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GENETIC STRUCTURE AND GENE FLOW OF FRAGMENTED BAT POPULATIONS

CONSEQUENCES FOR CONSERVATION

PATRÍCIA ISABEL ROSA SALGUEIRO

DOUTORAMENTO EM BIOLOGIA

(Biologia Evolutiva)

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À minha mãe

How the bat came to be

“Once upon a time, the sun entangled himself in a tree, and the earth plunged into cold darkness. A little squirrel lost its tail, had its fur burnt off and went blind, but gnawed at the tree's branches until the heavenly globe could rise again. As a reward, the sun changed the naked creature, who had always wanted to fly, into the first bat.”

A native american tale adapted from Caduto M. & Bruchac J. (1994) Caduto, Michael J. *Keepers of the night: Native American Stories and Nocturnal Activities for Children*. Golden, Colo. Fulcrum Pub.

Nota prévia

Na elaboração desta dissertação, e nos termos do *N.º* 1 do Artigo 40, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no *Diário da República – II Série N.º 153*, de 5 de Julho de 2003, foram usados resultados de trabalhos já publicados, aceites, submetidos, ou a submeter a publicação em revistas científicas internacionais indexadas. Estes integram alguns dos capítulos da presente tese, e tendo sido realizados em colaboração. A candidata esclarece que participou integralmente na concepção, obtenção, análise e discussão dos resultados, bem como na redacção dos manuscritos dos artigos I a IV. No artigo V, a candidata participou parcialmente na captura de espécimes no campo, em trabalho de laboratório, na análise e discussão de parte dos resultados e colaborou também parcialmente na escrita do trabalho.
Devido à integração de vários artigos científicos o padrão de formatação apresentado é variável conforme as normas de cada revista de publicação.

Lisboa, Outubro de 2007

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Table of Contents

Resumo

Os Morcegos (ordem Chiroptera) são os únicos mamíferos terrestres que conseguiram colonizar arquipélagos oceânicos remotos pelos seus meios. As poucas espécies que chegaram até estas ilhas encontram-se em geral divididas em pequenas populações isoladas. Esta situação de isolamento levanta problemas de conservação relacionados com a estrutura genética das populações. Por um lado, o isolamento das diferentes populações insulares pode conduzir ao surgimento de diferenças genéticas entre ilhas, diferenças essas que deverão ser tidas em conta no planeamento de medidas de conservação, de forma a preservar o máximo de variabilidade genética possível. Por outro lado, a variabilidade genética de cada população insular é determinada pelas perdas inerentes ao efeito fundador, pelo tamanho limitado da população, assim como por ganhos devidos a imigrações posteriores e eventuais mutações.

A variação genética é a base da flexibilidade adaptativa das populações, sem a qual vêem frequentemente reduzidos o seu sucesso reprodutor, a sua capacidade de resistir a novas doenças e a possibilidade de se adaptarem às permanentes alterações ambientais. Neste contexto, estudou-se a variabilidade genética intra- e inter-ilhas do Morcego dos Açores (*Nyctalus azoreum*) distribuído por quase todo o arquipélago açoriano, avaliando-se ainda as suas relações filogenéticas com as populações continentais do seu ancestral, Morcego-arborícola-pequeno (*N. leisleri*).

Em Portugal Continental, o Morcego-de-peluche (*Miniopterus schreibersii*) partilha com as espécies insulares de morcegos o facto de se encontrar dividida em pequenas populações funcionais (colónias). De igual modo, este nível de fragmentação pode gerar o mesmo tipo de problemas que as espécies distribuídas em arquipélagos, em termos de genética populacional e conservação.

Tal como no resto da Europa, em Portugal os quirópteros são um dos grupos animais mais ameaçados. Embora, na última década, algumas espécies tenham sido alvo de diversos estudos, no que se refere à sua distribuição, abundância e ecologia, abordagens relativas à variação genética de populações portuguesas de morcegos não tinham ainda sido levadas a cabo.

A presente tese constitui uma importante contribuição para o conhecimento de duas espécies de morcegos europeias (o Morcego dos Açores e o Morcego-de-peluche), ambas com uma distribuição fragmentada das suas populações. Neste trabalho, foram abordados aspectos

relativos à evolução, filogeografia, genética populacional e conservação das espécies em estudo, com recurso a vários marcadores moleculares neutros.

Inicialmente, centrou-se a atenção no género *Nyctalus*, de modo a melhor compreender a sua história evolutiva. Para tal, foram analisadas as relações filogenéticas entre seis espécies do género, recorrendo a três regiões mitocondriais com taxas evolutivas diferentes: NADH desidrogenase 1 (ND1), citocromo *b* (Cyt *b*) e região control (CR). Este trabalho permitiu a confirmação do taxon chinês *N. plancyi* (=*N. velutinus*) como espécie, bem como a atribuição de origens distintas às populações insulares de *N. leisleri.* À população madeirense foi sugerida uma origem Europeia, enquanto que a população das Canárias se pensa ter origem em populações do Norte de África.

Um dos pontos mais importantes deste estudo filogenético foi o esclarecimento da relação entre o Morcego dos Açores com o seu ancestral continental, o Morcego-arborícola-pequeno. Embora, os haplótipos de Cyt *b* e CR detectados nos espécimes do Morcego dos Açores fossem exclusivos dos Açores, o nível de divergência genética medido entre as duas espécies é muito baixo comparativamente com outros mamíferos. Este facto indicia um fenómeno de especiação recente para o Morcego dos Açores. De forma a clarificar o nível de especiação e consequente estatuto taxonómico, recorreu-se à análise de microssatélites (marcadores moleculares com elevada taxa de mutação). Confirmou-se o isolamento demográfico do Morcego dos Açores, devido à ausência de fluxo genético recente entre este e o Morcego-arborícola-pequeno.

Numa abordagem intra-específica do Morcego dos Açores, e utilizando as mesmas sequências de CR e microssatélites, verificou-se haver um elevado nível de estruturação nas suas populações. Observou-se ainda que as distâncias genéticas entre populações estão significativamente correlacionadas com as distâncias geográficas, evidenciando sinais de isolamento pela distância. Como o haplótipo de CR mais abundante se encontra espalhado por todo o arquipélago, depreende-se ter ocorrido uma colonização recente do mesmo, seguida de uma expansão demográfica. Os tempos de expansão estimados a partir do marcador CR sugerem que os Açores foram colonizados pelo ancestral do Morcego dos Açores entre o fim do Plistocénico e o início do Holocénico, pelo que se deduz ter havido uma colonização não mediada por humanos.

Tendo em conta os resultados de variabilidade genética obtidos para ambos os tipos de marcadores, o Morcego dos Açores, não se encontra particularmente empobrecido em termos genéticos, tendo em atenção que se trata de uma espécie insular. Os maiores níveis de variabilidade genética, bem como os de haplótipos únicos e alelos privados foram detectados na ilha de S. Miguel. Esta é a ilha com a maior área e também a mais próxima de território

continental, tendo sido provavelmente a primeira a ser ocupada por esta espécie de morcego. A análise da estrutura das populações do Morcego dos Açores revelou duas subpopulações distintas: a da ilha de S. Miguel e a do Grupo Central (constituído pelas ilhas do Faial, Pico, S. Jorge, Terceira e Graciosa). Entre estas duas unidades populacionais, o nível de fluxo genético é reduzido, sendo mais provável que ocorra por dispersão passiva, tendo em conta a ocorrência frequente de tempestades no arquipélago. Ao contrário do que ocorre em outros mamíferos, em especial morcegos, não foi detectada dispersão diferenciada pelo sexo no Morcego dos Açores.

No que se refere ao estudo do Morcego-de-peluche continental, recorreu-se ao mesmo tipo de instrumentos moleculares usados para analisar o Morcego dos Açores. As populações portuguesas encontram-se estruturadas em quatro subpopulações: Nordeste, Centro, Sul e Marvão, e também nestas populações fragmentadas se detectou isolamento pela distância. Marvão, a maior colónia de criação de Morcegos-de-peluche conhecida no mundo, apresenta, como era esperado, os maiores valores de diversidade genética. Comparativamente às colónias de criação, as colónias de hibernação apresentam um maior nível de diversidade genética, o que se deve ao facto de estas abrigarem indivíduos com origem em diferentes colónias de criação.

Relativamente à distribuição geográfica, as colónias do Sul de Portugal apresentam maiores níveis de variabilidade genética que as do Norte do país. A informação acerca da diversidade genética desta espécie, contribuiu para aprofundar o conhecimento da sua história populacional. Nomeadamente, foi possível confirmar para o Morcego-de-peluche um processo de recolonização pós-glacial a partir do Sul da Península Ibérica ou do Norte de África. Outro aspecto a salientar é que apesar da actual disponibilidade de mais abrigos intermédios artificiais (e.g. minas) para o Morcego-de-peluche, a acentuada estrutura genética coincide com a distribuição de áreas calcárias, onde se encontram a maioria dos abrigos naturais originais da espécie.

O estudo da estrutura populacional permitiu compreender melhor alguns aspectos da biologia da espécie e tirar ilações acerca de comportamentos reprodutores e de dispersão. Apesar de ambos os tipos de marcadores (mitocondriais e nucleares) terem apresentado a mesma estrutura populacional sugerida, foi detectada uma grande discrepância entre os valores de diferenciação genética obtidos com os diferentes marcadores (10 vezes superior no DNA mitocondrial). Este facto veio corroborar os resultados de estudos demográficos anteriores que haviam reportado um forte comportamento filopátrico por parte das fêmeas do Morcego-de-peluche, sugerindo que grande parte do fluxo genético entre colónias é da responsabilidade dos machos.

Por fim, esta tese permitiu reafirmar a grande importância da conservação do Morcego dos Açores e do Morcego-de-peluche. Em ambos os casos, foi possível determinar a distribuição da

variabilidade genética, assim como compreender as causas da sua estruturação, quer fossem acontecimentos históricos, isolamento pela distância ou dispersão. Os resultados de estrutura populacional obtidos ajudaram a definir unidades de gestão, e a partir destas, várias medidas importantes para a conservação das duas espécies foram sugeridas.

Palavras-chave: conservação, *Miniopterus schreibersii*, morcegos, *Nyctalus azoreum*, populações fragmentadas

Abstract

Species distributed in fragmented populations have particular conservation problems that are related to population genetics. This applies both to species sub-divided in small isolated populations, such as insular species inhabiting archipelagos, and to mainland species that are fragmented in small functional populations, such as colonies. In both cases, genetic structure is one of the most fundamental pieces of information for their conservation and management. This is the case of the study species in the present thesis: the insular the Azorean bat (*Nyctalus azoreum*), distributed in most islands of the Azores archipelago, and the continental Schreiber's bent winged bat (*Miniopterus schereibersii*), with a population fragmented in several separate colonies.

Like in most of Europe, bats are one of the most threatened animal groups in Portugal, where some bat species have been studied regarding distribution, abundance and ecology. Yet, the application of molecular data to the knowledge and conservation of Portuguese bat populations has been unexplored until now.

In this thesis, mtDNA sequences and nuclear microsatellite loci were used to increase the knowledge about the fragmented populations of the two threatened bat species mentioned above.

This study included an examination of the phylogenetic relationships among several species within the genus *Nyctalus*, and of the genetic divergence between *N. azoreum* and its mainland ancestor, *N. leisleri*. It also involved the analysis of the genetic diversity within populations, and genetic differentiation among the studied fragmented populations, thus revealing new insights about their population structure, history and behavioural dispersal.

This thesis provided important information to define biologically meaningful conservation units for the studied species. For *N. azoreum*, two management units were suggested (S. Miguel island and the Central Group), while for *M. schreibersii* four units were defined (North, West Centre, Marvão and South). These units should be taken into account when planning conservation and management actions.

Keywords: bats, conservation, fragmented populations, *Miniopterus schreibersii*, *Nyctalus azoreum*

Chapter I General Introduction

I General Introduction

1.1. Overview

Bats (order Chiroptera) are the only terrestrial mammals that have been able to establish by themselves in many oceanic archipelagos. The few bat species that managed to reach these remote islands are often subdivided in small isolated populations, which have particular conservation problems related to the genetics of populations. This is the case of the Azorean bat (*Nyctalus azoreum*), which has populations on most islands of the Azorean archipelago.

In mainland Portugal, the Schreiber's bent-winged bat (*Miniopterus schreibersii*) shares with that insular species the fact of being subdivided in small functional populations, in this case colonies roosting in caves. To some extent this population subdivision makes them experience the same type of genetic and conservation consequences of species in archipelagos.

As much as in the rest of Europe, the Chiroptera are one of the most threatened animal groups in Portugal. Although a few bat species have been extensively studied regarding distribution, abundance and ecology in the last decade, an approach evaluating the genetic variation in Portuguese bat populations has never been carried out before.

The main aim of this thesis is to investigate patterns of genetic variation, genetic structure and gene flow in fragmented populations of the two bat species mentioned above. In particular, molecular tools were used to examine past and recent population processes affecting within and between species genetic diversity of the studied species. Ultimately, these findings were used to generate recommendations for their conservation and management.

The introduction starts with a review about phylogeography and speciation, reconsidering species concepts. Shifting from the species to the population level, population fragmentation was reassessed, approaching behavioural and geographic aspects, and focusing on island systems. After that, background information and novel perspectives on conservation genetics were provided. Subsequently, the molecular markers and methodological approaches used on the study are described. Finally, an introduction to the study organisms will be presented and the major molecular genetic studies on them will be appraised.

1.1.1. Phylogeography and speciation

Knowledge about the molecular basis of genes has transformed biological research. The common use of molecular markers has strongly facilitated the study of gene flow or migration

patterns and the reconstruction of demographic histories of both populations and species. The discipline related to the principles and historical processes behind the spatial distribution of genealogical lineages (within and among closely related species) is phylogeography (Avise 2000). This field was first introduced by Avise et al. (1987) and it usually requires integration of other subjects, such as population genetics, ethology, demography, evolutionary biology, palaeontology, geology, and historical geography; being usually seen as the bridge between micro- and macro-evolutionary processes (Avise 2000, Avise et al. 1987). Phylogeographic differentiation at the intra-specific level has been fully recognized by the theories of speciation and macroevolution (Avise et al. 1987). Thus, comparisons of phylogeographic patterns among species have been made so that general patterns of their evolutionary history may be inferred (Hewitt 2000, Zink 1996). These have been frequently used to deduce re-colonisations and range expansions of many animal groups, associated with the Ice Ages, and have led to the resurgence of interest in Pleistocene biogeographic influences on speciation processes (Hewitt 1996, Klicka and Zink 1997).

The origin of species remains one of the least understood and yet most important questions in evolutionary biology (Tregenza and Bridle 1997). Speciation is the essential process that gives rise to species diversity (Barraclough and Sean 2001); it is an incidental, non-adaptive consequence of divergence of populations (Futuyma 1998, Mayr 1963). Except for the so-called instantaneous speciation, for example by polyploidy (Mayr 1963), it is a gradual process, taking between a few thousand to more than 20 million years to occur, on average 3 million years (Futuyma 1998). Features like the abundance of geographic barriers, low species dispersal rates, strong sexual selection, population bottlenecks and ecological specialization may increase the rate of speciation (Futuyma 1998). For example, it is known that morphological evolution is accelerated among insular mammals (Millien 2006).

Mayr (1963) classified speciation by modes according to geography and level: 1) hybridization, 2) instantaneous speciation (through individuals), and 3) gradual speciation (through populations). This last mode refers to the evolution of reproductive barriers based on several allele substitutions, and comprises three geographic levels: a) sympatric (biological barriers prevent gene exchange within an initial randomly mating population), b) parapatric (divergence of spatially segregated populations connected by gene flow), and c) allopatric (physical barriers reduce gene flow between populations).

More recently, Templeton (1982) proposed another classification based on population genetic mode: 1) transilience (corresponding to Mayr's hybridization and instantaneous speciation), and 2) divergence (corresponding to Mayr's gradual speciation). The main

exceptions are the two modes of Mayr's allopatric speciation (vicariant and peripatric), which are differently classified by Templeton. Specifically, Mayr's vicariant speciation is considered an adaptive divergence mode, while the peripatric or founder effect speciation is placed on the genetic transilience mode by Templeton (1982).

Many authors support that allopatric speciation is predominant in animals (Barraclough and Vogler 2000, Coyne 1992, Futuyma and Mayer 1980, Mayr 1963, Turelli et al. 2001, Wiens 2004). A major mode of allopatric speciation is by founder effect, when a sample of few individuals from a widespread population diverges and becomes reproductively isolated (Futuyma 1998). Some authors consider that founding events lead to "genetic revolutions" through genetic drift (Carson and Templeton 1984, Mayr 1963, Templeton 1980). Drift activates speeding up of divergence, thus supporting a shift to a new adaptive peak. Although proofs for this process in nature are unclear, the fast emergence of multiple distinct lineages in isolated and restricted areas may reinforce the frequency of the founder effect mode. Monophyletic groups within oceanic archipelagos usually show high levels of allopatric species richness, mainly due to adaptive radiation, e.g. Darwin's finches (Grant 1986) and giant tortoises (Beheregaray et al. 2004) in the Galapagos; short-faced bats (Davalos 2007) and anoles in the Caribbean (Glor et al. 2005, Losos et al. 1998); sunbirds across Indian Ocean islands (Warren et al. 2003); spiders in Hawaii (Gillespie 2004). Nevertheless, this peak shift model of speciation by drift or Mayr's "genetic revolution" is rather controversial. It received many criticisms from Coyne and Orr (2004). These authors reject the importance of founder effects on island radiations, claiming that natural selection on allopatric populations in a new environment is the main cause. Either way, oceanic archipelagos offer an exceptional set for the study of speciation, being of most importance in the progress of evolutionary theory.

Currently, methods of molecular genetic and genomics are generally employed to study speciation. These have allowed improvements on inferences about speciation processes and the nature of species boundaries in natural populations. Moreover, they have contributed to the discovery of "barrier genes" (Noor and Feder 2006).

The view on how speciation occurs depends on the concept of what is a species (Cracraft 1983, Harrison 1998, Wiens 2004). Given the enormity of the biodiversity crisis (Frankham et al. 2002, Primack 1995), accurately describing species diversity is fundamental (Sites and Crandall 1997). Thus, the concept of species is essential to areas such as evolutionary biology, ecology and conservation biology (Templeton et al. 2000). Nevertheless, biologists do not always agree about what species are (Hey 2001, Wheeler and Meier 2000, Wiens 2004), and there are multiple

concepts in use. For detailed reviews on species concepts, see Mallet (2001), Sites and Marshall (2004) and Wheeler and Meier (2000).

Initially, taxonomists like Linnaeus supported a “typological” or “essentialist” view of species, which were distinguished by discrete morphological characteristics (Mayr 1942, Mayr 1963). Later, Darwin (1859) held a concept of "varieties between which there are no or few morphological intermediates”, therefore challenging the need for a species concept (Mallet 2001).

From the 1950s onwards, the most widely used and consensual concept among evolutionary biologists was the “Biological Species Concept” (BSC) (Futuyma 1998, Mallet 2001). This concept defines species as “a group of actually or potentially interbreeding natural populations that is reproductively isolated from other such groups” (Mayr 1942). BSC’s domain is, however, limited to sexual, out-crossing populations and to narrow time frames (Mayr and Ashlock 1991). Additionally, the interbreeding criterion can be difficult to apply when allopatric populations are involved, given that geographic isolation is not an intrinsic isolating mechanism (Mayr 1963, Mayr 2000).

Some critics state that reproductive isolation has no consistent genotypic or phenotypic correlates to predict reproductive compatibility of allopatric groups (McKitrick and Zink 1988), and that other traits are more important. This is the case with most of lineage-based species concepts, such as the "Cohesion" (CSC, Templeton 1989) or the “Phylogenetic” (PSC, Cracraft 1989) concepts, and in fact these can be more advantageous when classifying species, particularly allopatric taxa (Noor 2002). Following de Queiroz (1998), these lineage-based concepts concur on the principle of a species as an independent lineage, only disagreeing on the criteria applied to different stages of lineage divergence.

With the PSC, speciation is considered to occur when new independent lineages acquire fixed diagnostic characters, such as base-pairs in a mitochondrial DNA (Wiens 2004).

Currently, molecular biology has provided more evidence on which to base species detection, other than that based purely on morphology (Hillis 1987, Mayr 2000). Molecular data has been used to resolve many species boundaries (Tautz et al. 2003); large scale DNA sequencing of living species holds such great promise in taxonomy (Vogler and Monaghan 2007), that some authors have actually come to designate this moment as the “molecularisation of taxonomy” (Lee 2004). Thanks to this phenomenon, the PSC became quite popular in species delimitation, being more appropriate in biogeography and phylogeography studies (Mallet 2001). Cracraft (1989) championed the PSC, in which species are defined as “the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent”. However,

this may include paraphyletic species, and some taxa before categorized as sub-species are now accepted as separate species (Mallet 2001). This has led to taxonomic inflation compared with earlier taxonomies, and to a wave of taxonomic splitting, particularly in charismatic vertebrates such as birds and primates (Isaac et al. 2004).

Species concepts were, are, and probably will continue to be a controversial subject. There are authors (e.g. Hendry et al. 2000, Mishler 1999) that yet defend a taxonomy without species ranks. Simpson (1951) and Templeton (1989) have tried some consensus by suggesting the inclusion of morphological, ecological, phylogenetic and reproductive criteria (Mallet 2001). For example, these elements are combined in the CSC (Templeton 1989), which describes species as a distinct evolutionary lineage that also represents a reproductive community in either a genetic and / or adaptation and / or ecological sense (Templeton et al. 2000). Irrespective of all the concepts, most conservation biologists consider species as evolutionary units and therefore seek to understand how genetic variability is apportioned within and among populations so that evolutionary potential may be conserved (Avise and Hamrick 1996). This leads to a focus on individual populations as the primary conservation concerns because evolutionary mechanisms operate at this level and their conservation maximises adaptive potential and the possibility for speciation (Soulé 1989).

1.1.2. Population fragmentation

Under appropriate conditions, speciation may happen through population fragmentation, especially in populations with a large geographic range (Gavrilets and Vose 1998). On the other hand, fragmented populations may divide species into increasingly isolated and smaller pieces, more prone to extinction (Ehrlich and Wilson 1991). The genetic consequences of population fragmentation depend essentially on gene flow and population size, which, if reduced, can be extremely detrimental and result in smaller and more isolated populations with diminished genetic diversity (Frankham et al. 2002). Conditions such as these may restrain adaptive responses to selection (Stockwell et al. 2003, Templeton et al. 2001).

Given this dramatic scenario and the urgency to understand the evolutionary dynamics of small populations, it is not surprising that a great number of studies deal with population fragmentation in several biological groups over the last decade (e.g. Bergl and Vigilant 2007, Burkey and Reed 2006, Dayanandan et al. 1999, Johnson et al. 2003, Lippe et al. 2006, Martinez-Cruz et al. 2004, Mesquita et al. 2005, Reece et al. 2005, Rossiter et al. 2000, von Segesser et al. 1999, Wimmer et al. 2002).

Frankham et al. (2002) suggested numerous possible fragmented population structures, for example: stepping-stone models (neighbouring populations exchange migrants), mainland to island (migration from source to sink populations), metapopulations (regular extinction and colonisation events, Hanski and Gilpin 1997), or totally isolated fragments.

Due to the importance of this last model in the present study, it will be considered in more detail in the next section (Islands). Furthermore, the effects of fragmentation are influenced by behavioural aspects, characteristic of each species, particularly roost choice and dispersal ability associated with migration rates among fragments, a topic that will be reviewed bellow (section Behavioural factors).

Islands

The common characteristic uniting all island systems is isolation (Gillespie and Roderick 2002). Many lessons applied to continental fragmented populations have been learned from real island systems (e.g. Burkey 1995). In fact, these have long been important for the study of fundamental questions in evolution, ecology and conservation (Darwin 1859, Grant 1998, Ricklefs and Bermingham 2007, Whittaker 1998). MacArthur and Wilson (1967) with their "equilibrium theory of island biogeography", were the first to recognise the significance of island isolation and population size in generating and preserving species diversity. Later, this same association was also applied to genetic diversity (Jaenike 1973), stating that genetic variation on islands should be determined by the net effects of loss at foundation, subsequent loss caused by finite population size and gains arising from secondary immigration and mutations. With the recent progress of molecular markers and phylogenetic approaches, new paths of investigation of island systems have emerged (Emerson 2002, Grant et al. 2001), revealing mainland source populations and colonization patterns within archipelagos (e.g. Warren et al. 2003).

As a consequence of extreme fragmentation, the risk of genetic erosion is particularly high on islands. Although insular species represent a fairly small part of the World's biodiversity, more than 60% of recent documented vertebrate extinctions have occurred on islands (Diamond 1989, Steadman 1995). Furthermore, island species are often endemic having relatively higher extinction rates than non-endemic island populations (Frankham et al. 2002). High extinction rates, following disturbance on island *versus* mainland populations, are likely to reflect their susceptibility to stochastic effects, but also the lower levels of genetic diversity (Frankham

1998). In a review by Frankham (1997), the reduction in genetic variation was significantly greater in island endemic than in non-endemic island populations of mammals and birds.

Recently, there has been a heated debate over the role of genetic *versus* ecological factors in extinction of island endemics [review and commentaries by Groombridge (2007), Jamieson (2007a), Jamieson (2007b) and Reed (2007)]. The discussion opposed the views of Spielman et al. (2004) that emphasised the potential for genetic factors to influence extinction risk in a number of threatened species; and the ones of Blackburn et al. (2004) and Duncan and Blackburn (2004), which concluded that exotic predators have been the main drivers of extinction of birds on oceanic islands, disregarding genetic factors. Groombridge (2007), Jamieson (2007a), Jamieson (2007b) and Reed (2007) in their comprehensive reassessment agreed that any influence in survival among island endemics due to genetic factors (e.g. inbreeding) would be overpowered by the strong and rapid predation pressure. They have, however, recognised that genetic factors have played a key role in the recovery of several wild populations, as reported by Hale and Briskie (2007), Pimm et al. (2006) and Tallmon et al. (2004).

In order to better understand the different patterns of speciation, biodiversity and conservation on islands, Gillespie and Roderick (2002) has distinguished two types of islands: "darwinian" islands (formed *de novo*) and "fragment" islands. The former have never been directly connected to colonisation sources and hold many available ecological niches. Typical Darwinian islands are volcanic oceanic islands, colonised by external biota, which if isolation persists, can be subsequently enriched by “neo-endemics”. These islands can last for 10-20 million years before being eroded back into the ocean. Examples include the Azores and the Galapagos archipelagos.

By contrast, in fragment islands, ecological space is initially occupied by colonists, which, previously to isolation, are in contact with colonisation sources. In these islands, given enough time, speciation may occur by “relictualization” with the formation of "paleo-endemics”. Typical fragment islands are either: continental islands, geologically recent emergent fragments, separated from the continental shelf by shallow waters (e.g. the British Isles); or continental fragments that diverged millions of years ago from mainland by tectonic drift (e.g. Cyprus). Several isolated habitats within continents (e.g. mountain tops or desert springs) share these biological properties and may be designated as fragment islands. Likewise, due to their disconnection by extrinsic barriers to gene flow (Barr and Holsinger 1985), caves can represent islands within the terrestrial environment (Gillespie and Roderick 2002). Depending on the circumstances and species involved, these may be colonized by migration from other similar

subterranean systems (Howarth 1981), or from the surface. Species coming from the surface expect subsequent adaptations (Howarth 1993), which is particularly relevant in groups like insects, spiders or fishes, with several obligate cave-dwelling species (Barr and Holsinger 1985).

Continental islands tend to have higher levels of genetic diversity than comparable oceanic islands, and depending on their degree of isolation, may not be distinguishable from their mainland counterparts (Frankham 1997). The magnitude of these differences is dependent on the colonist species dispersal ability.

Behavioural factors

There are a number of behavioural aspects affecting the genetic vulnerability of a population or species to fragmentation. For example, dispersal behaviour and long distance migrations may strongly affect the level of gene flow among population fragments. Also, cooperative behaviour or roost selection may be strong determinants of population structure, as well as breeding behaviour, which has an influence on the effective population size of a species. All these factors may be connected in a complex interactive network. The remaining of this section will focus on subjects like roosting choice, dispersal and mating behaviour.

