QUANTIFICATION OF GENETIC VARIATION IN ISLAND-BREEDING POPULATIONS OF PROCELLARIIFORMES:

AN ASSESSMENT OF THE IMPACT OF THE LONGLINE FISHING INDUSTRY ON SEABIRDS.

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ABSTRACT

The number of albatrosses that are killed on longlines in the Southern ocean is conservatively estimated to be 44 000 birds per annum. These numbers are biologically significant since albatrosses are a prime example of an extreme K-selected species. Ongoing long line fishing in the Southern ocean could lead to a decrease in the size of breeding colonies, and is a cause for major concern as it may impact the long-term survival of these birds. Quantifying genetic variation in threatened populations is a valuable application of molecular biology in conservation. In this study genetic variation was quantified using microsatellite analysis in order to investigate the effects of the longline fisheries on seabird populations. In addition, the feasibility of developing diagnostic markers for determining the provenance of birds forming part of the bycatch was also investigated. The inter-population genetic variance of three species of albatross from four distinct breeding colonies is described. Microsatellite markers were found to be highly variable and provided an assessment of the heterozygosity in the distinct populations, and a measure of the gene flow between these populations. Despite the extreme fidelity that adult albatrosses show to their breeding colonies, relatively low levels of genetic differentiation were observed between the colonies. This suggests that an integrated conservation management strategy could be undertaken successfully.

White-chinned petrels are also threatened by the longline fishing industry. Microsatellite markers developed in the albatross were, however, found to be uninformative in these more distantly related petrels. A microsatellite library for the white-chinned petrel was constructed, though no informative markers have yet been isolated.

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LIST OF ABBREVIATIONS

$(CA)_n$	Represents the dinucleotide CA motif repeated n times. It also implies the corresponding motif on the complementary DNA strand (ie: $(GT)_n$).
A	Mean number of alleles per locus
Amp	Ampicillin
BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EtBr	Ethidium bromide
H_E	Average expected heterozygosity
H_O	Average observed heterozygosity
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Broth
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride: Sodium tri-citrate (2:1)
TE	Tris-EDTA
T_M	Melting temperature of a nucleic acid hybrid
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

LIST OF UNITS

µg	Micrograms
µl	Microlitres
bp	Base pairs
cpm	Counts per million
M	Molar
mg	Milligrams
min	Minute
ml	Millilitres
mm	Millimolar
ms	Millisecond
pg	Picogram
pm	Picomole
S	Second
W	Watts

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1 INTRODUCTION

The growth of commercial fisheries has placed increasing pressure on previously isolated seabird populations, both in terms of direct competition for food resources, and indirectly as a result of fisheries-associated mortality (Brothers, 1991). It has been estimated that well in excess of 250 000 seabirds (including more than 44 000 albatrosses) are killed annually as a result of interactions with fishing vessels (Brothers, 1991). The impact of this incidental seabird mortality at the population level is difficult to assess, largely due to a dearth of baseline data regarding population structure and dynamics (Croxall, 1987). Monitoring of seabird populations is complex since individuals cannot be observed all year round. Established monitoring programmes have not been consistent, or have not been in place long enough to have produced reliable, long-term data regarding population numbers and structure for these remote island-breeding species (Croxall and Rothery, 1991).

Genetic studies have proved a useful means to determine social structure, colony isolation and gene flow (Paetkau and Strobeck, 1994; Taylor *et al.*, 1994; Pope *et al.*, 1996) and may be particularly useful where typical monitoring programmes are not feasible. Microsatellite DNA has become the genetic marker of choice for population genetic studies due to its high levels of polymorphism and the relative ease of use (Queller *et al.*, 1993). Microsatellite markers have been used in population genetic studies in a variety of organisms, including birds (Ellegren, 1992; Primmer *et al.*, 1995; Primmer *et al.*, 1997). However, only relatively recently have microsatellite markers been described for procellariiform seabirds (Burg, 1999).

1.1 CONSERVATION GENETICS

Conservation genetics is a synthesis of the theory and practice of standard population genetics as they relate to the management of threatened and endangered species. A major goal of conservation biology is to maintain variation in natural populations (Soulé, 1980). Historically, variation has been assessed at a morphological level. Modern biochemical techniques are able to reveal variation at the molecular level and have, as such, proved to be a far more sensitive measure of natural variation.

The application of genetic techniques in conservation has progressed rapidly over the past decade and is no longer generally regarded as "an esoteric mystery divorced from applied ecology" (Greig, 1978), but has become an integral tool in conservation management.

Conservation genetics can be divided into two major areas. The first is concerned with measuring genetic variability at the population level. The second deals with defining population structure in order to determine units which require conservation. This includes the problems of defining species and recognising hybridisation (Burke, 1994). In order to address these issues from a molecular perspective heterozygosity is used as a quantitative measure of genetic diversity.

1.1.1 MEASURING GENETIC DIVERSITY

Heterozygosity (H), or the mean number of individuals heterozygous at a particular locus, is a common measure of genetic variation in natural populations (Hartl, 1988). Heterozygosity has been correlated with a species fitness and vigour in the short-term (Clegg and Brown, 1985). In the long-term heterozygosity has been correlated with the ability of a population to respond to the pressures of natural selection, and thereby with the evolutionary persistence of the population (Frankel and Soulé, 1981). Allelic richness (A) is second common measure of genetic diversity. Allelic richness refers to the number of alleles present at varying frequencies at a particular locus (Young *et al.*, 1996) and is also thought to contribute to the long-term fitness and survival of a population.

Habitat destruction and the subsequent fragmentation of ecosystems are major factors reducing genetic variation in natural populations. Typically, as populations become smaller, fragmented, isolated and less stable they become vulnerable to loss of genetic variation through stochastic events such as genetic drift and fluctuations in allele

frequency (Avise, 1994). Similarly, small populations are more susceptible to inbreeding depression which can be defined as a decline in fitness due to an increase in the number of homozygotes present in the population resulting in the exposure of deleterious recessive alleles (Storfer, 1996).

An example of the effect of the loss of genetic variability in populations is the low levels of variability detected in the cheetah, *Acinonyx jubatus*. The high incidence of sperm abnormalities, elevated cub mortality and compromised immunity are all thought to result from the high levels of homozygosity observed in this species (O'Brien *et al.*,1985). The levels of genetic variation in populations can elucidate the extent of inbreeding and hybridisation, and can be used to determine population structure.

Additionally, barriers to migration, and therefore to gene flow, subdivide natural populations into sub-populations which may differ genetically from one another (O'Ryan *et al.*, 1998). A major contribution of conservation genetics is to aid in the maintenance of as much of the natural genetic diversity as possible both within and between such populations.

Current methods in use to measure genetic variation include allozyme analyses of protein loci, molecular surveys of genomic and mitochondrial DNA, and quantitative genetic analyses (reviewed in Milligan *et al.*, 1994).

Most techniques used to quantify genetic variation use neutral molecular markers rather than using those related to specific adaptive traits. This use is based on the underlying assumption that the level of variation detected at the marker loci directly reflects overall levels of variation assumed to confer future adaptation. It has been argued that there is little evidence to assume a direct connection between variation seen at neutral marker loci and variation at loci conferring fitness (Milligan *et al.*, 1994). In fact, there are documented instances in which there appears to be no correlation between heterozygosity and fitness; such as that of the forked fungus beetle (Whitlock, 1993), the brook trout (Hutchings and Ferguson, 1992) and more recently the black robin which has recovered from a single breeding pair to produce a population of 55 pairs with breeding success comparable to that of other robins (Ardern and Lambert, 1997).

However, despite the controversy surrounding the accuracy of using molecular marker variability as a universal measure of long-term survival, molecular genetics can play an

important role in determining the demographics of small populations since the demographic history of a population is reflected in its genetic composition.

1.1.2 Defining Conservation Units

A major issue in conservation genetics is what constitutes a conservation unit, and how to quantify this entity. The definition of conservation units within species is crucial for informed conservation management (Daugherty *et al.*, 1990). It is fundamental that with the limited time and resources available, conservation efforts are targeted at those units most in need of management. Moritz (1994) has suggested that there are two types of conservation unit significant for conservation. The first being Evolutionarily Significant Units (ESUs) which represent historically isolated populations that together encompass the evolutionary diversity of a taxon. ESUs commonly result from marked contractions in the original range of a species - as in the case of the ghost bat, *Macroderma gigas* (Worthington Wilmer *et al.*, 1994) - resulting in disjunct populations each of which warrants a conservation effort in its own right. The second type of conservation units are Management Units (MUs), which represent demographically independent sets of populations (Moritz, 1995).

Assessing the genetic variation within and between populations has become crucial in the definition of conservation units. Together with complementary ecological and behavioural information, genetic assessment of conservation units has the potential to revolutionise the management of endangered species. However, inadequate resources and a lack of political will remain the most significant obstacles to successful management of significant conservation units.

1.2 STUDY ORGANISMS

The Procellariiformes are a group of marine birds that can be identified by the horny tubes near the base of the bill. They are abundant, distributed worldwide, and breeding populations vary markedly in size (Warham, 1996). The Procellariiformes are represented by four families: (i) Diomedeidae, the albatrosses and mollymawks (collectively referred to using the general term "albatrosses"); (ii) Procellariidae, the typical petrels; (iii) Hydrobatidae, the storm petrels; and (iv) Pelecanoididae, the diving petrels, (Alexander *et al.*, 1965).

It is of use to note that in the literature the common names "albatross" and "petrel" are used rather loosely to describe the Procellariiformes. To avoid confusion I have used the full genus and species names wherever possible. When the term "albatross" has been used here it refers to all members of the family Diomedeidae (specifically *Diomedea exulans*, *Thalassarche chrysostoma and Thalassarche melanophris*). When the term "petrel" has been applied it refers to members of the family Procellariidae (specifically *Procellaria aequinoctialis*).

There are 14 species of Diomedeidae, 10 of which are confined to the Southern Ocean. The taxonomy of the group is controversial. To date behavioural data (Paterson *et al.*, 1995), morphological data (Coues, 1866), DNA hybridisation (Sibley and Ahlquist, 1990) and mtDNA data (Nunn *et al.* 1996) have been used to resolve phylogenetic relationships between the Procellariiformes. Nunn *et al.* (1996) have used cytochrome *b* sequence data (GenBank accession numbers U48940 to U48955) to place extant albatrosses into four genera , *Diomedea*, *Thalassarche*, *Phoebetria* and *Phoebastria*. It is this most recent classification that has been utilised throughout this thesis.

A brief review of the biological and ecological characteristics of procellariiform seabirds is included to contextualise the impact of the longline fisheries on these species. In addition some general features of the biology of each of the four species studied – *Procellaria aequinoctialis*, *Diomedea exulans*, *Thalassarche chrysostoma* and *Thalassarche melanophris* - can be found in Table 1. Figures 1A-C provide representative pictures of *Diomedea exulans*, *Thalassarche chrysostoma* and *Thalassarche melanophris*.

Albatrosses have a life strategy combining high adult survival, deferred sexual maturity, low reproductive rates and a long life span (Croxall and Prince, 1990). Any factor that disturbs these parameters may severely threaten their survival.

Breeding colonies are largely located on the isolated sub Antarctic islands (Appendix A) and may range from a few pairs to thousands of pairs (Croxall, 1979; Croxall and Rothery, 1991; Warham, 1996). These oceans fall under the jurisdiction of a number of countries and protection of the fauna and flora is therefore a global responsibility. Procellariiformes are primarily scavengers of fish and cephalopods (Warham, 1996). Albatrosses forage widely making long pelagic and short inshore trips (Weimerskirsch *et al.*, 1992) especially during chick provisioning. The growth of the fishing industry has resulted in an abundant supply of easily available food in the form of discarded bait and offal (Cooper *et al.* 1992).

Behavioural data indicate that high levels of philopatry, are exhibited by Procellariiformes, particularly *D. exulans* (Croxall and Rothery, 1991). Philopatry refers to the extremely high tendency of these species to return to, and breed at, the natal colonies. This has implications for the genetic profiles of these populations. Philopatry tends to promote genetic similarity of individuals in isolated populations as kinship would be high within a particular breeding population, but low between that population and other breeding populations (Ridley, 1993).

The wide range of these marine birds means that factors within both the immediate terrestrial and extensive marine habitats have an effect on their survival.

Wandering albatross chick - Australian Antarctic Division photo by Christo Baars
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Figure 1a ***Diomedea exulans*** **chick. When fully grown the wandering albatross is the largest of the albatrosses.**

Grey headed albatross - Australian Antarctic Division photo
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Figure 1b **Adult** ***Thalassarche chrysostoma.*** **Note the grey head which gives rise to its common name – the grey-headed mollymawk.**

Figure 1c **Adult *Thalassarche melanophris* with chick. Note the dark brows which give rise to its common name – the black-browed mollymawk.**

Table 1 **A brief summary of some of the features of *P. aequinoctialis*, *D. exulans*, *T. melanophris* and *T. chrysostoma* biology. All data from, Marchant and Higgins (1990), Warham (1996) and Ryan (1997) unless otherwise stated.**

	Procellaria. aequinoctialis **(Linnaeus, 1758)**	***Diomedea exulans*** **(Linnaeus, 1758)**	***Thalassarche melanophris*** **(Temminck, 1828)**	***Thalassarche chrysostoma*** **(Forster, 1785)**
Classification Order	Procellariiformes	Procellariiformes	Procellariiformes	Procellariiformes
Family	Procellariidae	Diomedeidae	Diomedeidae	Diomedeidae
Common name	White-chinned petrel	Wandering albatross	Black-browed mollymawk	Grey-headed mollymawk
Average adult weight	1.2 kg	7-11 kg	3.7 kg	3.5 kg
Distribution	*Range:* Colder southern ocean and off western South America.	*Range:* Circumpolar, in Antarctic, subantarctic and subtropical waters of Atlantic, Pacific and Indian oceans.	*Range:* Circumpolar south of subtropical zone.	*Range:* Circumpolar in Antarctic, subantarctic and subtropical waters.
Sexual maturity	4-10 years	10-12 years	7-9 years	8-10 years
Average clutch size	1	1	1	1
Frequency of breeding	Annual	Biennial	Annual	Biennial
Breeding colonies	South Georgia, Falklands, Prince Edward, Marion, Crozet, Kerguelen, Auckland, Campbell, Antipodes, Gough Inaccessible, Tristan da Cunha islands	South Georgia, Marion, Prince Edward, Crozet, Kerguelen, Macquarie, Heard, Antipodes, Gough, Campbell and Auckland islands.	Crozet, Kerguelen, Macquarie, Antipodes, Heard, Campbell, South Georgia, Falklands, Southern Argentina, Southern Chile, Isles de McDonald, Bishop and Clerk islands.	Prince Edward, Isles de Crozet, Kerguelen, Macquarie, Campbell, South Georgia, Marion, and Diego Ramirez islands

During the 1980s population declines were reported in various albatross species (Weimerskirch and Jouventin, 1987). Population declines have been confirmed for six of the fourteen albatross species (including *D. exulans, T. chrysostoma* and *T. melanophris*), while the status of three species remains unknown (Gales, 1993). It has since become evident that these declines are primarily the result of incidental seabird bycatch, particularly during the course of longline fishing (Brothers, 1991; Murray *et al.*, 1993; Cherel *et al.*, 1996). Further, involvement of the longline fisheries in seabird mortality has been confirmed with 63% of the ringed albatrosses recovered between 1975 and 1988 being from longline fisheries bycatch, in contrast to 38% between 1960 and 1974 (Croxall and Prince, 1990).

Longline fishing is concentrated between 30°S and 60°S (Brothers, 1991; de la Mare and Kerry, 1994), which coincides with the major foraging areas of the wandering albatross, black-browed mollymawk and white-chinned petrel (Woehler, 1996). As scavengers, many seabirds are attracted to fishing vessels and will follow vessels to feed on discarded offal. The fisheries have therefore created an abundant food source for these seabirds and altered seabird distribution patterns (Ryan and Moloney, 1988), but not without associated risks. Where the natural foraging territories of the seabirds and the fishing zones overlap, and where seabird distributions have been altered to coincide with those of the fisheries, these birds are at significant risk of being caught by fishing vessels (Cherel *et al.*, 1996; Brothers *et al.*, 1998). Since bird bycatch rates peak during the chick provisioning period it has been recommended that fishing within the foraging range of breeding colonies be avoided over this period (Ryan, 1996).

The impact of incidental fisheries mortality in seabirds is of particular concern in light of their life strategies. Several of the seabird species, notably the albatrosses and large petrels, show delayed sexual maturity and low fecundity, breeding annually or even biennially (Warham, 1996) (Table 1). Longevity, wide foraging ranges and exclusively pelagic feeding patterns make albatross populations sensitive bioindicators of the state of ocean systems (Croxall and Rothery, 1991). However, low reproductive rates mean that by the time adverse effects on population size, breeding, and survival are evident, the factors driving these changes have already been in operation for some time. Low population recruitment rates mean that even with the institution of remediation measures,

1.4 MICROSATELLITES IN CONSERVATION

Commonly used biochemical techniques to quantify genetic variation and identify units for conservation include allozymes, restriction fragment length polymorphisms (RFLPs) mitochondrial DNA (mtDNA) analysis and genomic DNA fingerprinting. These methods, however, may reveal too little variation at too few loci to be of use in many population genetics applications (reviewed in Amos and Hoelzel, 1992). The ideal marker would be one which is able to detect loci which range from invariable to hypervariable and which are co-dominantly inherited (Lambert and Millar, 1995).

