Phylogeography of the *Calonectris* shearwaters using molecular and morphometric data

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Abstract

We investigated phylogenetic relationships and the biogeographic history of the *Calonectris* species complex, using both molecular and biometric data from one population of the Cape Verde shearwater *Calonectris edwardsii* (Cape Verde Islands), one from the streaked shearwater *C. leucomelas* (western Pacific Ocean) and 26 from Cory's shearwater populations distributed across the Atlantic (*C. d. borealis*) and the Mediterranean (*C. d. diomedea*). The streaked shearwater appeared as the most basal and distant clades, whereas the genetic divergences among the three main clades within the Palearctic were similar. Clock calibrations match the first speciation event within *Calonectris* to the Panama Isthmus formation, suggesting a vicariant scenario for the divergence of the Pacific and the Palearctic clades. The separation between the Atlantic and Mediterranean clades would have occurred in allopatry by range contraction followed by local adaptation during the major biogeographic events of the Pleistocene. The endemic form from Cape Verde probably evolved as a result of ecological divergence from the Mediterranean subspecies. Finally, one Mediterranean population (Almeria) was unexpectedly grouped into the Atlantic subspecies clade, both by genetic and by morphometric analyses, pointing out the Almeria-Oran oceanographic front (AOOF) as the actual divide between the two Cory's shearwater subspecies. Our results highlight the importance of oceanographic boundaries as potentially effective barriers shaping population and species phylogeographical structure in pelagic seabirds.
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1. Introduction

Recent phylogenetic studies highlight the role of Pliocene and Pleistocene geological and climate events in shaping most of the present seabird species distributions, as glacial cycles promoted geographic splitting and bottlenecking of populations (Ball and Avise, 1992; Klicka and Zink, 1997; Avise et al., 1998; Moum and Árnason, 2001; Steeves et al., 2003, 2005b). Although major paleogeographic events explain most genus or species level speciation processes, phylogeographic patterns among closely related species would also reflect more complex spatial and temporal ecological interactions. Indeed, both physical and non-physical

boundaries have been suggested to have an important role as speciation barriers. Thus, not only would landmasses promote divergence (Avise, 2000; Steeves et al., 2003, 2005b), but strong breeding fidelity and natal philopatry among seabirds would have favoured allopatric distributions (Dearborn et al., 2003; Burg and Croxall, 2004; Steeves et al., 2005a). However, little is known about the nature of those non-physical barriers and only recently, local adaptation hypothesis have been suggested to explain divergence among some low-latitude seabird populations (Dearborn et al., 2003). In this context, the relative roles of oceanographic features and local selection pressures in delineating the phylogeographic patterns of seabird species remain poorly understood.

Among seabirds, many closely related species and conspecific populations show obvious geographic differences in

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vocalizations, body size and plumage (Newton, 2003). Those diversification patterns can be further examined from an ecological perspective. Habitat heterogeneity may promote diversity depending on how closely seabird species would be tied to their particular marine environment. And climate and oceanography may act as effective barriers promoting ecological speciation by habitat specialization.

In addition, phylogeographic hypothesis can be tested by investigating not only the patterns, but also the times of divergence. Depending on the ages of species divergences and timing of major geological and climate events, assessments can be made as regards which speciation scenario better explains the observed distributions. Given that the distribution of the taxa, considered from either an ecological or historical perspective, is linked to their evolutionary history, phylogeographic analyses based on mitochondrial DNA can help to test biogeographic hypotheses. Indeed, mtDNA has been proved to be a useful tool for testing biogeographic hypotheses and estimating divergence times in a wide range of animal species (Avise et al., 1987; Avise, 2000) including birds (Ball and Avise, 1992; Klicka and Zink, 1997).

Since pelagic seabirds are usually associated with specific marine habitat features (Zotier et al., 1999; Weimerskirch, 2002), they represent a suitable model for assessing the importance of the oceanographic environment from both historical and ecological perspectives. The ecological and historical interplay between the Atlantic and the Mediterranean is particularly interesting for investigating phylogeographic patterns among marine species. Even though the Mediterranean communicates with the Atlantic Ocean through a 14-km wide channel, their physical and biological oceanography is clearly distinct (Longhurst, 1998) and species diversity greatly differs between them (Fredj et al., 1992; Naranjo et al., 1998; Almada et al., 2001), including the diversity of seabird communities (Zotier et al., 1999). In the past, the Mediterranean has been completely separated from the Atlantic Ocean on a number of occasions and in the Late Miocene the Mediterranean was a desiccated deep. When the Messinian salinity crisis ended (5.3 My ago), the Atlantic waters poured into the Mediterranean after the tectonic collapse of the Gibraltar Strait (Krijgsman et al., 1999). According to the fossil record, shearwater and storm petrel species colonized the Mediterranean by that time in the Pliocene (Alcover et al., 1992; Tyrberg, 1999). However, recent studies suggest that it would not be until the Pleistocene, during the biogeographic events of the last glaciations, when closely related species and subspecies began to diverge (Avise and Walker, 1998).

In the present study, we investigate the phylogeographic relationships of the *Calonectris* species complex comparing phylogenetic, morphologic and biogeographic patterns of differentiation. We aim (i) to investigate the relative importance of historical processes, i.e. geological and climate events, driving the speciation processes of seabirds and (ii) to evaluate the role of oceanographic features as effective barriers shaping the phylogeographic structure of pelagic seabirds.

2. Materials and methodology

2.1. The study species

Calonectris shearwaters are pelagic species of northern subtropical seas nesting on isolated islands. The two well-recognized species in this genus are the streaked shearwater (*Calonectris leucomelas*), which breeds around Japan, Taiwan and the east of China and Korea, and Cory's shearwater (*C. diomedea*) breeding on North Atlantic and Mediterranean islands (Warham, 1990). Currently, Cory's shearwater comprises two geographically separated subspecies: the Mediterranean Cory's shearwater (*C. d. diomedea*), nesting from the Iberian coast islands to the Adriatic and Aegean; and the Atlantic Cory's shearwater (*C. d. borealis*), breeding on the Madeira, Salvagens, Canary and Azores islands. Formerly, the endemic Cape Verde shearwater (*C. edwardsii*), breeding exclusively in the Cape Verde archipelago, was also considered a subspecies of Cory's shearwater but it is now regarded as a full species (Hazevoet, 1995), although genetic analyses have not been performed so far.

