Inter- and Intraspecific Variation of the Nucleotide Sequence of the Cytochrome *b* Gene in Cory's *(Calonectris diomedea)*, Manx Shearwater *(Puffinus puffinus)* and the Fulmar *(Fulmarus glacialis)*

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The cytochrome *b* gene of three European taxa of the family of Procellariidae was amplified from total DNA and sequenced. The sequence comparison shows that the Fulmar *(Fulmarus glacialis)* is significantly distinct from shearwaters, whereas Cory's *(Calonectris diomedea)* and Manx Shearwater *(Puffinus puffinus)* are closely related. Although the populations of *C. diomedea* can be distinguished morphologically, the sequences of *cyt b* differ only slightly between the Atlantic and Mediterranean subspecies *(i.e. C. d. borealis versus C. d. diomedea)* and do not reveal other population differences within subspecies.

Introduction

The petrels or Procellariiformes form a highly distinctive group of marine birds sharing many biological characters which are obviously adaptations to their pelagic way of life. Using morphological criteria, this order (consisting of about 103 species) is divided into the families of Diomedeidae (albatrosses), Procellariidae (fulmars, shearwaters), Hydrobatidae (storm-petrels), and Pelecanoididae (diving petrels) [1, 2]. Using morphological characters alone, it is very difficult to reconstruct the corresponding phylogeny since convergent traits obscure the overall character pattern.

In recent years a new biochemical method has been developed, the polymerase chain reaction (PCR), which allows the amplification and sequencing of marker genes from total DNA of any organism [3]. Sequence variations of these genes can be used to evaluate relationships between species, genera and higher taxa [4]. The ultimate goal is to reconstruct phylogenetic trees and to decipher the underlying evolutionary history or migration patterns. A useful marker is the mitochondrial cytochrome *b* gene *(cyt b)* which displays enough sequence variation to assess the phylogenetic relationships in birds and other vertebrates at the species, genus and family level [4, 5].

We have determined and compared partial sequences of the cytochrome *b* gene (corresponding to positions 14846–15145 of human mtDNA [12]) to analyze the phylogenetic relationships between some European taxa of the family of Procellariidae, which include the Fulmar *(Fulmarus glacialis)*, Manx Shearwater *(Puffinus puffinus)* and Cory's Shearwater *(Calonectris diomedea)*. Cory's Shearwaters are subdivided into three subspecies, *i.e. C. d. diomedea* (breeding in the Mediterranean), *C. d. borealis* (Salvages and other Atlantic Islands) and *C. d. edwardsii* (on Cape Verde Is.). Since these subspecies and even members of the different island populations of *C. d. diomedea* can be distinguished morphologically, we have analyzed, in addition, the degree of intraspecific variation of the cytochrome *b* sequence.

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Materials and Methods

Origin of the DNA

F. glacialis (blood) and *P. puffinus* (breast muscle was taken from a dead bird) were birds breeding in Ireland. Blood from *C. diomedea* was collected from breeding birds of 5 Mediterranean and 1 Atlantic population. The locations of the breeding colonies are given in Fig. 1.

Processing of DNA

Small quantities of blood (100–200 µl) were collected and stored in a special EDTA-buffer [6]. Total DNA was isolated after digestion of protein with proteinase K. The cytochrome *b* gene was partially amplified by PCR using PCR primers modified after Kocher *et al.* [5]. PCR products were separated by agarose gel electrophoresis, extracted and reamplified asymmetrically. A portion of 300 nucleotides was sequenced directly employing T7 DNA polymerase and the chain termination method [3].

Fig. 1. Geographic distribution of *Calonectris diomedea:* sites of blood sampling and morphometric measurements. 1 = Salvage Islands and Alegranza, 2 = Chafarinas, 3 = Cabrera and Columbretes, 4 = Marseille, 5 = Tunis and Zembra, 6 = Malta and Linosa, 7 = Aegean islands off Crete.

Processing of DNA sequences

Sequences were aligned and analyzed by the program package PAUP 3.0 [7] using the maximum parsimony method and bootstrap analysis [7]. We used the sequence of *Gallus domesticus* [8] as an outgroup to root the partial phylogenetic tree of Procellariidae.

