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

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Abstract. The Azorean bat (*Nyctalus azoreum*), the only endemic mammal of the Azores archipelago (Portugal), diverged recently from its mainland relative, the Leisler's bat (*N. leisleri*). Although the two species are phenotypically very different, mtDNA studies detected very low genetic divergence between them, which could question the validity of the species status of *N. azoreum*. In order to assess the genetic variability in each species and check for present levels of gene flow between the two taxa, eight microsatellite loci were genotyped and analysed. The results indicated lower genetic diversity in the insular species. Many unshared alleles were found between the two species and no evidence of migrants, which provides strong support against any contemporary gene flow between them. The species status of the Azorean bat is discussed in the light of the cohesion species concept, and we conclude that it is an isolated species with a high conservation value.

Key words: Chiroptera, cohesion species concept, microsatellites

Introduction

Ever since Darwin's observations on the Galapagos, islands have been recognised as "laboratories" for the study of evolution. Insular species often differ from their mainland counterparts in a wide variety of features (Grant 1998). Island species tend to have higher densities and survivorship (Stamps & Buechner 1985, Adler & Levins 1994), and broader ecological niches (Grant 1998). Their behaviour can also be affected, and island populations often show reduced aggressiveness and relaxed territory boundaries (Stamps & Buechner 1985). Finally, isolation often results in severe size changes (Lomolino 2005, Millien 2006). This is the case of the only endemic mammal from the Azores Archipelago, the threatened Azorean bat (*Nyctalus azoreum* Thomas, 1901), which is darker and considerably smaller than its continental ancestor, the Leisler's bat (*N. leisleri* Kuhl, 1817) (Palmeirim 1991, Speakman & Webb 1993). The island species presents a broad ecological niche, occupying a variety of roosts like buildings, coastal cliffs and trees (Salgueiro et al. 2004), whereas its mainland counterpart is predominantly a tree dweller (Shiel 1999). Finally, the Azorean bat has echolocation calls with a higher peak frequency than its continental ancestor (Rainho et al. 2002, Skiba 2003), and a very unusual high level of diurnal activity (Moore 1975, Speakman 1995).

This marked phenotypic and ecological distinction contrasts with the unexpectedly low levels of genetic divergence found between the two species at several mitochondrial DNA markers (Salgueiro et al. 2007). The

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maximum genetic divergence found was of 3.6% for the control region, a situation that may have resulted from a very recent speciation process. In fact, the two mtDNA studies on *N. azoreum* (Salgueiro et al. 2004, Salgueiro et al. 2007) suggest that the Azores were colonized as recently as the late Pleistocene or even early Holocene by a single bat matrilineage.

N. azoreum was first described as an independent species by Thomas (1901), but Corbet (1978) reclassified it as subspecies (*N. leisleri verrucosus*). More recently, Palmeirim (1991) and Speakman & Webb (1993) both supported its species status, based on morphological data. Indeed, at the morphological level, the separation between *N. leisleri* and *N. azoreum* is greater than between other species of the same genus (Palmeirim 1991). However, our recent genetic work (Salgueiro et al. 2007) demonstrated that the mtDNA distance between the two taxa was considerably lower than that usually found between mammalian species (around 10% of sequence divergence), thus raising some doubts about the specific status of *N. azoreum*. Such uncertainties in species definitions may be detrimental for biodiversity conservation, since the taxonomic rank is a decisive factor in assessing conservation priority of endangered taxa. Due to the reduced and fragmented distribution range, and the small global population size, the *N. azoreum* is classified as an endangered species in a recent evaluation of the status and distribution of European mammals (Temple & Terry 2007, 2009).

The mtDNA markers that showed a poor resolving ability to clearly separate *N. azoreum* from *N. leisleri* were too conservative to resolve recent speciation events. Due to the recent divergence of the two species, it was necessary to apply molecular markers with faster mutation rates. Petren et al. (2005) showed that microsatellites are very informative to compare among species that are less that 4% divergent in mtDNA sequences. Allele frequency data are useful for studying evolutionary relationships of closely related species (Takezaki & Nei 1996). In fact, owing to their high variability levels, microsatellites have clarified many species relationships, including in bats (Racey et al. 2007). Mayer & von Helversen (2001) pointed out that cases of unresolved bat species status based on mtDNA, should be subjected to detailed studies on morphology, ecology, echolocation, and nuclear gene flow. This is in line with the increasingly recognized need to join phenotypic and genetic data in an integrative taxonomy approach (Will et al. 2005).

