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Phylogenetic relationships in Mediterranean and North Atlantic shearwaters (Aves: Procellariidae) based on nucleotide sequences of mtDNA

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Abstract

The mitochondrial cytochrome b gene of Shearwaters (genera *Puffinus* and *Calonectris*) from the western, central, and eastern Mediterranean (*C. d. diomedea*, *P. y. yelkouan* and *P. y. mauretanicus*) and the North Atlantic (*C. d. borealis*, *P. puffinus*, *P. assimilis baroli*) was amplified by polymerase chain reaction (PCR) and sequenced directly. Phylogenetic relationships within the group of shearwaters [including published taxa of Austin (1996)] were evaluated with parsimony, likelihood and distance methods. The Balearic Shearwater (*P. y. mauretanicus*) is a sibling taxon of *P. y. yelkouan* of eastern and central Mediterranean. Since both taxa are distinguished by several morphological, behavioural and ecological traits, and by 2.2 to 2.9% nucleotide substitutions (which is in the same range as distances between "good" Shearwater species) we consider *P. yelkouan* and *P. mauretanicus* distinct species and not subspecies of *P. puffinus* as assumed earlier. Little Shearwaters of North Atlantic *P. assimilis barolis* do not share direct ancestry with *P. assimilis* subspecies of the Southern hemisphere but appear closely related to *P. lherminieri*.

Within the Procellariinae fulmars and shearwaters each represent a monophyletic assemblage. Of the shearwaters studied, *Puffinus* shows two main monophyletic lineages consisting of the *Puffinus* subgroup (phylogenetically associated with *Calonectris*) on the one hand and the subgroups Thyellodroma, Hemipuffinus, Ardenna, Neonectris of the southern hemisphere on the other. Apparently, *Puffinus* constitutes a paraphyletic taxon, because *Calonectris* falls between both *Puffinus* clades. In addition, cytochrome b sequence data show that the three subfamilies of the Procellariidae, i.e. Procellariinae, Hydrobatinae and

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Diomedeinae, can be recognized as monophyletic groups.

Keywords: Cytochrome b; Nucleotide sequences; Phylogeny; Procellariidae; Procellariinae; Hydrobatinae; Diomedeinae; *Puffinus*; *Calonectris*; Mediterranean; North Atlantic shearwaters

1. Introduction

The classification of the *Puffinus* Shearwaters breeding in the Mediterranean and North Atlantic has been a matter of debate over the last century. Originally, all birds from the Mediterranean were described as a subspecies of the Manx Shearwater *Puffinus puffinus* Brünnich 1764, namely "Levantine" Shearwater *P. p. yelkouan* Cerbi 1927. However, Witherby (1921) considered most specimens that were collected on the British coasts in late summer as a separate taxon, which was described as a third subspecies of the Manx Shearwater, *P. p. mauretanicus* by Lowe (1921). Mayaud (1932) realized that there were two Mediterranean taxa, *P. p. yelkouan* in the central and eastern basin, and *P. p. mauretanicus* in the western Mediterranean (breeding only on the Balearic Islands); he recognized the similarities between both Mediterranean subspecies and the clear-cut differences to the much smaller atlantic form, *P. p. puffinus*. Most handbooks still consider these taxa as subspecies of the Manx Shearwater (Cramp and Simmons, 1977; Bauer and Glutz, 1987; Murphy, 1952). Recently, it has been suggested to combine both Mediterranean taxa, *yelkouan* and *mauretanicus*, in a single distinct species, the Yelkouan Shearwater (*Puffinus yelkouan*) since it is well-separated from Manx Shearwater by significant differences in size, morphology, vocalizations, feeding habits, phenology, and migratory behaviour (Bourne et al., 1988; Sibley and Monroe, 1990; Yésou et al., 1990; Paterson and Yésou, 1994). This new classification is reflected also by DNA data; nucleotide sequences of the cytochrome b gene differ significantly between *P. p. puffinus* and east Mediterranean *P. p. yelkouan* (Wink et al., 1993b; Heidrich et al., 1996). At present, Balearic Shearwaters are usually considered as a subspecies, race or morph of Yelkouan Shearwaters (Yésou et al., 1990; Paterson and Yésou, 1994; Zotier et al., 1992; Del Hoyo et al., 1992; Pons and Palmer, 1996; Bretagnolle and Zotier, in press), whereas Walker et al. (1990), McMinn et al. (1990) and Altaba (1993) have treated the taxon as a distinct species.

The mitochondrial cytochrome b is a toolmark in avian phylogeny (but see Meyer, 1994), as it displays enough variation to assess phylogenetic relationships within genera or even families (Kocher et al., 1989; Wittmann et al., 1995; Wink, 1995; Wink et al., 1996), and is able to resolve the evolutionary past between approximately 100,000 and 30,000,000 years ago (Avise, 1994). Recently, nucleotide sequences of the cytochrome b gene were employed to study the molecular systematics and phylogenetic relationships of some Procellariidae (Wink et al., 1993a,b; Heidrich et al., 1996; Austin, 1996; Nunn et al., 1996).

The present paper aims to resolve the phylogeny and speciation of the Mediterranean *Puffinus* Shearwaters in comparison with the North Atlantic Shearwaters

P. puffinus, *P. assimilis baroli* and other *Puffinus* species. In addition, using own nucleotide sequences of the shearwater *Calonectris*, of the petrels *Bulweria*, *Fulmarus*, *Oceanodroma*, and *Hydrobates* as well as published sequences of the petrels *Procellaria*, *Macronectes*, and the albatrosses *Diomedea*, *Phoebastria*, *Phoebetria*, and *Thalassarche* (Nunn et al., 1996), phylogenetic relationships within and between the three subfamilies Procellariinae, Hydrobatinae and Diomedeinae were reconstructed.

2. Materials and methods

Origin of blood samples. Blood or feather samples originated from localities given in Table 1. Identification of the shearwaters was unequivocal since usually breeding birds of known taxonomic status were sampled by experienced ornithologists. Sequences from other Procellariidae were obtained from published sources (Austin, 1996; Nunn et al., 1996; Heidrich et al., 1996). Blood samples were taken either by puncture of the brachial or tarsal vein of the birds. Between 100 and 500 µl of blood were collected using a syringe, and transferred immediately either to a modified

Table 1
Origin of blood samples or nucleotide sequences

Species	Code	Locality	Collector
Bulweria bulwerii 1,2	Bulbul1/2	Madeira	F. Zino
Calonectris d. borealis 16/17	Cdb1/2	Selvage Isd., Portugal	F. Zino
Calonectris d. diomedea 5	K2(866)	Crete, GR	D. Ristow
Calonectris d. diomedea 6	IC3	Columbretes Isd, E	J. Dies–Jambrino
Fulmarus glacialis 1,2	Fg1/2	Saltee Isd, IRE	M. Wink
Fulmarus glacialoides 2	Fgl2	Concepcion, Chile	M. Wink
Hydrobates pelagicus 1–3	SsA/SsB/SsC	Malta	J. Borg
Oceanodroma castro	Occas1/2	Madeira, Portugal	F. Zino
P. assimilis baroli 1,2	Pass1/2	Madeira, Portugal	F. Zino
P. gravis 1	Pgr1	California, USA	J. Heinze
P. mauretanicus 1–21	402–422	Cabrera Isd, E	J.S. Aguiar
P. puffinus 1	Mal II/III	Cullenstown, IRE	M. Wink
P. puffinus 2–3	MaI I	Puffin Isd, IRE	M. Wink
P. tenuirostris 2	Pt2	Philys Isd, NZ	F. Heinze
P. yelkouan 1,2	I,II	Crete, GR	D. Ristow
P. yelkouan 3–6	243/279/200	Malta	J. Borg
P. yelkouan 8–10	2173–2175	Naxos, GR	D. Ristow
Phalacrocorax aristoteles	171	Crete, GR	D. Ristow
Sula bassana	Bt1	Saltee Isd, IRE	M. Wink

Sequences of *Diomedea amsterdamensis*, *D. epomophora*, *Macronectes gigantea*, *Phoebastria albatrus*, *Phoebetria fusca*, *Ph. immutabilis*, *Ph. palpebrata*, *Procellaria cinerea*, *Thalassarche bulleri*, and *Th. chrysostoma* originated from Nunn et al. (1996); *Puffinus a. assimilis* 1, *P. a. haurakiensis*, *P. auricularis*, *P. bulleri*, *P. creatopus*, *P. gavia*, *P. gravis* 2, *P. griseus*, *P. huttoni*, *P. lherminieri boydi*, *P. l. lherminieri*, *P. nativitatis* *P. pacificus*, and *P. tenuirostris* 3 were from Austin (1996).

EDTA/Tris-buffer (0.1 M Tris, pH 7.4, 10% EDTA, 1% NaF, 0.1% thymol) or to absolute ethanol. Samples were stored at 4°C for periods of up to 6 months, with negligible effects on the yield and quality of the DNA.

DNA extraction. Total DNA was extracted from the blood samples by an overnight incubation at 37°C in lysis buffer (10 mM Tris (pH 7.5), 25 mM EDTA, 75 mM NaCl, 1% SDS including 1 mg of proteinase K (Boehringer Mannheim), followed by a standard phenol/chloroform protein extraction. DNA was precipitated from the supernatant with 0.8 vol of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer (Sambrook et al., 1989).

