham *et al*. 2002). The combination of small population size, fragmented distribution and isolation often causes a reduction in genetic diversity, leading to the loss of potential to adapt to sudden environmental changes (Hoffmann *et al*. 2003).

The Azores consists of nine volcanic islands located in the North Atlantic about 1500 km west of mainland Portugal (Fig. 1). They are subdivided into three groups of islands, the Occidental Group (Corvo and Flores), the Central Group (Faial, Pico, São Jorge, Graciosa and Terceira) and the Oriental Group (São Miguel and Santa Maria). These islands were formed along spreading mid-oceanic ridges during relatively recent times. Santa Maria is believed to be the oldest, around 8 million years (Abdel-Monem *et al*. 1975), and Pico the youngest, around 0.3 million years (Chovelon 1982).

The Azorean bat *Nyctalus azoreum* (Thomas, 1901), the only known endemic land mammal in the Azores, has been reported from most of the islands (Palmeirim 1991; Speakman & Webb 1993). It is common on São Miguel and on islands of the Central Group, but is rare on Santa Maria, and is absent from the Occidental Group (Rainho *et al*. 2002). Because the Azorean bat is endemic to this small archipelago and lives in highly fragmented populations, it is considered to be a threatened species. It is thought to have evolved from a continental ancestor related to the Leisler's bat *Nyctalus leisleri* (Palmeirim 1991; Speakman & Webb 1993).

To understand the evolutionary significance of this insular species, gene diversity and population structure were examined throughout the archipelago. In particular, the current genetic variability was measured within and among islands, using a mitochondrial marker, to infer levels of

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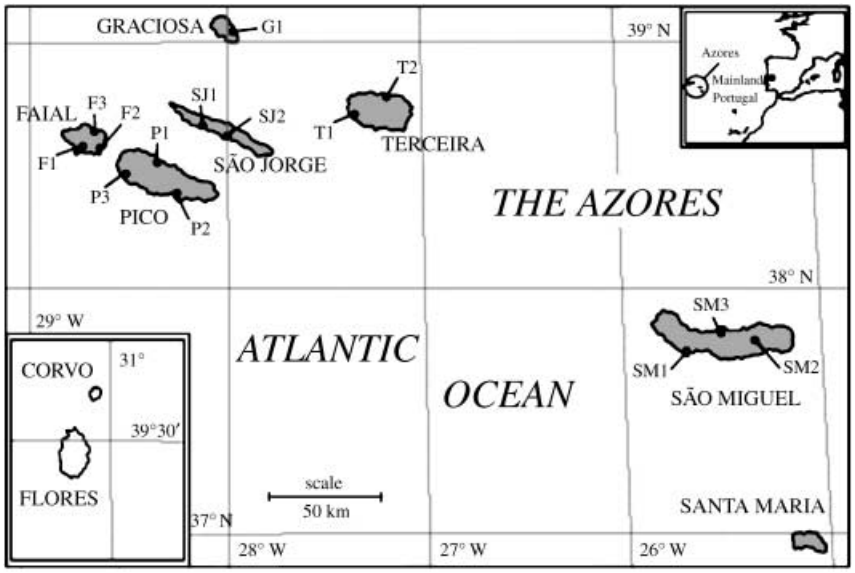

Fig. 1 Map of the Azores Archipelago with the localization of the 14 sampled colonies (see Table 1 for descriptions). Islands with confirmed presence of *Nyctalus azoreum* are shaded in grey.

population connectedness. Our results not only demonstrate that *N. azoreum* has unique haplotypes and a marked genetic structuring, but also suggest that the bat's ancestor colonized this remote archipelago in Holocene times. These results are relevant for the maintenance of the maximal evolutionary potential of the unique Azorean bat.