Roosting

Roost availability and choice have a determining role in the population structure of many animal communities. In choosing where to live, many animals actively select specific places. The ones able to occupy those sites of preference tend to gain in higher fitness (Futuyma 1998). The specificity of roost preferences and scarcity of roosting sites may lead to a fragmented distribution of populations. These factors can be especially important in bat species, since these animals spend most of their lives inside roosts (Altringham 1999). This is particularly relevant in the case of cave-dwelling species, given that caves are the most stable and persistent maternity and hibernating sites for bats (Racey and Entwistle 2003), and their number is considerably limited in nature.

Dispersal and mating behaviour

Dispersal behaviours can be quite dissimilar, with some species remaining in or returning to their places of birth, and others moving over very large distances. A strong negative correlation

was reported between genetic differentiation and dispersal ability, with the flying taxa analysed (birds and insects) having the lowest genetic differentiation values (Frankham et al. 2002). Nevertheless, different patterns of dispersal can be found within the same animal taxa. For example, in some archipelagos there are bat genera with high gene flow, thus showing high dispersal within the archipelago, whereas others are divided in divergent monophyletic units on each island, showing no gene flow and low dispersal (reviewed by Heaney 2007).

Furthermore, even in the case of species with confirmed dispersal ability, these may show behaviours that restrict dispersal, such as sex-biased philopatry and dispersal (Lowe et al. 2004). In many avian and mammalian species, one sex is philopatric and the other tends to disperse. The dispersing sex tends to be female in birds and male in mammals (Greenwood 1980).

Restricted dispersal usually causes reduced gene flow among populations, resulting in high genetic structuring. Therefore, sex biased dispersal may lead to differences in the genetic structure of populations between sexes (if individuals are sampled after dispersal), but also between differently inherited genomes (Prugnolle and de Meeûs 2002) depending, for example, on the breeding characteristics and mating system (Chesser and Baker 1996).

Dispersal behaviour is often related to the mating system of a species (Greenwood 1980). Cost-benefit studies have suggested that in a mate defence mating system, usually polygenic (as in the mammalian mating system), it would be more advantageous for males to disperse in search of new mates, thus reducing the costs of inbreeding (Futuyma 1998, Greenwood 1980). On the other hand, in a resource defence system, typical among birds, males benefit from philopatry because of familiarity with resources, while females benefit from dispersal, allowing them to choose among males and their defended resources (Clarke et al. 1997, Greenwood 1980).

In addition to the level of gene flow, mating greatly affects the effective population size of a species. One aspect of breeding behaviour, which is potentially important for effective population size and gene flow, is polygamy. Polygyny, where a single male mates with several females providing parental care (Krebs and Davies 1993), is the most frequent case of polygamy among mammals.

Sexual selection may lead to great differences in individuals' mating success. One of the ultimate bases of mate choice is suggested to be the genetic quality of the individual (Krebs and Davies 1993). In polygynous species this is particularly significant, since males do not provide any parental care, and sperm is their only contribution to offspring.

Male dispersal and female philopatry are very common features among bats, which, similarly to other mammals, tend to be polygynous (Burland et al. 2001, Kerth et al. 2000, Kerth et al.

2002, Kerth and Petit 2005, Miller-Butterworth et al. 2003, Palmeirim and Rodrigues 1995, Rivers et al. 2005, Thompson 1992, Veith et al. 2004, Worthington Wilmer et al. 1994).

1.1.3. Conservation Genetics

The biological diversity of the planet is being rapidly depleted as a consequence of both direct and indirect human actions (Centre 1992). Not only a significant number of species has become extinct, but many others had their population sizes drastically reduced, thus becoming susceptible to stochastic effects, whether environmental, catastrophic, demographic, or genetic, such as inbreeding depression, loss of genetic variation, and accumulation of deleterious mutations (Frankham 1995).

Many authors recognise the importance of genetic factors in conservation (Centre 1992, Falk and Holsinger 1991, Frankel and Soulé 1981, Frankham et al. 2002, Hoffmann and Parsons 1991, Loeschcke et al. 1994, Primack 1995, Schonewald-Cox et al. 1983, Soulé 1986, Soulé 1987, Soulé and Wilcox 1980). Genetic diversity is the raw material for evolutionary change (Hillis and Moritz 1990, Weir 1990). Its loss can contribute to a reduction in reproductive success, disease resistance (Allendorf and Leary 1986, O'Brien et al. 1985), and even in the ability of species to successfully adapt to long-term environmental changes (Templeton 1994). The IUCN (World Conservation Union) has recognized genetic diversity as one of the three levels of biological diversity requiring conservation (McNeely et al. 1990). The role of genetics in conservation has grown over the years, and the knowledge acquired is now a valuable tool in the management of endangered species.

Conservation genetics became a prominent discipline in the early 1980s (Schonewald-Cox et al. 1983), and it was defined by Frankham et al. (2002) as “the application of genetics to preserve species as dynamic entities capable of coping with environmental change”. Important issues on conservation genetics are the resolution of taxonomic uncertainties (Moritz 1995, O' Brien 1994, Sites and Marshall 2003), detection of introgressive hybridization (Gotelli et al. 1994, Vallender et al. 2007), and the definition of appropriate units for conservation (Moritz 1994, Paetkau 1999).

With the advent of the polymerase chain reaction (PCR) and popular use of molecular markers, new non-destructive techniques were available for a number of procedures such as: genotyping of endangered species (Morin and Woodruff 1995) or unique museum specimens (Baker 1994, Rosenbaum et al. 2000), determining paternity (Avise 1994, Primmer et al. 1995), inferring the relationship among founders (Geyer et al. 1993, Haig et al. 1994, Tarr et al. 1998),

identifying the best populations for reintroductions (Vrijenhoek 1994), and detecting illegal hunting (Avise 1994, Baker and Palumbi 1994).

Currently, there is a lively discussion over the development of centralized DNA barcodes (Hebert et al. 2003, Stoeckle 2003), which use DNA sequences as unique identifiers of species. On one hand, the supporters of the DNA barcoding (Hebert et al. 2003, Rubinoff 2006, Stoeckle 2003, Tautz et al. 2003) advocate that this is helpful in conservation biology for the identification of illegally imported biological products and for the rapid assessment of biodiversity studies, accelerating and optimising these processes. On the other hand, the main criticism of DNA barcoding has arisen from classical taxonomists (Dunn 2003, Lipscomb et al. 2003, Seberg et al. 2003) that support taxonomy based in morphology. De Salle and Amato (2004) suggested that DNA barcoding could be complemented by morphological or allozyme information. Others argue that the controversy will keep on until DNA methodology in taxonomy is better founded in the existing theory of evolutionary biology and phylogenetics (Vogler and Monaghan 2007). Nevertheless, several initiatives towards barcoding specific groups have already been launched [e.g. cetaceans (Baker et al. 2003) and bacteria (http://www.dsmz.de/bactnom/bactname)]; and several recent studies validate the effectiveness of barcoding in the identification of several animal groups (Clare et al. 2007, Ivanova et al. 2007, Kerr et al. 2007, Smith et al. 2006).

Following De Salle and Amato (2004), another main application of conservation genetics incorporates pattern and process into a cohesive approach to decision-making about endangered species. For this, introduction of nested clade analysis by Templeton (1998) was most relevant, since it can be applied to better comprehend ecological, demographic and genetic aspects of populations.

Conservation genetics and conservation biology haven't always had a "happy marriage" (Soulé and Mills 1992), which may have been due to an overemphasis of the importance of genetic variation and to a possible neglect of demographic and environmental factors (e.g. habitat threat, disease or predation) in species conservation (Caughley 1994). Lande (1988) was one of the first to argue the importance of genetics and demography in conservation biology, pointing out the primacy of demographic factors for understanding extinction. On the other hand, Avise (1989) countered by a defence of genetic approaches, and more recently Soulé and Mills (1992) and Westemeier et al. (1998) reinforced the relevance of genetic diversity loss. This matter has been recently discussed in a debate focused on the conservation of island endemics (Jamieson 2007a, section Islands). Although susceptibility of island populations to extinction has been interpreted as being mainly due to "non-genetic" causes (Frankham 1995), such as the impact of introduced predators (Blackburn et al. 2004, Duncan and Blackburn 2004), genetic

factors cannot be disregarded as another cause of the higher extinction rates of island populations (Frankham 2005, Frankham et al. 2002). Finally, Groombridge (2007) and Reed (2007) recommended a further integration of both factors into one evolutionary framework reinforcing the multidisciplinary character of the discipline Conservation Genetics.

1.2. Methods and approaches

1.2.1. Molecular methods

Nowadays, there are many genetic markers available, each with its own advantages and downsides. Usually, the combination of different molecular markers is the most appropriate decision.

In the present study, the molecular markers used were mitochondrial DNA (mtDNA) sequences (Papers I, III and V) and nuclear microsatellites (Papers II, IV and V). Both types of markers have the advantage of only requiring minute sample amounts, since they are amplified by PCR. An overview on the mentioned markers is given below.

Mitochondrial DNA

The animal mtDNA has a circular structure (Figure 1), generally coding for genes involved in the mitochondrial translation apparatus, electron transport, and oxidative phosphorylation (Ballard and Rand 2005). Avise et al. (1987) showed how mtDNA could be used to link population genetics and systematics. Since then, mtDNA sequences are one of the most widely used tools in phylogenetic and population genetic studies (Taberlet 1996), undoubtedly facilitated by the development of PCR technology and the use of universal primers (Kocher et al. 1989) for amplification of mtDNA segments.

MtDNA is haploid, maternal inherited (Giles et al. 1980) without recombination and has a fast evolutionary rate (Brown et al. 1979), thus providing ample information on evolutionary changes. Its effective population size is only one quarter of nuclear autosomal genes (Moore 1995).

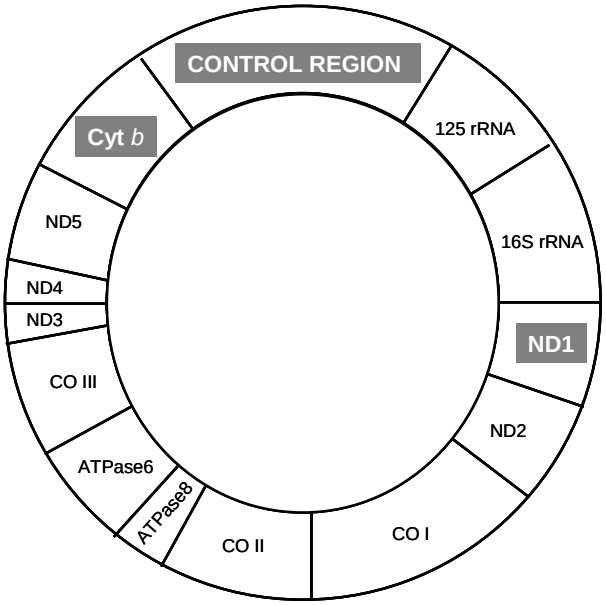

Figure 1 Schematic representation of mitochondrial DNA. Regions studied in the present thesis are labelled in grey.

Cytochrome b

The evolutionary dynamics of the mitochondrial cytochrome *b* (Cyt *b*) is very well known (Esposti et al. 1993). In animals, Cyt *b* is a highly conserved coding gene and therefore commonly employed in molecular phylogenetic studies (Nei and Kumar 2000).

It is among the most broadly sequenced genes in vertebrates (Irwin et al. 1991), and it is known to fit well the species boundaries based on classic taxonomy (Johns and Avise 1998). Cyt *b* has therefore become an adequate molecular marker to identify mammals at the species level (Baker and Bradley 2006) and has been widely used in bat studies (e.g. Barratt et al. 1997, Cardinal and Christidis 2000, Ditchfield 2000, Hoffmann and Baker 2003, Ibañez et al. 2006, Juste et al. 2004, Juste et al. 2003, Kawai et al. 2003, Miller-Butterworth et al. 2005, Pestano et al. 2003b).

NADH dehydrogenase 1

The nicotinamide adenine dinucleotide dehydrogenase 1 (ND1) is a highly conserved mitochondrial coding region, which has been widely used in phylogenetic studies in various animal groups (Cao et al. 1998, Garcia-Moreno et al. 2003, Leach and Reeder 2002, Macey et al. 1998, Tagliaro et al. 2005, Taylor et al. 1999, Zardoya and Meyer 1996).

Regarding bats, ND1 was applied to estimate the amount of cryptic diversity among European species (Ibañez et al. 2006, Kiefer et al. 2002, Mayer and von Helversen 2001), and to analyse the molecular systematics of several chiropteran groups (Kawai et al. 2002, Petit et al. 1999, Ruedi and Mayer 2001). Recently, it allowed the unambiguous genetic identification of 34

bat species (Mayer et al. 2007). Furthermore, following Petit (1998), ND1 was employed to calibrate the divergence rate of the hypervariable segment (HV II) of the control region.

Control region

Besides coding genes, there is also a non-coding zone in the mtDNA, known as the control region (CR), or displacement loop (D-loop). With around 1000 bp, it is responsible for the transcription of mitochondrial genes and contains the point of origin of heavy strand replication. Mammalian CR evolves 3-5 times faster than the rest of the mtDNA (Aquadro and Greenberg 1983, Cann et al. 1984) and shows strong transition/transversion bias (Nei and Kumar 2000).

The highest degree of polymorphism is observed within two hypervariable segments, HV I and HV II, which can be amplified and sequenced separately (Aquadro and Greenberg 1983). In the present study it was analysed the HV II.

CR has been used in many phylogeographic studies focusing intra-specific or micro-evolutionary patterns, and is rather informative in Chiroptera (e.g. Campbell et al. 2006, Castella et al. 2001, Chen et al. 2006, Hisheh et al. 1998, Juste et al. 2004, Juste et al. 2003, Kerth et al. 2000, Lloyd 2003, Petit 1998, Petit et al. 1999, Ruedi and Castella 2003, Spitzenberger et al. 2003, Worthington Wilmer et al. 1994).

Nuclear DNA

The DNA contained within the nucleus of eukaryotic organisms (nDNA), typically encodes more of the genome than mtDNA and unlike it, it is inherited bisexually, providing also information about the male population.

Microsatellites

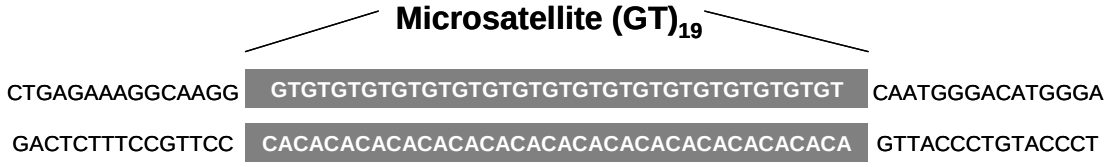

Figure 2 Schematic representation of a microsatellite tandem repeat.

The most commonly used nDNA markers are the microsatellites, which comprise simple repetitive nucleotide motifs (1–6bp in length, Figure 2). These are co-dominant, highly abundant among eukaryotes, and show high polymorphism levels (Goldstein and Schlötterer 1999), owing to their fast mutation rates (Schlötterer and Wiehe 1999). Given this set of characteristics, it is not surprising that microsatellites became a very powerful genetic marker, having contributed to great advances on the assessment of genetic diversity, detection of population genetic structure, parentage and relatedness tests and recent population history studies (Zhang and Hewitt 2003). In the last decade, microsatellites have been isolated for a wide variety of species, including chiropterans, and were applied in several population studies (Burland et al. 2001, Campbell et al. 2006, Kerth et al. 2002, Ortega et al. 2003, Petit and Mayer 1999, Petri et al. 1997, Rossiter et al. 2000, Ruedi and Castella 2003, Worthington Wilmer et al. 1999). Some have been specifically used in the study of cryptic species (Berthier et al. 2006, Racey et al. 2007, Vonhof et al. 2006).

1.2.2. Statistical methods

Genetic diversity

Genetic diversity in a population is crucial, since it provides the potential for the species to adapt to changes in the environment (Templeton 1994). Therefore, the amount of genetic diversity works as a "health indicator" for the studied populations. The measures used to describe mtDNA variation, in Papers III and V, were nucleotide diversity (π), and haplotype diversity (h). The former consists on the average number of nucleotide differences per site between pairs of sequences in a population (Nei and Li 1979), the latter is the probability of two haplotypes drawn at random being different, regardless of the sequence relationship (Nei and Tajima 1981). Following Avise (2000), "under normal population demographic conditions, coalescent processes ensure phylogenetic connections among genotypes within a species via vertical pathways of descent". If various haplotypes co-occur in the same population, this may be due to many colonization events, a single founder event entailing multiple lineages, or to accumulated mutations over time in that population. The mismatch distribution of haplotypes expected for each of the cases is distinct. A founder event followed by demographic expansion would result in a unimodal distribution of pairwise differences between haplotypes (Harpending 1994). Alternatively, if multiple colonisations had occurred, the admixture of different lineages is expected to generate a multimodal mismatch distribution, measured by the raggedness index (Harpending 1994). In the present thesis (Papers III and V), this methodology was used to investigate the demographic signature of mtDNA haplotype variation within both studied bat

species populations. Time since expansion was inferred from the demographic expansion parameter estimates (Rogers and Harpending 1992) with a calibrated rate of divergence of about 20% per million years (Petit et al. 1999).

Contrary to mitochondrial markers, which work as a single locus, microsatellites are co-dominant, thus allowing analysis based on allelic frequencies. Therefore, tests for deviations from Hardy–Weinberg equilibrium (HWE) are possible, as well as for fits to linkage equilibrium. In the present study (Papers II, IV and V), HWE was tested using exact tests based on contingency tables (Guo and Thompson 1992) and deviations from linkage equilibrium were tested with likelihood ratio tests (Slatkin and Excoffier 1996). Allelic frequencies and gene diversity were calculated, as well as allelic richness corrected by the rarefaction index (R), which accounts for differences in sample size (Paper IV and V). The occurrence of private (unique) alleles was used to describe population distinctiveness (Slatkin 1985b). Private allelic richness was estimated using the rarefaction method of Kalinowski (2005) in Paper II.

Bottleneck effect

Genetic variation may be lost when a population has continuing small sizes or when it experiences severe and rapid size reductions or bottlenecks (Garza and Williamson 2001). These events increase the likelihood of population extinction through genetic drift and possible inbreeding (Lowe et al. 2004). The measures of genetic variability loss can help to detect genetic bottlenecks, and this is often done using microsatellite loci (Luikart et al. 1998).

During bottlenecks, allelic richness decreases mainly due to rare alleles. Since these are lost randomly with respect to allele size population, the ratio of the number of alleles in each population to the total range in allele sizes (*M*) decreases with both the strength and duration of bottlenecks (Garza and Williamson 2001) This method remains sensitive to size reductions even up to 500 generations following the event (Garza and Williamson 2001), detecting older events relatively to other methods. It was used in Paper II, so as to detect possible past bottlenecks caused by old founder events.

Phylogenetic approaches

Phylogenetic analysis of DNA is an essential instrument for the study of evolutionary relationships of practically all levels of classification, including species or even interspecific populations (Nei and Kumar 2000). Phylogenetic methods include some based on discrete characters such as: maximum parsimony (MP) or maximum likelihood (ML); and also distance methods based on several mutational models. For example, the Kimura 2-parameters model

(Kimura 1980) takes into account different substitution rates between transitions and transversions in the sequences. Distance and MP methods were used in Paper I. Although less common, allele frequency based on microsatellite data is also useful for studying evolutionary relationships of closely related species (Takezaki and Nei 1996). With these markers, other genetic distances are usually calculated [e.g. chord distance Dc (Cavalli-Sforza and Edwards 1967), Nei's distance Da (Nei et al. 1983), in Paper II]. With any of the markers, the consistency of relationships is assessed by bootstrap. The distance methods employ clustering algorithms like the neighbour-joining (NJ, Saitou and Nei 1987).

Because genetic relationships among closely related species, or among populations within species, can be reticulate rather than bifurcated, networks are usually a better option. Multifurcating networks explain processes at these levels more efficiently and they are able to incorporate predictions from population genetics theory (Posada and Crandall 2001). In this thesis, haplotype networks were obtained in Papers I, III and V, both using a parsimony-based search of the missing haplotypes.

Genetic differentiation

Wright's *F*-statistics (Wright 1931) measures the differentiation at some levels of a (hierarchically) subdivided population, which is influenced by mutation and migration (Excoffier and Heckel 2006). F_{ST} is the fixation index that describes the reduction in heterozygosity within a population relative to the total population due to selection or drift. When considering microsatellites, several models of mutation should be taken into account, namely: the Infinite Allele Model (IAM) by Kimura and Crow (1964), where a mutation involves any number of tandem repeats, producing new alleles not previously present in the population; the Stepwise mutation Model (SMM) by Kimura and Otha (1978), in which mutations increase or decrease allele sizes by single units, alleles may possibly mutating towards alleles already existing in the population; and the Two-phased Model (TPM) by Di Rienzo et al. (1994), intermediate to the SMM and IAM, wherein mutations introduce a gain or loss of X nucleotides. In Papers II, IV and V, several genetic differentiation measures have been used: F_{ST} (based on IAM), R_{ST} (based on SMM), and a standardized measure of genetic differentiation, G_{ST} (Hedrick 2005).

Population structure

The genetic structure of a population is influenced by its history as well as by the species own characteristics. A weak genetic structure indicates species cohesion, while the opposite is linked to strong fragmentation and in some cases may suggest incipient speciation.

Following Pearse and Crandall (2004), several different methods were used to investigate and visualise the population genetic structure in this study: hierarchical analyses of molecular variance AMOVA (Excoffier et al. 1992, in Papers II III and IV), spatial analysis of molecular variance SAMOVA (Dupanloup et al. 2002, in Paper V), and a Factorial Correspondence Analysis (Escofier and Pagès 1988) over the populations in Paper IV and V. Furthermore, in Papers II and III, it was conducted a Bayesian analysis, which allows the incorporation of background information into data analysis (Beaumont and Rannala 2004). Bayesian methods allow the inference of the partitioning of individuals into subpopulations (Pritchard et al. 2000), and the presence of subpopulations within a larger population among and within the identified subpopulations (Corander et al. 2003).

Dispersal and gene flow

Species genetics and dynamics are strongly affected by dispersal. Thus, this life history trait has been an important issue in evolutionary biology research (Clobert et al. 2001). The estimation of levels of dispersal or gene flow in natural populations can be made by two types of methods: direct and indirect (Slatkin 1985a) The former include field observations and mark-release–recapture or radio-tracking methods, which are difficult to apply to certain organisms, and are frequently inadequate to investigate long-term dispersal (Frankham et al. 2002). Therefore, indirect estimates using genetic markers have become very common.

A classical way of measuring gene flow among populations is through the *N*m (number of migrants per generation) as a function derived from Wright's (1931) equation F_{ST} =1/(4Nm+1). Hartl and Clark (1989) suggested a qualitative evaluation of these measures: if F_{ST} is less than 0.05, gene flow is considered high, this is more limited at values of F_{ST} between 0.05 and 0.15, greatly reduced if F_{ST} ranges from 0.15 to 0.25, and extremely reduced when F_{ST} is greater than 0.25. Nevertheless, there are some caveats on this calculation of *N*m (Whitlock and McCauley 1999), since the model assumes that populations are at constant equilibrium between immigration and drift and populations contribute equally to each others' gene pool regardless of spatial scale. Slatkin (1993) applied Wright's (1943) model of isolation-by-distance (IBD) and showed that when equilibrium between drift and migration is achieved in populations, a positive association between genetic differentiation and geographical distance is expected. When dispersal is restricted due to geographical barriers, differentiation will increase due to genetic drift (Hutchison and Templeton 1999). IBD is used to infer the extent of genetic connectivity within and between populations. IBD was calculated for mitochondrial DNA (Papers III and V), and nuclear DNA (Papers IV and V), by means of a Mantel test (Mantel 1967) correlating

geographical distance and mitochondrial and nuclear differentiation. More recently, the use of methods to assign putative migrants to populations on the basis of multilocus genotypes (Bayesian assignment methods) has expanded. These methods were employed in Papers II and IV for the estimation of gene flow.

Sex-biased dispersal

Sex-biased dispersal is broadly spread in vertebrates. Under this dispersal pattern, individuals from one sex tend to disperse more, whereas individuals from the other sex tend to stay or return to their natal site (philopatry) (Prugnolle and de Meeûs 2002). Differences in the dispersal between the sexes are expected to result in significant dissimilarity in the population genetic parameters (Mossman and Waser 1999, Prugnolle and de Meeûs 2002). Generally, two methodologies are used to evaluate sex biased dispersal: 1) comparison between nuclear and mitochondrial markers; 2) analysis comparing both sexes based on bi-parentally inherited markers. Both were used in the present study in Papers IV and V. With the former, it is expected to find distinct population structures portrayed by the different markers and a sign of past sex biased dispersal, whereas with the latter, it is possible to detect contrasted population differentiation between sexes if individuals are sampled after dispersal, and detect current dispersal. For this analysis, it was followed the approach described by Goudet et al. (2002).

1.3. Study Organisms

1.3.1. Bats (Order Chiroptera)

Bats are unique among mammals not only due to their capacity for powered flight, but also because, despite their small size, they have life-history characteristics that are generally attributable to larger species (Barclay and Harder 2003). They develop and reproduce slowly and live long lives, being placed at the slow end of the fast-slow continuum of life-history traits (Read and Harvey 1989).

The Order Chiroptera is one of the most ubiquitous mammalian orders, the second most diverse (Wilson and Reeder. 2005), and it includes the most gregarious and colonial mammals known (Altringham 1999). Two sub-orders have been defined based on palaeontological and morphological data: the Old World Megachiroptera, or flying foxes (generally non-echolocating bats), and the more widespread and diverse echolocating bats, Microchiroptera (Miller 1907 in Jones and Teeling 2006).

With the dawn of molecular studies, and generalisation of phylogenetic approaches, bat systematics has been revised (e.g. Eick et al. 2005). Studies by Murphy et al. (2001), Nikaido et al. (2000) and Van den Bussche and Hoofer (2004) have supported a monophyletic origin of Chiroptera. On the other hand, the traditional division of bats in the two suborders, mentioned above, has been contested by several studies placing Megachiroptera bats and rhinolophoid microbats (excluding nycterids) in a single clade, and the remaining microbats in another (Hutcheon and Kirsch 2004, Springer et al. 2001, Teeling et al. 2005, Van den Bussche and Hoofer 2004).

Bats spend most of their lives in their roosts, which seems to have strongly influenced their evolution (Altringham 1999, Kunz and Fenton 2003). Bats show a wide variety of roost preferences, occupying both natural (e.g. caves, rock crevices, plants) and man-made structures (e.g. mines, tombs, buildings, bridges, Kunz and Lumsden 2003). Some bat species are roost specialists, selecting a unique type of roost. For example, *Lasiurus cinereus* only selects tree foliage roosts (Hutchinson and Lacki 2000), *Pipistrellus pipistrellus* and *Plecotus auritus* nowadays roost mostly in man-made constructions (respectively, Thompson 1992 and Entwistle et al. 1997) and *Miniopterus schreibersii* only roosts in caves and mines (Rodrigues 1999). Other bat species, such as *Eptesicus fuscus* (Agosta 2002) and *Nyctalus azoreum* (Paper III) are roost generalists.

Caves are considered the most stable and persistent roosts for bats, providing the most important maternity and hibernating sites for many species (Racey and Entwistle 2003). Their general scarcity, together with the thermoregulatory advantages of communal living, may have led to the formation of vast colonies by some species (Altringham 1999). However, such colonial behaviour in roosts may cause great vulnerability, since the destruction of a single roost may affect substantially the effective population size and genetic structure of a bat species.

Many chiropterans perform annual migratory movements, involving large numbers of individuals across long distances, when moving between summer nurseries and winter hibernacula (Fleming and Eby 2003). Bats are also the only terrestrial mammals to have managed to colonise most islands, even if remote (e.g. Galapagos, Hawaii, New Zealand, Madeira and Azores archipelagos), without human aid (Carlquist 1965, Daniel 1990, Darwin 1859, Palmeirim 1991, Santos-Reis and Mathias 1996). Some of the few species that managed to reach distant islands are now fragmented into relatively small isolated populations inhabiting each island. This situation creates particular conservation problems specifically related with the genetic diversity and structure of those populations.

According to Hilton-Taylor (2000), roughly 25% of bat species are considered threatened, with 12 species already being extinct (1%). All but one of these extinct Megachiropterans (eight species) were insular species (Racey and Entwistle 2003). One of the features found to predict extinction risk in bats is small geographic range (Jones et al. 2003). Overall, population fragmentation, due either to roosting behaviour, or island distribution, is a feature of bat species that often increases their vulnerability.