PCR and DNA-sequencing. Primer pairs used for PCR were modified from those given by Kocher et al. (1989) to match the seabird sequences as closely as possible: mt-A (L-14970) (positions refer to the cytochrome b gene of *Gallus*; Desjardin and Morais, 1990): CAA CAT CTC AGC ATG ATG AAA CTT CG and mt-Fr (H-16086): TCA GTT TTT GGT TTA CAA GAC CAA TG. For amplification, 1 μg of total DNA was used as a target, plus 25 pmol each of primers mt-A and mt-Fr, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 μl 10 × amplification buffer and 2 units Taq-Polymerase (AGS, Heidelberg) in a total volume of 100 μl. After an initial denaturation (4 min at 94°C), 30 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C were performed on a Biometra thermocycler. After 30 cycles the reaction temperature was maintained at 72°C for 4 min and then lowered to 4°C for further storage. Non-incorporated primers and nucleotides were inactivated enzymatically by shrimp alkaline phosphatase and exonuclease I; then 5–7 μl of the 1100 bp PCR products of the mitochondrial cytochrome b gene were sequenced directly according to the Sequenase PCR product sequencing kit protocoll (USB-Amersham). Alpha ^{35}S-dATP was used as a radioactive tracer. For the cytochrome b gene, the following sequencing primers were used to obtain overlapping sequences; mt-B (H-15318): TCA AAA TGA TAT TTG TCC TC, mt-G (L-15155): CWT CCT TMT TCT TCA TCT GCA TCT AC, mt-M ATG RGA CAA YCC AAC MCT A, mt-D (L-15546): ATC CCA TTC CAC CCC TAC TAC TCC, and mt-H (L-15699): CAA ACC MGA ATG ATA CTT C. Products of the sequencing reactions were separated on a 6% polyacrylamide/7 M urea gel by electrophoresis at 65 W. After drying, the gel was exposed to an X-ray film (Hyperfilm-MP, Amersham) for 3–4 days, and developed (X-ray developer and fixer, Kodak). Nucleotide sequences (1080 nt) of the cytochrome b gene (corresponding to positions 14992–16072 of mtDNA from *Gallus gallus*; Desjardins and Morais, 1990) will be documented at the EMBL databank. Variable characters of a representative set of taxa are illustrated in Table 2.

Sequence analysis. Sequences were aligned to the cytochrome b sequence of *Gallus gallus* (Desjardins and Morais, 1990) and analysed with Maximum Parsimony (MP; PAUP 3.1.1; Swofford, 1993), Maximum Likelihood (ML; PHYLIP 3.572c), and Neighbour Joining (NJ; MEGA 1.0; Kumar et al., 1993) which generate reasonably good phylogenetic trees (Nei, 1996). For NJ, Jukes-Cantor distance algorithm was employed for bootstrap analyses. With PAUP, heuristic algorithms (unweighted) were selected using both simple or random sample addition and tree-bisection-reconnection branch swapping options (results were identical). A strict consensus tree was generated for the most parsimonious tree. Bootstrap analyses (500 replicates) were

Table 2
Illustration of variable nucleotides of representative taxa in the 1043 nt data set of the cytochrome b gene. . = nuleotide identical to that in the top line; ? = missing information; complete sequences will be deposited in the embl nucleotide data bank

			11111111111	11111111111111111111
	1111112222223333	34444555556666777778	88899999900001122223	33444455556667888889
	35690245681245670367	92589127890369256891	24701367902581703693	58145703692581036792
Calonectris diomedea6	GCTCCAGTGATCACTACATT	ATCACACTATGCTCCCAAAC	CACTTACGTGCTACCAATTC	ATACCATACACACCCTCCCC
Calonectris borealis16	..C.................	....	....	....
Bulweria bulwerii1	A.C.....ACC..A..T.CC	.C....TC..A...T.....	........C.......GCC.	...............C.T..
Diomedea amsterdamensis	A...T...ACC..GC.A.CC	.C...G.C..A.CAA.....	...C...A...C....GCCT	.C...CC...A....C..T.
Diomedea epomophora sanfordi	A......CACC..GC.A.CC	GC...G.C..A.CAA.....	...C....C..C....GCCT	.C...CC...A....CT...
Fulmarus glacialis1	A.C.T...ACC..T....CC	.CT.T..C..A.CTT.....	T..C.......C....GCC.	C..T.........TT...AT
Fulmarus glacialoides2	A.CA...AACC..A....CC	....T...G.A.CAT.T...	...CA...CTT.......C.	CC...........T....AT
Hydrobates pelagicus1	..CA..CCCCCT.A..AGCC	..T....C....C.A.....	.GA...A...AC.....CC.	....A.........TC.AAT
Macronectes giganteus	A.C.T..CACC..A....CC	.C..T..C....CT......	..TC..........T...C.	CC.T....T...TT....A.
Oceanodroma castro1	..CATGCCCCC..GC....C	.C..T.AC....C.A.....	..AC....C.AC......C.	.C..A..C......TCTAAT
Phalacrocorax aristoteles	ATC....CACC..A..A.C.	.C..T..C...TCTA...C.	T.AC....CT.CC..C..C.	.CT...C..C?....C.T..
Phoebastria albatrus	A...TG.CACC..GC.A.CC	.......C...TCAAT....	...C.......C....GCC.	.C...CC...A.....T.T.
Phoebastria immutabilis	...TT..CACC..GC.A.CC	.......C..A.CAAT....	...C.......C....GCCT	.C...CC...A....CT...
Phoebetria fusca	A.C.T...ACC..AC.A.CC	GCT....C..A.CAAT..G.	...C.G.A...C.T...C.T	.CGT.C....A..T.C....
Phoebetria palpebrata	A.C.T...ACC..AC.A.CC	.CT....C..A.CAAT....	...C.G.A..AC.T...C.T	.C...C....A....C....
Procellaria cinerea	A.C.....ACC..A....CC	..T.......A.CTTT....	T.T.......TC.T...CCT	.C.T....T.....T...T.
Puffinus assimilis bar1	A......CACC.........	..........ATCTTT...T	........C.........C.	.........C....T...A.
Puffinus mauretanicus10	C......CACC.G..G....	.......C..ATCTTT...T	..........TC.T..GC..	.........C....T...A.
Puffinus puff puffinus1	C......CACC.........	..........A.CTTT...T	........C...........	.C.......T....T...G.
Puffinus yelkouan1	C......CCCC....G....	...G...C..AT.TTT.T.T	.............TT..CC.	.........C....T...A.
Puffinus yelkouan3	C......CACC....G....	...G...C..AT.TTT...T	.............TT..CC.	.........C....T...A.
Puffinus yelkouan9	.......CACC....G....	...G...C..AT.TTT...T	.............TT..CC.	.........C....T...A.
Sula bassana	A.A.T...ACC.TA....CG	.CAT.T.C.CC.CAA.....	..AC..A.C..CC.TT.CCT	.CC..C....AC..TC..AT
Thalassarche bulleri bulleri	A.C....CACC.....A.CC	TC.....C..A.CAAT...T	...C...A...C....GC.T	.C...TC...A...TC..T.
Thalassarche chrysostoma	A.C....CACC..A..A.CC	.C.....C..A.CAAT...T	...C...A........GC.T	.C...TC...A...TC..T.

—continued

Table 2—*continued*

	11222222222222222222	22222222222222222222	23333333333333333333	33333333333333444444
	99000011111222223344	44555555666667889999	90001233334444455556	66677777888999000111
	58145702369235684703	69023589145799281478	90398703692567814780	36925689147039258147
Calonectris diomedea6	CAAATCCATCTCCATCACAC	AGGCATCCACATTTCAATCT	CATCAATACCCCCTACATAT	CCCCAATCCAAAAAACAACC
Calonectris borealis16	....	C	T...................	......C....G........
Bulweria bulwerii1	T.....T.C.C..G...T..	.A.T.......C.C..G..C	TTC..G...........C..	.T....C.....CG......
Diomedea amsterdamensis	.C.C....C.A.........	.A..CA....TC.C..C.TC	T.AA.G...TT.......GC	A.T.G.C.....TC.T..T.
Diomedea epomophora sanfordi	.CC.....C.A..GCT....	.A..CA....T..C..C.TC	T.AA.....TT.......GC	A.T...C.....T..T..T.
Fulmarus glacialis1	T.........C..T......	.AAT.CT....C.C.....C	T....GC.A.TT.....C..	.TT...C.....C..TG.TT
Fulmarus glacialoides2	T.........C..T......	.AAT.C.....C.C.GG..C	T.C..GC.G.TT....GC..	ATTT..C...G.CG.T..T.
Hydrobates pelagicus1	.....T..C.C..CC.....	TCA.CC..T..C....C.AC	T.AA.T.C....A.G..A.C	G..AG.CAT..GCGCT....
Macronectes giganteus	T.........C..T...T..	.A..CA.....C..T....C	T.AA.GC....T....GC..	.TT.G.C....GCG.T..T.
Oceanodroma castro1	.........TC..CC....T	TA.TCCT..T.CC.TGC..C	T.A..C.....T.C...A.C	T.TA..C.T.GGTTC.....
Phalacrocorax aristoteles	.C..C..T..A......A..	.AAT..A..A.C.CT....C	T..ACC..A........C.C	A.....C..T..T.......
Phoebastria albatrus	.CC.....C.A..G......	.A..CA.....C.C..C.TC	TGAA.G...T.......CGC	A.T...C....GTG.T....
Phoebastria immutabilis	.CC.....C.A..G......	.A..CA.....C.C..C..C	TGAA.G...T.......CGC	A.TA..C.TG..TG.T....
Phoebetria fusca	.TT..T....G......T..	.A..CA.....C..T....C	T.AA.G...T.........C	ATT.G.C.T...TG.T..TT
Phoebetria palpebrata	.TT..T....G......T..	.A..CA.....C..T....C	T.AA.G...T.......C.C	ATT.G.C.T...TG.T..TT
Procellaria cinerea	T.......C.A..G..G...	.A.T..TT...C.....C..	T.C..G.....T...TG...	..T..GCTA...CGC..GT.
Puffinus assimilis bar1	....	.A...C.............C	T..................C	A.....C.....C.......
Puffinus mauretanicus10	..........C....TG.G.	.A...C...........C.C	T.C...C............C	A..T..C.....C.......
Puffinus puff puffinus1	........C......TG...	.A...C.............C	T.C...C..........C.C	A.....C.....CG......
Puffinus yelkouan1	..........C....TG...	.A...C..G........C.C	T.C...C............C	A.....C.....CG......
Puffinus yelkouan3	..........C....TG...	.A...C..G........C.C	T.C...C............C	A.....C.....CG......
Puffinus yelkouan9	..........C....TG...	.A...C..G........C.C	T.C...C............C	A.....C.....CG......
Sula bassana	....C.....CT.G......	.A.T.C.....C.A.....C	TTAGC...TT.T.....C.C	A.TT..C.T...CTG.TG.T
Thalassarche bulleri bulleri	.CT.......G.TGA.....	.AA.CG.............C	T.AG..C..TT........C	AT..G.C.....T..T..T.
Thalassarche chrysostoma	.CT.....C.G..GA.....	.AA.CA.............C	T.AA..C..TT......C.C	AT..G.C.....T..T..T.