Microsatellite DNA is a relatively recently discovered molecular marker that appears to be a prime contender for consideration as the universal genetic marker (Tautz and Renz, 1984; Litt and Luty, 1989; Tautz, 1989). Microsatellites are short segments of generally non-coding DNA in which a tandem repeat motif of between two and six bases is repeated a variable number of times eg: $(CA)_n$, where n is the number of times the motif is repeated. These motifs are evenly distributed throughout the eukaryotic genome (Hamada *et al.*, 1982). Microsatellites are exceptionally variable, with the potential for many alleles at a locus (Amos *et al.*, 1993) and require minute quantities of starting material due to their use of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) for amplification of the loci of interest (Weber and May, 1989). Variation at microsatellite loci is thought to arise as a result of intra-allelic polymerase slippage during DNA recombination (Schlötterer and Tautz, 1993). This results in length polymorphisms as expansion or contraction of the locus occurs by a single repeat unit over time. The mutation rates at microsatellite loci are relatively high, approximately 10^{-4} (Schlötterer and Tautz, 1993). For these reasons microsatellites are widely regarded as among the most powerful genetic markers available (Lambert and Millar, 1995; Goldstein and Pollock, 1997). The popularity of microsatellite DNA in current population genetics is not due to any breakthrough regarding its function in the genome, but rather due to advances in genetic techniques that have allowed microsatellites to be exploited as genetic markers. Although it was known as early as 1980 that the number of repeats at a microsatellite locus could vary between individuals (Slightom *et al.*, 1980) the cloning, amplification and sequencing technologies available at that time were inadequate to allow the utilisation of these polymorphisms. Following the advent of PCR it was realised that

1.5 THE AVIAN GENOME

Due to its domestication and commercial importance the chicken, *Gallus domesticus*, constitutes the most well characterised of the aves at present (Churkina *et al.*, 1995). Approximately 79% of the avian DNA sequences in Genbank have been derived from the Galliformes, and more than 75% of these are mitochondrial (cytochrome *b*, and D-loop) sequences. There is, therefore a serious lack of avian sequence data available for comparative analysis. However, various studies have indicated that the avian genome possesses a number of interesting features for investigation (Hamada *et al.*, 1982; Primmer *et al.*, 1997).

1.5.1 GENOME SIZE

The haploid DNA content (DNA C-value) varies widely between eukaryotes with no correlation between DNA content and organismic complexity or the number of protein-coding genes (Cavalier-Smith, 1978). DNA content, however, correlates with nuclear volume, cell volume, cell cycle length and minimum generation time. Thus it has been demonstrated that small-celled, rapidly growing and metabolising species such as birds have low C-values, whereas large-celled, sluggish species have high C-values (Cavalier-Smith, 1978) It has been hypothesized that in order to achieve a high metabolic rate a small genome size is required (Cavalier-Smith, 1985). This would appear to be borne out by the fact that, within the vertebrates, birds and bats have the highest metabolic rates and the lowest DNA C-values (Tiersch and Wachtel, 1991; Van Den Bussche *et al.*, 1995). In bats this appears to have been achieved through a reduction in the copy number of repetitive elements (Van Den Bussche *et al.*, 1995). The Chiroptera have the smallest genome size among the mammals (between 50 and 87% of that characteristic of other mammalian orders) (Burton *et al.*, 1989). The mean DNA content in birds is 2.83 ±0.33 pg per cell (Tiersch and Wachtel, 1991) compared to a mean of 8 pg per cell in mammals (Cavalier-Smith, 1978). It has been shown that this is, at least partly, due to a reduction in the amount of non-coding DNA, specifically repetitive elements (Epplen *et al.*,1978; Doolittle, 1985). Dot-blot hybridisation studies have revealed a low density of several microsatellite motifs (Hamada and Kakunaga, 1982). In addition Hughes and Hughes (1995) have evidence to suggest that DNA loss has also occurred from the introns, and that this has taken place disproportionally in long introns through multiple, scattered

2 GENERAL RESEARCH AIMS

The objective of this study was to investigate the use of microsatellite markers to quantify genetic variation within and between breeding populations of three procellariiformes; the wandering albatross *Diomedea exulans* (Linnaeus, 1758), the black-browed mollymawk *Thalassarche melanophris* (Temminck, 1828), and the grey-headed mollymawk *Thalassarche chrysostoma* (Forster, 1785).

The major research areas can be summarised as follows:

- To determine population structure for *D. exulans*, *T. chrysostoma* and *T. melanophris* from South Georgia, Marion and Prince Edward islands, with a view to developing a microsatellite based method for unambiguously assigning birds forming part of the longline fisheries bycatch to their breeding populations
- To explore the extent of successful cross-species amplification of microsatellite loci developed in one non-passerine avian species in related species using Procellariiformes as an example.
- To construct a genomic microsatellite library for use in the white-chinned petrel, *Procellaria aequinoctialis* (Linnaeus, 1758).

3 SECTION A: MICROSATELLITE ANALYSIS OF *D. EXULANS, T. MELANOPHRIS, T. CHRYSOSTOMA* AND *P. AEQUINOCTIALIS*

3.1 MATERIALS AND METHODS

3.1.1 SAMPLES

Samples were obtained from breeding populations of *D. exulans*, *T. melanophris*, *T. chrysostoma* and *P. aequinoctialis* on three southern ocean islands, as well as from birds forming part of the longline fisheries bycatch (Table 2). A complete list showing details of each sample is included in Appendix B.

Table 2 **Sample numbers analysed for *D. exulans*, *T.chrysostoma*, *T. melanophris* and *P. aequinoctialis* from four of the Southern Ocean breeding colonies**

Locality / Species	South. Georgia	Marion	Prince Edward (Upper colony)	Prince Edward (Lower colony)	Bycatch
Diomedea exulans	15	27	10	10	—
Thalassarche chrysostoma	16	18	—	—	—
Thalassarche melanophrys	15	—	—	—	—
Procellaria aequinoctialis	16	19	—	—	31

These included varying DNA template, primer and magnesium concentrations, temperature and PCR annealing and extension times. The five pairs of the 12 primer sets tested that yielded polymorphic products were chosen for amplification.

Since the primers were obtained from T. Burg in primer pair cocktails both the forward and reverse primer were labelled during the end-labelling procedure. 0.1 pM of primer pair cocktail was labelled with 0.8 μCi [γ-^{32}P]dATP using T_4 polynucleotide kinase at 37°C for 90 minutes (Sambrook *et al.*, 1989). The PCR was performed in a final volume of 10 μl containing 0.25 U BioTaq polymerase (Whitehead Scientific), 0.1 pM of the labelled primer pair, 10 ng sample DNA, 50 mM of each of the deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 1x $MgCl_2$-free NH_4 reaction buffer. PCR amplifications were performed in a Stratagene Robocycler 96 according to the following thermal profile: an initial denaturation step at +94°C for 2 min, followed by a series of 35 cycles at +94°C for 30 s, annealing temperature (T_M) (Table 3) for 45 s and +72°C for 50 s, and followed by a single extension at +72°C for 10 min. Following amplification 4 μl of formamide loading dye was added to the amplified product. Prior to loading 3 μl of the PCR product on a 6%, denaturing, polyacrylamide gel the sample was denatured at 90°C for 2 min. Samples were electrophoresed for at 55 Watts for 3-4 hours. The gels were dried and exposed to autoradiographic film for between 2 and 24 hours.

Genotypes were scored from the autoradiographs and allele lengths in base pairs (bp) were determined using an AT size marker made from DNA derived from the M13 polycloning site.

Hardy-Weinberg equilibrium was assessed using an Exact test (Guo and Thompson, 1992) and the overall significance was determined using a sequential Bonferroni test (Rice, 1988).

In order to assess the level of population subdivision, the genetic variation of one sub-population is compared to that within other sub-populations, or to that of the entire population using fixation indices. Wright (1978) developed the concept of fixation indices (F-statistics) that partition heterozygosity into components which reflect the genetic structure of a population or between populations. Fixation indices are used to measure the degree of inbreeding and differentiation between subpopulations. F_{IS} refers to the proportion of variation within individuals relative to the variation found in subpopulations, F_{IT} to the proportion of variation within individuals relative to that of the total population and F_{ST} to the proportion of variation occurring in subpopulations relative to the total variation found in the population.

Both F_{IS} and F_{IT} should be close to zero. Positive values for these values suggest inbreeding, while negative values suggest lower than expected rates of inbreeding (resulting in an excess of heterozygotes). Heterozygote excess may occur when the females within a group are closely related while their male offspring disperse prior to breeding, (Avise, 1994).

A strongly positive F_{ST} indicates genetic substructure. FST is therefore an "inbreeding coefficient" as the reduction in heterozygosity of a population due to random genetic drift is calculated in order to determine inbreeding in subpopulations relative to the total population (Hartl, 1988). F_{ST} determines the probability that two alleles chosen at random from within the same subpopulation are identical by descent (Hartl, 1988 pp. 90).

Values of F_{ST} less than 0.05 suggest little structuring, whereas values between 0.1 and 1.0 suggest increasing genetic structuring. Wright (1978) proposed the following guidelines to the interpretation of F_{ST}: F_{ST} values of 0 to 0.05 indicate little genetic differentiation; F_{ST} values of 0.05 to 0.15 indicates moderate genetic differentiation; F_{ST} values of 0.15 to 0.25 indicate significant genetic differentiation.

Nei (1973) developed G_{ST}, a fixation-index equivalent to F_{ST}, for use with molecular data.

3.2 RESULTS

Procellariidae

When tested in *P. aequinoctialis* the primers for locus HrU2, which was previously shown to amplify a product in *Calonectris* (Primmer *et al.*, 1996), consistently amplified a monomorphic locus (Figure 2) despite optimisation of template DNA, primer and magnesium concentrations under a wide range of temperature conditions. This locus was therefore determined to be uninformative in *P. aequinoctialis*.

The 26 primer pairs developed in *D. exulans, T. chrysostoma* and *T. melanophris* (Burg, 1999) were exhaustively tested under a range of PCR conditions and found to be uninformative for *P. aequinoctialis* (Table 4). Seven of these primer pairs did not amplify a product in *P. aequinoctialis*, the remaining 19 primer sets amplified monomorphic loci (Figure 3).

Table 4 **Primers developed in *D. exulans* (DE), *T. chrysostoma* (DC) and *T. melanophris* (Dm) were tested in *P. aequinoctialis*. The presence or absence of a product, and the number of alleles was determined.**

PRIMER	PRODUCT	ALLELES
DE7	Yes	1
DE8	Yes	1
DC9	Yes	1
DE11	Yes	1
DE17	No	-
DC19	No	-
DE20	Yes	1
DC22	Yes	1
DM27	Yes	1
DC25	Yes	1
DE3	Yes	1
DM30	Yes	1
DE1	Yes	1
DE2	No	-
DC16	Yes	1
DE18	No	-
DC21	Yes	1
DM23	Yes	1
DM24	Yes	1
DC26	No	-
DM28	Yes	1
DM29	No	-
DM31	Yes	1
DM6	No	-
DC10	Yes	1
DC12	Yes	1

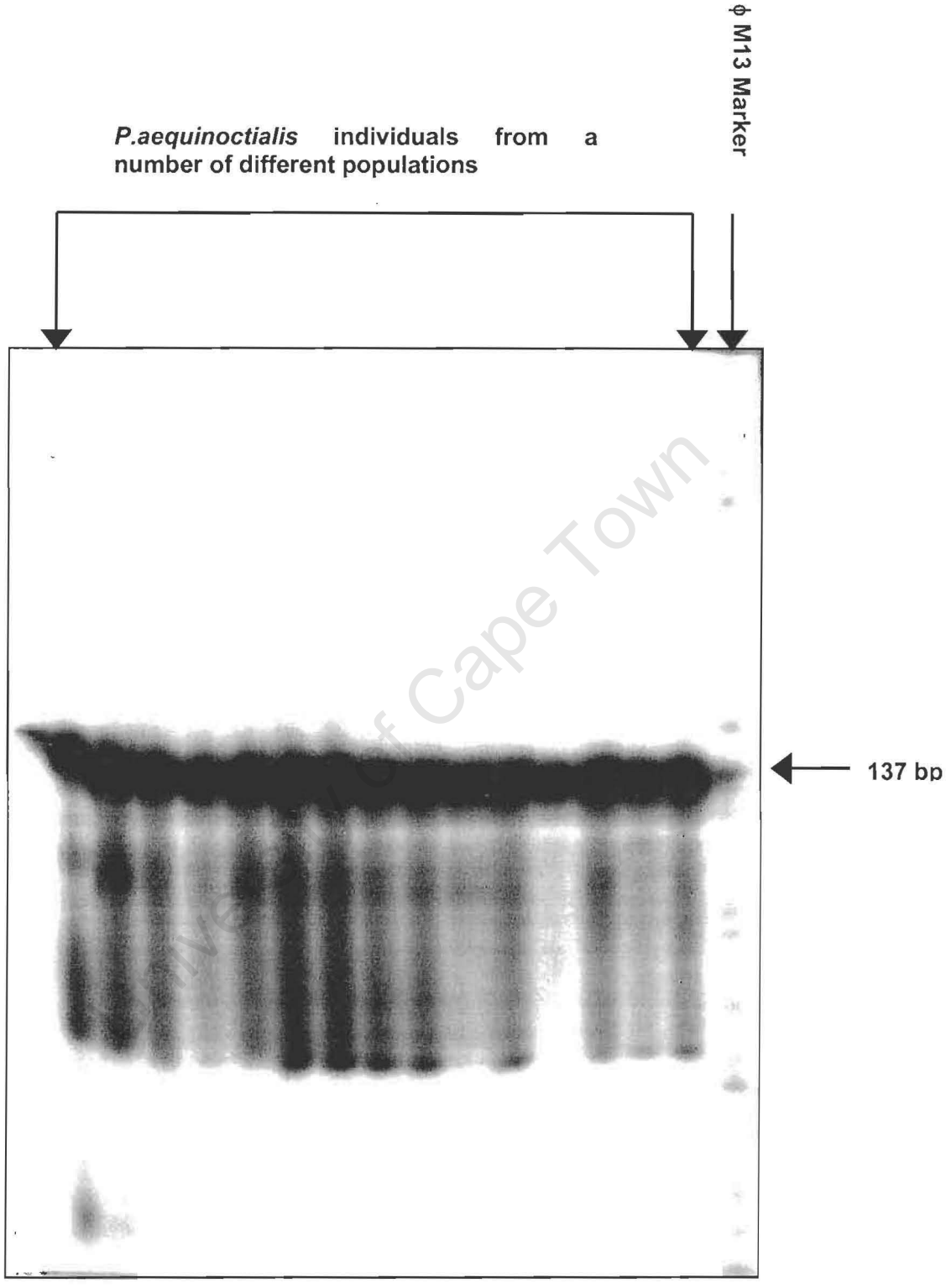

Figure 2 Complete lack of allelic diversity at HrU2 in *P. aequinoctialis* individuals from diverse bycatch localities. Amplified products were separated on a 6% polyacrylamide gel and exposed to autoradiographic film for between 30 minutes and 24 hours. Alleles were scored according to absolute length by comparison to a phage M13 size marker.

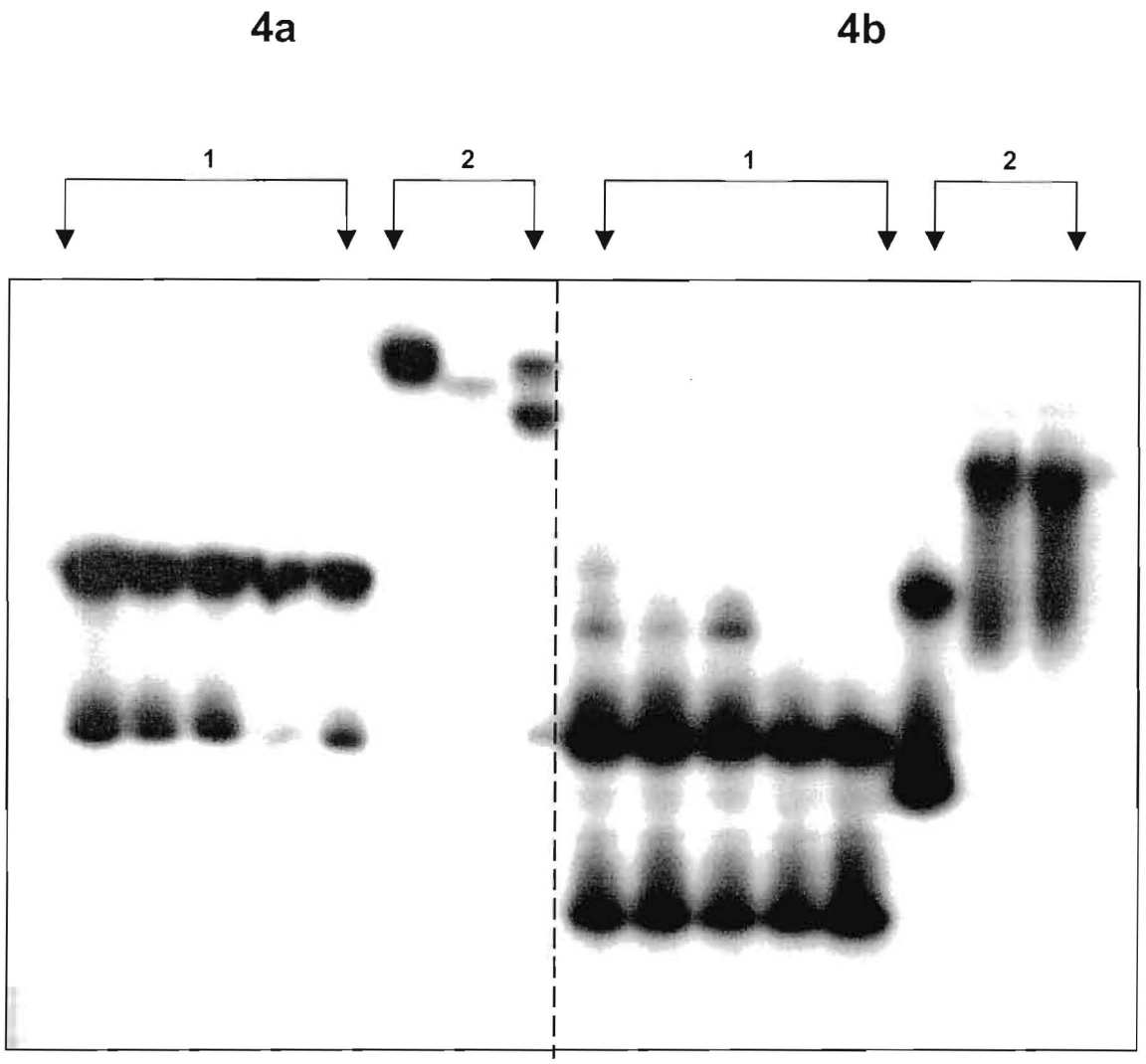

Figure 4a Allelic diversity in *D. exulans* (2) compared to complete lack of allelic diversity in *P. aequinoctialis* (1) at locus DE11. Amplified products were separated on a 6% polyacrylamide gel and exposed to autoradiographic film for between 30 minutes and 24 hours. Alleles were scored according to absolute length by comparison to a phage M13 size marker.

