



## Research Article

## Genetic identity of *Pipistrellus maderensis* from the Madeira archipelago: a first assessment, and implications for conservation

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### Keywords:

*Pipistrellus maderensis*  
Madeira archipelago  
cytochrome b  
phylogenetic relationships

### Article history:

Received: 5 January 2013  
Accepted: 11 January 2013

### Acknowledgements

The authors are very grateful to Christian Erard, Jiri Gaisler, Virginie Mézan-Muxart, Paul Racey, Danilo Russo and to two anonymous referees for their critical revision of our manuscript. Authors are also very grateful to Direcção Regional do Ambiente (Funchal: Madeira) that authorized the capture and handling of bats.

### Abstract

According to the IUCN global Red List, *Pipistrellus maderensis* is among the most endangered bat species in Europe. Its populations are scattered across some islands of the Atlantic Ocean, particularly Madeira and the Canary archipelagoes. This geographical pattern is likely to result in significant genetic differences between populations which would have important implications to set conservation priorities. To test this hypothesis, we analyze cytochrome b sequences and compared populations from Madeira and the Canary islands. Five sequences from Madeiran individuals were analysed and compared to 30 sequences extracted from GenBank from *Pipistrellus maderensis* from the Canary islands and *Pipistrellus kuhlii*.

Our results indicate a significant divergence between the two groups, smaller than between true species, but higher than intra-group divergence. However, further research on the Madeiran population is needed, including the use of sequences of other mitochondrial markers and nuclear marker and microsatellites.

## Introduction

As typical with oceanic islands (Findley, 1993), the bat fauna richness of the Madeira Archipelago (Portugal, Atlantic Ocean) is lower than that found on the closest mainland. Recent systematic surveys confirmed the current presence of three species, *Plecotus austriacus* and the two endemisms *Pipistrellus maderensis* and *Nyctalus leisleri verucosus* (Jesus et al., 2009; Teixeira and Jesus, 2009).

*P. maderensis* is found on Madeira and Porto Santo (Madeira archipelago), La Palma, La Gomera, El Hierro and Tenerife islands (Canary archipelago) and possibly on some islands of the Azores (Rainho et al., 2002; Trujillo and Gonzalez, 2011). Although it is found from sea level up to 2150 m a.s.l. on the Canary Islands, it is more common in lowlands (Fajardo and Benzal, 1999). Its taxonomic status has been discussed, and in the past some authors have considered it as an intraspecific form of Kuhl's pipistrelle *Pipistrellus kuhlii* (e.g. Corbet 1978). *P. maderensis* probably derives from its closest relative *P. kuhlii* (Pestano et al., 2003), a West-Palaearctic and Afro-tropical bat widespread in much of southern Europe (Vernier and Bogdanowicz, 1999) – or from ancestral forms common to both lineages. Besides being morphologically similar (Corbet, 1978; Trujillo, 1991), unlike other insular pipistrelles in which social calls markedly differ from the closest mainland relative (Georgiakakis and Russo, 2012), the two species also share very similar social calls broadcast during agonistic interactions (Russo et al., 2009), possibly as a result of stabilizing selection that has acted on the ancestral island colonizers. Such social calls may mutually elicit interspecific reactions, as seen with crossed playback experiments (Russo et al., 2009). Unlike social calls, echolocation calls markedly differ between such species, those of *P. maderensis* being more similar to those of *P. pipistrellus* (Russo et al., 2009; Teixeira and Jesus, 2009).

According to recent molecular work, *P. kuhlii* does not represent a species but a species complex (Stadelmann et al., 2004; Ibañez et al., 2006; Evin et al., 2011). Using cyt b and ND1 sequences, Ibañez et al. (2006) found 2 distinct groups, emphasizing the difficulty of carrying out a phylogenetic reconstruction of *P. maderensis* and *P. kuhlii* of Macaronesian islands.

Pestano et al. (2003) suggested that *P. maderensis* from the Canary islands probably consists of three evolutionary significant units (ESU) and found that sequence divergence was low. Although previous studies (Pestano et al., 2003; Mayer et al., 2007) analyzed mtDNA of *P. maderensis*, no published work has estimated the genetic divergence in mtDNA between Madeira and Canary populations, as well as their phylogenetic relationships.

