

# Genetic and Phenotypic Differentiation between the Critically Endangered Balearic Shearwater and Neighboring Colonies of Its Sibling Species

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## Abstract

Understanding the demographic and evolutionary processes within and between populations is essential for developing effective management strategies. Thus, for establishing good conservation policies both genetic and phenotypic studies are crucial. We carried out an integrated analysis of genetic and phenotypic characters of the critically endangered Balearic shearwater *Puffinus mauretanicus* (182 individuals) and compared them with those of 2 nearby colonies of Yelkouan shearwater *P. yelkouan* (40 individuals), a species for which hybridization has been hypothesized. The results of the microsatellite analyses were compared with previous mitochondrial DNA analyses. Genetic variability was low in the Balearic shearwater and high levels of inbreeding were revealed at local scale. Most dispersal in Balearic shearwaters was to neighboring sites, even though low levels of population structure were found. The admixture between the 2 species was much higher at nuclear than at mitochondrial level, but phenotypic characters would seem to indicate that a lower level of admixture exists. Individual nuclear DNA, mtDNA, and phenotype did not match at individual level, showing that migration alone cannot explain this phenomenon. We suggest that these 2 young shearwater species could have been involved in processes of divergence and admixing. However, due to the longer coalescence times in nuclear markers, incomplete lineage sorting cannot be ruled out.

**Key words:** conservation, hybridization, incomplete lineage sorting, population structure, *Puffinus mauretanicus*, *Puffinus yelkouan*

The evolution of genetic distinctiveness in populations is the result of the interaction between processes tending to produce local genetic differentiation (mutation, genetic drift, and natural selection) and forces tending to produce genetic homogeneity (gene flow) (Wright 1943; Slatkin 1985, 1987; Grant 1998; Bohonak 1999). Thus, gene flow may either constrain evolution by preventing adaptation to local conditions or promote evolution by spreading new genes and combinations of genes throughout a species' range. Therefore, the degree of genetic differentiation is determined by both historical demography and the amount of contemporary gene flow. Beyond the general aim of assessing the demographic and evolutionary processes in natural populations, assessing genetic distinctiveness is especially relevant in endangered species given that some genetic factors may affect species viability. On the one hand, genetic variation increases the ability of populations to

respond adaptively to future environmental change, while, on the other hand, gene flow may protect populations from processes such as the reduction in genetic diversity due to genetic drift and inbreeding (Frankham et al. 2002; Keller and Waller 2002), even though in some cases it may also prevent local adaptation (Mayr 1963).

Populations which have evolved separately over a long period of time may come into contact and produce a mixed population, thereby incorporating genes from one genetically distinct population into another (Futuyma 1998). This process may also occur between 2 different species, resulting in natural hybridization. In spite of its rarity at individual level, natural hybridization is quite common at specific level (Mallet 2005, 2007), being an important process in the shaping of the evolutionary trajectories of numerous animal and plant clades (e.g., Dowling and DeMarais 1993; Howard 1993; Barton 2001; Johnston et al. 2003; Rieseberg et al.

2003; Grant and Grant 2006). This seems to be particularly common in birds (Grant and Grant 1992; Arnold 1997), in which speciation may involve little genetic differentiation, and in which postmating barriers seem to evolve slower than in other vertebrates (Prager and Wilson 1975; Fitzpatrick 2004). The overall frequency of hybridization in birds is more than 9%, although the distribution of such events is not spread evenly across the 23 orders (Arnold 1997). Despite having been documented in several seabird species, including Procellariiformes (Kuroda 1967; Hunter 1983; Pierotti 1987; Bell 1996; Andersson 1999; Austin et al. 2004; Sternkopf et al. 2010; Brown et al. 2011), natural hybridization seems to be more frequent in terrestrial birds than in seabirds (Arnold 1997).

Natural hybridization can either promote evolutionary divergence between taxa, for example, by reinforcement (Servedio 2004; Hoskin et al. 2005; Urbanelli and Porretta 2008) or prevent it (Mayr 1963; Coyne and Orr 2004); species' integrity will depend on rates of dispersal and gene flow between taxa, as well as on the natural selection acting on the 2 species involved and on the resulting hybrids. Hybridization may then potentially lead to: 1) the establishment of a stable and localized hybrid zone that does not lead to the disappearance of the original species; 2) the disappearance of one of the two original species; or even 3) the appearance of a new species, resulting from hybrid speciation (Mallet 2007). One possible cause of the disappearance of one of the two original species may be due to genetic swamping: the interbreeding between the species can cause a "swamping" of the rarer species' gene pool, creating hybrids that drive the originally purebred native stock to complete extinction.

Thus, attention should be paid to hybridization in endangered species, in part as a way of understanding the factors that influence the evolutionary trajectory of a species and in part because hybridization could lead to extinction or genetic swamping (Rhymer and Simberloff 1996; Allendorf et al. 2001; Genovart 2009). Genetic studies are thus crucial for establishing good conservation policies. However, researchers should not use molecular markers to the exclusion of other phenotypic data that may reveal important information about ecological adaptations—which neutral markers fail to reveal.