Figure 4b Allelic diversity in *D.exulans* (2) compared to complete lack of allelic diversity in *P. aequinoctialis* (1) at locus DC21. Amplified products were separated on a 6% polyacrylamide gel and exposed to autoradiographic film for between 30 minutes and 24 hours. Alleles were scored according to absolute length by comparison to a phage M13 size marker.

3.2.1 HETEROZYGOSITY

Heterozygosity values for all five loci were calculated for each population in each species. Significance of the Hardy-Weinberg test was measured using the Exact test which takes into account low allele frequencies and small sample sizes. Deviations from Hardy-Weinberg equilibrium were considered to be significant at the 95% confidence interval (*ie*: if $p < 0.05$). In order to test the overall significance of deviations from Hardy-Weinberg equilibrium a sequential Bonferroni test (Rice, 1988) was also performed.

3.2.1.1 Diomedea exulans

Table 5a provides the heterozygosity values expected under conditions of Hardy-Weinberg equilibrium, and the values observed at each of the five loci for each of the four populations. Guo and Thompson's (1992) Markov chain method (as implemented in GENEPOP 3.1D, Raymond and Rousset, *pers. comm.*) was used to calculate Exact tests in order to observe whether there was deviation from Hardy-Weinberg equilibrium at any of the loci. Deviations from Hardy-Weinberg equilibrium were only considered to be significant at the 95% confidence interval (*ie*: if $p < 0.05$). Using the Exact test four loci were found to show significant deviation from the Hardy-Weinberg equilibrium: DE7 in the lower Prince Edward population, DE11 in the upper Prince Edward population, and DC21 in the South Georgia and Marion island populations. Although the Exact test may indicate a significant result at a particular locus, it is important to know the overall significance. To this end a sequential Bonferroni test was performed. The Bonferroni test showed that only locus DC21 in the Marion island population was, in fact, significant overall (Table 5b). In order to establish the reason for the departure from Hardy-Weinberg equilibrium the F_{IS} value was assessed. The negative F_{IS} value calculated for locus DC21 in the Marion island population implies that the departure from Hardy-Weinberg equilibrium is probably due to a heterozygote excess. All other loci showed no significant departure from Hardy-Weinberg equilibrium.

3.2.1.2 Thalassarche chrysostoma

Table 6 provides the heterozygosity values expected under conditions of Hardy-Weinberg equilibrium, and the values observed at each of the five loci for the two populations. Guo

Table 5a **Observed and expected microsatellite heterozygosity (Nei, 1978), in four island-breeding populations of the wandering albatross, *D. exulans*. Significance levels were calculated using the exact test. Mean values for H_E and H_O for all four loci are indicated at the base of the table.**

Locus	South Georgia	Marion	Prince Edward (Upper Colony)	Prince Edward (Lower Colony)
DE7				
H_E	0.474	0.561	0.568	0.521
H_O	0.500	0.682	0.800	0.900
P-value	1.000	0.386	0.173	0.045$^{\Theta}$
DE8				
H_E	0.521	0.499	0.485	0.527
H_O	0.500	0.471	0.333	0.571
P-value	1.000	1.000	0.970	1.000
DE11				
H_E	0.554	0.715	0.725	0.467
H_O	0.308	0.417	0.250	0.600
P-value	0.088	0.194	0.054$^{\Theta}$	1.000
DE20				
H_E	0.442	0.208	0.325	0.395
H_O	0.600	0.231	0.375	0.500
P-value	0.480	1.000	1.000	1.000
DC21				
H_E	0.607	0.655	0.582	0.611
H_O	0.400	0.885	0.857	0.800
P-value	0.007$^{\Theta}$	0.001$^{\Theta}$	0.161	1.000
All 5 Loci				
H_E	0.519	0.528	0.537	0.504
H_O	0.462	0.537	0.523	0.674

$^{\Theta}$ Significant deviation from Hardy-Weinberg equilibrium.

Table 6a **Observed and expected microsatellite heterozygosity (Nei, 1978), in two island-breeding populations of the grey-headed mollymawk, *T. chrysostoma*. Significance levels were calculated using the exact test. Mean values for H_E and H_O for all four loci are indicated at the base of the table.**

Locus	South Georgia	Marion
DE7		
H_E	0.402	0.114
H_O	0.400	0.118
P-value	1.000	1.000
DE8		
H_E	0.747	0.693
H_O	0.800	0.647
P-value	0.583	1.000
DE11		
H_E	0.758	0.785
H_O	0.727	0.538
P-value	1.000	0.597
DE20		
H_E	0.802	0.697
H_O	0.733	0.444
P-value	0.613	0.319
DC21		
H_E	0.635	0.816
H_O	0.750	0.529
P-value	0.119	0.608
All 5 Loci		
H_E	0.669	0.621
H_O	0.682	0.455

Table 6b **Overall significance of the Exact test-derived p values using a sequential Bonferroni test (Rice, 1988) at five loci in two island-breeding populations of the grey-headed mollymawk, *T. chrysostoma***

Locus/Locality	Exact test p-values	Bonferroni significance value (p)
DE7 / South Georgia	1	0.0500
DE7 / Marion Island	1	0.0250
DE8 / Marion Island	1	0.0167
DE11 / South Georgia	1	0.0125
DE20 / South Georgia	0.613	0.0100
DC21 / Marion Island	0.608	0.0083
DE11 / Marion Island	0.597	0.0071
DE8 / South Georgia	0.583	0.0063
DE20 / Marion Island	0.319	0.0056
DC21 / South Georgia	0.119	0.0050

3.2.2 Allelic Diversity

Allele size variations in *D. exulans*, *T. chrysostoma* and *T. melanophris* are detailed in Table 8. Loci DE8 and DE20, which were developed in *D. exulans*, were found to be more polymorphic in both *T. chrysostoma* and *T. melanophris* than either was in the source species. DE11, developed in *D. exulans*, was most polymorphic in T. chrysostoma, while loci DE7 and DC21 were most polymorphic in the source species, *D. exulans* and *T. chrysostoma* respectively.

3.2.2.1 Diomedea exulans

All of the five microsatellite alleles surveyed were considered to be polymorphic at the 99% confidence level since in no instance did the frequency of the most common allele exceed 0.95.

There were two alleles present at the least variable loci (DE8 and DE20), and seven alleles at the most variable locus (DE11). Jacknifing was used to correct for unequal sample sizes in order to generate (*A*), the mean number of alleles per locus (Table 9). Jacknifing was selected as bootstrap re-sampling may result in only a subset of the alleles in the larger populations being considered since bootstrapping effectively reduces the sizes of all populations to the size of the smallest population, while jacknifing randomly re-samples alleles from the entire data set. In this manner it was demonstrated that the allelic richness does not differ significantly among the four populations of *D. exulans* studied with all populations exhibiting an average of between 2.19 (+- 0.2) and 2.80 (+- 0.7) alleles per locus.

The allele frequency distributions at all five loci are given in Figures 5 A-E.

At locus DE7 (Figure 5A) there are three alleles. The most common allele (119) occurs at a frequency of more than 0.5 in all four populations. The upper and lower Prince Edward populations share allele 117 at an appreciable frequency, while South Georgia and Marion islands share allele 121 at appreciable frequency.

At locus DE8 (Figure 5B) there are only two alleles at similar frequencies. The upper Prince Edward population has allele 184 at a higher frequency than allele 182, whereas the lower Prince Edward population has allele 182 at a higher frequency than allele 184.

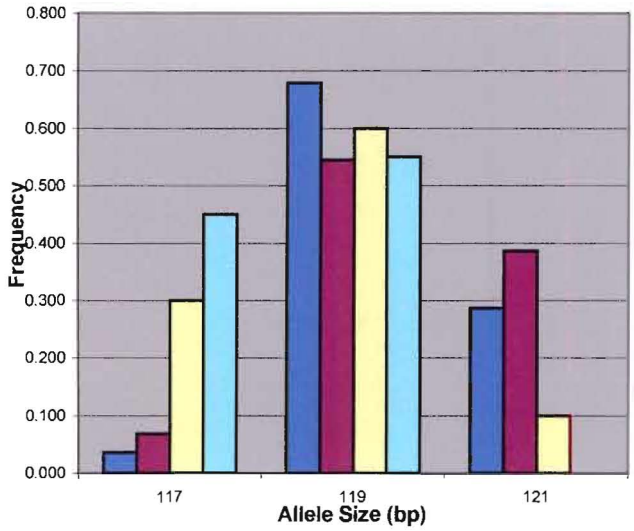

Figure 5a Locus DE7

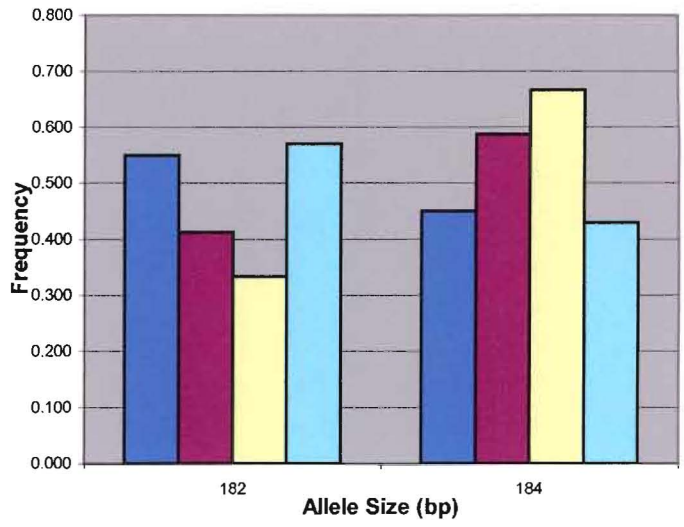

Figure 5b Locus DE8

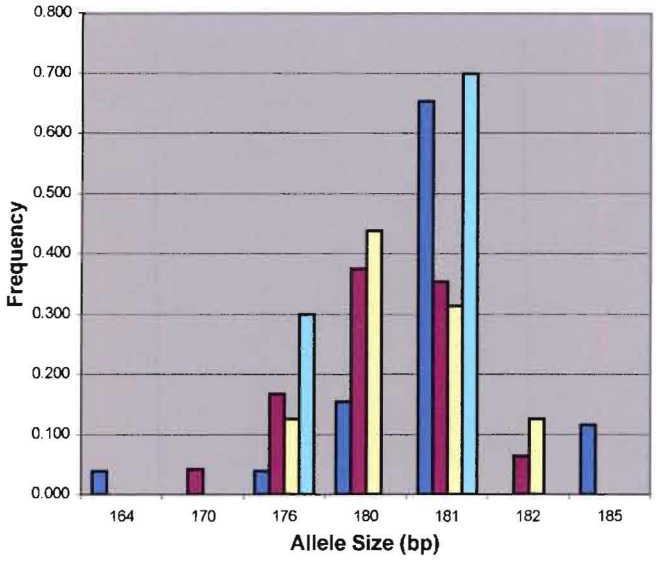

Figure 5c Locus DE11

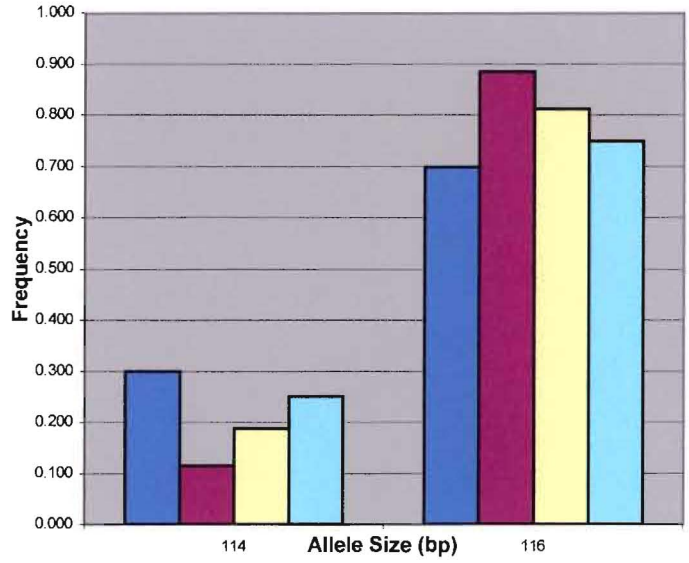

Figure 5d Locus DE20

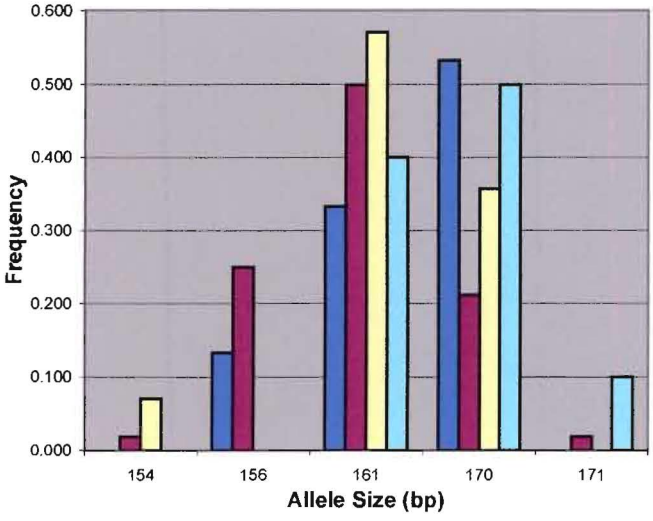

Figure 5e Locus DC21

Figure 5a-e Allele frequencies at five loci in *D. exulans* from Marion island, South Georgia island, and the upper and lower Prince Edward island populations

3.2.2.2 *Thalassarche chrysostoma*

All of the five microsatellite alleles surveyed were considered to be polymorphic at the 99% confidence level since in no instance did the frequency of the most common allele exceed 0.95.

There were four alleles present at the least variable locus (DE7), and six alleles at the most variable loci (DE11 and DC21). Jacknifing was used to correct for unequal sample sizes in order to generate (*A*), the mean number of alleles per locus (Table 10). This method was selected as bootstrap re-sampling could result in only a subset of the alleles in the larger populations being considered as bootstrap re-sampling effectively reduces the sizes of all populations to the size of the smallest population, while jacknifing randomly re-samples alleles from the entire data set. In this manner it was demonstrated that the South Georgia population has slightly more allelic diversity (A=5.0) than the Marion island group(A=4.3).

The allele frequency distributions at all five loci are given in Figure 6 A-E

At locus DE7 (Figure 6A) there are four alleles. The most common allele (119) occurs at a frequency of more than 0.7 in both populations. Both the South Georgia and Marion populations share allele 117 at low frequencies of 0.133 and 0.059 respectively. Alleles 118 and 121 are unique to the South Georgia population, with allele118 occurring at an appreciable frequency of 0.33,while allele 121 is rare, occurring at a frequency of 0.067.

There are five alleles present at locus DE8 (Figure 6B). Allele 184 and 186 are most common in both populations with 184 more common in the Marion population (F=0.0471) than in the South Georgia population (F=0.367). Allele 186 is marginally more common in the South Georgia population (F=0.333) than in the Marion population (F=0.265). Allele 182 is the only allele unique to South Georgia but it occurs at a low frequency of 0.067. Alleles 180 and 188 occur at similar frequencies in both populations.

