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Piecing the *punicus* Puzzle

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1. Introduction

The occurrence of *Myotis* species in the Mediterranean region has been documented for a very long time. At present, 15 *Myotis* species are known to inhabit the Mediterranean region (Temple & Cuttelod, 2009). However the classification of some of these species has been continuously shifting and somewhat difficult to determine. One such species has been what is now referred to as *Myotis punicus* Felten, 1977 (Castella *et al*., 2000). Until the late 1990s *Myotis punicus* was generally thought to be an insular variant of either *Myotis myotis* or *Myotis blythii*, mostly because both these species are distributed throughout the Mediterranean region. It was considered to be either a smaller variant of *Myotis myotis* (Gulia, 1913; Ellerman & Morrison-Scott, 1966; Benda & Horácek, 1995), or a larger variant of *Myotis blythii* (Lanza, 1959; Strelkov, 1972; Felten *et al*., 1977; Bogan *et al*., 1978; Corbet, 1978). In Malta, some authors also attributed particular individuals to other species including *Myotis daubentoni* (Gulia, 1913), *Myotis capaccinii* (Gulia, 1913) and *Myotis oxygnathus* (Lanfranco, 1969). However, several authors have commented on the differences observed from individuals of *Myotis myotis* and *Myotis blythii* across the rest of their distribution range and expressed doubt as to the correct classification (Strinati, 1951; Strelkov, 1972; Felten *et al*., 1977; Gaisler, 1983; Menu & Popelard, 1987; Borg *et al*., 1990; Courtois *et al*., 1992).

The distinguishing features of *Myotis punicus* were first reported through comparative analyses of morphometric data (Benda & Horácek, 1995; Arlettaz *et al*., 1997). Cranial morphometrics in conjunction with measurements of forearm and ear length presented a distinct cluster of individuals from the Mediterranean region intermediate in size between *Myotis myotis* and *Myotis blythii*. It was also noted that this intermediate cluster lacked the white spot of hair on the forehead, which is typical of *Myotis blythii* (Arlettaz *et al*., 1997). Among the distinctive features of *Myotis punicus* are its large size (comparable to *Myotis myotis*), the plagiopatagium (wing membrane) starting at the base of the toes, a lancet shaped tragus and distinct dorsal (light brown) and ventral (white) fur coloration (Dietz & von Helversen, 2004).

2. The appropriate sampling method

However, genetic analysis was required to solve this riddle and obtaining the samples required for such analyses was the first hurdle. In order to carry out research on a protected species such as *Myotis punicus*, which is protected, together with all other European bats

under the EUROBATS Agreement (The Agreement on the Conservation of Populations of European Bats, 1994), as well as under local legislation, a sampling permit is required. Permits issued for such research limit the type of sampling that can be carried out and the amount of tissue that can be taken from each individual bat. Most of the genetic analyses carried out on *Myotis* species around the Mediterranean would not have been possible had it not been for the development of a particular non-lethal sampling technique based on skin biopsies (Worthington Wilmer & Barratt, 1996). Before the advent of this technique, the most common methods of obtaining tissue samples from bats for genetic studies had been blood samples, toe clipping (the removal of the smallest digit) or muscle biopsies (Wilkinson and Chapman, 1991) but there were a number of ethical and technical issues associated with such sampling.

The use of this biopsy punch technique for sampling wing and tail membrane was shown to yield sufficient, good quality high molecular weight DNA to carry out most Polymerase Chain Reaction (PCR) based genetic analyses. The main advantages of this sampling technique are that it is quicker and simpler than the previously mentioned sampling methods, can be easily carried out in the field and is applicable to all chiropteran species regardless of size (Worthington Wilmer & Barratt, 1996). Using this method, tissue biopsies are taken from the wing membrane (plagiopatagium) or the tail membrane (uropatagium) using a sterile punch (Stiefel Laboratories) of a diameter that ranges from 2mm to 8mm, the size used being determined by the size and wing area of the bat species being studied. A 3mm punch was reported to yield approximately 15µg of genomic DNA (Worthington Wilmer & Barratt, 1996). This sampling method was originally tested on the species *Pipistrellus pipistrellus* (Barrett *et al.*, 1995) and *Macroderma gigas* (Worthington Wilmer *et al.*, 1994) because they cover most of the size range of michrochiropterans, weighing 5g and 150g respectively. In addition, megachiropteran species were also sampled using this technique (Worthington Wilmer & Barratt, 1996).

This technique was deemed to be safe through follow-up of the sampled bats. The holes in the wing or tail membranes resulting from such biopsies were observed to heal within four weeks in most species (Worthington Wilmer & Barratt, 1996). The presence of tears in bat wings which do not impair the flight capacity of the individual have been frequently observed in the wild, sometimes even as a result of copulation. However in order to be completely safe for the bat, particular attention must be made to select a region of the wing or tail membrane that contains few or no visible blood vessels so that bleeding does not occur and infection is avoided, resulting in faster healing.

3. Piecing the puzzle

With this sampling technique available and proven to be the safest and most effective method available for obtaining genomic DNA from bats, the search into the genetic structure of the Mediteranean *Myotis* species could progress a lot faster. In fact, *Myotis punicus* was proposed as warranting its separate classification at the beginning of the decade on account of studies based on the genetic analyses of cytochrome b (a mitochondrial respiratory gene) and microsatellites (Castella *et al.*, 2000). In this study the authors set out to test the effect of the Strait of Gibraltar as a geographical barrier to gene flow in colonies of *Myotis myotis* between Spain and Morocco. A section of the cytochrome b gene and six microsatellite loci were used in conjunction because, being of mitochondrial and nuclear

origin respectively, they provided information regarding the proportion of males and female migrants contributing to the gene pool of a population and shed light onto the phylogenetics of the populations across the Strait of Gibraltar.

The cytochrome b gene is part of the mitochondrial DNA (mtDNA), which means that it is inherited maternally (Avise, 1994) and as such can provide information about the inter-population movements pertinent solely to the females. On the other hand, microsatellites are nuclear markers, which means that they are inherited biparentally (Tautz & Renz, 1984) and thus can be used to follow the movements of both males and females. When comparing microsatellites between two populations, the exchange of mating individuals of just one sex, be it males or females, would be sufficient to homogenise both populations, even if no individuals of the other sex ever leave their native population.