*P. maderensis* is among the most threatened bats in Europe, being endangered B1ab(iii,v) according to the IUCN Global Red List (Juste et al., 2008) and critically endangered according to the Portuguese Red Book of Vertebrates (Queiroz et al., 2005; Jesus et al., 2009). Its extent of occurrence is less than 5000 km<sup>2</sup> and highly fragmented. According to the IUCN assessment (04 Apr 2012), its population trend is still decreasing (Juste et al., 2008). On Madeira, the maximum estimated number of *P. maderensis* was of ca. 1000 individuals based on the estimate by Queiroz et al. (2005). Given the human activities like large-scale land use change, the use of biocids/pesticides (as observed in *P. maderensis* on Canary islands – Trujillo 2008) and roost disturbance and destruction occurred on the island ever since the human settlement, as well as some natural events such as floods and wildfires (Queiroz et al., 2005; Jesus et al., 2009), the archipelago's population is now certainly smaller and even more at risk. Besides these factors, others such as predation by the introduced *Rattus rattus* and *Felis catus* can be important threats to madeiran pipistrelle, as it is also suggested for other island pipistrelles such as *Pipistrellus murrayi* of Christmas Island (Schulz and Lunsden, 2004). On the Canary islands, where *P.*

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*maderensis* occurs, it is the most frequently observed bat, but population size and trend have not yet been assessed (Palomo and Gisbert, 2002; Juste et al., 2008). On the Azores, pipistrelle bats are rare and provided they are actually *P. maderensis*, the total number of individuals is probably less than 300 (Queiroz et al., 2005).

The species is protected under Bern Convention, and is included in Annex IV of 92/43 EU Habitats and Species Directive.

A full understanding of the taxonomic status of *P. maderensis* populations on Madeira and the Canary Islands respectively is crucial to correctly assess the overall conservation status of this taxon and adopt reliable conservation measures. However, if such populations basically belong to a single Evolutionary Significant Unit (ESU) (Moritz, 1994), then despite the worrying conservation status of this bat on Madeira, the apparently more encouraging situation on the Canary archipelago would make the latter a stronghold to preserve the species on its global range. Otherwise, the Madeira population would represent an especially critical conservation issue deserving ad hoc management.

In the light of this consideration, appropriate knowledge is urgently needed. On such bases, in this study we present the results of a first assessment aimed to ascertain whether such populations can be regarded as a single ESU.

We used partial sequences of cytochrome b to examine the position of Madeiran population of *P. maderensis* in relation to the *P. kuhlii* complex and *P. maderensis* from the Canary islands, and also to estimate the levels of divergence between the forms of Madeira and the Canary islands.

## Materials and Methods

### Study area

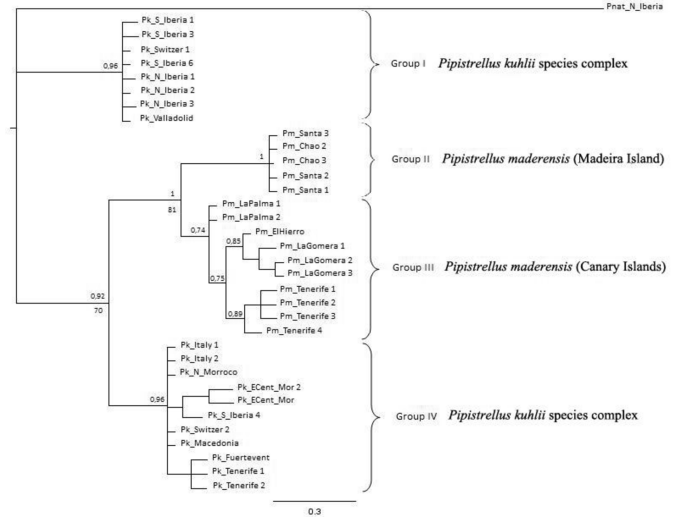
The Madeira Archipelago consists of Madeira, Porto Santo Islands, the Desertas Islands and the Selvagens Islands. Madeira is the largest island (750 km<sup>2</sup>) and lies ca. 700 km from the western coast of Africa (Morocco). The islands show marked ecological differences determined by different climatic patterns observed in their various regions (Báez and Sánchez-Pinto, 1983). Vegetation on Porto Santo and the Desertas is less developed than on Madeira Island. According to Geldmacher et al. (2000) and Galopim de Carvalho and Brandão (1991), such islands originated in different times: the Desertas 3.6 million years (MY) ago, Madeira 4.6 MY, the Selvagens 12 MY and Porto Santo 14 MY.

### Sampling localities and analysis procedures

The number and geographic locations of the specimens used in this study are given in Tab. 1. Bats were captured under license with mist nets and identified to species (Palmeirim, 1990; Dietz and Von Helversen, 2004) at foraging or drinking sites. A small wing tissue sample (< 2 mm in diameter) was collected from each subject with biopsy punch and preserved in 70% ethanol (Worthington Wilmer and Barratt, 1996). After such operations bats were released immediately at the capture site.