The aim of this study was to analyze variation in genetic and phenotypic characters in a critically endangered seabird, the Balearic shearwater *Puffinus mauretanicus*, and compare it to its closest sibling species, the Yelkouan shearwater *P. yelkouan*, with which it is thought to hybridize (Genovart et al. 2005). The taxonomy of this group is much debated and has undergone changes in recent years (Austin 1996); although the Balearic shearwater was once considered to be a subspecies of the Yelkouan shearwater (Sibley and Monroe 1990; Yésou et al. 1990), they are now considered to be different young species that are separable by morphometrics and coloration (Walker et al. 1990; Heidrich et al. 1998; Sangster et al. 2002; Ruiz and Martí 2004). Mitochondrial DNA (mtDNA) has been previously analyzed in the Balearic shearwater (Genovart et al. 2007). Variation in

mtDNA patterns suggest that, after a range expansion during the Pleistocene, the very recent demographic decline in this critically endangered species has not yet decreased genetic variability in its mtDNA. Furthermore, despite its observed philopatry (Aguilar 2000; Ruiz and Martí 2004), a weak population structure has been revealed. Both shearwater species clearly differ in mtDNA, and genetic evidence has been found for maternal introgression from Yelkouan shearwaters into a peripheral colony of Balearic shearwaters (Menorca Island; Figure 1) (Genovart et al. 2007). Multilocus microsatellite data analyses should allow us also to assess male-related patterns, which will be necessary to confirm the hybridization between these 2 species in Menorca.

In this study, we analyzed microsatellite loci and phenotypic characters in the critically endangered Balearic shearwater and in 2 neighboring colonies of Yelkouan shearwater in order to: 1) measure inbreeding and levels of variability in the endangered Balearic shearwater; 2) reveal ongoing demographic processes and population structure in these species; and 3) analyze the relationship between these 2 recently diverged species as a means of studying in more detail the introgression process previously detected in Menorca.

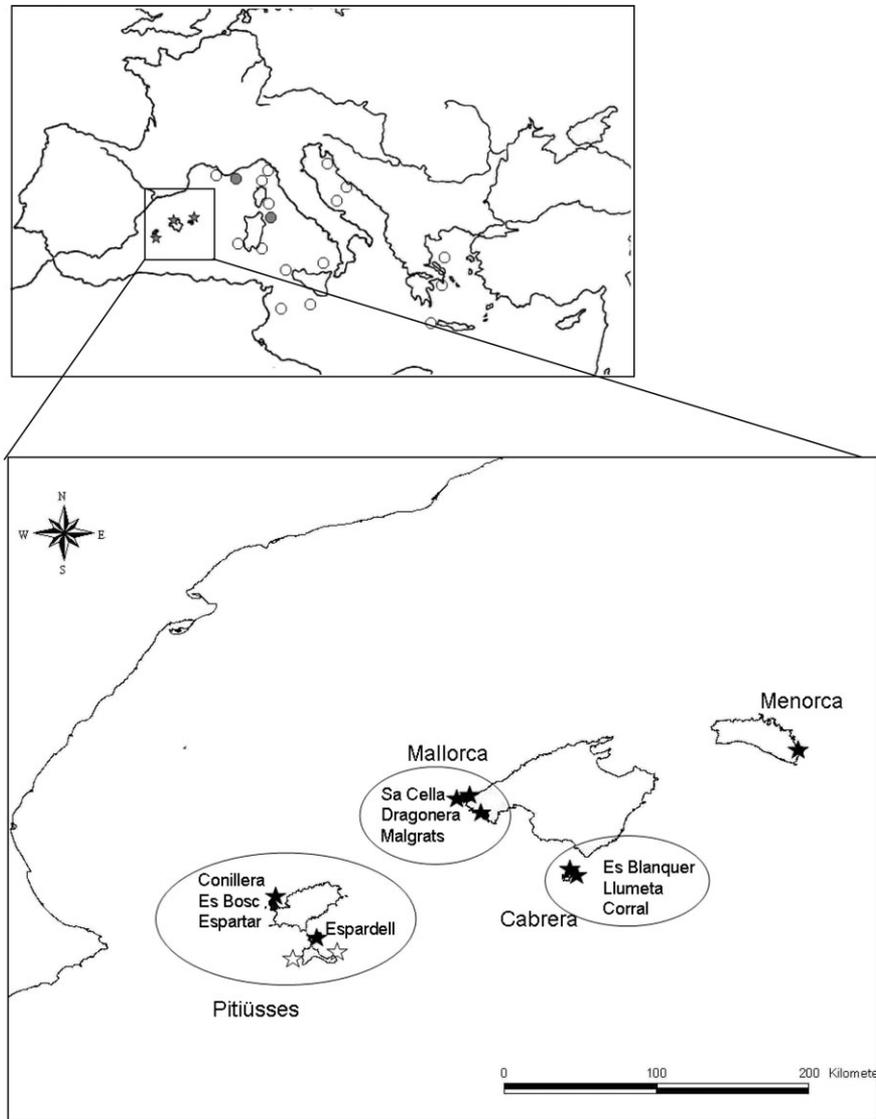
## Materials and Methods

### Species

The Balearic shearwater is a seabird endemic to the Balearic Archipelago, and its entire world population is estimated at just 1800–2000 breeding pairs. It is considered to be critically endangered and recent studies have suggested a mean extinction time for this species of approximately 40 years (Oro et al. 2004). The distribution of the Yelkouan shearwater is larger and includes the central and eastern Mediterranean, as well as the Black Sea (Bourgeois and Vidal 2008), and its population is estimated at approximately 14 000–50 000 breeding pairs (Zotier et al. 1992; BirdLife International 2004; IUCN 2007). Currently, its risk of extinction is considered to be low (BirdLife International 2006; IUCN 2007), although more data is needed as it is still a poorly known species and recent studies have suggested a serious population decline (Bourgeois and Vidal 2008) and very low adult survival rates (Oppel et al. 2011). Except in Menorca, where introgression has been detected (Genovart et al. 2007), no other mixed colonies have been detected and the 2 species can be clearly separated by morphometrics and coloration, since the Balearic shearwater is larger and darker than the Yelkouan (Sangster et al. 2002).