2.2. Sampling

From 2001 to 2005, blood samples as well as morphometric measures were collected from adult birds on 26 breeding colonies of Cory's shearwater across the Mediterranean and Atlantic region, one breeding colony of the Cape Verde shearwater and one of the streaked shearwater (Fig. 1). Genetic analyses were performed on 57 Cory's shearwaters, 10 Cape Verde shearwaters and 3 streaked shearwaters. In addition, an individual sample from the Manx shearwater *Puffinus puffinus* was included for outgroup comparisons as a sister taxon of the *Calonectris* species (Nunn and Stanley, 1998).

2.3. DNA isolation, amplification and sequencing

DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford et al. (1998). We checked DNA quality and concentration by 0.7% agarose gel electrophoresis, and only templates without degradation signals were included in the analyses. For the mitochondrial Cytochrome *b* gene, we designed four specific primers (L14987/H15685 and L15562/H16025) using previously published sequences of various shearwater species. The Cytochrome *b* gene was amplified in two fragments of approximate lengths of 420 and 680 bp using each of the two following pairs of primers: L14987 (5′CATCTC CGCCTGATGAAACT3′) and H15685 (5′TGCTGGAG TGAAGTTTTCTGG3′); L15562 (5′CCCATTTCACCC CTATTTCA3′) and H16025 (5′CTAGAGCTCCGATAA TGGGGA3′), respectively. We amplified Domain I of the mitochondrial control region of all three *Calonectris* species using the three specific primers that we designed using a few published sequences of various seabird species; either CAL2H (5′CATCCCATCCAACTTAAG3′) or CAL4H
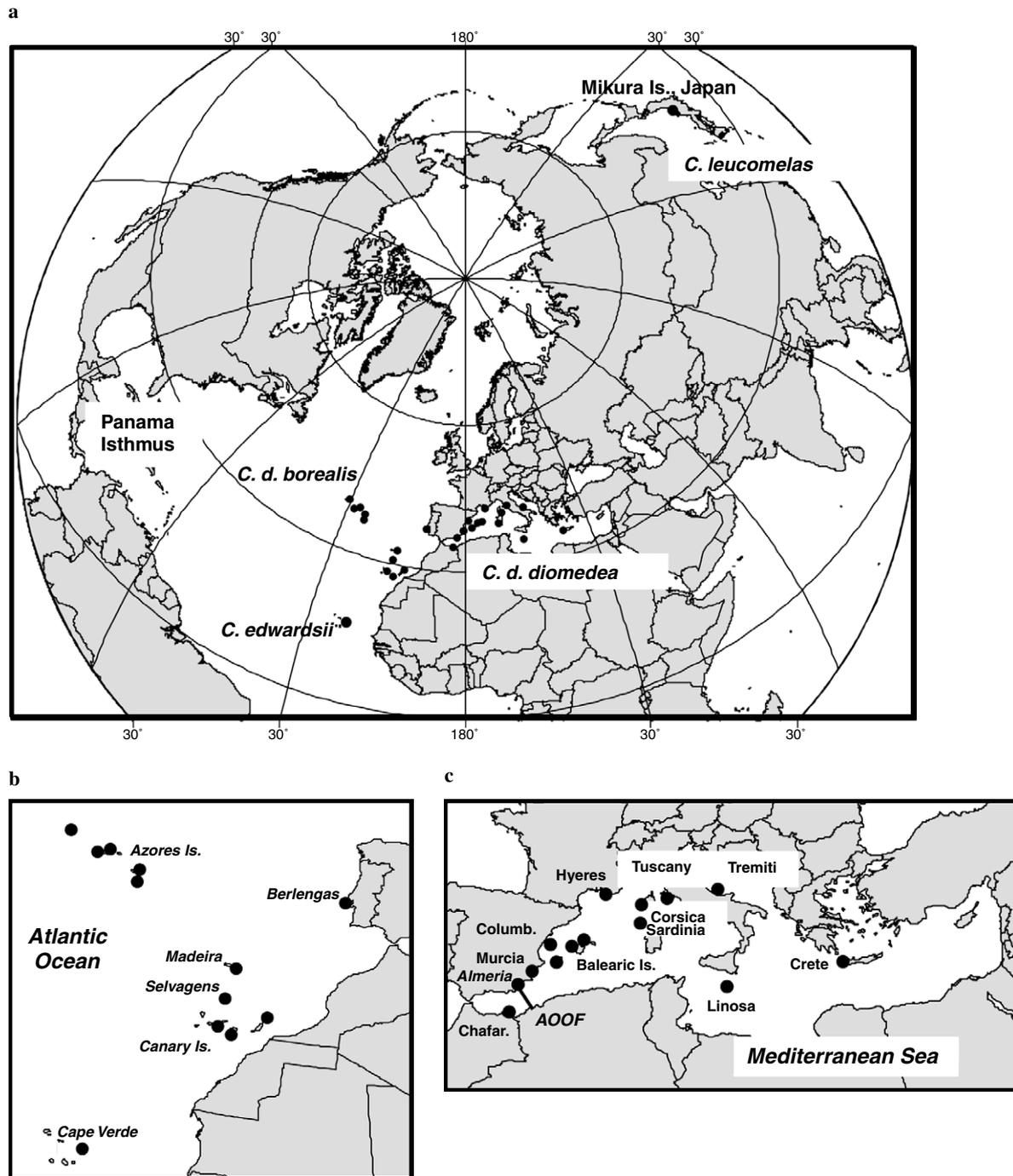
Fig. 1. (a) Geographic distributions of all three *Calonectris* species across the Mediterranean, the Atlantic and the Pacific Oceans. (b) Island populations of the Cory's shearwater and the Cape Verde shearwater sampled across the Atlantic. (c) Island populations of Cory's shearwater sampled in the Mediterranean; the AOOF (Almeria Oran Oceanographic Front) is showed as a more realistic divide between the Cory's shearwater subspecies. Almeria is also highlighted in italics. Balearic Islands include Mallorca, Cabrera, Menorca and Ibiza Islands; Azores Islands include Flores, Sao Miguel, Faial, Graciosa, Corvo and Sta.Maria; Canary Islands include Gran Canaria, Tenerife and Lanzarote Islands.