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                             44444444444444444444 44444444444444455555 55555555555555555555 55555555555555566666
                             22222333444555566666 67777888888899900011 11222333334455566677 77777888889999900000
                             03469258127036923458 94789012346925814703 69258145676925814702 34679567894578903467

Calonectris diomedea6        CACCCACCCCTCCTCCATAT GATACTCTAATTCACCCAAC ACTCTCTGTATCACATCCTT CACCACATCTCTTATATAAT
Calonectris borealis16       .................... ..........C..C...... .................... ..............C.....
Bulweria bulweriil           ...A...TT.C..C.....C ....GC....C..C...... ..C.ATC........C..C. .........C..C.C.....
Diomedea amsterdamensis      .C.AT..T..CT.CG....C .C.GTC..C.CC.CT..... T..GGAC...C.G..CT.C. .....T...CA.C.C....C
Diomedea epomophora sanfordi .C.AT..T..CT.CG....C .C.GTC..C.CCTCT..... C..AGAC...C.G..CT.C. .....T...CA.C.C....C
Fulmarus glacialis1          ...A...T..C....TGC.. .G...C....CCTC.T.... ..C.A.C........C..C. .........CT.C.C.C...
Fulmarus glacialoides2       ...A...T.TC....TGC.. .T...CT....CTC.T.G.. ..C.A.C........C..C. .........C..C.C.C...
Hydrobates pelagicus1        T..G...TT..........C .T........CCTC.T.GC. ..C.A.C.......GC..CA .T...T.....C..C..C.C
Macronectes giganteus        ...A...T..C..C.TGC.. .....C.....C.C.T.... ..C.A.C........CT.C. .........C..CGC.C..G
Oceanodroma castro1          G..A...T..C..C....C. .TC.......CCAC.A..C. ..C.A.C..G.T......CA .T....C.ACAC.TCGCCCC
Phalacrocorax aristoteles    A..AAC....C.A..T...C .CA..CAC..CC.C.G..TT C.CAA..TC.CT...C.A.. .T.A.T..TC..C.C.CC.C
Phoebastria albatrus         .T.AT.TT..CT..A....C AC.GTC..C.CC.CT...G. T..GAA......G...T.C. T....TGC.CA...C.C..C
Phoebastria immutabilis      .C.AT.TT..CT.CA....C AC.GTC..C.CCTCT...G. T..GGAC.....G.....C. T....TGC.CA....GC..C
Phoebetria fusca             .C.AT..T..C..CA....C .CC..A..C.CCAC.TT..A C..AAAC.........T.C. T....T..TCA.C.C.C.GC
Phoebetria palpebrata        .C.AT.....C..CA....C .C...A..C.CC.C.TTG.A C.CAGAC........CT.C. T....T...CA.C.C.C.GC
Procellaria cinerea          T..A...TT.C....T...C .G...C...GC..C.....T .TC.C.C....T...C..C. T........CA.C.C.C...
Puffinus assimilis bar1      ..TA......C..CT....C .....C.......C..T... ......C...C....C.... .....T...C......CT.C
Puffinus mauretanicus10      ..TA.......T..TTGC.. ..C..C.....C.C..T... ......C........C.... .....T..TC..C...CT.C
Puffinus puff puffinus1      ..TA..........T....C ..C..C.....C.C..T... ......C....T...C.... .....T..TC..C...CC.C
Puffinus yelkouan1           ..TA...T........G..C ..C..C.....C.C..T... ......C........C.... .....T..TC..C...CT.C
Puffinus yelkouan3           ..TA...T........G..C ..C..C.....C.C..T... ......C........C.... .....T..TC..C.......
Puffinus yelkouan9           ..TA...T........G..C ..C..C.....C.C..T... ......C........C.... .....T..TC..C...CT.C
Sula bassana                 A.TA...T.TC.ACAT..C. .GCG.CTAG?C..T....C. ...AA.CTC......C..C. .T.ACT..GCA.C..CAC.C
Thalassarche bulleri bulleri .CTAT.....C...A....C ACC..C..C.CCTC.TT..G C.CGAACC..C..T.C..C. T....T...CT.C.C.C...
Thalassarche chrysostoma     .CTAT.....C...A....C .C...C..C.CC.C.TT.GG C...AACC..C..TC...C. T.A..T...CG.C.C.C...
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—continued

Table 2 — *continued*

	66666666666666666666	66666666666666666666	66677777777777777777	77777777777777777777
	01111112222233333444	45555556677788888899	99900001112222333444	55556667777888889999
	90245891245701369258	91234576902545678903	67902581470349258127	03693581457013692569
Calonectris diomedea6	CCTCAAAAACCACTATCACA	CAGGAGCCCATAACTAATCA	CCTCTCGAAACTCCATCCTT	TCGCTACAATACCATAAACG
Calonectris borealis16	....	........T.......G...	....	.........C..........
Bulweria bulwerii1	..C.......T..C...T..	......T..TC.....G.T.	T.C...A...TC.TTC..CC	ATT.CTT..C........TA
Diomedea amsterdamensis	T.....G..A..TCCC.C..	T...C.....A..T..G.T.	T.C...A.G..C...C.TCC	A.CTC.GT.C......CC..
Diomedea epomophora sanfordi	T.....G....GTCCCAC..	T...T.....A..T..G.T.	T.C.C.A....C.T...TCC	A.CT..AC.C......CC..
Fulmarus glacialis1	........T....C.C.CT.	.G......T..C....G...	T.C...A.GG.CT.....CC	A.C........TT..CC...
Fulmarus glacialoides2	........T....C.C.CT.	.G......T.CT....G...	T.C..TA..G.C.....TCC	A.C.C.T....TT..CT...
Hydrobates pelagicus1	..A.C....AAG.CCCACT.	....G.....C....TG..C	T.C...A....C.T..T.CC	A.C.C.A..C.....C.G..
Macronectes giganteus	..C.....T...TC.C.T..	........T.CCG...G.T.	......A.GG.C...C..CC	A.CT..T....T..GCC...
Oceanodroma castro1	A.C..G....A.TC.CA..G	.C....T.T.C.G..TG.GT	T.A...A.G.TC...C..CC	ATATC.A..C.T...CC...
Phalacrocorax aristoteles	..CTC....AA........C	T.........C.....GC.C	A...CTA....C.T.CTTCC	A.C.C.A..C...G.CCC.A
Phoebastria albatrus	T...C.G......C.CAC..	....T.....C.GT..G...	T.C.C.A....C.T.C..CC	G.C.CGTT.C......CC..
Phoebastria immutabilis	....C.G......C.CAC..	....T.....C.....G...	T.C.C.A....C.T.C..CC	A.C.C.TTTC......CC..
Phoebetria fusca	.TA..........C.CAT..	....T.......GT..G..C	T....TA....C.T.CT.CC	A.C.C..C.C......CC..
Phoebetria palpebrata	.TA..........C.CAT..	....T.......GT..G..C	T.C..TA....C...CT.CC	A.C.C..C.C......CC..
Procellaria cinerea	..C..........C.C...T	T.......T.C..T..G...	A.C....C.......C.TC.	ATT.C.T..C.........A
Puffinus assimilis bar1	.............C......	T.....T.T.C.G...G...	T.C........CTT....CC	A.C...T..C........T.
Puffinus mauretanicus10	.............C......	......TTT.CGG...G...	T.C........CTT....CC	A.C...T..C..........
Puffinus puff puffinus1	.............C......	......TTT.C.G...G...	T.AT.......CTT....CC	A.C...T..C..........
Puffinus yelkouan1	...........G.C......	......TTT.CGG...G...	T.C........CTT....CC	A.C...T..C..........
Puffinus yelkouan3	....	..TT.T.TT.CGG...G...	T.C........CTT....CC	A.C...T..C..........
Puffinus yelkouan9	...........G.C......	......TTTTCGG...G...	T.C........CTT....CC	A.C...T..C..........
Sula bassana	T?CT.........C.C.T..	..........?..TC.G.TC	TTC...A....CTT...TCC	A.T.C.A........CC.TA
Thalassarche bulleri bulleri	..A....GG....C.CAC.G	....C.T......T..G..C	T...CT.....C.T.C..CC	A.C.C.TCGCG.....CC..
Thalassarche chrysostoma	..A.....G....C.CACTG	....C........T..G..C	T...CTA....C.T.C..CC	A.C.C.TC.CG..G..CC..