### Sampling and Laboratory Methods

Blood samples were taken from 182 Balearic shearwaters from throughout almost all of its breeding range and from 40 Yelkouan shearwaters from 2 neighboring colonies on the Hyères Islands ( $N = 25$ ) and Sardegna ( $N = 15$ ) (see Figure 1 and Table 1). Sampled adults included in the genetic analysis are also analyzed for morphology and



**Figure 1.** Location of breeding sites of Balearic and Yelkouan shearwaters shown by stars and dots, respectively. Sampled colonies of the 2 species are indicated by solid symbols.

plumage (see below). The total DNA was isolated from ethanol-preserved samples using the standard phenol–chloroform extraction method (Sambrook et al. 1989). As cytochrome b has proved to be a suitable marker for comparing these 2 species (Genovart et al. 2007), we sequenced 800 bp of the mtDNA cytochrome B gene from 3 Yelkouan shearwaters from Sardegna to be compared with previously found haplotypes ( $N = 112$ ) and with fragments obtained from GenBank (accession numbers: DQ230131–230316, AJ004216, AJ004217, AJ004222, AJ004224, and AY219971; see details in Genovart et al. 2007). For nuclear data, shearwaters were genotyped at 8 microsatellite loci (González et al. 2009). Polymerase chain reaction products were analyzed using an ABI3100 automatic sequencer and the ABI software GeneMapper v. 3.7. All the individuals used in the mitochondrial analysis ( $N = 115$ ) were included in the microsatellite analysis ( $N = 222$ ).

### Phylogenetic Analysis

A total of 115 sequences were analyzed, and we used the Manx shearwater (*Puffinus puffinus*) as an outgroup; the best-fitting substitution model was selected using hierarchical likelihood ratio tests implemented in Modeltest 3.06 (Posada and Crandall 1998). Using MEGA software v.4 (Tamura et al. 2007), we calculated the net distances between Balearic, Yelkouan, and Manx shearwaters. A Bayesian posterior probability approach was used to show relationships between taxa using MrBayes software v.3.01b (Huelsenbeck and Ronquist 2001). The Bayesian inference was obtained with random starting trees without constraints. Five simultaneous Markov chains were run for 2 000 000 generations and trees were sampled every 100 generations. We used 1 cold and 3 heated chains per replicate. The model parameters were treated as unknown, thereby allowing

**Table 1** Genetic diversity descriptors, globally and at population level in 11 colonies of Balearic shearwater and 2 colonies of Yelkouan shearwater

		<i>N</i>	<i>P</i>	<i>a</i>	<i>H<sub>e</sub></i> (SD)	<i>H<sub>o</sub></i> (SD)	<i>F<sub>IS</sub></i> (CI 95%)
Balearic shearwaters	Mallorca, Sa Cella	14	6	3.75	0.50 ± 0.32	0.36 ± 0.31	0.29 (0.06–0.43)*
	Mallorca, Malgrats	20	7	4.00	0.53 ± 0.29	0.33 ± 0.24	0.40 (0.21–0.52)*
	Mallorca, Dragonera	10	5	3.13	0.42 ± 0.32	0.30 ± 0.31	0.30 (–0.04 to 0.47)*
	Menorca	26	7	5.00	0.49 ± 0.29	0.34 ± 0.24	0.30 (0.16–0.42)*
	Cabrera, Es Blanquer	35	7	5.00	0.50 ± 0.30	0.37 ± 0.27	0.26 (0.13–0.35)*
	Cabrera, Llumeta	5	4	5.00	0.55 ± 0.35	0.57 ± 0.37	–0.04 (–0.43 to –0.04)
	Cabrera, Corral	9	5	2.5	0.34 ± 0.28	0.29 ± 0.29	0.16 (–0.11 to 0.23)
	Eivissa, Es Bosc	20	6	3.63	0.48 ± 0.30	0.35 ± 0.27	0.26 (0.09–0.38)*
	Eivissa, Conillera	20	6	3.75	0.51 ± 0.32	0.35 ± 0.24	0.31 (0.14–0.41)*
	Eivissa, Espartar	12	6	3.50	0.51 ± 0.32	0.36 ± 0.26	0.30 (0.07–0.40)*
	Formentera, Espardell	11	4	3.75	0.51 ± 0.30	0.43 ± 0.27	0.17 (–0.11 to 0.36)
	Mean			3.91	0.49	0.37	
Yelkouan shearwaters	Hyerès Islands	15	7	3.63	0.50 ± 0.32	0.34 ± 0.30	0.23 (0.11–0.31)*
	Sardegna	25	6	4.63	0.53 ± 0.30	0.41 ± 0.30	0.32 (0.10–0.45)*

*N*, individuals sampled; *P*, number of polymorphic loci; *a*, average number of alleles per locus; *H<sub>o</sub>* and *H<sub>e</sub>*, observed and unbiased expected heterozygosity (Nei 1978); and mean estimates of *F<sub>IS</sub>* (Weir and Cockerham 1984), followed by a 95% confidence interval (95 % CI). Global departure (*P* < 0.01) from Hardy–Weinberg equilibrium is indicated with asterisk.

variable values to be estimated by each analysis. Analyses were repeated in 2 separate runs to ensure that trees converged on the same topology and similar parameters.

All further genetic analysis refers to microsatellite data.

### Genetic Variability

We measured the mean number of alleles per locus, as well as the observed and unbiased expected heterozygosity, and the inbreeding coefficient (*F<sub>IS</sub>*), and tested for deviations from the Hardy–Weinberg equilibrium. We also estimated the variances of *F<sub>IT</sub>*, *F<sub>IS</sub>*, and *F<sub>ST</sub>* using the Jackknife resampling method (Weir 1990); this yields a confidence interval of estimates for each parameter. Using permutations (>1000), we tested for the occurrence of nonrandom associations of pairs of loci (linkage disequilibrium). All analyses were conducted with the program Genetix v. 4.05 (Belkhir et al. 1996).

