

**Genetic evidence for speciation of
the Manx Shearwater *Puffinus puffinus* and
Mediterranean Shearwater *Puffinus yelkouan****

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The Atlantic and Mediterranean populations of the Manx shearwater *Puffinus p. puffinus* and *P. p. yelkouan* are usually treated as subspecies, although they differ in morphology, vocalisations and geographical characters. To solve this taxonomic question we have employed methods of molecular biology such as polymerase chain reaction (PCR) and DNA sequencing. The cytochrome-b gene of the Atlantic and East Mediterranean Manx shearwater was amplified by (PCR) and partially sequenced (300 base pairs). Sequences differ substantially (6.6% nucleotide exchanges) between both taxa. For comparison Cory's shearwater *Calonectris diomedea* and *P. puffinus* differ by 7.7%, whereas the subspecies of *C. diomedea* are separated by 1.5% base substitutions. It is likely therefore, that the Manx and the Mediterranean shearwater have reached species level already. In consequence, both taxa should be considered as allospecies belonging to a common superspecies. Cytochrome-b sequences of *P. puffinus* and *P. yelkouan* are compared phylogenetically ("maximum parsimony"; PAUP 3.1.1) with those of other Procellariidae *Calonectris diomedea*, *Fulmarus glacialis* and other seabirds *Larus argentatus*, *Rissa tridactyla*, *Phalacrocorax aristotelis*. *Puffinus* appears paraphyletic to *Calonectris* whereas *Fulmarus*, although clearly a petrel, is only distantly related to shearwaters.

Keywords: *Puffinus puffinus*, *Puffinus yelkouan*, molecular systematics, PCR, cytochrome-b gene, Procellariidae.

Zusammenfassung

Wink, M., P. Heidrich & D. Ristow (1993): Molekulare Differenzierung zwischen Schwarzschnabelsturmtaucher *Puffinus puffinus* und Mittelmeersturmtaucher *Puffinus yelkouan* anhand von Nukleotidsequenzen des Cytochrom-b Gens. – Vogelwelt 114: 226-232.

Bislang sind die atlantischen und ostmediterranen Populationen des Schwarzschnabelsturmtauchers meist als Subspecies *Puffinus p. puffinus* und *P. p. yelkouan* betrachtet worden, obwohl sie sich morphologisch, stimmlich und biogeographisch deutlich unterscheiden. Um diese taxonomische Frage zu klären, haben wir molekularbiologische Methoden wie die Polymerase Kettenreaktion (PCR) und DNA-Sequenzierung eingesetzt. Aus Gesamt-DNA wurde das mitochondrielle Cytochrom-b Gen von beiden Taxa mittels PCR amplifiziert und teilweise sequenziert (300 Basenpaare). Die Nukleotidsequenzen unterscheiden sich durch 6,6% Basenaustausche. Zum Vergleich, Gelschnabelsturmtaucher und Schwarzschnabelsturmtaucher weisen 7,7% Unterschiede auf, während sich Unterarten des Gelschnabelsturmtauchers mit 1,5% Basenaustausche differenzieren lassen. *P. yelkouan* hat demnach bereits Artniveau erreicht. Deshalb sollten beide Taxa als getrennte Arten, Schwarzschnabelsturmtaucher *Puffinus puffinus* und Mittelmeersturmtaucher *P. yelkouan*, betrachtet werden, die einer Superspecies angehören. Die Phylogenie beider Taxa wird aufgrund von Sequenzvergleichen des

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Cytochrom-b Gens nach der „Maximum Parsimony“-Methode (PAUP 3.1.1.) in Relation zu anderen europäischen Sturmvögeln Gelbschnabelsturmtaucher *Calonectris diomedea*, Eissturmvogel *Fulmarus glacialis* und Seevögeln (Silbermöwe *Larus argentatus*, Dreizehenmöwe *Rissa tridactyla* und Krähenscharbe *Phalacrocorax aristotelis*) analysiert.