Materials and methods

Sampling

During the summer of 2001, 14 nursery colonies of *Nyctalus azoreum* were sampled throughout its current range (Fig. 1). Ten of the nurseries were located in abandoned buildings, while three were found in coastal cliffs and only one in a tree. Between six and 21 individuals were sampled in each colony. Tissue samples were obtained from a nonlethal, sterile biopsy punch of the wing membrane (Worthington Wilmer & Barratt 1996) and were preserved in 95% ethanol. To provide a comparative population of *Nyctalus* from the mainland, 27 individuals of *Nyctalus leisleri* were sampled in continental Portugal during June 2002. These continental bats were not captured in roosts, but were mist-netted over a water tank. This sample included 16 pregnant females and 11 adult males, which might have originated from several neighbouring colonies. The gene diversity measured in this continental sample might therefore be an overestimate of the diversity within breeding colonies.

DNA extraction

Genomic DNA was extracted from the wing punches following a salt/chloroform procedure modified from (Miller *et al*. 1988) by adding one step of chloroform : isoamyl alcohol (24 : 1) extraction to the original protocol. The purified DNA was re-suspended in 100 µL of sterile water.

Polymerase chain reaction amplification and sequencing

We amplified the hypervariable domain (HVII) of the mitochondrial D-loop via a polymerase chain reaction using the primers L16517 (Fumagalli *et al*. 1996) and sH651 (Castella *et al*. 2001). Amplifications were performed in 50-µL reaction volumes under the conditions described in Castella *et al*. (2001). Sequencing was carried out with primer L16517 and BigDye Sequencing mix (Applied Biosystems) following the manufacturer's instructions. Sequencing reaction products were electrophoresed on an ABI PRISM-377 automated sequencer (Applied Biosystems).

Data analysis

The HVII D-loop sequences were aligned with SEQUENCHER 3.0 (Gene Codes Corp.). Haplotypes were connected on a network obtained using the 95% parsimony criterion implemented in the program TCS (Clement *et al*. 2000). Levels of gene diversity within each island and colony were described as haplotype (h) and nucleotide (π) diversities using ARLEQUIN 2.0 (Schneider *et al*. 2000). Genetic differentiation among populations was quantified by performing a global test of differentiation among samples (Raymond & Rousset 1995) and by computing pairwise Φ_{ST}. These analyses take into account the presence of indels, which differentiate some haplotypes.

Some bats are known to be reluctant to fly over open bodies of water (Castella *et al*. 2000). Thus, to understand

the influence of geographical barriers on the population differentiation, we performed correlation analyses between the matrices of pairwise Φ_{ST} and two distinct measures of geographical distance. One was the straight geographical distance between pairs of colonies, measured in km (GeoDist). The other was the minimum sea-crossing distance (SeaDist) estimated by summing the shortest sea-crossing distance between island pairs using, where possible, intervening islands as stepping-stones (Hisheh *et al*. 1998). While the straight geographical distance might simply reflect an isolation-by-distance pattern of gene flow, the latter distance focuses on the sea distance as a barrier to dispersal. Since the two measures of geographical distance are highly correlated ($r = 0.99$), we performed partial Mantel tests to assess which of the geographical distances added the most significant effect to the comparison with Φ_{ST}, when the other was already taken into account (Smouse *et al*. 1986).

Moreover, we examined population structure within and among islands with two hierarchical analyses of molecular variance (AMOVA, Excoffier *et al*. 1992). The first analysis considered each island as a distinct group, and the second contrasted the most isolated island, São Miguel, with those of the Central Group (Fig. 1).

The coexistence of several haplotypes within the same island can result from multiple colonization events, from a founder event involving several lineages, or from mutations that accumulated *in situ* over time. If we assume that a population expansion has necessarily followed the successful colonization of an island, the mismatch distribution of haplotypes should be different in the first two situations compared to the third one. Indeed, a single founder event followed by demographic expansion would result in a unimodal distribution of pairwise differences between haplotypes (Harpending 1994). If populations resulted from several colonization episodes, the admixture of different lineages is expected to generate a multimodal mismatch distribution. This can be measured by the raggedness index (Harpending 1994). Therefore, a mismatch analysis of sequences for each island was carried out, using ARLEQUIN. To assess the significance of the observed values, 10 000 replicates were performed.