Locus DE11 (Figure 6C) has six alleles all of which are shared by both populations, albeit at varying frequencies. The most common allele in the South Georgia population is allele 180 at a frequency of 0.455. Allele 180 occurs at a lower frequency in the Marion population (F=0.192). In the Marion population allele 182 is most common at a

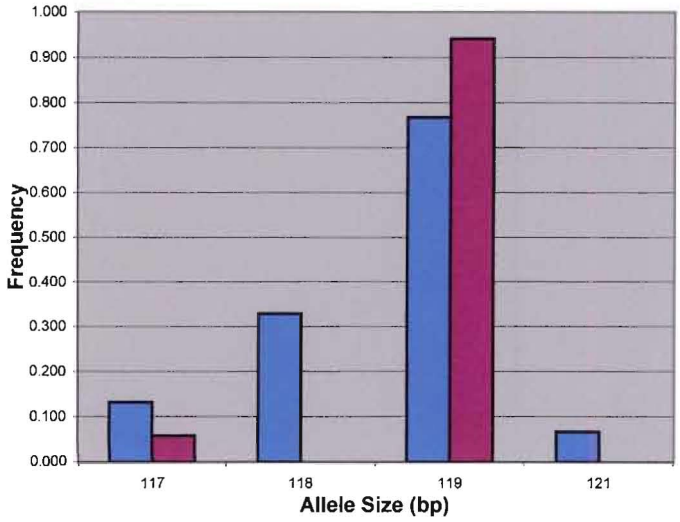

Figure 6a Locus DE7

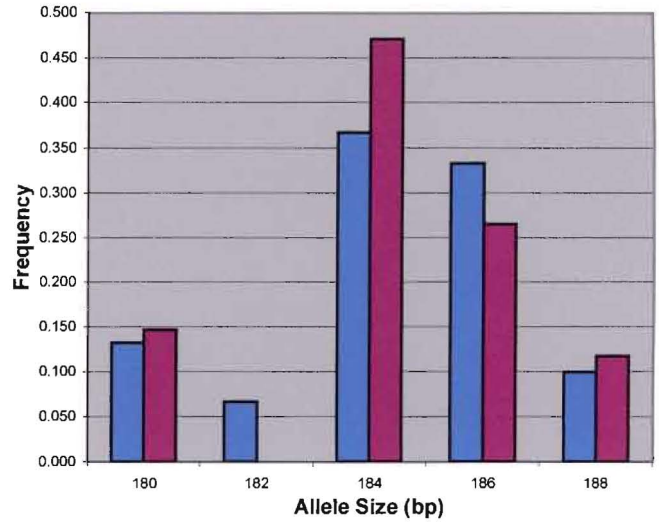

Figure 6b Locus DE8

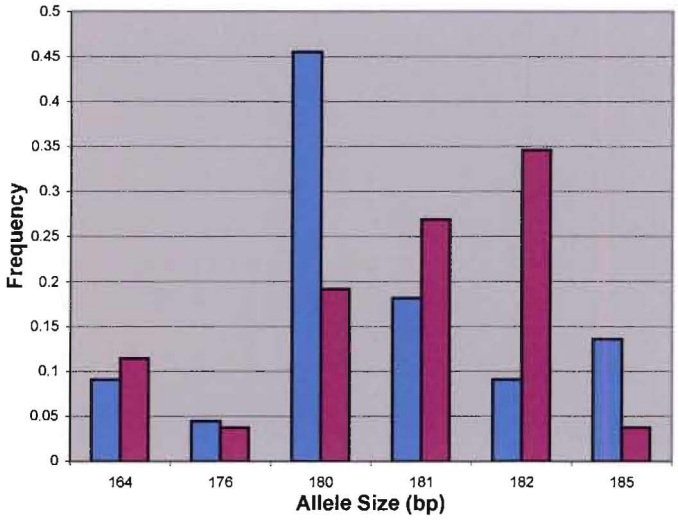

Figure 6c Locus DE11

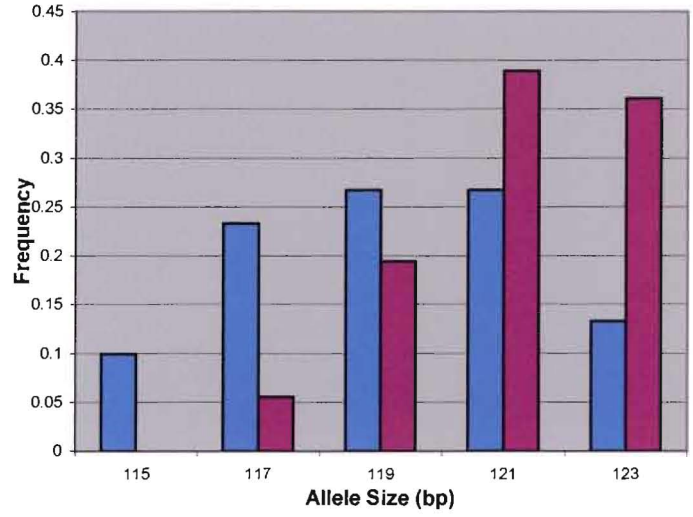

Figure 6d Locus DE20

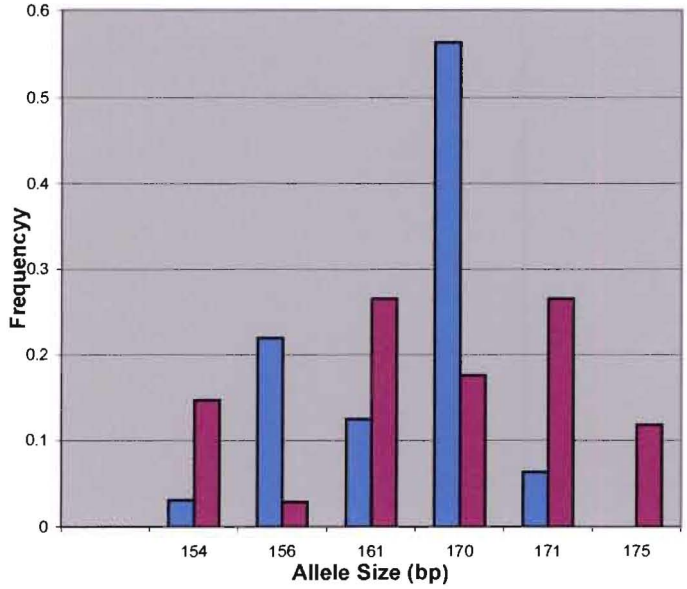

Figure 6e Locus DC21

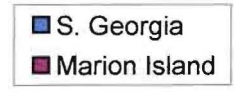

Figure 6a-e Allele frequencies at five loci in *T. chrysostoma* from Marion island, South Georgia island, and the upper and lower Prince Edward island populations.

3.2.2.3 *Thalassarche melanophris*

All of the five microsatellite alleles surveyed were considered to be polymorphic at the 99% confidence level since in no instance did the frequency of the most common allele exceed 0.95.

There were two alleles present at the least variable locus (DE7), and four alleles at the most variable loci (DE11 and DC21). Jacknifing was used to correct for unequal sample sizes in order to generate (*A*), the mean number of alleles per locus (Table 11) This method was selected as bootstrap re-sampling could result in only a subset of the alleles in the larger populations being considered as bootstrap re-sampling effectively reduces the sizes of all populations to the size of the smallest population, while jacknifing randomly re-samples alleles from the entire data set. In this manner it was demonstrated that the South Georgia population has an average of 3.2 alleles per locus.

The allele frequency distributions at all five loci for the one population analysed are given in Figure 7 A-E.

At locus DE7 (Figure 7A) there are two alleles. The most common allele (119) occurs at a frequency of 0.933 and the other allele is rare at a frequency of 0.067.

There are three alleles present at locus DE8 (Figure 7B). Allele 186 is most common at a frequency of 0.567, with alleles 182 and 184 at similar frequencies (0.233 and 0.200 respectively).

Locus DE11 (Figure 7C) has four alleles. Allele 182 is most common at a frequency of 0.333, and alleles176 and 180 have similar frequencies of 0.267 and 0.233 respectively. Allele 181 occurs at a slightly lower frequency of 0.167.

There are three alleles at locus DE20 (Figure 7D) with allele 124 being most common at a frequency of 0.433. Alleles 122 and 126 occur at similar frequencies of 0.267 and 0.300 respectively.

There are four loci present at locus DC21 (Figure 7E), allele 156 being most common (F=0.467). Alleles 154 and 161 occur at the same frequency (F=0.167) and alleles 170 occurs at a slightly higher frequency of 0.200.

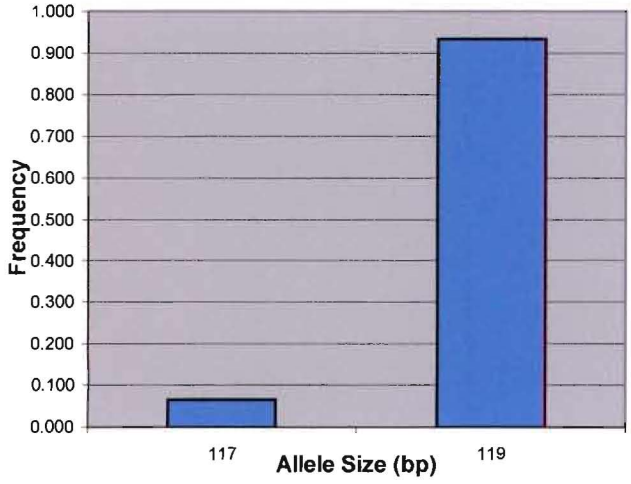

Figure 7a Locus DE7

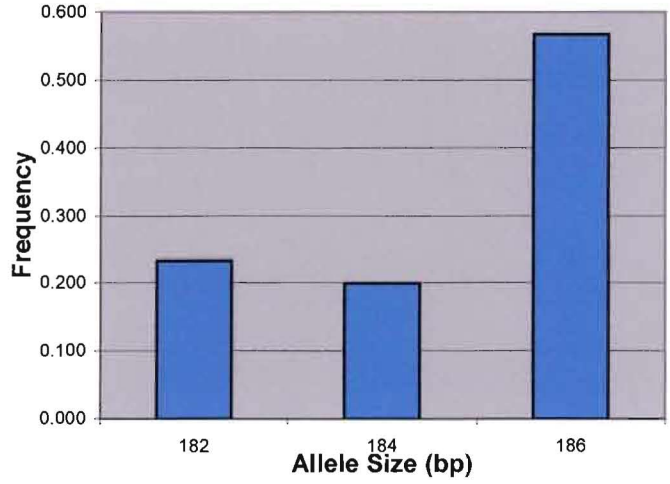

Figure 7b Locus DE8

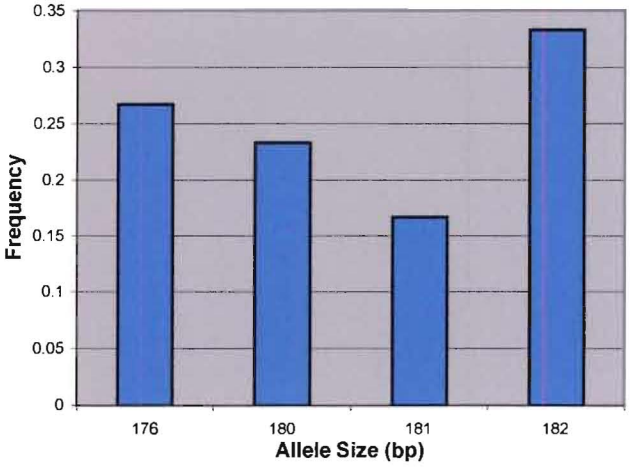

Figure 7c Locus DE11

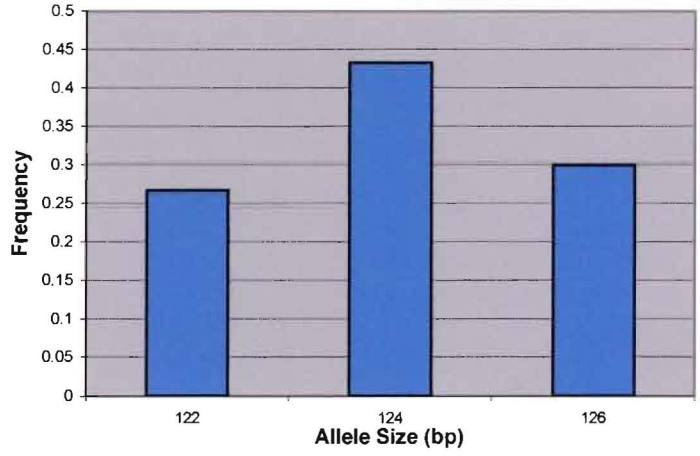

Figure 7d Locus DE20

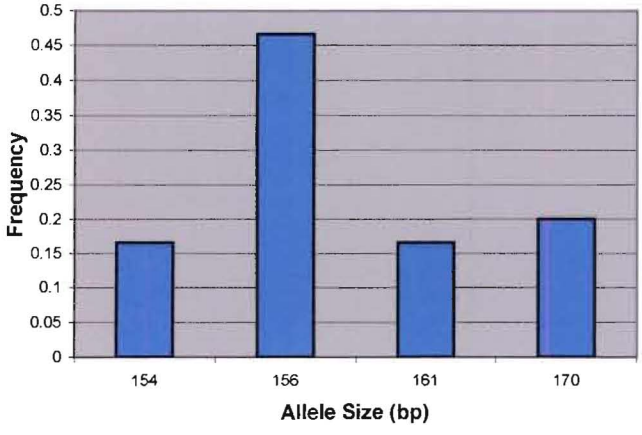

Figure 7e Locus DC21

Figure 7a-e Allele frequencies at five loci in *T. melanophris* from South Georgia island

Table 8 **Allele lengths (in base pairs) at five polymorphic loci in *D. exulans*, *T. chrysostoma* and *T. melanophris*.**

	DE7	DE8	DE11	DE20	DC21
D. exulans	117-121	182-184	164-185	114-116	154-171
T. chrysostoma	117-121	180-188	164-185	115-123	154-175
T. melanophris	117-119	182-186	176-182	122-126	154-170

Table 9 **Mean sample size per locus, polymorphism, mean number of alleles per locus (*A*) jacknifed to correct for unequal sample sizes, average observed (H_O) and expected (H_E) Hardy-Weinberg heterozygosities for *D. exulans* populations. Standard errors are given in parentheses.**

Population	Sample Size	% Loci Polymorphic	*A*	*He*	*Ho*
South Georgia	15	100.0	2.62 (0.5)	0.519 (0.029)	0.462 (0.050)
Marion Island	27	100.0	2.80 (0.7)	0.528 (0.088)	0.537 (0.113)
Prince Edward (Upper)	10	100.0	2.75 (0.4)	0.537 (0.066)	0.523 (0.127)
Prince Edward (Lower)	10	100.0	2.19 (0.2)	0.504 (0.036)	0.674 (0.075)

Table 10 **Mean sample size per locus, polymorphism, mean number of alleles per locus (*A*) jacknifed to correct for unequal sample sizes, mean observed (H_O) and expected (H_E) Hardy-Weinberg heterozygosities for *T. chrysostoma* populations. Standard errors are given in parentheses.**

Population	Sample Size	% Loci Polymorphic	*A*	*He*	*Ho*
South Georgia	16	100.0	5.0 (0.3)	0.669 (0.072)	0.682 (0.072)
Marion Island	18	100.0	4.31 (0.7)	0.621 (0.129)	0.455 (0.090)

Table 11 **Mean sample size per locus, polymorphism, mean number of alleles per locus (*A*) jacknifed to correct for unequal sample sizes, average observed (H_O) and expected (H_E) Hardy-Weinberg heterozygosities for *T. melanophris* populations. Standard errors are given in parentheses.**

Population	Sample Size	% Loci Polymorphic	*A*	*He*	*Ho*
South Georgia	15	100.0	3.2 (0.4)	0.576 (0.115)	0.533 (0.105)

3.2.3 GENETIC DIFFERENTIATION

Differentiation among populations was determined by calculating fixation and distance indices. Distance and fixation indices combine information on the frequency and identity of alleles in order to estimate population differentiation. Here fixation and distance measures based on both infinite alleles (F_{ST}) and stepwise (R_{ST}) mutation models are presented and compared. Software packages used to calculate these measures include GENEPOP V3.1D (Raymond and Rousset, 1995), R_{ST} CALC (Goodman, 1997), $AGAR_{ST}$ (Harley, *pers. comm.*).

R_{ST} is an F_{ST} analogue, equivalent to the fraction of the total variance in allele size (in terms of number of repeat units) between populations (Slatkin 1995) and is more suited to dealing with molecular data. R_{ST} analogues, Rho and $Rho\__{ST}$, were calculated between pairs of populations using three different software packages: GENEPOP V3.1D, R_{ST} CALC, and $AGAR_{ST}$. These are compared in order to demonstrate the differing results which are obtained using different software, and the complications this can cause in interpreting results.

Fixation (F_{ST}) values were calculated between pairs of populations using GENEPOP V3.1D which implements a "weighted" analysis of variance (Cockerham, 1973; Weir and Cockerham, 1984).

GENEPOP implements a "weighted" analysis of variance as described by Michalakis and Excoffier (1996) in order to generate an R_{ST} analogue called "Rho_st".

The Rho value generated by R_{ST} CALC is calculated by averaging variance components. Averaging variance components is used in order to standardise the contribution made by small and large population sample sizes to overall variance. The calculation is based on the weighting matrix developed by Michalakis and Excoffier (1996) in which loci are assumed to be independent, but are weighted individually. This works out to be equivalent to averaging variance components before calculating R_{ST} rather than averaging the R_{ST} values over loci.

3.2.3.1 *Diomedea exulans*

All three software packages generate R_{ST} analogues which concur that there is least population differentiation between the upper and lower populations on Prince Edward island (Table 12). The low levels of differentiation (Rho_ST = -0.002 to Rho = 0.0016) observed between these two colonies indicates that they are essentially a panmictic population. Since the populations occur on the same island in close proximity to one another this is not unreasonable. The number of migrants per generation estimated to be 13.54, indicating significant gene flow between these colonies. The F_{ST} value of 0.050 appears to overestimate the amount of population differentiation between the upper and lower Prince Edward populations.