### Genetic Structure

Using Arlequin v.2 (Schneider et al. 2000), we derived an *F<sub>IT</sub>* pairwise distance matrix between sampling localities (Weir and Cockerham 1984) and estimated their significance levels using permutation tests (>1000 times). We used the sequential Bonferroni correction in tests involving multiple comparisons (Rice 1989). Additionally, we computed a hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) that provides estimates of the percentage of total variance accounted for within and between populations. Statistical significance was determined by >1000 permutations of the genotypes. Given that we found no significant differences between neighboring colonies, we were able to pool colonies of Balearic shearwaters into 4 groups corresponding to 4 major geographical units—Mallorca, Cabrera, Menorca, and the Pitiuses Islands (Eivissa and Formentera) (see

Figure 1),—that were then used as population units. Using these groups, along with the 2 colonies of Yelkouan shearwater, we computed 2 different types of AMOVA analyses: 1) considering only Balearic shearwaters and 2) considering both Balearic and the 2 colonies of Yelkouan shearwaters.

We also examined the population structure and the extent of hybridization using an individual-based Bayesian clustering approach with Structure (Pritchard et al. 2000). This program assumes a model with a specific number of populations (*K*) and then assigns individual genotypes to all the different populations. To evaluate the population structure in Balearic shearwaters, only individuals from the Balearic archipelago were analyzed. We computed 20 runs with values of *K* ranging from 1 to 10. The estimation of *K* was based on Evanno's method (Evanno et al. 2005). To analyze the extent of hybridization between these 2 species, we analyzed individuals from both species, and we forced to run structure under the hypothesis of *K* = 2, that is, a 2-population model, where it is assumed that there are 2 populations (species) contributing to the gene pool of the sample. Putatively admixed individuals can be estimated by assuming that they inherit some fraction of their genome (*q*) from each parental population (Pritchard et al. 2000). Simulations have shown that the threshold *q* value of 0.20 performed better (Vähä and Primmer 2006); thus, if an individual had a *q* value between 0 and <0.20 or >0.80, it was classified as parental, while any individual with a *q* value between 0.20 and 0.80 was classified as a hybrid. In both analyses, the admixture ancestry model was run with the assumption of correlated allele frequencies (Falush et al. 2003) and each replicate was run for 1, 000, 000 iterations after a burn-in of 100, 000 runs. We used the most recent version of this program, which allows a weak population structure to be inferred with the assistance of sampling information (Hubisz et al. 2009).

Patterns of differentiation among individuals were visualized by a factorial correspondence analysis of multi-locus scores (MCA) computed using Genetix v.4.05 (8 loci, 2 factors) (Belkhir et al. 1996). Conventionally, the first axis contributes most to the total inertia and usually displays the differentiation between species. We also specifically looked at the position of individuals from Menorca with Yelkouan mtDNA haplotypes so as to further separate hybridization from simple migration processes.

### Phenotypic Information

Body measurements were collected from 362 adults from 7 colonies of Balearic shearwater and 1 colony of Yelkouan shearwater (Sardagna). All adults were captured in colonies during the breeding period. Head-plus-bill length, minimum bill depth and tarsus length were measured with a digital caliper ( $\pm 0.02$  mm) and wing length with a ruler ( $\pm 0.5$  mm) (for more details on measurements, see Genovart et al. 2003). Individual coloration patterns were registered for 456 individuals. Coloration was described on a scale (1–5) in terms of the extent of the pectoral collar, and the ventral and undertail feather coloration (see also Bretagnolle et al. 2000), with the whitest individuals being classed as 1 and the darkest as 5. More precisely, individuals classed as 1 had no complete collar, white abdomen, and almost white undertail feathers, whereas birds classed as 5 had a nearly complete collar, a darkly mottled abdomen, and dark or mostly dark undertail feathers. For subsequent analyses, we grouped individuals into 3 coloration groups for the sake of simplicity: mostly white (1 and 2), intermediate (3), and mostly dark (4 and 5). Individuals were sexed using either molecular techniques (Ellegren 1996) (ca. 20% of individuals) or applying a specific discriminant function (Genovart et al. 2003).

A principal component analysis (PCA) of morphometrics was used to obtain an index of individual body size (BSI) and a General Linear Model was applied to analyze the relationship between this BSI and coloration pattern, sex and colony.

Another PCA using morphometrics (Head-plus-bill length, minimum bill depth, and tarsus length) and the coloration index was performed to visualize patterns of phenotypic differentiation among individuals. Given that this is a sexually dimorphic species (Genovart et al. 2003), different PCAs were carried out for males and females. We used SPSS version 14.0 and R ([www.r-project.org](http://www.r-project.org)) to perform statistical analyses.

## Results

### Phylogenetic Analysis

The selected model that best fitted the data was a HKY85 with among-site mutation heterogeneity ( $G = 0.016$ ). Accordingly, the Bayesian design allowed for 2 parameters ( $N_{st} = 2$ ) and for the gamma distribution shape to be estimated. The consensus tree distinguished 2 major clades

with high posterior probabilities (Figure 2). Individuals from Sardegna clustered with the Yelkouan haplotypes obtained from GenBank and with the introgressed Balearic shearwaters. From the 16 individuals with introgressed mtDNA, 15 showed the same Yelkouan haplotype, corresponding to 1 haplotype obtained from GenBank (YG12; Figure 2); a single individual had a previously undescribed Yelkouan haplotype (B13; Figure 2). All individuals from Sardegna also had previously undescribed Yelkouan haplotypes (Y2, Y3, and Y4; Figure 2). The second major cluster included all remaining Balearic haplotypes grouped in a well-differentiated “Balearic clade” (Figure 2). The net uncorrected divergence between these 2 Mediterranean taxa was 1% and the p-distances of both taxa from the Manx shearwater were about 2.5%.