Field studies on C. d. diomedea

Up to now we have closely monitored a specific Aegean breeding colony for more than 20 years [9, 11]. Nestlings of approx. 100 pairs were ringed each year since 1979. In subsequent years, especially between 1985 and 1992 all breeding birds within a study plot were captured and checked if they were birds that had hatched on this island. Since we had recorded the exact location of birth and of later breeding, it was possible to evaluate the distance between both sites as a measure for natal philopatry.

Results and Discussion

DNA was isolated from *C. diomedea, P. puffinus* and *F. glacialis* and the *cyt b* gene was partially amplified by PCR. PCR products were isolated, purified and sequenced (Fig. 2). Fig. 3A illustrates the results of a heuristic analysis (PAUP 3.0) of the nucleotide sequences in form of a phylogram. Branch lengths are proportional to genetic distances between taxa. For comparison, Fig. 3B gives the results of a bootstrap analysis [7] (1000 replications) which provides probability values for each furcation (illustration as a cladogram), indicating that the phylogenetic tree obtained probably reveals the true phylogeny.

The molecular data (Fig. 2 and 3) confirm that the Fulmar is not a shearwater but obviously belongs to a separate genus or even family and that Cory's and Manx Shearwater are closely related. It should be recalled that their life styles and general biology are very similar [1, 2, 9]. These results suggest that *cyt b* is a good molecular marker (as already shown in other bird species [5]) to study the phylogeny of Procellariidae.

Our next question was whether we could detect intraspecific variation in Cory's shearwater. We have analyzed the size of specimens of *C. diomedea* which breed on Salvage Island *(C. d. borealis)* as compared to that from birds originating from dif-

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Gallus      CATAGCCCAC AAAGGCGGTG GCTATGAGTG TGAGGAGGAG GATTACTCCT GTGTTTCAGG
Manx        ?????????? ???...A..T ........A. ....T..... ...G..... ..........
Fulmar1     ?????????? ??....A..T ........G. ....T..A.. .....T.... ..........
Fulmar2     ?????????? ?.........T ........G. ....T..A.. .......... ..........
Cory.329    ????????.. G.....A..T ........A. ....T..... A..G..C... ..?.......
Cory.220    ?????????? ?????????? ???????.A. ....??.... A..G..C... ......?...
Cory.7865   ?????????? ???...A..T ........A. ....T..... A..G..C... ..........
Cory.62     ?????????? ?.....A..T ........A. ....T..... A..G..C... A.A...A...

Gallus      TTTCCTTGTA GAGGTAGGAG CCGTAGTATA GGCCTCGTCC GATGTGAAGG AAGATACAGA
Manx        .C..T..A.. T.....A... ........G. AT........ ......C... T.A.......
Fulmar1     ....T..... A.......... ..A.....G. AT........ A..A..T... T.A..G....
Fulmar2     ....T..... A.......... ..A.....G. AT........ A..A..T... T.A..G....
Cory.329    ....T..... T.....A... ..A.....G. AT........ ......G... T.G..G....
Cory.220    ....T..... T.....A... ..A.....G. AT........ ......T... ?.A.?G....
Cory.7865   ....T..... T.....A... ..A.....G. AT........ ......G... T.A..G....
Cory.62     ....T..... T.....A... ..A.....G. AT........ ......G... T.A..G....

Gallus      TGAAGAAGAA TGAGGCGCCG TTTGCGTGGA GATTCCGGAT TAGTCAGCCG TATTGTACGT
Manx        .......... ......A... .....A..T. .G..T..... ......A..A ..........
Fulmar1     ....A..... ......T... .....A..T. ....T..A.. G........G ..C.......
Fulmar2     ....A..... ......T... .....A..T. ....T..A.. G........G ..C.......
Cory.329    .......... ......T... .....A..T. .G..T..A.. ......A..A ........?.
Cory.220    .......... ......T... ...?.?..T. .G..T..A.. ......A..A ..........
Cory.7865   .......... ......T... .....A..T. .G..T..A.. ......A..A ..........
Cory.62     .......... ......T... .....A..T. .G..T..A.. ......A..A ..........