Two different approaches were used to estimate the time of colonization of the archipelago. One was based on the time-of-expansion parameter calculated in the mismatch distribution analysis (Harpending 1994), and the other was based on the mean sequence divergence as a measure of the time from the most common ancestor of insular lineages. Petit *et al*. (1999) calibrated the rate of divergence of HVII D-loop sequences for *Nyctalus noctula* at about 20% per million years. Although not ideal, this calibration is consistent with several other studies in mammals (see Petit *et al*. 1999) and can provide a rough idea of divergence time.

Results

A stretch of 396 base pairs (bp) from the hypervariable segment (HVII) of the D-loop was sequenced in all the individuals. This fragment starts at the 3′ end of the central conserved block and ends before the R2 tandem repeats (Fumagalli *et al*. 1996). In both *Nyctalus azoreum* and *Nyctalus leisleri*, the multiple R2 repeats were identical to the motif $(CGCATA)_n$ reported in *Nyctalus noctula* (Petit *et al*. 1999; Petit & Mayer 2000) or in *Myotis myotis* (Castella *et al*. 2000).

D-loop diversity

The alignment of 159 *N. azoreum* sequences resulted in 15 variable sites defining 15 distinct haplotypes (Tables 1 and 2). Most of the observed substitutions were transitions (nine), with only three transversions and two single base-pair deletions or insertions (indels). Remarkably, a third alignment gap consisted of an insertion of a stretch of 22 bp (GTT TAA TGG TTA CAG GAC ATT T), which corresponded to a duplication of positions 189–210. This insertion was unique to some colonies of the Central Group (Faial, Pico, São Jorge and Graciosa), and was absent from São Miguel, Terceira and from other species of *Nyctalus* sequenced so far (Petit *et al*. 1999; Petit & Mayer 2000; personal observations). To avoid overestimating differentiation because of this large insertion, it was treated as a single mutational event in all subsequent analyses.

The 27 continental *N. leisleri* sequenced for the same HVII segment presented only three distinct haplotypes (Table 1). These haplotypes differed by one or two mutations from each other (Fig. 2 and Table 2), but were distinct from Azorean lineages at least by six mutations. All sequences are available at GenBank (accession numbers AY756598–AY756615).

The parsimony network of haplotypes (Fig. 2) revealed a star-like topology for the Azores samples. The haplotype in the centre of that star (A7) was the most abundant and was the only lineage found in all the islands, while haplotypes observed on the lateral branches were usually specific to one or a few islands (Table 2). The other two widespread haplotypes (A4 and A10) showed very disjunct distributions, as A4 was restricted to the Central Group, while A10 was found only in Graciosa, Terceira and São Miguel. Graciosa was the only island where these two haplotypes coexisted. Overall, eight of the 15 insular haplotypes were found on a single island (Table 2) but four to eight haplotypes were found on each island (Table 1).

Population differentiation and geographical structure

As most haplotypes were shared among colonies within the same island, population differentiation at this level

Table 1 Molecular variability of 14 colonies of *Nyctalus azoreum* and one group of *N. leisleri* specimens from Portugal

