Ecological and Evolutionary Genetics of *Puffinus* spp.

Jeremy James Austin, BSc. (Hons.)

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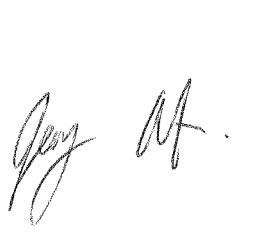
Signed 

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Summary

Three distinct molecular genetic techniques were applied at different levels in the evolutionary hierarchy to investigate the reproductive ecology, population biology and systematics of species in the shearwater genus *Puffinus*, with particular emphasis on the short-tailed shearwater, *P. tenuirostris*, or Tasmanian muttonbird.

Genetic relationships between mated pairs of adult short-tailed shearwaters and the single offspring in the nest were analysed by multilocus DNA fingerprinting. The human polycore minisatellite probe, 33.6, revealed sufficient variation in shearwater DNA to allow individual-specific identification. In addition this probe hybridised to a large minisatellite restriction-fragment derived from the female W chromosome, which allowed the identification of sex of adults and nestlings in this sexually monomorphic species. Analysis of DNA fingerprint profiles from 107 nestlings and one or both of the attendant adults in each case, in two independent studies, revealed 13 cases where a nestling was not related to one of the attendant adults. Although four of these unrelated adults could be accounted for by sampling errors, the remaining nine cases all involved the male in each nest and were more likely to have resulted from extra-pair copulations involving the attendant female and an unknown, extra-pair male. These results suggest that although short-tailed shearwaters exhibit strong pair fidelity and social monogamy, some birds are engaging in an alternative mating strategy that may substantially enhance both male and female reproductive success. Future estimates of life-time reproductive success in this species will have to allow for the small percentage of paired males that are unrelated to the nestlings that they are providing care for.

Restriction enzyme analysis of mitochondrial DNA (mtDNA) was used to examine mtDNA variation among 335 short-tailed shearwaters from 11 breeding colonies across southeastern Australia, and assess population genetic structure as a genetic test of the observed strict breeding and natal philopatry exhibited by this species. Eleven 6/5.33-base and four 4-base restriction enzymes revealed 25 and 48 mtDNA haplotypes in two overlapping surveys of 215 individuals from seven colonies and 231 individuals from eight colonies, respectively. A low mean sequence diversity among individuals (0.247%) and lack of spatial structuring of mtDNA haplotypes suggests a lack of population genetic structure and a reduced ancestral

population size during the Pleistocene glaciation, followed by a population and range expansion to current levels. Intracolony mtDNA diversities in three recently established colonies, and in one colony that has experienced a recent bottleneck were comparable to mtDNA diversities within larger and older colonies. This suggests that, despite strict philopatry in those colonies, colony founding and recovery from population reduction occurs via immigration of a large number of individuals.

Phylogenetic relationships among 19 extant species and subspecies within the genus *Puffinus* were examined using partial sequences from the mitochondrial cytochrome *b* gene. Nucleotide variation in a 307 bp fragment of this gene was sufficient to distinguish all taxa, except in one case, and contained phylogenetic information to resolve both shallow and deep phylogenetic relationships. Phylogenetic analysis of these sequences revealed a deep phylogenetic split amongst *Puffinus* species with one clade containing the larger, less aquatic and highly migratory Southern Hemisphere species and the second the smaller, more aquatic, less migratory and more northerly distributed forms. Within each clade, several currently recognised taxonomic subgroups were resolved, which have evolved via a polytomous or rapid series of speciations from a Southern Hemisphere or North Atlantic ancestor. Secondary dispersal has seen representatives of the second clade distributed widely throughout the major ocean basins. The phylogenetic hypothesis based on molecular data is generally concordant with trees based on morphological characters. Lack of congruence between the morphological and molecular trees and unexpected phylogenetic relationships among taxa were explained by introgressive hybridisation between two taxa or lineage sorting from a recent common ancestor, an error in one morphological tree, and a more parsimonious interpretation of a prevous evolutionary scenario for the genus. The phylogenetic results suggest a taxonomic revision of the subgroup Neonectris, which currently is a paraphyletic group, and supports previous suggestions that the two Mediterranean subspecies of the Manx shearwater, *P. puffinus. yelkouan* and *P. p. mauretanicus* should be elevated to the level of species and separate from the Atlantic form, *P. p. puffinus*.

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Chapter 1

General Introduction

1.1 Molecular phylogenetics: ecology and evolution

1.1.1 Introduction

Phylogeny is evolutionary history (Avise 1994). The ultimate goal of phylogenetics is to reconstruct the true evolutionary history of a species or group of species. Evolutionary history has a hierarchical nature such that individual pedigrees represent the fine-level structure of phylogenetic relationships between groups and populations within a species, reproductively isolated species, and phyla and other higher taxa (Fig. 1.1; Avise *et al.* 1987; Maddison & Maddison 1992). A wide range of behavioural, ecological, environmental and geological factors operating at different levels in the evolutionary hierarchy will affect phylogeny. Phylogenetic study at any level from the individual to phylum therefore requires an understanding of evolutionary history, organismal biology and biogeography, and incorporates a broad range of problems in ecology, population and evolutionary biology, and systematics.

Phylogenetic investigations require characters that accurately reflect organismal genealogy and evolutionary history. In the last several decades, advances in the understanding of the biochemistry of organisms and the genetic basis of inheritance have allowed for molecular level analyses that provide a broad range of heritable markers. These molecular markers have been applied to phylogenetic studies at many distinct levels in the evolutionary hierarchy to examine genetic relatedness between individuals, genetic structure of populations and to estimate relationships among taxa (Avise 1994). Phylogenetics, at the molecular level, focuses on an organisms genome (DNA) because it is the genetic sequence of an organism that is the ultimate record of its evolutionary history (Zuckerkandl & Pauling 1965). Genetic information finds expression at several different levels. At the first level, genetic information is expressed in the sequence of DNA and RNA, at the second level in the structure of protein molecules, at the third in the chemical structure of cell components and products and at the

fourth level in morphology, physiology and behaviour. The most complete expression of genetic information and highest information content is found in the DNA molecule. Recent molecular approaches to infer phylogenetic relationships have therefore become focused on the DNA molecule.

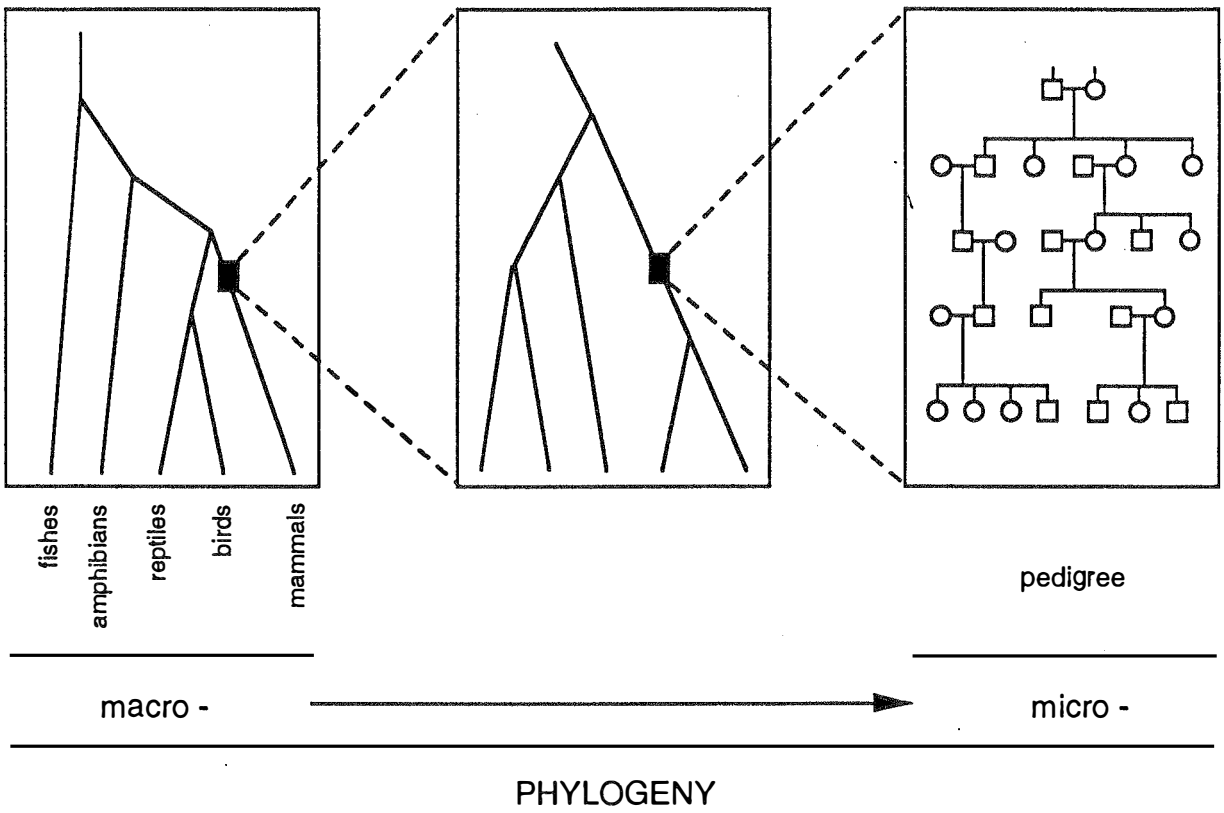

Figure 1.1. The hierarchical nature of evolutionary history and phylogeny. From Avise *et al.* (1987) and Avise (1994).

In addition to the unambiguously genetic, and therefore heritable, nature of molecular characters, there are a number of other general properties that are particularly useful in phylogenetic studies. The genome represents an almost limitless and universal set of molecular characters (Avise 1994). Thus large numbers of homologous characters can be compared between a diverse array of organisms, irrespective of gross morphology. Divergence of molecular characters is approximately linearly related with divergence time

(Zuckerkandl & Pauling 1962). This has lead to the proposal of constancy of evolutionary rates for molecular characters and a universal 'molecular clock'. Although it is now clear that evolutionary rate is not constant both among different classes of molecular characters and, in some cases, among different organisms for the same set of characters (Nei 1987; Rand 1994), assumptions of rate constancy can be made for specific molecular markers and taxonomic groups, and they form the basis of some methods of phylogeny estimation and calculating divergence times (Moritz & Hillis 1990). Finally, variation at the molecular level is generally assumed to be selectively neutral or nearly neutral. The neutral theory of evolution (Kimura 1968, 1983) has been and remains controversial but is now seen as a null hypothesis for most phylogenetic studies (Moritz & Hillis 1987, Avise 1994). Under neutrality, molecular variation is a function of the mutation rate and random genetic drift (Kimura 1983). Estimates of phylogeny, based on neutral molecular characters, will therefore more accurately reflect evolutionary history than characters biased by selection.

In animals, molecular characters fall into three general categories: gene products (e.g. proteins), nuclear DNA, and mitochondrial DNA (mtDNA). Within each category different techniques target different levels of variation in the molecule involved and are applicable at different levels in the evolutionary hierarchy from close familial relationships to phylogenetically distant ones. The characteristics, analysis and applicability of these molecular characters as applied to phylogeny have recently been reviewed by Hillis and Moritz (1990) and Avise (1994), and include immunological assays of proteins, allozyme electrophoresis, DNA-DNA hybridisation, restriction enzyme analysis of mtDNA, single-copy nuclear DNA and repetitive (satellite) nuclear DNA sequences, and DNA sequencing of both mitochondrial and nuclear segments. The specific molecular techniques used in the present study are discussed, in detail, in the following sections.

1.1.2 Repetitive nuclear DNA sequences

Characteristics

Nuclear DNA is a complex arrangement of coding and non-coding regions, single copy and repetitive elements, subdivided between a number of distinct chromosomes. A large proportion of the nuclear genome is made up of various types of repetitive DNA sequences, many of which have no direct role in encoding phenotypes (Turner *et al.* 1991). One particular class of repetitive DNA consists of minisatellites which are sequences of repetitive

DNA that share a common structure, comprising multiple (up to several hundred), tandem repeats of a short, 10-70 base pair unit (Jeffreys *et al.* 1985a) (Fig. 1.2). Minisatellite loci are dispersed throughout the genome, mainly on autosomal chromosomes (Jeffreys *et al.* 1985a) although some are sex-linked (Rabenhold *et al.* 1991; Millar *et al.* 1992; Graves *et al.* 1993). Minisatellites are among the most polymorphic sequences ever detected (Burke 1989) resulting in multiple alleles (6-80) and high heterozygosities at individual loci. Allelic variation is the result of differences in the number of tandem repeat units and is generated by a high rate of mutation to new length alleles, presumably through processes such as unequal sister-chromatid exchange or replication slippage (Jeffreys *et al.* 1988; Armour & Jeffreys 1992). The mutation rate increases with heterozygosity as predicted by the neutral mutation/random drift hypothesis (Jeffreys *et al.* 1988).

Analysis

The analysis of variation at minisatellite loci has become known as DNA fingerprinting (Jeffreys *et al.* 1985a) and utilises a number of basic molecular biology techniques (Fig. 1.3). Subsets of minisatellite loci share a common, highly conserved, core sequence within the tandem repeat unit (Fig. 1.2). Probes made up of tandem repeats of a sequence homologous to a particular core sequence can be used to detect different, non-exclusive sets of minisatellites at single or multiple loci. Multi-locus DNA fingerprinting utilises the commonality of the core sequence in minisatellites to assess simultaneously variation at multiple loci. Although there are a large number of probes that have been described (Bruford *et al.* 1992) the four most commonly used, which have general applicability across a wide range of taxa, are: 33.6 and 33.15, which are composed of tandem repeats of variants of a GC-rich human myoglobin minisatellite core sequence (Jeffreys *et al.* 1985a); M13, consisting of a 2900 bp fragment from wild type bacteriophage M13 containing 2 clusters of a GC-rich 156 bp repeat (Vassart *et al.* 1987); and 3'HVR, a GC-rich hypervariable region 3' to the human a-globin locus (Jarman *et al.* 1986; Fowler *et al.* 1988). Single-locus DNA fingerprinting sequentially assesses allelic variation at individual minisatellite loci. Because of the high degree of specificity involved, single-locus probes are often species specific. Each new study therefore requires the isolation and characterisation of a number of these locus-specific probes which has only recently become generally feasible (Burke *et al.* 1991).

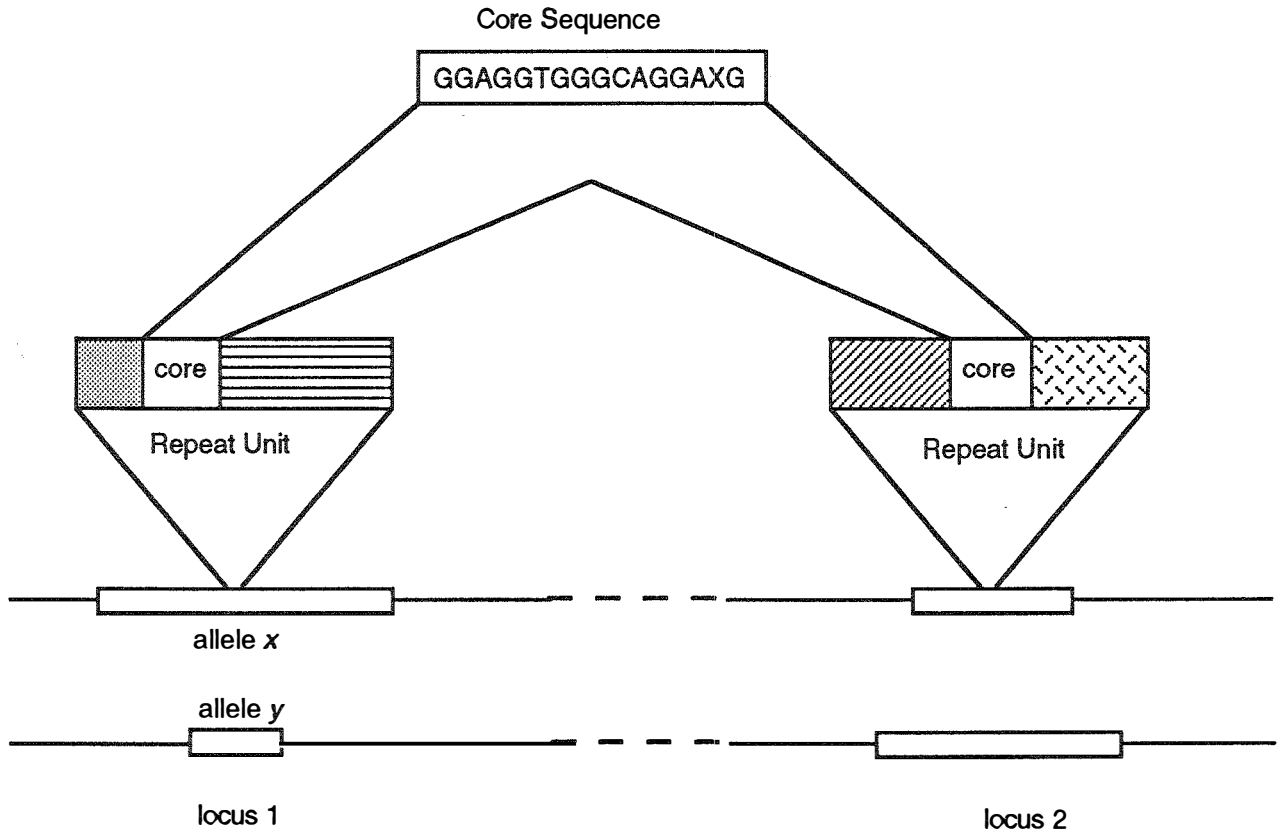

Figure 1.2. Structure and molecular basis of variation at minisatellite loci. Minisatellites consist of multiple, tandem repeat units that share a common core sequence. Minisatellite alleles on homologous chromosomes vary in the number of repeat units. Ministallite loci vary in the structure of the repeat unit. Adapted from Wetton (1990).

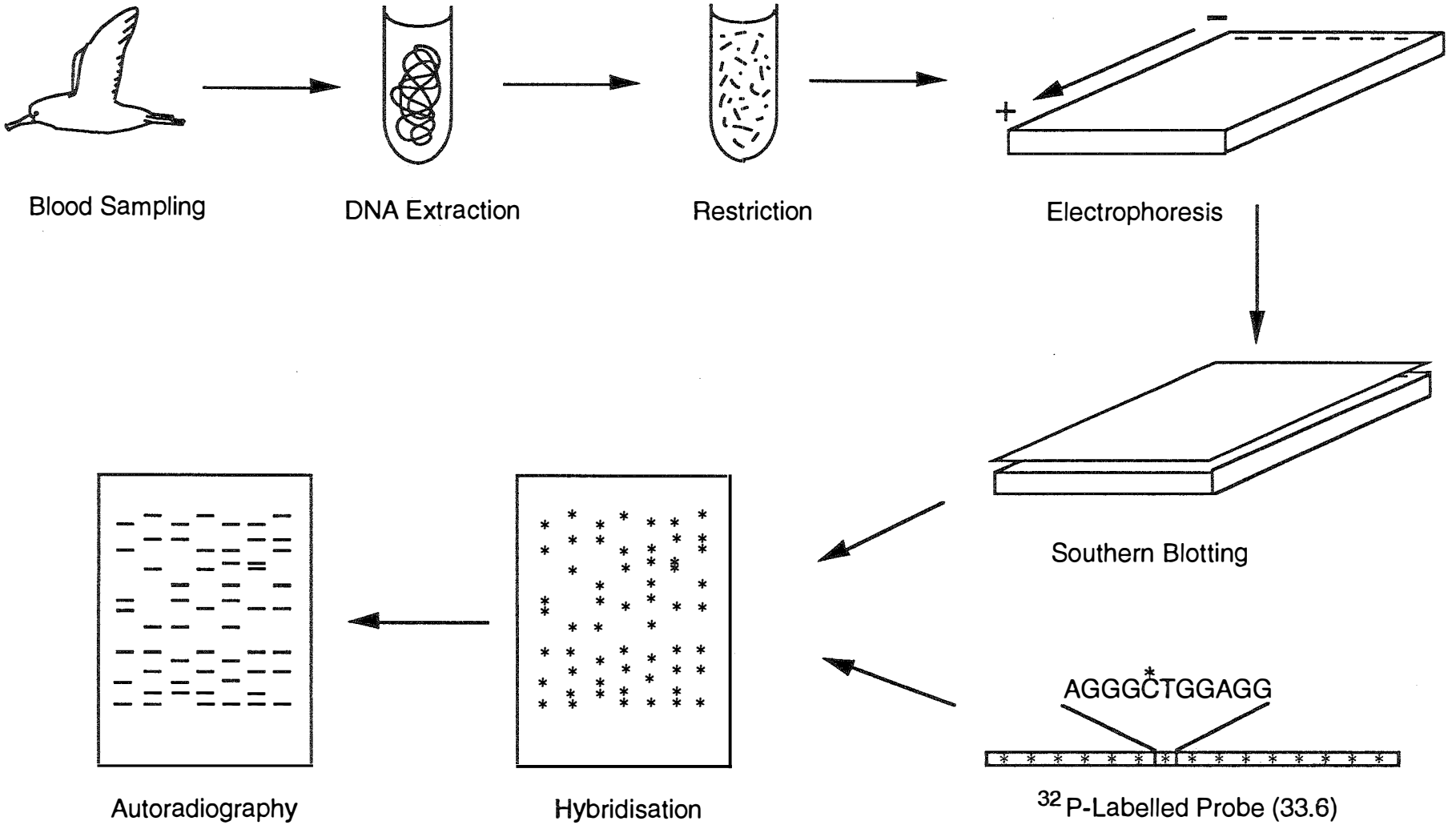

Figure 1.3. The DNA fingerprinting method. DNA is extracted from blood, digested with a 4-bp restriction enzyme, separated on a size basis by electrohporesis through an agarose gel, transferred to a nylon membrane by Southern blotting, and hybridised with a radioactively labelled probe. An autoradiograph is produced by exposure of the hybridised memebrane to X-ray film. Adapted from Wetton (1990).

Total genomic DNA is extracted from either blood or tissue samples taken from the target organism and then digested with a restriction enzyme. Restriction enzymes, isolated from bacteria, cleave double stranded DNA at specific, palindromic recognition sequences, usually 4, 5 or 6 base pairs in length. Several hundred different restriction enzymes have been characterised and most have unique recognition sequences. The length of the recognition sequence affects the frequency that it will occur in a DNA strand. If a DNA strand has equal proportions of the four nucleotides, and nucleotides occur at random, a 4-base pair recognition sequence should occur, on average, every $4^4 = 256$ base pairs, whereas a 6-base pair recognition sequence should occur every $4^6 = 4096$ base pairs. Variation in the number and size of fragments produced by digestion of DNA with restriction enzymes are known as restriction fragment length polymorphisms (RFLPs), and are the result of nucleotide substitutions creating or destroying recognition sequences, additions, deletions or duplications of DNA or inversions/rearrangements of DNA segments.

The choice of restriction enzyme for the analysis of minisatellite variation is important it must cut frequently enough to release restriction fragments of a size that closely reflects the size of the minisatellites they carry, but not cleave the minisatellite repeat sequences themselves. Restriction enzymes with 4-base recognition sequences are therefore the most commonly used because of the high number of expected restriction sites in DNA sequences flanking the minisatellite. RFLPs in this case reflect the number of tandem duplications of the repeat unit in the minisatellite-containing restriction fragment. Restriction fragments are separated on a size basis by electrophoresis through an agarose gel and then transferred and fixed to a nylon membrane by Southern blotting (Southern 1975). The filter is hybridised with a radioactively labelled minisatellite probe, which binds to DNA restriction fragments containing minisatellites with homologous core sequences, and then washed to remove excess probe and exposed to X-ray film. The autoradiograph consists of a number of bands indicating the relative position of the different sized minisatellite-containing restriction fragments, and represents the DNA fingerprint or DNA profile for that individual.

The characteristics of DNA profiles and the minisatellite loci they represent have been studied in a range of animals, including humans (Jeffreys *et al.* 1986; Fowler *et al.* 1988), mice (Jeffreys *et al.* 1987), dogs and cats (Jeffreys & Morton 1987) and birds (Burke & Bruford

1987; Wetton *et al.* 1987; Burke *et al.* 1989; Birkhead *et al.* 1990; Bruford *et al.* 1990; Gyllensten *et al.* 1990; Hanotte *et al.* 1992; Wetton *et al.*1992; Hartley *et al.* 1993; Lifjeld *et al.* 1993; Pinxten *et al.* 1993) and have been summarised below. First, a multi-locus DNA profile is usually composed of 10-30 individual bands, each representing a different minisatellite fragment from a similar number of different loci. A single-locus DNA profile for any particular individual will consist of only one or two fragments, depending on whether that individual is homozygous or heterozygous, respectively, at that particular minisatellite locus. Second, minisatellite fragments show Mendelian inheritance; heterozygous fragments are transmitted on average to half of the offspring. Third, minisatellite fragments are generally inherited independently. Non-independence of fragments can arise through allelism (the appearance of both alleles at a particular locus on the scorable region of a gel) and/or linkage (the cosegregation of two or more fragments which arises when either a minisatellite fragment is cleaved internally by the restriction enzyme or minisatellite loci are situated close together on the one chromosome), but is rarely observed. Hanotte *et al.* (1992) have reported both allelism and tight linkage for a large number of fragments in red grouse. Fourth, DNA profiles are somatically stable in different tissues and for cultured cell lines (Jeffreys *et al.* 1985b). Finally, as a consequence of the independent Mendelian inheritance of minisatellite fragments and the large number of highly polymorphic loci that are simultaneously identified, DNA profiles in many species are individual-specific. Only in cases of extreme inbreeding or for monozygotic twins will the DNA profiles of two individuals be identical.

Applications

Outside of human forensic and genetic applications, DNA fingerprinting has two main applications in population biology and ecology. The first, and most widespread, use is that of paternity analysis in wild populations. The assessment of genetic relationships between individuals is of particular importance in behavioural ecology to determine realised reproductive success and examine the evolutionary impact of alternative mating strategies. For example, no copulations outside of the pair bond were observed in a population of monogamous tree swallows (*Tachycineta bicolor*) but, based on multilocus DNA fingerprinting of birds from 16 nests, 38% of the nestlings were sired by an extra-pair male (Lifjeld *et al.* 1993). Estimates of male reproductive success based on behavioural

observations only are therefore substantially incorrect in this species. The genetic analysis provided an even greater level of resolution. The illegitimate offspring were not randomly distributed among broods, rather a small number of males were suffering most of the cuckoldry, and therefore loss of reproductive success. At the other extreme, Hunter *et al.* (1992) observed regular occurrences of copulations between birds from different pairs in the northern fulmar (*Fulmarus glacialis*) but did not detect any extra-pair paternity. Thus despite adopting an alternative mating strategy involving copulations with extra-pair females, male fulmars do not increase their reproductive success. Jarne *et al.* (1992) used multilocus DNA fingerprinting to distinguish self-fertilising and cross-fertilising mating systems in the hermaphroditic snail *Bulinus globosus*.

Two properties of genetic inheritance form the basis for assigning or excluding parentage by DNA fingerprinting. In diploid, sexually reproducing organisms an offspring inherits half of its autosomal DNA from each parent and its entire nuclear genome from both parents combined. In an ideal situation where the same set of homologous loci is assayed completely for all individuals; both alleles at each locus can be identified and all individuals are heterozygous at all loci; each allele is represented by only a single fragment and all fragments are independently inherited; there are an infinite number of alleles at each locus; and there is no mutation from one generation to the next; an offspring will share exactly 50% of its fragments with each parent and all fragments in the offspring's DNA profile will be present in one or the other of the parents' profile. Second-, third- and fourth-order and more distant relatives will share a percentage of their fragments in direct proportion to their level of genetic relatedness. Unrelated individuals will share no fragments.

In reality, a number of characteristics of minisatellite genetics and practical limitations of the fingerprinting technique mean that there can be considerable deviation from the expected levels of band-sharing for the different levels of relatedness. First, although the majority of studies have shown that minisatellite fragments are inherited independently, allelism and linkage do occur (Birkhead *et al.* 1990; Wetton *et al.* 1992). In the case of linkage, different fragments in a DNA profile may exhibit different degrees of linkage. The extent of allelism is also uncertain. One fragment might represent one allele at a locus, or even both alleles if the individual is homozygous. The other allele might be another fragment in the scorable region of the gel or it might be one of the unscorable mass of poorly resolved low molecular weight

fragments. A detailed segregation analysis of large families is necessary to demonstrate independence between fragments, but in many cases, this is not possible.

Second, although allelic diversity at minisatellite loci is high, there is only a finite number of alleles at each locus. Two individuals can therefore share fragments by chance rather than descent (Burke *et al.* 1991).

Third, at extremely variable loci the mutation rate to alleles containing a different number of repeat units is high enough to be observed directly in pedigree analyses (Jeffreys *et al.* 1988). As a result of this germ-line instability at minisatellite loci a significant number of offspring will display fragments in their DNA profiles that represent a newly arisen allele, which are not shared with either of the true parents.

Fourth, only a subset of all the loci (and alleles) belonging to a particular minisatellite family are scored. The remainder are either present and unscorable or have migrated off the scorable region of the gel. Therefore incomplete, and potentially different, sets of loci are compared between individuals. Also, in comparisons between related individuals, if inherited fragments are non-randomly distributed over the entire size range of fragments (scorable and unscored fragments) then the observed band-sharing (of scored fragments) will over or under estimate the genetic relatedness (Burke *et al.* 1991; Bruford *et al.* 1992).

The final problem involves co-migration of unrelated fragments. Separation of DNA fragments through agarose gels is based on size; however, the resolution of fragments of different sizes will depend on their size, the length of electrophoresis, gel concentration and buffer type and pH. Fragments that migrate the same distance in two lanes may therefore represent identical alleles at an homologous locus, indicating some degree of relatedness, or unrelated, non-homologous loci, which by chance are of a similar size. Co-migration will inflate the observed band-sharing between individuals relative to their actual level of genetic relatedness (Bruford *et al.* 1992). In practice, co-migration of unrelated, non-identical fragments increases as the size of fragments decreases. This places a lower limit on the size of scorable fragments on an autoradiograph.

These problems complicate the interpretation of DNA profiles because, on average, individuals share a higher proportion of their fragments than their coefficient of relatedness; the background band-sharing among non relatives is usually 10-30% and there can be considerable variation about the mean band-sharing similarity appropriate to any specific level of relatedness (Lynch 1988). This lack of genetic resolution severely limits the precision of DNA fingerprinting in establishing degrees of relatedness but does not prevent use of DNA profiles to examine parentage. First degree relatives (i.e. parent-offspring) will, on average, have more fragments in common than unrelated individuals. Depending on the background level of band-sharing in a population an offspring will share approximately half of its fragments with each true parent and all or most (where a mutation event has produced a new length allele) of the offspring's fragments will be present in one or the other parent's profile.

The second application of DNA fingerprinting has been to examine levels of relationships within and between groups of animals, to test hypotheses related to cooperative behaviour, kinship and population structure (Faulkes *et al.* 1990; Gilbert *et al.* 1990, 1991; Reeve *et al.* 1990; Hoelzel & Dover 1991; Coffroth *et al.*1992; Lehman *et al.* 1992; Baker *et al.* 1993; Degnan 1993; Haig *et al.* 1993). In general, the high levels of variability at minisatellite loci limit the application of DNA fingerprinting to population level comparisons to species in which effective population sizes are small and are relatively inbred (i.e. show dramatically reduced levels of genetic variation).

1.1.3 Mitochondrial DNA

Characteristics

Mitochondrial DNA (mtDNA) and the subcellular organelle in which it is found, the mitochondrion, are believed to have had an endosymbiotic origin (Gray 1989). The structural and evolutionary properties of the mitochondrial genome vary significantly between major taxonomic groups (Moritz *et al.* 1987; Gray 1989; Rand 1994), so the following discussion applies particularly to vertebrates and in some cases specific groups of vertebrates.

Mitochondrial DNA is a small (typically 15-20 kb), duplex, covalently closed circular molecule which represents a clearly homologous DNA sequence for comparison between individuals (Brown 1985; Moritz *et al.* 1987). The mtDNA genome exhibits a simple

sequence organisation and conserved gene content. The molecule includes 2 rRNA genes, 22 tRNA genes, 13 genes that code for subunits of enzymes functioning in electron transport or ATP synthesis and a control region which lacks structural genes but contains sequences that initiate replication and transcription (Anderson *et al.* 1981; Brown 1985; Chomyn *et al.* 1985; Moritz *et al.* 1987). In vertebrates the control region contains a displacement (D-loop) structure which functions in replication (Kasamatsu *et al.* 1971). Gene arrangement appears to be conserved within vertebrates, with the exception of birds (Desjardins & Morais 1990, 1991) and marsupials (Pääbo *et al.* 1991) which have unique gene arrangements relative to other mammals, amphibians and fish. Gene order differs also between vertebrates and invertebrates, and between different invertebrate groups (Gray 1989). The mtDNA molecule is exceptionally compact with no interrupted genes or introns (Anderson *et al.* 1981, 1982; Bibb *et al.* 1981; Roe *et al.* 1985), few intergenic sequences (Brown 1985; Moritz *et al.* 1987) and, except in the control region, no or few large insertions or duplications (Gray 1989; Harrison 1989).

In the majority of species studied so far, and particularly among endothermic vertebrates, somatic and germ cells of an individual exhibit homoplasmy of mtDNA, that is, a single detectable mtDNA nucleotide sequence (Avise & Lansman 1983; Rand 1994). Although lack of intra-individual sequence heterogeneity has not been fully explained, its absence may be attributable to sorting of mtDNA molecules into homogeneous populations by random genetic drift in intermediate germ cells, where mtDNA numbers might be small (Chapman *et al.* 1982; Takahata 1985). Heteroplasmy involving size differences in mtDNA have been reported, but appears to be a short-lived evolutionary state (Avise 1991).

In most animals mtDNA is maternally inherited (Avise & Lansman 1983; Lansman *et al.* 1983; Gyllensten *et al.* 1985; Avise & Vrijenhoek 1987; Avise 1991). Exceptions to the strict maternal mode of inheritance are known (Avise 1994) so that unless paternal contribution of mtDNA can be empirically disproved in the species under study, uniparental inheritance must be assumed. Mitochondrial DNA therefore represents a haploid genome which is clonally transmitted and recombination between molecules occurs rarely or not at all (Brown 1985; Wilson *et al.* 1985). With strict maternal and haploid inheritance and lack of recombination, separation of differentiated genomes is maintained during sexual reproduction. However, although mtDNA is a composition of a number of different genes,

evolutionarily it represents a single genetic unit. MtDNA therefore represents a set of completely linked markers which permit clear definition of maternal genealogies and allows discrimination between common ancestry and convergence (Wilson *et al.* 1985, Avise 1986).

Animal mtDNA is translated using a distinctive genetic code, which is slightly different from the 'universal' genetic code of nuclear DNA (Brown 1985). The mtDNA codon recognition pattern is simplified such that eight tRNAs can each decode the four different codons, differing in the third position, that specify a given amino acid (Gray 1989; Avise 1991). Third codon position changes are effectively silent in these cases.

Animal mtDNA exhibits a rapid rate of nucleotide sequence evolution, although the considerable variability among taxa in absolute evolutionary rate precludes the use of a universal mtDNA molecular clock (Harrison 1989; Rand 1994). Initial rates of sequence divergence have been estimated (0.5-1% per lineage per million years) at 5-10 times the rate observed for scnDNA in studies of mtDNA evolution in primates (Brown *et al.* 1979, Brown *et al.* 1982). Data from a variety of tetrapod groups appear to be consistent with the estimates from primates (Wilson *et al.* 1985, Moritz *et al.* 1987). Rand (1994) has recently reviewed a number of studies that suggest that thermal habit and/or metabolic rate of an organism can influence the rate of mtDNA evolution. Therefore the estimated 2% sequence divergence between species per million years can probably only be applied to endothermic vertebrates. Vertebrate ectotherms and invertebrates exhibit substantially different rates of mtDNA sequence divergence (Rand 1994).

The reasons for the enhanced rate of mtDNA sequence evolution relative to scnDNA are unclear, however higher rates of oxidative damage induced mutation, inefficient DNA replication and repair mechanisms, relaxed selection and/or functional constraints on the mtDNA molecule, variable organismal population sizes and the population biology of the germ line cytoplasm may be involved to different extents (Gray 1989; Rand 1994).

Rates of sequence evolution are not constant along the length of the mtDNA molecule. The control region rapidly accumulates changes in the form of base substitutions and

rearrangements, whereas the large and small rRNA genes are highly conserved, with some regions showing no sequence divergence across major phylogenetic boundaries (Brown 1985; Hixson & Brown 1986; Moritz *et al.* 1987). Because of the heterogenous distribution of base substitutions, relationships between taxa at very different levels of divergence can be established by comparing different regions of the molecule. In the initial rapid phase of sequence evolution, between recently diverged taxa, most base substitutions occur in intergenic sequences, in the control region, or at codon positions where they do not cause amino acid replacements (Brown 1983; Brown 1985; Moritz *et al.* 1987). Beyond about 15% overall sequence divergence the rate of nucleotide substitution appears to slow dramatically as these positions, subject to relatively weak functional constraints, become saturated. Most detectable changes beyond this point involve functionally important sites (Harrison 1989).

Analysis

Restriction Enzyme Analysis

Restriction enzyme analysis of mtDNA has been the most common method of assessing nucleotide variation in the mitochondrial genome and providing molecular markers in population biology and phylogenetic studies. Variation is detected as RFLPs using restriction enzymes which cut the circular mtDNA molecule into a number of linear fragments equal to the number of restriction sites for that particular enzyme (Fig. 1.4). Typically mtDNA is extracted and purified from a tissue source with a high mtDNA copy number (e.g. liver or oocytes). Following restriction enzyme digestion, mtDNA fragments are separated on the basis of size by electrophoresis through agarose or polyacrylamide gels. Depending on gel concentration and the specific buffer used, agarose gels can resolve fragments over the size range from 300 bp to 20 kb, whereas polyacrylamide gels provide suitable resolution in the range of 10 bp to 1 kb (Dowling *et al.* 1990). Restriction fragments, derived from highly purified mtDNA, can be visualised by a variety of methods including post-electrophoresis ethidium bromide staining (Dowling *et al.* 1990) or silver staining (Tegelström 1986), or pre-electrophoresis end-labelling of restriction fragments with ^{32}P- or ^{35}S-labelled nucleotides and subsequent post-electrophoresis autoradiography of dried gels (Ovenden *et al.* 1989). Alternatively, when small amounts of impure mtDNA are available restriction fragments may be visualised by hybridisation to a radioactively labelled mtDNA probe, either *in situ*

(Brasher 1992) or following Southern blot transfer to a nylon or nitrocellulose membrane (Dowling *et al.* 1990). An internal molecular size standard is included on each gel to enable estimation of the size of the restriction fragments in the sample DNAs.

Therefore, for each individual, an array of restriction fragments is detected, the number and size of which reflect the number and distribution of restriction sites, for a particular enzyme, in that individuals mtDNA. Because restriction enzymes vary in the length and the actual sequence of their recognition sites, sequential digestion of mtDNA with a number of different restriction enzymes is used to assess variation at multiple sites within the entire mitochondrial genome. The use of 10-20 5-base and 6-base restriction enzymes, each with a unique recognition sequence, will generally produce 50-100 restriction fragments per individual, equivalent to approximately 250-600 bp of sequence (Avise 1994). Restriction enzymes with 4-base recognition sequences cut DNA more frequently, and therefore assess more of the mtDNA sequence than enzymes with 6-base recognition sequences. However, the larger number of fragments produced complicates the analysis of these RFLPs. The raw data from a restriction enzyme analysis consist of a set of restriction fragment length profiles, for each individual, for each of several restriction enzymes.

The interpretation of RFLP data initially involves identifying the nature of the variation observed. The mutational source of the molecular variation (nucleotide substitution, addition/deletion, rearrangement) which results in RFLPs can be inferred from the characteristics of the fragment profiles themselves (Dowling *et al.* 1990; Avise 1994). The majority of RFLPs are the result of nucleotide substitutions creating or destroying restriction sites for particular enzymes; however length mutations and gene rearrangements become more common in comparisons between more distantly related taxa.

Where the number of single-enzyme restriction fragments identified per individual is small (< 10) the number and relative positions of the restriction sites separating unique fragment patterns can usually be deduced. For example, in Fig. 1.4 the loss of fragments b and c and gain of fragment e, equal in size to the sum of b and c, can be interpreted as the loss of a restriction site (site 3) in genome B. Alternatively, the positions of restriction sites from multiple enzymes may be physically mapped relative to one another using double-digest techniques (Sambrook *et al.* 1989; Ovenden *et al.* 1992; Smolenski *et al.* 1993). Either of

these methods can be used to construct a binary data set of presence and absence of restriction sites across restriction enzymes for subsequent analysis. However, where large numbers and/or complicated patterns of restriction fragments are produced (such as when a 4-base restriction enzyme is used), it is difficult to either infer restriction site changes from the observed patterns of the fragments, or map the positions of sites. In these cases analysis must be restricted to restriction fragment presence/absence.

Restriction site information is preferable to fragment data because the loss or gain of restriction fragments is not necessarily independent. For example, in Fig. 1.4 the loss of a single restriction (site 3) in genome B results in the loss of two smaller fragments (b and c) and the appearance of one larger one (e). Although methods of analysis of fragment data allow for non-independence, the assumptions necessary for such methods may not always be met (Avise 1994).

The advantages of the restriction enzyme approach to assess sequence variation in mtDNA are that the mitochondrial genomes of large numbers of individuals can be examined rapidly, easily and relatively inexpensively. Restriction enzymes assess variation in the entire mtDNA molecule and include different genes evolving at different rates. However restriction enzyme analysis has a number of disadvantages specific to particular applications. Restriction sites offer a biased sample of nucleotide sequences since they only cut DNA at palindromic sequences (Kocher *et al.* 1989). The restriction enzyme method, applied to the whole mtDNA molecule, has a limited phylogenetic range being reliable only among closely related taxa, mainly at or below the genus level (Kocher *et al.* 1989). Fragment comparisons become unreliable if there is length variation or if the overall sequence divergence exceeds 10-15% (Upholt 1977; Kessler & Avise 1985). Restriction enzyme analysis is limited to species that are large enough to provide milligram amounts of tissue. In addition, samples cannot be obtained from rare species and the requirement for fresh or cryopreserved samples restricts sampling to easily accessible species. Traces of short, modified DNA that survive in museum specimens or ancient remains cannot be analysed with restriction enzymes (Kocher *et al.* 1989).

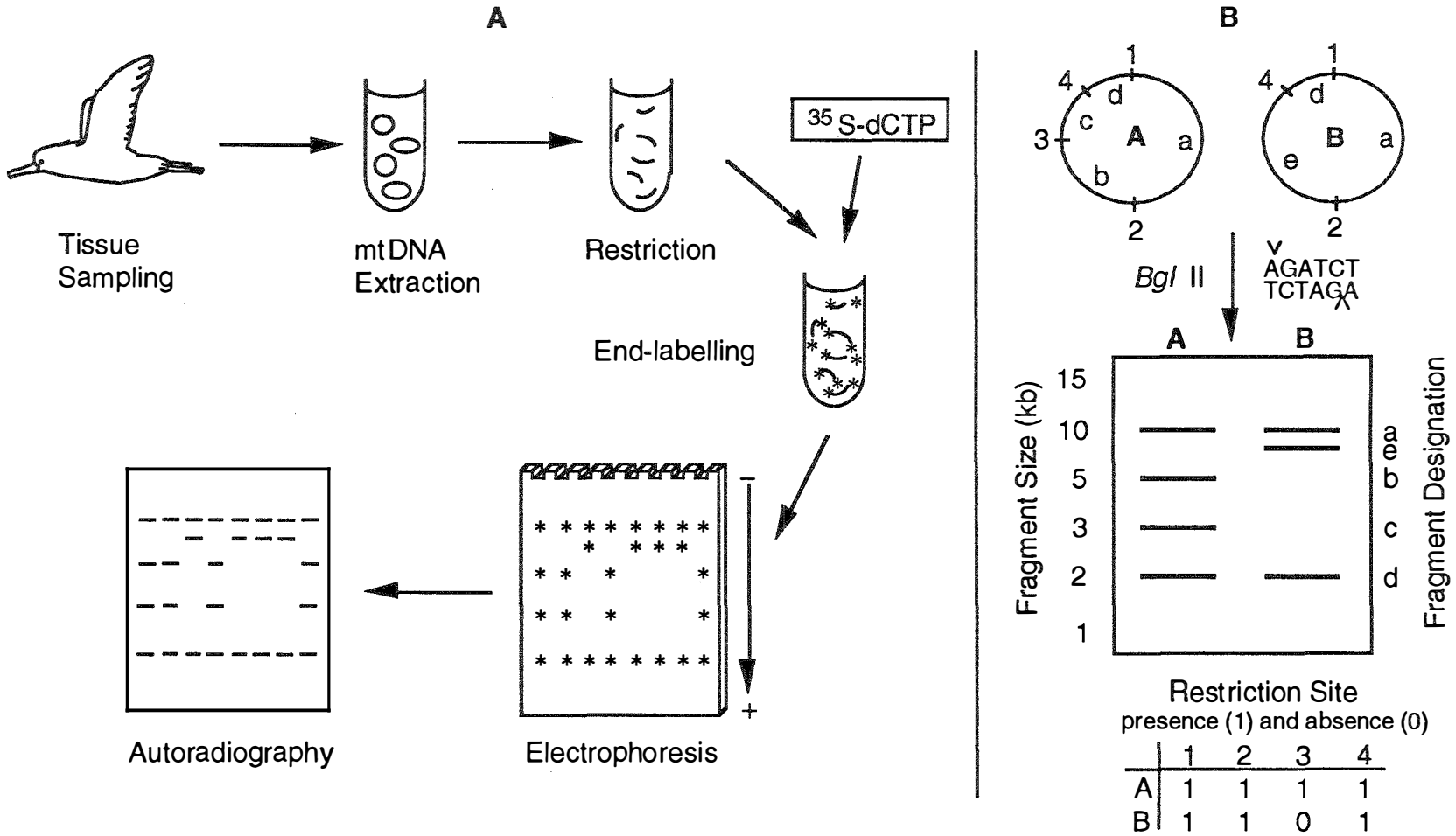

Figure 1.4. Restriction enzyme analysis of mtDNA. A. Methodology. Closed circular mtDNA is extracted from tissue rich in mitochondria and then digested with a restriction enzyme that cuts at one or more sites. Restriction fragments are radioactively end-labelled with e.g. ^{35}S-dCTP and electrophoresed through agarose or polyacrylamide gels. Autoradiography reveals the position of labelled mtDNA restriction frgaments.
B. Interpretation. Two mtDNA genomes, A and B, digested with *Bgl* II show different fragment patterns following electrophoresis. The sum of fragment lengths b and c equals the length of fragment e. This can be explained by the loss of a restriction site, 3, in genome B. Restriction site presence/absence data is used as characters for further analysis.

Direct Sequence Analysis

Restriction enzyme analysis and direct sequencing offer two ways of assessing sequence variation in and obtaining phylogenetic information from mtDNA. Complete sequencing of homologous DNA fragments provides a powerful, informative and direct method, with the highest possible resolution, for the reconstruction of evolutionary relationships. The advantages of DNA sequence determination include the facts that sequence differences among species are measured directly rather than by estimating such differences by using indirect methods (Arnheim *et al.* 1990), the characters (nucleotide base pairs) are the basic units of genetic information encoded in the DNA of organism, each character can exist in four discrete character states and that the potential extent of informative data sets are immense, being limited ultimately by the number of nucleotide pairs in an organisms DNA (Hillis 1987; Harrison 1989; Moritz & Hillis 1990). Short DNA sequences are a versatile source of phylogenetic information extending from the intraspecific level to the intergeneric level (Kocher *et al.* 1989). In addition, sequence data are readily accumulated creating a data base that can be referred to for further phylogenetic comparisons and analyses as new sequence data become available. Finally, perhaps the greatest advantage of direct sequencing, when combined with the recently developed polymerase chain reaction (PCR), is that it has enabled the retrieval of DNA sequences from very small amounts of fresh samples and the extensively degraded DNA found in museum specimens and archaeological remains. The limitations of direct sequence analysis include its expense, technical difficulty (although this has lessened significantly in recent years) and the fact that sequences from only a small segment (usually parts of one or two genes) of the mtDNA molecule are compared: variation in the remainder is not assessed.

Hillis *et al.* (1990) have identified four steps that are involved in obtaining DNA sequence data for phylogenetic study. Initially a suitable target sequence, exhibiting sufficient nucleotide variation appropriate to the particular level in the evolutionary hierarchy under study, must be identified. This process has been simplified by the characterisation of variation in a number of mitochondrial genes and the phylogenetic resolution provided by them. In general, the rate of nucleotide evolution decreases from the control region, to protein coding genes, to RNA genes. For example, Wenink *et al.* (1993) were able to detect genetic differentiation between geographically isolated, circumpolar populations of a shorebird, the dunlin (*Calidris alpina*) using cytochrome *b* and hypervariable mtDNA control-region

sequences. Edwards and Wilson (1990) investigated phylogenetic relationships between five species of a genus of Australian songbird (*Pomatostomus*) using partial sequences of the mitochondrial cytochrome *b* gene. Based on almost complete sequences of the mitochondrial cytochrome *b* gene, Edwards *et al.* (1991) were able to resolve a phylogenetically deep branch separating songbirds from other perching birds within the Order Passeriformes. Hedges (1994) used mitochondrially encoded rRNA and tRNA genes to investigate the evolutionary origins of birds and obtained support for a bird-crocodile relationship.

Second, the target sequence must be isolated and purified, in high copy number, from each individual examined. There are two methods for obtaining sequenceable amounts of DNA; the first involves cloning the target sequence, *in vivo*, into a suitable viral or bacterial vector, using recombinant DNA techniques, and the second uses the *in vitro* enzymatic amplification of the target sequence by the polymerase chain reaction. Cloning methods have had limited use in phylogenetic studies because of the excessive time and effort required to obtain homologous DNA sequences from multiple individuals (Hillis *et al.* 1990; Avise 1994). In addition, sequence analysis of the extensively damaged DNA found in old and ancient remains, using molecular cloning techniques, is both difficult and subject to errors such as cloning artifacts (Pääbo & Wilson 1988).

The polymerase chain reaction involves the enzymatic amplification of a specific DNA segment performed as a repeated series of a discrete cycle (Saiki *et al.* 1985, 1988). A single PCR cycle consists of 3 steps (Fig 1.5).

Template DNA denaturation The PCR reaction requires a single-stranded template. In the first step the double stranded template DNA is heat denatured into single strands. This allows the oligonucleotide primers to anneal to the single-stranded template DNA in the second step of the reaction.

Primer hybridisation Two oligonucleotide primers, short (15-30 nucleotides), single stranded pieces of DNA, are used that hybridise to complementary sequences, on opposite strands of the template DNA, that flank the target region to be amplified. Thus, following denaturation of the DNA, each primer hybridises to one of the two separated strands with the 3'-hydroxyl end of each primer orientated towards the target sequence.

DNA polymerase extension of the annealed primers The annealed primers are then extended

on the template strand with a DNA polymerase, thereby making copies of the target segment. The primers are necessary for the initiation of the extension reaction and the template DNA acts as a reference strand for the polymerase which adds complementary nucleotide bases to the free 3'-hydroxyl end of the primer sequence.

If the newly synthesised strand extends to or beyond the region complementary to the other primer it can serve as a template for a subsequent primer extension reaction. The primers are incorporated into all subsequently amplified DNA templates ensuring 100% homologous priming sites in subsequent PCR cycles. Each cycle of amplification can potentially double the amount of the target DNA present. Consequently, repetitions of the PCR cycle result in the exponential amplification of a discrete DNA fragment equal in length to the sum of the lengths of the two primers and the target DNA. The extreme sensitivity of the PCR lies in the fact that successful amplification of many millions of copies of a specific DNA fragment can be initiated from a single DNA molecule containing the target sequence.

Third, the sequence of the target DNA segment must be determined. There are two general methods for direct sequence determination of DNA, the chemical method (Maxam & Gilbert 1977, 1980) and the enzymatic dideoxy method (Sanger *et al.* 1977). In the chemical method single-stranded target DNA is end-labelled (radioactively or chemically) and then in each of four separate reactions, subjected to base-specific chemical cleavage, such that in each reaction all of the labelled fragments terminate in one or two of the four nucleotide bases corresponding to the occurrence of those bases in the original sequence.

In the dideoxy sequencing method, primer-directed, radioactively or chemically labelled, complementary copies of the target DNA are synthesised using a DNA polymerase (Fig. 1.6). Base specific, dideoxynucleotide termination of the synthesised strands in each of four separate reactions produces a set of discrete sized fragments, in each reaction, terminating in one of the four nucleotides and corresponding to the occurrence of the complementary nucleotide in the template sequence. In both methods the products of the four reactions are electrophoretically separated through denaturing polyacrylamide gels capable of resolving single nucleotide differences between fragments. Fragments from each of the four reactions are separated on adjacent lanes of the gel and visualised using standard

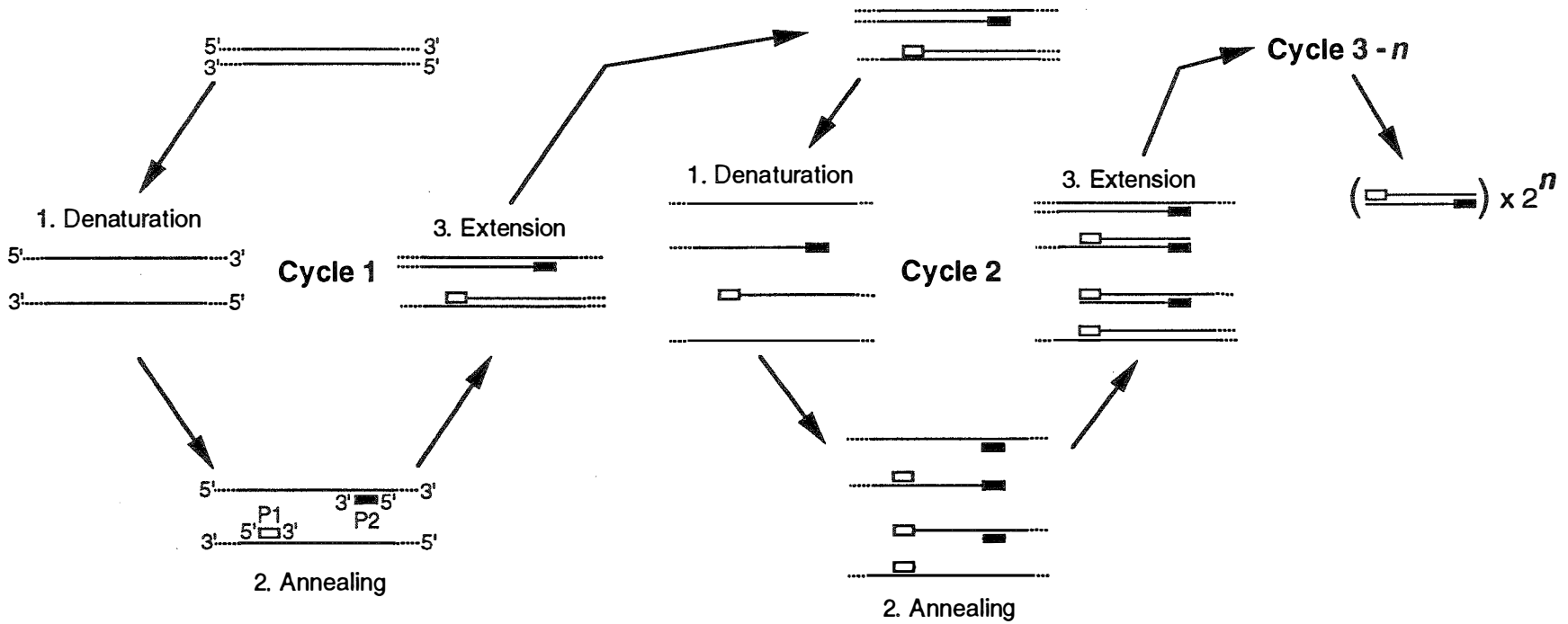

Figure 1.5. Procedure for the amplification of a specific DNA fragment by the polymerase chain reaction (PCR). Each cycle consists of a denaturation step where the double stranded template DNA is dissociated into single strands; an annealing step where two, single-stranded oligonucleotide primers (P1 and P2) are annealed to the template DNA, and an extension step where a thermostable DNA polymerase makes a complemantary copy of the template DNA strand, starting at the 3' end of each primer. This cycle is repeated many times to generate large numbers of copies of a discrete sized DNA fragment bounded at each end by the primer sequences.

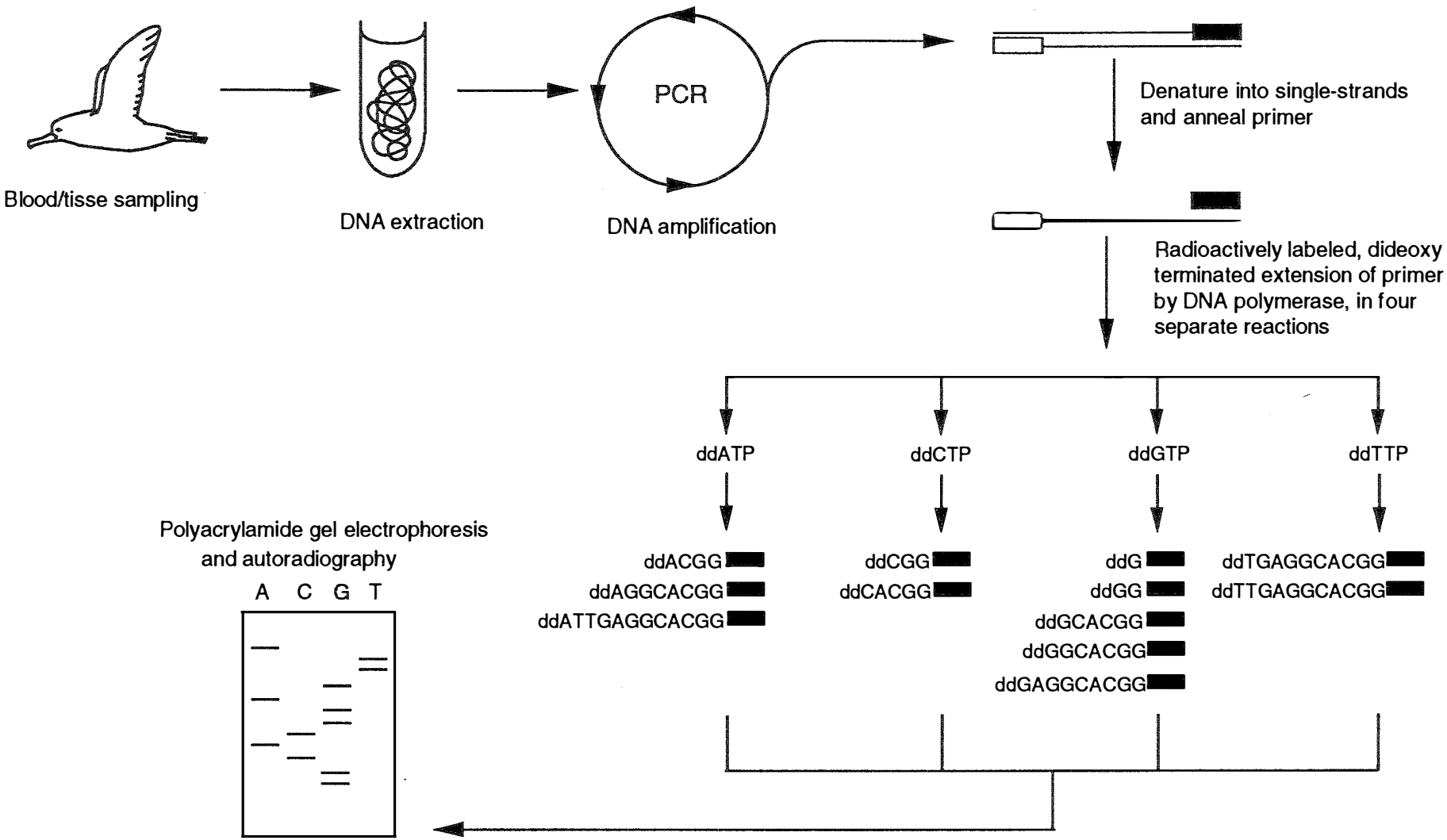

Figure 1.6. Procedure for dideoxy sequencing of PCR amplified DNA segments. See text for details.

autoradiographic techniques (for radioactively labelled fragments) or chemiluminescent techniques (for chemically labelled fragments). The DNA sequence is then read directly from the ladderlike banding pattern of the visualised fragments.

Fourth, sequences from different individuals must be aligned so that only homologous nucleotides are compared. There are a variety of methods for nucleotide sequence alignment and all attempt to maximise the similarity between sequences by invoking insertion/deletion (gap) events and nucleotide base substitutions (Mindell 1991). Alignment of sequences from protein coding genes is relatively simple because the tandemly arranged 3 bp codons provide readily distinguishable features. Deletions and insertions are rare because of the disruption to the reading frame that these events produce. Alignment of sequences from RNA genes and the mitochondrial control region is more difficult because there are no selective constraints to maintain reading frames and so insertion/deletion events occur more frequently (Mindell 1991).

Applications

Restriction enzyme and direct sequence analysis of mtDNA have been applied extensively, at the intra- and interspecific levels, to examine population genetic structure, gene flow, speciation, hybridisation and systematics (Moritz *et al.* 1987; Avise 1994). At the intraspecific level, the characteristics of mtDNA make it a particularly useful and sensitive molecule for investigating population level processes. The maternal inheritance, haploid transmission and absence of recombination of mtDNA result in an effective population size for the mitochondrial genome that is approximately one-quarter of that for nuclear genes. Mitochondrial genes, therefore, are more sensitive to the effects of stochastic processes (e.g. random genetic drift), founder events and population bottlenecks than are nuclear genes (Birky *et al.* 1983; Avise *et al.* 1984). At the intra- and interspecific levels genetic affinities between individuals, defined by mtDNA genotypes, will reflect matriarchal phylogeny (Avise & Lansman 1983) and can therefore be used to investigate evolutionary relationships among populations and closely related species. The relatively rapid rate of mtDNA nucleotide evolution results in a high level of polymorphism among individuals which provides the opportunity to discriminate between recently isolated populations and species.

Within species, the main focus of mtDNA analysis has been to characterise the genetic structure of wild populations. A large number of studies of intraspecific genetic variation in mtDNA have been made on a diverse range of organisms (reviewed by Wilson *et al.* 1985; Avise *et al.* 1987; Moritz *et al.* 1987; Avise 1994), revealing a generally high level of genetic polymorphism and geographical structuring of mtDNA. This primary information has been used, at a second level, to make inferences about the evolutionary implications of specific individual or group behaviour and life history characteristics, specific physical or ecological barriers to dispersal, the demographic and biogeographic histories of groups of organisms, and provide information for the conservation and management of commercially important biological resources (e.g. fisheries) and endangered species.

Particular individual or group behaviour may have potential genetic and subsequent ecological or evolutionary implications. These behavioural mechanisms include kinship, sociality, philopatry and behavioural niche adaptation and may result in genetic differentiation of sympatric populations. For example, based on tagging studies, female green turtles (*Chelonia mydas*) are known to exhibit strong breeding philopatry to particular islands that may be several thousands of kilometres from the feeding grounds. Restriction enzyme analysis of mtDNA of turtles from colonies in the Indian and Pacific Oceans revealed significant population differentiation among colonies within and between the two ocean basins. These data support the hypothesis of consistent and long term female natal philopatry to breeding islands (Bowen *et al.* 1989; Meylan *et al.* 1990; Bowen *et al.* 1992). In addition, Bowen *et al.* (1989) were able to test a biogeographic hypothesis for the origins of the Ascension Island colony that involved natal philopatry and drift of the continental plate containing Ascension Island away from the South American coast over millions of years. By comparing mtDNA genotypes of turtles from Ascension Island with those from other colonies Bowen *et al.* (1989) showed that the Ascension colony must have been colonised relatively recently.

Hoelzel and Dover (1991) found that two sympatric populations of killer whales (*Orcinus orca*) in the northeast Pacific Ocean were genetically distinct, based on direct sequence analysis of the D-loop region of mtDNA. These two populations use different foraging strategies and target different prey items, suggesting behavioural isolating mechanisms which have lead to the observed genetic differentiation. Similarly, an unusual and non-random

genetic (mtDNA) composition of a group of poisoned humpback whales (*Megaptera novaeangliae*) lead Baker *et al.* (1994) to suggest that these whales were a socially or ecologically distinct subgroup, isolated from other humpback whales by group specific behaviours. The prolonged association between a calf and its mother in the humpback whale may reinforce "group" migration destination, and specialised prey preference or foraging strategy, leading to concordance between maternally inherited genetic markers and specific behavioural traits.

