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What genetics tell us about the conservation of the critically endangered Balearic Shearwater?

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ABSTRACT

The Balearic shearwater *Puffinus mauretanicus* is one of the most critically endangered seabirds in the world. The species is endemic to the Balearic archipelago, and conservation concerns are the low number of breeding pairs, the low adult survival, and the possible hybridization with a sibling species, the morphologically smaller Yelkouan shearwater (*P. yelkouan*). We sampled almost the entire breeding range of the species and analyzed the genetic variation at two mitochondrial DNA regions. No genetic evidence of population decline was found. Despite the observed philopatry, we detected a weak population structure mainly due to connectivity among colonies higher than expected, but also to a Pleistocene demographic expansion. Some colonies showed a high imbalance between immigration and emigration rates, suggesting spatial heterogeneity in patch quality. Genetic evidence of maternal introgression from the sibling species was reinforced, but almost only in a peripheral colony and not followed, at least to date, by the spread of the introgressed mtDNA lineages. Morphometric differences were not correlated with mtDNA haplotypes and introgression is probably due to a secondary contact between the two species several generations ago. Overall, results suggested that the very recent demographic decline in this critically endangered species has not yet decreased its genetic variability, and connectivity found among most colonies should help to reduce species extinction risk. Spreading of introgression should be monitored, but the species is not jeopardized at the moment by genetic factors and the major conservation actions should concentrate at enhancing adult survival.

1. Introduction

The majority of recorded extinctions and a substantial proportion of currently threatened species occur on islands (Frankham et al., 2002). In particular, island avifauna is one of the most affected groups, with extinction rates showing a strong increase in recent times due to habitat degradation and especially predation by human-introduced species (Blackburn et al., 2004). Some avian orders, such as the Procellariformes, show particularly high extinction risks (Lockwood et al., 2000). Various ecological methods can be used to estimate the degree of connectivity between colonies, population size, and in general to quantify the risk of extinction. The analysis of genetic variation within and between colonies is considered an important tool for at least two reasons. First, ecological data, unless long temporal series are available,

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produce a temporally limited window of the demographic patterns, which, although important, can be misleading if extrapolated to the long-term. Second, the risk of extinction, due for example to low genetic variation or hybridization, should always be evaluated (Frankham, 2005). Here, we use a population genetic approach to understand the history and demography of one of the most critically endangered seabirds in the world, the Balearic shearwater, *Puffinus mauretanicus* (IUCN Red List, Oro et al., 2004). This species is an endemic seabird of the Balearic archipelago (Western Mediterranean). Once considered a subspecies of the Yelkouan (Levantine) shearwater, *P. yelkouan*, breeding in the Central and Eastern Mediterranean (Bourne et al., 1988; Yésou et al., 1990; Sibley and Monroe, 1990; Hamer, 2003), the Balearic shearwater is now classified as a distinct species on the basis of both morphological and genetic data (Heidrich et al., 1998; The British Ornithologist Union, 2005).

The evolution of the genus *Puffinus* is controversial. All species probably resulted from the fragmentation of an original North Atlantic ancestral population, followed by dispersal through sea connections (Austin, 1996; Heidrich et al., 1998; Zotier et al., 1999). Different authors, however, have suggested different hypotheses to explain the divergence in the Mediterranean area. Vous (1976), Heidrich et al. (1998) and Zotier et al. (1999) support the hypothesis that *Puffinus* shearwaters entered the Mediterranean Sea after the reopening of the strait of Gibraltar at the end of the Messinian crisis (about 5.3 MY ago), and that only after this event did the divergence of the different Mediterranean species occur. Alternatively, Bourne et al. (1988) and Austin (1996) suggest that the ancestors of Balearic and Yelkouan shearwaters diverged earlier, as a consequence of the establishment of shallow inland seas in central Europe. These taxa reached a sufficient degree of differentiation at that time to remain distinct when the inland seas joined to form the Mediterranean Sea and were reconnected to the Atlantic at the end of the Pliocene.

The history of Balearic shearwaters in the last decades is characterized by a rapid demographic decline (Oro et al., 2004). Current estimates for the whole species range between 1800 and 2000 reproductive pairs (Arcos and Oro, 2003), with a recent Population Viability Analysis suggesting that the mean extinction time for the Balearic shearwater could be as little as 40 years (Oro et al., 2004). The total number of colonies has drastically decreased in the last few decades. Additionally, the estimated mean adult survival probability is abnormally low (Oro et al., 2004). The reasons for this situation probably include at-sea factors (such as becoming caught in fishing gear), predation of adults by feral cats and other alien mammals (Louzao et al., 2006), and possibly also hybridization with the sibling species the Yelkouan shearwater. Recent studies have actually identified adults with phenotypic traits of Yelkouan shearwaters in Menorca, the most North-Eastern island of the Balearic archipelago (Alcover et al., 2003; Ruiz et al., 2003; Genovart et al., 2005), as well as several adults with mitochondrial haplotypes of the sibling species (Genovart et al., 2005). Beside the possible outbreeding depression effects (Edmonds, 1999; Marr et al., 2002), the simple genome dilution of the rare and declining Balearic shearwater due to the introgression of genomes from the more common Yelkouan shearwaters could potentially threaten the former species (Rhymer and Simberloff, 1996; Allendorf et al., 2001).

In this paper, we analyze the genetic variation, the phylogeography and the population structure of the Balearic shearwater. We will also jointly consider genetic data with some morphological measurements to better solve the question about the possible inter-specific introgression. The major questions we shall address are (1) Has the population decline produced a decrease of genetic variation? (2) What is the degree of genetic connectivity among colonies? (3) Has occurred inter-specific introgression in the endemic species, and how spread it is (4) What is the timing of the divergence between the two Mediterranean species? By solving these questions we should enhance the current conservation diagnosis of the species, and in turn better define the immediate priority actions for its conservation.