The R_{ST} analogues concur that there is little to moderate population differentiation between *D. exulans* populations on South Georgia, Marion and the upper colony on Prince Edward island (Table 12). The moderate to high population differentiation between the Marion island and lower Prince Edward island populations (Rho_ST = 0.182 to Rho = 0.143) is anomalous in the light of the fact that there appears to be significant gene flow between the two Prince Edward populations, and between the Marion and upper Prince Edward population, but little gene flow and more differentiation between the Marion and lower Prince Edward populations. This unusual result is consistent across all F_{ST} and R_{ST} calculations. This may reflect a biological phenomenon, though no such reason is immediately apparent. Surveying a larger set of microsatellite loci may indicate whether or not this is a real result or just a statistical abnormality.

3.2.3.2 *Thalassarche chrysostoma*

Pairwise comparison of the South Georgia and Marion populations indicates moderate levels of population differentiation (Table 13). The F_{ST} estimate of differentiation (F_{ST} = 0.052) was slightly higher than the Rho_ST and Rho (R_{ST} CALC) estimates. $AGAR_{ST}$ (Rho = 0.025) appears to underestimate the amount of population differentiation. There are an estimated 1.4 migrants per generation between the T. chrysostoma populations on Marion and South Georgia. This is a lower number of migrants than predicted for the *D. exulans* populations on the same islands (N_M = 3.74)(Table 12).

3.2.3.3 Thalassarche melanophris

Distance measures could not be calculated for *T. melanophris* as only a single population was analysed.

3.2.3.4 Differentiation between Species at South Georgia island

While measures of population differentiation are not designed to determine species level differentiation it is interesting to assess the performance of different software packages to this problem. Microsatellites are not generally used for species level differentiation given their homoplasy and the amount of noise present (Jarne and Lagoda, 1996). This is further compounded when complex repeats units are used in the assessment, as is the case in this investigation.

All three species were sampled at South Georgia. F_{ST} values (Table 14) indicate that *D. exulans* is most different from *T. melanophris* (F_{ST} = 0.267) and that *T. melanophris* and *T. chrysostoma* are most closely related (F_{ST} = 0.1298). It should be noted that these values indicate substantial differentiation (especially considering that F_{ST} values between populations of the same species are generally well below 0.1 in the populations used in this study) as would be expected when dealing with distinct species. However, given that these species have been separate for at least 10^5 years both the F_{ST} and R_{ST} values should be virtually 1.There are a number of reasons that this is not the case. Firstly in the case of F_{ST} it is due to the failure to comply with an infinite allele model which is accepted for microsatellites. It is also the restricted size range since F_{ST} can only go to 1 if only one (different) allele remains in each population. Even if all alleles are different in all the populations where there is more than one allele F_{ST} cannot reach 1. This is the reason that these measures of differentiation are useless beyond a certain time period, and why they are generally not useful for differentiating at the species level.

The trends seen using F_{ST} are reflected in the pairwise comparisons using Rho_st (GENEPOP) (Table 14) and Rho (R_{ST} CALC and $AGAR_{ST}$) (Table 14), though these values are higher than those calculated using F_{ST} *eg*: Comparing *D. exulans* and *T. melanophris* still gives the largest Rho_st value, but it is extremely high - 0.836 – indicating a high degree of differentiation. Rho (R_{ST} CALC) for the same pairwise comparison was also high at 0.380.

3.2.3.5 Differentiation between Species at Marion island

The Rho_st value of 0.567 for the pairwise comparison of *D. exulans* and *T. chrysostoma* at Marion Island (Table 15) was once again considerably higher than the F_{ST} (0.285) (Table 15), R_{ST} CALC Rho (0.300) (Table 15) or $AGAR_{ST}$ Rho (0.271) (Table 15) values obtained for the same pairwise comparison.

Table 12 Pairwise F_{ST}, pairwise R_{ST} analogues (Rho_{ST} and Rho) calculated using Genepop, RST Calc and $AGAR_{ST}$ for four *D. exulans* populations

Population	F_{ST} (Genepop)	Rho_{ST} (Genepop)	Rho (RSTCalc)	Rho ($AGAR_{ST}$)	N_M (p-value)
South Georgia /Marion	0.052	0.090	0.056	0.033	3.74 (0.03)
South Georgia /Prince Edward (Top)	0.048	0.033	0.044	0.021	5.74 (0.15)
South Georgia /Prince Edward (Bottom)	0.029	0.096	0.086	0.064	2.40 (0.01)
Marion /Prince Edward (Top)	0.006	0.034	0.033	0.033	5.13 (0.10)
Marion /Prince Edward (Bottom)	0.102	0.182	0.155	0.143	1.04 (0.00)
Prince Edward (Top) /Prince Edward (Bottom)	0.050	0.015	0.016	-0.002	13.54 (0.23)

Table 13 Pairwise F_{ST}, pairwise R_{ST} analogues (Rho_{ST} and Rho) calculated using Genepop, RST Calc and $AGAR_{ST}$ for two *T. chrysostoma* populations

Population	F_{ST} (Genepop)	Rho_{ST} (Genepop)	Rho (RSTCalc)	Rho ($AGAR_{ST}$)	N_M (p-value)
South Georgia /Marion	0.052	0.042	0.040	0.025	1.40 (0.08)

Table 14 **Pairwise F_{ST}, pairwise R_{ST} analogues (Rho_{ST} and Rho) calculated using Genepop, RST Calc and $AGAR_{ST}$ in South Georgia island populations.**

Species	F_{ST} (Genepop)	Rho_{ST} (Genepop)	Rho (RSTCalc)	Rho ($AGAR_{ST}$)
D. exulans / T. chrysostoma	0.186	0.406	0.204	0.182
D. exulans / T. melanophris	0.267	0.836	0.379	0.369
T. chrysostoma / T. melanophris	0.130	0.526	0.199	0.189

Table 15 **Pairwise F_{ST}, pairwise R_{ST} analogues (Rho_{ST} and Rho) calculated using Genepop, RST Calc and $AGAR_{ST}$ in Marion island populations.**

Species	F_{ST} (Genepop)	Rho_{ST} (Genepop)	Rho (RSTCalc)	Rho ($AGAR_{ST}$)
D. exulans / T. chrysostoma	0.285	0.564	0.300	0.271

3.3 DISCUSSION

3.3.1 CROSS-SPECIES PRIMER AMPLIFICATION

Primers developed in families other than Procellariidae consistently failed to amplify polymorphic loci in *P. aequinoctialis*. This result is not entirely unexpected since the accumulation of mutations in the sequences flanking the locus of interest could prevent adequate annealing of the primers (McDonald and Potts, 1996). The accumulation of mutations in the flanking regions is a function of the evolutionary time that has elapsed since divergence of the taxa. So, while primers have been reported to work in related species, the use of primers across some families may not be possible since the accumulation of mutations may be significant at higher levels of divergence. While Primmer *et al.* (1996) have reported cross-family amplification in passerine families, there is, as yet, little evidence that this holds for non-passerine families such as Diomedeidae and Procellariidae.

Levels of polymorphism at microsatellite loci may decrease with phylogenetic distance from the source species used to construct the genomic library (Moore *et al.*, 1991). Difficulty in the interpretation of results may therefore arise when primers developed in one species are applied to another species. Fitzsimmons *et al.* (1995) explain variation in allele size and polymorphism among taxa as a result of "bias in cloning and characterisation of microsatellite loci". Screening protocols favour the selection of microsatellites which are longer and more variable than the average. When these are applied in other taxa repeat lengths tend to be shorter and the loci are less polymorphic (Ellegren *et al.*, 1995; Crawford *et al.*, 1997). Consequently there was concern that loci developed in *D. exulans* may show lower levels of polymorphism in *T. chrysostoma* and *T. melanophris*.

The microsatellite loci used in this study did not consistently show higher levels of variation in the source species. While this finding contrasts with the majority of the literature, it is comparable with results using these loci in different Diomedea and Thalassarche populations (Burg, 1999).

3.3.2 Genetic Variation and Population Structure

3.3.2.1 Heterozygosity and Allelic Diversity

Genetic drift is a major factor influencing variation in all the populations studied. In addition, it is generally accepted that all Southern ocean populations have suffered declines during the previous two or three decades (Weimerskirch and Jouventin, 1987; Croxall, 1990; Tennyson, 1990; Woehler, 1996). This may explain slightly lower levels of allelic diversity in *D. exulans* at South Georgia (H_E= 0.462), which has experienced the most considerable population decreases, compared to *T. chrysostoma* (H_E=0.682). and *T. melanophris* (H_E=0.533) at the same location (Croxall, 1979; Croxall and Prince, 1990).

Queller and Goodnight (1989) propose that a locus is more informative when it has multiple alleles at similar frequencies, rather than few alleles at vastly disparate frequencies. By these criteria the five loci analysed in this study are moderately informative since the alleles are present at similar frequencies although there are often less than five alleles at any one locus. The average number of alleles at a locus in all three species was low to moderate with between two and seven alleles per locus in *D. exulans*, an average of three alleles per locus in *T. melanophris* and between four and six alleles per locus in *T. chrysostoma*. In *D. exulans* and *T. chrysostoma* locus DE20 is most informative as it shows a marked difference in the mean allele sizes between populations.

3.3.2.2 Null Alleles

Alleles which are not consistently amplified are known as "null" alleles (Pemberton *et al.*, 1995). An apparent deficiency of heterozygotes may result from the presence of null alleles. Exclusion of even a few non-amplifying alleles can seriously impact on the interpretation of genotype frequency distributions and lead to incorrect conclusions regarding the levels of inbreeding in a population (Pemberton *et al.*, 1995. Null alleles usually arise where point mutations in the flanking sequences prevent primer binding and amplification of the locus (Paetkau and Strobeck, 1995). Due to the accumulation of point mutations in the sequences flanking a microsatellite locus over time, null alleles are particularly prevalent during cross-species amplification (McDonald and Potts, 1996). It is not, however, uncommon for loci to have non-amplifying alleles in the species from which they were cloned. In theory, null alleles can be revealed by lowering the priming

stringency or by redesigning primers to avoid mismatches. Additionally, the number of non-amplifying alleles can be minimised by designing primers away from the sequences directly flanking the microsatellite as these regions are more prone to mutation as a result of DNA polymerase slippage near the microsatellite repeat (Koorey *et al.*, 1993). Based on the heterozygosity data the presence of null alleles at the studied loci is unlikely. Locus, DC21 in the Marion island population of *D. exulans*, the only locus to show departure from Hardy-Weinberg equilibrium, shows a heterozygote excess, rather than the homozygote excess which is expected if there are null alleles present.

3.3.2.3 Philopatry and Migration

Behavioural data indicate that many procellariiformes return to their natal island colonies to breed (Marchant and Higgins, 1990; Croxall and Rothery, 1991). Philopatry may result in low levels of genetic variation within populations, and high levels of differentiation between populations. Lambert and Millar (1995) have shown that in the case of New Zealand's Blue Duck, *Hymenolaimus malacorhynchos*, low levels of genetic variation, as detected by minisatellite DNA, is a result of philopatry, which leads to low levels of dispersal and inbreeding.

The level of migration between populations sets a limit to the amount of genetic differentiation that can occur. Very little migration is required to prevent significant genetic divergence among subpopulations resulting from random genetic drift (Hartl, 1988). There are three types of barrier to gene flow: (i) geographical distance (ii) ecological distance and (iii) behavioural distance (Chesser, 1983). Geographic distance is not a major consideration in these wide-ranging ocean species, however, ecological and behavioural distances may operate in these populations, albeit at low levels.

This study indicates there that there are approximately 13.5 migrants per generation between the upper and lower populations of Prince Edward island, indicating significant gene flow between these geographically proximate colonies. Levels of migration between geographically distinct islands such as Marion and South Georgia are 3.7 and 1.4 migrants per generation in *D. exulans* and *T. chrysostoma* respectively. These levels are low if it is taken into account that generation time in these birds is equal to the average age of reproducing adults, a value estimated to be eleven years in *D. exulans* and nine years in *T. chrysostoma* (Marchant and Higgins, 1990). Between one and four migrants

over a period of ten years is relatively low and since very few breeding populations of procellariiformes have been monitored at all, fewer still for continuous periods of more than a decade (Croxall and Prince, 1990). It is not unlikely, therefore, that such low levels of migration between populations may have gone unnoticed in behavioural studies. The levels of migration are, however, evidently sufficient to prevent significant population differentiation. The excess of heterozygotes seen at locus DC21 in *D. exulans* implies that if this low level of migration is occurring, it is through the dispersal of male birds as has been reported for oystercatchers in the Northern Netherlands (van Treuren *et al.*, 1999).

Even these low levels of migration are, however, sufficient to counter the diversifying effect of genetic drift. Marked population structuring was not observed for populations of any of the species studied. The low levels of genetic differentiation (Rho calculated using R_{ST} CALC =0.056) between the *D. exulans* populations on South Georgia and Marion islands is maintained through the migration of only 3.7 individuals per generation. In *T. chrysostoma* a similar level of genetic variation (Rho calculated using R_{ST} CALC =0.040) is maintained by 1.4 migrants per generation. The upper and lower Prince Edward *D. exulans* populations were essentially panmictic (R_{ST} =0.016) with approximately 13.5 migrants per generation ensuring the homogenising of these two populations. Migration is therefore a powerful force acting against genetic divergence resulting from genetic drift between populations.

Breeding-site fidelity does not appear to be sufficient to result in genetic differentiation between populations of *D. exulans*, *T. chrysostoma*, *T. melanophris* on Marion , South Georgia and the Prince Edward islands. It has been suggested that the lack of population structure on a worldwide scale seen in a shorebird, the turnstone, may be due to recent expansion from a bottlenecked population rather than from global gene flow (Wenink *et al.*, 1994). The high heterozygosities obtained in these species indicate that it is unlikely that there has been recent expansion from a bottlenecked population. An alternative is that there has been migration from an original large population in an isolated locality though it will be necessary to analyse population differentiation for a larger number of islands in order to determine whether the low levels of population structure are due to recent gene flow between islands or to migration.

To compound the debate Johns and Avise (1998) have suggested that avian species show lower genetic distance than mammals or reptiles, specifically, that they show significantly less genetic divergence than similar taxa in other genera. Bird taxa therefore appear to be shifted down approximately one taxonomic level relative to other vertebrate taxa (Kessler and Avise, 1985) *ie:* divergences between species within a mammalian genus are approximately the same as those between distinct avian genera. These lower levels of genetic distance occur in both nuclear and mitochondrial genes.

Since no previous population level analysis of genetic variation using mtDNA or allozymes have been carried out on procellariiformes it is difficult to determine the relative performance of microsatellites in addressing questions relating to seabird population structure. Molecular studies have been limited to the use of mtDNA for reconstructing phylogenies for the Procellariiformes (Nunn *et al.*, 1996).

3.3.3 Implications for Conservation Management

The success of management plans will be increased by an understanding of the level at which populations are genetically distinct. This can be established by assessing the levels of gene flow between populations. It has been suggested that if populations share so few alleles so as to be genetically distinct, they also warrant treatment as separate "management units" (Moritz, 1994). The microsatellite data obtained in this study indicate that there are low to moderate levels of gene flow between populations in each of the species. The geographically distinct populations share a large number of microsatellite alleles. This implies that they may not require treatment as separate management units, which will considerably simplify their conservation.

It would be recommended that fluctuations in population size and genetic diversity for each colony should continue to be monitored over the long term.

3.3.4 Assigning individuals to breeding populations

A novel application of molecular data is the ability to assign an individual of unknown provenance to the breeding population of origin. This promises to be of particular use in understanding population processes in migratory species which are difficult to observe (Haig *et al.*, 1997), and in identifying the origin of products from species for which there is an illegal commercial market (Baker and Palumbi, 1994). In order to identify breeding

population origins it is necessary to identify population-specific markers. Using mtDNA the origins of wintering dunlins (*Calidris alpina*) (Wenink *et al.*, 1993) and of various whale species (Baker and Palumbi, 1994) have been determined. This requires the availability of an array of loci at which alleles unique to specific breeding populations are represented at high enough frequencies to be diagnostic. This was not found to be the case for the loci examined in this study. The unique alleles found to be population-specific occurred at low frequencies (below 0.1) and were therefore considered to be rare, alleles, and not suitable as diagnostic markers. Since only five loci were tested it may be possible to uncover such high frequency diagnostic markers if a larger set of loci are surveyed. It is, however, likely that there is insufficient variation at most loci for the species studied, particularly if the levels of philopatry are not as high as has been suggested by behavioural studies.

In the absence of diagnostic markers that are restricted to specific geographic populations it is not possible to establish the origin of a specific sample. It may, however, be feasible to use a maximum likelihood method to attempt to determine the provenance of an individual using a "mixed stock model" (Baker *et al.*, 1996)

3.3.5 Problems with Currently Available Analysis Software

When dealing with inaccessible and endangered populations it is often difficult to obtain large samples and ideal exact sample sizes. The different ways in which small samples and different sized sample sets are handled by existing software packages appears to account for at least some of the variation observed in the measures of population differentiation.

Clear evidence that microsatellite data do not completely fit the assumptions followed by common models used to estimate genetic differentiation can be seen in the range of values obtained for the F_{ST} and R_{ST} values obtained for populations of *D. exulans* and *T. chrysostoma* using different published software packages.