Life history characteristics have a significant impact on the geographic structure of populations. McMillan *et al.* (1992) have shown that for two species of sea urchin inhabiting similar regions of Australasia, differences in the length of the planktonic larval stage result in substantial differences in the geographic structuring of the populations sampled. They used restriction enzyme analysis of mtDNA to show that populations of *Heliocidaris tuberculata*, which has a long larval stage and therefore high dispersal potential, were not genetically structured. In contrast, *H. erythrogramma*, which has a much shorter larval life and consequently a restricted dispersal capability, exhibited significant geographic structuring of populations.

The analysis of mtDNA can be used to provide an evolutionary perspective on demographic and biogeographic histories of species. Avise (1992) summarised the results of mtDNA restriction enzyme analyses of geographical population structure of 19 species of marine, freshwater and terrestrial vertebrates along the southeastern coast of the USA. In almost all assayed species there is significant genetic divergence between populations on the Atlantic and Gulf of Mexico Coasts, which are separated by the Florida 'panhandle'. The strong concordance in geographical population structure of these species implies similar biogeographic histories, probably related to environmental fluctuations during the Pleistocene glaciations which separated Atlantic and Gulf populations. In addition, most species exhibit much lower levels of mtDNA diversity than that predicted under neutrality theory and based on current population sizes. This suggests a much reduced female effective population size, relative to extant population size, which Avise (1992) argues could be a result of historical demographic factors such as variance in reproductive success among females, population bottlenecks and extinctions with subsequent recovery and recolonisation.

The northern elephant seal (*Mirounga angustirostrus*) was hunted to near extinction in the late 1800's but has since recovered to a population size of 120 000 individuals. Hoelzel *et al.* (1993) assessed genetic variation in populations of the northern elephant seal using direct sequence analysis of two regions of the mitochondrial genome and found low levels of mtDNA diversity relative to the southern elephant seal (*M.leonina*). In order to examine the severity of the population bottleneck, Hoelzel *et al.* (1993) used mathematical models to simulate the short term evolutionary dynamics of mtDNA in the northern elephant seal population following the bottleneck. The most likely scenario for the bottleneck event was either a 20-year population minimum of fewer than 30 seals or an extreme single year event involving less than 20 individuals. An important outcome of the modelling process is that in a substantial number of simulations the species became extinct due to demographic stochasticity alone.

The conservation and management of commercially important biological resources, such as marine and freshwater fisheries, requires the identification of stocks that have a sound genetic basis (Ovenden 1990). Different management strategies must be employed when a species is composed of a number of discrete, geographic subpopulations or is effectively a panmictic unit. Brasher *et al.* (1992a), using restriction site analysis of mtDNA, revealed genetic differentiation between Australian and New Zealand populations of the commercially important rock lobster *Jasus verreauxi*. This result suggests that, despite a long lived planktonic larval stage, genetic exchange between the two disjunct populations is limited. In contrast, Ovenden *et al.* (1992), using identical techniques, did not detect genetic subdivision between Australasian populations of the equally important lobster species, *Jasus edwardsii*, which occurs across a similar geographic range and was formerly recognised and managed as two separate species (*J.edwardsii* in New Zealand and *J.novaehollandii* in Australia). Thus, recruitment to adult populations of these two species occurs independently and nonindependently, respectively, in the two countries, and the species themselves will require different management strategies.

Characterising the levels of genetic variation and the degree of genetic distinctiveness of populations within species has relevance to conservation strategies. For example, regional populations of the African wild dog (*Lycaon pictus*), in eastern and western Africa, have

declined substantially in recent times, and are subject to conservation efforts. Girman *et al.* (1993) used restriction enzyme and direct sequence analysis of mtDNA to examine genetic variation in and among eastern and, less threatened, southern African populations. Genetic variability in both regional populations is as high as that found in other large canid species, suggesting that the decline in population size is not the result of inbreeding related problems in isolated dog packs. However, eastern and southern populations are genetically distinct, so that they require separate conservation programs which avoid augmentations and reintroductions from one region into another. In addition, Girman *et al.* (1993) recommended special conservation measures for eastern African populations since a survey of several zoos revealed that all but one of the African wild dogs in captivity were derived from the southern population.

At the interspecific level mtDNA analysis has been used to examine speciation, hybridisation, and systematic and phylogenetic relationships. The cichlid fishes of East African lakes provide a rare example of a massive speciation event which probably occurred over an evolutionarily short and recent period of time. There are almost 800 morphologically, ecologically and behaviourally diverse species of cichlids in the three large lakes of East Africa, Lakes Malawi, Victoria and Tanganyika, most of which are endemic to a particular lake (Kocher *et al.* 1993; Meyer 1993). Species from different lakes share specific morphological, ecological or behavioural characteristics and these have complicated attempts to resolve the evolutionary relationships among species within and between lakes, and the processes that lead to such an explosive radiation. Using direct sequence analysis of segments of the mitochondrial genome Meyer *et al.* (1990), Sturmbauer and Meyer (1992) and Kocher *et al.* (1993) assessed phylogenetic relationships among species from the East African lakes. The general conclusions from these studies are that the species flocks in Lakes Victoria and Malawi are each monophyletic, but the Tanganyika flock occupies an ancestral position in the mtDNA phylogeny and represents a reservoir of ancient cichlid lineages from which the species flocks in the other two lakes are derived. The independent evolutionary histories of species flocks from different lakes therefore show that speciation has occurred separately within each lake, with considerable convergence in morphological, ecological and behavioural traits in species from different lakes. When combined with geological information concerning the ages and histories of these lakes, the mtDNA data suggest that fluctuations in water level over evolutionary time scales leading to fragmentation of the

existing water masses may have influenced the process of genetic differentiation leading to speciation. Intraspecific analysis of mtDNA variability (Sturmbauer & Meyer 1992) revealed significant differentiation over small geographic scales, suggesting that the ecological specialisation of many species produces ecological barriers to dispersal (in the form of unsuitable habitat) within lakes which may lead to intralacustrine speciation by microallopatric processes (Meyer 1993). The low levels of genetic divergence between morphological species also reveal the extremely high rate of morphological diversification and rapid speciation that has occurred within species flocks over an evolutionarily short period of time.

Cooper *et al.* (1992) used mitochondrial 12S rRNA sequences to investigate phylogenetic relationships within the ratite group of birds. New Zealand was previously inhabited by two groups of these flightless birds, the moas (now extinct) and the kiwis. Based on biogeographic and morphological evidence, the moas and kiwis were believed to have evolved from a common ancestor within New Zealand. However, the molecular data show that kiwis are more closely related to Australian and African ratites, the emu, ostrich and cassowary, and that moas diverged earlier and independently within the ratite lineage. Thus these two groups of morphologically similar, flightless birds had independent origins within New Zealand.

Mitochondrial DNA has proved to be a useful genetic marker in studies of natural hybridisation events between closely related species. Using restriction enzyme analysis of mtDNA Tegelström (1987b) examined genetic diversity in Fennoscandian populations of the bank vole (*Clethrionomys glareolus*). An unusually high mtDNA sequence divergence between southern-central and northern populations suggested the possibility of introgression of the divergent mtDNA lineages into the northern population by hybridisation with a closely related species. By comparing the mtDNA haplotypes identified in *C. glareolus* with two other Fennoscandian species, *C. rutilus* and *C. rufocanus*, it was possible to identify *C. rutilus*, the northern red-backed vole, as the maternal species involved in the hybridisation. Allozyme data did not support the alternate hypothesis that the mtDNA similarity between northern *C. glareolus* and *C. rutilus* is due to a speciation event following separation from southern-central *C. glareolus*. The mtDNA data also suggest an evolutionarily recent

hybridisation event occurring during the postglacial recolonisation of Fennoscandia.

1.1.4 PCR and molecular phylogenetics

Until recently two restrictions have limited molecular approaches to phylogenetics. The need for relatively large amounts of undamaged DNA requires killing the animal and frequently cryopreservation of the samples in the field. Deep freeze facilities are however difficult to transport, expensive and often unavailable in certain areas. This presents problems where a species is extinct or virtually unknown and the only known specimens are represented merely by the holotype or type series, the species is rare, there is legal protection of the species or its habitat, the habitat is inaccessible, known collection localities have been destroyed or the costs of obtaining fresh material is prohibitive (Hillis 1987). The combination of the sensitivity of the PCR and stability of DNA mean that so little tissue is needed that samples (e.g. single plucked feathers (Taberlet & Bouvet 1991), or a drop of blood (Arctander 1988)) can be taken from live individuals and stored easily and cheaply without the need for freezing.

Molecular phylogenetics has also been limited by the amount of sequence information that could be obtained with the use of traditional recombinant DNA techniques. The simplicity, speed, sensitivity and versatility of the PCR make it more efficient than traditional cloning techniques and it can be applied to a much larger number of samples. PCR is therefore well suited to studies of large populations and diverse groups of organisms and is rapidly being applied to the analysis of phylogenetic relationships.

DNA can survive in tissues long after the death of the organism involved. DNA has been recovered from a variety of ancient tissues, including dry remains (museum skins, natural animal mummies and human mummies Higuchi *et al.* 1984; Houde & Braun 1988; Pääbo 1989), frozen remains (mammoth muscle, Higuchi & Wilson 1984), wet remains (ethanol preserved museum specimens and human brain preserved in peat, Doran *et al.* 1986) and amber preserved insects (DeSalle *et al.* 1992; Cano *et al.* 1993) ranging in age up to 120 million years.

Ancient DNA is however extensively modified with a reduction in size down to an average of

only a few hundred base pairs and an abundance of lesions such as baseless sites, oxidised pyrimidines, and cross-links, many of which are the result of oxidative processes (Pääbo *et al.* 1989). Ancient DNA is invariably degraded to an average molecular size of 100-200 bp with substantial amounts of DNA in the 40-500 bp range, although the extent of size reduction is not correlated to the age of the samples (Pääbo *et al.* 1989). The extent of these chemical modifications means that less than 1% of the DNA molecules recovered from museum specimens or archaeological remains are undamaged. Sequence analysis of this extensively damaged DNA, using molecular cloning techniques, is both difficult and subject to errors such as cloning artifacts (Pääbo & Wilson 1988).

The capacity of the PCR to amplify preferentially a specific DNA segment from a very small number of intact DNA molecules, containing the relevant sequence, in the presence of a vast excess of damaged molecules has enabled, for the first time, the retrieval of reliable DNA sequence information from many ancient tissue remains (Thomas *et al.* 1989; Pääbo & Wilson 1988). Intact molecules are preferentially amplified because most damaged molecules will either not be replicated at all, e.g. due to inter or intra molecular cross-links, or will be at a replicative disadvantage because the abundance of lesions slow down the DNA polymerase. The PCR also has the advantage of producing more reliable sequence data because it is an in vitro system with normally no capacity for repair or misrepair (Pääbo *et al.* 1989). Museum specimens, such as dried skins, can provide a source of samples to obtain sequence data, via the PCR, for phylogenetic analyses. The PCR therefore allows phylogenetic analysis of almost any group of organisms by providing access to fresh material, which can be easily and non-lethally collected in the field, and a vast amount of genetic material in museum collections, representing extant and extinct species and enormous amounts of time consuming field work.

1.2 The shearwater genus *Puffinus*.

The Order Procellariiformes is a monophyletic and apparently ancient group of morphologically distinct seabirds known as petrels (Olson 1985; Warham 1990). All petrels are characterised by a hooked bill, covered in horny plates, and the distinctive raised tubular nostrils arising near the base of the beak (Marchant & Higgins 1990; Warham 1990). The fossil record of this group extends over 50 million years (Olson 1985) and combined with the structural and behavioural uniqueness of the petrels suggests that the procellariiform ancestor

probably diverged from other seabirds during the late Cretaceous (Harper 1978). Subsequent evolution has resulted in one of the most numerous and diverse groups of birds, which is highly successful and specialised in the marine environment. This success is reflected in the almost three orders of magnitude range in body size (Harper 1978; Warham 1990), diversity of habits and feeding behaviour from aerial (surface feeding in flight) and surface (surface seizing) to aquatic (pursuit plunging and diving) (Ashmole 1971), and distribution throughout all oceans of the world, from the tropics to the poles, with some species sedentary and others undertaking transequatorial, transoceanic and circumpolar migrations (Harper 1978).

Although the monophyly of the Procellariiformes is accepted (Harper 1978; Sibley & Ahlquist 1990), the phylogeny and systematics of extant taxa within the group remains unclear, especially at the species level (Bourne 1987). Current taxonomy has the order consisting of some 103 species and divided into four well defined families: Diomedeidae (albatrosses), Procellariidae (fulmars and shearwaters), Hyrdobatidae (storm petrels) and Pelecanoididae (diving petrels) (Jouanin & Mougin 1979; Warham 1990). The family Procellariidae represents the main radiation of medium-sized petrels, consisting of the group of aerial and aquatic fulmars, gadfly petrels, prions, petrels and shearwaters (Marchant & Higgins 1990).

The shearwater genus Puffinus contains a morphologically and behaviourally heterogenous group of essentially pelagic, small to medium sized petrels with a world-wide distribution. Members of the genus are distributed and breed at all latitudes between the Arctic and Antarctic circles, in all oceans and the Caribbean and Mediterranean seas, but are most abundant and in the highest diversity in the cool temperate seas around Australasia (Marchant & Higgins 1990; Warham 1990). Shearwaters are among the worlds most abundant birds, with populations of *P. tenuirostris*, *P. griseus* and *P. gravis* numbering in the tens of millions (Harper 1978; Warham 1990).

Birds of this genus are distinguished from other Procellariiformes by their long slender bills with paired, bevelled nasal tubes separated by a thick septum, and a number of osteological features (Wragg 1985; Warham 1990). Plumage colour is generally dark brown to black on

the dorsal surface and brown or white ventrally, with considerable individual variation and intertaxon overlap in plumage characters. Some species show a distinct dark, light and intermediate plumage polymorphism. Size varies almost two-fold between the largest and heaviest species, *P. gravis*, *P. carneipes* and *P. creatopus*, with wingspans of 100-120 cm and weights up to 750 g, to the smallest, *P. lherminieri* and *P. assimilis*, which have wingspans of 60-75 cm and weigh about 200 g. There is little or no sexual dimorphism in external morphology or plumage (Warham 1990).

Puffinus shearwaters feed on crustaceans, cephalopods and fish obtained by aerial, surface and aquatic feeding methods. All members of the genus breed colonially in habitats ranging from small, low lying coastal islands to mountain slopes 1500 m above sea level and many kilometres inland. Most species nest in underground burrows or cavities on open ground or under a dense canopy of ferns, shrubs, tussocks grass or forest, and are normally only nocturnal on land (Warham 1990). Six species undertake substantial, annual, trans-equatorial migrations and five of these are southern hemisphere breeders that move north into the North Pacific and North Atlantic Oceans. Only *P. p. puffinus* migrates south from its breeding colonies in the North Atlantic to winter off the east coast of South America.

The systematics of the genus has been and remains controversial. The most recent classification by Warham (1990), based on the conservative list of Jouanin and Mougin (1979), includes 36 separate taxa in 15 recognised species. The taxonomic uncertainty is due to the widespread use of external morphology and plumage colours and patterns as taxonomic characters, which show considerable overlap between related species and have an unknown genetic component (Wragg 1985; Berruti 1990). This problem has been compounded by a general lack of comparative biological information for most species. Many breeding colonies are remote and inaccessible, and for at least one taxon, *P. lherminieri. heinrothi*, unknown. Most species spend a considerable proportion of their life cycle away from the breeding colonies, dispersed over large areas of oceanic water. Many specimens are beach washed birds of unknown origin, age or sex, and museum specimens suffer from shrinkage and colour changes as they dry. In addition, the systematic uncertainty is compounded by a general lack of phylogenetic information on intertaxon relationships.

1.3 The short-tailed shearwater, *Puffinus tenuirostris*

1.3.1 Life history

The short-tailed shearwater or muttonbird (*Puffinus tenuirostris*) is a medium sized shearwater, which in appearance has an overall dark brown-grey plumage, with paler underwings. Muttonbirds are the most abundant and the only member of the Procellariiformes that breed entirely in Australia. An estimated 23 million birds breed annually (Skira *et al.* 1985), nesting in densely packed burrows in about 250 colonies on inshore islands and headlands of southern Australia. Colony size varies from a few to several million breeding pairs. At present there are approximately 170 colonies in Tasmania and its near offshore islands (Skira *et al.* 1985), 30 colonies along the coastline of Victoria (Harris & Norman 1981), 33 colonies in South Australia (Skira 1991),13 colonies in New South Wales (NSW) (Lane 1979) and several small colonies in Western Australia (Johnstone *et al.* 1990a, b; Lane 1983). The breeding range of the species is therefore centred around Tasmania and along the coastline of Victoria. The extremes of the range are to the west at Figure of Eight Island in Western Australia, to the south at Maatsyker Island in Tasmania (Marchant & Higgins 1990) and to the north at Muttonbird Island in NSW (Lane 1991).

The biology and life history of *P. tenuirostris* is well known from a number of studies, including a 45 year study at a colony on Fisher Island in eastern Bass Strait (Serventy 1963, 1967a,b; Naarding 1980, 1981; Serventy & Curry 1984; Fitzherbert 1985; Bradley *et al.* 1989, 1990; Wooller *et al.* 1988, 1989, 1990) and has recently been reviewed by Marchant and Higgins (1990) and Skira (1991). Short-tailed shearwaters are trans-equatorial migrants, completing an annual return migration of approximately 30 000 km. Each year, in April and May, birds fly north, from the breeding areas in southern Australia, in the western half of the Pacific Ocean to spend the boreal summer in the north Pacific, Bering Strait and Arctic Ocean. A small number of birds have been recorded in the northeast Indian Ocean in May (Marchant & Higgins 1990) but it is unclear whether these birds remain in this area or continue into the Pacific and join the main body of the migration.The return migration begins in late August and crosses the central Pacific Ocean.

Breeding adult birds are present at the breeding colonies from September until April. Prebreeding birds arrive later and depart earlier than breeding birds. On land short-tailed shearwaters are almost entirely nocturnal, arriving at the colony only after dusk and leaving

before dawn. The breeding season of short-tailed shearwaters is both long (7 months) and highly synchronised between years and throughout the breeding range. Sexually mature birds arrive at the breeding colonies suddenly and in large numbers during the last week of September. Breeding birds are socially monogamous and exhibit strong nest site and mate fidelity, usually returning to breed with the same partner in the same burrow for many years. In addition to the strong breeding philopatry, short-tailed shearwaters exhibit a remarkably strong natal philopatry, returning to breed not only in the colony in which they were hatched, but frequently to a site very close to the natal burrow.

After arrival individuals indulge in noisy courtship behaviour during which established pairs are reunited or new pairs are formed. At the same time existing burrows are occupied and renovated or new ones excavated; each member of the pair sharing in the excavation and maintenance of the burrow. Burrows are up to 2 m deep and consist of the entrance at the surface, a tunnel of varying length and an enlarged nesting chamber which is roughly lined with dry grasses and other vegetation. Pairs are territorial and defend an area around the entrance and within the burrow. Pairing, mating, nesting and rearing young occur within this territory. The birds usually feed at sea during the day but may remain silent and inactive in the burrow. Copulation occurs on open ground in front of the burrow or in the burrow during the nest building period. Sexual behaviour and copulations begin 15-17 days after the first landfall and continue throughout the nest-building period and are frequently seen 3-4 days before the pre-laying exodus. Compared to other parts of the breeding season, relatively little is known about reproductive behaviour and in particular the sexual fidelity of paired birds. In studies of lifetime reproductive success (Bradley *et al.* 1989, 1990; Wooller *et al.* 1988, 1989, 1990) it has always been assumed that the pair bonded birds providing care for a nestling are the actual genetic parents of this nestling.

Almost all birds leave the colony in early November, in a pre-laying exodus of about 20 days, and remain at sea until 19-21 November. This exodus from the colony allows females and males to collect sufficient food to produce the egg, and to build up body reserves for the first incubation shift. Egg laying is highly synchronised within 13 days and occurs between 23-28 November in 85% of all known cases (Serventy *et al.* 1971). Only a single, large egg is laid and predated, damaged, infertile or unhatched eggs are not replaced. The egg is

incubated for 53-55 days with both male and female incubating alternatively in shifts of between 10-18 days. Incubation is usually started by the male and most eggs hatch during the males third incubation shift in mid-late January. The newly hatched chick is brooded by one parent for rarely longer than 2 days and is then left unattended by day. Both parents share in feeding which is usually nightly until the nestling is *c.*10 days old and thereafter at decreasing intervals. Nestlings spend approximately 95 days in the burrow during which they grow rapidly, forming large fat deposits, and reaching an average maximum weight of 860 g, 50% heavier than the adults. By the end of March the nestlings remain unfed for 3-15 days. The nestlings receive their last meal in early April, and are then deserted by their parents who begin their migration. The nestlings remain in the burrow for another 2-3 weeks while they fledge. Fledglings leave the colony to begin their first migration from late April to early May.

Mortality between fledging and the first breeding attempt is high and is approximately 50% in the first year (Serventy 1967b). Annual mortality of mature adults breeding for the first time is approximately 10% but then decreases to about 7% for the next 10 years, before increasing substantially for shearwaters breeding for more than 10 years (Bradley *et al.* 1989, 1991). Natural causes of mortality include predation by other species of birds and snakes, disease, starvation and flooding of low-lying burrows by heavy rain (Skira *et al.* 1985). Mass mortality due to starvation and exhaustion occurs in some years during either the northward or return migration, as large numbers of dead birds are periodically washed ashore in Japan and on the east coast of Australia (Serventy 1967b; Oka & Maruyama 1985; Oka 1986; Crowley 1991). Serventy (1967b) reported severe mortality on the east coast of Australia in 15 out of the 111 years between 1851 and 1962.

1.3.2 Human impact and exploitation

Prior to European settlement (*c.* 1800), Aborigines in Tasmania harvested eggs and chicks during the breeding season for food (Ryan 1981). Subsequently shearwaters have formed the basis of a muttonbird industry in Tasmania with pre-fledgling chicks taken for their meat, feathers and oil during an open season in March and April each year (Skira *et al.* 1985). The species is fully protected in New South Wales, Victoria, South Australia and Western Australia and is not harvested by humans. Eggs were taken prior to 1900 in Victoria and

Tasmania, but this practice was ceased at the turn of the century. Harvesting of muttonbirds has been separated into commercial and non-commercial operations. Commercial exploitation has been confined to the Hunter and Furneaux Groups of islands in western and eastern Bass Strait, respectively. Annual harvests of chicks in the Furneaux Group alone reached nearly one million by 1900. This level of harvesting continued until about 1925 (Skira 1987) but has steadily decreased to a level where the entire commercial harvest is now approximately 300 000 birds. For the last 30 years the annual harvest at commercial colonies has been 30% or less of the available birds (Skira *et al.* 1985).

Non-commercial harvesting involves an open season during the first two weeks of April during which individual operators are restricted to a small daily bag limit. Non-commercial exploitation was probably minimal until 40 years ago when motor and water transport became common and provided easy access to many colonies. The total annual harvest in the early 1980s was estimated to be 300 000 chicks, and between 1977 and 1981 90% or more of the nestlings were taken each season in 24 monitored colonies (Skira *et al.* 1985). The long lifespan and large pool of pre-breeding birds probably saved many of these colonies from extinction. In 1986, all non-commercial harvesting of muttonbirds in Tasmania, except in the Hunter and Furneaux Groups was stopped. However, poaching of birds continues and may represent a significant and continuing pressure on some colonies (Skira & Wapstra 1980).

In addition to direct harvesting of chicks, humans have had a significant impact on mortality of short-tailed shearwaters. It is estimated that at least 500 000 shearwaters, mostly fledglings of that year, are killed annually during the migration across the Pacific in gill net and other drift net fisheries (King 1984; Ogi 1984; Jones & DeGange 1988). Predation by feral cats, dogs and foxes poses a threat to both adults and nestlings, especially in colonies close to cities and towns, and may have a significant impact on annual breeding success (Naarding 1980). Human interference has had a marked effect on the physical condition of colonies, which in turn affects the ability of shearwaters to breed successfully. Large volumes of pedestrian traffic especially in non-commercially exploited colonies and colonies close to human habitation results in the collapse of many burrows, compaction of the soil which prevents birds digging burrows and destruction of vegetation leading to erosion of large areas within colonies (Naarding 1981). Non-commercial operators also intentionally

damaged burrows in attempts to dig out nestlings that are beyond reach from the surface (Naarding 1981). The grazing of cattle and sheep in areas containing shearwater colonies has similar effects: burrows are trampled, soil is compacted where stock make tracks through the colony, and vegetation is destroyed. Fire is a constant threat, with the potential to remove vegetation and promote erosion (Naarding 1981).

1.4 Aims

This thesis deals with the application of molecular genetic techniques to problems in the ecology, population biology, systematics and evolutionary biology of species in the genus *Puffinus* with particular focus on the short-tailed shearwater *P. tenuirostris*. The primary objectives of the study are given below.

To test the assumption of monogamy in *P. tenuirostris* by examining levels of genetic relatedness between pair-bonded birds and the single offspring in the nesting burrow for which they each provide considerable parental care. The extent of mate infidelity will be examined in relation to the species' biology and its effect on lifetime reproductive success and is a unique application of a molecular technique in a species whose nocturnal and subterranean breeding habit generally precludes the use of observational data to examine mate fidelity.

To assess levels of geographic population structure among distinct breeding colonies of *P. tenuirostris* from across its breeding range in southeastern Australia. In particular to test the hypothesis that natal and breeding philopatry has created and maintained genetic subdivision between colonies, and to examine the genetic effects of recent demographic fluctuations in particular colonies.

To examine molecular-based, phylogenetic relationships among extant taxa of *Puffinus*, and to use this phylogeny to evaluate the existing morphologically-based taxonomy, and evolutionary scenarios for the genus.

Chapter 2

Molecular Genetic Analysis of Mating Systems in the Short-tailed Shearwater, *Puffinus tenuirostris*.

Sections of this chapter have been published:

Austin, J. J., Carter, R. E. and Parkin, D. T. (1993) Genetic evidence for extra-pair fertilisations in socially monogamous short-tailed shearwaters, *Puffinus tenuirostris* (Procellariiformes: Procellariidae), using DNA fingerprinting. *Australian Journal of Zoology* **41:** 1-11.

Austin, J. and Edmunds, M. (1994) Hatching success of the short-tailed shearwater (*Puffinus tenuirostris*) in two Tasmanian colonies. *Corella* **18:** 44-46.

2.1 INTRODUCTION

2.1.1 Monogamy and extra-pair copulations in birds

At the micro-evolutionary scale, organismal phylogeny is a function of the generation to generation pattern of individual survival and reproduction. Phylogenetic relationships between populations, species and higher taxa can ultimately be traced through individual pedigrees. Two inter-related factors that influence phylogenetic structure at the micro-evolutionary scale are the mating system (the mating relationship between the sexes) and individual reproductive success (the number of offspring produced that survive to breed). The nature of the mating system (monogamy, polygamy or promiscuity) will determine the general pattern of gene transmission from one generation to the next. However, within any particular mating system, individual reproductive success may vary according to a number of factors including mating success (the number of copulations that result in fertilisation) and individual "quality". Individual quality comprises both phenotypic and genotypic quality as good parents can successfully raise more young and individuals with superior genotypes produce high quality offspring. Differential mating success will occur through two mechanisms of sexual selection: intrasexual selection where members of one sex (usually males) compete

to mate with the other sex; and intersexual selection, where members of one sex (usually females) choose mates (Darwin 1871). The intensity of sexual selection is determined by the relative investment in reproduction by each sex (Birkhead & Møller 1992).

Mating systems can be categorised using social or genetic associations between the sexes (Westneat *et al.* 1990). The monogamous pair is the most common of a wide variety of mating systems that has been recorded in birds and is almost universal among over 250 species of seabirds in 4 orders and 13 families (Lack 1968). Wittenberger and Tilson (1980) have defined monogamy, using both social and genetic criteria, as a '*prolonged association and essentially exclusive mating relationship between one male and one female*'. A prolonged association implies coordinated and consistent social interactions between pair bonded birds, but does not specify the frequency of these interactions. An essentially exclusive mating relationship defines the pattern, but not frequency, of copulations and gene transfer across generations, but does not preclude occasional, covert matings outside the pair bond. The definition of monogamy does not specify the degree of parental investment provided by either sex.

There are a number of factors that appear to have affected the evolution of mating systems and, in particular, monogamy in birds (Lack 1968; Trivers 1972; Emlen & Oring 1977; Wittenberger & Tilson 1980) . In colonial seabirds that lay one-egg clutches, such as emperor and king penguins, albatrosses, shearwaters, storm petrels and diving petrels, and where the sex ratio is approximately equal, monogamy appears to have evolved because male parental care is essential to reproductive success (Wittenberger & Tilson 1980). Females alone are not capable of rearing a chick and a pair cannot raise more than one young due to the limitations of distance and travelling time between breeding colonies and feeding grounds (Wittenberger & Tilson 1980) and other energetic constraints related to food availability and quality (Fitzherbert 1985). Individuals of each sex maximise their reproductive success by having only one mate and helping to rear the offspring, rather than having multiple mates and only one parent feed the young (Lack 1968). There are conflicting views on the extent of sexual selection in monogamous birds (Trivers 1972; Gladstone 1979; Birkhead & Møller 1992). Gladstone (1979) has argued that reproductive investment by each sex in some monogamous birds will be approximately equal despite anisogamy, because males often invest more than

females in construction, maintenance and defence of the nest, and female investment in egg production is small relative to both her total investment and the investment of the male. Therefore, assuming an unbiased sex ratio, sexual selection should act approximately equally on both sexes and both males and females should choose their mates carefully. The variance in reproductive success as a result of mating success would be small and equal for both sexes.

Although a particular mating system may appear to predominate, not all members of a species will adopt the same strategy (Krebs & Davies 1978). Individuals may increase their reproductive success relative to others of the same sex, within a monogamous system, by engaging in one of a number of alternative strategies. Intraspecific brood parasitism (ISBP) or egg dumping occurs when a female lays one or more eggs in the nest of another pair and does not assist with incubation or provide care for the nestling (Yom-Tov 1980). The male in the parasitised pair may or may not have fertilised the dumped egg(s). Rapid mate-switching occurs when a pair bond is broken and the male and/or female establishes a new pair bond with a different mate (McKinney *et al.* 1984; Birkhead *et al.* 1990). Mate-switching provides males with the opportunity to increase their mating success by fertilising the eggs of more than one female. Females may use mate-switching to ensure fertilisation of their eggs, or to produce higher quality offspring through processes of inter- and intrasexual selection.

Extra-pair copulations (EPCs) are copulations that occur between one member of a pair and another individual outside the pair bond (from another pair or unpaired). EPCs have been observed in at least 115 species of monogamous birds in 30 families (Gladstone 1979; Ford 1983; McKinney *et al.* 1984) suggesting that this alternative mating strategy is widespread among birds previously thought to be entirely monogamous. Among Procellariiformes, EPCs have been recorded in monogamous grey-faced petrels (*Pterodroma macroptera gouldi*, Imber 1976), Buller's albatross (*Diomedea bulleri*, Richdale 1949), wandering albatross (*Diomedea exulans*, Tickell 1968) and Laysan albatross (*Diomedea immutabilis*, Fisher 1971). The significance of EPCs as a reproductive strategy, within a monogamous system, and the ecological and social conditions determining its occurrence have been considered for both males (Trivers 1972; Beecher & Beecher 1979) and females (Fitch & Schugart 1984; Smith 1988; Colwell & Oring 1989). Trivers (1972) suggested that males of monogamous

where they help raise young with the pair bonded female but also pursue EPCs. If EPCs have a chance of leading to a successful fertilisation (an extra-pair fertilisation, EPF) then they could enhance male reproductive success at relatively little cost by parasitising the parental care offered by another male. Females may also adopt a mixed reproductive strategy in which they obtain both genetic and non-genetic benefits from copulations with extra-pair males (Westneat *et al.* 1990).

There are several mechanisms by which EPCs will result in sexual selection in monogamous birds (Birkhead & Møller 1992). Intrasexual competition between males may occur through direct physical fights for the opportunity to copulate with an extra-pair female. Sperm competition, the competition between sperm of different males to fertilise the egg(s) of a single female (Parker 1970), is a more subtle form of intrasexual competition and will occur whenever a female copulates with more than one male. The opportunity for sperm competition in birds is enhanced because sperm are stored in the female reproductive tract prior to fertilisation (Birkhead & Møller 1992). Intersexual selection can occur *via* female choice of EPC partners on the basis of non-resource attributes related to male genetic quality (Hamilton & Zuk 1982; Andersson 1986; Heywood 1989) . Females may obtain good genes which enhance the genetic quality of their offspring. Intra and intersexual selection can occur simultaneously because female choice of male copulation partners (intersexual selection) will result in sperm competition (intersexual selection) (Birkhead & Møller 1992). As males have the opportunity to greatly enhance their mating success, sexual selection through EPCs in monogamous birds is likely to result in a greater variance in reproductive success for males than females.

The opportunity for individuals to engage in EPCs is controlled by a number of factors including the social system (colonial or solitary), male behaviour (mate guarding), reproductive synchrony and female behaviour. EPCs are more frequent in colonially breeding birds (Møller & Birkhead 1993). The high density of birds in breeding colonies provides many more potential extra-pair mates and makes them easily accessible. EPCs are less frequent in species in which males guard their mates (Møller & Birkhead 1993). Mate guarding, where males remain close to their mates and actively deter other males during the females fertilisable period (Beecher & Beecher 1979; Birkhead 1979), will reduce

opportunities for EPCs for both the extra-pair male and pair female. Mate guarding and coloniality are negatively correlated because many colonially breeding birds are prevented from effective mate guarding. Breeding pairs are separated when one or both birds are feeding at sea and one member of the pair may have to remain at the colony to guard the nest, so females can remain unguarded for a substantial amount of time. Breeding synchrony confines the opportunities for EPCs to a small part of the breeding cycle and may may force males to make trade-offs in reproductive effort between pursuing EPC and ensuring paternity in their own mate (Westneat *et al.* 1990). It has been predicted that synchronously breeding species should show a lower frequency of EPC than asynchronously breeding species (Westneat *et al.* 1990). However, this prediction assumes a high and consistent level of mate guarding which, in many synchronously and colonially breeding species, is not the case (Møller & Birkhead 1993).

EPCs can occur in three ways each differing and defined by the female behavioural response. Unforced EPCs (UEPCs) occur when the female either actively initiates and solicits an EPC (e.g. northern fulmar [*Fulmarus glacialis*], Hatch 1987; black-capped chickadee [*Parus atricapillus*], Smith 1988; razorbill [*Alca torda*], Wagner 1992a), or accepts or offers no resistance to an EPC (e.g. white ibis [*Eudocimus albus*], Frederick 1987; barn swallow [*Hirundo rustica*], Møller 1988; tree swallow [*Tachycineta bicolor*], Venier *et al.* 1993). Forced EPCs (FEPCs) are actively or apparently resisted by the female (e.g. lesser snow goose [*Anser caerulescens caerulescens*], Mineau & Cooke 1979; common murre [*Uria aalge*], Birkhead *et al.* 1985; purple martin [*Progne subis*], Morton 1987). From a male perspective, the opportunity for EPC will be greatest when females solicit and least when they resist copulations.

The extent to which different individuals will engage in EPCs will depend on the opportunities for EPCs, trade-offs in reproductive effort and the relative costs and benefits of EPCs. Males must make trade-offs between mating effort (the acquisition of mates and EPCs, and paternity assurance) and parental effort based on the relative gain from each type of effort (Maynard Smith 1977; Low 1978). The factors that affect this trade-off include the value of paternal care and the opportunities for additional matings outside the pair bond (Westneat *et al.* 1990). There are a number of potential genetic and non-genetic benefits and costs to both males and females from engaging in EPCs (Westneat *et al.* 1990; Birkhead & Møller 1992).

Possible benefits and costs to both sexes include insurance against mate infertility and insurance mate acquisition, and risk of parasite or disease transmission, respectively. For males the main potential benefit is increased reproductive success (Trivers 1972) and costs are sperm depletion, risk of loss of within-pair paternity and risk of injury from the mate of the extra-pair female. The potential benefits for females include material benefits (food or parental care), increased genetic diversity of offspring, increased genetic quality of offspring and to insure against low sperm viability resulting from extended sperm storage. Possible costs to females are reduced genetic quality of offspring, a risk of physical injury and disturbance of breeding activities by harassment from extra-pair males. In addition females may apparently accept EPCs to avoid the costs.

2.1.2 Genetic markers in behavioural ecology

In studies of breeding success and mating systems of wild populations, it is necessary to identify reliably individuals and unambiguously establish the genetic relationships between them. However, observations of social organisation and mating behaviour have frequently failed to determine the true breeding structure of a population (Quinn *et al.* 1987; Gyllensten *et al.* 1990; Wetton 1990). There are a number of problems associated with observational studies, with respect to mixed reproductive strategies involving EPCs. First, estimates of reproductive success based on the number of fledged young may be biased if males have successfully achieved EPCs or have been cuckolded by another male (Burke 1989). Second, many birds copulate infrequently or in visually occluded areas (e.g. in vegetation, nesting burrows, or at night). Third, EPCs may not be as visible as within pair copulations. In many cases males must obtain EPCs without the knowledge of the pair male, which would also make it difficult for an observer to identify (Birkhead & Møller 1992). Fourth, it is very difficult to determine, by observation alone, whether a copulation has resulted in insemination and impossible to evaluate the ejaculate size and sperm number of within pair and extra pair copulations, and the eventual success in fertilisation (Ford 1983; Møller & Birkhead 1993).

Attempts to verify true biological relationships have focussed recently on genetically polymorphic markers which allow individual identification. Morphological characters have been used successfully on a limited number of bird species. Analysis of heritable morphological characters such as tarsus and wing length, and plumage markers suggest that

EPFs occur in pied flycatchers (*Ficedula hypoleuca*, Alatalo *et al.* 1984), barn swallows (Møller 1987) and indigo buntings (*Passerina cyanea*, Payne & Payne 1989), and in captive mallard ducks (*Anas platyrhynchos*, Burns *et al.* 1980) and lesser snow geese (Cooke & Mirsky 1972; Lank *et al.* 1989), respectively. The main disadvantages of these methods, which preclude their widespread use on wild birds, are that the genetic basis of the characteristic needs to be demonstrated, large sample sizes are often required and assignment of paternity to a particular individual is rarely, if ever possible (Birkhead 1988). Biochemical polymorphisms of proteins (i.e. allozymes) have been used widely to exclude paternity in indigo buntings (Westneat 1987), white-crowned sparrows (*Zonotrichia leucophrys*, Sherman & Morton 1988), acorn woodpeckers (*Melanerpes formicivorus*, Mumme *et al.* 1985), bobolinks (*Dolichonyx oryzivorus*, Gavin & Bollinger 1985), white-fronted bee-eaters (*Merops bullockoides*, Wrege & Emlen 1987), european starlings (*Sturnus vulgaris*, Hoffenberg *et al.* 1988) and house sparrows (*Passer domesticus*, Wetton 1990). However, protein electrophoresis relies on sufficient variation at a number of enzyme loci. In many birds protein polymorphisms do not provide sufficiently variable genetic markers to unambiguously determine parentage (Birkhead 1988). Molecular techniques have recently been developed which detect variation in the sequence of DNA and these provide a powerful method for determining genetic relationships.

Restriction fragment length polymorphisms (RFLPs) have been used to detect multiple maternity and paternity in nests of lesser snow geese (Quinn *et al.* 1987). Using this technique, DNA probes, derived from unique or single copy, non-coding sequences and specific to individual loci are used to identify polymorphisms (alleles) based on the presence and absence of restriction enzyme sites. By using a number of probes to examine variation at different loci , and given the high variability and Mendelian inheritance of these markers (Quinn & White 1987a), it was possible to confirm or exclude parentage of male and female nest attendants. One problem with RFLP analysis is that because the DNA probes are derived from unique sequences they are likely to be species specific. For each new species to be analysed a new set of DNA probes must be isolated and characterised (Burke 1989).

Multilocus DNA fingerprinting is a technique, originally developed for use in forensics, paternity and genetic analysis of humans by Jeffreys *et al.* (1985a, b), that can detect

hypervariable 'minisatellite' sequences at multiple loci in genomic DNA. The human minisatellite probes, 33.6 and 33.15, developed by Jeffreys *et al.* (1985a) have been shown to cross-hybridise with homologous minisatellites present in the genomes of many organisms including rice (Dallas 1988), snails (Jarne *et al.* 1992), gorgonian coral (Coffroth *et al.* 1992), fish (Carter *et al.* 1991), turtles (Parker & Whiteman 1993), mammals (Harris *et al.* 1991; Ribble 1991; Tegelström *et al.* 1991; Pemberton *et al.* 1992; Baker *et al.* 1993) and birds. DNA fingerprinting has been applied extensively to allow individual identification, paternity determination and detection of EPFs and cuckoldry in wild bird populations (Wetton *et al.* 1987; Burke *et al.* 1989; Birkhead *et al.* 1990; Gibbs *et al.* 1990; Morton *et al.* 1990; Westneat 1990; Graves *et al.* 1992). Numerous fingerprinting studies have shown that EPCs result in EPFs but the frequency of extra-pair paternity varies enormously. For example the percentage of EPFs detected was zero in the northern fulmar (*Fulmarus glacialis*, Hunter *et al.* 1992), willow warbler and wood warbler (Gyllensten *et al.* 1990), or close to zero in the european bee-eater (*Merops apiaster*, Jones *et al.* 1991), dunnock (Burke *et al.* 1989) and zebra finch (Birkhead *et al.* 1990), but high in the red-winged black bird (28%, *Agelaius phoeniceus*, Gibbs *et al.* 1990), indigo bunting (35%, Westneat 1990), tree swallow (38%, Lifjeld *et al.* 1993) and reed bunting (72%, *Emberiza schoeniclus*, Dixon A. and Burke T., unpublished, in Hartley *et al.* 1993). EPFs have been found to vary between different populations within one species. Norwegian and Swedish populations of the pied flycatcher had a low (4%, Lifjeld *et al.* 1991) and high (24%, Gelter & Tegelström 1992) rate of EPFs, respectively.

2.1.3 Short-tailed shearwater

Short-tailed shearwaters are socially monogamous. Adults form pair bonds for up to 15 years, with a maximum recorded breeding lifespan of 27 years (Bradley *et al.* 1990). Mated pairs undertake coordinated breeding activities from pair formation to burrow construction and defence, egg incubation and feeding and caring for the nestling. Apart from production of gametes, parental investment by both sexes is approximately equal (Serventy 1967b). Change of mate is known to occur and results from death of a mate or divorce of a living partner.

Short-tailed shearwaters are ecologically constrained to raising a single nestling each year, which requires a considerable and extended investment by both the male and female in each pair (Fitzherbert 1985). However, colonial breeding and synchronised fertility of this species

spatially and temporally concentrates fertile birds creating considerable opportunity for both males and females to seek alternative mating partners, and thus enhance their reproductive success. Breeding birds are present in in the colony in the greatest numbers, and are particularly active on the surface in the last days before the pre-laying exodus (Wooller *et al.* 1989). In addition, the pre-laying exodus creates a situation whereby copulation (which occurs prior to departure) and fertilisation (which occurs some time before return) are presumably separated by a period of two to three weeks (Fitzherbert 1985). This provides greater opportunity for sperm competition between pair and extra-pair males.

2.1.4 Aims

In the present study I assessed the applicability of multi-locus DNA fingerprinting, using probes derived from core sequences of human minisatellites, to allow individual identification and examine parentage in the short-tailed shearwater. In previous studies of lifetime reproductive success in the short-tailed shearwater, paternity has been assumed from an observed association between an egg or chick and the attendant adult bird (Bradley *et al.* 1989). Behavioural evidence for exclusive mating relationships between socially monogamous pairs is not available for this species. However, a number of characteristics of its breeding biology suggests that EPCs may be an important part of the mating system. In addition, a growing number of genetic studies have shown that the observed social monogamy of many species does not extend to an exclusive mating relationship. Paternity in the short-tailed shearwater is therefore uncertain. The results of this analysis are used to discuss the significance of and ecological and behavioural factors that affect the frequency and success of EPCs in this socially monogamous bird.

2.2 METHODS

2.2.1 Study sites

Two short-tailed shearwater colonies at Cape Direction (43°06'S, 147°25'E) on the South Arm Peninsula (Fig. 2.1) and Cape Queen Elizabeth (43°14'S, 147°25'E) on Bruny Island (Fig. 2.2), Tasmania, Australia were chosen because of their close proximity to the University of Tasmania and relative inaccessibility (thus reducing the risk of disturbance and damage to breeding birds and nesting burrows by members of the public).

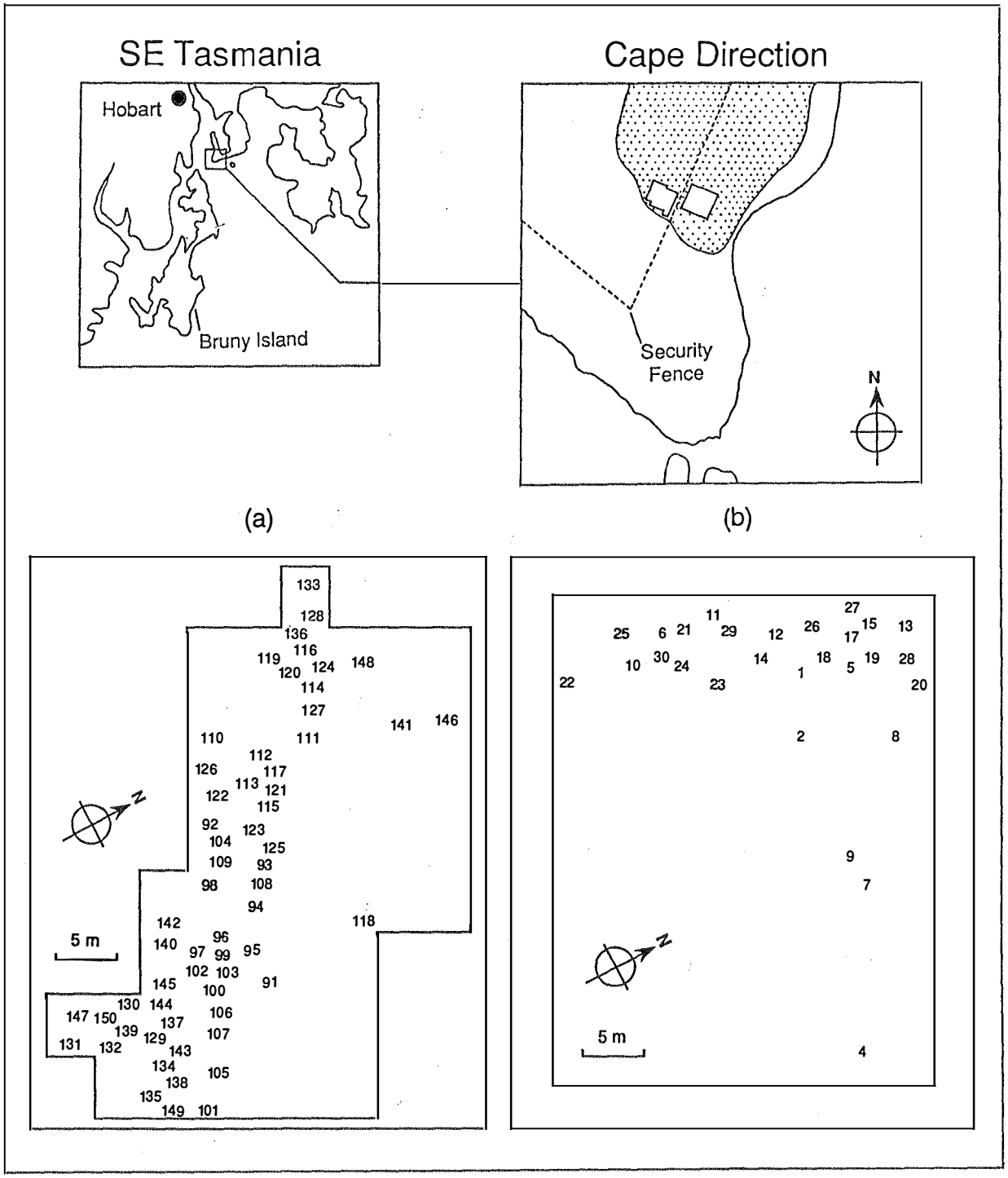

Figure 2.1. Location and orientation of short-tailed shearwater nesting burrows at Cape Direction within the (a) 1992/93 and (b) 1991 study sites.

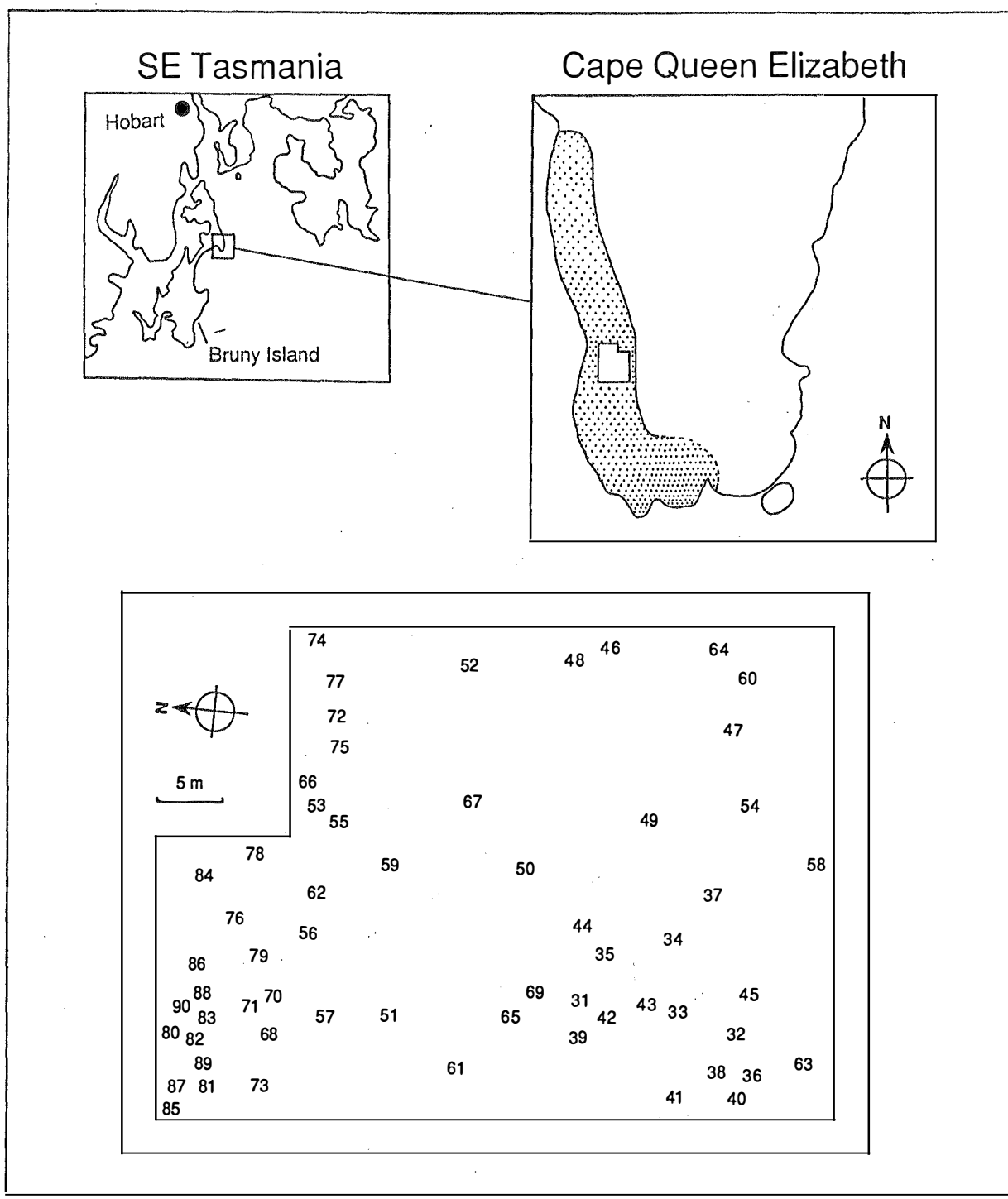

Figure 2.2. Location and orientation of short-tailed shearwater nesting burrows at Cape Queen Elizabeth in the 1992/93 study site.

The colony at Cape Direction consists of an estimated 10 000-20 000 occupied burrows (Naarding 1980) at a density of 0.65±0.11 burrows m^{-2} (Skira & Wapstra 1980) on the eastern side of a steeply sloping headland vegetated with short grass. Approximately half of the colony lies inside a defence force ammunitions compound and is enclosed by a high security barbed wire fence.

The Cape Queen Elizabeth colony comprises approximately 17 000 burrows (Skira *et al.* 1985) at a density of 0.47±0.18 burrows m^{-2} (Skira & Wapstra 1980). The colony runs the length of the western side of the headland and is vegetated almost entirely by succulents.

2.2.2 Sampling

An initial pilot study was conducted at Cape Direction during an 8-day period in March 1991. At this stage of the breeding season nestlings are approaching fledging and breeding adults return to feed them at 7-10 day intervals (Marchant & Higgins 1990). Thirty burrows, in which the nestling was accessible, were available within a 2 000 m^2 area near the southern edge of the colony, outside of the security fence (Fig. 2.1). Each burrow was marked with an individually numbered wooden stake. Adult birds, returning to the colony at dusk to feed their chicks, were trapped in their burrow using a simple one-way door set into the entrance. Traps were checked approximately 1 h after dusk and trapped adults removed from the burrow. A blood sample was taken (see below) and each bird was then marked with yellow spray paint, on one leg and the breast feathers, to allow identification if it was subsequently retrapped. Adults were released approximately 5 min after removal from the burrow. Nestlings were sampled at the end of the 8 day period and were replaced into the burrow within 5 min.

A second, more comprehensive, study was made at both Cape Direction and Cape Queen Elizabeth during the 1992/93 breeding season. Adults, incubating eggs in early December, were removed from burrows during the day and a blood sample taken (see below). The first adult captured from each burrow was marked with yellow spray paint, on one leg and the breast feathers, to distinguish it from its mate, and was returned to the burrow within 5 min. Incubating adults change shifts every 10-16 days (Serventy 1967a). By returning to the burrow at 8-9 day intervals (a maximum of 2 times) it was possible to capture the second

adult. Nestlings (when present) were sampled in late January and early February 1993 when they were less than 20 days old and were replaced into the burrow within 5 min.

At both colonies 60 burrows, in which the nesting chamber was accessible, were available within areas of 940 m^2 (Cape Direction, Fig. 2.1) and 1600 m^2 (Cape Queen Elizabeth, Fig. 2.2). Each burrow was marked with an individually numbered metal stake. At Cape Direction the 60 burrows were located inside the security fence (to reduce the possibility of disturbance) and did not include any of the burrows from the 1991 survey.

Blood samples were collected from adults and nestlings using an identical 2-person operation. Each bird was firmly restrained on its back with one wing extended. Ethanol was applied to the elbow region to dampen the feathers, allowing the brachial vein to be exposed, to disinfect the area and to increase blood flow. A 21 gauge needle was used to puncture the brachial vein at a point where it crosses the elbow at the distal end of the humerus. Approximately 200 µl of blood was collected using a capillary tube and immediately suspended in 1 ml of absolute ethanol. A tissue was held firmly over the wound until bleeding ceased. Blood samples were transported to the laboratory, on ice, on the day of collection, and subsequently stored at 4°C until required for DNA extraction.

Individuals and blood samples were identified by a code in which the first number refers to the burrow in which they were captured, the letter identifies them as either an adult (A) or nestling (C) and the second number (for adults only) refers to whether it was the first or second adult captured. In the 1991 survey burrows were numbered successively from 1 to 30. In the 1992/93 season burrows at Cape Queen Elizabeth and Cape Direction were numbered successively from 31 to 90 and 91 to 150, respectively.

2.2.3 DNA profiling methods

Restriction enzyme/multilocus DNA probe selection

The quality and information content of DNA profiles is, to a large extent, determined by the combination of restriction enzyme and multilocus probe used. Prior to the analysis of all samples, DNA from a group of 5 unrelated individuals was used to screen four 4-base recognition restriction enzymes (*Alu* I, *Hae* III, *Hinf* I, *Mbo* I) and two human

minisatellite derived RNA probes (pSPT 19.6 from 33.6, pSPT 18.15 from 33.15) to select an enzyme/probe combination that produced the most informative DNA profiles. Subsequently, all individuals were screened using the same single enzyme/probe combination.

DNA extraction

A 150-200 µl aliquot of the blood/ethanol mixture was centrifuged briefly and the ethanol supernatant poured off. The blood pellet (representing 20-30 µl of whole blood) was washed in 100 µl of SET (150 mM NaCl, 50 mM Tris.HCl, 1 mM EDTA pH 8.0), centrifuged briefly again and the supernatant poured off. The blood pellet was then resuspended in 650 µl SET. Blood cells were lysed and proteins digested by overnight incubation at 55°C with 15 µl of 10 mg ml^{-1} Proteinase K and 7.5 µl of 25% SDS (sodium dodecyl sulfate, w:v). Proteins and cellular debris were removed by 2 phenol (equilibrated with 1 M Tris pH 8.0), 1 phenol:chloroform:isoamyl alcohol (IAA) (25:24:1, v:v) and 1 chloroform:IAA (24:1) extractions. For each extraction the DNA solution was mixed gently with 0.5 ml of the organic solvent for 30 min on a rotary mixer, centrifuged at 8 000 G for 7 min and the upper, aqueous phase transferred to a fresh 1.5 ml centrifuge tube using a wide bore pipette tip (this reduces damage to the DNA which can become sheared by repetitive passage through narrow apertures). DNA was precipitated by addition of 950 µl cold (-20°C) absolute ethanol, mixed vigorously and stored at -20°C for 30 min. The DNA precipitate was centrifuged at 8 000 G for 7 min and the supernatant poured off. Pelleted DNA was washed with 750 µl of cold (–20°C) 75% ethanol, recentrifuged briefly and the supernatant poured off. The DNA pellet was air dried at 37°C for 15 min, resuspended in 30-100 µl of TE (10 mM Tris.HCl, 1 mM EDTA pH 8.0) by overnight incubation at 55°C, and subsequently stored at 4°C.

DNA restriction

Resuspended DNA (15 µl) was digested at 37°C for a minimum of 12 h with 10 Units (1 µl) of either *Alu* I, *Hae* III, *Hinf* I, *Mbo* I (Northumbria Biologicals Limited, U.K.) in the presence of 4 mM spermidine (2 µl of a 40 mM stock solution) and 2 µl of the appropriate 10x reaction buffer. The quality of the restricted DNA was assessed by a minigel assay in which 2 µl aliquots of the restriction reaction were mixed with 2 µl of 2x Bromophenol Blue (BPB) loading buffer (4% Ficoll 400 w:v, 0.05% bromophenol blue w:v, 0.05% xylene cyanol w:v, 40 mM EDTA pH 8.0) and electrophoresed through a 0.8% LE agarose minigel

in TBE buffer (89 mM Tris.HCl, 89 mM boric acid, 2 mM EDTA pH8.0) containing 0.05 mg ml^{-1} ethidium bromide for 40 min at 100 V. DNA fragments were visualised under UV light (354 nm) and incomplete digests were identified by the presence of an excess of high molecular weight fragments. A further 10 U of restriction enzyme were added to any partially digested samples, incubation continued and reassayed later by minigel.

The concentration of restricted DNA was determined with a Hoefer TKO-100 DNA fluorimeter. A 2 µl aliquot of each restriction digest was suspended in 2 ml of 1x TNE buffer (10 mM Tris.HCl, 100 mM NaCl, 1 mM EDTA pH7.4) containing 0.1 µg ml^{-1} Hoechst 33258 dye in a disposable plastic cuvette. The cuvette was inverted several times to disperse the DNA evenly before insertion into the fluorimeter. The dye binds DNA and emits light at 458 nm when exposed to UV light at 365 nm. The intensity of the emitted light is measured by a photo-sensitive cell and is linearly related to the concentration of DNA. The machine was calibrated relative to a reference sample of calf thymus DNA allowing an absolute estimation of the DNA concentration for each restricted sample. All restrictions were then diluted to a concentration of 150 µg ml^{-1} of DNA with 2x BPB loading buffer.

Electrophoresis

Cut genomic DNA (3 µg) was denatured at 65°C for 10 min and then placed on ice for 2 min before loading into the wells of a 20x24 cm horizontal, 1% LE agarose gel in TAE buffer (40 mM Tris.acetate, 1 mM EDTA pH~8.5). The samples were allowed to equilibrate with the running buffer for 10 min and then electrophoresed for 45-48 h at 40 V until fragments smaller than 2 kb had migrated off the end of the gel.

Blotting

The DNA was depurinated, denatured and neutralised *in situ* by soaking the gels for 20 min in 0.2 M HCl, followed by 35 min in 1.5 M NaCl, 0.5 M NaOH and 45 min in 3 M NaCl, 0.5 M Tris.HCl (pH 7.0). The weak acid damages some purine bases leaving single stranded nicks in the helix. This reduces the size of the larger minisatellite fragments sufficiently to permit efficient transfer from the gel. Denaturation of double stranded DNA releases single stranded fragments.

DNA was transferred onto BioRad Zeta-Probe GT nylon membrane by a modified Southern blotting technique. The gels were trimmed by removing a 4 cm strip from the well end, and placed on a wick of Whatman 3MM paper supported on a plastic plate above a reservoir of 20x SSC (3 M NaCl, 0.3 M sodium citrate). A 20x20 cm sheet of Zeta-Probe was placed onto the surface of the gel, followed by two 21.5x21.5 cm squares of Whatman 3MM paper. Each layer was pre-wetted in 2x SSC and care was taken to avoid trapping air bubbles between layers. A stack of dry absorbent paper towels 10 cm deep was layered on top of the blot and gently compressed by a 500 g weight. Blotting was allowed to proceed overnight.

DNA transfers out of the gel and onto the membrane as the buffer is drawn through the gel from the reservoir into the towel stack. On the completion of blotting, the blot was dismantled and the filter rinsed in 2x SSC for 2 min, then left to air dry on absorbent paper for 20 min. The DNA was fixed to the filter by baking *in vacuo* at 80°C for 2 h.

Probe preparation

The Jeffreys' polycore probes, 33.15 and 33.6, were available as pSPT derivatives, whereby inserts from the polycore probes had been subcloned into pSPT18 and pSPT19 transcription vectors, respectively, as described by Carter *et al.* (1989). Aliquots (20-50 µl) of the pSPT vectors were restricted with *Eco* RI (pSPT19.6) or *Hin* dIII (pSPT18.15). These restriction enzymes linearise the vectors by cutting the polylinker distal to the insert with respect to the T7 promoter. The digests were assayed fluorimetrically (as described) and diluted to 0.24 µg μl^{-1} of plasmid DNA with sterile TE.

Radioactively labelled RNA probes were prepared by transcription from the linearised pSPT vectors, using the following recipe:

4 µl (1 µg) restricted pSPT; 1 µl ATP (10 mM); 1 µl GTP (10 mM); 1 µl UTP (10 mM); 4 µl 5x transcription buffer; 2 µl DTT (100 mM); 1 µl RNase inhibitor (25 U); 1 µl T7 RNA polymerase (10 U); and 5 µl ^{32}P CTP (400 Ci $mmol^{-1}$). The reaction was incubated at 38°C for 1 h and then stopped by the addition of an equal volume of Nick-stop mix. Unincorporated nucleotides were removed by spun column chromatography (Ausubel 1994) through a 1.0 ml column of Biogel-P60 (BioRad) equilibrated with TE. The transcript is the first fraction to elute from the column. Aliquots (1 µl) of the reaction before and after

separation were mixed with Ecoscint fluid and assayed by scintillation counting to determine the efficiency of incorporation.

Prehybridisation and hybridisation

Filters were prehybridised in 500 ml of 1x SSC, 1% SDS, 1x Blotto (Johnson *et al.* 1984) in a 22 x 22 cm plastic box, gently agitated at 65°C for 6-8 h. Up to $6x10^7$ cpm (counts per minute) of the RNA probe was added and hybridisation allowed to proceed overnight at 65°C, with gentle agitation.

Washing and autoradiography

Filters were washed at a stringency of 1x SSC, 0.1% SDS at 65°C with 3-4 changes of solution, each wash lasting 25 min. A final, brief rinse in 2x SSC was made to remove excess SDS prior to autoradiography. Filters were allowed to air dry slightly, and then while still damp, wrapped in clingfilm.

Autoradiographs were produced with Fuji RX film by 1-4 days exposure using two intensifying screens at -80°C or 3-12 days without screens at room temperature.

Deprobing

Once autoradiography had been completed, filters were stripped of hybridised probe by two 30 min washes in boiling 1% SDS. The deprobed filters were rinsed briefly in 2x SSC and allowed to air dry.

