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ORIGINAL PAPER

Genetic divergence and phylogeography in the genus *Nyctalus* (Mammalia, Chiroptera): implications for population history of the insular bat *Nyctalus azoreum*

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Abstract We used three mitochondrial DNA fragments with different substitution rates (ND1, Cyt *b* and the CR) to infer phylogenetic relationships among six species of the genus *Nyctalus*, and compare levels of genetic divergence between the insular, vulnerable *Nyctalus azoreum* and its continental counterpart to assess the origins of the Azorean bat. The larger species found throughout the Palaearctic region (*N. lasiopterus, N. aviator* and *N. noctula*) share a unique chromosome formula ($2n = 42$) and form a monophyletic clade in our reconstructions. *Nyctalus plancyi* (= *velutinus*), a Chinese taxon with $2n = 36$ chromosomes, is sometimes included in *N. noctula*, but is genetically very divergent from the latter and deserves full species status. All Cyt *b* and CR haplotypes of *N. azoreum* are closely related and only found in the Azores archipelago, but when compared to continental sequences of *N. leisleri*, levels of mtDNA divergence are unusually low for mammalian species. This contrasts with the high level of differentiation that *N. azoreum* has attained in its morphology, ecology, and echolocation calls, suggesting a recent split followed by fast evolutionary change. The molecular data suggest that *N. azoreum* originated from a European population of *N. leisleri*, and that the colonisation of the Azores occurred at the end of the Pleistocene. The Madeiran populations of *N. leisleri* also appear to have a European origin, whereas those of the Canary Islands probably came from North Africa. In spite of its recent origin and low genetic divergence, the Azorean bat is well differentiated and consequently represents a unique evolutionary unit with great conservation value.

Keywords Azores · Bat · Colonisation · Mitochondrial DNA · Phylogeography · *Nyctalus azoreum* · *Nyctalus leisleri*

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Introduction

The origin of species remains one the least well-understood and most important questions in evolutionary biology (Tregenza and Bridle 1997; Tregenza 2002). Since the most common process of speciation is considered to be the allopatric mode (e.g. Wiens 2004; Mayr 1942), isolated oceanic islands have played a major role in the development of evolutionary theory by offering unique settings for the study of spatial and temporal patterns of biological diversification (Beheregaray et al. 2004).

The Azores archipelago comprises nine islands of recent origin (8–0.04 Myr), (Borges and Brown 1999), which lies about 1,500 km west of continental Portugal. The indigenous land fauna of vertebrates has no amphibians or reptiles, but includes 21 species of birds and two mammals, the bats *Nyctalus azoreum* (Thomas 1901) and a member of the genus *Pipistrellus* with an unclear taxonomic status (Skiba 1996; Rainho et al. 2002). The only endemic Azorean mammal is *N. azoreum*, which colonised all islands but Flores and Corvo (Fig. 1). Due to this restricted distribution area, it is

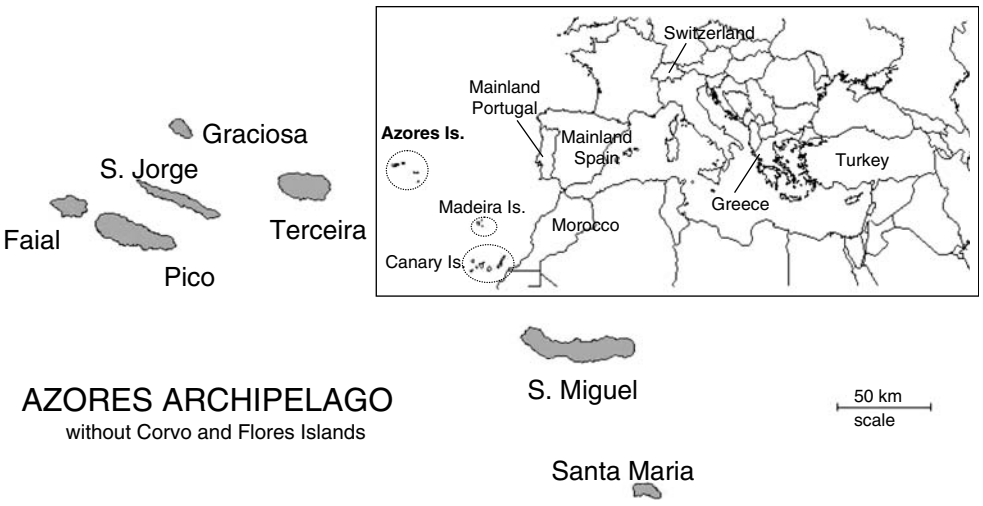

Fig. 1 Map of Europe and the Atlantic archipelagos of Canary, Madeira and Azores, showing in closer detail the Islands of the Azorean archipelago with confirmed presence of *Nyctalus azoreum*. (See Appendices 1 and 2 for further details). *N. azoreum* is absent from the islands of Flores and Corvo, so they are not shown

considered critically endangered in the Portuguese Red Data Book (Queiroz et al. 2006) and as vulnerable by the U.I.C.N. standards (Chiroptera Specialist Group 2000). Thomas (1901) described this taxon as a separate species, a view supported by authors like Miller (1912). Corbet (1978) considered it as a subspecies of *N. leisleri* (Kuhl, 1817). The latter is widespread in the Palaearctic region and occurs marginally in the western Himalayas (Simmons 2005). More recently, Palmeirim (1991) and Speakman and Webb (1993) reassessed the taxonomic status of the Azorean bat. Although they recognised a close phylogenetic relationship with *N. leisleri*, they judged the suite of morphological characters distinguishing both taxa sufficient to warrant full species status to *N. azoreum*. Externally, the Azorean bat (forearm length 35–42 mm; weight 6–15 g, personal observation) is indeed substantially smaller than Leisler's bat (forearm length 38–47 mm; weight 11–20 g; Macdonald and Barrett 1993). It also has a darker pelage, echolocation calls with a fundamental frequency 4–5 Hz higher (Rainho et al. 2002; Skiba 2003), and a strong tendency to fly and hunt during daytime (Moore 1975; Speakman 1995), which is unusual among chiroptera. The controversy about the taxonomic status of various other described subspecies or species within the genus *Nyctalus* is still ongoing (Simmons 2005).