The mtDNA variation observed across the Strait of Gibraltar showed a very weak differentiation between populations on the same side of the Strait because all the mtDNA haplotypes recorded within the Spainish or Moroccan populations were identical or very similar to each other. Concomitantly, almost all the sequence variation present (i.e. 54-59 observed base substitutions over the 600 base pairs of the cytochrome b gene sequenced) was observed when comparing populations across the Strait presenting two groups which are endemic to either side of the Strait of Gibraltar. This dichotomy suggested that this region was inhabited by two genetically distinct groups that have been reproductively isolated for millions of years (Castella *et al*., 2000). An interesting find was that some cytochrome b haplotypes were only found in one colony, suggesting that females may be more philopatric than males to their natal colonies. In fact, a similar bias of sex dispersal was also proposed as a result of mitochondrial Hypervariable Region I (a control region located within the D-loop of mitochondria) studies carried out on a population of *Myotis myotis* in Germany (Petri *et al*., 1997). This means that both *Myotis myotis* and *Myotis punicus* are known to exhibit this behaviour.

The findings of the microsatellite analysis supported those from the mtDNA with microsatellite variability being high and evenly distributed among populations from the side of the Strait indicating that colonies from the same side of the Strait were only weakly differentiated from each other. This suggested that there was high nuclear gene flow taking place between the colonies within either region over considerable geographical distances, with a range covering at least 770km, which lead to very weak genetic differentiation between such populations. In contrast, a strong genetic differentiation was apparent across the Strait of Gibraltar. Three of the six microsatellites analysed presented almost no overlap between alleles across the Strait, while the other three microsatellite loci analysed had a more overlapping allelic distribution across the Strait (Castella *et al*., 2000). The significance of this result to future diagnostic tests was that *Myotis myotis* and *Myotis punicus* could be distinguished using their three unique alleles as well as comparing the allele frequencies for the other three shared loci. Furthermore, the analysis of the same six microsatellite loci in *Myotis blythii* from various locations in Europe and Asia showed that *Myotis blythii* appears to be more closely related to the Spanish populations of *Myotis myotis* than to the Moroccan populations of *Myotis punicus* making it easier to eliminate the possible mix-up caused when using only morphometric comparisons.

Allozyme analysis had been originally used to uncover distinct allelic frequencies for *Myotis* populations from the Mediterranean region (Arlettaz *et al*., 1997) giving a clear indication

that the differences observed from mainland European populations of *Myotis myotis* and *Myotis blythii* where not simply phenotypic variations. Allozymes are allelic variants of enzymes encoded by structural genes. A total of 35 allozyme loci were essayed in these analyses but only 11 of these showed any variability within the three *Myotis* species, having in general either two or three alleles. Some allozymes can be diagnostic as in the case of the ADA and GOT-1 loci, which are fixed for alternative alleles for *Myotis myotis* and *Myotis blythii* in Europe and Asia (Arlettaz, 1995; Arlettaz *et al*., 1997b). The phylogenetic analysis of these three *Myotis* species in the Mediterranean region using allozymes suggested a closer phylogenetic relationship of *Myotis myotis* with *Myotis punicus* than with *Myotis blythii* although the association was not very strong. These contrast substantially with the phylogenetic results obtained from the combined use of cytochrome b and the six microsatellite loci in which *Myotis myotis* is more closely related to *Myotis blythii* than *Myotis punicus* by a very strong association (Castella *et al*., 2000).

Additionally, the gathered data was used to understand the process by which *Myotis punicus* established itself and spread in the Mediterranean region. For cytochrome b the authors applied a divergence rate in mammals of 2% per million years (Johns & Avise, 1998) and based on the difference observed between *Myotis myotis* and *Myotis punicus*, which was about 11%, determined that the divergence between these two species must date back to the Pliocene epoch. This means that these species have diverged from a common ancestor around that time and have remained isolated ever since, colonising and spreading along the two sides of the Mediterranean up to their meeting at the Strait of Gibraltar. This hypothesis is supported by the fossil record, given that fossils of typical *Myotis myotis* are known at least since the Pleistocene in Spain (Sevilla, 1989) and the Maltese Islands have been inhabited by *Myotis* species at least since the late Quaternary (Felten *et al*., 1977), as shown by the fossil records from Ghar Dalam (Storch, 1974). This coincides with the existence of the last land bridge between Europe and North Africa, which was during the last Messinian crisis of 5.5 million years ago, when the greater part of the present-day Mediterranean Sea dried up. Thus dispersal across the Strait of Gibraltar must have been severely limited since the Pliocene. This hypothesis was strengthened when another study of African *Myotis* species showed that the divergence between *Myotis punicus* from *Myotis myotis* and *Myotis blythii* can be traced back to the Pliocene (Stadelmann *et al*., 2004).

Taking into consideration the long distances *Myotis* species are capable of covering over relatively short periods of time, such as has been shown in *Myotis myotis* females, which are known to cover up to 25km daily between their nursery roosts and feeding grounds (Arlettaz, 1996; Arlettaz, 1999) and annual distances of several hundreds of kilometres between summer and winter roosts (Horácek, 1985; Paz *et al*., 1986), these species have had ample time to exchange mating individuals between Europe and North Africa especially considering that they have been able to successfully colonise all the major islands of the Mediterranean Sea which could act as stepping stones between the two continents. They have even managed to colonise Mallorca, which is about 200km away from Spain and yet the haplotypes on this island are identical or very similar to those of Spanish populations (Castella *et al*., 2000).

Both the temporal factor of over 5.5 million years since establishment and the physical ability of *Myotis* species to cover vast distances over both land and sea argue against the hypothesis that 14km of open sea separating Europe from North Africa could have been

sufficient as a lone factor to prevent gene flow. Two questions that still remain unanswered however are whether *Myotis myotis* and *Myotis punicus* ever exchange migrants across the Strait of Gibraltar and which routes have been used by these bats to colonise Europe and North Africa. Another more plausible explanation proposed was that of competitive exclusion between *Myotis myotis* and *Myotis punicus* since the niche occupied by *Myotis punicus* in North Africa is very similar to that occupied by *Myotis myotis* in Europe in that both have a diet based on ground-dwelling arthropods such as carabid beetles, ground crickets, scorpions, etc.) (Arlettaz *et al.*, 1997a; Arlettaz, 1999). This does not however explain why *Myotis punicus* is not sympatric with *Myotis blythii* since the latter exploits a completely different niche throughout its distribution range, with a diet that is based principally on grass-dwelling prey such as bush crickets (Arlettaz *et al.*, 1997a; Arlettaz, 1999). Thus, for the moment, the justification for the current distribution of these three sibling species remains open to debate with the historical processes of colonisation and competitive exclusion being the strongest contendants. The only certainty is that to maintain such high levels of genetic differentiation between the populations of the sibling species *Myotis myotis* and *Myotis punicus*, a strong, persistent and ancient barrier preventing gene flow has to be present (Castella *et al.*, 2000).