Molecular studies in bats

Studies on bat systematics, behaviour and ecology are still greatly needed to increase knowledge of evolutionary processes and for improving their conservation and management.

Due to their unique features, bats are particularly difficult to study using traditional ecological methods; the use of molecular tools has proved to be extremely helpful to obtain information on this animal group. Until 2001, population genetic studies focusing on bats were limited to approximately 60 publications, relating to less than 70 of the 963 bat species (Burland and Wilmer 2001). According to this same review, the chiropteran family with the highest number of studies was the Vespertilionidae (23 publications). Since then, the number of publications on this family has almost tripled (65 publications). This expansion has been visible in many research areas, such as systematics, species boundaries, and population genetics.

The reason for such increase seems to be related with several aspects. To begin with, many molecular investigations have been stimulated by the debate over bat systematics (see review by Eick et al. 2005). Presently, the monophyletic origin of bats has been corroborated, several relationships among families have been clarified (see references above), and the molecular systematics of several chiropteran genus have been analysed (e.g. Hoofer and Van den Bussche 2003, Kawai et al. 2002, Ruedi and Mayer 2001).

Another important aspect concerns species boundaries. Due to crypticism and/or hybridization, some species cannot be distinguished using morphological characters. In these cases, molecular genetics have revolutionised some aspects of traditional bat taxonomy. One of the first well-known cases was the European pipistrelle bat (*Pipistrellus pipistrellus*), regarded as a single species until Jones and van Parijs (1993) identified two echolocation “phonic types”, and Barratt et al. (1997) confirmed the occurrence of two corresponding distinct mtDNA lineages. Since then, several new cryptic species have been described, and their taxonomic position re-evaluated, using molecular systematics (Benda et al. 2004, Guillén-Servent and Francis 2006, Kiefer and Veith 2001, Mucedda et al. 2002, Spitzenberger et al. 2002, Spitzenberger et al. 2003, Thabah et al. 2006, von Helversen et al. 2001). Furthermore, cryptic diversity among bats has

been assessed with various markers (e.g. Berthier et al. 2006, Ibañez et al. 2006, Mayer and von Helversen 2001, Racey et al. 2007).

At the population level, molecular studies have generally confirmed low genetic differentiation among populations of migratory species (Burland et al. 2001, Russell et al. 2005), although an exception has been also presented (Miller-Butterworth et al. 2003). Mating behaviour also has a great impact on the population genetic structure of bats. Genetic studies of paternity seem to support a wide variety of mating behaviours (i.e., polygyny, multiple mating by females, or female choice) in bat species (Heckel and von Helversen 2003, Kerth et al. 2002, McCracken and Wilkinson 2000, Rivers et al. 2005, Veith et al. 2004, Vonhof et al. 2006).

Another important issue approaching chiropteran populations are the physical barriers to gene flow, especially important when considering insular species. In the review by Burland et al. (2001), only ten island species had been studied. Nowadays, many more insular bat populations have been surveyed in phylogeographic studies, and molecular markers have been invaluable in establishing the colonization history of many of them (e.g. Bunce et al. 2005, Carstens et al. 2004, Chen et al. 2006, Davalos 2007, Hisheh et al. 2004, Juste et al. 2004, Juste et al. 2003, Lloyd 2003, Maharadatunkamsi et al. 2003, Pestano et al. 2003a, Pestano et al. 2003b, Pulvers and Colgan 2007, Roberts 2006a, Roberts 2006b).

It is worth mentioning the relevance of molecular genetic approaches in the study of threatened species. In studies aiming at the determination of conservation status, detection of endangered populations or planning of management strategies, non-invasive methods are highly recommended. Since the middle 90's, the use of molecular markers in threatened bat studies was intensified (e.g. Barratt et al. 1997, Campbell et al. 2004, Corneaux and McCracken 1996, Davalos 2005, Lloyd 2003, Ramos Pereira et al. 2006, Rossiter et al. 2000, Russell et al. 2005).

To conclude, the use of several molecular markers in bat studies has become routine, and thanks to the generalisation of genetic studies, bat taxonomy has been clarified and facilitated (see Mayer et al. 2007) where 34 bat species were unambiguously identified). This tendency will probably expand with the use of the DNA barcoding system, which effectiveness has been recently assessed for species discrimination in Neotropical bats (Clare et al. 2007).

1.3.2. Azorean bat (*Nyctalus azoreum*)

The Azorean bat (*Nyctalus azoreum* Thomas, 1901) belongs to the family Vespertilionidae, genus *Nyctalus*. Koopman (1994) recognized seven species of this genus occurring in the entire Palaearctic region, and entering only marginally into the Indomalay region (Simmons 2005). *N.*

azoreum is the smallest representative of the genus in Europe (Palmeirim 1991). It is restricted to the archipelago of Azores, being its only endemic mammal. It has a dark pelage (Figure 3), measuring 53-55 mm head-body length, 34-36mm tail length, 35-42 mm forearm length, and weighting around 6-15 g (unpublished data). It is a bat species which presents great phonic plasticity, especially on the social calls. Its echolocation is characterised by an average peak frequency of 32.1 kHz (Rainho et al. 2002).

Figure 3 Drawing of *N. azoreum* by Luís Filipe Lopes © 2007

The Azores (37º - 40º N, 25º - 31º W) are situated in the middle Northern Atlantic Ocean, about 1500 Km away from the mainland, at the junction of three tectonic plates: North American, Eurasian and African plates. The archipelago comprises nine major inhabited islands, divided into three groups: the Western group of Corvo and Flores; the central group of Faial, Pico, S. Jorge, Graciosa and Terceira; and the Eastern group of S. Miguel and Santa Maria (Figure 4). The islands of Azores are totally volcanic and of recent origin, the oldest island, Santa Maria, being around 8 Myr BP, (Abdel-Monem et al. 1975), and the youngest, Pico, around 0.3 Myr BP (Chovelon 1982). The Azorean bat is present in seven of the nine islands of the archipelago, corresponding to the Central and Eastern groups (i.e. it is absent in the Western group). It is considerably common in most of these islands, but is rare on Santa Maria and to a least extent on Graciosa (Rainho et al. 2002).

N. azoreum is thought to have evolved from a continental ancestor related to the Leisler's bat (*Nyctalus leisleri*) and it can be distinguished from the later by its smaller size, darker pelage and higher peak frequency calls (Rainho et al. 2002, Skiba 2003). Although mainly nocturnal, this species is probably the most active microchiropteran during daytime worldwide (Moore 1975, Speakman 1995). In terms of roosting preferences, this species is considered a generalist, since it roosts in crevices, rocks, buildings, coastal cliffs, as well as in trees (Paper III).

N. azoreum was first described as a distinct species by Thomas (1901), a status equally sustained by Miller (1912), but Corbet (1978) reduced its status to that of a subspecies of *N. leisleri* (Kuhl 1817). More recently, the systematic position of the Azorean bat was re-examined by Palmeirim (1991) and Speakman and Webb (1993) and both agreed on the species status of *N. azoreum*. Given the restricted distribution and small global population size the Azorean bat has a global status of "Endangered" (IUCN 2007b). In the most recent Portuguese Red Data Book it is classified as "Critically Endangered" (Queiroz et al. 2006b).

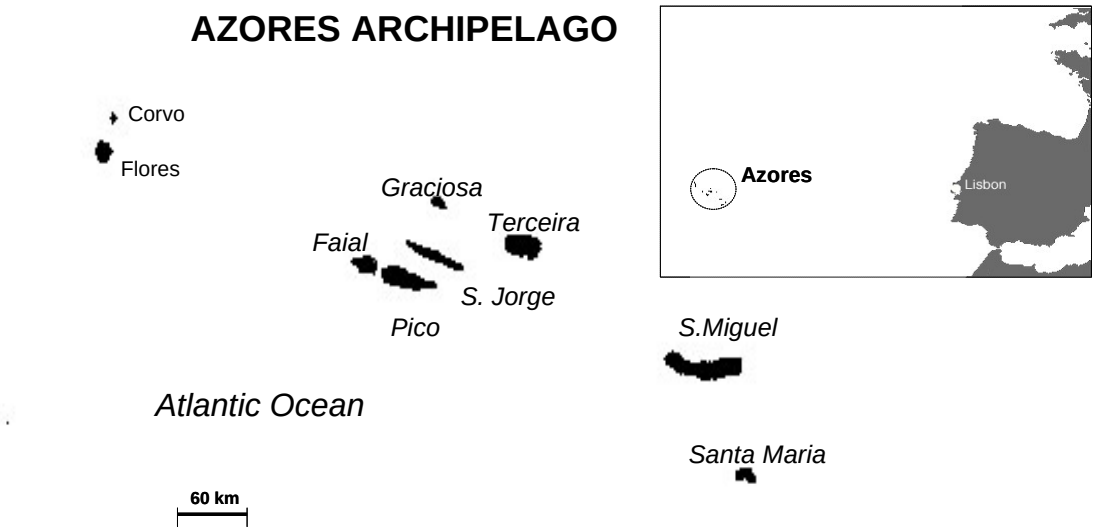

Figure 4 Map of the Azores archipelago, *N. azoreum* is found in the islands typed in italic

1.3.3. Schreiber's bent-winged bat (*Miniopterus schreibersii*)

Generally considered as part of the family Vespertilionidae, subfamily Miniopterinae (Corbet and Hill 1991, Koopman 1994), the taxonomic position of the genus *Miniopterus* has been debated for long (Gopalakrishna and Chari 1983, Mein and Tupinier 1977). Recent phylogenetic studies have, however, supported its status as a separate family, Miniopteridae, given the extreme lineage divergence in relation to the vespertilionids (Eick et al. 2005, Hoofer and Van den Bussche 2003, Hutcheon and Kirsch 2004, Kawai et al. 2002, Van den Bussche and Hoofer 2004).

The Schreiber's bent-winged bat (*Miniopterus schereibersii* Kuhl, 1817) has a grey brownish pelage (Figure 5), measuring 50-62 mm head-body length, 56-64 mm tail length, and 42-48 mm forearm length, and weighing between 9-16 g (MacDonald and Barrett 1993). In its echolocation calls the frequency of most energy averages 54.2 kHz (Russo and Jones 2002). It is a highly gregarious cave-dwelling species and forms large colonies during most of the year. It makes

regional migrations, the most important of which are between wintering roosts (hibernacula) and the roosts of summer maternity colonies, flying along hundreds of kilometres (Rodrigues 1999). Ringing studies by Palmeirim and Rodrigues (1995) indicate that females present strong philopatric behaviour, returning to give birth in the colonies in which they were born. Throughout the mating and hibernation periods, bats from diverse nurseries share roosts (Palmeirim and Rodrigues 1995, Serra-Cobo et al. 1998).

Figure 5 Drawing of *M. schereibersii* by Jean Chevallier © (http: //www.cons-dev.org/GVS)

M. schreibersii is a polytypic species with one of the widest distributions among mammals (Hutson et al. 2001), ranging from southern Eurasia (Figure 6) to Africa, Australia, and the Solomon Islands (Rodrigues 1999). The taxonomy of the *M. schreibersii* complex has been highly debated and is not consensual (Appleton et al. 2004, Koopman 1994). The molecular study of Tian et al. (2004) has suggested the division of *M. schreibersii* into three species in Europe, Asia and Australia, confirming the previous viewpoint of Maeda (1982) based on morphometric data. Overall, several recent phylogenetic surveys have confirmed the presence of the nominate form *M. schreibersii* in western Turkey, extending into continental Europe and northern Africa (Appleton et al. 2004, Bilgin et al. 2006, Miller-Butterworth et al. 2005, Tian et al. 2004).

Although considered as a relatively abundant species, the Schreiber's bent-winged bat population size is currently declining in some areas of Europe (IUCN 2007a).

Figure 6 European distribution of *M. schreibersii* (striped area), adapted from (IUCN 2007a)

In Portugal, it is the most abundant cave-dwelling bat (Palmeirim and Rodrigues 1992), with a few tens of thousands of individuals distributed by less than 20 roosts (Queiroz et al. 2006a). The fact that *M. schreibersii* congregates in a reduced number of colonies makes this species vulnerable. Important potential threats to this bat species include roost destruction or disturbance, particularly during the breeding or hibernation seasons, loss of feeding areas, and excessive use of pesticides (Queiroz et al. 2006a). The Schreiber's bent-winged bat was classified as "Vulnerable" in the Portuguese Red Data Book (Queiroz et al. 2006a) and as "Near Threatened" by the IUCN (2007a).

1.4. Aims and Thesis structure

The major focus of this thesis was the genetic structure and gene flow in fragmented populations (insular and continental) of two threatened bat species (*N. azoreum* and *M. schreibersii*). Employing molecular tools, the genetic diversity of those populations and the current and historical processes influencing their structure were studied. The results were used to suggest some conservation guidelines.

The specific objectives of this thesis were:

1. To analyse the phylogenetic relationships among several species within the *Nyctalus* genus, using mtDNA sequences..

2. To determine the level of genetic divergence between the insular Azorean bat and its continental ancestor, the Leisler's bat, either through mtDNA sequences (CR, Cyt *b* and ND1) or genotyping microsatellite markers, and therefore clarify the taxonomic status of the Azorean bat.

3. To determine the level of genetic structuring, to infer the colonization history and measure levels of gene flow among the isolated populations of the Azorean bat, by investigating the levels of genetic diversity and genetic differentiation at the mtDNA CR and microsatellites.

4. To determine the level of genetic structuring, to infer the post-glacial re-colonization route and measure levels of gene flow among the fragmented populations of the Schreiber's bent-winged bat, by examining the levels of genetic diversity and genetic differentiation at the mtDNA CR and microsatellites.

5. To define priority units for the conservation of the studied species, based on the previous genetic insights, and to suggest management measures.

In order to address these specific objectives, the dissertation is organized in five chapters. Specifically, Chapter I corresponds to the General Introduction and it is followed by two main results chapters (II and III). Chapter II concerns the genetic relationships between the insular bat *Nyctalus azoreum* and its mainland ancestral *N. leisleri*. It comprises two studies, each dealing with specific sets of molecular markers. In the first study (Paper I), mtDNA sequences of three regions with different substitution rates (ND1, Cyt *b* and CR) were used to review phylogenetic relationships among six species of the genus *Nyctalus*, and measure the levels of genetic divergence between the Azorean bat and its continental counterpart, therefore inferring the origin of the insular bat. In the second study (Paper II), microsatellite loci were analysed in the same populations and individuals used in the Paper I (the Azorean and Leisler's bats) to assist in the detection of contemporary gene flow between the two species, as well as to confirm their taxonomic status. Also, the colonization path of the insular species inside the archipelago was deduced through the examination of past founder events.

Chapter III focused on the genetic variation in species with fragmented populations. One of the target species is the above referred insular *N. azoreum.* In this chapter, its patterns of genetic diversity and population structure throughout the archipelago were examined to infer the possible date of colonisation and levels of population connectedness on islands (past and present), using mitochondrial and nuclear markers (Papers III and IV, respectively).

Another bat species studied in this thesis was *M. schreibersii*, which shares with the island species the fact that they are subdivided in small functional populations, in this case, cave colonies. The effects of migration, philopatry and post-glacial events on the species genetic structure were examined, evaluating the different factors for species conservation. Specifically,

the patterns of genetic variation and gene flow among roosts were analysed, checking if mitochondrial (CR) and microsatellite markers recapitulate distinct genetic scenarios (Paper V).

In Chapter IV, a general discussion with an integrated overview of the results from Chapters II and III is presented. Finally, with Chapter V a synthesis of the findings is provided, pointing out some key limitations to this study and suggesting future avenues of research.

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Chapter II Genetic relationships between bat species

2.1. Island *versus* continental bat species

2.1.1 Paper I

Genetic divergence and phylogeography in the genus *Nyctalus* (Mammalia, Chiroptera): implications for population history of the insular bat *Nyctalus azoreum*. *Genetica* 130, 169-181.

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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

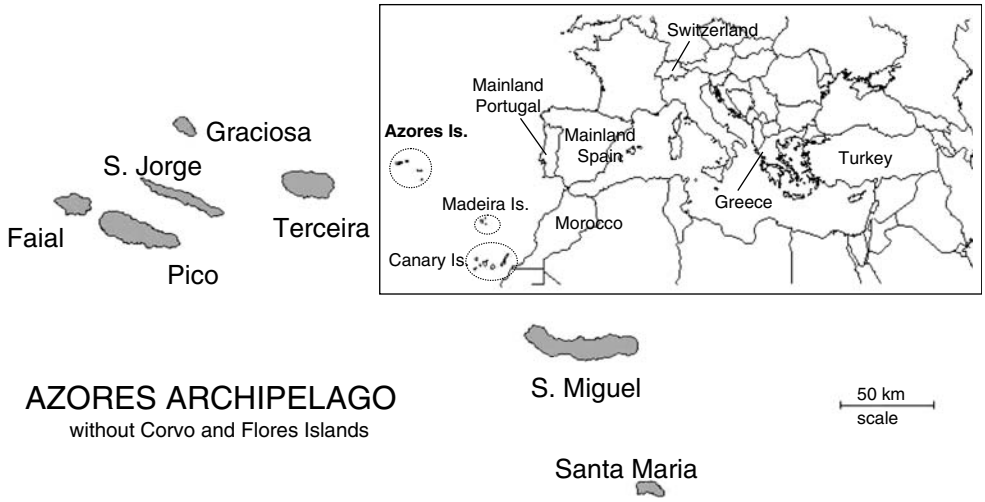

Fig. 1 Map of Europe and the Atlantic archipelagos of Canary, Madeira and Azores, showing in closer detail the Islands of the Azorean archipelago with confirmed presence of *Nyctalus azoreum*. (See Appendices 1 and 2 for further details). *N. azoreum* is absent from the islands of Flores and Corvo, so 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

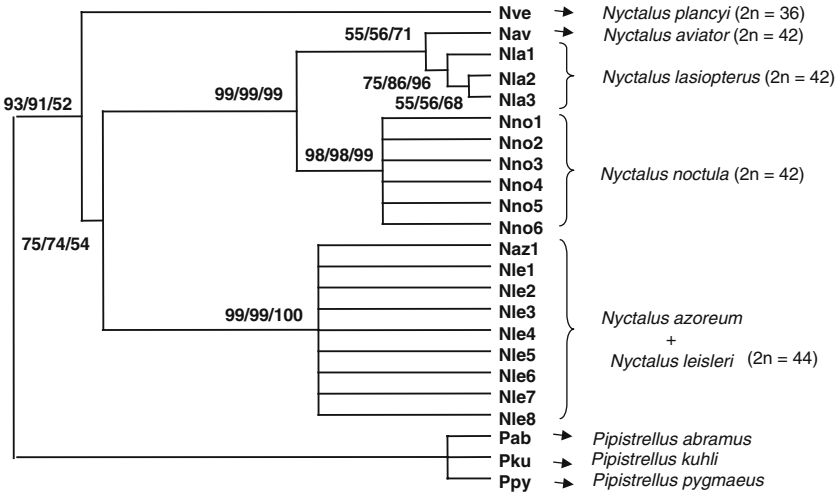

Fig. 2 Phylogenetic tree (see text for details) of relationships among *Nyctalus* species, based on a 639 bp ND1 fragment and using three species of *Pipistrellus* as outgroups (*P. pygmaeus*, *P. abramus* and *P. kuhlii*). The bootstrap values correspond respectively to the results of the following 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

Genetica (2007) 130:169–181

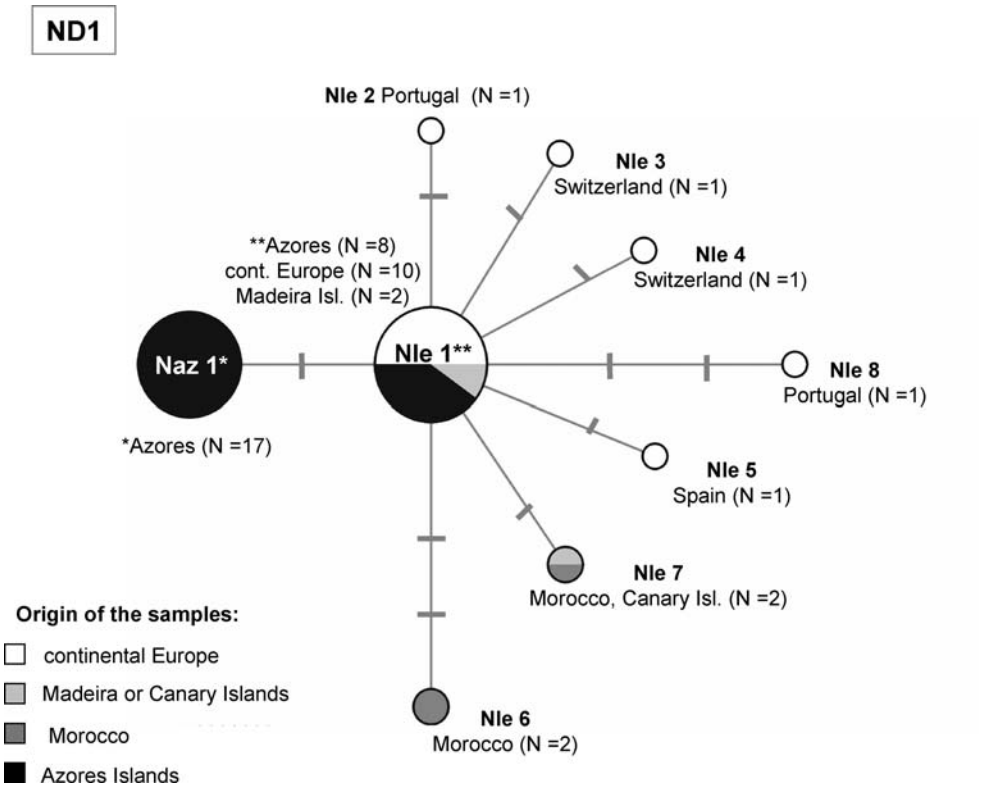

Fig. 3 Median-joining network based on nine haplotypes from 639 bp of the ND1 gene sequenced over 48 individuals of *N. azoreum* and *N. leisleri* (see Appendices 1, 2). The area of circles is proportional to the frequency of the haplotypes. Each *grey bar* in between the lines connecting the haplotypes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

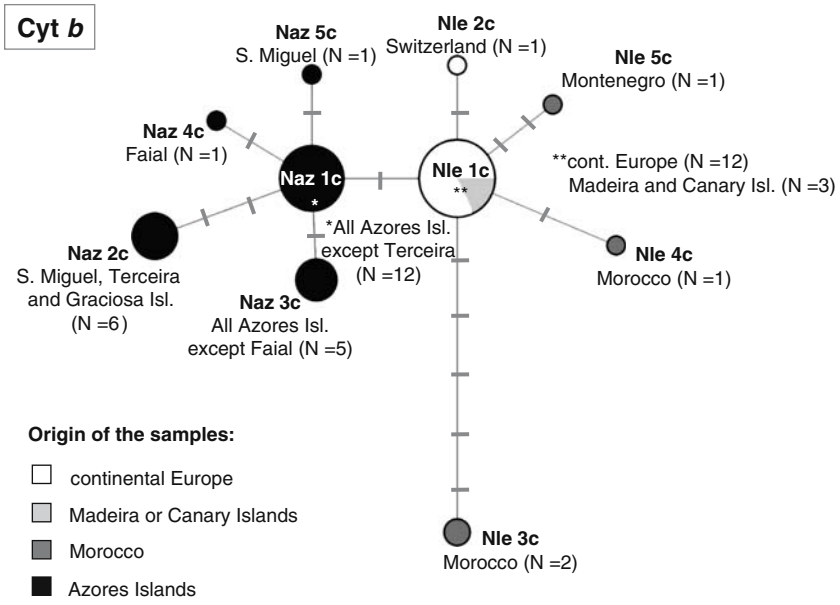

Fig. 4 Median-joining network based on 17 haplotypes evidenced in 692 bp of the Cyt *b* gene sequenced over 47 individuals of *N. azoreum* and *N. leisleri* (see Appendices 1, 2). The area of circles is proportional to the frequency of the haplotypes. Each *grey bar* in between the lines connecting the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

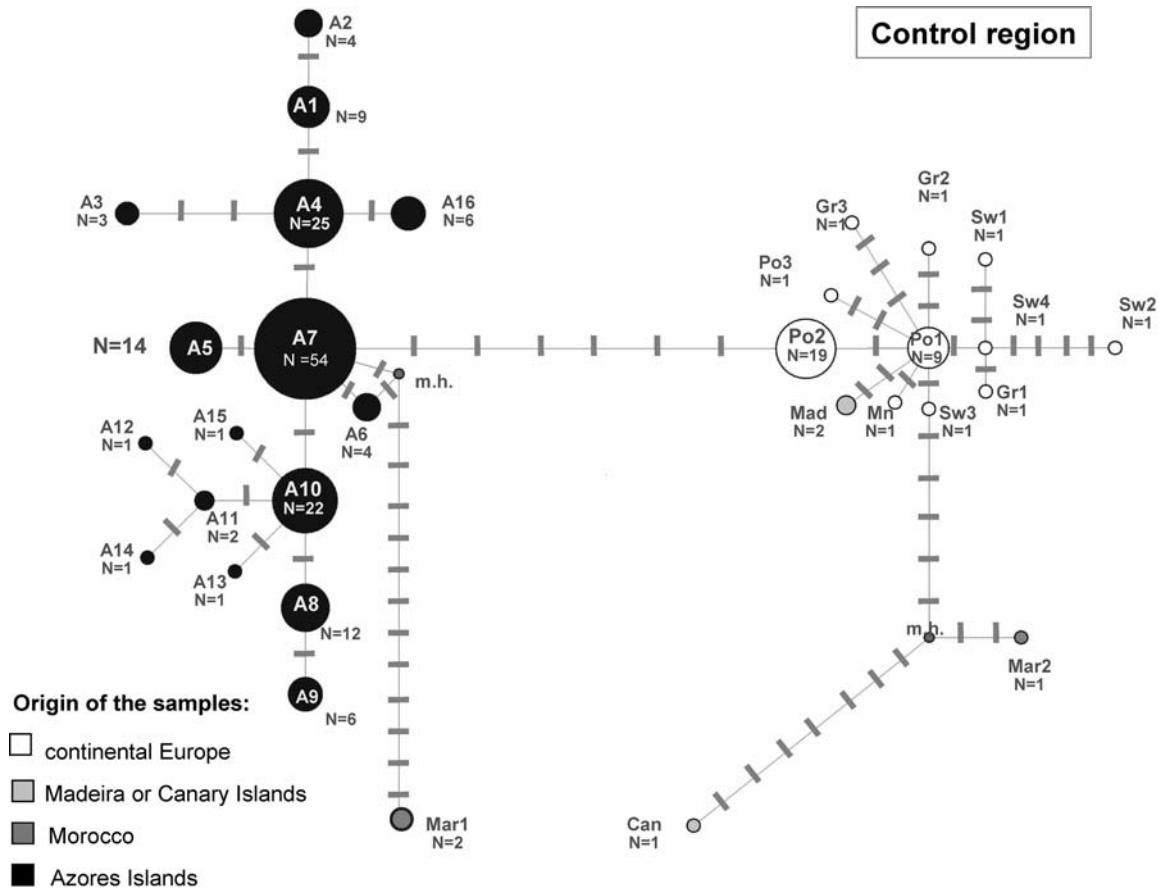

Fig. 5 Median-joining network based on 30 haplotypes shown by sequencing 422 bp of CR in 159 Azorean bats and 43 Leisler's bats (see Appendices 1, 2). Each *grey bar* in between the lines connecting the haplotypes represents one mutation. The frequency and geographic distribution of each haplotype are 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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2.1.2 Paper II

Lack of gene flow between the insular bat *Nyctalus azoreum* and its closest mainland ancestor *Nyctalus leisleri*: evidence from microsatellites.

Salgueiro P (in preparation)

Lack of gene flow between the insular bat *Nyctalus azoreum* and its mainland ancestor *Nyctalus leisleri*: evidence from microsatellites

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Abstract

The Azorean bat (*Nyctalus azoreum*), the only endemic mammal of the Azores archipelago (Portugal), recently diverged from its mainland relative, the Leisler's bat (*N. leisleri*). In order to assess the genetic variability in each species and check for present levels of gene flow between the two taxa, eight microsatellite loci were genotyped and analysed. The results indicated lower genetic diversity in the insular bat. We found no evidence of migrants and many unshared alleles between the two species, which provides strong evidence against any contemporary gene flow between them. S. Miguel was the only island with no historical genetic signatures of population reduction, thus suggesting that it was the first island of the Azores to be colonised and the source of colonisation to the other islands. Overall, our findings support that the Azorean bat is a reproductively isolated species with a high conservation value.