(5′AGCCTATGTATGGATGTGCAT3′) was used in conjunction with CAL1L (5′GGTCCTGAAGCTAGTAA TAC3′). The PCR was carried out in a total volume of 25 µL containing 40 mM Tris (pH 8.0), 200 mM KCl, 6 mM MgCl$_2$, 0.01% gelatin, 0.4 mM of each primer, 0.15 mM of each dNTP, 2 mM of MgCl$_2$, 0.5 U Bio*Taq* DNA polymerase (Bio-Rad Laboratories) and 40–60 ng of DNA template. Each reaction started with 4 min at 94 °C, then the plate.

amplification was carried out for 40 cycles of denaturation at 94 °C for 45 s, annealing at 60 or 56 °C (for L14987/ H15685 or L15562/H16025 Cytochrome *b* primer pairs) or 58 or 60 °C (for CAL1L/CAL2R or CAL1L/CAL4R control region primer pairs) for 45 s, and extension at 72 °C for 1 min 30 s. A final extension step at 72 °C for 5 min was performed. Amplification products were separated by electrophoresis in 30% acrylamide gels, stained using ethidium

bromide and visualized under UV. PCR products were purified using the JETquick PCR Product Purification Spin Kit (Genomed, Inc., St. Louis, USA). PCR products were sequenced with the same amplification primers on an automated ABI-301 DNA Sequencher (Applied Biosystems, Foster city, USA) using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems). We used Bioedit version 5.0.1 (Hall, 1999) to assemble, edit and align sequences. To assess the reliability of the data, we compared the sequences with previously published data on the studied species. For the Cytochrome *b* gene, two sequences from Cory's shearwater (AY139626 and CDU74356) and one from the streaked shearwater (AF076045) were obtained from GenBank and included in the alignment as a reference sequence for each species, and all variable sites were confirmed by visual inspections of the chromatograms. No genetic data for the *Calonectris* species were available for the control region, and no genetic data were available for the Cape Verde shearwater for either of the two mitochondrial genes. All sequences have been submitted to GenBank (GenBank Accession Nos. DQ372022–DQ372050 and DQ371968–DQ372021).

2.4. Sequence data analyses

For the population level analyses, we defined three groups based on the clustered haplotypes in the phylogenetic analysis corresponding to the Mediterranean, the Atlantic and the Cape Verde populations, respectively (see Results). We calculated genetic statistics at the intraspecific level as the gene diversity index, the number of haplotypes and the number of polymorphic sites using DNAsp 4.0 package (Rozas and Rozas, 1999). Genetic distances for both intraspecific and interspecific levels were calculated using MEGA version 3.0 (Kumar et al., 2005).

2.5. Phylogenetic analyses

We used the partition homogeneity test (Farris et al., 1994; Swofford, 2002) to examine whether there was evidence for different phylogenetic signals between Cytochrome *b* and the control region. No significant differences were found between mitochondrial markers ($P = 0.123$). Thus, we performed all the phylogenetic analysis for a composite sequence of 1250 bp (Appendix 1). However, to test reliability of the phylogenetic signal we also performed independent phylogenetic analysis in each partition set (Page, 1996; Cunningham, 1997).

First, we tested for neutrality for the entire data set and for each gene partition separately using the Tajima's test included in the DNAsp package (Rozas and Rozas, 1999). For both genes and for the combined data set, we used MODELTEST 3.6 (Posada and Crandall, 1998) to search for the best-fit model of nucleotide substitution for our sequence data. Given the likelihood scores of the hierarchical likelihood ratio test, for both genes we selected the model TrN + G using the empirically determined base fre-

quencies, the discrete gamma distribution and the substitution model for among site variation. In the case of the combined data set, the selected model was TrN + I + G. Such models were applied in a maximum likelihood phylogenetic analysis using PAUP*4.0b10 (Swofford, 2002). We also performed a Bayesian analysis using MrBayes v3.0B4 (Huelsenbeck and Ronquist, 2001) for Markov-chain Monte–Carlo Bayesian posterior probabilities. The maximum likelihood model employed six substitution types (Nst = 6). Rate variation across sites was modelled using a gamma distribution, with a proportion of sites being invariant (rates invgamma). The Markov-chain Monte–Carlo search was run with four chains for 2,000,000 generations. Finally, we carried out a parsimony analysis using a heuristic search, TBR branch swapping and random addition of taxa for 100 replicates. For both gene partitions, we differentially weighted substitution types based on inverse of their observed frequency in the original data set. Reliability of the MP trees was assessed by bootstrap analysis (Felsenstein, 1985), involving 1000 replications.

In the case of the Cytochrome *b* gene, to estimate approximate times of cladogenic events in the phylogeny, we first performed a maximum likelihood ratio test to determine whether the sequences were evolving according to a molecular clock. We compared the log likelihood of the tree without enforcing the molecular clock with the log likelihood of the tree constructed under molecular clock assumptions. The likelihood ratio test ($-2(\log \text{Lik1} - \log \text{Lik0})$) was used to test for significance of the difference between the likelihoods of the two trees. We then calculated 'net' sequence divergence (Kimura two parameters model; Kimura, 1980) between main clades using MEGA 3.0 (Kumar et al., 2005). Divergence rates were converted to absolute times by using a rate of 0.90% sequence divergence per My estimated for the Cytochrome *b* gene for the intermediate-sized *Procellaridae* (Nunn and Stanley, 1998). This rate was estimated from first-appearance fossils and the maximum divergence within the group which would make their rate estimates biased in the direction of slower rates. Nevertheless, dates based on this calibration should be the most useful approximations to estimate the time between branching points on the phylogeny (García-Moreno, 2004).

