DNA AMPLIFICATION AND SEQUENCING OF UNIDENTIFIED DARK-RUMPED *OCEANODROMA* STORM-PETRELS (AVES) IN THE NORTH ATLANTIC OCEAN

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With 1 figure and 2 tables

ABSTRACT. DNA sequence data from the mitochondrial cytochrome-b gene are presented for two samples taken from dark-rumped *Oceanodoma* storm-petrels in the North Atlantic Ocean. These are compared with data from *Oceanodroma castro*, *O. leucorrhoa* and *O. monorhis*. It is shown that the sequences are indistinguishable from *O. monorhis*, supporting the identification based upon morphological and vocalisation data.

RUNNING HEAD: DNA sequences of *Oceanodroma* storm-petrels.

INTRODUCTION

Since 1983, there have been eleven captures of an unknown storm-petrel (Hydrobatidae: *Oceanodroma* sp.) in the northeastern Atlantic. All these records have been from three localities between June and July (BRETAGNOLLE *et al* 1990; CUBITT *et al.*, 1992), and appear to concern representatives of the same taxon. In some instances, the same individual has been recaptured in the same or successive years.

DNA analysis has proved to be a valuable tool for investigating evolutionary relationships among various avian taxa (AVISE & ZINK, 1988; SMITH *et al.*, 1991), and we were invited to assist with the determination of these mystery birds. The possibility existed that they were representatives of one of the dark-rumped Pacific species of *Oceanodroma* or even a new and hitherto unsuspected taxon. We used blood samples from two of the birds

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captured in 1991 to compare DNA sequences of the unknown birds with other storm-petrels. Samples were collected by FJAZ on Selvagem Grande and. DTP from Tynemouth in July 1990. Samples of other species were collected and contributed by these and other workers, including P-OW.

DNA sequencing and analysis

The technique that we have used is one that has become established as an excellent method for comparing homologous DNA sequences, both within and between populations and species. The Polymerase Chain Reaction (MULLIS & FALOONA, 1986; SAIKI *et al.*, 1988) has many uses in molecular biology, but provides evolutionary and population geneticists with an ideal tool for handling the large sample sizes required to analyse DNA variation in natural populations.

The gene chosen for analysis in the storm-petrels was cytochrome *b* (cyt-*b*). This is one of 13 protein-encoding genes in the circular mitochondrial DNA (mtDNA) genome. The mtDNA genome of chicken is 16,775 base-pairs in length (DESJARDINS & MORAIS 1988), and the cyt-*b* gene itself is 1000 base-pairs long. We amplified and sequenced a part of this using the "universal" primers of KOCHER *et al.* (1989), which specifically target a section of the gene for amplification.

The sequence of these newly synthesised fragments was determined using the method of Sanger, incorporating the use of chain-terminating dideoxynucleotides, and ^{35}S-dATP as a radio-label. Four reactions are done - one for each base G, A, T and C - and the pattern of bands following autoradiography is read in accordance with these.

DNA sequences were entered into *DNA-STAR*, a computer-based library and handling/analysis package. The sequence data can be analysed on the basis of the presence or absence of shared sites among aligned sequences. In these alignments, base substitutions are scored as transitions or transversions. Transitions are exchanges of one purine base for another, or one pyrimidine base for another. Transversions are the exchange of a purine for a pyrimidine, or vice versa. The former are more frequent, but there is a saturation point above which the observed increase in the number of transitions is slower than that in the number of transversions. For this reason, it is the ratio of the two kinds of substitution that is used when examining closely-related species, rather than the gross number of substitutions. However, the percentage divergence can provide an indication of the degree of saturation, or likely loss of information owing to multiple substitutions at the same site ('multiple hits').