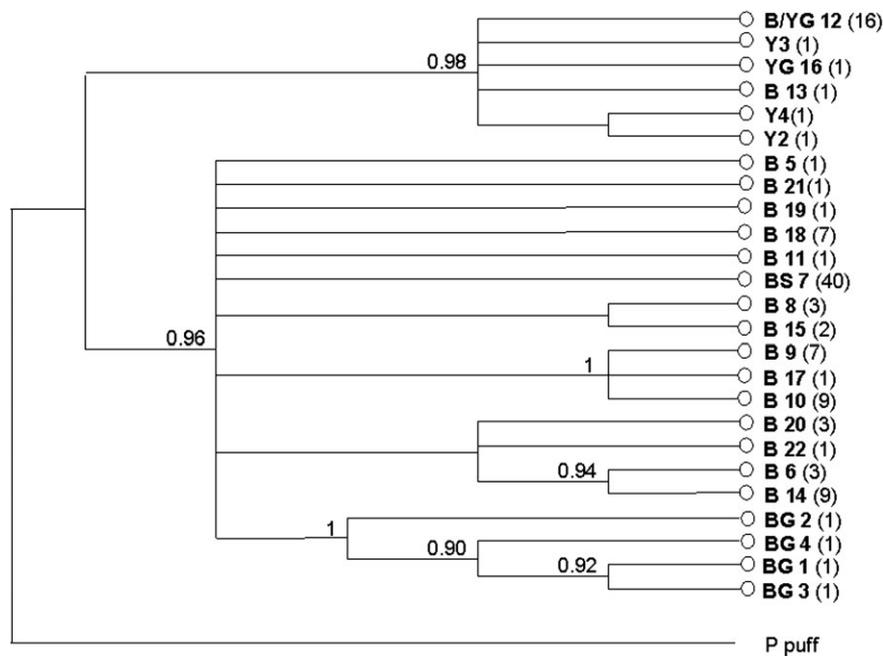
All further genetic results refer to microsatellite data.

### Genetic Variability

The mean number of alleles per locus ranged from 2 to 15, with a mean of 3.91 ( $\pm 0.81$ ) and 4.13 ( $\pm 0.71$ ) for Balearic and Yelkouan shearwater, respectively. The observed and unbiased expected heterozygosity at the 8 microsatellite loci in different colonies are shown in Table 1. We detected a departure from the Hardy–Weinberg expectations of a heterozygote deficiency in all colonies except for the 1 on the island of Espardell and 2 on Cabrera (Llumeta and Es Corral), however, it should be noted that these 3 colonies had the smallest sample sizes. A deficit in heterozygotes may be mimicked by null alleles (Pemberton et al. 1995). This was checked by assuming that some of the homozygotes were heterozygotes for the null allele and that individuals failing to amplify were homozygous for the null allele. This explanation was not supported since the loci with the highest proportions of heterozygote deficiency did not show higher proportions of nonamplifications. Additionally, when the Jackknife procedure was applied, the  $F_{IS}$  values, ranging from 0.21 to 0.32 (global  $F_{IS} = 0.28$ ), did not significantly differ. None of the pairs of loci showed linkage disequilibrium after Bonferroni corrections.

### Population Structure in Balearic Shearwaters

Pairwise genetic comparisons between colonies of Balearic shearwaters had low levels of differentiation and there were no significant differences between neighboring colonies. Significant differences only appeared between Malgrats (Mallorca) and Menorca ( $F_{ST} = 0.28$ ), Malgrats and Llumeta (Cabrera) ( $F_{ST} = 0.32$ ), and Dragonera (Mallorca) and Es Bosc (Pitiüses) ( $F_{ST} = 0.43$ ). However, if the individuals are grouped into the 4 major geographical units (thereby avoiding the highly restrictive sequential Bonferroni corrections), with the exception of Cabrera and Mallorca, and Cabrera and Menorca, the 4 main groups all differ significantly from each other (Mallorca–Menorca  $F_{ST} = 0.039$ , Pitiüses–Menorca  $F_{ST} = 0.030$ , Pitiüses–Mallorca  $F_{ST} = 0.025$ , Pitiüses–Cabrera  $F_{ST} = 0.019$ ; Table 2). Thus, in agreement with previous mtDNA results (Genovart et al.



**Figure 2.** Majority rule consensus tree obtained after the Bayesian inference and posterior probabilities of the relationship between mtDNA haplotypes of the 2 shearwater species (model:  $N_{st} = 2$  and gamma-distributed mutation rates across sites). B: indicate haplotypes observed in shearwaters from the Balearic Islands, BG: haplotypes of Balearic shearwaters obtained from GenBank, Y: haplotypes obtained from shearwaters from Sardegna, YG: haplotypes of Yelkouan shearwaters obtained from GenBank. Only clades supported by posterior probabilities  $>0.90$  are shown. Number between parentheses represents the number of individuals sharing the same haplotype.

2007), Cabrera was found to be the most connected island of all the islands and Menorca the most isolated. In the AMOVA analysis, the overall  $F_{ST}$  value was small but highly significant ( $F_{ST} = 0.031$ ,  $P < 0.0001$ ). The hierarchical model that explained the most variance grouped individuals into 3 main groups corresponding to: Menorca, Mallorca, and Cabrera, and the Pitiüses Islands. Although significant ( $P = 0.02$ ), the variation explained by these 3 groups was low (1.36%) and about 96% of the variation was assigned to variability within populations.

When applying the Bayesian clustering approach, we also chose the model with 3 population clusters as the best fit for our data (Supplementary Figure S1). However, Evanno's

method cannot assess whether or not  $K = 1$  is the best  $K$  and so we cannot reject  $K = 1$  as the uppermost level of structuring.