2.6. Morphometric analysis

For the morphometric analysis, we performed a clustering analysis on the biometric data to assess the population structure and the degree of morphologic differentiation among Cory's shearwater populations as well as with the Cape Verde and the streaked shearwaters. The average value of four biometric measures (bill depth at nostril and tarsus, bill and wing length) from 27 island populations was included in the analysis, combining our data with those compiled from literature (Appendix 2). Male and female means were corrected by subtracting the species mean for each sex from the corresponding mean for each population.

Then, to allow for the comparison of different biometric measures, we standardized each variable in the whole data set by subtracting the overall mean for each variable and dividing by the standard deviation. Using NTSYSpc version 2.1 (Rohlf, 1997), we calculated the average taxonomic distance for all pairwise combinations of populations and constructed a cladogram from the dissimilarity matrix using a neighbour-joining clustering analysis. To investigate the existence of latitudinal and longitudinal patterns in morphology, we tested the correlation among the population mean values for each biometric measure with the corresponding latitude and longitude coordinates of each population. We performed the correlation analyses by sex and considering each Cory's subspecies separately using the SPSS 12.0 package.

3. Results

3.1. Sequence data

For the control region data set, among the 69 sequences of 28 populations, 59 out of 79 (74.6%) variable sites were parsimony informative (Appendix 3b). We found 52 different haplotypes for the *Calonectris* species analysed: 25 for the Mediterranean Cory's shearwater; 19 for the Atlantic Cory's shearwater; 6 for the Cape Verde shearwater and 2 for the streaked shearwater. For the Cytochrome *b* gene sequences, 52 out of 114 (45.6%) variable sites were parsimony informative (Appendix 3a), resulting in 27 different haplotypes: 11 from the Mediterranean and 11 from the Atlantic Cory's shearwaters; 5 from the Cape Verde and 2 from the streaked shearwaters. In both the genes, no haplotype was shared among the two Cory's shearwater subspecies, the Cape Verde and the streaked shearwater. However, remarkable differences were found in the degree of variability obtained for each gene partition. In the control region, the average pairwise sequence divergence of all *Calonectris* populations (Kimura two parameters model; Kimura 1980) was 4.8% (S.E. 0.007; Bootstrap 1000 replicates). The streaked shearwater appeared as the most distant clade showing 12% (S.E. 1.1%) sequence divergence from the Cape Verde shearwater and 11% sequence divergence (S.E. 1.8–1.9%) from the Atlantic and the Mediterranean Cory's shearwater. The sequence divergence estimated between the Atlantic and Mediterranean shearwaters was the lowest, 5.4% (S.E. 1%); but similar to the divergence from the Mediterranean subspecies to the Cape Verde shearwater (5.7%; S.E. 1.2%), whereas the divergence between the Atlantic subspecies and the Cape Verde shearwater was slightly greater (6.6%; S.E. 1.4%). In the Cytochrome *b* gene, the average pairwise sequence divergence of all *Calonectris* populations (Kimura two parameters model; Kimura 1980) was 1.1% (S.E. 0.002; Bootstrap 1000 replicates). According to the Cytochrome *b* estimates, the streaked shearwater appeared as the most divergent clade showing 3.2% (S.E. 0.6%) sequence divergence from the Cape Verde shearwater and the Mediterranean Cory's shearwater, and a slightly greater sequence divergence (3.4%, S.E. 0.06%) from the Atlantic Cory's shearwater. The sequence divergence estimated between the Atlantic and Mediterranean shearwaters was 1.1% (S.E. 0.1%) similar to the divergence from the two Cory's shearwater subspecies to the Cape Verde shearwater (1.1%; S.E. 0.3%).

3.2. Phylogeography

Bayesian, parsimony and maximum likelihood analyses on the combined data set grouped haplotypes into four main clades corresponding to the four major taxa conventionally accepted (Fig. 2). Besides the maximum likelihood analyses for either, the Cytochrome *b* gene (Appendix 4b) and the mitochondrial control region (Appendix 4a), supported a similar tree topology. Moreover, all the analyses consistently grouped populations into four clades that were statistically well supported. The main division of the trees separated a Pacific clade (the streaked shearwater) (BS 100%; MC Bayesian posterior probabilities) from a Palearctic clade (BS 100%), which included the two Cory's shearwater subspecies together with the Cape Verde species. Within the Palearctic clade, the Mediterranean Cory's shearwater grouped together with the Cape Verde shearwater in a single clade (BS 100%), separated from the Atlantic Cory's shearwater populations in a second clade (BS 100%). Although independent phylogenetic analyses on the control region agreed with those on the combined data set, phylogenies based on Cytochrome *b* grouped the Cape Verde species, the Mediterranean and the Atlantic Cory's shearwater subspecies in three distinct poorly supported clades (BS 84%, BS 62% and BS 55%, respectively) (Appendix 4b). As regards population structuring, within the Palearctic clades a few other internal nodes were well supported (Fig. 2). For the Mediterranean clade (BS 100%), two subclades were differentiated and four groups of haplotypes (BS 100%, BS 100%, BS 99% and BS 96%) grouped apart from the main clade. The Atlantic clade (BS 100%) was slightly less structured and some Atlantic haplotypes grouped into a well-supported subclade (BS 100%). Finally, haplotypes within the Cape Verde clade (BS 100%) also appeared slightly substructured and split into two distinct subgroups (BS 100 and 98%) (Fig. 2).