By examining the number of shared base substitutions across all species, it is possible

to construct a tree of relationships. Where such a tree is derived from the sequence comparisons of a single gene, it is more correctly referred to as a "gene tree" rather than a species tree, which may be an important distinction. The most common method is based on the principle of 'maximum parsimony'. This relies upon constructing a tree that describes the relationships among the sequences by finding the shortest number of steps required to generate the observed differences. This is achieved using 'phylogenetically informative sites', those at which at least two different bases are each shared by at least two sequences. We have constructed gene trees for the cyt-*b* gene section of storm-petrels using the software package PAUP version 3.0g (SWOFFORD 1990). We used the sequence of a Short-tailed Shearwater *Puffinus tenuirostris* as an outgroup species to root this tree. A statistical test that can be applied to such a tree is the bootstrap. This permits the placement of confidence limits upon the internal branches of the tree, and gives a measure of its robustness.

Sequences from Swinhoe's and Atlantic dark-rumped storm-petrels

Partial cytochrome b gene sequences obtained from the dark-rumped petrels captured at Tynemouth and on the Salvages in 1991 were fully aligned against the chicken reference sequence, and there were no insertions or deletions. The petrel sequences are identical across 302 bases; full details will be published elsewhere (DAWSON *et al.*, in prep.). This strongly suggests that these birds represent the same taxon. The sequences were markedly different from those obtained from Leach's *Oceanodroma leucorrhoa*, Madeiran *O. castro*, Galapagos *O. tethys* and British Storm-Petrels Hydrobates *pelagicus*. Sequences from museum skins of Swinhoe's Storm-Petrels O. monorhis collected in Korea by P-OW matched those obtained for the unknown birds. Subsequently, equally similar sequences were obtained from fresh feathers donated by Dr. NATALIA LITVINENKO (Vladivostock), and fresh tissue samples from Kùgùl Islet (P-OW).

Thus, our findings support the morphological and behavioural data and indicate that the unknown birds of the northeastern Atlantic are indeed *O. monorhis*. However, a further question is to assess the taxonomic position of *O. monorhis* relative to the other *Oceanodroma* species, and in particular *O. leucorrhoa* with which the former has been taxonomically associated (e.g. AUSTIN, 1952). By comparing differences within and among populations, and relating these to differences found among species, we hope to elucidate the evolutionary relationships of this problematic group.

Within-population variation

Although the sample sizes are small, there is very little variation in *cyt-b* sequence within a population, and identical sequences are found in different individuals. Thus, in

Madeiran Storm-Petrels from Bugio (Madeira), there are four distinct types (Table 1) that are distinguished by differences at only three sites. Haplotype 1 (H1) has the bases C...C...C at these positions and is shared by six out of ten individuals. H4 has the bases T...T...T at the equivalent positions, and is shared by two individuals. H2 and H3 have combinations of C and T at these positions. As a minimum of three substitution events are required to get from H1 to H4, it is likely that we do not have a representative view of the overall haplotype frequencies within the population. This will also be discussed in more detail elsewhere (DAWSON *et al.*, in prep.). However, we have fairly convincing evidence of the similarity of individuals within a population.

We have found similarly low levels of intra-population variation among those individuals that we have examined for *O. leucorrhoa* from the Shetland and Flannan Islands in the north-east Atlantic and at sea in the Pacific, for *O. monorhis* from Korea and Vladivostock, and for *H. pelagicus* captured at Tynemouth (DAWSON *et al.* in prep.).

Between population variation

There is little material available for examining differences in sequence between populations. As yet, we have been unable to obtain fresh samples from the darker-rumped races of Leach's Storm-Petrel from the eastern Pacific for comparison with nominate Pacific and Atlantic birds, while feather samples kindly donated by the Natural History Museum, Tring UK yielded ambiguous sequences. No variation has been found among the representatives of the populations of *O. monorhis.*, and the Atlantic records may involve birds from an as yet unlocated breeding population. The data from the nominate *O. l. leucorrhoa* provide some evidence of population divergence; birds examined from the northeastern Atlantic can apparently be distinguished from that from Canada and the Pacific by a single base change. However, this is a transversion - an unusual substitution event to find within a subspecies. As it occurs within a region that sometimes proves difficult to resolve, this difference is being re-checked.