A population genetic study based on the highly variable control region (CR) of the mitochondrial DNA revealed high genetic diversity of haplotypes within the Azorean bat (Salgueiro et al. 2004). However, all insular haplotypes were closely related to each other, suggesting that the populations of *N. azoreum* result from a single colonisation event. The strong geographic partitioning of gene diversity in the archipelago further indicates limited inter-island female gene flow. This initial study focused on patterns of gene flow among islands, but comparisons with the continental counterparts were restricted to a single population of *N. leisleri* from central Portugal.

In the present paper, we expanded the genetic comparisons to many more continental populations of *N. leisleri* sampled in Europe and North Africa and to other species in the genus *Nyctalus*. Three mitochondrial genes with different evolutionary rates were considered: nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1), cytochrome *b* (Cyt *b*), and CR. This will give new insights into the evolution of *Nyctalus* species and provide further information about the phylogenetic relationships and origins of *N. azoreum*.

Materials and methods

To assess the phylogenetic position of the Azorean bat within the *Nyctalus* radiation, we included all species of this genus recognised by Koopman (1994), except the Himalayan *N. montanus* (Barrett-Hamilton, 1906). New samples from various tissue collections were analysed together with sequences of other *Nyctalus* species already deposited in GenBank (Appendices 1, 2).

Initially, we sequenced two mtDNA genes, ND1 and Cyt *b*, which are usually very informative at the generic level (Ruedi and Mayer 2001; Avise and Walker 1999). For ND1, in addition to the tissue samples already mentioned, we used one sequence of *N. leisleri* (Ruedi and Mayer 2001), five from *N. lasiopterus* (Mayer and von Helversen 2001), 19 from *N. noctula* (Petit et al. 1999), one from *N. plancyi* (= *N. velutinus*) and one from *N. aviator* (Kawai et al. 2002). Sequences from

three species of the genus *Pipistrellus*, (*P. pygmaeus*, *P. kuhlii*) sequenced by Mayer and von Helversen (2001) and *P. abramus* sequenced by Nikaido et al. (2001), were used as outgroups in the ND1 phylogeny (see Appendix 2 for details on the origin of samples). This broader phylogenetic analysis was restricted to the ND1 data set analysis because this was the only gene for which sequences from other *Nyctalus* species are available on Genbank.

For the Cyt *b* data set, published sequences of *Nyctalus* species were scant and analyses were therefore only used for the comparison between *N. leisleri* and *N. azoreum*.

In order to increase phylogenetic resolution between the latter two taxa, a more rapidly evolving segment of the mitochondrial CR (HVII, see Salgueiro et al. 2004) was also sequenced for a subset of specimens. These bats included 25 tissue samples of *N. azoreum*, representative of most lineages revealed by CR variation in a previous study (Salgueiro et al. 2004) (Appendix 1) and 23 samples of *N. leisleri* from Portugal, Spain, Switzerland, Greece, Turkey, Czech Republic, Montenegro and Morocco (Appendix 1). We also used two samples of the insular subspecies *N. leisleri verrucosus* (Bowditch, 1825) from Madeira Island and one of *N. leisleri ssp.* from La Palma Island (Canaries archipelago). All new sequences obtained in this study are available at GenBank (accession numbers DQ887579–DQ887608).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from tissue samples 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.

The more conserved mtDNA regions were amplified via PCR with the primer pairs L14724 and H15574 (Smith and Patton 1993) for the Cyt *b*, and ER65 and ER66 (Petit et al. 1999) for the ND1 gene. Likewise, we amplified the hypervariable domain (HVII) of the mitochondrial CR using the primers L16517 (Fumagalli et al. 1996) and sH651 (Castella et al. 2001). Amplifications were performed in 50 μl reaction volumes following the conditions described in Castella et al. (2001) for the CR. The PCR profile for Cyt *b* consisted of 3 min of initial denaturation at 94°C, followed by 37 cycles of 45 s at 93°C, 45 s at 45°C, 1 min at 72°C and a final extension step of 5 min at 72°C. For the ND1 gene, the annealing temperature was 50°C, the number of cycles was increased to 40, and the intermediate step of 72°C lasted for 1.5 min.

PCR products were purified and sequenced using the primers L16517 for the CR (three individuals were also sequenced with sH651), L14724 for Cyt *b* (three individuals were also sequenced with H15574) and ER70 (Petit et al. 1999) for ND1. Sequencing reaction products were electrophoresed on an ABI PRISM-377 automated sequencer (Applied Biosystems).

Sequence and phylogenetic analysis

Sequences were edited and aligned with SEQUENCHER 4.2 (Gene Codes Corp.). The nucleotide substitutions and translation into amino acids were determined with MACCLADE 4.0, (Maddison and Maddison 1992). The alignment of the non-coding CR sequences was further adjusted by eye to minimise gaps (indels).

For each data set, an appropriate model of nucleotide substitutions was determined with the program MODELTEST 3.06 (Posada and Crandall 1998). Neighbour-Joining (NJ) trees were constructed using the probability model identified above. Phylogenetic relationships were also reconstructed with the maximum parsimony (MP) approach applying a heuristic search and TBR branch swapping. The nodal support for the resulting topologies was evaluated by 5,000 bootstrap replicates. Analyses were performed on the haplotypes with PAUP 4.0b10 (Swofford 1998). To be comparable with other studies (e.g. Bradley and Baker 2001), we have also calculated the corrected genetic divergence based on K2P (Kimura 1980) pairwise distances.