We extracted total genomic DNA using standard methods (Sambrook et al., 1989). Primers used in both amplification and sequencing of cytochrome b were Molcit-F (Ibañez et al., 2006) and MVZ-16 (Smith and Patton, 1993). The PCR cycling procedure was according to Ibañez et al. (2006). PCR fragments were sequenced in an ABI 310 sequencer (Applied Biosystem DNA Sequencing Apparatus).

DNA sequences were aligned using Clustal W (Thompson et al., 1994). We considered one dataset of about 478 bp. Cytochrome b revealed no indels. The alignment is available on request from the corresponding author. We sequenced the cyt b fragments in 5 *P. maderensis* from two localities on Madeira island (see Tab. 1), and compared them for phylogenetic analysis, with 30 partial sequences of mainland and Canary islands *P. maderensis* and *P. kuhlii*, from GenBank, that fully overlapped with our 478 bp sequences. We also included in this analysis a partial sequence from *P. nathusii* as an outgroup (see Tab. 1).



**Figure 1** – Tree derived from Bayesian analysis of cytochrome b fragment for *Pipistrellus maderensis* and *P. kuhlii*. Average posterior probabilities are shown near nodes. Above nodes, the posterior probability indicates the probability that the clade is correct under the model. Bootstrap values are given below nodes (only the most important values for this study are given). Polytomous nodes indicate that the resolutions of the nodes involved have posterior probabilities less than 0.5. The tree was rooted using *Pipistrellus nathusii* from Northern Iberia.

**Table 1** – Details of material and sequences used in the present study; Locality/origin of sequences and samples, GenBank Accession Nos and codes given in Fig. 1 and 2.

Species	Locality	Accession number	Codes
<i>P. maderensis</i>	Madeira - Santa do Porto Moniz	KC520774	Pm_Santa 1
	Madeira - Santa do Porto Moniz	KC520773	Pm_Santa 2
	Madeira - Santa do Porto Moniz	KC520770	Pm_Santa 3
	Madeira - Chão da Ribeira	KC520771	Pm_Chao 2
	Madeira - Chão da Ribeira	KC520772	Pm_Chao 3
	Canary Isl. - La Palma	AJ426632	Pm_LaPalma 1
	Canary Isl. - La Palma	AJ426618	Pm_LaPalma 2
	Canary Isl. - El Hierro	AJ426617	Pm_ElHierro
	Canary Isl. - La Gomera	AJ426616	Pm_LaGomera 1
	Canary Isl. - La Gomera	AJ426615	Pm_LaGomera 2
<i>P. kuhlii</i> species complex	Canary Isl. - La Gomera	AJ426614	Pm_LaGomera 3
	Canary Isl. - Tenerife	AJ426613	Pm_Tenerife 1
	Canary Isl. - Tenerife	AJ426612	Pm_Tenerife 2
	Canary Isl. - Tenerife	AJ426611	Pm_Tenerife 3
	Canary Isl. - Tenerife	AJ426610	Pm_Tenerife 4
	Italy	EU360660	Pk_Italy 1
	Italy	EU360659	Pk_Italy 2
	Northern Morocco	EU360653	Pk_N_Morocco
	Morocco - East/center	EU360651	Pk_ECent_Mor
	Morocco - East/center	EU360650	Pk_ECent_Mor 2
	Southern Iberia	EU360658	Pk_S_Iberia 1
	Southern Iberia	EU360654	Pk_S_Iberia 3
	Southern Iberia	DQ120846	Pk_S_Iberia 4
	Southern Iberia	DQ120844	Pk_S_Iberia 6
	Northern Iberia	DQ120843	Pk_N_Iberia 1
	Northern Iberia	DQ120842	Pk_N_Iberia 2
	Northern Iberia	DQ120841	Pk_N_Iberia 3
	Iberia-Spain-Valladolid	AJ426619	Pk_Valladolid
	Switzerland	DQ120848	Pk_Switzer 1
	Switzerland	DQ120847	Pk_Switzer 2
Macedonia	AJ504444	Pk_Macedonia	
Canary Isl. - Fuerteventura	AJ426609	Pk_Fuertevent	
<i>P. nathusii</i>	Canary Isl. - Tenerife	AJ426608	Pk_Tenerife 1
	Canary Isl. - Tenerife	AJ426607	Pk_Tenerife 2
<i>P. nathusii</i>	Northern Iberia	DQ120849	Pnat_N_Iberia