Scoring

Autoradiographs were scored by eye. Comparison of DNA profiles were made only for individuals that were adjacent or adjacent but one on the same gel, and for bands representing fragments larger than 3-3.5 kb. A band in one individual was scored as matching that in another if they differed in migration by less than 0.5 mm and were of similar intensity. Nestlings were always run between their putative parents and so comparisons between individuals included: pairs of unrelated adults from different burrows, pairs composed of a nestling and an adult from another burrow, and either complete families (putative parent-

nestling-putative parent), where samples from both putative parents were available, or incomplete families (putative parent-nestling), where a sample from only one putative parent had been obtained.

For all inter-individual comparisons the proportion of fragments shared or band-sharing coefficient (D),

$$D = 2N_{AB}/[N_A + N_B]$$ (Wetton *et al.* 1987, Lynch 1988)

was calculated, where N_A and N_B are the number of fragments in individual A and B, and N_{AB} is the number of fragments that they share. For comparisons involving a nestlings and both putative parents the number of fragments unique to the nestling, termed novel fragments, was also noted.

2.2.4 Assignment of parentage

Clutch size in short-tailed shearwaters is restricted to one, making a segregation analysis of minisatellite fragments impossible. It was therefore assumed that non-independence (due to allelism, linkage or linkage disequilibrium) is negligible. Without detailed evidence to support this assumption, examination of parentage was based on the following, empirical criteria, and is essentially as described by Westneat (1990).

Novel fragments

Novel fragments in a nestlings DNA profile were used to identify families in which at least one of the putative parents could be excluded as a true parent. Novel fragments can be explained either by mutation or if the nestling is not the progeny of one or both putative parents.

The high rate of mutation at minisatellite loci is expected to produce novel fragments in some offspring when compared with their true genetic parents. Depending on the mutation rate and the number of fragments scored, mutations generally can only account for a low frequency of novel fragments. Nestlings with no novel fragments, or no more than could be explained by mutation, were assumed to be descended from both putative parents.

A higher frequency of novel fragments is expected if at least one of the putative parents is not

related to the nestling. The number of novel fragments will depend on the number of fragments inherited from each true parent (approximately 50% each) and the level of band-sharing between unrelated individuals. Nestlings with many novel fragments were assumed to be descended from at least one adult other than the putative parents.

Band -sharing coefficient

The band-sharing coefficient was used to identify the excluded (i.e. unrelated) parent in families of nestlings with many novel fragments because, providing that relatives are not involved, any adult that is not an actual parent should have a band-sharing coefficient with the nestling similar to that for unrelated individuals. All band-sharing summary statistics were calculated using angular transformed data (Sokal & Rohlf 1981; Hunter *et al.* 1992), which is appropriate for proportions, such as band-sharing data, which form a binomial, rather than a normal, distribution.

Observed distributions of band-sharing values between non-relatives and between nestlings and true parents were constructed. Band-sharing values for unrelated individuals were calculated for pairs of randomly chosen adults (1991 samples) or nestling and an adult from another burrow (1992/93 samples), thus representing an unrelated adult-nestling comparison. The parent-nestling distributions were obtained from families in which the observed number of novel fragments did not exclude either putative parent. Band-sharing values between excluded offspring (identified by many novel fragments) and each putative parent were compared with these empirical distributions to identify the unrelated parent.

Band-sharing values within the distribution for non-relatives indicated that the putative parent was not an actual parent. Band-sharing values within the distribution for parent-nestling were considered sufficient to assign parentage.

Probability analysis

Due to the small sample size and relatively large number of incomplete families in the Cape Direction 1991 sample an additional test of relationships between nestlings and their putative parents was made. This test compared the number of fragments unique to each individual and shared among them with those expected under models of either no relationship (i.e. the adult is not the parent) or first degree relationship (i.e. the adult is the parent) using a modification

of the model described by Brookfield (1989). This technique calculates the probability of obtaining the observed pattern of DNA profiles, for comparisons between nestling and putative parent, under the two models. The most likely model has the greatest probability and is considered significant if it is twenty times, or more, greater than the probability of the alternative model. The model assumes that different bands in the DNA profile represent unlinked loci and are non-allelic, that no co-migration occurs, all bands occur equally frequently in the population and that the mutation rate is constant for all loci. However, as these assumptions could not be tested in this study, the results from the probability analysis must not be treated as absolute.

2.2.5 Behavioural observations

To obtain behavioural information to assist in interpreting the genetic data, casual observations of birds were made on the 21 and 29 October 1993, at the Cape Deslacs colony (42°59'S, 147°34'E), which corresponded to the last 10-14 days before the pre-laying exodus. Cape Deslacs was chosen because of the presence of an observation deck from which it was relatively easy to observe a small area of the colony from above, and is less than 30 km from the Cape Direction and Cape Queen Elizabeth colonies. Observations were made by two observers either from the deck or from two other areas approximately 100 m away. In each area a small number of focal burrows, less than 5 m from the observer, were watched, birds and burrows were not individually marked. On the first visit observations were made from 2000 to 2200 h (EDST, eastern daylight savings time) and on the second visit from 2000 to 2300 h and 0400 to 0700 h (EDST). During observation periods the number and sequence of birds entering and leaving a burrow, the response of birds in or at the entrance of burrows to the approach of others, and the pattern of copulatory behaviour was recorded.

2.3 RESULTS

2.3.1 Enzyme/probe selection

Probing of DNA from a group of 5 unrelated individuals, restricted with one of four 4-base restriction enzymes (*Alu* I, *Hae* III, *Hinf* I, *Mbo* I), with two human minisatellite derived RNA probes (pSPT 19.6, pSPT 18.15) revealed considerable differences in the quality of the DNA profiles obtained. The enzyme *Hae* III and minisatellite probe 19.6 were selected for the survey of all families as this combination produced DNA profiles of the highest quality

and resolution, with the best compromise between maximising the number of resolvable fragments and minimising the proportion of shared fragments (Fig. 2.3).

2.3.2 Variability of DNA profiles

DNA profiles obtained from 30 randomly chosen adult birds, sampled from the Cape Direction colony in 1991, exhibited a high level of polymorphism, suggesting that the restriction-fragment patterns revealed by pSPT 19.6 are individual specific. The mean band-sharing between unrelated birds (x) was determined from the mean of band-sharing values (D) for pairwise comparisons between adjacent individuals on the same gel. The mean probability of band-sharing for short-tailed shearwaters was estimated to be x=0.298 (n=30). Assuming independent segregation of fragments, all alleles to be of equal frequency and comigrating bands between 2 individuals always to be identical alleles, then the mean allele frequency at minisatellite loci can be estimated as:

$q=1-(1-x)^{0.5}$ (Jeffreys *et al.* 1985b).

The mean allele frequency for shearwaters was 0.162, indicating multiple alleles (n=6) at minisatellite loci in shearwater DNA, and a high level of heterozygosity h=0.91 where:

$h=2(1-q)/(2-q)$ (Georges *et al.* 1988).

The probability that two individuals have identical DNA profiles can be estimated as:

$P=(1-2x+2x^2)^{n/x}$ (Jeffreys *et al.* 1985b).

For short-tailed shearwaters, $P=9.83\times10^{-16}$, confirming that shearwater minisatellites are sufficiently variable to be individual specific.

The number of scorable fragments per individual was high enough to allow comparisons between individuals (Table 2.1). The difference between the two groups of individuals for the Cape Direction 1991 sample was due to variation in the quality of hybridisation patterns obtained.

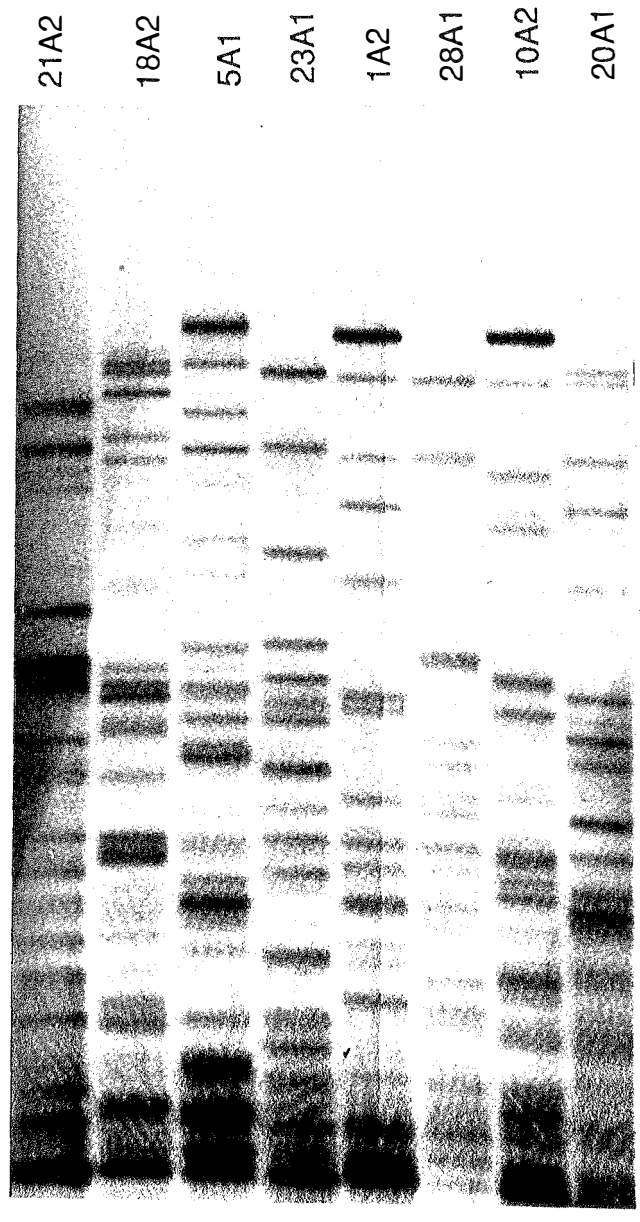

Figure 2.3. DNA fingerprints revealed by the human multilocus minisatellite probe pSPT 19.6 for eight unrelated short-tailed shearwaters, *P. tenuirostris*. Profiles were sufficiently variable to allow individual-specific identification.

Table 2.1. Summary statistics for the number of bands scored in DNA profiles for comparisons between nestlings and putative parents, unrelated adults and nestlings and unrelated adults of short-tailed shearwaters at two breeding colonies. CD91: Cape Direction 1991, CD93: Cape Direction 1992/93, CQE93: Cape Queen Elizabeth 1992/93.

	CD91	CD91	CD93	CD93	CQE93	CQE93
	Nestling - Putative Parent	Unrelated Adults	Nestling - Putative Parent	Nestling - Unrelated Adult	Nestling - Putative Parent	Nestling - Unrelated Adult
mean	15.7	18.8	16.4	16.4	19.3	19.4
n	54	30	134	112	119	92
SE	0.51	0.33	0.21	0.26	0.30	0.34
min.	6	14	11	11	9	9
max.	22	23	23	24	28	27

2.3.3 Sex-specific fragments

In the process of scoring the DNA profiles obtained for the large number of complete families during 1993 an apparently sex-specific restriction fragment was detected (Fig. 2.4). In all 83 complete families sampled from Cape Direction and Cape Queen Elizabeth in 1993 only one of each pair of adults exhibited this particular restriction fragment. The characteristics of the fragment made it easy to identify: it was generally the largest fragment present, showed almost no detectable variation in size and hybridised very strongly to the probe pSPT 19.6. Circumstantial evidence that the fragment was female-specific came from the fact that in most cases the first adult sampled in each burrow did not possess the fragment. Male short-tailed shearwaters usually take the first incubation shift of the egg (Marchant & Higgins 1990).

To test this hypothesis blood samples were collected from 10 breeding pairs of shearwaters on 30 November and 8 December 1993 at the Cape Direction 1991 study site. Each bird was sexed by cloacal examination (Serventy 1956), a technique which can identify females by their dilated, reddened and sometimes bleeding cloaca immediately after egg-laying. I was previously unaware of this technique, which could have been used to sex most birds in the 1992/93 study at Cape Direction and Cape Queen Elizabeth. Serventy's (1956) description of the female cloaca after egg laying differed quite considerably from those examined at Cape Direction in November and December 1993. Although blood samples were collected in the

days immediately after the egg-laying period (and therefore most females would have laid in the 14 days previously) no damage or blood-staining to the cloacal mucosa was evident on any of the birds examined. The only apparent difference between members of a pair was the degree of dilation of the cloaca, and in three out of the ten pairs this was ambiguous with both birds exhibiting dilated cloacas to some degree.

DNA profiles were generated for 8 pairs of the sampled birds using identical techniques to those described in Section 2.2.3. A bacteriophage lambda DNA (New England Biolabs, Ca.) digested with *Hin* dIII was used as a molecular weight size standard during electrophoresis, and lambda fragments were identified by ethidium bromide/UV light visualisation. Seven of the pairs contained birds that had been unambiguously sexed by cloacal dilation. In the eighth pair, the second bird sampled had an obviously dilated cloaca and was therefore a female. The first bird sampled exhibited a slightly dilated cloaca and was not identified as male or female. Only a single individual in each of the 8 pairs of birds displayed the large (approximately 8.5 kb) restriction fragment which hybridised intensely with the pSPT 19.6 probe and in all cases this was the female member of the pair identified by cloacal examination. For the eighth pair, the second bird possessed the fragment and the first did not, confirming the female and male, respectively, status of these individuals. Subsequently, the presence or absence of the female-specific restriction fragment was used to identify the sex of all birds (adults and nestlings) included in both the 1991 and 1992/93 studies (Fig. 2.4).

2.3.4 Assignment of parentage-Cape Direction 1991

From the initial 30 nesting burrows, blood samples were collected from 22 nestlings and 32 adults, comprising 10 complete families (both putative parents and the nestling) and 12 incomplete families (one putative parent and the nestling). No adults were trapped in 7 of the remaining 8 burrows and one nestling disappeared from its burrow during the study period.

In the initial analysis of the 1991 data, knowledge of the female-specific restriction fragment was not available. Therefore in examining parentage of each nestling maternity was assumed to be correct (Austin *et al.* 1993). However this assumption was shown to be incorrect when the sex of each putative parent was identified using the presence or absence of the sex-specific restriction fragment.

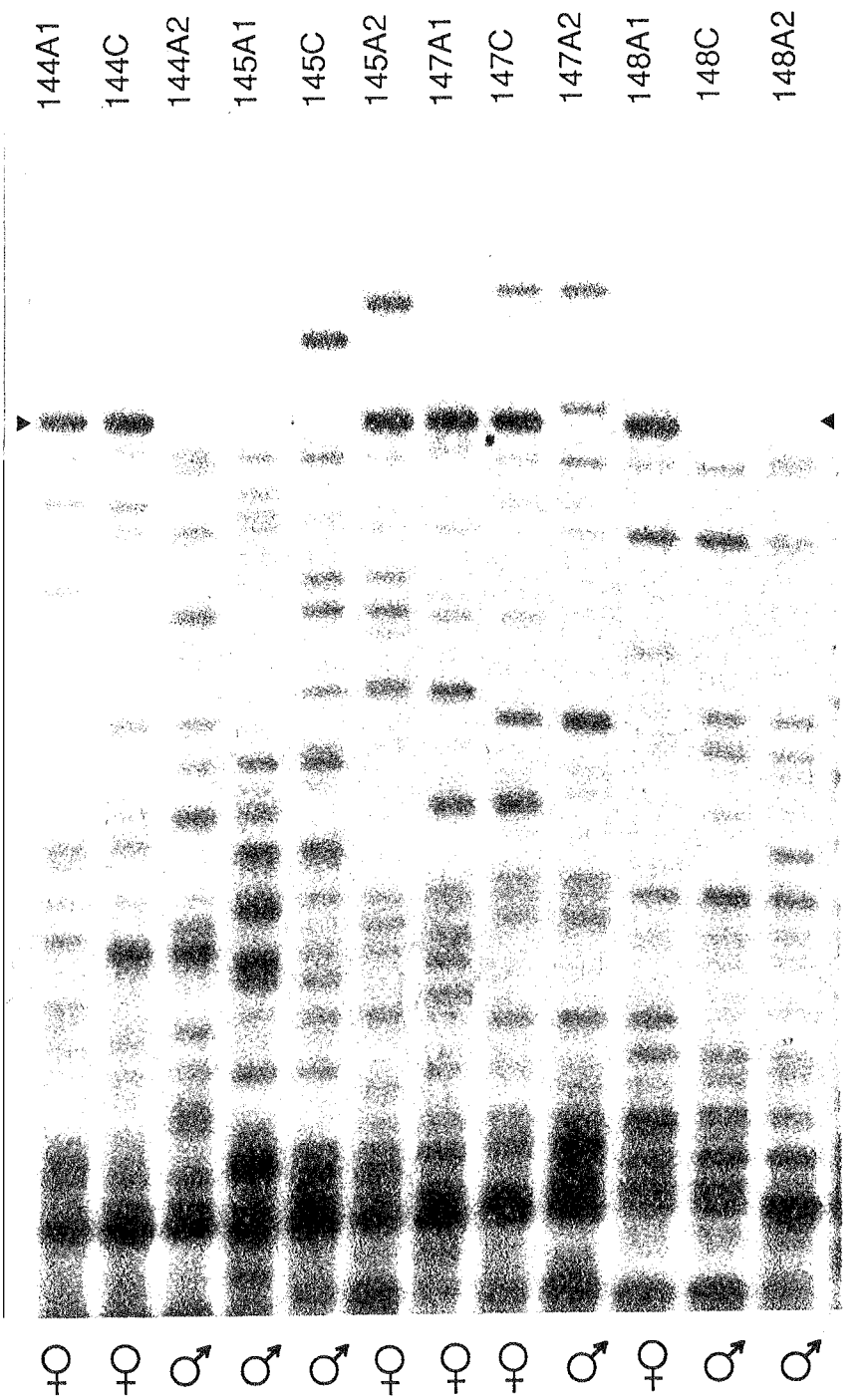

Figure 2.4. DNA fingerprints for four short-tailed shearwater families. Arrows show the female-specific minisatellite DNA fragment used to identify the sex of adults and nestlings.

Of the 10 nestlings for which both attendant adults were sampled, three (5C, 15C, 21C) had between four and seven fragments that could not be assigned to either of the putative parents (Table 2.2, see Fig. 2.5 for example), suggesting that these nestlings were not descended from at least one of the putative parents. The proportion of fragments shared, in pairwise comparisons, between 30 adults was used to provide a measure of the background band-sharing between unrelated birds. These comparisons revealed a mean (±SD) and range of band-sharing coefficients of 0.298 (±0.011) and 0.130-0.450, respectively.

The frequency distribution of band-sharing coefficients between unrelated adults and between nestlings with no novel bands and their parents is shown in Fig. 2.6a. The distribution is markedly bimodal with a distinction in similarity coefficients for unrelated individuals and parent-offspring comparisons. The mean (±SD) and range of band-sharing coefficients between the seven nestlings with no novel bands and their parents was 0.622 (±0.0082) and 0.455-0.800, respectively. Examination of band-sharing coefficients between the three excluded nestlings, in burrows 5, 15 and 21, and each putative parent (Table 2.2, Fig. 2.6b) revealed very low values (less than 0.2) with the female attendant in each pair. In addition, the one adult sampled in burrow 28 (a male bird) showed a low band-sharing coefficient with the nestling (Fig. 2.6c). As these band-sharing values fall within the range for unrelated birds, the female adult birds in burrows 5, 15, 21 cannot be a parent of the nestling in their respective burrows. Based on band-sharing values alone, the attendant male bird in burrow 28 is unlikely to be related to the nestling but without a sample from the female attendant this cannot be confirmed (see discussion for burrow 101 at Cape Direction in 1992/93; Section 2.3.5). Parentage of the remaining 11 nestlings, for which only one putative parent was sampled, was confirmed. The male attendant adult in burrow 5 also has a low band-sharing coefficient with the nestling, falling in the zone between unrelated individuals and parent-offspring values and below the 99% confidence range for parent-offspring values. Positive assignment or exclusion of parentage for this bird is therefore not possible.

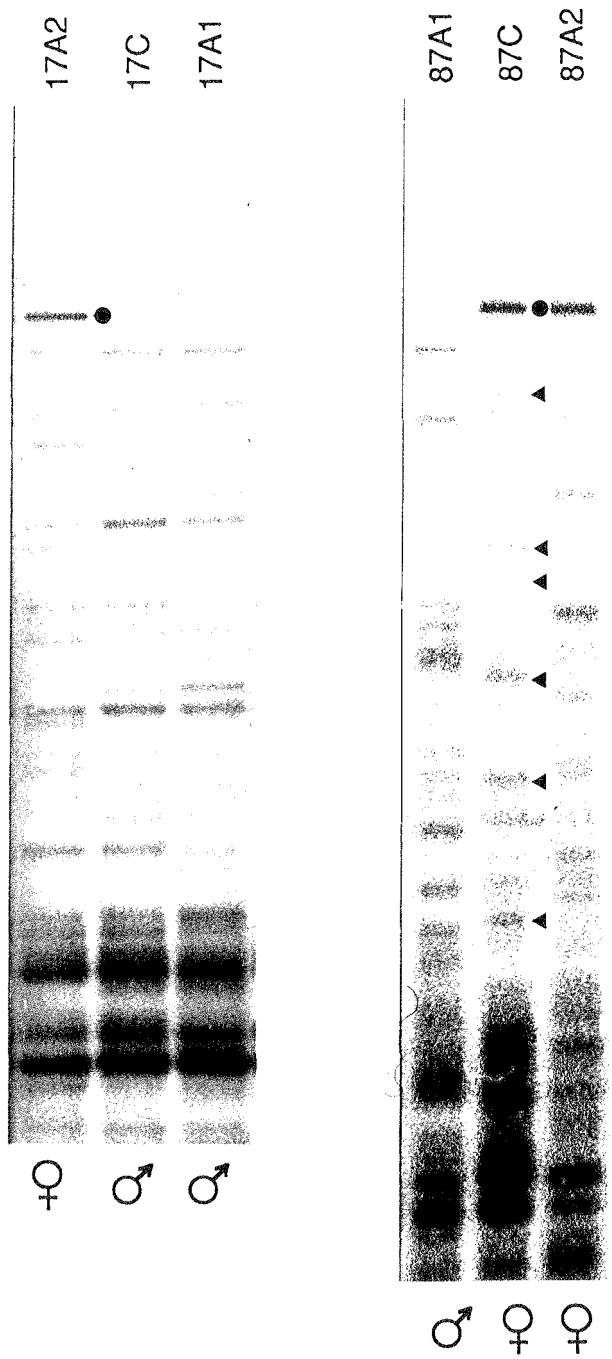

Figure 2.5. DNA fingerprints of two *P. tenuirostris* families (2 adults and nestling) obtained with the multilocus human minisatellite probe pSPT 19.6. Every fragment in the DNA profile of the nestling in burrow 17 (17C) was present in the fingerprints of one or both adults (17A1, 17A2). Both adults are therefore a genetic parent of this nestling. In burrow 87 the nestling (87C) had several novel DNA fragments which were not found in either adult (indicated by solid arrowheads). This nestling is related to the female adult (87A2) but not the male attendant (87A1). The female-specific DNA fragment, used to identify the sex of the individuals concerned, is indicated by the solid circles.

Table 2.2. Sex of nestlings and the number of bands shared, the band sharing coefficient (D) and the number of novel bands for comparisons between DNA profiles of nestlings and their putative parent(s) (female and male) for 22 short-tailed shearwater burrows at Cape Direction in 1991.

		# bands scored			# bands shared		D		
Burrow	Sex	Nestling	Female	Male	Female	Male	Female	Male	Novel bands
1	F	18	17	18	10	12	0.571	0.667	0
5	F	13	17	12	2	5	0.133	0.400	6
9	F	11	10	13	8	7	0.762	0.583	0
10	M	11	11	15	5	9	0.455	0.692	0
12	F	18	18	17	9	13	0.500	0.743	0
15	M	18	14	17	3	14	0.188	0.800	4
17	M	21	15	18	12	13	0.667	0.667	0
18	M	17	18	15	10	10	0.571	0.625	0
21	F	18	13	16	3	10	0.194	0.588	7
27	M	17	15	19	9	11	0.563	0.611	0
2	F	20	20		10		0.500		
4	M	6		6		4		0.667	
11	M	17		14		8		0.516	
13	M	17		21		14		0.737	
14	F	18		21		14		0.718	
19	F	13		13		9		0.692	
20	F	10		11		8		0.762	
22	M	17	21		14		0.737		
23	F	21		17		14		0.737	
24	M	18		15		10		0.606	
25	M	17	22		13		0.667		
28	F	15		9		4		0.333	

Probability analysis of DNA profiles between nestlings and putative parents produce similar results (Table 2.3). In burrows for which only one adult was sampled, the male attendant in burrow 28 was significantly more likely not to be the parent of the nestling, the attendant male in burrow 11 and female in burrow 2 are more likely to be a parent, but not significantly so, and all other adults (n=9) were significantly more likely to be a parent. The probability of obtaining the observed pattern of band-sharing between individuals was highest under the model of both adults being parents for 7 of 10 nestlings for which both putative parents were sampled. Of the remaining three, a significantly more likely model for two nestlings (15C and 21C) was that one of the attendant adults was not a parent. In burrow 5 the most likely model is that neither adult is a parent; however, the probability is not significantly greater than that for the hypothesis that attendant male is a parent and attendant female is not.

Table 2.3. Probabilities associated with four models of relatedness for comparisons of DNA fingerprints between short-tailed shearwater nestlings and their putative parents. Model 1=the adult(s) is/are the parent(s); Model 2=adult 1 is a parent, adult 2 is not; Model 3=adult 2 is a parent, adult 1 is not; Model 4= the adult(s) is/are not the parent(s).

Nestling and adults	Model 1	Model 2	Model 3	Model 4	Most likely
18C/18A1/18A2	3.64×10^{-9}	1.57×10^{-14}	1.65×10^{-13}	1.43×10^{-16}	1[A]
15C/15A1/15A2	6.97×10^{-19}	5.61×10^{-11}	3.85×10^{-20}	1.71×10^{-16}	2[A]
21C/21A1/21A2	1.97×10^{-26}	1.42×10^{-19}	4.10×10^{-14}	4.87×10^{-16}	3[A]
5C/5A1/5A2	2.10×10^{-26}	2.96×10^{-16}	2.33×10^{-12}	3.46×10^{-11}	4[B]
14C/14A1	1.37×10^{-3}	-	-	5.68×10^{-8}	1[A]
28C/28A1	5.35×10^{-5}	-	-	1.74×10^{-3}	4[A]
2C/2A1	5.96×10^{-4}	-	-	2.74×10^{-4}	1
11C/11A1	1.13×10^{-3}	-	-	1.99×10^{-4}	1

[A] The most likely model is significantly more so than the other models being tested.

[B] Model 4 is only significantly more likely than models 1 and 2, but not model 3.

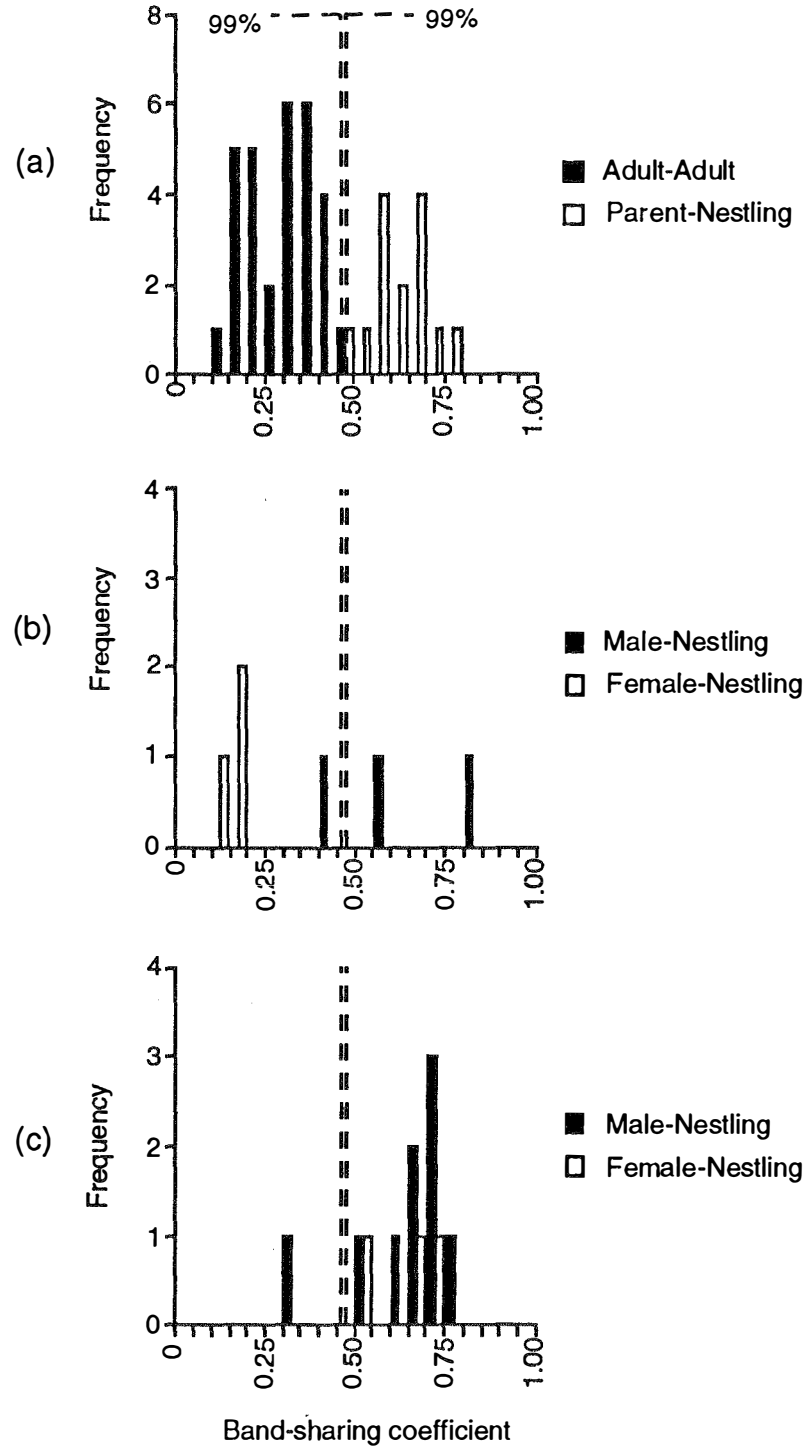

Fig 2.6. Frequency distribution of band-sharing coefficients between: (a) unrelated adults and between parents and nestlings with no novel fragments; (b) nestlings with many novel fragments and both putative parents; and (c) nestlings and the putative parent for burrows in which only one adult was sampled.Dashed lined represent one-tailed 99% confidence limits calculated, assuming a normal distribution, for the distributions in (a).

2.3.5 Assignment of parentage - Cape Direction and Cape Queen Elizabeth 1992/3

Of the 60 marked burrows at Cape Direction (CD), blood samples from 44 complete families and one incomplete family were collected. Thirteen of the remaining 15 burrows contained no egg or chick, and in 2 burrows the egg had been abandoned (Appendix 2a; Austin & Edmunds 1994). At Cape Queen Elizabeth (CQE) blood samples from 39 complete families (both putative parents and the nestling) and one incomplete family (one putative parent and the nestling) were obtained from the 60 marked burrows. In the remaining 20 burrows the egg had either been abandoned (n=10), the burrow contained neither egg nor chick (n=9, i.e. had presumably suffered from predation) or, in one case, had been inhabited by a fairy penguin (*Eudyptula minor*) (Appendix 2a; Austin & Edmunds 1994). In total, 83 complete and 2 incomplete families were available for examination of parentage. The two colonies were treated independently during the analysis of parentage to control for any intercolony differences in minisatellite variation and band-sharing.

Many of the nestlings (19 of 44 at CD, Table 2.4; 18 of 39 at CQE, Table 2.5) exhibited novel fragments not present in either of the putative parents. The distribution of the number of novel fragments per nestling is not uniform (Fig. 2.7). The majority of nestlings present 0, 1 or 2 novel fragments, a small number have between 4 and 8 fragments that cannot be assigned to either putative parent. Assuming that up to two novel fragments are the result of mutation, the mean number of novel fragments per nestling (m) was estimated to be 0.40 (CD) and 0.41 (CQE). The mutation rate (μ=m/N) was calculated from the mean number of novel fragments and the average number of fragments scored (N) to be 0.024 (CD) and 0.021 (CQE) per fragment per meiotic event. These values are within the order of magnitude range found in other species of birds (Burke & Bruford 1987; Burke *et al.* 1989; Rabenhold *et al.* 1990; Westneat 1990; Lifjeld *et al.* 1991; Gelter & Tegelström 1992; Decker *et al.* 1993; Pinxten *et al.* 1993).The observed number of offspring having 0, 1 or 2 novel fragments did not differ significantly from the Poison distribution (CD χ^2=1.74, 0.10<P<0.25; CQE χ^2=1.34, 0.10<P<0.25). From the Poisson distribution, the probability of nestlings having four or more novel fragments through mutation alone is less than 0.001. There were four nestlings at Cape Direction in burrows 98, 122, 127 and 140, and five nestlings at Cape Queen Elizabeth in burrows 39, 42, 75, 86 and 87 that had between 4 and 8 fragments that could not be assigned to either of the putative parents. This number of novel fragments can

Table 2.4. Sex of nestlings and the number of bands shared, the band sharing coefficient (D) and the number of novel bands for comparisons between DNA profiles of nestlings and their putative parent(s) (female and male) for 45 short-tailed shearwater burrows at Cape Direction in 1992/93.

		# bands scored			# bands shared		D		
Burrow	Sex	Nestling	Female	Male	Female	Male	Female	Male	Novel bands
91	F	15	15	15	10	9	0.667	0.600	0
92	F	20	17	19	8	13	0.432	0.667	1
93	M	19	19	22	11	12	0.579	0.585	2
94	M	21	16	19	12	14	0.649	0.700	0
96	M	23	22	20	14	13	0.622	0.605	1
97	M	17	14	16	10	9	0.645	0.545	0
98	M	21	21	21	13	6	0.619	0.286	6
99	F	17	15	21	8	13	0.500	0.684	0
100	M	16	17	19	10	12	0.606	0.686	0
101	M	12	15	18	10	4	0.741	0.267	1
103	M	18	15	16	11	11	0.667	0.647	0
105	F	15	14	18	8	11	0.552	0.667	0
109	F	13	16	16	8	8	0.552	0.552	1
110	M	14	14	16	9	10	0.643	0.667	0
112	M	15	13	13	10	10	0.714	0.714	0
113	F	14	16	14	8	8	0.533	0.571	0
114	M	18	13	16	9	12	0.581	0.706	0
115	F	18	17	17	12	11	0.686	0.629	0
116	F	18	18	13	10	11	0.556	0.710	0
117	F	19	16	17	9	11	0.514	0.611	1
118	M	20	17	20	11	13	0.595	0.650	0
120	M	15	17	13	9	10	0.563	0.714	0
121	F	15	16	16	10	8	0.645	0.516	0
122	M	20	17	21	13	7	0.703	0.341	6
123	M	17	16	19	12	14	0.727	0.778	0
125	F	16	15	15	8	11	0.516	0.710	0
127	M	23	18	20	16	7	0.780	0.326	5
128	M	16	14	16	10	8	0.667	0.500	1
129	M	16	16	18	8	9	0.500	0.529	0
132	M	15	13	16	10	9	0.714	0.581	1
133	F	16	16	12	11	8	0.688	0.571	1
134	M	16	18	16	9	12	0.529	0.750	0
136	M	16	19	15	8	10	0.457	0.645	0
137	M	15	16	14	8	9	0.516	0.621	1
138	M	17	17	11	11	10	0.647	0.714	0
140	F	15	19	17	8	4	0.471	0.250	6
141	M	19	15	17	11	13	0.647	0.722	1
142	M	15	12	15	9	11	0.667	0.733	0
143	F	18	15	16	12	8	0.727	0.471	1
144	F	16	18	16	8	9	0.471	0.563	1
145	M	19	15	18	11	12	0.647	0.649	1
147	F	14	13	14	6	10	0.444	0.714	0
148	M	11	13	14	5	9	0.417	0.720	1
150	M	17	15	15	11	12	0.688	0.750	0
149	M	15	14		9		0.621		

Table 2.5. Sex of nestlings and the number of bands shared, the band sharing coefficient (D) and the number of novel bands for comparisons between DNA profiles of nestlings and their putative parent(s) (female and male) for 40 short-tailed shearwater burrows at Cape Queen Elizabeth in 1992/93.

		# bands scored			# bands shared		D		Novel
Burrow	Sex	Nestling	Female	Male	Female	Male	Female	Male	bands
31	M	18	19	20	9	11	0.486	0.579	0
32	M	15	14	15	8	8	0.552	0.533	0
33	M	15	17	24	9	10	0.563	0.513	0
34	M	18	22	20	9	13	0.450	0.684	1
35	M	16	18	18	11	9	0.647	0.529	0
37	M	18	16	16	10	11	0.588	0.647	1
38	F	22	21	23	12	14	0.558	0.622	0
39	M	21	18	21	10	8	0.513	0.381	4
40	M	19	21	20	10	11	0.500	0.564	1
42	F	21	18	24	10	7	0.513	0.311	7
43	F	21	20	25	12	16	0.585	0.696	0
44	F	20	21	18	12	12	0.585	0.632	0
45	F	23	23	23	15	12	0.652	0.522	0
49	M	21	17	21	14	11	0.737	0.524	0
50	F	28	28	22	20	14	0.714	0.560	0
51	M	25	23	20	14	13	0.583	0.578	1
54	F	21	22	21	10	13	0.465	0.619	1
55	F	19	20	22	8	14	0.410	0.683	0
56	F	20	20	20	13	10	0.650	0.500	1
57	M	25	21	21	13	14	0.565	0.609	0
59	F	17	19	17	9	10	0.500	0.588	1
60	M	17	21	22	10	12	0.526	0.615	0
61	F	15	17	20	9	9	0.563	0.514	0
63	F	12	20	9	9	6	0.563	0.571	1
64	M	18	19	14	16	6	0.865	0.375	0
69	M	17	18	14	10	9	0.571	0.581	1
70	F	17	14	18	10	11	0.645	0.629	1
72	F	21	18	16	14	12	0.718	0.649	0
75	F	20	22	21	11	3	0.524	0.146	8
77	F	18	19	19	9	13	0.486	0.703	0
78	F	22	20	20	11	13	0.524	0.619	1
79	F	19	18	22	12	11	0.649	0.537	0
80	M	22	18	17	16	10	0.800	0.513	0
81	M	13	11	16	10	8	0.833	0.552	0
85	M	24	18	20	13	12	0.619	0.545	2
86	M	16	18	18	10	4	0.588	0.235	5
87	F	19	20	21	12	2	0.615	0.100	6
88	F	18	25	21	9	12	0.419	0.615	0
90	M	21	18	24	13	14	0.667	0.622	1
53C	F	15	13		9		0.643		

not be explained by mutation alone suggesting that these nestlings were unrelated to at least one of the putative parents.

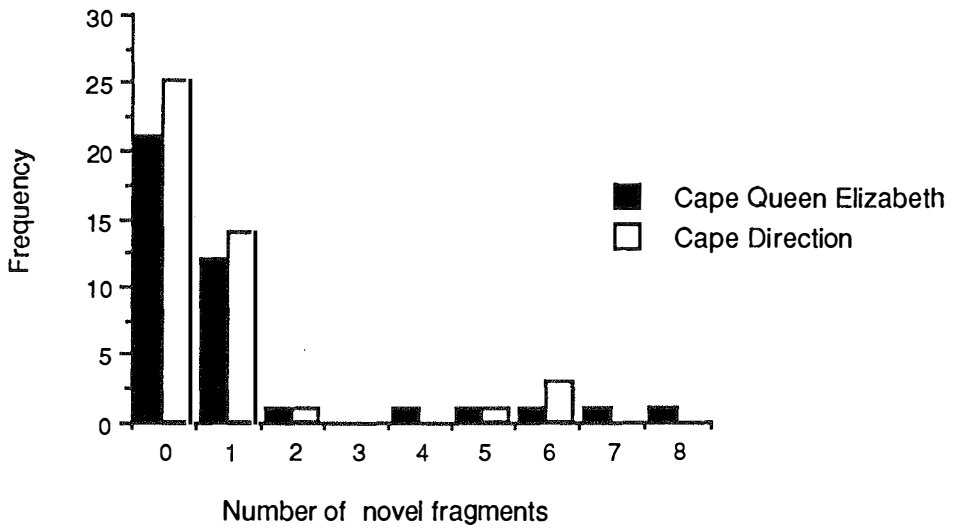

Figure 2.7. Frequency distribution of the number of novel fragments observed in comparisons of DNA profiles between nestlings and both putative parents of the short-tailed shearwater at Cape Direction and Cape Queen Elizabeth.

The band-sharing distributions for unrelated adult-nestling and parent-nestling comparisons, for both samples (Fig. 2.8a and Fig. 2.9a), are nearly distinct with only minimal overlap. The mean (±SD) and range of band-sharing coefficients between unrelated adults and nestlings was 0.229 (±0.0100) and 0.061-0.485, respectively, at Cape Direction and 0.206 (±0.0130) and 0-0.400, respectively, at Cape Queen Elizabeth. The mean (±SD) and range of band-sharing coefficients between nestlings with two or less novel bands and their parents was 0.618 (±0.0096) and 0.267-0.778, respectively, at Cape Direction and 0.591 (±0.0093) and 0.375-0.865, respectively, at Cape Queen Elizabeth. In the Cape Direction sample the band-sharing coefficient between nestling 101 and the male parent was only 0.267 despite only a single novel fragment. The proportion of fragments shared by this nestling and the female parent was high (0.741). Westneat (1990) found a similar situation in indigo buntings (*Passerina cyanea*), and suggested that the high band-sharing between the female parent and nestling may contribute to the low band-sharing with the male parent. Also, the chances of observing one case in which many of the fragments inherited from the male are smaller than the scorable size range are increased when a large number of nestlings are examined.

Examination of the band-sharing coefficient between the 9 excluded nestlings and each putative parent revealed a strong asymmetry between parents (Fig. 2.8b and Fig. 2.9b). In all cases, band-sharing proportions between the nestling and the putative male parent were less than 0.4, within the range for non-relatives and below the 98% confidence limit for the parent-nestling distribution. In contrast, band-sharing values between the nestling and putative female parent were high (>0.45) and within the range for parent-nestling comparisons. This pattern was clearer when band-sharing coefficients with each putative parent were plotted against the number of novel fragments (Fig. 2.10). Nestlings with many novel fragments shared a similar proportion of bands with the putative female parent as did nestlings with few novel fragments. In contrast, band-sharing coefficients with the putative male parents were either high, for nestlings with two or fewer novel fragments, and low, for nestlings with greater than three novel fragments. The two distributions are clearly distinct.

It was therefore concluded that the attendant males (and assumed mate of the attendant female) in burrows 98, 122, 127, 140 at Cape Direction and 39, 42, 75, 86, 87 at Cape Queen Elizabeth were not a true genetic parent of the respective nestlings. Maternity, in all cases, was confirmed. Parentage of the nestlings in burrows 53 and 149, where only one putative parent was sampled, was also confirmed. Band-sharing coefficients between the nestling and the putative female parent were high and within the range for parent-nestling comparisons.

2.3.6 Behavioural observations

Darkness and the inability to individually identify birds made observations very difficult and as a result somewhat subjective. Birds began arriving at the colony at dusk, shortly after the commencement of observations, and continued for the duration of the dusk observations, although the apparent number of arriving birds was very low by the end of the dusk observation period. The arrival of birds was not direct but instead they spent sometime circling over the colony before landing. Upon landing most birds moved straight to their (assumed) burrow and either entered or sat outside the entrance. Pairs of birds were often seen sitting together at the entrance to a burrow. As dawn approached birds began emerging from their burrows, and much more surface activity was evident. Birds began taking to the air before dawn and continued until it was light. The following observations were made during the dusk and dawn periods:

1.There was a 30 min time difference between the first and second bird entering one burrow,

the sex of both birds was unknown.

2. A bird entered a burrow already occupied by another bird. Vocalisations were heard and a bird quickly left the burrow and moved out of the observed area.

3. Copulations were witnessed on four occasions between different pairs of birds at the entrance to their burrow. The sequence of behaviour was essentially as described by Marchant and Higgins (1990). "The male squats on the crouched female, with feet flattened along female's wings; male moves head from side to side causing bills of both birds to lap constantly over each other; soft low call given. After cloacal contact, male extends wings slightly and slides off female."

4. Numerous agonistic interactions were observed. These varied from vocalisations (the "territorial " call of Marchant & Higgins 1990), to one bird of a pair at a burrow entrance chasing off another that approached too close, to fights where two birds locked bills and, with wings outstretched, pushed and pulled each other along the ground.

5. One pair of birds were seen to be particularly aggressive in chasing off any others that approached within 1-2 m of the burrow entrance.

6. At least four birds were seen entering a single burrow and then leaving and reentering over a half hour period. Subsequent inspection of the burrow revealed only one tunnel and nesting chamber (i.e. two burrows did not share the same entrance).

7. Numerous individual birds were seen moving through the observed areas and occasionally stopping at and "looking" inside the entrance of a burrow.

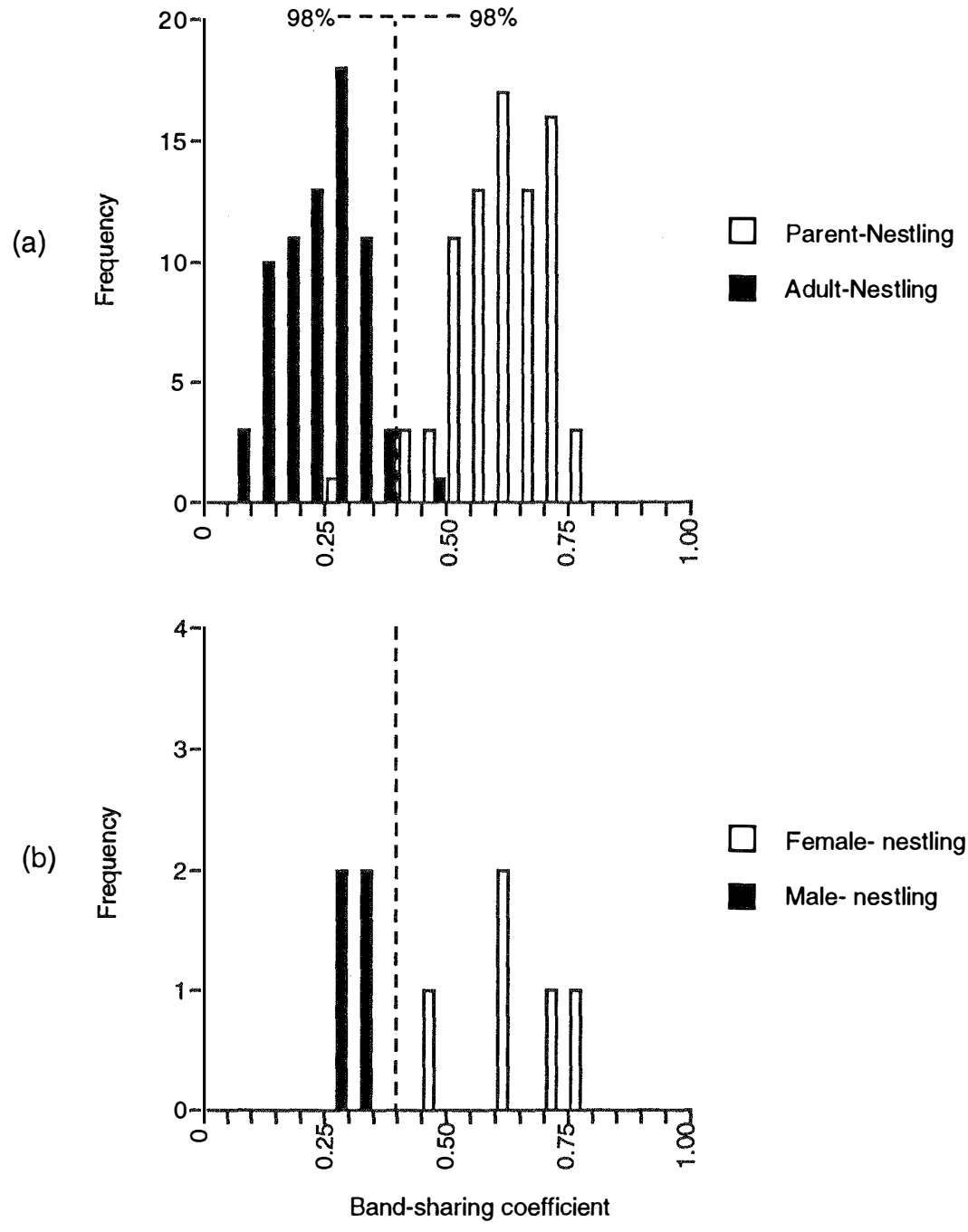

Fig 2.8. Frequency distribution of band-sharing coefficients between: (a) nestlings with less than two novel fragments and both putative parents and between nestlings and an unrelated adult; and (b) nestlings with many novel fragments and both putative parents, for short-tailed shearwaters at Cape Direction. Dashed lined represent maximally non-overlapping, one-tailed 98% confidence limits calculated, assuming a normal distribution, for the distributions in (a).

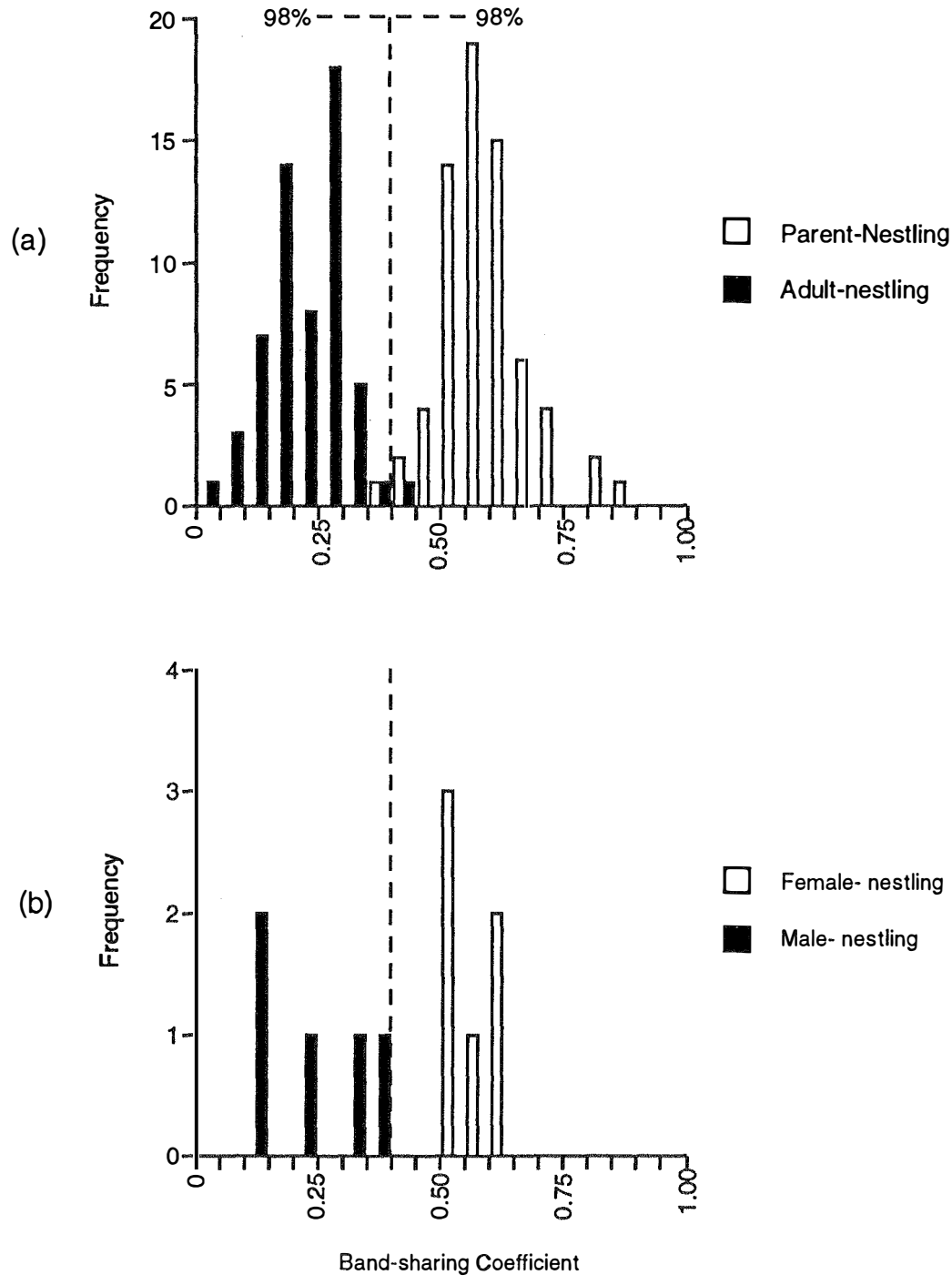

Fig 2.9. Frequency distribution of band-sharing coefficients between: (a) nestlings with less than two novel fragments and both putative parents and between nestlings and an unrelated adult; and (b) nestlings with many novel fragments and both putative parents, for short-tailed shearwaters at Cape Queen Elizabeth. Dashed lined represent maximally non-overlapping, one-tailed 98% confidence limits calculated, assuming a normal distribution, for the distributions in (a).

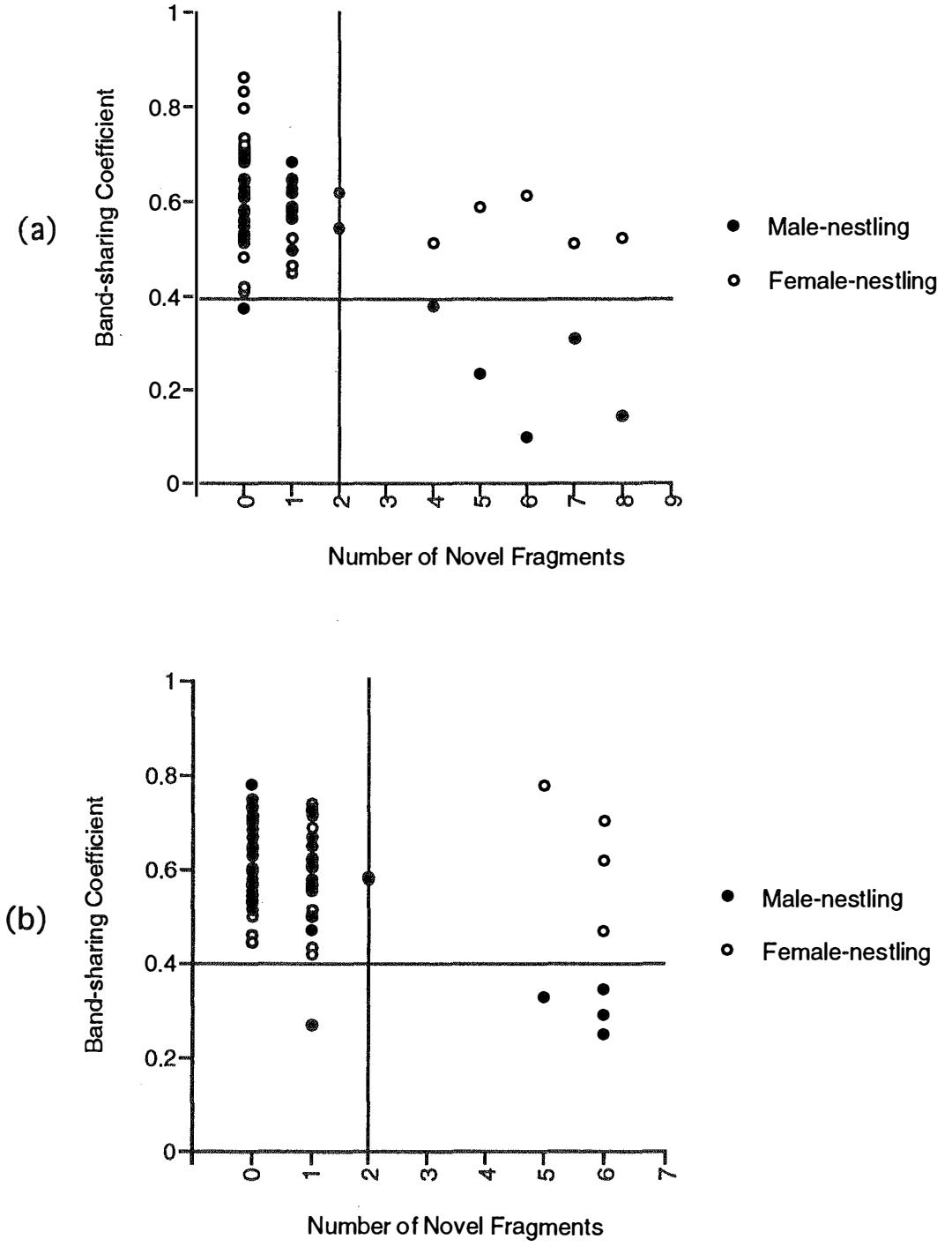

Fig 2.10. Plot of band-sharing coefficients between nestlings and their putative parents and the number of novel fragments present in the nestling's DNA profile for birds breeding at: (a) Cape Direction and (b) Cape Queen Elizabeth.

2.4 DISCUSSION

2.4.1 Variability of DNA profiles

The RNA probe derived from the human minisatellite 33.6 core sequence effectively hybridised with shearwater DNA to reveal individual-specific profiles, indicating high levels of polymorphism at minisatellite loci with homologous sequences to those discovered in man (Jeffreys *et al.* 1985a). Variability was sufficiently high to either confirm or exclude parentage in 107 complete and incomplete families of short-tailed shearwaters from two breeding colonies.

2.4.2 Sex-specific restriction fragments

Unambiguous gender identification in sexually monomorphic birds is difficult. Techniques that have been used for the identification of sex in monomorphic birds include observations of sexual behaviour (Hatch 1990), surgical methods (Prus & Schmutz 1987; Goldizen *et al.* 1993), and genetic markers based on chromosomal analysis (Biederman *et al.* 1982; Nakamura *et al.* 1990; Parker *et al.* 1991), biochemical markers (Baverstock *et al.* 1982) and specific DNA sequences (Quinn *et al.* 1990; Rabenhold *et al.* 1991; Millar *et al.* 1992; Graves *et al.* 1993; Longmire *et al.* 1993).

Genetic methods of sex identification in birds are based on the heteromorphic sex chromosomes, Z and W. Males are homogametic (ZZ) and females heterogametic (ZW) (Bloom 1974). The differences in size and morphology of the Z and W chromosomes allows relatively simple assignment of sex based on an individual's sex chromosome complement (Biederman *et al.* 1982; Parker *et al.* 1991) or total DNA content (Nakamura *et al.* 1990). Baverstock *et al.* (1982) reported a cytoplasmic isozyme that was linked to the Z chromosome in five species of birds. Allozyme electrophoresis could distinguish males (which were heterozygous) and females (which were hemizygous) for the particular enzyme.

DNA sequences that are unique to one or both of the sex chromosomes or that exhibit some unique and distinguishable property (usually size) relative to similar sequences on autosomal chromosomes have provided molecular based methods for identifying sex in many bird species. Most of these methods have concentrated on repetitive sequences on the W chromosome, however Quinn *et al.* (1990) isolated a single copy segment of the Z

chromosome that shows homology to a unique-sized restriction fragment from the W chromosome in snow geese (*Chen caerulescens caerulescens*). Sex identification in this species can therefore be made either on the presence (female) or absence (male) of the W chromosome derived fragment, or the haploid (female) or diploid (male) presence of other Z chromosome derived restriction fragments identified by the same probe.

A number of studies, using different multilocus minisatellite probes, have reported female-specific restriction fragments in addition to the highly variable fragments typical of multilocus DNA fingerprinting profiles. Rabenhold *et al.* (1991) detected female-specific restriction fragments in the DNA of stripe-backed wrens (*Campylorhynchus nuchalis*) using the probe 33.15. The size of the sex-specific fragments (6-47 kb) depended on the restriction enzyme used, but in general they were near the upper size limit of the more variable autosomal-derived fragments. The same probe did not detect similar sex-specific restriction fragments in a closely related species, *C. griseus*. Millar *et al.* (1992) found high molecular weight (28-45 kb) female-specific restriction fragments with homology to the multilocus minisatellite probe pV47-2 (Longmire *et al.* 1990) in brown skuas (*Catharacta lonnbergi*). The female-specific fragments showed no homology to the probe 33.6. Graves *et al.* (1993) reported a large (27 kb) female-specific restriction fragment in shags (*Phalacrocorax aristotelis*) that hybridised intensely to the probe 33.6, but not 33.15. Longmire *et al.* (1993) detected female-specific restriction fragments using three microsatellite probes, consisting of di- or trinucleotide repeats, in six of nine bird species examined. Similar to the results using minisatellite probes, the female-specific restriction fragments identified using the microsatellite probes were very large (50 kb).

These results show that in several species of birds a region of highly repetitive DNA occurs on the avian W chromosome belonging to a family of mini- or microsatellite DNA that is spread throughout the genome. The particular family of satellite DNA appears to be species specific, as different probes detect female-specific restriction fragments in different species, and probes that detect sex-specific fragments in one species, do not detect them in others. Similarly, this study has identified a female-specific minisatellite fragment, homologous to the human probe 33.6, which must be located on the W chromosome. Although the female-specific restriction fragment in short-tailed shearwaters was considerably smaller than sizes for similar fragments reported in other studies, it was generally one of the largest detected by

the 33.6 probe.

Rabenhold *et al.* (1991) have discussed the characteristics of avian W chromosomes with regard to the properties of female-specific repetitive DNA. The W chromosome is nearly entirely heterochromatic and consists largely of repetitive DNA (Tone *et al.* 1982; Bloom 1974). Rabenhold *et al.* (1991) have argued that repetitive DNA on the W chromosome, homologous to mini- and microsatellite probes, derives from the Z chromosome by crossing over or transposition. Subsequently, this W chromosome repetitive segment is free of its function in the autosomes, is less exposed to recombination and so is amplified into much longer tandem repetitive arrays. The W chromosome repetitive regions exhibit an intraspecific conservation of size, relative to the highly variable loci on autosomal chromosomes. This suggests that the W chromosome sequences are protected from the mechanisms that produce size variation in satellite DNA, either because of the heterochromatic nature of the W chromosome or the absence of recombination (Rabenhold *et al.* 1991).

Short-tailed shearwaters can only be reliably sexed in the days immediately after egg laying. The identification of an easily distinguishable female-specific restriction fragment in the DNA profiles of this species has allowed the sex of all birds in this study to be accurately determined. Without this information, an assumption of gender was made (Austin *et al.* 1993) for the 1991 study which has been shown to be incorrect. Gender identification in the 1992/93 survey has provided strong support for the conclusion of mate infidelity in the short-tailed shearwater. Identification of the female-specific minisatellite fragment in shearwaters may be applied to additional areas of the species' biology. For example, sex ratios at hatching and of adult birds at breeding colonies, and sex-biased mortality during the nestling phase and during migration may be examined using this method.

2.4.3 Unrelated attendant adults

At Cape Direction in 1991 four adult birds captured when entering a burrow were not related to the nestling in that burrow. In 1992/93, four and five adult birds incubating eggs at Cape Direction and Cape Queen Elizabeth, respectively, were not related to the nestling that hatched in that burrow. The presence of these unrelated birds can be explained either by an error in sampling or alternative reproductive strategies operating in this species.

The nature of the trapping method employed in the 1991 study meant that should an adult enter the incorrect burrow there was no chance for it to move to the correct one. These false captures could be breeding adults entering the wrong burrow. Alternatively prebreeding and possibly unpaired or failed-breeding birds may be present in the colony late in the breeding season and may arrive with the breeders at dusk and enter burrows at random (Serventy 1967b; Serventy & Curry 1984; Fitzherbert 1985). Brooke (1990) reviewed a series of experiments on Manx shearwaters, *Puffinus puffinus*, and concluded that vision is the primary sense used in locating the nesting burrow. The ability to locate the burrow appears to be strong but there are few data on the frequency of breeding birds entering the wrong burrow.

Without knowledge of the sex of the 4 unrelated adults identified in the 1991 study it was argued (Austin *et al.* 1993) that the frequency of birds entering an incorrect burrow was low enough to not account for the presence of these unrelated birds. First, during a 40-year study of the short-tailed shearwater, Bradley *et al.* (1990) recorded only rare instances of three individuals in one burrow. In these cases only two birds were regular occupants. In the 1991 study no more than two adult birds were trapped in one burrow. Second, at the time when blood samples were collected approximately half the burrows in the colony were unoccupied (resulting from either failure to lay or hatch an egg, or death of the nestling). Once vacated, such burrows have never subsequently been found to contain adult birds (I. Skira. personal communication). Therefore, assuming maternity to be correct the unrelated birds were assumed to be males. However, identification of the sex of the 4 unrelated birds in 1991 revealed that three were females and only one a male. These females were more likely to be the result of a mistrap than a consequence of a more complex, alternative mating strategy (see below). In the 1992/93 study, only birds incubating eggs were sampled so potential errors in sampling were eliminated. Therefore unrelated attendant adults identified in the 1992/93 study were meaningful.