Between species variation

When comparisons are made between species, a clear difference is observed. Sequences among storm-petrels differ by from eight to 12 per cent (8 - 10% within the *Oceanodroma*). On this basis, *O. monorhis* is most similar to *O. leucorrhoa*. However, in order to examine the relationships more closely, it is necessary to examine the nature and number of base substitutions.

The number of transitions (shown below the diagonal in Table 2) observed among

pairs of storm-petrel species is between 20 and 31, far more than the 1-3 seen when comparing species or populations. The number of transversions is rather lower, and it is the ratio of these different kinds of substitution events that becomes rather more informative. Thus, the ratio is greatest for *O. monorhis* vs. *O. leucorrhoa* (20:1), lower for *O.monorhis* vs. *O. castro* (6:1) and *O. leucorrhoa* vs. *O. castro* (5:1), and lowest for *O. castro* vs. *H. pelagicus* (4:1). These ratios are guides to the degree of divergence among the genes, taken to reflect the divergence among species. However, it also illustrates the speed at which genes begin to lose some of their resolving power. In particular, the position of *H. pelagicus* is not clearly different from the *Oceanodroma*, this branch being only weakly supported by parsimony analysis (Fig 1). What is clear is that the close relationship of *O. monorhis* and *O. leucorrhoa* is accentuated. Earlier studies (EDWARDS *et al.*, 1991) suggested that a 20:1 ratio was typical of closely related species. As more data have accumulated, it has become clear that this is by no means a 'magic number', for good species may differ by far fewer substitutions in their cytochrome b sequences. However, on the basis of our cytochrome b data, it seems probable that *O. monorhis* and *O. leucorrhoa* are sister species, supporting the current classification. We shall not know the true extent of molecular divergence of the *monorhis-leucorrhoa* complex until samples from the eastern Pacific races of *O. leucorrhoa* are examined.

Conclusions

1. Cytochrome *b* gene sequences from the two unknown birds were identical and matched homologous sequences obtained from Swinhoe's Storm-Petrel tissue from three separate sources. This supports the identification of BRETAGNOLLE, CUBITT and co-workers, and raises the exciting possibility of the existence in the Atlantic of a breeding population of this otherwise exclusively Pacific seabird.

2. Intra- and inter-population sequence variation among storm-petrel species has been examined. Where sufficient samples have been analysed for Madeiran Storm-Petrel, it is clear that sequence differences are in the region of 1% among individuals. Similar population variation is suggested by comparisons of Swinhoe's, Leach's and British Storm-Petrels. Further study of the frequency and distribution of haplotypes should provide clearer information about the extent of variations.

3. Between species variation among storm-petrels is clear, and is similar to that found among passerine species. Sequences differ by approximately 10%. Transition: transversion ratios suggest that *O. monorhis* and *O. leucorrhoa* are sister species. There is one amino acid substitution between these two species following translation into the corresponding protein sequence; this involves two transition events.

4. The inclusion of *H. pelagicus* does not resolve the monophyly of the *Oceanodroma* using maximum parsimony analysis. This is likely to be the combined result of the short length of sequence analysed, the saturation of a rapidly evolving gene sequence owing to multiple hits, and the parameters adopted for the analysis rather than a misclassification of this species.

5. The cytochrome *b* gene sequences used in this study have proved to be useful in the identification of an unknown species of storm-petrel. However, there are limitations in using this "universal" fragment of the gene when carrying out phylogenetic analyses. Further investigation into the *O. leucorrhoa* complex of storm-petrels shouould provide a fuller account of the evolution of this enigmatic group of seabirds.