Over the past ten years the above knowledge about the genetics of *Myotis punicus* has been used to further expand on these analyses and confirm its segregation from *Myotis myotis* and *Myotis blythii* as well as confirm the range of its distribution, which covers the greater part of the Maghreb region from Morocco, through Algeria and Tunisia, up to Tripolitania in north-west Libya and northwards to the European islands of Malta, Corsica and Sardinia (Castella *et al.*, 2000; Mucedda & Nuvoli, 2000; Topál & Ruedi, 2001; Beuneux, 2004; Baron and Vella, 2010; Biollaz *et al.*, 2010).

On the Maltese Islands, *Myotis punicus* has a unique ecological niche because it is the only *Myotis* species and currently their largest resident bat species (Borg, 1998). The Maltese archipelago consists of seven islands covering an area of 316 square kilometres of which only the largest three islands, Malta (245 km^2), Gozo (67 km^2) and Comino (2.8 km^2), are inhabited. The deep karstic caves and extensive garigue spread throughout the archipelago provided the ideal habitat combination for the colonisation of *Myotis punicus*. However, in depth studies to better understand this species in Malta were fuelled by the realisation that incessant human disturbance as a result of urbanisation was leading to dwindling population numbers (Borg, 1998; Baron, 2007; Baron & Vella, 2010).

An allozyme study of the Maltese *Myotis punicus* population was undertaken to compliment data available for *Myotis myotis* and *Myotis blythii* (Ruedi *et al.*, 1990; Arlettaz *et al.* 1997). Using the novel combination of cellulose acetate allozyme electrophoresis with a non-lethal sampling technique (wing biopsy punches), enzyme biochemistry was used to shed light on the allele frequencies at six loci. This study showed that Nei's (1978) Genetic Distance (D) ranged from 0 to 0.047 indicating that the population on the Maltese Islands is a single panmictic unit with an tendency towards becoming isolated mating systems (overall F_{ST} = 0.272) across the territory due to inbreeding as a result of diminishing population numbers. Another interesting outcome of this study was the identification of gene duplication in Glucose Phosphate Isomerase (GPI - 5.3.1.9), which was never reported in *Myotis myotis* and *Myotis blythii* making it a unique species identifier for *Myotis punicus* within this three species complex (Baron & Vella, 2010).

Subsequently the morphometric data collected during the sampling sessions across the Maltese Islands for the allozyme study were amalgamated with those of the previous 20 years to explore the premise of niche expansion in *Myotis punicus* following the extinction of *Rhinolophus ferrumequinum* and shed light onto whether the increase in human disturbance has restricted or promoted variation within the Maltese population. Although the statistics carried out on external characters such as ear length and forearm length showed significant broadening in the value ranges of body size, it was proposed that other more immutable features such as cranial and dentition measurements should be included into such statistical considerations (Baron & Borg, 2011).

Concurrently other researchers were looking in detail at the cranial morphometrics of *Myotis punicus* samples from across the distribution range in greater detail (Evin *et al.*, 2008) and these strengthened the mitochondrial data for *Myotis punicus* (Castella *et al.*, 2000). Using 19 lateral and 29 ventral curvatures and tips present on the skull of *Myotis punicus*, which were mapped as three dimensional co-ordinates, it was possible to obtain a means of identifying *Myotis punicus* from *Myotis myotis* and *Myotis blythii* solely by cranial measurements. The results of this study revealed that the skull shape of *Myotis punicus* completely differs from that of any other *Myotis* in Europe and North Africa (Evin *et al.*, 2008). Apart from that, it was observed that there were morphological differences in the skull shape and size of *Myotis punicus* populations inhabiting the Mediterranean Islands compared to those inhabiting North Africa. This was interpreted as being in accordance with the genetic data available (Castella *et al.*, 2000) which had already indicated the presence of two distinct evolutionary lineages within *Myotis punicus*. The suggested reason for these morphological differences was a strong enough restriction of gene flow between the *Myotis punicus* populations of North Africa and those on the Mediterranean Islands to bring about morphological segregation (also known as demographic independence) (Evin *et al.*, 2008).

However, genetic isolation on its own is not a valid reason for the observed cranial differences. Each phenotypic change is generally driven by a selective pressure presented by the different environments inhabited by the two populations. In bats, diet is known to be an important selective factor acting upon the evolution of cranial morphology (Freeman, 1979; Reduker, 1983; Van Cakenberghe, Herrel & Aguirre, 2002; Aguirre et al., 2003; Dumont & Herrel, 2003). The differences in cranium, teeth and the associated muscles presented by different species are only in part due to the different prey types forming part of a species' diet (Reduker, 1983). Thus when two species have a similar diet it is expected that the cranial morphologies would be similar. This was shown to be the case in *Myotis myotis* which presents greater morphological similarities to *Myotis punicus* than to *Myotis blythii*, which could be the result of morphological convergence due to their similarity in feeding habits (Evin *et al.*, 2008), even though the genetic data had shown *Myotis myotis* to be more closely related to *Myotis blythii*.

Once it was determined that the insular populations of *Myotis punicus* were distinct from those of North Africa, the question arose as to how different the populations on the separate islands were from each other. The cytochrome b gene was sequenced for individuals from roosts across the Maltese Islands in an attempt to isolate SNPs unique to the Maltese population of *Myotis punicus*. Through PCR of the Second Hypervariable Domain (HVII) of the mitochondrial D-loop followed by sequencing, it was determined that only a single haplotype is present on the Maltese Islands (Baron, unpublished). It was recently possible to

compare this data with haplotypes isolated from all over the Mediterranean Basin (Biollaz *et al.*, 2010). Interestingly the closest haplotype was found in Tunisia showing that Malta could have been used as a route to the other Mediterranean islands.