Keywords: Chiroptera, cohesion species concept, *Nyctalus azoreum*, microsatellites

Introduction

Ever since Darwin's observations on the Galapagos, islands have been recognised as "laboratories" for the study of evolution. Insular species are often adaptively divergent in a wide variety of features (Grant 1998). Compared with their mainland counterparts, island species tend to have higher densities and survivorship (Adler and Levins 1994, Stamps and Buechner 1985), and broader ecological niches (Grant 1998). Aspects of their behaviour are affected, and island populations often show reduced aggressiveness and relaxed territory boundaries (Stamps and Buechner 1985). The species' isolation often results in severe body size changes (Lomolino 2005, Millien 2006). This is the case of the only endemic mammal from the Azores Archipelago, the Azorean bat (*Nyctalus azoreum*), which is considerably smaller (Palmeirim 1991, Speakman and Webb 1993) and darker than its continental ancestor, the Leisler's bat (*N. leisleri*). It presents a broader ecological niche, occupying a variety of roosts like buildings, coastal cliffs and trees (Salgueiro et al. 2004) whereas its mainland counterpart is predominantly a tree dweller (Shiel 1999). Furthermore, the Azorean bat presents echolocation calls with a higher peak frequency than its continental ancestor (Rainho et al. 2002, Skiba 2003), and an unusually high level of diurnal activity (Moore 1975, Speakman 1995), probably a consequence of predator scarcity (Grant 1998).

This marked phenotypic distinction contrasts with the unexpectedly low levels of genetic divergence found between the two species at several mitochondrial DNA markers (Salgueiro et al. 2007). The maximum genetic divergence found was of 3.6% for the control region. This may have resulted from a very recent speciation process. In fact, the two mtDNA studies done with *N. azoreum* (Salgueiro et al. 2004, Salgueiro et al. 2007) have confirmed this, suggesting that the Azores were colonised naturally sometime between the late Pleistocene and the early Holocene by a single bat matrilineage. *N. azoreum* was first described by Thomas (1901). Later, Corbet (1978) classified it as subspecies (*N. leisleri verrucosus*) and more recently, Palmeirim (1991) and Speakman and Webb (1993) confirmed its species status. These studies were based on morphometric data. Our recent genetic insights confused again the taxonomic status of the Azorean bat.

Uncertainties in species definitions may be detrimental for biodiversity conservation, since taxonomic rank is a decisive factor in assessing conservation priority of endangered taxa. Sequencing parts of the mtDNA only supply the lower limit of true species diversity (Mayer et al. 2007). Besides, only the sympatric occurrence of both taxa can finally proof the existence of a true species under the biological species concept (Mayr 1963). Avoiding problems with species concepts, the evolutionarily significant unit (ESU) was proposed as the minimal unit of

conservation by Ryder (1986). Moritz (1994) defined ESUs as morphologically and genetically populations distinct from other similar ones with a distinct evolutionary history, and showing mitochondrial or nuclear evidence for cessation of gene flow. Crandall et al. (2000) and Templeton (1998) proposed a Cohesion Species Concept, in which speciation requires significant or adaptive isolation and ecological divergence. Accordingly, Mayer and von Helversen (2001) highlighted that cases of unresolved bat species status based on mtDNA, should be subjected to detailed studies on morphology, ecology, echolocation, and nuclear gene flow. This agrees with the present necessity of joining phenotypic and genetic data in an integrative taxonomy approach (Will et al. 2005).

Some mtDNA markers showed a poor resolving ability to clearly separate *N. azoreum* from *N. leisleri*, due to the compressed time frame of divergence. Therefore, it was necessary to apply more suitable molecular markers to resolve recent speciation events.

Petren et al. (2005) showed that microsatellites are informative when compared among species that are less that 4% divergent based on mtDNA sequence. Allele frequency data are useful for studying evolutionary relationships of closely related species (Takezaki and Nei 1996). Owing to their high variability levels, microsatellites have clarified many species relationships (Chirhart et al. 2005, Kruger et al. 2005, Petren et al. 1999), including bats (Racey et al. 2007).

Aspects of inter-island population structure in the Azorean bat were investigated with microsatellites (Salgueiro et al. in press). However, *N. azoreum* and *N. leisleri* have not yet been compared with nuclear microsatellite markers, which will be achieved in the current study. We aim at examining genetic diversity and genetic divergence between species. These data will assist in evaluating the level of contemporary gene flow between the two species in order to support their taxonomic status. We also seek to infer the colonisation path inside the archipelago, through the analysis of past founder events.

Methods

Study area and sampling

The Azores is possibly the most isolated archipelago in the North Atlantic, positioned on the junction of the tectonic plates of Europe, Africa, and America. It is located about 1500 km west of mainland Europe, and 3900 km east of North America. It encompasses nine islands that extend along 600 km the Atlantic Ocean (38º30' N 28º00' W, Figure 1). These islands were formed along the oceanic ridge at fairly recent times, Santa Maria being the oldest, around 8 Myr (Abdel-Monem et al. 1975) and Pico the youngest, with 0.3 Myr (Chovelon 1982). We searched for bats in the whole archipelago, but only caught specimens in the five islands of the Central

Group, Faial, Pico, S. Jorge, Terceira, Graciosa, and in S. Miguel, which is part of the Eastern Group (Figure 1).

Our sampling included 279 individuals of *N. azoreum* as reported in Salgueiro et al. (in press) and 29 specimens of *N. leisleri* from one forest site in continental Portugal, plus 10 from other regions: Spain (1), Switzerland (4), Greece (2), Turkey (1), Czech Republic (1), Montenegro (1). Part of this sample set was previously typed for mitochondrial genes by Salgueiro et al. (2007). We performed non-lethal sterile biopsy punches of the wing membrane (Worthington Wilmer and Barratt 1996).

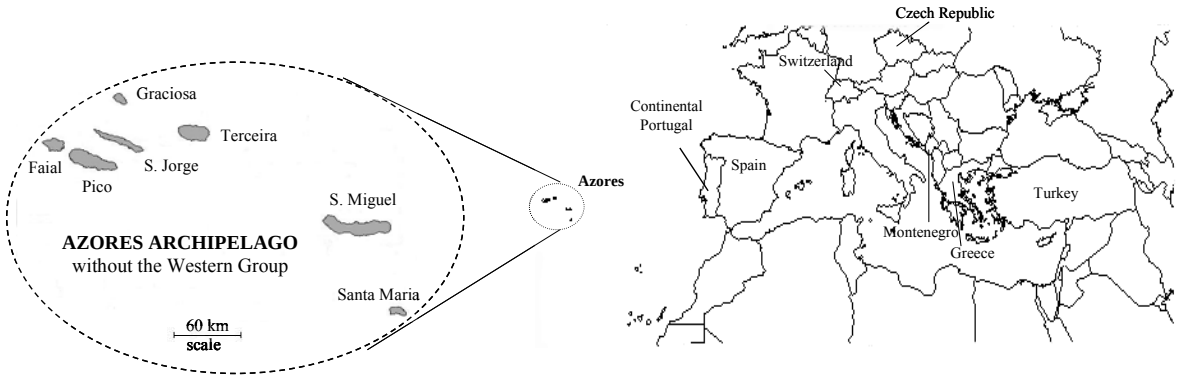


Figure 1
Map of Europe, showing in closer detail the islands of the Azorean archipelago with *Nyctalus azoreum* (the species is absent from the islands of Flores and Corvo, not shown).

Microsatellites genotyping

We extracted total genomic DNA from wing membrane tissue preserved in 95% ethanol, following a standard salt–chloroform procedure modified from Miller et al. (1988). DNA was re-suspended in 100 µl of pure water and stored at -20°C.

Eight microsatellite loci originally isolated from other bat species (Mayer et al. 2000, Miller-Butterworth et al. 2002, Moore et al. 1998, Petri et al. 1997) amplified reliably and were polymorphic in both species. Primers, labelling, PCR conditions and scoring protocols are reported in Salgueiro et al. (in press).

Data analysis

Departure from Hardy–Weinberg equilibrium, heterozygote deficits and linkage equilibrium were tested in ARLEQUIN 3.01 (Schneider et al. 2000). Levels of significance for multiple tests were determined using sequential Bonferroni corrections for multiple comparisons to minimize type I errors (Rice 1989).

Intra-specific and intra-population gene diversity and number of alleles per locus were calculated in FSTAT 2.9.3 (Goudet 1995). In this same software we grouped the samples per species and compared them (randomization tests with 15000 permutations). Private alleles were calculated using CONVERT (Glaubitz 2004). Allelic richness and private allelic richness was estimated using the rarefaction method of Kalinowski (2005) by sampling 76 gene copies per species and 64 per sampling site. We compared the number of alleles per locus without (*A*) and with (*R*) rarefaction using two-sample *t*-test.

In order to detect population size reductions in each island, we used the *M* ratio from Garza and Williamson (2001). It has been suggested that different methods, relying on different statistics, exhibit different timescales for the detection of bottlenecks. The *M* ratio is supposed to remain sensitive to bottlenecks even up to 500 generations following the event, detecting older events than other methods. This method calculates the ratio (*M*) of the number of alleles in each population to the total range in allele sizes. Since rare microsatellite alleles are lost randomly with respect to allele size during population size reductions, the *M* ratio decreases with both the strength and duration of bottlenecks (Garza and Williamson 2001). The observed value is compared to a distribution obtained by simulating 10,000 times a population at equilibrium. The test is significant if more than 95% of the simulated values are superior to the observed value. The value of *M* and its significance level were computed using the software M_P_VAL and CRITICAL_M (Garza and Williamson 2001). Following the authors' suggestions, it was assumed a two-phase model (TPM) with 88% of mutations involved the addition or deletion of one repeat unit (p_s), and the mean size of larger mutations (Δ_g) was set to 2.8 microsatellite-repeat units.

In order to use similar sample sizes, for the comparisons among populations, we restricted the *N. leisleri* sample to the individuals collected in Portugal.

With the purpose of weighing up the contribution of stepwise-like mutations on the genetic differentiation between the two species, we performed a permutation test using SPAGEDI 1.2g (Hardy and Vekemans 2002). In this test, different allele sizes at each locus were randomly permuted among allelic states (2000 permutations) generating a simulated distribution of R_{ST} values (pR_{ST}). As the observed R_{ST} was not significantly larger than its simulated value (results not shown), then there is no support for a mutational component to differentiation, and F_{ST} is considered a better estimator of genetic differentiation among populations (Hardy et al. 2003).

An analysis of molecular variance AMOVA (Excoffier et al. 1992) was performed using all seven populations to estimate the total percentage variance attributable to differences between species, among populations within species, and among individuals within populations. These calculations were performed also in ARLEQUIN.

For a visual representation of genetic patterns, we have applied a clustering of groups of individuals available at the program BAPS 4 (Corander et al. 2004, Corander et al. 2003), which employs stochastic optimization. The tested groups corresponded to the sampled populations (7) or species (2), to detect genetically divergent clusters. The number of clusters (K) was set to 2, 3, 4, 5, 6, 7 and 8, and for each K the analysis was replicated 10 times.

To evaluate the relationships among populations, several genetic distances were calculated: chord distance Dc (Cavalli-Sforza and Edwards 1967), Nei's distance Da (Nei et al. 1983), Ds (Nei 1972). Given that the allele size permutation test (Hardy et al. 2003) did not show significance, the distance $(\delta\mu)^2$ (Goldstein et al. 1995) based on allele size information was excluded from the analysis. Un-rooted Neighbour-Joining trees (Saitou and Nei 1987) were built. The consistency of relationships was evaluated by bootstrapping over loci with 5000 permutations. These calculations were performed with POPULATIONS 1.2.28 software (Langella 2002), which also provided estimates of the above mentioned distances between species. Phenograms were visualized using TREEVIEW (Page 1996) and NJPLOT (Perrière and Gouy 1996).

We checked if there were first generation (F0) immigrants using the Bayesian assignment procedure of (Rannala and Mountain 1997) as implemented in GENECLASS 2 (Piry et al. 2004). We used Paetkau's et al. (2004) method to compute probabilities from 10000 simulated genotypes. This creates a test distribution of simulated individuals by drawing haplotypes, rather than alleles, from the observed data and thus preserves the partial linkage disequilibrium present in genotypes that have immigrant ancestry, but are not F0 immigrants (Paetkau et al. 2004).

Results

Genetic variability and bottleneck analysis

The Azorean bats had less microsatellite variation than the Leisler's bats. After Bonferroni corrections, all loci were found to be in Hardy-Weinberg equilibrium for both species. In the Portuguese sample of *N. leisleri*, we detected significant linkage disequilibrium in the pair of loci P217 and NN8'. Nevertheless, there is no strong evidence for linked loci, and we considered that overall these two loci provided independent information.

Following the correction for unequal sample size, the number of alleles per locus per species with rarefying correction (*R*) was significantly different from the one without it (*A*) (t= 2.94; d.f.= 14; $P \leq 0.05$). Therefore, we will refer mainly to *R*, instead of the usual *A*, when comparing the two species. Although the values were close, the average corrected allelic richness and private alleles (*PR*) over all loci in the Azorean bat (mean R= 10, Σ_R= 76; mean PR= 1, Σ_{PR}= 9) was significantly lower than in the Leisler's bat (mean R= 12, Σ_R= 100; mean PR= 4, Σ_{PR}= 32;

Wilcoxon signed ranks tests Z= 2.52, one-sided *P*= 0.006; Appendix). In addition, the expected heterozygosity of the insular species (H_E = 0.82, SD= 0.007) was significantly lower than that of the mainland (H_E = 0.88, SD= 0.012; Wilcoxon signed ranks test Z= 2.38, one-sided *P*= 0.009). These results were confirmed by the permutations test for difference between groups (here each group was a species) for the *R*, H_E, H_O implemented in FSTAT (one-sided $P \leq 0.05$).

We compared the allelic frequencies of the private alleles in Leisler's bats with the frequencies of those that were shared with Azorean bats for evidence that founder effects may have eliminated low frequency alleles in the latter. The mean frequency of shared alleles (0.09) was significantly higher than the mean frequency of alleles unique to Leislers's bats (0.03) (Wilcoxon signed ranks test Z= 2.37, one-sided *P*= 0.009).

Regarding bottleneck analysis, the *M* ratio ranged from 0.66 for the island of Graciosa to 0.86 for S. Miguel. This method detected possible bottleneck effects in three islands (Pico, S. Jorge and Graciosa, Table 1). These results are similar for both tested values of different μ and *N*e values.

Genetic structure and gene flow

Strong genetic structuring between *N. azoreum* and *N. leisleri* was evident by highly significant estimates of (F_{ST}= 0.061, P< 0.0001). Similarly, all pairwise cross-species population comparisons showed high and significant levels of genetic differentiation (Table 2). Genetic distances between species were: Da= 0.209, Dc= 0.485, and Ds= 0.485.

Not surprisingly, the hierarchical locus by locus AMOVA showed that the between-species component of variance (4.5%, *P*< 0.0001) was higher than that among populations within species (3.4%, *P*< 0.0001). Nevertheless, when a new structure in three groups (Central Group, S. Miguel and *N. leisleri*) was suggested, the percentage of variance among groups increased to 6.3% (*P*< 0.0001) and among populations decreased to 0.9% (*P*< 0.0001). Similarly, using BAPS, we found the highest probability with three distinct clusters (K=3, Log of optimal partition: -9885.8342), corresponding to *N. leisleri* sample and the two groups of islands within the Azores archipelago (Central Group and S. Miguel).

Although the number of loci used in this study is far from that recommended to infer proper genealogical relationships between species (Schlötterer 2001, Takezaki and Nei 1996), the phylograms obtained from different distances suggest a clear separation among these same groups (100% bootstrap for Dc and Da, and 93% for Ds, Figure 2). Both Dc and Da distance estimators generally show the highest probability of obtaining the correct tree topology (Takezaki and Nei 1996). However, we chose to show the Ds tree (Figure 2), because it

presented the same topology as the previous ones, and Ds is more appropriate to infer evolutionary times than the other distances (Takezaki and Nei 1996). Analysing the islands of Central Group as one, the *N. leisleri* population is almost equidistant from both Central Group and S. Miguel (0.57 and 0.60, respectively). The distance Ds between Central Group and S. Miguel is nearly half of that (0.34).

GENECLASS identified no migrants ($P< 0.01$) between Azores and the mainland. When the assignment test was performed with all *N. leisleri* samples, three bats from Portugal were allocated as migrants from the sample containing individuals from the rest of Europe.

Table 1 Bottleneck analysis: *M* ratio tests

	M ratio reductions	
Islands	***M***	**TPM 88**
Faial	0.794	N.S.
Pico	0.729	0.03
S. Jorge	0.731	0.03
Terceira	0.780	N.S.
Graciosa	0.661	0.009
S. Miguel	0.862	N.S.

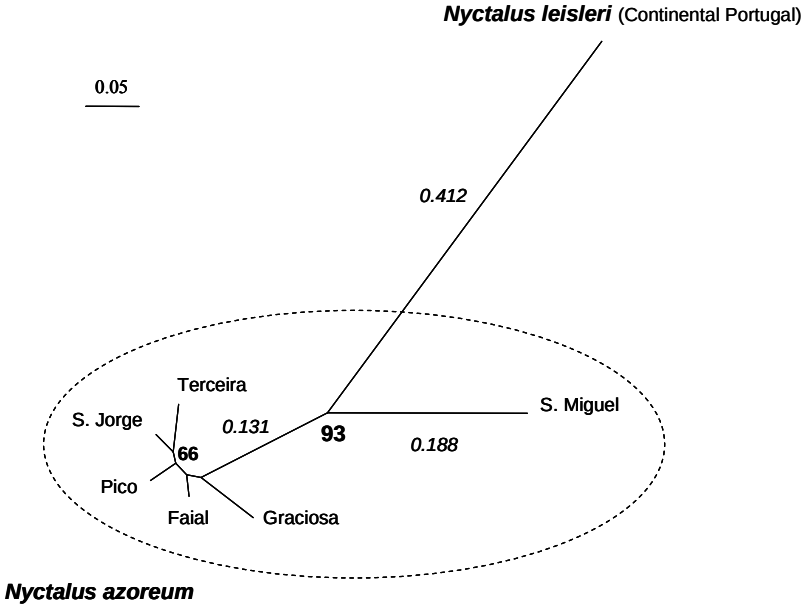
M ratio probability values calculated under a TPM

Table 2 Matrix of pairwise comparisons of F_{ST} for the two island groups of Azorean bat populations and one Leisler's population

		Azorean bats	
Species	Population	Central Group	S. Miguel
Azorean bats	Central Group	-	
	S. Miguel	0.064	-
Leisler's bats	Continental Portugal	0.075	0.079

All comparisons were significant after a sequential Bonferroni correction ($P < 0.001$)

Figure 2
Neighbour-joining tree based on the standard Nei's distance (Ds) of six Azorean bat populations and one Leisler's bat population, constructed from allelic frequencies of eight microsatellite loci. Numbers represent the reliability of the branches based on 5000 bootstrap re-sampling. Branch length is shown in italic.



Discussion

Gene flow between Azorean bats and Leisler's bats

Our results showed no evidence of contemporary gene flow between Azorean bats and Leisler's bats, thus corroborating the demographic isolation of the two species. Clear genetic differentiation between the species was shown by highly significant F_{ST} values, by the phylogenetic and by the Bayesian analyses performed. Furthermore, no migrants between species were identified, and circa 15 species-specific alleles were detected, although this value is underestimated for the continental species.

Island populations tend to show reduced genetic variation as reported for some insular mammals (Eldridge et al. 1999, Hinten et al. 2003, Paetkau and Strobeck 1994, Wang et al. 2005). Our finding of lower genetic diversity in *N. azoreum* than *N. leisleri* support the scenario of a unique colonisation event reported in Salgueiro et al. (2004).

Founder events

The combination of the spatial distribution of genetic diversity with methods for detecting historical variation of population size improves the understanding of the colonisation process.

The *M* ratio method detected a significant reduction in population size for three islands of the Central Group (assuming TPM). But in fact, only Graciosa (M=0.661) presented a value in the range of those observed for the data sets known to have suffered a reduction in size analysed by Garza and Williamson (2001). On the other hand, only S. Miguel (M=0.862) exhibited a value similar to that of natural populations that have not experienced reductions (Garza and Williamson 2001). Abdelkrim et al. (2005) suggested that even when genetic diversity and numbers of loci were low (eight, like in the present study), one could reasonably expect to distinguish between potentially old and more recent reduction in population size and founder effects. Therefore, since *M* ratio is more sensitive to older bottleneck events (Garza and Williamson 2001), we may suggest that the bottlenecked islands detected by *M* ratio could match to the remaining signature of old founder events, probably corresponding to the late arrival of the bat in those islands and suggesting that Pico, S. Jorge and Graciosa were the last islands to be colonised. Overall, these results show that S. Miguel has had a very stable population for a longer time with no historical genetic signatures of bottlenecks, implying that this was the first island to be colonised, and becoming a source for invasion of the neighbouring islands. These findings are in agreement with the greatest expansion time previously detected in S. Miguel (Salgueiro et al. 2004).

Taxonomic and evolutionary relevance

Evolution is a work in progress of changing modes and time affecting genetic composition, life history and other biological parameters. Species are complex to identify, since species concepts are often not mutually exclusive and are controversial (Goldstein et al. 2000).

Crandall et al. (2000) and Templeton et al. (2000) recommended that species should be supported from an ecological and genetic perspective as testable hypotheses, which incorporate both pattern and process into species inference (Templeton et al. 2000). Finally, both genetic and ecological exchangeability are determined for recent and ancient times (Crandall et al. 2000). Rejecting any of these hypotheses allows the definition of distinct cohesion species (Templeton et al. 2000). Ecological exchangeability is analysed through characters related with life-history, morphology, behaviour or habitat.

With regard to the Azorean bat, it is demographically isolated from the continental Leisler's bat by a distance of 1500 km, and it has experienced a fast adaptation revealed by morphological, ecological and behavioural characters (see Introduction). On the other hand, genetic exchangeability comes primarily from molecular data showing an absence of gene flow. The two species share no control region mtDNA haplotypes (Salgueiro et al. 2004), defining reciprocally monophyletic clades indicative of historic isolation. Also, the present microsatellite study warrants the rejection of recent gene flow.

Consequently, our results support species status for the Azorean bat since they provide evidence for recent and long-term isolation and due to previous studies that confirm divergence in fitness-related traits. The very low levels of genetic divergence detected in the more conserved mtDNA genes (ND1 and Cyt *b*, Salgueiro et al. 2007), are possibly a sign of old shared ancestry that remained after a recent speciation phenomenon followed by fast morphological evolution.

The example of *N. azoreum* is in line with the results of Millien (2006), which suggest that "rates of morphological evolution are significantly greater for islands than for mainland mammal populations, due to their intrinsic capacity to evolve faster when confronted with a rapid change in their environment".

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Appendix Individual alleles (represented by their sizes), number of alleles (*A*), allelic richness corrected for the minimum sample size per species (38 individuals) (*R*), number of species-private alleles (*P*), and private allelic richness corrected for the minimum sample size (*NPR*), for eight microsatellite loci in Leisler's bat (*Nle*) and Azorean bat (*Na*)

Locus	**Species**	**Alleles**																							***A***	***R***	***P***	***PR***
P217	*Nle*	172		188			200		218	222	225	229	233	236	240	245	249	253	257	261	265	269	273		18	18	5	10
	Na	**172**	**176**		**192**	**196**	**200**	**204**	**218**	**222**	**225**	**229**	**233**	**236**	**240**				**257**			**269**	**273**		**16**	**10**	**4**	**2**
NN8`	*Nle*		80	82	84	86	88	90	92	94	96	98	100	102											12	12	1	4
	Na	**78**	**80**	**82**	**84**	**86**	**88**	**90**	**92**	**94**	**96**	**98**		**102**											**12**	**8**	**1**	**0**
NN18	*Nle*	272	274	276	278	280	282	284	286	288	290	292	294	296	298	300									15	15	2	4
	Na	**272**	**274**	**276**	**278**	**280**	**282**	**284**	**286**	**288**	**290**	**292**	**294**	**296**											**13**	**11**	**0**	**0**
P223	*Nle*	98	102	106	110	114	118	122																	7	7	0	1
	Na	**98**	**102**	**106**	**110**	**114**	**118**	**122**	**126**																**8**	**7**	**1**	**1**
P20	*Nle*	156		164	166	168	170	172	174	176	178	180	182	184	186	188		192							15	15	2	5
	Na		**162**	**164**		**168**	**170**	**172**	**174**	**176**	**178**	**180**	**182**	**184**	**186**	**188**	**190**	**192**	**194**						**16**	**11**	**3**	**1**
P13	*Nle*	140	142	144	148	150	152	154	156	158	160	162	164	166	168	172									14	14	2	5
	Na	**140**	**142**	**144**	**148**	**150**	**152**	**154**	**156**	**158**	**160**	**162**	**164**	**166**											**13**	**10**	**1**	**1**
MS2	*Nle*	192		200	202	204	206	208	210	212	214														9	9	1	2
	Na		**198**	**200**	**202**	**204**	**206**	**208**	**210**	**212**	**214**	**216**	**218**	**220**											**12**	**9**	**4**	**2**
EM1	*Nle*	239			245	247	249	251	253	255	257	259	261												10	10	1	2
	Na		**241**	**243**	**245**	**247**	**249**	**251**	**253**	**255**	**257**	**259**	**261**												**11**	**10**	**2**	**2**
Mean over all *loci*	*Nle*																								13	12	2	4
	Na																								**13**	**10**	**2**	**1**
Totals	*Nle*																								100	100	14	32
	Na																								**101**	**76**	**16**	**9**

Chapter III Genetics of fragmented bat populations

3.1. Island populations

3.1.1. Paper III

Mitochondrial DNA variation and population structure of the island endemic Azorean bat (*Nyctalus azoreum*). *Molecular Ecology* 13, 3357-3366.

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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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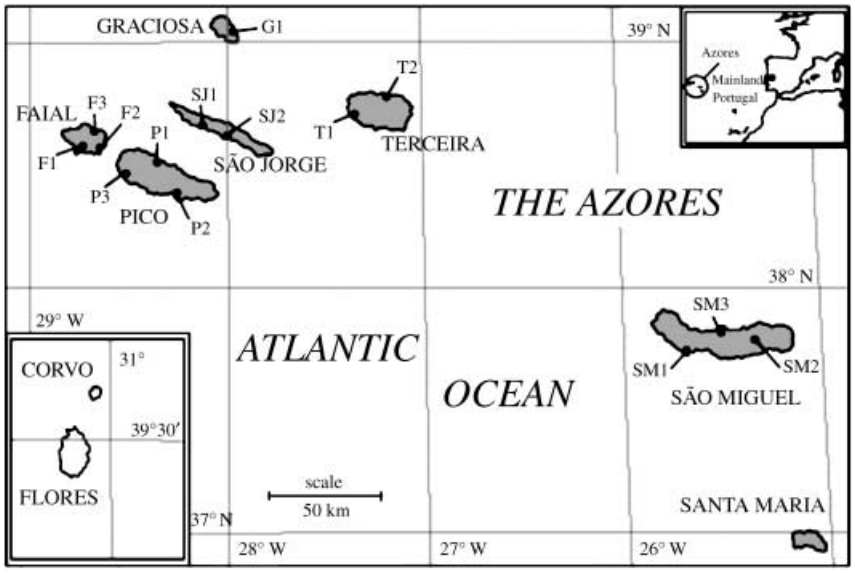

Fig. 1 Map of the Azores Archipelago with the localization of the 14 sampled colonies (see Table 1 for descriptions). Islands with confirmed presence of *Nyctalus azoreum* are shaded in grey.

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			Continental
Haplotype	111111122212367 8800159138090 20	211/ 232	222222233333 333469901478 349987873689	F1	F2	F3	P1	P2	P3	SJ1	SJ2	T1	T2	G	SM1	SM2	SM3	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.