Gallus      TCCGGCAAGT GTGGGCTACG GAGGAGAAGG CTAGGGATGT GTCTGCTGTG TAGTGCATGG
Manx        .T.....T.. A.....G... ..T....... ....A.T... A..A..A... .....T..A.
Fulmar1     .T.....T.. ......A... ..T....... ...A..T... ...A..A... ..........
Fulmar2     .T.....T.. ......A... ..T....... ...A..T... ...A..A... ..........
Cory.329    .T.....T.. A.....A... ..T..A.... ......T... ..TG..G..A ??????????
Cory.220    .T.....T.. A.....A... ..T..A.... ......T... ..T...T..A ........A.
Cory.7865   .T.....T.. A.....A... ..T..A.... ......T... ...G..G..A ........A.
Cory.62     .T.....T.. A.....A... ..T..A.... ......T... ...G..G..A ........A.

Gallus      CTAGTAGTAG GCCGGTGAGG ATTTGGGTCA TGAGGCAGAC TGCTAATAGG AAGCCGAAAT
Manx        .......... ...A..T.AA ........T. GT.......T GC...GG..A G.......G.
Fulmar1     ......A... A.....T... ........T. .T.......T GC....G..G G.T.....G.
Fulmar2     ......A... A.....T... ........T. .T.......T GC....G..G G.T.....G.
Cory.329    .......... ...A..T.AA ........T. GT..A..T.. AC...GG... ??????????
Cory.220    .......... ...A..T.AA .....??.T. GT.??????? ?????????? ??????????
Cory.7865   .......... ...A..T.AA ........T. GT..A..T.. ?G...GG..A G.C.....G.
Cory.62     .......... ...A..T.AA ........T. GT..A..T.. AC...GG..A G.C.....GC
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. = nucleotide identical to that in the first line, ? = nucleotide unknown.

Fig. 2. Partial nucleotide sequences of the cytochrome *b* gene of the Fulmar *(Fulmarus glacialis)*, Manx *(Puffinus puffinus)* and Cory's Shearwater *(Calonectris diomedea)*, *C. d. borealis:* Cory.329 and 220 are from Alegranza; *C. d. diomedea:* Cory.7865 came from Linosa and Cory.62 from Tunis.

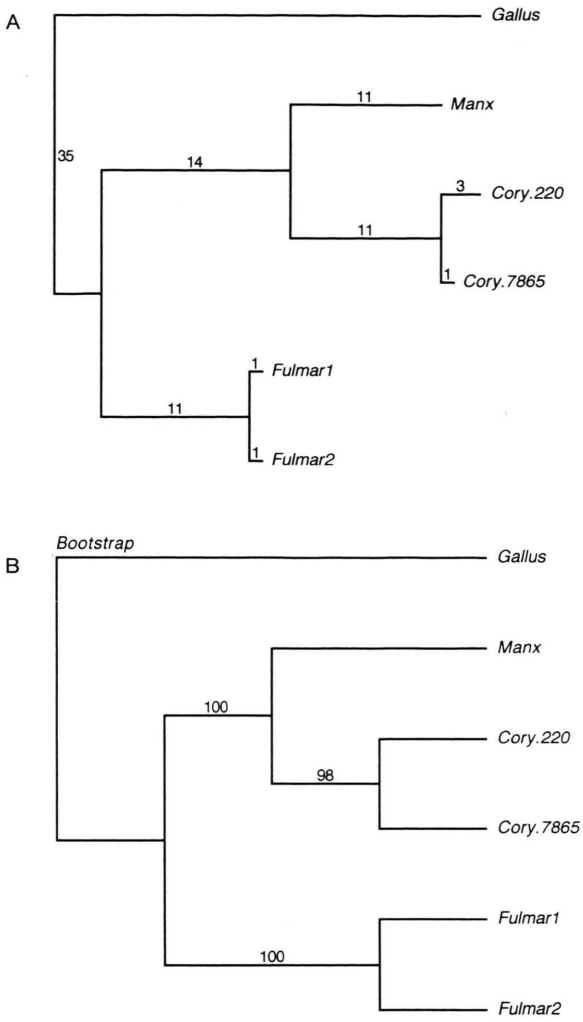

Fig. 3. Analysis of the nucleotide sequences of the cytochrome *b* gene by the maximum parsimony method [7]. A. Illustration as a phylogram. Numbers refer to nucleotide substitutions between related taxa. B. Results of a bootstrap analysis (1000 replications). Values are probabilities (in %) for each furcation. The following options were employed: heuristic search; sequence addition: closest; branch swapping algorithm: tree bisection-reconnection (TBR).