In conjunction with the HVII amplification and sequencing, 13 microsatellite loci previously described for *Myotis myotis* (Castella & Ruedi, 2000) were analysed for the Maltese population of *Myotis punicus*. It was thus possible to obtain a reliable data set for a representative number of individuals from across Malta. However, until recently, only limited microsatellite data for *Myotis punicus* was available (Castella *et al*., 2000). With the availability of microsatellite data from the other Mediterranean Islands to compare with (Biollaz *et al.*, 2010), the microsatellite data collected in Malta could be put to more rigorous evaluation. A permit application has recently been approved to expand this study to sample individuals from as many roosts as possible and obtain a clearer and more complete picture of the variability throughout the Maltese archipelago in an attempt to answer questions related to allele frequency distribution and possible inferences of roost movements.

The mitochondrial and microsatellite haplotypes from the Maltese Islands would not have any meaning had it not been for the detailed work carried out across the Mediterranean region by Biollaz *et al.* (2010). In this study the authors set out to determine the population genetic structure of *Myotis punicus* and current patterns of gene flow between the islands of Corsica and Sardinia and their relationships with North African populations. A combination of mitochondrial and nuclear markers was used to compare levels of gene flow within and between Corsica, Sardinia and North Africa by estimating the contributions of both sexes to the migrant gene pool. Due to the different evolution rates of the selected markers (Chesser & Baker, 1996), it was possible to investigate both recent demographic processes and more remote events in the population history of *Myotis punicus* (Bertorelle & Barbujani, 1995). Based on the proximity between the islands of Corsica and Sardinia and their distance from North Africa, it was expected that a higher genetic differentiation would be present between the populations of North Africa and the two islands than between the insular populations of Corsica and Sardinia.

The theoretical basis of this study is that colonisation of adjacent islands by bats depends in part on the ecological attributes such as dispersal and colonisation abilities of the species and due to this, bat populations on such islands would probably have similar phylogeographical histories as a result of identical colonisation strategies and most probably similar insular geomorphological factors (Trujillo *et al.*, 2002; Pestano *et al.*, 2003; Juste *et al.*, 2004; Salgueiro *et al.*, 2007). However, since the geological history of a region influences the ecology and pattern of diversification of the species, it is the combination of ecological and historical factors of a particular species on a specific island that generates the intraspecific genetic diversity observed between populations on neighbouring islands (Heaney *et al.*, 2005; Roberts, 2006; Heaney, 2007).

The islands of Corsica and Sardinia offer a very interesting view into the dispersal of *Myotis punicus* because they have common geological (Meulenkamp & Sissingh, 2003) and faunal assemblage histories (Vigne, 1992; Ferrandini & Salotti, 1995; van der Made, 1999; Marra, 2005; Sondaar & Van der Geer, 2005). In addition to this, after the Messinian salinity crisis, which occurred 5.5 million years ago (Krijgsman *et al.*, 1999), Corsica and Sardinia were isolated from the mainland by Pliocene flooding (van der Made et al., 2006), which gave rise to endemic species (Carranza & Amat, 2005) but then during the Pleistocene glaciations, the

lowering in sea level provided periods of intermittent contact during which faunal exchanges could have possibly occurred (Lanza, 1972; Lanza, 1983; Caloi *et al.*, 1986; van Andel & Tzedakis, 1996).

The mitochondrial analysis of part of the HVII of the mitochondrial D-loop was carried out using primers previously tested on *Myotis myotis* (Fumagalli *et al.*, 1996; Castella *et al.*, 2001). Sequencing of the HVII region revealed 26 different haplotypes (3 haplotypes in Corsica, 13 in Sardinia, 3 in Morocco and 7 in Tunisia). The sequenced region contained a total of 38 variables sites, of which 31 were present more than once. The haplotypes segregated into three main groups - corresponding to the combined samples from the islands of Corsica and Sardinia, the samples from the region around Tunisia and the samples from across Morocco. About 15 mutations separated Corsica and Sardinia from Tunisia and Morocco and the latter two between themselves. Interestingly, no haplotypes were shared between the islands since the insular populations were separated by at least one mutation. The results also suggested that the Corsican haplotypes are derived from the most represented Sardinian haplotype, which was found in almost half the sampled Sardinian individuals. This means that the population inhabiting Corsica most probably crossed over from Sardinia. Similarly in Morocco, the great majority of samples were represented by a single haplotype. Thus overall, haplotype diversity and nucleotide diversity were lower in the populations of Morocco and Corsica than in those of Tunisia and Sardinia (Biollaz *et al.*, 2010). The mitochondrial data was also used to estimate the time of divergence of the insular populations. These analyses indicated that the Sardinian population separated from the common ancestor population in North Africa during the early Pleistocene while the Corsican populations diverged much later, during the mid-Pleistocene. These results support the hypothesis that the colonisation of the Mediterranean islands by *Myotis punicus* occurred in a stepping-stone manner.

The microsatellite analyses involving the use of seven loci were amplified and analysed using primers originally designed for *Myotis myotis* (Castella & Ruedi, 2000). The microsatellite results confirmed the segregation obtained through the mitochondrial analysis, with no differentiation being observed for all seven microsatellite loci between the insular populations or between the populations of North Africa. On the other hand, there were no shared haplotypes between the populations of North Africa, Sardinia and Corsica (Biollaz et al., 2010).

The data from these two sets of molecular markers was used to understand the exchange of individual between the islands of Corsica and Sardinia. The authors focused solely on the exchange of individuals between the islands because of the geographical distances involved. While Sardinia is separated from North Africa by 200km of open water, the islands of Corsica and Sardinia are separated by the Strait of Bonifacio, which is just 11km. Mitochondrial and nuclear analyses both suggested that male and female *Myotis punicus* moved freely within Corsica and Sardinia and thus appeared to be strong dispersers compared with the populations in North Africa. The authors suggest that the discrepancy between the populations of North Africa and those on Corsica and Sardinia could be due to a non-equilibrium situation on the islands with contemporary gene flow being masked by the fact that these populations are expanding or recently established from a common source population (Whitlock, 1992). On the other hand, despite the apparent high dispersal ability, dispersal between Corsica and Sardinia is virtually non-existent. Open water seems to

represent an almost unsurpassable barrier that drastically hampers gene flow between Corsica, Sardinia and North Africa irrespective of the distance. As a result of this, *Myotis punicus* populations inhabiting Corsica and Sardinia appear to be completely isolated (Biollaz *et al.*, 2010).

