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A PRELIMINARY ANALYSIS OF THE POPULATION GENETICS OF *MYOTIS PUNICUS* IN THE MALTESE ISLANDS

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ABSTRACT - By combining molecular genetic techniques with a non-lethal sampling technique, it was possible to undertake a preliminary study of the population structure of *Myotis punicus* Felten, 1977 in the Maltese Islands. Twelve sites spread around the Maltese Islands were investigated and a total of 36 individuals found in four of these sites were sampled over a period of 6 months. Tissues for analysis were obtained by 4 mm biopsy punches and morphometric data (forearm length, ear length, tragus length, wing-span and weight) were collected. The comparison of average values for these measurements showed sexual dimorphism, with females being the larger sex. A recapture rate of 19% was achieved, and population size was estimated by Lincoln-Petersen technique to be ca. 200 individuals. Nei's Genetic Identity (I) showed values from 0.954 to 0.686, while Genetic Distance (D) values ranged from 0 to 0.047. The results obtained in this study suggest that the population on the Maltese Islands represents a single panmictic unit, even though the overall value of the Fixation Index, 0.272, indicates that breeding groups are relatively isolated, probably as a consequence of high roost fidelity.

Keywords: bat, Chiroptera, Vespertilionidae, non-lethal sampling, electrophoresis, conservation

RIASSUNTO - ***Analisi preliminare della genetica di popolazione di* Myotis punicus *(Chiroptera, Vespertilionidae) nelle isole maltesi***. Alcune indicazioni preliminari sulla struttura della popolazione di *Myotis punicus* Felten, 1977 delle isole maltesi sono state ottenute tramite tecniche di genetica molecolare non invasiva. I campionamenti hanno interessato 12 roost potenziali, monitorando per un periodo di 6 mesi un totale di 36 animali. I tessuti per le analisi sono stati ottenuti tramite punzoni da biopsia di 4 mm di diametro e sono state raccolte alcune misure morfometriche standard (lunghezza dell'avambraccio, dell'orecchio e del trago, apertura alare e peso). Il confronto delle misure nei due sessi indica che le femmine sono in media più grandi dei maschi. Il tasso di ricattura è stato pari al 19%, mentre, tramite il metodo di Lincoln-Petersen, la popolazione è stata stimata in circa 200 individui. L'indice di Nei varia da 0.954 a 0.686, mentre la distanza genetica è compresa tra 0 e 0.047. I risultati ottenuti suggeriscono che la popolazione maltese rappresenta un'unità panmittica, anche se il valore complessivo dell'indice di fissazione, 0.272, indica che le diverse sotto-popolazioni sono relativamente isolate, probabilmente a causa dell'elevata fedeltà ai roost riproduttivi.

Parole chiave: pipistrelli, Chiroptera, Vespertilionidae, campionamento non ivasivo, elettroforesi, conservazione

INTRODUCTION

The Maltese Islands have been inhabited by bats, including *Myotis* spp., at least since the late Quaternary (Felten *et al*., 1977), as shown by the fossil records from Ghar Dalam (Storch, 1974). Over the last century, several authors have proposed and debated the occurrence on these islands of a variety of *Myotis* species, including *M. blythii* (Lanza, 1959), *M. blythii omari* (Strelkow, 1972), *M. daubentoni* (Gulia, 1913), *M. capaccinii* (Gulia, 1913), *M. myotis* (Gulia, 1913) and *M. oxygnathus* (Lanfranco, 1969). More recent reports (Felten *et al*., 1977; Borg *et al*., 1997) indicate that the only *Myotis* species presently inhabiting the Maltese Islands is *M. punicus* (previously called *M. blythii punicus* by Felten *et al*., 1977), which in the past was a relatively common resident species, with reports of large swarms leaving their roosts in summer (Gulia, 1913; Lanfranco, 1969). Nonetheless, in the second half of the century, a severe decline of its population has been reported, as a consequence of roost disturbance, persecution and vandalism (Borg *et al.*, 1997).

In the Maltese Islands, only a small number of caves or equivalent man-made structures offer suitable roosting conditions for bats. Moreover, in recent years the two largest colonies (one of which was the only known nursery) of the island have been lost (Borg, 1998). The status, movements, morphometrics, roosts and diet of *M. punicus* on Malta have been recently investigated (Borg, 1998), whilst a population genetic study has never been carried out. Thus, the aim of this study was to obtain insight into the genetic variation, structure and differentiation within the current local population, since a robust conservation effort is promptly required.