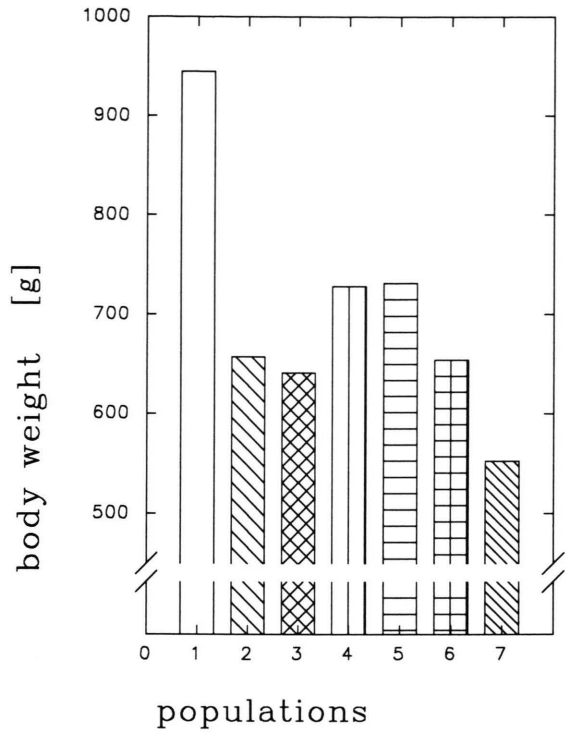

Fig. 4. Body weights of *C. diomedea* of different breeding populations. *C. d. borealis:* 1 = Salvage Islands; *C. d. diomedea:* 2 = Chafarinas*, 3 = Cabrera, 4 = Marseille, 5 = Zembra, 6 = Malta, 7 = Crete*.

* Data from our own studies; other data from [1].

ferent Mediterranean colonies (Fig. 1 and 5): the body weights differ significantly between both subspecies (Fig. 4); *C. d. borealis* is almost two times heavier than Aegean shearwaters. In addition, a size-cline can be seen from West to East for the Mediterranean birds. A significant ($p < 0.05$) linear correlation exists between body weight, wing length and ambient temperatures at the breeding grounds (Fig. 5) suggesting that size variation in *C. diomedea* may be of adaptive nature.

Does the sequence of the *cyt b* gene reflect respective differences, too? For this purpose we have sequenced part of *cyt b* gene from 2–3 birds of 6 populations (Fig. 1). As can be seen from Fig. 6 birds belonging to the subspecies *C. d. borealis* are clearly distinguished by 2 common nucleotide substitutions, whereas birds from the Mediterranean colonies are almost identical. This means that these populations are either still connected through gene flow or that isolation is a recent phenomenon. This problem has been studied before on a more limited base with two Mediterranean and one Atlantic population by allozyme analysis [13]: Whereas subspecies *C. d. borealis* and *C. d. diomedea* can be readily distinguished no differences were found between the populations from Sicily and from Sardinia indicating a certain de-

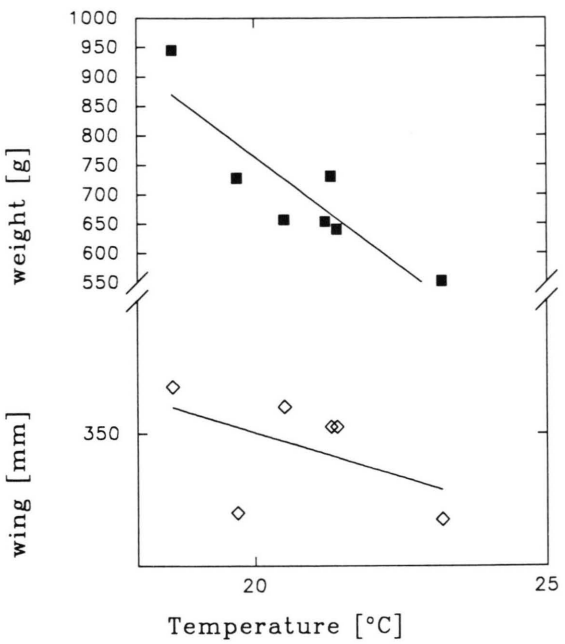

Fig. 5. Body weights and wing lengths of the different populations (according to [1] and own data) in relation to ambient temperatures (mean values) at the breeding grounds (months V–VII).