	88888888888888888888 00000111111122223334 12478012367925891470	88888888888888888888 44444555556666777788 14679012581478013601	88888888889999999999 88888999990000001111 23689145780134692358	99999999999999999999 22333444455566666778 47369256814806789580
Calonectris diomedea6	CTACTAGCCATCCCAGTAAA	GAAACTTCCCTCACTCACAT	CCGCGCCCATCACCGAAGGT	AAATACCACCTCACCTTCCC
Calonectris borealis16	....C...............	....	....	..................T.
Bulweria bulwerii1	TC..C.A.T...T...C...	A.........CA.TC.....	...T.T........A...A.	...C....TT.T....C...
Diomedea amsterdamensis	TC..C.AGTCC.A..TC..C	A..G...T.ACT......C.	.T.T.....C.......ACC	...C.....T..GTA.C...
Diomedea epomophora sanfordi	TC..C.AGTCC.A..TC..C	A..G...T.ACT..C...C.	.T.......C......GAC.	...C...G....GTA.C...
Fulmarus glacialis1	.A..C.T....T.T..C...	A.........C...A..T..	....AT........A...AA	..........C...A.C.T.
Fulmarus glacialoides2	.C..C.T.T...T.G.C...	A.........C...C.....	....AT.T......A...AA	..........C...A.C.T.
Hydrobates pelagicus1	.C..CGAG.CC.A..TC..C	A.GG......C...C....C	.....T...C....A..ATC	.CGCG....?C..TACC..T
Macronectes giganteus	.C..C.T......T..C.G.	A.......T.C...C..T..	....AT........A....G	..G.......CT..A.C.T.
Oceanodroma castro1	.C..C.AG.CC..T.TC...	A.G.TCC..TCTG.C....C	.....TT..CA......A?C	G..C.....T...AACC.TT
Phalacrocorax aristoteles	.CC.C.A..CC..T.TC..C	T...A..TTACT.AC.G.GC	A..G...T..T...A..ATC	...C......C.G.A.AT..
Phoebastria albatrus	TC..C.AG.CC.A.GTC.GC	A..G.....GCT.TC...C.	.T.T.T.T.C.......AC.	.........T....A.C...
Phoebastria immutabilis	TC..C.AGTTC.A..TC.GC	A..G.....ACT..C...C.	.T...T.T.C.......ACC	..G..T...TC...A.C...
Phoebetria fusca	TC..C.AG.CCTA..TC..T	A...T..T.AC...C..TC.	....AT...C.......AT.	...C....TTC...A.C...
Phoebetria palpebrata	TC..C.AG.CC.A..TC..T	A...T..T.AC......TC.	..A.AT...CT......AT.	...C.....TC...A.C...
Procellaria cinerea	.C..C...............	....T.....C...C...G.	.....T........A....C	..GC....T.CT..T.CT..
Puffinus assimilis bar1	.C.TC...............	........T.....CT....	.....T.......TA...A.	...C.T....C.....CT..
Puffinus mauretanicus10	.C..C...............	........T.C....T....	..............A...AC	..GC......C.....CT..
Puffinus puff puffinus1	.C..C...............	........T.C....T....	.............TA...A.	..GC......C.....CT?.
Puffinus yelkouan1	.C..C...............	........T.C....T....	..............A...AC	..GC......C.....CT..
Puffinus yelkouan3	.C..C...............	........T.C....T....	...T..........A...AC	..GC......C.....CT..
Puffinus yelkouan9	.C..C...............	........T.C....T....	..............A...AC	..GC......C.....CT..
Sula bassana	TCC.CCAG.TC....TC..T	A......TGA....C....C	T.......CCTG..AC..CC	...C.....TC.????????
Thalassarche bulleri bulleri	.C..C.AG.CCT...TC..T	ACG.T....AC...C..TC.	....AT...C..T....ACC	...C..T..TCT.TA.C...
Thalassarche chrysostoma	.C..C.AGTCCT...TCG.T	ACG.T....A....C..TC.	....AT......T....ACC	...C..T..TCT.TA.C...

—continued

Table 2 — *continued*

	111111111111	111
	99999999000000000000	000
	88889999000000111122	233
	14781369234578012436	727
Calonectris diomedea6	CCCCATCCCATTTCGAGTCA	ATA
Calonectris borealis16	....	...
Bulweria bulwerii1	TT..GCT.TGCC.T...C..	G..
Diomedea amsterdamensis	...T.C....CC..C..C..	...
Diomedea epomophora sanfordi	...TGC....C...C.AC..	...
Fulmarus glacialis1	T....C..T..C.....C..	..T
Fulmarus glacialoides2	.....C..T..C....AC..	.CT
Hydrobates pelagicus1	.....CT..G.ACA.G.C..	.?T
Macronectes giganteus	.....C.....C....AC..	.CT
Oceanodroma castro1	T....C......CA...C..	.A.
Phalacrocorax aristoteles	..A..C.T.C.......C..	.C.
Phoebastria albatrus	T..T.C.T..C...C..C..	...
Phoebastria immutabilis	...T.C.TT.CC..C.AC..	...
Phoebetria fusca	....GC....C...C..CTG	...
Phoebetria palpebrata	....GC....C...C..CTG	...
Procellaria cinerea	T....C............T.	...
Puffinus assimilis bar1	.....C......CT......	...
Puffinus mauretanicus10	T....C..T...CT....T.	G..
Puffinus puff puffinus1	T....C......CT....T.	...
Puffinus yelkouan1	T....C......CT....T.	...
Puffinus yelkouan3	T....C......CT....T.	.C.
Puffinus yelkouan9	T....C......CT....T.	...
Sula bassana	???????????????????.	.?.
Thalassarche bulleri bulleri	....GC....CC..C..C.G	...
Thalassarche chrysostoma	....GC....CC..C..C.G	...

performed to test the robustness of the furcations obtained (Felsenstein, 1985). A maximum likelihood analysis (ML) was performed with DNAML in PHYLIP (version 3.572c) to test the significance of branch lengths (Felsenstein, 1993).

3. Results and discussion

3.1. Reconstruction of phylogenetic relationships in shearwaters

Nucleotide sequences of *P. puffinus, P. y. yelkouan, P. y. mauretanicus, P. assimilis baroli, P. gravis, P. tenuirostris, Calonectris d. diomedea, Calonectris d. borealis* were determined in our own laboratory and further *Puffinus* sequences were selected from published data (Austin, 1996) (Table 1). Intraspecific sequence variation was found within *P. yelkouan* which was correlated with geographic origin, i.e. birds from Crete, Naxos and Malta have colony-specific haplotypes (Figs. 1 and 2). Since philopatry is extremely high in shearwaters (Swatschek et al., 1994), the evolution of specific haplotypes is favoured. Twenty-one specimens of *P. y. mauretanicus* were collected from different colonies of the Balearic Islands. A few variable nucleotides were found, but haplotypes did not correspond to individual colonies.

For the aligned data set of 21 shearwater taxa only 300 nt of the cytochrome b gene (with 90 variable and 65 parsimony informative sites) were available for a phylogenetic analysis, which was carried out by character state and distance methods. NJ, MP, and ML trees were reconstructed using the sequence of the Fulmar (*Fulmarus glacialis*) as an outgroup (Fig. 1A–C). However, other more distant outgroups did hardly affect tree topology. The three methods of phylogenetic reconstruction produced trees with a similar topology (Fig. 1 A–C); especially, MP and ML trees produced a very similar branching pattern within the Puffinus subgroup. Differences between ML/MP and NJ are probably due to the comparably short sequences (Nei, 1996) but these differences do not affect the following interpretations.

Austin (1996) had studied sequences of 19 *Puffinus* species/subspecies and had recognized two main monophyletic clades, one with *P. pacificus, P. bulleri, P. tenuirostris, P. griseus, P. gravis, P. carneipes* and *P. creatopus* (subgroups Thyellodroma, Hemipuffinus, Ardenna, Neonectris) showing an essentially southern hemisphere distribution; the other consisting of small dark-backed and white-bellied shear-waters of the subgroup Puffinus of worldwide distribution (*P. nativitatis, P. p. yelkouan, P. p. mauretanicus, P. huttoni, P. gavia, P. a. assimilis, P.a. haurakiensis, P. a. baroli, P. l. lherminieri, P. l. boydi, P. auricularis,* and *P. p. puffinus*). The separation of the subgroup *Puffinus* from the other subgroups is also evident from our analyses (Fig. 1A–C). This separation agrees with the traditional grouping of *Puffinus* shearwaters which was based on osteological and external morphological characters (Kuroda, 1954; Warham, 1990). Only the position of *P. nativitatis*, which was placed in the subgroup Neonectris but now turns up in the *Puffinus* clade (Fig. 1A–C) differs from the classical concept [see Austin (1996) for a detailed discussion]. Relationships within and between the subgroups Thyellodroma, Hemipuffinus, Ardenna and Neonectris which show an essentially southern hemisphere distribution were already