In monogamous birds the presence of an unrelated attendant adult at the nest can be the result of one of several alternative reproductive strategies. Depending on paternity of the dumped egg, intraspecific brood parasitism (ISBP) will result in either the attendant female (e.g. zebra

finches, Birkhead *et al.* 1990; barnacle geese [*Branta leucopsis*], Choudhury *et al.* 1993) or both the attendant female and male (e.g. lesser snow goose, Quinn *et al.* 1987; zebra finches, Birkhead *et al.* 1990; starlings, Pinxten *et al.* 1993) being genetically unrelated to the nestling.

Although ISBP has been recorded in birds with multiple egg clutches (Quinn *et al.* 1987, Birkhead *et al.* 1990, Choudhury *et al.* 1993, Pinxten *et al.* 1993) it is extremely unlikely in species which lay only a single egg. Clutch augmentation experiments in Laysan albatrosses (Rice & Kenyon 1962), Manx shearwaters (Harris 1966), Leach's storm petrels (*Oceanodroma leucorhoa*, Lack 1968) and short-tailed shearwaters (Norman & Gottsch 1969) have shown that breeding pairs cannot raise two nestlings and in short-tailed shearwaters many pairs failed to hatch both eggs (Norman & Gottsch 1969). In fact, energetic constraints on breeding short-tailed shearwaters appear to be so severe that many pairs are unable to fledge even a single young (Fitzherbert 1985). Intraspecific brood parasitism cannot therefore operate in the short-tailed shearwater.

Polyandry occurs where a female mates with two males both of whom feed the young (Krebs & Davies 1987). If polyandry is not detected and the paternal male is not sampled a situation is created in which a male in an apparently monogamous pair is unrelated to the offspring. Cooperative breeding occurs where offspring from previous breeding seasons assist their parent(s) with incubating the egg and feeding the nestling (Woolfenden & Fitzpatrick 1984). Both of these explanations are unlikely because only rare occurrences of more than two birds in a burrow have been recorded and no birds have ever been recorded as forming more than one pair bond in a season (Bradley *et al.* 1990). Rapid mate switching/replacement occurring prior to egg laying (McKinney *et al.* 1984; Birkhead *et al.* 1990; Pinxten *et al.* 1993) could result in the second male being unrelated to the offspring. However, there are no records of short-tailed shearwaters changing mates within a season (Wooller *et al.* 1988). Mate switching or replacement that results in the new male mate being unrelated to the nestling is extremely unlikely as it would have to occur during or just prior to the pre-laying exodus when all birds are at sea. Pair bonding and successful breeding attempts require a sustained and coordinated effort from both members of a pair beginning nine weeks before egg laying. The presence of unrelated female birds in three of four burrows in 1991 must be due to the capture of these females entering the wrong burrow.

The final and most likely reason for an attendant adult male and nestling to be unrelated is that the nestling is the result of an extra-pair fertilisation (EPF) by a male outside the pair bond. In nine burrows at Cape Direction and Cape Queen Elizabeth in 1992/93, respectively, maternity of the nestling was confirmed, but paternity by the attendant male was excluded. These nestlings must be the result of an extra-pair fertilisation, implicating one or more extra-pair copulations (EPCs) between the attendant female and an unknown, extra-pair male.

2.4.4. Extra-pair copulations

Although there are at least 115 species of monogamous birds in 30 families in which EPCs have been recorded (Ford 1983), the genetic consequences or success of this behaviour has only been revealed in a relatively small number of species. The occurrence of successful EPFs in short-tailed shearwaters suggests that some individuals may be adopting the mixed reproductive strategy of Trivers (1972) in which males of monogamous species assist the pair-bonded female raise young but also take advantage of opportunities to inseminate other females by EPCs. Alternatively, unpaired males may be responsible for the observed EPFs. Although DNA profiling can provide evidence that EPCs are occurring, the genetic data alone cannot answer questions of why and how EPCs occur, and the type of individuals that are involved. These behavioural and biological questions must be addressed using observational and long-term data. Attempts to observe breeding birds during the fertilisable period, prior to the pre-egg laying exodus, provided few answers and confirmed that quantitative observations of breeding behaviour in this species are extremely difficult to obtain. However EPFs in the short-tailed shearwater can be interpreted based on current knowledge from a long-term study of the breeding biology of this species at Fisher Island (Bradley *et al.* 1991) and the extensive literature on EPCs in monogamous birds (Westneat *et al.* 1990; Birkhead & Møller 1992).

2.4.4.1 Sexual selection

The existence of EPFs in short-tailed shearwaters suggests that sexual selection through differential mating success may be occurring, despite a predominantly monogamous mating system. There must be strong selection for males to ensure paternity at their own nest in birds that lay single egg clutches (Birkhead *et al.* 1985), and especially where the egg is laid without replacement. The two main paternity assurance mechanisms available to males are

mate guarding and frequent within pair copulations and will result in intrasexual competition by direct male confrontation or sperm competition. Intersexual selection may occur via female choice of extra-pair partners.

Mate guarding

Mate guarding, where males remain close to their mates and actively deter other males during the females fertilisable period (Beecher & Beecher 1979; Birkhead 1979), is important in protecting the pair bonded female from EPC attempts. The extent to which males guard their mates varies greatly, both inter- and intraspecifically (Morton 1987; Møller & Birkhead 1993) and will affect the opportunities for EPCs by other males.

Short-tailed shearwaters differ from most other colonially breeding birds in that all individuals arrive and depart from the colony at approximately the same time each day (Marshall & Serventy 1956; personal observations). There is no constraint on one member of a pair to remain at the colony and defend the nest while the other is away feeding, as is the case for most other colonially breeding birds (Møller & Birkhead 1993). Therefore there is the potential for efficient mate guarding by male short-tailed shearwaters because they can be present in the colony at the same time as the female. Behavioural data suggest that at least some birds vigorously defend either the area around the burrow entrance or their partner (Marchant & Higgins 1990; personal observations), perhaps implicating mate guarding. In addition, males could effectively guard their mates once both birds are in the burrow.

However, male short-tailed shearwaters will be ecologically constrained in their ability to mate guard, because birds must leave the colony each day to feed. There are a number of consequences of this separation between feeding areas and the breeding colony. First, in Manx shearwaters, attendance at the colony by males and females known to have commenced breeding is less than 100% on any particular night during the copulatory phase (Perrins & Brooke 1976). Although males may attempt to coordinate their colony attendances with those of the mate, females are probably alone on at least some nights, or at least parts of some nights. Some male grey-faced petrels also fail to attend the colony when their female is present (Imber 1976). A similar situation probably exists in short-tailed shearwaters (I. Skira, personal communication). Birds travel considerable distances during the day to feed (Marchant & Higgins 1990), and a combination of distance and weather, and perhaps

inexperience may result in a failure to return to or late arrival at the colony on some nights. A second, closely associated factor is that unless males follow their mates all day the female may land in a part of the colony away from the breeding burrow. A male cannot guard a female if he hasn't witnessed her arrival and she is in another part of the colony.

In addition to the constraint on mate guarding imposed by the daily movement of birds to and from the colony, the presence of large numbers of birds above ground at dusk and before dawn would make efficient mate guarding very difficult. Fights and other distractions can occur during which females remain unattended. Shearwater colonies are also visually occluded habitats. It may be very difficult for a male to follow or keep his mate in sight at all times because of darkness and dense vegetation in the colony. The opportunities for mate guarding are therefore likely to be restricted. Male shearwaters are unable to guard their mates for the entire copulatory period.

Although male short-tailed shearwaters are unable to mate guard continuously, intraspecific studies of purple martins (Morton 1987) and large cactus finches (Grant & Grant 1989) suggest that the extent of mate guarding by males varies and will affect the chance that their mate is involved in EPCs. Only short breaks in mate guarding are necessary for other males to capitalise on EPC opportunities, and it appears that males of many species target unguarded females, when the chances of EPC success are greatest (Birkhead *et al.* 1985). Male shearwaters that fail to optimise their opportunities to mate guard by being absent from the colony on some nights, arriving at the colony after the female mate or who are regularly distracted in fights are more likely to be cuckolded through EPCs.

Sperm competition

Male short-tailed shearwaters will be uncertain of paternity with their mate because of the constraints on mate guarding. Males of many bird species use frequent within pair copulations as a paternity assurance mechanism, in an attempt to devalue inseminations by other males (Birkhead *et al.* 1987; Møller & Birkhead 1993). There is great variation in the extent and success of observed EPCs resulting in EPFs suggesting that the outcome of sperm competition is highly variable. In willow warblers and wood warblers (Gyllensten *et al.*

1990), northern fulmars (Hunter *et al.* 1992) and merlins (David Parkin, personal communication) none of the observed EPCs resulted in EPFs. At the other extreme no EPCS were observed in a study of monogamous tree swallows but 38% of offspring resulted from EPFs (Lifjeld *et al.* 1993). There are a number of behavioural and physiological factors that affect success of EPCs through sperm competition. These include the timing of EPCs, the success of EPCs in transferring sperm and the success of EPC sperm in fertilising the egg.

An EPC must occur during the females fertilisation period, the interval when an insemination could result in fertilisation (Minneau & Cooke 1979; Westneat *et al.* 1990). The fertilisation period ends shortly after ovulation, and in domesticated birds this occurs 24 hours before the egg is laid (Howarth 1971). Nothing is known about the interval between fertilisation and egg laying in birds that lay single egg clutches. The fertilisation period may begin a considerable length of time before egg laying if there is extended sperm viability and prolonged sperm storage in the female reproductive tract (Birkhead 1988). In seabirds, particularly Procellariiformes, sperm storage is thought to be well developed as females of most species produce a single large egg, which requires the female to spend a large amount of time away from the colony feeding prior to egg laying (e.g. 30 days in Buller's shearwater [*Puffinus bulleri*], Harper 1983; 60 days in grey-faced petrels, Imber 1976).

Assuming that fertilisation occurs close to the egg laying date in short-tailed shearwaters, the females fertilisation period and the viability of sperm in the oviduct must be approximately three weeks long because the pre-laying exodus is 20-21 days (Serventy 1967b) and there are no records of any species of Procellariiformes copulating at sea. Observational data that copulations are most frequently seen 3-4 days before departure (Fitzherbert 1985), and that spermatogenesis reaches its peak during this time (Marshall & Serventy 1956), suggest that females are fertilisable only in the last few days before the pre-laying exodus. However, the effective fertilisation period may be longer than this as copulations are known to occur in the 20 days before the pre-laying exodus (Norman 1969), i.e. 6-7 weeks prior to egg laying, and active sperm are present in ejaculates at least 10 days before the prelaying exodus (Fitzherbert 1985). Due to breeding synchrony, all breeding female short-tailed shearwaters are fertilisable at the same time, so every EPC attempt during this period will involve a potentially fertilisable female. Successful EPC attempts in shearwaters are therefore made in the last few days

before the pre-laying exodus.

A successful EPC is one in which cloacal contact and sperm transfer occurs (Birkhead 1988). Although this can be difficult to determine by observation, a number of studies have shown that the success rate of EPCs is generally less than 20%, and in many cases approaches zero (Birkhead 1988).

The success of an EPC is determined by the behaviour of the female (Hatch 1987; Wagner 1991b; Venier *et al.* 1993) and presence and behaviour of the pair male (Birkhead *et al.* 1985). Many species of birds lack intromittant organs, and some degree of female cooperation is necessary to enable cloacal contact and insemination (Ford 1983; Fitch & Schugart 1984; Venier & Robertson 1991). Whether copulations are forced or unforced, females may control their success by resisting attempts by the male to mount and preventing cloacal contact by holding the tail over the cloaca (e.g. razorbills, Wagner 1991b). Forced EPC attempts are the most frequently observed in studies of EPCs in birds (Birkhead 1988) probably due to the fact that forced copulations and female resistance are more obvious to human observers than either type of UEPC. FEPCs are rarely successful because of female resistance and the ability to prevent cloacal contact (Ford 1983; Birkhead 1988; Wagner 1991b). FEPCs are only successful if the female submits to the extra-pair male and allows cloacal contact so that she can escape from him or avoid physical injury (Birkhead 1988), if the male can effectively restrain the female and force cloacal contact (e.g. lesser scaup [*Aythya affinis*], Afton 1985), or if more than one male attempts to copulate with a single female and she is overpowered (e.g.common murre, Birkhead *et al.* 1985). UEPCs, whether passively accepted or solicited, are more likely to be and have generally been found to be more successful (Birkhead 1988). The presence of the male partner reduces the success rate of EPCs as he can effectively interrupt or prevent any such attempts (Birkhead 1988).

Female short-tailed shearwaters are likely to have considerable control over the success of copulations as substantial coordination is required to enable mounting by the male, and the tail could effectively be used to prevent cloacal contact. The EPFs observed in the short-tailed shearwater are most likely to have resulted from UEPCs, either passively accepted or solicited, in which the pair male was not present because of his absence from the colony or

involvement in a fight or defence of the nesting burrow. However, the possibility of successful FEPCs either from the female accepting a copulation attempt to avoid the costs of resisting or from several males simultaneously attempting to copulate with a single female cannot be ruled out.

Sperm competition within the female reproductive tract will ultimately determine the success of fertilisation. Little is known about sperm competition in wild birds, but studies of domesticated species have revealed a number of factors that may influence the outcome of inseminations by more than one male.

First, opportunities for sperm competition are greatest in species with extended sperm viability and prolonged sperm storage (Birkhead 1988). In short-tailed shearwaters sperm must be stored and remain viable in the female reproductive tract for at least the three weeks of the pre-laying exodus, thus introducing the potential for sperm competition. Sperm viability and fertility decreases with increasing storage duration (McKinney *et al.* 1984), so inseminations late in the fertilisation period should have the greatest chance of fertilising an egg and producing a viable offspring. The implication for EPCs in short-tailed shearwaters is that males should attempt EPCs as close as possible to the beginning of the pre-laying exodus, so that sperm retain maximum viability and fertility at fertilisation.

Second, the relative number of within-pair copulations and EPCs will determine the relative number of competing sperm from pair and extra-pair males (Parker 1984). An EPC is more likely to lead to an EPF if the proportion of viable sperm from the EPC is high relative to that of the pair male (Birkhead 1988). This may result if the pair male is infertile or produces sperm of low viability or poor fertilising efficiency, or if within-pair copulation rates are low. A successful EPC strategy would involve multiple copulations with extra-pair mates to maximise the proportion of extra-pair sperm competing for fertilisation.

Third, the chronological order of within-pair and extra-pair inseminations may affect the probability of fertilisation as, in several species of captive birds, the last male to mate with a female has a greater chance of fertilising the egg (Birkhead *et al.* 1988; Birkhead & Hunter 1990). The last male advantage appears to be related to the way sperm is stored in the female

reproductive tract. In domestic chickens sperm from the most recent insemination sits on top of and leaves the sperm storage tubules before sperm from previous inseminations (Compton *et al.* 1978). The effect of sperm precedence is greatest when competing inseminations are well separated in time. If competing copulations are very close together the relative numbers of sperm from the two males will more strongly affect the probability of fertilisation (McKinney *et al.* 1984). Sperm precedence may also occur if sperm from one insemination are flushed out of sperm storage areas by a subsequent insemination (Birkhead & Hunter 1990). Hunter *et al.* (1992) have suggested that in birds that lay only a single egg all stored sperm may be released simultaneously, thus preventing any last male sperm precedence, and there is some evidence that this does occur in northern fulmars (Hatch 1983).

Frequent within pair copulations may be an effective paternity guard because they will increase the proportion of sperm relative to extra-pair males, and increase the probability that the pair male is the last to copulate with the female. Copulation rates within pairs are known to vary enormously both inter- and intraspecifically (Birkhead 1988) and appear to be related to the frequency of EPCs (Møller & Birkhead 1993). Although copulations in short-tailed shearwaters are known to occur over several weeks, the frequency within pairs is unknown. In addition, nothing is known about the dynamics of sperm storage and competition in this species. In a study of the northern fulmar, a monogamous, colonially nesting, breeding-site faithful seabird, pair males obtain the greatest number of (and the last) copulations relative to extra-pair males (Hunter *et al.* 1992). The number of within pair and extra pair copulations ranged from one to 54 and none to 17, respectively. Multiple EPCs occurred in many pairs; EPCs represented 4/9 and 6/18 copulations in 2 pairs, and in one of these 6 EPCS were followed by only 3 pair copulations prior to egg laying. No EPFs were detected in any pairs suggesting that pair males were not infertile or did not have low sperm viability. Within pair paternity was assured by frequent copulations and was more likely to occur through a higher proportion of pair sperm relative to sperm from EPCs, than the last male precedence.

Sperm storage and some form of sperm competition must occur in short-tailed shearwaters. If, as in domesticated birds, the number and order of inseminations by extra-pair males relative to pair males is important in determining fertilisation success, then the observed EPFs must have resulted from EPCs involving pairs in which the male had low sperm viability or fertility, the number of within-pair copulations was very low and/or the extra-pair male

achieved the last insemination before the prelaying exodus. As in other colonially breeding seabirds, male short-tailed shearwaters probably attempt to ensure within-pair paternity by copulating frequently, and as close to the pre-laying exodus as possible, to increase the proportion of sperm relative to any extra-pair inseminations and to obtain the last insemination before fertilisation. As a consequence of intrasexual selection, the observed EPFs in shearwaters represent minimum estimates of EPCs in the two colonies studied. Many more females may have been involved in EPCs which did not result in an EPF.

Intersexual selection

Møller (1992) has argued that male phenotypic and genotypic quality may vary significantly and that female mate choice may be constrained in monogamous birds because of pair bonding by previous females. Female mate choice will result in the first females to mate pairing with the best quality males, but every female cannot be mated to the best male. Subsequent females will be constrained in their mate choice to the extent that only lower quality males will be available. In addition there is only a subset of the complete group of males that will be available to each female as, in a large colony, females would not come into contact with or be able to appraise every male (Gladstone 1979). In short-tailed shearwaters, mate choice is severely constrained by strong pair and site fidelity, low annual mortality of breeding adults (~10%, Wooller *et al.* 1988) and low frequency of pairing (81% of completed breeders had three or fewer mates, Wooller *et al.* 1988) . Alternatively, females may pair with males for reasons which may increase the chances of raising offspring to independence, but are unrelated to male genetic quality; territory quality, the quality and extent of male parental care and breeding site familiarity and fidelity may lead females to pair with males of lower genetic quality (Westneat *et al.* 1990). For these reasons female choice of a breeding partner may not reflect the best choice of mating partner. Females paired with low quality males may use EPCs to modify their choice of mating partner by engaging in copulations with higher quality males.

The EPFs observed in the short-tailed shearwater may have occurred via female choice for higher quality males. There is some evidence to support female choice via EPCs in birds. Females may be able to appraise male quality by resisting copulation attempts to test male vigour (Westneat *et al.* 1990; Wagner 1991a); males that are more persistent in their pursuit

of EPCs or can overcome female resistance may be of high quality. Alternatively females may solicit EPCs to stimulate male-male competition in order to identify the dominant male (Cox & LeBoeuf 1977; Montgomerie & Thornhill 1989).

Male black-capped chickadees (Smith 1988), cattle egrets (*Bubulcus ibis*, Fujioka & Yamagishi 1981) and white ibises (Frederick 1987) of high dominance rank are more likely to be involved in EPCs than subordinate males. Male dominance may reflect fighting ability and endurance (Møller 1992). In all three species, female solicitation of EPCs are involved to some extent, and in black-capped chickadees and white ibises most of the females involved in EPCs are paired with low-ranking males.

Females may accept extra-pair copulations from older males because survival ability is a measure of male quality (Manning 1985). Female tree swallows mated to young and less experienced males are more likely to engage in EPCs which resulted in EPFs than female mates of older males (Lifjeld *et al.* 1993). Older males achieve greater EPC success than younger males in lesser scaups (Afton 1985), bam swallows (Møller 1985), indigo buntings (Westneat 1987, 1990) and purple martins (Morton *et al.* 1990). However in all of these species most EPCs are forced, and so it was difficult to demonstrate female choice.

Male secondary sexual characteristics may reflect male quality and have been shown to influence female choice. Males with enhanced secondary sexual characteristics (badge size in house sparrows, Møller 1990; bill redness in zebra finches, Burley & Price 1991) relative to other males are more likely to engage in EPCs. Females are more likely to be involved in EPCs with males having enhanced characteristics relative to their mates. Female choice based on secondary sexual characteristics is unlikely in short-tailed shearwaters as there are no sexual dimorphisms in this species (Marchant & Higgins 1990).

Alternatively, Wagner (1992b) has proposed a 'status hypothesis' for migratory, long-lived and high breeding-site fidelity birds to explain why males do not reduce parental care when they are uncertain of their paternity, based on an idea originally developed by Zahavi (1976) to explain cooperative breeding. The basis of this hypothesis is that male social status is affected by a number of behaviours performed in view of other members of the colony, and

that, because of breeding site fidelity, individuals have the opportunity to assess each other over many years. In many birds, including short-tailed shearwaters, providing parental care is an energetically expensive behaviour performed over several months. Performance as a parent affects divorce rates in shearwaters (Bradley *et al.* 1990) and may influence the social status of males. One consequence of this is that females may use male performance as a parent (i.e. parental effort) to assess male quality which could affect female willingness to engage in EPCs with particular males.

2.4.4.2 Opportunities for extra-pair copulations

Opportunities for EPCs are largely determined by the spatial and temporal availability of females, and male and female behaviour. EPCs are more frequent in colonial than solitary breeding birds supporting the hypothesis that EPCs (and the risk of lowered reproductive success through EPFs) are a cost of social living to males (Møller & Birkhead 1993). Coloniality also appears to be costly to male short-tailed shearwaters; the high density of fertile females in shearwater colonies provides many, easily accessible opportunities for EPCs.

Møller & Birkhead (1993) have proposed that the costs and benefits of EPCs are not shared equally among males in a colony as a consequence of a differential success in obtaining EPCs and ensuring paternity in their own nest. This differential success may be related to age, if significant annual male breeding mortality occurs, or some other male attribute, such as phenotypic quality. In the colonial breeding swallow older mated males gain more successful EPCs than younger mated males suggesting that the potential reproductive benefits of colonial life are restricted to males that survive to an older age (Møller 1987). Morton *et al.* (1990) have shown age-biased EPFs in colonial breeding purple martins. Older males can almost double their mating success through FEPCs with several different females mated to younger males. A few older males in the colony obtain most of the benefits of EPCs while a larger number of younger males share the cost. In tree swallows the distribution of EPFs is not random between pairs (Lifjeld *et al.* 1993). Nine out of 16 males had one or no extra-pair offspring in their broods, whereas the remaining 7 broods contained more than 50% extra-pair offspring. The costs of EPCs are therefore not shared equally among all males.

A test of this hypothesis in short-tailed shearwaters would require accurate estimates of

lifetime reproductive success, coupled with estimates of male "quality", to determine whether the costs and benefits of EPCs are shared equally or unequally among all males. The low annual breeding mortality of short-tailed shearwaters would suggest that, over a males lifetime, the loss of mating success through loss of within-pair paternity when young would be balanced by gains obtained through successful EPFs when older. However this would depend on the relative loss and gain of paternity between the first and last breeding attempt. Young males may be cuckolded only once or twice but older males may obtain many EPFs (e.g. purple martins, Morton *et al.* 1990). Birkhead & Møller (1992) have suggested that effects of male quality will modify age-related effects. For example, male red-winged blackbirds can significantly increase their reproductive success through EPFs, and those that do are also more likely to gain paternity with their own mate (Gibbs *et al.* 1990). Therefore males that suffered cuckoldry were less likely to make up this loss through EPFs.

The highly synchronous breeding season of short-tailed shearwaters imposes severe constraints on the opportunities for males to pursue EPCs. Within-pair paternity assurance (mate guarding and frequent copulations) and coordination of breeding activities between members of a pair (parental effort) must take priority over the pursuit of EPCs for most males to ensure reproductive success. Birkhead and Biggins (1987) have shown that the risk to females of being involved in an EPC for colonial and synchronously breeding snow geese and common guillemots (*Uria aalge*) is greatest for females that lay latest. A similar scenario can be proposed for short-tailed shearwaters. The pre-egglaying exodus from breeding colonies occurs over several days, at which time all females are potentially fertilisable. If males paired to females that leave early continue to return to the colony, the number of males relative to females (i.e. the sex ratio) will increase. These males will be released from the constraint of mate guarding and other duties and will therefore be free to pursue EPCs. Females that delay their departure from the colony will suffer an increased risk of involvement in EPC. Either her mate will have difficulty protecting her from the increasing number of unconstrained males seeking copulations, or she will have a greater choice of males from which to solicit an EPC.

Male and female behaviour will also affect the opportunities for EPCs. Mate guarding by males and the effect of female behaviour on EPC success in short-tailed shearwaters has

already been discussed.

2.4.4.3 Benefits of extra-pair copulations

At least 10% of female and an unknown proportion of male short-tailed shearwaters were involved in EPCs in the 1992/93 breeding season at Cape Queen Elizabeth and Cape Direction. The extent to which both male and female shearwaters engage in EPCs is dependent on the relative costs and benefits of this behaviour. These costs and benefits are difficult to evaluate without detailed behavioural data.

There are two benefits which, in shearwaters, may apply equally to males and females. EPCs may provide insurance against mate infertility (Gibson & Jewell 1982; Buitron 1983; Wetton & Parkin 1991; Wagner 1992a). Although this benefit has generally been expressed as an advantage to females, insurance against infertility in the short-tailed shearwater is a major potential to both sexes as only a single egg is laid without replacement and it can be argued that male parental investment is equal to or only slightly less than that of the female (Serventy 1967b; Fitzherbert 1985). Females mated to males with low fertility could ensure that their egg is fertilised, whereas males mated to infertile females could attempt to fertilise eggs of another more fertile female. At least 10% of all eggs fail to hatch despite complete incubation implying infertility is relatively frequent (Serventy & Curry 1984). Of shearwater pairs that fail to produce an egg in the preceding season, 33% change partner by divorce (i.e. while their previous partner is still alive) (Wooller *et al.* 1990). Divorce rates are significantly lower following breeding success (Bradley *et al.* 1990), suggesting infertility is an important cause of divorce, and it appears that birds are capable of recognising this in their partner. The infertility hypothesis predicts a low frequency of EPC which should consistently involve the mates of the same males.

A second, closely aligned benefit of EPCs is that of mate assessment, either for future pair bonding or EPCs (Colwell & Oring 1989). Females may use EPCs to assess the quality of potential EPC partners (Westneat *et al.* 1990). Alternatively, birds of either sex may use EPCs in one season as a means of initiating a pair bond for the next season (Wagner 1991a). Incompatibility between mates has been suggested as a cause of breeding failure in short-tailed shearwaters leading to divorce (Wooller *et al.* 1988), so that EPCs may represent a

first stage in the divorce process when the pair-bond weakens or partners begin to seek more compatible or fertile mates. Mortality between one breeding season and the next may leave birds unpaired (Bradley *et al.* 1990). EPCs may provide some birds with a potential alternative mate as an insurance against failure of their present mate returning in the subsequent season (Wagner 1991a).

The main benefit to breeding males is that EPCs, if successful, will enhance their reproductive success at relatively little cost by parasitising the parental care of another male (Trivers 1972). This benefit may be great in short-tailed shearwaters because a relatively small proportion (8%) of the breeding population is responsible for most (53%) of the next generation (Wooller *et al.* 1989). In fact, 29% of shearwaters that have completed their reproductive careers are responsible for producing all of the offspring that return to breed (Wooller *et al.* 1988). In a breeding lifespan with a maximum of 27 years (Bradley *et al.* 1989) and a mean of 9.3 years (Wooller *et al.* 1988) shearwaters produce on average 4.7 eggs and fledge 2.7 young (Wooller *et al.* 1988). Any male that can adopt a strategy of EPCs thus has the opportunity to greatly enhance his contribution to the next generation.

The length of the breeding lifespan appears to be a major factor influencing lifetime reproductive success. Shearwaters that delay breeding live longer and show a higher fecundity than early breeders (Wooller *et al.* 1988). There are several strategies, involving EPCs, that male shearwaters can adopt to increase offspring production and extend their breeding lifespan. A substantial percentage (28%) of birds spend at least two years in the colony 'prospecting' before forming a pair-bond and attempting to raise young (Wooller *et al.* 1989). During this time, males that can effect fertilisations with an already paired female may gain the double advantage of parasitising the parental care of the pair-bonded male and, by delaying breeding (in the sense of finding a mate and raising young), increase their chances of survival and higher fecundity.

About 18% of males known to have bred at least once on Fisher Island can be expected to be present in the colony but not associated with an egg (Wooller *et al.* 1990). Although some are unable to secure a mate that year or have a mate whose egg has failed after laying, many are in pairs that appear not to lay. Once again, if the male can obtain one or more successful EPCs it may enhance his long-term survival by not rearing a chick that year, whilst still siring

an offspring.

Females may gain genetic and non-genetic benefits from EPCs by pursuing a mixed reproductive strategy in which they raise offspring with the best mate that they can attract as a breeding partner, but copulate with males outside the pair bond (Westneat *et al.* 1990; Wagner 1992a). Although it is impossible for females to lay more than one egg per year, they can take measures, in addition to providing parental care, to increase the chances of their offspring returning to breed. Given the biased reproductive success and high mortality between fledging and the first breeding attempt (Wooller *et al.* 1988) EPCs may provide two ways by which females can increase the survival chances of their offspring.

Females may increase the genetic quality of their offspring by mating with males that are genetically superior to their own mates (Mineau & Cooke 1979; Buitron 1983; Fitch & Schugart 1984; Møller 1988; Smith 1988; Lifjeld *et al.* 1993). Møller (1992) has proposed that females in monogamous species are constrained by the mating system in their choice of mate, and Westneat *et al.* (1990) suggest several situations in which females may be paired with low quality males. If males do vary in their genetic quality then females paired with low quality males will have most to gain from EPCs. It is predicted that the probability of female involvement in EPCs is inversely related to the genetic quality of their mates - EPCs should occur in a small percentage of pairs, and that the mates of some males should show consistent involvement in EPC activity (Westneat *et al.* 1990; Møller 1992).

Females may increase the genetic diversity of their offspring by mating with genetically dissimilar mates (Williams 1975; Gladstone 1979). Short-tailed shearwaters, and other birds that lay one egg clutches, can only achieve genetic diversity across years, but could be important as individuals can remain paired with the same mate for more than 10 years (Bradley *et al.* 1990). Genetic diversity of offspring is important in unpredictable environments and especially with coevolving diseases or parasites (Westneat *et al.* 1990). Mortality between fledging and the first breeding attempt is high (60% averaged over 20 years, range 40-85%, Serventy & Curry 1984) and is a function of the long (3-15 years, Wooller *et al.* 1988) pre-breeding period. Significant mortality of migrating birds occurs in some years from starvation and exhaustion, probably due to adverse weather and low food

availability (Wooller *et al.* 1989; Crowley 1991). Offspring diversity may reduce the variance of female reproductive success by increasing the chances that at least some young will return to breed. If this hypothesis is true then EPCs should be evenly distributed among pairs from one year to the next (Westneat *et al.* 1990).

A third benefit is to insure against low sperm viability as a result of extended sperm storage. Because females are forced to spend a long time at sea feeding prior to egg laying, during which they cannot receive copulations, sperm viability may be reduced at the time of fertilisation (McKinney *et al.* 1984) which will reduce the chances of producing viable offspring (Birkhead 1988). It would be advantageous for females to receive inseminations as close to their departure date as possible to ensure maximum sperm viability at fertilisation. If the pair male is absent from the colony during the last days before departure, the female may insure against low sperm viability from previous within pair copulations by accepting EPCs in her mates absence. Approximately 10% of female grey-faced petrels appear to adopt this strategy when their mate cannot coordinate his colony attendance with hers at the critical time (Imber 1976).

Other non-genetic benefits to females of EPC such as courtship feeding and additional parental care from extra-pair males are unlikely in short-tailed shearwaters, or in monogamous birds in general (Westneat *et al.* 1990; Birkhead & Møller 1992) as neither type of male behaviour has been recorded in this species.

2.4.4.4 Costs of extra-pair copulations

Although both male and female short-tailed shearwaters may gain substantial benefits from EPCs, the rate of EPFs in this species is low, suggesting that there are a number of potential costs and other factors which will influence the frequency and success of EPCs. One cost applicable to both sexes is an increased exposure to parasites or diseases carried by the extra-pair mate (Hamilton 1990); however little is known about disease transmission during sexual behaviour.

Frequent copulations by males outside the pair bond may lead to sperm depletion and thus a risk of losing within pair paternity through insufficient sperm supplies for pair copulations

maintain their social status in the long term and to strengthen the pair bond so that the female might increase his confidence of paternity in the next breeding season (Wagner 1992b).

Females may also risk injury if EPCs are forced or involve attempts by many males (McKinney *et al.* 1984). EPCs may also represent a cost to females if continued harassment from extra-pair males disturbs important within-pair breeding activities such as copulations and nesting burrow construction and maintenance.

(Birkhead & Møller 1992). Males that pursue EPCs may experience a higher risk of cuckoldry because mateguarding and the pursuit of EPCs are mutually exclusive activities. This cost has yet to be demonstrated in any synchronously breeding bird. Males may also risk physical injury from the pair male when attempting EPCs.

Female shearwaters engaging in EPCs risk retaliation by their mate in the form of physical attack, desertion and subsequent breaking of the pair bond, or a reduction in parental care from the male (Trivers 1972; Gladstone 1979; Westneat *et al.* 1990). Physical punishment by the male mate is a rare behaviour in birds (Westneat *et al.* 1990) and is therefore unlikely in the short-tailed shearwater. In shearwaters and other seabirds reproductive success increases with breeding experience and the length of the pair bond, and decreases after a change of mate (Bradley *et al.* 1990; Wooller *et al.* 1990). Desertion by the male would reduce both the current and future reproductive success of the female (Fitch & Schugart 1984; Bradley *et al.* 1990) and may constrain the female in her willingness to engage in EPCs because of the value of the pair bond. Males that reduce or withhold parental care would sacrifice the current reproductive success of the female and may increase their chances of survival to another year when they would be older and less likely to be cuckolded (Westneat 1987).

However males themselves may be constrained in their response to EPC by the female mate. Desertion may reduce the males' reproductive success as it is unlikely, due to breeding synchrony, that he will be able to re-pair that year and because of the deleterious effect on his own breeding success (Bradley *et al.* 1990). As male parental care is critical to offspring survival, the benefits of withholding care are unlikely to outweigh the cost of failure to fledge a nestling that might be their own. A second reason why males may lose more than they gain by withholding parental care is that they risk lowering their social status, and subsequently their reproductive success. The 'status hypothesis' proposed by Wagner (1992b) may apply to short-tailed shearwaters because performance as a parent affects divorce rates in shearwaters (Bradley *et al.* 1990) and may influence the social status of males. If social status affects a males ability to obtain a mate, EPC, breeding site or win fights with other males, then a male that reduces or withholds parental care may reduce his social status and suffer future reductions in reproductive success. Males that are uncertain of their paternity should therefore maintain their parental effort, even if the nestling is not related to them, to

Chapter 3

Population Genetic Structure of the Short-tailed Shearwater, *Puffinus tenuirostris*.

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3.1 INTRODUCTION

3.1.1 Gene flow and genetic population structure

An intraspecific phylogeny represents the evolutionary history of individuals and populations within a species (Avise 1989). At the whole-organism level the structure of these population trees is affected by various biogeographic processes, historical demographic factors and taxon-specific dispersal characteristics, which in turn will influence the patterns of intraspecific genetic variation. The extent to which this genetic variation is geographically structured among populations will also be dependent on a number of evolutionary forces including mutation, random genetic drift due to finite population size and natural selection (Slatkin 1987). Gene flow, the movement of gametes, individuals, populations or extranuclear DNA (e.g. mtDNA) (Slatkin 1985a), will constrain the genetic differentiation of geographically separate populations occurring through mutation, random genetic drift and/or natural selection (Slatkin 1987). In terms of species' biology, mobility or dispersal capability appears to be an important factor influencing the extent of genetic differentiation of animal populations (Ball *et al.* 1988; Baker *et al.* 1990b; Ward *et al.* 1992).

Long-distance dispersal of planktonic gametes and larvae of many marine species of fish and invertebrates provides the opportunity for extensive gene flow between populations. For example, the lack of mtDNA differentiation among populations of the red rock lobster (*Jasus*

edwardsii), separated by distances of up to 4600 km, in Australia and New Zealand is probably due to the widespread dispersal of the long-lived planktonic larval stage (Ovenden *et al.* 1992). Among vertebrates, adult vagility is a major potential source of gene flow between populations. The ability to fly makes birds a group of highly vagile animals in which the potential for gene flow appears to be frequently realised (Barrowclough 1983; Tegelström 1987a; Ball *et al.* 1988; Burson 1990; Stangel *et al.* 1991). Limited mtDNA and allozyme genetic differentiation between geographically widespread populations of great tits (*Parus major*, Tegelström 1987a) and red-winged blackbirds (Ball *et al.* 1988), and common terns (Burson 1990) and white ibises (*Eudocimus albus*, Stangel *et al.* 1991), respectively, was attributed to the common and/or long distance dispersal observed in these species.

Realised gene flow may be significantly less than that predicted on the basis of a species' intrinsic dispersal capability for several reasons. Physical or ecological barriers may prevent or reduce dispersal between populations (Ball *et al.* 1988; Avise 1994). The Cook Strait separating the north and south islands of New Zealand appears to act as an effective barrier to dispersal between these two islands for the introduced common chaffinch (Baker *et al.* 1990b). The significant geographic structuring of allozyme variation in the chaffinch is primarily due to differentiation between populations on the north and south islands. Similarly chaffinches on isolated oceanic islands in the Atlantic are well differentiated genetically, based on allozyme variation, but continental populations, with no barriers to dispersal, are only weakly differentiated (Baker *et al.* 1990a).

Behavioural constraints may restrict the dispersal of one or both sexes from natal site or social group in many species. Philopatry, faithfulness to a site or group, has been recorded in many species of birds and mammals (Greenwood 1980) and may be natal (faithfulness to birth site or group) or breeding (faithfulness to the first breeding site or group). The extent of philopatry may also be sex biased. In mammals females tend to be more philopatric than males, but in birds females are usually the dispersing sex (Greenwood 1980; Greenwood & Harvey 1982). However, some birds exhibit unbiased or female biased philopatry which may lead to genetic structuring of mtDNA haplotypes. For example, fairy prions (*Pachyptila turtur*) are a colonially nesting seabird that are believed to be strongly philopatric: individuals that hatch in one colony return there to breed. Ovenden *et al.* (1991) compared and

contrasted genetic variation within and between three colonies of the fairy prion using restriction enzyme analysis of mtDNA. The genetic differentiation between the breeding colonies provided strong evidence for philopatry acting in this species. In addition, the genetic analysis revealed an absence of mtDNA variation in one colony which allowed Ovenden *et al.* (1991) to infer a founder event or population bottleneck involving no more than four female birds in that colony.

Many avian species exhibit strong natal and breeding philopatry, which is expected to promote genetic differentiation among populations (Quinn & White 1987b; Shields 1990; Avise & Ball 1991). In at least four species, Canada goose (*Branta canadensis*, Van Wagner & Baker 1986; Shields & Wilson 1987), black brant (*Branta bernicla*, Shields 1990), fairy prions (*Pachyptila turtur*, Ovenden *et al.* 1991) and dunlins (*Calidris alpina*, Wenink *et al.* 1993) genetic differentiation between some populations appears to be maintained by strong natal philopatry. Molecular techniques have now been applied to many species of birds to examine the effect of philopatry on intraspecific population genetic structure and phylogeny (Shields 1990; Moum *et al.* 1991; Ovenden *et al.* 1991; Avise *et al.* 1992; Birt-Friesen *et al.* 1992; Wenink *et al.* 1993).

In philopatric colonially nesting birds population sub-division is expected to conform to a stepping-stone model (Kimura 1953; Crow & Kimura 1970) in which each colony is treated as a deme and gene flow, when present, occurs between adjacent colonies. If philopatry acts to promote and maintain genetic differentiation between breeding colonies it may be possible to characterise individual colonies or geographically localised groups of colonies using appropriate molecular markers. These molecular markers could be used to identify the natal origins of captured birds (Quinn & White 1987b) which could provide information on the relationships between birds in social and feeding aggregations relative to their breeding colony and the origins of founding members of recently established colonies. Using sequences from the control region and cytochrome *b* gene in mtDNA, Wenink *et al.* (1993) were able to genetically characterise individual, geographically distinct populations of dunlins (*Caldris alpina*) and therefore distinguish subpopulations within migrating and wintering flocks of these birds.

Direct (genetic) and indirect (observational) estimates of the extent of population genetic structure within a species can produce conflicting results (Moum *et al.* 1991; Avise *et al.* 1992; Birt-Friesen *et al.* 1992) due to the fundamentally different components of gene flow that these methods assess. Indirect methods of estimating population genetic structure assess the frequency and distance of dispersal of birds based on the recovery of banded individuals. These methods can only assess contemporary levels of gene flow because they are limited in terms of the number of populations or colonies (space) and the number of years (time) from which data can be collected (Rockwell & Barrowclough 1987). Indirect methods cannot asses the evolutionary component of gene flow, which will be reflected in an intraspecific phylogeny. Direct estimates of the type of genetic structure of populations are based on the geographic distribution of molecular genetic markers. These markers retain a phylogenetic record of population connectedness and historical demographic changes and, therefore, reflect past as well as contemporary gene flow (Avise *et al.* 1992).

3.1.2 Phylogeography

The analysis of mitochondrial DNA (mtDNA) has proved to be an informative and useful method of examining population genetic structure in a variety of organisms, including birds (Ball *et al.* 1988; Ovenden *et al.* 1991; Avise *et al.* 1992; Birt-Friesen *et al.* 1992). The maternal and haploid inheritance, relatively rapid evolution and extensive inter-individual polymorphism of mtDNA provides opportunities to investigate intraspecific phylogenies. Maternal inheritance and haploid transmission result in an effective population size for the mitochondrial genome about one-quarter of that for nuclear genes. Mitochondrial genes, therefore, are more sensitive to the effects of random genetic drift, founder events and bottlenecks than are nuclear genes (Birky *et al.* 1983).

The relationship between an intraspecific gene phylogeny and the geographic distribution of extant genotypes comprises the phylogeographic structure of a species and can provide valuable information on the evolutionary circumstances that have lead to the observed pattern (Avise *et al.* 1987). Avise (1992) has summarised mtDNA phylogeographic patterns for an array of regional species and concluded that the evolutionary depth of population structure varies widely and that shallow intraspecific subdivisions reflect evolutionary recent population separations related to dispersal and gene flow patterns. The magnitude of mtDNA phylogenetic divergence and the extent of geographic structuring of mtDNA haplotypes may

vary independently, such that a number of phylogeographic patterns are possible (Fig. 3.1). At one extreme (Category I of Avise *et al.* 1987) there may be major phylogenetic discontinuities between mtDNA haplotypes which occupy discrete geographic regions within a species' range, suggesting long term zoogeographic barriers to gene flow. For example, regional populations of some birds exhibit deep phylogenetic differentiation suggesting historical population separation. A continent-wide survey of mtDNA diversity in the white-eye (*Zosterops lateralis*) revealed a major (2.3 % sequence divergence) phylogenetic break between eastern and western populations (Degnan & Moritz 1993).

Category	Genetic Divergence Pattern	Geographic Distribution			Likely Evolutionary Circumstance
		Region 1	Region 2	Region 3	
I	discontinuous	A B C	L M N	X Y Z	A. Long-term extrinsic (e.g. zoogeographic) barriers to gene flow; and/or B. Extinctions of intermediate genotypes in species with limited gene flow.
II	discontinuous	A B C L M N X Y Z			A. Recent, secondary admixture zones; or B. Intrinsic (e.g. reproductive isolation) barriers among sympatric sibling species.
III	continuous	L	M	N	Limited gene flow in a species not subdivided by long-term zoogeographic barriers.
IV	continuous	L M N			Very extensive gene flow in a species not subdivided by long-term zoogeographic barriers.
V	continuous	N	L	M	Intermediate gene flow in a species not subdivided by long-term zoogeographic barriers.

Figure 3.1. Possible intraspecific phylogeographic patterns observed in mtDNA studies. From Avise *et al.* (1987).

Similar geographically defined, phylogenetically divergent groups of mtDNA haplotypes have been reported for the seaside sparrow (*Ammodramus maritimus nigrescens*, Avise &

Nelson 1989) and fox sparrow (*Passerella iliaca*, Zink 1991a). At the other extreme there may be minimal divergence in a mtDNA phylogeny with a widespread geographic distribution of closely related mtDNA clones (Category IV of Avise *et al.* 1987), suggesting an absence of long-term barriers to dispersal and extensive gene flow.

Of particular relevance to genetic population structures in philopatric, colonially nesting birds is phylogeographic category III of Avise *et al.* (1987). This phylogeographic pattern (Fig. 3.1), in which all mtDNA haplotypes are phylogenetically closely related but are spatially separated, can be explained by historically limited gene flow (e.g. mediated by strong natal philopatry) between populations which have not been subdivided by long-term zoogeographic barriers to dispersal, and is phylogenetically equivalent to "island" and "stepping stone" models of genetic population structure (Avise *et al.* 1987).

3.1.3 Short-tailed shearwater

Although short-tailed shearwaters are transequatorial migrants, long-term banding studies suggest strong natal and breeding philopatry, with only very limited natal dispersal to adjacent colonies (Wooller *et al.* 1990). For example, of 23 banded adults found in a survey of 4 573 birds breeding on Little Green Island in Bass Strait during 1988, 17 had been banded on Little Green Island as adults or nestlings, three as nestlings on Great Dog Island and three as surface adults on Fisher Island (I. Skira personal communication). The latter two colonies are less than 5 km from Little Green Island. In addition, approximately 73 000 individuals were banded at various colonies in Victoria, New South Wales (NSW), South Australia and elsewhere in Tasmania over 18 years, but none have been recovered in a number of large surveys on Little Green Island or in annual surveys on Fisher Island (Serventy & Curry 1984). Approximately 4 800 short-tailed shearwaters have been banded since 1984: 193 have been recovered and 115 of these were recovered at a breeding colony. All of these birds were banded in the same colony in which they were subsequently recovered (Australian Bird and Bat Banding Schemes, personal communication). No breeding dispersal of short-tailed shearwaters has been recorded.

Populations of the short-tailed shearwater must have experienced considerable flux within the last 15 000 years. Most current breeding sites would have been unsuitable breeding habitat during the last glaciation, when the sea level was at least 100 m lower than it is today

(Milliman & Emery 1968), as they would have been up to 100 km from the coast. Most colonies must therefore have been colonised within the last several thousand years. Humans have had a substantial impact on shearwater populations, particularly since European settlement of Australia (Section 1.3.2) In the last 100 years many new colonies have been established both within and outside the historical range of the species, representing both a major range, and possible population, expansion (Davies 1959; Harris & Bode 1981). Individual colonies have become extinct, fluctuated in size and suffered severe bottleneck events (Gillham 1962; Harris & Bode 1981). There are well documented examples of colony size fluctuation on Phillip Island in Victoria during this century (Norman and Gottsch 1969), and the colony at Gabo Island, on the eastern Victorian coast, was reduced to approximately 100 pairs in 1959 (Gillham 1962). Thus, although breeding colonies may be relatively isolated on a generation-to-generation scale, they may be closely related through recent historical connections.

3.1.4 Aims

This study applied restriction-enzyme analysis of mtDNA to examine the population genetic structure of short-tailed shearwaters, representing 11 distinct nesting colonies, throughout southeastern Australia. One colony that had been subjected to a recent population bottleneck (Gabo Island; Gillham 1962), and several recently established colonies (Cape Direction, Cape Deslacs and Montague Island; Bryden 1966; Lane 1979) were included. The remaining eight colonies were all known or presumed to have predated European settlement. Philopatry may be acting to maintain genetic differentiation between breeding colonies in this species, as has been shown in colonial nesting fairy prions (*Pachyptila turtur*) occupying a similar breeding range (Ovenden *et al.* 1991). If individual colonies represent distinct intraspecific assemblages of mtDNA haplotypes, colony-specific mtDNA markers could be used to identify the natal origins of captured birds. This could provide valuable information on the relationship between birds during migration and foraging, on wintering grounds and during the breeding season. In addition, the natal origins of founding females of the recently established colonies could be determined. However, although breeding colonies may be genetically isolated through contemporary philopatry, patterns of mtDNA differentiation may be complicated by the unstable demographic history of this species.

3.2 METHODS

3.2.1 Sample collection

Liver samples were collected from 335 shearwater nestlings during the southern summer breeding seasons from 1989 to 1992 from 11 colonies in southeastern Australia (Table 3.1, Fig. 3.2), comprising six colonies in Tasmania at Whalebone Point, Bruny Island, Cape Deslacs, Cape Direction, Trial Harbour, Little Green Island and Great Dog Island; four colonies in Victoria at Port Fairy, Cape Woolamai, Phillip Island, Doughboy Island and Gabo Island; and Montague Island in NSW. At all colonies samples were collected from as large an area as possible to reduce possible sampling errors associated with birds breeding in close proximity to their natal burrow. Nestlings were removed from burrows by hand and killed by chloroform overdose or cervical dislocation. Liver tissue was dissected from dead birds and stored in liquid nitrogen within 30 min.

3.2.2 Extraction and purification of mtDNA

Mitochondrial DNA was extracted from thawed liver samples by a modification (Brasher *et al.* 1992b) of the method of Chapman and Powers (1984). Approximately 2 g of liver tissue was homogenised in five volumes of ice cold TEK buffer (50 mM Tris.HCl, 10 mM EDTA, 1.5% KCl (w:v), pH 7.5) with two strokes of a glass/Teflon homogeniser. The homogenate was underlayered with 5 ml of 15% sucrose-TEK (w:v) in a 50 ml centrifuge tube and centrifuged at 1 000 G for 10 min to remove cellular debris. The supernatant was carefully removed from above the sucrose layer, transferred to another 50 ml tube, underlayered with another 5 ml of 15% sucrose-TEK and centrifugation repeated. The supernatant was transferred to a 50 ml centrifuge tube, made to approximately 25 ml with ice cold TEK buffer and centrifuged at 18 000 G for 30 min at 4° C to pellet mitochondria. The supernatant was removed, the mitochondrial pellet resuspended in 25 ml of TEK buffer and centrifugation repeated. The final mitochondrial pellet was resuspended in 400 µl of TEK and transferred to a 1.5 ml centrifuge tube.

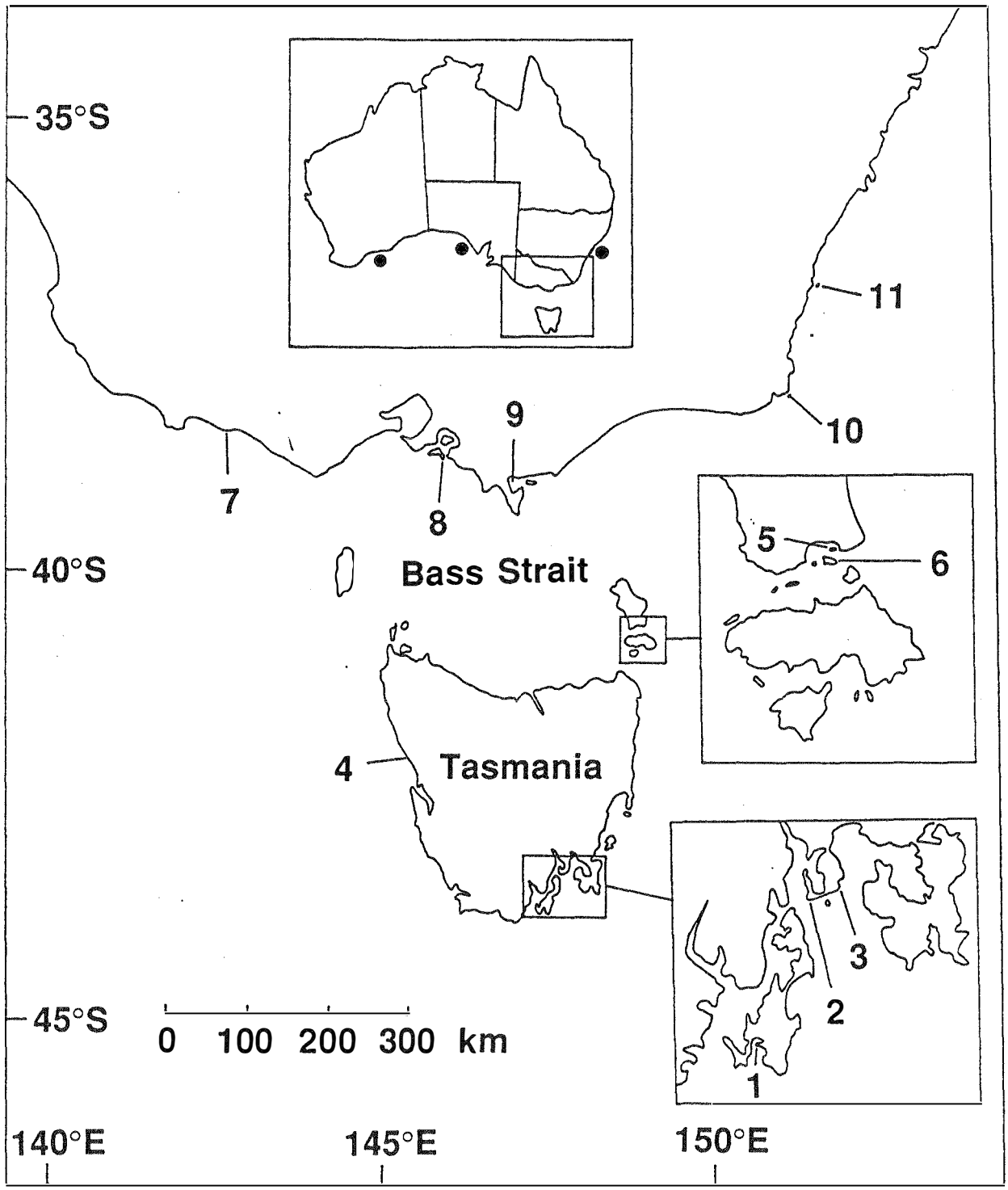

Figure 3.2. Locations of breeding colonies of short-tailed shearwaters: (1) Whalebone Point; (2) Cape Direction; (3) Cape Deslacs; (4) Trial Harbour; (5) Little Green Island; (6) Great Dog Island; (7) Port Fairy; (8) Cape Woolamai; (9) Doughboy Island; (10) Gabo Island; (11) Montague Island. Dots on map inset indicate positions of colonies at extremes of breeding range.

Table 3.1. Location, approximate size (b = number of burrows, p = number of pairs), sample size and history (approximate age of colony) for 11 short-tailed shearwater colonies included in this study.

Colony	Location	Size	Sample Size	History
(1) Whalebone Point	43°28'S 147°14'E	3 000 b[1]	29	Presumed pre-1800
(2) Cape Direction	43°06'S 147°25'E	20 000 b[1]	30	*c.* 1900[2]
(3) Cape Deslacs	42°59'S 147°33'E	20 000 b[1]	30	*c.* 1900[2]
(4) Trial Harbour	41°55'S 145°09'E	15 000 b[1]	30	Presumed pre-1800
(5) Little Green Island	40°13'S 148°15'S	150 000 b[1]	35	Pre-1800[3]
(6) Great Dog Island	40°15'S 148°14'E	375 000 p[3]	39	Pre-1800[3]
(7) Port Fairy	38°24'S 142°15'E	52 100 b[4]	30	Presumed pre-1800
(8) Cape Woolamai	38°34'S 145°22'E	356 000 b[5]	30	Presumed pre-1800
(9) Doughboy Island	38°46'S 146°17'E	2 000 b[4]	30	Presumed pre-1800
(10) Gabo Island	37°34'S 149°55'E	40 000 p[6]	30	Presumed pre-1800 Bottleneck 1959[8]
(11) Montague Island	36°15'S 150°13'E	15 000 p[7]	22	*c.* 1958[7]

[1] Naarding (1980); [2] Bryden (1966); [3] Skira *et al.* (1985); [4] Harris and Norman (1981); [5] Harris and Bode (1981); [6] Reilly (1977); [7] Lane (1979).

Mitochondrial membranes were lysed by the addition of 1/10 volume of 10% (v:v) non-idet P40 in TEK and incubated at room temperature for 10 min. The resulting lysate was centrifuged at 12 000 G for 10 min to pellet mitochondrial membranes. The supernatant was removed and proteins and remaining cellular debris were removed by 2 phenol (equilibrated with 1M Tris pH 8.0), 1 chloroform:IAA (24:1, v:v) extractions. For each phenol extraction the mtDNA solution was mixed gently by inversion for 1 min with 0.5 ml of the organic solvent, allowed to stand for 5 min and centrifuged at 12 000 G for 10 min and the upper, aqueous phase transferred to a fresh 1.5 ml centrifuge tube. For the chloroform:IAA extraction the supernatant was mixed by inversion for 1 min with 0.5 ml of the solvent and centrifuged at 12 000 G for 5 sec.

The final supernatant was transferred to a 1.5 ml centrifuge tube and mtDNA was precipitated by addition of two volumes of cold (-20° C) absolute ethanol, mixed vigorously and stored at -20° C overnight. The mtDNA precipitate was centrifuged at 12 000 G for 15 min at 4° C and the supernatant removed with a stretched Pasteur pipette. Pelleted mtDNA was dried under vacuum at room temperature for 20 min, resuspended in 100 µl of sterile water for every gram of starting tissue and subsequently stored at -20° C.

3.2.3 Restriction enzyme digestion and identification of mtDNA fragments

The mtDNA of 215 individuals collected from Whalebone Point, Cape Deslacs, Cape Direction, Trial Harbour, Little Green Island, Great Dog Island and Montague Island was digested with eight 6-base restriction enzymes (*Afl* II, *Apa* LI, *Bcl* I, *Bgl* II, *Bst* XI, *Eco* RV, *Hin* dIII, *Pvu* II) and three 5.33-base restriction enzymes (*Ava* I, *Ban* I, *Hae* II). For sample sizes see Table 1. Subsequently, with the availability of samples from more colonies and based on the results of the 6/5.33-base enzyme survey, the mtDNA of 231 individuals from Whalebone Point, Trial Harbour, Great Dog Island, Port Fairy, Cape Woolamai, Doughboy Island, Gabo Island and Montague Island were analysed with four 4–base restriction enzymes (*Hha* I, *Hinf* I, *Msp* I, *Taq* I). All of the birds from Whalebone Point, Trial Harbour, Great Dog Island and Montague Island analysed with 4–base restriction enzymes were included in the 6/5.33-base enzyme survey.

Each sample was digested in a reaction mix containing 3 µl of mtDNA solution, 2 µl of the

appropriate 10x restriction buffer and 1µl (5-20 U) of restriction enzyme in a final volume of 20 µl made up with sterile distilled water, according to the supplier's (New England Biolabs, USA) instructions. Restriction fragments produced by 6/5.33-base and 4-base restriction enzymes were radioactively labelled with ^{35}S-dCTP using the exonuclease and polymerase activity of Klenow fragment DNA polymerase I (Bresatec, South Australia) as described by Ovenden *et al.* (1989). The radioactively labelled restriction fragments produced by 6/5.33–base restriction enzymes were separated by molecular weight through 3 mm thick, 16x16 cm vertical, 1.4% agarose gels in TPE buffer (80mM Tris.phosphate, 2mM EDTA pH 8.0). The gels were electrophoresed at 15 mA for approximately 24 h or until the bromophenol blue marker dye had migrated more than two-thirds the length of the gel (Ovenden *et al.* 1988). A bacteriophage lambda DNA (New England Biolabs, Ca.) digested with *Hin* dIII was used as a molecular weight size standard.

The radioactively labelled restriction fragments produced by 4-base restriction enzymes were separated through 500 mm long, 0.25 mm thick 5% polyacrylamide gels in TBE buffer (89 mM Tris.HCl, 89 mM boric acid, 25 mM EDTA pH 8.2). The gels were electrophoresed for 1-2 h at 25 mA or until the bromophenol blue marker dye had migrated more than two-thirds the length of the gel (Smolenski *et al.* 1993). A size standard was provided by lambda DNA digested with *Bgl* I.

On the completion of electrophoresis, gels were dried using a fan heater and autoradiographed for 1-3 days.

3.2.4 Data analysis

Interpretation of mtDNA fragments

The 6/5.33-base and 4-base restriction-enzyme data were analysed separately. Unique fragment patterns identified with each enzyme were assigned an uppercase letter, the most common fragment pattern, or morph, for each enzyme being designated "A." For the 6/5.33–base restriction enzymes different gel fragment patterns between individuals for specific restriction enzymes can identify which fragments are joined when particular restriction sites are lost. Therefore although restriction sites for all enzymes were not mapped relative to one another, the loss or gain of restriction sites for each enzyme could be inferred

from the loss or gain of appropriately sized fragments. It was difficult to determine 4-base restriction-site gains and/or losses between individuals due to the large number of fragments. Subsequent analyses of the 4-base data, therefore, were based on restriction-fragment presence and absence. Analysis based on fragment homology can be unreliable if there is length variation, or if the overall sequence diversity exceeds 10-15% (Upholt 1977; Kessler & Avise 1985); these limitations were not approached in this study. Each individual in the 6/5.33-base and 4-base enzyme surveys was assigned a composite 11 or 4 letter code or haplotype, respectively, corresponding to the fragment patterns produced from that individual for each restriction-enzyme. Restriction site (6/5.33-base enzymes) or fragment (4-base enzymes) presence and absence was converted to binary form and entered into a computer for analysis.

Nucleotide sequence diversity

For the 6/5.33-base enzyme data, nucleotide sequence diversity was calculated between pairs of mitochondrial genomes, with standard errors, using the maximum-likelihood method of Nei and Tajima (1983). The number of nucleotide substitutions per base pair (∂) is given by

$$\partial = -\ln (1-\pi)$$ (equation 25 in Nei & Tajima 1983).

The value π, represents the probability that sequences X and Y will have different nucleotides at a given nucleotide position, and is calculated from the number of restriction sites present in X (m_X) and Y (m_Y), the number of sites shared by X and Y (m_{XY}) and the 'r' value of the enzyme (5.33 or 6). The standard deviation of ∂ ($V\partial$) is given by

$$V\partial=(2-S)(1-S)/(2r^2mS)$$ (equation 11 in Nei & Tajima 1983),

where

$$S = 2m_{XY}/(m_X + m_Y) \text{ and } m=(m_X + m_Y)/2.$$

An estimate of the number of nucleotide substitutions per base pair was calculated from the presence or absence of 4-base restriction-fragment data using an iterative method based on the equation

$$F\approx P^4/(3-2P)$$ (equation 20 in Nei & Li 1979),

where

$$F=2n_{XY}/(n_X + n_Y),$$

n_X and n_Y are the number of fragments for individuals X and Y, respectively, n_{XY} is the number of fragments shared by X and Y, and $P=e^{-r\partial/2}$.

Nucleotide sequence diversity within populations was calculated as

$$\pi=\Sigma_{i\neq j}\, d_{ij}/[n(n-1)]$$ (equation 2 in Nei & Jin 1989),

where d_{ij} is the estimate of the number of nucleotide substitutions between sequences i and j, n is the number of individuals sampled and $\Sigma_{i\neq j}$ represents the summation of all pairwise comparisons. The variance of π was calculated using equation 3 of Nei and Jin (1989),

$$V(\pi) = 1/[n(n-1)]^2\, [\Sigma_{i\neq j}V(d_{ij}) + \Sigma_{i\neq j}\Sigma_{k\neq 1}\mathrm{Cov}(d_{ij}.d_{kl})].$$

Net nucleotide sequence divergences between pairs of populations was calculated using equation 16 of Nei and Jin (1989),

$$d_A = d_{XY} - (d_X + d_Y)/2,$$

where d_X and d_Y are the π values in populations X and Y and d_{XY} is the average number of nucleotide substitutions per site between X and Y. The variance of d_A was calculated using equation 17 of Nei and Jin (1989). By calculating the variance of the net interpopulational sequence divergence it is possible to statistically test whether population X is significantly different from population Y using the null hypothesis that $d_A=0$. A Student's *t*-test, with Bonferroni correction for the large number of pairwise comparisons (Rice 1989), was used to determine whether the net divergence estimates were significantly different from zero. Because of the large number of pair wise comparisons made, a small number are expected to yield a significant result by chance alone (Ovenden *et al.* 1992). The sequential Bonferroni correction eliminates this bias by testing for significance at the table-wide level.