2. Materials and methods

2.1. Sampling

A total of 113 individuals were sampled from 10 different colonies covering most (77%) of the distribution range of the Balearic Shearwater (see Table 1 and Fig. 1). Small blood samples (ca. 25 μl) were taken from a leg vein puncture, captured with a syringe and transferred to a tube containing absolute ethanol. In Menorca, we obtained blood samples from both members of nine breeding couples to determine whether mating also occurs between individuals with mitochondrial haplotypes attributed to the two different species.

2.2. DNA extraction, amplification and sequencing

Total DNA was isolated from blood samples by an overnight incubation at 55 °C in SET buffer (Sambrook et al., 1989) with 30 μl SDS 10% and 2.5 U/ml of proteinase K followed by a standard phenol/chloroform protocol (Sambrook et al., 1989). DNA was resuspended in TE buffer (Sambrook et al., 1989). Two mitochondrial regions were analyzed in this study: a cytochrome *b* gene fragment of 880 bp (cyt *b*) and a control region fragment of 300 bp (CR). The primer pairs mt-A (L-14970) and mt-Fr (H-16086) given in (Heidrich et al. (1998)) were used to amplify and sequence the DNA fragment of the mitochondrial cyt b gene. Amplification reactions were performed in a total volume of 25 μl with 1 μM of each primer, 0.25 mM dNTP, 1× Taq buffer, 1 U of Taq DNA polymerase (Bioline), 0.01% gelatine (USB), 2.5 mM of $MgCl_2$ and 1–2 μl of template DNA. The thermocycling conditions were 94 °C for 1 min, followed by 35 ccycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 5 min. For the control region amplification, we used the primers ND6F (3′ATTTGATGCAACCGCTAACC 5′), designed specifically for this species, and H505 (Burg and Croxall, 2001). Amplification reactions were performed in a total volume of 20 μl with 0.5 μM of each primer, 0.2 mM dNTP, 1× Taq buffer, 1 U of Taq DNA polymerase (Bioline), 2 mM of MgCl2 and 2 μl of template DNA. The thermocycling conditions were 94 °C for 2 min, followed by 34 cycles of 94 °C for 2 min, 56 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min. PCR product was purified and concentrated by centrifugal dialysis with a commercial

Table 1 – Sampling localities, number of individuals analyzed for cytochrome b (Cyt b) and control region (CR) analysis, and haplotypes detected in each breeding locality

Locality	Cyt *b*	CR	Haplotypes
Menorca	24	22	**Cytb**: CB7(2),CB8,CB10(3),CB11,CB12(14),CB13, CB14,CB15 **CR**: DL6(2), DL7(3), DL8(4), DL9 (2), DL26, DL27, DL28, DL30(2), DL35, DL38, DL43 (2), 2 IND
Malgrats, Mallorca	19	17	**Cytb**: CB5, CB6(3), CB7(3), CB8, CB9(4), CB10(3), CB14(3),CB19 **CR**: DL2(2), DL3, DL4, DL5, DL6(3), DL7, DL22, DL29, DL31, DL36, DL37, DL43, 2 IND
Sa Cella, Mallorca	16	13	**Cytb**: CB7(12), CB9, CB10(2), CB14 **CR**: DL1(2), DL2, DL23, DL33, DL34 (2), DL39, DL42, 4 IND
Blanquer, Cabrera	11	12	**Cytb**: CB7(5), CB9(2), CB12,CB14, CB17(2) **CR**: DL1, DL6, DL15,DL16, DL17, DL18(2), DL39(2), DL41, 2 IND
Llumeta, Cabrera	5	5	**Cytb**: CB7(5) **CR**: DL10, DL12, DL13,DL14, 1 IND
Es Corral, Cabrera	1	1	**Cytb**: CB14 **CR**:1 IND
Conillera, Pitiüses	20	17	**Cytb**:CB7(9), CB8, CB9, CB14, CB15, CB17(5), CB19(2) **CR**: DL6(2),DL10(4), DL19, DL20, DL21, DL24, DL25(2), DL31, DL39, 3 IND
Es Bosc, Pitiüses	2	3	**Cytb**:CB14, CB18 **CR**: DL6, DL13, DL19
Espardell, Pitiüses	5	5	**Cytb**:CB7(3), CB20, CB21 **CR**: DL5, DL10, DL11, DL18, DL32
Dragonera, Mallorca	2	3	**Cytb**:CB7, CB10 **CR**: DL2, DL6, DL40
Total	105	98	

Individuals with no haplotype assignment due to heteroplasmy are coded as IND.

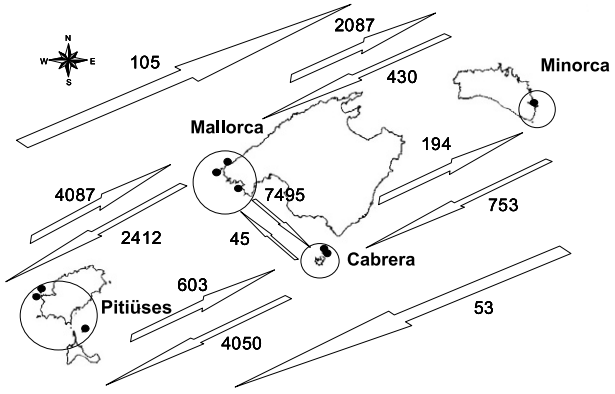

Fig. 1 – Map of breeding colonies of Balearic Shearwater, indicated by solid dots. The four main groups of colonies, Menorca, Mallorca, Cabrera and Pitiüses, are also shown by open circles. Arrows with numbers indicate the average of estimates of gene flow between colonies obtained with two mtDNA markers (cytochrome *b*/control region) using a coalescence approach (see text for details).