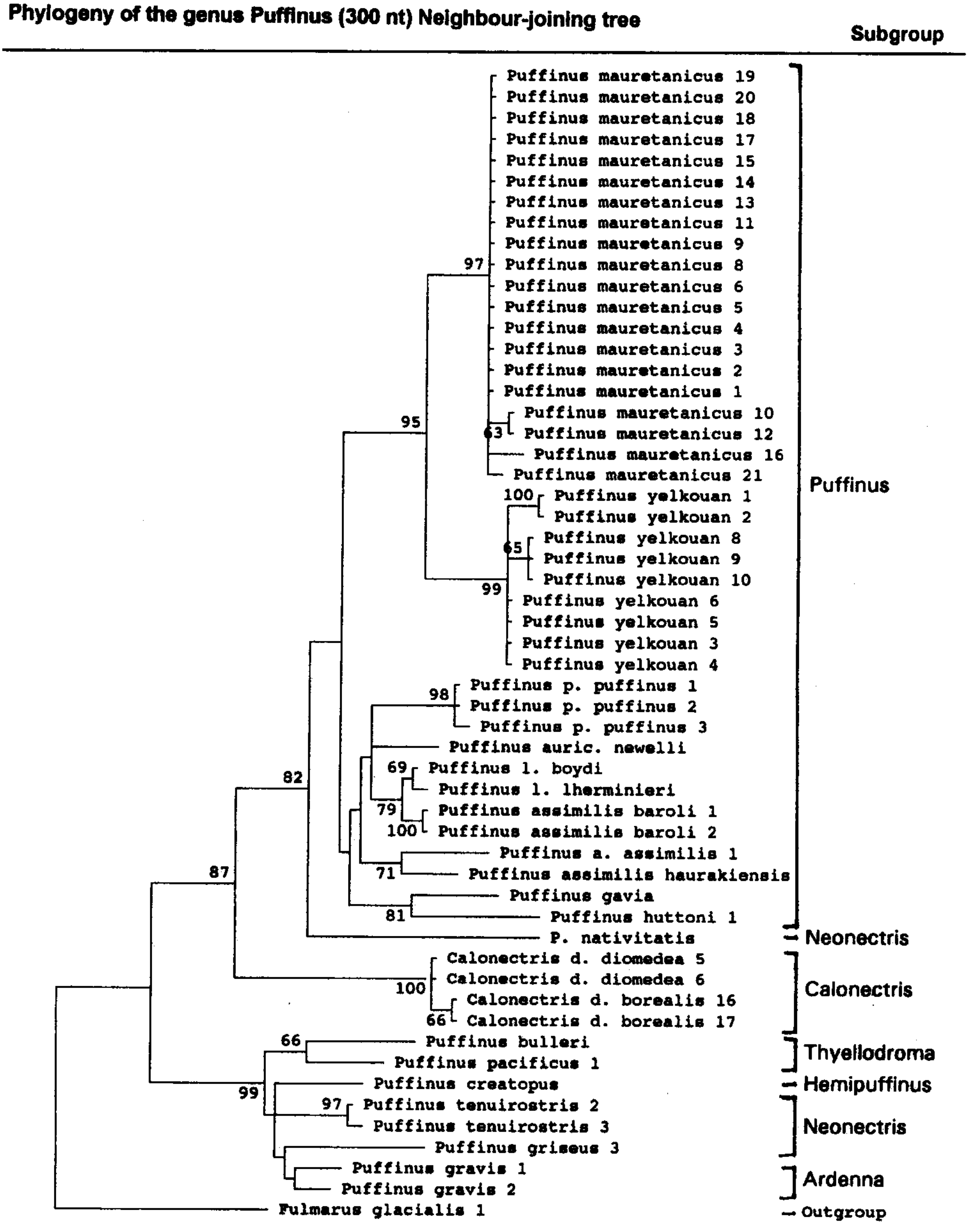

Fig. 1. Phylogenetic relationships in *Puffinus* shearwaters based on 300 bp of the mitochondrial cytochrome B gene using *Fulmarus glacialis* as an outgroup. (A) NJ-bootstrap tree (Jukes-Cantor; 500 replicates) Bootstrap confidence levels (> 60%) are given at each furcation. (B) A strict MP tree reconstructed from 53 equally parsimonious trees; length 188 steps (min 101, max. 704 steps); CI = 0.537, HI = 0.463, RI = 0.856). (C) Maximum likelihood tree (ln = − 1413.14). All branch lengths (except those within *P. mauretanicus* and within *P. yelkouan*) are significantly positive ($p < 0.01$; Felsenstein, 1993).

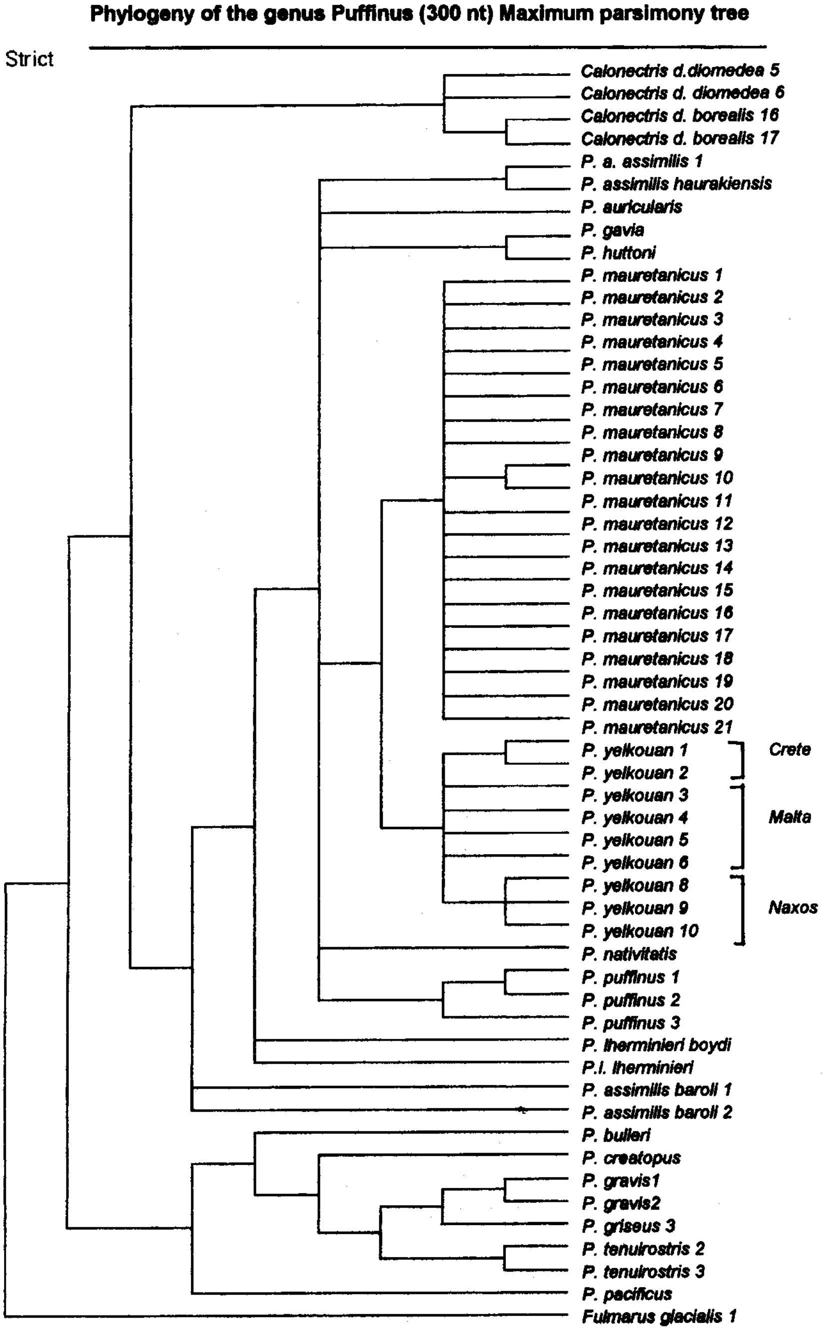

Fig. 1 — *continued*

Phylogeny of the genus Puffinus (300 nt) Maximum Likelihood tree

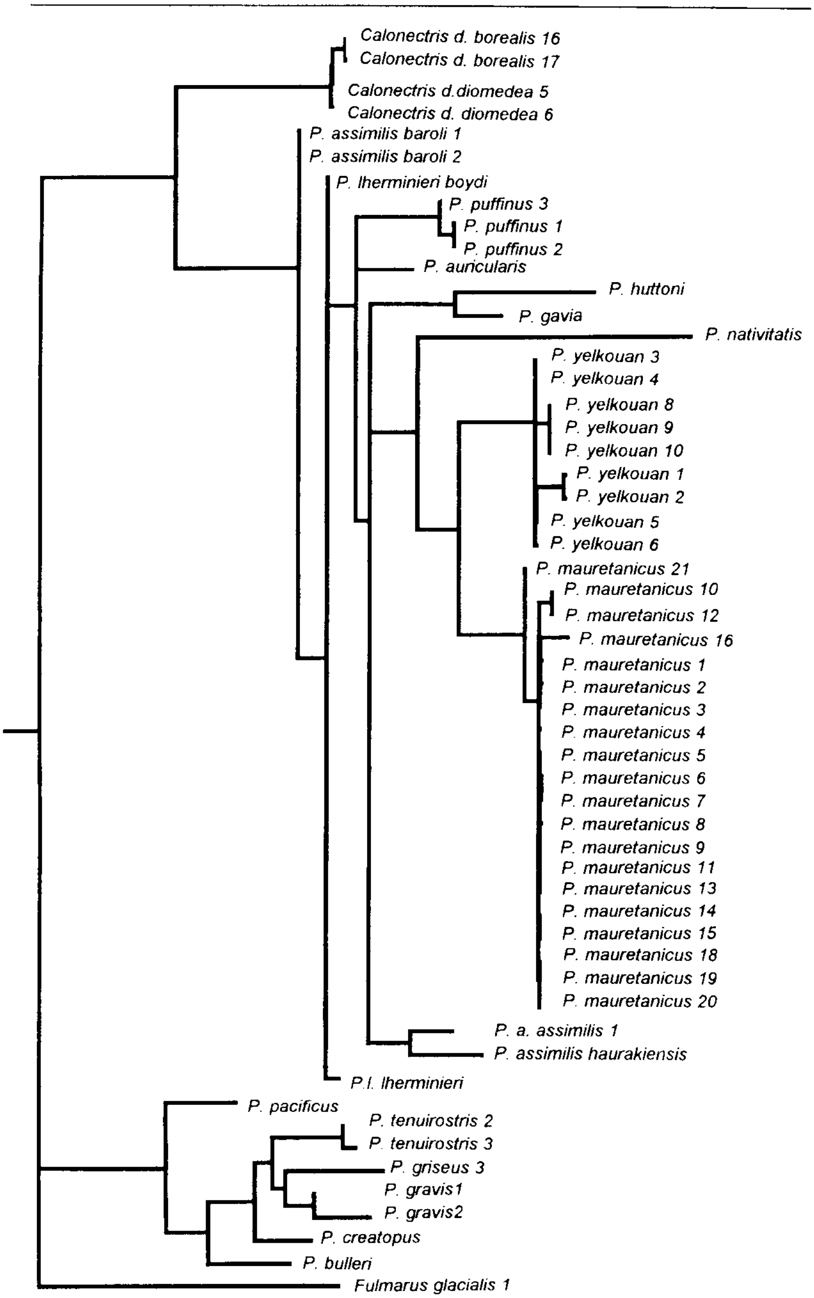

Fig. 1 — *continued*

treated by Austin (1996) and will not be dealt with in this communication. Within the subgroup Puffinus a few relationships are elaborated in more detail here.