1. Introduction

Manx shearwater *Puffinus puffinus* Brünnich, 1764 represents a polytypic taxon and breeds mainly in the east temperate North Atlantic, the Mediterranean and locally in the Pacific (Cramp & Simmons 1977). In Europe three subspecies are usually distinguished on account of geographical and morphological differences: The nominate *P. p. puffinus* (Brünnich, 1764), *P. p. mauretanicus* Lowe 1921 and *P. p. yelkouan* (Cerbi, 1927) (Cramp & Simmons 1977; Bauer & Glutz 1987; Brooke 1990; Warham 1990). *P. p. mauretanicus* and *P. p. yelkouan* occur in the Mediteranean: Whereas *P. p. mauretanicus* breeds on the Balearics, *P. p. yelkouan* is a bird of the eastern Mediterranean.

While most handbooks consider these taxa as subspecies (Cramp & Simmons 1977; Bauer & Glutz 1987), *P. p. yelkouan* has been treated as a distinct species by some authors (Bourne et al. 1988; Sibley & Monroe 1990). In order to solve this question we have employed techniques of molecular genetics, such as polymerase chain reaction and DNA sequencing (Ehrlich 1989; Innis et al. 1990; Hoelzel 1992; Hillis & Moritz 1991). The mitochondrial cytochrome-b gene has been selected as a marker gene since its sequence has been found informative for many phylogenetic and taxonomic problems of animals, especially of birds (Kocher et al. 1989; Richman & Price 1992; Wink et al. 1993, 1994; Helbig et al. 1993, 1994; Seibold et al. 1993, 1994a, b; Heidrich & Wink 1994).

In this communication the cytochrome-b nucleotide sequences of the Manx and Mediterranean Shearwater were used to evaluate their degree of speciation and to reconstruct a phylogenetic relationship between other European seabirds (*Calonectris diomedea*, *Fulmarus glacialis*, *Larus argentatus*, *Rissa tridactyla*, *Phalacrocorax aristotelis*).

2. Material and Methods

Origin of birds: An adult *P. p. puffinus* was found dead near Puffin Island, Ireland in Aug. 1992. *P. yelkouan* (adult and 1 nestling) were from an island off Crete (Greece).

DNA-methods: Blood or tissues were stored in a modified EDTA-buffer (Arcander 1988) at ambient temperatures in the field. DNA was extracted after digestion with proteinase K (Boehringer) according to Swatschek et al. (1993). Using PCR the cytochrome-b gene was amplified and purified by agarose gelelectrophoresis and followed by Quiagen extraction (Diagen, Düsseldorf) (Wink et al. 1993; Helbig et al. 1994). Double-stranded PCR products were directly sequenced by the chain termination method (Sambrook et al. 1989) using ³⁵S- α -dATP (NEN Dupont) as a tracer. Sequences were determined manually from autoradiograms and aligned

with the cytochrome-b sequence of *Gallus domesticus* (Desjardin & Morais 1990). Sequence data were evaluated according to the "maximum parsimony" principle with the phylogeny program PAUP 3.1.1 (Swofford 1993) considered to be very reliable and useful for this purpose (Steward 1993).

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3. Results and Discussion

DNA from both subspecies of Manx Shearwater was amplified using primers specific for the mitochondrial cytochrome-b gene (Kocher et al. 1989). This gene was only partially sequenced since enough information could be obtained from a 300 bp fragment to answer the given taxonomic problems (Fig. 1). Both *Puffinus* sequences were evaluated using PAUP 3.1.1. and compared with homologous sequences of other Procellariidae, such as the Cory's shearwater *Calonectris diomedea* and the Fulmar *Fulmarus glacialis* and other seabirds (Herring gull, *Larus argentatus*, Kittiwake *Rissa tridactyla* and Shag *Phalacrocorax aristotelis*). These sequences had been produced by identical procedures in our laboratory (Wink et al. 1993, 1994).