Islands/Colonies	*n*	*nh*	*h* ± SD	π ± SD	τ (95% CI)
Nyctalus azoreum					
Faial Island					
F1 — Feteiras	10	5	0.822 ± 0.097	0.004 ± 0.003	
F2 — Horta	10	5	0.844 ± 0.080	0.004 ± 0.003	
F3 — Espalhafatos	11	4	0.673 ± 0.123	0.003 ± 0.002	
Total	**31**	**6**	**0.751 ± 0.053**	**0.004 ± 0.003**	**1.4 (0.5–2.5)**
Pico Island					
P1 — São Roque	11	3	0.655 ± 0.112	0.002 ± 0.002	
P2 — Lajes	11	4	0.709 ± 0.099	0.002 ± 0.002	
P3 — Mirateca	10	2	0.556 ± 0.075	0.001 ± 0.001	
Total	**32**	**5**	**0.796 ± 0.031**	**0.003 ± 0.002**	**1.5 (0.2–2.1)**
São Jorge Island					
SJ1 — Manadas	14	5	0.769 ± 0.076	0.003 ± 0.002	
SJ2 — Boa Hora	11	3	0.473 ± 0.162	0.002 ± 0.002	
Total	**25**	**5**	**0.660 ± 0.074**	**0.003 ± 0.002**	**1.1 (0–1.8)**
Terceira Island					
T1 — Cinco Ribeiras	6	2	0.333 ± 0.215	0.001 ± 0.001	
T2 — Quatro Ribeiras	14	4	0.659 ± 0.090	0.002 ± 0.002	
Total	**20**	**4**	**0.616 ± 0.067**	**0.002 ± 0.002**	**0.9 (0–1.5)**
Graciosa Island					
G1 — Praia	**21**	**4**	**0.695 ± 0.070**	**0.003 ± 0.002**	**1.5 (0–2)**
São Miguel Island					
SM1 — Ponta Delgada	9	3	0.639 ± 0.126	0.003 ± 0.002	
SM2 — Furnas	11	6	0.836 ± 0.089	0.005 ± 0.003	
SM3 — Ribeirinha	10	4	0.711 ± 0.118	0.004 ± 0.003	
Total	**30**	**8**	**0.777 ± 0.053**	**0.004 ± 0.003**	**1.9 (0.7–3.7)**
Nyctalus leisleri					
Serra do Açor (mainland)	**27**	**3**	**0.416 ± 0.095**	**0.002 ± 0.001**	**—**

Total and mean values are given in bold for each island. Letters correspond to the colonies located in Fig. 1. *n* is the number of individuals sequenced; *nh*, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; SD, standard deviation. τ is the parameter of time of expansion inferred from mismatch distributions with 95% of confidence interval (CI).

was generally weak (Φ_{ST} between −0.08 and 0.16) and nonsignificant.

One major exception was P3 on Pico (Fig. 1). This colony shared two haplotypes (A1 and A4) with all colonies from the adjacent island of Faial, while none of them were found in the other two colonies from Pico. Accordingly, P3 was highly differentiated from the other two colonies on Pico (Φ_{ST} = 0.66 and 0.62, $P < 0.001$), but only marginally so from the colonies of Faial (Φ_{ST} between 0.21 and 0.29, $P \leq 0.05$). Looking at a broader geographical scale, the Central Group islands of Faial, Pico and São Jorge, and those of Graciosa and Terceira, were only weakly differentiated from each other (Table 3). All other pairwise comparisons suggested a highly significant structure among islands, and thus very restricted interisland gene flow. To obtain quantitative estimates of female gene flow among islands, we intended to use a maximum likelihood approach based on the coalescent theory, as implemented, for example in MIGRATE 1.7.3 (Beerli & Felsenstein 2001). However, as many haplotypes differed by very few mutations, or just by indels which cannot be modelled in these likelihood approaches (Abdo *et al*. 2004), our Bayesian estimates failed to converge and gave inconsistent results from one run to another (results available from the senior author). We await the implementation of more sophisticated models of DNA evolution to undertake more quantitative estimates of gene flow.

In the two designs explored (all islands treated separately or the Central Group vs. São Miguel), the partition of molecular variance revealed by the hierarchical AMOVA showed that most variance was contained within colonies (62 and 47%, respectively), while the effect of the groups accounted for an additional 23 and 39% of total variance, respectively (Table 4).

Pairwise genetic distance (Φ_{ST}) was strongly correlated with both the straight geographical distance (GeoDist) and

Table 2 Variable nucleotide positions within the 396-bp sequence of D-loop analysed in 186 bats