Kit (Millipore and Quiagen). We checked the amplification and purification results by loading 1 μl of product in a 1.5% agarose gel. The fragment was sequenced directly from purified PCR product using an ABI 3100 automated sequencer (Applied Biosystems, Warrington, UK).

In this study, we also used four Balearic Shearwater sequences, four Yelkouan Shearwater sequences, and one Manx Shearwater *P. puffinus* sequence, available on Genbank (accession numbers AJ004208-AJ004211, AJ004216, AJ004217, AJ004222, AJ004224, AY219971) (Heidrich et al., 1998).

2.3. *Sequence alignment and variation*

Sequences were assembled and easily aligned by eye using Chromas Pro (version 1.21) with no regions of ambiguous alignment. For both mitochondrial regions analyzed, Modeltest v 3.06 was used to select the substitution model that best fit our data (Posada and Crandall, 1998) by performing a hierarchical test of likelihood. Phylogenetic relationships among haplotypes were analyzed by Neighbour-joining (NJ) and Maximum Likelihood (ML) approaches using PAUP 4.0b10 (Swofford, 2003) and assuming the selected substitution model. Robustness of the nodes was assessed through bootstrapping (Felsenstein, 1985) and quartet puzzling, respectively (Strimmer and Von Haeseler, 1996).

Genetic variation was described using the number of different sequences (i.e., the number of haplotypes), gene diversity (i.e., the expected heterozygosity) and nucleotide diversity (the average number of pairwise differences per nucleotide site). All these indices were calculated with the software package Arlequin (v.2.0.1.1.) (Schneider et al., 2000) for each DNA fragment using the corrected distance based on the adequate model of sequence evolution. Several individuals showed heteroplasmy in the CR fragment, probably due to true mitochondrial heteroplasmy, and we did not

assign them any haplotype (see Table 1). Those mtDNA sequences clearly belonging to the Yelkouan shearwater clade were used to tentatively estimate the genetic variation and divergence between the Balearic shearwater and its sibling species.

2.4. Demographic inferences

Several approaches were used to understand the level of connectivity between populations and the possible variation in population size over time. We started working with colonies as population units. We then pooled neighbouring colonies, creating four groups corresponding to the four major geographic units (Mallorca, Cabrera, Menorca, and Pitiüses, the last one corresponding to Eivissa plus Formentera, Fig. 1), and these groups were used as population units.

Pairwise *Fst* statistics and global AMOVA (Analysis of Molecular Variance, Excoffier et al., 1992) were computed using Arlequin with corrected sequence distances. Three AMOVA analyzes were performed as follows: (a) including all individuals; (b) excluding individuals with haplotypes attributed to the sibling species; and (c) excluding all individuals from Menorca, where the evidence of hybridization is stronger.

In order to better understand the migration patterns, the genetic relationships between groups were then analyzed in more detail using Migrate (Beerli and Felsenstein, 1999, 2001) that utilize Monte Carlo Markov Chains and the coalescent approach to obtain Maximum Likelihood estimates of migration rates in both directions. This method uses all the information in the data, and it should therefore provide better estimates of genetic divergence, especially when migration rates are high (and *Fst* values are consequently low). Each MCMC run consisted of 10 short chains (sampling 10,000 trees) and two long chains (sampling 5,000,000 trees) with a burn-in period of 10,000 trees. In some cases, larger sampling and an adaptive heating regime with four parallel chains and initial relative temperatures of 1, 1.2, 1.5 and 3.0 were used. The robustness of the estimates across different runs of the Markov Chains was finally verified (see Beerli and Felsenstein, 1999, 2001). With this method, we get the estimates of the migration parameter *M* (migration rate scaled by the mutation rate) between any pair of groups, in both directions. To attain some additional information, we estimated the total immigration and emigration rates in each group using the estimated M values and also their relative scores (to avoid strong outlier effects).

The deviation from the genetic variation expected under neutrality and demographic stability was tested using Tajima's *D* (1989) and Fu's F_S(1997) statistics, as implemented in Arlequin (v.2.0.1.1.). Fu's F_S is particularly sensitive to population growth. Negative values of both statistics are interpreted as evidence for population expansion. The population expansion hypothesis was further tested with the mismatch distribution analysis computed with Arlequin and graphically represented with DNASP (Rozas et al., 2003). The frequency distribution of the pairwise differences between DNA sequences (the mismatch distribution) is usually multimodal in samples from populations at demographic equilibrium but unimodal in populations that have experienced a demographic expansion (Rogers and Harpending, 1992). Statistical testing of the mismatch distribution was based on the Schneider and Excoffier (1999) approach, which compares the observed distribution with that expected under a null model of population expansion. If the null hypothesis of expansion cannot be rejected, the parameters of the expansion (age and population size before and after the event) can be estimated if a substitution rate is available. The evolution rate of 0.9%/MY for cytochrome *b* based on fossil calibration in medium-size Procellaridae (Nunn and Stanley, 1998), and a generation time for the species of 11 years (Oro et al., 2004) was used for that purpose. Finally, the phylogeographic pattern was further investigated using the Nested Clade Phylogeographic Analysis (NCPA) (Templeton, 1998). This analysis can be used to test the hypothesis of geographical association between haplotypes, and to suggest possible interpretations. Though criticized (Knowles and Maddison (2002), but see Templeton (2004)), the NCPA provides a useful empirical framework to understand and explain the geographic distribution of different sequences. The network of mitochondrial shearwater haplotypes was inferred using statistical parsimony implemented in TCS v 1.13 (Clement et al., 2000), according to Templeton (1998). The nesting clade analysis design used for the NCPA was constructed by hand (Templeton, 1998). Then we create an input file for Geodis (Posada et al., 2000) with the corresponding locations of the populations as longitudes and latitudes. Geodis (Posada et al., 2000) was then used to compute NCPA distance measures: the clade distance (D_c) and the nested clade distance (D_n), and their statistical significance. D_c measures the geographical range of a particular clade and D_n compares the geographical range of a particular clade to that of its sister clades. The difference between these statistics, computed in a tip clade and its interior clade, is calculated and their significance tested by permutations. Only those clades with significant geographic association are analyzed following the inference key (Templeton, 2004).