Overall, the phylogeographic structure obtained for the two gene partitions and the combined data set agree with the spatially segregated distributions of the *Calonectris* clades. However, the haplotypes corresponding to one Mediterranean population (Terreros Is., Almeria, Spain) were placed within the Atlantic cluster.

The results of the Tajima's test were not significant considering both Cytochrome *b* and the control region (Tajima's $D = -1.02$; $P > 0.10$ and $D = -0.89$; $P > 0.10$, respectively) providing evidence that the DNA sequences of the three *Calonectris* species agree with neutrality expectations. As regards the mode of evolution, the results of the maximum likelihood ratio test for the Cytochrome *b* gene confirmed that sequences were evolving according to a
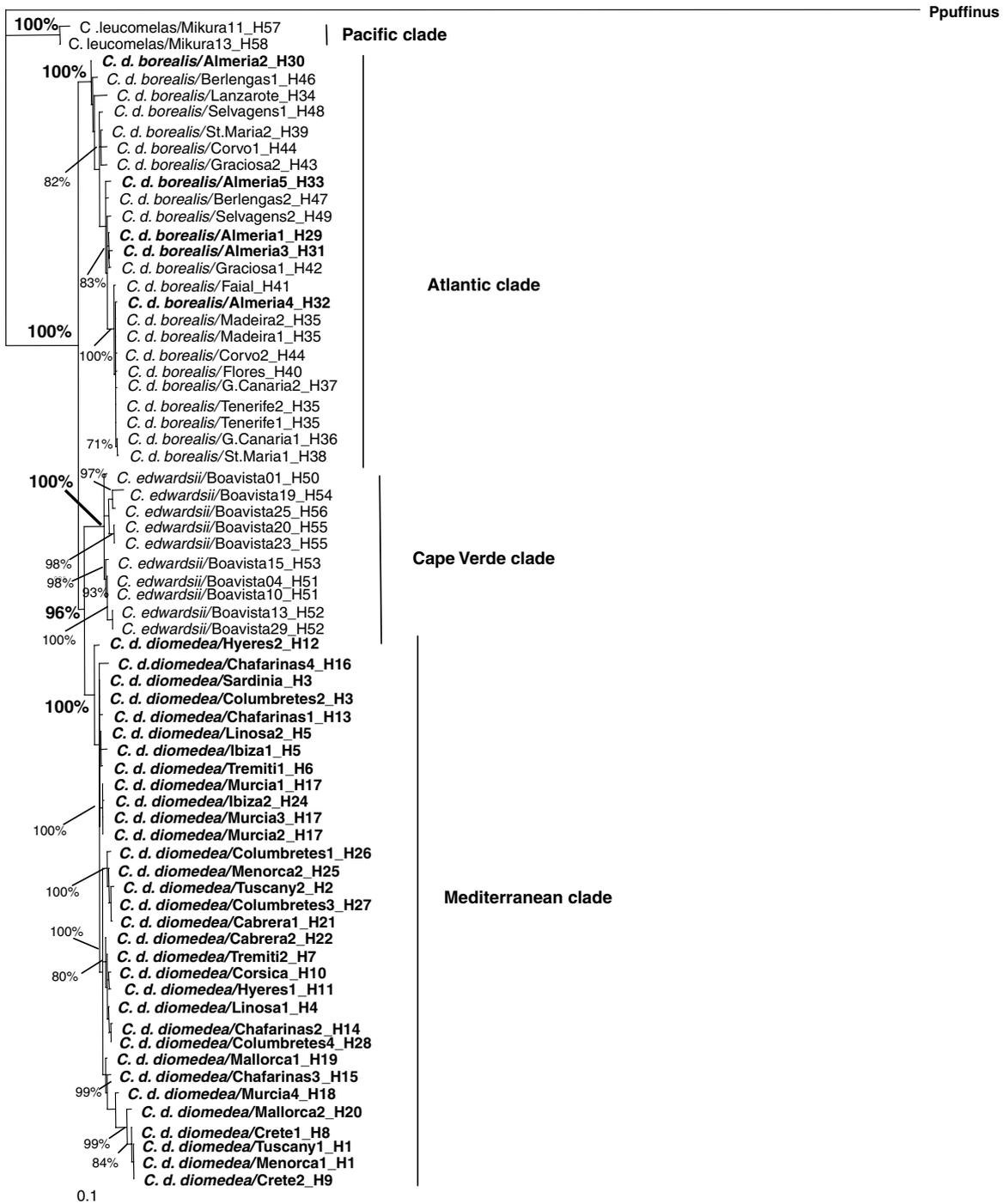
Fig. 2. Maximum likelihood phylogenetic tree for *Calonectris* shearwaters based on 1250 combined bp of the mtDNA Cytochrome *b* gene and the control region. Numbers adjacent to branches are Monte–Carlo posterior probabilities greater than 70%, and main clade support is indicated in bold. Each sequence is labelled with the species and the geographic location. Mediterranean, Atlantic and Pacific populations are indicated in bold, italics and normal font, respectively. Note that individuals from Almeria (Mediterranean) were classified within the Atlantic clade.

molecular clock. The comparison between the log likelihood value (−2154.22) of the tree without enforcing the molecular clock and the log value of the same tree constructed under molecular clock assumptions (−2170.51) did not differ significantly (likelihood ratio test statistic = 32.56; DF = 70; *P* = 0.95). For each subspecies that was phylogeographically subdivided, we estimated net sequence divergence between major phylogroups thus attempting to

correct for within-group diversity (Avise et al., 1998). Then, we converted Cytochrome *b* net sequence divergence to an estimate of population separation time using the calibration rate for the *Procellariidae* family. The separation between the *Calonectris* species and its *Puffinus* sister clade would have occurred 9 My ago. The first speciation event within the *Calonectris* species complex which separated the Pacific and the Palearctic clades took place approximately

3.44 My ago. Within the Palearctic clade, the Atlantic, the Mediterranean Cory's shearwater subspecies and the Cape Verde shearwater coalesced into a common ancestor much more recently, between 900,000 and 700,000 years ago. Slight differences in divergence times among the three Palearctic subclades together with the outgroup comparisons suggest that the Atlantic Cory's shearwater was the ancient clade, whereas the Cape Verde shearwater and the Mediterranean Cory's subspecies seemed to be more recently derived clades. Nevertheless, the estimated dates based on the Nunn and Stanley (1998) fossil-calibration rate should be considered as approximate taking into account the bias associated with the method for calibrating the evolutionary rates (as is described in Methods) as well as the errors associated with the fossil record, both phylogenetic uncertainties and dating geologic errors (García-Moreno, 2004).