The hypothesis that Corsica might have been colonised from Sardinia and the strong bottleneck resulting from such a colonisation event could explain the lower mitochondrial diversity observed in the *Myotis punicus* population of Corsica. Small insular populations, due to the limited carrying capacity of such islands, tend to be more susceptible to extinction and drift and as a result show less variability than on larger islands which can support a more extensive genetic variability (Johnson *et al.*, 2000). Corsica is smaller than Sardinia and most distant from the North African source population. Moreover, caves are a rare habitat which can be found exclusively in the north of the island (Courtois *et al.*, 1997), while Sardinia is larger and more karstic, with more potentially suitable caves and foraging habitats. Also, while the population of *Myotis punicus* in Corsica is currently estimated at around 3000 individuals with four nursery colonies (Beuneux, 2004), that in Sardinia consists of 19 large nursery colonies (Mucedda *et al.*, 1999). Therefore, the smaller population size of Corsica contains a lower genetic diversity, especially since there is no immigration from Sardinia. Compared with the pooled population of North Africa, Corsica and Sardinia harbour significantly lower allelic richness as well as observed and expected heterozygosities (Biollaz *et al.*, 2010). The authors suggest that such genetic features could reflect recent population crashes or a bottleneck during the colonisation of these islands, reducing the effective population size (Frankham, 1997; Knopp *et al.*, 2007).

The reasoning behind the colonisation of Corsica from Sardinia is based on the availability of land bridges during subsequent Pleistocene glaciations which brought about the lowering of sea level and the exposure of previously submerged land (Rohling *et al.*, 1998). The geographical distances between Mediterranean islands and the surrounding mainland were thus reduced and with the emergence of land bridges between some islands, it became easier for species to explore and colonise new territories and one of the species that took advantage of this situation was *Myotis punicus*. The population spread out slowly from North Africa and extended all the way up to Corsica in stages. Once the glaciation periods ended and the water levels rose again the colonising populations were isolated and this would explain the strong reduction of gene flow observed in both mitochondrial and genomic markers.

Interestingly, despite the presence of no water barriers in North Africa, a strong mitochondrial differentiation was revealed between the nursery colonies of *Myotis punicus* in Tunisia and Morocco. This contrasts strongly with the phylogeographical pattern observed in European *Myotis myotis*, which present a main haplotype spanning from the south of Spain to Poland and Greece. The pattern of mitochondrial haplotype uniformity across Europe in *Myotis myotis* was explained by a post-glacial recolonisation from a single Spanish glacial refugium (Ruedi & Castella, 2003). The huge divergence between the populations of Tunisia and Morocco suggests that these two populations have in some way been isolated since the Pleistocene. In fact, despite the current nuclear gene flow (which is due to male-biased dispersal), no female exchange seems to have occurred since then. Thus it was proposed that the low haplotype diversity due to isolation could have been enhanced by a combination of the populations in Morocco being confined to the High Atlas Mountains

and the philopatric behaviour of *Myotis punicus* females (Biollaz *et al.*, 2010). This is not an isolated case of divergence in North Africa. Similar divergence between eastern and western lineages in North Africa have been previously documented in species such as in white-toothed shrews, *Crocidura russula* (Brändli *et al.*, 2005), in tree frogs, *Hyla* spp. (Stöck *et al.*, 2008), and in spur-thighed tortoises, *Testudo graeca* (Fritz *et al.*, 2009). This demonstrates that a strong barrier, possibly driven by climatic fluctuations during the Pleistocene, has affected the distribution of a number of species lineages in this region (Biollaz *et al.*, 2010).

A by-product of the research into the *Myotis punicus* population of the Maltese Islands was the setting up of a technique for the preparation of *Myotis punicus* cell lines. The testing of three mitochondrial regions and thirteen microsatellites for each bat sampled required more DNA than was being collected per individual as stipulated by the legal permit for protected species issued for the project, especially in the cases where sequencing did not give conclusive results and the sample had to be retested for one or more loci. To supplement the need for more DNA the two options available were either to bulk up the DNA extracted from each biopsy punch using whole genome amplification or else increase the amount of cellular material used for the DNA extraction. The latter was opted for and a cell culture project was set up in which transient cell lines were created for as many individuals as possible. The success rate of this culture effort was 37% due to a number of limiting factors. The prime difficulty was antibiotic resistant fungal infections that had survived the short wash step in 70% ethanol and that had transferred into the culture medium from the wing membrane. The second most common setback was that samples did not present a large enough seeding surface and died before enough cells had grown out, onto the plastic surface, to be able to sustain a cell population. In addition to growing primary cultures of fibroblasts several attempts were made to obtain an immortalised (permanent) *Myotis punicus* cell line. The difficulty in transfecting and immortalising primary cells is well known and although the transfection and selection processes were successful, no immortalised cell line has as yet been achieved. The benefit of having available such cell cultures greatly outweighs the effort put into the set up, optimisation and maintenance required and the use of this technique for the production of transient cultures in vitro can be applied to any line of chiropteran genetic conservation research (Baron, in preparation).

4. Conclusion

Thus, over the past eleven years, the resident *Myotis* species of the Maltese Islands has gone from being considered a small, unimportant population of either *Myotis myotis* or *Myotis blythii*, about which very little was known, to a key population in the understanding of how a species unique to the Mediteranean has spread from North Africa towards the European islands by a stepping-stone mechanism through allozyme, mitochondrial and microsatellite analyses and has served as a driving force in the development of a cell culture technique for chiropteran conservation genetics.

In the end, every research question answered adds another piece to this puzzle but there are dozens of questions still unanswered regarding the *Myotis punicus* population on the Maltese Islands such as: Is there an exchange of individuals with other populations of the Mediterranean region? If yes, where from and where to? how often? and what is the driving force for these migrations? If not, is the aquatic barrier the only factor limiting this exchange? Are there any unique genetic markers to this insular population of *Myotis*

punicus? If inbreeding becomes a critical issue, would it be possible to bring in individuals to boost numbers? and which would be the best population to bring them from?

As more advanced laboratory techniques become available, more questions will be answered, adding even more pieces to this puzzle and other questions as yet unasked might eventually find themselves forming part of this ever-growing puzzle for future scientists to solve.

5. References

Aguirre, L.F., Herrel, A., van Damme, R., & Matthysen, E. (2003). The implications of food hardness for diet in bats. *Functional Ecology* 17: 201–212.