2.5. Morphometric analysis

Body measures were collected from 120 breeding adults. Head-plus-bill length, minimum bill depth and tarsus were measured with a digital calliper (±0.02 mm) and wing length with a ruler. Principal component analysis (PCA) was used to obtain a body size index factor. Using this index, a General Linear Model (GLM, as implemented in SPSS, version 12.0) was applied to analyze the relationship between body size, mitochondrial haplotype, geographic origin and sex (the genus *Puffinus* shows sexual dimorphism; Genovart et al., 2003).

3. Results

3.1. Sequence alignment and variation

In the analysis of 113 individuals, 29 segregating sites (24 of which were parsimony informative) were observed in the cyt *b* region. It was only possible to sequence the CR in 98 individuals: this revealed 34 polymorphic sites (30 of which were parsimony informative). The GenBank Accession numbers for the sequences are Nos. DQ230131–230316.

According to the hierarchical likelihood ratio test, the HKY model (Hasegawa et al., 1985) was identified as the most appropriate for both mitochondrial regions. For cyt *b*, we found equal evolution rates for all sites. In the CR, the estimated value of the parameter α of the gamma distribution of the substitution rates was 0.3102.

NJ and ML trees for the two regions were mostly concordant. The trees distinguished two major clades with high bootstrap support (see NJ haplotype trees in Fig. 2), and Manx shearwater used as outgroup. According to the reference cyt *b* sequences in GenBank, the two clades can be attributed to mtDNA haplotypes of two different species, the Yelkouan and the Balearic shearwater, respectively. At least 10 substitutions separated the two clades in the less polymorphic cyt *b* fragment. With the exception of one chick sampled in the island of Cabrera, all the other individuals with the unexpected Yelkouan cyt *b* haplotypes come from the island of Menorca. In Menorca, in fact, 15 out of 24 sampled individuals had Yelkouan haplotypes, 14 of which were identical to a sequence deposited in GenBank from one individual of Crete (AJ004216) (Haplotype CB12). As expected, due to the largely non-recombining nature of mtDNA, concordant results are provided by the analysis of the CR: individuals with Yelkouan cyt *b* haplotypes also had CR sequences classified in a different clade.

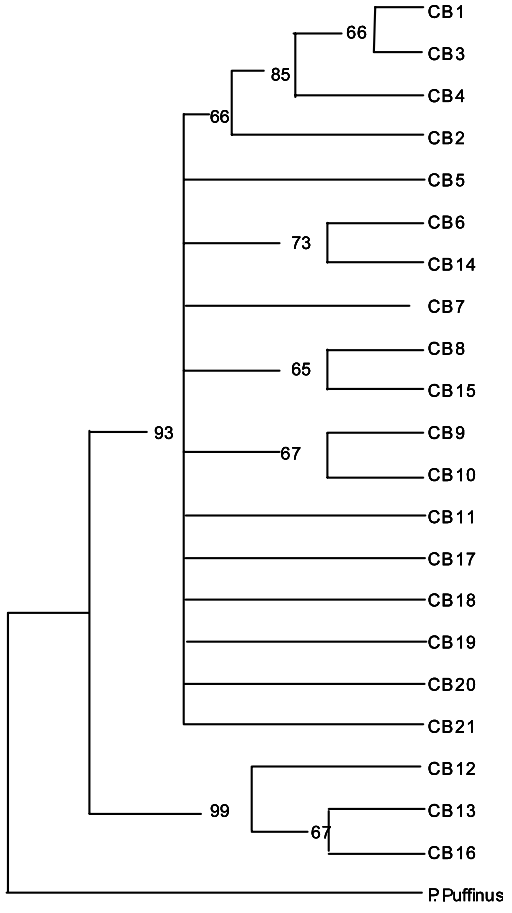

Fig. 2 – Neighbour-joining reconstruction using corrected distances inferred from Cytochrome *b* haplotypes. Nodes with bootstrap support above 50% are shown.

The average distance between Balearic and Yelkouan cyt *b* haplotypes was equal to 0.94%, which translates into an approximate age of the most recent common ancestor of about 1 million years if the specific rate for Procellaridae available for this DNA region is used.

The level of genetic variation in the Balearic Shearwaters was obviously higher when all individuals, including those with Yelkouan haplotypes, were analyzed (Table 2). However, even when the Yelkouan haplotypes were excluded, both gene and nucleotide diversities were comparable with or higher than those values observed in several non-endangered bird species as *Oporornis tolmiei* (Mila et al., 2000), *Sterna fuscata* (Qu et al., 2005), *Calidris alpina* (Wenink et al., 1993), *Alca torda* and *Uria aalgae* (Moum and Arnason, 2001) *Somateria mollissima* (Tiedemann et al., 2004), *Aquila heliaca* and *Milvus milvus* (Roques and Negro, 2005). We did not observe any evident reduction of genetic variation even within colonies (Table 2). The only exceptions are Sa Cella and one colony from Cabrera with small sample size. In both colonies, low diversities were observed, though only at the cyt *b* fragment.