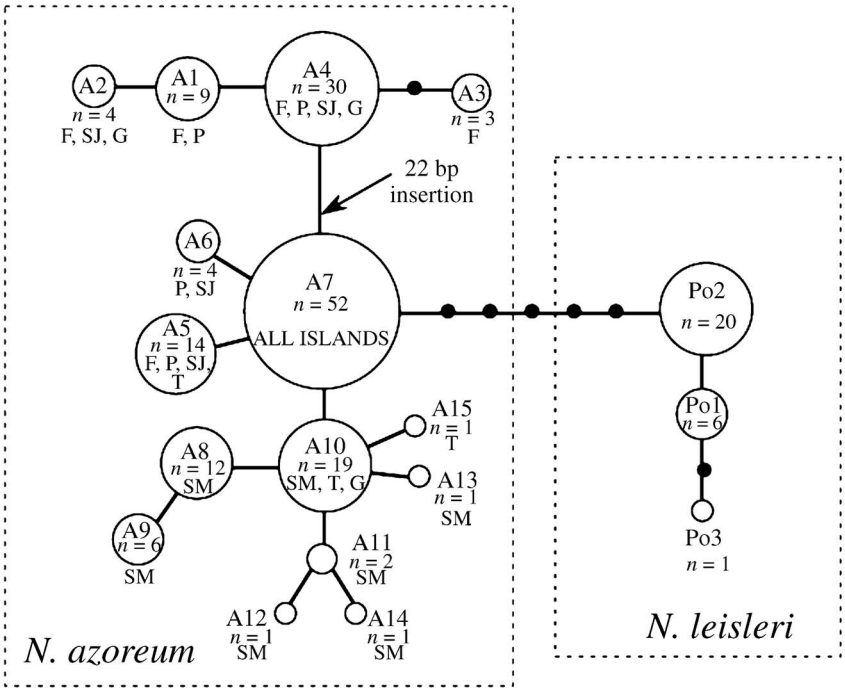

Fig. 2 Parsimony network of the 18 haplotypes (white circles) obtained by sequencing 396 bp of D-loop in 159 Azorean bats and 27 Leisler's bats (see Table 2). Filled circles represent missing (or unsampled) haplotypes. The area of each circle is proportional to the frequency of the haplotype. Each segment 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

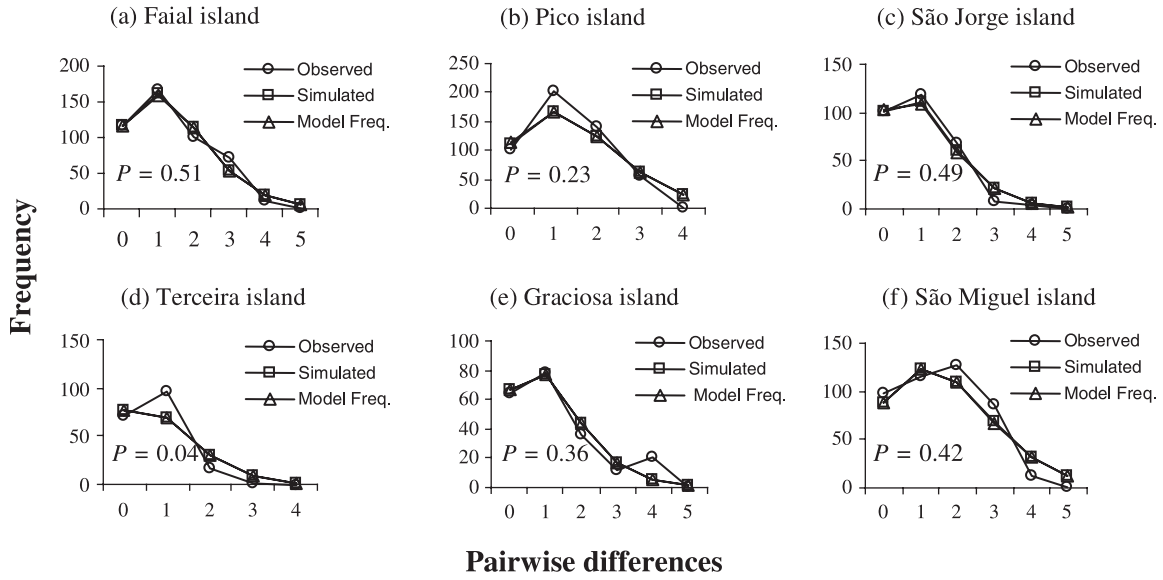

Fig. 3 Mismatch distributions of the D-loop region in six island populations of *Nyctalus azoreum*. These curves represent the frequency distribution of pairwise differences. *P*-values represent the probability that the variance of the simulated data set is equal to or greater than the observed data 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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3.1.2 Paper IV

Gene flow and population structure of the endemic Azorean bat (*Nyctalus azoreum*) based on microsatellites: implications for conservation. *Conservation Genetics*. DOI: 10.1007/s10592-007-9430-z

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RESEARCH ARTICLE

Gene flow and population structure of the endemic Azorean bat (*Nyctalus azoreum*) based on microsatellites: implications for conservation

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Abstract The Azorean bat (*Nyctalus azoreum*) is endemic to the Azores archipelago and is listed as endangered due to its reduced and fragmented distribution range. We assessed genetic diversity at eight microsatellite loci in 280 individuals from 14 locations throughout six islands. Overall, we found that the Azorean bat populations are not genetically impoverished. Indeed, the number of alleles per locus ranged from 8 to 10 and the observed heterozygosity ranged from 0.77 in Terceira to 0.83 in Faial. The highest genetic diversity and level of private alleles was observed in S. Miguel, the largest island, and the closest to the mainland. Private alleles occurred at all islands except in Graciosa. Global and pairwise FST among islands were all statistically significant, suggesting restricted gene flow. These results, together with those of factorial correspondence analysis, Bayesian clustering method, and individual assignment tests, corroborate the conclusions of a previous mtDNA based study, providing strong support for the existence of two major subpopulations: one includes all islands of the Central Group and the other corresponds to S. Miguel. Gene flow between them is very limited, suggesting that management plans should avoid translocations between these subpopulations.

Keywords Azores · Chiroptera · Gene flow · Microsatellites · Management unit

Introduction

Archipelagos provide good opportunities to study population subdivision (Hewitt 2001; Emerson 2002), because if water represents a barrier to gene flow, different islands are expected to be inhabited by relatively differentiated populations. Island populations are prone to be genetically impoverished, due to possible founder effects (Wildt et al. 1987; Hoezel et al. 1993), isolation from the source population, and stochastic processes attributable to limited size of the population (Wilcox 1980). Thus genetic factors play an important role in small populations (Reed and Frankham 2003) and may impact negatively on the long-term persistence of insular species (Frankham 1997, 1998). Therefore, genetic assessment of island populations not only provides information on current and historic levels of gene diversity, but may also help to develop appropriate conservation strategies (Frankham et al. 2002; Frankham 2003).

The Azores archipelago (37°–40° N, 25°–31° W) is situated about 1,500 km west of mainland Europe and 3,900 km east of America, which makes it the most remote archipelago in the North Atlantic. It ranges across seven degrees of longitude and more than 600 km, and includes nine islands of volcanic origin roughly aligned from west to east: Flores, Corvo, Faial, Pico, S. Jorge, Graciosa, Terceira, S. Miguel and Santa Maria (Fig. 1).

As a result of their isolation, most of the native plant species on the Azores are phylogenetically primitive, and related to the pre-glacial flora of Europe. However,

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Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | ☐ LE | ☐ TYPESET
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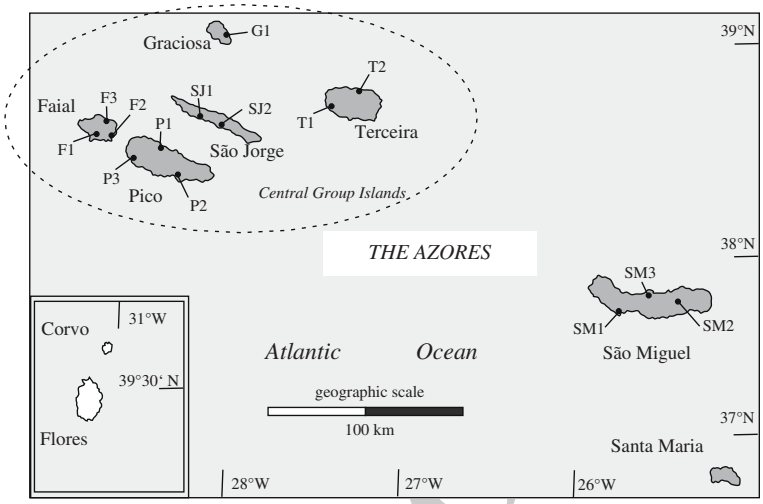

Fig. 1 Map of the Azores archipelago with the location of the 14 sites where *N. azoreum* were sampled: Faial (F1–F3), Pico (P1–P3), S. Jorge (SJ1–SJ2), Graciosa (G1), Terceira (T1–T2) and S. Miguel (SM1–SM3). The islands of Corvo and Flores lie outside the range of the Azorean bat. The islands of the Central Group are enclosed by the broken line

compared to other Macaronesian archipelagos, the Azores has a relatively low faunistic diversity and only 6% of its vertebrates are endemic. Most of the nine species of mammals present appear to have been introduced to the islands by humans (Mathias et al. 1998). The exceptions are two species of bats: an unidentified pipistrelle species *Pipistrellus sp.* (Rainho et al. 2002; Skiba 2003) and the Azorean bat *Nyctalus azoreum* (Thomas 1901).

Nyctalus azoreum is found in all but the westernmost islands of Flores and Corvo (Fig. 1). While relatively common throughout most of its range, it is rare on Santa Maria. Given its reduced and fragmented distribution range, and small global population size, the Azorean bat is considered a critically endangered species in the recent assessment for the Portuguese Red Data Book (Queiroz et al. 2006).

Previous genetic studies based on mitochondrial DNA (mtDNA) identified unique lineages in the Azores archipelago, but revealed low levels of haplotype divergence between *N. azoreum* and its continental relative, *N. leisleri* (Salgueiro et al. 2007). Population genetics analyses based on control region data further showed high levels of variability and a strong geographic partitioning of genetic variance in Azorean bats, suggesting limited gene flow among islands (Salgueiro et al. 2004). However, as mtDNA is transmitted clonally through the mother, these characteristics may apply to females only. Several other studies on bats have shown that despite strong female philopatry, extensive gene flow can occur through the males (Petit and Mayer 1999; Worthington Wilmer et al. 1999; Castella et al. 2001).

Here we investigate population structure and patterns of gene flow of the Azorean bat using bi-parentally inherited nuclear markers (i.e. microsatellites). The highly variable microsatellite *loci* can also provide a finer resolution of population-level dynamics, suitable for conservation genetics (Goldstein and Schlötterer 1999). These markers are here used (a) to compare the genetic structure obtained with nuclear *versus* mitochondrial markers, and (b) to identify possible factors that could have influenced population structure. The implications of these results for the conservation of this threatened species are discussed.

Methods

Tissue collection and DNA extraction

During the summer of 2001, we sampled with the aid of non-lethal sterile biopsy-punches (Worthington Wilmer and Barratt 1996) a total of 280 Azorean bats. Specimens were captured at 14 colonies inhabiting six of the seven islands occupied by *N. azoreum* (Fig. 1), as described in Salgueiro et al. (2004). Total sample sizes for each island varied from 32 to 66 (see details in Appendix I). All individuals already sequenced for the control region in our previous study (Salgueiro et al. 2004) were also genotyped here.

A small piece of wing membrane from each bat was preserved in 95% ethanol until DNA extraction. These ethanol-preserved tissues were then treated with SDS and proteinase-K, and DNA was extracted using a standard salt-chloroform procedure modified from Miller et al. (1988). The purified DNA was re-suspended in 100 µl of sterile water.

Microsatellite analyses

As microsatellite primers specific for *N. azoreum* were not available, we used seven *loci* developed in other vespertilionid bats (P13, P20, P217, P223 from Mayer et al. 2000;

Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | LE | TYPESET
MS Code : COGE-07-86 | CP | DISK

NN8' from Castella et al. 2001, NN18 from Petri et al. 1997; MS2 from Miller-Butterworth et al. 2002) and one *locus* conserved across many eutherian mammals (EM, Moore et al. 1998).

PCR amplifications were conducted in 10 µl reaction volumes following the conditions described in the original studies and further optimised to fit in four multiplex reactions (using the Multiplex PCR kit of QIAGEN). These multiplex reactions consisted of an initial denaturation step at 95°C for 15 min., followed by 30 cycles of the series: 95°C for 30 s, annealing temperature (Ta: 52°C for P217, NN8', NN18; 40°C for P13; 49°C for P20, P223; 58°C for MS2 and EM) for 90 s, 72°C for 60 s; and a final amplification step of 20 min. at 72°C. These amplifications were performed with primers labelled with Beckman dyes and PCR products were run in CEQ 2000XL-BECKMAN COULTER equipment. Scoring of allelic size was aided by using the CEQ 8000 genetic analysis system.

Statistical analyses

Microsatellite variation was tested for deviations from Hardy–Weinberg equilibrium (HWE) using exact tests based on contingency tables (Guo and Thompson 1992), and for deviations from linkage equilibrium with likelihood ratio tests (Slatkin and Excoffier 1996) available in ARLEQUIN version 3.01 (Excoffier et al. 2005). Allelic frequencies and gene diversity were computed using FSTAT v. 2.9.3 (Goudet 1995). A measure of allelic diversity correcting for differences in sample size was also calculated using rarefaction (*R*), available in the same software. The occurrence of private (unique) alleles was used to describe population distinctiveness (Slatkin 1985). In order to detect potential differences in dispersal between females and males, F_{IS}, F_{ST}, gene diversity (H_S), relatedness (Relat), mean assignment index (*mAIc*) and variance of the assignment index (*vAIc*) were quantified separately for both sexes in all the archipelago and in each island, following the approach described by Goudet et al. (2002). Statistical significance of differences was determined using the randomization method implemented in FSTAT (10,000 permutations).

The extent of genetic differentiation among islands and colonies was quantified by computing pairwise F_{ST} (based on the infinite allele model) and R_{ST} (taking into account allele size), using ARLEQUIN. In order to assess the influence of stepwise-like mutations versus drift on genetic differentiation for each pair of populations, we performed a permutation test available in the software SPAGEDI v. 1.2 g (Hardy and Vekemans 2002). Allele size at each *locus* was randomly permuted among allelic states (2,000 permutations) to simulate a distribution of R_{ST} values (pR_{ST}) and 95% confidence intervals (CI) under the null hypothesis that differences in allele sizes do not contribute to population differentiation (Hardy et al. 2003).

For highly variable *loci* such as microsatellites, the maximum F_{ST} value among populations may be low and may not be directly comparable with other classes of markers (Hedrick 2005). In order to compare population structure at microsatellites and mtDNA data, we therefore calculated a standardized measure of genetic differentiation, G'_{ST} (Hedrick 2005), by dividing the observed F_{ST} value by its theoretical maximum, F_{max} using the program RECODEDATA v. 0.1 (Meirmans 2006).

Sequential Bonferroni corrections adjusted critical probability values for multiple tests to minimize type I errors (Rice 1989) in the HWE, linkage disequilibrium and F_{ST} comparisons.

With the aim of checking for isolation by distance (IBD), pairwise estimates of F_{ST} and G'_{ST} among sampling sites were tested for correlation with straight geographic distances through Mantel tests, using MANTEL v. 2.0 (Liedloff 1999). To investigate the genetic structure of the Azorean bat populations in the archipelago we analysed their molecular variance (AMOVA, Excoffier et al. 1992) using ARLEQUIN. We also performed a factorial correspondence analysis (FCA) on the multilocus genotype of each individual, using the option that takes into account the population of origin (FCA over populations), as implemented in GENETIX v. 4.05.2 (Belkhir et al. 2004). In addition, we used the program BAPS v. 4 (Corander et al. 2003; Corander et al. 2004), which jointly estimates the posterior probabilities for the number of populations, the partition of individuals among the inferred populations, and the relative allele frequencies. BAPS uses stochastic optimization to infer the posterior mode of genetic structure. The number of clusters (K, i.e. island populations or group of islands) was set to 2, 3, 4, 5 and 6, and for each K the analysis was replicated 10 times.

In order to estimate levels of contemporary gene flow among islands, effective migrants were detected with assignment tests applied on multilocus genotypes. First generation migrants were identified using the Bayesian likelihood criterion of Rannala and Mountain (1997) and the re-sampling algorithm of Paetkau et al. (2004). We used as assignment criterion L = L_home/L_max, which is the ratio of the likelihood computed from the population where the individual was sampled (L_home) over the highest likelihood value among all population samples, including the population where the individual was sampled (L_max, see Paetkau et al. 2004). These calculations were performed with GENECLASS v. 2.0 (Piry et al. 2004) using 10,000 simulations, and a probability threshold α of 0.01 to accept a first generation migrant.

Results

Within-population genetic variability

The eight microsatellite *loci* were polymorphic and the number of alleles per *locus* ranged from eight for P223 to 16 for P217 and P20 (Appendix I). Mean number of alleles per *locus* (*A*) ranged from 8.1 in Graciosa and 8.4 in S. Jorge to 10.1 in S. Miguel. These estimates are very similar to those calculated with the rarefaction index, suggesting that there is little or no bias introduced by using unequal sample size of populations (Appendix I).

Private alleles were found in all sampled islands except Graciosa, and occurred at frequencies ranging from 0.01 to 0.04. From the total of 21 private alleles found, 13 were unique to S. Miguel, representing 16% of the whole array of alleles detected in this island. The presence of these unique alleles is indicative of a long history of isolation of S. Miguel populations. On the other islands, only 1–3 of the observed alleles were island-specific (Appendix I).

One significant deviation from Hardy–Weinberg expectations was found (colony F2 at *locus* P13), but no consistent pattern occurred either across *loci* or across populations. Thus, there was no evidence for null alleles, Wahlund effects, or strong linkage disequilibrium among the microsatellite *loci* in *N. azoreum*. Observed heterozygosity was high, ranging from 0.769 in Terceira to 0.829 in Faial. Of the several statistical descriptors (F_{IS}, F_{ST}, H_S, Relat, *mAIc* and *vAIc*) calculated separately for the 90 males and 176 females, none showed significant differences between sexes (Table 1). This was also true when the divergent populations from S. Miguel were excluded from the calculation.

Population differentiation

Global multilocus pairwise R_{ST} estimates among islands (Table 2) and colonies were smaller than the 95% range of simulated pR_{ST} values, indicating that allele size or stepwise mutations are not strongly influencing population differentiation of the Azorean bat. Hardy et al. (2003) suggest that, in this situation, F_{ST} should be preferred over R_{ST} for estimating population differentiation. In addition, inferences derived from R_{ST} (e.g. IBD, etc.) were very similar to those obtained from F_{ST}, so we only report the latter.

The global population differentiation calculated over all islands was highly significant ($F_{ST} = 0.036$; $P \leq 0.001$; see Table 3 for pairwise comparisons). These differentiation values were, however, small to moderate, with higher values being found for the comparisons between S. Miguel and the islands of the Central Group (Fig. 1 and Table 4). At the scale of the entire archipelago, pairwise F_{ST} among colonies was strongly correlated with the linear geographic distance ($r = 0.87$, $P < 0.01$). However, when S. Miguel was removed from the calculations, genetic and geographic distances within the Central Group Islands were no more correlated ($r = 0.09$, $P > 0.05$), suggesting that at this scale gene flow among populations was not simply dependant on geographic proximity.

The standardized genetic differentiation measure for these nuclear markers ($G'_{ST} = 0.175$) indicates that the F_{ST} value reaches only 17% of its theoretical maximum. The same differentiation parameter calculated at the mtDNA marker analyzed in Salgueiro et al. (2004), corresponds to 44% of the maximum theoretical value ($G'_{ST} = 0.442$). This suggests that Azorean bat populations are about 2.5 times more structured at mitochondrial markers than at nuclear markers. This difference between the two classes of markers is also pronounced at a smaller scale, i.e. between islands within the archipelago (Table 4), with most pairwise comparisons being 2–20 times smaller for microsatellites than for haplotypes. The partition of genetic structure of the Azorean bat populations in the archipelago is thus qualitatively similar, but quantitatively different for microsatellites and mtDNA data (Table 5).

Table 1 Results of the sex-biased dispersal tests: estimated F_{IS}, F_{ST}, gene diversity (H_S), relatedness (Relat), mean assignment index (*mAIc*) and variance of the assignment index (*vAIc*), and correspondent significance levels

		N	mAIc	vAIc	F_{IS}	F_{ST}	Relat	H_S
Azores Archipelago	Females ♀	176	–0.041	7.199	–0.006	0.036	0.069	0.796
	Males ♂	90	0.080	6.610	0.010	0.034	0.064	0.801
	P one-sided test	–	0.634	0.551	0.218	0.223	0.203	0.299
	P two-sided test	–	0.726	0.681	0.488	0.797	0.745	0.469
Central Group	Females ♀	137	–0.004	7.668	0.009	0.009	0.017	0.796
	Males ♂	66	0.008	6.788	0.013	0.007	0.013	0.806
	P one-sided test	–	0.508	0.598	0.167	0.416	0.406	0.202
	P two-sided test	–	0.977	0.616	0.421	0.801	0.786	0.286

The first lines correspond to bats sampled over the entire archipelago, and the last ones to bats sampled only in the Central Group of islands

Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | LE | TYPESET
MS Code : COGE-07-86 | CP | DISK

Table 2 Mean F_{ST}, R_{ST} and permutated pR_{ST} (95% confidence interval in parentheses) values of genetic differentiation among Azorean bat populations measured at eight microsatellite loci

Locus	F_{ST}	R_{ST}	pR_{ST} (95%CI))
P217	0.036	0.031	0.035 (–0.007–0.106)
NN8′	0.026	0.000	0.025 (–0.005–0.083)
NN18	0.023	0.004	0.021 (–0.007–0.068)
P223	0.053	0.034	0.048 (–0.007–0.156)
P20	0.029	0.054	0.026 (–0.001–0.064)
P13	0.030	0.002	0.026 (–0.006–0.085)
MS2	0.015	0.053*	0.015 (–0.006–0.057)
EM1	0.024	0.019	0.022 (–0.005–0.072)
All loci	0.030	0.026	0.034 (0.004–0.084)

*Significance values at the $P = 0.05$ level (observed > expected)

Figure 2 illustrates the position of the 279 genotyped Azorean bats onto the factorial space based on microsatellite allele frequencies. The first axis of variation (67.2% on the FC I) clearly separates the individuals from S. Miguel from those from the other islands (Fig. 2). No further subgroups appeared on this factorial analysis. Consistent with these results, the number of clusters inferred with the Bayesian clustering method implemented in BAPS suggested the existence of only two clusters in the optimal partition ($P = 1$), one corresponding to bats of the Central Group and the other to S. Miguel.

Table 5 Comparison of mitochondrial (data in Salgueiro et al. 2004) and nuclear markers partitions of genetic variation in the Azorean bat

	Percentage of variation	
	mtDNA Φ_{ST}	Microsatellites F_{ST}
Among groups	30.52	5.87
Among islands within groups	10.22	0.91
Within islands	59.26	93.22

The two groups include S. Miguel and the islands of the Central Group, respectively

Assignment tests

GENECLASS assigned 271 of 279 individuals to the island from which they were sampled (Table 6), suggesting that very few bats are likely to have moved recently among islands. The three closest islands in the Central Group (Faial, Pico and S. Jorge, Fig. 1) accounted for three of the eight potential migrants identified by GENECLASS (Table 6). Overall six potential migrants were moving within the Central Group, while the remaining two could be recent emigrants from the Central Group to S. Miguel. However, compared to the other potential migrants identified in GENECLASS, these two putative long-distance emigrants had only a slightly lower likelihood (–1,173 and –1,259) of being local (i.e. non-migratory) individuals from S. Miguel. The differences in likelihood of the other putative migrants

Table 3 Estimated pairwise F_{ST} values among island populations of *Nyctalus azoreum*

Populations	Faial	Pico	S. Jorge	Terceira	Graciosa	S. Miguel
Faial	–					
Pico	0.006*	–				
S. Jorge	0.006*	0.006*	–			
Terceira	**0.009****	**0.010****	**0.006***	–		
Graciosa	**0.012*****	**0.013*****	**0.014*****	**0.021*****	–	
S. Miguel	**0.056*****	**0.070*****	**0.073*****	**0.076*****	**0.069*****	–

In bold: significant values after a sequential Bonferroni correction

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 4 Estimated pairwise standardized G'_{ST} values (Hedrick 2005) among islands for nuclear DNA (below diagonal) and for mtDNA (above diagonal) data set

Populations	Faial	Pico	S. Jorge	Terceira	Graciosa	S. Miguel
Faial	–	0.053	0.161	0.321	0.457	0.586
Pico	0.030	–	0.143	0.498	0.604	0.724
S. Jorge	0.029	0.029	–	0.587	0.512	0.761
Terceira	0.044	0.046	0.027	–	0.038	0.542
Graciosa	0.068	0.067	0.073	0.101	–	0.609
S. Miguel	0.280	0.350	0.347	0.356	0.343	–

Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | LE | TYPESET
MS Code : COGE-07-86 | CP | DISK

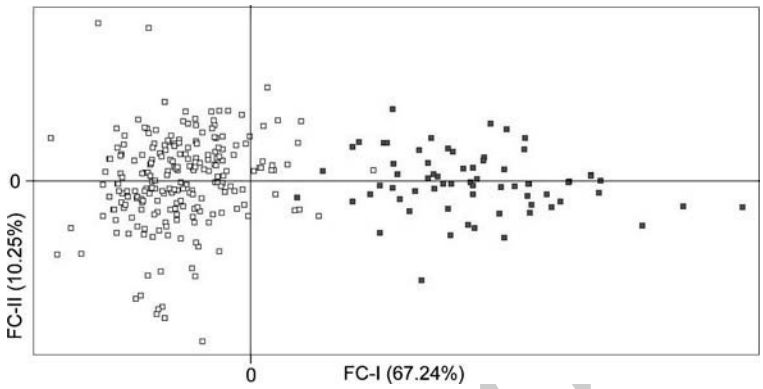

Fig. 2 Projection of 279 individual microsatellite genotypes of Azorean bats on the first axes of a factorial component analysis. Bats from the Central Group are shown in white and those from S. Miguel Island in black. Inertia percentage values are presented for each factorial component (FC-I and FC-II)

Table 6 Results of the assignment tests: number of potential first generation migrants ($P < 0.01$) and the most likely population of origin (in row) assigned to each population indicated in the column

To	From						
	Faial	Pico	S. Jorge	Terceira	Graciosa	S. Miguel	Total immigrants
Faial	*42*	1			1		**2**
Pico	1	*48*					**1**
S. Jorge	1		*47*				**1**
Terceira		1		*38*			**1**
Graciosa		1			*32*		**1**
S. Miguel	1			1		*64*	**2**
Total emigrants	**3**	**3**	**0**	**1**	**1**	**0**	

In italic are the individuals assigned to the correct sample population and in bold, the total number of immigrants and emigrants inferred for each island

inferred in GENECLASS were greater (–2,907 to –3,482). Given the probability threshold of 0.01 we chose in these assignment tests, two to three of the 280 tested individuals might also be "false positive" migrants (Manel et al. 2005), including those two from S. Miguel. This indicates that the genetic signature of these two putative emigrants to S. Miguel is best considered as untypical of any source population.

Just one of the eight individuals identified as potential first generation migrants was a male. As recommended by Lawson Handley and Perrin (2007) to avoid bias due to unequal representation of sexes, the same analysis was performed separately for each sex over the entire archipelago, but this did not alter the pattern of low inter-island gene flow (results not shown).

Discussion

Genetic diversity and population differentiation

The gene diversity measured at eight microsatellite *loci* in each of the islands inhabited by the Azorean bat indicates that these populations are not genetically impoverished. Indeed, with a mean heterozygosity ranging from 0.79 to 0.81, the six island populations of *N. azoreum* compare well with estimates made on other vespertilionids in continental Europe (e.g. 0.77–0.83 in *N. noctula*, Petit and Mayer 1999, 0.75 in *Myotis myotis*, Ruedi and Castella 2003, 0.79 in *M. nattereri*, Rivers et al. 2005, or 0.79–0.83 in *Plecotus auritus*, Veith et al. 2004). These results thus confirm earlier findings based on a highly variable portion of the mtDNA, which Azorean bat populations have retained or accumulated substantial genetic diversity (Salgueiro et al. 2004).