Evidence that the available models and algorithms do not adequately deal with microsatellite data has previously been reported in grey seals (Allen *et al.*, 1995). Using Fst, the number of migrants per generation (N*m*) was found to be 41, using Slatkin's Rst (1995) it was 13.8, and using Slatkin's private allele method (1993) it was 5.6. Forbes *et*

al., (1995) on examination of variation within and between sheep species concluded that classic methods such as Fst and Nei's *D* differed considerably from allele-size based methods such as those of Goldstein *et al.* (1997) and Slatkin (1995). According to Allen *et al.* (1995) Allele frequency methods were more sensitive to population differences within species, while size-based methods gave results consistent with biogeographical and other genetic data.

Since the application of the inappropriate model can lead to false conclusions regarding the patterns of genetic differentiation in natural populations it is important that new models, algorithms, and ultimately computer packages, capable of analysing microsatellite data be developed.

3.4 FUTURE PROSPECTS

3.4.1 TECHNICAL ADVANCES

A recent advance in microsatellite genotyping is the development of fluorescent semi-automated genotyping techniques and allele binning (Ghosh *et al.*, 1997). Increased general application of these techniques in the future will save time and decrease the potential for incorrect genotyping due to human error. Given the number of loci and populations required to allow meaningful population level analyses it is possible that automated genotyping will be useful in conservation genetics. However, the ongoing necessity to design primers for the each new species studied, and the amount of time required to optimise PCR conditions mean that automation will most likely be restricted to well-characterised species of commercial interest.

3.4.2 PROCELLARIIFORM POPULATION GENETICS

Using the current set of microsatellite markers and population samples we have been able to demonstrate the potential of microsatellite analysis for determining the natal origin of Procellariiformes forming part of the longline bycatch, although more loci and samples from a broader range of breeding populations would be useful in improving the accuracy of the final statistics.

4 SECTION B: CONSTRUCTION OF A GENOMIC LIBRARY FOR *P. AEQUINOCTIALIS*

4.1 INTRODUCTION

While microsatellites are regarded as excellent polymorphic markers for population genetic studies (Bruford *et al.*, 1996) their use is currently restricted because a microsatellite library must be developed for each new species studied (McDonald and Potts, 1997). It is possible that loci developed for one species may be informative in closely related species. For example, primers developed in one whale species successfully amplify synonymous microsatellite loci in other whale species (Schlötterer *et al.*, 1991), and primers developed to polymorphic loci in cattle are informative in sheep (Moore *et al.*, 1991). Should this generally be the case it will reduce the time and expense required to develop microsatellite primers for each new species studied. However, this does not always hold true and in many cases novel microsatellite libraries have to be made (Moore *et al.*, 1991).

Phylogenetic reconstruction based on mtDNA sequences and morphology would indicate that the families Diomedeidae and Procellariidae are separated by at least 10 million years (Nunn *et al.*, 1996). I tested whether the informative loci present in Diomedeidae were suitably conserved to be amplified in Procellariidae (represented by *P. aequinoctialis*). Since no polymorphic loci could be amplified , the development of a microsatellite library specifically for *P. aequinoctialis* was undertaken. It is likely that the loci developed for *P. aequinoctialis* will prove useful in studying population structure, kinship and mating systems in the other Procellariidae.

4.2 MATERIALS AND METHODS

4.2.1 DNA EXTRACTIONS

Genomic DNA was extracted from 500 mg muscle samples taken from eight white-chinned petrels caught during longline fishing in the southern ocean (A1-A8, Appendix B). A standard overnight proteinase K (10 mg/ml) digestion followed by phenol:chloroform-ethanol precipitation (Ausubel *et al.*, 1989) was used. The DNA

pellets were subsequently washed in 70% ethanol, vacuum dried and resuspended in 300 µl Tris-EDTA, pH 8.

4.2.2 Restriction Enzyme Digestion, Size Selection and Legation of genomic DNA

Approximately 10 µg of genomic DNA pooled from eight individuals was digested to completion at 37°C with an excess of a combination of three 4-base pair recognition restriction enzymes: *Alu*I, *Rsa*I and *Hae*III (5 U/µg DNA). The digested DNA was electrophoresed through a 2% agarose gel and lambda DNA digested with *Dra*I was used as a size marker. DNA fragments in the 250-500 base pair range were excised from the gel and purified using Genelute™ agarose spin columns (Supelco) (Figure 8).

The size selected fragments were ligated into pUC18 plasmid vectors (Appendix C) which was linearised with *Sma*I (20 U/µg DNA). Ligations were performed in a final volume of 10 µl containing 1x ligation buffer, 1.34 U Fast-link™ DNA ligase (Epicentre Technologies), 120 ng of size selected DNA, 20 ng of *Sma*I-digested pUC18 DNA and 0.5 mM ATP. This ratio of DNA to vector was empirically determined to yield the best ligation success. Ligations were performed in the presence of *Sma*I to prevent recircularisation of the plasmid. The reaction was incubated at 25°C for 15 minutes, followed by incubation at 70°C for 15 minutes as recommended by the manufacturer (Epicentre Technologies). The efficacy of the ligation reaction was confirmed by running a sample of the product on a 1.5% agarose gel for comparison with native pUC18.

Ligation products were introduced into electro-competent *E.coli* XL1-blue cells by electroporation (1.5kV, R= 7.5 ms) using an Electroporator II apparatus (Invitrogen). The electro-competent cells were prepared according to a standard protocol (Invitrogen), and transformation efficiencies were calculated as $1x10^8$ CFU (colony forming units) per µg of DNA which is within the $1x10^7$ to $1x10^9$ range expected for electroporations (Sambrook *et al.*, 1989).

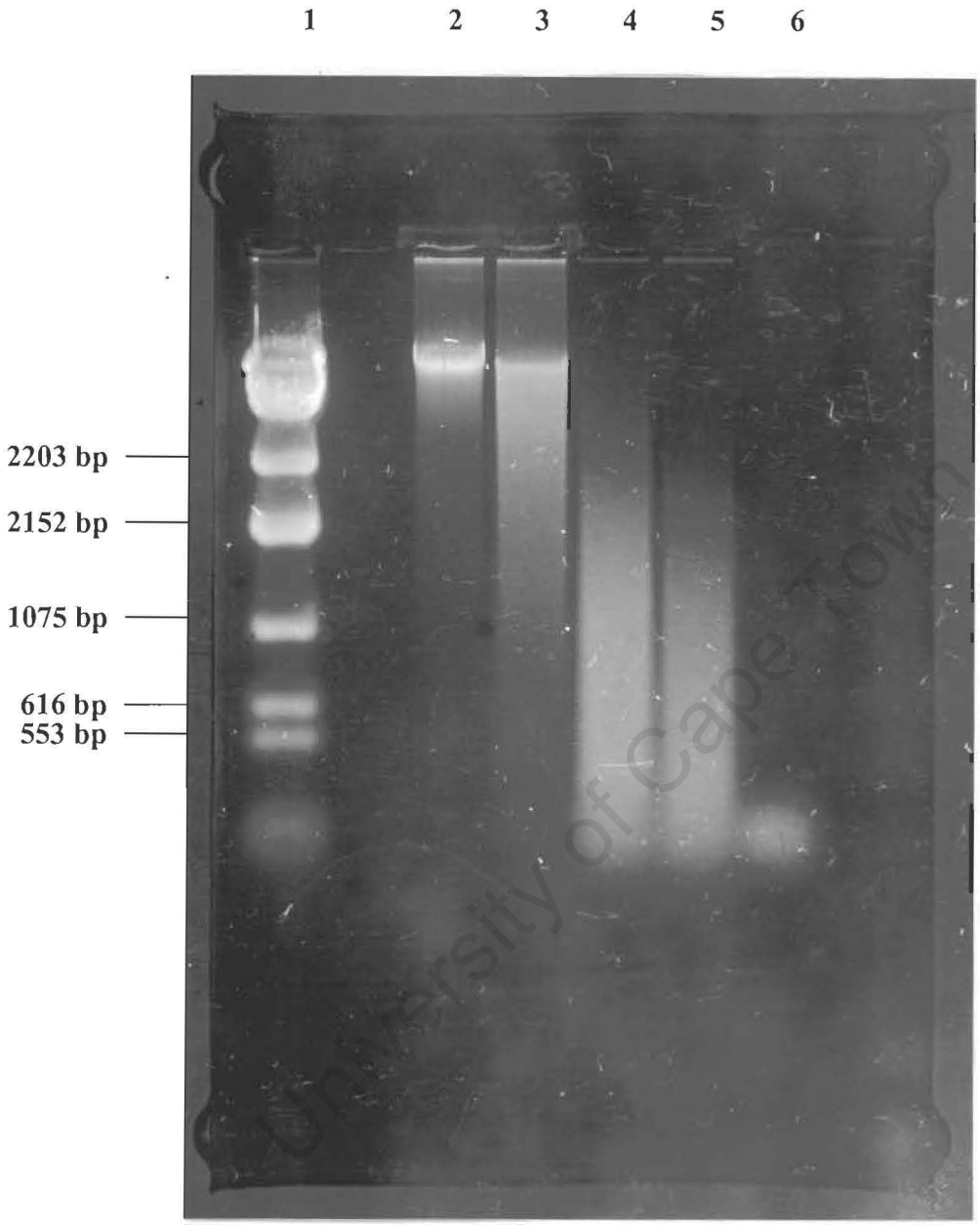

Figure 8 10μg of genomic *P. aequinoctialis* DNA (lane 2) was digested with a combination of 3 restriction enzymes; AluI, RsaI and HaeIII, for 30 seconds (lane 3), 15 minutes (lane 4) and 4 hours (lane 5). Digests were run alongside a λDNA marker cut with DraI (lane 1). The 250-500bp region was extracted from the gel and purified using a Genelute agarose spin column (lane 6). DNA was visualized by staining with EtBr.

4.2.3 MICROSATELLITE SCREENING

Aliquots from the transformation mixtures were plated at an appropriate density for screening - approximately 3000 colonies per 245 mm x 245 mm agar plate (Nunc). The Luria-broth (LB) agar plates contained 60 μg/ml ampicillin , 50 mM IPTG and 20 mg/ml X-gal. Insert-containing recombinants were identified using insertional inactivation of the β-galactosidase gene, based on a blue/white colour selection. Replica Hybond-N^+ (Amersham) filters of the bacterial colonies were produced by laying dry Hybond-N^+ membrane over the surface of the colonies and gently lifting the membrane. The agar plates were re-incubated at 37°C for 3 hours in order to allow regrowth of the colonies for subsequent picking of the positive clones.

The replica filters were laid colony-side up on Whatmanns No. 1 filter paper pre-soaked in 2x SSC / 5% SDS, and left for 2 minutes at room temperature in order to lyse the bacterial cells. Filters were then baked in a microwave oven at 650 W for 10 minutes in order to denature and fix the bacterial DNA to the filter (Bulewela *et al.*, 1979). Filters were pre-hybridised in a 1 M Na_2HPO_4, 0.5 M EDTA, 7% SDS and 1% BSA. in a rotisserie hybridisation oven at 65°C for 2 hours.

50 ng of a synthetic oligonucleotide probe; poly(CA/GT)$_n$ (n=15) (Pharmacia Biotech) was labelled to a specific activity of $5x10^6$ cpm/μg of DNA with [α-^{32}P]dCTP (10 μCi/μl) by random priming using a random primed DNA labelling kit (Roche Molecular Biochemicals). Hybridisation took place overnight, following which the filters were washed in 2x SSC; 0.1% SDS at 65°C for 15 minutes, repeated at least three times until the background radioactivity was at low levels.

Filters were exposed to autoradiographic film for between five and 48 hours (Figure 9). Colonies giving strong positive signals were individually picked from the original plates and transferred to separate wells of microtitre plates containing liquid LB with 60μg/ml ampicillin, and 10% glycerol. These microtitre plates were incubated at 37°C for 2 hours to promote growth of the putative positive clones. These discrete colonies were re-screened using the same probe and protocol as previously described (Figure 10).

Colonies giving a strong signal after secondary screening were cultured overnight in 3 ml aliquots of LB containing 60μg/ml ampicillin. Plasmid DNA was extracted from these

bacterial cultures using a standard plasmid mini-prep purification protocol (Biotechniques, 1988) modified slightly to include precipitation in polyethylene glycol prior to ethanol precipitation in order to facilitate automated sequencing. The presence of insert DNA was confirmed by electrophoresis of the plasmid DNA on a 1.5% agarose gel and comparing the profile with that of native pUC18 digested with *Hind*III (Figure 11). Clones confirmed to contain an insert were sequenced with an Applied Biosystems automated sequencer (Model 373A, version 2.0.1S) using M13 forward and reverse primers.

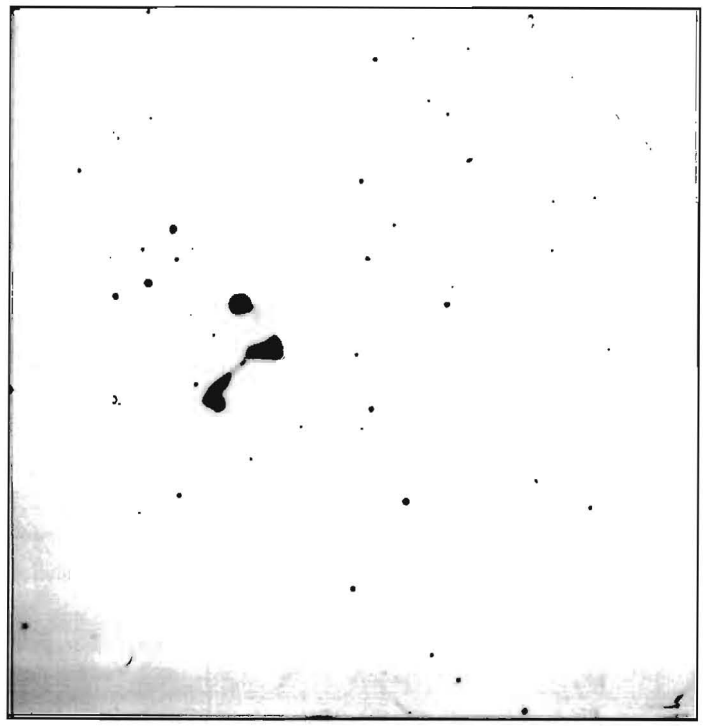

Figure 9 Primary screening of recombinant clones from a size-selected genomic library in *P. aequinoctialis*. Filters were hybridised with a radioactively labelled poly(CA) probe and exposed to autoradiographic film for between five and 48 hours.

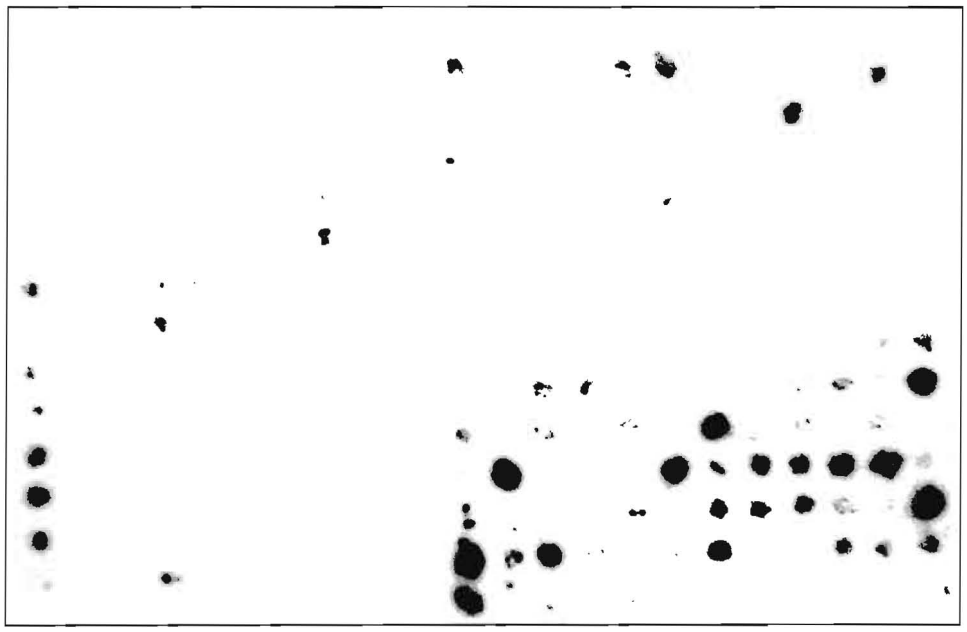

Figure 10 Secondary screening of positive clones selected during the primary screening step (Figure 9). Positive clones were picked after a primary hybridisation and transferred to microtitre plates following which they were reprobed using a radioactively labelled poly(CA) probe.