Because genetic relationships among closely related species or among populations within species can be reticulate rather than bifurcating, we also connected the haplotypes on a median-joining network (Bandelt et al. 1999) obtained with the software NETWORK 4.1.0.9 (Röhl 2004). This method combines the topology of a minimum spanning tree (Excoffier and Smouse 1994) with a parsimony—based search of the missing haplotypes (Posada and Crandall 2001). For the genes ND1 and Cyt *b* all mutations were treated with an equal weight. For the CR segment, the network obtained with all mutations equally weighted was very complex due to multiple equally parsimonious connections. We thus also applied a 5:1 weight to transversion:transition, (which corresponds to the empirical ratio observed in the CR dataset (see Results). This reduced considerably the number of possible connections and resulted in a more tractable network.

We have also estimated the pairwise distances based on HKY (Hasegawa et al. 1985) to calculate the divergence time between *N. azoreum* and *N. leisleri*. According to Ruedi and Mayer (2001) the divergence rate in the genus *Myotis* was of 4.7% Myr^{-1} for the sequences of Cyt *b* and ND1. Our data showed that the CR evolves 7.3 times faster than ND1 and 5.3 times faster than Cyt *b* (average 6.3). So, we estimated the rate of divergence of the CR of the Leisler's bat and Azorean bat at 29.61% Myr^{-1}.

Results

Divergence within the genus *Nyctalus*

The alignment of 639 bp of the ND1 gene sequenced in 75 bats from seven species resulted in 20 different haplotypes (Appendix 2). This alignment includes 218 variable characters, 152 of which are parsimony informative. According to MODELTEST, the best fit model based on hierarchical likelihood ratio tests was HKY (Hasegawa et al. 1985) with site heterogeneity, gamma shape parameter ($G = 1.503$), proportion of invariable sites ($I = 0.582$) and T_i/T_v ratio = 13.99. On the other hand, the best-fit model selected by the Akaike Information Criterion was TrN (Rodriguez et al. 1990) with gamma shape parameter ($G = 2.087$) and proportion of invariable sites ($I = 0.601$).

The NJ tree based on both distances and the MP tree were identical (Fig. 2). Except for *N. aviator*, *N. azoreum* and *N. leisleri*, the phylogenetic trees clearly separate the different species into monophyletic groups supported by strong bootstrap values. *N. azoreum* and *N. leisleri* grouped in a polytomic clade, confirming their close phylogenetic relationship. Bats from Madeira or from Canary Islands have no distinct sequences compared to those from continental *N. leisleri*.

Within any given clade (Fig. 2), sequence divergence was less than 1%. Corrected sequence divergence between *N. lasiopterus* and *N. aviator* was 4%, while comparisons between the *N. noctula* and *N. lasiopterus* were up to 6% of divergence. These three species of large *Nyctalus* are united in a clade supported strongly by all phylogenetic methods.

The comparison of the *N. leisleri/azoreum* clade with the large *Nyctalus* clade showed 15% of divergence. This was similar to the divergence found between the *N. leisleri/azoreum* clade and *N. plancyi* (17%) or the outgroup (17%).

Comparisons between *N. azoreum* and *N. leisleri*

Focusing on *N. azoreum* and *N. leisleri* alone, 48 ND1 sequences of 639 bp were aligned. This alignment showed ten polymorphic sites and defined nine different haplotypes (Appendix 1). All substitutions were synonymous transitions and none were parsimony informative.

The median-joining network based on the ND1 haplotypes of *N. azoreum* and *N. leisleri* (Fig. 3) revealed that most Azorean bats possessed a unique

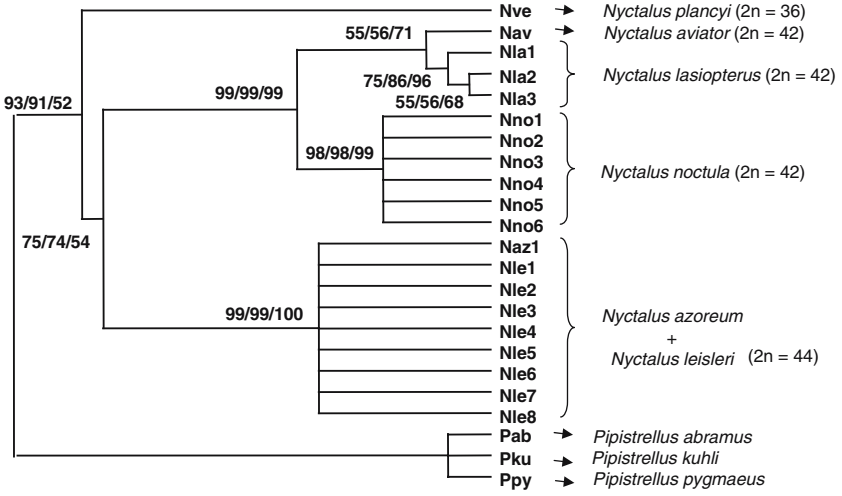

Fig. 2 Phylogenetic tree (see text for details) of relationships among *Nyctalus* species, based on a 639 bp ND1 fragment and using three species of *Pipistrellus* as outgroups (*P. pygmaeus*, *P. abramus* and *P. kuhlii*). The bootstrap values correspond respectively to the results of the following analyses: NJ (TrN and HKY distance models) and MP (heuristic search, TBR branch swapping). The haplotype codes and their origins are indicated in Appendices 1 and 2. Species karyotypes are also indicated