Variable sites		
	1112222 3333333344	
	5770121278 0023367946	
	0582160305 3974792048	
Pm_Santa 3	GCCTGATGAA CCTACGCATT	} Madeira Island
Pm_Chao 2	.....	
Pm_Chao 3	.....T...	
Pm_Santa 2	.....	} Canary Islands
Pm_Santa 1	.....	
Pm_LaPalma 1	.T..AGC... TTCG.A..AC	
Pm_LaPalma 2	.T..AGC... TTCG.A..AC	
Pm_ElHierro	.T.CAGC..G TTCG.A..AC	
Pm_LaGomera 1	.T.CAGC..G TTC..A.TAC	
Pm_LaGomera 2	AT.CAGC..G TTC..A..AC	
Pm_LaGomera 3	AT.CAGC..G TTC..A..AC	
Pm_Tenerife 1	.TTC.GC.G. TTCG.A..AC	
Pm_Tenerife 2	.TTC.GC... TTCG.A..AC	
Pm_Tenerife 3	.TTC.GCA.. TTCG.A..AC	
Pm_Tenerife 4	.TTCAGC... TTCGTA..AC	

Figure 2 – Variable sites of the cytochrome b sequences in *Pipistrellus maderensis*.

The true evolutionary relationships may be obscured in DNA sequence data sets if sites are saturated by multiple substitutions (Swofford et al., 1996). To test for saturation, observed pairwise proportions of transitions and transversions were plotted against sequence divergence and calculated using DAMBE version 4.2.13 (Xia and Xie, 2001).

For phylogenetic analysis, we employed PAUP\* 4.0b10 (Swofford, 2002) and MEGA version 3.1 (Kumar et al., 2004) software packages. We used maximum likelihood (ML) and Bayesian inference. We followed the approach outlined by Huelsenbeck and Crandall (1997) to test for 56 alternative models of evolution, employing PAUP\* 4.0b10 and Modeltest 3.7 (Posada and Crandall, 1998). Once a model of evolution was chosen according to the Akaike information criterion (Posada and Buckley, 2004), it was used to estimate a tree using ML criteria (Felsenstein, 1985). A heuristic search with tree bisection reconnection (TBR) and 10 replicates of random addition of taxa was performed to estimate a tree. The steepest descent option was ineffective, so we used the MULPARS option. The relative robustness of each dichotomy was established by bootstrap analysis. Non-parametric bootstrap support for nodes was estimated using the “fast” option with 100 heuristic bootstrap replicates implemented in PAUP\* 4.0b10. The Bayesian analysis was implemented using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001), which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) analysis. Bayesian analysis was conducted with random starting trees, four MCMC chains (one cold, three heated), run  $1.0 \times 10^7$  generations, and sampled every 100 generations using a General-Time-Reversible model of evolution with a gamma model of among-site rate variation. In all searches stationarity of Markov Chain was determined as the point when sampled negative log-likelihood values plotted against the number of generations reached a stable mean equilibrium value; “burn-in” data sampled from generations preceding this point were discarded. The burn-in value was 10000.

Table 2 – Descriptive statistics of cytochrome b K2P pairwise distances between the four main groups of *P. maderensis* and *P. kuhlii* as defined in Fig. 1. In each cell, the upper line gives mean  $\pm$  standard deviation; the lower line gives the maximum and minimum. Gaps/Missing Data were treated with the “Complete Deletion” option (465 bp were considered) as implemented in MEGA version 4.0 (Kumar et al., 2004).

	Group I	Group II	Group III	Group IV
Group I	0.0019 $\pm$ 0.0013 [0; 0.0040]	0.0590 $\pm$ 0.0019 [0.0540; 0.0610]	0.0611+0.0040 [0.0520; 0.0660]	0.0529+0.0019 [0.0490; 0.0570]
Group II		0.0008 $\pm$ 0.0010 [0; 0.0020]	<b>0.0283<math>\pm</math>0.0024</b> <b>[0.0240; 0.0330]</b>	0.0478 $\pm$ 0.0031 [0.0450; 0.0540]
Group III			0.0074 $\pm$ 0.0358 [0; 0.0130]	0.0361 $\pm$ 0.0045 [0.0290; 0.0450]
Group IV				0.0059 $\pm$ 0.0046 [0; 0.0180]

## Results and Discussion

In the 5 newly sequenced individuals we recovered two haplotypes that differed by only a single transition change (Fig.2).