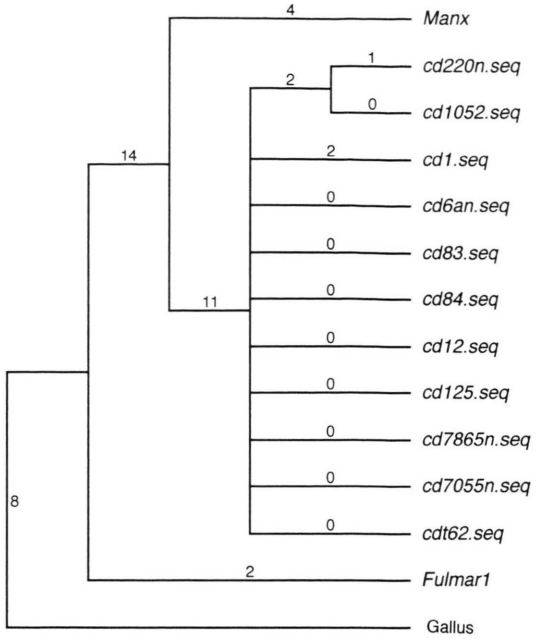

Fig. 6. Intraspecific variation of *cyt b* sequences of *C. diomedea*. Analysis was performed with PAUP 3.0 (heuristic search; informative characters, random addition; branch swapping algorithm: tree bisection-reconnection (TBR); consensus tree according to majority rule (50%) [7]). Origin of Cory's shearwaters: *C. d. borealis:* Alegranza: cd220n.seq, cd1052.seq; *C. d. diomedea:* Crete: cd1.seq, cd6an.seq; Columbretes: cd83.seq, cd84.seq; Malta: cd12.seq, cd125.seq; Linosa: cd7865n.seq, cd7055n.seq; Tunis: cdt62.seq.

gree of panmixia. It was suggested that perhaps 4–19 individuals could be exchanged among colonies per generation [13].

Studying the dispersal of *C. d. diomedea* in the Aegean colony (No. 7 in Fig. 1) we found a high degree of natal philopatry especially in young males which settle very closely to their site of birth when they start breeding at the age of 5–8 years (Fig. 7). The dispersal data and the morphological cline provide some evidence for the existence of a genetic isolation mechanism. In addition, our ringing/recovery data did not reveal any exchange between colonies [11]. Therefore, we suggest that the island populations are genetically isolated to some degree but that limited gene flow still exists and that the time of separation has been too short to allow a divergence at the level of the *cyt b* gene.

We intend to extend the *cyt b* sequences and add more species in order to approximate as closely as possible the true molecular phylogeny of Procellariiformes. Although the present results are, in general, in excellent agreement with traditional morphological and biological data, it should not be forgotten that our molecular analysis, too, has

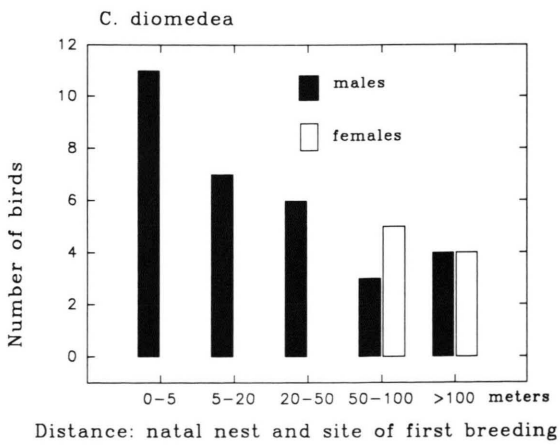

Fig. 7. Natal philopatry of Cory's shearwater. Distances (in meters) between the site of birth and the place of first breeding (results of 1985–1992); results from 31 male and 9 female birds of the Aegean colony (Fig. 1, No. 7).

its inherent shortcomings. Since mitochondrial genes are inherited maternally, any hybridization which took place in the past [10] may distort the phylogenetic picture. In the long run, we certainly need the information of several genes, including mitochondrial and nuclear ones, to fully understand the evolutionary history of a taxon. In order to resolve the population differences between Mediterranean shearwaters we need to analyze a more variable gene. We expect that the mitochondrial D-loop will be useful to solve this question [4].

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