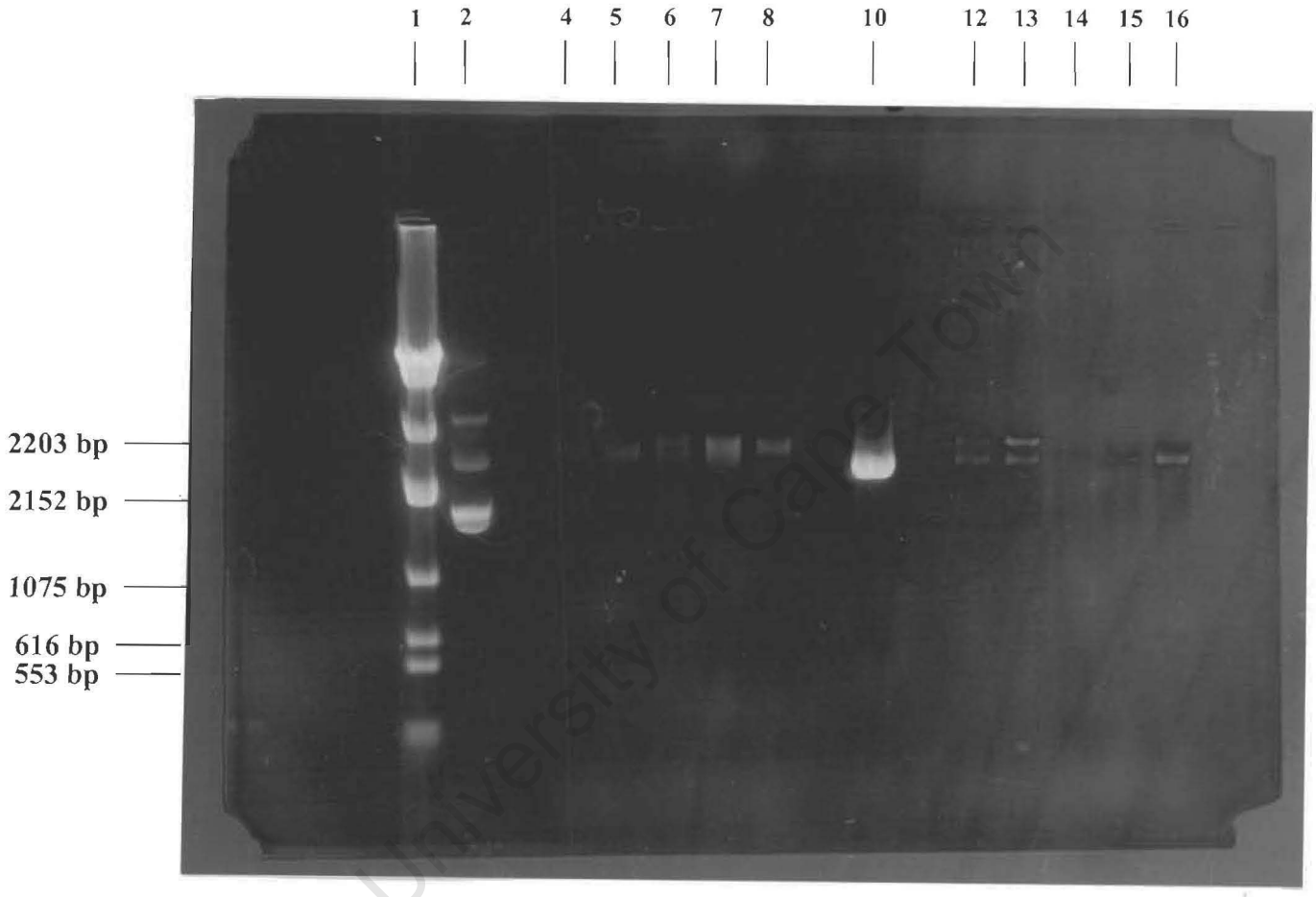

Figure 11 Positive clones (lanes 4-8 and 12-16) isolated from the *P. aequinoctialis* genome were checked for inserts by comparison to the native pUC18 plasmid vector that had been linearised through digestion with Hind III (lane 10). Lane 1 shows a λ-DraI size marker. Lane 2 shows the three forms of native pUC18 (circular, linear and nicked circular forms).

4.3 RESULTS

4.3.1 ABUNDANCE OF MICROSATELLITE LOCI

A total of 90 000 clones were screened for $(CA)_n$ repeats and 12 000 clones for $(TC)_n$ repeats. Blue/white colour selection indicated that approximately 10% of these recombinant clones did not contain inserts. Based on the mean avian genome size of 1.96 x 10^6 kb (Cavalier-Smith, 1985), and assuming an average insert size of 300 bp with no insert being present in more than one clone, 24 300 kb of the *P. aequinoctialis* genome was screened. This corresponds to 1.24% of the diploid genome.

Following primary screening 500 positive clones were identified. This number was reduced to 49 clones (10% of the original potential positives) following a secondary round of screening. (Table 16).

Table 16 **Abundance of $(CA)_n$ microsatellite loci in Procellariiformes**

Total	1° Screening	2° Screening	Sequenced	Perfect Repeats n=3	Imperfect Repeats	Repeats in pUC18
90 000	500	49	42	5	12	21
	0.56% of total clones	9.8% of 1° screening	86% of 2° screening	12% of sequenced clones	29% of sequenced clones	50% of sequenced clones

4.3.2 CHARACTERISATION OF MICROSATELLITE LOCI

DNA was isolated from 49 of the putative positive clones, and 42 were sequenced. Thirty-eight (88%) of these clones were found to contain microsatellite loci with $n > 3$. These microsatellite loci could be divided into three groups as described by Weber (1990): None of the clones contained pure $(CA)_n$ repeats where $n > 3$, or compound repeats. Twelve clones contained imperfect repeats motifs. Twenty-one instances of the

microsatellite repeat $[CA]_3A[CA]_1$ were found to occur within the pUC18 vector DNA. Five clones contained $(CA)_n$ where n=3.

Of the 42 microsatellite loci isolated and sequenced none were considered suitable for the development of primer sets for PCR amplification and genotyping. Imperfect and compound repeats are generally considered inferior to pure repeats in terms of the polymorphism displayed in population genetic studies (Ellegren, 1993). In addition, in largely because of the five perfect repeat loci which may have proved useful, two had fewer than 6 repeat units, and in the remaining three the inserts had been cut too close the 3' or 5' end of the motif, resulting in insufficient sequence information with which to develop primer pairs.

PCR primers were optimally designed to amplify one of the interrupted repeats $[(CA)_2$ ATTC $(CA)_3$ A $(CA)_1]$ and one of the short pure repeats $[(CA)_3]$, but both consistently failed to amplify products in *P. aequinoctialis*.

4.4 DISCUSSION

Birds have been shown to have a relatively small genome size compared to mammals, largely due to a reduction in the amount of non-coding DNA (Epplen *et al.*,1978; Doolittle, 1985). Since microsatellites are non-coding DNA, microsatellite abundance is similarly reduced (Hamada *et al.*, 1982; Manor *et al.*, 1988 and Primmer *et al.*, 1997) with dinucleotide motifs more common in humans than in birds (Primmer *et al.*, 1997). There are approximately 1 500 (CA) ≥ 14 repeat motifs in the chicken genome, compared to 16 000 and 17 000 in the human and pig genomes respectively (Primmer *et al.*, 1997). This provides evidence for a 10 to 15 -fold reduction in the number of dinucleotide repeat motifs in the avian genome. The reduced density of microsatellites in the avian genome has practical implications for the isolation and characterisation of novel loci.

While we have followed standard methods for cloning, isolation and characterisation of microsatellites (Rassmann *et al.*, 1991), recent publications have described protocols that enrich the yield of microsatellite-containing clones (Ostrander *et al.*, 1992; Armour *et al.* 1994 and Kandpal *et al.*, 1994). These "enriched" methods can result in a 50-fold enrichment for microsatellite repeats (Ostrander *et al.*, 1992) as compared to the conventional genomic library making protocols. There are different approaches to

generating microsatellite enriched libraries using either hybridisation selection (Armour *et al.*, 1994; Kandpal *et al.*, 1994), primer amplification prior to cloning (Ostrander *et al.*, 1992), or triplex affinity capture (Nishikawa *et al.*, 1995). In addition, there exist a number of methods which amplify anonymous microsatellite loci using RAPD (Ender *et al.*, 1996) or anchored PCR–based techniques (Fisher *et al.*, 1996). We did not attempt to use the hybridisation selection approach largely due to the difficulty in obtaining the specialised reagents required for these techniques. In addition it was felt that the time taken to establish these new protocols could be used more productively in screening a larger number of clones for rare repeat motifs. The isolation of anonymous microsatellite loci using RAPD based techniques was attempted but found to be so irreproducible as to be of little use.

In general the construction of marker-enriched libraries is necessary to avoid screening extensive numbers of clones. In the case of birds, where it has been demonstrated that microsatellite are up to 40% more rare than in mammals (Primmer *et al.*, 1997), these methods are increasing in popularity (Piertney *et al.*, 1998; Burg, 1999). Future work in developing a microsatellite library for use in *P. aequinoctialis* would be well advised to use enriched techniques in order to reduce the number of clones which need to be screened in order to isolate informative loci.

Most microsatellite studies have focussed on $(CA)_n$ /$(GT)_n$ repeat motifs as these have been shown to be the most common repeats in eukaryotic genomes studied to date (Beckmann and Weber, 1992). In birds there does not appear to be a single microsatellite motif as common as $(CA)_n$ in mammals (Primmer *et al.*, 1997). It is therefore probably advisable to screen with a cocktail of oligonucleotide probes including di- and tri-nucleotides.

The level of polymorphism at microsatellite loci has been shown to be proportional to the length of the repeat motif, with longer repeat motifs ($n > 16$) having polymorphic information content (PIC) values of 0.5 or more (Weber, 1990). It is therefore preferable to utilise the longest uninterrupted repeats possible for population genetic studies as these are most informative.

We have reported here the development of a microsatellite library for the white-chinned petrel, *P. aequinoctialis*. Since the avian genome contains approximately one-third the

total number of base pairs of that in the human genome (Bloom *et al.*, 1993) a large number of clones had to be sequenced in order to attempt to isolate a sufficient number of microsatellite loci for primer development. The 90% decrease in the numbers of putative following secondary screening illustrates the importance of a secondary round of screening in reducing the high background obtained due to non-specific hybridisation of the probe. In this case the high percentage of false positives even subsequent to secondary screening can be attributed to a short repeat sequence $[(CA)_2A(CA)_1]$ present in pUC18 which was identified using the computer program DAPSA (Harley, *pers. comm.*). This suggests that either the hybridisation or washing conditions were not stringent enough to prevent binding of the probe to short repeat motifs. This could be remediated by a combination of the following conditions: lengthening the washing time, increasing the hybridisation and washing temperatures, or by changing the probe selected for screening the library.

While we isolated 42 $(CA)_n$ microsatellite containing clones, only 2 of these were suitable for primer development, and of these neither produced a polymorphic amplification product when tested in *P. aequinoctialis*. Therefore, no informative microsatellite markers could be developed for use in Procellariiform population genetics. It is recommended that enriched library making techniques be used for the development of a microsatellite library for *P. aequinoctialis*.

5 CONCLUSIONS

The value of using microsatellites in determining population level structure, gene flow and variation for conservation purposes, as demonstrated in this study, remains undisputed.

Despite significant losses to the fisheries moderate to good heterozygosities have been observed in *D. exulans*, *T. chrysostoma* and *T. melanophris*. The diversity evident in these populations can be preserved in these populations provided a conservation management strategy to minimise fisheries bycatch is implemented and enforced.

Low levels of population differentiation were revealed between the breeding colonies of each of the three albatross species at the five loci tested. Thus, the natal origin of birds killed by the longline fisheries cannot be determined using these five markers. However, these low levels of differentiation imply that a "one-stock" management strategy may be an appropriate conservation measure. Implementation of a such a strategy would require that these low levels of population differentiation are confirmed by testing a larger array of microsatellite markers across a wider range of breeding populations from diverse breeding localities.

We have demonstrated the potential of heterologous microsatellite amplification to at least partially alleviate the need to make new microsatellite libraries for each new species of non-passerine studied. The extent of cross-species amplification for Procellariiformes appears to extend to the family level. Loci isolated in the genus *Diomedea* are informative in all tested members of the family Diomedidae, but not in the related family Procellariidae. This gives some evidence towards determining the evolutionary distances across which microsatellites can provide useful information.

Attempts to identify informative microsatellite loci in the genus *Procellaria* have indicated, in agreement with the literature, that microsatellites are less frequent in birds. This places emphasis on the value of using microsatellite enrichment techniques when cloning markers in birds, and on the value of screening for a range of microsatellite motifs.

This study complements previous behavioural observations by providing the first molecular perspective on parameters such as population structure and migration in the

Diomedidae. As such it provides the basis for future population genetic studies in this family.

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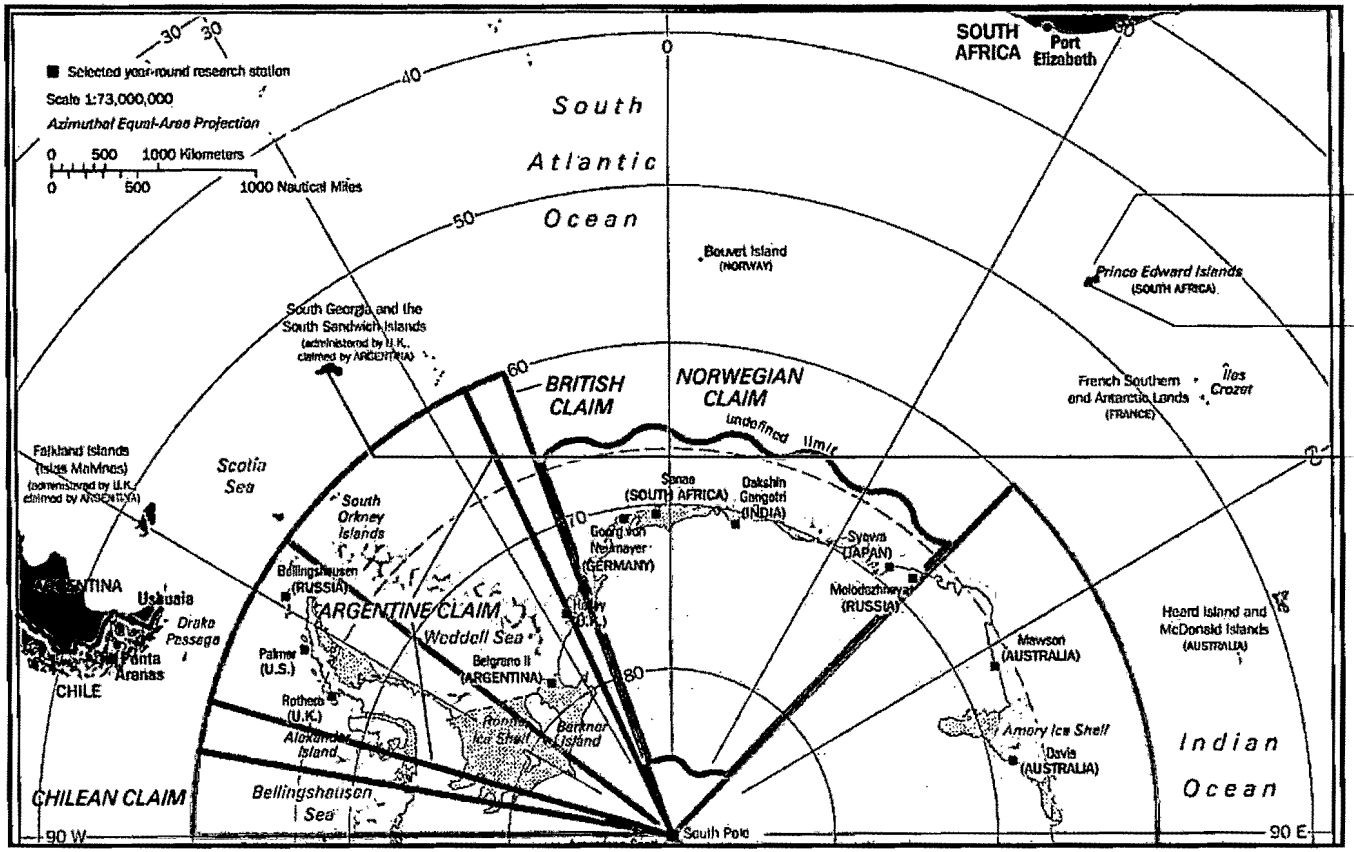
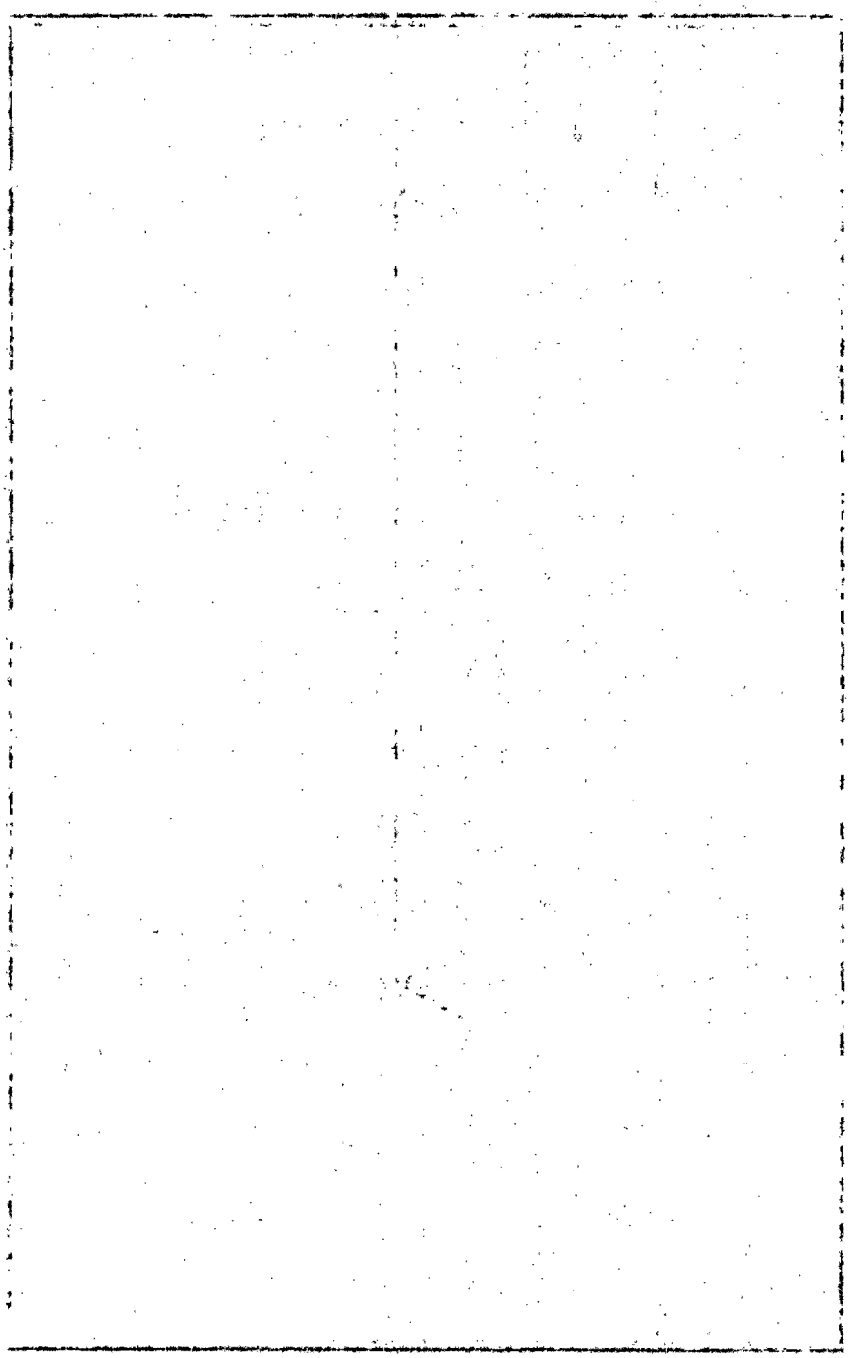
7 APPENDICES

7.1 APPENDIX A

The relative locations of South Georgia island and the Prince Edward island group, which includes Marion island and Prince Edward island. (Reproduced from `http://www.lib.utexas.edu/Libs/PCL/Map_collection/islands_oceans_poles/Antarctic_pol97.jpg`





7.2 APPENDIX B

Sample identification information, source, location and date of collection for *P. aequinoctialis*, *D. exulans*, *T. chrysostoma* and *T. melanophris* samples used in this study.