Arlettaz, R. (1995). Ecology of the Sibling Mouse-Eared Bats (*Myotis myotis* and *Myotis blythii*): Zoogeography, Niche, Competition, and Foraging. Horus Publishers, Martigny, Switzerland.

Arlettaz, R. (1996). Feeding behaviour and foraging strategy of free-living Mouse-eared bats, *Myotis myotis* and *Myotis blythii*. *Animal Behaviour*, 51: 1-11.

Arlettaz, R. (1999). Habitat selection as a major resource partitioning mechanism between the two sympatric sibling bat species *Myotis myotis* and *Myotis blythii*. *Journal of Animal Ecology*, 68: 460-471.

Arlettaz, R., Perrin, N., & Hausser, J. (1997a). Trophic resource partitioning and competition between the two sibling bat species *Myotis myotis* and *Myotis blythii*. *Journal of Animal Ecology*, 66: 897-911.

Arlettaz, R., Ruedi, M., Ibañez, C., Pameirim, J., & Hausser, J. (1997b). A new perspective on the zoogeography of the sibling mouse-eared bat species *Myotis myotis* and *Myotis blythii* : morphological, genetical and ecological evidence. *J. Zool., Lond.* 242: 45-62.

Avise, J.C. (1994). Molecular markers, natural history and evolution. Chapman & Hall, New York.

Baron, B. (2007). A look at the Chiropteran Fauna of the Maltese Islands: Towards an effective Action Plan for their conservation. *Xjenza* 12 (2007): 1-9.

Baron, B., & Borg, J.J. (2011). Evidence of niche expansion in the *Myotispunicus*(*Mammalia Chiroptera*) of the Maltese Islands. *Naturalista sicil.*, S. IV, 35 (2): 3-13.

Baron, B., & Vella, A. (2010). A preliminary analysis of the population genetics of *Myotis punicus* in the Maltese Islands. *Hystrix It. J. Mamm.* (n.s.) 21(1) (2010): 65-72.

Barrett, E.M., Bruford, M.W., Burland, T.M., Jones, G., Racey, P.A., & Wayne, R.K. (1995). Characterization of mitochondrial DNA variability within the michrochiropteran genus *Pipistrellus*: Approaches and applications. *Symposium of the Zoological Society of London*, 67: 377-386.

Barrett, E.M., Deaville, R., Burland, T.M., Bruford, M.W., Jones, G., Racey, P.A., & Wayne, R.K. (1997). DNA answers the call of pipistrelle bat species. *Nature*, 387: 138-139.

Benda, P., & Horácek, I. (1995). Biometrics of *Myotis myotis* and *Myotis blythi*. *Myotis* 32-33: 45-55.

Bertorelle, G. & Barbujani, G. (1995) Analysis of DNA diversity by spatial autocorrelation. *Genetics*, 140: 811–819.

Beuneux, G. (2004). Morphometrics and ecology of *Myotis* cf. *punicus* (Chiroptera, Vespertilionidae) in Corsica. *Mammalia* 68 (4): 269-273.

Biollaz, F., Bruyndonckx, N., Beuneux, G., Mucedda, M., Goudet, J., & Christe, P. (2010). Genetic isolation of insular populations of the Maghrebian bat, *Myotis punicus*, in the Mediterranean Basin. *Journal of Biogeography*, Volume 37, Issue 8, 1557-1569.

Bogan, M.A., Setzer, H.W., Findley, J.S., & Wilson, D.E. (1978). Phenetics of *Myotis blythii* in Morocco. In: Proceedings of the Fourth International Bat Research Conference, Nairobi, pp. 217-230.

Borg, J., Fiore, M., Violani, C., & Zava, B. (1990). Observations on the Chiropterofauna of Gozo, Maltese Islands. *Boll. Mus. Reg. Nat. Torino* 8: 501-515.

Borg, J.J. (1998). The Lesser Mouse-eared Bat *Myotis blythii punicus* Felten, 1977 in Malta. Notes on status, morphometrics, movements, and diet (Chiroptera, Vespertilionidae). *Naturalista Siciliano* 22 (3-4): 365-374.

Brändli, L., Handley, L.J.L., Vogel, P., & Perrin, N. (2005). Evolutionary history of the greater white-toothed shrew (Crocidura russula) inferred from analysis of mtDNA, Y, and X chromosome markers. *Molecular Phylogenetics and Evolution*, 37: 832–844.

Caloi, L.T., Kotsakis, M., & Palombo, R. (1986). La fauna vertebrati terrestri del Pleistocene delle isole del Mediterraneo. *Geologica Romana*, 25: 235–256.

Carranza, S., & Amat, F. (2005). Taxonomy, biogeography and evolution of Euproctus (Amphibia: Salamandridae), with the resurrection of the genus Calotriton and the description of a new endemic species from the Iberian Peninsula. *Zoological Journal of the Linnean Society*, 145: 555–582.

Castella, V., & Ruedi, M. (2000). Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). *Molecular Ecology*, 9, 993-1011.

Castella, V., & Ruedi, M. (2000). Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). *Molecular Ecology*, 9: 1000– 1002.

Castella, V., Ruedi, M., & Excoffier, L. (2001). Contrasted patterns of mitochondrial and nuclear structure among nursery colonies of the bat *Myotis myotis*. *Journal of Evolutionary Biology*, 14: 708–720.

Castella, V., Ruedi, M., Excoffier, L., Ibanez, C., Arlettez, R., & Hausser, J. (2000). Is the Gibraltar Strait a barrier to gene flow for the bat *Myotis myotis* (Chiroptera: Vespertilionidae). *Molecular Ecology*, 9, 1761-1772.

Chesser, R.K. & Baker, R.J. (1996) Effective sizes and dynamics of uniparentally and diparentally inherited genes. *Genetics*, 144: 1225–1235.

Corbet, G.B. (1978). The mammals of the Palaearctic Region: a taxonomic review. Cornell University Press, London.

Courtois, J.Y., Faggio, G., & Salotti, M. (1992). Chiroptères de Corse. Actualisation des cartes de repartition et revision du statut des espèces troglophiles. Biguglia: Corsica Stampa.

Courtois, J.Y., Mucedda, M., Salotti, M., & Casale, A. (1997). Deux îles, deux peuplements: comparaison des populations de Chiropte`res troglophiles de Corse et de Sardaigne. *Arvicola*, 9: 15–18.