Chi-squared analysis of haplotype frequencies

A chi-square test was used to compare observed and expected haplotype frequencies, for haplotypes occurring more than once, to detect possible population subdivision among colonies. The significance of the chi-square value was tested using a Monte-Carlo method (Roff & Bentzen 1989) as expected class sizes were often less than five.

Gene diversity analysis

An estimate of genotypic diversity, the observed probability that a pair of individuals in a population possess a different mtDNA haplotype, was calculated as

$$G=(n/n-1)(1-\Sigma f_i^2),$$

where f_i is the frequency of the ith mtDNA haplotype in a population of size n (Nei 1987). G is the mtDNA equivalent of single-locus heterozygosity for nuclear genes (Zink 1991a).

An estimate of genetic variability due to geographic subdivision was gained using the population statistic G_{ST} (Takahata & Palumbi 1985). G_{ST} measures the proportion of genetic variation that is due to genetic differences between populations and uses restriction sites or fragments as alternate alleles. Equations 17 and 19 of Takahata and Palumbi (1985) were used to estimate the intrademe (I) and interdeme (J) identity probability using restriction-site (6/5.33-base enzyme data) or restriction-fragment (4-base enzyme data) presence/absence data from each population. The identity probability is the probability that two randomly sampled homologous DNA molecules will be identical. The magnitude of these identity probabilities are primarily determined by the effective migration rate between populations (Ovenden & White 1990).

$$I=1/[l(n-1)]\ \Sigma Ci(Ci-1) \text{ and}$$

$$J=1/l\ n.n'\ \Sigma Ci.Ci',$$

where n and n′ are the number of mitochondrial genomes sampled from each deme, l is the number of restriction sites/fragments identified within the n genomes from each deme, Ci and Ci′ are the numbers of genomes cut at restriction site i/numbers of genomes possessing restriction fragment i. Unlike the loss or gain of a restriction site, the loss or gain of a restriction fragment is not an independent event, as the gain of a restriction site will result in the appearance of two new restriction fragments. Kessler and Avise (1984) have argued that the non-independence of fragments is problematic only when data from single enzymes are analysed one at a time. In addition, a given site gain can result in the formation of one of a large number of possible new fragment pairs depending on the particular position of the site gain.

The large number of rare haplotypes (i.e. haplotypes found only once) identified in most mtDNA surveys of natural populations artificially inflates the proportion of genetic variation that occurs among demes (Palumbi *et al.* 1991a). The significance of the G_{ST} value obtained was, therefore, evaluated by bootstrapping (Palumbi & Wilson 1990), in which the original data set was sampled, with replacement, to generate a series of bootstrap samples which were the same size as the original data set. The true G_{ST} value was compared to the distribution of 1 000 bootstrap G_{ST} values, obtained from random rearrangements of the raw data, to estimate the error involved in calculating the true G_{ST} from the original data set.. Significant population subdivision was deemed to be present if the true G_{ST} value was

greater than 95% of the bootstrap G_{ST} values.

Phylogenetic reconstruction

Phylogenetic relationships between the observed 6/5.33-base and 4-base restriction enzyme mtDNA haplotypes were estimated using the unweighted pair-group method with arithmetic mean (UPGMA) (Sneath & Sokal 1973) which groups operational taxonomic units (OTUs) according to overall similarity or distance based on genetic distance values that have been computed for all pairs of OTUs. The UPGMA dendrogram was constructed from a nucleotide sequence diversity distance matrix between all pairs of haplotypes. The validity of the nodes in the UPGMA tree were assessed by the magnitude of standard errors on branch points relative to the distance between adjacent branch points (Nei *et al.* 1985). The major assumption of the UPGMA is that the rate of nucleotide substitution, along all branches of the tree, is equal. Despite this assumption, UPGMA has often been shown, in computer simulations to be superior in recovering the true species tree (Tatento *et al.* 1982; Nei *et al.* 1983).

Wagner parsimony analysis was applied to the the 6/5.33-base restriction-enzyme site binary character data using the tree bisection-reconnection branch swapping algorithm of version 3.1 of the computer program PAUP (Swofford 1993). Wagner parsimony (Kluge & Farris 1969; Farris 1970) is appropriate to restriction site data because it permits free reversibility of characters (i.e. change of character states in either direction is assumed to be equally probable, and character states may transform from one state to another and back again). A 50% majortity rule consensus network was produced from the most parsimonious networks identified and characters regulating the topologies of the consensus network were identified.

3.3 RESULTS

3.3.1 6/5.33-base Enzyme Survey

The 11 restriction enzymes identified a total of 63 restriction sites among 215 shearwater mitochondrial genomes, with 44 to 52 restriction sites assayed per mtDNA clone (Fig. 3.3). For four enzymes, an approximately 200 bp insert in two individuals compared to all other mtDNA genomes was observed. These individuals were omitted from subsequent analyses.

The size (x±SE) of the shearwater genome was estimated to be 20,768±62 base pairs from the sum of all fragments for a single individual representing each enzyme morph on all gels. The 11 restriction enzymes therefore assayed approximately 1.73% of the mtDNA genome in this species.

Each restriction-enzyme yielded one to six morphs (1: *Hin* dIII and *Pvu* II, 2: *Afl* II, *Apa* LI, *Ava* I, *Bgl* II and *Eco* RV, 4: *Ban* I, *Bcl* I and *Bst* XI, 6: *Hae* II) (Fig. 3.3) which, in combination, identified 25 haplotypes, differing by one to ten restriction-site gains or losses. There were 40 monomorphic restriction sites (present in all haplotypes) and 23 polymorphic sites (occurring in some but not all haplotypes). The three most common haplotypes (a, b and c) occurred in all seven colonies and collectively accounted for 84% of all individuals surveyed (Table 3.2). The remaining 22 haplotypes were represented by only one to nine individuals and, with one exception (haplotype d), were shared between two colonies (haplotypes e, f and g) or exclusive to single colonies (haplotypes h-y). The genotypic diversity (G) of mtDNA haplotypes identified in the 213 individuals was 0.61.

The mean mtDNA sequence diversity among the 213 shearwater genomes was 0.247 ± 0.100%, with a range of 0 to 1.850%. Two shearwaters, one from Whalebone Point (haplotype i) and the other from Trial Harbour (haplotype q) had the most divergent mitochondrial genomes (1.85 ± 0.62%). No variation in the intrapopulational sequence diversities were detected between the seven shearwater colonies (Table 3.3), which were all similar to the overall population nucleotide sequence diversity. The net interpopulational nucleotide sequence divergence between birds from pairs of colonies was zero (n=17) or not significantly different from zero (P>0.2; n=4) in all 21 possible pairwise comparisons. Haplotype frequencies for individuals sampled from the seven colonies were also not

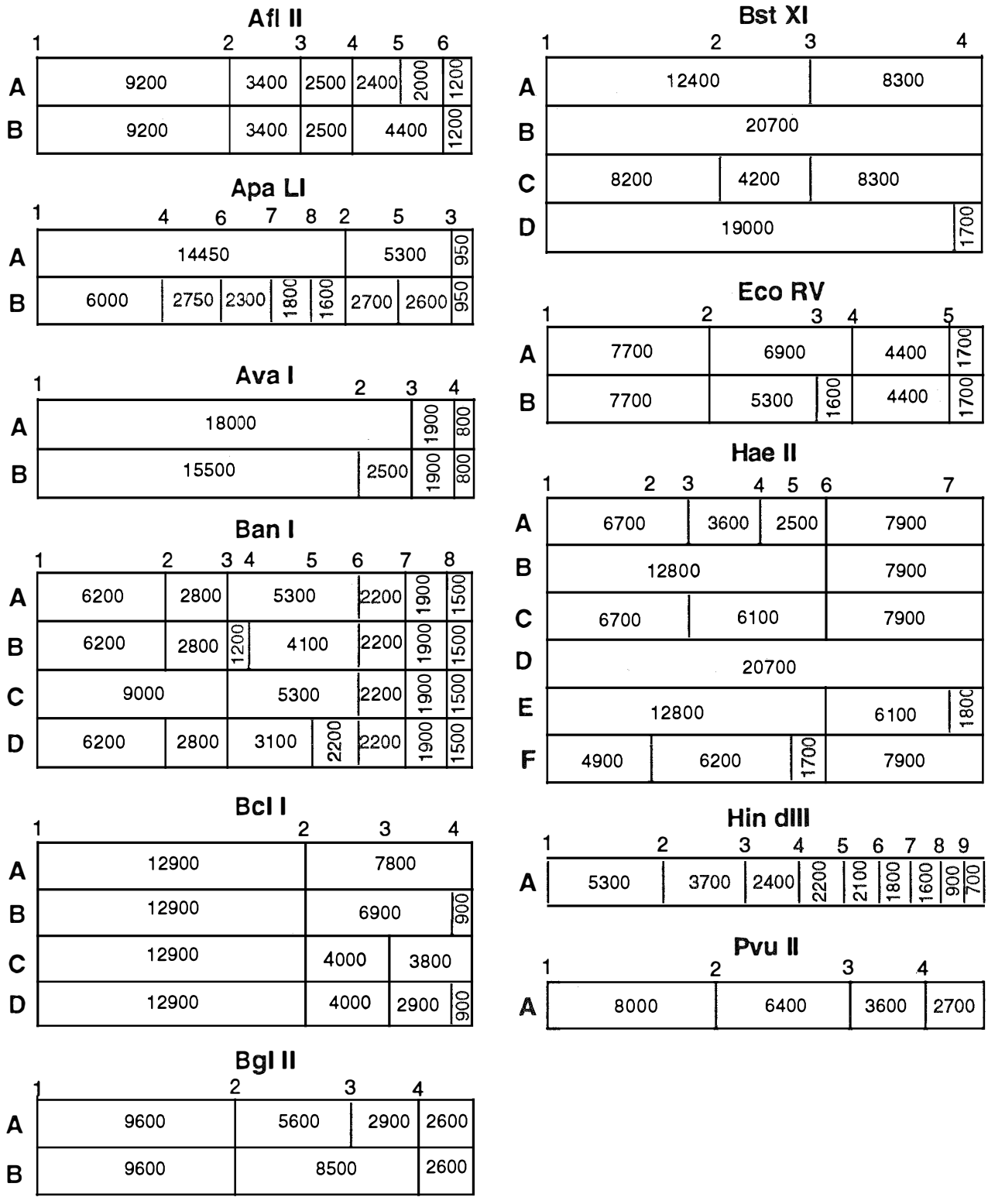

Figure 3.3. The fragment composition of the restriction site morphs identified among short-tailed shearwater mitochondrial genomes by 11 6/5.33-base restriction enzymes. Approximate size of fragments and the numbering of restriction sites are given.

Table 3.2. Numbers of 6/5.33-base restriction enzyme haplotypes scored from each of seven short-tailed shearwater colonies. Each haplotype composed of morph designations for restriction enzymes *Afl* II, *Apa* LI, *Ava* I, *Ban* I, *Bcl* I, *Bgl* II, *Bst* XI, *Eco* RV, *Hae* II, *Hin* dIII and *Pvu* II, respectively.

		Locality (*n*)							
	Haplotype	1 Whalebone Point (29)	3 Cape Deslacs (29)	2 Cape Direction (30)	4 Trial Harbour (30)	5 Little Green Island (34)	6 Great Dog Island (39)	11 Montague Island (22)	Total
a	AAAAAAAAAAA	14	19	17	17	23	25	13	128
b	AAAAAAAABAA	6	5	6	5	4	7	4	37
c	AAAAAAAACAA	2	1	2	4	1	2	1	13
d	AABAAAAABAA	1	0	2	0	2	2	2	9
e	AAAAAABAAAA	1	0	2	0	0	0	0	3
f	AAAAABAABAA	0	0	0	1	0	1	0	2
g	AAAACAAAAAA	0	1	0	0	1	0	0	2
h	AAAAAAAADAA	2	0	0	0	0	0	0	2
i	AABAAABAEAA	1	0	0	0	0	0	0	1
j	AAAADAAABAA	1	0	0	0	0	0	0	1
k	AAAAAADAAAA	1	0	0	0	0	0	0	1
l	AAAAAAAAFAA	0	1	0	0	0	0	0	1
m	AAAAAABABAA	0	1	0	0	0	0	0	1
n	AAAAAACAAAA	0	1	0	0	0	0	0	1
o	AABABAABBAA	0	0	1	0	0	0	0	1
p	AAABAAAAAAA	0	0	0	1	0	0	0	1
q	ABAAAAAAAAA	0	0	0	1	0	0	0	1
r	BABAAAAABAA	0	0	0	1	0	0	0	1
s	AAAAABAACAA	0	0	0	0	1	0	0	1
t	AABABAAABAA	0	0	0	0	1	0	0	1
u	BAAAAAAAAAA	0	0	0	0	1	0	0	1
v	AABAAACABAA	0	0	0	0	0	1	0	1
w	AAAAABAAAAA	0	0	0	0	0	1	0	1
x	AAADAAAAAAA	0	0	0	0	0	0	1	1
y	AABCAAAABAA	0	0	0	0	0	0	1	1

Table 3.3. Percent intrapopulational mtDNA nucleotide sequence diversity (x±SE) for 11 short-tailed shearwater colonies.

Locality	6/5.33-base enzyme	4-base enzyme
1 Whalebone Point	0.3286±0.1396	0.2038±0.1104
4 Trial Harbour	0.2710±0.1342	0.2304±0.0980
2 Cape Direction	0.2565±0.1312	—
3 Cape Deslacs	0.2142±0.1132	—
6 Great Dog Island	0.2205±0.1215	0.2639±0.1004
5 Little Green Island	0.2142±0.1026	—
7 Port Fairy	—	0.2455±0.1000
8 Cape Woolamai	—	0.2573±0.0943
9 Doughboy Island	—	0.2952±0.1078
10 Gabo Island	—	0.2145±0.0948
11 Montague Island	0.2581±0.1300	0.2462±0.1081

significantly different. The observed chi-square value for haplotypes occurring more than once was 41.24. This value was less than 494 of 1 000 random rearrangements of the data set giving a probability of obtaining a value of chi-square as large or larger than the observed chi-square of P=0.49±0.032. The null hypothesis of homogeneity of haplotype frequencies between colonies could not be rejected. The amount of genetic variation among colonies due to geographic subdivision (G_{ST}) was 0.284. This value does not demonstrate significant population differentiation as it greater than only 46% of the 1 000 bootstrapped G_{ST} values (range 0.264 to 0.323).

The 25 haplotypes were clustered by UPGMA (Fig. 3.4) into two major groups, the first containing the three most common haplotypes a, b and c, and the second containing the fourth most common haplotype, d. However these groups were not supported strongly because of considerable overlap in the standard errors on the branch points separating each group. Within each group, common and rare haplotypes were geographically widespread and there was no tendency for closely related haplotypes to be geographically localised.

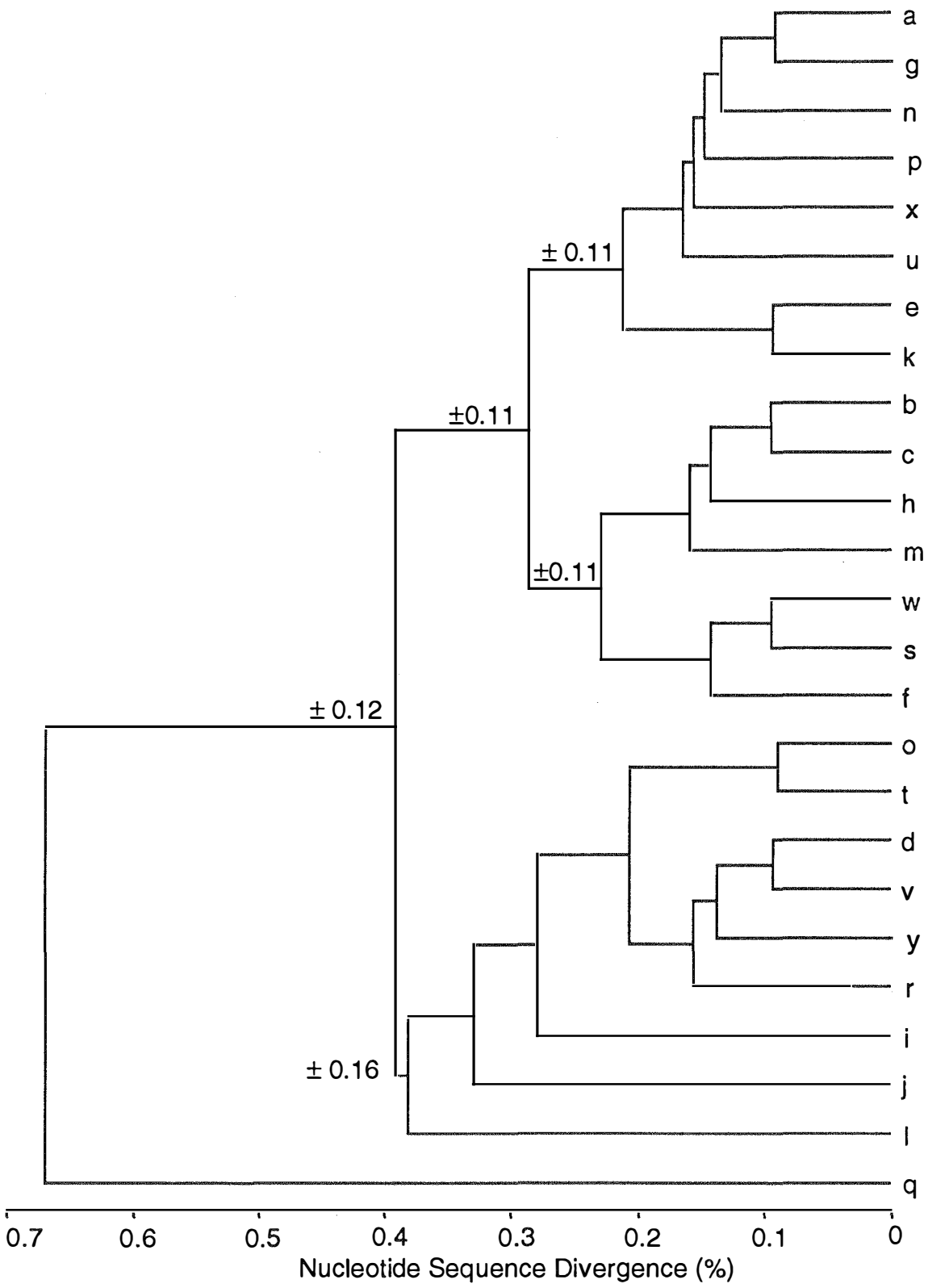

Figure 3.4. UPGMA dendrogram describing relationships between 25 short-tailed shearwater mtDNA haplotypes, identified with 11 6/5.33-base restriction enzymes, based on pairwise mtDNA nucleotide sequence divergence. Size of standard error for selected branch points are indicated.

Parsimony analysis of the substitutional relationships among the 25 haplotypes produced 675 most parsimonious networks of length 31 steps. A 50% majority-rule consensus network, of length 34 steps (consistency index 0.676), revealed two clades centred on common haplotypes a and d, separated by five mutational steps (Fig. 3.5). Two of these changes involved a single *Bgl* II restriction site (i.e. a reversal) and a third (*Ava* I site 2) exhibited considerable homoplasy in the group centred on haplotype d. Within each group most haplotypes were separated by only two or three restriction-site changes. The consensus network, as with the UPGMA dendrogram, was geographically uninformative, because haplotypes from geographically close colonies were widely distributed throughout the network. In comparison to the UPGMA dendrogram the the topology of the parsimony network exhibited considerable differences in the relationships between common haplotypes b, c and d and the associated rare haplotypes. These appear to be a result of the large number of convergent character changes controlling the topology of the parsimony network. For example, although haplotypes b and c were separated by 5 site changes, they differed by only a single *Hae* II site change. The remaining 4 changes involved reversals of *Bgl* III site 3 and *Ava* 2 site 2.

3.3.2 4-base enzyme survey

The four restriction-enzymes produced 117 restriction fragments from the 231 shearwater mitochondrial genomes (Appendix 3a). Almost twice as many fragments (83-86) were identified per mtDNA clone, compared to the 6/5.33-base enzyme survey. The four enzymes assayed 2.27% of the shearwater mitochondrial genome.

A total of 48 composite haplotypes were identified, consisting of 6 to 15 morphs per restriction enzyme (6: *Hinf* I, 8: *Taq* I, 11: *Msp* I, 15: *Hha* I). Three haplotypes (1, 2 and 3) were common to all eight colonies sampled and represented 39% of all individuals (Table 3.4). Three haplotypes (4, 5 and 6) were found in seven colonies. The remaining 42 haplotypes were represented by eight or less individuals and occurred in fewer than five of the eight colonies.

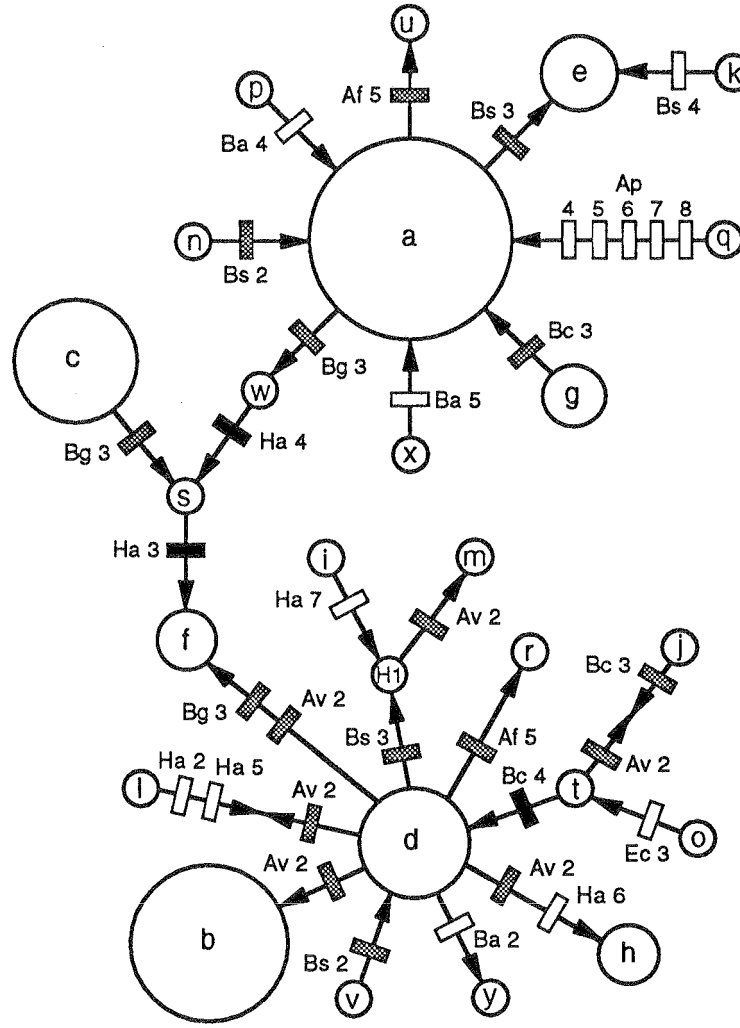

Figure 3.5. Unrooted consensus network describing relationships among 25 short-tailed shearwater mt DNA haplotypes, identified with 11 6/5.33-base restriction enzymes. Values in boxes alongside branches represent the percentage of times that the group above that branch (assuming haplotype d represents the bottom of the network) occurred in 675 parsimonious 31 step trees. Haplotype designations are as in Table 3.2. Haplotype H1 represents a hypothetical haplotype that was not observed. Labelled bars and arrows on lines joining haplotypes indicate the direction of loss of restriction sites. Each restriction site is denoted by the first two letters of the enzyme and the site number for that enzyme (eg. Bg 3 = *Bgl* I Site 3). Open and solid bars represent site changes that were autapomorphic with respect to haplotypes and clades, respectively. Shaded bars represent convergent synapomorphic site changes. The size of each circle is proportional to the frequency of that haplotype.

Table 3.4. Numbers of 4-base restriction enzyme haplotypes scored from each of eight short-tailed shearwater colonies. Each haplotype composed of morph designations for restriction enzymes *Hha* I, *Msp* I, *Hinf* I and *Taq* I, respectively.

	Locality (*n*)								
Haplotype	1 Whalebone Point (29)	4 Trial Harbour (30)	6 Great Dog Island (30)	7 Port Fairy (30)	8 Cape Woolamai (30)	9 Dough-Boy Island (30)	10 Gabo Island (30)	11 Montague Island (22)	Total
1 AAAA	11	7	3	5	10	2	5	3	46
2 ABAA	3	3	5	4	1	3	5	2	26
3 CAAA	4	3	2	2	1	2	3	1	18
4 BAAA	0	4	4	3	4	2	6	5	28
5 BBAA	1	2	2	5	2	1	3	0	16
6 DAAA	3	2	1	2	2	0	1	2	13
7 CBAA	3	0	1	0	0	2	0	2	8
8 CDAA	1	1	0	0	2	1	0	1	6
9 DBAA	0	2	0	0	1	2	1	0	6
10 EAAA	1	0	0	2	0	2	0	0	5
11 FAAA	0	0	3	0	0	1	0	1	5
12 CAAB	0	1	2	1	0	0	1	0	5
13 FBAA	0	0	0	1	0	3	1	0	5
14 CCAA	0	0	0	1	0	1	1	2	5
15 EBAA	0	1	1	0	0	0	1	0	3
16 AAAC	0	0	1	0	1	0	0	0	2
17 EAAB	0	0	1	0	0	1	0	0	2
18 CFAA	0	0	0	0	1	1	0	0	2
19 IAAA	1	0	0	0	0	0	0	0	1
20 BGAA	1	0	0	0	0	0	0	0	1
21 AADA	0	1	0	0	0	0	0	0	1
22 ABCA	0	1	0	0	0	0	0	0	1
23 GAAA	0	1	0	0	0	0	0	0	1
24 BAAE	0	1	0	0	0	0	0	0	1
25 BCAA	0	0	1	0	0	0	0	0	1
26 HDAD	0	0	1	0	0	0	0	0	1
27 JBBA	0	0	1	0	0	0	0	0	1
28 BBAF	0	0	1	0	0	0	0	0	1
29 BIAA	0	0	0	1	0	0	0	0	1
30 KAAA	0	0	0	1	0	0	0	0	1
31 BEAA	0	0	0	1	0	0	0	0	1
32 CAAC	0	0	0	1	0	0	0	0	1
33 CCAG	0	0	0	0	1	0	0	0	1
34 CAAH	0	0	0	0	1	0	0	0	1
35 DDAA	0	0	0	0	1	0	0	0	1
36 LAAC	0	0	0	0	1	0	0	0	1
37 AHAA	0	0	0	0	1	0	0	0	1
38 AJAA	0	0	0	0	0	1	0	0	1
39 AEAA	0	0	0	0	0	1	0	0	1
40 DKAA	0	0	0	0	0	1	0	0	1
41 ECEA	0	0	0	0	0	1	0	0	1
42 EAFA	0	0	0	0	0	1	0	0	1
43 MCBA	0	0	0	0	0	1	0	0	1
44 DAAB	0	0	0	0	0	0	1	0	1
45 AAAB	0	0	0	0	0	0	1	0	1
46 NAAA	0	0	0	0	0	0	0	1	1
47 ACAA	0	0	0	0	0	0	0	1	1
48 OAAA	0	0	0	0	0	0	0	1	1

Thirty haplotypes were exclusive to a single colony. The genotypic diversity (G) of the 231 shearwater mtDNA genomes in this survey was 0.92.

The mean mtDNA sequence diversity among the 231 shearwater genomes was 0.247±0.060%, with a range of 0 to 0.890%. Mean intrapopulational diversities were similar both among colonies and with estimates derived from the 6/5.33-base enzyme survey (Table 3.3). Pairwise comparisons of all eight colonies revealed 27 net interpopulational mtDNA divergence estimates that were either zero (n=13) or not significantly different from zero (n=14) (Table 3.5).The one remaining comparison between the Doughboy Island and Trial Harbour colonies yielded a significantly large net interpopulational mtDNA divergence of 0.0158±0.0048% (0.002>P>0.001). A chi-squared test of homogeneity of morphs for all haplotypes occurring more than once was nonsignificant. The observed chi-square was 130.65 which was less than 195/1000 random rearrangements of the data set (P=0.195±0.025). The G_{ST} value for the eight populations was 0.1922, being greater than only 42% of the 1 000 bootstrapped G_{ST} values (range 0.172 to 0.209).

Table 3.5. Mean percent interpopulational mtDNA divergence estimates ($x10^{-2}$); (above diagonal) and standard errors ($x10^{-2}$); (below diagonal) for short-tailed shearwater genomes analysed with 4-base restriction enzymes. Figure in bold type denotes a significant difference in mtDNA divergence.

	Locality							
Locality	1	4	6	7	8	9	10	11
1 Whalebone Pt.	-	0.078	0.767	0.696	0.146	1.169	0.688	0
4 Trial Harbour	0.209	-	0	0	0	**1.580**	0	0
6 Great Dog Island	0.676	0	-	0	0.338	0.729	0	0
7 Port Fairy	0.565	0	0	-	0.271	1.056	0	0
8 Cape Woolamai	0.577	0	0.560	0.216	-	2.120	0.151	0
9 Doughboy Island	1.07	0.481	0.235	0.356	0.993	-	1.400	0.322
10 Gabo Island	0.651	0	0	0	0.311	0.753	-	0
11 Montague Island	0	0	0	0	0	0.331	0	-

The UPGMA dendrogram (Fig. 3.6) clustered the 48 haplotypes into two major groups, the first containing five of the six most common haplotypes (1, 2, 4, 5, 6) and the second common haplotype 3. The standard errors on all branch points were considerably larger than the length of the branches separating the respective groups, so this grouping of haplotypes was not strongly supported. Closely related haplotypes within each group were not geographically localised. Due to the large number of haplotypes and characters assayed it was deemed impractical to undertake a parsimony analysis of the 4-base restriction enzyme data.

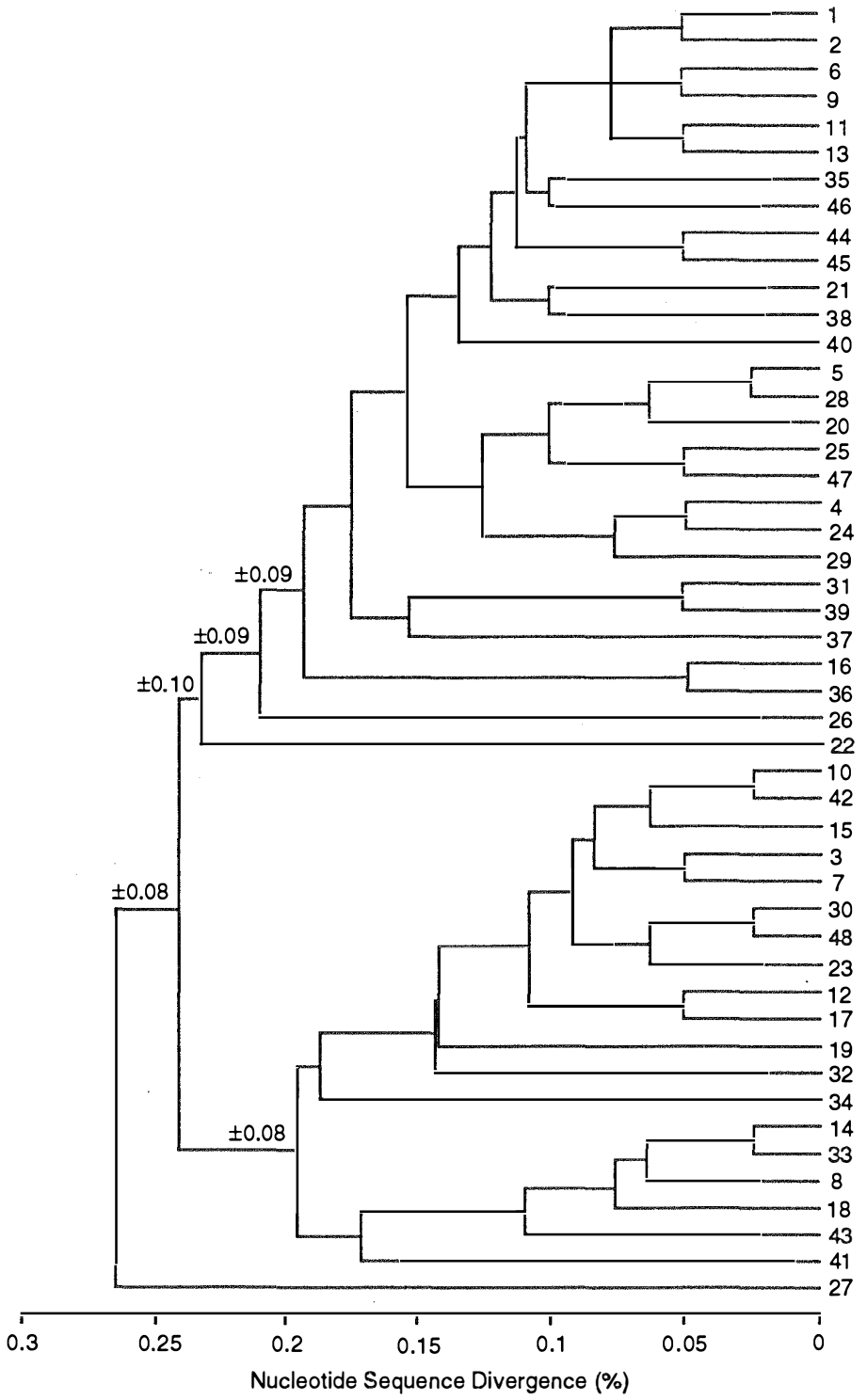

Figure 3.6. UPGMA dendrogram describing relationships between 48 short-tailed shearwater mtDNA haplotypes, identified with four 4-base restriction enzymes, based on pairwise mtDNA nucleotide sequence divergence. Size of standard error for selected branch points are indicated.

3.4 DISCUSSION

3.4.1 Characteristics of shearwater mtDNA

The size of the short-tailed shearwater mitochondrial genome (20.7 kb) is larger than the range of 16.3-17.3 reported by Shields and Helm-Bychowski (1988) for birds, but is equivalent to the upper size limit reported for mourning doves (*Zenaida macroura*, Ball & Avise 1992) and is only 1-2 kb larger than the fairy prion (*Pachyptila turtur*, Ovenden *et al.* 1991) and 10 species of shore bird (Dittman & Zink 1991). Two individuals appeared to have a genome size approximately 200 bp larger than all other birds surveyed. Although not common in birds (Avise & Zink 1988; Ball & Avise 1992), variation in mtDNA size between individuals has been reported for a wide range of organisms (Moritz *et al.* 1987). Small length variation commonly occurs in the control region and can arise from duplication or deletion of DNA segments or replication slippage (Moritz *et al.* 1987).

Short-tailed shearwater mtDNA exhibits a low level of variability which is generally comparable to the mean nucleotide sequence diversities for other species of birds that lack significant population genetic structure such as great tits, *Parus major* (Tegelström 1987a), red-winged blackbirds, *Agelaius phoeniceus* (Ball *et al.* 1988), pied flycatchers, *Ficedula hypoleuca* (Tegelström *et al.* 1990), common murres, *Uria aalge* (Moum *et al.* 1991), common grackles, *Quiscalus quiscula* (Zink *et al.* 1991c), song sparrows, *Melospiza melodia* (Zink 1991a; Ball & Avise 1992), downy woodpeckers, *Picoides pubescens*, mourning dove, *Zenaida macroura*, brown-headed cowbird, *Molothrus ater* (Ball & Avise 1992), chickadees, *Parus* spp. (Gill *et al.* 1993), chipping sparrow, *Spizella passerina* (Zink & Dittman 1993a). The low level of intraspecific mtDNA diversity in short-tailed shearwaters reflects the absence of divergent haplotypes in this species.

3.4.2 6/5.33-base versus 4-base restriction enzyme data

The rationale for using 4-base restriction enzymes in the second part of this study is related to the degree of resolution provided by 6/5.33-base enzymes for detecting population differentiation. Ovenden (1990) has discussed the use of 6-base and 4-base restriction enzymes in relation to the extent of intraspecific mtDNA sequence diversity. Because of the shorter recognition sequence, 4-base restriction enzymes cut mtDNA more frequently and therefore can sample a greater proportion of nucleotides within the genome, than 6-base enzymes. Ovenden (1990) suggested that 4-base enzymes provide a greater level of

resolution and are, therefore, more likely to detect population subdivision if it is present, than 6-base enzymes when intraspecific mtDNA diversity is low (less than 0.15%). The increased resolving power of 4-base enzymes, relative to 6-base enzymes, has been empirically demonstrated by Smolenski *et al.* (1993) in a restriction enzyme survey of stock separation in a marine fish, the orange roughy (*Hoplostethus atlanticus*). Ten 6-base enzymes revealed a low (0.126%) intraspecific mtDNA diversity and no evidence for reproductively isolated populations. However, an analysis of a subset of the same group of fish with three 4-base restriction enzymes identified genetic subdivision between two geographic regions.

The low mean mtDNA sequence diversity assessed with the 6/5.33-base restriction enzymes suggested that the resolution of these enzymes may be insufficient to detect real genetic differences between shearwater colonies (although according to Ovenden (1990) for a mtDNA genome size of 20768 bp and intraspecific diversity of 0.247% only thirteen 6-base restriction enzymes are theoretically required to detect at least one variant nucleotide per genome). The four 4-base restriction enzymes assayed approximately 100 base pairs more of the mtDNA genome, over almost twice as many restriction sites, as the eleven 6/5.33-base restriction enzymes. The high mtDNA genotypic diversity (G=0.92) revealed by the 4-base restriction enzymes, relative to the 6/5.33-base mtDNA haplotypes (G=0.61), suggests that the 4-base enzymes resolved most of the mtDNA variation present in the short-tailed shearwater. The 4-base restriction enzyme survey included colonies from a wider geographic range which encompassed all of the colonies examined with the 6/5.33-base restriction enzymes. However the 4-base enzyme survey detected an identical level of intraspecific mtDNA sequence diversity and similar levels of diversity within populations. Apart from two colonies (Doughboy Island and Trial Harbour) the 4-base enzymes did not resolve population differentiation.

3.4.3 Philopatry, gene flow and genetic population structure

The genetic population structure of a species comprises two, partly interdependent components: the magnitude of mtDNA sequence diversity and the extent to which this diversity is subdivided within and between geographically separate populations (Avise 1989). Short-tailed shearwaters across southeastern Australia exhibited a low estimated mean sequence diversity among pairs of mtDNA genomes. There was no evidence, as assessed by net nucleotide sequence divergence, gene diversity or haplotype frequency, that this mtDNA diversity was geographically structured among shearwater breeding colonies. Most

individuals possessed one of a small number of closely related haplotypes. Most rare haplotypes differed from the common haplotypes by only one or two restriction site changes. The small phylogenetic divergence between mtDNA haplotypes which were geographically widespread among sampled colonies corresponds to a phylogeographic category of organisms (Category IV of Avise *et al.* 1987, Fig. 3.1) characterised by high levels of gene flow and which have not been subdivided by long-term biogeographic barriers. Together, these aspects of shearwater mtDNA variability demonstrate a lack of detectable genetic population structure which consists of a group of geographically undifferentiated, closely related haplotypes.

This result is, in part, unexpected for a number of reasons. First, the colonies included in this study encompass the majority (in terms of numbers of birds) of the species breeding range; individual colonies were separated by distances of up to 860 km. Second, no long- distance natal or breeding dispersal has been recorded in this species despite large-scale and long-term banding and recovery surveys. Over a 40-year period, only 23 successful natal dispersals have been recorded in surveys of tens of thousands of burrows; each dispersal event involved a distance of less than 5 km between natal and breeding colony (I. Skira personal communication; Serventy & Curry 1984; Wooller *et al.* 1990). Third, strict philopatry has been demonstrated, by restriction-enzyme analysis of mtDNA, in at least one colony of the fairy prion, a colonially nesting seabird occupying a breeding range similar to that of the short-tailed shearwater (Ovenden *et al.* 1991).

The significantly large net interpopulational sequence divergence between the Doughboy Island and Trial Harbour colonies suggests limited gene flow between them. This result is difficult to understand since the Doughboy Island colony is situated near the centre of the species' range, neither colony display any apparently unique characters in terms of its size, age or history compared to any other colony; and neither colony appears to be genetically isolated from the three more geographically distant colonies at Montague Island, Gabo Island and Whalebone Point.

The limited geographic structuring among mtDNA haplotypes suggests contemporary gene flow and/or recent evolutionary connections between populations (Slatkin 1987; Slatkin & Maddison 1989). Indeed, the geographically widespread occurrence of both the common and rare haplotypes, observed in more than one individual, indicates an extensive and recent

intercolony exchange. This phylogeographic pattern is similar to those found in other species of colonially nesting birds, such as common murres (*Uria aalge*, Moum *et al.* 1991), white ibises (*Eudocimus albus*, Stangel *et al.* 1991), and thick-billed murres (*Uria lomvia*, Birt-Friesen *et al.* 1992), but is different from the geographically structured pattern found in Canada geese (*Branta canadensis*, Van Wagner & Baker 1986; Shields & Wilson 1987), black brant (*Branta bernicla nigricans*, Shields 1990), fairy prions (*Pachyptila turtur*, Ovenden *et al.* 1991) and dunlins (*Calidris alpina*, Wenink *et al.* 1993).

The relatively conservative magnitude of intraspecific genetic divergence in shearwaters and birds in general (Tegelström 1987a; Ball *et al.* 1988) is consistent with extensive contemporary gene flow among populations, and reflects a high, flight-mediated, dispersal potential. Birky *et al.* (1983) and Slatkin (1987) have shown that an exchange of as few as one or two females per generation would be sufficient to counteract genetic drift in the mitochondrial genome. Estimates of gene flow between populations involve estimating Nm, where N is the local population size and m is the migration rate between populations. The magnitude of Nm indicates the importance of gene flow relative to genetic drift. Where $Nm>1$ high gene flow is indicated and genetic divergence between populations will be minimal. Values of $Nm<1$ imply low levels of gene flow, such that random genetic drift will result in substantial genetic differentiation. Assuming equilibrium between gene flow and genetic drift among populations, the effective gene flow, or number of females exchanged per generation (Nm), among colonies can be estimated using gene diversity data (Takahata & Palumbi 1985) or the observed frequency of private alleles within samples (Slatkin 1985b). Both methods produce similar estimates of gene flow under a wide variety of population conditions (Slatkin & Barton 1989). Nm can be calculated from G_{ST} as

$$Nm=(1/G_{ST}-1)/2$$ (Takahata & Palumbi 1985).

The G_{ST} values calculated from the 6/5.33-base and 4-base enzyme data give estimates of $Nm=1.4$ and 2.1, respectively. This suggests a low level of gene flow which would be sufficient to maintain the observed genetic homogeneity of mtDNA haplotypes. However the G_{ST}-based estimates of gene flow are unreliable when there are relatively few or no common haplotypes (Avise *et al.* 1992), as is the case for short-tailed shearwaters. Also, due to recent fluctuations in overall population and individual colony size and colony number (see below) it is unlikely that short-tailed shearwater populations have reached genetic equilibrium. Estimates of Nm greater than one must be interpreted as indicating sufficient

contemporary gene flow to prevent genetic divergence of populations or a recent historical association between the sampled populations and, therefore, these methods cannot distinguish between contemporary gene flow and historical associations of colonies (Slatkin & Maddison 1989). Banding studies suggest strong philopatry, although a small number of natal dispersals to geographically very close colonies have been recorded (Wooller *et al.* 1990). However, no long-distance dispersal has been reported. Thus, contemporary estimates of gene flow between existing breeding colonies cannot account for their genetic similarity. Avise *et al.* (1992) have discussed the problems raised by such contrast between band-return studies and the geographic distributions of mtDNA. They suggested that "an understanding of population structure requires the integration of both evolutionary (genetic) and contemporary (direct observational) perspectives." Based on this evidence, it is unlikely that a low level of contemporary intercolony movement by females is responsible for the observed homogeneity of mtDNA haplotypes. However the current patterns of mtDNA differentiation can be explained by two types of gene flow involving recent evolutionary connections between breeding colonies (Slatkin 1985a).

Gene flow between colonies may involve rare, large-scale movements of individuals associated with extinction and recolonisation of local populations (Slatkin 1985a; Slatkin 1987; Avise *et al.* 1992). Circumstantial evidence of this type of gene flow in short-tailed shearwaters is available, given that the low intrinsic population growth through reproduction (Wooller *et al.* 1988) cannot account for the rapid growth in size observed in several colonies. The Montague Island colony and others on the NSW coast were founded in the last 35 years (Lane 1979) and have grown rapidly. Similarly, colonies at Cape Direction and Cape Deslacs appeared early this century (Bryden 1966). Learmonth (1965) recorded the reestablishment of a breeding colony at Portland on the southwest coast of Victoria after a lapse of 70 years. The Gabo Island colony suffered a severe population bottleneck in 1959 in which colony size was reduced to approximately 100 pairs (Gillham 1962) from which it has rapidly recovered to a size of 30 to 40 000 pairs (Reilly 1977).

The genetic data support this hypothesis. The intrapopulational sequence diversities (Table 3.2) in the recently established colonies at Cape Direction, Cape Deslacs and Montague Island, and in the Gabo Island colony following the bottleneck event are similar to the genetic diversity for the population overall. Seutin *et al.* (1993) report a similar absence of reduced

mtDNA nucleotide diversity for island populations of the streaked saltator (*Saltator albicollis*) that were likely to have experienced population bottlenecks and founder events during island colonisation. This suggests large founder populations and population recovery via a substantial, and probably opportunistic, immigration of birds (Slatkin & Maddison 1989). Birt-Friesen *et al.* (1992) have suggested a similar scenario for philopatric, colonially nesting thick-billed murres (*Uria lomvia*). In contrast, a recently formed fairy prion colony was found to be devoid of mtDNA restriction site variation, suggesting a very small founder population and a lack of large scale gene flow (Ovenden *et al.* 1991).

On an evolutionarily more distant scale, gene flow may be associated with connections among colonies as a result of a major range expansion, from a small number of ancestral populations, which has produced the current geographic distribution of breeding colonies (Slatkin 1985a). Most current breeding colonies must have originated in the last 10 000 years and may have been derived from a restricted breeding population during the Pleistocene glaciation. The last glaciation reduced sea-levels by up to 100 m (Milliman & Emery 1968) so most current breeding sites would have been inland and unsuitable breeding habitat. The current breeding strong-hold of the species, Bass Strait was entirely exposed and formed a land bridge between Tasmania and mainland Australia (Davies 1974). Glaciation may have forced the species' breeding range north where, if the number of suitable breeding islands was similar to the present, the population size may have been substantially reduced. Most of the current breeding range must have been colonised since the retreat of the Pleistocene glaciers and subsequent rise in sea level between 12 000 and 6 000 years ago (Dartnall 1974), and it is also probable that population size has expanded dramatically in the recent past and appears to have continued until present times. Similar scenarios involving postglacial range expansions have been invoked to explain the limited geographic structuring of mtDNA haplotypes in pied flycatchers (*Ficedula hypoleuca*, Tegelström *et al.* 1990), common murres (*Uria aalge*, Moum *et al.* 1991), common grackles (*Quiscalus quiscula*, Zink *et al.* 1991c), chipping sparrows (*Spizella passerina*, Zink & Dittman 1993a) and song sparrows (*Melospiza melodia*, Zink & Dittman 1993b).

The lack of significant population structure as determined by mtDNA analysis does not necessarily prove a complete absence of genetic differentiation in this species. First,

restriction enzyme analysis of mtDNA may provide insufficient resolution to detect slight genetic differentiation over relatively short evolutionary time scales (Palumbi *et al.* 1991a). Although the high rate of mtDNA evolution accelerates the genetic differentiation among populations, the temporal resolution of restriction enzyme surveys is dependent upon the number of restriction sites examined (Ovenden 1990; Palumbi *et al.* 1991a). Populations that have diverged only recently, on an evolutionary time scale, may not have accumulated sufficient mtDNA nucleotide divergence to be detected by restriction enzyme techniques. The 6/5.33-base and 4-base restriction enzymes examined only 1.73% and 2.27% of the shearwater mitochondrial genome, respectively. Tegelström (1987a) has shown that a reduction in the number of restriction sites analysed will reduce the number of mtDNA haplotypes identified. The number of restriction sites examined using the 6/5.33-base and 4-base enzymes may have been insufficient to reveal a large proportion of the variation present. If most shearwater colonies formed in recent evolutionary time this level of resolution may be too coarse to detect real but small genetic differences between breeding colonies. However, as has already been discussed, the high genotypic diversity of 4-base mtDNA haplotypes suggests that most of the mtDNA variation in this species was revealed, at least by the 4-base restriction enzymes.

Second, mtDNA surveys assess only one gene tree within an organismal pedigree, and this gene tree represents the matriarchal component of a species phylogeny (Avise 1989; Ball & Avise 1992). Sex-biased dispersal will lead to differences in genetic population structure for biparentally inherited nuclear genes and maternally inherited mtDNA. Male biased philopatry and female biased dispersal are common in birds (Greenwood 1980; Greenwood & Harvey 1982). There is no available information on the ratio of female and male dispersal in short-tailed shearwaters but if females are the dispersing sex then the absence of population differentiation in maternally inherited mtDNA may not be reflected in the geographic structuring of nuclear genes.

3.4.4 Historical demographic considerations

Avise (1989) has reviewed a number of theoretical and empirical studies which have used probability models of gene lineage sorting to interpret evolutionary relationships of mtDNA haplotypes within and between populations. The results of these analyses revealed that the

observed mtDNA phylogeographic structure for many species is consistent with unstable demographic histories of populations over space and recent evolutionary time. Historical demographic factors appear to have had a significant impact on existing mtDNA divergence.

Neigel and Avise (1986) have modelled the evolutionary dynamics of mtDNA lineages in subdivided populations derived from a common ancestral population, under various demographic scenarios. In the absence of gene flow, phylogenetic relationships of mtDNA haplotypes between populations are likely to be polyphyletic, paraphyletic and monophyletic with increasing time since population separation. As a consequence of stochastic lineage sorting, the probability of separated populations reaching a phylogenetic status of monophyly with respect to mtDNA haplotypes is high only after approximately $4N_f$ generations, where N_f is the female population size. At times less than this, mtDNA haplotypes from separate populations may be more closely related than mtDNA lineages within each population. The exact time to expected monophyly for isolated populations is influenced by the size of, and geographic distribution of mtDNA lineages among founding populations, population growth rate and variance in the number of offspring (Avise *et al.* 1984; Neigel & Avise 1986). Stochastic lineage sorting of mtDNA haplotypes, and therefore the expected time to monophyly, will be longer for large founding populations and/or expanding populations (Avise *et al.* 1984) representing random samples from the geographic range of the ancestral population. The results of these simulations show that, for populations that have been separated relatively recently, the phylogenetic relationships of mtDNA haplotypes among populations are unlikely to be concordant with the actual population separations themselves. If most of the current shearwater colonies were formed after the end of the last glaciation, and founding populations were large and have expanded to current levels, it is unlikely that there has been sufficient time for mtDNA lineage sorting in philopatrically isolated colonies to produce concordance between mtDNA phylogeny and population isolation.

Avise *et al.* (1988) have compared empirically estimated times to shared mtDNA haplotype ancestry with expected times generated under neutrality theory as a function of evolutionary effective size of female populations ($N_{f(e)}$), based on an mtDNA evolutionary rate of 2% sequence divergence between lineages per million years. These and subsequent comparisons (Zink *et al.* 1991c; Avise 1992; Ball & Avise 1992) have included a number of bird species and the general conclusions are that intraspecific mtDNA sequence diversities of extant

mtDNA haplotypes are much smaller than the expected diversities under neutrality theory for current population sizes and that observed times to common ancestry for mtDNA haplotypes are consistent with evolutionary effective female population sizes several orders of magnitude smaller than current population census sizes. Barrowclough and Shields (1984) reached a similar conclusion based on rates of karyotypic change in birds.

A reduced long-term effective population size ($N_{f(e)}$) relative to the current breeding population size can be attributed to a restricted number of female ancestors that have contributed to the current gene pool (Avise *et al.* 1988; Avise 1989). There are a number of biologically plausible scenarios, involving historical demographic factors, that have been proposed to explain reduced evolutionary effective size of female populations: large variances in progeny survival among females, periodic fluctuations in female population size including population bottlenecks, periodic extinctions and recolonisations of local populations, or rapid colonisation from a limited population source, perhaps including very recent expansions to current levels (Avise *et al.* 1988; Avise 1989, 1992; Ball & Avise 1992).

The phylogeographic pattern of shearwater mtDNA suggests similar demographic influences. Direct observations of contemporary shearwater populations provide substantial evidence for fluctuations in population size and geographic range. Climatic changes over recent evolutionary time are also likely to have affected the size and distribution of populations (Avise 1989). The low mean mtDNA sequence diversity among pairs of shearwater mitochondrial genomes suggests a reduced long-term effective female population size relative to the current breeding population size. Wooller *et al.* (1988) have shown that reproductive success of short-tailed shearwaters is strongly biased towards a small percentage of breeding pairs. Only 29 % of breeding birds successfully produce young that return to breed. A large variance in reproductive success will act to reduce evolutionary effective female population size. Population fluctuations in individual breeding colonies have been reported. Apart from the severe bottleneck in the Gabo Island colony (Gillham 1962), the Cape Woolamai colony has expanded, in area, considerably since the turn of the century (Harris & Bode 1981). Shearwaters may have experienced a very recent and rapid population expansion given that a number of new colonies have been formed both inside and outside the historical breeding range (Bryden 1966; Harris & Bode 1981).

The last glacial period appears to have had a major role in influencing mtDNA diversity in many avian species currently occupying previously glaciated ranges, through population bottlenecks and founder events during subsequent population expansion from a restricted number of breeding refugia (Ball *et al.* 1988; Tegelström *et al.* 1990; Moore *et al.* 1991; Mourn *et al.* 1991; Ovenden *et al.* 1991; Avise *et al.* 1992; Birt-Friesen *et al.* 1992; Zink & Dittman 1993a). A similar scenario is likely for short-tailed shearwaters as has already been discussed; glaciation restricting both population size and distribution with a rapid, and apparently continuing, post-glacial expansion to present levels.

Alternatively, selection acting on a favourable mtDNA haplotype in the recent past and the subsequent rapid transfer of this advantageous mutation throughout populations in a species would remove most pre-existing mtDNA genetic diversity (Nei & Graur 1984; Avise *et al.* 1988) and would funnel mtDNA lineages through a small number of females. A third possibility involves a reduced rate of mtDNA evolution compared to the 0.5-1% divergence per million years per lineage that has been reported for several vertebrate groups (Shields & Wilson 1987). However, Avise (1992) argued that these two alternatives to historical demographic considerations are unlikely given the correlation between $N_{f(e)}$ and N_f reported for 16 species of fish, birds and invertebrates. For a reduced mtDNA evolutionary rate to produce the observed discrepancies between current population sizes and estimated long-term effective population size, mtDNA would have had to evolve as much as three orders of magnitude more slowly than the reported rates for vertebrates (Avise *et al.* 1988).

3.4.5 Genetic population structure of a regional fauna

Avise (1992) has shown that concordant patterns of mtDNA population structure in a number of species occupying a similar geographic range provide evidence of similar vicariant histories. However the genetic effect of historical events may vary between species. The contrast between the genetic structure of populations of fairy prions and short-tailed shearwaters, with similar life-histories and occupying a similar geographic range is not unique to this study. Zink (1991a) found a significant mtDNA divergence between eastern and western populations of fox sparrows (*Passerella iliaca*) in the western United States, but in the sympatric song sparrow (*Melospiza melodia*) no geographic differentiation of mtDNA was detected (also Zink & Dittmann 1993b). Although the two species are presently

sympatric, they appear to have experienced different evolutionary histories. The contrast between the fairy prion and short-tailed shearwater can be extended to include other regional species. McMillan *et al.* (1992) surveyed restriction enzyme variation in mtDNA of the sea urchin (*Heliocidaris erythrogramma*) which occupies a geographic range encompassing the entire southern coastline of Australia, including Tasmania, and has restricted dispersal capabilities as a consequence of a short (3-4 days) larval stage. Although there was only limited phylogeographic structuring of mtDNA haplotypes, gene diversity (G_{ST}) analysis of the mtDNA data revealed a significant differentiation between populations in Tasmania and those on the south coast of Victoria which have been separated since the flooding of Bass Strait at the end of the last ice age. Similarly, mtDNA restriction enzyme variation in the southern cardinalfish (*Vincentia conspersa*) revealed significant genetic divergence of populations that had been historically separated by the Bass Strait land bridge during the last glacial period (T. J. Krasnicki, J. R. Ovenden and R. W. G. White, unpublished data). The southern cardinal fish is a sedentary, mouth brooding species in which larval and adult dispersal are minimal. From these comparisons it appears that recent biogeographic events have had different affects on intraspecific population structure in four different species, probably modulated by different demographic histories and dispersal capabilities.

Chapter 4

Molecular Systematics of Shearwaters in the Genus *Puffinus*.

4.1 INTRODUCTION

4.1.1 Mitochondrial DNA and avian systematics

The characteristics of the mitochondrial genome that make it useful for determining intraspecific genetic structure also enable reconstructions of matriarchal phylogeny among taxa, at a broad range of phylogenetic levels. Mitochondrial DNA analysis has proved to be a powerful technique to investigate avian systematics at the intra- and intergeneric level. Restriction enzyme analysis of mtDNA has provided information that suggests a unique pattern of hybridisation involving two closely related species of flycatcher, *Ficedula albicollis* and *F. hyploleuca* and a third unknown species (Tegelström & Gelter 1990). Restriction enzyme studies have also been used extensively to test previous evolutionary hypotheses, based on morphological or allozyme data, and to evaluate the phylogenetic significance of taxonomic classifications for a wide range of avian taxa (Kessler & Avise 1984; Ovenden *et al.* 1987; Avise *et al.* 1990; Zink & Avise 1990; Dittmann & Zink 1991; Zink *et al.* 1991a, b, c; Ball & Avise 1992; Zink & Dittmann 1993a).

Direct sequence analyses of segments of the mitochondrial genome have been used to verify the specific status of a recently discovered bush-shrike (*Laniarius liberatus*, Smith *et al.* 1991), to interpret the biogeographic history of kiwis and moas in New Zealand (Cooper *et al.* 1992), to examine the evolution of male display characters in bowerbirds (Kusmierski *et al.* 1993) and to resolve a phylogenetically deep split between songbirds and other perching birds (Edwards *et al.* 1991). Studies combining both restriction enzyme and direct sequence analysis have investigated the phylogenetic relationships among Australian songbirds (Edwards & Wilson 1990) and the systematic affinities of a number of species and genera of geese (Quinn *et al.* 1991).

Polymerase chain reaction (PCR) amplification and direct sequencing is possible using unpurified mtDNA from nanogram samples of fresh specimens and microgram amounts of old tissue, preserved wet or dried (Higuchi *et al.* 1984; Kocher *et al.* 1989; Thomas *et al.* 1989). Sufficient quantities of DNA can, therefore, be obtained from a wide variety of sources including fresh, frozen, ethanol or chemically preserved tissue and blood (Kocher *et al.* 1989; Thomas *et al.* 1989; Edwards & Wilson 1990; Quinn *et al.* 1991; Arctander 1988), freshly plucked or ethanol preserved hairs (Vigilant *et al.* 1989) and feathers (Smith *et al.* 1991; Taberlet & Bouvet 1991), and dried skins and soft tissue (e.g. muscle) (Higuchi *et al.* 1984; Houde & Braun 1988; Pääbo 1989; Thomas *et al.* 1989; Leeton *et al.* 1993). One advantage of amplifying mtDNA, as opposed to nuclear DNA, from ancient and old remains is that the high mtDNA copy number per nucleated cell facilitates its survival and retrieval (Pääbo *et al.* 1989).

4.1.2 The utility of short mtDNA sequences

For practical reasons only small segments (a few hundred base pairs) of the mitochondrial genome can be sequenced and compared for phylogenetic analyses. Short mtDNA sequences have a proven utility in phylogenetic studies at a range of levels in the evolutionary hierarchy, from the population level (Birt-Friesen *et al.* 1992; Wenink *et al.* 1993) to phylogenetically deep relationships within and among the major vertebrate groups (Meyer & Wilson 1990; Edwards *et al.* 1991; Irwin *et al.* 1991) . However, it is crucial to choose a gene, or part of, that will allow reliable resolution at the systematic level being studied.

The mitochondrial cytochrome *b* gene has proved valuable for phylogenetic analysis at or near the genus level in birds (Edwards & Wilson 1990; Smith *et al.* 1991; Arctander 1991; Kusmierski *et al.* 1993). The cytochrome *b* gene is among the most conserved of the 13 protein coding genes in animal mtDNA (Brown 1985). Specific segments of the cytochrome *b* gene show little variation across a wide taxonomic range. 'Universal' oligonucleotide primers are available that target these conserved regions and allow the amplification of homologous segments of the mitochondrial cytochrome *b* gene, flanked by the conserved sequences, from diverse species via the PCR (Kocher *et al.* 1989; Palumbi *et al.* 1991b). This fragment of the cytochrome *b* gene is a versatile source of phylogenetic information, providing a high power of phylogenetic resolution (Kocher *et al.* 1989; Edwards *et al.* 1991; Arctander 1991). The conservative amino acid composition and pattern of variation in

all studied organisms (Howel 1989; Edwards *et al.* 1991; Irwin *et al.* 1991) indicates, importantly, an apparent functional invariance in this gene. This supports the idea that the selective pressure, and therefore rates of substitution, on this gene will not vary much between different taxa especially when closely related forms are compared (Arctander 1991).

Although mtDNA and in particular cytochrome *b* sequences show general phylogenetic utility, they are not universally applicable (Graybeal 1993; Meyer 1994). The range over which cytochrome *b* gene sequences (and mtDNA in general) are useful for phylogenetic analysis is bounded at one extreme by ancestral polymorphism and stochastic lineage sorting among recently diverged taxa (Neigel & Avise 1986; Pamilo & Nei 1988) and at the other by saturation of rapidly evolving sequences (Moritz *et al.* 1987). Phylogenetic analysis of cytochrome *b* sequences is complicated by the observed variation in evolutionary rates among first, second and third codon positions, among transition and transversion substitutions and silent versus replacement substitutions (Brown *et al.* 1982; Kocher *et al.* 1989; Edwards *et al.* 1991; Irwin *et al.* 1991; Graybeal 1993). Third codon position substitutions, transition substitutions and silent substitutions all occur considerably more frequently than the other types of substitutions, and may become saturated relatively rapidly in diverged lineages. This reduces the phylogenetic depth that cytochrome *b* sequences can resolve because on the one hand rapidly evolving sequences become saturated and phylogenetically uninformative, whereas more slowly evolving categories of change may not have accumulated sufficient change in lineages that have been separated for a long period of time (Graybeal 1993; Meyer 1994). Therefore it is important to examine the evolutionary properties of the cytochrome *b* gene segment in the group under study to ensure that phylogenetic information is present in the observed sequence variation.

4.1.3 Phylogenetic inference

Similarities and differences in nucleotide sequences form the basis for reconstructing phylogenetic relationships among taxa. Overall similarity consists of shared-ancestral characters (symplesiomorphies) which are retained from a common ancestor, shared-derived characters (synapomorphies) which are newly evolved characters in more recent lineages that share common descent, and homoplasies (convergences, parallelisms and reversals) which are newly evolved characters that have been derived independently along different lineages

(Stewart 1993; Avise 1994). There are many methods of inferring phylogenies from molecular sequences (Nei 1987; Felsenstein 1988; Swofford & Olson 1990) and they can be divided into distance methods and discrete character methods.

Distance methods reconstruct phylogenetic relationships from a matrix of pairwise distances between taxa. This is a two stage process. First, actual pairwise distances between all taxa are estimated under a particular model of sequence evolution (e.g. Kimura two parameter model, Kimura 1980; six parameter or maximum likelihood model, Felsenstein 1981; Hasegawa *et al.* 1985; Kishino & Hasegawa 1989) to correct for multiple substitutions. Second, the taxa are phylogenetically clustered, using one of a variety of algorithms (Fitch & Margoliash 1967; Farris 1972; Sneath & Sokal 1973; Saitou & Nei 1987), such that the topology and branch lengths of the inferred tree represent a best fit to the values in the distance matrix. The neighbor-joining (NJ) method (Saitou & Nei 1987) is one of the most widely used to infer phylogenetic relationships from sequence data (von Haeseler *et al.* 1993). This method does not assume equal rates of nucleotide evolution among lineages and has the advantage over some other distance methods of being computationally fast (Nei 1991; von Haeseler 1993). The use of distance methods for phylogenetic analysis of sequence data have been criticised because of an apparent loss of information when nucleotide sequences are transformed to distances (Farris 1985; Penny 1982; but see Nei 1991).