Given the important stretches of open water separating the islands of the Azores (Fig. 1), one expects genetic diversity to be highly structured among islands. Global measures indicate clear population structure within the archipelago ($G'_{ST} = 0.036$) but a substantial part of this global structure is due to the genetic distinctness of bats inhabiting S. Miguel (Fig. 2). When F_{ST} estimates are restricted to the Central Group (i.e. by excluding S. Miguel), then the population structure becomes close to zero ($F_{ST} = 0.01$), which suggests that nuclear gene flow is close to panmixia at this geographical scale. As a comparison, another species of *Nyctalus*, *N. noctula*, exhibits similar low levels of nuclear genetic structure ($F_{ST} = 0.006$) but they

Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | LE | TYPESET
MS Code : COGE-07-86 | CP | DISK

were sampled over vast areas of contiguous land in continental Europe (Petit et al. 2001). Two hypotheses may explain the surprising low population structure of Azorean bats within the Central Group islands. Populations on these islands may have originated from a common source population (e.g. S. Miguel) but did not have time to reach mutation-drift equilibrium or to differentiate on each island. In this case, the lack of differentiation would be merely due to a non-equilibrium situation, not to current gene flow. Alternatively, populations from the Central Group are in mutation-drift equilibrium and maintain significant contemporaneous inter-island gene flow that counteracts differentiation. It is notoriously difficult to disentangle these two processes (Templeton et al. 1995) but we favour the hypothesis of ongoing gene flow because both the assignment tests of multilocus genotypes (Table 6) and previous estimates of gene flow based on shared haplotypes of mtDNA (Salgueiro et al. 2004) suggest effective inter-island migrations. In the future, a test of this interpretation could be conducted with direct observations of ringed or radio-tagged bats.

Together, these results suggest that open sea, at least for nearby islands within the Central Group (Fig. 1), is not acting as an absolute barrier for gene flow in *N. azoreum*. However, the distinctness of bats from S. Miguel (Fig. 2), which have more private alleles at microsatellite *loci* and unique haplotypes (Salgueiro et al. 2004), suggests that beyond a few tens of km of open sea, over water dispersal is much rarer.

Mammals in general show strong bias for male-dispersal (Greenwood 1980) that is also typical for several European vespertilionid bats (e.g. Petit and Mayer 1999; Castella et al. 2001; Kerth et al. 2002). Although we did not conduct specific analyses to quantify the male and female contributions to gene flow, several lines of evidence suggest that this bias, if present, is not as strong as in other bat species studied so far. First, the assignment tests of genotypes (Table 6) suggest that eight bats could be recent migrants. Yet, seven of those potential migrants are females, indicating clearly that this sex is involved in these inter-island movements. Second, the overall difference of population structure measured at the nuclear genes (G'_{ST}=0.175) is only about 2.5 times smaller than that measured for mtDNA haplotypes (G'_{ST}=0.442). This is close to the value expected in a panmictic breeding system with no strong sex bias in gene flow (Birky et al. 1989). Other species of bats with strong male-biased dispersal (e.g. *N. noctula*, *M. myotis* or *M. bechsteinii*) exhibited much more contrasted levels of differentiation at these two classes of markers (Petit and Mayer 1999; Castella et al. 2001; Kerth et al. 2002). Third, when the analysis of some population parameters (like F_{IS}, F_{ST}, H_S, Relat, *mAIc* and *vAIc*) was compared between sexes, none of them was significantly different, whereas at least a significant higher F_{ST} and *mAIc* in females would be expected in breeding systems with a male-biased dispersal intensity of at least 80:20 (Goudet et al. 2002). The lack of strong evidence for male-biased dispersal in *N. azoreum* compared to other continental species might suggest a greater role played by passive dispersal. The strong winds blowing in this oceanic archipelago may promote passive dispersal (see e.g. Barcia et al. 2005), thus affecting both male and female bats. This scenario would be in line with the indirect estimates of gene flow performed here.

Conservation implications

Contrary to what would be expected for insular fragmented populations, all genetic information indicates that the Azorean bat is not genetically impoverished or particularly inbred (Appendix I and Salgueiro et al. 2004). However, the data gathered reveal a significant genetic discontinuity between populations from the Central Group of islands and from S. Miguel (Fig. 2), which indicates that these two entities are exchanging few or no migrants. This scenario has been corroborated by markers with different modes of inheritance (bi-parental versus female-transmitted). Indeed, results based on both nuclear and mitochondrial markers (Salgueiro et al. 2004) suggest that the species *N. azoreum* is comprised of at least two major subpopulations, one occurring on the island of S. Miguel, the other occupying the islands of the Central Group. Demographic independence is therefore very likely between these two subgroups, and they should be considered as distinct management units, MUs (Moritz 1994) that require separate monitoring and management.

The present density of *N. azoreum* on most islands can be quite high, but on Santa Maria it is low (Rainho et al. 2002, IUCN 2007) and genetic information for that island is still lacking. However, none of the islands of the archipelago is large and consequently, even at high local densities, the total population size on each island is small. In addition, the colonial behaviour of the species makes it particularly vulnerable, because a large proportion of the breeding population of each island is concentrated in a reduced number of roosts. Since many of these breeding colonies are located in buildings (see e.g. Salgueiro et al. 2004), single destructive actions could wipe out an important part of the population of an island.

Genetic data further suggest that translocation of animals within the Central Group could be recommended if it becomes necessary to boost the population of one of its islands, since natural inter-island migrations within this group appear to occur at least occasionally. In contrast, translocations between the Central Group and S. Miguel

Journal : Large 10592 | Dispatch : 29-9-2007 | Pages : 9
Article No. : 9430 | LE | TYPESET
MS Code : COGE-07-86 | CP | DISK

should be avoided due to their longer history of isolation and concomitant likely development of local adaptations. Unfortunately, no comprehensive study on behaviour and ecology of Azorean bats has been conducted to date. Together with ringing investigations aimed at estimating the individual dispersal ability, such studies could be useful in the future to indicate whether this genetic discontinuity is correlated with other traits relevant to the evolutionary potential of this species of conservation concern.

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Supplementary material

Appendix I Summary statistics for eight microsatellite loci from the Azorean bat populations

Islands	Sampling sites		P217	NN8'	NN18	P223	P20	P13	MS2	EM1	All *loci* Total	All *loci* Mean
Faial	F1 Feteiras *N*=13	*A*	7	5	7	6	5	7	7	7	51	6.4
		R	6	5	6	5	4	6	6	6	44	5.5
		S	172-236	82-98	278-296	98-122	172-186	152-166	200-214	241-257		
		C(F)	236 (0.42)	98 (0.35)	290 (0.35)	98,114, 118 (0.27)	180, 182 (0.38)	156 (0.38)	212 (0.50)	241 (0.30)		
		H_O	0.769	0.692	0.923	0.692	0.615	0.917	0.769	1.000		0.797
		H_E	0.782	0.803	0.797	0.818	0.732	0.819	0.720	0.837		0.789
		P(F)	-	-	-	-	-	-	-	-	0	
	F2 Horta *N*=16	*A*	8	9	7	6	9	7	7	8	61	7.6
		R	7	7	6	5	7	6	6	7	50	6.3
		S	172-236	78-98	278-292	98-122	162-186	152-166	200-216	241-259		
		C(F)	236 (0.31)	90, 96 98 (0.19)	290 (0.44)	98 (0.34)	182 (0.28)	156 (0.31)	212 (0.28)	241 (0.28)		
		H_O	0.875	0.875	0.750	0.813	1.000	**0.750**	0.688	1.000		0.844
		H_E	0.839	0.877	0.776	0.774	0.857	0.808	0.839	0.843		0.827
		P(F)	-	78 (0.03)	-	-	164 (0.03)	-	-	-	2	
	F3 Espalhafatos *N*=15	*A*	8	7	8	5	7	9	6	8	58	7.3
		R	6	6	7	5	6	6	5	7	48	6.1
		S	172-236	80-98	272-292	98-122	174-188	140-166	200-216	241-259		
		C(F)	229 (0.27)	90, 92 (0.27)	290 (0.27)	118 (0.37)	182 (0.37)	156 (0.43)	212 (0.40)	241, 247 (0.27)		
		H_O	0.933	0.800	0.867	0.800	0.867	0.800	0.667	1.000		0.842
		H_E	0.844	0.823	0.871	0.759	0.798	0.772	0.800	0.844		0.814
		P(F)	-	-	-	-	-	-	-	-	0	
	Faial Island *N*=44	*A*	10	9	9	6	11	10	9	9	73	9.1
		R	9	8	9	6	10	9	9	9	68	8.5
		S	172-236	78-98	272-296	98-122	162-188	140-166	200-216	241-259		
		C(F)	236 (0.32)	98 (0.23)	290 (0.35)	118 (0.32)	182 (0.34)	156 (0.37)	212 (0.39)	241 0.28		
		H_O	0.864	0.795	0.841	0.773	0.841	0.814	0.705	1		0.829
		H_E	0.831	0.842	0.813	0.781	0.795	0.793	0.780	0.839		0.809
		P(F)	-	78 (0.01)	-	-	164 (0.01)	-	-	-	2	

Appendix I Summary statistics for eight microsatellite loci from the Azorean bat populations (cont.)

Islands	Sampling sites		P217	NN8'	NN18	P223	P20	P13	MS2	EM1	All *loci*	
Pico	P1 São Roque *N*=15	*A*	8	7	9	5	8	9	7	7	60	7.5
		R	7	6	6	5	6	7	5	6	49	6.1
		S	172-236	82-98	272-296	98-122	176-188	140-166	198-214	241-259		
		C(F)	229 (0.30)	92 (0.30)	290, 292 (0.27)	118 (0.37)	180 (0.27)	156 (0.27)	212 (0.40)	241 (0.27)		
		H_O	0.867	0.933	0.800	0.733	0.800	0.733	0.800	0.867		0.817
		H_E	0.860	0.837	0.821	0.747	0.846	0.876	0.736	0.839		0.820
		P(F)	-	-	-	-	-	-	-	-	0	
	P2 Lajes *N*=22	*A*	9	8	10	7	8	9	8	7	66	8.3
		R	6	6	7	5	6	7	6	6	49	6.2
		S	172-236	80-102	274-296	98-126	176-194	140-166	200-216	241-259		
		C (F)	236 (0.32)	92 (0.23)	278, 290 (0.23)	98 (0.45)	180 (0.45)	156 (0.43)	206 (0.39)	241 (0.36)		
		H_O	0.818	0.864	0.864	0.727	0.864	0.714	0.909	0.773		0.817
		H_E	0.812	0.852	0.868	0.726	0.746	0.801	0.794	0.789		0.799
		P(F)	-	102 (0.02)	-	-	194 (0.02)	-	-	-	2	
	P3 Mirateca *N*=13	*A*	5	8	7	5	7	6	8	7	53	6.6
		R	4	7	6	4	6	5	7	6	45	5.6
		S	196-236	80-98	278-296	98-122	162-188	152-166	198-216	241-259		
		C(F)	229 (0.31)	82 (0.27)	290 (0.31)	98 (0.38)	180 (0.38)	156 (0.31)	212 (0.27)	255 (0.31)		
		H_O	0.615	0.846	0.692	0.692	0.923	0.923	0.846	0.923		0.808
		H_E	0.634	0.846	0.831	0.729	0.800	0.815	0.855	0.825		0.792
		P(F)	-	-	-	-	-	-	-	-	0	
	Pico Island *N*=50	*A*	9	9	11	7	10	9	9	7	71	8.9
		R	9	9	10	7	9	9	9	7	67	8.4
		S	172-236	80-102	272-296	98-126	162-194	140-166	198-216	241-259		
		C(F)	229 (0.33)	92 (0.24)	290 (0.26)	98 (0.40)	180 (0.38)	156 (0.35)	212 (0.24)	255 (0.20)		
		H_O	0.780	0.880	0.800	0.720	0.860	0.776	0.860	0.840		0.814
		H_E	0.800	0.844	0.842	0.734	0.792	0.823	0.804	0.815		0.807
		P(F)	-	102 (0.01)	-	-	194 (0.01)	-	198 (0.02)	-	3	

Appendix I Summary statistics for eight microsatellite loci from the Azorean bat populations (cont.)

Islands	Sampling sites		P217	NN8'	NN18	P223	P20	P13	MS2	EM1	All *loci*	
São Jorge	SJ1 Manadas *N*=29	*A*	8	7	9	6	7	7	7	8	59	7.4
		R	5	6	7	5	5	5	6	6	45	5.7
		S	172-236	80-98	274-296	98-126	162-188	152-166	200-216	241-259		
		C(F)	236 (0.45)	98 (0.26)	290 (0.26)	98 (0.38)	180 (0.41)	156 (0.39)	206 (0.36)	241 (0.29)		
		H_O	0.828	0.759	0.897	0.690	0.586	0.630	0.931	0.759		0.760
		H_E	0.744	0.831	0.848	0.732	0.764	0.767	0.802	0.829		0.790
		P(F)	176 (0.02)	-	-	-	-	-	-	-	1	
	SJ2 Boa Hora *N*=19	*A*	7	8	10	5	6	7	6	8	57	7.1
		R	5	7	8	4	5	6	5	7	46	5.8
		S	172-236	80-98	272-296	98-122	176-186	144-166	200-214	241-261		
		C(F)	236 (0.42)	92 (0.26)	290 (0.40)	98 (0.63)	180 (0.39)	156 (0.31)	212 (0.39)	241, 247 (0.21)		
		H_O	0.684	0.947	0.842	0.579	0.895	0.833	0.737	0.684		0.808
		H_E	0.727	0.862	0.821	0.595	0.762	0.825	0.771	0.727		0.761
		P(F)	-	-	-	-	-	-	-	-	0	
	São Jorge Island *N*=48	*A*	9	8	11	6	8	8	7	10	67	8.4
		R	8	8	10	6	7	8	7	9	63	7.9
		S	172-236	80-98	272-296	98-126	162-188	144-166	200-216	241-261		
		C(F)	236 (0.44)	98 (0.23)	290 (0.31)	98 (0.48)	180 (0.41)	156 (0.36)	206,212 (0.28)	241 (0.26)		
		H_O	0.771	0.833	0.875	0.646	0.708	0.711	0.854	0.833		0.779
		H_E	0.741	0.837	0.843	0.696	0.762	0.781	0.806	0.845		0.789
		P(F)	176 (0.01)	-	-	-	-	-	-	-	1	

Appendix I Summary statistics for eight microsatellite loci from the Azorean bat populations (cont.)

Islands	Sampling sites		P217	NN8'	NN18	P223	P20	P13	MS2	EM1	All *loci*	
Terceira	T1 Sta Bárbara/ Cinco Ribeiras *N*=8	*A*	6	5	7	5	4	6	6	6	45	5.6
		R	6	5	7	5	4	6	6	6	45	5.6
		S	172-257	80-98	278-296	98-122	180-188	140-166	200-214	241-261		
		C(F)	236 (0.44)	98 (0.38)	278 (0.25)	118 (0.38)	180 (0.50)	156 (0.50)	210 (0.31)	247 (0.44)		
		H_O	0.625	0.875	0.750	0.750	0.625	0.750	0.750	0.625		0.734
		H_E	0.850	0.808	0.883	0.792	0.675	0.792	0.867	0.850		0.815
		P(F)	257 (0.06)	-	-	-	-	-	-	-	1	
	T2 Quatro Ribeiras *N*=30	*A*	9	8	10	7	9	8	7	9	67	8.4
		R	6	6	7	5	6	5	6	6	47	5.9
		S	172-240	80-98	272-296	98-126	174-190	140-166	200-216	241-261		
		C(F)	236 (0.42)	98 (0.33)	290 (0.30)	98 (0.40)	180 (0.38)	156 (0.47)	206 (0.33)	243 (0.25)		
		H_O	0.667	0.867	0.867	0.667	0.833	0.733	0.833	0.667		0.775
		H_E	0.766	0.818	0.854	0.723	0.776	0.722	0.803	0.766		0.779
		P(F)	240 (0.02)	-	-	-	-	-	-	-	1	T
	Terceira Island *N*=39	*A*	10	9	10	7	9	9	7	9	70	8.8
		R	9	9	10	7	9	9	7	9	68	8.5
		S	172-257	80-98	272-296	98-126	174-188	140-166	200-216	241-261		
		C(F)	236 (0.43)	98 (0.33)	290 (0.27)	98 (0.37)	180 (0.41)	156 (0.49)	206 (0.40)	247 (0.23)		
		H_O	0.667	0.872	0.846	0.692	0.795	0.718	0.821	0.744		0.769
		H_E	0.766	0.817	0.861	0.728	0.751	0.721	0.813	0.834		0.786
		P(F)	240, 257 (0.01)	-	-	-	-	-	-	-	2	
Graciosa	G Graciosa Island *N*=32	*A*	8	7	11	6	7	10	8	8	65	8.1
		**R*	6	6	8	4	5	7	6	6	48	6
		S	172-236	80-98	272-296	98-122	176-190	140-166	200-216	241-259		
		C(F)	236 (0.33)	90 (0.27)	278 (0.19)	98 (0.41)	184 (0.36)	156 (0.23)	212 (0.27)	255 (0.34)		
		H_O	0.781	0.969	0.938	0.6875	0.656	0.875	0.844	0.750		0.813
		H_E	0.801	0.831	0.893	0.69593	0.785	0.867	0.806	0.821		0.812
		P(F)	-	-	-	-	-	-	-	-	0	

Appendix I Summary statistics for eight microsatellite loci from the Azorean bat populations (cont.)

Islands	Sampling sites		P217	NN8'	NN18	P223	P20	P13	MS2	EM1	All *loci*	
											Total	Mean
São Miguel	SM1 Ponta Delgada *N*=23	*A*	8	6	7	7	11	9	7	8	63	7.9
		R	6	5	5	5	7	7	5	6	47	5.8
		S	218-273	80-94	272-292	98-126	168-192	144-166	204-220	241-259		
		C(F)	225 (0.30)	92 (0.35)	290 (0.54)	110 (0.33)	182 (0.26)	162 (0.34)	206 (0.39)	243 (0.24)		
		H_O	0.826	0.739	0.739	0.609	0.696	0.818	0.783	0.826		0.760
		H_E	0.842	0.788	0.674	0.781	0.842	0.825	0.758	0.842		0.794
		P(F)	273 (0.02)	-	-	-	-	-	218 (0.02)	-	2	
	SM2 Furnas *N*=19	*A*	6	5	9	7	9	7	7	9	59	7.4
		R	5	4	7	6	7	6	6	7	46	5.8
		S	218-236	82-98	272-296	102-126	168-186	142-166	204-216	241-261		
		C(F)	222 (0.34)	92 (0.55)	290 (0.45)	114 (0.39)	182 (0.37)	154 (0.29)	206 (0.21)	243 (0.24)		
		H_O	0.789	0.684	0.737	0.842	0.895	0.737	0.842	0.789		0.789
		H_E	0.780	0.639	0.777	0.777	0.815	0.832	0.792	0.780		0.774
		P(F)	-	-	-	106 (0.05)	-	142 (0.05)	-	-	2	
	SM3 Ribeirinha *N*=22	*A*	8	9	9	5	9	7	8	10	65	8.1
		R	6	6	6	4	7	5	6	7	48	6.0
		S	200-269	80-98	272-294	98-122	170-192	144-166	204-220	241-261		
		C(F)	222 (0.30)	90 (0.30)	290 (0.55)	114 (0.39)	182 (0.31)	166 (0.31)	210 (0.25)	247 (0.18)		
		H_O	0.909	0.773	0.727	0.773	0.762	0.762	0.909	0.909		0.821
		H_E	0.826	0.808	0.706	0.699	0.856	0.826	0.841	0.826		0.799
		P(F)	-	86 (0.02)	-	-	-	-	-	-	1	
	São Miguel Island *N*=66	*A*	9	9	11	8	13	10	10	11	81	10.1
		R	8	7	9	7	11	9	9	10	71	8.8
		S	200-273	80-98	272-296	98-126	168-192	142-166	200-220	241-261		
		C(F)	222 (0.27)	92 (0.38)	290 (0.51)	114 (0.34)	182 (0.30)	162 (0.27)	206 (0.25)	243 (0.21)		
		H_O	0.818	0.738	0.738	0.742	0.769	0.778	0.846	0.877		0.788
		H_E	0.816	0.760	0.708	0.756	0.842	0.825	0.815	0.853		0.797
		P(F)	218. 269 (0.02). 273 (0.01)	86 (0.01)	-	106 (0.02)	168 (0.02). 170 (0.04). 192 (0.03)	142. 150 (0.02)	218 (0.01). 220 (0.02)	253 (0.04)	13	

N Number of specimens genotyped; *A* number of alleles; *R* Allelic Richness per *locus* and population based on minimum sample size (32 individuals for islands and *8 individuals for colonies) calculated in FSTAT; *S* Allelic size range in bp; *F* Frequency of the allele; *C* size in bp of the most common allele; *P* size in bp of the unique allele in a population; *H*O observed heterozygosity; *H*E expected heterozygosity. $P < 0.007$. deviation from HWE using Arlequin 3.0. after a sequential Bonferroni correction in bold

Appendix II Estimated pairwise G'_{ST} (below diagonal) and F_{ST} (above diagonal) values among sampling sites.

	F1	F2	F3	P1	P2	P3	SJ1	SJ2	T1	T2	G	SM1	SM2	SM3
F1	-	0.006	0.000	0.004	0.014*	0.019*	0.002	0.019*	0.000	0.015*	**0.026**	**0.062*****	**0.083*****	**0.060*****
F2	0.035	-	-0.003	0.015*	0.009	0.010	0.004	0.022**	0.021*	0.009	0.012*	**0.044*****	**0.076*****	**0.045*****
F3	0.005	-0.011	-	0.004	0.007	0.005	0.005	0.021**	-0.004	0.007	0.003	**0.052*****	**0.067*****	**0.050*****
P1	0.024	0.086	0.027	-	0.009	0.005	0.010	**0.031*****	0.024*	**0.025*****	0.012*	**0.056*****	**0.076*****	**0.067*****
P2	0.065	0.051	0.037	0.051	-	0.016*	-0.003	**0.025*****	0.004	0.003	**0.021*****	**0.073*****	**0.089*****	**0.079*****
P3	0.091	0.057	0.029	0.025	0.079	-	0.006	**0.032*****	0.019	0.014*	0.012*	**0.070*****	**0.097*****	**0.080*****
SJ1	0.005	0.018	0.021	0.050	-0.015	0.027	-	0.017**	-0.002	0.000	0.011*	**0.067*****	**0.095*****	**0.078*****
SJ2	0.086	0.114	0.104	0.155	0.120	0.148	0.075	-	0.023*	**0.024*****	**0.028*****	**0.076*****	**0.090*****	**0.072*****
T1	-0.010	0.091	-0.040	0.101	-0.004	0.074	-0.029	0.091	-	-0.007	0.019*	**0.079*****	**0.084*****	**0.075*****
T2	0.068	0.043	0.034	0.122	0.014	0.064	0.000	0.109	-0.035	-	**0.020*****	**0.068*****	**0.088*****	**0.072*****
G	0.131	0.064	0.017	0.066	0.108	0.063	0.058	0.139	0.092	0.096	-	**0.066*****	**0.082*****	**0.065*****
SM1	0.290	0.226	0.259	0.281	0.352	0.329	0.318	0.348	0.374	0.320	0.331	-	0.010	0.004
SM2	0.383	0.389	0.331	0.377	0.425	0.457	0.442	0.407	0.406	0.406	0.400	0.047	-	0.008
SM3	0.285	0.240	0.254	0.344	0.390	0.386	0.371	0.332	0.367	0.344	0.329	0.019	0.040	-

In bold: significant F_{ST} values. after a sequential Bonferroni correction. ***P < 0.001. ** P < 0.01. * P < 0.05

3.2. Continental fragmented populations

3.2.1 Paper V

Population structure of a cave-dwelling bat, *Miniopterus schreibersii*: Does it reflect history and social organization?

Ramos Pereira MJ, Salgueiro P, Rodrigues L, Coelho MM, Palmeirim JM (submitted)

Population structure of a cave-dwelling bat, *Miniopterus schreibersii*: Does it reflect history and social organization?

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Abstract

Many colonial bat species make regional migrations, and the consequent gene flow may eliminate geographic genetic structure resulting from history of colonization, and prevent structuring due to social organization. We verified if history and social organization have detectable impacts on the genetic structure of *Miniopterus schreibersii*, a cave dwelling bat with high female philopatry, in spite of its strong migratory behaviour. After studying virtually all the nursing colonies in Portugal, we concluded that there is a significant geographic structure and that the overall pattern is similar for mitochondrial and nuclear DNA. Genetic differentiation was significantly correlated with geographical distance, suggesting isolation by distance. However, structuring of mitochondrial DNA was much more marked than that of nuclear DNA, a consequence of the strong female philopatry and a bias for male-mediated gene flow. Wintering colonies were more genetically diverse than nursing colonies because the former receive individuals from distinct breeding populations. Haplotype diversity of the northern colonies, the youngest according to mismatch analyses, is only about half of that of the central and southern colonies. This is most likely a consequence of the colonization history of *M. schreibersii*, which presumably expanded northward after the last glacial age.

Keywords: Chiroptera, Iberian Peninsula, microsatellites, mitochondrial DNA, post-glacial expansion, male-mediated gene flow

Introduction

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

Like many other cave-dwelling bats, *M. schereibersii* is a social species that forms large colonies throughout most of the year, which in Portugal can include up to 20 thousand individuals (Palmeirim and Rodrigues 1995). Males and females migrate extensively across hundreds of kilometres and, during the mating season, animals from different nursing populations share roosts, so intercolony fertilizations are to be expected. However, females almost always return to the colony where they were born to give birth, whereas males are less attached to their birth sites (Palmeirim and Rodrigues 1995).

If this social organization affects the genetic structure of the population of *M. schereibersii* and of socially similar bat species, strong female philopatry may result in a marked structure at the maternally transmitted mitochondrial DNA. In contrast, bi-parentally inherited nuclear markers may not evince strong structuring due to generalized male-mediated gene flow (Castella et al. 2001, Petit et al. 2001, Petit and Mayer 1999, Worthington Wilmer et al. 1999).

Hibernation colonies often congregate individuals from different nurseries (Palmeirim and Rodrigues 1995, Serra-Cobo et al. 1998). Consequently, if social organization is relevant for genetic structuring of M. schereibersii populations, nurseries and hibernacula may present different genetic compositions.

Recent phylogenetic studies have shown that *M. schereibersii* is probably restricted to Europe and North Africa (Appleton et al. 2004, Bilgin et al. 2006, Miller-Butterworth et al. 2005, Tian et al. 2004). In Europe, this species shows Mediterranean climatic preferences, occurring mostly in the south of the continent, including all of the Iberian Peninsula. However, during the glacial periods of the Pleistocene, northern Iberia was certainly too cold for this species, which may have survived in the south of the Peninsula, as suggested by the fossil record of the Würm (Telles Antunes 1993). The range of *M. schereibersii* certainly expanded northward after the last glacial period. Such range expansions are often important in shaping the genetic structure of populations, especially in species with low mobility (Hewitt 1996), but their impact

in highly mobile species, like *M. schreibersii*, is less predictable.

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

Our aim is to determine if social organization and historical events have detectable impacts on the genetic structuring of the populations of cave-dwelling bats at countrywide scales, using *M. schereibersii* in Portugal as a model. We studied the influence of social organization on the genetic structure of the populations by determining if (i) separate colonies are genetically distinct, (ii) the stronger philopatry of the females results in a more marked structure at the mitochondrial DNA than at the nuclear DNA, and (iii) hibernation colonies are genetically more diverse than nurseries. In addition, we determined if genetic structure reflects the history of expansion of *M. schereibersii* in the Iberian Peninsula, checking if there is a lower genetic diversity in the regions that became available with the warming that followed the last glacial age.

Materials and Methods

Sampling

During the late nursing seasons (15-25 July) of 1998 and 2004, when all the juveniles are already flying, we obtained tissue samples from 407 adult females at eleven of the twelve nursing colonies of *M. schreibersii* known in Portugal (Figure 1, Table 1). Bats were caught by hand inside the daytime roosts or with harp traps during the emergence. Nursing colonies consist mainly of females and their young, which sometimes form separate clusters. Samples of the two years were pooled since it is very unlikely that genetic structure and diversity suffered significant changes in that interval, as these bats can live more than nineteen years (Avril 1997) and generation time is about nine years (calculated, using unpublished capture data, as the age of maturity + ½ the length of reproductive period in life cycle). The size of the studied colonies is monitored annually, and it remained quite stable between the two sampling periods (unpublished data). During the winter of 2004 we also sampled 96 individuals at four of the 15 known hibernation colonies, which harbour both males and females. Tissue samples were obtained from a non-lethal sterile biopsy punch of the wing membrane (Worthington Wilmer and Barratt 1996) and preserved in 100% ethanol.