Dietz, C., & von Helversen, O. (2004). Illustrated identification key to the bats of Europe. Electronic Publication. Version 1.0. released 15.12.2004. Tuebingen & Erlangen (Germany).

Dumont, E.R., & Herrel, A. (2003). The effects of gape angle and bite point on bite force in bats. *The Journal of Experimental Biology* 206: 2117–2123.

Ellerman, J.R., & Morrison-Scott, T.C.S. (1966). Checklist of Palaearctic and Indian Mammals, 1758-1946. Alden Press, Oxford.

Evin, A., Baylac, M., Ruedi, M., Mucedda, M., & Pons, J.M. (2008). Taxonomy, skull diversity and evolution in a species complex of *Myotis* (Chiroptera: Vespertilionidae): a geometric morphometric appraisal. *Biological Journal of the Linnean Society*, 95: 529–538.

Felten, H., Spitzenberger, F., & Storch, G. (1977). Zur Kleinsäugerfauna West-Anatoliens. Teil IIIa. *Senckenberg. Biol*. 58: 1-44.

Ferrandini, J., & Salotti, M. (1995). Discovery of considerable upper Pleistocene and Holocene fossil fillings in the karst of Oletta region (Corsica). *Geobios*, 28: 117–124.

Frankham, R. (1997). Do island populations have less genetic variation than mainland populations? *Heredity*, 78: 311–327.

Freeman, P. (1979). Specialized insectivory: beetle-eating and moth-eating molossid bats. *Journal of Mammalogy* 60: 467– 479.

Fritz, U., Harris, D.J., Fahd, S., Rouag, R., Martínez, E.G., Casalduero, A.G., Široký, P., Kalboussi, M., Jdeidi, T.B., & Hundsdörfer, A.K. (2009). Mitochondrial phylogeography of Testudo graeca in the Western Mediterranean: old complex divergence in North Africa and recent arrival in Europe. *Amphibia–Reptilia*, 30: 63–80.

Fumagalli, L., Taberlet, P., Favre, L., & Hausser, J. (1996). Origin and evolution of homologous repeated sequences in the mitochondrial DNA control region of shrews. *Molecular Biology and Evolution*, 13: 31–46.

Gaisler, J. (1983). Nouvelles données sur les Chiropteres du nord algérien. Mammalia 47: 359-369.

Gulia, G. (1913). Uno Sguardo alla Zoologia delle Isole Maltesi. IX International Congress of Zoology, Monaco, March 1913, Pages: 545-555.

Heaney, L.R. (2007). Is a new paradigm emerging for oceanic island biogeography? *Journal of Biogeography*, 34: 753–757.

Heaney, L.R., Walsh, J.S., & Peterson, A.T. (2005). The roles of geological history and colonization abilities in genetic differentiation between mammalian populations in the Philippine archipelago. *Journal of Biogeography*, 32: 229–247.

Horácek, I. (1985). Population ecology of *Myotis myotis* in Central Bohemia (Mammalia: Chiroptera). Acta Universitas Carolinae – Biologica, 8: 161-267.

Johns, G.C., & Avise, J.C. (1998). A comparative summary of genetic distances in the vertebrates from the mitochondrial Cytochrome b gene. *Molecular Biology and Evolution*, 15: 1481-1490.

Johnson, K.P., Adler, F.R., & Cherry, J.L. (2000). Genetic and phylogenetic consequences of island biogeography. *Evolution*, 54: 387–396.

Juste, J., Ibáñez, C., Muñoz, J., Trujillo, D., Benda, P., Karatas, A., & Ruedi, M. (2004). Mitochondrial phylogeography of the long-eared bats (*Plecotus*) in the Mediterranean Palaearctic and Atlantic Islands. *Molecular Phylogenetics and Evolution*, 31: 1114–1126.

Knopp, T., Cano, J.M., Crochet, P.A., & Merila, J. (2007). Contrasting levels of variation in neutral and quantitative genetic loci on island populations of moor frogs (*Rana arvalis*). *Conservation Genetics*, 8: 45–56.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., & Wilson, D.S. (1999). Chronology, causes and progression of the Messinian salinity crisis. *Nature*, 400: 652–655.

Lanfranco, G. (1969). Maltese Mammals (Central Mediterranean). Progress press, Malta, Pp: 1-28.

Lanza, B. (1959). Chiroptera Blumenbach, 1774 (pp. 187-473). In: Toschi A., and Lanza B. Fauna d'Italia, Vol. IV, Mammalia, generalità, Insectivora, Chiroptera; Bologna; Ed. Calderini, pp. 485.

Lanza, B. (1972). The natural history of the Cerbicale Islands (southeastern Corsica) with particular reference to their herpetofauna. *Natura Bresciana*, 63, 185–202.

Lanza, B. (1983). Ipotesi sulle origini del popolamento erpetologico della Sardegna. *Lavori della Societa Italiana di Biogeografia*, 8: 723–744.

Marra, A.C. (2005). Pleistocene mammals of Mediterranean islands. *Quaternary International*, 129: 5–14.

Menu, H. & Popelard, J.B. (1987). Utilisation des caractères dentaires pour la déterermination des vespertilionidés de l'ouest européen. *Rinolophe* 4: 1-88.

Meulenkamp, J.E., & Sissingh, W. (2003). Tertiary palaeogeography and tectonostratigraphic evolution of the Northern and Southern Peri-Tethys platforms and the intermediate domains of the African–Eurasian convergent plate boundary zone. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 196: 209–228.

Mucedda, M., & Nuvoli, T. (2000). Indagine biometrica sul "grande Myotis" (Chiroptera, Vespertilionidae) della Grotta Sa Rocca Ulari (Borutta) e di alter località della Sardegna. *Sardegna Speleol.* 17: 46-51.

Mucedda, M., Bertelli, M.L., & Pidinchedda, E. (1999). Risultati di 6 anni di censimento dei pipistrelli in Sardegna. Atti del primo convegno italiano sui chirotteri (ed. by G. Dondini, O. Papalini and S. Vergari), pp. 105–114. Proceedings of the First Italian Bat Congress, Castell'Azzara (Grosseto).