Microsatellites have been used to study inter-island population structure in the Azorean bat (Salgueiro et al. 2008), but not to compare the insular species with its relative on the European mainland. In the present study, we make this comparison, examining genetic diversity and divergence between the two species. The main objective is to evaluate the level of contemporary gene flow between them, thus contributing to the clarification of their taxonomic status.

Study Area

The nine islands of the Azores Archipelago extend along 600 km in the Atlantic Ocean, about 1 500 km west of mainland Europe, and 3 900 km east of North America (Fig. 1). We searched for bats in the seven islands where *N. azoreum* is present, but only managed to capture specimens in the five islands of the Central Group (Faial, Pico, S. Jorge, Terceira, Graciosa) and in S. Miguel, which is part of the Eastern Group (Fig. 1).

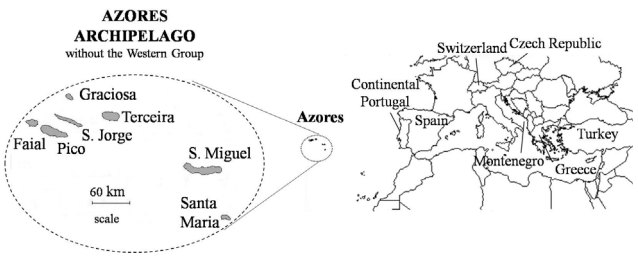


Fig. 1. Map of Europe, showing in closer detail the islands of the Azorean archipelago with *Nyctalus azoreum* (the species is absent from the islands of Flores and Corvo, not shown).

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Material and Methods

Sampling

The sampling included 279 individuals of *N. azoreum* (Salgueiro et al. 2008), 29 specimens of *N. leisleri* from one forest site in continental Portugal, and 10 from other regions: Spain (1), Switzerland (4), Greece (2), Turkey (1), Czech Republic (1), Montenegro (1). Part of this sample set was previously genotyped for mitochondrial genes by Salgueiro et al. (2007). We performed non-lethal sterile biopsy punches of the wing membrane (Worthington Wilmer & Barratt 1996), and released the animals.

Genotyping with microsatellites

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

Eight microsatellite loci originally isolated from other bat species (Petri et al. 1997, Moore et al. 1998, Mayer et al. 2000, Miller-Butterworth et al. 2002) amplified reliably and were polymorphic in both species. Primers, labelling, PCR conditions and scoring protocols are reported in Salgueiro et al. (2008).

Data analysis

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

Intra-specific and intra-population gene diversity and number of alleles per locus were calculated in Fstat 2.9.3 (Goudet 1995). In this same software, samples were grouped per species and compared (randomization tests with 15 000 permutations). Private alleles were calculated using Convert (Glaubitz 2004). Allelic richness and private allelic richness was estimated using the rarefaction method of Kalinowski (2005) by sampling 76 gene copies per species and 64 per sampling site. The number of alleles per locus with (*A*) and without (*R*) rarefying was compared using a two-sample *t*-test.

In order to use similar sample sizes, for the comparisons among populations the *N. leisleri* sample was restricted to the individuals collected in continental Portugal, which is the region of mainland Europe closest to the Azores.

To determine the contribution of stepwise-like mutations on the genetic differentiation between the two species, a permutation test was performed using Spagedi 1.2g (Hardy & Vekemans 2002). Different allele sizes at each locus were randomly permuted among allelic states (2 000 permutations) generating a simulated distribution of R_{ST} values (pR_{ST}). As the observed R_{ST} was not significantly larger than its simulated value (results not shown), then there is no support for a mutational component to differentiation, and F_{ST} is considered a better estimator of genetic differentiation among such populations (Hardy et al. 2003).

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

For a visual representation of genetic patterns, we performed a factorial correspondence analysis (FCA) over populations on the multilocus genotype of each individual, as implemented in Genetix v. 4.05.2 (Belkhir et al. 2004). We have also applied a clustering of groups of individuals, available at the program Baps 4 (Corander et al. 2004), which employs stochastic optimization. The tested groups corresponded to the sampled populations (7) or species (2), to detect genetically divergent clusters. The number of clusters (K) was set to 2, 3, 4, 5, 6, 7 and 8, and for each K the analysis was replicated 10 times.