Gallus	AGGCGGTGGC	TATGAGTGTG	AGGAGGAGGA	TTACTCCTGT	GTTTCAGGTT	TCCTTGTAGA
<i>P. yelkouan</i>	??????????	??????A.C.	..C.....	.G.....C	..T.A..T.
<i>P. puffinus</i>	...A..T..A...	..T.....	.G.....C	..T.A..T.
Gallus	GGTAGGAGCC	GTAGTATAGG	CCTCGTCCGA	TGTGAAGGAA	GATACAGATG	AAGAAGATG
<i>P. yelkouan</i>	A.....G.AT	T...T.	A.....
<i>P. puffinus</i>	...A.....G.AT	C...T.	A.....
Gallus	AGGCGCCTTT	CGGTGGAGAT	TCCGGATGAG	TCAGCCGTAT	TGTACGTTCC	GGCAAGTGTG
<i>P. yelkouan</i>A..T..G	..T..A..T.A..T	..A..T..A.
<i>P. puffinus</i>	...A.....	...A..T..G	..T...T.A..A..TT..A.
Gallus	GGCTACGGAG	GAGAAGGCTA	GGGATGTGTC	TGCTGTGTAG	TGCATGGCTA	GTAGTAGGCC
<i>P. yelkouan</i>	...A.....T	..A.....	..A.T...A.	A..A..A..A	..T.....C.....
<i>P. puffinus</i>	...G.....T	..A.....	..A.T...A.	A..A.....	..T..A....
Gallus	GGTGAGGATT	TGGGTCATGA	GGCAGACTGC	TAATAGGAAG	CCGAAATTTC	ATCATGCTGA
<i>P. yelkouan</i>	A..T.AA...GT.TGC.	..GG..AG..G....
<i>P. puffinus</i>	A..T.AA...T.GT.TGC.	..GG..AG..G....

Fig. 1: Nucleotide sequence of cytochrome-b (H-strand) of *P. puffinus* and *P. yelkouan*. . = base identical to that in the first line; ? = base could not be identified on the autoradiogram. - Nucleotidsequenz des Cytochrom-b Gens (H-Strang) von *Puffinus puffinus* und *P. yelkouan*. . = Base identisch mit derjenigen in der jeweils ersten Zeile eines Blockes; ? = Base konnte auf dem Autoradiogram nicht gelesen werden.

Both sequences of the Mediterranean shearwater were identical. Therefore, only one sequence was used for the following evaluations. Variation in the nucleotide sequences of *Gallus* and both shearwaters (Fig. 1) often concern the third base in a triplet codon resulting in "silent" mutations only, i.e. this substitution does not lead to any alteration of the amino acid

sequence of the protein although it is highly informative for studying molecular taxonomy.

Tab. 1 contains a distance matrix of all taxa studied as a measure of similarity. The cytochrome-b gene of *P. p. puffinus* differs from that of *P. p. yelkouan* by 18 nucleotide exchanges (= 6.6%). Considering the differences between other taxa from Procellariidae, Laridae and Phalacrocoracidae this value is of the same magnitude as corresponding values between established species, which are between 7.7% and 17%. In *C. diomedea* we have evaluated the genetic differences between two subspecies, e.g. *C. d. diomedea* from the Mediterranean and *C. d. borealis* from the Atlantic. Although both taxa differ significantly in size, their cytochrome-b sequences are very similar, differing only by 0.7-1.5% (Wink et al. 1993). In contrast, the differences between the two *Puffinus* taxa are typical for species and much too high for subspecies level. Therefore, we suggest that both taxa represent "true" species, i.e. *Puffinus puffinus* and *P. yelkouan* acknowledging the substantial morphological, acoustical and geographical differences, described in Bourne et al. 1988; Cramp & Simmons (1977) and Bauer & Glutz (1987).

Tab. 1: Evaluation of nucleotide exchanges in cytochrome-b sequences of shearwaters, gulls and shag. Below diagonal: absolute distances; above diagonal: mean distances in %. – Zusammenstellung der Basenaustausche im Cytochrom-b Gen von Sturmtauchern, Krähenscharbe und Möwen. Unterhalb der Diagonale: Absolute Distanzen (Anzahl der Substitutionen); oberhalb der Diagonale: mittlere Distanzen in %.