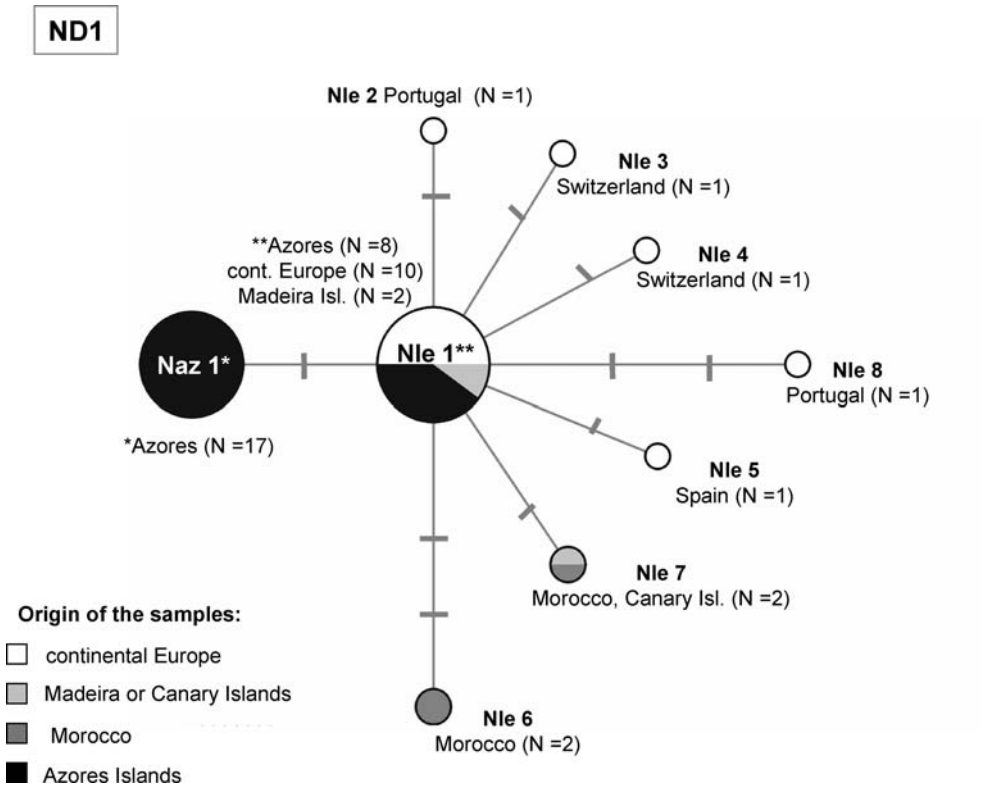

Fig. 3 Median-joining network based on nine haplotypes from 639 bp of the ND1 gene sequenced over 48 individuals of *N. azoreum* and *N. leisleri* (see Appendices 1, 2). The area of circles is proportional to the frequency of the haplotypes. Each *grey bar* in between the lines connecting the haplotypes represents one mutation. The frequency and geographic distribution of each haplotype are also presented

haplotype (Naz1), while eight others had the haplotype (Nle1) that is widespread in continental Europe. This Nle1 haplotype was also present in Madeira (Fig. 3). These two ND1 haplotypes differ by a single mutation. The Canary island haplotype (Nle7) was also found in Morocco and differed by one mutation from the widespread Nle1.

The partial segment (692 bp) of Cyt *b* was sequenced for 47 bats. We found a total of 15 polymorphic sites (one parsimony informative), yielding 10 different haplotypes (Appendix 1). All inferred substitutions were transitions, 11 were synonymous and four were at the second position. The median-joining network based on Cyt *b* haplotypes revealed two star-like structures (Fig. 4). The former corresponded to the five European and Moroccan haplotypes that radiated from the Nle1c lineage, and was shared by European, Madeiran and Canarian samples. The latter coincided with the five Azorean haplotypes, radiating from Naz1c, which also derived from the continental Nle1c lineage. No lineage was shared between mainland and Azorean samples. The maximal genetic divergence was found between a sample from Morocco (Nle3c) and an Azorean bat (Naz2c), 1.2%. The haplotype in the centre of the Azorean star-like net (Naz1c) was also the most common haplotype found in the Azores, as it is found in all islands except Terceira. The island with the highest haplotypic diversity was S. Miguel, where four of the Azorean lineages co-occurred.

As expected for a faster, non-coding segment, the alignment of *N. azoreum* and *N. leisleri* CR sequences identified more haplotypes. Forty-four variable sites, of which 17 were parsimony informative, defined 13 distinct haplotypes (Appendix 1). Most substitutions were transitions (35), with only four transversions and five deletions or insertions (indels). One of the indels was an insertion of 46 bp found in one sample of *N. leisleri* from Switzerland. The initial 22 bp of this large insertion were identical to the sequence of another insertion detected in Azorean bats (Salgueiro et al. 2004), while the remaining 24 bp were unique to the Swiss bat. This suggest that the 46 bp indel might actually result from two independent mutational events (two insertions) acquired independently in both lineages. We thus treated the 22 bp and the 24 bp indels as two independent mutations in the analyses. The median-joining network (Fig. 5) based on CR haplotypes from Azorean and Leisler's bats showed 16 exclusive Azorean lineages distributed over two star-like structures. These differed from the closest continental haplotype (Po2) by only six substitutions (2% of sequence divergence).

As for the other mitochondrial markers, the island with the highest CR haplotypic diversity was S. Miguel, which supports eight of the 16 insular haplotypes (see