STUDY AREA AND METHODS

In the Maltese Islands *M. punicus* is protected by law and thus sampling was carried out in accordance with the issued MEPA permit NP00026/05. A total of 12 sites (8 in Malta and 4 in Gozo; Tab. 1 and Fig. 1), were investigated once a month between June and November 2005. Eight sites were natural caves (which may have minor man-made modifications), while four were man-made structures.

Bats were captured inside their roosts by hand nets or from known paths by mist netting. Captured individuals were identified according to their large body size, the wing membrane starting at the base of the toes, the lancet-shaped tragus and the clear line of demarcation between the dorsal and ventral fur coloration (Dietz and von Helversen, 2004). All sampled individuals were ringed and sexed. Forearm length, ear length, tragus length and wing span were measured. Bat counts were also carried out during the surveys using a night vision camera at caves' entrances. Tissue samples were taken from the lower end of each wing membrane using sterile Stiefel® Biopsy Punches with diameter of 4 mm, according to the method outlined in Worthington Wilmer and Barratt (1996). All bats were released at the site of capture within a few minutes.

Tissue samples were transported in dry ice (-20° C) to the laboratory, where they were then stored at –80° C. According to Ruedi *et al.* (1990) and Arlettaz *et al.* (1997), the chosen loci were: Glutamate Oxaloacetate Transaminase (GOT - 2.6.1.1), Mannose Phosphate Isomerase (MPI - 5.3.1.8), Glucose Phosphate Isomerase (GPI - 5.3.1.9), Esterases (ES - 3.1.1.1), Phosphogluconate

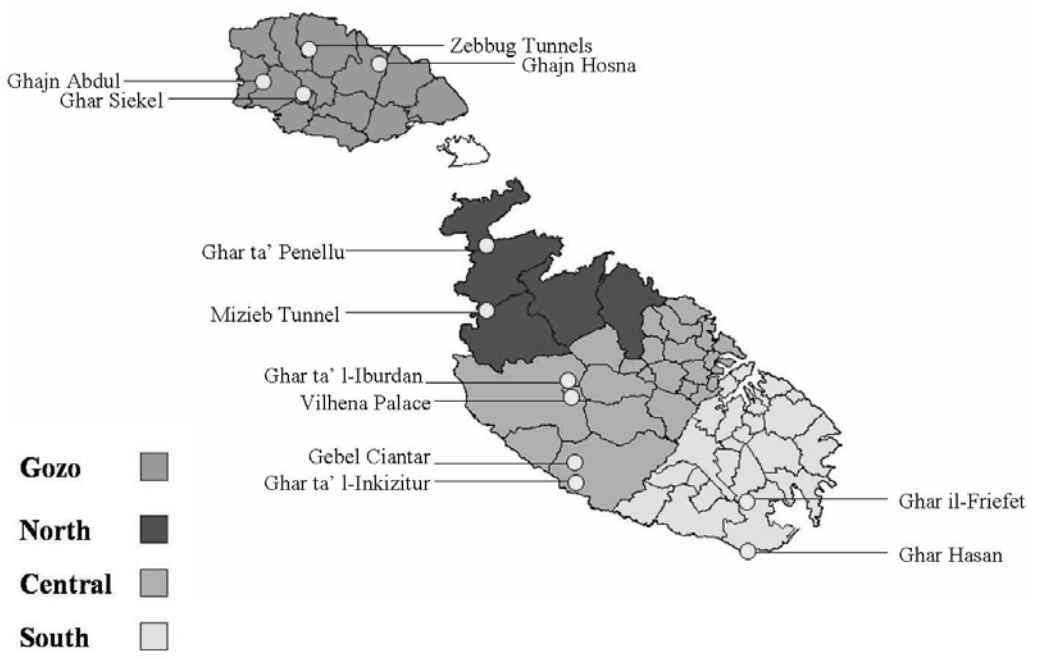

Figure 1 - Study area (Maltese Islands) and sampling sites.

dehydrogenase (6-PGD - 1.1.1.44) and Glucose-6-phosphate dehydrogenase (G-6-PD - 1.1.1.49). For each locus, the most common allele was arbitrarily labelled as "1", while the less common form was "2". Each tissue sample was macerated in a 0.5ml PCR tube, using a rounded-off capillary tube, in 4 µl of deionised water. This procedure was performed on an ice block to avoid the heating of the tissue. Successively, the tubes were centrifuged at 11500 rpm for 5 minutes at 4°C to obtain the supernatant. Helena Laboratories Titan® III (94 x 76mm) cellulose acetate plates were used for this electrophoretic procedure. The buffer solutions and electrophoretic conditions used for the specific enzymes are described in Richardson *et al.* (1986). The gels were run at 120V and 50mA for 45 minutes. The staining recipes used for each enzyme are detailed in Richardson *et al.* (1986). The stain solutions were prepared in 5 ml volumes and added drop-wise to the gels, followed by incubation at 35°C.