Discrete character methods, which use the original sequence data, include maximum parsimony (Fitch 1971) and maximum likelihood procedures (Felsenstein 1981). Methods based on parsimony reconstruct phylogenetic relationships that require the minimum number of character-state (nucleotide) changes to account for the observed distribution of characters at each nucleotide position among taxa. Parsimony analysis incorporates a cladistic approach (Hennig 1966) to phylogenetic inference because phylogenetic reconstructions are made on the assumption that shared characters represent synapomorhpies (i.e are shared-derived). Conflict among characters requires extra character state changes (homoplasies) to be inferred. The parsimony method would always retrieve the true tree if there was no homoplasy (i.e. parallelisms and reversals) in nucleotide evolution because shared characters would define common ancestry (Sidow & Wilson 1991). The advantages of using a maximum parsimony approach for phylogenetic analysis are that this method is less dependent on assumptions of

sequence evolution than other methods (Cracraft & Helm-Bychowski 1991; but see Felsenstein 1988), and that currently available parsimony algorithms are fast, easy to use, and allow a detailed investigation of the properties of and support for the most parsimonious and suboptimal trees (Swofford & Olson 1990).

Maximum likelihood methods reconstruct phylogenetic relationships based on a particular probabilistic model of sequence evolution (Felsenstein 1981). For each nucleotide site a log likelihood is calculated for each of all possible tree topologies, which represents the probability of the observed distribution of characters among taxa, given the evolutionary model, for that particular topology. The log likelihood for each topology is summed for all nucleotide sites and the tree with the highest likelihood represents the best estimate of phylogenetic relationships. The maximum likelihood approach therefore reconstructs a topology and branch lengths which maximises the probability that the observed data would have occurred, given the model of sequence evolution.

The maximum likelihood method is potentially the most powerful and reliable method of phylogenetic inference (Sidow & Wilson 1991) but its main limitation is that it requires a precise model of sequence evolution (Felsenstein 1988). As a result, maximum likelihood methods make a number of explicit assumptions about sequence evolution, whereas assumptions for most other methods are implicit. The most realistic model, the six parameter or maximum likelihood model (Felsenstein 1981; Hasegawa *et al.* 1985; Kishino & Hasegawa 1989), is implemented in the maximum likelihood algorithm in the PHYLIP package (Felsenstein 1993), and assumes that each site in the sequence evolves independently, different lineages evolve independently, the base composition is at equilibrium but permits different frequencies of the four nucleotides, no insertions or deletions occur, and the rate of change to each base is proportional to its equilibrium frequency but is independent of the identity of the base that is being replaced and allows different rates of substitution at different sites and independent rates for transition and transversion substitutions (Swofford & Olson 1990; Felsenstein 1993).

The relative accuracy and reliability of the different methods in recovering the correct phylogenetic tree is controversial. Nei (1991) has summarised results of many simulation studies which are designed to test the efficiency of different phylogenetic methods using

sequence data generated for a specific tree under a particular model of nucleotide evolution. The results from these simulation studies do not support any one single method of analysis for all applications but suggest that the reliability and accuracy of different methods depends on many factors, including the topology of the true tree, the overall rate of nucleotide substitutions and the number of nucleotide substitutions per site, and the transition/transversion ratios (Nei 1991). The maximum parsimony method becomes unreliable when there are many nucleotide substitutions per site (i.e. a high level of homoplasy) and small numbers of nucleotides are examined (Nei 1991). In addition, the maximum parsimony method is inefficient when there are differences in evolutionary rate among lineages (Felsenstein 1978). Maximum parsimony is, however, reliable for closely related taxa where levels of homoplasy are low (Sidow & Wilson 1991).

The neighbor-joining method is generally more efficient than most other distance methods, and compared to discrete character methods is equally reliable providing that pairwise distances are accurately estimated to correct for multiple substitutions (Nei 1991). The maximum likelihood method is also efficient providing the assumptions of the model of sequence evolution are satisfied. Hillis *et al.* (1994) used both simulations and real experimental phylogenies to show that, provided that data sets do not contain extensive homoplasy (i.e. variable sites are not saturated), most methods of phylogenetic inference appear to be robust to deviations from the assumed simple models of evolution and are efficient at estimating phylogenies over a range of evolutionary conditions. Therefore, it would seem advisable to analyse sequence data using each of the three methods of phylogenetic inference. A lack of congruence between the trees obtained may indicate deviations from general models of sequence evolution in the group under study.

4.1.4 The shearwater genus *Puffinus*

The shearwater genus *Puffinus* currently comprises 15 recognised species, five of which contain between two and twelve subspecies, although the taxonomic status of many of these remains controversial (Jouanin & Mougin 1979; Bourne 1987; Bourne *et al.* 1988; Berruti 1990; Walker *et al.* 1990; Warham 1990). The species have been grouped into a number of subgroups on the basis of osteological and external morphological characters (Kuroda 1954; Jouanin & Mougin 1979). The taxa, with general details on their distribution and migration, taken from Jouanin and Mougin (1979), Marchant and Higgins (1990) and Warham (1990), are as below (see Fig. 4.1).

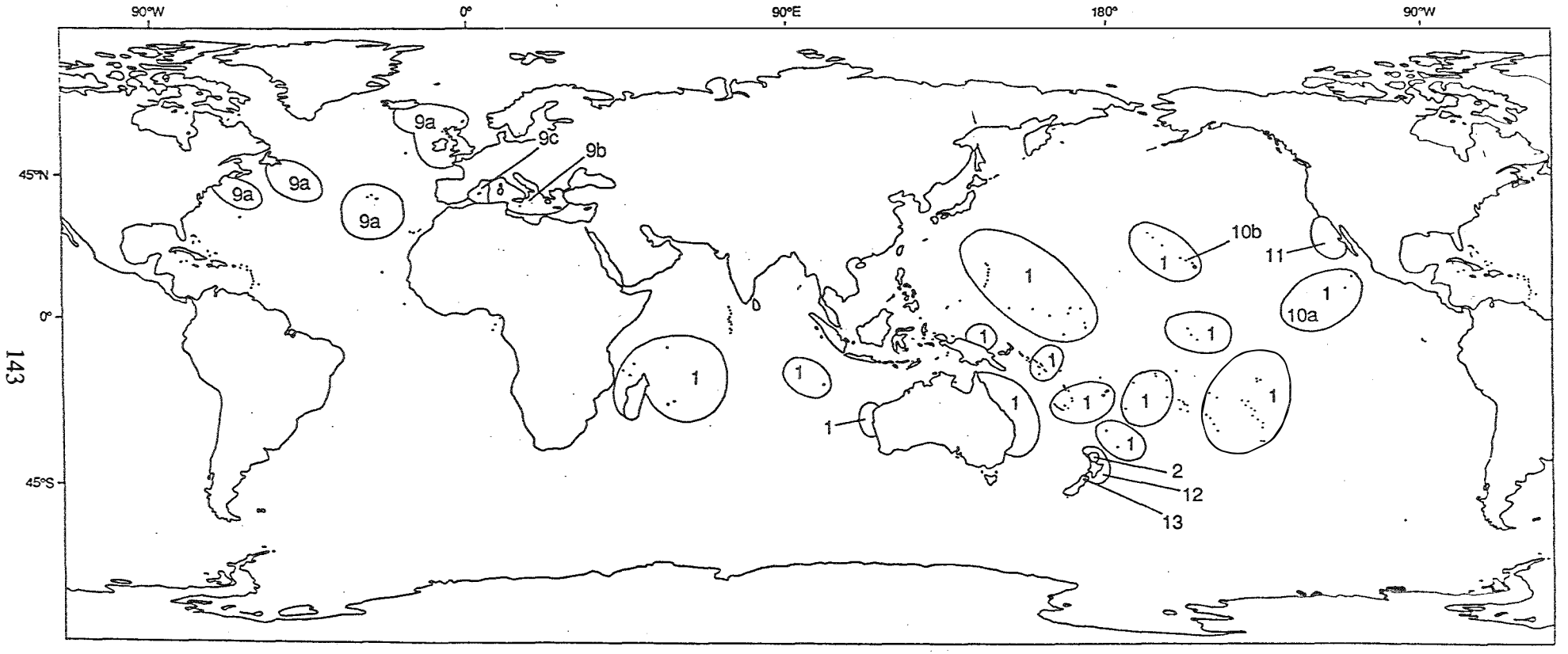

Figure 4. 1. Breeding distribution of *Puffinus* shearwaters (redrawn from Warham 1990). 1. *P. pacificus*; 2. *P. bulleri*; 9. *P. puffinus*, 9a. *P. p. puffinus*, 9b. *P. p. yelkouan*, 9c. *P. p. mauretanicus*; 10. *P. auricularis*, 10a. *P. a. auricularis*, 10b. *P. a. newelli*; 11. *P. opisthomelas*; 12. *P. gavia*; 13. *P. huttoni*.

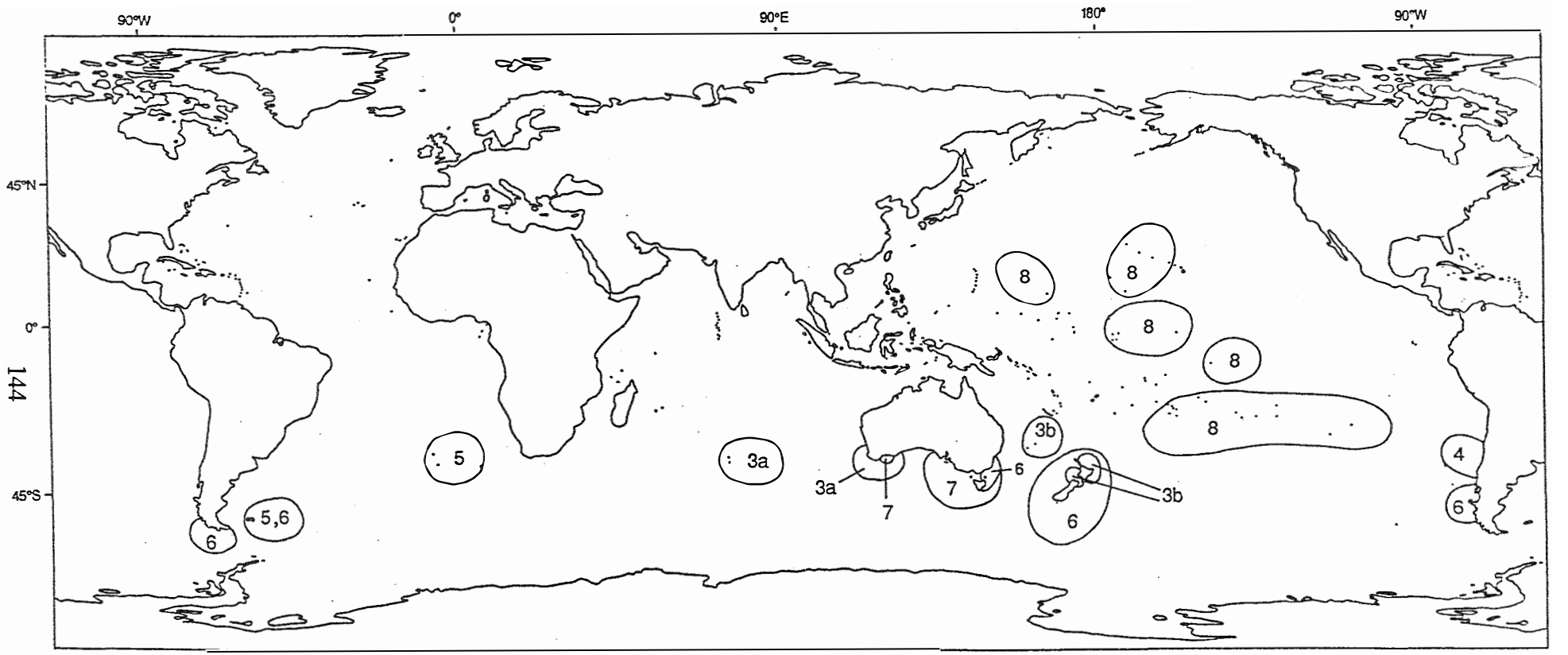

Figure 4.1. (continued). 3. *P. carneipes*, 3a. *P. c. carneipes*, 3b. *P. c. hullianus*; 4. *P. creatopus*; 5. *P. gravis*; 6. *P. griseus*; 7. *P.tenuirostris*; 8. *P. nativitatis.*

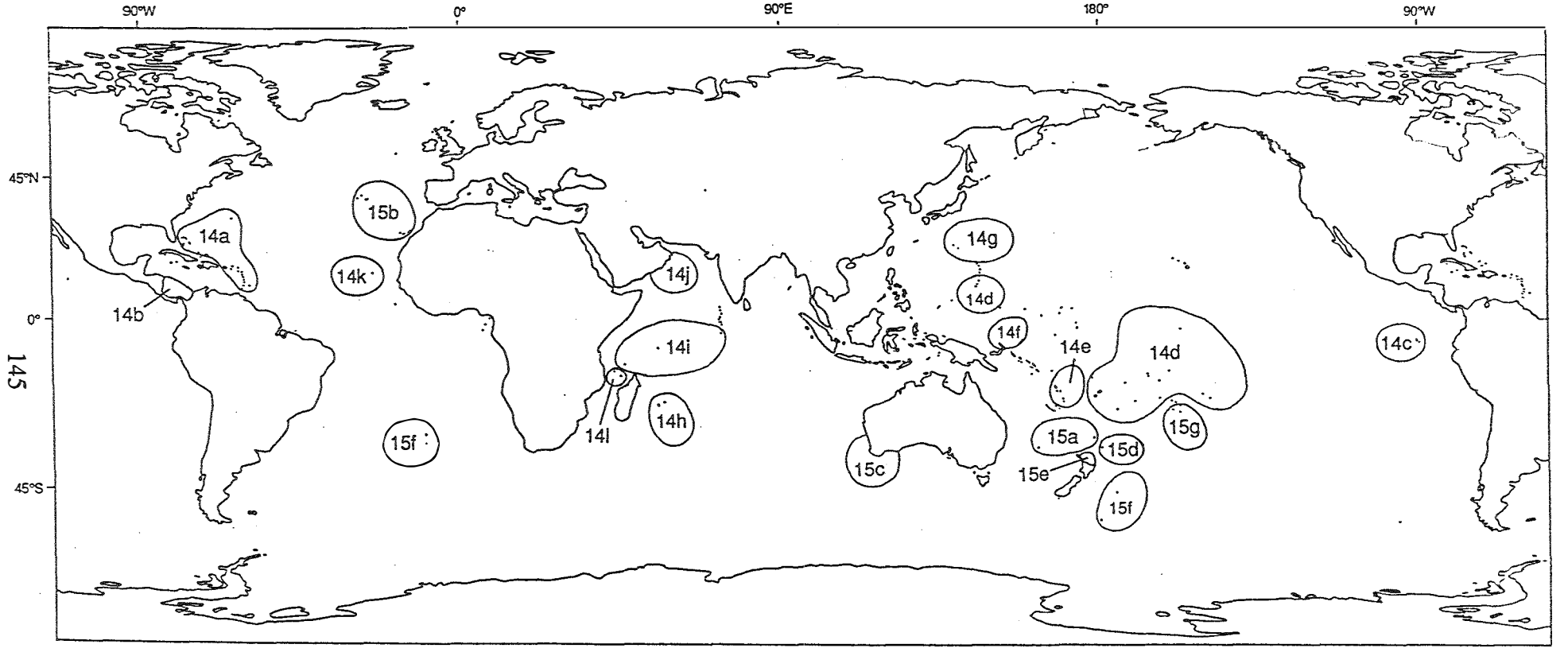

Figure 4.1 (continued). 14. *P. lherminieri*, 14a. *P. l. lherminieri*, 14b. *P. l. loyemilleri*, 14c. *P. l. subalaris*, 14d. *P. l. dichrous*, 14e. *P. l. gunax*, 14f. *P. l. heinrothi* (suspected breeding locality only), 14g. *P. l. bannermani*, 14h. *P. l. bailloni*, 14i. *P. l. nicolae*, 14j. *P. l. persicus*, 14k. *P. l. boydi*, 14l. *P. l. temptator*; 15. *P. assimilis*, 15a. *P. a. assimilis*, 15b. *P. a. baroli*, 15c. *P. a. tunneyi*, 15d. *P. a. kermadecensis*, 15e. *P. a. haurakiensis*, 15f. *P. a. elegans*, 15g. *P. a. myrtae*.

Subgroup Thyellodroma

P. pacificus - Wedge-tailed shearwater

A large, lightly built shearwater with light, dark and intermediate plumage polymorphism. Widely distributed throughout the tropical and subtropical Pacific and Indian Oceans, breeding on islands in the central, western and southwestern sectors of the Pacific Ocean and eastern, central and western sectors of the Indian Ocean. Some subtropical populations migrate extensively, while tropical populations appear to be non migratory.

P. bulleri - Buller's shearwater

Closely resembles *P. pacificus* in size and shape. Breeds only on islands off the North Island of New Zealand and is a transequatorial migrant to the North Pacific.

Subgroup Hemipuffinus

P. carneipes - Flesh-footed shearwater

A large all dark species composed of two subspecies with distinct distributions in the Pacific and Indian Oceans.

P. c. carneipes. Breeds in the Indian Ocean on St. Paul Island and on islands along the south coast of Western Australia, and migrates north and west in the Indian Ocean.

P. c. hullianus. Breeds in the Australasian region of the Pacific Ocean on Lord Howe Island and islands off the North Island of New Zealand. A transequatorial migrant to the North Pacific.

P. creatopus - Pale-footed shearwater

Large shearwater with a light, dark and intermediate plumage polymorphism on the underparts. Breeds on the Juan Fernandez Islands in the southeast Pacific and is a transequatorial migrant to the North Pacific.

Subgroup Ardenna

P. gravis - Great shearwater

A large shearwater with a characteristic plumage pattern consisting of a dark belly patch on mostly white underparts, and a white collar. This species breeds in the South Atlantic at Tristan da Cunha Island, Gough Island and the Falklands Islands and is a transequatorial migrant to the North Atlantic.

Subgroup Neonectris

P. griseus - Sooty shearwater

A large, dark plumaged shearwater with distinct breeding locations in the southwest Pacific, on islands along the southeastern coastline of Australia and around the coast of New Zealand and on subantarctic islands southeast of New Zealand, and in the South Atlantic and South Pacific around the southern tip of South America. A transequatorial migrant to the North Pacific and North Atlantic oceans.

P. tenuirostris - Short-tailed shearwater

A medium-large sized, all dark shearwater which breeds along the southern coastline of Australia and migrates to the North Pacific.

P. nativitatis - Christmas shearwater

A medium sized shearwater with dark plumage, distributed through the tropical Pacific Ocean. The species breeds on tropical and subtropical islands in the central Pacific, north and south of the equator and is apparently non-migratory.

Subgroup Puffinus

All of the taxa in this subgroup have a distinctive dark dorsal surface and lighter or white ventral colouration. There is considerable overlap in plumage patterns and characteristics between species and subspecies.

P. puffinus - Manx, Levantine and Balearic shearwaters

A species of controversial taxonomic status. Previously included all species within the subgroup Puffinus. The specific status of *P.p. yelkouan* and *P. p. mauretanicus* has recently been proposed (Bourne *et al.* 1988; Walker *et al.* 1990).

P. p. puffinus - Manx shearwater

A medium sized shearwater distributed in the North Atlantic. Breeding sites range from Iceland in the north, to islands off the coast of the British Isles and France, the Azores and Canary islands in the south and on the western side of the Atlantic at NewFoundland and the northeast coast of USA. This subspecies is a transequatorial migrant to the South Atlantic.

P. p. yelkouan - Levantine shearwater

A medium sized shearwater geographically confined to the Mediterranean Sea where it breeds on islands in the central and eastern sectors from the south coast of France to the Aegean and Adriatic Seas. This subspecies is a coastal group (as opposed to

pelagic) and although not strictly migratory, it does appear to disperse from the breeding areas, generally in a north and east direction toward the Black Sea.

P. p. mauretanicus - Balearic shearwater

A slightly larger subspecies with a predominantly coastal, Mediterranean habitat. However, this group breeds near the western entrance of the Mediterranean at Pityusae and the Balearic Islands, and most individuals migrate west out of the Mediterranean and north into the English Channel, rarely as far north as Scotland and Scandinavia.

P. auricularis - Townsend's and Newell's shearwaters

P. a. auricularis - Townsend's shearwater

A medium sized shearwater that breeds on the Revillagigedo Islands off the west coast of Mexico. Although there are very little data on movements and distribution, this group appears to be non-migratory.

P. a. newelli - Newell's shearwater

A medium sized shearwater that once bred on all the main Hawaiian islands but now only in the mountains of Kauai Island. It is probably a migratory group, although the direction and distance of migration is unknown. Bourne *et al.* (1988) suggested a migration to the eastern tropical Pacific, while Jouanin and Mougin (1979) proposed a location to the south and west.

P. opisthomelas - Black-vented shearwater

A medium sized shearwater that breeds only on islands off the Pacific coast of Baja California, and migrates north as far as the northeast Pacific Ocean.

P. gavia - Fluttering shearwater

A small shearwater that breeds on islands around the North Island of New Zealand. The species is a partial migrant west to the east coast of Australia and southeast to areas off the coast off the South Island of New Zealand.

P. huttoni - Hutton's shearwater

A small shearwater, almost identical in external morphology and plumage to *P. gavia.* The only known breeding site is in the Seaward Kaikura Mountains on the South Island of New Zealand. Most birds remain in the adjacent coastal seas however some disperse around the entire coastline of Australia.

P. lherminieri - Audubon's shearwater

A group of small shearwaters comprising 12 subspecies with a pantropical distribution. Although little is known about the range and dispersal of most subspecies the extent of geographic variation and the number of recognised taxa suggests that most are sedentary around the breeding areas. The following is a list of subspecies and breeding locations.

P. l. lherminieri - islands throughout the northeastern edge of the Carribean Sea and into the western Atlantic.

P. l. loyemilleri - islands off the coast of Panama, in the southwestern corner of the Caribbean Sea.

P. l. subalaris - Galapagos Islands

P. l. dichrous - islands throughout the central Pacific Ocean.

P. l. gunax - islands of the Banks Group in the New Hebrides (western Pacific).

P. l. heinrothi - unknown breeding location, probably around New Britain in the western Pacific, where the only known specimens have been collected.

P. l. bannermani - Bonon and Volvano Islands in the northwest Pacific Ocean.

P. l. bailloni - Mascarene Island in the western Indian Ocean, off the east coast of Madagascar.

P. l. nicolae - islands across the northwestern Indian Ocean.

P. l. persicus - island in the Arabian Sea.

P. l. boydi - Cape Verde Islands off the northwest coast of Africa.

P. l. temptator - Comoro Islands, in the western Indian Ocean.

P. assimilis - Little shearwater

A group of seven subspecies representing the smallest shearwaters, which apart from *P. a. baroli* has an essentially sub-tropical and subantarctic distribution in the southern hemisphere. Although little is known about range over which most subspecies disperse, most are considered non-migratory. The following is a list of subspecies and breeding locations.

P. a. assimilis - Lord Howe and Norfolk Islands in the southwestern Pacific Ocean.

P. a. baroli - the Azores, Desertas, Salvages and Canary islands in the eastern Atlantic.

P. a. tunneyi - islands off the coast of southwestern Western Australia.

P. a. kermadecensis - Kermadec Island in the southwestern PAcific Ocean.

P. a. haurakiensis - islands off the east coast of the North Island of New Zealand.

P. a. elegans - in the South Atlantic at Tristan da Cunha and Gough Islands, and in the southwest Pacific at Antipodes, Chatham and Auckland Islands.

P. a. myrtae - Austral Group in the south-central Pacific Ocean.

The Manx group of shearwaters, consisting of all species historically included as subspecies of *P. puffinus* (Murphy 1952) (*P. au. newelli*, *P. au. auricularis*, *P. p. puffinus*, *P. p. yelkouan*, *P. p. mauretanicus*, *P. opisthomelas*, *P. gavia* and *P. huttoni*) can be divided into three groups based on current distribution, external morphology, breeding season and migration patterns (Bourne *et al.* 1988). These groups are *P. gavia* and *P. huttoni* which are extralimital southern hemisphere representatives of the Manx group; *P. p. puffinus*, *P. au. auricularis* and *P. au. newelli* which are northern hemisphere, black-backed forms which lay late in the spring and tend to migrate south in winter; and *P. p. yelkouan*, *P. p. mauretanicus* and *P. opisthomelas* which are northern Hemisphere brown-backed forms which lay early in the year and migrate north from the breeding areas. *P. huttoni* and *P. gavia* show affinities with the black-backed and brown-backed forms, respectively..

There have been two phylogenetic studies that have focused specifically on the *Puffinus* shearwaters. Kuroda (1954) examined habit differentiation, osteology, external morphology, distribution and the fossil record to propose phylogenetic relationships among species in the genus *Puffinus*. In this phylogeny, each subgroup of taxa forms a monophyletic clade, with the subgroup Thyellodroma the most ancestral and the Puffinus subgroup the most derived (Fig. 4.2A). Kuroda (1954) did not examine any material from the Manx group (*P. puffinus*. sspp, *P. auricularis*. sspp, *P. opisthomelas*, *P. huttoni*, *P. gavia*) but followed the work of Murphy (1952) for these species. Wragg (1985) produced an almost identical phylogeny for the genus using a largely independent set of comparative osteological characters (Fig. 4.2B). One area of conflict between these two studies is the phylogenetic placement of *P. nativitatis* which Kuroda (1954) grouped with *P. griseus* and *P. tenuirostris* in the subgroup Neonectris, while Wragg (1985) showed that *P. nativitatis* is a sister group of the Puffinus subgroup, distinct from the Neonectris subgroup of *P. griseus* and *P. tenuirostris*.

Kuroda (1954) proposed a scenario for the evolutionary history of the Procellariiformes, including the shearwaters. The fossil record for Procellariiformes is relatively scarce and is

centred around western Europe and North America, although the main distributional centre of extant taxa is presently in the southern hemisphere. This led Kuroda (1954) to suggest that the North Atlantic was the original ancestral area where most of the main Procellariiform

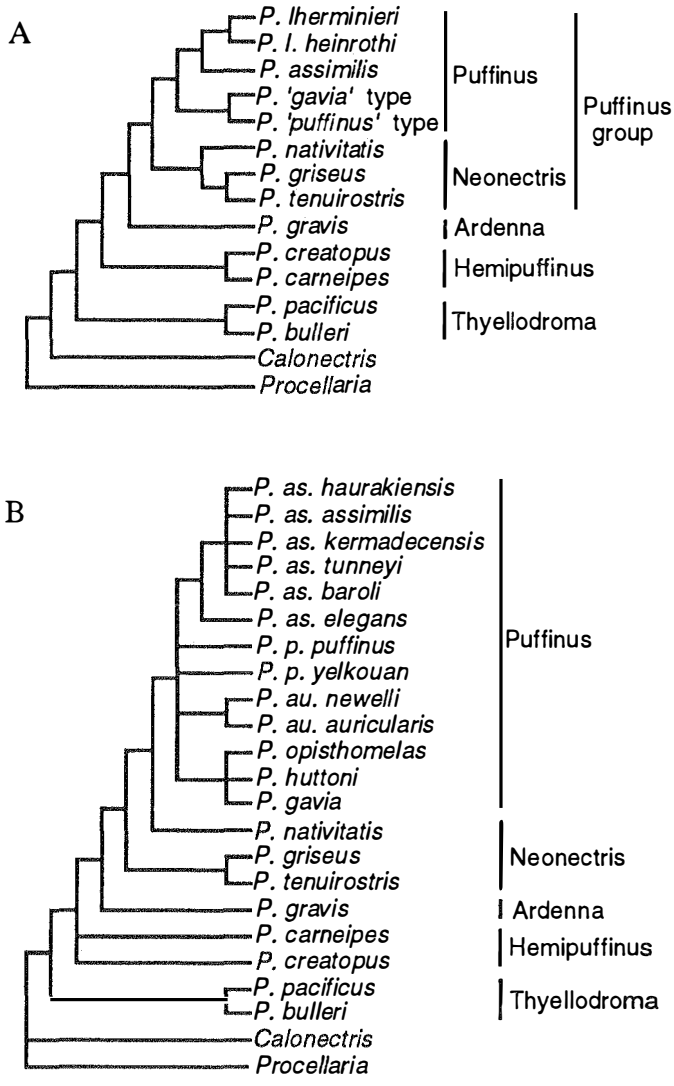

Figure 4.2. Hypothesised phylogenetic relationships among *Puffinus* species and subspecies based on: (A) osteology, external morphology, distribution and habit differentiation (redrawn from Kuroda 1954); and (B) osteology (redrawn from Wragg 1985). Specific and subspecific names have been changed in the Kuroda tree to reflect current taxonomy. The five subgroups of species (Kuroda 1954; Jouanin & Mougin 1979) are indicated. In addition Kuroda (1954) proposed that the Puffinus and Neonectris subgroups belong to a Puffinus group.

groups, including the *Puffinus* subgroups, differentiated. Olson (1985) stated that most of the *Puffinus* subgroups had evolved by the middle Miocene, approximately 15 million years ago, which is supported by a sudden increase in the diversity of procellariid fossils in the eastern North Pacific about this time (reviewed by Warheit 1992). These ancestral groups would then have moved independently into the southern hemisphere *via* a westward migration to form a secondary evolutionary centre from which the present species differentiated and distributed throughout the Southern Ocean and Indo-Atlantic waters and later into the tropical Pacific. Some groups (e.g. Puffinus subgroup) would have remained in the original ancestral area where they differentiated and dispersed west into the Pacific Ocean across the flooded Panamanian landbridge (*P. auricularis* sspp, *P. lherminieri* sspp) and east into the Indian and Pacific Oceans through the Mediterranean (*P. gavia*, *P. huttoni*, *P. opisthomelas*) which may have had connections with both the Atlantic and Indian Oceans or via the southern tip of Africa (*P. assimilis* sspp). The phylogenetic relationships among *Puffinus* taxa proposed by Kuroda (1954) and Wragg (1985) are consistent with this evolutionary scenario involving multiple invasions into the Pacific and Indian Oceans from an ancestral stock which differentiated in the North Atlantic.

Bourne *et al.* (1988) proposed a similar scenario for the Puffinus subgroup. They argued that based on current distribution and representation in the fossil record the Puffinus subgroup ancestor originated in the North Atlantic which was then part of the Tethys Ocean. Evolution of extant taxa occurred as a consequence of the disruption of that ocean during the middle Tertiary into a series of temporarily landlocked seas and the separation from the Pacific Ocean when the Panamanian landbridge formed at the end of the Tertiary. The sequence of events suggested by Bourne *et al.* (1988) are as follows. The ancestor of *P. assimilis* and *P. lherminieri* was the first to diverge *via* a migration into the Indian Ocean, with a subsequent dispersal throughout the tropical and subtropical latitudes of all three major oceans. The original ancestral population was then fragmented by the formation of shallow inland seas in central Europe during the Miocene and Pliocene (Busson *et al.* 1980 in Bourne *et al.* 1988; King 1983; Walker *et al.* 1990) in which the ancestor of the brown-backed, coastal species, *P. p. yelkouan*, *P. p. mauretanicus* and possibly *P. opisthomelas* and *P. gavia* evolved. The ancestor of the pelagic black-backed species *P. p. puffinus*, *P. au. auricularis*, *P. au. newelli* and *P. huttoni* may have had a subtropical distribution with a continuous range from the North Atlantic to the Pacific across the flooded Panamanian

landbridge. These taxa were separated by dispersal into the southwestern Pacific (*P. huttoni*) and by the formation of the Panamanian landbridge some 2 x 10^6 years ago. The ancestors of the Mediterranean and Atlantic Manx shearwaters were sufficiently diverged to remain distinct when the inland seas and the Atlantic were reconnected near the end of the Pliocene (King 1983). *P. opisthomelas* and *P. gavia* may have evolved from the coastal ancestor which managed to reach the eastern and southern Pacific before the appearance of the Panamanian landbridge, or alternatively morphologically similar forms may have evolved independently in these two areas from the ancestor of the black-backed forms. Bourne *et al.* (1988) therefore believe that extant taxa representing the Puffinus subgroup are sibling species which diverged as a result of separation and reconnection of seas and oceans around the North Atlantic during the late Tertiary. These taxa have secondarily spread into the Pacific where they may or may not have evolved into morphologically similar forms.

A number of molecular genetic studies involving Procellariiform birds have included representatives from the genus *Puffinus*. Harper (1978) examined electrophoretic variability of blood plasma proteins among 29 species of Procellariiformes, including six species of *Puffinus*: *P. bulleri*, *P. carneipes*, *P. griseus*, *P. gavia*, *P. huttoni*, and *P. assimilis*. He concluded that *P. carneipes*, *P. griseus*, *P. gavia* and *P. huttoni* are all closely related, but that *P. bulleri* exhibits a number of unique forms of plasma proteins that make it less closely related to the former. *P. assimilis* exhibits even more unusual plasma proteins, which led Harper (1978) to speculate that it may be incorrectly assigned to the *Puffinus* genus. Barrowclough *et al.* (1981) investigated genetic divergence among 12 species of Procellariiformes based on allozyme variation at 16 protein coding loci. However, they included only one species of *Puffinus*, *P. griseus*, and did not examine phylogenetic relationships among the taxa involved. Wragg (1985) compared red blood cell enzymes from *P. gavia*, *P. huttoni* and *P. griseus* and found the former two species to be closely related, with *P. griseus* genetically more distant. Similar to the findings of Barrowclough *et al.* (1981) genetic identity values among the three species were intermediate between mean values for Passeriform birds and mammals. Kuroda *et al.* (1990) examined allozyme variation at 23 protein coding loci among 15 species of Procellariiforms, including *P. pacificus*, *P. griseus*, *P. tenuirostris* and *P. lherminieri* (ssp?). They found no genetic divergence between *P. griseus* and *P. tenuirostris*, and concluded that these two taxa form a clade with

P. lherminieri. The genetic data indicate that *P. pacificus* is more closely related to the *Pterodroma* petrels, but Kuroda *et al.* (1990) argued that this incongruent relationship was probably due to the low levels of divergence among the taxa involved and the patterns of allelic variation among *Puffinus* and *Pterodroma*. Wink *et al.* (1993) used mtDNA cytochrome *b* gene sequences to investigate intra and interspecific variation in Cory's shearwater (*Calonectris diomedea*), *P. puffinus* and the fulmar (*Fulmarus glacialis*). Phylogenetic analysis of these sequences showed that the two shearwaters, *C. diomedea* and *P. puffinus*, are closely related and are distinct from the fulmar.

Taxonomic controversy is centred around species in the Puffinus subgroup. In particular, the specific and subspecific status of taxa in the *lherminieri/assimilis* complex are uncertain. There have been suggestions that the *lherminieri/assimilis* complex represents a single variable species (Bourne 1986), two species consisting of numerous subspecies (Jouanin & Mougin 1979), or more than two species where a number of current subspecies (*P. l. heinrothi*, *P. l. bannermani*, and *P. l. persicus*) are assigned specific rank (Jouanin & Mougin 1979). *P. l. boydi* is considered by some to be incorrectly assigned to this species and should instead be placed in *assimilis* because of its closer morphological affinities with the latter (Cramp & Simmons 1977). The taxonomy of the Manx group has been treated inconsistently in the three regions where it occurs, as two species in the Southern Hemisphere, two species in the North Pacific, but only a single species in the Atlantic and Mediterranean (Bourne *et al.* 1988). Bourne et al. (1988) and Walker et al. (1990) have both suggested, on the basis of comparative biology and osteology, that the two Mediterranean subspecies of *P. puffinus* should be recognised as one or two distinct species, *P. yelkouan yelkouan* and *P. y. mauretanicus* or *P. yelkouan* and *P. mauretanicus*, respectively.

4.1.5 Aims

I obtained DNA sequences from a 307 bp segment of the mitochondrial cytochrome *b* gene from species and subspecies in the genus *Puffinus*. PCR amplification of this cytochrome *b* fragment using 'universal' primers was attempted from samples collected invasively and non-invasively from live birds, and retrospectively from deceased individuals, museum skins and tissue collections. The sequences obtained were used to examine phylogenetic relationships among extant *Puffinus* taxa and to test phylogenetic hypotheses proposed by Kuroda (1954) and Wragg (1985). The high level of systematic resolution provided by mitochondrial

cytochrome *b* gene sequences may provide a phylogenetic background in which to rationalise current taxonomic controversy surrounding species and subspecies groups in the shearwater genus *Puffinus*.

4.2 METHODS

4.2.1 PCR hygiene

The extreme sensitivity of the PCR to amplify small amounts of DNA requires special precautions to minimise the opportunities for contamination of samples with unwanted DNA, and to detect and eliminate these contaminations wherever possible. This study followed the general recommendations for PCR "hygiene" made by Kwok and Higuchi (1989) and Pääbo *et al.* (1989). All pre-PCR work (storage of tissue samples, extraction and storage of DNA, storage of PCR reagents and dedicated equipment and disposables, and set-up of PCR reactions) was performed in rooms well separated from the main laboratory where PCR amplification and post-PCR work (electrophoresis, storage and purification of PCR product and DNA sequencing) was performed.

Refrigerators, freezers, water baths, centrifuge and other equipment dedicated for this task were stored in the pre-PCR work areas. All glassware used in pre-PCR work was routinely heat treated to 200°C for at least 5 h to destroy surface contaminating DNA. All disposable plasticware (pipette tips, 1.5 ml and 0.5 ml centrifuge tubes), pipettors and reagents were purchased independently of the main laboratory and dedicated to pre-PCR work. DNA solutions were only pipetted using disposable, positive displacement PCR pipettors (Drumond Scientific Company), which were heat treated to 200°C for at least 5 h prior to use. All other pipetting of solutions used standard laboratory adjustable pipettors and sterile BioGard pipette tips (1-160 µl, Edwards Instrument Company) fitted with filter tips to prevent aerosol contamination of the barrel of the pipettor. All non-sterile disposable plasticware (microcentrifuge tubes), distilled water, and buffers were sterilised by autoclaving. All reagents (Proteinase K, water, buffers, primer solutions, *Taq* DNA polymerase, nucleotide mixes) were aliqouted into appropriately sized lots and stored at –20°C. Prior to any work, the pre-PCR work bench was wiped with 1M HCl to denature any surface contaminating DNA. Disposable gloves were worn at all times when handling any pre-PCR equipment or solutions, and were changed frequently.

A control extraction was performed alongside all extractions of DNA from tissue samples. The control contained the same reagents and underwent identical manipulations as the authentic extractions, but contained no tissue sample. Similarly a control tube was included alongside all PCR amplifications, and contained identical reagents to authentic amplifications but no tissue extract was added. The control extract controlled for contamination of extraction solutions or contamination during the extraction procedure. The control amplification controlled for contamination of PCR reagents or contamination during PCR-setup. DNA sequencing of the amplified cytochrome *b* fragment was only performed when both the control extract and negative PCR control did not show evidence of contamination (i.e. no amplification of any product).

4.2.2 Sample collection

I attempted to obtain samples from every species and subspecies of *Puffinus*, and, for taxa with widespread distributions, multiple samples from across the geographic range. Tissue samples (blood, feathers, kidney, liver or muscle) for this study were either from material collected from live or dead birds by the author or authorised persons acting on behalf of the author, or from museum material (whole dried skins or preserved tissue collections). A complete list of all samples available is given in Appendix 4a, and the subset from which sequence data was obtained is presented in Table 4.1.

The majority of samples from which sequence data was obtained were collected on request from the author so that there was some degree of uniformity and control over collection conditions. Collectors were provided with disposable gloves, sterile needles and capillary tubes (for blood collections), or sterile tweezers (for feather collections) and tubes containing high grade absolute ethanol. Using gloved hands to prevent human DNA contamination of the samples, collectors were instructed to either collect a small blood sample using an identical technique to that described in Section 2.2.2, or using the tweezers pluck 3-4 breast feathers from each bird, and to place the sample into a separate tube. These were stored at ambient temperature or 4°C until shipment to the University of Tasmania *via* normal airmail postal services.

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Table 4.1. *Puffinus* taxa, their collection details and methods of preservation of samples used in a phylogenetic study of mitochondrial cytochrome b gene sequence variation. [1] Underlined numbers in sample code refers to the Australian Museum identification number for this specimen; [2] Underlined alphanumerics in sample code refers to the University of Washington, Burke Museum identification code for this specimen; [3] Underlined numbers in sample code refers to the Universiteit van Amsterdam, Instituut voor Taxonomische Zoölogie (Zoölogisch Mueum) identification number for this specimen; [4] Underlined alphanumerics in sample code refer to the Louisiana State University, Museum of Natural Sciences identification code for these specimens.

Specimen	Common Name	Code	Locality	Lat., Long.	Tissue	Date Collected	Collector	Storage
Puffinus pacificus	Wedge-tailed shearwater	Ppac3	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac5	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac8	Montague I., Australia	36°15'S, 150°13'E	Feather	March 1992	J. Austin	Liquid Nitrogen
P.pacificus	Wedge-tailed shearwater	Ppac10	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac79	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	L. Ballance/	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac80	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	D. O'Daniel	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac96	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac97	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac99	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac100	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul26	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul29	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul041804[1]	NSW, Australia	35°S, 151°E	Feather	November 1962	P. Strong	Dried museum skin
P.carneipes.hullianus	Flesh-footed shearwater	Pcar16	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes.hullianus	Flesh-footed shearwater	Pcar40	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P. creatopus	Pale-footed shearwater	PcreJMB942[2]	North Pacific Ocean	44°09'N, 159°40'W	Kidney	August 1991	Unknown	70% EtOH, 4°C
P.gravis	Greater shearwater	Pgra154	Emerald Isle, North Carolina, USA	34°41'N, 76°57'W	Feather	June 1993	Unknown	70% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri21	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri25	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri41	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P. tenuirostris	Short-tailed shearwater	Pten5	Bruny I, Australia	43°28'S, 147°14'E	Liver	March 1991	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten200	Port Fairy, Australia	38°24'S, 142°15'E	Liver	March 1992	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten323	Montague I., Australia	36°15'S, 150°13'E	Liver	March 1992	J. Austin	Liquid Nitrogen
P.nativitatis	Christmas shearwater	Pnat81	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.nativitatis	Christmas shearwater	Pnat83	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf86	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	July 1993	J. L. R. Luengo	100% EtOH, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf117	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk61	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk63	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma112	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma116	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw106	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw108	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva1	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva2	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut7	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut9	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.lherminieri.boydi	Audubon's shearwater	Plby39.756[3]	Razo I., Cape Verde Islands	16°N,24°W	Feather	October 1988	T. Prins	Dried museum skin
P.lherminieri.lherminieri	Audubon's shearwater	PlB-20893[4]	near Oregon Inlet, USA	35°30'N, 75°W	Kidney	Unknown	D. L. Dittmann	-20°C freezer
P.lherminieri.lherminieri	Audubon's shearwater	PlB-20918[4]	near Oregon Inlet, USA	35°30'N, 75°W	Kidney	Unknown	D. L. Dittmann	-20°C freezer
P.assimilis.assimilis	Little shearwater	Paas161	Lord Howe Island, Australia	31°28'S, 159°09'E	Feather	July 1994	D. Hiscox	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha31	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha33	Poor Knights Islands., New Zealand	35°30'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr91	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr93	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C

Upon receipt all samples were assigned a unique code consisting of four letters designating the genus and species (e.g. Ppac for *Puffinus pacificus*), and in some cases subspecies, followed by a variable length string of letters and or numbers representing a number assigned by the author (e.g. 77) or, for museum derived samples, the museums' identification code for that particular specimen (e.g. 030233 for a specimen of *P.pacificus* from the Australian Museum).

4.2.3 DNA extraction

Extraction of DNA for PCR amplification requires methods that minimise the number of steps involved, and thus minimise the opportunity for cross transfer of samples or introduction of contaminating DNA, and maximise the removal of substances that inhibit the enzymatic activity of the thermostable *Taq* DNA polymerase.

Total DNA was extracted from blood, feather and tissue samples using a modification of the method described by Walsh *et al.* (1991). For feathers taken from museum skins, approximately 5 mm of the feather tip was cut off and washed in 500 µl of sterile 70% ethanol for 5-10 min. The feather tip was then transferred to 500 µl of sterile distilled water and washed for another 5-10 min. This treatment acts to remove any dust and surface contaminating DNA from the sample. An additional advantage is that it softens the feather tip making it easier to cut up. Subsequently, all samples were treated identically.

Approximately 5 mm of a single feather tip, or 1-2 µl of preserved blood, or 1–2 mm^3 of tissue was cut into small (< 1 mm) pieces using a sterile scalpel blade on a clean surface (the inside of a scalpel blade packet) and then transferred to a 1.5 ml tube containing 200 µl of a 6% Chelex 100 solution (Instagene Purification Matrix, BIORAD) and 0.1-0.3 µg/µl Proteinase K. A control extract was performed for each set of extractions and contained the Chelex/Proteinase K solution but no tissue was added. The mixture was incubated at 56°C for 2-16 h with occasional gentle mixing. Samples were then vortex mixed for 10 sec, placed in a boiling water bath for 8 min, vortexed again for 10 sec and centrifuged at 12 000 G for 2-3 min. The final extract was stored at -20°C. Prior to PCR amplification, extracts were allowed to thaw at room temperature, mixed vigorously for 10 sec and centrifuged at 12 000 G for 2-3 min.

An alternative protocol, which was used on some museum material, is a slight modification of that described by Leeton *et al.* (1993). Using a sterile scalpel blade the terminal 2-5 mm of a feather shaft was cut off and washed in 500 µl of 70% ethanol for 5-10 min. The feather tip was transferred to 500 µl of sterile distilled water and washed for another 5-10 min. The tip was then cut into <1mm sized pieces and placed into a 1.5 ml microcentrifuge tube containing 0.5 ml of digestion buffer (0.05 M Tris.HCL; 0.01 M EDTA; 0.1 M NaCl; pH 8.0), 0.04 M DTT, 10% SDS (w:v) and 100 µg Proteinase K. The mixture was incubated at 37°C for 12–18 h, and then extracted twice with an equal volume of phenol:chloroform:IAA (25:24:1), in which the organic solvent was mixed gently with the aqueous solution for 5 min, centrifuged at 10 000 G for 5 min, and the aqueous phase transferred to a fresh tube. Following the second extraction the aqueous phase was transferred to a Millipore Ultrafree-MC filter microcentrifuge tube and centrifuged at 4 000 G for 20 min. Approximately 400 µl of sterile distilled water was added to the filter unit and centrifugation repeated. Washing was repeated two more times and the final centrifugation was continued until an approximately 50 µl final volume was achieved. The final extract was transferred to a 0.5 ml microcentrifuge tube and stored at -20°C.

4.2.4 DNA amplification and sequencing methods

PCR amplification

For each of the specimens in Table 4.1 a 307 bp segment (excluding primer sequences) of the mitochondrial cytochrome *b* gene was amplified by the polymerase chain reaction using shortened versions of the 'universal' primers of Kocher *et al.* (1989): L14841 (5'–CCATCCAACATCTCAGCATGATGAAA-3' = CB1-L of Palumbi *et al.* 1991b) and H15149 (5'-CCCTCAGAATGATATTTGTCCTCA-3' = CB2-H of Palumbi *et al.* 1991b). The letters L and H refer to the light and heavy strands respectively and the numbers refer to the base position of the 3' end of the primer in the human mtDNA sequence (Anderson *et al.* 1981).

A flow chart showing the various methods used in amplifying and sequencing the target segment of the cytochrome *b* gene is presented in Fig. 4.3. Primary, double-stranded amplifications were carried out in a 50 µl volume containing 67 mM Tris.HCl (pH 8.8), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 2 µg/ml Bovine Serum Albumin

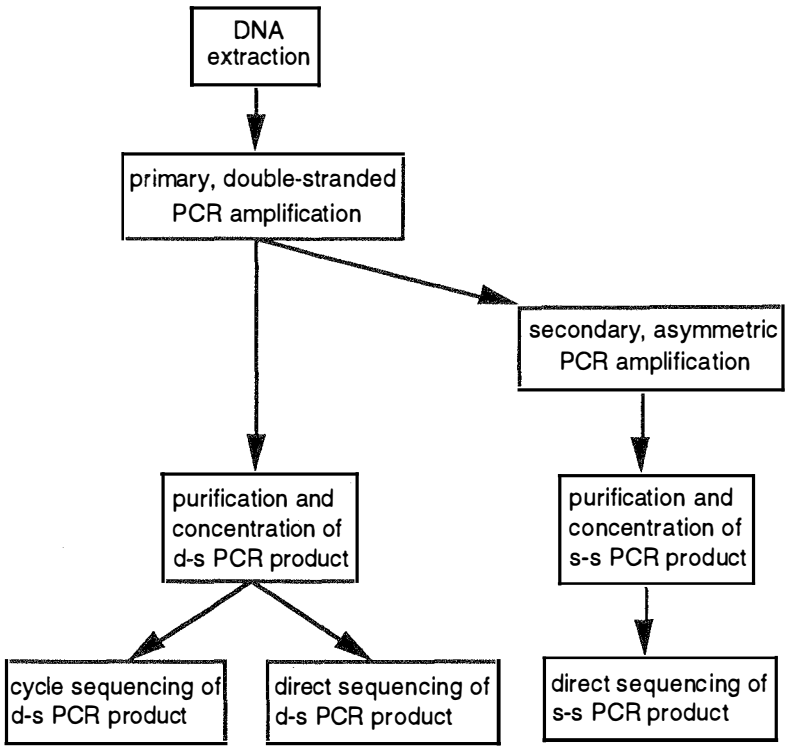

Figure 4.3. Flowchart showing the three methods of obtaining sequence data from the mitochondrial cytochrome *b* gene of *Puffinus*. Direct sequencing of double-stranded (d-s) PCR product was the main method used, although some sequences were obtained by sequencing of single-stranded (s-s) template following a secondary, asymmetric PCR amplification. Cycle sequencing consistently produced poor quality results.

(BSA, Sigma, fraction V), 1-2 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus). The presence of BSA in the reaction mix acts to stabilise the *Taq* DNA polymerase and prevent inhibition of amplification of DNA from both modern and museum specimens (Pääbo 1990; Cooper 1993). The reaction mix was overlaid with two drops of sterile mineral oil and then 1-10 µl of the template extract was added. Amplifications were performed in a Corbett Research FTS-320 Thermal Sequencer, using 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 50-55°C for 1 min and extension at 72°C for 2 min. The initial denaturing step of 94°C in cycle 1 was extended to 3 min to ensure complete denaturation of all initial template strands and the final extension step in cycle 40 was extended to 10 min to allow complete extension of all synthesised strands. Modifications of the basic cycling protocol involved increasing or decreasing the number of cycles (30 or 50 respectively) and variation in the length of each step; however these modifications generally did not enhance the yield or quality of the amplified product.

To assess the quality and quantity of the amplified product a 3 µl aliquot of each PCR amplification was mixed with 2 µl of 2x Bromophenol Blue (BPB) loading buffer and electrophoresed through a 1% agarose minigel in 1xTAE buffer (0.04 M Tris-acetate, 1 mM EDTA pH 7.8) for 40 min at 70 V. The minigel was stained in 150 ml of the running buffer containing 0.5 $\mu g.ml^{-1}$ ethidium bromide for 15-20 min and examined under UV light. A SPP-1 phage DNA restricted with *Eco RI* (Bresatec) was used as a molecular weight size standard.

Secondary, asymmetric amplifications were carried out on some samples, to generate single-stranded DNA, using the unbalanced priming method (Gyllensten & Erlich 1988) and as described by Palumbi *et al.* (1991b). Two separate reactions were performed for each template to generate both light- and heavy-strand single-stranded product. Amplifications were performed in a 50 µl volume containing 67 mM Tris.HCl (pH 8.8), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of one primer, 2 µg/ml BSA, 1-2 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus). The reaction mix was overlaid with two drops of sterile mineral oil and then 2 µl of the undiluted primary (double-stranded) PCR product was added. There are sufficient amounts of the second primer in the primary PCR product to effect amplification in the secondary reaction. Amplifications were performed using 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 57°C for 1 min and extension at 72°C for 2 min. The initial denaturing step of 94°C in cycle 1 was extended to 3 min and the final extension step in cycle 40 was extended to 10 min. Amplifications were assessed by minigel electrophoresis as described above.

Purification and concentration of PCR product

Double-stranded PCR product was purified and concentrated using one of two commercially available kits, GeneClean II (BIO 101) and Bresa-Clean (Bresatec). The protocol for the use of both kits is similar. The remaining 47 µl of each PCR reaction was electrophoresed through a 1.4% agarose minigel gel in 1xTAE buffer (pH7.8) for 40 min at 70 V, to separate the product from primers and excess nucleotides. The minigel was stained, in a light-proof box, in 150 ml of the running buffer containing 0.5 $\mu g.ml^{-1}$ ethidium bromide fo 15-20 min. The minigel was examined under UV light and the band containing the amplified sequence

was excised from the gel, cut into small pieces and placed into a preweighed 1.5 ml microcentrifuge tube. The mass of each gel slice was determined.

For the Geneclean kit, 3 volumes (w:v) of the sodium iodide stock solution was added and the tube incubated at 55°C, with occasional mixing, for 5 min or until all the agarose had melted. Five microlitres of resuspended Glassmilk was added to the solution which was then mixed well by inversion and placed on ice for 5-10 min, with occasional mixing. The Glassmilk was pelleted by centrifugation at 12 000 G for 5 sec and the supernatant removed and discarded with a drawn Pasteur pipette. The Glassmilk was washed three times with 0.5 ml of cold (<0°C) New Wash. For each wash the Glassmilk pellet was resuspended by repeatedly pipetting the New Wash solution up and down in the tube; the suspension was then centrifuged at 12 000 G for 5 sec and the supernatant drawn off and discarded. The final centrifugation was extended to 1 min and the washed Glassmilk pellet was then dried *in vacuo* for 5-10 min. DNA was eluted from the Glassmilk by resuspending the pellet in 10 µl of sterile distilled water, incubation at 50°C for 3 min, and centrifugation at 12 000 G for 1 min. The supernatant, containing the eluted DNA was carefully removed and placed in a 0.5 ml microcentrifuge tube. The elution was repeated using another 6-10 µl of sterile distilled water, to enhance recovery of DNA. The two eluants were combined to give a final volume of approximately 16 µl.

For the Bresa-Clean kit, 3 volumes (w:v) of the Bresa-Salt stock solution was added and the tube incubated at 55°C, with occasional mixing, for 5 min or until all the agarose had melted. Five microlitres of resuspended Bresa-Bind matrix was added to the solution which was then mixed well by inversion and incubated at room temperature for 5 min, with occasional mixing. The matrix was pelleted by centrifugation at 12 000 G for 5 sec and the supernatant removed and discarded with a drawn Pasteur pipette. The matrix was washed twice with 0.75 ml of cold (4°C) Bresa-Wash. For each wash the pelleted matrix was resuspended by repeatedly pipetting the Bresa-Wash solution up and down in the tube, the suspension was centrifuged at 12 000 G for 5 sec and the supernatant drawn off and discarded. The final washed matrix pellet was then dried *in vacuo* for 5-10 min. DNA was eluted from the matrix by resuspending the pellet in 10 µl of sterile distilled water, incubation at 50°C for 5 min, and centrifugation at 12 000 G for 1 min. The supernatant, containing the eluted DNA was

carefully removed and placed in a 0.5 ml microcentrifuge tube. The elution was repeated using another 6-10 µl of sterile distilled water, to enhance recovery of DNA. The two eluants were combined to give a final volume of approximately 16 µl.

Using these methods up to 1.5 µg of double-stranded DNA could be recovered from a 50 µl PCR reaction volume, although yields were dependent on the strength of the initial amplification. Purified and concentrated double-stranded DNA was stored at -20°C.

Single-stranded DNA was purified and concentrated using a protocol described by Palumbi *et al.* (1991b). The remaining 47 µl of each PCR reaction was transferred to a fresh 0.5 ml microcentrifuge tube. Transfer of mineral oil was avoided by wiping the pipette tip with a Kimwipe prior to pipetting into the fresh tube. To the tube was added 25 µl of 7.5 M ammonium acetate (pH 5.2) and 75 µl of isopropanol. The solution was mixed well and incubated at room temperature for 15 min with occasional mixing. Precipitated DNA was pelleted by centrifugation at 12 000 G for 10 min and the supernatant drawn off with a pasteur pipette. The pellet was washed with 0.5 ml ice-cold 70% ethanol and then centrifuged at 12 000 G for 2 min. The supernatant was removed and the pellet was dried *in vacuo* for 15 min and resuspended in 7 µl of 0.1xTE buffer (pH 7.5). Purified and concentrated single-stranded DNA was stored at -20°C.

DNA sequencing

Three sequencing strategies were utilised in this study. All were based on the dideoxy sequencing method (Sanger *et al.* 1977) with radioactive (^{35}S-dATP) labelling of the sequence product. Both the light and heavy strands were sequenced for each individual. This allowed the complete sequence of the 307 bp target region to be determined and provided authentication of sequence from one strand in the overlapping sequence from the complementary strand. Oligonucleotide primers used for sequencing were identical to those used in the PCR amplification of the target sequence.

The first method used the commercially available CircumVent Thermal Cycle DNA Sequencing Kit (New England Biolabs) and double-stranded DNA template in which a thermostable DNA polymerase (Vent$_R$[exo$^-$]) is used to generate sequence product through repeated cycles of a distinct temperature profile, similar to a PCR reaction. The advantages of

this method are that very little template DNA is required, separate annealing, labelling and termination steps are not necessary and the sequencing reactions are performed at high temperature, potentially eliminating problems associated with secondary structure in the DNA template. The protocol for sequencing with labelled dATP incorporation, described in the instruction manual, was followed (New England Biolabs 1993). This method was introduced following problems with the two alternate protocols (below), however sequence data obtained were consistently of poor quality and so this method was abandoned.

All sequences reported in this study were obtained by direct sequencing of either double-stranded or single-stranded DNA template using the commercially available Sequenase Version 2.0 DNA sequencing kit (United States Biochemicals). Direct sequencing of double-stranded template was the initial, and main, method of generating sequence data. The switch to single-stranded (and cycle) sequencing was made when difficulties arose in generating consistent and high quality sequence data from the light strand of the template. Subsequently, evidence was obtained suggesting that most of the sequencing problems were related to some form of degradation of the primers, either through repeated freezing and thawing or the length of storage. Given the relative speed with which both strands could be sequenced from a single, primary amplification of the template, double-stranded sequencing remained the method of choice.

Direct sequencing of double-stranded DNA was performed in 0.5 ml microcentrifuge tubes using the following protocol, which is a modification of that described in the Sequenase Version 2.0 instruction manual (United States Biochemical 1993). The primer (light- or heavy-strand) was annealed to the template in an annealing reaction containing 1 µl of a 10 µM primer solution, 2 µl of the 5x Sequenase Reaction Buffer, 1 µl of 20% (v:v) Non-idet P40 and 7 µl of DNA template solution. The reaction was placed in a boiling water bath or metal heating block set to 99°C for 3 min, then snap-frozen in a dry ice/ethanol bath or liquid nitrogen bath and placed on ice. Prior to the labelling reaction, tubes were centrifuged briefly to collect condensation. Extension and radioactive labelling of the annealed primer were performed in a labelling reaction in which 1 µl of 0.1 M DTT, 2 µl of the Sequenase dGTP Labelling Mix diluted 1 in 5 with sterile distilled water, 0.5-1.5 µl of α-^{35}S-dATP (12.5 $\mu Ci.\mu l^{-1}$), 2 µl of Sequenase T7 DNA polymerase diluted 1 in 8 with Enzyme Dilution

Buffer was added to the annealed primer-template solution, mixed and incubated at room temperature for 2-5 min. Four base–specific termination reactions were performed for each labelling reaction, in each of which 3.5 µl of the labelling reaction was mixed with 2.5 µl of the dGTP Termination Mix (ddATP, ddCTP, ddGTP or ddTTP) and incubated at 37°C for 3–5 min. At the completion of the termination reactions, 4 µl of Stop Mix was added to each tube. Completed sequencing reactions were stored at -20°C. The quality of the sequence produced using this protocol is significantly improved by the inclusion of the detergent Non-idet P40 in the annealing reaction (Bachmann *et al.* 1990).

Direct sequencing of single-stranded template was performed using a similar protocol except that the Non-idet P40 was omitted from the annealing reaction, the annealing reaction was heated to 65°C for 5 min in a heat block and then allowed to cool to room temperature over 15-30 min followed by a brief centrifugation and storage on ice prior to the labelling reaction, and the Labelling Mix was omitted from the labelling reaction (replaced with sterile distilled water only). This protocol is as described by Palumbi *et al.* (1991b).

Electrophoresis and autoradiography

Sequencing products were separated through 500 mm long, 6% polyacrylamide wedge shaped gels (0.25-0.75 mm thick) in TBE buffer (89 mM Tris.HCl, 89 mM boric acid, 25 mM EDTA pH 8.2). The gels were fitted with a standard 48 tooth sharks-tooth comb and were pre-electrophoresed at 60W (~2300 V) for 45 min to obtain a gel temperature of approximately 50°C. Sequencing products were heated to 80°C for 2 min and then 2-3 µl loaded onto the gel in the order ACGT. The gels were electrophoresed for 15-30 min at 60 W (~2 300 V), and then 55 W (~2 000 V) for 75-90 min until the bromophenol blue marker dye had migrated to the bottom edge of the gel. On completion of electrophoresis the sequencing apparatus was dismantled and the gel washed in 10% (v:v) glacial acetic acid for 1 h, with gentle agitation. The gel was dried in a 37°C oven overnight and an autoradiograph produced by exposure to X-ray film for 24 to 120 h. Sequences from both the light and heavy strands of each template were read, over a light box, and recorded in a 5' to 3' direction as the light strand sequence.

4.2.5 Sequence alignments and phylogenetic analyses

Sequences were aligned by eye, a simple procedure given that no gaps or deletions were detected in any of the sequences. Two incomplete sequences of 288- and 289-bp, missing the last 19 and 18 bases, respectively, of the 3' end of the cytochrome *b* fragment from the northern fulmar (*Fulmarus glacialis*) were obtained from the literature (Wink *et al.* 1993) and included as an outgroup taxa.

Three methods of phylogenetic analysis were applied to the aligned sequence data; these are the most commonly used in phylogenetic studies based on molecular sequences.

Maximum parsimony, maximum likelihood and neighbour-joining distance matrix methods were used, on Apple Macintosh computers, to evaluate phylogenetic relationships among taxa. To decrease computational load and because of the small amount of intra-taxon sequence variation, a consensus sequence was produced for each taxon (species or subspecies). At variable sites within species the consensus sequence was assigned either the most common nucleotide at that site among individuals (i.e. majority rule, 5/9 cases), or the nucleotide that required the least amount of change relative to all other sequences (i.e. most parsimonious, 4/9 cases). In the latter cases, 3/4 of the variant nucleotides were unique to that individual. The 18 unknown nucleotides from the 3'-end of the fulmar consensus sequence were coded as missing data. In all analyses the fulmar sequence was assigned as an outgroup to root trees.

Maximum likelihood analysis was performed on the entire data set using the DNAML program in the phylogeny inference package, PHYLIP Version 3.5c (Felsenstein 1993). The optimal value for the transition/transversion (TS/TV) ratio was determined empirically (Kishino & Hasegawa 1989) using a subset of the 11 taxa and maximising the likelihood. It was necessary to reduce the number of taxa to decrease computer run time, but this does not reduce the efficiency of the technique in finding the optimal ratio (A. Cooper, R. Kusmierski, personal communications). The subset of taxa was chosen from an initial tree, generated using maximum parsimony, by eliminating ingroup taxa that clustered closely and included *P. pacificus*, *P. c. hullianus*, *P. griseus*, *P. nativitatis*, *P. p. puffinus*, *P. p. mauretanicus*, *P. au. newelli*, *P. huttoni*, *P. l. boydi*, *P. as. assimilis* and *F. glacialis*. Transition/transversion ratios of between 1 and 20 were trialled in global searches for the best

tree among the subset of taxa, using empirical base frequencies, one category of substitution rates and random input of taxa. The optimal TS/TV ratio was then applied to a global search using the same options as for the trial trees, and a tree of maximum likelihood recovered. The significance of branch lengths was tested using an approximate likelihood ratio test, implemented in the DNAML program, although this test may overestimate the significance of branch lengths (Felsenstein 1993).

Parsimony analysis was performed on phylogenetically informative sites using PAUP 3.1 (Swofford 1993). The large number of terminal taxa, and particularly the application of various weighting schemes to the data, prevented the use of the exhaustive or branch-and-bound search options which are guaranteed to find the shortest tree(s). The heuristic search option was therefore used with the random stepwise addition and tree-bisection-reconnection branch swapping options, repeated 100 times. The random stepwise addition procedure decreases the chance that the heuristic search becomes trapped in a local optima by providing different initial trees on which to search for the optimal tree (Swofford & Begle 1993). Results were the same irrespective of the addition sequence of taxa suggesting that the optimal trees recovered represented all of the optimal trees for that particular data set (Swofford & Begle 1993). Strict consensus trees were generated for all most parsimonious trees found in each search.