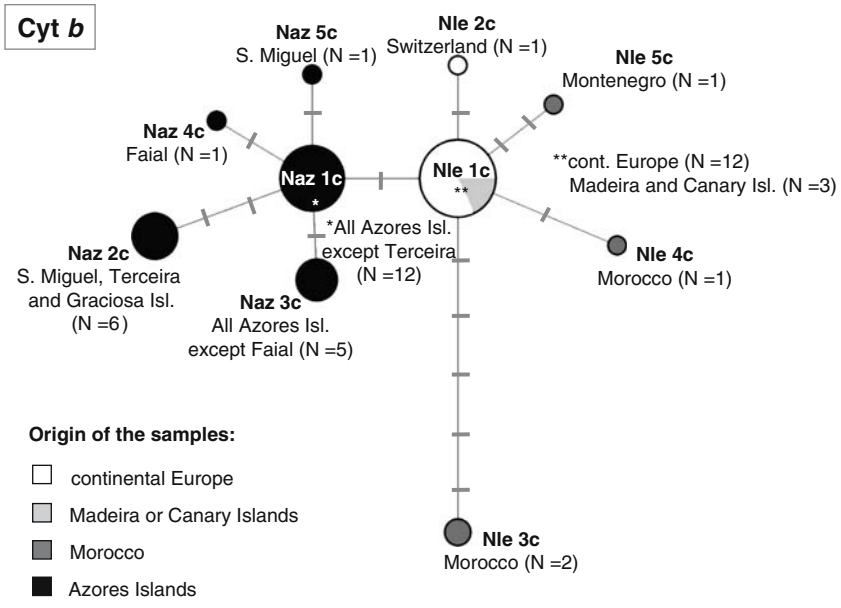

Fig. 4 Median-joining network based on 17 haplotypes evidenced in 692 bp of the Cyt *b* gene sequenced over 47 individuals of *N. azoreum* and *N. leisleri* (see Appendices 1, 2). The area of circles is proportional to the frequency of the haplotypes. Each *grey bar* in between the lines connecting the haplotypes represents one mutation. The frequency and geographic distribution of each haplotype are also presented

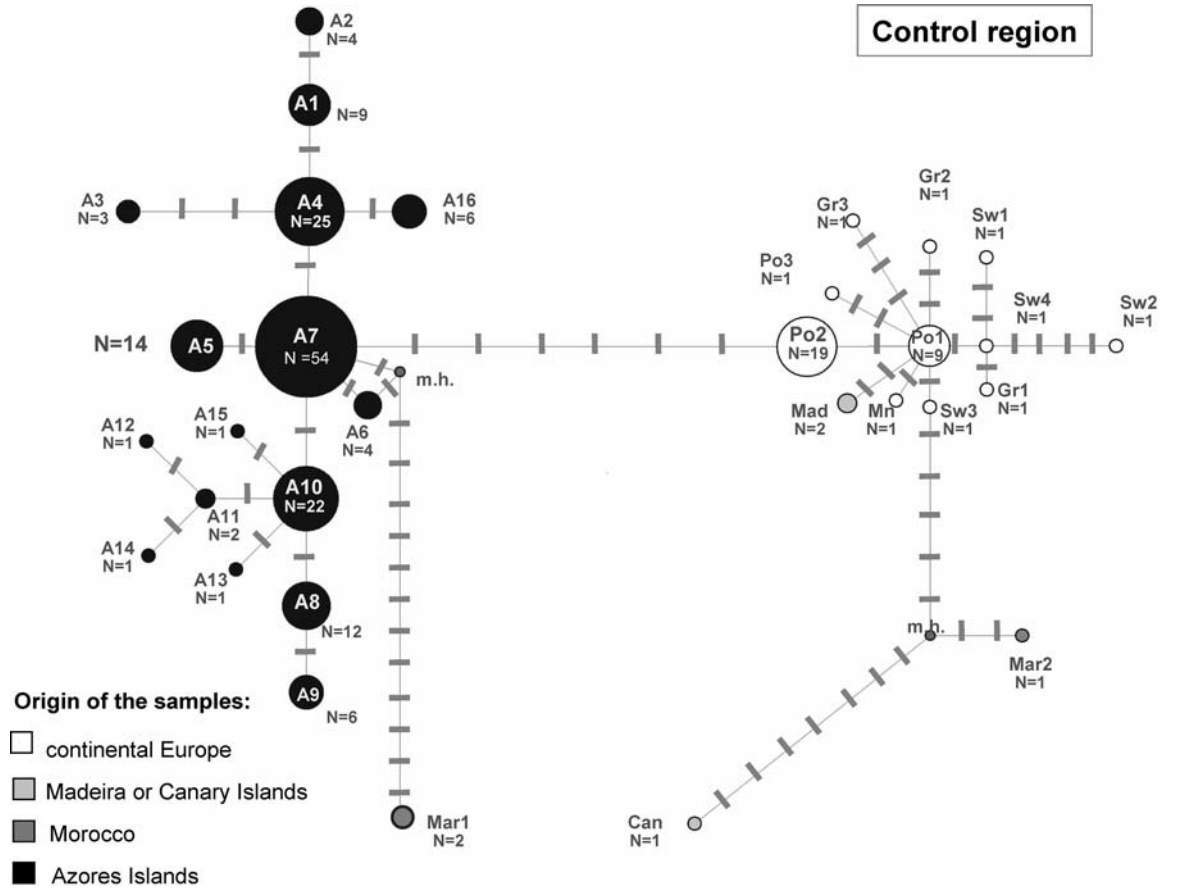

Fig. 5 Median-joining network based on 30 haplotypes shown by sequencing 422 bp of CR in 159 Azorean bats and 43 Leisler's bats (see Appendices 1, 2). Each *grey bar* in between the lines connecting the haplotypes represents one mutation. The frequency and geographic distribution of each haplotype are also presented. *m.h.* is the abbreviation for missing haplotype

Salgueiro et al. 2004 for more details). In the centre of the star-like network of the Leisler's bat was the haplotype Po1 (common to Portugal, Spain, Czech Republic and Turkey, Fig. 5). The two Madeiran samples differed from the widespread Po1 haplotype by two substitutions. The lineages Can from the Canary Islands and Mar2 from Morocco differed from Po1 by 15 and 10 substitutions respectively. The other Moroccan lineage (Mar1) was linked to the Azorean lineages by 14 mutations (3% of sequence divergence). The other European haplotypes were distinct from Po1 by less than six mutations.