All body measures were grouped by sex and locality. For each colony, descriptive statistics - One-Sample Kolmogorov-Smirnov test and Paired-Sample t-tests - were calculated using SPSS v13.0. Allele frequency, variation (polymorphism and heterozygozity), Nei's (1978) Genetic Distance (D) and Identity (I) as well as the Fixation Index, F_{ST} (Nei, 1972; Nei, 1973; Wright, 1978) were calculated using Tools for Population Genetic Analyses (TFPGA) v1.3 and subsequently worked out manually in Microsoft Excel. F_{ST} was used to describe the level of heterozygosity and determine the level of genetic differentiation between the local colonies, *i.e.* the relative divergence of each colony from the ideal situation of a single breeding population. Population size was estimated using the Lincoln-Petersen capture-mark-recapture

technique (Petersen, 1896; Lincoln, 1930) at the four active roosts, since the Maltese Islands can be considered a closed system (i.e. there is no exchange of individuals with mainland populations). The total population was estimated as: (total N° of bats ringed x total N° of bats recaptured) / (total N° of ringed bats recaptured).
Births did not occur during the study period and a 10% mortality (based on that of other *Myotis* species) was applied to the adult population.

RESULTS

A total of 36 bats were sampled from four colonies (Tab. 1). All enzymes showed the expected number of loci except GPI, which showed two loci.

Table 1 - Details of all traditionally known roosting sites of *Myotis punicus* in the Maltese Islands with their respective GPS positions, sampling effort, historical records and status. No v. = number of visits; No c. = number of captures; No rec. = number of re-captures; No ob. = max number observed; Borg = max number observed by Borg 1998, 2002; RCS = roost current status: Ab (abandoned), St (stable), Re (reduced), Bl (blocked).

Sampling site	Locality	GPS coord.	No v.	No c.	No rec.	No ob.	Borg	RCS
Ghar il-Friefet	Birzebbuga	35°50.382'N 14°31.108'E	1	0	0	0	100	Ab
Ghar Hasan	Birzebbuga	35°48.421'N 14°31.055'E	3	0	0	0	150	Ab
Vilhena Palace (M1)	Mdina	35°53.137'N 14°24.289'E	9	17	2	26	35	St
Ghar ta' l-Iburdan	Rabat	35°52.515'N 14°23.301'E	2	0	0	0	5	Re
Gebel Ciantar (M4)	Dingli	35°50.753'N 14°24.076'E	4	6	2	5	15	Re
Ghar ta' l-Inkizitur (M3)	Girgenti	35°51.220'N 14°24.386'E	2	4	1	4	40	Re
Tunnel (M2)	Mizieb	35°57.379'N 14°22.425'E	5	9	2	5	10	St
Ghar ta' Penellu	Mellieha	35°58.831'N 14°21.144'E	2	0	0	6	12	St
Ghajn Hosna	Xaghra (Gozo)	36°03.630'N 14°16.635'E	2	0	0	0	2	Re
Ghar Siekel	Fontana (Gozo)	36°02.237'N 14°13.714'E	1	0	0	0	20	Bl
Tunnels	Zebbug (Gozo)	36°03.990'N 14°14.113'E	2	0	0	0	5	Bl
Ghajn Abdul	S. Lawrenz (Gozo)	36°02.310'N 14°11.922'E	2	0	0	0	2	Re

Table 2 - Allele frequencies for the 9 loci investigated at the four active roosts (see Table 1).

		Locality			
Locus	Allele	M1	M2	M3	M4
GOT-1	1	0.9412	0.8889	0.5000	0.8333
	2	0.0588	0.1111	0.5000	0.1667
GOT-2	1	1.0000	1.0000	1.0000	1.0000
	2	0.0000	0.0000	0.0000	0.0000
MPI	1	0.9375	0.7778	0.0000	0.5000
	2	0.0625	0.2222	1.0000	0.5000
GPI-1	1	1.0000	1.0000	1.0000	1.0000
	2	0.0000	0.0000	0.0000	0.0000
GPI-2	1	1.0000	0.8889	0.0000	0.5000
	2	0.0000	0.1111	1.0000	0.5000
ES-1	1	0.9118	0.9444	1.0000	1.0000
	2	0.0882	0.0556	0.0000	0.0000
ES-2	1	0.4118	1.0000	0.7500	1.0000
	2	0.5882	0.0000	0.2500	0.0000
6-PGD	1	0.6176	0.3889	0.3750	0.4167
	2	0.3824	0.6111	0.6250	0.5833
G-6-PD	1	0.6250	0.6111	0.7500	0.9167
	2	0.3750	0.3889	0.2500	0.0833

What is noteworthy is the difference in activity between the two loci, with the unexpected GPI-2 locus showing fainter bands than GPI-1. Out of the overall 9 investigated loci, two - GOT-2 and GPI-1 -, were monomorphic for all the sampled individuals (Tab. 2).