#### Differentiation between Balearic and Yelkouan Shearwaters

As with mtDNA,  $F_{ST}$  pairwise differences revealed statistically significant differences between the 2 Mediterranean species, albeit of a lower magnitude than expected (Table 2, Supplementary Table S1). Apart for those from Menorca, individuals from the Hyères Islands were significantly different from the Balearic shearwaters ( $F_{ST}$  between 0.04 and 0.067; Table 2). Birds from Sardegna had

**Table 2** Microsatellite DNA  $F_{ST}$  pairwise distance matrices (Weir and Cockerham 1984)

	Balearic shearwater colonies				Yelkouan shearwater colonies	
	Menorca	Mallorca	Cabrera	Pitiüsses	Sardegna	Hyères Isl.
Balearic shearwater colonies						
Menorca	0.000					
Mallorca	<b>0.039</b>	0.000				
Cabrera	0.015	0.013	0.000			
Pitiüsses	<b>0.030</b>	<b>0.025</b>	<b>0.019</b>	0.000		
Yelkouan shearwater colonies						
Sardegna	0.039	<b>0.067</b>	<b>0.064</b>	<b>0.042</b>	0.000	
Hyères Isl.	0.031	<b>0.066</b>	<b>0.058</b>	<b>0.064</b>	<b>0.052</b>	0.000

Distance is calculated between shearwater colonies grouped in the major geographic units (for details, see text). Significant values after permutation test ( $>1000$  times) are shown in bold.

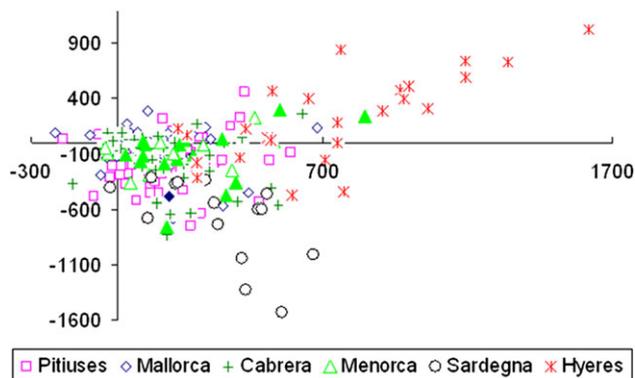
**Table 3** Pure parental individuals (both Yelkouan and Balearic shearwater) and proportion of membership number of each shearwater colonies grouped in the major geographic units in each of the 2 clusters (for details see text)

	Proportion of membership		Pure parental individuals	
	Cluster 1	Cluster 2	Balearic/Yelkouan	N
Menorca	0.57	0.43	3/2	26
Mallorca	0.60	0.40	15/3	44
Cabrera	0.70	0.30	14/0	50
Pitiüsses	0.50	0.50	6/3	63
Sardegna	0.22	0.78	0/8	15
Hyerès Isl.	0.19	0.81	0/17	25

greater differences from individuals from Mallorca and Cabrera than from birds breeding on the Pitiüsses Islands and were not significantly different from those from Menorca (Table 2). In the AMOVA analysis, the overall  $F_{ST}$  value for all populations was also unexpectedly low ( $F_{ST} = 0.042$ ,  $P < 0.0001$ ) and, even though the best models do separate Balearic shearwaters from Yelkouan shearwaters, surprisingly, the main part of the variance (about 95 %) was still explained by differences within populations.

When applying the Bayesian clustering approach forcing  $k = 2$ , one of the clusters was mostly present in Balearic shearwaters and another in Yelkouan shearwaters; nevertheless, most individuals did have mixed ancestry (Table 3).

Representation of the MCA on the 2 principal axes is shown in Figure 3. The first axis of the MCA captured about 38% of the total inertia contained in the data set, while the second captured about 22%. The analysis not only showed some differentiation between Balearic and Yelkouan shearwaters but also between Yelkouan shearwaters from the Hyères Islands and those from Sardegna. When we specifically looked at the position of individuals from Menorca with Yelkouan mtDNA haplotypes, we observed that some



**Figure 3.** Patterns of differentiation among individuals visualized by factorial correspondence analysis of multilocus scores (8 loci, 2 factors). Each colony is represented by a different symbol. Those Balearic shearwaters with mtDNA haplotypes corresponding to Yelkouan shearwater haplotypes are represented by closed symbols.

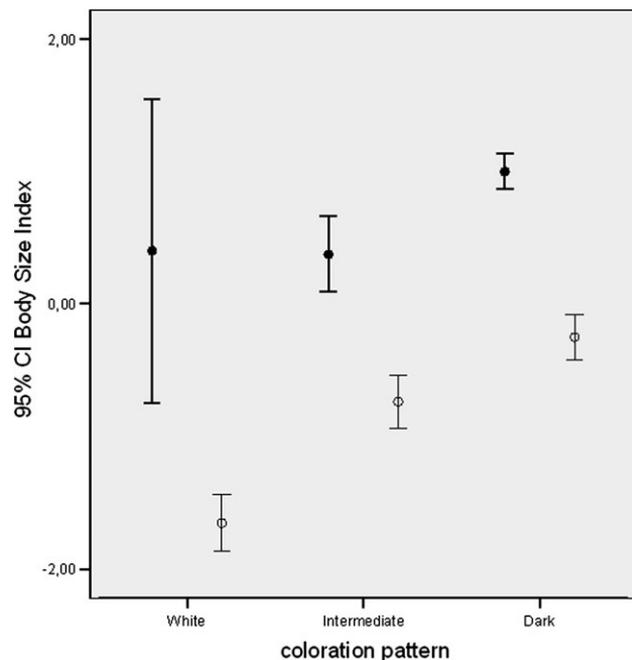
clustered with Yelkouan shearwaters, while others clearly fall into the Balearic cluster (open and closed symbols in Figure 3).