The minimum divergence times estimated from the three mitochondrial segments are relatively concordant, but given the usual limitations of such molecular estimates, these data should be considered with caution. For the ND1 dataset, the minimum age include 0 years, since the Nle1 haplotype is shared by Azorean and European bats (see Fig. 3). For the Cyt *b* dataset, the minimum divergence time (i.e. calculated between Naz1c and Nle1c) is about 31,000 years, while that for the fast evolving CR, the divergence between A1 and Po2 (see Fig. 5) is about 55,000 years.

MODELTEST selected a K80 model (Kimura 1980) with site heterogeneity (gamma shape parameter = 0.2125) and T_i/T_v ratio = 5.28 as the best fit for CR sequences. This model was used to estimate NJ trees based on pairwise distances among CR haplotypes.

The NJ and the MP trees were identical (data not shown). Both separated the Azorean haplotypes in a monophyletic group distinct from *N. leisleri* with 76 or 82% of bootstrap support (NJ and MP, respectively). All the other haplotypes, except Can, Mar1 and Mar2, formed another clade with 62 or 66% of bootstrap support (NJ and MP). The Canarian and the more divergent Moroccan haplotypes were the outliers of these phylogenetic reconstructions, confirming the topology obtained in the CR median-joining network (Fig. 5).

Discussion

Phylogenetic relationships within the genus *Nyctalus*

Species of the genus *Nyctalus* are distributed across the entire Palaearctic region, and enter only marginally into the Indomalay region (Simmons 2005; Koopman 1994). The taxonomy of the species in this genus have been, however, highly controversial. For instance, species referred by Koopman (1994) in the *Stenopterus* group are placed into *Pipistrellus* or *Hypsugo* by Corbet and Hill (1992) or Simmons (2005). Other species like *N. azoreum* or *N. plancyi*, were variously treated as full species or as subspecies of other widespread taxa (Tate 1942; Corbet 1978; Palmeirim 1991). If we follow the most recent review by Simmons (2005), the genus *Nyctalus* now contains eight species. *N. lasiopterus* occurs in the West Palaearctic region, while *N. aviator* is its East Palaearctic vicariant; both are the larger species in the genus (Koopman 1994). Three allopatric taxa of intermediate size are further recognized as distinct species; *N. noctula* occurs over most of the West Palaearctic, east to Central Asia, and south to Vietnam and Malaysia; *N. furvus* is endemic to Japan; *N. plancyi* occurs in eastern China and Taiwan. The last three species currently recognized are the smallest: the West Palaearctic *N. leisleri* (including *verrucosus* as a subspecies from Madeira), the Indomalay *N. montanus* (Barrett-Hamilton, 1906), and *N. azoreum*, which is endemic to the Azores archipelago.

Karyotypic analyses (Volleth 1992; Lin et al. 2002) support several of these taxonomic separations. For instance, *N. plancyi* (Zhang 1990) from China and Taiwan share a unique chromosome formula of $2n = 36$ chromosomes (Lin et al. 2002), while *N. noctula*, *N. lasiopterus* and *N. aviator* have $2n = 42$ chromosomes. *N. leisleri* and *N. furvus* have $2n = 44$ chromosomes as shown by Volleth (1992). Although detailed banding pattern of karyotypes are not yet available for all species, Volleth (1992) suggested that the $2n = 42$ karyotype of *N. lasiopterus*, *N. aviator* and *N. noctula* can be regarded as a synapomorphy defining a monophyletic group. Our phylogenetic reconstructions confirm Volleth's hypothesis as they strongly support the monophyly of that group (bootstrap support of 99–100%; Fig. 2). In this group, the largest species *N. lasiopterus* and *N. aviator* are found on opposite sides of the Palaearctic region and were previously considered as conspecific (Tate 1942; Imaizumi 1970), but are now mostly recognized as separate species (Corbet 1978; Maeda 1983; Simmons 2005). They appear as related species in the phylogenetic tree based on ND1, although with low bootstrap values (Fig. 2) and their corrected sequence divergence (4%) is low for distinct species in vespertilionid bats (Mayer and von Helversen 2001; Ruedi and Mayer 2001; Bradley and Baker 2001). Clearly, samples from intermediate geographic locations between Europe and Japan (e.g. from China) are needed to determine if these two taxa should be treated as separate species or vicariant populations of the same species.

N. plancyi from China, which used to be considered an Asian subspecies of *N. noctula* (Corbet 1978; Corbet and Hill 1992), appears separated by 17% of sequence divergence at the ND1 gene from *N. noctula*. None of the ND1 reconstructions indicates a sister group relationship between these two taxa, which is in line with their chromosomal distinctiveness (Lin et al. 2002), thus supporting species status for *N. plancyi*.

As the karyotypes for *N. azoreum* and *N. montanus* are currently unknown, and no ND1 sequence is accessible for *N. furvus*, these taxa cannot be included in this combined karyological and molecular appraisal of phylogenetic relationships. However, in the ND1 reconstruction, *N. leisleri*, *N. l. verrucosus* and *N. azoreum* are very closely related to each other, which is in agreement with the results of similar analyses based on morphological characters (Palmeirim 1991; Speakman and Webb 1993).

Divergence between *N. azoreum* and *N. leisleri*

Despite the remoteness of the Azores (1,500 km), ND1 lineages sampled on this archipelago only differ by zero or one mutation (0–0.2% of sequence divergence) from the common, continental lineage of *N. leisleri*. This pattern of low genetic divergence between Azorean bats and continental or other insular Leisler's bat is consistent with the results obtained with the Cyt *b* dataset (Fig. 4), although in this gene at least one mutation (over 692 bp) distinguishes all Azorean bats from *N. leisleri* (0.1–1.2% of divergence). These divergence values are very low, but comparable to the ones found between the sympatric European species pairs with the lowest levels of interspecific sequence difference: less than 2% between *Eptesicus serotinus*/*E. nilssonii* or less than 2.6% between *Myotis myotis*/*M. blythii* for Cyt *b* and ND1 (Mayer and von Helversen 2001; Ruedi and Mayer 2001).