3.3. Biometric structure of the Calonectris species

We examined differences in body measurements among Cory's shearwater populations, the Cape Verde shearwater and the streaked shearwater as a separate species. The cladogram represents the similarity pattern of the 27 island populations included in the analysis, considering four biometric measures (Fig. 3). Agreeing with the phylogenetic analysis, four groups were clearly defined corresponding to each of the two subspecies of Cory's shearwater, the Cape Verde shearwater and the streaked shearwater. The first cluster grouped populations corresponding to the clade with the largest body size, the Atlantic Cory's shearwater. In line with the genetic analyses, one Mediterranean population, Almeria, appeared as morphologically similar to the Atlantic populations being set within the Atlantic cluster. Apart from Almeria, all Mediterranean populations grouped together in a single cluster corresponding to the intermediate in body size, the Mediterranean Cory's shearwater. Finally, the medium-sized streaked shearwater and the smallest in size Cape Verde shearwater were grouped separately. The Cape Verde and the streaked shearwaters appeared as a different morphospecies, whereas the Atlantic and the Mediterranean subspecies turned out as morphologically more similar. Moreover, the neighbour-joining
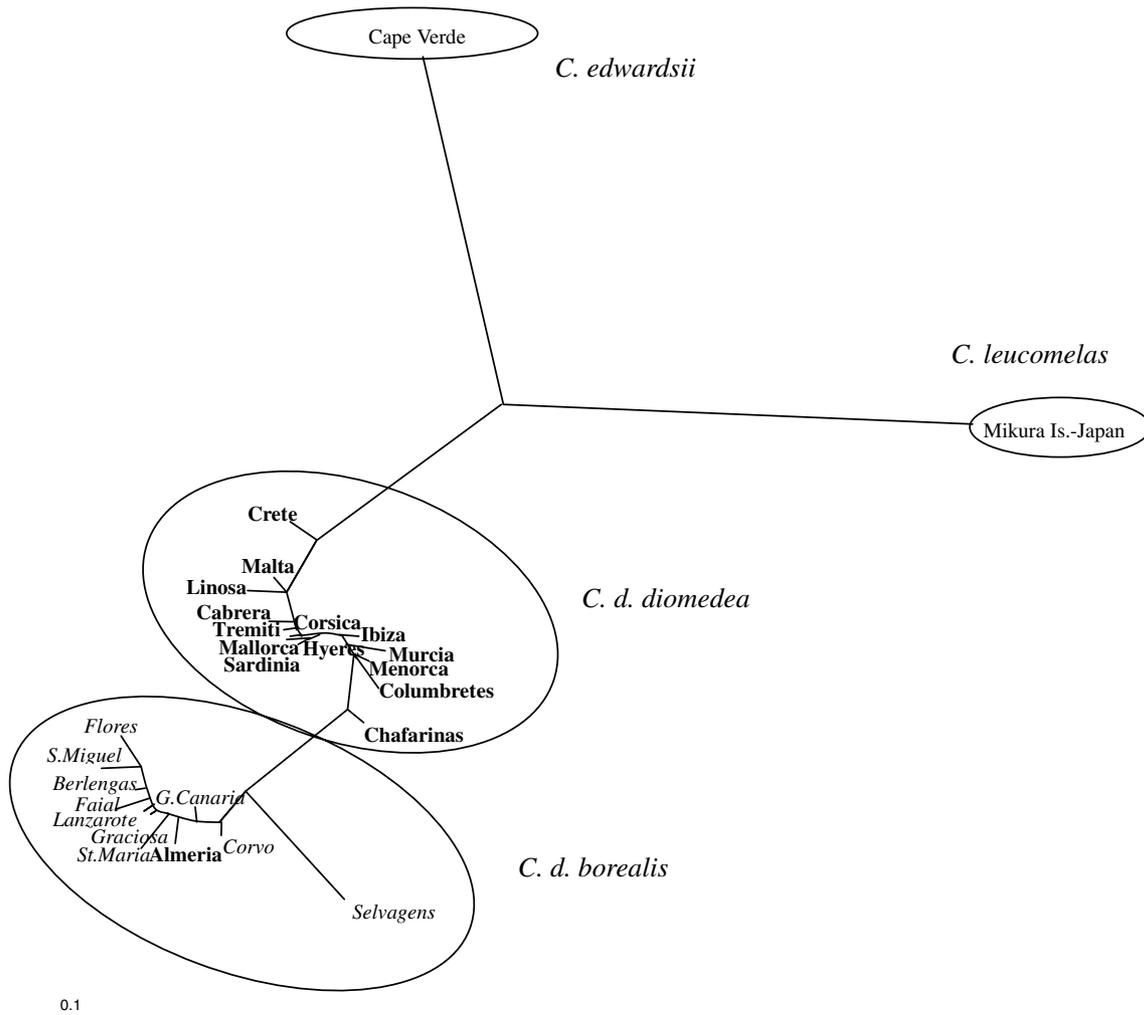

Fig. 3. Neighbour-joining tree showing the similarity pattern among the 27 populations included in the biometric analysis. Grouped populations representing different morphospecies are shown. Mediterranean populations are indicated in bold and Atlantic populations in italics.

clustering analysis suggested the existence of a biometric structure within Cory's shearwater across its geographical distribution (Fig. 3), not manifested in the phylogenetic analyses (Fig. 2). That is, we could detect a geographic gradient in morphometrics within the Mediterranean region as a slight increase in body size from east to west, that is, from the most eastern population, Crete, to the most western population, Chafarinas.