To assess the phylogenetic information content of the sequence data, the skewness of the tree-length distribution for 10 000 random trees generated using the Random Trees option in PAUP was examined with the *g1* statistic. Hillis and Huelsenbeck (1992) have shown that the tree-length distribution of random trees from a sequence data set that contains a significant phylogenetic signal is strongly skewed to the left. Tree-length distributions for data sets that have been saturated with nucleotide changes and therefore contain substantial random "noise" are more symmetrical. The *g1* statistic provides an estimate of the skewness of tree-length distributions and can be used to test for significant phylogenetic information content in sequence data matrices.

In the main parsimony analysis, all nucleotide sites were uniformly weighted, with a differential weighting scheme applied to transition and transversion character state changes to

reflect the observed transition bias in shearwater nucleotide evolution. Symmetrical TS/TV ratios between 1 (i.e.equally weighted) and 20 (transversions weighted 20 times as much as transitions) were examined using the stepmatrix option in PAUP. The relative significance of particular clades was estimated from a majority-rule consensus of trees obtained by heuristic searches of 500 bootstrap samples of the original data set (Felsenstein 1985). In addition, near most parsimonious trees were examined by constructing strict consensus trees for each step greater than the most parsimonious trees. The number of steps greater than parsimony that a clade remains resolved provides an indication of the phylogenetic strength of that clade (Desalle 1992).

Nucleotide sequence divergence values for all pairwise comparisons of taxa were calculated using a maximum-likelihood model, in the DNADIST program of PHYLIP, which assumes independent evolution at all sites, and allows for different rates of transitions and transversions and different frequencies of the four nucleotides (Felsenstein 1993). The following settings were used for the DNADIST program: one category of substitution rates, empirical base frequencies were used and the optimal TS/TV ratio determined under maximum likelihood analysis. The neighbour-joining algorithm (Saitou & Nei 1987), NEIGHBOR in PHYLIP, was used to fit a tree to the distance matrix of sequence divergence values, without the assumption of equal rates of evolution among taxa.

Trees obtained using parsimony and distance matrix methods, and alternative tree topologies based on the results of Kuroda (1954) and Wragg (1985) were compared to the tree of maximum likelihood using the statistical test of Kishino and Hasegawa (1989) which estimates the variance of the difference in likelihood between competing trees, taken across sequence positions. The test was implemented using the DNAML program in PHYLIP in which competing tree topologies were input as user defined trees and their respective log likelihoods, and the variance of the difference between likelihoods calculated. The alternate tree was considered significantly worse than the tree of maximum likelihood if the difference in their log likelihoods was greater than 1.96 standard deviations of the difference.

4.3 RESULTS

4.3.1 DNA sequences

Complete DNA sequences were obtained from a 307 bp segment of the cytochrome *b* gene for 46 shearwaters representing 19 extant taxa (Table 4.1 and Fig. 4.4). Two partial sequences of 288 and 287 bp of this segment were obtained from the literature for *F. glacialis* (Wink *et al.* 1993). The shearwater sequences were validated by a number of criteria. Firstly, both the light and heavy strands were sequenced to allow authentication in the region of overlap. In all cases, the sequences from both strands for each individual were 100% complementary. Secondly, when the cytochrome *b* fragment from an individual was sequenced more than once (e.g. to resolve an ambiguity in the original sequencing attempt), this was done from an independent PCR amplification and in all cases the second sequence was identical to the first. Third, the sequences were validated as being of shearwater origin by comparison with a published sequence for *P. p. puffinus* (Wink *et al.* 1993), and their phylogenetic affinity with other Procellariiform cytochrome *b* gene sequences (R. Dawson, personal communication).

DNA sequences were obtained from every tissue type (blood, feather, liver and kidney) preserved and stored under a variety of conditions ranging from deep frozen, refrigerated and room temperature storage, to ethanol preservation and simple air-drying, and ranging in age since collection from several months to 32 years. Attempts to obtain sequence data from additional specimens failed because of problems with extracting DNA from tissue samples (i.e. repeated attempts to amplify from separate extractions failed), an inability to amplify sufficient DNA to warrant attempts to sequence (i.e. weak amplifications or no amplifications), or ambiguous/non avian sequences obtained from apparently authentic amplifications. These problems were generally restricted to the use of feathers plucked from dried museum skins. There were 15 such specimens ranging in age from six years (Plby39.756) to 99 years (Pten032171). Authenticated sequences were obtained for only two of these, Plby39.756 and Pbul041804 (32 years old). For each of these specimens, two independent extractions, PCR amplifications and DNA sequencing reactions yielded identical sequences. A DNA fragment of the appropriate size was amplified from DNA extractions of two other specimens, Pten032171 and Popi31353, however when sequenced these proved to be of human origin. For the remaining 11 museum specimens, no DNA could be amplified despite a number of different extraction techniques and rigorous attempts to optimise PCR conditions.

```
Ppac3        C TTT GGG TCT CTC CTA GGC ATC TGC CTA CTA ACT CAA ATC TTA ACC GGC CTA TTA CTA
Ppac5        . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac8        . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac10       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac79       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac80       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac96       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac97       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac99       . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac100      . ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ...
Pbul26       . ... ..A ... ... ... ..T ... ... ... ... ... ... ... C.. ... ... ... ... ...
Pbul29       . ... ..A ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul041804   . ... ..A ... ... ... ..T ... ... ... ... ... ... ... C.. ... ... ... ... ...
Pcar16       . ... ..A ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ..G ...
Pcar40       . ... ..A ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ..G ...
PcreJMB942   . ... ..A ... ... ... ... ... ... ... ... ..C G.. ... ... ... ... ... ... ...
Pgra154      . ... ..A ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ...
Pgri21       . ... ..A ... ... ... ... ... ... ... ..G ..C ... ... ... ... ... ... ..G ..G
Pgri25       . ... ..A ... ... ... ... ... ... ... ..G ..C ... ... ... ... ... ... ..G ..G
Pgri41       . ... ..A ... ... ... ... ... ... ... ..G ..C ... ... ... ... ... ... ..G ..G
Pten5        . ... ..A ... ... ... ... ... ... ... ... ..C ... ... C.. ... ... ... ... ...
Pten200      . ... ..A ... ... ... ... ... ... ... ... ..C ... ... C.. ... ... ... ... ...
Pten323      . ... ..A ... ... ... ... ... ... ... ... ..C ... ... C.. ... ... ... ... ...
Pnat81       . ... ..C ... ..T ... ..T ... ... ... ... ... ... ... ... ..T ... ... C.. ...
Pnat83       . ... ..C ... ..T ... ..T ... ... ... ... ... ... ... ... ..T ... ... C.. ...
Pppf86       . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Pppf117      . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Ppyk61       . ... ..C ... ... ... ... ... ... ... ..G ..C ... ..T ... ..T ... ..G C.. ...
Ppyk63       . ... ..C ... ... ... ... ... ... ... ..G ..C ... ..T ... ..T ... ..G C.. ...
Ppma112      . ... ..C ... ... ... ... ... ... ..G ..G ..C ... ..T ... ..T ... ... C.. ...
Ppma116      . ... ..C ... ... ... ... ... ... ..G ..G ..C ... ..T ... ..T ... ... C.. ...
Panw106      . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Panw108      . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Pgva1        . ... ..C ... ... ... ... ... ... ... T.. ..C ... ..T ... ..T ... ... C.. ...
Pgva2        . ... ..C ... ... ... ... ... ... ... T.. ..C ... ..T ... ..T ... ... C.. ...
Pgva46       . ... ..? ... ... ... ... ... ... ... G.. ..C ... ..T ... ..T ... ... C.. ...
Phut7        . ... ..C ... ... ... ... ... ... ... T.. ..C ... ..T ... ..T ... ... C.. ...
Phut9        . ... ..C ... ... ... ... ... ... ... T.. ..C ... ..T ... ..T ... ... C.. ...
Plby39.756   . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
PllB-20893   . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
PllB-20918   . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Paas161      . ... ..C ... ... ... ..T ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Paha31       . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... ... ...
Paha33       T ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... ... ...
Pabr91       . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Pabr93       . ... ..C ... ... ... ... ... ... ... ... ..C ... ..T ... ..T ... ... C.. ...
Fglac1       . ..C ..A ..C ... T.. ... ... ... ... A.. ..C ... ... C.. ... ..T ... ... ...
Fglac2       . ..C ..A ..C ... T.. ... ... ... ... A.. ..C ... ... C.. ... ..T ... ... ...
          14842      14850
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Figure 4.4. Partial nucleotide sequences of the mitochondrial cytochrome *b* gene for 19 species and subspecies of the genus *Puffinus*, and the the northern fulmar (*Fulmarus glacialis*, Wink *et al.* 1993). Dots indicate identity with the Ppac3 sequence. Numbers refer to corresponding positions in the human mtDNA sequence. ? indicates unknown base.

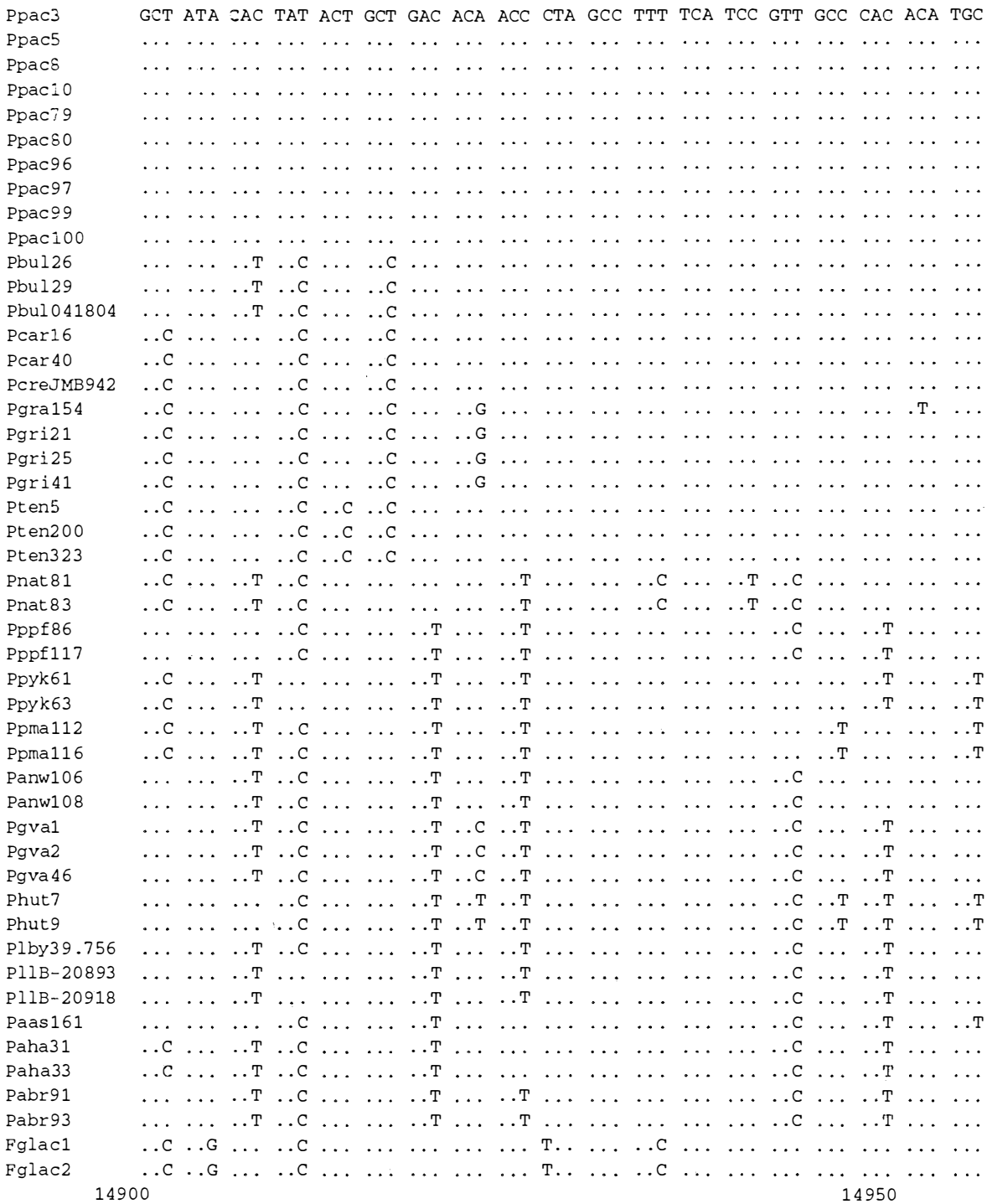

Figure 4.4.-continued

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Ppac3        CGA AAC GTA CAA TAT GGT TGA CTA ATC CGG AAC CTA CAT GCA AAT GGA GCC TCA TTC
Ppac5        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac8        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac10       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac79       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac80       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac96       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac97       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac99       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac100      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul26       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pbul29       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pbul041804   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pcar16       ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pcar40       ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C ... ... ...
PcreJMB942   ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pgra154      ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pgri21       ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pgri25       ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pgri41       ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pten5        ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pten200      ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pten323      ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ...
Pnat81       ... ... ... ... ..C ... ... ... ... ..A ... T.. ... ... ..C ... ... ..C ...
Pnat83       ... ... ... ... ..C ... ... ... ... ..A ... T.. ... ... ..C ... ... ..C ...
Pppf86       ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ..C ..T ... ... ...
Pppf117      ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ..C ..T ... ... ...
Ppyk61       ... ..T ... ... ..C ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Ppyk63       ... ..T ... ... ..C ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Ppma112      ... ... ... ... ..C ... ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Ppma116      ... ... ... ... ..C ... ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Panw106      ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ..C ..C ..C ... ... ...
Panw108      ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ..C ..C ..C ... ... ...
Pgva1        ... ... ... ... ... ... ... ... ..T ..A ... T.. ... ... ..C ... ... ... ...
Pgva2        ... ... ... ... ... ... ... ... ..T ..A ... T.. ... ... ..C ... ... ... ...
Pgva46       ... ... ... ... ... ... ... ... ..T ..A ... T.. ... ... ..C ... ... ... ...
Phut7        ... ... ... ... ..C ..C ... ... ..T ... ... T.. ... ... ..C ... ... ... ...
Phut9        ... ... ... ... ..C ..C ... ... ..T ... ... T.. ... ... ..C ... ... ... ...
Plby39.756   ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
PllB-20893   ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
PllB-20918   ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Paas161      ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..T ... ... ...
Paha31       ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ... ..C ... ... ...
Paha33       ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ... ..C ... ... ...
Pabr91       ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Pabr93       ... ... ... ... ... ..C ... ... ..T ..A ... ... ... ... ..C ..C ... ... ...
Fglac1       ... ... ... ..G ..C ..C ... ..C ..T ..A ..T ... ... ... ..C ... ... ... ...
Fglac2       ... ... ... ..G ..C ..C ... ..C ..T ..A ..T ... ... ... ..C ... ... ... ...
                                                                  15000
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Figure 4.4.-continued

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Ppac3        TTT TTC ATC TGC ATT TAT CTC CAC ATT GGA CGA GGA TTC TAC TAT GGC TCC TAC CTA
Ppac5        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac8        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac10       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac79       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac80       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac96       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac97       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac99       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac100      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul26       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ..G
Pbul29       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ..G
Pbul041804   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ..G
Pcar16       ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
Pcar40       ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
PcreJMB942   ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
Pgra154      ... ... ... ..T ... ... ... ... ..C ... ... ... ... ... ..C ... ... ... ...
Pgri21       ... ... ... ..T ... ... ... ..T ... ... ... ... ... ... ..C ... ... ... ...
Pgri25       ... ... ... ..T ... ... ... ..T ... ... ... ... ... ... ..C ... ... ... ...
Pgri41       ... ... ... ..T ... ... ... ..T ... ... ... ... ... ... ..C ... ... ... ...
Pten5        ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
Pten200      ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
Pten323      ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ...
Pnat81       ..C ... ... ..T ..C ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Pnat83       ..C ... ... ..T ..C ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Pppf86       ..C ... ... ..T ... ..C ..G ... ..C ... ... ... ... ... ..C ... ..T ... ...
Pppf117      ..C ... ... ..T ... ..C ..G ... ..C ... ... ... ... ... ..C ... ..T ... ...
Ppyk61       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Ppyk63       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Ppma112      ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Ppma116      ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ... ... ...
Panw106      ..C ... ... ..T ... ..C ... ... ..C ... ... ... ... ... ... ... ..T ... ...
Panw108      ..C ... ... ..T ... ..C ... ... ..C ... ... ... ... ... ... ... ..T ... ...
Pgva1        ..C ... ... ... ... ..C ..A ... ..C ... ..G ... ... ... ... ... ..T ... ..G
Pgva2        ..C ... ... ... ... ..C ..A ... ..C ... ..G ... ... ... ... ... ..T ... ..G
Pgva46       ..C ... ... ... ... ..C ..A ... ..C ... ..G ... ... ... ... ... ..T ... ..G
Phut7        ..C ... ... ... ... ..C T.A ... ..C ... ... ..G ... ... ... ... ..T ... ..G
Phut9        ..C ... ... ... ... ..C T.A ... ..C ... ... ..G ... ... ... ... ..T ... ..G
Plby39.756   ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ..T ... ...
PllB-20893   ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ..T ... ...
PllB-20918   ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ..T ... ...
Paas161      ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ..T ... ... ..T ..T ... ...
Paha31       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ..T ..T ... ...
Paha33       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ..T ..T ... ...
Pabr91       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ..T ... ...
Pabr93       ..C ... ... ..T ... ..C ..A ... ..C ... ... ... ... ... ... ... ..T ... ...
Fglac1       ... ... ... ... ... ..C ..A ..T ... ... ... ... ... ... ... ... ... ... ..T
Fglac2       ... ... ... ... ... ..C ..A ..T ... ... ... ... ... ... ... ... ... ... ..T
                                                          15050
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Figure 4.4.-continued

```
Ppac3       TAC AAA GAA ACC TGA AAC ACA GGA GTC ATT CTT CTA CTT ACC CTC ATG GCA ACT GCC
Ppac5       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac8       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac10      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac79      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac80      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac96      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac97      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac99      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Ppac100     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul26      ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul29      ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pbul041804  ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pcar16      ..T ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ...
Pcar40      ..T ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ...
PcreJMB942  ..T ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ...
Pgra154     ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Pgri21      .CT ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ...
Pgri25      ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ...
Pgri41      ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ...
Pten5       ..T ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ..C ...
Pten200     ..T ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ..C ...
Pten323     ..T ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ..C ...
Pnat81      ..T ... ... ..T ... ..T ... ... ... ... ..C ... ..C ..T ... ..A ... ... ...
Pnat83      ..T ... ... ..T ... ..T ... ... ... ... ..C ... ..C ..T ... ..A ... ... ...
Pppf86      ..T ... ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pppf117     ..T ... ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Ppyk61      ..T ... ..G ... ... ... ... ... ... ..C ..C ..G ..C ..T ... ..A ... ... ...
Ppyk63      ..T ... ..G ... ... ... ... ... ... ..C ..C ..G ..C ..T ... ..A ... ... ...
Ppma112     ..T ... ..G ... ..G ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Ppma116     ..T ... ..G ... ..G ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Panw106     ..T ..G ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Panw108     ..T ..G ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pgva1       ... ... ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pgva2       ... ... ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pgva46      ... ... ..G ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Phut7       ..T ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Phut9       ..T ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Plby39.756  ... ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
PllB-20893  ... ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
PllB-20918  ... ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Paas161     ..T ... ..G ... ... ..T ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Paha31      ..T ... ..G ... ... ..T ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Paha33      ..T ... ..G ... ... ..T ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pabr91      ... ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Pabr93      ... ... ... ... ... ... ... ... ... ..C ..C ... ..C ..T ... ..A ... ... ...
Fglac1      ... ... ... ... ... ... ... ... A.A ..C ... ... ..C ... ... ..A ... ... ...
Fglac2      ... ... ... ... ... ... ... ... G.A ..C ... ... ..C ... ... ..A ... ..C ...
                                            15100
```

Figure 4.4.-continued

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Ppac3          TTC GTG GGA TAT GTT CTG CCC
Ppac5          ... ... ... ... ... ... ...
Ppac8          ... ... ... ... ... ... ...
Ppac10         ... ... ... ... ... ... ...
Ppac79         ... ... ... ... ... ... ...
Ppac80         ... ... ... ... ... ... ...
Ppac96         ... ... ... ... ... ... ...
Ppac97         ... ... ... ... ... ... ...
Ppac99         ... ... ... ... ... ... ...
Ppac100        ... ... ... ... ... ... ...
Pbul26         ... ... ... ... ... ..A ...
Pbul29         ... ... ... ... ... ... ...
Pbul041804     ... ... ... ... ... ..A ...
Pcar16         ... ..A ... ... ..C ... ...
Pcar40         ... ..A ... ... ..C ... ...
PcreJMB942     ... ..A ... ... ..C ... ...
Pgra154        ... ..A ... ... ..C ... ...
Pgri21         ... ... ... ... ..C ..A ...
Pgri25         ... ... ... ... ..C ..A ...
Pgri41         ... ... ... ... ..C ..A ...
Pten5          ... ..A ... ... ..C ..A ..T
Pten200        ... ..A ... ... ..C ..A ..T
Pten323        ... ..A ... ... ..C ..A ..T
Pnat81         ... ..A ..G ..C ..C T.A ..T
Pnat83         ... ..A ..G ..C ..C T.A ..T
Pppf86         ... ..A ... ... ..C ..A ...
Pppf117        ... ..A ... ... ..C ..A ...
Ppyk61         ... ..A ... ..C ..C ..A ...
Ppyk63         ... ..A ... ..C ..C ..A ...
Ppma112        ... ..A ... ..C ..C ..A ...
Ppma116        ... ..A ... ..C ..C ..A ...
Panw106        ... ..A ... ... ..C ..A ...
Panw108        ... ..A ... ... ..C ..A ...
Pgva1          ... ..A ... ..C ..C ..A ...
Pgva2          ... ..A ... ..C ..C ..A ...
Pgva46         ... ..A ... ..C ..C ..A ...
Phut7          ... ... ... ..C ..C ..A ...
Phut9          ..A ... ... ..C ..C ..A ...
Plby39.756     ... ..A ... ... ..C ..A ..T
PllB-20893     ... ..A ... ... ..C ... ...
PllB-20918     ... ..A ... ... ..C ... ...
Paas161        ... ..A ... ..C ..C ..A ..T
Paha31         ... ..A ... ..C ..C ..A ..T
Paha33         ... ..A ... ..C ..C ..A ..T
Pabr91         ... ..A ... ... ..C ..A ..T
Pabr93         ... ..A ... ... ..C ..A ..T
Fglac1         .?? ??? ??? ??? ??? ??? ???
Fglac2         ..? ??? ??? ??? ??? ??? ???
                                    15148
```

Figure 4.4.-continued

Despite numerous attempts, no samples were able to be obtained from *P. c. carneipes*, *P. a. auricularis*, and most of the subspecies of *P. lherminieri* and *P. assimilis*. Feathers from one museum specimen of *P. opisthomelas* were made available but no cytochrome *b* sequences could be obtained from this specimen.

4.3.2 Sequence variation in the cytochrome *b* gene

Intrataxon variation

Cytochrome *b* sequences were obtained from two or more individuals for 15 of the 19 *Puffinus* species and subspecies examined, and in five of these taxa (*P.pacificus*, *P.griseus*, *P.tenuirostris*, *P.p.puffinus*, *P.gavia*) the individual birds originated from different and geographically separate breeding colonies. Samples from *P.pacificus* and *P.tenuirostris*, in particular, represented a major part of the respective species' breeding range. Thus it was possible to assess the extent of intraspecific and intrasubspecific variation in this segment of the mitochondrial cytochrome *b* gene. Nine taxa showed no intrataxon variation, five taxa (*P.pacificus*, *P.griseus*, *P.gavia*, *P.huttoni*, and *P.as.haurakiensis*) were variable at only one nucleotide position, and *P.bulleri* showed intraspecific variation at two sites (Fig. 4.4). At all seven of these variable sites, the variant nucleotide was present in only one individual, and in five cases the variant was unique among all 48 sequences. Intrataxon sequence variation in this segment of the cytochrome *b* gene was therefore negligible.

Intertaxon variation

In comparisons between taxa, 222 (72%) of the 307 nucleotide sites examined were invariant and 85 (28%) sites were variable, of which nine (11%) were at first codon positions, one (1%) at a second codon position and 75 (88%) at third codon positions (Fig. 4.4). Of the 85 variable sites, 53 (62.4%) were phylogenetically informative, with four (8%) at first codon positions and 49 (92%) at third codon positions. Eight of the nine variable first codon positions and all of the phylogenetically informative first codon positions occurred in leucine codons and were silent substitutions. No insertions or deletions were observed in any taxa, relative to other *Puffinus* taxa, or the chicken or human cytochrome *b* sequences. There was a strong observed bias in transition substitutions relative to transversion substitutions . In pairwise comparisons between *Puffinus* taxa the number of transitions varied between zero and 40, and the number of transversions between zero and 4 (Table 4.2). When transitions were plotted against transversions a steep linear relationship was found with an observed

initial TS/TV ratio between closely related taxa of approximately 10-15:1 (Fig. 4.5A).

Table 4.2. Number of transitions (above the diagonal) and number of transversions (below the diagonal) for pairwise comparisons of 307 bp of mtDNA cytochrome *b* gene between 19 species and subspecies of *Puffinus* shearwaters and 288 bp of mtDNA cytochrome *b* gene of the fulmar (*Fulmarus glacialis*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Puffinus pacificus*	-	11	14	13	15	17	18	34	27	34	33	29	31	33	28	25	37	31	28	24
2. *P. bulleri*	0	-	13	12	14	14	13	32	27	36	33	29	31	33	30	31	37	33	30	25
3. *P. c. hullianus*	0	0	-	1	7	9	10	31	24	31	30	26	34	34	27	26	32	26	27	24
4. *P. creatopus*	1	1	1	-	6	10	9	30	23	30	29	25	33	33	26	25	32	25	26	23
5. *P. gravis*	0	0	0	1	-	8	9	32	23	30	29	27	32	34	28	27	35	29	28	27
6. *P. griseus*	1	1	1	2	1	-	10	36	27	32	31	31	36	36	32	33	40	33	32	27
7. *P. tenuirostris*	1	1	1	2	1	2	-	31	24	30	30	28	34	36	27	30	35	28	27	25
8. *P. nativitatis*	3	3	3	4	3	4	4	-	24	26	23	23	25	29	22	25	26	23	22	32
9. *P. p. puffinus*	3	3	3	4	3	4	4	2	-	17	16	7	11	17	9	10	12	14	9	32
10. *P. p. yelkouan*	3	3	3	4	3	4	4	2	0	-	9	13	17	19	14	13	17	15	14	31
11. *P. p. mauretanicus*	3	3	3	4	3	4	4	2	0	0	-	12	16	18	15	16	18	16	15	30
12. *P. au. newelli*	3	3	3	4	3	4	4	4	2	2	2	-	10	16	5	6	14	10	5	29
13. *P. gavia*	3	3	3	4	3	4	4	2	2	2	2	4	-	13	9	10	17	14	9	30
14. *P. huttoni*	3	3	3	4	3	4	4	2	2	2	2	4	0	-	15	16	19	20	15	31
15. *P. l. boydi*	3	3	3	4	3	4	4	2	0	0	0	2	2	2	-	3	11	9	0	27
16. *P. l. lherminieri*	3	3	3	4	3	4	4	2	0	0	0	2	2	2	0	-	14	12	3	28
17. *P. as. assimilis*	3	3	3	4	3	4	4	2	0	0	0	2	2	2	0	0	-	8	11	38
18. *P. as. haurakiensis*	3	3	3	4	3	4	4	2	0	0	0	2	2	2	0	0	0	-	9	29
19. *P. as. baroli*	3	3	3	4	3	4	4	2	0	0	0	2	2	2	0	0	0	0	-	27
20. *Fulmarus glacialis*	5	5	5	6	5	6	6	6	6	6	6	8	6	6	6	6	6	6	6	-

Net sequence divergence between pairs of *Puffinus* taxa, estimated under a maximum likelihood model, ranged from zero (*P. l. boydi* v *P.as. baroli*) to 14.85% (*P. tenuirostris* v *P. huttoni*) (Table 4.3). Sequence divergence averaged 3.52% (range 0.99-5.81%, n=7) among currently recognised subspecies. The two distance matrices (transitions/transversions Table 4.2; corrected sequence divergence Table 4.3) show that the taxa cluster into two distinct groups. The mean and range of sequence divergence among species in the group containing taxa 1-7 was 4.04% and 0.66-6.54% (n=21), among species in the group containing taxa 8-19 was 5.56% and 0.0-11.12% (n=59) , and for comparisons of taxa between each of these groups was 12.18% and 9.22-14.85% (n=84). Overall, the mean sequence divergence between species of *Puffinus* was 8.75%. Between *Puffinus* taxa and the outgroup, *F. glacialis*, the mean and range was 13.48% and 11.25-15.3%, respectively.

Table 4.3. Percent sequence divergence, calculated using a maximum likelihood model (Felsenstein 1993), in the mitochondrial cytochrome *b* gene of *Puffinus* and the outgroup taxa, *Fulmarus glacialis*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *P. pacificus*	-																			
2. *P. bulleri*	3.68	-																		
3. *P. c. hullianus*	4.73	4.39	-																	
4. *P. creatopus*	4.75	4.40	0.66	-																
5. *P. gravis*	5.08	4.74	2.32	2.33	-															
6. *P. griseus*	6.16	5.09	3.35	4.06	3.00	-														
7. *P. tenuirostris*	6.54	4.76	3.71	3.72	3.36	4.06	-													
8. *P. nativitatis*	13.57	12.76	12.36	12.41	12.76	14.83	12.83	-												
9. *P. p. puffinus*	10.77	10.77	9.60	9.65	9.22	11.17	10.04	9.20	-											
10. *P. p. yelkouan*	13.53	14.36	12.32	12.38	11.92	13.14	12.40	9.95	5.81	-										
11. *P. p. mauretanicus*	13.13	13.13	11.92	11.98	11.53	12.73	12.39	8.80	5.45	3.00	-									
12. *P. au. newelli*	11.53	11.54	10.36	10.41	10.75	12.75	11.60	9.63	3.03	5.12	4.76	-								
13. *P. gavia*	12.33	12.33	13.55	13.61	12.75	14.82	14.03	9.58	4.42	6.56	6.20	4.79	-							
14. *P. huttoni*	13.12	13.12	13.54	13.60	13.55	14.80	14.85	11.12	6.57	7.29	6.92	6.97	4.38	-						
15. *P. l. boydi*	11.17	11.97	10.78	10.83	11.17	13.19	11.23	8.46	3.01	4.75	5.10	2.34	3.72	5.85	-					
16. *P. l. lherminieri*	9.98	12.36	10.38	10.42	10.77	13.59	12.42	9.59	3.36	4.39	5.45	2.68	4.07	6.21	0.99	-				
17. *P. as. assimilis*	13.57	13.57	11.56	11.61	12.76	14.83	12.82	9.21	4.05	5.81	6.17	4.77	5.85	6.57	3.71	4.76	-			
18. *P. as. haurakiensis*	12.35	13.16	10.38	10.42	11.56	13.58	11.61	8.83	4.75	5.10	5.45	4.07	5.49	7.67	3.02	4.05	2.67	-		
19. *P. as. baroli*	11.17	11.97	10.78	10.83	11.17	13.19	11.23	8.46	3.01	4.75	5.10	2.34	3.72	5.85	0.00	0.99	3.71	3.02	-	
20. *Fulmarus glacialis*	11.25	11.69	11.25	11.30	12.54	12.99	12.60	15.30	15.27	14.78	14.33	14.91	14.36	14.81	13.06	13.50	15.28	13.93	13.06	-

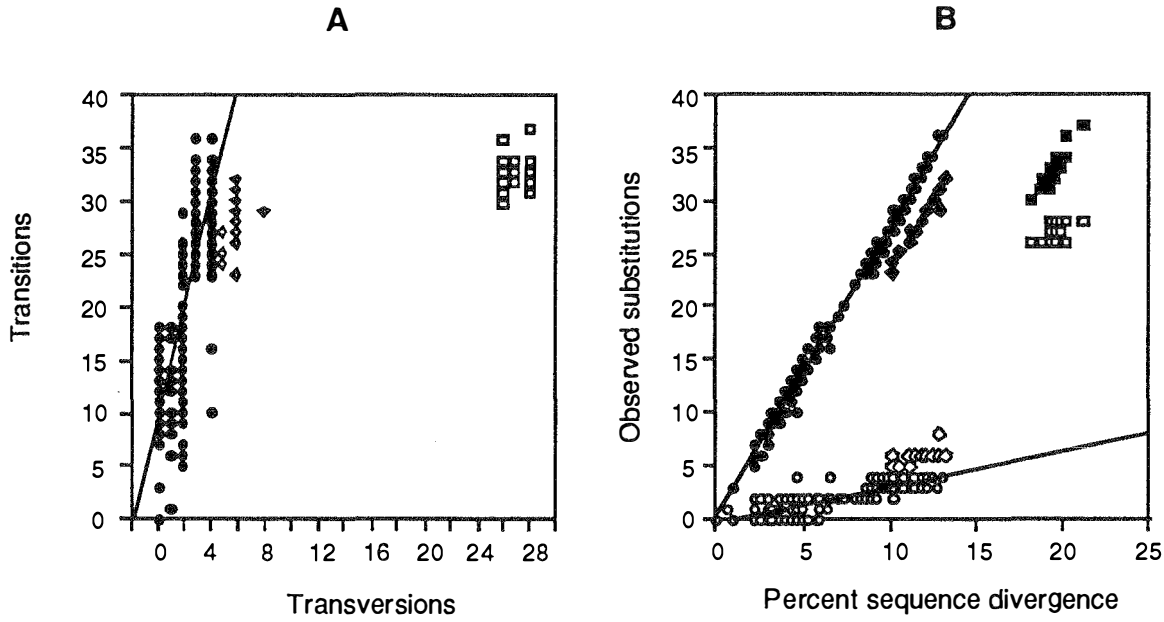

Figure 4.5. Patterns of nucleotide substitution among shearwater cytochrome *b* gene sequences. (A) Mean and range of number of transitions against number of transversions for pairwise comparisons of *Puffinus* taxa with: other *Puffinus* taxa (solid circles); the fulmar, *F.glacialis,* (open diamonds); and the chicken, *G.domesticus*, (open squares). Linear regression line for comparsions between *Puffinus* taxa is shown ($r^2 = 0.60$). (B) Transitions (solid symbols) and transversions (open symbols) against uncorrected percent sequence divergence for pairwise comparisons of *Puffinus* taxa with: other *Puffinus* taxa (circles), the fulmar (diamonds) and the chicken (squares). Linear regression lines for comparisons between *Puffinus* taxa are shown (transitions: $r^2=0.99$; transversions: $r^2=0.68$). Comparisons with the fulmar are underestimates as only 288-bp of cytochrome *b* sequence for this species were available.

Base composition was not constant across codon positions (Fig. 4.6). At first positions of codons there were almost equal frequencies of the four bases. At second codon positions a distinct bias towards thymines was evident, while at third codon positions there was a strong bias against guanine and thymine (Fig. 4.6).

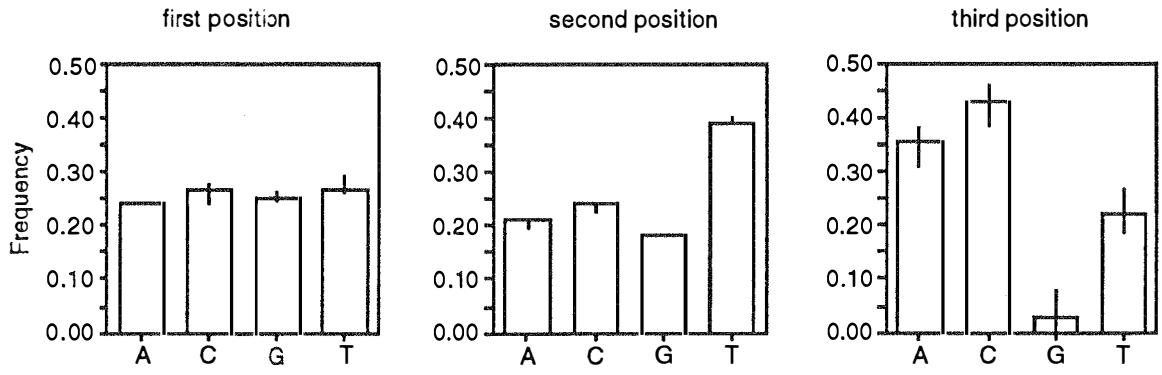

Figure 4.6. Mean and range of base composition at first, second and third codon positions in a 307 bp segment of the cytochrome *b* gene from 19 *Puffinus* taxa. A = adenine, C = cytosine, G = guanine, T = thymine.

4.3.3 Phylogenetic analyses

Phylogenetic information content

The shearwater cytochrome *b* sequences generated in this study contain phylogenetic information at the intrageneric level. The relative lack of multiple substitutions (homoplasy) at variable and phylogenetically informative sites in the DNA sequences was demonstrated by two properties of the data. First, the tree-length distribution of 10 000 random parsimony trees was significantly skewed to the left ($g1$ = -0.53, $P<0.01$) indicating a strong phylogenetic signal in the data set (Hillis & Huelsenbeck 1992).

Second, examination of patterns of transition and transversion substitutions showed that the threshold of sequence divergence, where multiple substitutions at variable sites erase the record of previous substitutions, had not been reached. Pairwise comparisons of nucleotide changes between *Puffinus* taxa revealed a linear relationship between transitions and transversions (Fig. 4.5 A) and between transitions or transversions and overall, uncorrected sequence divergence (Fig. 4.5B). For all intra-*Puffinus* pairwise comparisons transversions make up a small proportion of the total pairwise difference (TS + TV), even between the most divergent taxa (Fig. 4.7). This indicates that transversions had not yet begun to erase the record of transitions, and that transitions are informative. In more distant comparisons between *Puffinus* taxa and the chicken, transversion substitutions were relatively more frequent (Fig. 4.5.A, B; Fig. 4.7) to the extent that previous transition changes would be erased by transversions and transition changes themselves had reached saturation. Patterns of

nucleotide substitution between *Puffinus* taxa and the outgroup, *Fulmarus glacialis*, were difficult to properly diagnose because of the shorter sequences compared (288 bp *cf* 307 bp).

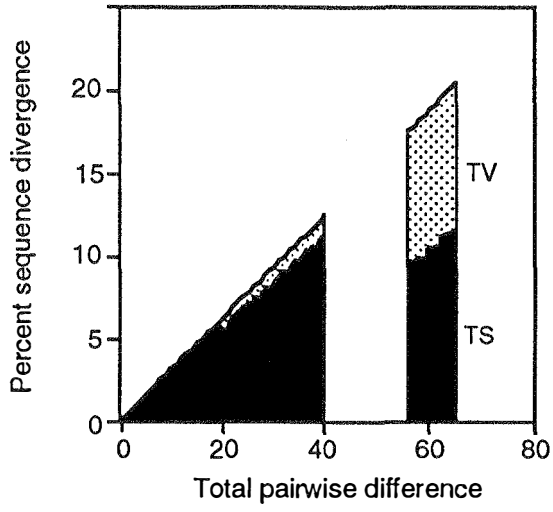

Figure 4. 7. The proportion of the total pairwise difference (TS + TV) contributed by transitions (black) and transversions (hatched) for different levels of total sequence divergence, for comparisons among *Puffinus* taxa (left segment of graph) and between *Puffinus* taxa and the chicken (right segment of graph).

Tree construction

Under maximum likelihood (ML) analysis, the optimal TS/TV ratio for a subset of 11 taxa was 9 (Fig. 4.8). Over a range of TS/TV ratios from 5 to 20 the topology of the tree of maximum likelihood for these 11 taxa did not vary. For a TS/TV ratio of 1 the topology of the recovered tree differed from the tree for TS/TV ratios greater than 5 only in the arrangement of two adjacent taxa (i.e. [*P. p. puffinus*,[*P. au. newelli*, *P. l. boydi*]] *cf* [*P. au. newelli*,[*P. p. puffinus*, *P. l. boydi*]]). The difference in log likelihood between trees with TS/TV ratios of between 5 and 20 was less than 2.5 (Fig. 4.8), so it is difficult to place confidence on any one value in this range as an accurate estimate of the absolute TS/TV bias. This range encompasses the range of TS/TV bias observed in pairwise comparisons between the least diverged *Puffinus* taxa (Fig.4.5A). The tree of maximum likelihood, including all taxa and all sites, had a log likelihood of -1239.55 and is shown in Fig. 4.9.

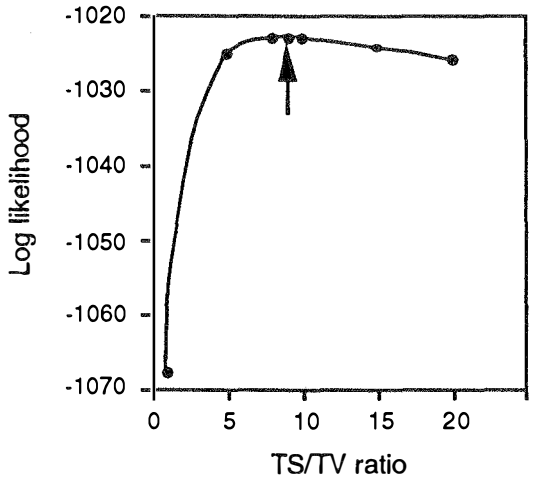

Figure 4.8. Variation in log likelihood for trees of maximum likelihood calculated for a subset of 11 *Puffinus* taxa using different transition/transversion (TS/TV) ratios. The tree with the greatest log likelihood (ln = -1023.1) was found for a TS/TV ratio of 9, as indicated by the arrow.

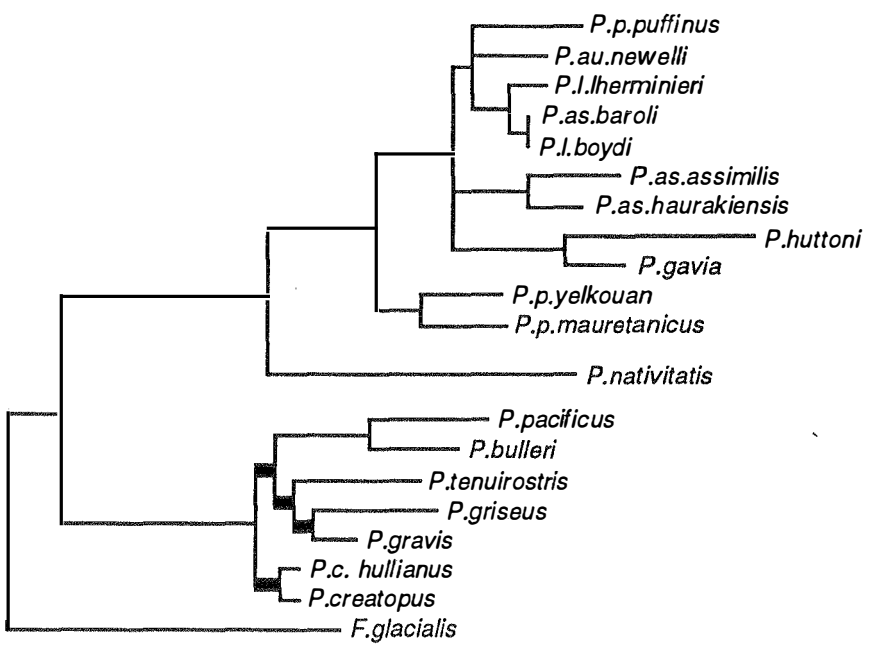

Figure 4.9. The maximum likelihood tree (ln = -1239.55) for 19 *Puffinus* taxa, rooted using the fulmar (*F. glacialis*) as an outgroup. Branch lengths are proportional to the expected number of nucleotide substitutions per site. Branches of zero length were collapsed to yield polytomies. The four bold branches are not significantly greater than zero at the 0.01 level. All other branch lengths are significantly positive ($P < 0.01$).

Maximum parsimony (MP) analysis using only phylogenetically informative sites produced 12 most parsimonious trees, 125 steps in length, when transitions and transversions were weighted equally, and two most parsimonious trees when transversions were weighted between 3 and 20 times more than transitions. Different weights were not applied to codon positions because all of the first codon substitutions were silent mutations in leucine codons and no second codon position variation was phylogenetically informative. Using the optimal TS/TV ratio from the maximum likelihood analysis of 9, these two trees had a length of 202 steps. When transversions were weighted twice as much as transitions 14 equally parsimonious, 139 step trees were recovered. The 12 most parsimonious trees using equally weighted transitions and transversions had a consistency index (CI) of 0.48 and a retention index (RI) of 0.75. PAUP cannot calculate tree statistics when character state changes are weighted differently, but trees with the same topology as the two most parsimonious transversion weighted trees had a CI = 0.48 and RI = 0.74, when transitions and transversions were equally weighted. Strict consensus trees for equally weighted and 9:1 weighted transversions and transitions are shown in Fig. 4.10 A and B, respectively. The strict consensus of trees with a 2:1 TV/TS weighting was identical in topology to the unweighted consensus tree, except that the branch leading from the *P. p. yelkouan* and *P. p. mauretanicus* group to the other Puffinus-subgroup taxa was not resolved.

The neighbour-joining (NJ) tree, produced from a phenetic analysis of nucleotide sequence divergence among taxa is shown in Fig. 4.11.

Phylogenetic relationships among Puffinus taxa

The three methods of phylogenetic reconstruction produced trees with a similar topology. In general, two monophyletic groups of taxa are clearly distinguished. One clade consists of taxa from the subgroups Thyellodroma (*P. pacificus* and *P. bulleri*), Hemipuffinus (*P. carneipes* and *P. creatopus*), Ardenna (*P. gravis*) and two species of Neonectris (*P. griseus and P. tenuirostris*). The other clade consists of the the third species from the subgroup Neonectris (*P. nativitatis*) and all of the Puffinus subgroup taxa (*P. au. newelli*, *P. puffinus* sspp., *P.gavia*, *P. huttoni*, *P.lherminieri* sspp. and *P. assimilis* sspp.).

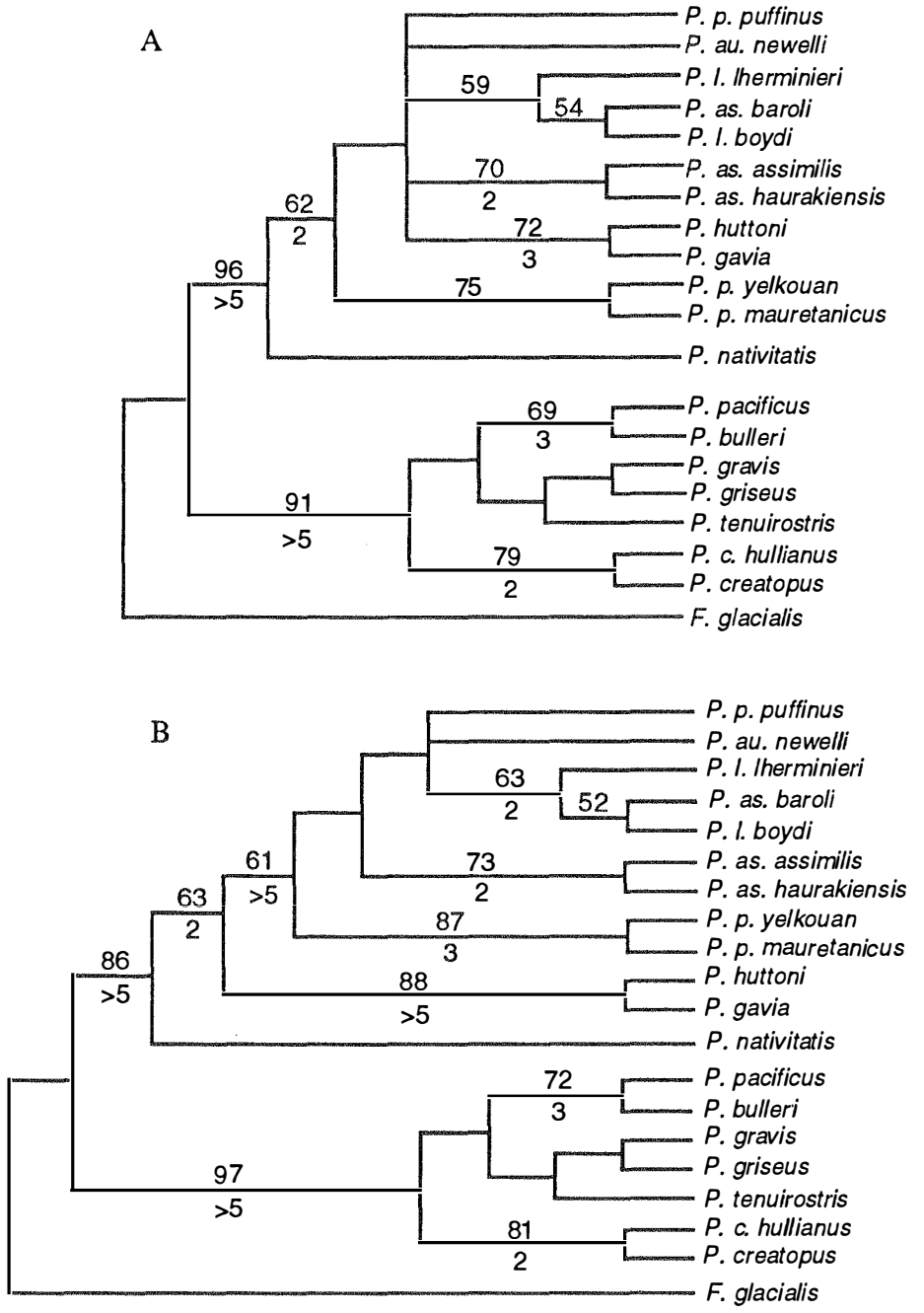

Figure 4.10. Maximum parsimony cladograms showing the phylogenetic relationships among 19 *Puffinus* taxa and the outgroup taxon, *F. glacialis*. Numbers above branches indicate the bootstrap percentages, greater than 50%, from 500 replicates. Numbers below branches indicate the number of steps longer than the most parsimonious trees before that branch was not supported (other than one). (A) Strict consensus of 12 most parsimonious 125-step trees, with all characters uniformly weighted. (B) Strict consensus of the two most parsimonious, 202-step trees with transversions weighted 9 and transitions 1. The same two trees were recovered for TS/TV ratios between 3 and 20. This tree is identical to one of the most parsimonious trees. The other tree differed only in the placement of *P. p. puffinus* and *P. au. newelli* as a monophyletic group.

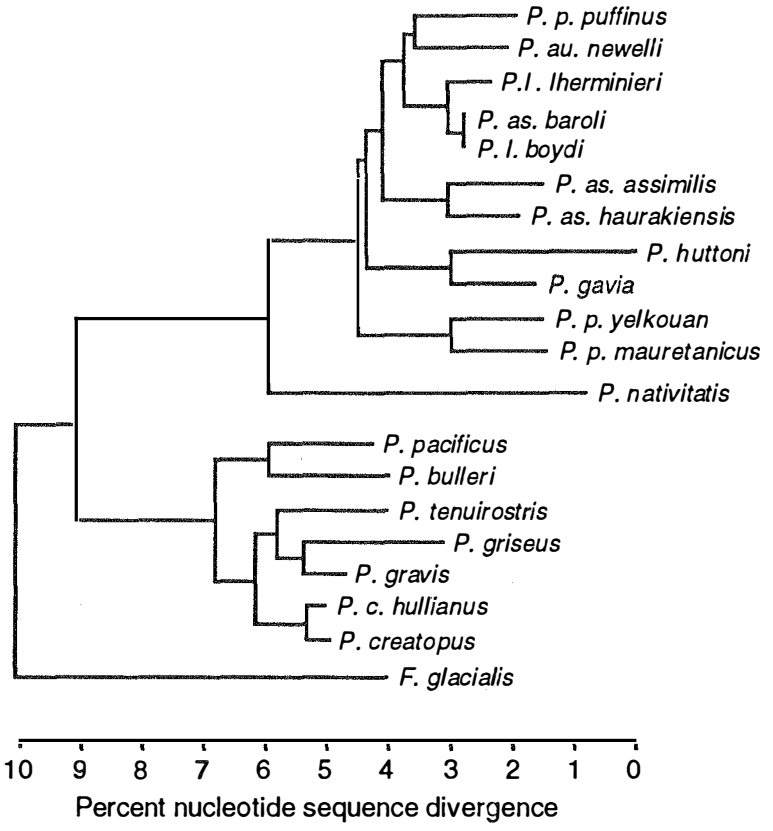

Figure 4.11. The neighbor-joining tree describing the results of a phenetic analysis of nucleotide sequence divergence among 19 *Puffinus* taxa, without the assumption of equal rates of evolution. Branch lengths are proportional to the percent nucleotide sequence divergence as indicated by the scale bar.

Within each clade, pairs and trios of taxa show consistent phylogenetic association. In the first clade, *pacificus* and *bulleri* (Thyellodroma), *carneipes* and *creatopus* (Hemipuffinus), and *griseus*, *gravis* and *tenuirostris* form three phylogenetically related groups. In the ML and MP trees the latter group is a sister group with the Thyellodroma clade, but in the NJ tree this group is more closely associated with Hemipuffinus. In the other major clade, the Puffinus-subgroup taxa form a clade to the exclusion of *P. nativitatis*, which is more distantly related. Within the Puffinus-subgroup, *yelkouan* and *mauretanicus*, *huttoni* and *gavia*, *assimilis* and *haurakiensis*, and *lherminieri*, *boydi* and *baroli* form four phylogenetically related groups. The relationship among these groups and with *puffinus* and *newelli* varies between the three methods of tree construction.

In the ML tree, *puffinus* and *newelli* form a clade with the *lherminieri /baroli /boydi* group, and these form a sister group with two other groups, *assimilis/haurakiensis* and *huttoni/gavia*. These taxa form a clade which, in turn, is a sister group to *yelkouan/mauretanicus*. In the equally weighted MP tree, relationships among the groups of taxa are unresolved, except for the *yelkouan/mauretanicus* group which forms a sister group to the remainder. In the weighted MP tree, relationships among the (sub)species groups are more resolved with the *lherminieri/baroli/boydi* clade as a sister group with *puffinus* and *newelli*, and this clade is in turn a sister group with *assimilis/haurakiensis*, and then *yelkouan/mauretanicus*, with *huttoni/gavia* the most distantly related. In the NJ tree, the cladistic structure in the Puffinus-subgroup is identical to the weighted ML tree except that the positions of *huttoni/gavia* and *yelkouan/mauretanicus* are reversed.

Support (or the lack of it) for particular cladistic patterns revealed by the various phylogenetic analyses of the data was demonstrated by a number of techniques. The weighted MP tree and the NJ tree were not significantly worse representations of the true tree, compared to the ML tree, according to the likelihood variance test of Kishino and Hasegawa (1989) (Table 4.4). The equally weighted MP tree could not be examined because, to implement the test in PHYLIP, requires that trees be fully dichotomous at all nodes except the root. However, given its similarity to the weighted MP tree and the NJ tree it is highly unlikely that the equally weighted tree would be significantly worse than the ML tree.

These results suggest that no one cladistic arrangement of the pairs and trios of taxa within the two major clades is a significantly better arrangement than competing hypotheses identified by the various methods. However, when the monophyly of the two major *Puffinus* groups was disrupted by moving *P. griseus* and *P. tenuirostris* onto the branch leading to *P. nativitatis* (Fig. 4.12), as implied by the current taxonomy and based on the phylogenetic hypotheses of Kuroda (1954), then this tree was significantly worse than the ML tree according to the likelihood variance test (Table 4.4). A modified ML tree with the outgroup rooted at the node where the *pacificus/bulleri* clade joins the tree, as proposed by Kuroda (1954) and Wragg (1985), had a much lower log likelihood than the ML tree but was not significantly worse according to the Kishino-Hasegawa test (Table 4.4).

Table 4.4. Log likelihood values for different trees describing the phylogenetic relationships of 19 species and subspecies of *Puffinus*. A particular tree topology was considered a significantly worse representation of the true tree than the tree of maximum likelihood (ML, Fig. 4.9) if the S.D of the difference in log likelihood was more than 1.96 times the difference itself. Trees compared were the maximum parsimony tree with transversions weighted relative to transitions (MP-weighted, Fig. 4.10B), the neighbour-joining tree (NJ, Fig. 4.11), a tree in which *P.griseus*, *P. tenuirostris* and *P. nativitatis* formed a monophyletic group within the Puffinus-subgroup clade (Kuroda (Neonectris), Fig. 4.12) and a tree in which the *F. glacialis* was rooted to the node joining the *pacificus/bulleri* clade to the rest of the tree (Kuroda/Wragg (root), Fig. 4.2).

Tree	Log likelihood	Δ log likelihood	S.D	Significant
ML	-1239.55			
MP -weighted	-1242.00	-2.45	7.35	No
NJ	-1240.60	-1.05	1.80	No
Kuroda (Neonectris)	-1292.10	-52.55	16.18	Yes
Kuroda/Wragg (root)	-1254.06	-14.51	9.16	No

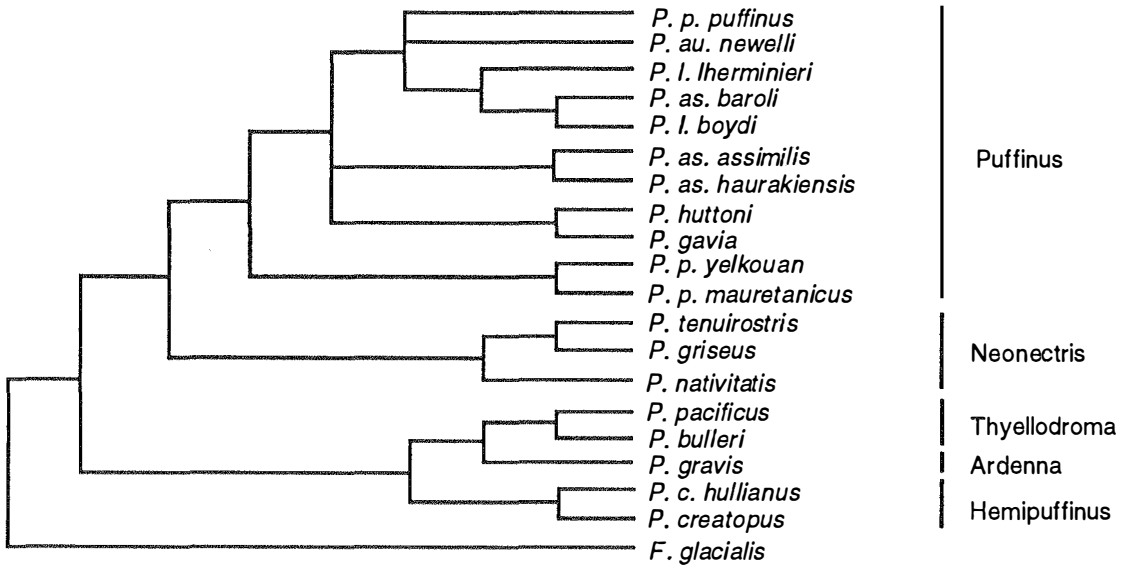

Figure 4.12. The maximum likelihood tree in which the toplogy has been disrupted to support the current taxonomy of the genus *Puffinus* and the phylogenetic hypotheses of Kuroda (1954) and Wragg (1985) in which *P. griseus*, *P. tenuirostris* and *P. nativitatis* form a subgroup, Neonectris, with closer phylogenetic affinities to the Puffinus subgroup than to the other species subgroups. The toplogy of this tree is a significantly worse representation of the true tree compared to the tree of maximum likelihood, according to the likelihood variance test of Kishino and Hasegawa (1989) (see Table 4.4).

Bootstrap proportions for both the equally weighted and transversion weighted MP trees were similar (Fig. 4.10). The highest level of bootstrap support (i.e. a clade occurring in greater than 90% of 500 bootstrap replicates) was for the monophyly of the two major clades within the genus *Puffinus*. The only other groups to appear in more than 70% of the bootstrap replicates were the *pacificus/bulleri*, *carneipes/creatopus*, *yelkouan/mauretanicus*, *huttoni/gavia* and *assimilis/haurakiensis* clades. Lower bootstrap proportions of around 60% supported the monophyly of the Puffinus-subgroup within the clade containing this group and *P. nativitatis*, the *lherminieri/baroli/boydi* group, and in the weighted MP tree, the monophyly of the Puffinus-subgroup taxa to the exclusion of the *huttoni/gavia* clade.

The clades that received the highest bootstrap support were generally also the ones that remained resolved in trees longer than parsimony. Finite computer memory prevented the examination of trees greater than five steps longer than the most parsimonious trees. For the equally weighted MP tree, the two major clades remained resolved in trees at least 5 steps longer than the 125 step, most parsimonious trees. Support for other clades was lost in trees three steps longer (*huttoni/gavia*, *pacificus/bulleri*), two steps longer (*assimilis/haurakiensis*, *carneipes/creatopus*, Puffinus-subgroup), and one step longer (all other clades) than the most parsimonious trees. Under weighted parsimony, the two major clades as well as the *huttoni/gavia* group and the clade containing all other Puffinus-subgroup taxa were supported in trees at least five steps longer than the 202 step, most parsimonious trees. Other clades lost support for trees three steps longer (*pacificus/bulleri*), two steps longer (*carneipes/creatopus*, *lherminieri/baroli/boydi*, Puffinus-subgroup) and one step longer (all other clades) than the most parsimonious trees.

4.4 DISCUSSION

4.4.1 Sources of DNA for systematic studies

This study clearly demonstrates the compatibility of a wide range of tissue sources, preservation and storage conditions and specimen ages, with PCR-based methods of obtaining DNA sequence data for systematic studies of birds (Houde & Braun 1988; Taberlet & Bouvet 1991; Leeton *et al.* 1993). DNA was successfully amplified from a range of tissues, collected both invasively (blood) and non-invasively (feathers) from live birds, and

retrospectively (kidney, muscle and feathers) from dead specimens. Sequence data were retrieved from samples that had been preserved under a range of conditions from those which maintain the structural integrity of DNA for long periods (liquid nitrogen, -20°C frozen and ethanol preservation), to those where DNA is expected to be highly modified and degraded (dried museum skins).

In particular, the demonstrated utility of feathers collected non-invasively from live birds, preserved and stored under readily available conditions, has important implications for studies of avian systematics (Taberlet & Bouvet 1991). Samples from taxa under study can be collected with minimal disturbance to the birds themselves, without the need for killing any individuals or access to special preservation and storage facilities, such as freezers or liquid nitrogen dewars. PCR-based methods, using non-invasively collected material should, therefore, provide avian systematicists access to a much broader range of taxa on which to study evolutionary processes.

The use of museum material in phylogenetic studies has received much attention and interest. Ancient DNA is obviously preserved in, and can be retrieved from museum material ranging in age up to many millions of years (Higuchi *et al.* 1984; Kocher *et al.* 1989; Pääbo 1989; Thomas *et al.* 1989; Smith *et al.* 1991; DeSalle *et al.* 1992; Cano *et al.* 1993). Houde and Braun (1988) and Leeton *et al.* (1993) have both recommended museum skins as sources of DNA for phylogenetic studies of birds. However, the success rate in obtaining authentic DNA sequences from such specimens has not been discussed. In this study DNA was successfully amplified from feathers taken from only two of the 15 dried museum skins available. The remaining specimens either yielded human contaminated sequences, or no amplifiable DNA. Problems with routinely amplifying segments of DNA from avian museum material appear to be common (R. Dawson, S. Trewick, G. Nunn, L. Christidis personal communications). The low success rate with such material appears to be related to the material itself, rather than methods of extracting or amplifying DNA.

These problems suggest caution in using museum material for systematic studies. Museum specimens are often rare and/or have value to multiple users. Molecular based studies are necessarily destructive, so that the chance of success in retrieving amplifiable DNA from specimens must be weighed against damage to the specimen itself (see Graves & Braun

1992). Given the extra time, effort and cost involved in extracting and amplifying DNA from museum material, and the generally low success rate, I reiterate the recommendations made by Graves and Braun (1992) and Pääbo *et al.* (1992) that wherever possible museum specimens be used as a last resort, and that considerable care should be taken to avoid excessive damage to specimens that are used. These recommendations were followed in the present study. Techniques were trialled on less valuable museum specimens, before attempts were made to obtain sequence data from those taxa which it had proved impossible to obtain fresh material. Given the relative ease with which tissue samples can be non-invasively collected from live animals, and preserved and stored under readily available conditions, attempts to obtain such samples should take priority over the use of museums as sources of material. Obviously, where the species of interest is extinct, or it is impossible to make collections from live individuals, then museum collections become a potentially valuable source of material for genetic studies.