The observed heterozygosity (H_o) in each site was not significantly different from the Hardy-Weinberg expected heterozygosity (H_e) (1 d.f., $P < 0.05$). Nei's (1978) Genetic Identity (I) and Genetic Distance (D) were calculated for each pair-wise site. Values for I ranged from 0.954 to 0.686 and those for D ranged from 0 to 0.047 (Tab. 3).

Table 3 - Nei's genetic distance for the four sampled roosts.

D	M1	M2	M3	M4
M1	0.0000	0.0631	0.3766	0.1400
M2		0.0000	0.2552	0.0471
M3			0.0000	0.1029
M4				0.0000

Average F_{ST} was 0.272. Sexual dimorphism was shown for forearm length ($t = 4.84$, 8 d.f., $P < 0.05$), ear length ($t = 1.96$, 8 d.f., $P < 0.05$) and wing span ($t = 2.16$, 8 d.f., $P < 0.05$), which showed

normality when tested by a One-Sample Kolmogorov-Smirnov Test.
Seven individuals (19%) were recaptured, of which five were found in the same roost from which they had been previously captured. The other two bats had moved for wintering about 9 km away from the first capture locality. The roost count surveys aimed at assessing the overall population size gave an estimate of ca. 200 individuals. Both the number of roosts and colonies' size were lower than those previously recorded by Borg (Tab. 1; Borg *et al.*, 1997; Borg, 1998; Borg, 2002).

DISCUSSION

The presence of two loci for GPI has been reported by Richardson *et al.* (1986) as a rare occurrence in a few species, e.g. *Gambusia affinis*, where two loci form through gene duplication and in some cases even present weak hybrid heterodimers. GPI-1 is known to give very bold bands, which show that it is present in high concentrations in the tissues sampled. Nonetheless, neither Ruedi *et al.* (1990) nor Arlettaz *et al.* (1997), working on the closely related species *M. myotis* and *M. blythii* reported such a phenomenon. It is thus possible that GPI duplication is a peculiarity of *M. punicus* and may be used to easily distinguish it from the other species of the genus.
Heterozygosity showed that the overall population of *M. punicus* in the Maltese Islands represents a single panmictic unit, with no deficiency in heterozygosity within the colonies. With regards to allele frequency, polymorphism was higher than in *M. myotis* and *M. blythii* (Ruedi *et al.*, 1990). Nei's (1972) Genetic Distance (D) showed a high proportion of genes being identical between the colonies, although there is evidence of a certain degree of differentiation (divergence from the value of 0). Finally, F_{ST} confirmed the hypothesis of low gene flow between the sites sampled, suggesting that breeding groups are relatively isolated. Low gene flow may be a result of high roost fidelity in Maltese *M. punicus*. At the end of autumn mating often occurs in the same roosts where clubs of males dwell throughout the year, while, as soon as the juveniles can fly, the females with their litter return to the roost from which females had left prior to wintering in the nursery. These females effectively return to the same clubs of males every year, impeding gene flow between different roosting sites Borg (pers. comm.).
The total sample obtained suggests that the Maltese population is very small and still declining. For comparison, data collected by Borg (pers. comm.) between 2000 and 2005 included 47 individuals newly ringed and 11 recaptures (23%), a recapture rate comparable to that of the present study.
This reduction in population size, together with roost fidelity, may have a significant impact on the conservation of this species. Moreover, as shown for *M. myotis* (Castella *et al.*, 2000), individuals do not move over distances longer than a few kilometres, dramatically limiting the possibility of population restocking through immigration from neighbouring countries. Our results state that immediate action needs to be taken in order to preserve as much as possible the present level of polymorphism and heterozygosity in

what remains of the local *M. punicus* population. Bat populations are dependent on very specific areas for roosting and feeding, which may be deeply altered by human activities such as quarrying, urbanisation, tourism, and agricultural practices. As observed throughout the sites, disturbance is the main cause of the local population decline. For this reason the conservation strategy for *M. punicus* should involve an action plan, providing for the conservation and protection of roosting and feeding grounds (Baron, 2007). Locally, the status of *M. punicus* should be changed from vulnerable (as listed in the Red Data Book for the Maltese Islands - Schembri and Sultana, 1989) to Critically Endangered, since evidence indicates that *M. punicus* meets criteria B and C (IUCN 2001) and faces a high risk of extinction.

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