The biometric structure within the Mediterranean was also pointed out in the correlation analysis. Indeed, we found a significant positive longitudinal pattern within the Mediterranean subspecies in two of the four biometric measures, for both males and females (tarsus: $R^2 = 0.29$, $n = 14$, $P = 0.047$ and $R^2 = 0.74$, $n = 14$, $P < 0.001$; wing: $R^2 = 0.27$, $n = 14$, $P = 0.055$ and $R^2 = 0.70$, $n = 14$, $P < 0.001$, for males and females, respectively). For the Atlantic subspecies, only one significant correlation was found between the four measures and longitude. However, we found a clear trend to increase the size with latitude in the Atlantic (tarsus: $R^2 = 0.35$, $n = 11$, $P = 0.056$ and $R^2 = 0.31$, $n = 11$, $P = 0.073$; wing: $R^2 = 0.40$, $n = 11$, $P = 0.038$ and $R^2 = 0.36$, $n = 11$, $P = 0.053$, for males and females, respectively).

4. Discussion

4.1. Phylogenetic structure

Not only morphologic but also all the phylogenetic analyses performed grouped populations into four main clusters. Furthermore, general agreement among trees obtained from Cytochrome b and the control region separately and from combining both data partitions would give confidence to the phylogenetic accuracy of the tree topology obtained (Cunningham, 1997). Although both independent data sets supported all four *Calonectris* clades, there were some inconsistencies resolving evolutionary relationships within the Palearctic clade. Although for the Cytochrome b gene, phylogenetic relationships among the three main subclades within the Palearctic appeared unresolved, the control region provided a better estimate for the *Calonectris* shearwater phylogeny, elucidating inner relationships. Conflicting phylogenetic signals between the gene partitions would result from the lack of resolution in the Cytochrome b gene. It could be explained by a faster divergence rate of the control region compared to the Cytochrome b gene. Nevertheless, combining both sequence sets into a single analysis provided the greatest number of relationships receiving strong support. Moreover, the phylogenetic relationships among the three main subclades within the Palearctic agree with those suggested from morphology (Bourne, 1955; Cramp and Simmons, 1977; Porter et al., 1997; this study). Thus, congruence between gene trees together with concurrence in morphology should provide evidences that the phylogeny suggested corresponds to the species tree for the *Calonectris* species complex (Slowinksi and Page, 1999). However, as both sequence sets belong to the mitochondrial genome and could be considered as linked genes, fur-

ther investigations based on nuclear markers are needed to confirm our results (Ballard and Whitlock, 2004).

The phylogenetic structure obtained agreed with the spatially segregated distributions of all four *Calonectris* clades and corresponded to the four major taxa conventionally accepted. Within the Palearctic clade, Atlantic and Mediterranean haplotypes differed from each other as much as from the Cape Verde haplotypes, suggesting long-term geographic isolation and gene flow barriers among all three clades. Besides, biometric patterns of the Mediterranean subspecies suggested an additional morphologic structure across its distribution, previously recognized by other authors (Massa and Lo Valvo, 1986; Granadeiro, 1993). That is, there is a significant increase in body size from the east to the west within the Mediterranean subspecies and a less marked pattern from south to north within the Atlantic subspecies. This pattern is probably related to the complex longitudinal and latitudinal oceanographic subzonation of the Mediterranean and the Atlantic, respectively (Longhurst, 1998). Alternatively, the morphometric gradient within the Mediterranean could be interpreted as a transition cline between the smaller Mediterranean and the larger Atlantic Cory's shearwater forms, but this hypothesis is not supported by the lack of subspecies substructure in the genetic analyses. Nevertheless, further research on population genetic structure is needed to determine the degree of isolation among geographically closer Mediterranean and Atlantic populations of Cory's shearwaters.

Although there was a major correspondence between genetic clades and geographic distributions, interestingly all sequences from five individuals analysed from Almeria (Mediterranean) corresponded to the Atlantic clade (Fig. 2). This assignment is well supported by the multiple fixed differences in the Cytochrome b gene differentiating the Atlantic and the Mediterranean subspecies. The Atlantic identity of the shearwaters from Almeria is further supported by the morphological analyses based on 31 individuals from this locality (Fig. 3). There are some previous reports of Atlantic pairs breeding in the Mediterranean (Lo Valvo and Massa, 1988; Sánchez, 1997; Thibault and Bretagnolle, 1998; Martínez-Abraín et al., 2002), and at least one case of hybridization between the two subspecies (Martínez-Abraín et al., 2002). Nevertheless, we found no evidence of hybridization in Almeria, and given that genetic and morphologic analyses are based on a substantial number of individuals from this colony, we can unequivocally diagnose this breeding population as the first Atlantic population found to breed in the Mediterranean. This result challenges the current view assuming the Gibraltar Strait as the distribution barrier between the two Cory's shearwater subspecies and suggests that the divide between the subspecies better corresponds to the Almeria-Oran Oceanographic Front (AOOF). In fact, the AOOF represents a major oceanographic discontinuity in the Mediterranean, being the real boundary between Atlantic and Mediterranean surface waters (Beckers et al., 1997). The biogeographic importance of this boundary is well reflected in

several genetic studies on marine species with Atlantic–Mediterranean distributions, which exhibit a phylogenetic break in open waters at the AOOF (Pannacciulli et al., 1997; Quesada et al., 1995). Although the Atlantic closest breeding colony is Chafarinas Is., which most breeders actually belong to the Mediterranean subspecies (Molina et al., 2005), Almeria would have a stronger Atlantic influence by the AOOF. Furthermore, Almeria would probably be the first breeding locality reached if we take into account the dominant winds at the Mediterranean entrance. Since Procellariforms, including *Calonectris* shearwaters, largely use winds for their oceanic movements (Schreiber and Burger, 2002), this feature is relevant because prevailing winds probably reflect connectivity among geographic locations better than absolute distances. Thus, our results underline the importance of oceanographic features and prevailing winds on the marine surface as important factors explaining the phylogeographic patterns in seabirds.