3.2. *Demographic inferences*

The presence of Yelkouan haplotypes in more than half of the Menorca individuals, but almost absent beyond, clearly introduces a strong geographic structure of the genetic variation. In fact, the molecular *Fst* value (estimated considering both allele frequencies and sequences) was significant and equal to 0.36 and 0.16 for cyt *b* and CR, respectively (see Table 3). The co-occurrence of highly divergent haplotypes from two species almost exclusively in Menorca is responsible for this result, and it also suggests that emigration from this island to the other islands, at least recently and for individuals with yelkouan haplotypes, was low. However, when either the Yelkouan haplotypes or all the Menorca individuals were excluded, *Fst* dropped to non-significant values (see Table 3). Also, pairwise *Fst* comparisons, computed considering the molecular distance between alleles, revealed low differentiation between colonies, with only the most southern group (Pitiüses) showing significant *Fst*values of 10.0% ($P < 0.05$) and 6.5 % ($P < 0.05$) in the cyt *b* comparison with Menorca and Mallorca, respectively. With the maximum likelihood method, we can derive estimates of the migration parameter *M* (see supporting material), and since the confidence intervals of most estimates were large, we tried to reduce them by combining the estimates obtained from the different mtDNA regions. The values reported in Fig. 1 were simply obtained by averaging the two estimates, after multiplying by 5 the *M* values observed for the CR (where the mutation rate is about five times larger than in cyt *b*, as suggested by the mismatch analyzes on the complete data sets: see below). These figures should be considered in a relative, rather than absolute, way. However, an idea of their absolute meaning can be achieved after multiplying them by 10^{-3}. This is the order of magnitude of the $N\mu$ parameter (cyt *b*, per site) as estimated by Migrate, and the product of this value and *M* will give to the more usual *Nm* parameter. The patterns of migration were not symmetric; for example, the gene flow from Cabrera to Mallorca colonies was much lower than that from Mallorca to Cabrera. As expected, Menorca was the most isolated

Table 2 – Genetic diversity indices global and at population level in Balearic shearwater (excluding those individuals with introgressed haplotypes)

	N	Hapl	S	$H^{\wedge}$	Nuc.div (%)	Mean pairwise diff
Cytochrome b						
Menorca	9	6	7	0.89 ± 0.09	0.26 ± 0.00	2.29 ± 1.38
Malgrats	19	8	9	0.89 ± 0.03	0.298 ± 0.02	2.610 ± 1.46
Sa cella	16	4	4	0.44 ± 0.14	0.092 ± 0.00	0.81 ± 0.61
Cabrera,Blanquer	10	4	4	0.73 ± 0.12	0.127 ± 0.10	1.11 ± 0.79
Cabrera,Llumeta	5	1	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eivissa, Conillera	20	7	7	0.75 ± 0.08	0.137 ± 0.10	1.26 ± 0.83
Espardell	5	3	3	0.70 ± 0.22	0.135 ± 0.12	1.20 ± 0.91
All Balearic Shearwaters	105	16	23	0.81 ± 0.03	0.4 ± 0.00	3.52 ± 1.81
B. s no introgressed	89	15	15	0.77 ± 0.04	0.18 ± 0.00	1.62 ± 0.97
Control region						
Menorca	8	4	17	0.82 ± 0.10	2.4 ± 1.44	7.25 ± 3.80
Malgrats	14	11	28	0.96 ± 0.04	2.87 ± 1.58	8.61 ± 4.23
Sa Cella	9	7	25	0.94 ± 0.07	3.56 ± 2.1	9.7 ± 4.91
Cabrera,Blanquer	9	7	26	0.94 ± 0.07	3.06 ± 1.8	9.29 ± 4.72
Cabrera,Llumeta	4	4	14	1 ± 0.18	2.4 ± 1.7	7.18 ± 4.27
Eivissa, Conillera	14	9	31	0.91 ± 0.06	2.56 ± 1.4	7.96 ± 3.94
Espardell	5	5	14	1 ± 0.13	2.2 ± 1.43	6.55 ± 3.73
All Balearic Shearwaters	84	43	50	0.97 ± 0.01	3.72 ± 0.02	11.03 ± 5.06
B. s no introgressed	70	34	43	0.96 ± 0.01	2.8 ± 0.014	8.33 ± 3.91

N, individuals sampled; Hapl., haplotypes found, S, segregating sites, $H^{\wedge}$, gene diversity (Nei, 1987), Nuc.div (%), nucleotide diversity in percentage and mean number of pairwise differences.

Table 3 – Percentage of Variance detected within and among populations and global Fstvalues (P < 0.01 indicated by *) in three different AMOVA analyzes including or excluding individuals with introgressed DNA or excluding all individuals from Menorca

	Within pop (%)	Among pop (%)	Fst
Cytochrome b			
All individuals	64.21	35.79	0.358*
Without introgressed DNA	94.28	5.72	0.057
Without Menorca	93.78	6.22	0.062
Control region			
All individuals	83.95	16.05	0.161*
Without introgressed DNA	100.69	−0.69	−0.007
Without Menorca	101.57	−1.57	−0.016

colony, with both emigration and immigration rates being smaller than in the other islands (see Table 4). Additionally we found a high imbalance between immigration and emigration rates in some groups; Cabrera behaved differently than Mallorca, with the former probably acting as a sink of individuals, and the latter as a source.

The genetic variation in the Balearic shearwater is compatible with a model of sudden demographic expansion. Both Tajima's D and Fu's F_S statistics were negative and significant in the cyt b analysis ($D = -1.47$, $P < 0.05$; $F_S = -8.39$, $P < 0.005$). They were also negative when the CR fragment was considered, but only Fu's F was statistically significant ($D = -0.4696$, n.s., $F_S = -8.889$, $P < 0.001$). The mismatch distributions for both mtDNA regions were compatible ($P > 0.05$) with an expansion model (Fig. 3). The shape of the distribution for the CR looked more ragged, but only two non-contiguous points (at 2 and 10 differences, respectively) were actually responsible for this irregular shape. The expansion parameters estimated from these distributions are the effective population sizes before (θ_0) and after (θ_1) the expansion, scaled by the mutation rate, and the age of the expansion (τ), again scaled by the mutation rate. For the cyt b data, estimates were $\theta_0 = 0.412$, $\theta_1 = 6.149$ and $\tau = 1.68$, whereas for the CR we obtained $\theta_0 = 2.728$, $\theta_1 = 36.464$ and $\tau = 6.664$. Comparisons between the same parameter estimated in both DNA regions