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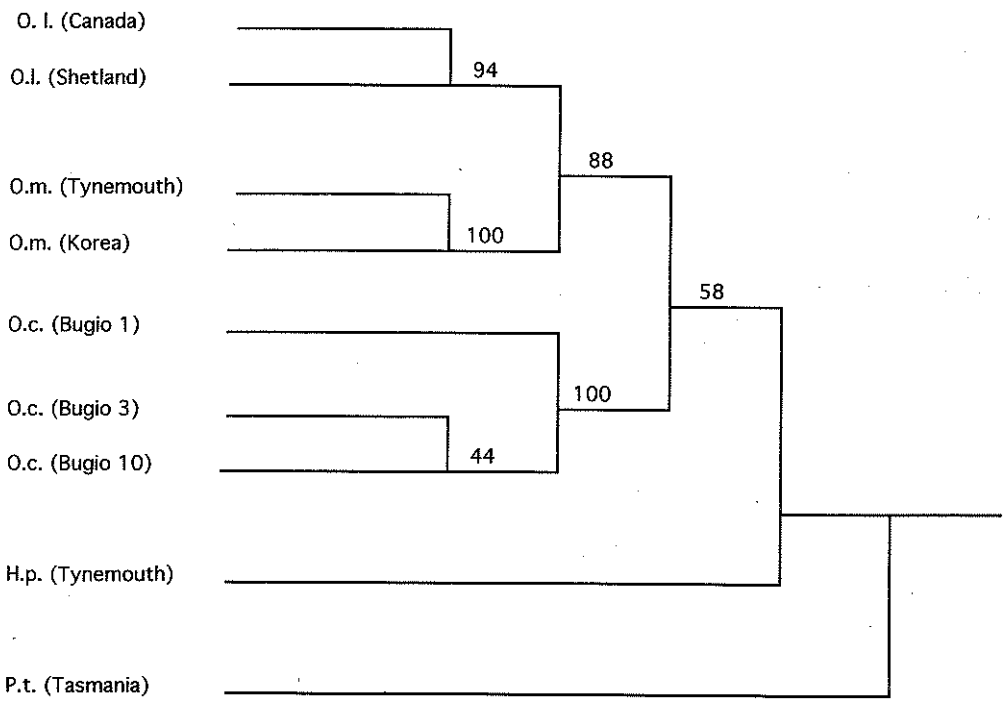

Figure 1 - Shortest length parsimony tree generated for Storm-Petrels and Short-tailed Shearwater (transversions weighted as 10x transitions).

TABLE 1 - Base composition at three sites in the sequence of cytochrome-b from 10 random Madeiran Petrels captured at Bugio, Madeira.

	130	220	250	No Indivs
Haplotype 1	C	C	C	6
Haplotype 2	C	T	C	1
Haplotype 3	T	C	T	1
Haplotype 4	T	T	T	2

TABLE 2 - Transitions (above diagonal) and transversions (below) detected in the sequence of cytochrome-b from 9 oceanic seabirds:

	O.l. C	*O.l.* S	*O.m.*T	*O.m.*K	*O.c.*1	*O.c.*2	*O.c.*10	*H.p.*T	*P.t.*T
O.l. C	-	2	23	25	25	19	18	25	31
O.l. S	1	-	25	27	27	21	20	27	33
*O.m.*T	5	4	-	2	3	30	29	24	31
*O.m.*K	5	4	0	-	1	30	29	24	30
*O.c.*1	5	4	0	0	-	30	29	25	31
*O.c.*2	2	1	5	5	5	-	0	31	28
*O.c.*10	2	1	5	5	5	0	-	31	27
*H.p.*T	8	7	9	9	9	8	7	-	28
*P.t.*T	17	18	18	18	18	19	19	21	-

O.l. C - *Oceanodroma leucorrhoa* from Canada
O.l. S - *Oceanodroma leucorrhoa* from Shetland
*O.m.*T - *O. monorhis* from Tynemouth
*O.m.*K - *O. monorhis* from Korea
*O.c.*1 - *O. castro* from Madeira
O.c. 2- *O. castro* from Madeira
*O.c.*10- *O. castro* from Madeira
H.p. T - *Hydrobates pelagicus* from Tynemouth
P.t. T - *Puffinus tenuirostris*. from Tasmania