	Positions of variable nucleotides in base pairs (bp)			Populations															
				Faial			Pico			São Jorge		Terceira		Graciosa	São Miguel				
Haplotype	111111122212367 880015913809020	211/ 232	222222233333 333469901478 349987873689	F1	F2	F3	P1	P2	P3	SJ1	SJ2	T1	T2	G	SM1	SM2	SM3	Continental Portugal
A1	TAGTGGGAGT	22 bp	TAAAGGGA-AGC	2	1	1			5									
A2	....	.	...G.....			1				1				2				
A3	...C....A.	.	C..........	1	2													
A4	........A.	.	....	2	3	3		1	5	5	8			3				
A5	........A.	–	..G.....	1	1		3	5		2	1		1					
A6	........AC	–	....				2	1		1								
A7	........A.	–	....	4	3	6	6	4		5	2	5	5	6	5	1		
A8	......T.A.	–	.......-....												3	4	5	
A9	......T.A.	–	....A..-....													3	3	
A10	......T.A.	–	....									1	7	10	1	1		
A11	......T.A.	–	........C...														1	
A12	.T....T.A.	–	........C...														1	
A13	..A...T.A.	–	....													1		
A14	......T.A.	–	......A.C...													1		
A15	......T.A.	–	..........A										1					
Po1	C...AA..A.	–	C....A...CA.															6
Po2	C....A..A.	–	C....A...CA.															20
Po3	C...AA.GA.	–	CG...A...CA.															1

Dots indicate that the same nucleotide is present in haplotype A1. Dashes represent single base-pair indels, except between positions 211 and 232 which is an insertion of 22 bp. These variable positions define 15 haplotypes in *Nyctalus azoreum* (A1–A15) and three in *Nyctalus leisleri* (Po1–Po3 from continental Portugal). The second part of the table indicates the distribution and frequency of these haplotypes in the different islands; letter codes under each island represent three different colonies sampled and correspond to locations in Fig. 1.

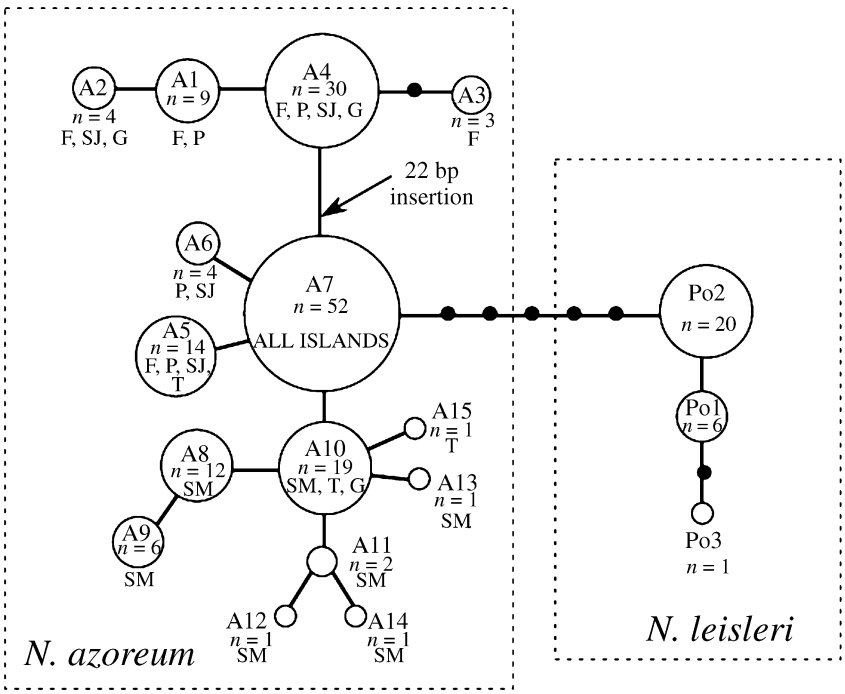

Fig. 2 Parsimony network of the 18 haplotypes (white circles) obtained by sequencing 396 bp of D-loop in 159 Azorean bats and 27 Leisler's bats (see Table 2). Filled circles represent missing (or unsampled) haplotypes. The area of each circle is proportional to the frequency of the haplotype. Each segment connecting haplotypes represents one mutation, except for the 22-bp insertion indicated by an arrow. Abbreviated island names under each haplotype indicate their location (F, Faial; P, Pico; SJ, São Jorge; T, Terceira; G, Graciosa; SM, São Miguel; Po, continental Portugal).