The Yelkouan Shearwaters *P. y. yelkouan* and *P. y. mauretanicus* represent sibling taxa and their common clade is supported by a bootstrap value of 95–100% (Figs. 1A

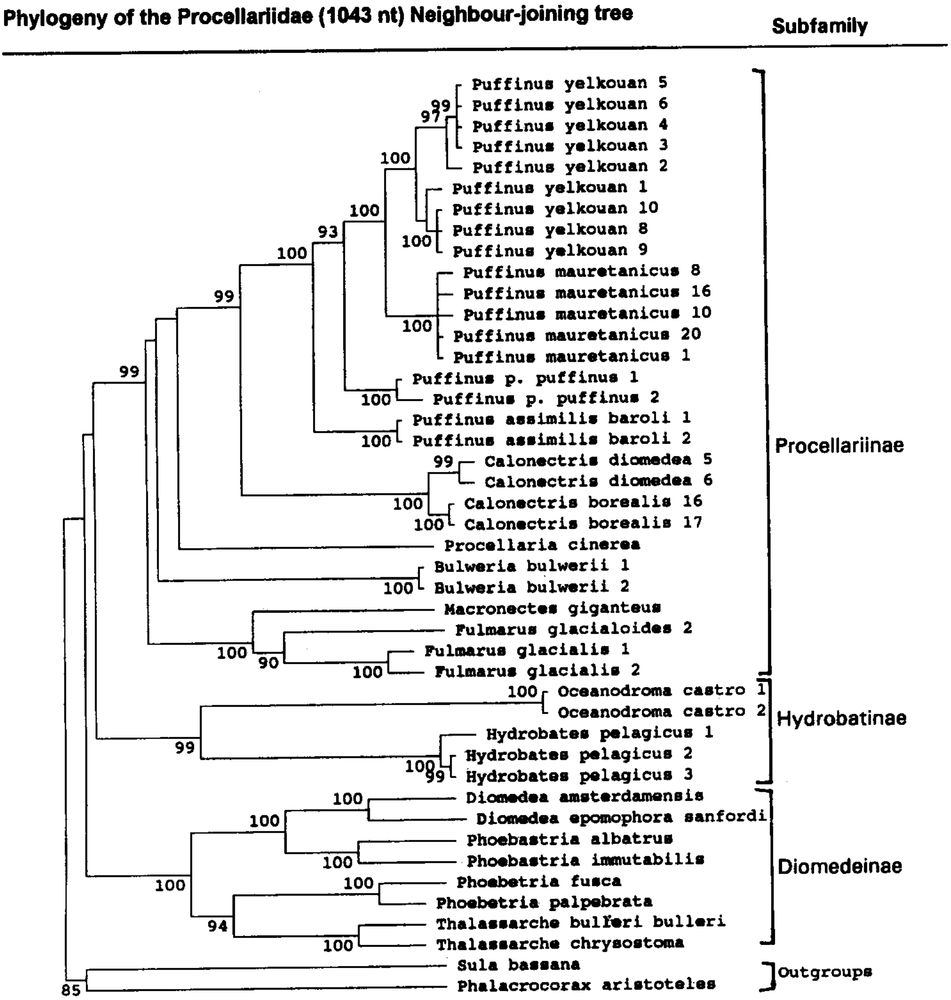

Fig. 2. Phylogenetic relationships of the procellariidae based on 1043 nt of the cytochrome b gene. (A) NJ-bootstrap tree (Jukes-Cantor; 500 replicates) with *Sula bassana* and *Phalacrocorax aristoteles* as outgroups. Illustration as a phylogram in which branch lengths are proportional to genetic distances between taxa. Bootstrap confidence levels ($>$ 80%) are given at each furcation. (B) MP bootstrap tree using *Sula bassana* and *Phalacrocorax aristoteles* as outgroups; heuristic search, simple sample addition; length 1235 steps (min 612, max. 3624 steps); CI = 0.496, HI = 0.504, RI = 0.793); the data set contains 428 variable and 378 parsimony informative characters. (C) Maximum likelihood tree (ln = − 7563.09). All branch lengths are significantly positive ($p < 0.01$; Felsenstein, 1993).

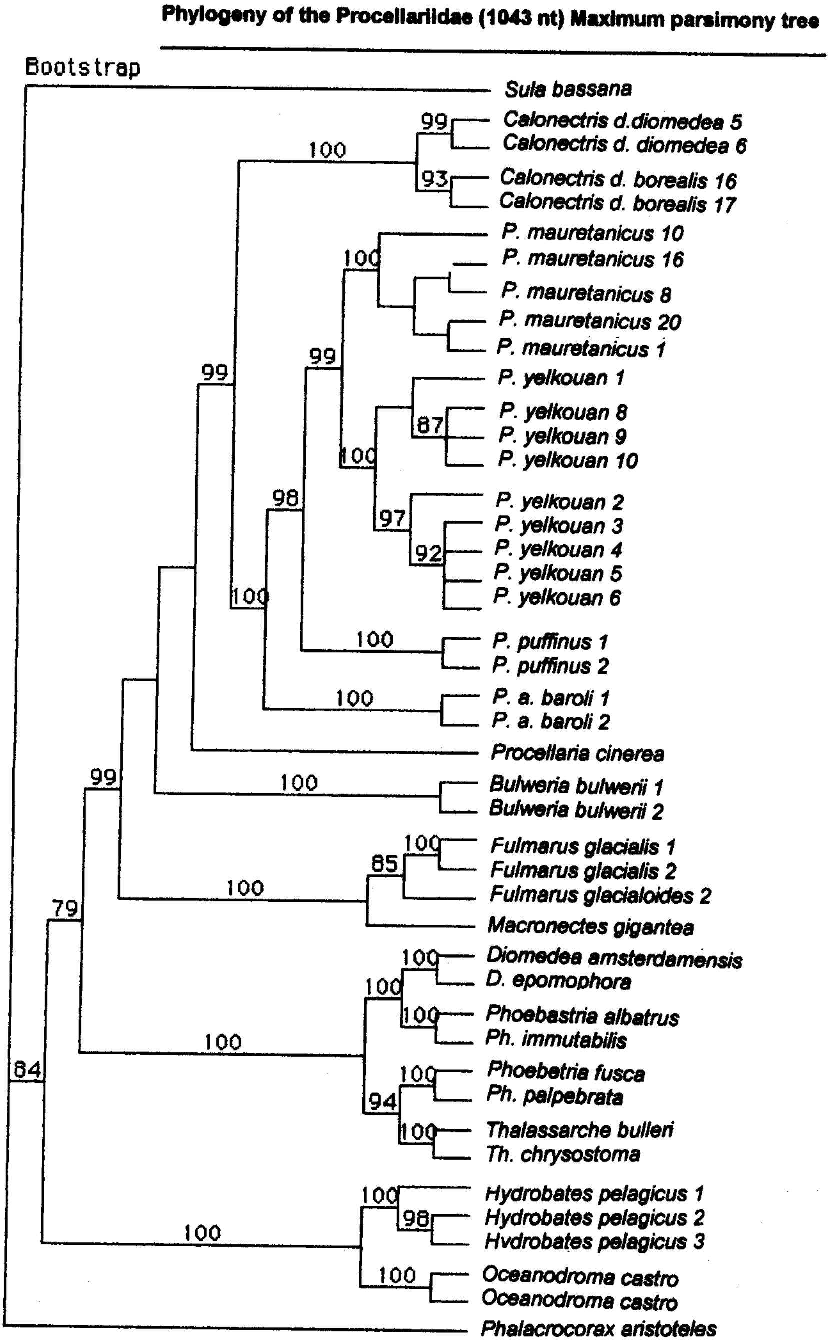

Fig. 2 — *continued*

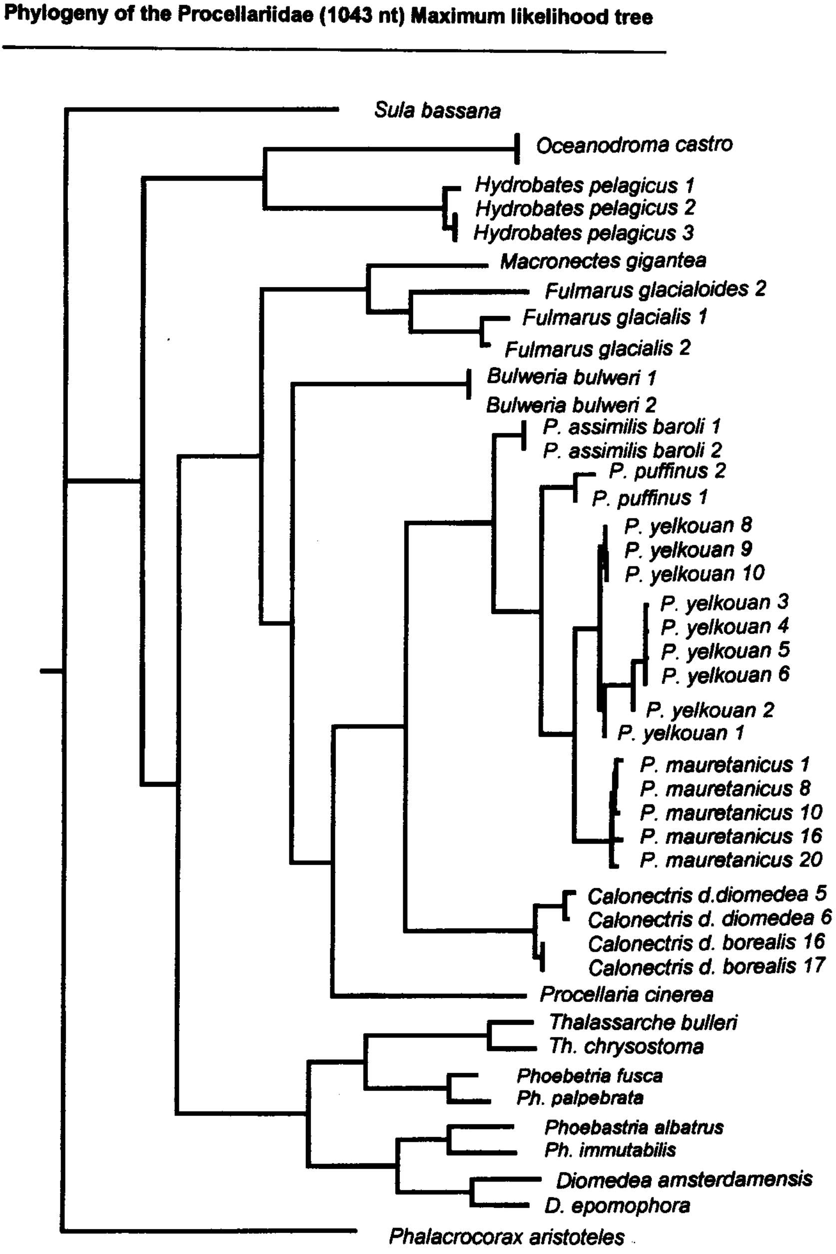
Phylogeny of the Procellariidae (1043 nt) Maximum likelihood tree