Nei, M. (1978). Estimation of Average Heterozygosity and genetic Distance from a small number of individuals. *Genetics* 89: 583-590 July, 1978

Paz, de O., Fernandez, R., & Benzal, J. (1986). El anillamiento de Quiropteros en el centro de la peninsula iberica durante el period 1977-86. *Boletin de la Estacion Central de Ecologia*, 30: 113-138.

Pestano, J., Brown, R.P., Suárez, N.M., Benzal, J., & Fajardo, S. (2003). Intraspecific evolution of Canary Island Plecotine bats, based on mtDNA sequences. *Heredity*, 90: 302–307.

Petri, B., Pääbo, S., Von Haeseler, A., & Tautz, D. (1997). Paternity assessment and population subdivision in a natural population of the Larger Mouse eared bat *Myotis myotis*. *Ecology*, 6: 235-242.

Reduker, D.W. (1983). Functional analysis of the masticatory apparatus in two species of *Myotis*. *Journal of Mammogy* 64: 277–286.

Roberts, T.E. (2006). Multiple levels of allopatric divergence in the endemic Philippine fruit bat Haplonycteris fischeri (Pteropodidae). *Biological Journal of the Linnean Society*, 88: 329–349.

Rohling, E.J., Fenton, M., Jorissen, F.J., Bertrand, P., Ganssen, G., & Caulet, J.P. (1998). Magnitudes of sea-level low stands of the past 500,000 years. *Nature*, 394: 162–165.

Ruedi, M., & Castella, V. (2003). Genetic consequences of the ice ages on nurseries of the bat Myotis myotis: a mitochondrial and nuclear survey. *Molecular Ecology*, 12: 1527–1540.

Ruedi, M., Arlettaz, R., & Maddalena, T. (1990). Distinction morphologique et biochimique de deux espèce jumelles de chauves souris: *Myotis myotis* (Bork.) et *Myotis blythii* (Tomes) (Mammalia; Vespertilionidae). *Mammalia* 54: 3, 415-429.

Salgueiro, P., Ruedi, M., Coelho, M.M., & Palmeirim, J.M. (2007). Genetic divergence and phylogeography in the genus *Nyctalus* (Mammalia, Chiroptera): implications for population history of the insular bat *Nyctalus azoreum*. *Genetica*, 130: 169–181.

Sevilla, P. (1989). Quaternary fauna of bats in Spain: Paleoecologic and biogeographic interest. In: *European Bat Research 1987* (eds. Hanak, V., Horácek, I., & Gaisler, J.), pp. 349-355. Charles University Press, Praha, Tchechia.

Sondaar, P.Y., & Van der Geer, A.A.E. (2005). Evolution and extinction of Plio-Pleistocene island ungulates. *Les ongulés holarctiques du Pliocène et du Pléistocene* (ed. by E. Crégut-Bonnoure), pp. 241–256. Maison de la Géologie, Paris.

Stadelmann, B., Jacobs, D.S., Schoeman, C., & Ruedi, M. (2004). Phylogeny of African *Myotis* bats (Chiroptera, Vespertilionidae) inferred from cytochrome b sequences. *Acta Chiropterologica*, 6: 177–192.

Storch G. (1974). Quartare Fledermaus-Faunen von der Insel Malta. *Senckenbergiana lethaea* 55 (1/5): 407-434.

Stöck, M., Dubey, S., Klütsch, C., Litvinchuk, S.N., Scheidt, U., & Perrin, N. (2008). Mitochondrial and nuclear phylogeny of circum-Mediterranean tree frogs from the Hyla arborea group. *Molecular Phylogenetics and Evolution*, 49: 1019– 1024.

Strelkov, P.P. (1972). Myotis blythii (Tomes, 1857): Distribution, geographical variability and differences from *Myotis myotis* (Borkhausen, 1797). *Acta Theriol.* 17: 355-380. (In Russian).

Strinati, P. (1951). Note sure les chauves-souris du Maroc. *Mammalia* 15: 23-31.

Tautz D., & Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acid Research*, 12: 4127-4138.

Temple, H.J., & Cuttelod, A. (Compilers). 2009. *The Status and Distribution of Mediterranean Mammals*. Gland, Switzerland and Cambridge, UK: IUCN. vii+32pp. Available at: http://cmsdata.iucn.org/downloads/mediteranean_mammals_web2.pdf (cited on 13th August 2011).

Topál, G., & Ruedi, M. (2001). *Myotis blythii* (Tomes, 1857) - Kleines Mausohr, in Handbuch der Säugetiere Europas. Band 4/I (Fledertiere). Ed. F. Krapp, Aula-Verlag, Wiebelsheim: 209-215.

Trujillo, D., Ibáñez, C., & Juste, J. (2002). A new subspecies of *Barbastella barbastellus* (Mammalia: Chiroptera: Vespertilionidae) from the Canary Islands. *Revue Suisse de Zoologie*, 109: 543–550.

van Andel, T.H., & Tzedakis, P.C. (1996). Palaeolithic landscapes of Europe and environs, 150,000–25,000 years ago. *Quaternary Science Reviews*, 15: 481–500.

Van Cakenberghe, V., Herrel, A., & Aguirre, L.F. (2002). Evolutionary relationships between cranial shape and diet in bats (Mammalia: Chiroptera). In: Aerts P, ed. *Topics in functional and ecological vertebrate morphology*. Maastricht: Shaker Publishing, 205–236.

van der Made, J. (1999). Biogeography and stratigraphy of the Mio-Pleistocene mammals of Sardinia and the description of some fossils. *Deinsea* (Rotterdam), 7: 337–360.

Vigne, J.D. (1992). Zooarchaeology and the biogeographical history of the mammals of Corsica and Sardinia since the last ice age. *Mammal Review*, 22: 87–96.

Whitlock, M.C. (1992) Temporal fluctuations in demographic parameters and the genetic variance among populations. *Evolution*, 46: 608–615.

Wilkinson, G.S., & Chapman, A.M. (1991). Length and sequence variation in evening bat d-loop mtDNA. *Genetics*, 128: 607-617.

Worthington Wilmer, J., & Barratt, E. (1996). A non-lethal method of tissue sampling for genetic studies of chiropterans. *Bat Research News*, 37:1–3.

Worthington Wilmer, J.M., Moritz, C., Hall, L., & Toop, J. (1994). Extreme population structuring in the threatened ghost bat, *Macroderma gigas*: Evidence from mitochondrial DNA. *Proceedings of the Royal Society, London*. Series B, 257: 193-198.

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