Even for the more rapidly evolving CR, *N. azoreum* presents 1.6–3.6% of sequence divergence from European haplotypes of *N. leisleri*. The single Canarian *N. leisleri* presents about the same level of genetic difference from its conspecific in Europe. Other species of bats examined in the Canary Islands (*Barbastella barbastellus*, Juste et al. 2003; *Hypsugo savii*, *Pipistrellus maderensis*, Pestano et al. 2003; and *Plecotus teneriffae* Juste et al. 2004) are more divergent (>2%) from their continental counterparts than is *N. azoreum*. Likewise, the two Leisler's bats from Madeira representing *N. l. verrucosus* are genetically very similar to their continental congeners. They share the same Cyt *b* and ND1 haplotypes that are widespread in continental Europe (Figs. 3, 4) and diverge by only two mutations (0.5% of sequence divergence) from the closest European CR lineage (Fig. 5).

Overall, given the wide geographic extent of our sampling, Azorean and Leisler's bats have remarkably conservative mitochondrial sequences. This conservatism is even more marked than that reported by Petit et al. (1999) for the common noctule (*N. noctula*), using the same segment of the CR. The lack of strong genetic structure over wide geographic areas in *N. noctula* was interpreted as evidence of recent range expansion and/or extensive gene flow (Petit et al. 1999), factors that may also apply in *N. leisleri*.

The low level of genetic differentiation between *N. azoreum* and *N. leisleri* measured at several mitochondrial markers contrasts with their marked phenotypic differences (smaller size, darker pelage, higher frequency echolocation call and day time flight). The markedly divergent phenotypic traits are adaptive features that can evolve fast in response to selection, especially in small, insular populations, while the neutral mitochondrial markers are not expected to evolve like genes under selection (e.g. Polly 2001; Grant and Grant 1997). This decoupled evolution between phenotypic and mitochondrial genetic markers has been detected in other insular taxa (Bunce et al. 2005; Glor et al. 2003; Zwartjes 2003), including in bats (Maharadatunkamsi et al. 2003; Schmitt Kitchener and How 1995).

Sources and timing of island colonisation

Because genetic differences among lineages of *N. leisleri* are small, it is difficult to determine precisely the mainland source of the insular taxa. However, we found that both the Azorean and Madeiran *Nyctalus* bear the commonest European ND1 haplotype. Also, for Cyt *b* and CR, the closest continental haplotype to these insular taxa was a widespread lineage found in mainland Europe. Overall, these results clearly point to a continental European origin for both the Azorean and Madeiran *Nyctalus*. Juste et al. (2004) also suggested a European origin for the Madeiran populations of the bat *Plecotus austriacus*, and Europe is also the origin of the Azorean land birds (Le Grand 1984; Hounsome 1993). In contrast, we found some evidence that *N. leisleri* from the Canary Islands have a North African origin. The only studied *N. leisleri* from these

islands shares the ND1 haplotype with a sample from Morocco. In addition, it presents a CR haplotype differentiated by 14 substitutions from the closest European lineage, while seven of these mutations are shared with a Moroccan sample. The Canary Islands are the geographically closest to Africa, and several other flying vertebrates of its fauna seem to have originated in Africa rather than in Europe [e.g. the bat *Plecotus teneriffae* (Juste et al. 2004) and the bird *Parus caeruleus teneriffae*-group (Kvist et al. 2005)].

The minimum divergence time of lineages estimated from genetic data is often contentious as it depends both on a rate calibration and of an overall measure of genetic divergence. Depending on which mitochondrial gene is considered here, the minimum divergence time of the separation of insular and continental lineages is comprised between 0 and 55,000 years BP. This corresponds roughly to a late Pleistocene/Holocene divergence, that is consistent with previous estimates based on demographic parameters (12,000 and 25,000 years BP) calculated by Salgueiro et al. (2004). As humans only colonised the Azores less than 600 years ago, these molecular estimates of divergence times suggest that the first bats who arrived on the archipelago were unlikely brought by men.

Taxonomic and conservation implications for the Azorean bat

With the rapid development of sequencing methods, it is tempting to make decisions on bat taxonomy at species level based on molecular markers (Barratt et al. 1997; Bradley and Baker 2001; Kiefer and Veith 2002; Spitzenberger et al. 2002), which is problematic for allopatric populations (Mayr and Ashlock 1991; Reed and Frankham 2001). Discrepancies between the results of studies based on morphology and on neutral genetic markers are not unexpected (Ruedi and McCracken 2006), since those markers rarely code for the phenotypic traits expressed by the morphology, or because the history recovered from a given gene is not necessarily reflecting the history of the entire organism (Pamilo and Nei 1988). *N. azoreum* is an interesting example of such a disagreement as this insular bat reached a high level of phenotypic differentiation from its continental ancestor, while observed low genetic differences implicate recent common ancestry of mitochondrial lineages. As referred above, the situation of the pair *N. azoreum/N. leisleri* is quite similar to that of two pairs of well established sympatric European species; *E. serotinus/E. nilssonii* and *M. myotis/M. blythii* that are also phenetically divergent but genetically very similar. In fact, Mayer and von Helversen (2001) found that even the fast evolving CR marker, which clearly separated *N. azoreum* from its ancestor, failed to show reciprocal monophyly between *E. serotinus* and *E. nilssonii*. They suggest that the surprisingly low levels of genetic divergence between these phenetically very distinct species are most likely the result of recent splits and rapid morphological divergence. Mayer and von Helversen (2001) point out that in such cases mitochondrial DNA sequence analysis can be insufficient to separate all bats, and that the species status can only be resolved with detailed studies on morphology, ecology, echolocation, and nuclear gene flow. As referred above, there are marked differences between the two species at the first three levels. We are currently studying potential male-mediated gene flow between *N. leisleri* and *N. azoreum* in order to contribute to the clarification of the taxonomic status of the Azorean bat.