Table 3 Pairwise genetic differentiation (Φ_{ST}) among insular populations of *Nyctalus azoreum*

	Faial	Pico	São Jorge	Terceira	Graciosa	São Miguel
Faial	0					
Pico	0.04*	0				
São Jorge	0.02	0.02	0			
Terceira	0.22***	0.24***	0.25***	0		
Graciosa	0.21***	0.24***	0.25***	−0.02	0	
São Miguel	0.45***	0.47***	0.50***	0.15**	0.13*	0

Values are based on the Kimura two-parameter distance.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; probability that observed heterozygosity differed from expectation.

Table 4 Apportionment of molecular variance measured among populations of *Nyctalus azoreum* from the entire archipelago, or between populations from the Central Group and São Miguel

Groups	Total variance	% among groups	% among colonies within groups	% within colonies	*P*-value
Each island	0.872	22.8	14.8	62.4	0.01173
Central Group vs São Miguel	1.152	39.1	13.7	47.2	0.00961

P represents the significance of the variation among groups.

the minimum sea-crossing distance (SeaDist) ($r = 0.59$ and 0.56, $P < 0.001$). However, partial Mantel tests indicated that, once GeoDist was taken into account, SeaDist did not add any significant effect to the correlation with Φ_{ST} ($r = -0.288$, $P = 0.973$). In the reverse situation, GeoDist still added significant variance when SeaDist was primarily considered ($r = 0.375$, $P = 0.006$).

Population expansion

The unimodal distribution of pairwise differences among haplotypes (Fig. 3) and the nonsignificant raggedness index (results not shown) were consistent with a model of sudden expansion for each island. The parameters of expansion τ estimated for each distribution (Table 1) were

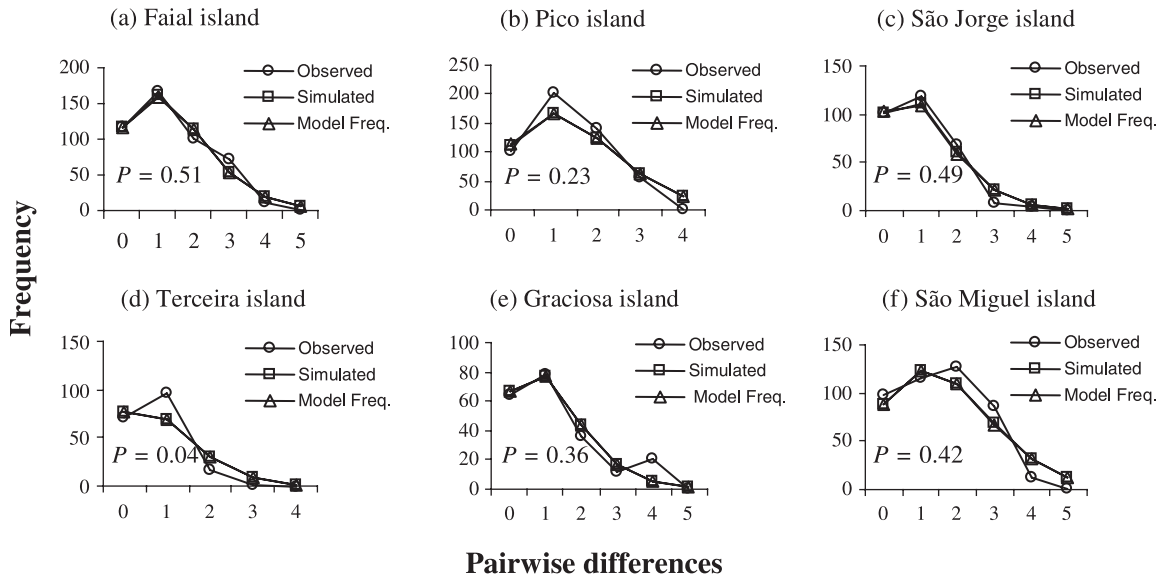

Fig. 3 Mismatch distributions of the D-loop region in six island populations of *Nyctalus azoreum*. These curves represent the frequency distribution of pairwise differences. *P*-values represent the probability that the variance of the simulated data set is equal to or greater than the observed data set.

consistent among islands and varied between 0.9 (Terceira) and 1.9 (São Miguel). Assuming a mutation rate of 20% per million years, i.e. equal to that calibrated for the HVII of *N. noctula* (Petit *et al*. 1999), and a generation time of 2 years, these expansion times would correspond approximately to 12 032 and 25 400 years, respectively.