To evaluate the relationships among populations, several genetic distances were calculated: chord distance Dc (Cavalli-Sforza & Edwards 1967), Nei's distance Da (Nei et al.

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1983), Ds (Nei 1972). Given that the allele size permutation test (Hardy et al. 2003) did not show significance, the distance $(\delta\mu)^2$ (Goldstein et al. 1995) based on allele size information was excluded from the analysis. Unrooted Neighbour-Joining trees (Saitou & Nei 1987) were built. The consistency of relationships was evaluated by bootstrapping over loci with 5 000 permutations. These calculations were performed with Populations 1.2.28 (Langella 2002), which also provided estimates of the above mentioned distances between species. Phenograms were visualized using Treeview (Page 1996) and NJPlot (Perrière & Gouy 1996).

We checked if there were first generation (F0) immigrants using the Bayesian assignment procedure of Rannala & Mountain (1997) as implemented in Geneclass 2 (Piry et al. 2004). The Paetkau's et al. (2004) method was used to compute probabilities from 10 000 simulated genotypes. This creates a test distribution of simulated individuals by drawing haplotypes, rather than alleles, from the observed data and thus preserves the partial linkage disequilibrium present in genotypes that have immigrant ancestry, but are not F0 immigrants (Paetkau et al. 2004).

Results

Genetic variability

After Bonferroni corrections, all loci were found to be in Hardy-Weinberg equilibrium for both species. In the Portuguese sample of *N. leisleri*, significant linkage disequilibrium was detected in the pair of loci P217 and NN8'. Nevertheless, there is no strong evidence for linked loci, and it was considered that overall these two loci provided independent information.

Following the correction for unequal sample size, the number of alleles per locus per species with rarefying correction (R) was significantly different from the one without it (A) ($t = 2.94$; d.f. = 14; $P \leq 0.05$). Therefore, we will refer mainly to R, instead of the usual A, when comparing the two species.

Overall, the Azorean bats had less microsatellite variation than the mainland Leisler's bats. The average corrected allelic richness and private alleles (PR) over all loci in the Azorean bat (mean $R = 10$, $\Sigma_R = 76$; mean $PR = 1$, $\Sigma_{PR} = 9$) was significantly lower than in the Leisler's bat (mean $R = 12$, $\Sigma_R = 100$; mean $PR = 4$, $\Sigma_{PR} = 32$) (Wilcoxon signed ranks tests $Z = 2.52$, one-sided $P = 0.006$) (Table 1). In addition, the expected heterozygosity of the insular species ($H_E = 0.82$, SD = 0.007) was significantly lower than that of the mainland ($H_E = 0.88$, SD = 0.012) (Wilcoxon signed ranks test $Z = 2.38$, one-sided $P = 0.009$). These results were confirmed by the permutations test for difference between groups (here each group was a species) for the R, H_E, H_O implemented in Fstat (one-sided $P \leq 0.05$).

A considerable percentage of unshared alleles have been found in each species (32% in *N. leisleri* and 12% in *N. azoreum*, Table 1).

Table 1. Individual alleles (represented by their sizes), number of alleles (A), allelic richness corrected for the minimum sample size per species (38 individuals) (R), number of species-private alleles (P), private allelic richness corrected for the minimum sample size (NPR), for eight microsatellite loci in Leisler's bat (*Nle*) and Azorean bat (*Na*).

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

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Genetic structure and gene flow

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

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

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

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

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

After a factorial components analysis, we obtained the projection of the 318 genotyped bats onto the factorial space represented in Fig. 2. The first axis of variation clearly separates the two species (100% of inertia on the FC-1).