Regardless of its taxonomic status the Azorean bat clearly represents an evolutionary unit integrating unique lineages with great conservation value. Since evolutionary mechanisms operate at the level of local populations, the Azorean bat should keep its conservation status, maximising its adaptive potential and the possibility for further differentiation (Soulé 1989).

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Appendix 1

Table 1 List of 25 specimens of *Nyctalus azoreum* and 23 specimens of *Nyctalus leisleri*, their localities of origin and corresponding haplotypes amplified for the three genes: CR, Cyt *b* and ND1

Species	Origin	Haplotypes			Voucher or source
		CR	Cyt *b*	ND1	
Nyctalus azoreum	Faial	A7	Naz1c	Naz1	Wing punch
	Faial	A1	Naz1c	Naz1	Wing punch
	Faial	A4	Naz1c	Naz1	Wing punch
	Faial	A7	Naz1c	Naz1	Wing punch
	Pico	A4	Naz1c	Naz1	Wing punch
	Pico	A7	Naz1c	Naz1	Wing punch
	Pico	A7	Naz3c	Naz1	Wing punch
	Pico	A1	Naz1c	Naz1	Wing punch
	Pico	A1	Naz1c	Naz1	Wing punch
	S. Jorge	A5	Naz1c	Naz1	Wing punch
	S. Jorge	A4	Naz1c	Naz1	Wing punch
	S. Jorge	A7	Naz3c	Naz1	Wing punch
	Terceira	A7	Naz3c	Naz1	Wing punch
	Terceira	A15	Naz2c	Nle1	Wing punch
	Terceira	A10	Naz2c	Nle1	Wing punch
	Terceira	A10	Naz2c	Nle1	Wing punch
	Graciosa	A7	Naz3c	Naz1	Wing punch
	Graciosa	A2	Naz1c	Naz1	Wing punch
	Graciosa	A10	Naz2c	Nle1	Wing punch
	Graciosa	A10	Naz2c	Nle1	Wing punch
	S. Miguel	A7	Naz3c	Naz1	Wing punch
	S. Miguel	A8	Naz4c	Nle1	Wing punch
	S. Miguel	A7	Naz1c	Naz1	Wing punch
	S. Miguel	A11	Naz5c	Nle1	Wing punch
	S. Miguel	A10	Naz2c	Nle1	Wing punch
Nyctalus leisleri	Serra do Açor, Portugal	Po1	Nle1c	Nle8	Wing punch
	Serra do Açor, Portugal	Po2	Nle1c	Nle1	Wing punch
	Serra do Açor, Portugal	Po2	Nle1c	Nle1	Wing punch
	Serra do Açor, Portugal	Po3	Nle1c	Nle2	Wing punch
	Cadiz, Spain	Po1	Nle1c	Nle5	EBD15255
	Argovie, Switzerland	Sw1	Nle1c	Nle3	HPS 1
	Luzern, Switzerland	Sw2	Nle1c	Nle4	HPS 2
	Obwald, Switzerland	Sw3	Nle2c (GenBank: AF376832)	Nle1 (GenBank: AY033949)	HPS 2639
	Geneva, Switzerland	Sw4	Nle1c	Nle1	Wing punch
	Sterea Ellada, Greece	Gr1	Nle1c	Nle1	NMP48724
	Thessaly, Greece	Gr2	Nle1c	Nle1	NMP49035
	Macedonia, Greece	Gr3	Nle1c	Nle1	NMP49042
	Anatolia, Turkey	Po1	-	Nle1	NMP47979
	Bohemia, Czech Republic	Po1	Nle1c	Nle1	NMP(pb 1/04)
	Montenegro	Mn	Nle5c	Nle1	NMP(pb 2405)
	Tetouan, Morocco	Po1	Nle1c	Nle5	EBD 020714Nle4
	Tetouan, Morocco	Mar1	Nle3c	Nle6	EBD 020714Nle5
	Rif, Morocco	Mar2	Nle4c	Nle7	NMP90026
	Middle Atlas, Morocco	Sw4	Nle1c	Nle1	NMP90034
	Middle Atlas, Morocco	Mar1	Nle3c	Nle6	NMP90100
	La Palma, Canary Isl., Spain	Can	Nle1c	Nle7	D. Trujillo (27-11-00)
N. l. verrucosus	Madeira Isl., Portugal	Mad	Nle1c	Nle1	D. Trujillo (31-06-96)
	Madeira Isl., Portugal	Mad	Nle1c	Nle1	Rainho et al. (2002)

The sequences obtained from GenBank are referenced

EBD Estación Biológica de Doñana, Spain; *NMP* National Museum of Prague, Czech Republic; *HPS* H.-P. Stutz, Zürich Museum, Switzerland

Appendix 2

Table 2 List of ND1 sequences from species of the genus *Nyctalus* and *Pipistrellus*, from GenBank and the corresponding haplotypes designation in the present study

Species	Origin	ND1	Haplotypes
Nyctalus lasiopterus	Greece or Hungary	AF401432	Nla1
	Greece or Hungary	AF401433	Nla2
	Greece or Hungary	AF401436	Nla3
Nyctalus noctula	Germany	AF065103	Nno1
	United Kingdom	AF065109	Nno2
	Russia	AF065105	Nno3
	Austria, Germany, France, Poland and Russia	AF065106	Nno4
	Germany	AF065107	Nno5
	France	AF065108	Nno6
Nyctalus plancyi	China	AB079820	Nve
Nyctalus aviator	Japan	AB079819	Nav
Pipistrellus abramus	Japan	AB061528	Pab
Pipistrellus kuhlii	Greece	AF401416	Pku
Pipistrellus pygmaeus	Germany, Greece, Russia, Sweden and Ukraine	AF401413	Ppy

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