Fig. 2 — *continued*

and 2A,B). Both taxa are separated by a genetic distance (i.e. number of nucleotide substitutions) of 2.2–2.9% which is in the same range as distances (Table 3) between established species (see below for a detailed discussion). *P. yelkouan* and

P. mauretanicus do not appear in a same direct clade with *P. puffinus* indicating that they are not subspecies of *P. puffinus* as assumed earlier (Bauer and Glutz, 1987). However, the phylogenetic analyses clearly show that the shearwaters of the Puffinus subgroup (to which *P. puffinus, P. yelkouan* and *P. mauretanicus* belong) are monophyletic, i.e. they share common ancestry.

The Little Shearwater *P. assimilis* apparently represents a polyphyletic taxon (Fig. 1A–C): *P. assimilis baroli* which breeds on Atlantic Islands off the northwest coast of Africa differs significantly from *P. a. assimilis* and *P. a. haurakiensis* which breed in the subantarctic and subtropical southern hemisphere (Del Hoyo et al., 1992; Austin 1996). The sequence of *P. a. baroli* given in Austin (1996) was identical to that of *P. lherminieri boydi*; our two birds differ at two positions from the sequence given in Austin (1996). Since our birds came from Madeira and his birds were from Tenerife geographic differences could explain the discrepancy. On the other hand, *P. l. boydi* which breeds on the Cape Verde Islands is morphologically similar to *P. assimilis* and therefore, has been treated as a subspecies of *P. assimilis* (Bauer and Glutz, 1987; Del Hoyo et al., 1992). Since *P. a. baroli* appears closer related to *P. lherminieri* than to *P. a. assimilis* and *P. a. haurakiensis* (Fig. 1A–C) it needs to be analysed in more detail whether *P. a. baroli* represents a subspecies of Audubon's Shearwater (*P. lherminieri*) or a distinct species of its own. A past introgressive hybridisation between *P. l. boydi* and *P. a. baroli* as discussed by Austin (1996) cannot be ruled out (by studying a mitochondrial, i.e. maternally transmitted gene) but appears to be less likely.

Cory's shearwater breeds either in the Mediterranean (*C. d. diomedea*) or on Atlantic Islands (*C. d. borealis*). Both subspecies differ by distinct nucleotide substitutions. Our phylogenetic reconstructions (NJ, MP, and ML) show that *Calonectris* falls between both subgroups of *Puffinus* (Fig. 1A–C) making *Puffinus* a paraphyletic taxon. Previously, it had been assumed that *Calonectris* is a sister taxon to *Puffinus* (Kuroda, 1954; Jouanin and Mougin, 1979). If our result is correct, it needs to be discussed whether the two *Puffinus* clades ought to be splitted into two genera or whether *Calonectris* and *Puffinus* can to be lumped into a single genus. But further genetic data, preferably from a nuclear gene (not being inherited maternally as mitochondrial genes), are necessary to decide and to corroborate this issue.

3.2. Phylogenetic relationships between shearwaters, petrels and albatrosses

Shearwaters, petrels, and albatrosses are classified either as the subfamilies Procellariinae, Hydrobatinae and Diomedeinae of the Procellariidae (Sibley and Munroe, 1990) or as families Procellariidae, Hydrobatidae and Diomedeidae within the Procellariiformes (Del Hoyo et al., 1992). For a first phylogenetic analysis we have se- lected those taxa for which long cytochrome b gene sequences were available either from our laboratory or from the literature (e.g. for albatrosses; Nunn et al., 1996). As can be seen from Fig. 2, we could analyse the relationships between 8 taxa of the Diomedeinae (Nunn et al., 1996), 2 taxa of the Hydrobatinae, and 11 taxa of the Procellariinae. Taking *Sula bassana* and *Phalacrocorax aristoteles* as outgroups (other outgroups, even the non-related *Gallus* produced identical trees), trees reconstructed by MP, NJ or ML had almost identical topologies (Fig. 2A–C). The three subfamilies are unequivocally recognized as monophyletic groups (bootstrap values 99 or 100%). The only difference between NJ and MP/ML trees was the position of Hydrobatinae and Diomedeinae. Whereas the Diomedeinae were at the base of the NJ tree, this position was taken by the Hydrobatinae in MP and ML trees. Four monophyletic groups within albatrosses (southern mollymawks, sooty albatrosses, north Pacific albatrosses and "great" albatrosses) are evident as already published by Nunn et al. (1996).

The Southern Giant Petrel (*M. giganteus*) and the Northern and Southern Fulmar (*F. glacialis, F. glacialoides*) form a monophyletic clade as expected from morphological evidence (Del Hoyo et al., 1992). The Petrels *Bulweria* and *Procellaria* cluster between the Fulmars and the shearwater of the *Calonectris/Puffinus* group which appear as a monophyletic assemblage (supported by bootstrap values of 99 and 100%).

3.3. Balearic shearwater is a distinct species

A number of palaeonthological (Mayaud and Schaub, 1950; Weesie, 1987; M. McMinn, pers. comm.), morphological, behavioural and ecological differences would imply that *P. yelkouan* and *P. mauretanicus* represent distinct species: Both taxa are allopatric during the breeding season, and ringing recoveries provide no evidence for any exchange between *P. yelkouan* and *P. mauretanicus* breeding colonies (Garcias and Cardona, 1995). Balearic Shearwater is slightly bigger in size and more robust than Yelkouan Shearwater. It is darker in colour and shows less contrasted light underparts; furthermore, *P. mauretanicus* bears a broad and dark breast band, which is diagnostic in the field, although much variation exists (Yésou et al., 1990; Paterson and Yésou, 1994). Apart from the reproductive segregation, both species show a remarkable different post-breeding migratory behaviour: while most of the Balearic Shearwaters leave the Mediterranean in summer for a post-breeding moult in the waters of the Bay of Biscay – although some individuals can remain in the Alboran sea all through the summer (Le Mao and Yésou, 1993) – Yelkouan Shearwaters diverge towards the east during the same period, with important numbers entering the Black sea through the Bosporus (Yésou, 1991). Both species occur together in the Balearic sea and on the NE Spanish coasts in winter, just prior to the breeding season. In the November–February period most if not all of the Balearic Shearwater population concentrates on the coast from Valencia to Catalonia, often assembled in big foraging monospecific flocks in the vicinity of the continental shelf (Gutiérrez and Figuerola, 1995).

The divergence detected in the cytochrome b sequence between Balearic Shearwater and Yelkouan Shearwater accounts for 2.2–2.9%. This divergence is slightly smaller than that between established Shearwater species, such as *P. puffinus* to *P. assimilis* (3.3%) or *P. puffinus* to *P. yelkouan* (2.9 –3.5%) but higher than between the two subspecies of *C. diomedea* (1.2%) (Table 3). In addition, similar or even smaller divergence values were obtained by Austin (1996) between other "good" *Puffinus* species. Thus, also the divergence data would support the separation of *P. yelkouan* and *P. mauretanicus* as distinct phylogenetic species. Also in many other instances

Table 3

Divergence (i.e. nucleotide substitutions (1 = 100%) in the 1043 bp data set) between selected pairs of shearwater, petrel and albatros taxa

	1	2	3	4	5	6	7	8	9	10	11	12
1. Calonectris diomedea 6		0.0951	0.0913	0.0893	0.1162	0.1220	0.1316	0.1578	0.1731	0.0760	0.0837	0.1740
2. Puffinus mauretanicus 10			0.0221	0.0288	0.1191	0.1114	0.1220	0.1550	0.1702	0.0452	0.0327	0.1566
3. Puffinus yelkouan 1				0.0125	0.1143	0.1162	0.1249	0.1453	0.1663	0.0423	0.0298	0.1566
4. Puffinus yelkouan 3					0.1134	0.1172	0.1239	0.1501	0.1731	0.0490	0.0356	0.1586
5. Bulweria bulwerii 1						0.1105	0.1114	0.1444	0.1587	0.1029	0.1106	0.1474
6. Fulmarus glacialis 1							0.0576	0.1482	0.1519	0.1135	0.1183	0.1515
7. Fulmaus glacialofies 2								0.1473	0.1577	0.1173	0.1192	0.1597
8. Hydrobates pelagicus 1									0.1252	0.1464	0.1493	0.1660
9. Oceanodoma castro 1										0.1588	0.1588	0.1660
10. Puffinus a. baroli 1											0.0337	0.1475
11. Puffinus puffinus 1												0.1546
12. Sula bassana												
13. Diomedea amsterdamensis												
14. Diomedea epomophora												
15. Macronectes giganteus												
16. Phoebastria albatrus												
17. Procellaria cinerea												
18. Phoebetria fusca												
19. Phoebastria immutabilis												
20. Phoebetria palpebrata												
21. Thalassarche bulleri												
22. Thalasarche chrysostoma												
23. Calonectris d. borealis 16												
24. Phalacrocorax aristoteles												
25. Puffinus yelkouan 9												