Although the number of loci used in this study is far from that recommended to infer proper genealogical relationships between species (Takezaki & Nei 1996, Schlötterer 2001), the phylograms obtained from different distances suggest a clear separation among these same groups (100% bootstrap for Dc and Da and 93% for Ds, Fig. 3). Both Dc and

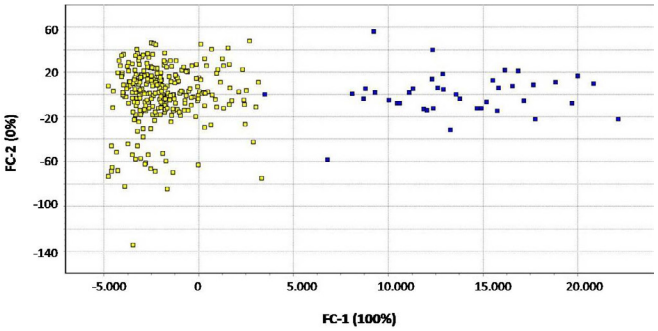


Fig. 2. Graphic representation of microsatellite genotypes of *N. leisleri* (in black) and *N. azoreum* (in white) after a factorial component analysis. For each factorial component (FC-1 and FC-2) inertia percentage values are shown.

Da distance estimators generally show the highest probability of obtaining the correct tree topology (Takezaki & Nei 1996). However, we chose to show the Ds tree (Fig. 3), because it is more appropriate to infer evolutionary times (Takezaki & Nei 1996) and presented the same topology as the other distances. Pooling the islands of Central Group in one unit, the *N. leisleri* population is almost equidistant from the Central Group and S. Miguel (0.57 and 0.60, respectively). The distance Ds between the Central Group and S. Miguel (0.34) is nearly half of that distance.

Geneclass identified no migrants ($P < 0.01$) between Azores and the mainland. When

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the assignment test was performed with all *N. leisleri* samples, three bats from Portugal were allocated as migrants from the sample containing individuals from the rest of Europe.

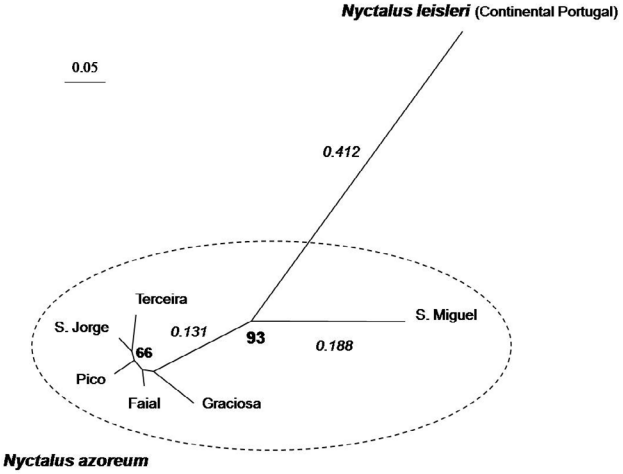


Fig. 3. Neighbour-joining tree based on the standard Nei's distance (Ds) of six Azorean bat populations and one Leisler's bat population, constructed from allelic frequencies of eight microsatellite loci. Numbers represent the reliability of the branches based on 5 000 bootstrap re-sampling. Branch length is shown in italic.

Discussion

Gene flow between Azorean bats and Leisler's bats

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

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

Taxonomic and evolutionary relevance

Under the biological species concept (Mayr 1963) only the sympatric occurrence of taxa allows the confirmation of species level differentiation, which makes it difficult to apply in the case of allopatric taxa. Templeton (1998) and Crandall et al. (2000) defended the use of the Cohesion Species Concept, in which recognition of speciation requires both significant or adaptive isolation and ecological divergence. These authors recommended that species should be supported from an ecological and genetic perspective as testable hypotheses. Both genetic and ecological exchangeability are determined for recent and ancient times (Crandall et al. 2000). Rejecting any of these hypotheses will allow the definition of distinct cohesion species (Templeton et al. 2000). Ecological exchangeability is analysed through characters related with life-history, morphology, behaviour or habitat.

The Azorean bat is demographically isolated from the continental Leisler's bat by a distance of 1 500 km, and it has experienced a fast adaptation revealed by morphological, ecological and behavioural characters (see Introduction). The two species share no control region mtDNA haplotypes (Salgueiro et al. 2004), defining reciprocally monophyletic clades indicative of historic isolation. In addition, this study demonstrates that there are many unshared microsatellite alleles and no recent gene flow.

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

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In fact, a few other well established bat species are known to have very low levels of mtDNA divergence (Mayer & von Helversen 2001). As Mayer et al. (2007) pointed out, sequencing of parts of the mitochondrial genome can only provide the lower limit of true species diversity.

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

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