### Phenotypic Variation

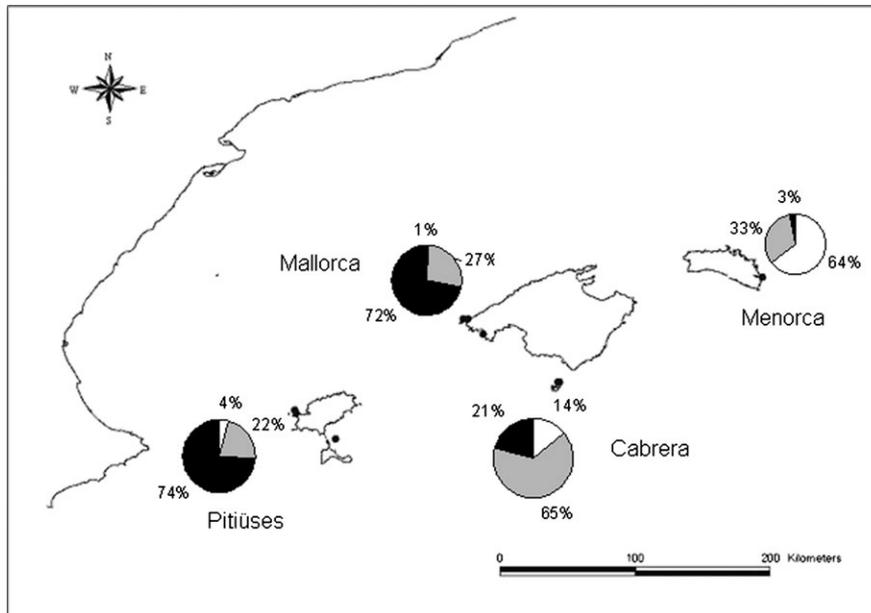
As expected in a sexually dimorphic species, body size was strongly influenced by sex ( $F_{237} = 101.1$ ,  $P < 0.001$ ). We also detected a high correlation between BSI and coloration patterns in both sexes ( $F_{237} = 8.9$ ,  $P < 0.001$ ) (Figure 4), and between BSI and breeding colony ( $F_{237} = 7.47$ ,  $P < 0.001$ ). Coloration pattern also clearly differed depending on the breeding colony (Figure 5), with individuals from Mallorca and the Pitiüsses colonies being darker and those from Menorca whiter (64% of the individuals had Yelkouan shearwater coloration).

PCA allowed us to visualize patterns of phenotypic variation in males and females (Figure 6). Clear differences in the phenotype appear between Balearic shearwaters (excluding those from Menorca) and Yelkouan shearwaters. In males, the first axis of the PCA captured about 47% of the total inertia contained in the data set, whereas the second axis captured about 20%. In females, the differentiation between taxa was more apparent and the first axis of the PCA captured about 56% of the total inertia, whereas the second captured about 18%.

In Menorca, individual phenotypic variation was not correlated with genetics because some of the individuals with Yelkouan phenotype had Balearic-type nuclear or mtDNA, whereas others had Yelkouan-type nuclear or mtDNA.



**Figure 4.** A PCA of morphometrics was used to obtain an index of body size (BSI). Individuals were grouped into 3 coloration groups: white, intermediate, and dark. We detected a high correlation between BSI and coloration patterns in both males (solid dots) and females (open dots).



**Figure 5.** Percentage of individual Balearic shearwaters with white, intermediate, and dark coloration patterns (shown by white, gray, and black, respectively) at different breeding colonies (for details, see Figure 1). Coloration pattern clearly differed depending on the breeding colony.

## Discussion

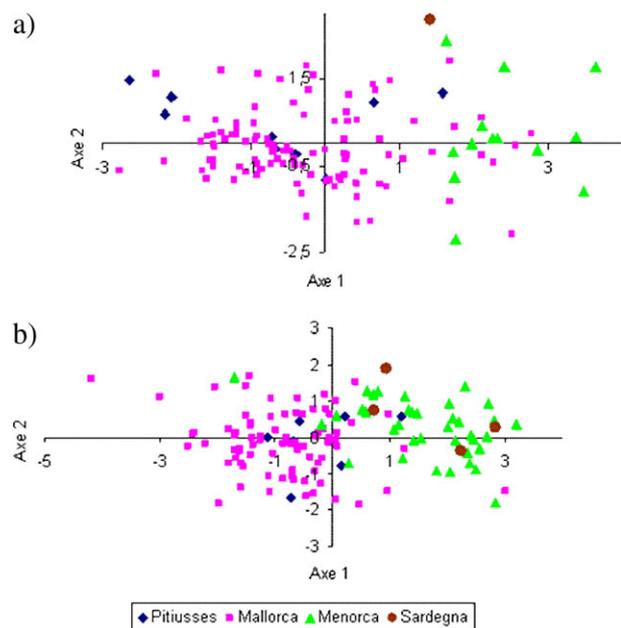
### Genetic Variability in Balearic Shearwaters

Genovart et al. (2007) found high levels of genetic variability in the mtDNA of Balearic shearwaters and concluded that sufficient time had not elapsed since the onset of the critical population decline to leave a genetic signature. In our study, microsatellite data clearly show a lower genetic variability; however, since we have no measure of genetic variability before the recent population decline, we cannot affirm whether or not any loss of genetic variability has occurred. Additionally, the low levels of genetic variability in nuclear DNA may also be linked to Procellariiformes' patterns of philopatry and dispersal, since similar genetic variability has recently been found in 3 other species on the same group (Bried et al. 2007; Milot et al. 2007; but see Genovart Thibault JC, Igual JM, Bauzá-Ribot MM, Rabouam C, Bretagnolle V, unpublished data). High levels of inbreeding at local scale probably reflect not only the effects of philopatry but also the relatively few available breeding sites and the low density of breeders in most colonies. The high levels of inbreeding and possibly, the low genetic variability found in the Balearic shearwater may both have negative effects on the viability of this species and would appear to be a further and an as yet unformulated conservation threat for this species.