4.2. Historical biogeography and speciation mode

According to previous phylogenies (Nunn and Stanley, 1998; Kennedy and Page, 2002), the Pacific clade appeared in the base of the *Calonectris* phylogeny, closer to its *Puffinus* sister clade. Since ancient members of a taxon are expected to be found closer to its geographic origin (Crisci et al., 2003), the obtained phylogeny supports a Pacific origin for *Calonectris*. However, from a biogeographic perspective, the area of highest species diversity has been traditionally considered the centre of origin of the group (Blackburn and Gaston, 1996; Jetz et al., 2004). Thus, the greater diversity in the Atlantic compared to the Pacific Ocean, that is, the presence of two clades in geographically disjunct areas (the Atlantic Cory's and the Cape Verde shearwaters) as well as the presence of a peripheral clade in the Mediterranean, supports an Atlantic origin for the *Calonectris* group. In addition, fossil records and estimates of divergence dates can help to disentangle the biogeographic origin of the *Calonectris* group. We estimated the first speciation event between the Pacific and the Palearctic clade to about 3 My ago. However, *Calonectris* fossils have been described from South Africa (Olson, 1983) and from South Carolina (Olson and Rasmussen, 2001) dating back to the late Miocene and early Pliocene, about 5 My ago, that is, prior to the molecular clock estimate of the Pacific and the Palearctic species separation. Since fossil remains have been identified as belonging to Cory's shearwater, the *Calonectris* ancestor would have been more similar to the Palearctic form and would have been present in the Atlantic since 5 My ago, supporting an Atlantic origin for this group. Indeed, fossil records suggest an Atlantic origin for most procellariform species and subspecies with allopatric forms in the Pacific and the Atlantic oceans (Austin, 2004). However, a Pacific origin cannot be completely ruled out since few data are available as regards for fossil procellariforms in the Pacific.

The estimated separation between the Pacific and the Palearctic *Calonectris* clades dated back to 3 My ago, which corresponds to a major geological event: the separation of the Atlantic and Pacific Oceans by the Panama Land Bridge (Coates et al., 1992). These matching events suggest that vicariance played a major role explaining the speciation between the Palearctic and Pacific *Calonectris* clades. Indeed, a number of genetic studies indicate that the emergence of the Panama Isthmus led to the vicariant speciation of many tropical and south-temperate marine taxa (Avise, 2000). Furthermore, a similar study on the related *Puffinus* shearwaters also suggested the separation between Pacific and North Atlantic species took place when the Panama Land Bridge formed (Austin, 2004).

Regarding the separation between the Atlantic and the Mediterranean clades, we estimated the separation between the two Cory's shearwater subspecies at 1 Mya. Clock calibrations in the present study do not support the two former hypotheses suggesting either the colonization of the Mediterranean by an Atlantic ancestor just 10,000 years ago (Voous, 1976), or an older separation up to 5 My ago (Olson and Rasmussen, 2001). Indeed, congruent patterns and divergence times dating back to the Pleistocene ages have been found among seabirds related to the *Calonectris* clade (*Hydrobates pelagicus*, *Puffinus* spp.), also showing a similar phylogeographic boundary between Atlantic and Mediterranean regions (Austin, 2004; Cagnon et al., 2004). Within the Palearctic clade, the separation among the three main subclades since the late Pleistocene matches major paleogeographic events during the last ice ages. Although Cory's shearwater would have colonized the Mediterranean in the early Pliocene, range contractions into Pleistocene refuges (Azores, Madeira and Canary Islands, as well as various Mediterranean Islands and coastlines) may have limited gene flow among populations. In such a scenario, allopatry during the Pleistocene ages together with the highly philopatric behaviour of the species (Rabouam et al., 1998) could have favoured isolation by distance. Furthermore, it is known that Pleistocene glacial cycles and associated ecological changes probably promoted changes in climate and oceanography, those that characterise the Mediterranean today (Longhurst, 1998). In this context, the geographic pattern of genetic differentiation among populations would have been reinforced by local adaptation to each of the two basins.

According to this study, the separation of the Cape Verde lineage occurred about 700,000 years ago, providing evidence that the clade has been in the archipelago at least this long. However, the apparently recent divergence between Cory's and the Cape Verde shearwater species contrasts with the noticeable morphological differentiation. It follows that once the islands were colonized, the species may have been differentiated through local adaptation to the new oceanographic environment. In general, body size among the procellariform species decreases towards the tropical areas, probably due to the lower productivity and the lower wind speed (Newton, 2003). Indeed, the Cape

Verde waters belongs to the Afro-tropical biogeographical region and exhibits subtropical oceanographic features clearly distinct from other Macaronesic Islands where the Atlantic Cory's shearwaters breed (Longhurst, 1998). The distinct oceanographic environment may explain incongruence between genetic and morphological patterns of differentiation as regards the two Cory's shearwater subspecies.

To summarize, the phylogeographic history of the *Calonectris* species complex suggests a strong geographic component to the cladogenesis among the main species and subspecies clades. Phylogeny indicates that *Calonectris* species evolutionary history involves a vicariant speciation resulting in the Pacific and Palearctic clades after the Panama Land Bridge formed. Within the Palearctic clade, the Cape Verde shearwater probably evolved as an endemic form by ecological divergence. Similarly, the Atlantic and Mediterranean clades would have evolved in allopatry by range contraction followed by a local adaptation to specific oceanographic conditions, during the Pleistocene ice ages. Indeed, the Almeria Oran Oceanographic Front apparently represents a more significant barrier between the two Cory's subspecies than the Gibraltar strait. Overall, these results suggest that marine environment and oceanographic fronts play a major role in the phylogeography of pelagic seabirds.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.05.006.

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