Table 3—*continued*

	13	14	15	16	17	18	19	20	21	22	23	24	25
1. Calonectris diomedea 6	0.1556	0.1575	0.1249	0.1556	0.1172	0.1575	0.1595	0.1527	0.1585	0.1556	0.0125	0.1646	0.0903
2. Puffinus mauretanicus 10	0.1566	0.1566	0.1239	0.1537	0.1095	0.1527	0.1518	0.1489	0.1422	0.1489	0.0922	0.1598	0.0221
3. Puffinus yelkouan 1	0.1556	0.1537	0.1191	0.1518	0.1066	0.1489	0.1527	0.1479	0.1441	0.1489	0.0884	0.1588	0.0038
4. Puffinus yelkouan 3	0.1547	0.1566	0.1191	0.1527	0.1076	0.1518	0.1556	0.1508	0.1470	0.1449	0.0865	0.1578	0.0125
5. Bulweria bulwerii 1	0.1316	0.1383	0.1143	0.1345	0.1057	0.1383	0.1354	0.1345	0.1441	0.1393	0.1095	0.1569	0.1114
6. Fulmarus glacialis 1	0.1431	0.1527	0.0682	0.1518	0.1105	0.1364	0.1499	0.1345	0.1345	0.1354	0.1134	0.1616	0.1153
7. Fulmaus glacialofies 2	0.1527	0.1556	0.0759	0.1547	0.1201	0.1537	0.1470	0.1460	0.1499	0.1451	0.1249	0.1617	0.1239
8. Hydrobates pelagicus 1	0.1530	0.1540	0.1521	0.1530	0.1578	0.1511	0.1463	0.1434	0.1540	0.1511	0.1492	0.1668	0.1453
9. Oceanodoma castro 1	0.1673	0.1712	0.1442	0.1625	0.1654	0.1635	0.1635	0.1615	0.1740	0.1788	0.1606	0.1734	0.1663
10. Puffinus a. baroli 1	0.1442	0.1394	0.1163	0.1413	0.1058	0.1442	0.1413	0.1394	0.1337	0.1346	0.0731	0.1435	0.0413
11. Puffinus puffinus 1	0.1519	0.1481	0.1183	0.1462	0.1038	0.1442	0.1471	0.1404	0.1375	0.1385	0.0798	0.1493	0.0298
12. Sula bassana	0.1484	0.1515	0.1627	0.1648	0.1586	0.1484	0.1546	0.1464	0.1658	0.1658	0.1720	0.1475	0.1556
13. Diomedea amsterdamensis		0.0375	0.1412	0.0682	0.1518	0.0980	0.0692	0.0903	0.1057	0.1037	0.1508	0.1569	0.1547
14. Diomedea epomophora			0.1527	0.0730	0.1623	0.1009	0.0701	0.0999	0.1047	0.1047	0.1527	0.1578	0.1527
15. Macronectes giganteus				0.1460	0.1153	0.1297	0.1479	0.1278	0.1451	0.1403	0.1162	0.1588	0.1182
16. Phoebastria albatrus					0.1556	0.1037	0.0394	0.1018	0.1153	0.1114	0.1489	0.1588	0.1508
17. Procellaria cinerea						0.1431	0.1556	0.1393	0.1499	0.1508	0.1105	0.1588	0.1057
18. Phoebetria fusca							0.1018	0.0211	0.0788	0.0807	0.1508	0.1550	0.1479
19. Phoebastria immutabilis								0.0989	0.1095	0.1066	0.1566	0.1636	0.1508
20. Phoebetria palpebrata									0.0807	0.0807	0.1460	0.1521	0.1470
21. Thalassarche bulleri										0.0269	0.1518	0.1578	0.1431
22. Thalasarche chrysostoma											0.1489	0.1550	0.1479
23. Calonectris d. borealis 16												0.1578	0.0874
24. Phalacrocorax aristoteles													0.1578
25. Puffinus yelkouan 9													

mtDNA data have been applied to define species status on account of differences in cytochrome b genes (Barrat et al., 1997). Since mitochondrial genes are inherited maternally, it has been controversially debated whether mtDNA may be used to assess species limits (Zink and McKitrick, 1995). If two taxa differ morphologically, anatomically, geographically, ecologically or behaviourly and if nucleotide differences are in the same range as between established species in the same systematic group, these DNA differences would support their separation as phylogenetic species in our view (Heidrich et al., 1996). Keeping the constraints in mind, mtDNA data are not 100% conclusive but nevertheless valuable systematic tools.

Bretagnolle and Zotier (1996) have recently proposed to maintain the subspecific status for the Balearic Shearwater, a conclusion based on the similarities in vocalizations and the absence of differential responses to non-specific calls between the oriental and the balearic Shearwaters. Their argument can be challenged for two reasons. First, positive responses to heterospecific vocalizations are not rare in Procellariiformes (Mínguez, 1994), and secondly, both populations have never breed sympatrically, which would diminish the need of a strong acoustic differentiation. Furthermore, the importance of vocalizations for petrel systematics has been questioned (James and Robertson, 1986).

A calibration of a “molecular clock” for mtDNA in *Puffinus* or in other Procellariiformes is not available (but see discussion in Nunn et al., 1996). However, if we use the crude calibration of the mtDNA sequence divergence rate obtained for geese (Shields and Wilson, 1987; Wilson et al., 1987) which equals 2% nucleotide substitutions per million year, the divergence between *mauretanicus* and *yelkouan* has occurred approximately 1 million years ago. Voous (1976) suggested that *Puffinus* was the first Shearwater entering the Mediterranean after the reopening of the Strait of Gibraltar, which took place during the “messinian crisis” about 5 million years ago (Alcover et al., 1981; Maldonado, 1989). In consequence, the evolution and speciation of the Mediterranean *Puffinus* species should have taken place after this event. Balearic Shearwater have been found in the fossil record of the Balearic Islands at least from the Middle Pleistocene onwards. The present species shows strong similarities to *P. nestori*, an extinct species from the Lower Pleistocene of Ca Na Reina cave in Ibiza (Alcover, 1989a; Sondaar et al., 1995), found in levels dated approximately 2 million years (M. McMinn, pers. comm.). The absence of *P. nestori* from the Plio-Pleistocene fossil record of the eastern Mediterranean suggests that this taxon was an ancestor of the Balearic Shearwater, and that the divergence from the clade leading to Yelkouan Shearwater happened even earlier. The fossil register of *Puffinus* of the eastern part of the Mediterranean is scarce and not properly dated (Weesie, 1987), thus this hypothesis still awaits confirmation. Nevertheless, the fossil data imply that the molecular divergence time estimate of 1 million appears to be rather conservative and that the real value could be even higher. Since the speed of the molecular clock is correlated with reproduction time – Shearwater only breed when 3 to 5 years old and produce a single chick – we should expect that the clock is slower in Shearwater than in geese. This would agree with the conclusion drawn from albatrosses, that cytochrome b evolutionary rates are slow compared with those of most mammals (Nunn et al., 1996). Concluding, the Mediterranean shearwaters

P. mauretanicus and *P. yelkouan* are “good” species which appear to have evolved from allopatric populations of an ancestral member of the *Puffinus* clade about 1–2 million years ago.

3.4. Conservation of the balearic shearwater

Balearic Shearwaters only breed on the Balearic archipelago. The effective population size is in the range of 1800–4000 breeding pairs, most of them (ca. 70%) concentrated on the island of Formentera (Del Hoyo et al., 1992; Aguilar, unpubl. data). Numbers of Balearic Shearwaters wintering on the NE coast of Spain are between 8000–11,000 birds (Gutièrrez and Figuerola, 1995). These numbers are in agreement with size of flocks of post-breeding moulting birds detected in the Bay of Biscay before November (Le Mao and Yésou, 1993) indicating that the figure of 15,000 birds (breeders and non-breeders) published by Yésou et al. (1990) appears to be an overestimation.

The species only breeds on isolated islets around the main islands of the archipelago, or in inaccessible caves and small to medium size cavities in sea cliffs of Formentera, one locality in Mallorca, on islets of Cabrera archipelago and Menorca. The former breeding range of the species was much broader than at present, with huge colonies in Ibiza (Alcover et al., 1994); colonies were also found on Cabrera gran (M. McMinn, pers. comm.) which are deserted at present. Two factors have been cited as the most likely cause responsible for the steep decrease of the Balearic populations: Direct human consumption (Mayol, 1985), which although less intense, still continues on Formentera, and the introduction of alien species, particularly rats *Rattus rattus* (Alcover, 1989b), and most probably feral cats *Felis catus* and genets *Genetta genetta*. These threats appear to exist worldwide for members of the whole genus: at least three insular species have become extinct soon after the arrival of man to the respective breeding islands (Walker et al., 1990; McMinn et al., 1990; Holdaway and Worthy, 1994); and other species have suffered strong reductions in their numbers. Local extinctions are quite common (Vigne et al., 1991). Since *P. mauretanicus* breeds in such a small area it is highly vulnerable and needs special attention and protection.

In view of our results, the conservation status of the Balearic Shearwater needs to be revised: In Spain, the Balearic Shearwater is officially classified as a subspecies of *P. yelkouan*. Since *P. yelkouan* is in *SPEC Category* 4 which implies that the species has a “favourable conservation status in Europe” (Tucker and Heath, 1994) its status is only “*vulnerable*” (Blanco and González, 1992). Since our data imply that *P. mauretanicus* is a “good” species, its conservation status would have to change to “*endangered*” in Spain, with a *SPEC Category* 2 in Europe.

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