### Population Structure in Balearic Shearwaters

Even though the species is assumed to be highly philopatric (Warham 1990), high connectivity does exist between colonies of Balearic shearwaters, as has been previously found in other seabird species (Burg and Croxall 2001; Moum and Arnason 2001). Additionally, in agreement with

other studies of seabirds (Burg and Croxall 2001; Inchausti and Weimerskirch 2002), our results support the idea that most dispersal is to neighboring sites. However, this



**Figure 6.** Plot of phenotypic characters (head-plus-bill length, minimum bill depth, tarsus length, and coloration pattern) from individual shearwaters by first- and second-factor scores derived from Principal Components Analysis (PCA); as shearwaters are sexually dimorphic, a separate analysis was made for males (a) and females (b).

connectivity contrasts with the high levels of inbreeding at local scale, which probably suggests that, while philopatry may exist at local level, emigration acts as a homogenizing force at a larger geographical scale. Similarly, Friesen et al. (1996) found genetic substructuring in thick-billed murres *Uria lomvia* at local level, which contrasted with its genetic homogeneity at a macrogeographical level.

From a conservation point of view, the unexpectedly high levels of gene flow may counteract the effects of genetic drift in small local populations (Slatkin 1985, 1987), as well as facilitating range expansion (Kokko and Lopez-Sepulcre 2006) and helping to resist range contraction (Channell and Lomolino 2000), and as such may potentially play a significant role in maintaining species viability.

### Comparison of Nuclear versus Mitochondrial Data

Even though we detected population structure with both markers, the level of structuring detected with microsatellites was much lower than that previously detected with mtDNA ( $F_{ST} = 0.031$  and  $F_{ST} = 0.36$ , respectively; see Genovart et al. 2007), even when corrections for differences in effective population sizes between markers were made (Brito 2007) ( $F_{ST} = 0.12$ ). The discrepancy observed between both markers in levels of population structure may be linked to several factors. A first possibility would be size homoplasy of alleles leading to an underestimation of between-population differentiation. A second hypothesis would be that due to longer coalescence times, nuclear markers are a more lagging indicators of changes in population structure for populations with relatively short periods of isolation (Zink and Barrowclough 2008). A third possibility is that differential gene flow may occur in males and females. In our case, given the relatively low levels of allelic diversity, microsatellites did not seem to be saturated and so the first of these 3 possibilities can be rejected, and so either nuclear genes are not able to detect a recent fragmentation event, or sex-biased gene flow exists.

### Natural Hybridization

Previous mtDNA analysis has suggested that natural hybridization between Balearic and Yelkouan shearwaters occurs on Menorca (Genovart et al. 2007). Additionally, given that, other than on Menorca, mtDNA haplotypes from the 2 species were reciprocally monophyletic on the gene trees and were separated by a minimum of 10 mutational steps, it would seem likely that historically little or no hybridization has occurred between the 2 species outside Menorca, and that their gene pools have been independent for a long period (at least in the matrilineal line). As well, phenotypic results and some differential behavioral patterns (Ruiz and Martí 2004; Curé et al. 2010) seem to indicate a separation between both taxa for a long period. Unexpectedly, microsatellite markers revealed little differentiation between the 2 species and suggest high levels of gene flow between them, at least between Balearic shearwater and the 2 nearby colonies of Yelkouan shearwaters. It is

noticeable that on Menorca, individual nuclear DNA, mtDNA, and phenotype did not match, suggesting that migration alone cannot explain such phenomenon. We suggest 2 possible explanations: the first is as discussed above, that is, the longer coalescence times in nuclear genes may be disguising a simple diverging process between two recently diverged species. Yet, a second and more plausible explanation is that these 2 young shearwater species could have been involved in processes of divergence and admixing. At present, the secondary contact between the two recently diverging clades would result in gene exchange, leading to increased genetic similarity and thus to an evolutionary web rather than a diverging tree. Nevertheless, if we are to understand fully the relationship between these taxa, genetic and ecological studies of more colonies of Yelkouan shearwater (especially in the eastern Mediterranean Basin) are still required. This would allow us to assess whether the admixture between the 2 species is a general and widespread process or whether it is specific to a limited zone of contact.

Thus, a new issue arises that is of interest above all to conservation managers: are these Mediterranean shearwaters two different species or just one? Similar or even greater levels of divergence have been found between named subspecies (e.g., Cory's shearwaters) (Gómez-Díaz et al. 2006); yet, the same levels of divergence and admixing have been found to occur within named bird species (Crochet et al. 2003; Grant et al. 2004; Helbig et al. 2005). We believe that further studies should be carried out to definitely resolve these taxonomic questions, and that more colonies throughout the breeding range of the Yelkouan shearwater should be genetically analyzed. Before any such additional studies are able to provide more pertinent data, we advise caution and propose that these two taxa, which have been isolated for a long period, should be maintained as separate species, thereby guaranteeing a maximum level of protection for the critically endangered Balearic shearwater.

### Conservation Remarks

New data have emerged from this study that are relevant to the conservation of Balearic shearwaters. On one hand, a possible decrease in genetic variability and high levels of inbreeding at a local scale pose a new potential threat for the species. Although on the other, the high connectivity detected between colonies could improve the species' ability to overcome critical periods, but may also prevent local adaptation. Additionally, we believe that natural hybridization between these two young species is a natural process; yet, despite the fact that some anthropogenic factors, such as a lack of suitable habitat for breeding, may promote hybridization between these two species, hybridization should not be treated as a threat, but as a natural evolutionary process, and no conservation measures need be implemented to prevent it. Nevertheless, due to the critically endangered situation of the Balearic shearwater, immediate conservation efforts should concentrate on enhancing adult survival and protecting breeding areas as a means to inverting the dramatic decline that is occurring in this species.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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