Discussion

This paper represents, to our knowledge, the first population genetic study of an endemic vertebrate from the Azores. Mitochondrial DNA haplotypes suggest that the various Azorean bat populations surveyed originated from a single natural colonization event. Their current population structure throughout the archipelago is therefore shaped by both historical and current factors limiting gene flow among islands.

Genetic diversity and levels of gene flow

Most island populations show reduced genetic diversity because of founder effects. *Nyctalus azoreum* is not an exception as the mean nucleotide diversity (mean $\pi = 0.003$) measured at the highly variable D-loop is low compared to the mean values found in other mainland European species such as *Nyctalus noctula* ($\pi = 0.009$; Petit *et al*. 1999) or *Myotis myotis* ($\pi = 0.006$; Ruedi & Castella 2003). However, the mean haplotypic diversity found in breeding colonies of Azorean bats ($h = 0.67$, Table 1) is not impoverished when compared to that of these continental vespertilionids ($h = 0.74$ and 0.49, respectively). The haplotypic diversity of *N. azoreum* is even higher than that of the reference population of *N. leisleri* from continental Portugal ($h = 0.42$, Table 1). Although this reference population might in fact be unusually uniform in terms of mitochondrial diversity (more continental samples should be checked for this), this comparison indicates that current populations of *N. azoreum* have maintained substantial genetic variability in spite of their insular and highly fragmented distribution.

Furthermore, the Azorean bats showed a strong genetic discontinuity between colonies found on São Miguel and those sampled in the Central Group ($\Phi_{ST} = 0.13–0.50$, Table 3). This differentiation accounts for 39% of the total variance measured over the archipelago (Table 4). Indeed, six of the eight haplotypes found on São Miguel are unique to this island, while seven of the nine haplotypes are unique to the Central Group (Table 2 and Fig. 2). This strong differentiation indicates that new, local mutations have accumulated in these two groups of islands with very little or no subsequent gene exchange. The insertion of a unique stretch of 22 bp present in the haplotypes A1 to A4 further supports this interpretation, as it was found only in islands of the Central Group (Fig. 2). Likewise, the single base pair indels at position 307 and at position 313 (Table 2) are found only on São Miguel. The few haplotypes shared between the Central and the Oriental Groups (A7 and A10) are probably the result of their common historical ancestry (shared ancestral polymorphism) or to convergent mutation, rather than to current gene flow. A higher proportion of haplotypes is shared among islands within the Central Group (Fig. 2), regardless of whether indels are taken into account or not (Table 2). This is supported by lower levels

of pairwise genetic differentiation (Table 3), which suggests that females do, at least occasionally, migrate to neighbouring islands. Most populations within islands also exchange breeding females, as the genetic differentiation among colonies is usually not significant (results not shown). This interpretation of ongoing gene flow within and among neighbouring islands is further supported by the significant pattern of isolation by distance displayed by the breeding colonies, regardless of whether any open sea separates them. Thus, the open sea is not a barrier *per se* to *N. azoreum*, although when combined with large geographical distances (e.g. between São Miguel and the Central Group), over-water dispersal becomes very unlikely. We stress that these conclusions are valid for females only, which are usually known to be more philopatric than males in temperate bats (Palmeirim & Rodrigues 1995; Kerth *et al*. 2000; Castella *et al*. 2001; Kerth *et al*. 2002). We are currently evaluating, with nuclear DNA markers, whether male-mediated gene flow in *N. azoreum* is similar to the patterns observed in this initial study based on mtDNA markers only.