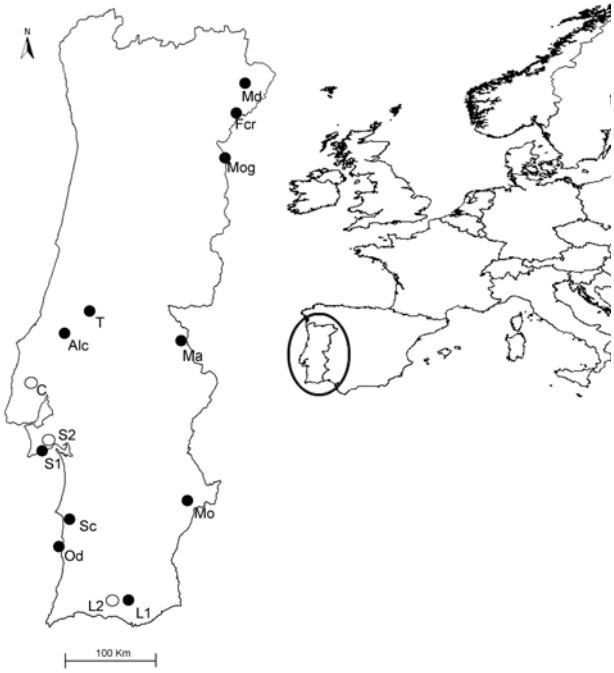

Figure 1
General location of sampling sites. Filled dots correspond to nurseries (*Md* – Miranda do Douro I, *Mog* – Mogadouro II, *Fcr* – Figueira de Castelo Rodrigo I, *T* – Tomar I, *Alc* – Alcanena I, *S1* – Sesimbra I, *Mo* – Moura I, *Sc* – Santiago do Cacém, *Od* – Odemira I, *L1* – Loulé I),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

Table 1 Sample size, year of sampling and molecular variability of the 11 nursing colonies and the 4 hibernation colonies of *M. schreibersii* studied. *nh*, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *A*, allele richness per locus; H_E, expected heterozygosity; *SD*, standard deviation.

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

*Ma is both a nursing and a hibernation roost

DNA extraction and amplification

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

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

We amplified the microsatellite loci in 10 µl PCR reaction volumes, using the five primer pairs *Mschreib1-5* described by Miller-Butterworth et al. (2002). *NCAM,* described by Moore et al. (1998), was also tested but revealed to be monomorphic. The PCR conditions were optimised in order to use the Multiplex PCR Kit (QIAGEN), which facilitates the simultaneous amplification of several loci. The multiplex PCR profile consists of an initial denaturation and hot DNA polymerase activation at 95°C for 15 min, followed by 30 cycles of the series: 95°C for 30 s, annealing temperature (55ºC for *MS1* and *MS3*, 58ºC for *MS2* and *MS4*, 53ºC for *MS5*) for 90 s, 72°C for 60 s; a final amplification step of 10 – 20 min. at 72°C was performed. After detecting the polymorphic loci, primers were labelled with Beckman dyes, and PCR products were run in a CEQ 2000XL-Beckman Coulter equipment. Allele calling was performed using a CEQ 8000 GENETIC ANALYSIS SYSTEM.

Phylogenetic reconstruction

MtDNA sequences were aligned and edited with SEQUENCHER 3.0 (Gene Codes Corp.). Haplotypes were connected on a network obtained using the 95% parsimony criterion implemented in NETWORK 4.0.01 (Röhl 2003).

Genetic diversity within colonies

Mitochondrial diversity was represented by haplotype (h) and nucleotide (π) diversities calculated in ARLEQUIN 2.0 (Schneider et al. 2000). According to the classical models of range expansion a post-glacial expansion from southern refugia may result in a northward decrease in genetic polymorphism (Hewitt 1996, 2000). We investigated the existence of such a geographic trend in the levels of gene diversity of nurseries applying a bootstrap analysis to the haplotypes (*P*-value was calculated by counting the number of times when the number of haplotypes of the

northern colonies was inferior to the average and dividing by 10,000, the number of resampling procedures).

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

To test the hypothesis that hibernation colonies are genetically more diverse than nurseries, we compared mitochondrial genetic diversity (h and π) between hibernacula and nurseries using bootstrap. To control any bias caused by the fact that few males were sampled in nurseries, we performed two analyses: one with females only, and one pooling males and females.

Differentiation among nursing colonies

To understand if separate colonies are genetically distinct, the differentiation among nursing populations at the mtDNA level was calculated performing a global test of differentiation between samples (Raymond and Rousset 1995), and computing both pairwise F_{ST} based on haplotype frequencies only, and $\Phi_{ST,}$ based on haplotype frequencies and genetic distances among haplotypes. These analyses take into account the presence of indels, which differentiate some haplotypes. For microsatellites, we estimated R-statistics according to Rousset (1996) and F-statistics, between pairs of localities according to Weir and Cockerham (1984). We performed the computations in ARLEQUIN 2.0 (Schneider et al. 2000) and FSTAT 2.9.3.2 (Goudet 1995).

To test the hypothesis of isolation by distance, we calculated the correlation between geographical distance and mitochondrial and nuclear F_{ST} by means of a Mantel test in APE package for R (Paradis et al. 2004). Isolation by distance was considered to be present if, after 10,000 permutations, the probability of obtaining the observed correlation by chance was below 1%.

To define geographically homogeneous groups of populations we performed a spatial analysis of molecular variance (SAMOVA, Dupanloup et al. 2002) with mitochondrial and nuclear markers, using the program SAMOVA 1.0., which determines the partitioning of

populations that maximizes the Φ_{CT} value when a certain number of groups are specified (Dupanloup et al. 2002). It was used to identify the most likely number of groups within the data set from repeated analyses, specifying two to eleven groups, and to choose the partitioning of populations that maximizes the Φ_{CT} value. Individual multilocus genotypes were also subjected to a Factorial Correspondence Analysis over the populations using GENETIX (Belkhir et al. 1996).

Sex-biased dispersal

We investigated for sex-biased dispersal by testing if F_{IS}, F_{ST}, mean assignment index (*AI*), and *AI* variance differed significantly between the two sexes (Goudet et al. 2002). The most dispersing sex is expected to have a positive $F_{IS,}$ a lower F_{ST} and mean *AI*, and a larger variance of *AI* (Goudet et al. 2002). All calculations were conducted in FSTAT 2.9.3.2 (Goudet 1995).

Population expansion

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

Results

Mitochondrial and nuclear polymorphism

A stretch of 518 bp from the mtDNA control region was sequenced in 312 individuals (GenBank accession numbers to be added at proof stage). The alignment of the sequences resulted in 24 variable sites defining 39 distinct haplotypes (Table 2). Of the observed substitutions 19 were transitions, 4 were transversions, and 2 single base-pair deletions or insertions (indels). Genotyping the total of 503 bats at the five microsatellite loci scored 56 distinct alleles. No null alleles or large allele drop-out were identified. No pair of loci was

significantly associated at the colony level after Bonferroni corrections (Rice 1989) and no departures from Hardy-Weinberg equilibrium (Guo and Thompson 1992) were detected, except for locus *Mschreiber4* in Alcanena and Marvão populations. Independent segregation of the alleles at the five loci was therefore assumed in subsequent analyses. Allele frequencies of the five microsatellite loci are presented as supplementary material.

Table 2 Variable nucleotide positions within the 518-bp sequence of the D-loop *a*nalysed in 312 specimens of *M. schreibersii*.

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

Phylogenetic analyses

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

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

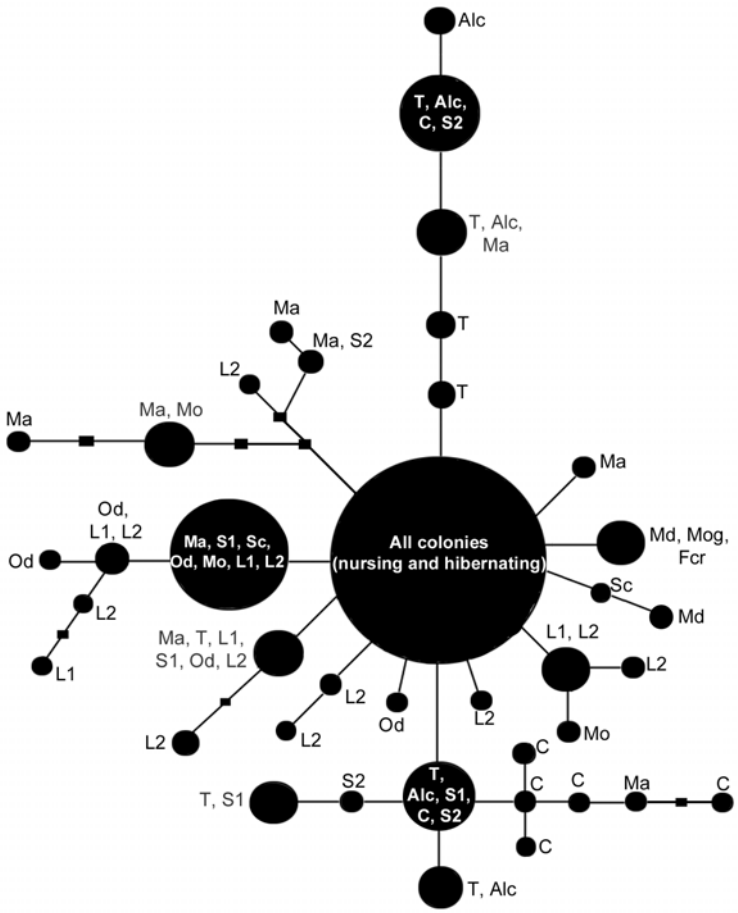

Figure 2
Minimum spanning tree (MST) of the 39 different haplotypes found by sequencing 518 bp of mtDNA D-loop of 312 *M. schreibersii*. Haplotypes are represented by circles, and the line between two circles corresponds to a single mutation. Small squares are for haplotypes inferred from the minimum spanning tree, but which were not observed in the sample (missing haplotypes).

Genetic diversity within colonies

Molecular variability of the nursing and hibernation colonies is shown in Table 1. Haplotype diversity of mtDNA in the northern nursing colonies (Md, Fcr and Mog) was approximately half of the diversity held by the southern nursing colonies. A northwards decrease in nucleotide diversity was also evident. This north-south decline in both haplotype and nucleotide diversities was shown to be significant (P<0.01). For the nuclear markers, mean allele richness was 5.97 and mean expected heterozygosity was 0.64. Alleles were roughly evenly distributed among the 15 colonies sampled. A few low-frequency alleles were specific to one or a few colonies. A north-south decline in allele richness and in expected heterozygosity, was also shown to be significant (P<0.01). Resampling using just females or both males and females showed that hibernacula had significantly higher haplotype and nucleotide diversities values than nurseries (P<0.01).

Differentiation among colonies

Differentiation between colonies at the mtDNA level was generally moderate (Table 3), but low within the same geographical region. In this latter case Φ_{ST} and F_{ST} values were usually not significant. The correlation between geographical distance and Φ_{ST} was significant (r=0.38; P<0.01).

At the nuclear level, R_{ST} over loci (0.0268) and mean pairwise F_{ST} (0.022 ± 0.003) revealed a weak structure among colonies, although several pairwise R_{ST} and F_{ST} values were still significantly different from zero (results not shown), particularly between colonies of distinct geographical regions. The correlation between pairwise R_{ST} and geographical distance, investigated by Mantels' test, was also significant (r=0.31; P<0.05).

In SAMOVA, Φ_{CT} approach a maximum as the number of groups increased, reaching 95% of the maximum value with four groups (Figure 3a), both for the mitochondrial and nuclear data sets. When more groups are defined, the Φ_{CT} value increases but some groups begin to consist of single colonies. This division of the nursing colonies in four groups explained about 80% of overall genetic variation, both at the mtDNA and the microsatellite level (p<0.01). This arrangement is coherent with the factorial correspondence analysis results. However, in this case Marvão, which appears as a separate unit in the four-group SAMOVA, is here particularly close to the Central-West colonies (Figure 3b).

Table 3 Pairwise genetic differentiation (mtDNA Φ_{ST}) among the nursing colonies of *M. schereibersii*

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

All *P* values < 0.01, except those marked with $^{+}$, which are non-significant at α= 0.01.

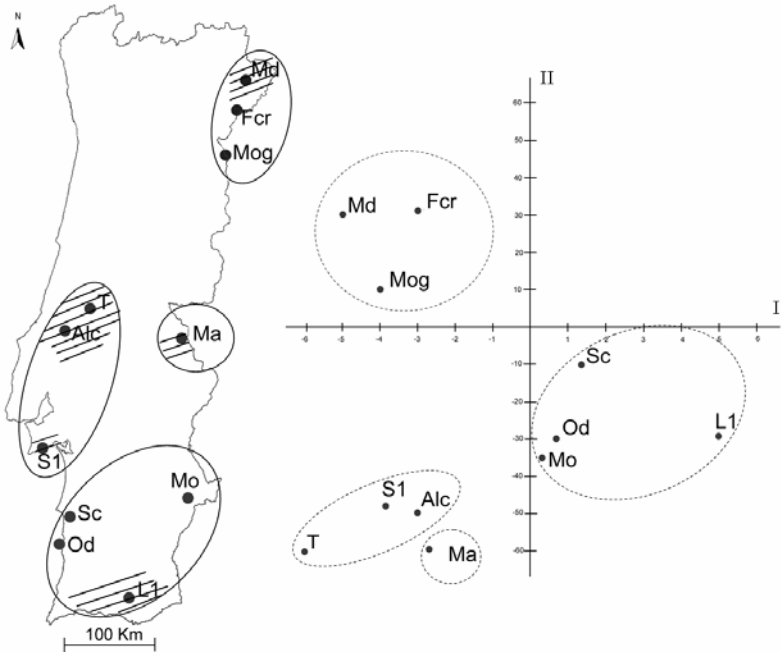

Figure 3
a) Map of the groups of populations identified by the SAMOVA, and b) plot with the results of the factorial correspondence analysis of the populations, using the individual microsatellite genotypes. Dashed areas represent major limestone regions according to Ribeiro *et al.* (1987).

Sex-biased dispersal

Tests for F_{IS} and mean *AI* were significant (P<0.05) confirming the expectation that males are the key dispersing sex. Globally males presented a positive F_{IS} against the negative value of the females (male F_{IS}=0.0765; female F_{IS}=-0.0004), lower F_{ST} (male F_{ST}= 0.0351; female F_{ST}= 0.0408), lower mean *A*I and a slightly larger variance of *AI* (male *AI*= -0.0806, var*AI*=3.9499;

female *AI*= 0.3938, var*AI*=3.1428).

Population expansion

As colonies showed low differentiation within the same geographical region we chose to treat as populations the four groups revealed by the SAMOVA. This approach minimises the possible effects of dispersal between colonies, which is more likely to occur within the same region. The hypothesis of expansion is not rejected by the SSD tests (all *P*-values>0.05). The Harpending raggedness index was non-significant in all nursing colonies and in the 4 groups (Table 4). The combined data set showed unimodal smooth curves, with single major peaks at around 1, 1, and 4 differences in Central-West (CW), South (S), and Central-East (CE) respectively (Figure 4). The North-East (NE) group reached the maximum value around 0. These results may suggest expansion of the regional populations, starting at about 20,000 years (during the late Pleistocene) in Marvão (CE), the oldest population, and at about 4,300 years (Holocene) in the more recent NE population (Table 4). However, confidence intervals for θ_0 and θ_1 are very large, leading to some overlap between them and consequently challenging the model of expansion.

Table 4 Values of τ, θ_0, θ_1, time since expansion (*t*) and raggedness index *P*-values

Population	τ (95% CI)	θ_0 (95% CI)	θ_1 (95% CI)	t (max-min)	*P*-value
NE(Md+Fcr+Mog)	0.89 (0.66 - 2.31)	0.00 (0.00 - 1.88)	0.67 (0.00 - 4005.67)	4 280 (3 204 - 11 158)	0.20
CW(T+Alc+S1)	1.93 (0.69 - 3.52)	0.00 (0.00 - 2.23)	11.92 (1.29 - 7319.42)	9 300 (3 344 - 16 973)	0.55
CE(Ma)	4.14(1.14 - 11.86)	0.00 (0.00 - 12.17)	4.91 (0.57 - 4975.91)	19 985 (5 482 - 57 254)	0.25
S(Mo+Sc+Od+L1)	1.63 (0.89 - 2.23)	0.00 (0.00 - 1.095)	18.14 (3.089 - 5699.69	7857 (4 280 - 10 772)	0.90

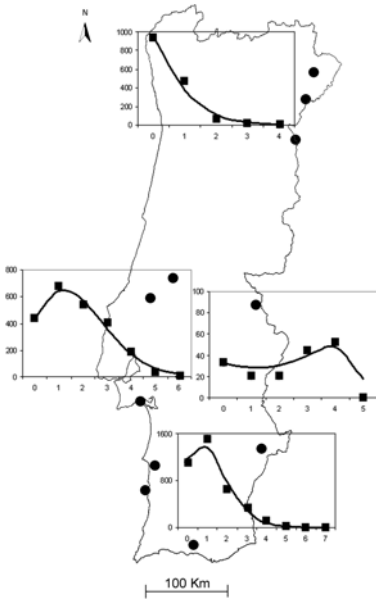

Figure 4
Mismatch distributions for groups of nursing colonies. X-axis - number of pairwise differences between sequences; Y-axis - frequency; black squares indicate observed values; lines show model distribution.

Discussion

Miniopterus schreibersii shows clear population subdivision and male-biased gene flow

Mitochondrial and nuclear DNA structure revealed similar geographic patterns in spite of the distinct spatial behaviour of male and female *M. schreibersii*. Both pairwise Φ_{ST} and R_{ST} were significantly correlated with geographical distance, suggesting that isolation by distance is relevant for the studied mitochondrial and nuclear markers.

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

The observed structuring at the mitochondrial DNA level was much more marked than that for nuclear DNA. Additionally, the tests for sex-biased dispersal suggested that males are responsible for more gene flow than females. However, these results should be interpreted with caution because these tests are mainly indicated for non-overlapping generations, which is not the case in our study.

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

Since females are philopatric and the structuring of the mitochondrial DNA is more marked than that of nuclear DNA, we conclude that males are responsible for most of the gene flow. Female philopatry and male-mediated gene flow are characteristics of many mammal species (Greenwood 1980) and have been shown for several bat species such as *Macroderma gigas* (Worthington Wilmer et al. 1999), *Nyctalus noctula* (Petit et al. 2001, Petit and Mayer 1999, 2000), *Myotis myotis* (Castella et al. 2001, Ruedi and Castella 2003), *Myotis bechsteinii* (Kerth et al. 2002) and *Plecotus auritus* (Veith et al. 2004).

Mating occurs in underground roosts that gather males and females from several nursing colonies (Palmeirim and Rodrigues 1995). This fact, associated with the extensive movements

that these bats make during the mating season, suggests widespread gene-flow and consequently a near panmitic situation at the scale of the Portuguese territory. However, we found a clear structure at the nuclear DNA, even though it is quite weak. How to explain the persistence of this structure at the nuclear level? *M. schreibersii* is dependent on underground roosts throughout the yearly cycle. Mining activities created a continuous network of roosts for these species in the Portuguese territory. Only a few of these roosts are used by nursing colonies but many are used as temporary shelters, where presumably mating takes place during the autumn. However, in the past, before the dissemination of underground mining activities, the availability of suitable roosts was far more discontinuous. In Portugal, the great majority of potential roosts were concentrated in two large and three small limestone areas (Figure 3a). The genetic units revealed by nuclear DNA are, to a great extent, coincident with these limestone areas, which are separated by vast regions without natural caves, even if we consider the bordering Spanish territory. Consequently, the observed geographic structure in nuclear DNA markers may be a remnant of times when connectivity between populations was much lower than that existing today. The increased connectivity between populations of strict cave-dwelling bat species due to the creation of man-made roosts may enhance gene-flow and, in the long term, reduce their geographic genetic structuring in the nuclear DNA. At the mitochondrial level this is less likely to occur due to the great philopatry of females to their birth colonies. Our results can, in the future, be used as a baseline reference to determine if this phenomenon is indeed occurring.

Winter roosts are genetically more diverse because they gather bats from multiple nurseries

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

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

animals. The results suggest that summer and winter roosts may be separated by several hundred kilometres, but bats tend to hibernate in colonies located in the same region of their nurseries. A more complete sampling scheme that includes nursing, mating and hibernation roosts, and the analysis of more microsatellite loci would give a better picture of the migration patterns of *M. schreibersii*.

Post-glacial northward expansion is reflected in genetic structure

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

The population of *M. schreibersii* in northern Portugal and adjacent areas in Spain, includes both nursing and hibernation colonies, and is about as large as those of central and southern Portugal. This excludes the possibility that the observed lower diversity is a result of a smaller population size.

Mismatch distribution also indicate that the northernmost Portuguese colonies are the youngest ones. Estimates of population time expansion show very large confidence intervals and have to be interpreted with care. These estimates may be somewhat inaccurate, not only due to sample size but also as a consequence of the migration of individuals into some colonies, after these became established.

It has been demonstrated that Southern Iberia served as a glacial refuge for a variety of thermophilic organisms, such as the woodmouse, *Apodemus sylvaticus* (Michaux et al. 2003), the European rabbit, *Oryctolagus cunniculus* (Branco et al. 2002), the pygmy marbled newt, *Triturus pygmaeus* (Wallis and Arntzen 1989) and deciduous oak species, *Quercus* spp. (Brewer et al. 2002). The presence of *M. schreibersii* in fossil material from the Würm from southern Portugal (Telles Antunes 1993) and our estimates of expansion times, suggest that the species survived there during the last glacial period. Our results strongly support that there was a post-glacial northward expansion of *M. schreibersii* but this could have occurred regardless of the survival of the species in southern Iberia during the glacial periods. A scenario of disappearance from Iberia, followed by a post-glacial recolonisation from North Africa across the Strait of Gibraltar, is also compatible with our results. The sampling of North African colonies is essential to fully

understand the history of post-glacial colonisation of the Iberian Peninsula by *M. schreibersii*.

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Chapter IV General Discussion

IV General Discussion

In the two preceding chapters, the main results obtained from the five papers were discussed separately. The present chapter consists of an integrated discussion of all the results, following the issues indicated in the General Introduction (Chapter I) and allowing the comparison between the two studied species.

4.1. Phylogeography of the genus *Nyctalus*

In the last decade there has been an increasing number of phylogeographic studies in chiropteran taxa from all over the world (Bilgin et al. 2006, Campbell et al. 2004, Ditchfield 2000, Hoffmann and Baker 2003, Juste et al. 1999, Lloyd 2003, Pulvers and Colgan 2007, Russell et al. 2005, Weyandt and Van Den Bussche 2007), including Europe (Hulva et al. 2004, Ibañez et al. 2006, Mayer and von Helversen 2001, Petit et al. 1999, Ruedi and Castella 2003). Several of these reports have focused on insular species (Campbell et al. 2006, Carstens et al. 2004, Hisheh et al. 1998, Roberts 2006a, Roberts 2006b), particularly from the Atlantic islands (Juste et al. 2004, Juste et al. 2003, Pestano et al. 2003a, Pestano et al. 2003b).

Although a pioneer molecular phylogeographic study has been carried on the European population of *Nyctalus noctula* (Schreber, 1774) by Petit et al. (1999), studies on the Paleartic genus *Nyctalus* have been mostly based on morphological and chromosomal data (Harada 1988, Koopman 1994). In this thesis, it is presented, for the first time, a comprehensive study of the phylogenetic relationships among most of the recognized species of the genus *Nyctalus*. This phylogeographic appraisal, based on molecular data, has allowed a better understanding of the evolution of the Azorean bat.

We analysed 75 sequences, with 639 bp, of the mtDNA ND1 gene from six species of the genus *Nyctalus* (Paper I). The complete concordance at all major nodes among ND1 trees, based on different reconstruction methods (maximum parsimony and genetic distances, Paper I, Figure 2), strongly supports the phylogenetic branching pattern obtained. Overall, the topology of the phylogenetic trees concurs with previous karyologic studies on the genus (Lin et al. 2002, Volleth 1992), and the genetic data from Mayer and von Helversen (2001).

Specifically, the Chinese sample of *N. plancyi* = *N. velutinus* (Gerbe, 1880), with 2n = 36, showed a very high genetic divergence (17%) from *N. noctula* (2n = 36). This corroborates the specific status of *N. plancyi* attributed by Ao et al. (2006), Lin et al. (2002), Wu et al. (2004) and

Zhang (1990), based on the difference in chromosome numbers and karyotypic relationships. It also contradicts its previous classification as a subspecies of *N. noctula* by Corbet (1978) and Corbet and Hill (1992).

The present findings confirmed the monophyly of a clade comprising species with 2n = 42 karyotype: *N. lasiopterus* (Schreber, 1780), *N. aviator* (Thomas, 1911) and *N. noctula*, with a bootstrap value of 99%, Figure 2 in Paper I. This is in agreement with Volleth's (1992) point of view. Within this clade, *N. noctula* appears as a well supported monophyletic lineage separated by 6% of sequence divergence from *N. lasiopterus*. In contrast, the resolution of the relationship between *N. lasiopterus* and *N. aviator* was not so successful. These have been described as one species by Imaizumi (1970) and Tate (1942) but, more recently, other investigators recognized them as two distinct species (Corbet 1978, Maeda 1983, Simmons 2005). The present results corroborate the close relationship between them, showing a sequence divergence of 4%. This value is, however, considered low when compared to other separate vespertilionid bat species (Ibañez et al. 2006, Mayer and von Helversen 2001, Ruedi and Mayer 2001). Therefore, in order to clarify this issue it is recommended an analysis that includes specimens from the intermediate distribution area of the two species, which were not available to us.

Finally, this phylogenetic analysis lends support to a very close relationship between *N. leisleri* (Kuhl, 1817) and *N. azoreum* (Thomas, 1901), placing them jointly in a monophyletic clade with 99-100% of bootstrap replicates. This concurs with the idea of *N. azoreum* being derived from *N. leisleri*, based on morphometric data (Palmeirim 1991, Speakman and Webb 1993).

4.2. Speciation of the Azorean bat (*Nyctalus azoreum*)

In order to better understand the phylogenetic relationships between the Azorean bat and its continental ancestor, more rapidly evolving mitochondrial segments were added to the study (Cyt *b* and CR, Paper I and III). In total, 51 specimens from both species were sequenced, including samples from Europe, North Africa and the subspecies of Madeira and Canary Islands (Figure 1, Paper I).

Examination of all three mtDNA regions indicates that differences between these two species are very low. Starting with ND1, the results showed two haplotypes within all Azorean bat samples. One was shared with the European and the Madeiran Leisler's bats; the other was unique to the species, as well as the most common (Figure 3, Paper I). Sequence divergence levels in this gene varied between 0 and 0.2 %. Results from CR and Cyt *b* showed unique

phylogenetic lineages to each species. Regarding the gene Cyt *b*, Azorean lineages only differed by one to four mutations (0.1% - 1.2% of sequence divergence) from the mainland lineages (Figure 4, Paper I).

Adding up the faster-evolving CR helped to reveal further phylogeographic structure within the *azoreum / leisleri* clade. In the Azores, a total of 16 specific haplotypes were found, and at least six mutations distinguished all Azorean bats from Leisler's bats, with mean genetic divergences ranging from 1.6% to 3.6%. This level of difference was similar to that found between the Canarian *N. leisleri* sample and its Moroccan conspecific. The two Leisler's bats from Madeira, representing *N. l. verrucosus*, were also genetically very close to European samples, only differing by two substitutions (0.5% of sequence divergence, see Figure 5 in Paper I).