The plots of observed pairwise divergences of haplotypes for transitions and transversions revealed negligible saturation (data not shown), so our analyses included all sites. The amplified cytochrome b fragments yielded unambiguous sequences of 478 bp in length, with 93 variable sites of which 44 were parsimony informative and 49 singletons. We observed no insertions or deletions.

ML and Bayesian analyses gave similar overall estimates of relationships (Fig. 1). The most appropriate model for the data was the TVM model ( $-\ln(L) = 1242.6558$ ) with a discreet approximation of the gamma distribution (0.1988). The ML heuristic search using this model found one best tree ( $-\ln(L) = 1241.5535$ ).

The node that separates Madeira from the Canary islands bats is better supported in the Bayesian topology (posterior probabilities = 1.0) than in the ML tree (bootstrap value = 81), yet the values of support remain high and the node well-supported (Fig. 1).

Based on Kimura’s 2-parameter distance (Kimura, 1980), the average divergence between Madeira and the Canary islands group is approximately 2.83% (minimum 2.4%; maximum 3.3%), which is significantly higher than intra-group variation (Tab/ 2).

Considerably, no haplotypes are shared between Madeira and the Canary islands populations (Fig/ 2).

Low guanine content, lack of alignment problems and the similarity with cytochrome b sequences of the same genus or species suggest that nucleotide sequences represent mitochondrial genes rather than nuclear pseudogenes (Zhang and Hewitt, 1996).

The analysis of the cytochrome b sequences produced a robust estimate of relationships for the populations from the Canary islands and the Madeira Archipelago. Clearly, the taxon is monophyletic, and the populations from the two archipelagoes represent “sister populations”.

Despite their morphological similarity (Trujillo 1991, 2008; unpublished data), the two populations show a much higher sequence divergence than that observed within groups. The two groups are thus well separated. Although we acknowledge that our sample size was limited, our data suggest that no gene flow occurs between Madeira and the Canary Islands. However, further investigation is needed to confirm this, using mitochondrial and nuclear markers, and including microsatellites.

Hulva et al. (2004) considered for different bat species levels of divergence of 6-7%. For Ibañez et al. (2006), well-recognized species among bats show intra-specific genetic divergence lower than 2.5% whereas values over 5% indicate the existence of cryptic taxonomic diversity. They also suggest that a conservative approach (based on morphology, ecology and bioacoustics) is needed to provide a reliable species description. Although our data are preliminary, they point at the existence of two subspecies in *P. maderensis* because of the observed level of divergence, the apparent lack of gene flow, the haplotype differences and the different geographical distribution.

As expected according to Pestano et al. (2003) and Ibañez et al. (2006), the topology we obtained (Fig. 1) confirms the paraphyletic nature of the *P. kuhlii* complex.

Following Pestano et al. (2003), postulating the existence of three possible ESUs, we may in fact add a fourth ESU to this framework, restricted to the Madeiran population of *P. maderensis*.

Future work will ascertain whether *P. kuhlii* is also present on Madeira, as it seems according to the rarely recorded echolocation calls (our observation) and also consider pipistrelle populations from the Azores and Porto Santo Islands, where more ESUs could be observed. Our recent surveys on Porto Santo suggests that bats no longer occur there. *P. kuhlii*, unlike the related *P. nathusii*, is a non-migratory species (Hutterer et al., 2005). According to Serangeli et al. (2012) *P. kuhlii* travels less than 5 km in total each night, which suggests small home ranges. Thus, the sea-crossing in *P. kuhlii* complex will be, certainly, almost absent between the Canary Islands and Madeira (distance between the Canary Islands and Madeira is about 460 km). Probably, this will be the case for the sister species *P. maderensis*.

Probably, *P. maderensis* would face great difficulties in crossing the stretch of sea separating Madeira from Porto Santo, as the minimum distances between these two islands is about 40 km. These facts and the results of this study suggest a very interesting evolutionary scenario. Also, the population of Porto Santo (if not extinct), may deserve high conservation efforts.

Bat populations on islands are especially vulnerable and exposed to sudden demographic crises due to intrinsic or extrinsic factors (e.g. Del Vaglio et al. 2011). The *P. maderensis* global population is made of strongly isolated components (Pestano et al. 2003; this study) and this condition highlights the especially threatened conservation status of this species. The quick and large-scale land use change occurred on the island of Madeira, coupled with recent catastrophic events such as massive landslides and wildfires which destroyed large forest areas make this situation especially critical. Also based on the first evidence that we present, we propose that Madeiran *P. maderensis* should be managed as a separate evolutionary unit and urge conservation authorities to deal with the crucial task of preserving it. ☺

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