Table 4 – Total immigration and emigration rates estimated for the four major groups of colonies

	Sum of M values		Sum of M scores	
	Immigration	Emigration	Immigration	Emigration
Menorca	2386	1235	15	14
Mallorca	4561	11993	17	29
Cabrera	8850	4289	25	15
Pitiüses	6514	4794	21	20

The last two columns refer to the sum of M scores, where the score is just an increasing number attributed to an M value after ordering.

suggest that the mutation rate in cyt *b* is between 4 and 6.6 times smaller than that in the CR. Both sets of estimates support an approximately 13–14-fold demographic expansion. Using the calibrated rate of 0.9%/MY for cyt *b*, and a generation time for this species of 11 years, these estimates would imply a change in effective population size (number of females) from about 4700 to about 70,500 after the population expansion, which would have taken place about 212,000 years ago (Confidence Interval, $\alpha = 0.05$: 74,000–546,000 years).

Finally, the NCPA was applied to the cyt *b* haplotype network. Using this region, the statistical parsimony network was able to connect all haplotypes (including those attributed to the Yelkouan species) without loops corresponding to unresolved mutation paths (see Fig. 4). This network among haplotypes showed the clear separation between Balearic and Yelkouan shearwaters haplotypes corresponding with the two major clades detected with the NJ analysis (see Fig. 2). However, inside each species clade (3-1 and 3-2 in Fig. 4), haplotypes were separated by only one or two base substitutions. As expected, in the total cladogram clade, the NCPA rejected the null hypothesis of no geographical association of haplotypes ($P < 0.0001$). The reason was the occurrence almost exclusively in Menorca of individuals with Yelkouan haplotypes. The method could not discriminate between long distance colonization coupled with subsequent fragmentation and past fragmentation followed by range expansion. Based on previous results (see above) and knowledge of the species, we obviously favour the second hypothesis of a secondary contact between species in Menorca. When the Balearic-specific part of the cladogram was analyzed, contiguous range expansion was detected in the larger clades 1-6 and 2-3, while restricted gene flow with isolation by distance was detected in clade 2.2.

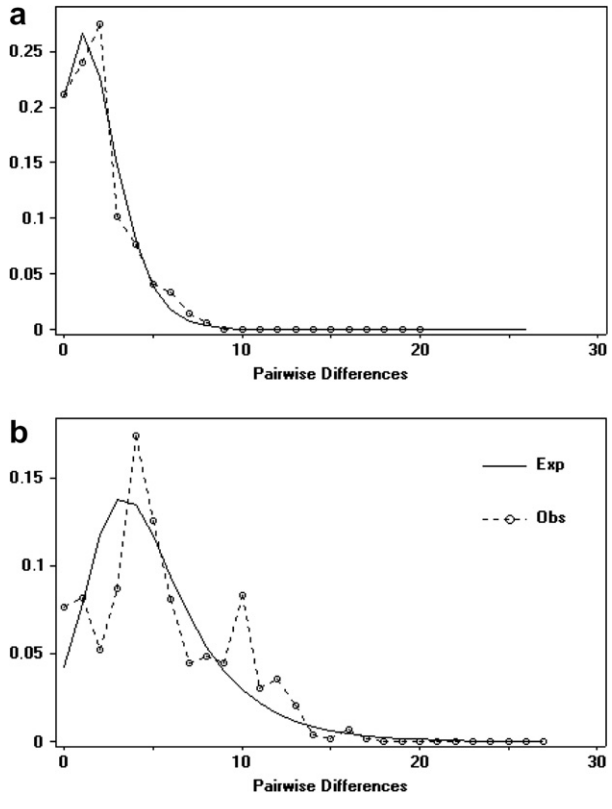

Fig. 3 – Mismatch distributions for mtDNA from Balearic shearwater in (a) cytochrome *b* and (b) partial control region. The expected model is based on a population growth-decline model: (a) ($\theta_0 = 0.412$, $\theta_1 = 6.149$ and $\tau = 1.68$; $P = 0.54$); (b) ($\theta_0 = 2.795$, $\theta_1 = 36.489$ and $\tau = 2.646$; $P = 0.087$) and represented by a continuous line. The observed frequency is represented by a dotted line.

3.3. *Hybridization and morphometric analysis*

Direct evidence of hybridization between individuals with mtDNA haplotypes belonging to the two major clades arose from four mixed couples sampled in Menorca: two males with Yelkouan haplotypes coupled with two females with Balearic haplotypes and two females with Yelkouan haplotypes coupled with males with Balearic haplotypes. The inter-specific hybridization process was further analyzed by comparing the body size index in different groups of birds. The emerging pattern, keeping in mind that "pure" Yelkouan shearwater are smaller than "pure" Balearic shearwater (Bull et al., 2004), can be summarized as follows (see Fig. 5): (i) individuals from Menorca were significantly smaller, but more variable, than individuals from the other islands ($F_{81.3} = 72.63$ $P < 0.001$); (ii) individuals with Yelkouan haplotypes were not significantly different from individuals with Balearic haplotypes from Menorca ($F_{20.3} = 0.120$, n.s.); (iii) confirming results from a previous study (Genovart et al., 2003), males were significantly larger than females in all groups ($F_{81.3} = 44.50$ $P < 0.001$).