These findings differ from studies on other bat species from the Canary Islands, which showed higher divergence levels (> 2%) towards their continental congeners (e.g. *Barbastella barbastellus*, Juste et al. 2003; *Hypsugo savii* and *Pipistrellus maderensis*, Pestano et al. 2003b; and *Plecotus teneriffae* Juste et al. 2004). The divergence values between *N. azoreum* and *N. leisleri* are very low and within the range of some intra-specific comparisons (Bradley and Baker 2001, Mayer and von Helversen 2001, Ruedi and Mayer 2001). Nevertheless, these are still close to the values found between two cryptic species pairs, with divergence values being less than 2% between *Eptesicus serotinus / E. nilssonii*, and less than 2.6% between *Myotis myotis / M. blythii,* for Cyt *b* and ND1 (Mayer and von Helversen 2001, Ruedi and Mayer 2001). However, there was no reciprocal monophyly between *E. serotinus* and *E. nilssonii* with the CR marker (Mayer and von Helversen 2001), which, in the present study, clearly separated *N. azoreum* from its ancestor.

The low genetic divergence at several mitochondrial markers (Paper I) deeply contrasts with the clear phenotypic distinction between the *N. azoreum* and *N. leisleri*. These results called into question the specific status conferred upon the Azorean bat, which has been controversial over the years. *N. azoreum* was described, for the first time, as a species by Thomas (1901). Later, it was classed as a sub-species of *N. leisleri* by Corbet (1978). More recently, Palmeirim (1991) and Speakman and Webb (1993) confirmed its species rank, by virtue of significant divergence in size, being smaller then its continental ancestor. Besides the reduced size, other aspects distinguish the Azorean bat from its mainland counterpart: the dark pelage colour; the higher peak frequency echolocation calls (Rainho et al. 2002, Skiba 2003); the unusual diurnal activity (Moore 1975, Speakman 1995); and the distinct roost preferences, occupying a great variety of roosts (e.g. buildings, coastal cliffs and trees, Paper I).

This disagreement between divergence based on morphology and on neutral genetic markers is not unusual (Ruedi and McCracken in press). In general, morphologically distinct species, with very low levels of genetic divergence between them, are probably the result of recent separation followed by fast morphological divergence (Mayer and von Helversen 2001), as it was observed in the Vancouver Island marmot (Cardini et al. 2007). Likewise, the present findings concur with the recent study from Millien (2006) by showing the Azorean bat as another example of accelerated morphological evolution on insular mammals.

Resources for the conservation of endangered species are, to some extent, prioritized according to their taxonomic status (Haig 1998), so it is important to clarify their status. However, the taxonomic status of the Azorean bat was still unclear. Indeed, decisions on taxonomy, at species level, are problematic for allopatric taxa (Mayr and Ashlock 1991, Reed and Frankham 2003). As referred by Mayr (1963), only the sympatric occurrence of both taxa can finally proof the existence of true biological species. Even when considering lineage-based species concepts, supposedly more adequate for classifying allopatric taxa (Noor 2002), the doubts persist. The Azorean bat was separated by exclusive monophyletic lineages only when considering the CR and Cyt *b*, but not with the more conservative ND1 gene (Figure 2, Paper I).

These findings confirm that molecular measures of genetic diversity may not adequately reflect adaptive differences among populations (Reed and Frankham 2003). Therefore, a concept integrating morphological, ecological, phylogenetic and reproductive criteria was followed. Crandall et al. (2000) and Templeton (1998) defend the Cohesion Species Concept, in which species are supported by the absence of ecological and genetic exchangeability (recent or ancient) with other species. This concept is testable by two hypotheses. The first, concerning ecological exchangeability, is analysed through characters related with life-history, morphology, behaviour and habitat. Regarding the Azorean bat, this hypothesis can be clearly rejected, given all the phenotypic differentiation described above. The second hypothesis, concerning genetic exchangeability, is assessed primarily from molecular data showing an absence of gene flow. The present results showed that the Azorean bat did not share any CR and Cyt *b* haplotypes with the Leisler's bat (Paper I), defining reciprocally monophyletic clades that could be indicative of historic isolation at these genes. On the other hand, the two taxa share one ND1 lineage, which is possibly due to old common ancestry that remained after a recent speciation. Owing to the compressed time frame of divergence, some mtDNA data showed a small resolving ability to reject genetic exchangeability between the two species. Therefore, supplementary data from a more variable type of markers should help to clarify this issue. In order to test the null hypothesis of current gene flow between the Azorean bat and the Leisler`s bat, microsatellites were used,

which have helped to resolve many species relationships (Chirhart et al. 2005, Kruger et al. 2005, Petren et al. 1999, Racey et al. 2007).

This study based on microsatellite markers (Paper II) showed levels of genetic diversity significantly different between the two species, and circa 15 species-specific alleles were detected in *N. azoreum*. Phylogenetic analysis based on the distances (Dc, Da, Ds, Paper II) was able to partition species into distinct clades with strong bootstrap values (93-100%). Moreover, no putative migrants between species were found with the assignment tests. Overall, these results confirm the rejection of the recent gene flow hypothesis, which, together with previously demonstrated ecological isolation, supports species status for the Azorean bat. Like it was stated by Cardini et al. (2007), species like the Azorean bat, i.e. genetically similar but showing strong phenotypic divergence, are the opposite of cryptic species, with a great genetic distance and morphologically indistinguishable. These results reinforce the need for multidisciplinary projects in order to truly understand all aspects of biological diversity and its evolution.

Besides taxonomic issues, the results presented in this thesis provided important insights into the genetic history of the species. Firstly, the European origin of *N. azoreum* and *N. leisleri verrucosus* was confirmed. This is similar to the situation reported for the Madeiran populations of the bat *Plecotus austriacus* (Juste et al. 2004), and most Azorean birds (Hounsome 1993, Le Grand 1984). Conversely, the Leisler's bat sample from the Canary Islands seems to be more closely related to the sample from Morocco, suggesting a North African source for the Canary populations. This concurs with the findings about the bat *Plecotus teneriffae* (Juste et al. 2004) and the bird *Parus caeruleus teneriffae* (Kvist et al. 2005), and is not surprising because Africa is the closest continental mass.

We were also able to estimate the timing of separation of the Azorean bat from the Leisler's bat, based on sequence divergence data (Paper I) and expansion time calculations (Paper III). Both estimates indicated that bats arrived to the Azores in late Pleistocene/Holocene (up to 55000 years ago). These estimates exclude the hypothesis of a human mediated introduction, because men only occupied the archipelago from the 15th century on. Furthermore, it counters Ulfstrand's (1961) view point that bats have been on the archipelago for less than two centuries.

Accomplishing this 1500 km trip would require flying over the ocean without rest for many days, which is probably above the capacity of microchiropteran bats. Therefore, probably individuals of the Leisler's bat ancestor might have been picked up by storms moving across the Atlantic Ocean, and thus arrived in the Azores.

Furthermore, in Paper II, lower levels of allelic diversity and heterozygosity within Azorean bats when compared with Leisler's bats were found. These presented the same mean and total

number of alleles and private alleles as *N. azoreum*, yet only about one tenth of individuals were sampled for *N. leisleri* (Table I, Paper II). The present findings concur with other studies on insular mammals, which reported that island populations tend to show reduced genetic variation (Eldridge et al. 1999, Hinten et al. 2003, Paetkau and Strobeck 1994, Wang et al. 2005). Overall, results based on both nuclear and mitochondrial markers are consistent with a peripatric speciation (Mayr 1963) resulting from a unique colonization event (Paper II and III).

4.3. Fragmented populations of bat species

Genetic measures provide detailed information about the past and current population structure of the species, and whether or not this has changed significantly over time. In the present chapter, the results of genetic studies in two fragmented populations of bats species: the Azorean bat (*N. azoreum*) and Schreiber's bent-winged bat (*M. schreibersii*) will be examined and compared.

4.3.1. Genetic variation

Genetic variation is an important indicator of the condition of populations. In fragmented populations, when population size and gene flow have diminished, a reduction in genetic diversity is expected (Frankham et al. 2002), and this may decrease the capacity of populations to adapt to environmental changes (Lacy 1997, Stockwell et al. 2003, Templeton et al. 2001).

On islands, levels of genetic diversity are expected to be lower than those found on comparable mainland sites due to isolation and limited population size (Jaenike 1973). In the case of the Azorean bat, it was found lower mean nucleotide diversity in the CR (Paper III) relatively to other continental species (Petit et al. 1999, Ruedi and Castella 2003). Microsatellite allelic diversity and heterozygosity were also significantly lower than for the continental Leisler's bat (Paper II). Nevertheless, the mean haplotypic diversity at the same mtDNA region (Paper III) and the mean heterozygosity of nuclear markers (Paper II) is not that much reduced, when compared to other continental vespertilionids (Petit et al. 1999, Petit and Mayer 1999, Rivers et al. 2005, Ruedi and Castella 2003, Veith et al. 2004). The present results indicate that this insular endemic species is not genetically depleted and no signs of inbreeding depression were found. Within the archipelago, there are relevant differences in genetic variation. From the 16 CR haplotypes detected in *N. azoreum*, only two were shared between the S. Miguel Island and the Central Group of islands (Paper III). S. Miguel, the largest island of the archipelago and

the closest to the mainland, showed the highest genetic variability at all markers, therefore being one of the key representatives of the Azorean bat genetic variability.

Regarding the continental Schreiber's bent-winged bat (Paper V), nearly all the maternity colonies in Portugal were studied. The present results revealed levels of CR haplotype diversity comparable to the ones in Eastern European populations of the same species (Bilgin et al. 2006). Microsatellite heterozygosity was also in the range of *Miniopterus natalensis* (Miller-Butterworth et al. 2003). In the present study, there was an unequal geographic distribution of genetic variability, with colonies located in the North of the country (Figure 1, Paper V) showing the lowest levels of variability for both types of markers. The haplotype diversity found in the colonies located in the central and southern regions of the country was nearly twice than that found on the northern colonies. The former were also the only colonies containing private alleles and unique haplotypes. Moreover, the population at Marvão (Ma, Figure 1 in Paper V) showed the highest level of haplotype diversity, concurring with the fact that is the largest known breeding colony of *M. schreibersii* (Hutson et al. 2001). Also, comparing hibernacula with nurseries, the former are genetically more diverse than the later, because they harbour individuals from several nurseries, as observed in *N. noctula* (Petit and Mayer 2000).

4.3.2. Population structure

The representation of the population structure obtained in the present studies was most important in the definition of conservation units (see section 4.4. Implications for conservation).

Concerning *N. azoreum*, its genetic variability is highly structured over the archipelago. According to results from both mitochondrial and nuclear markers, there is high genetic differentiation between the islands of the Central Group and S. Miguel, which is likely due to limited, or no gene flow, between them. When the Azorean bat was compared to its mainland ancestral at nuclear markers, the S. Miguel population was closer to the continental species than the Central Group populations (Figure 2, Paper II). On the other hand, lower levels of differentiation among islands of the Central Group were detected, possibly a consequence of recent common ancestry but also of some current genetic exchangeability among them (Paper III and IV). Overall, these results imply that the ocean constitutes a strong barrier to gene flow further than a few tens of km, but that the shorter distances among the islands of the Central Group seem to be insufficient to block gene flow.

The genetic variability of *M. schreibersii* is also significantly structured into four major subpopulations: North (Md, Fcr, Mog), East Centre (Ma), West Centre (T+Alc+S1), and South (Mo+Sc+Od+L1), at both types of markers (Figure 1, Paper V).

Presently, due to mining activities and the consequent creation of a continuous network of underground roosts throughout Portugal, it is expected the detection of a substantial level of genetic exchangeability among colonies, at least with the most variable markers like microsatellites. This was not the case, and even with these markers, the population structure of *M. schreibersii* was still very clear. This may result from a trace of an old bat distribution, where roosts were less available, and bats were totally dependent on natural caves. Indeed, the genetic sub-populations revealed by nuclear DNA match the limestone areas where the majority of potential natural roosts are concentrated. Although nowadays there are more man-made roosts available (e.g. abandoned mine tunnels), the behavioural dependence on underground roosts, originally restricted to some parts of the country, have left a mark on the present population structure.

4.3.3. Population history

The unequal distribution of genetic variability has given indications for population history inference. Using mismatch analysis, colonisation and expansion events were identified and dated for both species.

In the insular *N. azoreum*, the population from S. Miguel Island showed the highest expansion time, thus suggesting that it was the first island of the archipelago to have been colonised by bats (Paper III). These results were also corroborated by past bottleneck detection (Paper II), which indicated S. Miguel as the only population with no historical genetic signatures of population reduction. From the same previous mismatch analysis, it was deduced that the next island to be occupied in the Azores was probably Pico (Figure 1, Table 1, Paper III). However, the mark of old founder events was probably masked by current gene flow, which was detected among islands of the Central Group (Paper IV). Nevertheless, in order to accurately infer the colonisation path of the Azorean bat, samples from Santa Maria Island, the only where no bats were captured, should be added to the analysis (see section 5.2. Limitations to work).

In the Schreiber's bent-winged bat, the inferred age of demographic expansions together with the northward decline in genetic diversity are consistent with the northern colonies being the most recently occupied in the last glacial age (Paper V). This pattern of genetic variability distribution is present in other bat species, such as *Myotis myotis* (Ruedi and Castella 2003) and

Plecotus austriacus (Juste et al. 2004), which also performed a northward post-glacial colonisation in Europe. Glacial fossils of *M. schreibersii* were reported for the South of Portugal (Telles Antunes 1993), which corroborates the estimates of expansion times, thus suggesting the South of the Iberian Peninsula as a possible glacial refuge for this bat species. These findings concur with (Branco et al. 2002, Brewer et al. 2002, Michaux et al. 2003, Wallis and Arntzen 1989), who have also revealed southern Iberia as a glacial refuge for a variety of thermophilic organisms. Even so, these results do not exclude the possibility that the Schreiber's bent-winged bat disappeared from Iberia, having its glacial refuge in North Africa. The sampling of North African colonies is therefore essential to fully understand the history of postglacial colonisation of the Iberian Peninsula by *M. schreibersii*.

4.3.4. Dispersal

As shown by the previous example, population structure is directly influenced by behavioural factors like roosting preferences, which in turn affects the dispersal of species.

In both studied species, the genetic distances among populations were significantly correlated with geographic distances at both types of markers, thus suggesting a pattern of isolation by distance. However, as shown in Papers IV and V, the way of dispersal is distinct for each species.

The present results suggest that the dispersal of the Azorean bat is mainly passive, and equal in both sexes. When genetic differentiation was compared between differently inherited markers, slightly more structure was found in mitochondrial than in nuclear DNA (Paper IV). As the effective population size differs between the markers used (Birky et al. 1989), the genetic structure of both data was compared using a standardized measure of genetic differentiation, G_{ST}. The difference was within the four fold range expected in the comparison between the two markers. The observed difference in structure between nuclear and mitochondrial markers for the Azorean bat is smaller than the same comparison for other bat species, where sex-biased dispersal has been reported (e.g. Castella et al. 2001, Kerth et al. 2002, Petit and Mayer 1999, Worthington Wilmer et al. 1999, and Paper V). Also, tests for biased dispersal using only microsatellites did not show any significant difference between sexes. Moreover, the assignment tests indicated the majority of putative migrants in the Azores archipelago as females (Paper IV). On the whole, these findings suggest a greater role played by passive dispersal in the Azores archipelago, probably influenced by the strong winds blowing in the Atlantic Ocean.

A strong bias in male-dispersal is the norm in mammals (Greenwood 1980) and this pattern has been reported in many bat species (Castella et al. 2001, Kerth et al. 2002, Petit and Mayer 1999, Worthington Wilmer et al. 1999). Sex-biased dispersal usually causes discrepancies between the pattern of genetic differentiation obtained from both sexes or from differently inherited markers (Prugnolle and de Meeûs 2002). Such is the case of the Schreiber's bent-winged bat, in which it was observed a ten times higher level of structuring at the mtDNA than at nuclear DNA. This, together with the results from tests for sex-biased dispersal, supports female philopatry and male-biased dispersal (Paper V). The present findings concur with the demographic study by Palmeirim and Rodrigues (1995) that reported a great majority of females returning to the cave in which they were born to give birth.

4.4. Implications for conservation and management

In order to accomplish a value in nature conservation, the acquired genetic data information should be used to create outcomes of management actions. The results presented in this thesis have practical implications for the conservation perspective of the bat species in study. Particularly, the present findings may assist in determining fundamental units for conservation purposes, as either Evolutionary Significant Units (ESUs), or Management Units (MUs).

Moritz (1994) defined ESUs as geographically discrete sets of populations that have evolved separately for a substantial period of time. Thus, ESUs represent units reciprocally monophyletic for mtDNA and possess significantly divergent allele frequencies at nuclear loci. On the other hand, MUs are demographically independent populations, corresponding to the ecological components of the ESUs that must be monitored and managed (Moritz 1999). If population size diminishes dramatically in part of the range of a species, translocations between MUs within an ESU may be considered (Moritz 1999). MUs are more appropriate in short-term conservation situations, and so far have been attributed to several populations of many species (Friesen et al. 2005, Holycross and Douglas 2007, Michaux et al. 2004, Paquin et al. 2006), including insular ones (Friesen et al. 2006, Fumagalli et al. 1999, Kanthaswamy et al. 2006, Kretzmann et al. 2001). The present work has allowed the definition of several MUs in the studied species.

4.4.1. Insular Azorean bat (*Nyctalus azoreum*)

The present study has identified monophyletic lineages at several mtDNA markers within the Azorean bat. This has confirmed an evolutionary split from its continental ancestor that

conducted to speciation. Therefore, *N. azoreum* forms a distinct evolutionary unit integrating an irreplaceable genetic heritage, with great conservation value.

Most island populations show a great impoverishment in genetic variation, particularly in the case of endemic species (Frankham 1997). Nevertheless, no signs of dramatic reduction in genetic variation or inbreeding depression were found in the Azorean bat. Overall, the present genetic information accumulated about the Azorean bat confer a fairly positive scenario for the species, which concurs with the present high density of *N. azoreum* in most of its range, especially in the island of S. Miguel (Rainho et al. 2002). The main exceptions to this encouraging situation are the islands of Graciosa and Santa Maria, where the Azorean bat appears to be rare (IUCN 2007b, Rainho et al. 2002).

We have detected a strong genetic separation between the islands of the Central Group and S. Miguel, evidencing limited or no gene flow between them. Therefore, the Central Group and the S. Miguel Island should be considered as discrete MUs (Moritz 1994) and receive priority for conservation. If necessary in the future, translocation of animals among the islands of the Central Group could be possible, given that natural migrations take place within the group. Nevertheless, such translocations should only take place in a situation of dramatic drop of population size or local extirpation (e.g. via alterations to habitat, pollution, earthquakes, volcanic activity, diseases, etc.). In any case, the individuals to be moved should come from the closest island with a potential donor population.

In a recent assessment the Azorean bat population size was estimated at 2000-5000 individuals, and fewer than 1000 individuals on S. Miguel (IUCN 2007b). Since historical information about the Azorean bat past distribution is missing, and because there is no accurate quantitative information on its population trends, its decline has been supposed, but not confirmed (IUCN 2007b).

The major threats to *N. azoreum* are its extreme level of isolation, the fragmentation inherent to its distribution in an archipelago, and its small restricted range of distribution (less than 2000 km^2). Together, such factors make this species particularly sensitive to natural disasters and human disturbance (Queiroz et al. 2006b). The human threats with most impact are direct persecution and destruction of roost sites, to which the Azorean bat is particularly susceptible due to generalised use of man-made roosts, colonial behaviour, and diurnal flights, which make it more conspicuous and accessible to people than most other bats (IUCN 2007b). The loss and degradation of natural and semi-natural habitats, pesticide use and water pollution may also result in a decline in this endemic species (IUCN 2007b, Queiroz et al. 2006b). As a result of the combination of these factors, it has been categorized as “Critically Endangered” in the

Portuguese Red Data Book (Queiroz et al. 2006b), and "Endangered" by the IUCN (IUCN 2007b). The Azorean bat is included in Annex IV of the EU Habitats Directive, and its conservation is also required by the Bern Convention. Besides these legislation actions, no other specific conservation efforts have been implemented. Nevertheless, specific conservation measures have been suggested by IUCN (2007b) and Rainho et al. (2002), namely: the protection and monitoring of roosts, a public education program, the reduction of adverse agricultural practices, preservation and restoration of natural habitat, and the use of street lights that attract insects. Additionally, it is important to emphasise the need for regular surveys, along with long-term monitoring programs. A comprehensive study of the biology of this species, including its ecology and behaviour, is also required if its conservation is to be ensured.

4.4.2. Continental Schreiber's bent-winged bat (*Miniopterus schreibersii*)

Four major groups, with a relatively high differentiation level among them, have been identified within the Portuguese population of the Schreiber's bent-winged bat: North East, West-Centre, East-Centre (Marvão) and South (Figure 4, Paper V). In order to preserve genetic variability, these groups should be treated as distinct MUs. Such units require separate monitoring and management due to the inferred restricted gene flow and contemporary demographic independence (Moritz 1994). As mentioned for *N. azoreum*, these results are relevant in case re-introductions or translocations are carried out to recover from a regional catastrophic population reduction (e.g. due to man actions, or diseases). Because of the observed geographic structuring of the Portuguese population of the species any such translocations would have to be planned with care in order to maintain current levels of genetic diversity. Nurseries, in particular, would require special attention, since female philopatry defines distinct reproductive populations regardless of male behaviour.

In spite of its dramatic condition in central Europe, being extinct in Germany and Ukraine (IUCN 2007a), the situation of *M. schreibersii* in Portugal is considered relatively stable (Rodrigues et al. 2003). With a population size of some tens of thousand individuals, it is restricted to less than twenty colonies (Queiroz et al. 2006a). This dependence of a few roosts that harbour great percentages of the total population (e.g. Marvão) makes the Schreiber's bent-winged bat especially susceptible to disturbance and loss of underground habitats (IUCN 2007a, Queiroz et al. 2006a). Pesticide use is also considered a major threat to this species (IUCN 2007a, Queiroz et al. 2006a). Recently, in Southwestern Europe there have been mass mortality

events of this species with unknown causes (IUCN 2007a). The Schreiber's bent-winged bat is classed as "Vulnerable" in the Portuguese Red Data Book (Queiroz et al. 2006b) and "Near Threatened" by the IUCN (IUCN 2007a). Accordingly, it is protected internationally by the Bonn Convention (Eurobats), Bern Convention, and the EU Habitats Directive. In Portugal, some of its main roosts are protected as Natura 2000 sites. Unlike the Azorean bat, some conservation measures have already been implemented, such as long-term bi-annual monitoring, ringing and surveillance of nurseries and hibernacula, as well as communication and education programs concerning this bat species (ICN 2006). This work has provided many interesting results and should be carried on.

Recently, it has been stressed the need for proactive conservation based on integrative approaches, where ecological and genetic studies are combined in an effort to develop proper management plans (Reed 2007). With this in mind, the genetic insights into the present studied populations should be complemented by studies using non-neutral molecular markers and research about the levels of adaptive/ecological divergence among the proposed conservation units. As a final point, conservation policy makers and managers should incorporate considerations based on genetic information into management plans, so as to allow the preservation of viable and genetically diverse populations of both the Azorean and the Schreiber's bent-winged bats.

4.5. References

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Chapter V Conclusions

V Conclusions

5.1. Final Remarks

This thesis has given an important input to the knowledge of two European bat species (the Azorean bat and the Schreiber's bent-winged bat), with fragmented populations. Several questions were answered in respect to their phylogeography, evolution, population history and conservation. These issues were clarified with the aid of several neutral molecular markers and the major results are condensed as follows:

The phylogenetic relationships among six species of the genus *Nyctalus* were studied using three mitochondrial DNA segments with different evolutionary rates (ND1, Cyt *b* and CR) allowing for an increased knowledge about evolutionary history of this Chiropteran genus. This study has confirmed the full species status of the Chinese taxon *N. plancy*i, and has suggested distinct origins for the insular populations of *N. leisleri* from the Madeira (European origin) and the Canary Islands (North African origin).

In this phylogenetic appraisal, the main focus was on the Azorean bat, and the relationship with its continental ancestor, the Leisler's bat. Although all Cyt *b* and CR lineages of *N. azoreum* were exclusive of the Azores archipelago, mtDNA divergence between the two species was very small, when compared with other bats. This suggests a very recent speciation for the Azorean bat. In order to clarify this issue both species were compared using microsatellite markers. As no support was found of recent gene flow between Azorean bats and Leisler's bats, the demographic isolation of the two species was confirmed.

In an intra-specific approach using the same CR sequences and microsatellites, the populations of the Azorean bat showed a highly structured genetic variability, with evidences of isolation by distance. The most abundant haplotype was spread throughout the archipelago, suggesting a recent single colonisation event followed by expansion. The estimated expansion times suggest that the Azores were colonised between the end of the Pleistocene and beginning of the Holocene periods, well before the arrival of humans in the 15th century. Considering results from both kinds of markers, the Azorean bat populations are not genetically very impoverished, when compared with continental species. The genetic diversity, the level of unique haplotypes and private alleles was highest in S. Miguel, the largest of the islands, the closest to mainland, and most probably the first one to have been colonised by bats. Strong support for two major subpopulations in the Azores was found: one includes all islands of the

Central Group and the other corresponds to the island of S. Miguel. Gene flow between these subpopulations appears to be very limited, probably occurring due to passive dispersal promoted by storms in the archipelago. Against the norm in mammals, there were no strong evidences of sex-biased dispersal in the Azorean bat.

As regards the continental Schreiber's bent-winged bat, the same type of markers was used as for the previous intra-specific studies on the insular Azorean bat. Similarly, as detected with statistically significant differences in both nuclear and mtDNA, the studied populations are genetically structured into four subpopulations (North East, West Centre, South and Marvão). Genetic distances among the colonies were significantly correlated with geographical separation, thus suggesting isolation by distance.

The highest levels of genetic diversity were found in the colony of Marvão (the most numerous known nursing colony of this species in the world). Hibernacula were also genetically more variable than nursing colonies, because they harbour bats from distinct breeding populations. Moreover, southern colonies presented higher genetic variation than northern ones.

This information was used to understand some of the history of the species. Specifically, a northward post-glacial re-colonisation of *M. schreibersii* from a possible refuge in southern Iberia or northern Africa was confirmed. Also, the marked genetic structure matched the limestone areas where most of the natural caves are found, suggesting that the distribution of nursing colonies of Schreiber's bent-winged bat in the past was more restricted than today.

The population structure also helped to understand some aspects of the biology of the species, such as its dispersal and breeding behaviours. Although both types of markers suggested the same geographic structure, there was a disparity between the levels of differentiation between the markers (10 times greater for the mtDNA). This is consistent with strong female philopatry and male biased gene flow among colonies, which concurred with previous demographic studies.

Overall, this thesis stressed the high conservation value of both Azorean bat and Schreiber's bent-winged bat. This allowed the understanding of the distribution of genetic variability and the causes of the observed population genetic structure, such as historical events, isolation by distance or dispersal. By examining genetic structure in both species, it was possible to define biological meaningful conservation units and contribute with management suggestions.

5.2. Limitations of the work

In spite of all new insights to both history and conservation of the studied bat species, usually based on good samples, this study would have benefited much from access to some additional samples. Although samples from most of the distribution of the Azorean bat were obtained, the capture of bats from the island of Santa Maria was not possible (Figure 4, General Introduction). There were several attempts to capture them in two consecutive years (2001 and 2002), but these were unsuccessful mostly due to the rarity of the species on the island.

The phylogenetic analysis of the genus *Nyctalus* would benefit with the inclusion of samples from *N. montanus*, and samples from intermediate distribution areas for *N. aviator* and *N. lasiopterus.*

Finally, the phylogeographic study of the Schreiber's bent-winged bat would benefit from northern African samples, in order to fully understand this species' post-glacial colonisation pattern.

5.3. Future work

This study has deepened the knowledge about the studied bat species, as well as showed that there is a lot more to be learnt. Hopefully, this will stimulate the implementation of conservation projects as well as future research on bat ecology and behaviour.

These are the suggestions for additional research:

- To put into practice a long-term field study in order to capture and identify bats from the Western Group and from the Santa Maria Island.

- To implement a demographic, ecological and ethological study of the two species found in the Azores archipelago, focusing on their habitat preferences, breeding system, dispersal and feeding habits.

- To study all populations of the Azorean bat using non-neutral molecular markers and/or quantitative genetic approaches in order to understand the evolutionary importance of local adaptations and detect signatures of selection in this endemic species. Special attention should be paid to the Santa Maria Island, where no genetic information is yet available.

- To clarify the relationship between *N. lasiopterus* and *N. aviator*, adding samples from intermediate regions of their distribution to the previous analysis.

- To better understand the post-glacial colonisation of the Schreiber's bent-winged bat in Western Europe, by trying to obtain Northern African samples and performing a combined analysis using the present data.