Colonization scenario and divergence time

The Azores are isolated from any potential source area by at least 1500 km of open sea. Thus, the probability of colonization by most land mammals under natural conditions is very low. In other less isolated Macaronesian islands, man was responsible for several introductions of mammals, such as rodents and shrews (Gündüz *et al*. 2001; Vogel *et al*. 2003). Several lines of evidence support the hypothesis that the Azorean bat colonized the archipelago from a single, unidentified source of colonists and without the aid of man. First, the uniqueness of all *N. azoreum* haplotypes and their close phylogenetic relationships (within four mutations from the common A7, Fig. 2) strongly suggest that they derive from a single ancestral sequence. Second, predictions from the coalescence theory (Watterson & Guess 1977) identify the A7 haplotype as the closest to this putative ancestor. A7 is by far the commonest and the only ubiquitous sequence in the Azores. It is thus likely that the widespread occurrence of A7 results from this initial phase of colonization. Third, the star-like tree of haplotypes of *N. azoreum* (Fig. 2) and the concordant unimodal mismatch distribution of mutations among all insular populations (Fig. 3) are typical signatures of populations that underwent a sudden demographic expansion (Harpending 1994). Using a demographic model and a substitution rate of HVII calibrated for *Nyctalus* (Petit *et al*. 1999), we estimated that this expansion occurred during the early Holocene (12 032–25 400 years). This date would largely predate the first arrival of humans in the Azores in the 15th century AD. The current genetic evidence thus strongly suggests that the Azores were colonized naturally during the Holocene by a single matrilineage. During this initial stage of colonization, the ancestral lineage reached both the Oriental and the Central Groups. Subsequent movements of bats among these groups of islands were sufficiently rare to impede the spread of new mutations, which thus remained endemic to a single or to a few neighbouring islands (e.g. the large 22-bp insertion). Although a continental origin of Azorean bats is very likely, the single continental population of *N. leisleri* sampled in this study is inappropriate to identify the possible source area of the ancestors of *N. azoreum.* This question will be addressed in a forthcoming paper, using a more comprehensive sampling of European and North African *Nyctalus*.

Implications for conservation

Nyctalus azoreum is fairly abundant on several islands of the Central Group and on São Miguel (Palmeirim 1999), but its status is not well known in the smaller islands, particularly on Santa Maria, where these bats appear to be much rarer (Rainho *et al*. 2002). None of the islands of the archipelago is large, and consequently even at relatively high densities any local population is necessarily small in absolute number. In addition, the colonial behaviour of these bats, and hence the concentration of many reproductive individuals in few roosts, increases their vulnerability to direct destruction by man. This is particularly acute in the Azores since most of the known breeding colonies are found in buildings (10 of 14 sampled roosts) and bats are considered disturbing by some local people. Azorean bats are therefore at demographic risk, which justifies their current status as vulnerable in IUCN red lists (Hutson *et al*. 2001).

Besides these demographic risks, the Azorean bats bear a set of unique characteristics compared to their continental relative, *N. leisleri*. These include phenotypic differences such as: smaller size (Palmeirim 1991), higher peak frequency (32.1 kHz) of echolocation calls (Rainho *et al*. 2002; Skiba 2003) and a more diurnal behaviour (Moore 1975; Irwin & Speakman 2003); plus, highly localized genetic lineages (Fig. 2). It is not known whether the peculiar morphological or behavioural features of the Azorean bat vary within the archipelago or whether they are associated with the main genetic units underlined in this paper. However, the strong genetic discontinuity between the Central and Oriental Groups suggests that populations from these two areas are demographically autonomous, having evolved in relative isolation for a long time. Therefore, they should qualify as distinct management units (Avise 2000; Moritz 1994; Fraser & Bernatchez 2001) for conservation purposes. If translocation of bats is necessary to re-establish locally extinct populations, it should be limited to neighbouring islands, to avoid disrupting potential local adaptations. With the original vegetation cover of the islands almost completely replaced by agro-systems, which tend to evolve quickly in response to marked demands, the survival of *N.*

azoreum may depend, to some extent, on the maintenance of the species' genetic diversity and its adaptive flexibility.

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