4. Discussion

The mitochondrial DNA data, integrated with a morphometric analysis, allow us to suggest possible answers to the questions addressed in this study:

4.1. *Did the recorded decline of census size translate into reduced genetic variation?*

Despite the drastic census decline recorded in the Balearic shearwater, with an estimated mean extinction time of only 40 years (Oro et al., 2004), the genetic diversity indices were similar to those observed in many other non-endangered birds. This is not so surprising if we take into account that a lag time occurs following recent population declines and loss of genetic variation; assuming the actual population size and with a simple computation of the formulae of heterozygosity loss we should wait more than 5000 years to lose half of the heterozygosity in the population. The fossil record suggests that large seabird populations inhabited the Balearic Archipelago in the past, especially in the Pitiüses islands (Florit et al., 1989). The demographic decline of this species probably started after human introduction of alien species, approximately 1500–2000 years ago, but only in much more recent times (last 100 years) have accidental deaths at sea, probably caused by fishing gear, triggered a severe bottleneck (Oro et al., 2004). So it seems that in Balearic shearwater, not sufficient time has elapsed since the critical population decline to leave a genetic signature.

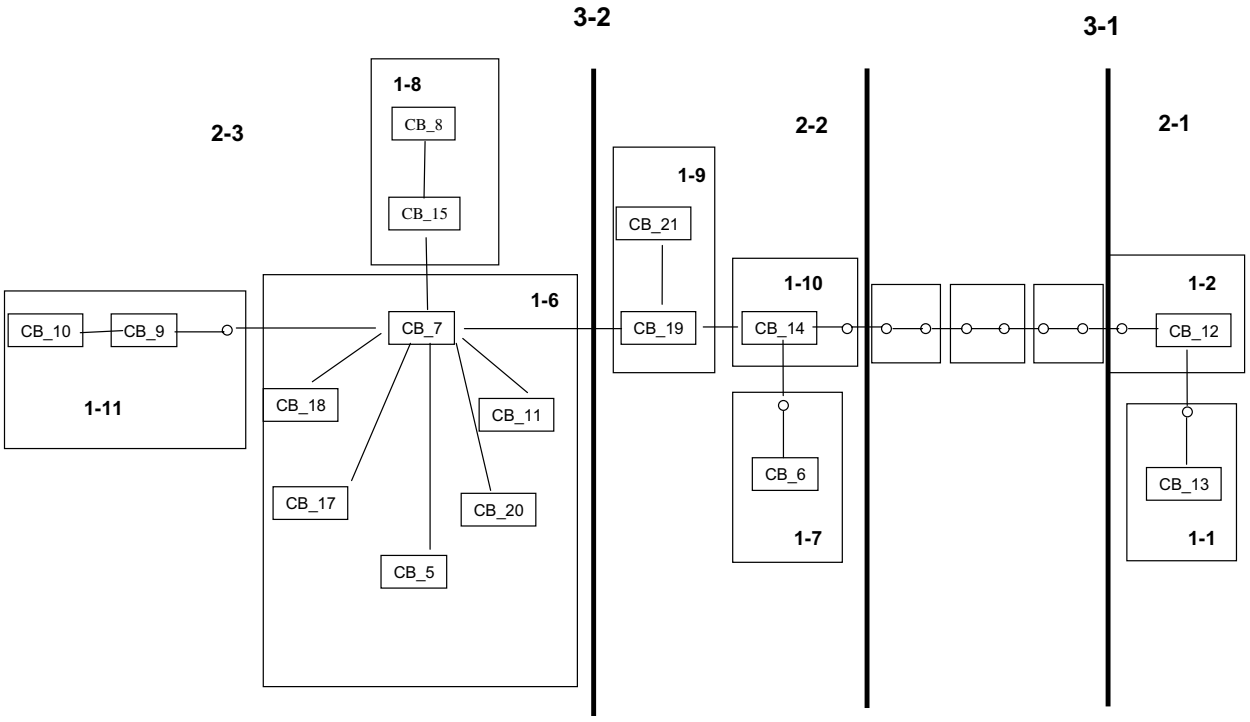

Fig. 4 – Nested clade analysis design derived from parsimony network among haplotypes of Balearic Shearwater for cytochrome *b*.

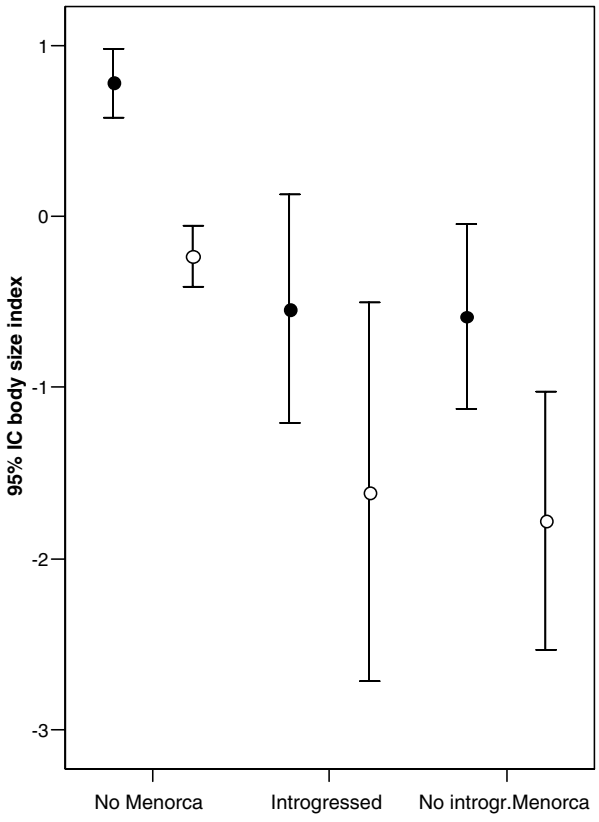

Fig. 5 – Morphometric comparison of body size index of Balearic shearwater between males (solid dots) and females (open dots) and between birds from Menorca and the other Balearic Islands with or without introgressed mtDNA.