Species	Individual	Age	Ring	Source	Location	Date
P. aequinoctialis	A1	U	U	Bycatch A	46° 10'S 37° 37'E	21-11-96
P. aequinoctialis	A2	U	U	Bycatch A	46° 18'S 37° 22'E	22-11-96
P. aequinoctialis	A3	U	U	Bycatch A	46° 19'S 37° 25'E	23-11-96
P. aequinoctialis	A4	U	U	Bycatch A	46° 17'S 37° 32'E	25-11-96
P. aequinoctialis	A5	U	U	Bycatch A	46° 17'S 37° 32'E	25-11-96
P. aequinoctialis	A6	U	U	Bycatch A	46° 16'S 37° 33'E	03-12-96
P. aequinoctialis	A7	U	U	Bycatch A	46° 21'S 38° 29'E	09-12-96
P. aequinoctialis	A8	U	U	Bycatch A	47° 02'S 37° 56'E	14-12-96
P. aequinoctialis	A9	U	U	Bycatch A	47° 06'S 37° 53'E	17-12-96
P. aequinoctialis	A10	U	U	Bycatch A	47° 06'S 37° 53'E	17-12-96
P. aequinoctialis	A11	U	U	Bycatch A	46° 48'S 38° 32'E	18-12-96
P. aequinoctialis	A12	U	U	Bycatch A	46° 48'S 38° 32'E	18-12-96
P. aequinoctialis	A13	U	U	Bycatch A	46° 45'S 38° 29'E	23-12-96
P. aequinoctialis	A14	U	U	Bycatch A	46° 45'S 38° 29'E	23-12-96
P. aequinoctialis	A15	U	U	Bycatch A	46° 45'S 38° 29'E	23-12-96
P. aequinoctialis	A16	U	U	Bycatch A	46° 47'S 38° 28'E	24-12-96
P. aequinoctialis	A17	U	U	Bycatch A	46° 47'S 38° 28'E	24-12-96
P. aequinoctialis	A18	U	U	Bycatch A	46° 47'S 38° 28'E	24-12-96
P. aequinoctialis	A19	U	U	Bycatch A	46° 44'S 38° 30'E	24-12-96
P. aequinoctialis	A20	U	U	Bycatch A	46° 47'S 38° 28'E	25-12-96
P. aequinoctialis	A21	U	U	Bycatch A	46° 47'S 38° 28'E	25-12-96
P. aequinoctialis	A22	U	U	Bycatch A	47° 06'S 38° 27'E	27-12-96
P. aequinoctialis	B23	U	U	Bycatch B	46° 20'S 39° 44'E	06-11-96
P. aequinoctialis	B24	U	U	Bycatch B	46° 19'S 39° 45'E	07-11-96
P. aequinoctialis	B25	U	U	Bycatch B	46° 21'S 39° 54'E	08-11-96
P. aequinoctialis	B26	U	U	Bycatch B	46° 21'S 39° 54'E	08-11-96
P. aequinoctialis	B27	U	U	Bycatch B	46° 19'S 39° 48'E	09-11-96
P. aequinoctialis	B28	U	U	Bycatch B	46° 20'S 39° 52'E	10-11-96
P. aequinoctialis	B29	U	U	Bycatch B	46° 38'S 37° 44'E	13-11-96
P. aequinoctialis	B30	U	U	Bycatch B	46° 33'S 37° 48'E	14-11-96
P. aequinoctialis	B31	U	U	Bycatch B	46° 33'S 37° 48'E	14-11-96
P. aequinoctialis	1	U	U	South Georgia	Bird Island	U
P. aequinoctialis	2	U	U	South Georgia	Bird Island	U
P. aequinoctialis	3	U	U	South Georgia	Bird Island	U
P. aequinoctialis	4	U	U	South Georgia	Bird Island	U
P. aequinoctialis	5	U	U	South Georgia	Bird Island	U
P. aequinoctialis	6	U	U	South Georgia	Bird Island	U
P. aequinoctialis	7	U	U	South Georgia	Bird Island	U
P. aequinoctialis	8	U	U	South Georgia	Bird Island	U

P. aequinoctialis	9	U	U	South Georgia	Bird Island	U
P. aequinoctialis	10	U	U	South Georgia	Bird Island	U
P. aequinoctialis	11	U	U	South Georgia	Bird Island	U
P. aequinoctialis	12	U	U	South Georgia	Bird Island	U
P. aequinoctialis	13	U	U	South Georgia	Bird Island	U
P. aequinoctialis	14	U	U	South Georgia	Bird Island	U
P. aequinoctialis	15	U	U	South Georgia	Bird Island	U
P. aequinoctialis	16	U	U	South Georgia	Bird Island	U
P. aequinoctialis	17	Adult	U	Marion Island	Base	06-10-96
P. aequinoctialis	18	Adult	U	Marion Island	Base	06-10-96
P. aequinoctialis	19	Adult	U	Marion Island	Base	06-10-96
P. aequinoctialis	20	Adult	U	Marion Island	Base	06-10-96
P. aequinoctialis	21	Adult	7-50863	Marion Island	Base	11-10-96
P. aequinoctialis	22	Adult	7-50872	Marion Island	Base	04-11-96
P. aequinoctialis	23	Adult	U	Marion Island	Base	01-12-96
P. aequinoctialis	24	Adult	7-50876	Marion Island	Prion Valley	13-12-96
P. aequinoctialis	25	Adult	7-50882	Marion Island	Base-vd Boogaart	14-12-96
P. aequinoctialis	26	Adult	7-50883	Marion Island	Base-vd Boogaart	14-12-96
P. aequinoctialis	27	Adult	7-50884	Marion Island	Prion Valley	19-12-96
P. aequinoctialis	28	Adult	7-50885	Marion Island	Prion Valley	19-12-96
P. aequinoctialis	29	Adult	7-50886	Marion Island	Prion Valley	19-12-96
P. aequinoctialis	30	Adult	7-50888	Marion Island	Base South	03-01-97
P. aequinoctialis	31	Adult	7-50590	Marion Island	Base South	03-01-97
P. aequinoctialis	32	Adult	7-50890	Marion Island	Prion Valley	07-01-97
P. aequinoctialis	33	Adult	7-50891	Marion Island	Prion Valley	07-01-97
P. aequinoctialis	34	Adult	7-50892	Marion Island	Prion Valley	12-01-97
P. aequinoctialis	35	Adult	7-50893	Marion Island	Prion Valley	28-01-97
P. aequinoctialis	36	Adult	7-50894	Marion Island	Prion Valley	28-01-97
P. aequinoctialis	37	Adult	7-50895	Marion Island	Prion Valley	01-02-97
Species	**Individual**	**Age**	**Ring**	**Source**	**Location**	**Date**
D. exulans	1	U	U	South Georgia	Bird Island	U
D. exulans	2	U	U	South Georgia	Bird Island	U
D. exulans	3	U	U	South Georgia	Bird Island	U
D. exulans	4	U	U	South Georgia	Bird Island	U
D. exulans	5	U	U	South Georgia	Bird Island	U
D. exulans	6	U	U	South Georgia	Bird Island	U
D. exulans	7	U	U	South Georgia	Bird Island	U
D. exulans	8	U	U	South Georgia	Bird Island	U
D. exulans	9	U	U	South Georgia	Bird Island	U
D. exulans	10	U	U	South Georgia	Bird Island	U
D. exulans	11	U	U	South Georgia	Bird Island	U
D. exulans	12	U	U	South Georgia	Bird Island	U
D. exulans	13	U	U	South Georgia	Bird Island	U
D. exulans	14	U	U	South Georgia	Bird Island	U
D. exulans	15	U	U	South Georgia	Bird Island	15-05-96
D. exulans	16	Chick	U	Marion Island	Base to Skua Ridge	15-05-96
D. exulans	17	Chick	U	Marion Island	Base to Skua Ridge	15-05-96
D. exulans	18	Chick	U	Marion Island	Maccie Bay MB015	96-02-08
D. exulans	19	Chick	U	Marion Island	Maccie Bay MB014	96-02-08
D. exulans	20	Chick	U	Marion Island	Maccie Bay MB005	96-02-08

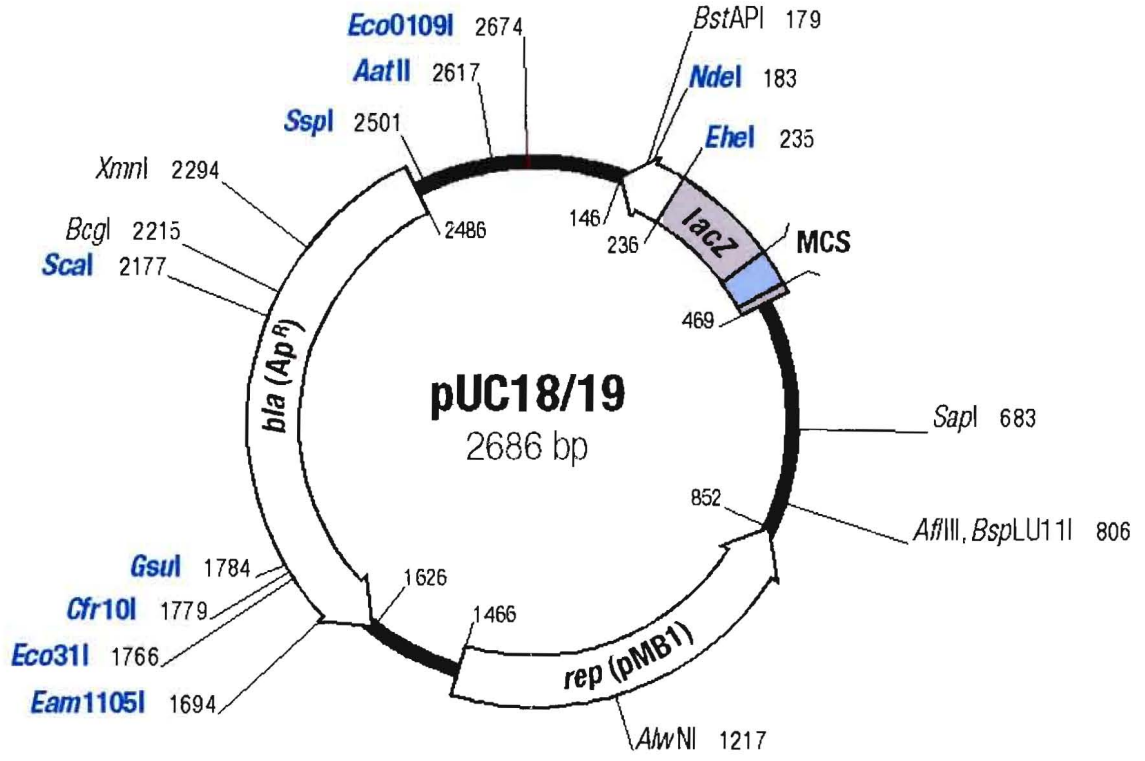
D. exulans	21	Chick	U	Marion Island	Maccie Bay MB006	96-02-08
D. exulans	22	Chick	U	Marion Island	Maccie Bay MB016	96-02-08
D. exulans	23	Chick	U	Marion Island	Maccie Bay MB002	96-04-08
D. exulans	24	Chick	U	Marion Island	Maccie Bay MB007	96-04-08
D. exulans	25	Chick	U	Marion Island	Maccie Bay MB017	96-04-08
D. exulans	26	Chick	U	Marion Island	Maccie Bay MB008	96-04-08
D. exulans	27	Chick	U	Marion Island	Maccie Bay MB001	96-04-08
D. exulans	28	Chick	U	Marion Island	Maccie Bay MB009	96-04-08
D. exulans	29	Chick	U	Marion Island	Maccie Bay MB010	96-04-08
D. exulans	30	Chick	U	Marion Island	Maccie Bay MB011	96-04-08
D. exulans	31	Chick	U	Marion Island	Maccie Bay MB012	96-04-08
D. exulans	32	Chick	U	Marion Island	Maccie Bay MB003	96-04-08
D. exulans	33	Chick	U	Marion Island	Maccie Bay MB018	96-04-08
D. exulans	34	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	35	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	36	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	37	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	38	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	39	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	40	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	41	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	42	Chick	U	Marion Island	Swartkop	26-11-96
D. exulans	43	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	44	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	45	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	46	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	47	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	48	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	49	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	50	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	51	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	52	U	U	Prince Edward	Top of Alby Valley	19-05-96
D. exulans	53	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	54	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	55	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	56	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	57	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	58	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	59	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	60	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	61	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
D. exulans	62	U	U	Prince Edward	Bottom of Alby Valley	19-05-96
Species	**Individual**	**Age**	**Ring**	**Source**	**Location**	**Date**
T. chrysostoma	1	U	U	South Georgia	Bird Island	U
T. chrysostoma	2	U	U	South Georgia	Bird Island	U
T. chrysostoma	3	U	U	South Georgia	Bird Island	U
T. chrysostoma	4	U	U	South Georgia	Bird Island	U
T. chrysostoma	5	U	U	South Georgia	Bird Island	U
T. chrysostoma	6	U	U	South Georgia	Bird Island	U
T. chrysostoma	7	U	U	South Georgia	Bird Island	U

T. chrysostoma	8	U	U	South Georgia	Bird Island	U
T. chrysostoma	9	U	U	South Georgia	Bird Island	U
T. chrysostoma	10	U	U	South Georgia	Bird Island	U
T. chrysostoma	11	U	U	South Georgia	Bird Island	U
T. chrysostoma	12	U	U	South Georgia	Bird Island	U
T. chrysostoma	13	U	U	South Georgia	Bird Island	U
T. chrysostoma	14	U	U	South Georgia	Bird Island	U
T. chrysostoma	15	U	U	South Georgia	Bird Island	U
T. chrysostoma	16	U	U	South Georgia	Bird Island	U
T. chrysostoma	17	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	18	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	19	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	20	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	21	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	22	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	23	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	24	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	25	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	26	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	27	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	28	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	29	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	30	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	31	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	32	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	33	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
T. chrysostoma	34	Chick	U	Marion Island	Greyheaded Ridge	24-03-97
Species	**Individual**	**Age**	**Ring**	**Source**	**Location**	**Date**
T. melanophris	1	U	U	South Georgia	Bird Island	U
T. melanophris	2	U	U	South Georgia	Bird Island	U
T. melanophris	3	U	U	South Georgia	Bird Island	U
T. melanophris	4	U	U	South Georgia	Bird Island	U
T. melanophris	5	U	U	South Georgia	Bird Island	U
T. melanophris	6	U	U	South Georgia	Bird Island	U
T. melanophris	7	U	U	South Georgia	Bird Island	U
T. melanophris	8	U	U	South Georgia	Bird Island	U
T. melanophris	9	U	U	South Georgia	Bird Island	U
T. melanophris	10	U	U	South Georgia	Bird Island	U
T. melanophris	11	U	U	South Georgia	Bird Island	U
T. melanophris	12	U	U	South Georgia	Bird Island	U
T. melanophris	13	U	U	South Georgia	Bird Island	U
T. melanophris	14	U	U	South Georgia	Bird Island	U
T. melanophris	15	U	U	South Georgia	Bird Island	U

* U – details unknown

8.2 APPENDIX C

Map of the pUC18 plasmid used as the cloning vector during the library making procedure.



pUC18