Taxon	1	2	3	4	5	6	7	8
1. <i>P. yelkouan</i>	–	6.6	14.0	10.4	10.3	16.6	15.4	15.1
2. <i>P. puffinus</i>	18	–	12.9	8.7	7.7	14.0	12.9	16.1
3. <i>F. glacialis</i>	38	37	–	13.5	12.9	12.5	15.3	15.7
4. <i>C. d. borealis</i>	26	23	36	–	1.5	14.0	14.2	15.9
5. <i>C. d. diomedea</i>	28	22	37	4	–	14.4	13.6	16.5
6. <i>P. aristotelis</i>	45	39	35	36	40	–	14.0	14.0
7. <i>L. argentatus</i>	42	37	44	38	39	39	–	7.9
8. <i>R. tridactyla</i>	41	45	44	41	46	39	22	–

Although we have not analyzed yet *P. p. mauretanicus* genetically it seems plausible to accept *mauretanicus* as a subspecies of *P. yelkouan* (Sibley & Monroe 1990). Because of differences in vocalizations and morphology Sibley & Monroe (1990) consider all subspecies of *P. puffinus* as allospecies [such as *P. yelkouan*, *P. auricularis* (from Hawaii and Revillagigedo Is.), *P. opisthomelas* (Baja California), *P. gavia* (from New Zealand) and *P. huttoni* from New Zealand] belonging to a superspecies and not as conspecific as do Cramp & Simmons (1977) or Bauer & Glutz (1987). It would be interesting to evaluate these taxa genetically whether they are as distinct from *P. puffinus* as *P. yelkouan*.

As a next step we have analyzed the phylogenetic relationship between Manx shearwater and other European seabird species by the maximum parsimony method employing exact algorithms ("Branch & Bound"). Fig. 2A illustrates a phylogram in which *Larus* and *Rissa* served as outgroups. Figures correspond to the number of nucleotide substitutions between species. Fig. 2B represents a cladogram calculated by a "bootstrap" procedure (Swofford 1993). Confidence estimates (in %) show the robustness of each furcation. It is evident that *Puffinus puffinus*, *P. yelkouan* and *C. diomedea* fall into one clade (true Procellariidae), whereas the fulmar is more distantly related. The position *Phalacrocorax* indicates that the respective taxa had a common ancestor in the far past. These relationships are in agreement with current taxonomic knowledge (Sibley & Monroe 1990).

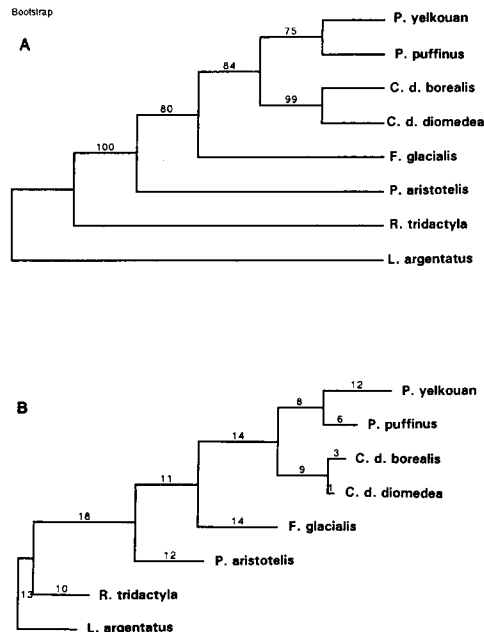


Fig. 2: Reconstruction of a phylogenetic tree by the maximum parsimony method using PAUP 3.1.1 Algorithm: Branch & Bound; bootstrap procedure: 100 replicates. *L. argentatus* and *R. tridactyla* served as "outgroups". A. Phylogram, B. cladogram. – Rekonstruktion der Phylogenie ausgewählter Sturmvögel nach der „Maximum parsimony“-Methode (Sparsamkeitsprinzip) mittels des Phylogenieprogramms PAUP 3.1.1.-Bedingungen: Exakter Algorithmus: Branch & Bound; Bootstrap-Verfahren mit *L. argentatus* und *tridactyla* als Außengruppen. A. Phylogram; Zahlen geben die Basensubstitutionen zwischen zwei Taxa an; B. Kladogram; Zahlen (in%) geben die statistische Wahrscheinlichkeit für das Vorliegen der angegebenen Verzweigung an.

Our study shows that methods of molecular biology can be helpful to elucidate and interpret taxonomic and ecological problems which were a matter of dispute using traditional methods. The molecular approach does not oppose traditional methods but rather complements them.

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