The ease with which samples can be collected for PCR-based studies, without the need for killing individuals, creates potential problems for the authenticity of particular samples assigned to a specific taxon, especially where the taxonomy of the group under study is controversial, as is the case for *Puffinus*. Unexpected results, such as the identical cytochrome *b* sequence obtained for *P. as. baroli* and *P. l. boydi* could have been the result of a sampling error where a sample was inadvertently assigned to an incorrect species. These sampling errors are difficult to detect because there is no permanent record of the specimens to compare morphological or other characteristics to establish the validity of the taxonomic assignment.

However, I believe that all of the samples represented in this study were taxonomically correctly assigned. All museum material had been identified and catalogued by experts at the respective institutions. All other material was collected from live birds at breeding colonies, by wildlife officers and research scientists with expertise in the particular species or subspecies inhabiting their region. For the specific case of the *P. as. baroli* and *P. l. boydi* identical sequences, the *baroli* material originated from the Canary Islands, while the *boydi* specimen was collected in the Cape Verde Islands, which is the only breeding location known for this taxon. In addition, the two *P. as. baroli* specimens from which sequence data were

obtained were collected from different locations on the island of Tenerife, making incorrect assignment of these samples even less likely. The *boydi* specimen was from a museum and is therefore unlikely to have been incorrectly assigned.

4.4.2 Properties of shearwater cytochrome *b* gene

Avian cytochrome *b* sequences show similar evolutionary dynamics as functional mitochondrial protein-coding genes in other vertebrates (Wilson *et al.* 1985; Kocher *et al.* 1989; Bartlett & Davidson 1991), and the shearwater cytochrome *b* gene is no exception. As expected for a gene evolving rapidly under strong functional constraints, the pattern of nucleotide substitution in the cytochrome*b* gene was biased. According to codon positions, third position substitutions, which are often silent, predominated over first and second position changes in a ratio of 88:1:11. Similar biases have been found in other intrageneric comparisons of birds, including murres (*Uria*, Birt-Friesen *et al.* 1992), geese (*Branta*, Quinn *et al.* 1991), sparrows (*Amphispiza*, Johnson & Cicero 1991), babblers (*Pomatostomus*, Edwards & Wilson 1990; Edwards *et al.* 1991) and shrikes (*Laniarius*, Smith *et al.* 1991).

All substitutions were not equally likely and in the genus *Puffinus* transversions (pyrimidine - purine) were approximately 10 times less likely than transitions (purine - purine, pyrimidine - pyrimidine) which is comparable to the generally large transition bias reported for birds (Kocher *et al.* 1989; Arctander 1991; Edwards *et al.* 1991; Johnson & Cicero 1991; Quinn *et al.* 1991; Smith *et al.* 1991; Birt-Friesen *et al.* 1992). Transitions accumulate so rapidly in some groups of birds that multiple hits at the most variable positions are likely to lead to saturation with a subsequent loss of phylogenetic information (e.g. Passeriformes, Edwards *et al.* 1991). Third codon positions are likely to reach saturation first because of their high rate of substitution. Therefore, substitutions at first and second codon positions and transversions will be phylogenetically informative for a much longer period of time (Edwards *et al.* 1991). However, this study has demonstrated that significant phylogenetic information was present in the sequences from the shearwater cytochrome *b* gene and that saturation effects, from multiple transition substitutions, or transversions erasing the record of previous transitions, had not yet randomised the phylogenetic signal present in third positions of codons.

The consistency index (CI, Kluge & Farris 1969) and retention index (RI, Farris 1989) provide a measure of fit of the character data to phylogenetic hypotheses proposed by maximum parsimony methods and therefore indicate the levels of homoplasy in the data. However the low consistency indices for the equally weighted and transversion weighted MP trees cannot be taken as a direct measure of homoplasy in the cytochrome *b* sequences because the CI is negatively correlated with the number of taxa included in a study (Sanderson & Donoghue 1989) and is strongly influenced by the distribution of character-state frequencies (Swofford 1991). The low CIs for the two sets of MP trees are comparable with the low CIs for other large morphological and molecular data sets (Sanderson & Donoghue 1989; Van Zandt Brower 1994). The retention index is a more reliable measure of the fit of character data to phylogenetic trees and is more important in assessing levels of homoplasy (DeSalle 1992), but there is also a negative correlation with the number of taxa involved (Archie 1990a,b). However, the much larger retention indices for the equally weighted and transversion weighted parsimonious trees indicates a high degree of character congruence, given the large number of taxa included in this study.

The strong compositional bias against guanine and thymine at third codon positions observed in *Puffinus* shearwaters, is typical of birds. The bias against guanine is a general feature of vertebrate mtDNA (Brown 1985; Kocher *et al.* 1989). However , the deficiency of thymine has been reported as an unusual characteristic of avian mtDNA (Kocher *et al.* 1989; Edwards *et al.* 1991; Johnson & Cicero 1991). This bias suggests that in addition to a transition bias in sequence evolution, the relative rates of specific transition and transversion changes are not constant (i.e. a T>C transition is more frequent than a C>T substitution).

4.4.3 Genetic divergence in *Puffinus* shearwaters

Genetic divergence among avian taxa at protein-coding loci in nuclear DNA is generally conservative relative to other vertebrate taxa at comparable taxonomic levels (reviewed by Avise 1983). Several hypotheses have been suggested to explain the low levels of molecular divergence observed in birds. First, the rate of evolution of avian nuclear DNA may be slower than in other animal groups (Zink 1982; Avise 1983). Second, avian taxa may share more recent common ancestors and may be taxonomically oversplit relative to other non-avian vertebrates (Zink 1982; Avise 1983; Tegelström & Gelter 1990). Finally, avian speciation

may occur without extensive genetic changes (Tegelström & Gelter 1990). Genetic distances among Procellariiform taxa, based on protein-coding loci, are intermediate between those of other birds and non-avian taxa (Barrowclough *et al.* 1981; Kuroda *et al.* 1990). Barrowclough *et al.* (1981) suggest that this reflects the greater evolutionary age of the Procellariiformes relative to the Passeriform taxa for which most estimates of genetic divergence have been made.

Kessler and Avise (1985) reported conservative patterns of mtDNA sequence divergence among species in five genera of birds, relative to interspecific comparisons for two non-avian genera (*Lepomis*, fish; *Hyla*, frog). They reiterated the hypotheses for the low nuclear divergence in birds, that either the rates of mtDNA nucleotide evolution in birds is decelerated or that avian congeners share more recent common ancestors than nonavian taxonomic equivalents. Shields and Helm-Bychowski (1988) reviewed mtDNA divergence among avian taxa and compared these to a much greater number of interspecific values for other vertebrate taxa. The average sequence divergence for interspecific comparisons involving nine genera of birds is 4.5% (range 1.4-7.3%), and is not considerably lower than values for other vertebrate and invertebrate groups (range of mean values for each genera 3.6-10.8%). Tegelström and Gelter (1990) summarised mtDNA sequence divergence among avian species, incorporating many of the genera and species reviewed by Shields and Helm-Bychowski (1988), and found a mean interspecific mtDNA sequence divergence of 5.2% (range 0.6-10%). The two genera used for comparison by Kessler and Avise (1985) and another genus of frogs (*Xenopus*) show much greater levels of interspecific sequence divergence (Shields & Helm-Bychowski 1988). Therefore avian congeners do not exhibit a substantially reduced mtDNA sequence divergence relative to other non avian taxa. The conclusions of Kessler and Avise (1985) appear to have been biased by the unusually large mtDNA divergences among the fish and frog taxa that they chose for comparison.

There is no evidence to suggest that the rate of mtDNA evolution in birds is decelerated compared to that of other vertebrates. Kocher *et al.* (1989) found similar rates of amino acid replacement in a segment of the cytochrome *b* gene in mammals and birds. Shields and Wilson (1987) estimated a rate of sequence divergence in mtDNA of 2% per million years in a comparison between two genera of geese, which is similar to the rate found in other

vertebrates (Brown *et al.* 1979).

Genetic divergence among *Puffinus* shearwaters based on the mitochondrial cytochrome *b* sequences are not directly comparable with estimates from other genes or with restriction enzyme studies of the entire mtDNA molecule. However, the range of interspecific sequence divergences within *Puffinus* (0-14.85%) incorporates the range for restriction enzyme studies of birds (Shields & Helm-Bychowski 1988; Tegelström & Gelter 1990). These values are also similar to interspecific sequence divergences calculated from cytochrome *b* sequences for other genera of birds, Australian babblers (*Pomatostomus*, 6.2-12%, Edwards & Wilson 1990) and geese (*Branta*, 6.7-6.9%, Quinn *et al.* 1991); mammals, pocket gophers (*Orthogeomys*, 2.77-16.3%, Sudman & Hafner 1992; *Cratogeomys*, 8.1–16.8%, Spradling Dewalt *et al.* 1993) and echimyid rodents (*Mesomys*, *Isothrix*, *Makalata*, *Dactylomys*, 8.6-18.7%, da Silva & Patton 1993); and toads (*Bufo*, 5-20%, Graybeal 1993).

4.4.4 Phylogenetic reconstructions

Methods of phylogenetic analysis using DNA sequence data attempt to reconstruct the phylogenetic history of the group of taxa from which the sequences were derived. A particular phylogenetic reconstruction must be examined in terms of how well the data support the recovered tree(s) and how accurately these tree(s) reflect the true phylogeny. Maximum likelihood, maximum parsimony and neighbor-joining distance methods of phylogenetic reconstruction, applied to mtDNA cytochrome *b* gene sequences from the 19 *Puffinus* taxa, produced trees with similar topologies. However concordance among different methods does not imply statistical significance for the observed relationships (Felsenstein 1993). Taken together the trees suggest that the genus *Puffinus* consists of two monophyletic clades, the first containing species representing the subgroups Thyellodroma, Hemipuffinus, Ardenna, and Neonectris, and the second comprising all of the species and subspecies of the subgroup Puffinus plus *P. nativitatis* from the Neonectris subgroup. Within each of these major clades, pairs and trios of taxa form monophyletic groups which vary in their cladistic arrangement between the different methods of tree construction.

Support from the data

Bootstrap proportions are usually treated as absolute probability values for particular topologies representing the true phylogeny. Hillis and Bull (1993) have examined the performance of the bootstrap proportion under a wide variety of conditions of phylogenetic analyses, as an estimate of the accuracy of phylogenetic reconstructions. They concluded that bootstrapping provides a conservative estimate of phylogenetic accuracy, and that bootstrap proportions should not be used as absolute estimates of the probability of accurately inferring particular clades. Felsenstein and Kishino (1993) do not dispute these findings, but suggest that the bootstrap proportion (P) can be interpreted as follows. If a bootstrap proportion favouring a group is calculated, then 'the probability that this much evidence will favour the group when it is not in fact on the true tree is less than or equal to 1-P.' This probability is conservative.

If bootstrap proportions are interpreted as conservative estimates of phylogenetic accuracy in the maximum parsimony trees, then there was substantial support for the major phylogenetic division within the genus *Puffinus*, and to a lesser extent the phylogenetic association of particular pairs and trios of terminal taxa. There was little support for alternative arrangements of these groups within each major clade. Examination of trees longer than the most parsimonious, and comparison of alternate topologies under maximum likelihood revealed similar support for generally the same set of clades. However, rerooting of the ancestral node, to reflect the phylogenetic hypotheses proposed by Kuroda (1954) and Wragg (1985), did not produce a significantly worse topology according to the Kishino-Hasegawa test. Kusmierski *et al.* (1993) have criticised this test as being overconservative, a claim supported by the following evidence. Under weighted parsimony analysis, a tree rooted at the node joining the *pacificus/bulleri* clade to the rest of the tree (i.e. an identical rooting arrangement used in the Kishino-Hasegawa test) was 218 steps long, 16 steps longer than the two most parsimonious trees. I therefore believe that this represents strong evidence to support the position of the ancestral root for the current data set.

A consensus tree based on molecular data

A consensus tree based on a conservative interpretation of these results is therefore proposed in which only those branches that received consistent support are resolved (Figure 4. 13). Branches which did not receive bootstrap support or were not resolved in trees longer than

the most parsimonious, or which were not significantly greater than zero in the maximum likelihood tree were collapsed in this consensus tree. In this tree, *Puffinus* taxa are split into two monophyletic species groups or clades, each containing a number of unresolved sister groups consisting of one, two or three sibling species.

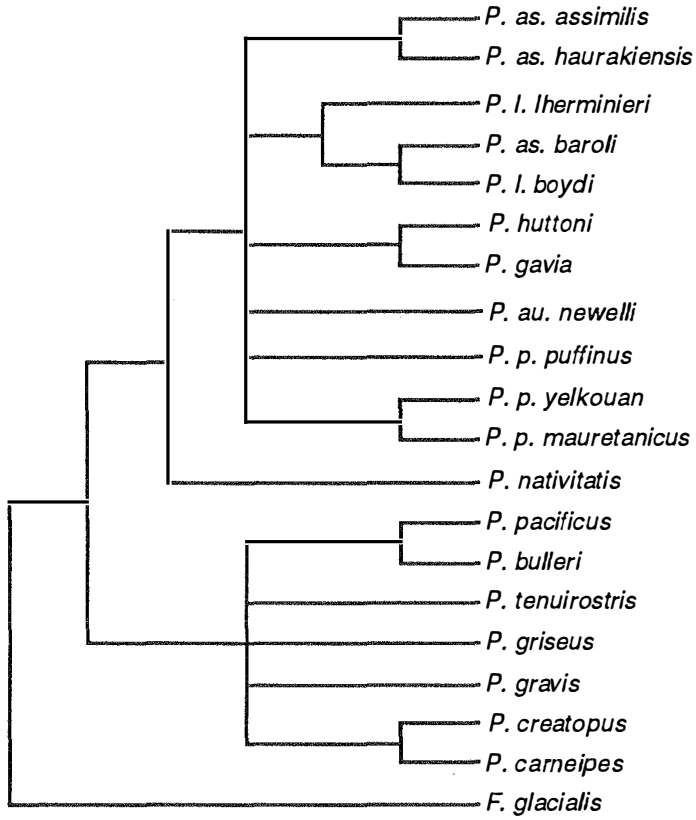

Figure 4.13. A conservative phylogenetic hypotheses for the evolutionary relationships among 19 taxa of *Puffinus* shearwaters, based on three methods of phylogenetic analysis of DNA sequence variation in a 307 bp segment of the mitochondrial cytochrome *b* gene. Branches leading to clades that did not receive a moderate level of support from the data in the original analyses were collapsed to yield polytomies in this final consensus tree.

Polytomies can be interpreted either as a failure of the data set to resolve a truly bifurcating topology, or alternatively may represent the best estimate of the true evolutionary history of the group (Hoelzer & Melnick 1994, but see DeSalle *et al.* 1994). In the former, insufficient sequence data from a gene evolving too slowly would not provide enough characters to accurately resolve phylogenetic relationships, whereas in the latter the phylogenetic reconstruction accurately reflects either a truly polytomous speciation event (a hard polytomy), or a rapid succession of speciations (a soft polytomy). Pamilo and Nei (1988)

have shown that the probability of accurately resolving speciation events that occur rapidly in evolutionary time from a polymorphic ancestral species is considerably less than one, even with large amounts of sequence data from a single gene. The cytochrome *b* sequences were able to resolve both phylogenetically deep and shallow relationships among clades and taxa within the genus *Puffinus*, but at an intermediate evolutionary 'depth', relationships were not resolved. The lack of resolution is therefore unlikely to be due to insufficient data, but instead provides evidence for a rapid phyletic radiation within each of the two major phylogenetic clades. The rapid origin and radiation of the respective lineages within each major clade, as implied by the polytomous branching pattern, is further supported by the short internode lengths compared to the long terminal branches and similar levels of sequence divergence among taxa in the ML and NJ trees, as suggested by Kraus and Miyamoto (1991). Given the evolutionary scale over which these cytochrome *b* sequences have resolved phylogenetic relationships these results support a phylogenetic hypothesis that the polytomous branching pattern within each clade represents the best resolution of a polytomous or a rapid bifurcating speciation event within two ancestral *Puffinus* groups.

Congruence and conflict between molecular and morphological trees

The molecular and morphological (Kuroda 1954; Wragg 1985) trees show a remarkable level of congruence, but also a number of conflicts which require further investigation. Congruence among independent data sets results in a high level of confidence that the true phylogeny has been recovered (Miyamoto & Cracraft 1991; Wheeler 1991). Congruent results include the monophyly of the subgroups Thyellodroma, Hemipuffinus and Puffinus, and the sister group relationship of clades within the Puffinus subgroup. The three conflicting arrangements, from the perspective of the molecular tree, are the placement of *P. as. baroli* in the *P. lherminieri* clade, and separate from the clade containing the two southern hemisphere *P. assimilis* taxa; the polyphyly of the Neonectris subgroup with *P. nativitatis* in one major clade and *P. griseus* and *P. tenuirostris* in the other; and the placement of the ancestral node (root) for the genus.

Mitochondrial DNA represents, in an evolutionary sense, a single genetic unit (Pamilo & Nei 1988; Crozier 1990). Therefore the phylogenetic reconstruction based on cytochrome *b* gene sequences represents a single gene tree, the matriarchal phylogeny, describing the

evolutionary relationships of that segment of DNA itself (Tatento *et al.* 1982; Nei 1987; Pamilo & Nei 1988; Avise 1989). The extent to which a gene tree is an accurate representation of the true species tree, which describes the evolutionary relationships of the actual species and subspecies, is influenced by a number of aspects of mtDNA genetics.

Estimations of mtDNA lineage survival through time (Avise *et al.* 1984) and subsequent computer simulations of branching processes (Neigel & Avise 1986) have demonstrated that, for recently diverged species, phylogenetic analysis may be confounded by random sorting of ancestral sequence polymorphism in mtDNA lineages. Stochastic lineage sorting in isolated species derived from a recent common ancestor will result in reciprocal monophyly of mtDNA lineages only after about $4N_f$ generations, where N_f is the female population size (Neigel & Avise 1986). At times less than this, gene trees for the daughter species evolve through two stages of evolutionary relationship, polyphyly and paraphyly, which may obscure the speciation event. Therefore, in recently diverged species and at times less than $4N_f$ generations some individuals in one species may be more closely related, in terms of their mtDNA, to individuals in another species than they are to members of their own species, as a result of the stochastic sorting of lineages from the ancestral species (Neigel & Avise 1986; Pamilo & Nei 1988; Avise 1989). An example of a mtDNA gene tree that is paraphyletic with respect to taxonomic boundaries involves the mallard (*Anas platyrhynchos*) and black (*A. rubripes*) ducks (Avise *et al.* 1990). Restriction enzyme analysis of mtDNA from mallards, which are widespread in the northern hemisphere, and black ducks, which are restricted to eastern North America, revealed that some mallards are more closely related to black ducks than they were to other mallards. Avise *et al.* (1990) suggest that this paraphyletic relationship of mallards is a result of lineage sorting during the recent speciation of black ducks. This phenomenon is unlikely to have obscured phylogenetic relationships in the genus *Puffinus* based on cytochrome *b* gene sequences for two reasons. First genetic divergence between most taxa is large enough that lineage sorting should have reached an endpoint where the gene tree and species tree are concordant (Felsenstein 1988). Second, sequence variation within species and subspecies is zero or low relative to inter-taxon comparisons. Thus each taxa has reached a stage of reciprocal monophyly with respect to mtDNA.

The second problem that may confuse the phylogenetic analyses of mtDNA sequences is

historical introgressive hybridisation between species or subspecies. Mitochondrial DNA is more likely to cross species boundaries during rare hybridisation events because, unlike nuclear genes, it is not closely linked to genes responsible for maintaining reproductive isolation (Barton & Jones 1983). Consequently, the distribution of mtDNA clones among taxa may conflict with taxonomic boundaries established on morphological and reproductive criteria (Barton & Jones 1983) as has been found in two species of Fennoscandian voles (Tegelström 1987b; see Section 1.1.3).

The vagility of shearwaters, the sympatric breeding ranges of many species and a lack of any demonstrated reproductive isolation among species and subspecies may have lead to hybridisation between populations at discrete breeding localities in the past, yielding a mtDNA phylogeny which obscures the evolutionary history of *Puffinus*. Introgressive hybridisation between *P. l. boydi* and *P. as. baroli* may explain the discordant position of *P. as. baroli* in the *Puffinus* cytochrome *b* phylogeny as these two subspecies breed on adjacent island groups in the eastern North Atlantic Ocean. Alternatively, the observed cytochrome *b* haplotypes for *P. as. baroli* may be the result of mtDNA lineage sorting during the speciation of the *assimilis/lherminieri* complex from a genetically polymorphic ancestor. At present it is difficult to distinguish between these possibilities because so few of the *assimilis* and *lherminieri* subspecies were available for phylogenetic analysis and because of the taxonomic uncertainty surrounding these two species.

Kuroda (1954) proposed the Neonectris subgroup as a monophyletic clade, with close association to the the Puffinus subgroup. However Kuroda (1954) placed *P. nativitatis* in Neonectris based only on a small number of external morphological characters and similarities in habit differentiation with *P. griseus* and *P. tenuirostris*. No osteological characters were obtained for this species. Subsequently, Wragg's (1985) osteological analysis, which incorporated skeletal material from *P. nativitatis*, found strong support for the monophyly of *P. nativitatis* and the Puffinus subgroup. The molecular data therefore are congruent with the more comprehensive data of Wragg (1985) and suggest that Kuroda's phylogenetic placement of *P. nativitatis* within the subgroup Neonectris is incorrect, and that the three taxa currently placed in this subgroup are polyphyletic.

The conflicting placement of the ancestral node on the molecular and morphological trees is difficult to reconcile. The morphological hypotheses suggest that a single ancestral taxon has sequentially given rise to each phylogenetic subgroup, with increasing adaptation to the aquatic environment. *P. pacificus* and *P. bulleri* represent relatively ancestral taxa, whereas the Puffinus subgroup is derived. The molecular data suggest an early split in the *Puffinus* ancestor with one lineage leading to the highly aquatic clade containing *P. nativitatis* and the Puffinus subgroup, and the other leading to the remaining subgroups in which the aquatic habit has evolved to varying degrees. This hypothesis requires convergent evolution in morphology and osteology to reflect similarities in these characters between the two clades. These competing hypotheses suggest different evolutionary histories for the genus and these will be discussed and compared in Section 4.4.4.

4.4.5 Implications for the phylogeny of *Puffinus*

The *Puffinus* cytochrome *b* phylogeny conflicts with the evolutionary scenario proposed by Kuroda (1954) and Wragg (1985). Both Kuroda's (1954) and Wragg's (1985) phylogenetic hypotheses suggest that the evolution of the five species subgroups of *Puffinus* occurred in a single geographic region (the North Atlantic according to Kuroda 1954) and that four of these migrated independently to the Southern Hemisphere where secondary speciation and dispersal occurred to give rise to the seven larger, migratory species now present. The ancestor of the Puffinus subgroup remained in the Europe-American area from which dispersal and speciation of the taxa in this subgroup occurred, as suggested by Bourne *et al.* (1988).

A much simpler interpretation of the Kuroda evolutionary hypothesis is supported by the molecular data. It does not dispute the *Puffinus* ancestral area as being in the North Atlantic, nor that a secondary evolutionary centre occurred in the Southern Hemisphere. However the deep phylogenetic split revealed by the molecular analysis suggests that extant *Puffinus* taxa are derived from two distinct ancestral groups, one in the North Atlantic and one in the Southern Hemisphere, that separated soon after the divergence of *Puffinus* from the shearwater ancestral stock. Therefore, rather than multiple invasions into the Southern Hemisphere by each of four *Puffinus* subgroups, the molecular data suggest that early in the evolutionary history of this genus a single ancestral population moved to the Southern

Hemisphere, where secondary evolution and dispersal lead to the divergence of the subgroups Thyellodroma, Hemipuffinus, Ardenna and Neonectris (without *P. nativitatis*). Within these lineages subsequent speciation has resulted in the seven extant taxa, all of which exhibit similar migratory patterns and have evolved an aquatic habit to varying degrees.

Within the other major clade, a *P. nativitatis* ancestor diverged from the Puffinus subgroup relatively early, perhaps by migration into the Pacific where it was isolated from the North Atlantic stock. The Puffinus subgroup, as suggested by Bourne *et al.* (1988), appears to be a group of old European sibling species that differentiated during the fragmentation of water bodies in the region of the North Atlantic and central Europe in the late Tertiary and via secondary dispersal into the Pacific and Indian Oceans. The molecular data suggest that at the same time as the Mediterranean taxa of *P. puffinus* were isolated in inland seas, the pelagic black-backed form dispersed into the Pacific resulting in the differentiation of *P. p. puffinus* in the Atlantic, and *P. au. newelli* and *P. huttoni* in the Pacific. The Pacific forms of the brown-backed Manx shearwaters, *P. opisthomelas* and *P. gavia*, appear to have evolved from a black-backed ancestor as Wragg's (1985) study placed both *P. opisthomelas* and *P. gavia* in a clade with *P. huttoni*, and the present study showed *P. huttoni* and *P. gavia* to be a strong monophyletic clade.

Thus, morphologically and ecologically similar brown-backed forms of the Manx shearwater group have evolved from at least two distinct ancestors, one in the Mediterranean and the other in the Pacific. The coastal Mediterranean species therefore have had an evolutionarily restricted distribution, whereas the black-backed group has spread from the Atlantic across the Pacific where it has differentiated into two distinct forms. It could be envisaged that the ancestor of the two Mediterranean shearwaters was isolated when the connection with the Atlantic closed, and that speciation occurred when the population became isolated in different water bodies as the inland sea dried and fragmented. Given the current breeding distribution and migration patterns of the two taxa, it is likely that *P. p. yelkouan* became isolated in the eastern sector, while *P.p. mauretanicus* was restricted to the western sector, and that the two have only come into contact after the connection with the Atlantic was restored during the Pliocene.

At the same time as the Manx group of shearwaters was differentiating, the ancestors of the

smaller groups of species, evolved through a process of secondary dispersal and the fragmentation of oceans and adjacent seas. This process saw members of the two phylogenetically distinct clades distributed worldwide and with broadly overlapping distributional ranges. Finally, speciation in many of these lineages has occurred within regions, perhaps via biogeographic processes and intrinsic mechanisms such as local adaptation and specialisation, which may have been maintained by long term philopatry to natal breeding areas.

4.4.6 Implications for the taxonomy of *Puffinus*

The degree of molecular sequence divergence cannot, by itself, define taxonomic boundaries (Ball & Avise 1992; Spradling Dewalt *et al.* 1993). However, the taxonomic hierarchy should reflect different levels of phylogenetic relationship. Phylogenetic information, combined with a broad set of comparative biological data, provides a powerful method of defining the taxonomic status and systematic relationships of specific taxa.

In general the phylogenetic relationships defined by the cytochrome *b* data support the current morphologically based taxonomy of *Puffinus*. The taxonomic subgroups Thyellodroma, Hemipuffinus and Puffinus were all supported as monophyletic clades in the *Puffinus* phylogeny. The Ardenna subgroup was not resolved in the phylogenetic analysis. The Neonectris subgroup which taxonomically contains *P. griseus*, *P. tenuirostris* and *P. nativitatis* is, phylogenetically, a polyphyletic group. This subgroup should be reviewed so that *P. griseus* and *P. tenuirostris* are treated separately from *P. nativitatis*, to reflect their separate phylogenetic affinities. Although not strongly supported by the phylogenetic analysis, both *P. griseus* and *P. tenuirostris* appear to be more closely related to *P. gravis*. A more comprehensive examination of the systematic relationships of these three species, at the subgeneric level, is therefore required.

From a phylogenetic perspective, the subgroup Puffinus is not equivalent to the other subgroups which are oversplit relative to it. For these taxonomic groups to become phylogenetically equivalent, the Puffinus subgroup must be split into a number of smaller groups or, alternatively, the species in the other major clade should be grouped into a single subgroup. Monophyletic clades within the Puffinus subgroup, such as the Mediterranean

shearwaters *P. p. yelkouan* and *P. p. mauretanicus*, the Southern Hemisphere *P. huttoni* and *P. gavia*, the Southern Hemisphere *P. assimilis* ssp, the Atlantic region *P. lherminieri* and *P. assimilis*, are as divergent from each other as are the remaining subgroups. This is perhaps a legacy of the inconsistent taxonomy applied to the Puffinus subgroup relative to the other species of shearwaters. At one stage all of the Mmanx shearwaters were considered as subspecies of *P. puffinus* and even in recent times the Manx group has been treated inconsistently between the three geographic areas where it occurs. Distinct species are recognised in the southwest and northeast Pacific, but in the Atlantic three distinct forms are still treated as subspecies of *P. puffinus*.

Bourne *et al.* (1988) and Walker *et al.* (1990) have argued that because of differences in their osteology and external morphology, and their discrete breeding ranges and migration patterns the Mediterranean shearwaters should be considered distinct from *P. p. puffinus* and therefore elevated to species level, as one species consisting of two subspecies (*P. yelkouan yelkouan* and *P. y. mauretanicus*) or two distinct species (*P. yelkouan* and *P. mauretanicus*), respectively. The genetic data strongly support these proposals, and in particular the specific status of both Mediterranean forms. Genetic divergence between the two Mediterranean shearwaters is as great as that between other currently recognised species, and it is obvious that they are sister taxa that are phylogenetically quite distinct from *P. p. puffinus*. Such a taxonomic revision of the Atlantic Manx shearwaters would produce a more phylogenetically hierarchical taxonomy.

The small number of *P. lherminieri* and *P. assimilis* taxa included in this study is unfortunate, given the taxonomic controversy surrounding these groups. Without additional phylogenetic information from the remaining taxa it is difficult to conclude whether the current taxonomy accurately reflects phylogeny, or whether the phylogenetic relationships have been obscured by introgressive hybridisation or a non-equilibrium state in lineage sorting of mtDNA following recent speciations. However, the extent of sequence divergence between *P. as. assimilis* and *P. as. haurakiensis*, and to a lesser extent between *P. l. lherminieri* and *baroli/boydi*, suggests a long term historical separation between the respective populations. This separation is of a magnitude similar to that between other currently recognised species and subspecies in the genus and supports the evolutionary

significance (Ball & Avise 1992) of currently recognised subspecies that were examined from the *assimilis/lherminieri* complex.

Chapter 5

General Discussion and Conclusions.

5.1 Conclusions

This thesis has examined the ecological and evolutionary genetics of species in the shearwater genus *Puffinus*, with particular emphasis on the short-tailed shearwater, *P. tenuirostris*. A range of molecular techniques were applied at three distinct levels in the evolutionary hierarchy (Avise *et al.* 1987; Maddison & Maddison 1992), from close familial relationships, to intraspecific population-level relationships and genetic divergence among subspecies and species within the genus. These studies demonstrate the applicability of different molecular techniques to different levels in the evolutionary hierarchy (Hillis & Moritz 1990; Zink 1991; Avise 1994) and to problems in avian systematics, and particularly *Puffinus* shearwaters, that traditionally have been difficult to understand (Zink 1991). The DNA fingerprinting study identified the existence of a reproductive strategy in short-tailed shearwaters that would have been difficult to identify by observational studies alone. The population-genetic study revealed an historical perspective on the population biology of short-tailed shearwaters which would be impossible to appreciate through contemporary banding studies. The phylogenetic study of *Puffinus* shearwaters provided a genetic perspective to the systematics of this genus, which had previously been lacking, with implications for interpretation of both the phylogeny and taxonomy of species in the genus.

A molecular-genetic examination of mating systems has shown the general applicability of multi-locus DNA fingerprinting, using probes derived from core sequences of human minisatellites, to allow individual identification and examine parentage in the short-tailed shearwater. A female-specific minisatellite DNA fragment, presumably located on the avian W chromosome, was identified which allows gender identification of both adults and nestlings in this sexually monomorphic species. Approximately 10% of males which could be unambiguously assigned as authentic nest attendants were unrelated to the nestling in their particular nesting burrow. The presence of these unrelated males can be explained by the

occurrence of extra-pair fertilisations involving one or more extra-pair copulations between the pair female and an unknown extra-pair male.

These results do not dispute the classification of short-tailed shearwaters as monogamous birds, because the definition of monogamy (Wittenberger & Tilson 1980) does allow occasional copulations outside of the pair bond. Instead, they indicate that the shearwater mating system is dynamic and that both males and females attempt to maximise their reproductive success within the limitations of a necessarily monogamous system. Some breeding short-tailed shearwaters are adopting a mixed mating strategy where they maintain a socially monogamous pair bond but also engage in EPCs. This may result in conflict at both an intra- and intersexual level as paired males must attempt to maintain an exclusive mating relationship with their mate, by deterring or disrupting copulation attempts from extra-pair males, or by competing at the level of sperm by frequent within-pair copulations. This male-orientated conflict must occur against a background of female willingness, indifference or resistance to engage in copulations with extra-pair males. EPCs are a cost of colonial breeding to short-tailed shearwaters and other social birds, because the high density of individuals within a colony provide many opportunities for copulations outside the pair bond. Mate guarding is likely to be inefficient and variable among breeding male shearwaters which creates greater opportunities for EPCs. Individual-specific factors such as mate-guarding efficiency, copulation frequency, sperm fertility and viability, male genotypic and phenotypic quality, female fertility and mate compatibility, will have a major influence on the relative costs and benefits of EPCs, the opportunities for EPCs and the realised success in copulating with extra-pair partners.

The genetic results indicate a number of important consequences for studies of the reproductive ecology of short-tailed shearwaters and estimates of reproductive success in this species. First, this study has *a posteriori* shown that EPCs occur. The occurrence and success of EPCs is highly variable in birds, suggesting that the costs and benefits of this behaviour and the effectiveness with which males and females can influence the outcome of fertilisation is species specific. EPCs has never been recorded in short-tailed shearwaters, but the genetic evidence suggests that a detailed study of shearwater copulatory behaviour should be attempted to better understand the role of EPCs in the shearwater mating system.

Second, estimates of lifetime reproductive success will be in error if about 10% of males are not related to the nestling for whom they provide parental care. The magnitude of the difference between actual fertilisation success and the number of offspring raised to fledging will depend on the patterns of EPFs, and may be great if specific individuals are consistently involved in EPCs. Studies of avian mating systems and reproductive success are at their most powerful when they combine behavioural data with accurate information on genetic relationships at a generation-to-generation scale (Burke *et al.* 1991). Studies of reproductive success in the short-tailed shearwater, which previously have been based on observational data only, should utilise these molecular-genetic techniques to obtain accurate data on fertilisation success for breeding male birds. Such information may lead to the reinterpretation of the large amount of data (Bradley *et al.* 1990; Wooller *et al.* 1988, 1989, 1990) already available on the effects of age, breeding experience, and duration of the pair bond on reproductive success in short-tailed shearwaters.

The ability to identify sex on the basis of the presence or absence of a large, easily identifiable minisatellite DNA fragment with homology to the human minisatellite probe 33.6 has many potential applications for studies of short-tailed shearwater ecology. If the female-specific fragment could be isolated and characterised, and a locus-specific probe developed, then sex identification in short-tailed shearwaters could be simplified into a dot-blot procedure (e.g. Griffiths 1992). Large numbers of individuals of any age could be sexed, at any time of the year, to examine sex ratios of breeding and nonbreeding birds in the colonies, as has been performed for communally breeding skuas (*Catharacta lonnbergi*, Millar *et al.* 1992), and changes in sex ratios of nestlings from hatching to fledging, similar to the studies of Graves *et al.* (1993) on shags (*Phalacrocorax aristotelis*) and Griffiths (1992) on lesser black-backed gulls (*Larus fuscus*). A male-biased sex ratio in breeding colonies would mean that females are a limiting resource and could explain the occurrence of EPCs, if unmated males attempt copulations with already mated females.

Population genetic structure among 11 short-tailed shearwater colonies was examined by analysis of restriction fragment length polymorphisms of mtDNA, using both 6-base and more sensitive 4-base restriction enzyme assays. This study represents one of the largest mtDNA population-genetic studies of birds, both in terms of the number of individuals and the geographic range of populations surveyed. Short-tailed shearwater mtDNA exhibited a

low level of intraspecific sequence diversity and there was no evidence to suggest phylogeographic structuring of this variation, contrary to expectations based on the observed strict natal and breeding philopatry of this species. The pattern of population genetic structure in the short-tailed shearwater indicates that shearwater colonies have had extensive and evolutionarily recent genetic contact. These results do not refute observational data indicating strong contemporary philopatry nor do they imply present-day panmixia. The apparently high contemporary gene flow, implied by the mtDNA data, is more likely to be an artifact of evolutionary connections among populations through a recent and extensive range expansion. Thus the mtDNA data can be interpreted as suggesting a scenario involving a population bottleneck during the last glaciation and subsequent population and range expansion, continuing until present times. In combination with observational data, the genetic data also suggest gene flow occurring via rare, large scale dispersal events. In contrast to the fairy prion which occupies a similar geographic range and where very small founding populations are implicated (Ovenden *et al.* 1991), establishment of short-tailed shearwater colonies and recovery from population bottlenecks appears to involve an immigration of large numbers of individuals.

The phylogenetic study demonstrated the utility of short mtDNA cytochrome *b* sequences for examining evolutionary relationships among *Puffinus* taxa. The mitochondrial cytochrome *b* gene was phylogenetically informative and interspecific sequence variation was sufficient to distinguish all taxa except in one case, which may be the result of introgressive hybridisation between *P. as. baroli* and *P. l. boydi* or lineage sorting of ancestral polymorphism in recently diverged taxa of the *assimilis/lherminieri* species complex. Sequence data were obtained from a wide range of source material, which reflects the power and versatility of the polymerase chain reaction in systematic studies. DNA was retrievable from plucked feathers, small blood samples and tissue samples taken from live and deceased birds, feathers plucked from dried museum skins, and frozen and ethanol preserved museum tissue collections. The ability to obtain DNA samples non-invasively from live birds and store them under readily available conditions should greatly enhance opportunities to study the phylogeny of avian groups and further the study of *Puffinus*.

The mtDNA cytochrome *b* phylogeny is generally concordant with hypotheses based on external morphology, osteology and habit proposed by Kuroda (1954) and Wragg (1985). The molecular data suggest three general stages in the evolutionary history of the genus *Puffinus*, which represents a much simpler interpretation of the scenario proposed by Kuroda (1954). The *Puffinus* ancestor diverged relatively early into two stocks, possibly one in the North Atlantic and the other in the Southern Hemisphere. The North Atlantic stock was the ancestor of the less migratory, more aquatic forms of the generally smaller *Puffinus* species. The Southern Hemisphere stock contained the ancestor of the larger and highly migratory species, which have developed the aquatic habit to different degrees. A rapid series of speciations or a polytomous speciation occurred within each ancestral group via dispersal and vicariant processes which led to secondary contact and range overlap between members of the two major clades. Subsequently, local speciation, perhaps via local adaptation (Wragg 1985) or biogeographic processes (Bourne *et al.* 1988), led to the divergence of currently extant taxa. The mtDNA phylogeny represents a single matriarchal gene tree within the overall *Puffinus* species tree. Congruence among different gene trees, using nuclear encoded DNA sequences, is therefore required to confirm the mtDNA phylogeny.

The phylogenetic relationships defined by mtDNA cytochrome *b* sequences reveal a number of taxonomic inconsistencies. First, the subgroup Neonectris is a paraphyletic group and therefore should be reviewed to reflect the different phylogenetic affinities of *P. nativitatis* (to the Puffinus subgroup) and *P. griseus*/ *P. tenuirostris* (to the other subgroups). Wragg's (1985) osteological analysis of *Puffinus* shearwaters supports this finding. Second, the extent of genetic divergence between subspecies of *P. puffinus* (*P. p. puffinus* v *P. p. yelkouan*/*P. p. mauretanicus*) parallels that between other full species of *Puffinus*. Although genetic divergence cannot be used as a sole taxonomic criteria, these data in combination with the phylogenetic relationships among *P. p. puffinus*, *P. p. yelkouan*/*P. p. mauretanicus* and the remaining taxa in the Puffinus subgroup support the proposal by Bourne *et al.* (1988) and Walker *et al.* (1990) that the two Mediterranean shearwaters should at least be assigned to a new species, and perhaps each elevated to species rank. The systematics of the *lherminieri*/*assimilis* species complex could not be resolved in the present study as too few subspecies were available. The extent of genetic divergence among those taxa that were included suggests that sequences from the mitochondrial cytochrome *b* gene

have the potential to resolve systematic relationships in this group. Attempts should therefore be made to obtain comprehensive genetic and biological information on all of these subspecies to review both their taxonomy and phylogenetic history.

5.2 Evolution of *Puffinus* shearwaters

A complete understanding of the evolutionary process requires a synthesis of both macro- and microevolutionary change (Moritz & Hillis 1990). A phylogeny can be resolved at successive levels in the evolutionary hierarchy from relationships among species and higher taxa, to intraspecific population separation and ultimately generation-to-generation individual pedigrees (Avise et al. 1987; Maddison & Maddison 1992). Although the three molecular-genetic studies addressed particular questions in the ecology, population biology and systematics of species in the genus *Puffinus*, they are all linked through a phylogenetic tree whose terminal twigs, representing contemporary generation-to-generation pedigrees, trace back to fine branches, representing patterns of historical population subdivision, biogeography and demography, and ultimately the deeper internal branches which represent the divergence of species and species groups.

These studies are perhaps unique in having resolved phylogenetic relationships at three distinct levels in the evolutionary hierarchy of a single group of birds. The integration of these three studies onto a single phylogeny clearly demonstrates the need for both an historical and contemporary perspective when interpreting phylogenetic patterns at any level in the evolutionary hierarchy. For example, population differentiation in the short-tailed shearwater has been influenced by both historical and recent ecological and biogeographic processes. Variation in reproductive success as a result of extra-pair copulations on a generation-to-generation scale will ultimately influence population genetic structure because of the restricted number of individuals through which genes are transferred. Interpreting an interspecific phylogeny, requires an understanding of the patterns of population subdivision and demographic factors at the intraspecific level. The genetic characteristics of extant taxa, used to reconstruct their phylogenetic relationships, are not only the result of the speciation events themselves, but subsequent population level, microevolutionary processes such as population bottlenecks, migrations, and population and range expansions. The evolutionary history of *Puffinus* shearwaters has and continues to be a dynamic process from the level of the individual, to patterns of intraspecific population history and the evolutionary divergence of the species themselves.

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Appendix 2a. Hatching success and fate of incubated eggs of short-tailed shearwaters at Cape Direction and Cape Queen Elizabeth in the 1992/93 study.

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HATCHING SUCCESS OF THE SHORT-TAILED SHEARWATER *Puffinus tenuirostris* IN TWO TASMANIAN COLONIES

JEREMY AUSTIN and MATTHEW EDMUNDS

Department of Zoology, University of Tasmania, GPO Box 252C, Hobart, Tasmania 7001

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INTRODUCTION

The Short-tailed Shearwater *Puffinus tenuirostris* is a colonially nesting seabird exhibiting one of the best documented cases of an invariable and highly synchronized breeding season. The breeding biology is well known from a number of studies, including a long-term study at a single colony in Bass Strait (Bradley *et al.* 1991). Egg-laying is constant from year to year and 85 per cent of eggs are laid between 23 and 28 November, with extremes of egg-laying from 20 November to 3 December (Serventy 1963). Only a single egg is laid and is not replaced if lost. The egg is incubated by both male and female parents in alternate shifts, and hatching occurs from mid-January (Serventy 1967). The chick is brooded for the first 2–3 days after hatching and is then left unattended during the day (Fitzherbert 1985).

Although overall reproductive success in the Short-tailed Shearwater has been well documented (e.g. Bradley *et al.* 1990; Wooller *et al.* 1990), relatively little is known about the hatching success in this species. Hatching success is likely to be an important variable influencing reproductive success and survival in K-selected seabird species, such as the Short-tailed Shearwater, which has a long incubation period. As part of a blood sampling programme, we monitored hatching success of breeding shearwaters in two Tasmanian colonies in a single nesting season.

METHODS

Sixty nesting burrows were individually marked in each of two colonies at Cape Queen Elizabeth, Bruny Island (43°14′S, 147°25′E) and Cape Direction (43°06′S, 147°25′E) in south-east Tasmania. Only burrows which were short enough to reach the occupants and in which an egg was present on the first visit were selected. The colony at Cape Queen Elizabeth was visited on five occasions — 4, 13 and 21 December 1992, 29 January and 7 February 1993. The Cape Direction colony was visited four times — on 7, 15 and 24 December 1992, and 2 February 1993. On each visit the presence or absence of each adult, the egg or a hatched chick was noted.

RESULTS

Details on the hatching success at both colonies are presented in Table 1. In one burrow at Cape Queen Elizabeth, the egg was cold and no adult was incubating on 13 December. However, on 21 December the egg was being reincubated and was subsequently successfully hatched. Thirty-nine eggs had hatched by 29 January at Cape Queen Elizabeth and three chicks were being attended by an adult at this time. The remaining 36 chicks were not being brooded. Three eggs were still being incubated on 29 January and on 7 February, one egg had hatched and the other two had been abandoned. At Cape Direction 45 eggs had hatched by 2 February and no chicks were being attended by an adult.

Appendix 2a. - continued

DISCUSSION

Hatching success at Cape Queen Elizabeth was estimated at 93 per cent, 95 per cent and 92 per cent in three consecutive seasons, and 68 per cent and 59 per cent at Hunter Island and Clifton Bluff, respectively, by Naarding (1980, 1981), and was based on chick occupancy in late January. The lower hatching success rate at the latter two colonies was attributed to a higher level of predation, mainly by feral cats (Naarding 1980). On Fisher Island, 25–83 per cent (mean 61%) of all eggs laid resulted in fledged young (Serventy and Curry 1984) and almost all young hatched are raised successfully to fledging (Wooller *et al.* 1988). These values therefore represent a lower estimate of hatching success at the Fisher Island colony. At Great Dog Island, Meathrel *et al.* (1993) recorded a hatching success of 55 per cent in a single season, with a large percentage of burrows that either collapsed or were empty. Hatching success at both Cape Queen Elizabeth and Cape Direction, in the 1992/93 season, are comparable with previous estimates for this species and represent minimum values since predation of some chicks may have occurred in the days between hatching and checking the contents of the burrows.

The reduced hatching success at Cape Queen Elizabeth relative to previous estimates may reflect an increased predation pressure at this colony in recent times. At both colonies, the majority of burrows which did not contain a chick appear to have suffered from predation of either the egg or the newly hatched nestling. Feral cats represent the major predation pressure on both adults and chicks and few colonies are completely free of cats (Naarding 1980). Predation appears to have been a significant cause of mortality at Cape Queen Elizabeth since carcasses of dead adults were a common sight during this study.

Serventy and Curry (1984) found that unhatched or deserted eggs were as common as the disappearance of the egg or chick from the burrow. A relatively large number of eggs also failed to hatch at Cape Queen Elizabeth. Unhatched eggs may have been either infertile, or abandoned due to disturbance of the incubating bird during burrow inspection (Serventy *et al.* 1971), depletion of energy reserves or death of one or both parents, or behavioural traits of the parents associated with a lack of breeding experience such as incubation attentiveness, pair-bond strength and co-ordination of incubation schedules (Meathrel *et al.* 1993).

The single chick hatched at Cape Queen Elizabeth after 29 January represents an extreme of incubation length and hatching date. Incubation lasts for 52–55 days (Serventy 1967). However, the Cape Queen Elizabeth egg was incubated for a minimum of 58 days, assuming that it was laid on 3 December and hatched on 30 January. Most eggs hatch between 10 and 23 January (Serventy and Curry 1984), but hatching may continue until 29 January (Lill and Baldwin 1983). This study has extended this date to at least 30 January.

Hatching success in the Short-tailed Shearwater shows considerable variation both between colonies and years indicating that the incubation period is important in determining overall reproductive success and mortality. More detailed investigations are necessary to establish the importance of the various factors influencing hatching success.

TABLE 1

Hatching success and the fate of unhatched eggs of Short-tailed Shearwaters breeding at Cape Queen Elizabeth and Cape Direction in 1992/93.

	Colony	
	Cape Queen Elizabeth	Cape Direction
No. burrows	60	60
No. eggs hatched (%)	40 (67%)	45 (75%)
Abandoned eggs	9	2
'Empty' burrows[1]	11	13

[1]'Empty' burrows refers to burrows that contained neither an unhatched egg or a chick.

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Appendix 2a. - continued

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BOOK REVIEWS

Co-operative Mechanisms for the Conservation of Albatrosses
R. Gales, 1993.
Government Printer, Hobart. 132 pp.

This report was commissioned by the Australian Nature Conservation Agency, Canberra, from where copies may be obtained. An account is given of the status of each of the 14 species. Three factors became apparent to the author.

'1. There is an alarming paucity of information regarding such basic parameters as size of breeding populations and demographic data.

2. For species where population size has been monitored, many of the populations are showing dramatic declines in numbers.

3. Direct mortality associated with fishing practices represents the major threats to the survival of albatrosses.'

Direct mortality associated with commercial fishing is reported for 12 of the 14 species of albatrosses. The future of albatrosses is bleak unless there is a co-operative effort by all nations involved to develop and apply measures to reduce this mortality. Albatrosses are not the only seabirds killed, but they like others with a low productive rate (the Wandering Albatross only breeds every second year and does not commence to breed until over 10 years old) are highly vulnerable.

Any persons concerned about the status of albatrosses should read this report.

M. D. Murray, Pymble

Review and Analysis of Albatross Banding Data held by the Australian Bird and Bat Banding Schemes
H. Battam and L. E. Smith, 1993.
Australian National Parks and Wildlife, Canberra. 163 pp.

This report (Research and Consultancy Agreement No. 138) gives recovery data for Wandering, Black-browed, Shy, Yellow Nosed, Grey-headed and Light-mantled Albatrosses banded on various islands in the Southern Ocean. More detailed data is given from the study of the Wandering Albatross which commenced in 1956 off the New South Wales coast at Bellambi. Over the last 20 years, a decline in numbers has occurred and immature and young birds are now rarely seen. Reasons for the decline are discussed. Information on the Japanese long-line fishing effort in the Tasman Sea is given. This is a report which all concerned with the declining status of albatrosses should read.

M. D. Murray, Pymble

Atlas of the Southern Hemisphere Albatrosses
W. L. N. Tickell, 1993. 9 pp.

A series of 10 maps is presented of the Southern Hemisphere centred around Antarctica with the oceans divided into a 5 by 5 degree grid, shaded to indicate that the albatross species has been sighted in the grid. The breeding islands of each species are marked. Maps are given for *Diomedea exulans, D. epomophora, D. melanophyrys, D. chrysostoma, D. chlororynchos, D. bulleri, D. cauta, Phoebetria fusca* and *P. palpebrata*. The maps are produced on A4 paper and are obtainable from the author (Department of Zoology, University of Bristol, Bristol, BS8 1UG, UK).

M. D. Murray, Pymble

Appendix 3a. Short-tailed shearwater restriction fragment presence (1) and absence (0) for mtDNA morphs identified with *Hha* I, *Msp* I, *Hinf* I and *Taq* 1 among 231 individuals.

Morph	Fragment Number																																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Hha I																																							
A	0	1	0	1	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	0	1	0	1	1	0	0	0	1	1	0	0	0	1	1	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	1	0	0	1	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	0	1	0	1	1	1	0	0	0	0	1	0	0	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	1	0	0	1	1	1	0	0	1	0	0	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F	0	1	0	1	1	1	0	0	1	0	0	0	0	1	1	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G	1	0	0	1	1	0	0	0	1	1	0	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H	0	1	0	1	1	0	0	0	1	0	1	0	0	1	1	1	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	1	0	0	1	0	1	1	1	0	0	1	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J	0	0	1	1	1	1	0	0	1	0	0	1	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K	1	0	0	1	1	0	0	0	1	0	1	0	0	1	1	0	1	0	0	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L	0	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M	1	0	0	1	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	0	1	0	1	1	1	0	0	0	0	1	0	1	0	1	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O	1	0	0	1	1	0	0	0	1	0	1	0	0	1	1	0	1	0	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Msp I																																							
A	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	0	1	0	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
B	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
C	0	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
D	0	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	0	1	1	1	0	1	0	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
E	1	0	0	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
F	0	1	1	1	1	0	1	1	1	1	0	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
G	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
H	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
I	0	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1	0	1	1	1	0	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
J	0	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	0	1	0	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
K	0	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Hinf I																																							
A	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
B	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1
C	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
D	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1
E	0	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
F	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1
Taq I																																							
A	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	0	0	-	-	-	-	-	-	-
B	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	0	-	-	-	-	-	-	-
C	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	1	-	-	-	-	-	-	-
D	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	0	0	-	-	-	-	-	-	-
E	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	0	0	-	-	-	-	-	-	-
F	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0	-	-	-	-	-	-	-
G	0	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	0	-	-	-	-	-	-	-
H	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	0	-	-	-	-	-	-	-

Appendix 4a. Collection and preservation details for all specimens collected for a molecular examination of phylogenetic relationships among species in the genus *Puffinus*.

Specimen	Common Name	Code	Locality	Lat., Long.	Tissue	Date Collected	Collector	Storage
Puffinus pacificus	Wedge-tailed shearwater	Ppac3	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac5	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac8	Montague I., Australia	36°15'S, 150°13'E	Feather	March 1992	J. Austin	Liquid Nitrogen
P.pacificus	Wedge-tailed shearwater	Ppac10	Montague I., Australia	36°15'S, 150°13'E	Blood	March 1992	J. Austin	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac76	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	L. Ballance/	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac77	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	D. O'Daniel	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac78	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	L.B/D.O'D	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac79	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	L.B/D.O'D	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac80	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	September 1992	L.B/D.O'D	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac96	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac97	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac98	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac99	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac100	Heron I., Australia	23°25'S, 151°55'E	Blood	January 1993	M. Preker	100% EtOH, 4°C
P.pacificus	Wedge-tailed shearwater	Ppac030233[1]	Cabbage Tree I., Australia	?	Feather	July 1926	Unknown	Dried museum skin
P.bulleri	Buller's shearwater	Pbul26	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul27	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul28	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul29	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul30	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.bulleri	Buller's shearwater	Pbul041804[1]	NSW South Coast, Australia	~35°S 151°E	Feather	November 1962	P. Strong	Dried museum skin
P.bulleri	Buller's shearwater	Pbul1041[2]	New Zealand	-	Muscle	Unknown	Unknown	-70°C freezer
P.bulleri	Buller's shearwater	Pbul1044[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.carneipes. hullianus	Flesh-footed shearwater	Pcar16	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar17	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar18	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar19	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar20	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar40	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.carneipes. hullianus	Flesh-footed shearwater	Pcar027168[1]	Lord Howe I., Australia	31°28'S, 159°09'E	Feather	February 1922	Unknown	Dried museum skin
P. creatopus	Pale-footed shearwater	PcreJMB942[3]	North Pacific Ocean	44°09'N, 159°40'W	Kidney	August 1991	Unknown	70% EtOH, 4°C
P.gravis	Greater shearwater	Pgra154[6]	Emerald Isle,North Carolina, USA	34°41'N, 76°57'W	Feather	June 1993	Unknown	70% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri21	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri22	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri23	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri24	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri25	Chatham I., New Zealand	44°S, 176°30'W	Feather	March 1993	R. Parish	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri41	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri42	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri43	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri44	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri45	Kauwahai I., New Zealand	37°S, 174°30'E	Feather	October 1992	G. Taylor	100% EtOH, 4°C
P.griseus	Sooty shearwater	Pgri1040[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.griseus	Sooty shearwater	Pgri1052[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.griseus	Sooty shearwater	Pgri1053[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.griseus	Sooty shearwater	Pgri1055[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.griseus	Sooty shearwater	Pgri1056[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.griseus	Sooty shearwater	Pgri059408[1]	Sydney, Australia	33°55'S, 151°10'E	Feather	January 1982	K. Lisser	Dried museum skin
P.griseus	Sooty shearwater	Pgri158	Falkland Islands	51°45'S, 59°W	Feather	March 1994	M. Bingham	4°C
P.griseus	Sooty shearwater	Pgri159	Falkland Islands	51°45'S, 59°W	Feather	March 1994	M. Bingham	4°C

Appendix 4a - continued

Specimen	Common Name	Code	Locality	Lat., Long.	Tissue	Date Collected	Collector	Storage
P. tenuirostris	Short-tailed shearwater	Pten5	Bruny I, Australia	43°28'S, 147°14'E	Liver	March 1991	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten50	Trial Harbour, Australia	41°55'S, 145°09'E	Liver	March 1991	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten200	Port Fairy, Australia	38°24'S, 142°15'E	Liver	March 1992	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten323	Montague I., Australia	36°15'S, 150°13'E	Liver	March 1992	J. Austin	Liquid Nitrogen
P. tenuirostris	Short-tailed shearwater	Pten032171[1]	Sydney, Australia	33°55'S, 151°10'E	Feather	October 1895	H. Grant	Dried museum skin
P.nativitatis	Christmas shearwater	Pnat81	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.nativitatis	Christmas shearwater	Pnat82	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.nativitatis	Christmas shearwater	Pnat83	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.nativitatis	Christmas shearwater	Pnat84	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.nativitatis	Christmas shearwater	Pnat85	Sand I., Johnston Atoll	16°45'N, 169°32'W	Blood	April 1993	B. A. Schreiber	100% EtOH, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf86	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	July 1993	J. L. R. Luengo	100% EtOH, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf117	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf118	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf119	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf120	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf121	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf122	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf123	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf124	Bardsey I., Wales	52°46'N, 4°48'W	Feather	November 1978	Unknown	Whole bird, -20°C
P.puffinus.puffinus	Manx shearwater	Pppf125	Skomer I., Wales	51°45'N, 5°18'W	Blood	1989	R. Griffiths	Unknown buffer, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf126	Skomer I., Wales	51°45'N, 5°18'W	Blood	1989	R. Griffiths	Unknown buffer, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf127	Skomer I., Wales	51°45'N, 5°18'W	Blood	1989	R. Griffiths	Unknown buffer, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf128	Skomer I., Wales	51°45'N, 5°18'W	Blood	1989	R. Griffiths	Unknown buffer, 4°C
P.puffinus.puffinus	Manx shearwater	Pppf129	Skomer I., Wales	51°45'N, 5°18'W	Blood	1989	R. Griffiths	Unknown buffer, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk61	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk62	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk63	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk64	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.yelkouan	Levantine shearwater	Ppyk65	Ile de Port-Cros, France	43°N, 6°23'E	Feather	July 1993	R. Zotier	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma111	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma112	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma113	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma114	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma115	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.puffinus.mauretanicus	Balearic shearwater	Ppma116	Cabrera Archipelago, Balearic Islands	39°09'N, 2°56'E	Blood	April 1993	J. S. A. Gonzalez	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw106	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw107	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw108	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw109	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.auricularis.newelli	Newell's shearwater	Panw110	Kauai I., Hawaii	22°N, 159°30'W	Blood	November 1993	T. Telfer	100% EtOH, 4°C
P.opisthomelas	Black-vented shearwater	Popi31353[3]	Channel Islands, USA	32°45'N, 118°30'W	Feather	December 1977	Unknown	Dried museum skin
P.gavia	Fluttering shearwater	Pgva1	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva2	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva3	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva4	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva5	Bream Islands, New Zealand	35°45'S, 174°30'E	Feather	January 1993	R. Parish	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva46	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva47	Long I., Marlborough Sound, New Zealand	41°S, 174°E	Feather	January 1993	B. Bell	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva48	Long I., Marlborough Sound, New Zealand	41°S, 174°E	Feather	January 1993	B. Bell	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva49	Long I., Marlborough Sound, New Zealand	41°S, 174°E	Feather	January 1993	B. Bell	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva50	Long I., Marlborough Sound, New Zealand	41°S, 174°E	Feather	January 1993	B. Bell	100% EtOH, 4°C
P.gavia	Fluttering shearwater	Pgva1030[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer

Appendix 4. a - continued

Specimen	Common Name	Code	Locality	Lat., Long.	Tissue	Date Collected	Collector	Storage
P.gavia	Fluttering shearwater	Pgva1034[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.gavia	Fluttering shearwater	Pgva1035[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.gavia	Fluttering shearwater	Pgva1036[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.gavia	Fluttering shearwater	Pgva1037[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.huttoni	Hutton's shearwater	Phut6	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut7	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut8	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut9	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut10	Seaward Kaikoura Mountains, New Zealand	42°15'S, 173°45'E	Feather	March 1992	I. Miller	100% EtOH, 4°C
P.huttoni	Hutton's shearwater	Phut1079[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.huttoni	Hutton's shearwater	Phut1085[2]	New Zealand	-	Liver	Unknown	Unknown	-70°C freezer
P.huttoni	Hutton's shearwater	Phut058237[1]	Byron Bay, Australia	28°43S, 153°34'E	Feather	February 1983	J. Willows	Dried museum skin
P.lherminieri.boydi	Audubon's shearwater	Plby39.756[4]	Razo I., Cape Verde Islands	16°N, 24°W	Feather	October 1988	Unknown	Dried museum skin
P.lherminieri.lherminieri	Audubon's shearwater	Pllh33.685[4]	Bonaire, Netherlands Antilles	12°15'N, 68°27'W	Feather	April 1980	Unknown	Dried museum skin
P.lherminieri.lherminieri	Audubon's shearwater	Pllh27.301[4]	Bonaire,Netherlands Antilles	12°15'N, 68°27'W	Feather	May 1960	Unknown	Dried museum skin
P.lherminieri.nicolae	Audubon's shearwater	Plnc5.686[4]	Cousine I., Seychelles	4°30'S, 56°W	Feather	May 1908	Unknown	Dried museum skin
P.lherminieri.subalaris	Audubon's shearwater	Plsb20.077[4]	Galapagos Islands	0°30'S, 90°30'W	Feather	October 1966	Unknown	Dried museum skin
P.lherminieri.lherminieri	Audubon's shearwater	PlB-20893[5]	near Oregon Inlet, USA	35°30'N, 75°W	Kidney	Unknown	D. L. Dittmann	-20°C freezer
P.lherminieri.lhermineir	Audubon's shearwater	PlB-20918[5]	near Oregon Inlet, USA	35°30'N, 75°W	Kidney	Unknown	D. L. Dittmann	-20°C freezer
P.lherminieri.lherminieri	Audubon's shearwater	Pll153[6]	Atlantic Beach, North Carolina, USA	34°42'N, 76°44'W	Feather	August 1993	Unknown	70% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha31	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha32	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	R. Parish	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha33	Poor Knights Islands, New Zealand	35°30'S, 174°45'E	Feather	November 1992	R. Parish	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha51	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha52	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha53	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha54	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.assimilis.haurakiensis	Little shearwater	Paha55	Marotiri I., New Zealand	35°50'S, 174°45'E	Feather	October 1992	M. Imber	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr91	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr92	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr93	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr94	Tenerife, Canary Islands	28°15'N, 16°35'W	Blood	June 1993	J. L. R. Luengo	100% EtOH, 4°C
P.assimilis.baroli	Little shearwater	Pabr44.472[4]	Madeira Islands, Portugal	32°45'N, 17°W	Feather	February 1905	Unknown	Dried museum skin
P.assimilis.elegans	Little shearwater	Pael14.483[4]	Tristan da Cunha Islands	37°S, 12°30'W	Feather	May 1959	Unknown	Dried museum skin
P.assimilis.baroli	Little shearwater	Pabr44.472[4]	Madeira Islands, Portugal	32°45'N, 17°W	Feather	February 1905	Unknown	Dried museum skin
P.assimilis.assimilis	Little shearwater	Paas160	Lord Howe Island, Australia	31°28'S, 159°09'E	Feather	July 1994	D. Hiscox	100% EtOH, 4°C
P.assimilis.assimilis	Little shearwater	Paas161	Lord Howe Island, Australia	31°28'S, 159°09'E	Feather	July 1994	D. Hiscox	100% EtOH, 4°C
P.assimilis.assimilis	Little shearwater	Paas162	Lord Howe Island, Australia	31°28'S, 159°09'E	Feather	July 1994	D. Hiscox	100% EtOH, 4°C

[1] Underlined numbers in sample code refers to the Australian Museum identification number for these specimens. Donated by W. E. Boles.

[2] Underlined numbers in sample code refers to the National Art Gallery and Museum (New Zealand) identification number for these specimens. Collection localities and dates were not available for these specimens. Donated by J. A. Bartle, N. Hyde and C. Daugherty.

[3] Underlined alphanumerics in sample code refers to the University of Washington, Burke Museum identification code for these specimens. Donated by G. A. Voelker.

[4] Underlined numbers in sample code refers to the Universiteit van Amsterdam, Instituut voor Taxonomische Zoologie (Zoologisch Mueum) identification number for these specimens. Donated by T. Prins.

[5] Underlined alphanumerics in sample code refer to the Louisiana State University, Museum of Natural Sciences identification code for these specimens. Donated by D. R. Reynolds.

[6] Donated by John A. Gerwin, North Carolina State Museum of Natural Sciences.