4.2. *What are the degree and the origin of genetic connectivity among colonies?*

The introgression of mtDNA haplotypes belonging to a different species, the Yelkouan shearwater, in the most Northern Island of the archipelago prevents a simple answer to this question. When all individuals were considered, the population structure was high. However, when we excluded the highly divergent Yelkouan haplotypes, and considered only the "real" Balearic haplotypes, very little population structure was detected. The island-specific introgression process artificially increased the population structure, but the very similar distributions of Balearic haplotypes in different islands suggest that the average connectivity among colonies is high. This result was unexpected, since different studies (Warham, 1990; Ruiz et al., 2003; Oro et al., 2004) estimated high levels of breeding philopatry in this species. A possible explanation for this connectivity among colonies could be a natal dispersal rate that is higher than expected (especially for females; Greenwood and Harvey, 1982), as suggested for other Procellariformes (Mougin et al., 1999; Martínez-Abraín et al., 2002). Additionally, the analysis presented in this study also pointed to an ancient range and population size expansion, possibly occurring ca. 200,000 years ago, which could have homogenized the genetic variation among the different islands. In agreement with this view, palaeontological data support the idea of a range expansion from South to North: Balearic shearwater fossils from the middle Pleistocene were found only in Eivissa, whereas less abundant and more recent fossils from the Upper Pleistocene were found further north, in Mallorca and Menorca (Florit et al., 1989; Seguí, 1996). Although results could not be explained without some further

gene flow between colonies, this large population size reached in the past (Florit et al., 1989) would have reduced the effects of genetic drift. Finally, when a more sophisticated approach was employed to detect migration rates between groups of colonies, gene flow between colonies was also detected and asymmetries in the dispersal patterns seemed to emerge. Again Menorca was clearly the more isolated colony. Additionally, some colonies showed a high imbalance between immigration and emigration rates, suggesting spatial heterogeneity in the quality of patches and the likely existence of sources and sinks at metapopulation level. For example, Cabrera seemed to act as a sink, principally having immigration from the other colonies but producing few dispersers, whereas the role of Mallorca as a source of recruits (i.e. emigrants) was also clear.

4.3. *How common is inter-specific hybridization?*

Two distantly related mtDNA lineages were detected in the Balearic shearwater, clearly corresponding to the two *Puffinus* species breeding in the Mediterranean region. Whereas almost all the individuals sampled in the central and southern islands of the archipelago showed typical Balearic shearwater mtDNA, the most Northern island (Menorca) presented clear evidence of introgression, with more than half of the birds having Yelkouan lineages. Shearwaters from Menorca had smaller and more variable body sizes than that observed in the other islands, although this difference was not related to the mtDNA lineage of the individuals. This wider variation in Menorca in males and females carrying Balearic mtDNA suggests reciprocal hybridization such that some of the birds are backcrosses or products of hybridizations. Additionally, due to the degree of connectivity found among colonies these morphological features maintained in a single island, would probably not be explained by very ancient contacts, or the stochastic persistence of ancestral polymorphisms. All these results pointed out that the most likely event was an historical secondary contact between the two species several generations ago in Menorca, the colony more close to the range of the sister species, and not followed, at least to date, by the spread of the introgressed mtDNA lineages towards the South. Since the level of genetic variation observed in the Yelkouan haplotypes was very small, the introgression could have involved only a small number of successfully dispersing females. The apparent absence of reproductive barriers between the Yelkouan and the Balearic shearwater is not atypical: several species of Procellariiforms are known to interbreed (Kuroda, 1967; Hunter, 1983; Pierotti, 1987), even in the genus *Puffinus* (Austin, 1996). The fate of species integrity upon natural hybridization is uncertain and depends on the interaction between selection and dispersal (Johannesen et al., 2006) and in our case assortative dispersal between individuals with different mitochondrial lineages could be playing a role.

4.4. *Which is the origin of the divergence between the two Mediterranean Puffinus species?*

The time since the most recent common ancestor between Yelkouan and Balearic mitochondrial lineages was dated to about 1 MY, in agreement with the estimate of Heidrich et al. (1998). This age is clearly incompatible with a very ancient divergence of these species, and can be easily reconciled with a speciation process that occurred in the Mediterranean Sea after the reopening of the Gibraltar Strait (dated at about 5 MY ago) (Heidrich et al., 1998; Zotier et al., 1999). *P. nestori*, now extinct, was found to inhabit Eivissa about 2 MY ago (Alcover, 1988), suggesting that several *Puffinus* species could have originated as a consequence of the colonization of the Mediterranean Sea by Atlantic ancestors.

5. Conclusions and conservation remarks

We suggest that the species diverged from the related Yelkouan shearwater around 1 million years ago, experienced an intense demographic expansion during the middle Pleistocene, and a secondary, probably historical contact with the Yelkouan species produced an inter-specific hybridization in a peripheral area of its range distribution. Thus, the fate of species integrity is uncertain and the possible diffusion of hybrid animals from Menorca should be monitored in the near future. However, a more precise quantification of the origin, timing and possibly evolution of this process could be obtained only by analysing other independent markers and evaluating the genetic variation in Yelkouan shearwaters. An additional conservation goal due to the dramatic situation of the species should be to assure the protection of colonies, not only those acting as population sources (such as Mallorca colonies), but also those detected as historical sinks (such as Cabrera). From a positive point of view, the study shows that the very recent demographic decline in this critically endangered species has not yet decreased its genetic variability, and connectivity found among most colonies should help to reduce species extinction risk. Thus the species is not jeopardized at the moment by genetic factors and the major conservation actions should concentrate at enhancing adult survival (see also Louzao et al., 2006). Although in many cases genetic factors may be playing an important role in the extinction of species, in some others, as in our study, the main threats are rather environmental, at which immediate management efforts should concentrate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2007.02.016.

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